The Role of Thyroid Hormone in the Ventromedial Nucleus of the Hypothalamus

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Abstract

The hypothalamic arcuate and paraventricular nuclei play important roles in the neuroendocrine regulation of systemic thyroid hormone homeostasis. However, the roles of other hypothalamic regions are poorly understood. Triiodothyronine (T3) is the active form of thyroid hormone. T3 administration to the hypothalamic ventromedial nucleus (VMN) of rats stimulates feeding, although the mechanism remains unclear.

Activation and inactivation of thyroid hormones is mediated by the iodothyronine deiodinases, where D2 is the activating enzyme and D3 the inactivating enzyme. Recombinant adeno-associated virus (rAAV) vectors were designed to modulate the local activity of thyroid hormones by over-expressing D2 (rAAV-D2) or D3 (rAAV-D3).

Two initial investigations were carried out employing these viruses in vivo. Initially, rats received bilateral injections of either rAAV-D2 or rAAV-GFP into the VMN. Cumulative food intake and body weight were unaffected, despite a significant increase D2 enzyme activity. However, fasted rAAV-D2 treated rats consumed significantly more food than controls over the initial 2 hours of refeeding following a 12 fast. In the second investigation, rats received bilateral injections of either rAAV-D3 or rAAV-GFP into the VMN. Hypothalamic D3 mRNA and brown adipose tissue activity were both significantly increased in the rAAV-D3 group, which was not associated with any change in systemic thyroid hormone levels. This suggested that sympathetic activity had been increased, independent of peripheral thyroid status.

In a final investigation, rats were challenged with a high fat diet (HFD) 17 days after iVMN injection of either rAAV-D3 or rAAV-GFP. The rAAV-D3 treated group gained significantly more weight and consumed significantly more energy than controls, per day of HFD treatment. The increased HFD consumption was matched with a significant increase in hypothalamic fatty acid synthase mRNA.

This thesis highlights putative mechanisms by which thyroid hormones affect peripheral metabolism and appetite via the VMN, through interplay with neuronal fatty acid homeostasis.
Declaration of Originality

I, John Counsell, declare that the research for this thesis is original and that the ideas were developed by me in conjunction with my supervisors. Where information has been derived from other sources; I confirm that this has been indicated in the thesis.
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<tbody>
<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>αMHC</td>
<td>alpha myosin heavy chain</td>
</tr>
<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
</tr>
<tr>
<td>ACC</td>
<td>acetyl CoA carboxylase</td>
</tr>
<tr>
<td>AgRP</td>
<td>agouti-related peptide</td>
</tr>
<tr>
<td>AICAR</td>
<td>aminoimidazole carboxamide ribonucleotide</td>
</tr>
<tr>
<td>AMPK</td>
<td>adenosine monophosphate kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ARC</td>
<td>arcuate nucleus</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BMR</td>
<td>basal metabolic rate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>CART</td>
<td>cocaine-and amphetamine-related transcript</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
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CNS, central nervous system
CPT-1, carnitine palmitoyl transferase
CRE, cAMP response element
CSF, cerebrospinal fluid
CREB, cAMP response element-binding protein
D1, iodothyronine deiodinase I
D2, iodothyronine deiodinase II
D3, iodothyronine deiodinase III
DIO, diet-induced obesity
DIT, diiodotyrosine
DMN, dorsomedial nucleus
DMEM, dulbecco’s modified eagle medium
DNA, deoxynucleic acid
dNTP, deoxynucleotide phosphates
ddNTP, dideoxynucleotides
dsDNA, double-stranded DNA
DTT, dithiotreitol
eEFSec/eSelB, eukaryotic elongation factor, selenocysteine-tRNA-specific
EGR-1, growth response factor 1
ELISA, enzyme-linked immunosorbant assay
ER, endoplasmic reticulum
FADH2, flavin adenine dinucleotide
FAS, fatty acid synthase
FBS, foetal bovine serum
FSH, follicle-stimulating hormone
fT3, free triiodothyronine
fT4, free thyroxine
FXR, farsenoid-X-receptor
G6PDH, glucose-6-phosphate dehydrogenase
GDW, glass-distilled water
GEE, generalised estimating equation
GFP, green fluorescent protein
GH, growth hormone
GLUT4, glucose transporter 4
GSIS, glucose-stimulated insulin secretion
GTP, guanosine nucleotide triphosphate
HCG, human chorionic gonadotropin
HEBS, hepes-buffered saline
HEK293T, human embryonic kidney cells
HFD, high fat diet
HIF-1α, hypoxia-inducible factor 1 alpha
HIV, human immunodeficiency virus
HPA, hypothalamic-pituitary-adrenal axis
HPLC, high performance liquid chromatography
HPT, hypothalamopituitary thyroid axis
HSL, hormone-sensitive lipase
HSV, herpes simplex virus
ICV, intracerebroventricular
IEE, integration efficiency element
ITR, inverted terminal repeat
iVMN, intraVMN
JEG3, Human placental choriocarcinoma cell line
KO, knock-out
LB, luria broth
LEPR, leptin receptor
LH, luteinising hormone
LTR, long terminal repeat
LXR, liver-X-receptor
MAPK, mitogen-activated protein kinase
MBH, mediobasal hypothalamus
MC4R, melanocortin receptor-4
MCD, malonyl CoA decarboxylase
MCT8, monocarboxylate transporter 8
ME, median eminence
MIT, monoiodotyrosine
mRNA, messenger RNA
MMTV, mouse mammary tumor virus
NA, noradrenaline
NADH, nicotinamide adenine dinucleotide
NCD, normal chow diet
NPY, neuropeptide Y
NSE, neurone-specific enolase
OATP1C1, organic anion transport protein 1C1
PACAP, pituitary adenylate cyclase-activating peptide
pACC, phosphorylated ACC
pAMPK, phosphorylated AMPK
PCR, polymerase chain reaction
PEI, polyethylenimine
PEPCK, phosphoenol pyruvate carboxykinase
PGC1, peroxisome proliferator-activated receptor γ co-activator 1
PNS, peripheral nervous system
POAH, preoptic anterior hypothalamus
POMC, pro-opiomelanocortin
PPAR, peroxisome proliferator-activated receptor
PTU, polythiouracil
PVN, paraventricular nucleus
qPCR, quantitative PCR
rAAV, recombinant AAV
RIA, radioimmunoassay

RNA, ribonucleic acid

rT3, reverse triiodothyronine

RXR, retinoid X receptor

SAP, shrimp alkaline phosphatise

SBP2, secis binding protein 2

SCN, suprachiasmatic nucleus

SDS, sodium dodecyl sulphate

SECIS, selonocysteine insertion sequence

SEM, standard error of mean

SNS, sympathetic nervous system

SF-1, steroidogenic factor-1

SON, supraoptic nucleus

SRE, sterol response element

SREBP, sterol regulatory element binding protein

ssRNA, single-stranded RNA

SXR, steroid xenobiotic receptor

T1AM, 3-iodothyronamine

T3, triiodothyronine

T4, thyroxine

TBG, thyroxine-binding globulin

TOFA, 5-tetradecyloxy-2-furoic acid
TR, thyroid hormone receptor
TRE, thyroid response element
TRH, thyrotropin-releasing hormone
TSH, thyroid-stimulating hormone
UCP, uncoupling protein
USP, ubiquitin-specific peptidases
UTP, uracil triphosphate
UTR, untranslated region
VMN, ventromedial nucleus
WPRE, woodchuck post-transcriptional regulatory element
WSB1, WD repeat and SOCS box-containing protein 1
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Finally, I send my appreciation to the maxillofacial team at Kings College Hospital for resetting my face.
Chapter 1

Introduction
1.1 Energy Homeostasis

Energy homeostasis is the process of balancing energy input with energy expenditure. Imbalances in the process result in either obesity or weight loss. An essential component of this system is the hypothalamus, which responds to circulating hormones to control energy consumption and utilisation via a complex network of neural circuits.

Hormones exist within the circulation in order for the central nervous system (CNS) to appropriately respond to changes in the peripheral state. Compelling evidence implicates at least two peripheral hormones as afferent signalling molecules to the CNS which circulate in relation to the level of adiposity.

1.1.1 Insulin

Insulin is produced by pancreatic islets in response to elevated blood glucose levels. Its primary function is to coordinate efficient utilisation and storage of nutritional constituents following a meal. Levels of plasma insulin have been shown to directly correlate with body weight and adiposity (Bagdade, 1967). Obese animals have higher basal insulin levels and secrete more insulin in response to a meal than lean subjects (Woods et al., 1974). Insulin levels increase during periods of positive energy balance, such as during meals, and decrease in states of negative energy balance, such as fasting. Insulin receptor mRNA (messenger) and protein is found in CNS regions involved in the regulation of appetite and energy homeostasis (Schwartz et al., 2000).

1.1.2 Leptin

Since its discovery leptin has proven to be a key metabolic hormone with actions throughout the body. Leptin is secreted from adipocytes and circulates in plasma in proportion to the level of adiposity (Maffei et al., 1995). This is conveyed to the brain via leptin receptors (LEPR) which are expressed in the hypothalamus, where leptin acts to decrease appetite and increase energy (Wang, 1997). A lack of leptin in circulation signals a state of starvation to the hypothalamus to instigate feeding. Disruption of leptin signalling either by knock-out (KO) of its gene (Ob) or LEPR results in hyperphagia and extreme obesity (Chua Jr et al., 1996, Zhang et al., 1994).
1.2 The Hypothalamus

The hypothalamus links the nervous and endocrine systems by responding to plasma hormones that reflect the metabolic state. It is present in the brain of all mammals including humans, where it lies inferior to the thalamus and superior to the brainstem in the ventral diencephalon. It can be anatomically divided into functional regions (nuclei), as illustrated in figure 1.1, which interact to modulate various processes, including food intake and energy expenditure. The hypothalamic area containing both the ventromedial and arcuate nuclei is an important area for energy homeostasis that may be referred to as the mediobasal hypothalamus (MBH).
Figure 1.1: Three dimensional diagrammatic representation of the relative positioning of the hypothalamic nuclei in the rat as viewed from the dorsal and caudal hypothalamus in the right hemisphere. Abbreviations: AHA, anterior hypothalamic area; ARC, arcuate nucleus; AV3V, anteroventral area of third ventricle; CI, capsula interna; DP, dorsal parvocellular subnucleus of paraventricular nucleus (PVN); DMH, dorsomedial nucleus; F, fornix; LHA, lateral hypothalamic area; LM, lateral magnocellular subnucleus of paraventricular nucleus; LPOA, lateral preoptic area; ME, median eminence; MP, medial parvocellular PVN; MPO, medial preoptic area; OT, optic tract; SCh, suprachiasmatic nucleus; SON, supraoptic nucleus; SI, substantia innominata; ST, subthalamic nucleus; VMN, ventromedial nucleus; VP, ventral parvocellular subnucleus of paraventricular nucleus (Berthoud, 2002).
1.2.1 Arcuate Nucleus

The arcuate nucleus (ARC) encircles the third ventricle and lies superior to the median eminence. It extends anterior from the optic chiasm and posterior to the mamillary bodies. The ARC has a semi-permeable blood brain barrier, which allows the passage of signalling molecules from the circulation, including leptin, insulin and ghrelin (Cone et al., 2001). In terms of energy homeostasis, the two most studied neuronal populations in the ARC are neurones co-expressing anorectic peptides cocaine-and amphetamine-related transcript (CART) and pro-opiomelanocortin (POMC), in addition to neurones co-expressing orexigenic peptides agouti related peptide (AgRP) and neuropeptide Y (NPY).

1.2.2 Paraventricular Nucleus

The paraventricular nucleus (PVN) is a discrete triangular cluster of neurones surrounding the third ventricle in the anterior hypothalamus. A large amount of evidence suggests that the PVN is important in the regulation of appetite control, lesions to the area result in hyperphagia and obesity (Fukushima et al., 1987). Microinjection studies have shown that administration of orexigenic peptides to the area, including NPY (Stanley et al., 1985) and AgRP (Kim et al., 2000), stimulates food intake whilst anorexigenic peptide administration inhibits fast-induced feeding. Intracerebroventricular (ICV) administration of NPY into the third or fourth ventricle induces the expression of the immediate early gene c-fos in the PVN, indicating neuronal activation in this area (Xu et al., 1995).

1.2.3 Ventromedial Nucleus

The ventromedial nucleus (VMN) lies medial to the third ventricle. The VMN has been considered an important nucleus in energy homeostasis since the finding that lesioning the VMN of rats resulted in marked hyperphagia and obesity (Albert et al., 1971) which has later been associated with hypertriglyceridemia, hyperglycaemia and hyperleptinemia (Satoh et al., 1997). This led to its initial definition as a ‘satiety centre’, although its functional role in energy homeostasis has since shown to be more complex.

The VMN is an important nucleus in the control of glucose homeostasis. A widely documented feature of the VMN is the presence of glucose-sensitive neurones which exhibit altered action potential frequency in response to glucose exposure. As extracellular glucose increases from 0.1 to 2.5 mM, the action potential frequency of glucose-excited neurons increases, whereas for glucose-inhibited neurons it decreases (Song et al., 2001).
The VMN is associated with several CNS regions important in energy homeostasis regulation. It receives input from the ARC, lateral hypothalamus, and amygdala (Elias et al., 1998, Luiten et al., 1983, Saper et al., 1976). The VMN also contains efferent projections to ARC, PVN, lateral hypothalamus, DMN, amygdala, ventral tegmental area, nucleus accumbens, and nucleus of the solitary tract (Ter Horst and Luiten, 1987, Mcclellan et al., 2006, Saper et al., 1976, Canteras et al., 1994, Krieger et al., 1979). It has been reported that ARC POMC neurones receive strong excitatory input from VMN neurones, which is diminished during fasting (Sternson et al., 2005).

The VMN contains neuronal populations which differ in their biochemical properties and functional identities. It contains hormone receptors and neurones regulating energy homeostasis, including those of leptin, insulin, and melanocortins (Dhillon et al., 2006, Kang et al., 2004). The VMN is also marked by expression of the receptor for pituitary adenylate cyclase-activating peptide (PACAP) (Hawke et al., 2009), brain-derived neurotrophic factor (BDNF) (Wang et al., 2007), and steroidogenic factor-1 (SF-1) (Ikeda et al., 1995).

SF-1 is a member of the nuclear hormone receptor family of transcriptional regulators, which demonstrates activity at multiple levels of the hypothalamo-pituitary-steroidogenic axis (Ikeda et al., 1995, Lynch et al., 1993). CNS expression of SF-1 is limited to and essential for the development of the VMN (Ikeda et al., 1995). Homozygous SF-1 KO models produce normal litter sizes, but die by eight days due to acute glucocorticoid deficiency and exhibit developmental abnormalities such as the absence of adrenal glands, gonads, and VMN structure (Dellovade et al., 2000, Luo et al., 1994). The effects of SF1 may be partly mediated by altered BDNF expression, which is colocalised with SF-1 in mouse ventrolateral VMN neurones (Tran et al., 2006).

BDNF is a neurotrophin that demonstrates an essential role in hypothalamic control of energy homeostasis. Heterozygotic and homozygotic BDNF KO models are associated with reduced BDNF protein in the PVN and VMN with a concomitant increase in body weight and plasma leptin (Kernie et al., 2000). The BDNF receptor TrkB is also expressed in the VMN (Yan et al., 1997) and human patients with a de novo mutation in the TrkB gene exhibit hyperphagia and obesity (Yeo et al., 2004), whilst chronic overexpression of BDNF in the MBH stimulates energy expenditure in adult rats without affecting food intake (Cao et al., 2009). Recent studies have suggested that BDNF expression in the VMN is regulated by leptin and the melanocortins (Komori et al., 2006, Xu et al., 2003).
1.3 The Hypothalamopituitary Axes

The hypothalamus interacts with the pituitary gland to regulate several physiological processes. Stimulation of the pituitary by hypothalamic afferents stimulates pituitary secretion of hormones that regulate major organ function. Two important targets regulated in this way are the adrenal gland and the thyroid gland, via the hypothalamo-pituitary adrenal (HPA) and the hypothalamo-pituitary thyroid (HPT) axes, respectively. These endocrine pathways regulate metabolic function both independently and synergistically.

1.3.1 The Pituitary Gland

The pituitary is anatomically divided into anterior and posterior sections and also the intermediate lobe whose function is unclear. The posterior pituitary is innervated by hypophyseal neurones whose cell bodies originate in the PVN and supra optic nucleus (SON). In contrast, the anterior pituitary is composed of a number of different endocrine cells including, thyrotrophs, gonadotrophs, and somatotrophs. The secretory activity of these cells is principally controlled by peptide-releasing hormones, which are secreted from the hypothalamus into the adenohypophyseal portal vasculature, which flows into the pituitary via the pituitary stalk.

1.3.2 The Hypothalamo-Pituitary Thyroid Axis

Thyrotropin-releasing hormone (TRH) is a tripeptide (Glu-His-Pro) synthesised in the parvicellular PVN. Following synthesis, TRH is transported to the median eminence en route to the anterior pituitary via the portal capillary plexus. Interaction of TRH with its receptor on pituitary thyrotropes induces an intracellular cascade, which triggers the synthesis, and release of thyroid-stimulating hormone (TSH) into the circulation.

TSH is a 28kDa glycoprotein consisting of α and β subunits. The α subunit is common to other pituitary hormones, such as luteinising hormone (LH), and follicle stimulating hormone (FSH). Plasma TSH exerts effects in a number of tissues, although its primary action is on thyroid follicles, which express the TSH receptor. Upon TSH interaction with its receptor, intracellular cyclic adenosine monophosphate (cAMP) levels and protein kinase A activity are elevated. This stimulates the expression of genes involved in thyroid hormone synthesis and release.

Thyroid hormones regulate pituitary TSH synthesis and release by direct and indirect mechanisms, with the latter being through TRH expressing hypophysiotropic neurones of the
parvicellular PVN (Kakucska et al., 1992). Thyroid hormones may also influence the synthesis of other pituitary hormones, such as growth hormone (GH) and prolactin, by modulating transcription of their common glycoprotein α-subunit (Madison et al., 1993).

Figure 1.2 Diagrammatic representation of the HPT Axis. TRH is released from the hypothalamus and stimulates pituitary secretion of TSH, which increases thyroidal production and secretion of thyroid hormones (T3 and T4). Plasma T3 and T4 form a negative feedback loop to the hypothalamus and pituitary where they inhibit expression of TRH and TSH, respectively. The hypothalamopituitary axis is subject to an additional negative feedback pathway, whereby TSH inhibits TRH production.
1.4 The Thyroid Gland

The thyroid gland is essential to endocrine function on a number of levels. In addition to its primary effect on the basal metabolic rate (BMR), it is permissive for multiple hormonal pathways, particularly the adrenal axis, and is becoming increasingly recognised as an essential molecule for brain function.

1.4.1 Anatomy

The thyroid gland lies caudal to the larynx and adheres to the trachea. Its functional unit is the follicle, whose lumen is colloidal, composed almost entirely of thyroglobulin. The major products of the thyroid are thyroxine and triiodothyronine, which are synthesised and released following hypothalamo-pituitary stimulation. It is these products which feedback to the hypothalamus and pituitary gland to regulate their own circulating levels.

1.4.2 Hormone Synthesis

Inorganic iodine (I\(^-\)) is transported into follicle cells via a basolateral membrane Na\(^+\) symporter and oxidised (I\(^+\)) by thyroperoxidase at the lumen, in the presence of H\(_2\)O\(_2\). I\(^+\) is used to iodinate tyrosine residues of colloidal thyroglobulin. Monoiodotyrosine (MIT) and diiodotyrosine (DIT) are formed after single or double iodination respectively. These residues are then coupled to yield precursors of biologically active thyroid hormone. Thyroid hormones are then liberated from thyroglobulin following lysosomal hydrolysis. T4 is generated from DIT-DIT coupling, and MIT-DIT formation yields T3.

1.4.3 Plasma Thyroid Hormone

Thyroid hormones have widespread importance in early life, being critical for differentiation, growth, and development. In adulthood, these hormones are major regulators of the BMR, oxygen consumption and modification of adenosine triphosphate (ATP) turnover.

Thyroxine (T4) was first isolated in 1915. Thirty-seven years later, triiodo-L-thyronine (T3) was detected in human plasma (Gross and Pitt-Rivers, 1952). Its ability to control goitre in thiouracil-treated rats brought thyroid hormone into focus and in the subsequent years, the roles of thyroid hormone in energy homeostasis and development were discovered with its functional significance receiving greater appreciation.

In peripheral tissues, thyroid hormone activity is regulated by local enzymatic conversion, and exerted via interaction with nuclear receptor complexes. The most physiologically
significant thyroid hormone molecules are T4 and T3. Both consist of two covalently linked tyrosine molecules giving a double aromatic ring structure to which iodide (I) ions are added. Thyroid hormone nomenclature depends on the number of iodine atoms present on the iodothyronine molecule, i.e. the possession of four I is what defines the T4 molecule.

In the circulation, thyroid hormone is bound to serum proteins. Approximately 0.015% of T4 and 0.33% of T3 are free in plasma, with the majority bound to thyroxine-binding globulin (TBG). Healthy (euthyroid) plasma concentrations range from 2.5 – 5.3 pmol/L free T3 (fT3), and 10-23 pmol/L free T4 (fT4). The euthyroid range of plasma TSH is 0.27 - 4.2 mU/L. Disease of the thyroid gland is generally indicated when circulating levels of these hormones lie outside of their respective euthyroid range.

1.4.4 Thyroid Gland Pathophysiology

Elevation of plasma thyroid hormone above the euthyroid range is termed as thyrotoxicosis. Over-production of thyroid hormone by the thyroid gland denotes hyperthyroidism, where patients often present with heat intolerance, tachycardia, hyperphagia and weight loss. Hypothyroidism, conversely, is caused by thyroid under-activity and is principally associated with weight gain, cold intolerance, and fatigue.

Thyroid disease states are generally associated with disruption of HPT feedback regulation. Primary thyroid disease defines improper activation of the thyroid gland, secondary thyroid disease concerns pituitary release of TSH, and tertiary thyroid disease describes inappropriate signalling by the thyroid hormone-responsive hypothalamus.

Thyroid hormone resistance is a syndrome of hyposensitivity to thyroid hormone. Plasma TSH, T3, and T4 are normally elevated, although patients present symptoms of both hypothyroidism and hyperthyroidism. The level of thyroid hormone resistance may vary across tissues. Pituitary resistance to thyroid hormone induces signs of hyperthyroidism in peripheral tissues, suggesting that the impairment of thyroid hormone signalling is limited to the pituitary gland (Refetoff et al., 1993), which inappropriately stimulates thyroid gland activity.
1.5 Intracellular Thyroid Hormone

Steroid hormones are known to exert their effects via interactions with intracellular receptors. The classical steroid hormone receptors include the glucocorticoid, mineralocorticoid, oestrogen, androgen, and progesterone receptors. The resulting receptor-ligand complexes either translocate to the nucleus to exert slow, genomic effects or mediate rapid, non-genomic effects via intracellular signalling pathways.

The primary intracellular effect of thyroid hormone occurs in a similar fashion to that of the steroid hormones, whereby it is achieved through the interaction of T3 with the intracellular thyroid hormone receptor (TR).

1.5.1 Thyroid Hormone Receptors

Two major TR isoforms, encoded on separate genes, were identified and designated as TRα and TRβ. Additional heterogeneity of TRs occurs due to alternative splicing.

Splicing of the initial TRα mRNA transcript generates two mature mRNAs which code for two distinct proteins: TRα1 and TRα2. This splice variation is critical to thyroid hormone function as it renders c-erbAα2 unable to bind its T3 ligand, despite maintaining affinity for target genes (Lazar et al., 1988). This highlights a mechanism of antagonism in TR arising from the same genetic locus and is an important physiological feature of this system.

TRβ1 and TRβ2 are both derived from the TRβ gene. This gene contains two promoter regions whose activation dictates whether either or both receptor variants are synthesised. Both receptors bind T3 and target genes with equal affinity and are regulated by pituitary-specific transcription factors (Wood et al., 1996).

All forms of thyroid hormone receptor are ubiquitously expressed in rat tissues (Hodin et al., 1990). TRα-1 has highest expression levels in skeletal muscle and adipose tissue, whereas TRβ-1 is dominant in the brain, liver, and kidney. TRβ-2 has tissue specific expression in the anterior pituitary and specific areas of the hypothalamus (Cook et al., 1992).

1.5.2 Genomic Actions of Thyroid Hormone

Thyroid hormone enters the cell before translocating to the nucleus to interact with its nuclear receptor. Active TR is found in the nucleus either as a homodimer or as a heterodimer, bound to the retinoid X receptor (RXR). T3 efficaciously binds to this heterodimer complex which sheds corepressor factors and recruits coactivators following ligand binding. This TR-T3
complex (holoTR) then interacts with the thyroid-response element (TRE) which is present in
the promoter of genes targeted by thyroid hormone (Brent, 1994).

HoloTR functions like other steroid hormone receptors by stimulating or suppressing the
transcription of target genes, however, the TR mechanism is unique in that unliganded TR
(aapoTR) also interacts with TRE to modulate transcription of T3-responsive genes. It has
been reported that holoTR and apoTR modulate transcription in opposite fashions to enable
differential regulation of gene expression, depending on the temporal level of T3 activity
(Brent et al., 1989).

1.5.3 Non-Genomic Actions of Thyroid Hormone
In addition to its well characterised effects on gene expression, thyroid hormone also elicits a
number of non-genomic effects to rapidly alter cellular BMR. The non-genomic roles for
thyroid hormone are concerned with modulation of cellular basal activity and are mediated
through its interactions with plasma membrane receptors and signal transduction pathways.

The cell surface receptor, integrin αVβ3, is a heterodimeric protein which, following its
stimulation by T3, activates mitogen-activated protein kinase (MAPK). Activated MAPK
then translocates to the nucleus to dissociate TRβ1 from its co-repressors (Bergh et al., 2005).
Optimal activation of TRβ1 requires its T3 ligand, however, evidence of this MAPK
interaction suggests that nuclear thyroid hormone is not always required to elicit a response
(Davis et al., 2008).

Further to its phosphorylation and activation of TRβ1, this T3-activated MAPK pathway also
leads to the phosphorylation and nuclear translocation of other nuclear proteins (Davis et al.,
2008). In this manner, it is hypothesised that thyroid hormone acts permissively in preparing
other nuclear hormone receptors for interactions with their natural ligands.

Another important non-genomic effect of T3 is its modulation of Na+/K+ ATPase insertion
into the plasma membrane, which has been highlighted in rat alveolar cells (Lei et al., 2003).
This is particularly important in the case of neurophysiology as these proton pumps are the
point of regulation in generation of the axonal action potential.

1.5.4 Other RXR Partners
Cholesterol, fatty acids, fat-soluble vitamins and various lipids are absorbed from the diet and
serve as precursors for nuclear receptor ligands. These lipids require activation by enzymes in
the gastrointestinal tract before being transported to tissues where they mediate their effects (Chawla et al., 2001).

The receptors for these ligands constitute a nuclear receptor paradigm whereby adopted orphan nuclear receptors heterodimerise with RXR to modulate cellular function. Peroxisome proliferator-activated receptors (PPAR) interact with fatty acids, liver-X-receptors (LXR) interact with oxysterols, farsenoid-X-receptors (FXR) interact with bile acids and steroid xenobiotic receptors (SXR) interact with xenobiotics (Chawla et al., 2001). Increased activity of these RXR partners has been linked with impaired thyroid hormone signalling, owing to competition for the RXR heterodimer, genomic binding sites and transcriptional cofactors (Liu and Brent, 2010).

1.5.5 Iodothyronine Deiodinases

Although one would assume that thyroid hormone effect should be relative to its plasma concentration, such potent molecules require strict regulation in order to control metabolic rate. Plasma levels of thyroid hormones do not exhibit diurnal patterns of secretion like other essential hormones. Instead, a physiological mechanism exists to control the metabolic activity of specific tissues, without the need to drastically increase thyroid gland output.

The thyroid hormone T4 requires a molecular modification before it can efficiently interact with its receptor. An iodide molecule can be removed from specific sites on the aromatic rings of T4 with the targeted site dictating the biological activity of the product. Outer ring deiodination of T4 molecule yields biologically active T3, whereas inner ring deiodination produces reverse T3 (rT3) molecule, which exhibits lower affinity for TR (Braverman, 1990).

These catabolic reactions are mediated by a group of selenoenzymes, known as the iodothyronine deiodinases, which may be up or down-regulated in specific tissues in response to metabolic requirements, such as cold exposure and fasting (Alkemade, 2010). Further to their transcriptional regulation, the deiodinases undergo post-translational modifications. There are three classes of deiodinases, all of which have unique genetic origins (Dio1, Dio2, Dio3) and vary in their iodide targets and kinetic properties (Figure 1.3).
Figure 1.3: The products of iodothyronine deiodination. Removal of iodide from the outer phenolic ring by D2 produces active T3 (A), whereas removal of iodide from the inner phenolic ring by D3 produces inactive rT3 (B). Rings highlighted blue indicate those which have been deiodinated.

1.5.5.1 Iodothyronine Deiodinase I

Iodothyronine deiodinase I (D1) can deiodinate either the inner or outer ring of iodothyronines and is thought to be responsible for the majority of T4 to T3 conversion in circulation. Tissues with a high level of D1 expression include the liver, kidney, lung, pituitary gland and thyroid gland. The preferential substrates for D1 are rT3 and conjugated thyroid hormone, which highlights its important role in the degradation of circulating thyroid hormone metabolites (Chopra, 1996). D1-generated T3 in the liver and kidney rapidly equilibrates with plasma levels, which is primarily due to residence of D1 at the cell membrane (Toyoda et al., 1995). D1 is expressed in the rat, but not human CNS.

D1 is sensitive to inhibition by high concentrations of iodothyronine substrates (of which rT3 is the most potent), polythiouracil (PTU), catecholamine agonists and several other polyphenol molecules, including flavonols, isoflavones, aurones and calcones.
D1 KO models exhibit elevated plasma T4 and rT3 with euthyroid levels of plasma TSH and T3. This suggests compensatory mechanisms exist for the regulation of plasma thyroid hormone. Notable features of these models include increased faecal excretion of iodothyronines, indicating impaired clearance of thyroid hormone metabolites for which D1 is principally responsible (Schneider et al., 2006).

1.5.5.2 Iodothyronine Deiodinase II
The metabolically active T3 molecular structure is 3,3’5 triiodo-L-thyronine, which is formed following deiodination of T4 at the C5’ position of its outer ring. This reaction is catalysed by iodothyronine deiodinase II (D2) which is expressed across a range of tissues to regulate intracellular T3 levels. Where D1 is important in producing T3 for export into the plasma, D2 is considered responsible for maintaining specific tissue levels given that its affinity for T4 is approximately 3 fold higher than that of D1 (Richard et al., 1998).

D2 is retained in the ER and does not reach the golgo apparatus during its trafficking. This provides an ideal locus for T3 reduction where it is in close proximity to the nucleus (Zeöld et al., 2006). D2-generated T3 has a much longer cellular residence time than T3 generated by D1, which highlights its importance to intracellular thyroid hormone activity. This is illustrated by the finding that almost total thyroid hormone receptor saturation is witnessed in cells of the cerebral cortex that express D2 (Burmeister et al., 1997).

1.5.5.3 Iodothyronine Deiodinase III
Iodothyronine deiodinase III (D3) selectively dehalogenates the inner ring at its C5 position to produce inert hormones rT3 and T2 from T4 and T3, respectively. D3 displays 3 orders of magnitude higher affinity for thyroid hormones than D1 underlining its role as the principal thyroid hormone inactivator (Richard et al., 1998). It has been reported that D3 functions as a homodimer (Sagar et al., 2008). Following its translation, D3 is internalised at the plasma membrane and integrates with endosomal vesicles in a process of constant recycling between endosomal storage and membrane-association (Baqui et al., 2003).

D3 is highly expressed in the CNS in adulthood, in addition to the skin, ear, eye, placenta and foetal tissues. During early postnatal life, alterations in the expression of D3 are critical for maturation of the HPT axis (Hernandez et al., 2006a). D3 activity is affected by substrate inhibition in the rat brain (Tu et al., 1999), where it is up-regulated in response to thyrotoxic doses of T3 (Esfandiari et al., 1992). D3 is resistant to PTU inhibition and exhibits higher affinity for T3 than for T4.
1.5.5.4 Physiological Regulation of Iodothyronine Deiodinases

It is not surprising that the activities of the deiodinases are subject to tight physiological regulation given the profound effects of their thyroid hormone products. The substrate affinities of D2 and D3 are approximately three-fold higher than that of D1, which underlines the instrumental role that these deiodinases play in regulating TH activity and the efficiency by which they achieve this.

Static retention of D2 at the endoplasmic reticulum (ER) enables T3 to be generated in the cytosol with optimal access to the nucleus, where it mediates many of its effects. It has been reported that homodimerisation of D2 is critical for catalytic activity (Sagar et al., 2008). Conversely, the subcellular localisation of D3 at the plasma membrane allows instant deiodination and consequential inactivation of thyroid hormone upon its entry into the cell (Baqui et al., 2003). These respective cellular localisations provide an efficient mechanism in which incoming thyroid hormones are readily inactivated by the cell in cases of D3 upregulation, whilst D2 is in adequate proximity to the nucleus to offer its T3 product to its receptor and allow entry of the heterodimeric complex.

The half-lives of D1 and D3 have been reported as being several hours whereas the D2 half-life is much less at around twenty minutes (Gereben et al., 2000). This highlights a key feature of the D2 pathway as being strict physiological regulation of its activity. This is mediated by a variety of factors, such as feedback from thyroid hormones themselves (Sagar et al., 2007). Whilst D1 and D3 are primarily regulated by thyroid hormones at the transcriptional level, D2 is subject to post-translational regulation by ubiquitination (Tu et al., 1999, Koenig, 2005, Gereben et al., 2000). This ubiquitination pathway involves complex interplay between a number of elements, such as the hypoxic cascade and the hedgehog family of transcription factors, as outlined in figure 1.4 which summarises the key features of the iodothyronine deiodinases.

In addition to thyroid hormones, the expression of the deiodinases is regulated by numerous factors. Catecholamines and glucocorticoids are known modulators of D2 and D3 (Araki et al., 2003, Coppola et al., 2005, Song and Oka, 2003, Darras et al., 1996, Van der Geyten and Darras, 2005, Van der Geyten et al., 1999), which highlights an important and deep association between the thyroid pathway and other steroidogenic factors. The deiodinases are also regulated by the physiological state, where D2 is upregulated by cold exposure and food
consumption, whilst D3 is upregulated by inflammatory pathways and the hypoxic response (Simonides et al., 2008, Boelen et al., 2005, Boelen et al., 2008).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Weight (Da)</td>
<td>29000</td>
<td>30500</td>
<td>31500</td>
</tr>
<tr>
<td>Preferred Substrates</td>
<td>rT3, T3</td>
<td>T4, rT3</td>
<td>T3, T4</td>
</tr>
<tr>
<td>Km</td>
<td>$10^{-7}$ - $10^{-6}$</td>
<td>$10^{-9}$</td>
<td>$10^{-9}$</td>
</tr>
<tr>
<td>Half-life</td>
<td>Hours</td>
<td>~20 minutes</td>
<td>Hours</td>
</tr>
<tr>
<td>Sub-cellular Location</td>
<td>Plasma membrane</td>
<td>Endoplasmic reticulum</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>Response to T3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transcriptional</td>
<td>↑↑</td>
<td>↓</td>
<td>↑↑</td>
</tr>
<tr>
<td>Post-translational</td>
<td>?</td>
<td>↓↓↓</td>
<td>?</td>
</tr>
<tr>
<td>Induced by:</td>
<td>T3</td>
<td>Cold, hyperphagia, catecholamines, bile acids, cAMP</td>
<td>Tissue injury, infection, growth factors, MAPK activators, HIF-1α</td>
</tr>
<tr>
<td>Repressed by:</td>
<td>Fasting, illness</td>
<td>T3, hedgehog family, WSB1</td>
<td>Growth hormone, glucocorticoids,</td>
</tr>
<tr>
<td>Physiological Role</td>
<td>Clearance of metabolites</td>
<td>Thermogenesis, development, intracellular and plasma T3 source</td>
<td>Development, T3 and T4 clearance, avoidance of intracellular T3 production</td>
</tr>
</tbody>
</table>

*Figure 1.4: Summary of features of the iodothyronine deiodinases, adapted from (Bianco and Kim, 2006). T3, triiodothyronine; rT3, reverse T3; T4, thyroxine; cAMP, cyclic adenosine-monophosphate; MAPK, mitogen-activated protein kinase; HIF-1α, hypoxia-inducible factor 1 alpha; WSB1, WD repeat and SOCS box-containing protein 1*
1.5.5.4 Selenocysteine Insertion

The deiodinases are all selenozymes, which means that they require selenium at their active sites for efficacy. Selenium is an essential trace element that is incorporated into proteins as selenocysteine (Sec), the 21st amino acid. Selenocysteine is similar to cysteine in terms of its structure, where the principal difference is the replacement of a thiol group with a selenol group. This selenol group is advantageous in offering greater catalytic activity to selenoenzymes by virtue of its higher ionisation state at physiological pH.

Sec is encoded by a UGA codon, which would normally signal the termination of translation. In order to translate Sec from this codon, rather than a termination signal, the selenocysteine insertion sequence (SECIS), an RNA element of around sixty nucleotides in length, is required to decode UGA with Sec-tRNA (Hoffmann and Berry, 2005). In the iodothyronine deiodinases genes, this element is found in the 3’ untranslated region (UTR) where it forms the secondary hairpin-loop structure which interacts with the selenocysteine insertion machinery (Buettner et al., 1998). The advantage of having SECIS in the 3’ UTR is that this avoids the need to create such a structure in the mRNA itself.

Mammalian Sec incorporation requires a Sec-specific translation elongation factor (eEFSec/eSelB) which specifically binds to the Sec-tRNA only (Fagegaltier et al., 2000). Two binding proteins have also been identified as being important for Sec insertion, which are SECIS-binding protein 2 (SBP2) and ribosomal protein L 30 (rpl30) (Kinzy et al., 2005, Copeland et al., 2000). It has been proposed that the Sec-insertion machinery involves a complex of Sec-tRNA, the SECIS element, eEFSec and SBP2 (Driscoll and Copeland, 2003). The precise model for selenocysteine insertion remains to be elucidated, although a basic representation is illustrated in figure 1.5.

Selenoprotein synthesis in vivo is highly selenium-dependent, which can limit deiodinase overexpression when selenium availability is limited (Driscoll and Copeland, 2003, Hawkes and Keim, 2003). Selenocysteine incorporation is not an efficient process and the replacement of the UGA codon in D1 with a cysteine codon yields up to 400 fold more D1 protein (Berry et al., 1992). Mutations in the SECIS machinery have been reported in humans who display abnormal thyroid hormone metabolism owing to reduced D2 activity (Dumitrescu et al., 2005).
Figure 1.5: The selenocystein insertion machine. The SECIS element in the 3’UTR forms a hairpin-loop structure containing a ‘core’ of four quarternary non Watson-Crick base pairings, which is bound by SBP2. The eEFSec-associated Sec-tRNA interacts with this complex and its insertion at the UGA site is chaperoned by this complex. Adapted from (Driscoll and Copeland, 2003).
1.5.5.5 Deiodinase Polymorphisms

Two D1 polymorphisms (D1-C785T and D1-A1814G), affecting serum T3:rT3 ratio in healthy subjects, have been identified (Peeters et al., 2003). Both polymorphisms occur in the 3’ UTR which indicates that the diseases result from impaired mRNA stability. Opposing clinical presentations have been observed in these polymorphisms where D1-C785T results in decreased serum T3:rT3 ratio, whilst the opposite has been noted with D1-A1814G (Peeters et al., 2003). This suggests that D1-C785T results in decreased D1 activity and D1-A1814G results in increased D1 activity. The involvement of the SECIS has been implicated here, which lies downstream of the Dio1 coding region (Bianco et al., 2002).

An interesting observation has been noted in studies of deiodinase polymorphisms, regarding the varying contributions of D1 and D2 to serum T3 with age. The D1-C785T polymorphism has been associated with reduced serum T3:rT3 ratio in the aged, but is not as effective in younger patients (Peeters et al., 2006). Conversely, a D2-ORFa-Gly3Asp polymorphism in the Dio2 gene affects serum T3:T4 ratio young, but not elderly patients (Peeters et al., 2005). This indicates that, in addition to its important role in maintaining local T3 concentrations, D2 is also important for maintain serum T3 concentrations in early life, but D1 becomes more important with age.

The first D2 polymorphism to be detected was D2-Thr92Ala which is associated with insulin resistance rather than plasma thyroid hormone levels (Mentuccia et al., 2002). The mechanism is not well established, but is thought to involve D2 activity in skeletal muscle and brown adipocytes in humans (Bianco et al., 2002). The pathways proposed to be involved include the regulation of T3-stimulated lipolysis and sympathetic innervation of white adipose tissue, originating from hypothalamic D2 (Viguerie et al., 2002, Romijn and Fliers, 2005).

The only D3 polymorphism to be identified, D3-T1546G, is located in the 3’ UTR of Dio3 (Peeters et al., 2003). This does not appear to manifest in thyroid hormone homeostasis and the specific effects are not well characterised.

1.5.6 Thyroid Hormone Transporters

Thyroid hormone must be transported across the plasma membrane before it may interact with deiodinases in the cytosol and receptors of the nucleus. The hydrophobic nature of thyroid hormone molecules may account for some of its entry into the cell by passive
diffusion, although specific carriers belonging to organic anion and amino acid transporter families have been identified as significant mediators of thyroid hormone entry into the cell.

1.5.6.1 Organic Anion Transporters

Organic anion transporters (OAT) mediate the uptake of iodothyronines and their sulphonated derivatives. OATs are members of the Na+/tauchlorate cotransporting polypeptide and Na⁺ independent organic anion transporting polypeptide (OATP) families.

Since other NTCP members are exclusively expressed in the liver, it is OATP transporters that have a prominent role in the transport of thyroid hormone. The particularly interesting OATP members with respect to thyroid hormone transport are OATP14 and OATP1C1, the latter of which is highly expressed in human brain (Pizzagalli et al., 2002). It is thought to preferentially transport the T4 molecule and based on its wide expression in CNS capillaries, it is assumed to play an important role in T4 penetration of the blood-brain barrier (BBB) (Jansen et al., 2005).

1.5.6.2 Amino acid Transporters

With thyroid hormone being composed of covalently bound amino acids it is logical that amino acid transporters facilitate their membrane passage. The monocarboxylate transporter (MCT) family comprise 14 identified members, with MCT8 being important in thyroid hormone transport. MCT8 is predominantly expressed in neuronal cells and T3 appears to be its preferential ligand (Friesema et al., 2008).

In humans, mutations of the MCT8 gene result in resistance to thyroid hormone and impaired CNS development due to central hypothyroidism (Schwartz et al., 2005). This X-linked condition is termed Allan-Herndon-Dudley Syndrome with which six large families have been identified (Allan et al., 1944, Bialer et al., 1992, Friesema et al., 2004). The abnormal transport of thyroid hormones in these patients is highlighted by the presence of elevated plasma fT3 levels with reduced plasma fT4 levels. This underlines the importance of MCT8 for T3 transport and the extent of its preference for this iodothyronine molecule.
Figure 1.6: The process of thyroid hormone entry into the cell, subsequent deiodination and eventual genomic interaction within the nucleus. Thyroid hormones T4 and T3 enter the cell via their respective transporters OATP1C1 and MCT8. D3 is localised to the plasma membrane and readily inactivates entering iodothyronines upon entry into the cell. D2 is localised to the endoplasmic reticulum, which allows it to produce active T3 ligand in close proximity to the nucleus. T3 enters the nucleus, where it binds either TR or TR-RXR heterodimer. The T3-TR-RXR heterotrimeric complex efficaciously mediates its effects on thyroid-responsive genes through interaction with the thyroid response element. T4, Thyroxine; T3, triiodothyronine; T2, diiodothyronine; D2, iodothyronine deiodinase II; D3, iodothyronine deiodinase III; OATP1C1, organic anion transporter 1C1; MCT8, monocarboxylate transporter 8; ER, endoplasmic reticulum; TR, thyroid hormone receptor; RXR, retinoid X receptor; TRE, thyroid response element.
1.6 Thyroid Hormone and the Metabolic Rate

In a fully relaxed subject at room temperature, energy expenditure equals the basal metabolic rate. The BMR is the amount of energy expended while at rest in a neutrally temperate environment with the resultant heat defined as obligatory thermogenesis. In states of thyroid dysfunction, alterations in energy homeostasis occur. It has long been accepted that thyroid hormone affects the BMR by modulating cellular metabolic cycles, through targets such as Na\(^+\)/K\(^+\) symporters and permissive interactions with steroid hormone receptors. However, in addition to this well defined metabolic role in peripheral organs, there is growing interest in how thyroid hormone regulates energy homeostasis via the CNS, with much focus on the hypothalamus.

1.6.1 Metabolic Pathways

T3 is an essential regulator of metabolic pathways with varying effects across peripheral tissues. The iodothyronine deiodinases locally control thyroid hormone effects by influencing its intracellular concentration, whilst expression of specific TR isoforms may vary across tissues. These features enable the metabolic rate in specific tissues to be modulated independent of plasma thyroid hormone levels. Two important metabolic pathways affected by thyroid hormone include fatty acid metabolism and glucose metabolism, which culminate in the synthesis of ATP.

1.6.1.1 Glucose Metabolism

Glucose homeostasis is influenced by thyroid hormone where an excess of T3 stimulates glucose uptake in muscle, glucose production in the liver and impairs glucose-stimulated insulin secretion.

T3 principally affects muscle glucose metabolism by stimulating glycolysis and the insertion of glucose transporter 4 (GLUT4) into the plasma membrane of myocytes (Leijendekker et al., 1987), although ATP production remains constant due to upregulation of UCP2 and UCP3 (Short et al., 2001). T3 is further linked to glucose metabolism through its regulation of carbohydrate response element binding protein, a basic helix-loop-helix leucine zipper transcription factor, which upregulates glycolytic and lipolytic enzymes in response to glucose and insulin. The net effect of these processes is a potent increase in oxygen consumption and thermogenesis.
The hyperthyroid state is generally associated with impaired glucose tolerance despite the characteristic reduction in body fat increased fuel oxidation. This may be, in part, due to the effect of holoTR on pancreatic islets, which impairs glucose-stimulated insulin secretion. T3 enhances hepatic glucose production by promoting glycogenolysis and gluconeogenesis. In thyrotoxic rats, endogenous glucose production was increased by 45%, with concomitant upregulation of hepatic phosphoenol pyruvate carboxykinase (PEPCK), a rate-limiting enzyme of hepatic gluconeogenesis (Klieverik et al., 2008).

1.6.1.1 Fatty Acid Metabolism

T3 treatment in rats stimulates lipolysis as a result of increased fatty acid β oxidation, where it augments catecholamine stimulation of lipolysis. T3 upregulates hormone-sensitive lipase (HSL) and inhibits the expression of cAMP phosphodiesterase, thereby increasing intracellular cAMP signalling to lipolytic pathways (Conti et al., 1995, Oppenheimer et al., 1991).

Lipogenesis is also stimulated by T3, which is important in the control of cholesterol homeostasis. T3 treatment stimulates the expression of the low density lipoprotein receptor and sterol regulatory element binding proteins (SREBPs), which interact with a number of lipogenic enzymes (Brenta et al., 2007, Baxter and Webb, 2009). Hypercholesterolaemia is a common symptom of hypothyroidism, marked by elevated serum LDLs and decreased hepatic lipase activity, which can be ameliorated by thyroid hormone replacement therapy (Brent, 1994, Packard et al., 1993).

Fatty acid metabolism is modulated by thyroid hormone, where the expression of several enzymes of this metabolic pathway is affected, including malic enzyme, glucose-6-phosphate dehydrogenase (G6PDH), acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS) (Oppenheimer et al., 1995).

The expression of the SREBP enzymes is regulated by thyroid hormones, where SREBP-1c is repressed and SREBP2 is induced by holoTR signalling (Shin and Osborne, 2003, Hashimoto et al., 2006). The SREBPs affect the expression of a range of enzymes involved in cholesterol and fatty acid synthesis, including FAS and ACC. SREBP function is also regulated by thyroid hormone in an indirect manner. SREBP homodimers recognise the sterol-response element (SRE) on target genes, some of which have tandem arrangement of the SRE and TRE, which positions them in close proximity (Bengoechea-Alonso and Ericsson, 2007). This is an important feature in ACCA expression, which is enhanced by the formation of a
tetrameric complex between SREBP-1 and TR-RXR, which stabilises SREBP-1 on the SRE (Zhang et al., 2003).

Thyroid hormone signalling has been implicated in the negative regulation of PPAR signalling. T3 and PPARs combine to modulate a variety of processes in fatty acid metabolism, such as the stimulation of β oxidation by carnitine palmitoyl transferase 1 alpha (CPT-1α) (Liu et al., 2007). Unliganded TR can inhibit the effects of PPAR by competing for RXR and occupying the PPAR-response element (PPRE) present on its target genes (Lemberger et al., 1996). Furthermore, treatment with a PPARα agonist can ameliorate the impaired glucose-stimulated insulin secretion associated with the hyperthyroid state, which highlights the complex interplay between thyroid hormone, fatty acid signalling and glucose homeostasis (Holness et al., 2008).

1.6.1.3 Adenylate Charge

T3 modulates cellular adenylate charge through the stimulation of ATP consuming proteins, such as the Na⁺/K⁺ or Ca²⁺ ATPases (Davis and Blas, 1981), and also stimulation of ATP synthesising machinery. Oxidation of fuels, such as glucose and fatty acids, yields electrons in the form of reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂). These molecules serves as carriers for electrons to enter the mitochondria, where they feed into the electron transport chain a series of protein complexes located in the mitochondrial inner-membrane. The movement of electrons along the electron transport chain produces the proton motive force. This electrochemical proton gradient is harnessed by ATP synthase to drive ATP synthesis (Lehninger et al., 2005).

1.6.2 Brown Adipocyte Thermogenesis

Thyroid hormone also plays a fundamental role in ‘adaptive’ thermogenesis (Lowell and Spiegelman, 2000). Adaptive thermogenesis in larger mammals was historically thought to be mediated by muscle-shivering and responses to dietary intake, with smaller mammals demonstrating a higher level of dependence on a specialised fat cell lineage, known as brown adipose tissue (BAT). However, recent evidence has suggested that BAT thermogenesis is also physiologically significant to humans and may protect against obesity (van Marken Lichtenbelt et al., 2009, Cypess et al., 2009, Virtanen et al., 2009).

The net effect of BAT thermogenesis is a decline in metabolic efficiency, causing cellular respiration to produce heat rather than replenish energy supplies. This serves a physiological
purpose to provide thermal energy to distant tissues via the circulatory system. BAT activity depends on nutrient supply and its activation by the sympathetic nervous system.

The uncoupling proteins (UCPs) are members of the mitochondrial anion-carrier proteins. There are five UCP homologues (UCP1-5) of which UCP2 and UCP3 share high sequence identity with UCP1. Whilst UCP1 is unique to BAT, UCP2 and UCP3 demonstrate a diverse expression pattern. The physiological role of UCP1 in adaptive thermogenesis is well defined, however, the specific roles of the other UCP homologues is not entirely clear.

Uncoupling protein 1 (UCP1) can be upregulated, to interfere with ATP synthesis, by peroxisome proliferator-activated receptor γ (PPARγ), co-activator 1 (PGC1), noradrenaline-stimulated cAMP response element-binding protein (CREB) phosphorylation (Thonberg et al., 2002, Puigserver et al., 1998) and thyroid hormone. Transcribed UCP1 translocates to the mitochondrial membrane, where it allows protons to re-enter the matrix for further respiration as opposed to driving ATP synthase activity. This dissociates the electron transport chain from ATP synthesis, which dissipates electrochemical energy as heat.
1.7 Hypothalamic Thyroid Hormone

Thyroid hormone has major effects on the developing brain in utero and during neonatal development. Studies in hypothyroid rats have shown that absence of thyroid hormone causes diminished axon and dendritic growth in various the neocortex, hippocampus, and cochlea. However, in adult mammals the central role of thyroid hormone is primarily concerned with the control of energy homeostasis owing to its variety of interactions with the hypothalamus.

Hypothalamic thyroid hormone is critical to the regulation of energy homeostasis, for which it provides important regulatory signals across a number of pathways. D2 is highly expressed in specialised glial cells, known as tanycytes, which line the floor and intralateral walls of the third ventricle (Lechan and Fekete, 2007). This location is of physiological relevance with regards to the third ventricle cerebrospinal fluid (CSF) being a source of T4 from which the thyroid hormone signal is transduced to the hypothalamus or pituitary. Following its activation by D2 in tanycytes, T3 enters neighbouring cells in a paracrine fashion to interact with its nuclear TR (Freitas et al., 2010). D3 expression in the CNS is primarily neuronal (Tu et al., 1999), thus intraneuronal thyroid hormone is readily inactivated upon upregulation of D3 (figure 1.5).

The myriad effects of thyroid hormone on peripheral tissues require localised regulation by the deiodinases and this is also relevant to the CNS. Hypothalamic thyroid hormone has been identified as a critical regulator of various regulatory processes including; the HPT axis, torpor, the gonadal axis and appetite.
Figure 1.8: Mechanism of thyroid hormone action in the brain. T4 is transported into tanycytes by OATP1C1, where it is deiodinated to give the active T3 molecule. T3 is then exported from the glial cell by MCT8 into extracellular space before entering neighbouring neurones via MCT8. D3 enzyme present at the plasma membrane is able to inactivate T3, therefore upregulation of this deiodinase can block the neuronal effects of T3 (Freitas et al., 2010).
1.7.1 HPT Feedback
Plasma levels of thyroid hormone are maintained within a physiological range by negative feedback to the hypothalamus, whereby T3 represses the transcription of TRH to reduce thyroid gland activity. In the fasted state, D2 is upregulated in the hypothalamus approximately 2-fold to increase local T3 availability and downregulate TRH (Diano et al., 1998), which ultimately reduces energy utilisation in the periphery. The precise hypophysiotropic pathway controlling T3 feedback to the PVN is unclear. TRβ2 is generally accepted as the dominant TR isoform in T3 stimulated TRH expression. However, wild-type and TRβ2 knock-outs show similarly reduced TRH expression during fasting, which suggests involvement of additional pathways (Abel et al., 2001).

ARC neurones projecting to the PVN are thought to play a role in HPT axis regulation, whereby POMC/CART expressing neurones stimulate and NPY/AgRP populations inhibit TRH synthesis in the PVN (Mihaly et al., 2000). This is supported by studies that have localised melanocortin receptor-4 (MC4R) on almost all TRH-expressing neurones of the caudal-medial parvocellular PVN in addition to recent evidence also suggests that T3 directly regulates the expression of MC4R (Decherf et al., 2010). Further to these effects on the melanocortin pathway, T3 has also been found to modulate ARC NPY/AgRP neurones that project to the PVN. In this investigation, it was reported that T3 stimulates the expression of UCP2 in this ARC neuronal population, in turn elevating mitochondrial density and modulating the metabolic rate of these neurons (Coppola et al., 2007).

1.7.1.1 The Role of the Deiodinases in HPT Feedback
Deiodinase-mediated local control of thyroid hormone signalling is an important feature of the HPT negative feedback pathway. In the hypothalamus, D2 expression is highest in the MBH, where it is upregulated in the fasted state state (Diano et al., 1998, Tu et al., 1997). D2 is not present in the PVN (Tu et al., 1997), which suggests that locally generated T3 arrives at the TRH-expressing neurones of the PVN from other hypothalamic nuclei or 3rd ventricular CSF (Freitas et al., 2010).

In the rat pituitary gland, D2 expression has been widely detected in TSH secreting cells (Christoffolete et al., 2006). In addition, D2 mRNA was also detected in anterior pituitary cells that were TSH-negative, suggesting that D2 is also expressed in non-TSH secreting cells (Christoffolete et al., 2006). The accepted pathway is that, through the coordinated expression of D2, the thyrotroph integrates circulating levels of T3 and T4 to direct TSH secretion. D2
expression in the thyrotroph is inversely correlated with serum TSH levels. This is because T4 binding to the D2 active site potentiates D2 ubiquitination (Sagar et al., 2007).

Through these pathways, serum fT4 concentration regulates HPT feedback, where reduced fT4 promotes a two to three-fold increase in serum TSH (Larsen et al., 1982, Riesco et al., 1977). This regulation has been shown to be dependent on D2 given that serum TSH levels are not suppressed by T4 administration in D2 knock-out mice (Schneider, 2001). However, PVN TRH levels were suppressed by T4 in this mouse model, despite the lack of effect on serum levels, indicating that resistance to T4 occurs primarily in the pituitary gland rather than the hypothalamus.

During early postnatal life, alterations in the expression of D3 are critical for maturation of the HPT axis (Hernandez et al., 2006b, Hernandez et al., 2006a). This is highlighted by the phenotype of the D3KO mouse, which undergoes extensive changes in thyroid homeostasis during the neonatal period before developing central hypothyroidism (Hernandez et al., 2007, Hernandez et al., 2006a). At birth, the expression of D3 is localised to the bed nucleus of the stria terminalis and hypothalamic preoptic nuclei, but by postnatal day 10 its expression is diffuse. It has been suggested that this neonatal expression pattern is linked with the establishment of the set-point for TRH secretion, which is essential for proper development of the HPT axis (Hernandez et al., 2006a).

1.7.2 Regulation of Sympathetic Outflow
The interplay between T3 and catecholamines is an essential, ubiquitous feature of physiology. An effect of this nature has been identified in the hypothalamus, where T3 appears to modulate hepatic gluconeogenesis via sympathetic outflow from the hypothalamic PVN.

Thyrotoxicosis is associated with elevated endogenous glucose production with associated hepatic insulin resistance. Sympathetic and parasympathetic denervation of the liver attenuated the increased glucose output, which suggests that hepatic glucose output could be affected by thyroid hormone at the level of the hypothalamus (Klieverik et al., 2008). In euthyroid rats, T3 microdialysis in the PVN stimulated endogenous glucose production by 11%, which highlights hypothalamic thyroid hormone as a potential activator of sympathetic outflow to the liver (Klieverik et al., 2009).
It remains to be ascertained whether this is a sole pathway of sympathetic activity under the control of thyroid hormone, although it is possible that additional organs may be regulated by thyroid hormone concentration in specific hypothalamic nuclei.

A role for thyroid hormone in sympathetic signalling was also highlighted in mice expressing TRα mutations (Sjögren et al., 2007, Pelletier et al., 2008). Despite the disruption to thyroid hormone signalling in peripheral tissues, which would normally hinder catabolic processes, these mice presented with a lean phenotype at an ambient temperature of 21°C. When housed at 30°C, these mice had a suppressed response to a norepinephrine challenge, suggesting modulated sympathetic signalling. Given the effect on body weight, it was perceived that the phenotype was due, in part, to an increased sympathetic drive from the CNS.

1.7.3 Photoperiodic Regulation

In mammals, nocturnal melatonin secretion by the pineal gland conveys temporal information to the hypothalamus to modulate the activity of its neural circuits and conserve energy-demanding processes. There is evidence from several species that the iodothyronine deiodinases are regulated by photoperiod to modulate energy homeostasis via hypothalamic energy circuits. In the Syrian hamster MBH, D2 is highly expressed in reproductively active hamsters during long day periods and downregulated during short days (Revel et al., 2006). Pinealectomy demonstrated that this regulation is melatonin dependent and injections of long day hamsters with melatonin for just one week were sufficient to reduce Dio2 expression to that of short day levels (Yasuo et al., 2007). In Siberian hamsters, D3 shows temporal regulation by photoperiod in the MBH during periods of hibernation. Upregulation of D3 in short-day periods was associated with a state of reduced metabolic activity, outlined by decreased body weight, and an inactive reproductive system, marked by significantly reduced testicular weight (Barrett et al., 2007).

1.7.4 Appetite and Energy Homeostasis

Hyperthyroidism is generally associated with increased energy expenditure and reduced body weight. The increased appetite is generally thought to compensate for the increase in metabolic rate, however roughly 5-10% of hyperthyroid individuals have a sufficiently increased appetite to gain weight despite the catabolic processes (Gurney, 1970). This suggests that thyroid hormone may directly affect hypothalamic energy circuits, with several regulators of appetite and energy expenditure implicated.
Intramuscular administration of T3 to rats has been shown to stimulate food intake, without affecting energy expenditure, and increase early growth response factor 1 (EGR-1) expression, a marker of neuronal activity, in VMN neurones (Kong et al., 2004). Dio2 mRNA upregulation in this region has been demonstrated within 12 and 24 hour of fasting, which suggests that an increased level of T3 production may function to promote refeeding. Furthermore, third ventricle α1 tanycytes are known to project to the VMN (Lechan and Fekete, 2007), which implicates this nucleus as a key hypothalamic target of thyroid hormone. Direct administration of T3 (50pmol) to the VMN caused a 4-fold increase in food intake without affecting systemic levels of thyroid hormone, an effect that was not witnessed in the ARC (Kong et al., 2004).

T3 has been linked to the genomic regulation of several mediators of hypothalamic neuropeptides, which present as potential players in its regulation of food intake. In addition to the direct regulation of MC4R (section 1.7.1), T3 is also associated with negative control over other anorectic factors, such as TRH, BDNF and POMC, and disinhibition of the hyperphagic neuropeptide AgRP via downregulation of LEPR (Byerly et al., 2009). The common end product of these mechanisms is that T3 stimulates hypothalamic pathways which promote feeding.

It has been mentioned that nutritional status can be affected by thyroid hormone homeostasis, however there is also evidence to suggest that regulation also occurs in the opposite direction. Low levels of leptin stimulate feeding via hypothalamic appetite centres. During fasting, circulating levels of leptin decrease, permitting an increase in plasma corticosterone, which has been shown to increase hypothalamic D2 expression both in vitro and in vivo (Coppola et al., 2005, Germain, 1986).

1.7.5 Controversy of Theories Regarding Hypothalamic T3 Action
Recent investigations into the role of thyroid hormone in the hypothalamus have brought about various conclusions, some of which contradict others. The theories surround the mechanisms by which hypothalamic T3 affects appetite and BAT stimulation, with respect to AMPK activity.

It has been proposed that an unliganded TR can induce hypermetabolism, as a result of elevated BMR, in mice lacking TRα1 (Sjögren et al., 2007, Pelletier et al., 2008). The investigators concluded that this phenotype was caused by increased sympathetic drive from the CNS due to increased incidence of apoTR, which was driving the process. This indicates
that holoTR, that is T3-bound TR, would drive the opposite phenotype in which T3 reduces systemic hypermetabolism as a consequence of reduced sympathetic activity.

However, this is counterintuitive to the findings of Lopez et al, who concluded that T3 stimulates peripheral metabolism by increasing sympathetic drive to BAT. In this study, rats were rendered hyperthyroid by chronic subcutaneous administration of T4, which induced an increase in food intake. Central administration of T3 to the 3rd ventricle resulted in a rapid decrease in activated AMPK (pAMPK) levels. (López et al., 2010). The role of AMPK in T3 signalling in the VMN was further explored in this investigation by employing an adenovirus encoding a constitutively active AMPKα, which was administered to the VMN of hyperthyroid Sprague-Dawley rats. The effect was body weight gain in hyperthyroid, but not in euthyroid rats. There were no reported effects on food intake in euthyroid rats despite an increase in markers of BAT activation (López et al., 2010).

The finding that T3 does not affect food intake via the hypothalamus has also been demonstrated in another study which investigated the effect of T3 on glucose homeostasis (Klieverik et al., 2009). This provides further cause for controversy when considering that two publications have identified a role for T3 in the VMN where it was shown to stimulate food intake (Ishii et al., 2008, Kong et al., 2004). Furthermore, Ishi et al demonstrated that AMPK is activated in this process, which also contradicts recent findings (López et al., 2010).

In summary of these contrasting findings, we have reports that: T3 stimulates feeding in the VMN (Ishii et al., 2008, Kong et al., 2004), with contradicting reports from other sources (Klieverik et al., 2009, López et al., 2010); T3 stimulates the BMR via the CNS (Sjögren et al., 2007, Pelletier et al., 2008), with contradicting reports from other sources (López et al., 2010); AMPK is activated by T3 in the VMN (Ishii et al., 2008), with contradicting reports from other sources (López et al., 2010). This illustrates the opacity of our current understanding of how T3 affects these hypothalamic energy circuits, with regards to appetite and energy expenditure.
1.8 Hypothesis and Purpose of Study

T3 is known to have a widespread effect on energy homeostasis. Its role in the developed central nervous system is to regulate activity of the thyroid gland via the HPT axis, although increasing evidence suggests that it may regulate other metabolic pathways via the CNS. Administration of a thyrotoxic dose of T3 directly to the VMN induced hyperphagia in adult rats. This highlights a potential role for thyroid hormone in the VMN in the regulation of energy homeostasis.

This research will examine the role of thyroid hormones in the VMN with respect to their regulation of energy homeostasis. Recombinant viral vectors have previously been used in the investigation of hypothalamic function with much success. In this investigation, recombinant viruses will be designed to overexpress the iodothyronine deiodinases for use in this investigation of the role of thyroid hormone in the VMN.

Initially, plasmids will be constructed to overexpress the dominant rat CNS deiodinases D2 and D3, with green fluorescent protein (GFP) packaged into the control plasmid. These plasmids will be tested for efficacy in vitro before being used for production of viral vectors, which will subsequently be administered to the VMN of male Wistar rats. The levels of food intake and body weight will be measured in these rat models, which will be analysed for body composition after termination of the experiment. Plasma will be analysed for various metabolic parameters, such as leptin, insulin and free thyroid hormones. Hypothalami will be assayed for the expression of various genes, in addition to deiodinase enzyme activity.

Given the hyperphagic association of T3 with the VMN, it is expected that D2 overexpression will increase food intake and body weight following rAAV-D2 treatment, whereas D3 overexpression by rAAV-D3 treatment will produce the opposite phenotype.
Chapter 2

Materials & Methods

All reagents were supplied by Invitrogen Life Technologies, Paisley, UK, unless otherwise stated.
2.1 Construction of rAAV Plasmids

Plasmids vectors were prepared for the transfer of constitutively expressed \textit{Dio2} and \textit{Dio3} transgenes. These plasmids (pTR-CGW-D2, pTR-CGW-D3) were then employed for the production of rAAV for overexpression of the relevant transgene.

2.1.1 Reverse Transcription

Materials

- Rat hypothalamic RNA (5mg/ml)
- 10mM dNTPs (GE Healthcare, Little Chalfont, UK)
- 5x reverse transcriptase buffer (Promega, Madison, WI)
- Avian myoblastoma virus reverse transcriptase (RT) 10U/µl (Promega, Madison, WI)
- Oligo dT (12-18) 200ng/µl (GE Healthcare, UK)

Method

The reaction was set up in a final volume of 20µl containing the following: 1mg/ml RNA, 1x RNA buffer, 1mM dNTPs (deoxynucleotide phosphates) and 10mg/ml oligo dT. The solution was heated to 65°C for five minutes and allowed to cool at room temperature for thirty minutes. Ten units of reverse transcriptase were then added and the reaction was incubated at 42°C for one hour. The reaction was then used in PCR.

2.1.2 Polymerase Chain Reaction

Materials

- 10x Taq buffer
- 20µM oligonucleotide primers (Oswel DNA service, Southampton, UK) (appendix I)
- Taq DNA polymerase (5U/µl)
- 10mM dNTPs (GE Healthcare, UK)
- Reverse-transcribed cDNA template

Method

Complementary DNA (cDNA) from the RT reaction was amplified using primers corresponding to sequences on the target gene. Half of the reverse transcription reaction was added to a tube containing 1x Taq® buffer, 0.2mM dNTPs, 200nM oligonucleotide primers. The reaction was heated to 95°C for five minutes and then 5U Taq DNA polymerase added. The reaction was then cycled 30 times through the following temperatures: 95°C for 30
seconds, 60°C for 30 seconds and 72°C for 45 seconds. After completion of the reaction, 10µl of the PCR products were visualised by gel electrophoresis on a 1% TAE/agarose gel.

2.1.2.1 Visualisation of PCR Amplicon

Materials
Agarose, type II-A medium EEO
50x TAE: (appendix I)
Ethidium Bromide (10mg/ml) (VWR International Ltd, Poole, UK)
DNA marker (BRL 1Kb plus ladder)
Gel loading buffer (appendix I)

Method
A 1% (w/v) agarose gel was prepared by dissolving the agarose in 1x TAE using a microwave oven. The gel was cooled to 45°C and ethidium bromide added to a final concentration of 0.5µg/ml. Once set the gel was placed into an electrophoresis tank containing 0.5x TAE with 0.5µg/ml ethidium bromide. Three microlitres of loading buffer were added to 10µl PCR product. In a separate tube, 1µl of DNA marker was added to 9µl GDW and treated in the same way. The samples were loaded onto the gel and electrophoresed at 10V/cm. The DNA was visualised by illumination with UV light (300nm).

2.1.3 Insertion of Amplicon into pTR-CGW

One of the most important advances in the field of molecular biology was the ability to cut DNA at specific sites and to ligate the cut ends. Restriction endonucleases (isolated from bacteria) recognise specific target sites within double stranded DNA and cleave it. Ligating cut fragments relies on the use of a virus-derived enzyme that catalyses the formation of phosphodiester bonds between a free 5’ phosphate and a free 3’ hydroxyl group.

2.1.3.1 Restriction Endonuclease Digestion of PCR Products and AAV Plasmid

Materials
PCR amplicon
pTR-CGW (rAAV plasmid) (gift from Dr. Verhaggen, Amsterdam)
Restriction endonucleases: BamHI, AgeI and BsrGI (New England Biolabs, Hitchin, Hertfordshire, UK)
10x restriction buffer (New England Biolabs, Hitchin, Hertfordshire, UK)
10x bovine serum albumin (BSA) (New England Biolabs, Hitchin, Hertfordshire, UK)
Phenol/Chloroform
3M sodium acetate, pH 5.2
Shrimp alkali phosphatase (SAP) 4U/µl (GE Healthcare)

Method
In separate reactions the amplicon and pTR-CGW were diluted in autoclaved glass distilled water (GDW). Restriction buffer, BSA and equal volumes of each restriction endonuclease (\(Dio3\) – BamHI and BsrGI; \(Dio2\) – BsrGI and AgeI; SECIS – BamHI and AgeI) were added to give a final concentration of 1x. The total volume of enzyme added was kept below 10% of the final volume. The reaction was incubated for at least one hour at 37°C. In the digestion of pTR-CGW, 8U SAP was added 30 minutes before the end of the incubation. SAP removes 5’ phosphate groups preventing self-ligation of the plasmid. The reactions were extracted with an equal volume of phenol/chloroform and the phases separated by centrifugation for three minutes at 13,000g. The DNA was ethanol precipitated with 0.1 volumes 3M sodium acetate pH5.2 and 2.5 volumes absolute ethanol and was incubated at -20°C for at least one hour. The DNA was recovered by centrifugation for seven minutes at 13,000g.

2.1.3.2 Electroelution of DNA Fragments
Following restriction digestion it is advantageous to purify the DNA fragment of interest and remove any contaminating fragments. This is especially true for plasmids as restriction digests are not 100% efficient and a small amount of closed plasmid DNA can produce a high background during transformations. The DNA was therefore size fractionated by electrophoresis on an agarose gel and the bands of interest electroeluted.

Materials
50x TAE (appendix I)
Dialysis tubing (New England Biolabs Ltd, Hitchin, Hertfordshire, UK)
Gel loading buffer (appendix I)
DNA marker

Method
A 1% agarose gel was prepared (as described in section 3.1.2.1). The precipitate DNA was dissolved in twenty microlitres of GDW and six microlitres of loading buffer added. One microlitre of DNA marker was added to 9µl GDW and 3µl loading buffer added. Samples were loaded onto the gel and electrophoresed at 10V/cm.
The DNA was visualised by illumination with 302nm ultraviolet light whilst the band of interest was cut out from the gel using a scalpel and placed into dialysis tubing sealed at one end with a clip. Four hundred microlitres of 0.5x TAE were added to the gel slice, air excluded from the end of the tubing and the other end sealed with a clip. The DNA was eluted from the gel by electrophoresis at 20V/cm for 20 minutes. The TAE was removed from the tubing, phenol/chloroform extracted and ethanol precipitated (as described in section 3.1.2.5). The DNA was dissolved in GDW and quantified visually by running 1µl on a 1% agarose gel.

2.1.3.3 Ligation of Amplicon into pTR-CGW

Materials
T4 DNA ligase, 6U/µl (New England Biolabs Ltd, Hitchin, Hertfordshire, UK)
10x Ligase buffer (as supplied)
Digested PCR product
Digested pTR-CGW

Method
DNA ligation was used to insert the digested Dio3 cDNA into pTR-CGW plasmid. Twenty nanograms of plasmid were dissolved in GDW and a four-fold molar excess of PCR product added. Reaction buffer was added to a final concentration of 1x and 6U T4 DNA ligase added to give a final volume of 10µl. The reaction was incubated at 16°C overnight.

2.1.4 Preparation and Purification of Plasmid Constructs

2.1.4.1 Transformation of Competent Bacteria by Electroporation
Competent bacteria can be induced to take up DNA via the process of electroporation. Passing a voltage across a bacteria/plasmid mixture opens transient pores in the bacteria plasma membrane allowing the plasmid to move into the cell. pTR-CGW contain an ampicillin resistance gene. Subsequent incubation allows the expression of the resistance genes before exposure to antibiotic.

Materials
Ligation reaction
SOB (appendix I)
LB_{(amp)} plates (appendix I)
JC8111 bacteria (ATCC, Middlesex, UK)
Glycercol (VWR International Ltd, Poole, UK)
Method
Prior to transformation, all salts must be removed from the reaction mixture to prevent arcing during electroporation. One microlitre of glycogen was added to the ligation reaction, to act as a carrier and the reaction ethanol precipitated for at least one hour. DNA was recovered by centrifugation for seven minutes at 13000g. The ligation reaction was then dissolved in 10 µl GDW.

An aliquot of frozen bacteria (JC8111 cells) was thawed on ice, 10ng of plasmid (5µl ligation reaction) added and the mixture transferred to an electrowvettewith an intra-electrode distance of 1mm. A voltage of 1.2 kilovolts was then passed across the cuvette. Eight hundred microlitres of warmed SOB was then added and the reaction incubated at 37°C for an hour. Agar plates (supplemented with 100µg/ml ampicillin) were warmed and 150µl of the transformed bacteria added to the plate. The bacteria were spread over the surface of the agar, the plate inverted and incubated at 37°C overnight.

2.1.4.2 Small Scale Plasmid Preparation (Sambrook and Russell, 2001)
Plasmids are isolated from several bacterial colonies on an agar plate to allow several clones to be analysed simultaneously. To isolate the plasmids from bacteria it is necessary to disrupt the cell wall to release the plasmid and at the same time contaminants (proteins, genomic DNA and RNA) must be removed. Treatment with alkaline SDS (sodium dodecyl sulphate) disrupts the cell wall and bacterial debris is then precipitated using potassium acetate. This step also removes most of the genomic DNA; RNA is removed later by treatment with RNase A.

Materials
LB(amp) (appendix I)
GTE (appendix I)
0.2M NaOH 1% SDS (w/v) (appendix I)
5M potassium acetate (appendix I)
Phenol/Chloroform
Propan-2-ol (VWR International Ltd, Poole, UK

Method
A single bacterial colony was placed in 2ml LB supplemented with 0.05mg/ml ampicillin. This was incubated overnight at 37°C with vigorous shaking. From this, 1.5ml was removed and centrifuged at 13,000g for 3 minutes, the remainder was stored at 4°C for future use.
Supernatant was removed and the bacterial pellet dissolved in 100µl GTE. Two hundred microlitres of alkaline SDS were added to the bacteria and the mixture left on ice for 5 minutes. One hundred and fifty microlitres of 5M KAc were then added and the mixture left on ice for a further 5 minutes. The mixture was centrifuged at 13,000g for 5 minutes and 350µl supernatant was removed to a fresh tube. The supernatant was purified by the addition of 350µl of phenol-chloroform to each sample before their centrifugation for 4 minutes. The upper aqueous phase, containing the genetic material, was then carefully removed using a sterile Pasteur pipette. DNA was precipitated out of the solution by the addition of 0.6 volume propan-2-ol and incubation at room temperature for 10 minutes. This was centrifuged at 13,000g for 7 minutes to pellet the DNA. Following removal of the supernatant, DNA was dissolved in 100µl GDW. An ethanol precipitation was carried out (as described in section 2.1.3.1) to further purify the recovered DNA. Restriction endonuclease digestion was carried out (as described in section 2.1.3.1) to verify which clones contained the correct plasmid insert. Purified DNA was dissolved in 10µl GDW. XmaI was used to check for presence of inverted terminal repeats (ITRs) and visualised by gel electrophoresis. Clones containing the correct insert and ITRs were selected for large scale purification.

2.1.4.3 Large Scale Plasmid Preparation(Sambrook and Russell, 2001)

Materials

LB\textsubscript{(amp)} (appendix I)
GTE (appendix I)
Lysozyme
0.2M sodium hydroxide/1% (w/v) SDS (appendix I)
5M potassium acetate (KAc)
Propan-2-ol (VWR International Ltd, Poole, UK)
100x TE (appendix I)
DNase free RNase A 10mg/ml in GDW (GE Healthcare)
Phenol/Chloroform

Method

A small quantity of bacteria containing pTR-CGW with the correct size insert was inoculated into 500ml LB\textsubscript{(amp)} and incubated at 37°C overnight with vigorous shaking. The bacteria were recovered by centrifugation for eight minutes at 3000g (4000rpm in HS-4 rotor in RC-5B superspeed centrifuge, Du Pont) at 4°C. The pellet was dissolved in 25ml GTE supplemented with 2mg/ml lysozyme and the sample incubated at room temperature for 5 minutes. Fifty
millilitres of NaOH/SDS were added, the sample mixed by inversion and incubated on ice for 5 minutes. Then 38ml 5M KAc were added, the sample mixed by inversion and incubated on ice for 10 minutes. Bacterial debris was removed by centrifugation for 15 minutes at 9000g (7000rpm in HS-4 rotor in RC-5B superspeed centrifuge, Du Pont) at 4°C. The supernatant was transferred to a clean tube and 0.6 volumes propan-2-ol added. The sample was incubated on ice for 15 minutes and the DNA recovered by centrifugation for 15 minutes at 9000g at 4°C. The pellet was dissolved in 10ml GDW to which 100µl 100x TE was added. RNase A was added at a concentration of 0.1mg/ml and the reaction incubated at 37°C for 30 minutes. The reaction was extracted with an equal volume of phenol/chloroform and the phases separated by centrifugation for 20 minutes at 10000g and 4°C. The DNA was recovered by addition of 0.1 volumes 2M sodium acetate pH 5.2 and one volume propan-2-ol and incubation at -20°C for at least one hour.

2.1.4.4 Caesium Chloride Gradient Purification

Large scale plasmid purification was carried out using a caesium chloride gradient. This purification method depends on the decrease in density of nucleic acids when bound to ethidium bromide. Because ethidium bromide binds by intercalation into the DNA, it causes unwinding of the helix. In closed circular DNA this increases supercoiling of the plasmid so binding of ethidium bromide is limited and the plasmid has a higher buoyant density than linear or nicked plasmids. The difference in buoyant density allows separation of the plasmid on a caesium chloride density gradient.

Materials

TES (appendix I)
Caesium chloride
10mg/ml ethidium bromide (VWR International Ltd, Poole, UK)
Propan-2-ol, caesium chloride saturated (appendix I)
Polyallomer tubes (Ultracrimp, Du Pont)

Method

DNA obtained from large scale plasmid purification was recovered by centrifugation for 20 minutes at 24,000g (12,000rpm in HBS rotor in RC5B superspeed centrifuge, Du Pont) and 4°C. DNA was then dissolved in 8.25ml TES. Eight-point-four grams of caesium chloride were dissolved in the DNA solution and 150µl of ethidium bromide added and the solution mixed. The sample was divided between two polyallomer tubes, balanced, and overlaid with
mineral oil. The tubes were sealed and centrifuged for 16 hours at 20°C and 60,000rpm (in an
A1256 rotor in a Sorvall 100SE centrifuge, Fisher Scientific, Loughborough, UK).

After centrifugation, DNA bands were visualised by 302nm ultraviolet illumination and the
band containing the closed pTR-CGW DNA removed using a 20-gauge needle and a 2ml
syringe. Ethidium bromide was removed from the plasmid by repeated extraction with an
equal volume of caesium chloride saturated propan-2-ol, until both phases were colourless.
The DNA was precipitated by addition of two volumes of GDW and six volumes of room
temperature absolute ethanol. DNA was removed by centrifugation for 15minutes and
24,000g at 20°C. The DNA pellet was dissolved in 0.4ml GDW, ethanol precipitated and
recovered by centrifugation for 7 minutes at 13,000g. The DNA was then dissolved in 1ml
GDW.

pTR-CGW was then quantified spectrophotometrically, 10µl of DNA solution was diluted
1:100 and placed into a quartz cuvette. Absorbance was read at 260 and 280nm UV 1101
spectrophotometer (WPA, Cambridgeshire, UK). The reading at 280nm gives an indication of
the purity of the sample as phenol absorbs more strongly at 280nm than DNA. The
concentration of the DNA was calculated using the following formula:

Concentration (µg/ml) = (A_{260} x dilution factor) x 50

Sample was digested using specific endonucleases BamHI and BsrGI to check insert size and
XmaI to check for ITR integrity.

2.1.4.5 Sequence Analysis of Large Scale Plasmid Preparation

Insert sequences were determined by DNA sequencing (Advanced Biotechnology Centre,
Imperial College London, UK). A dye-based chain termination method was utilised, which
involves a PCR-based reaction in which dideoxynucleotides (ddNTPs) are included. These
ddNTPs lack the ability to form phosphodiester bonds, which results in the production of
multiple fragments of different lengths depending on where each of the dNTPs are located in
the sequence.

In dye-termination sequencing, each of the four dideoxynucleotide terminators is labelled
with a different fluorescent dye that emits light at a certain wavelength. The resulting
amplicons are then separated by size and analysed photometrically to determine the sequence
of dNTPs based on where each dye is detected.
Method

Sequencing reactions were performed using PCR-based dye termination, where dilutions of each primer and the DNA sample were prepared in advance. Primers were designed to sequence the constructs in both directions. One 20µl aliquot of DNA (100ng/µl) and 20µl aliquots of each primer (3.2pmol/µl) were prepared in 0.5ml eppendorf tubes.

Sequencing reactions are processed using the following PCR programme: 96°C for 2 minutes; followed by 35 cycles of 96°C for 10 seconds, 50°C for 10 seconds, 60°C for 4 minutes. Reactions are then loaded onto an agarose gel to separate bands by size. The sequence of dyes is then determined photometrically by loading the gel onto a sequence analyser (Advanced Biotechnology Centre, Imperial College London, UK).

2.1.5 In Vitro Preparation of rAAV in HEK293T Cells

Adeno-associated virus was produced by using the two plasmid transfection system, which is illustrated in figure 2.1 (Grimm et al., 1998). Briefly, host cells (HEK293T) are co-transfected with the rAAV plasmid (figure 2.1) and a helper plasmid pDG (figure 2.2) using calcium phosphate. There is no homology between the two plasmids so they cannot recombine and form wild-type virus. This results in the production of rAAV particles. To release the particles cells are lysed by freeze thawing and the rAAV produced is purified.

2.1.5.1 Cell Maintenance

Materials
DMEM
Trypsin
Versene (appendix I)

Method

Cells were cultured in 75cm² flasks with 15ml DMEM (Gibco, Paisley, UK) containing DMEM and supplemented with 10% fetal bovine serum (FBS) and 5% penicillin and streptomycin. The cells were incubated at 37°C in a 5% CO₂ atmosphere. Medium was changed every 2 to 3 days. Cells were passaged 1:10 when confluent, approximately every 6 days. Briefly, cells were washed in 10ml versene. To detach cells from the flask, 0.25% trypsin in versene was added and cells left for several minutes until all appeared to have detached from the flask. The suspension was then added to 10ml DMEM to inactivate the trypsin and centrifuged for 5 minutes at 100g. The resulting pellet of cells was dissolved in 10ml DMEM and 1ml was used to seed two 75cm² flasks.
2.1.5.2 Calcium Phosphate Transfection

Materials
HEK293T cells
pTRCGW (including transgene)
pDG
1x HEBS buffer (appendix 1)
2M CaCl$_2$

Method
Cells were plated into a 10 chamber cell factory (L x W x H (mm): 335 x 205 x 190, culture area: 6320cm$^2$). Cells were incubated in 2000ml DMEM with 10% FBS. After 24 hours, when cells were 50% confluent, the culture medium was replaced (250ml culture medium was retained for transfection) and transfection performed 2 hours later.

Transfection mix was prepared for the cell factory, consisting of 560µg pTRCGW, 1.68mg pDG, 7.2ml 2M CaCl$_2$ and 52ml GDW. Immediately before transfection, 12ml of HEBS buffer in 48ml GDW was added and mixed gently. After standing at room temperature for 1 minute the mixture was added slowly to 250ml DMEM with 10% FBS and added to the cell factory.

Figure 2.1: Transfection systems for rAAV production (Grimm et al., 1998). Cells are transfected with the vector plasmid and an AAV/adenovirus hybrid helper plasmid. X, transgene; ITR, inverted terminal repeats; Ad5, adenovirus helper genes.
2.1.6 Recovery and Purification of rAAV Titre

Materials
0.01M PBS
DNaseI
Sepharose column (GE Healthcare, Little Chalfont, Buckinghamshire, UK)
Iodixanol (Life Sciences Technology, Eggenstein, Germany)
0.3x PBS/0.1M NaCl
0.3x PBS/2mM EDTA
2M MgCl₂
Benzonase (Novagen, Nottingham, UK)
Lysis buffer (appendix I)

Method
Forty-eight hours after transfection, cell medium was removed and cells were washed using 500ml PBS and then harvested using 500ml PBS/2mM EDTA. Cells were collected by centrifugation at 5000g for 10 minutes, resuspended in 150ml PBS and then re-centrifuged at 5000g for 10 minutes. Cells were then resuspended in a total of 60ml lysis buffer in falcon tubes. Cells were exposed to 3 freeze-thaw cycles, ten minutes in dry ice/ethanol followed by 10 minutes in a 37°C waterbath. 7.3µl 2M MgCl₂ and 1.2µl Benzonase were then added to each falcon tube and incubated at 37°C for 30 minutes. The solutions were then centrifuged at 2000g for 10 minutes to pellet cell debris. The supernatant was equally loaded into 4 polyallomer tubes (Sorvall). A preformed iodixanol gradient composed of 15%, 25%, 40% and 60% iodixanol was layered underneath. The tubes were then topped up with lysis buffer, heat sealed and centrifuged at 69,000g for 1 hour at 18°C (Sorvall 100 SE). To remove the rAAV particles the top of each tube was punctured with an 18G needle and rAAV collected by puncturing the bottom of the tube and collecting 5ml of iodixanol gradient from the 60/40% interface. The fractions from the 4 tubes were pooled, added to 20ml PBS and loaded onto a Sepharose column (pre-washed with 25ml 0.1M NaCl/0.3x PBS and 50ml 0.3x PBS). The column was washed with 50ml 0.3x PBS and the virus eluted with 15ml 350mM NaCl/0.3xPBS. The eluant was transferred to an Apollo Concentrator containing 10ml PBS and centrifuged at 4000g for 5 minutes. The flow through was discarded with a further 5ml PBS added below and 19.5ml PBS above the filter. The tube was again centrifuged at 4000g for 5 minutes with the remaining 0.5ml rAAV aliquoted into eppendorf tubes. Viral titre was quantified by dot blot analysis (section 2.1.7).
Figure 2.2: Structure of pTR-CGW plasmid vector.

Figure 2.3: The structure of helper/packaging plasmid pDG. Contains AAV and adenovirus genes required for amplification and packaging of rAAV. RTR – right terminal repeat, MMTV-Rep-Cap – promoter region encoding Rep and Cap proteins, expression is driven by mouse mammary tumour virus (MMTV) and long terminal repeat (LTR), VA, E2A and E4 – adenovirus helper genes required for replication.
2.1.7 Determination of Total Viral Titre by Dot Blot Analysis

The level of transgene expression is affected by the rAAV titre so total viral particle number was determined by dot blot analysis. Dot blot analysis is based on similar principles to Southern and Northern blots; a specific probe can be used to quantify a specific DNA or RNA species in a sample. Rather than being separated by size and transferred to a membrane, the sample being analysed is placed directly onto a membrane. The DNA or RNA species present in the sample can be detected and quantified using radiolabelled probes.

Materials

rAAV viral preparation
Solution A (appendix I)
Solution B (appendix I)
1mg/ml proteinase K
Phenol/chloroform
3M sodium acetate (appendix I)
Ethanol (VWR International Ltd, Poole, UK)
Glycogen
Denaturing solution: 1.5M NaCl 0.5M NaOH
Neutralising solution: 1M Tris/HCl pH7.4 1.5M NaCl
pTRCGW
Hybridisation buffer (appendix I)
Amasino wash buffer (appendix I)
Universal wash buffer (appendix I)

Method

Forty-five microlitres of solution A was added to 5µl rAAV virus stock and incubated for 30 minutes at 37°C. Two hundred microlitres of solution B was added and the sample was incubated at 55°C for 1 hour. DNA was extracted by adding an equal volume of phenol/chloroform. Ethanol precipitation of the DNA was performed by adding 1/10 volume 3M sodium acetate pH 5.2, 40µg glycogen and 2.5 volumes ice cold ethanol. After 1 hour at -20°C the solution was centrifuged at 8000g at 4°C, the supernatant removed, the pellet washed in 75% cold ethanol and air-dried. The pellet was dissolved in 10µl GDW and 1µl applied to a nylon membrane (Hybond-N). A series of dilutions of pTRCGW (50 to 0.1ng) were also applied to the membrane to act as a standard curve. The membrane washed for 5
minutes in denaturing solution, then washed twice for 5 minutes in neutralising solution. The membrane was then baked at 80°C for 2 hours.

A cDNA fragment of WPRE was used as a probe for rAAV transgenes. The probe was radio labelled with $^{32}$P dCTP as described in section 2.4.6.4. Half of the labelled probe was boiled and added to 10ml hybridisation buffer. Hybridisation was carried out overnight at 60°C. The following day the membrane was washed as described in section 2.4.6.5. The membrane was then placed on a phosphorimager screen overnight. Radiolabelled areas were visualised and quantified by image densitometry using ImageQuant software (Molecular Dynamics). Quantification was performed by comparing viral DNA to known amounts of pTR-CGW in the standard curve.

**2.2 In Vitro Investigations**

Deiodinase activity was measured in collaboration with the Department of Endocrinology and Metabolism of the Academic Medical Centre at the University of Amsterdam. Activity was measured *in vitro* following transfection of JEG3 cells with plasmid constructs.

**2.2.1 Cell Culture Maintenance**

**Materials**

- JEG3 Cells
- DMEM/F12 Media
- Foetal Bovine Serum
- Sodium Selenite (Sigma-Aldrich, Poole, UK)
- Trypsin
- Versene (appendix I)

**Method**

1 x $10^6$ cells were cultured in 75cm$^2$ flasks with 10ml DMEM/F12 (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS) and 100nM sodium selenite. The cells were incubated at 37°C in a 5% CO$_2$ atmosphere. Medium was changed every 2 to 3 days. Cells were passaged 1:10 when confluent, approximately every 4 days. The cells were washed in 10ml versene and detached from the flask using versene with 0.25% trypsin. The suspension was then added to 10ml DMEM/F12 to inactivate the trypsin and spun for 5 minutes at 100g. The resulting pellet of cells was dissolved in 10ml DMEM/F12 and 1ml was used to seed two 75cm$^2$ flasks.
2.2.2 Transient Plasmid Transfection Using Calcium Phosphate

Materials
JEG3 cells
pTR-CGW
1x HBS buffer (appendix I):
2M CaCl₂

Method
1 x 10⁶ cells were grown to 70% confluence in 60mm plates. Transfection mixes for each well were prepared in cryotubes with the following constituents: 5µl HBS, 1µg pTR-CGW and GDW (to give a final volume of 50µl). Three microlitres of CaCl₂ were then slowly added to each tube to initiate precipitation of the plasmid DNA. After 5 minutes, the contents of the tube were spotted into its respective well, containing 6ml of DMEM/F12 media.

2.2.3 Measuring Extracellular Thyroid Hormone Content

The concentration of fT3 and fT4 in DMEM/F12 media was analysed by radioimmunassay (RIA). Owing to the presence of specific thyroid hormone transporters and the lipophillic structure of iodothyronines, thyroid hormones are able to cross the plasma membrane of JEG3 cells. Therefore, measuring the content of fT3 and fT4 in culture media gives some indication of the level of deiodination following transfection of the constructs.

Materials
Transfected JEG3 Cells
DMEM/F12 Media

Method
Two hours prior to transfection (see 2.2.2), media was replaced. Cells were transfected with either pTR-CGW (n=4), pTR-CGW-D2 (n=4), or pTR-CGW-D3 (n=4). Fresh media was introduced to each well 48 hours after transfection. Twenty-four hours later, media was sampled for analysis of fT4 and fT3 content by radioimmunoassay (see 2.4.6.3).
2.2.4 Measurement of Deiodinase Activity in vitro

Iodothyronine deiodinase type III activity was measured in cells transfected with either pTR-CGW or pTR-CGW-D3. The JEG3 cell lines were harvested and delivered to collaborators at the Department of Endocrinology and Metabolism of the Academic Medical Centre at the University of Amsterdam.

Materials

1x PBS (appendix I)

PE buffer (100 mM phosphate buffer containing 2 mM EDTA and 50mM DTT)

Polytron homogeniser

Method

Cells were transfected with either pTR-CGW (n=5) or pTR-CGW-D3 (n=5) as described in section 2.2.2). After 72 hours, JEG3 cells were harvested in 1x PBS, using a cell-scraper, and centrifuged at 10,000g for 3min. The pellet was dissolved in PE buffer before homogenisation on ice using a polytron homogeniser. Sample protein concentrations were limited to 0.1 mg/ml for D3 activity. Cell lysates were stored at -80°C until assaying for D3 activity.

Deiodinase activity assays were performed as described previously (Boelen et al., 2005, Boelen et al., 2004a). Duplicate incubations of homogenates (~100 µg protein) were incubated for 1 hour at 37 C with 1 nM [3'-125I]T3/T4 (200,000 cpm) in a final volume of 0.1 ml PE buffer. Reactions were terminated by adding 0.1 ml ice-cold methanol. After centrifugation, 0.1 ml of the supernatant was added to an equal volume of 0.02 M ammonium acetate (pH 4). One hundred microlitres of this mixture were applied to a 4.6 x 250 mm Symmetry C18 column connected to an Alliance HPLC system. The column was eluted with acetonitrile (28–42% in 15 min) in 0.02M ammonium acetate (pH 4.0). Eluate radioactivity was measured using a Radiomatic Z-500 flow scintillation detector.
2.3 Analysis of Tissue Samples
Several metabolic parameters were analysed in male Wistar rat carcasses following intra-VMN administration of rAAV-D2, rAAV-D3, or rAAV-GFP.

2.3.1 Calculation of Rat Body Composition
Body composition was analysed to establish the ratio of fat mass to lean body mass. Rat carcasses were dissolved (saponified) in a strong organic solvent before glycerol and protein concentrations were measured using standard assay procedures.

2.3.1.1 Saponification of Carcass

Materials
3M KOH 65% ethanol (v/v) (Appendix I)
Ethanol (VWR International Ltd, Poole, UK)

Method
Animals were weighed and placed in separate 1L plastic containers. Carcasses were dissolved using 3M KOH in 65% ethanol in equal volume to carcass weight (1ml/g). Vessels were then incubated at 70°C for one hour to initiate liquefaction. Following this, the containers were placed in an oven at 70°C for 5 days. The resultant liquid was made up to 1.7L with absolute ethanol and stored at room temperature until required.

2.3.1.2 Glycerol Assay
In order to determine total fat content in rats, a glycerol assay was used. Whole carcass glycerol concentrations were determined using reagents and methods from Randox laboratories Ltd, Crumlin, Co. Antrim.

Materials
1M Glycerol (VWR International Ltd, Poole, UK)
Glycerol Assay Kit (Randox Laboratories Ltd, Crumlin, Co. Antrim)
Buffer R (appendix 1)
Reagent R (appendix 1)

Method
In order to determine the total fat content per rat a glycerol assay was used. All buffers were supplied ready to use and one vial of reagent R was reconstituted with 15 ml of buffer R. One molar glycerol stock solution was diluted in GDW to provide standard glycerol
concentrations of 5mM, 2mM, 1mM, 0.5mM, 0.2mM, 0.1mM, and 0.05mM. Ten microlitres of dissolved carcass sample was diluted 1:100 with GDW in separate microtubes ready for use in the assay. To begin the assay 1 ml reagent R was added to a new set of tubes. To this 30μl of sample, standard, or buffer R (for blank value) was added and left to incubate at room temperature for 10 minutes. Each tube was then read using a spectrophotometer at an absorbance of 520 nm. The carcass fat content was calculated from the glycerol reading by assuming a molecular weight of 885 per triglyceride molecule (Baginski et al., 1974).

2.3.1.3 Lowry Protein Assay

Carcass lean mass was determined by analysis of the protein content using a well established modified Lowry protein assay (Lowry et al., 1951). This method involves reaction of proteins with cupric sulphate and tartrate in an alkaline solution to form tetradentate copper-protein complexes. When a certain reagent is added (Folin-Ciocalteu) it is reduced in proportion to these copper complexes producing a product blue in colour that can be measured at 750 nm. The relative protein concentration can be calculated using the formula: $A_{750} = \varepsilon_{750} \times c \times l$

Materials

Biuret Reagent (Sigma-Aldrich, Poole, UK)
Folin-Ciocalteu Reagent (Sigma-Aldrich, Poole, UK)
Bovine Serum Albumin

Method

A bovine serum albumin (BSA) stock solution that was diluted in GDW to make up nine standard solutions of 1, 5, 25, 125, 250, 500, 750, 1,000, and 1,500 μg/ml. Ten microlitres of dissolved carcass was diluted in 1 ml GDW. 1X Folin-Ciocalteu reagent was prepared by diluting the supplied 2X reagent 1:1 with ultra pure water. All other reagents were supplied ready to use. 200 μl of standard and unknown sample were added in duplicate to microfuge tubes. 1ml modified Lowry reagent was then added to each tube at 15 second intervals, mixed well, and incubated for exactly 10 minutes. 100 μl of the prepared 1X Folin-Ciocalteu reagent was then added and vortexed. The tubes were covered and incubated at room temperature for 30 minutes. The samples were then immediately measured in a spectrophotometer at a wavelength of 750 nm. Absorbance values of the blank readings were subtracted from the absorbance readings of all samples and a standard curve plotted. The protein concentration of each sample was extrapolated from the standard curve.
2.3.2 In Situ Hybridisation for WPRE mRNA

*In situ* hybridisation is a method for the detection and quantification of mRNA whilst maintaining the structural integrity of the tissue. It relies on the hybridisation of a complimentary probe, usually radiolabelled, to the target mRNA in tissue mounted on poly-lysine coated slides. Rats received either bilateral injections of rAAV-D2 or unilateral injections of iVMN rAAV-D3. Three weeks after surgery *in situ* hybridisation was performed to confirm transgene expression and verify localisation of the virus.

An *in situ* hybridization reaction was performed to confirm expression of rAAV-plasmids in brain parenchyma. Coronal sections of 12μm were used for 35S-labeled antisense RNA probes complementary to the woodchuck posttranscriptional regulatory element (WPRE). The WPRE sequence is part of the expression cassette of all vectors used in this study to enhance expression. A 35S-labelled sense probe was also used as a control to validate that there was no non-specific binding of the antisense probe.

### 2.3.2.1 Production of Radiolabelled RNA Probe for In Situ Hybridisation

**Materials**

- Linearised template (for WPRE *in situ* pBluescript-WPRE (200ng/µl) linearised by digestion with Bam HI for antisense probe or EcoRI for sense)
- 100M DTT (appendix I)
- RNase inhibitor, 30U/µl
- 10x nucleotide mix: 10mM of each ATP, UTP, GTP
- T7 RNA polymerase, 20U/µl (Promega, Madison, WI, USA)
- T3 RNA polymerase (Promega, Madison, WI, USA)
- 10x RNApolymerase buffer (Promega, Madison, WI, USA)
- DNase I, 7.5 U/µl (GE Healthcare, Little Chalfont, Buckinghamshire, UK)
- 10x DNase buffer
- Ammonium acetate (appendix I)
- [35S]CTPαS, 30TBq/mmol, 1.5GBq/ml (GE Healthcare, Little Chalfont, Buckinghamshire, UK)

**Method**

Radioactively labelled RNA was produced using an *in vitro* transcription reaction. Two hundred nanograms of template were added to a reaction buffer containing 10mM DTT, 1x nucleotide mix, 1x polymerase buffer, 30U RNase inhibitor and 3.75MBq [35S]CTPαS in a
total volume of 9µl. To this 20U of RNA polymerase was added (T3 to the anti-sense reaction and T7 to the sense reaction) and the reaction incubated for at least 2 hours at 37°C. After incubation, 6µl GDW, 1µl DNase I and 3µl DNase buffer was added. This was followed by a 15 minute incubation at 37°C. Ammonium acetate was added to a final concentration of 2M and 2.5 volumes ice cold absolute ethanol was added and the mixture left at -20°C for at least an hour for the RNA to precipitate. RNA was recovered by centrifugation at 8,000g for 7 minutes and the pellet was dissolved in 100µl GDW. To measure the activity or the radiolabelled probe, 1µl was counted on a microbeta counter (Wallac, Waltham, MA).

2.3.2.2 Preparation of Slides

Materials
4% formaldehyde (VWR International Ltd, Poole, UK) in 0.01M PBS (Appendix I)
0.01M PBS (Appendix I)
100% acetic anhydride (VWR International Ltd, Poole, UK)
0.1M Triethanolamine pH 8.0 (TEA)
20x SSC (Appendix I)
3M sodium chloride
70% ethanol in distilled water
100% ethanol (VWR International Ltd, Poole, UK)
Chloroform (VWR International Ltd, Poole, UK)

Method
Twelve micrometre sections of rat brain were cut on a cryostat at -25°C (Bright Instrument Company, Huntingdon, Cambridge, UK). The sections were mounted onto poly-lysine coated slides (VWR) and stored at -70°C until hybridisation. *In situ* hybridisation was performed on every 3rd slide. The selected slides were placed in a rack and fixed in 4% formaldehyde in 0.01M PBS on ice for 20 minutes. Slides were then washed twice in 0.01M PBS for 5 minutes. Slides were subsequently washed in 0.1M TEA for 2 minutes and acetylated in 0.25% (v/v) acetic anhydride in 0.1M TEA for 10 minutes, they were then rinsed twice in 0.01M PBS for 2 minutes each time. Slides were dehydrated by sequential immersion in 70%, 95% and 100% ethanol solutions before washing in distilled water for 3 minutes. Finally slides were delipidated in chloroform for 5 minutes before rehydration in 95% and then 70% ethanol and finally being left to air dry.
2.3.2.3 Hybridisation of Radiolabelled Probe to Slides

Materials
1x RNAse Buffer (appendix I)
Hybridisation Buffer (appendix I)
2% Dextran sulphate (Appendix I)

Biomax film (Kodak, Hemel Hempstead, Hearts, UK)
Developer (Jet X-Ray, London, UK)
Fixer (Jet X-Ray, London, UK)

Method

Enough $^{35}$S labelled probe for 1 million counts per slide was added to the hybridisation buffer and 70µl was applied to each slide. A coverslip was placed on each slide, the slides were hybridised overnight at 60°C.

The slides were soaked in 4x SSC with gentle agitation to remove coverslips and then washed four times in 4x SSC for 5 minutes. Slides were RNase treated by incubation for 30 minutes at 37°C in 1x RNase buffer containing 100µg/ml RNase, followed by two 5 minute washes in 10mM DTT/2x SSC, followed by a 10 minute wash in 10mM DTT/1x SSC and a 10 minute wash in 10mM DTT/0.5x SSC, all at room temperature. Finally, slides were washed in 10mM DTT/0.1x SSC at 60°C for 30 minutes, rinsed briefly in 10mM DTT/0.1x SSC at room temperature and then washed for 3 minutes in 70% ethanol and 3 minutes in 100% ethanol and air dried. After washing and dehydration the slides were exposed to Bio-Max film at room temperature. After 3 days’ exposure the film was developed and WPRE distribution determined by observation of specific hybridisation.

2.3.3 Northern Blot Analysis of mRNA

Northern blotting was used to analyse the size and quantity of UCP-1 RNA in BAT. The RNA is size fractioned on a denaturing agarose gel by electrophoresis, transferred by capillary action to a nylon filter and covalently linked to it by baking. The RNA is denatured to remove secondary structure ensuring the rate of migration of the RNA is proportional to its total length.

2.3.3.1 Total RNA Extraction

Materials
Tri-reagent (Helena Biosciences, Sunderland, Tyne and Wear, UK)
Bromo-Chloro-Propane (Sigma-Aldrich, Poole, UK)
Isopropanol (VWR International Ltd, Poole, UK)
Ethanol (VWR International Ltd, Poole, UK)
GDW

Method
Total RNA was extracted using the Tri-reagent method according to the manufacturer’s protocol. Brown adipose tissue was ground under liquid nitrogen with a pestle and mortar before its homogenisation in 1ml Tri-reagent and subsequent transfer to 1.5ml eppendorfs to incubate at room temperature for five minutes. One hundred microlitres of bromo-chloro-propane were then added, mixed vigorously for 15 seconds, and then incubated for a further five minutes at room temperature. The emulsion was then centrifuged at 4°C, for 15 minutes, at 12000g, with the brake off. The upper aqueous phase was transferred to a clean eppendorf, precipitated with a half volume (300μl) room temperature isopropanol for five minutes at room temperature, and then centrifuged at 12000g for ten minutes at 4°C. This resulted in a pellet which was washed with 1 volume (1ml) 75% ethanol in GDW, centrifuged at 12000g for a further 10 minutes at 4°C, the pellet was then air dried, and dissolved in 400μl autoclaved GDW.

RNA concentration was determined spectrophotometrically. The RNA was diluted 1:100 in GDW and 1ml placed in a quartz cuvette. The absorbance was read at 260 and 280nm (UV-160 spectrophotometer, Shimadzu, Kyoto, Japan). The 280nm reading gives an indication of the purity of the sample. The concentration of RNA was calculated using the following formula:

RNA (µg) = dilution factor x 40 x OD260

Following quantification on the spectrophotometer the RNA samples were ethanol precipitated with 0.1 volumes sodium acetate pH 5.2 and 2.5 volumes ice cold absolute ethanol. The samples were precipitated at -20°C for a minimum of one hour and centrifuged at 12000g for 20 minutes, the supernatant was discarded and the pellet vacuum dried for five minutes. The pellets were re-suspended in the appropriate volume of GDW to give a 5mg/ml solution.

2.3.3.2 Integrity Analysis of the Total RNA

Materials
Agarose, type II-A medium EEO
Formaldehyde (VWR International Ltd, Poole, UK)
20x MOPS pH 7.0 (appendix 1)
Ethidium bromide (VWR International Ltd, Poole, UK)
DENAT (appendix I)
Gel loading buffer (appendix I)
100x TE pH 7.5 (appendix 1)
2M Sodium acetate pH 5.2
GDW

Method
The RNA secondary structure was denatured before confirming the integrity of the samples using a formaldehyde gel. One microlitre of this solution was added to 12μl DENAT. The samples were denatured at 65°C for five minutes and 3μl gel loading buffer added. The samples were loaded onto the gel and run in 1x MOPS buffer with 7.5 % formaldehyde (v/v) at 10 V/cm. The gel was stained in 1x TE with 0.1 mg/ml ethidium bromide on a shaking platform for thirty minutes and de-stained overnight in 1x TE to allow visualisation of the 18S and 28S ribosomal bands under UV light. The presence of intact ribosomal bands indicated the RNA was not degraded.

2.3.3.3 Transfer of RNA to Nylon Filters (Lehrach et al., 1977)

Materials
Formaldehyde (VWR International Ltd, Poole, UK)
20x MOPS pH 7.0 (appendix 1)
Hybond-N (GE Healthcare, Little Chalfont, Buckinghamshire, UK)

Method
An agarose gel was made by dissolving 1g agarose in GDW, 20x MOPS was added to a final concentration of 1x and formaldehyde added to 3% (v/v). Once set, the gel was transferred to an electrophoresis tank containing 1x MOPS, and 3% formaldehyde (v/v). Before loading, 50μg of total RNA was denatured by addition of 10μl denaturing buffer and incubation at 60°C for 5 minutes. After denaturation, 3μl loading buffer was added and the samples loaded onto the gel. The gel was electrophoresed at a constant voltage of 10V/cm. After electrophoresis, the RNA in the gel was transferred to Hybond-N by capillary action (Figure 2.4). After transfer, the filter was baked at 80°C for 1-2 hours.
Figure 2.4: Diagrammatic representation of the apparatus used for capillary transfer of RNA from a gel onto the Hybond nylon membrane prior to Northern blot analysis.
2.3.3.4 Random Primer Labelling of DNA Fragments

Materials
DNA fragment
5x ABC Buffer (appendix I)
12.5mM magnesium chloride
125mM Tris-HCl, pH8.0
23μM 2-mercaptoethanol
50μM dATP (GE Healthcare, Little Chalfont, Buckinghamshire, UK)
20x SSC (appendix I)
Filter paper
Saran wrap
Inverted gel
Hybond-N (GE Healthcare, Little Chalfont, Buckinghamshire, UK)
Paper towels
Glass plate
Weight (approx 200g)
50μM dGTP (GE Healthcare, Little Chalfont, Buckinghamshire, UK)
50μM dTTP (GE Healthcare, Little Chalfont, Buckinghamshire, UK)
1M N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid (HEPES) (Sigma Aldrich, Poole, UK)
34μg/ml random deoxynucleotide hexamers (GE Healthcare, Little Chalfont, Buckinghamshire, UK)
10mg/ml BSA, fraction V (Sigma Aldrich, Poole, UK)
[α-32P]-dCTP: 10Ci/ml, 3000Ci/mmol (GE Healthcare, Little Chalfont, Buckinghamshire, UK)
DNA polymerase I, Klenow fragment 9U/μl (GE Healthcare, Little Chalfont, Buckinghamshire, UK)
Sephadex G50 (appendix I)
1x TE (appendix I)

Method
20ng DNA in a total volume of 15μl GDW was boiled for five minutes. After boiling, the solution was made up to a total volume of 25μl containing 1x ABC buffer, 5mg/ml BSA, 10μCi dCTP and 1U Klenow. The reaction was incubated at 37°C for one hour and
incorporation calculated. Incorporation of radionucleotide into the oligonucleotide was measured using a mini Sephadex G50 column. The column was prepared by plugging a glass Pasteur pipette with glass wool and adding Sephadex G50. The labelling reaction volume was made up to 200μl with 1x TE and this was loaded onto the column. The column was eluted with 1x TE and 200μl fractions collected. The fractions were counted and the percentage incorporation calculated. The hottest two fractions were pooled and half added to the hybridisation mix.

2.3.3.5 Hybridisation of Northern Filters

Materials
Hybridisation/pre-hybridisation buffer (appendix I)
Amasino wash (appendix I)
Universal wash buffer (appendix I)

Methods
The baked nylon filter was pre-hybridised in a clean plastic bag with 20ml of pre-hybridisation buffer at a 60°C for at least two hours. Before hybridisation of the nylon filter, the radio-labelled probe was denatured for five minutes in a boiling water bath. The pre-hybridisation mix was then removed and replaced with 20ml fresh hybridisation buffer and 200μl probe was added to this and sealed. The sealed filter was then left to hybridise with the radio-labelled probe overnight at 60°C. The following day, the filter was washed to remove any non-specifically bound probe. The filter was washed three times in 50ml Amasino wash buffer for 20 minutes each time at 60°C. The filter was washed a further three times in Universal wash; each wash lasted for 20 minutes at 60°C. Finally the filter was exposed to a phosphor screen for two days and quantified using the imagequant computer package (GE Healthcare, Little Chalfont, UK).

2.3.3.6 Filter Stripping

Following hybridisation with a radiolabelled probe, northern filters can be stripped of radioactivity and re-used with minimal loss of RNA. Most northern filters can be stripped and re-probed five or six times depending on the abundance of the transcript of interest.

Materials
100 x TE (appendix I)
20% SDS
Method
The filter was stripped in 100-200 ml of 1xTE/0.5% SDS for 30 minutes in an 80°C water bath. The filter was then exposed overnight to a phosphoimager plate to ensure that all radioactive probe had been removed.

2.3.3.7 Oligo dT Correction (Sambrook and Russell, 2001)
To correct for variation in gel loading and RNA transfer when performing northern analysis, total mRNA on filters was quantified by oligo dT probing. The radiolabelled oligo dT probe hybridises with the poly A tail of mRNA species.

Materials
20ng Oligo dT template
5x Reaction buffer:
500mM cacodylate, pH6.8
5mM cobalt (II) chloride
0.5mM dithiothreitol (DTT)
0.5mg/ml Bovine serum albumin (BSA)
Terminal deoxytransferase, 20U/µl (Promega, Madison, WI, USA)
[α-32P]-dATP: 10ci/ml (GE Healthcare, Little Chalfont, Buckinghamshire, UK)
20x SSPE: (appendix 1)
3.6M sodium chloride
120mM di-sodium hydrogen orthophosphate
20mM EDTA
Sephadex G50 (appendix 1)
1x TE (appendix 1)

Method
Twenty nanograms of oligonucleotide was added to 1x reaction buffer that contained 70µCi of dATP and 20U of terminal transferase, in a final volume of 20µl. The reaction was incubated for one hour at 37°C. Incorporation of nucleotide into the oligonucleotide was measured using a mini Sephadex G50 column. The column was prepared by plugging a glass Pasteur pipette, with glass wool, and adding Sephadex G50. The labelling reaction volume was made up to 200µl with TE and this was loaded onto the column. The column was eluted with 1x TE and 200µl fractions collected. The fractions were counted and the percentage incorporation calculated. The hottest two fractions were pooled and one tenth added to the hybridisation mix. Filters were prehybridised in 20ml of prehybridisation buffer (5x SSPE,
0.2% (w/w) milk powder, 0.2% (v/v) nonidet) for a minimum of two hours at room temperature in a polythene bag on a shaking platform. Hybridisation solution was made by adding one tenth of the labelled probe to 10ml of the prehybridisation buffer. The prehybridisation solution was removed and replaced with the hybridisation solution. Hybridisation was carried out overnight at room temperature. Nonspecifically bound probe was removed by increasingly stringent washes. All wash steps were performed at room temperature. The first washes were carried out in; 5x SSPE; 0.2% SDS. The hybridisation solution was removed and replaced with 20ml of washing buffer and washed for five minutes at room temperature. This was repeated and then followed by two washes for thirty minutes each in 2x SSPE/0.2% SDS. Following this, the filter was exposed to a phosphoimager screen and radiolabelled bands quantified by image densitometry using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

2.3.4 Quantification of mRNA by qPCR

Quantitative PCR (qPCR) was used to analyse the expression of a range of mRNA sequences in dissected hypothalami using TaqMan® gene expression assays. Total RNA was extracted from tissue samples. From this RNA, mRNA was amplified by reverse transcription and used for qPCR analysis. All materials for qPCR were supplied by Applied Biosystems, Warrington, UK, unless otherwise stated.

2.3.4.1 RNA Extraction

Materials
Tri-reagent (Helena Biosciences, Sunderland, Tyne and Wear, UK)

Method
RNA was extracted from hypothalami using the Tri-reagent method according to the manufacturer’s protocol. Hypothalamic tissue was homogenised in 1ml Tri-reagent before subsequent transfer to 1.5ml eppendorfs and incubation at room temperature for five minutes.

2.3.4.2 RNA Purification
Sample RNA needed to be devoid of any genomic DNA contamination in order to accurately analyse mRNA levels by TaqMan® probe assay. This was achieved by treating the RNA samples with DNase solution.
Materials
Purelink RNA Mini kit
Chloroform (VWR International Ltd, Poole, UK)
Purelink DNase

Method
Following nucleoprotein dissolution with Tri-reagent, nucleic acids were extracted by adding 200μl of chloroform per 1ml of Tri-reagent used. RNA samples were processed for DNA removal using the Purelink RNA mini kit according to the manufacturer’s protocol (figure 2.5) (Gardiner et al., 2010).

The RNA was then dissolved and stored in RNase-free water before its yield was determined spectrophotometrically according to the following equation:

RNA (μg) = dilution factor x 40 x OD260 x sample volume (ml)
Figure 2.5: Extraction of total RNA using Invitrogen RNA minikit. Following incubation in Tri-reagent, samples were extracted in chloroform before ‘on-column’ digestion with Purelink DNase to leave ‘pure’ RNA.
2.3.4.3 Reverse Transcription of Hypothalamic mRNA

Messenger RNA was converted into its complementary cDNA sequence by reverse transcription in order to allow its amplification by PCR. The High Capacity cDNA Reverse Transcription Kit uses the random primer scheme for initiating cDNA synthesis.

Materials
High Capacity cDNA Reverse Transcription Kit
96 Well Assay Plate

Method
500ng of each RNA sample was added in triplicate to reaction wells containing 1X RT buffer, 4mM dNTP mix, 1X RT random primers, and Multiscribe reverse transcriptase. The plate was then foil-sealed before its incubation in a thermal cycler programmed to the following conditions: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes. Following cycle completion, cDNA templates were stored at -20°C.

Endogenous control cDNA templates were prepared by adding 50ng of each RNA sample before following the above procedure.

2.3.4.4 qPCR Assays from Hypothalamic cDNA

Each qPCR TaqMan® assay allows accurate quantification of gene expression by incorporating a reporter-tagged probe, which is complementary to part of the targeted cDNA sequence, into the amplification process. Probes were designed to cross exon-exon junctions to ensure amplification was limited to mRNA only. During PCR, the minor-groove binding probe anneals specifically to a complementary sequence between the forward and reverse primer sites. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence, primarily by Förster-type energy transfer. The DNA polymerase cleaves only probes that are hybridized to the target. Cleavage separates the reporter dye from the quencher dye to allow fluorescence of the reporter.

Materials
Sample cDNA template (25µg/ml)
Endogenous control cDNA template (2.5µg/ml)
TaqMan® Gene Expression Assay
TaqMan® Gene Expression Master Mix
384 Well Assay Plate
Method

The following constituents were mixed in each well for each qPCR reaction: 1μl of cDNA template (25ng), 10μl master mix, and GDW to give a 20μl reaction volume. For total RNA quantification, a gene expression assay targeting the 18S ribosomal subunit and 2μl of cDNA template (5ng) were used. Each reaction was mixed before the plate was sealed.

The sealed plate was loaded onto a ‘7900HT Fast Real-Time PCR System’ (Applied Biosystems, Warrington, UK) thermal cycler, on the ΔΔCt programme, under the following conditions: Held at 50°C for 2 minutes before 95°C for 10 minutes. Thermal cycling then proceeded where 15 seconds incubation at 95°C followed by 1 minute incubation at 60°C was repeated for 40 cycles. The amplification data for the target gene was normalised to the expression of the 18S endogenous control and expressed relative to an internal calibrator (control group sample), using the formula: $2^{-\Delta\Delta Ct}$.

2.3.4.5 Validation of qPCR Assays

Each qPCR assay was validated for use in a particular tissue prior to analysis, to assess suitability of 18S as an endogenous control target. This involved generating standard curves from hypothalamic RNA (at concentrations covering 3 logs) using both the 18S assay and target assay.

Materials

High Capacity cDNA Reverse Transcription Kit
96 Well Assay Plate
TaqMan® Gene Expression Assay
TaqMan® Gene Expression Master Mix
384 Well Assay Plate

Method

Standard curves of RNA mass were prepared, in triplicate, for reverse transcription (see 2.4.3.3) in the following manner:

<table>
<thead>
<tr>
<th>Assay</th>
<th>Standards (ng/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>50 25 10 5 1 0.5 0.1</td>
</tr>
<tr>
<td>Target</td>
<td>100 75 50 25 10 - -</td>
</tr>
</tbody>
</table>

Figure 2.6: Standards for validation of qPCR assays.
qPCR was then performed on each replicate (see 2.4.3.4) using the absolute quantification programme. The mean Ct values were plotted versus RNA mass to give standard curves. The assay efficiency was determined by the slope, where a value of 3.32 indicated 100% efficiency.

2.3.5 Measurement of Deiodinase Activity in Vivo

Deiodinase activity was measured in MBH punches in collaboration with Dr Anita Boelen of Department of Endocrinology and Metabolism of the Academic Medical Centre at the University of Amsterdam.

Materials

1.0mm Micron Biopsy Punch (Harvard Apparatus, Kent, UK)
PE Buffer (appendix 1)
4.6 x 250 mm Symmetry C18 column (Waters UK, Hertfordshire, UK)
Alliance HPLC system (Waters, Etten-Leur, The Netherlands)
Radiomatic Z-500 flow scintillation detector (Packard, Meriden, CT)

Method

Dissected brains were sliced in the coronal plane to the anterior commissure using a sled microtome. Three 800µm slices were then sequentially taken and placed onto separate poly-lysine slides on dry ice. Punch biopsies of the MBH were sampled under a light microscope, using a 1mm micron punch, and stored in an eppendorf vial at -80°C. These vials were packaged on dry ice and posted to the University of Amsterdam where the following methodology was used.

Samples were homogenised in 10x PE buffer to give approximately 500µl suspensions. Fifty microlitres of each sample was used in a reaction in duplicate to allow measurement of D2 and D3 activity in the same hypothalamic block. Deiodinase activity assays were performed as described previously (Boelen et al., 2005, Boelen et al., 2004a). Duplicate incubations of homogenates (~100 µg protein) were incubated for 1 hour at 37 C with 1 nM [3'-125I]T3/T4 (200,000 cpm) in a final volume of 0.1 ml PE buffer. Reactions were terminated by adding 0.1 ml ice-cold methanol. After centrifugation, 0.1 ml of the supernatant was added to an equal volume of 0.02 M ammonium acetate (pH 4). One hundred microlitres of this mixture were applied to a 4.6 x 250 mm Symmetry C18 column connected to an Alliance HPLC system. The column was eluted with acetonitrile (28–42% in 15 min) in
0.02M ammonium acetate (pH 4.0). Eluate radioactivity was measured using a Radiomatic Z-500 flow scintillation detector.

2.3.6 Radioimmunoassays

Radioimmunassay (RIA) was used to determine the concentrations of plasma hormones. The principle of RIA is based on the competitive nature of the binding of radiolabelled ligand and cold ligand for limited antibody binding sites.

Plasma insulin, TSH and luteinising hormone (LH) were measured by ‘in-house’ RIA methods. Natural porcine insulin, TSH and LH were iodinated using the iodogen method by Professor Mohammed Ghatel (Wood et al., 1981) and purified by HPLC.

2.3.6.1 Insulin Radioimmunoassay

Materials
Phosphate Buffer (appendix I)
$^{125}$I labelled insulin
Rabbit anti-insulin (MP Biomedicals LLC, Orangeburg, NY)
Polyethylene glycol (PEG)

Method
Plasma samples were vortexed and analyzed in duplicate using a specific rabbit antiserum (primary antibody) and synthetic hormone derivatives labelled with $^{125}$I as a tracer. Labelled insulin was reconstituted in phosphate buffer. Reaction tubes were incubated for 2 hours (22-25°C), with 200μl of $^{125}$I tracer prior to 200μl antiserum. The fractions of insulin were separated using charcoal precipitation where the pellet contained antibody-bound substrate and the supernatant contained free substrate. These free and bound fractions were counted for 100 seconds in a gamma scintillation counter and sample concentrations were calculated using a data reduction program (NE1600, NE Technology, UK).

2.3.6.2 Thyroid Stimulating Hormone Radioimmunoassay

Plasma samples were vortexed and analyzed in duplicate using a specific rabbit antiserum (MP Biomedicals LLC, Orangeburg, NY) and synthetic hormone derivatives labelled with $^{125}$I as a tracer.

Materials
Kemteck Buffer (appendix I)
$^{125}$I labelled TSH
Rabbit anti-TSH (MP Biomedicals LLC, Orangeburg, NY)
Goat anti-rabbit γ-globulins

Method
Plasma samples were vortexed and analyzed in duplicate using a specific rabbit antiserum and synthetic hormone derivatives labelled with $^{125}$I as a tracer. Labelled TSH was suspended in kemteck buffer. Samples were incubated for 2 hours (22-25°C), with 200μl of $^{125}$I tracer prior to 200μl antiserum. Free and bound molecules of TSH were separated using a secondary antibody; 500μl of PEG and Goat anti-rabbit γ-globulin in TRIS solution were added to each tube prior to centrifugation at 2500rpm for 15 minutes. The pellet and the supernatant were counted separately for 100 seconds in a gamma scintillation counter. Sample concentrations were calculated using a data reduction program (NE1600, NE Technology, UK).

2.3.6.3 Luteinising Hormone Radioimmunoassay
Plasma samples were vortexed and analyzed in duplicate using a specific rabbit antiserum (MP Biomedicals LLC, Orangeburg, NY) and synthetic hormone derivatives labelled with $^{125}$I as a tracer.

Materials
Kemteck Buffer (appendix I)
$^{125}$I labelled LH
Rabbit anti-LH (MP Biomedicals LLC, Orangeburg, NY)
Goat anti-rabbit γ-globulins

Method
Plasma samples were vortexed and analyzed in duplicate using rabbit anti-LH. Labelled LH was suspended in kemteck buffer. Free and bound molecules of LH were separated using secondary antibody. Samples were incubated for 2 hours (22-25°C), with 200μl of $^{125}$I tracer prior to 200μl antiserum. Secondly, 500μl of PEG and goat anti-rabbit γ-globulin in TRIS solution were added to each tube prior to centrifugation at 2500rpm for 15 minutes. Bound and free fractions were then separated and counted for 100 seconds in a gamma scintillation counter and sample concentrations were calculated using a data reduction program (NE1600, NE Technology, UK).
2.3.6.2 Leptin Radioimmunoassay

Materials
Rat Leptin RIA Kit (Millipore, Watford, UK)

Method
Plasma samples were vortexed and were then analyzed in duplicate using a specific rabbit antiserum and a synthetic leptin derivative labelled with $^{125}$I as a tracer. Free and bound molecules were separated using secondary antibody. For this, 100µl sample was incubated for 24 hours (20-25°C), with 100µl antiserum prior to 100µl of $^{125}$I tracer. Secondly, 1ml of precipitating reagent was added to each tube and incubated for 20mins prior to centrifugation at 2500rpm for 20 minutes. Bound and free fractions were then separated and both the pellet and the supernatant counted for 1min in a gamma scintillation counter and sample concentrations were calculated using a data reduction program (NE1600, NE Technology, UK).

2.3.6.3 fT3 and fT4 RIA

Materials
fT4 RIA Kit (Siemens Medical Solutions, Surrey, UK)
fT3 RIA Kit (Siemens Medical Solutions, Surrey, UK)

Method
Solid phase RIA relies on a specific antibody immobilised to the wall of a polypropylene tube. The antibody is then incubated with $^{125}$I labelled antigen and unlabelled antigen, from standards included in the kit, or sample, for a short fixed time. The bound and free fractions are separated by decanting and the bound fraction counted on a γ-counter. The non-linear calibration curve is determined and the amount of fT3 and fT4 calculated in terms of standard preparations.

The calibrators and $^{125}$I-labelled antigen were supplied in ready to use liquid form. Both calibrator and sample were added in the appropriate volume (fT3: 100µl, fT4 50µl) before addition of 1ml labelled antigen. The tubes were vortexed before being incubated at 37°C for three hours. Free and bound fractions were separated by decanting and the remaining activity in the tubes counted for sixty seconds. The mean counts per minute were calculated for standard and samples and then plotted against the standards to construct a standard curve. The standard curve was used to interpolate the fT3 and fT4 concentrations for the unknown samples.
2.4 In Vivo Techniques

2.4.1 Animals

Male Wistar rats (Charles River, Bicester, UK) were maintained in individual cages under a controlled temperature of 21-23°C with 12 hour light cycles and *ad libitum* access to food and water. All animal procedures were conducted under the British Home Office Animals (Scientific Procedures) Act 1986 (Project License 70/6377).

2.4.2 Intranuclear rAAV Administration Technique

Materials

- Isoflurane system (Vet-Tech Solutions Ltd., UK)
- Betadine (Seton Healthcare, Oldham, UK)
- Stereotaxic Frame (Clark electromedical instruments, Pangbourne, UK)
- 23.5 Gauge Steel Cannula (Plastics One Inc., Roanoke, VA, USA)
- 33 gauge steel injector (Plastics One Inc., Roanoke, VA, USA)
- 0.9% sodium chloride (Schering-Plough Ltd., UK)
- Buprenorphine (Reckitt-Benckiser Healthcare, Hull, UK)

Method

Male Wistar rats, of body weights ranging from 230 to 260g, were anaesthetised with an isoflurane system and immobilised on a stereotaxic frame. The incisor bar was set at 3.0mm below the interaural plane. The surgical area was shaved with an electronic razor and cleaned with 10% w/v povidine-iodine solution (Betadine). A small (<2cm) rostro-caudal incision was made in the scalp, which was cleared of subcutaneous tissue. Animals were injected bilaterally into the VMN using coordinates -2.8mm caudal, +/-0.7mm lateral to the bregma and 9.6mm below the skull, determined from the rat brain atlas of Paxinos and Watson (126). A stereotaxically mounted drill was used to make a 0.65mm burr hole in the skull. A 23.5 gauge steel cannula was placed, using a cannula arm, bilaterally into the VMN. Each animal was injected via a 33 gauge steel injector, which projected 1mm below the tip of the guide cannula. Either 1µl rAAV-D3 (titre: 5.56x10^{12} genome particles/ml, 1µl rAAV-D2 (titre: 1.12 x 10^{14}), or rAAV-GFP (titre: 5.04x10^{12}) genome particles/ml was administered bilaterally into the VMN of each rat. The pressure was removed from the injector and the cannula/injector unit was maintained *in situ* for 5 minutes before being slowly extracted with the aid of the stereotaxic frame to minimise backflow. The incision was sutured with two
mattress sutures with 6.0 proline thread. All animals received peri-operative care including: antibiotics (floxicillin:amoxicillin equal volumes, 37.5 mg/kg, i.p. 1 ml/rat), analgesia (buprenorphine, 45 mg/kg s.c. 0.05 ml/rat) and rehydration therapy (0.9% sodium chloride, i.p. 2.5 ml/rat). All surgical procedures were performed under aseptic conditions.

Injection coordinates were initially established and tested by following above procedure on euthanised Wistar rats, where Indian ink was administered to the injection site. Rat brains were then dissected (see 2.4.5) and mounted on corkboard, from which sections were taken and analysed for injection accuracy.

2.4.3 Cumulative Food Intake and Body Mass Change

Male Wistar rats were injected intra-VMN bilaterally with either rAAV-GFP, rAAV-D3 or rAAV-D2. After a 7 day recovery period, food intake and body mass were measured three times per week (between 09.00-11.00 hours). Animals were maintained on either a normal chow diet (NCD) (Research Diets) or a high fat diet (HFD) (Research Diets).

2.4.5 Dark Phase Refeeding Profile

Food was removed from animal cages either 12 hours or 24 hours prior to replacement of measured food quantity. Food was reweighed at 1, 2, 4, 8 and 24 hours post-injection. Body weight was measured prior to fasting, post-fasting and post-refeeding.

2.4.5 Collection of Tissue Samples

Rats were killed by carbon dioxide asphyxiation or decapitation in the early light phase. Brains were rapidly removed and lateral sections cut away using a razorblade to isolate hypothalami, which were frozen in liquid nitrogen before storage at -80°C.

Blood was taken by cardiac puncture and collected in heparinised or EDTA-chelated tubes containing 100μl aprotinin, and was immediately placed on ice before centrifugation at 4°C to obtain plasma. Plasma samples were stored at -80°C until performance of RIA. Interscapular BAT was dissected, weighed, frozen in liquid nitrogen and stored at -80°C until RNA extraction.

The contents of rat gastrointestinal tracts were removed before storing carcasses at -20°C in preparation for saponification.
2.5 Statistical Analysis

Data are presented as arithmetic means ± standard error of mean (SEM). Differences in body mass and cumulative food intake were assessed using a Generalised Estimation Equation (GEE) for repeated measures, using Stata 9 software (Stata, StataCorp LP, USA). The GEE is used to analyse longitudinal data and highlight correlation between data sets where random fluctuations could occur.

Group differences in body composition, RIA results, and tissue expression were analysed using unpaired t-tests, and were considered statistically significant at the 95% confidence level (p<0.05). Time-controlled feeding studies and qPCR data were analysed by one-way analysis of variance (ANOVA) and corrected for multiple testing using a Bonferroni post-hoc test. Data analysis was performed using Graphpad Prism 5 software.
Chapter 3

Production and Analysis of rAAV-D2 and rAAV-D3
3.1 Introduction

The investigations in this research involve the manipulation of D2 and D3 mRNA levels to modulate the local levels of T3. D1 enzyme was not utilised as it lacks specificity in its locus of deiodination, thus could either activate or inactivate thyroid hormones. The exploitation of local enzymatic regulation of hormone activity is advantageous over administration of exogenous hormones which would be rapidly degraded \textit{in vivo}.

Investigations have previously been carried out to look at the roles of D1 and D2 using genetic knock-out (KO) models. D2 KO models exhibit disrupted feedback regulation of pituitary TSH secretion (Schneider, 2001), CNS T3 content (Galton et al., 2007), and adaptive thermogenesis (De Jesus et al., 2001). Interestingly, combined D1/D2 KO models demonstrate only mild phenotypes and are not unlike euthyroid wild-types (Galton et al., 2009). This indicates that additional mechanisms could exist to compensate for the absence of systemic T4 to T3 conversion. D3 KO models are characterised by neonatal thyrotoxicosis followed by central hypothyroidism from postnatal day 15 to adulthood with decreased levels of hypothalamic T3 and elevated TRH (Hernandez et al., 2006a). This phenotype is counterintuitive to what would be expected and suggests that D3 is important in defining the set-point of HPT feedback regulation by T3 during early postnatal life.

The complex phenotypes of deiodinase KO models make the extrapolation of the role of thyroid hormones an arduous process, given the potential for compensatory changes in the expression of other isoforms and the critical role of these hormones in CNS development. DNA transfer allows localised expression of genetic material via infection of somatic cells. Stereotaxic injection of viral vectors is highly advantageous in the investigation of brain function as it allows specific nuclei to be directed in developed animals.

In this work, recombinant adeno-associated virus (rAAV) has been designed to overexpress either the \textit{Dio2} gene (rAAV-D2) or \textit{Dio3} gene (rAAV-D3) as a means of manipulating thyroid hormone activity.

3.1.1 Non-Viral Gene Transfer Methods

Gene transfer allows the transduction of genetic material to a cell population. There are a number of methods that can be employed for this, with calcium phosphate being one of the earliest methods described. A number of techniques have since been developed, including liposomes, cationic polymers and electroporation (Davis, 2002). It has also been reported that
naked DNA can be injected directly into tissues to overexpress a gene (Zhang et al., 2004, Zhang et al., 1997).

### 3.1.1.1 Calcium Phosphate Transfection

Transfection with calcium phosphate relies on precipitates of DNA forming through interaction of DNA phosphate groups with calcium ions (Chen and Okayama, 1987). DNA and calcium chloride are mixed before the addition of phosphate-buffered saline (PBS). The resulting DNA precipitates are thought to enter the cell by endocytosis. The principal benefits of this process are its low cost and technical simplicity. Despite its advantages, this method is hindered by difficulties in its in vivo application and variation in sizes of DNA precipitates, which reduces experimental consistency (Luo and Saltzman, 2000).

### 3.1.1.2 Polyethylenimine

Polyethylenimine (PEI) is a cationic polymer, which can be used as a delivery vector both in vitro and in vivo (Chemin et al., 1998). The molecule can exist in two different conformations, being either branched or linear. Branched PEI is a more efficient transducer and is more commonly used for gene delivery, as it efficiently complexes with DNA. Its amine-rich structure neutralises the net negative charge of DNA phosphate groups, which facilitates its transport across the plasma membrane. The transduction efficiency depends on the molar ratio of PEI nitrogens to DNA phosphate groups, which is an aspect that must be optimised to minimise the level of protein binding to the PEI/DNA complex. The optimal molar ratio varies across different cell types and DNA constructs (Boussif et al., 1995).

### 3.1.2 Recombinant Viruses for Gene Transfer

There is a wide choice of vector for gene transfer, with each exhibiting different properties that make them more or less advantageous for the intended effect. The advantages and disadvantages of each vector depend on its method of production, period of expression, immune response, tissue tropism, packaging capacity, chromosomal integration and ability to infect quiescent cells. These characteristics have been reviewed in a number of publications (Thomas et al., 2003, Walthier and Stein, 2000, Kay et al., 2001).

#### 3.1.2.1 Adenovirus

The principal advantages of adenovirus vectors are their broad range of tissue tropism and large packaging capacity (36kb). They are able to infect both quiescent and non-quiescent cells, in which the transduced genetic material exists episomally within the nucleus. A principal disadvantage is the short duration of overexpression, which is generally limited to 2
weeks due to their strong induction of a host immune response. This makes them suitable for obtaining acute, transient expression of the genetic material, although chronic investigations would be difficult to sustain (Yang et al., 1994).

3.1.2.2 Herpesvirus

Herpesviruses are linear, double-stranded DNA (dsDNA) viruses that have a large genomic packaging capacity and provide long-term infection. Their natural tropism for neuronal cells makes them a useful tool for CNS research. Following infection, the herpesvirus enters a latent infection cycle whereby its genetic material does not integrate with the chromosome, but rather exists episomally. Herpes Simplex Virus (HSV) has more than 70 gene products, some of which encode toxic proteins (McGeoch et al., 1988). Removing these products from HSV vectors enables their use in gene transfer (Samaniego et al., 1998), however, retaining its efficiency in the face of these deletions complicates its use as a recombinant virus (Marconi et al., 1996).

3.1.2.3 Retrovirus

Retroviruses are small, single-stranded RNA (ssRNA) viruses. They have 3 essential genes flanked by long terminal repeats that are required for integration into the host genome. Following infection, transduced RNA is reverse transcribed into dsDNA, which then randomly integrates into the host genome. Retroviruses have a modest packaging capacity (7-8kb), which can be a limiting factor. A major limitation of retroviral vectors is their inability to transduce quiescent cells due to the requirement for chromosomal exposure during cell division (Miller et al., 1990).

A member of the retrovirus family is the lentivirus, which is termed so on account of its long incubation period. The lentiviruses, often derived from Human Immunodeficiency Viruses (HIV), differ from other retroviruses as they are able to gain access to the nucleus independently. This enables their transduction of non-dividing cells, such as neurones (Carlotti et al., 2004). Stringent measures must be taken in order to prevent recombinant lentiviruses from forming a potentially pathogenic strain (Buchschacher Jr and Wong-Staal, 2000).

3.1.2.4 Adeno-Associated Virus

Adeno-associated viruses are small, ssDNA dependoviruses. They are termed ‘dependoviruses’ as propagation is dependent upon coinfection with an unrelated virus, such as adenovirus. More than 11 serotypes of AAV have been characterised of which the most
common are types 2, 3 and 5. Despite more than 80% of adults possessing neutralising antibodies against AAV (Russell and Kay, 1999), its residence within the host is generally commensal. AAV type 2 (AAV2) was the first characterised serotype and has natural neurotropism which allows specific transduction of brain parenchyma with minimal expression in neighbouring astroglial cells (Bartlett JS, 1998).

An AAV particle is approximately 20nm in diameter and is generally stable to changes in temperature and pH. The wild-type viral genome consists of two open reading frames; a rep (replication) gene, controlled by the promoters P5 and P9, and the cap (capsid) gene, controlled by the promoter P40. The ssAAV genome is flanked by inverted terminal repeats (ITR), which are 140-170 nucleotides in length and vary across different serotypes. The ITRs are essential cis elements of the rAAV genome, important for packaging, self-priming and integration of the vector.

Following successful infection of a cell, the ssAAV genome must be converted into dsDNA. This is achieved using the 3’ ITR, which forms a hairpin loop to prime second strand DNA synthesis of the remaining AAV genome. The 3’ ITR from the ‘parental’ strand is transferred to the ‘daughter’ strand where it used as a template for the original parent strand. The dsAAV genome then either integrates into the host genome or exists as a circular episomal element.

Wild-type AAV (wtAAV) integrates into host chromosome 19q13.42 in the absence of a helper virus in its latent state (Kotin et al., 1990). In the presence of a helper virus, wtAAV proceeds by replicating and generating new infectious particles. The targeted region of chromosomal integration is termed AAVS1 and is located within the MBS85 gene, which is a constitutive mediator of actin remodelling (Tan et al., 2001). The estimated efficiency of AAVS1-targeted integration by wtAAV is approximately 40–70% (Daya and Berns, 2008). Rep 68 and Rep 78, wtAAV proteins produced from transcripts under the control of the p5 promoter, are trans-acting elements which appear to be required for AAVS1 targeting (Weitzman et al., 1994).

### 3.1.2.5 Recombinant AAV

Recombinant AAV (rAAV) is employed as a viral vector system for the delivery of genetic material to host cells. The recombinant virus is devoid of its Rep and Cap genes with only ITRs remaining, which act as primers for auto-regulation of its transcription. When administered directly to brain parenchyma, rAAV does not prime an immune response and allows sustained overexpression of a gene after a single surgical session.
Genetic material transduced by the recombinant virus may either incorporate into the host genome or exist extrachromosomally as an episomal genome in vivo. Research to date suggests that AAV integration is largely dependent on the presence of the integration efficiency element (IEE) of the p5 promoter and certain regions of the Rep gene in addition the ITRs (Philpott et al., 2002, Weitzman et al., 1994). Therefore, chromosomal integration of the rAAV genome is relatively inefficient, with an estimated 85 - 95% of rAAV genetic material existing episomally (Schnepp et al., 2005, Afione et al., 1996). One investigation highlighted that, following rAAV transduction of hepatic tissue, partial hepatectomy (to induce cell division) resulted in approximately 85% reduction in vector genome between 12 weeks and 12 months post-infection (Nakai et al., 2001). In studies of rAAV transduction of mouse muscle tissue, it was demonstrated that the majority of vector DNA existed as double-stranded circular DNA, the conformation that is adopted by extrachromosomal episomes (Schnepp et al., 2003). This does not confound the efficiency of rAAV vectors given that, unlike other vectors, they do not require chromosomal integration for efficient replication (Flotte et al., 1994). However, it is estimated that rAAV infection of quiescent cells would be stable for long term investigations, although its persistence in mitotic cells could be relatively short-lived (McCown et al., 1996).

The efficiency of rAAV vectors can be improved by a variety of transcriptional elements. The cytomegalovirus (CMV) promoter has been frequently used to drive strong expression in the CNS, where high expression is witnessed 3 weeks after rAAV injection, but reduced by 4 months post-injection (Kaplitt et al., 1994). This silencing of the CMV promoter has been linked to its susceptibility to transcriptional activation by methylation of cysteine on CpG (cytosine per guanine) dinucleotides (Prösch et al., 1996). However, additional modifications to the rAAV construct can be made to optimise the persistence of vectors under the control of the CMV promoter. The woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) can be inserted, downstream of transgene sequence, to improve 3’ RNA processing, increase vector titre, and ultimately stabilise mRNA accumulation within infected cells (Schambach et al., 2005).

There are a number of disadvantages of using AAV for gene transfer. Being derived from one of the smallest DNA viruses, AAV constructs larger than 4.7kb are difficult to integrate into the viral genome. The AAV viral genome consists of ssDNA which must be converted to dsDNA before transgene expression reaches an optimal level (Ferrari et al., 1996). This process may reduce rAAV efficiency during the first few weeks after infection (Afione et al., 1996).
This limitation may be overcome by the use of self-complementary AAV vectors (scAAV), which are designed to bypass this phase of ssAAV to dsAAV conversion, by virtue of its palindromic dsDNA structure (McCarty et al., 2001). However, this reduces the rAAV vector capacity to approximately 2.4kb, which limits its use in research (Wu et al., 2007).

3.2 Aims of Study
To produce and evaluate the efficacy of plasmid constructs and rAAV titres that overexpress Dio2 and Dio3.
3.3 Experimental Results

Plasmid vectors were designed to overexpress the iodothyroinine deiodinase genes \textit{Dio2} and \textit{Dio3} (figure 3.1). Both enzymes require the SECIS element for optimal efficacy in deiodination. In the case of the \textit{Dio2} gene, the SECIS coding region lies 7.4kb downstream of the D2 coding sequence, in the 3’ UTR (Buettner et al., 1998). Incorporation of this full 7.4kb fragment into rAAV would be extremely difficult, given this vector’s packaging limitations (Le Bec and Douar, 2006). When designing the rAAV-D2 plasmid construct, the SECIS was manually inserted downstream of the \textit{Dio2} in much closer proximity. It has been reported that closer association of the D2 and SECIS coding sequences by deletion of a 3.7kb UTR sequence can in fact increase D2 activity (Gereben et al., 2002).

The \textit{Dio2} coding sequence was subcloned into pTR-CGW, before subsequent downstream ligation of the SECIS element. This was not required for the rAAV-D3 construct, as the SECIS locus is within suitable proximity of the \textit{Dio3} gene.

JEG3 cells selected for transfection as they exhibit very little endogenous deiodinase expression (Friesema et al., 2006). The prepared plasmid constructs were used to generate in vitro models of D2 and D3 overexpression for the analysis of deiodinase activity and cellular secretion of thyroid hormone molecules. Following these tests of efficacy, the plasmids were packaged into AAV virions with titre stored for the subsequent in vivo experiments.
Figure 3.1 Plasmid maps of pTR-CGW constructs. A, rAAV-D2 expression cassette; B, rAAV-D3 expression cassette. Restriction sites are indicated with arrows and number of nucleotides from the first base of the ITR is indicated in parentheses.
3.3.1 Amplification of *Dio3*, *Dio2*, and SECIS cDNA by RT-PCR

The PCR products from each reaction were analysed on a 1% TAE/Agarose gel. Each band corresponded to the expected size of the amplicon when compared to a 1kb DNA ladder, where *Dio2* was 959bp, SECIS 600bp and *Dio3* 1495bp (figure 3.2).

Figure 3.2: Agarose gel to show relative sizes of PCR amplicons. Lane 1; 1KB ladder (Invitrogen, UK) with bands marked for 500bp, 1000bp, 1500bp and 2000bp. Lane2; Dio2 amplicon of 959bp. Lane 3; SECIS amplicon of 600bp. Lane 4; Dio3 amplicon of 1495bp.
3.3.2 Small-Scale Preparation of Transformed Bacteria

Each ligated product was used to transform competent JC8111 bacteria, which were selected for the antibiotic resistance marker present in the pTR backbone by plating the bacteria on ampicillin-positive plates. Ten individual colonies were subsequently cultured and analysed for the presence of the correct insert by restriction digest. Example given shows pTR-CGW-D2-SECIS transformed colonies in lanes 3, 4, 6, 7, 8, 9, 10, and 11 possessing the correct 1559bp Dio2-SECIS ligated product (figure 3.3A).

The integrity of AAV ITRs was checked by XmaI restriction digest on each sample, as the ITR sequence contains the XmaI restriction site. The intensity of the 2 smaller sized fragments (2905bp, 3323bp), representing plasmid with ITRs successfully digested by XmaI, was compared to the intensity of the largest, undigested fragment (6228bp), representing plasmid deficient of ITRs. Figure 3.3B shows recovered DNA from pTR-CGW-D2-SECIS transformed colonies following XmaI restriction digest. The band profile visualised in lanes 3, 4, 7 and 9 indicates a good ITR ratio).

From this array of transformants, the colony represented by lane 4 was selected for large scale preparation and processing for rAAV production, as it was deemed to show the best overall profile.
Figure 3.3: Agarose gels demonstrating restriction digests of small-scale preparations of transformed bacteria. A: BamHI and BsrGI double-digest to reveal presence of Dio2-SECIS. B: XmaI digest to reveal presence of AAV ITRs in transformed Ptr plasmid. Lane 1, 1KB+ DNA ladder (Invitrogen, UK); Lanes 2-11, DNA samples from small-scale preparations of transformants.
3.3.3 Sequencing of pTR-CGW Plasmids

Sequencing of pTR-CGW plasmids was performed in both directions using a dye-termination protocol in collaboration with the Advanced Biotechnology Centre at Hammersmith Hospital, London. Sequence data obtained from the resulting chromatogram was uploaded onto NCBI nucleotide blast to compare the plasmid sequence homology with the NCBI gene database.
Figure 3.4: Sequencing data for pTR-CGW-D2-SECIS and pTR-CGW-D3. The submitted queries were 100% homologous to the Dio2 (A) and Dio3 (B) cDNA gene sequences (NCBI Refs: NM_172119.2, NM_017210.3 respectively). Areas highlighted blue indicate location of oligos used for sequencing reactions; areas highlighted yellow indicate coding sequences; areas highlighted black with yellow text indicate location of Sec codon; area highlighted black with red text indicates region containing conserved SECIS elements.
3.3.4 Effect of Plasmid Transfection on Deiodination *in vitro*

The efficacies of pTR-CGW-D2 and pTR-CGW-D3 were assessed by transfection of these plasmids into JEG3 cells, where pTR-CGW was used as a control plasmid. The fT3 and fT4 concentrations of DMEM/F12 (10% FBS) media were used as markers of deiodination by the transgenes. RIA was used to measure the concentrations of fT3 and fT4 in the culture medium twenty-four hours after replenishment with fresh media, which was added approximately 48 hours after transfection (see section 2.2.3). Data are expressed as percentage of pTR-CGW values for each hormone.

The RIA results (figure 3.5) demonstrated that, following pTR-CGW transfection, both fT3 and fT4 levels were at 8.1 +/- 0.03pmol/L and 7.5 +/- 0.43pmol/L, respectively. In the case of fT4, this was not significantly affected by pTR-CGW-D2-SECIS (96.1 +/- 9.2% of pTR-CGW value) or pTR-CGW-D3 transfection (112.1 +/- 5.1% of pTR-CGW value).

However, fT3 concentrations were affected, whereby pTR-CGW-D2-SECIS treated cells showed significantly (P=0.01) higher levels of fT3 (110.6 +/- 2.3% of pTR-CGW value) and pTR-CGW-D3 transfected cells showed significantly (P=0.002) lower levels of fT3 (94.3 +/- 0.8% of pTR-CGW value).

**Figure 3.5:** Free T3 and free T4 concentrations 24 hours after replacement of DMEM/F12, 10% FBS. Media content of fT3 and fT4 was measured by RIA 48 hours after calcium phosphate transfection of either pTR-CGW (green bars) (n=4), pTR-CGW-D2-SECIS (blue bars) (n=4), or pTR-CGW-D3 (black bars) (n=4). Data are expressed as percentage of pTR-CGW values for each hormone. *P<0.05, **P<0.01
3.3.5 Effect of Plasmid Transfection on Deiodinase Activity *in vitro*

The level of deiodinase activity was analysed and compared in JEG3 cells transfected with either pTR-CGW or pTR-CGW-D3. The effect of pTR-CGW-D2 was not investigated as D2 enzyme activity must be measured in fresh cell homogenate, which would be unlikely to maintain viability through transport to Amsterdam (Department of Endocrinology and Metabolism of the Academic Medical Centre at the University of Amsterdam).

Activity measurements demonstrated that pTR-CGW-D3 produced a higher level of D3 activity (51.28 +/- 8.54 fmol/min/mg) than pTR-CGW (5.23 +/- 0.86 fmol/min/mg), which was a statistically significant difference (P < 0.001) (figure 3.6).

![Figure 3.6: Iodothyronine deiodinase III activity in transfected JEG3 cells. D3 activity was measured in thawed JEG3 cell homogenate, which had been harvested 72 hours post calcium phosphate transfection of either pTR-CGW (green bar) (n=5) or pTR-CGW-D3 (black bar) (n=5). Data are expressed in fmol/min/mg. ***P<0.001](figure3.6.png)
3.3.6 Dot Blot Analysis of Purified rAAV Titres

Dot blot analysis was performed to determine the yield of the purified rAAV titres. A standard curve was prepared covering three logs of pTR-CGW quantity (figures 3.7A and 3.7B). The phosphorescence of the rAAV preparation (rAAV-D3 shown here as an example) was interpolated into this standard curve, in neat and 1:10 dilutions (figure 3.7C), with the quantity of plasmid then extrapolated. The rAAV-GFP titre yielded $1.5 \times 10^{14}$ vg/ml, the rAAV-D2 titre was $1.7 \times 10^{14}$ vg/ml and the rAAV-D3 titre was $1.8 \times 10^{14}$ vg/ml.

![Figure 3.7: Dot Blot Analysis of rAAV Titres. Phosphorescence obtained from standard curve serial dilutions of rAAV-GFP (A). Quantity of pTR-CGW plotted versus phosphorescence (B). Neat and 1:10 dilutions of rAAV-D3 (n=3) (C).](image)
3.4 Discussion

Gene transfer was chosen as a method for manipulating thyroid hormone levels within the hypothalamus. KO models are confounded by developmental compensation; therefore it was important to choose a strategy that allows physiological manipulation of a developed animal model. Previously, direct administration of T3 to the hypothalamus has been used to investigate its role within this brain region. However, this technique is limited by the duration of T3 persistence. An alternative strategy to combat this limitation would be the use of osmotic minipumps, which have been used to prolong administration of T3 to the PVN in more long-term investigations. However, this method is limited by the safety of having these minipumps secured to the rat in vivo for an extended period of time, whereas rAAV can be administered and expressed indefinitely after a single neurosurgical session. Therefore, gene transfer of the iodothyronine deiodinases was deemed the optimal research strategy for the investigations.

Recombinant AAV was the vector of choice for these investigations amid the availability of a range of other viral vectors. For the long-term transduction of the CNS, lentiviral vectors and AAV are most frequently used given their ability to transduce quiescent cells and their lack of immunogenicity. AAV has been identified as the optimal vector for the transduction of the hypothalamus and amygdala, when it was demonstrated that more cells can be efficiently transduced using rAAV in comparison to lentiviral vectors (de Backer Marijke et al., 2010).

The basic design of the pTR-CGW packaging constructs was to overexpress the appropriate gene through the combined action of the CMV promoter and the WPRE, which is known to achieve a sustained, high-level of transgene expression. In the case of rAAV-D2, the process was slightly more complex due to the necessity for a manual insertion of the SECIS. The SECIS was placed in closer proximity to the Dio2 coding region, which has been shown to have a positive impact on D2 activity (Ferrari et al., 1996). However, the effect of situating the SECIS element in such close proximity to the WPRE is not known and could influence the efficacy of either element. Thus, given that the manual insertion of SECIS was not optimised and evaluated, it is not certain that this vector system was of maximal efficiency.

Each pTR-CGW construct was transfected into JEG3 cells in order to assess its respective efficacy on thyroid hormone metabolism prior to its use in rAAV production. This was achieved by the measurement of fT3 and fT4 concentrations in cell media samples after 24 hours incubation. The results indicated that pTR-CGW-D2-SECIS increased the level of
cellular T3 secretion and pTR-CGW-D3 decreased T3 secretion. This was expected given the respective roles of D2 and D3. However, T4 levels were not of a detectable difference between each group.

A more comprehensive profile would be gathered if a time-course experiment were undertaken. This would involve sampling culture medium at various time points over a twenty-four hour period following the introduction of cell media with a known starting concentration of hormones, rather than simply relying on FBS for the fT3 and fT4 pool. This would provide greater detail of the kinetics of each construct in addition to a more quantitative set of data, rather than proportional differences.

The efficacies of the constructs were tested further by measuring iodothyronine deiodinase enzyme activity in transfected JEG3 cells. This was carried out in collaboration with Dr Anita Boelen at the University of Amsterdam who was able to measure D3 enzyme activity in frozen homogenates. D2 enzyme activity was not measured in JEG3 cells as the enzyme is must be measured in fresh cell homogenate, which would be unlikely to survive transport to Amsterdam. The D3 activity measurements demonstrated that the pTR-CGW-D3 construct produced an almost ten-fold higher level of JEG3 cell D3 activity than pTR-CGW. This indicated that the pTR-CGW-D3 construct was effective in vitro and was suitable for use in production of rAAV.

The production method for the rAAV titres used in these investigations involved a two plasmid transfection system, where the adenoviral helper genes were present on a single vector (pDG) and transfected simultaneously with the packaging vector (pTR-CGW) (Grimm et al., 1998). This improved method is identified as a safer and more efficient alternative to the adenovirus-dependent transduction method for the production of high quality AAV serotype 2 vectors that efficiently infect rat brain cells (Grimm et al., 2003).

The vector stocks of rAAV-GFP, rAAV-D2 and rAAV-D3 were all quantified and demonstrated high viral particle numbers. The infectious titres were not measured subsequent to this, although it has been reported that purification of rAAV using a NHS-activated HiTrap-Sepharose column yields infectious titres of more than 65% of the total particle number (Grimm et al., 1998). Therefore, the infectious titres would be expected to be in the region of $7 \times 10^{13}$ vg/ml, which would be sufficient for use in the intended 1µl injection volumes in vivo.
Chapter 4

The Effect of rAAV-D2 to the VMN of Rats

Maintained on a Normal Chow Diet
4.1 Introduction
Thyroid hormones are important for the hypothalamic regulation of several homeostatic pathways, such as; the HPT axis, torpor, the gonadal axis and appetite. Their activity within the CNS is controlled through the coordinated regulation of the iodothyronine deiodinases. Acute administration of T3 to the VMN of male Wistar rats induced a rapid hyperphagic response. D2 is the principal thyroid hormone activator, which produces active T3 hormone from the T4 precursor.

In this chapter, the physiological role of T3 in the VMN has been investigated in chronic investigations whereby rAAV-D2 has administered to achieve a long-term increase in Dio2 mRNA levels. D2 has a very short half-life and is extremely labile in vivo, therefore the mechanism of its rapid clearance by ubiquitination has been reviewed.

4.1.1 Iodothyronine deiodinase II in the Hypothalamus
As previously mentioned, D2 expression in the hypothalamus is limited to astrocytes and a subset of glial cells known as tanycytes (Lechan and Fekete, 2007), where Dio2 mRNA is expressed at a high concentration relative to other CNS regions (Fekete et al., 2000, Guadaño-Ferraz et al., 1997, Tu et al., 1999). The tanycytes can be classified as either α-tanycytes or β-tanycytes, which are then further organised into α-1, α-2, β-1 and β-2 subtypes. α-1 and α2 tanycytes line the walls of the third ventricle and can be distinguished by their endpoints. α1 tanycytes project to the VMN and appear to terminate on neurones, whereas α2 tanycytes project to the ARC ending either on a single capillary vessel or a single neurone, or extend to join the basal processes of β-1 tanycytes (Rodriguez et al., 1979, Akmayev et al., 1973).

T4 is transported into a third ventricle tanycyte, both passively and by OATP1C1, before undergoing deiodination. The product T3 is then transported out of the tanycyte, facilitated by the transporter MCT8, which effluxes the molecule into the extracellular space. From here, T3 can enter neighbouring neuronal cells to mediate its effects on neuronal metabolism (Freitas et al., 2010). In neurones, T3 has been shown to mediate its effects via both genomic and extragenomic interactions, generally via the TR, of which both the TRα and TRβ isoforms are expressed throughout the hypothalamus in rats and humans (Alkemade et al., 2005, Bradley et al., 1989).
D2 mRNA in the brain is transcriptionally regulated by numerous effects, such as stress, trauma, light, infection and hypothyroidism (Fekete and Lechan, 2007, Fliers et al., 2006, Lechan and Fekete, 2005). It has been reported that D2-expressing tanycytes are in direct opposition to NPY/AgRP neurones in the MBH (Coppola et al., 2007). In some rodent species, hypothalamic D2 activity exhibits a 12hour light/dark cycle which coincides with their metabolic activity. Furthermore, hypothalamic D2 activity increased in the fasted state (Diano et al., 1998). This increase in D2 activity has been linked to the relationship between circulating leptin levels and corticosterone (Coppola et al., 2005). It was reported that D2 activity is not affected by leptin in the fed state, but interestingly leptin administration attenuated the fasting-induced increase in D2. Furthermore, adrenalectomized rats did not show fasting-induced induction of D2 activity until they were treated with exogenous corticosterone. Leptin administration to these animals attenuated the rise in D2 activity. This presents an interesting pathway in which fasting-associated reductions in plasma leptin plays a role in D2 regulation via modulation of corticosterone.

4.1.1.1 Triiodothyronine in Hypothalamic Regulation of Food Intake

T3 serves essential roles in hypothalamic pathways of appetite regulation. It has already been mentioned that peripheral administration of T3 increases Egr1 immnuoreactivity in the VMN and direct administration of T3 to the VMN potently increases food intake in male Wistar rats, (Kong et al., 2004). These findings were not associated with any changes in locomotion or hypothalamic neuropeptide levels, therefore the effect was perceived to be solely on appetite.

The downstream mediators of T3 signalling within the CNS have been the focus of investigation, where several hypothalamic neuropeptides and adenosine monophosphate kinase (AMPK) have been presented as potential contributors to T3-associated hyperphagia. It has been reported that Sprague-Dawley rats with T3-induced thyrotoxicosis display hyperphagia with suppressed circulating leptin levels, increased hypothalamic NPY mRNA levels and decreased hypothalamic POMC mRNA levels (Ishii et al., 2000). Furthermore, it was reported that subcutaneous administration of 4.5nmol/kg T3 induced a 70% increase in hypothalamic AMPK activity complete with a 90% increase in phosphorylated ACC, the inhibited form of this enzyme (Ishii et al., 2008). Inhibition of ACC reduces the local concentration of malonyl CoA, which is known to promote food intake. Therefore, given that AMPK is known to inhibit ACC by phosphorylation, it was suggested that T3 stimulates feeding via activation of AMPK and modulation of hypothalamic fatty acid metabolism.
4.1.3 Post-Translational Regulation of Iodothyronine Deiodinase II

It is essential that D2 protein activity is regulated tightly given the potency of its product T3. In addition to its transcriptional regulation, important post-translational modifications of D2 exist to ensure that its activity is maintained at a safe level (Burmeister et al., 1997). This is reflected by its half-life of approximately 40 minutes. In comparison, the half-life of D1 is 12 hours (Gereben et al., 2000). One post-translational modification to D2 enzyme is its ubiquitination, which results in its degradation. This complicates efforts to locally overexpress this enzyme. The ubiquitination of D2 can be instigated by substrate inhibition from physiological concentrations of T4, rT3 and high concentrations of T3 (Sagar et al., 2007, Obregon et al., 1986). This potent and rapid feedback loop ensures that T3 levels are maintained within an appropriate physiological range and protects the cell from a potentially damaging hyperthyroid state.

Proteins with short half-lives, such as D2, are conjugated to ubiquitin prior to their proteasomal degradation. There are three essential components of this mechanism: E1, which activate the ubiquitin enzyme; E2, which conjugate the target; and E3, the ubiquitin ligases that facilitate assembly of the ubiquitination complex (Varshavsky, 1997). The E2 enzymes that have been identified as regulators of D2 ubiquitination include UBC6 and UBC7, where yeast strains lacking these enzymes exhibit stabilised D2 activity and impaired sensitivity to substrate-induced degradation (Botero et al., 2002). An E3 enzyme, WSB1 (WD repeat and SOCS box-containing protein 1), has been identified as a D2-specific ubiquitin ligase (Callebaut et al., 1997, Dentice et al., 2005). TEB4 is another E3 ligase that has been co-localised with WSB1 in D2-expressing cells, suggesting that D2-specific ubiquitination involves the interaction of a number of proteins.

It has been demonstrated that the WSB1 gene locus of chromosome 11 undergoes a chromosomal interaction with an imprinted Igf2/H19 locus on chromosome 7, which is required for appropriate WSB1 expression (Krueger and Osborne, 2006, Ling et al., 2006, Spilianakis and Flavell, 2006). This Igf2/H19 locus is part of a large network that also regulates Dio3 expression, thus it is possible that the regulation of both D2 and D3 are linked genomically (Da Rocha and Ferguson-Smith, 2004, Hernandez et al., 2002).

The ubiquitination pathway can be reversed in a process known as deubiquitination. The ubiquitination complex merely disrupts D2 homodimerisation without causing terminal
disassembly and is reversible (Sagar et al., 2007). Deubiquitination is mediated by ubiquitin-specific peptidases (USPs), of which USP20 and USP33 have been linked to D2 modifications, prolonging both the half-life and activity of D2 (Curcio-Morelli et al., 2003). Expression of both WSB1 and USP33 has been demonstrated in the hypothalamus and BAT, which highlights the importance of D2 ubiquitination to the role of T3 in homeostatic regulation.

4.1.3.1 Previous Attempts to Overexpress Deiodinase II
The D2 enzyme is subject to tight post-translational regulation, which limits its overexpression in vivo. However, D2 overexpression has been attempted in previous investigations. The most promising attempt to date was achieved by a transgenic method where D2 was overexpressed in the heart under the control of a αMHC receptor (Pachucki et al., 2001). This model presented with a minimal increase in myocardial T3 concentration, despite high myocardial D2 activity, which was increased approximately 100 fold. It was hypothesised that the lack of an increase in T3 concentration was owing to diffusion of T3 from myocardium into the blood stream, although this was not supported by the finding that similar levels of plasma thyroid hormones were detected in the control group. Despite absence of a significant effect on thyroid-responsive genes in the heart, an increased heart rate was detected in animals overexpressing D2. This suggests that the increased level of ectopic D2 activity was affecting heart rate, although no clear transcriptional pathway was determined.

4.2 Aims of Study
Until now, no investigation has been undertaken to examine the effect of increasing the local pool of T3 in hypothalamic networks. Work in this chapter was designed to increase T3 availability in the VMN by overexpressing D2 enzyme following iVMN administration of rAAV-D2.
4.3 Experimental Results

Male Wistar rats were bilaterally injected with either 1μl rAAV-D2 or 1μl rAAV-GFP into the VMN as described in chapter 2.3.2. Their phenotypes were analysed using the molecular biological techniques described in chapter 2.

4.3.1 Confirmation of Vector Expression in vivo

An initial investigation was undertaken, whereby 5 male Wistar rats received bilateral injections of rAAV-D2 to localise the transcript. Successful expression of rAAV in the VMN was verified by radioactive WPRE in situ hybridization. An in situ hybridisation picture is shown in figure 4.1.
Figure 4.1A: In situ hybridisation with Wpre-anti-sense probe. Demonstrates Wpre mRNA expression in rats with bilateral injection of rAAV-D2 in the VMN (white arrows)

Figure 4.1B: In situ hybridisation with Wpre-sense probe, to show non-specific binding in rats with bilateral intra-VMN injection of rAAV-D2.

Figure 4.1C: Schematic representation of coronal sections through the rat hypothalamus depicting the injection site. -2.76mm indicates caudal distance from bregma. Reprinted from “The Rat Brain in Stereotaxic Coordinates” (Paxinos and Watson, 2007)
4.3.2 Effect of iVMN rAAV-D2 Administration on Food Intake and Body Weight

Male Wistar rats received bilateral iVMN injections of either 1µl rAAV-GFP or 1µl AAV-D2. All animals were allowed to recover for 7 days after surgery. After this recovery period, consumption of normal chow and body weight gain were measured regularly.

4.3.2.1 Cumulative Body Weight Gain

Prior to rAAV injection there was no difference in body weight between the rAAV-GFP (260.8 +/- 1.9g) and rAAV-D2 (262.7 +/- 2.5g) groups. At day 64, when the experiment was terminated, there was still no difference in body weight between the rAAV-GFP controls (547.4 +/- 10.9g) and rAAV-D3 treated animals (555.1 +/- 17.7g) (Figure 4.2).

![Figure 4.2: Body weight profile following iVMN administration of either rAAV-GFP (green line) (n=12) or rAAV-D2 (black line) (n=13). Data are expressed as mean +/- SEM for both groups.](image)
4.3.2.2 Cumulative Food Intake
At day 64, there was no difference in the mass of food consumed by the rAAV-GFP group (1859.7 +/- 42.2g) compared to the rAAV-D2 group (1888.9 +/- 65.9g) (figure 4.3).

Figure 4.3: Cumulative food intake following bilateral iVMN injection of either rAAV-GFP (green line) (n=12) or rAAV-D2 (black line) (n=13). Data are expressed as mean +/- SEM for both groups.
4.3.2.3 Refeeding and Body Weight Profiles Following 24 Hour Fast

On day 64, the collection of longitudinal data was terminated. At this point, the level of food intake in rAAV-treated rats was examined following a 24 hour fast. There were no significant differences in food intake over any time period starting from the point of refeeding (figure 4.4A).

When analysing feeding over incremental time periods, it was noted that a significantly higher level of food intake was detected in the rAAV-D2 treated group (3.64 +/- 0.4g) compared to the rAAV-GFP controls (2.0 +/- 0.4g) during the period from 1 to 2 hours after refeeding (P = 0.01). There were no other differences detected at any other time increment. (figure 4.4B). There were no differences in body weight detected at any point over the investigation.
Figure 4.4: The effect of rAAV-D2 (black bars) (n=13) treatment on refeeding following a 24 hour fast compared to rAAV-GFP controls (green bars) (n=12). Refeeding data were measured at 1 hour, 2 hours, 4 hours, 8 hours, 12 hours and 24 hours after replacement of food (A). Refeeding data were analysed during the increments between each time point (B). Data are expressed as mean +/- SEM. * P<0.05
4.3.2.4 Refeeding and Body Weight Profiles Following 12 Hour Fast

One week after analysing the effects of a 24 hour fast on acute food intake, the effect of a 12 hour fast was investigated in rAAV-treated rats was examined following a 12 hour fast. There was a significantly greater level of food intake in the rAAV-D2 treatment group (11.1 +/- 0.6g) compared to the rAAV-GFP controls (8.8 +/- 0.7g) during the first 2 hours of refeeding (p=0.02). There were no other statistical differences when data were analysed in this way (figure 4.5A).

When feeding over the incremental time points was analysed, the mean level of food consumed by the rAAV-D2 group during the 0-1 and 1-2 hours of refeeding was not significantly greater. However, a significantly lower level of food intake was detected in the rAAV-D2 treated group compared to the rAAV-GFP controls during the time point increments 2-4 hours (2.4 +/- 0.6g vs 0.7 +/- 0.5g) and 4-8 hours (4.1 +/- 0.5g vs 2.2 +/- 0.5g) after refeeding (P = 0.03 and 0.02, respectively). These periods of reduced food intake were followed by an increased level of food intake in the rAAV-D2 treated group (4.8 +/- 0.6g) compared to the rAAV-GFP controls (3.0 +/- 0.4g) between 8 and 12 hours post-refeeding (P = 0.03) (figure 4.5B).

There were no differences in body weight detected at any point over the investigation (data not presented).
Figure 4.5: The effect of rAAV-D2 (black bars) (n=13) treatment on refeeding following a 12 hour fast, compared to that of rAAV-GFP (green bars) (n=12). Refeeding data were measured at 1 hour, 2 hours, 4 hours, 8 hours, 12 hours and 24 hours after replacement of food (A). Refeeding data were analysed during the increments between each time point (B). Data are expressed as mean +/- SEM. * P<0.05
4.3.3 Hypothalamic Expression Profile Following rAAV-D2 Treatment

Dissected hypothalami were analysed for specific mRNA levels by qPCR. There was a 3.5 fold upregulation of Dio2 mRNA in the rAAV-D2 treated group compared to rAAV-GFP controls (P < 0.001). A 1.6 fold increase in Dio3 mRNA was also detected in the rAAV-D2 treated group, although this failed to reach statistical significance (P = 0.08). There was no apparent difference in the level of hypothalamic WSB1 mRNA between the experimental groups (figure 4.6).

Figure 4.6: Hypothalamic mRNA profile was analysed by qPCR. mRNA was quantified for Dio2, Dio3 and Wsb1. qPCR assays were performed 78 days after bilateral administration of rAAV-D2 (black bars) (n=13) or rAAV-GFP (green bars) (n=12). Data are expressed as fold change relative to the mean ΔΔCt value for the rAAV-GFP controls +/- SEM. *** P<0.001
4.3.4 MBH Deiodinase Activity Following rAAV-D2 Treatment

A group of male Wistar rats were injected with either rAAV-D2 (n=8) or rAAV-GFP (n=9) and housed for 38 days before being decapitated with their brains collected. MBH punch biopsies were collected and assayed for D2 and D3 activity in collaboration with Dr Anita Boelen (Department of Endocrinology and Metabolism of the Academic Medical Centre at the University of Amsterdam).

The mean level of D2 enzyme activity was significantly higher (P = 0.03) in the rAAV-D2 group (0.12 +/- 0.05 fmol/min/mg) compared to controls (0.01 +/- 0.001 fmol/min/mg) (figure 4.7). However, a large increase in D2 activity was detected in only four of eight rAAV-D2 treated animals, which is reflected by the large standard error value for the rAAV-D2 treated group.

The mean level of D3 enzyme activity was several orders of magnitude higher than that of D2 activity, in both treatment groups. This was calculated as being approximately double the normal level of MBH D3 enzyme activity (9.55 +/- 0.2 fmol/min/mg). However, there was no statistical difference between D3 activity in rAAV-D2 treated animals (18.96 +/- 3.2 fmol/min/mg) compared with rAAV-GFP treated controls (23.33 +/- 3.4 fmol/min/mg).

![Figure 4.7: Hypothalamic D2 activity following rAAV-D2 treatment. Dissected hypothalami were assayed for D2 activity 38 days after bilateral iVMN injection of either rAAV-GFP (n=9) (green bars) or rAAV-D2 (n=8) (black bars). Non-injected MBH samples are included as controls (n=2) (white bars). Data are expressed as fmol/min/mg +/- SEM. * P<0.05](image-url)
4.3.5 Energy Homeostasis in Animals with Increased D2 Activity

Given that an increase in D2 activity was only detected in four of the eight rAAV-D2 treated rats, energy homeostasis parameters were re-analysed to include only those with increased activity. The mean enzyme activity in this group of animals was calculated to be 2.4 +/- 1.2 fmol/min/mg, which was approximately 25 fold higher than that of rAAV-GFP treated animals (0.009 +/- 0.001 fmol/min/mg) (P<0.001). However, this profound increase in D2 activity did not bring any noticeable differences in cumulative food intake or body weight gain upon reanalysis of these data sets (figure 4.8).

![Graph](image)

**Figure 4.8:** The effect of increased D2 activity on food intake and body weight. **Left axis:** cumulative body weight gain and food intake were calculated from the date of either rAAV-GFP (green bars) (n=9) or rAAV-D2 (black bars) (n=4) until the final time point (decapitation at day 38). Data are expressed as kg +/- SEM. **Right axis:** D2 enzyme activity was analysed in MBH punches 38 days after administration of either rAAV-GFP (green bars) or rAAV-D2 (black bars). Data are expressed as fmol/min/mg +/- SEM. *** P<0.001
4.4 Discussion

Previous studies examining the effect of thyroid hormones in the hypothalamus have involved either their chronic administration via a peripheral route (Klieverik et al., 2008, Klieverik et al., 2009, Ishii et al., 2008, Ishii et al., 2000) or acute administration of T3 centrally (Klieverik et al., 2009, Kong et al., 2004). rAAV-mediated overexpression of D2 is exploited here to modulate the local metabolism of T4 to T3, which enables chronic manipulation of the local T3 pool without the need for chronic administration. Work in this investigation looks specifically at the role of T3 within the VMN with respect to its regulation of energy homeostasis.

An initial experiment was conducted to analyse whether the rAAV cassette was successfully expressed in a region limited to the VMN, given that migration of the AAV into surrounding areas would complicate the separation of the VMN-specific effects from surrounding nuclei and hinder the reproducibility of the investigations. WPRE was the probe-target for in-situ hybridisation to coronal slices through the region encompassing the MBH. The results demonstrated a strong signal localised to the area containing the VMN at approximately 2.7mm caudal to bregma. The relative brain region was determined by its similarity to the surrounding architecture observed in the coronal slices published in “The Rat Brain in Stereotaxic Coordinates” (Paxinos and Watson, 2007).

One limitation when analysing the data is the lack of information regarding the specific site of virus injection. In this work, initial experiments were undertaken whereby euthanised animals were used for stereotaxic injection of Indian ink using the specified coordinates. Rat brains were then dissected and coronal sections were examined to determine the site of injection with respect to the rat brain atlas. However, the injection accuracy was not determined in the animals whose phenotypes were extensively analysed. This could be overcome by including a fluorescent marker, such as GFP, in the transgene construct, which could then be used to assess injection accuracy before processing tissues. Specifically, this could be carried out using laser dissection, where coronal slices of hypothalami are visualised under a blue 488nm argon laser to obtain green fluorescence, from which the transduced tissue can be identified anatomically and dissected for further analysis.

In a separate experiment, rats once again received either rAAV-D2. It has previously been reported that acute administration of T3 to the VMN of male Wistar rats induces a hyperphagic response (Kong et al., 2004). Again, rAAV-D2 administration to the VMN of
male Wistar rats gave a weak phenotype without notable differences in cumulative body weight gain or cumulative food intake. D2 enzyme is subject to tight post-translational regulation in order to prevent the development of a potentially damaging hyperthyroid environment (Gereben et al., 2000, Zavacki et al., 2009). However, both hypothalamic D2 expression and activity are increased in response to a fast (Diano et al., 1998). In light of this, it was hypothesised that fasting could reverse any hindrance in the efficacy of rAAV-D2-generated D2 enzyme.

The level of Dio2 mRNA detected by qPCR was significantly greater in the rAAV-D2 experimental group, in line with results from the cohort that demonstrated an increase in D2 activity. WSB1 mRNA was analysed as a marker of whether the D2 ubiquitination pathway had been activated by increased D2 expression, however, there was no significant change detected. A small change was witnessed in the level of Dio3 mRNA in the hypothalamus, whereby it was higher in the rAAV-D2 group, yet this was not statistically significant. This suggests that post-translational modification of D2 had not been greatly affected, although this would need clarification by quantifying the expression of other markers of the D2 ubiquitination pathway, such as TEB4, UBC6 and UBC7.

In an attempt to potentiate the increase in D2 expression, rats were fasted before having food intake measured at various time-points over a 24 hour period after reintroduction of food. The effect of a 24 hour fast was minimal with the only significant effect being an increase in food intake during the second hour after refeeding. A slight increase in food intake was also detected in rAAV-D2 treated animals during the first two hours of refeeding after a 12 hour fast. This was followed by a brief period of reduced food intake, which was presumably a compensatory response to the hyperphagia witnessed over the initial 2 hours of refeeding. This goes some way to supporting the findings made by Kong et al., whereby an increased local T3 pool in the VMN has an effect on food intake, albeit a modest one. This could be due to a lower level of T3 being produced by rAAV-D2 than the 1 to 5 nmol/kg dose range administered by Kong et al. In order to evaluate this, T3 could be measured by radioimmunassay in rAAV-D2 treated hypothalamic samples following acid extraction of hormones. Analysis could then be made to determine whether the rAAV-D2 model provides a comparatively low level of T3 production, in line with the phenotype.

The most significant finding in this chapter is arguably the promising ability for rAAV-D2 to successfully increase D2 activity in a localised region by viral gene transfer. D2 plays
important roles in many physiological systems; therefore, the rAAV-D2 virus could be
exploited as a useful tool for examining the effects of D2 in other species, additional CNS
regions, or even in additional tissues. The D2 enzyme is subject to tight post-translational
regulation (reviewed in section 4.1.2), which limits its overexpression in vivo. Until now, this
had only been achieved by transgenic methods in the heart, where D2 was overexpressed by
almost 1000 fold (Pachucki et al., 2001). However, this was without significant physiological
effects, as thyroid-responsive genes were only modestly upregulated and the overall
presentation was reminiscent of a relatively mildly thyrotoxic patient. It was hypothesised in
this publication that the weak effect was owing to some diffusion of T3 from the heart into
circulation, although was not supported by plasma assays. With this considered, it is possible
that the lack of physiological effects in the rAAV-D2 model, despite an increase in D2
activity, could be due to similar reasons to those experienced by Pachucki et al.

Deiodinase enzyme activity was assayed, in collaboration with the University of Amsterdam,
in MBH punch biopsies sampled from rats treated with either rAAV-D2 or rAAV-GFP. Despite a ten-fold increase in mean D2 enzyme activity by rAAV-D2 treatment compared to
rAAV-GFP, the effect was only statistically significant to the 97th percentile. This was due to
a disparity between the rAAV-D2 samples, in which only half of the subjects demonstrated a
large increase in D2 activity, with the rest showing a level similar to controls. Given that D2
enzyme is extremely labile, prolonged tissue dissection and subsequent punch biopsy may
have allowed the D2 activity of some samples to have been diminished. In order to examine
whether an increase in D2 activity exerts any effect on energy homeostasis parameters, the
cumulative body weight and food intake profiles of rAAV-D2 treated animals were
reanalysed to include only those exhibiting an increase in D2 enzyme activity. However,
there remained a lack of effect on either parameter, suggesting that the long-term effects of
elevated T3 in the VMN are modest in comparison to the effect of acute administration.

It is possible, however, that the local T3 pool was only modestly changed, despite a
significantly increased level of D2 enzyme activity. This was also suggested by authors who
achieved increased D2 activity in the heart (Pachucki et al., 2001). The influence of the
manual insertion of SECIS into the rAAV-D2 cassette was not investigated either in vivo or
in vitro. Without functional SECIS, D2 activity would be reduced; therefore it may have been
possible to drive higher D2 activity than witnessed. This could be investigated initially in
vitro by transfecting pTR-CGW-D2 without SECIS and comparing its effects on cultured
cells to pTR-CGW-D2 constructs containing the SECIS at various insertion points. Evidence
form D1 polymorphisms suggests that mutations in the region of the SECIS can have both positive and negative effects on deiodinase activity (Dumitrescu et al., 2005), which suggests that its location could be rate limiting. Even assuming that the manual insertion of SECIS was effective, the availability of selenium in vivo could have been an influential factor for both investigations, as the activity of all selenoenzymes is selenium-dependent. This could be overcome by supplementing in vivo models with selenium in the diet, which was achieved in vitro with the addition of sodium selenite.

An alternative theory for the lack of physiological effect in D2-overexpressing animals lies in the finding that D3 enzyme activity was increased in the MBH punches of both treatment groups. This increase in D3 activity could have suppressed any increase in local T3 availability and hindered the physiological response. These non-injected samples demonstrated approximately half of the level of D3 activity that was recorded in the rAAV-D2 and rAAV-GFP treated animals. These data were compared to results from a separate batch of non-injected MBH punches, which were analysed on a different day. A number of conclusions can be drawn from this.

Firstly, it is possible that D3 activity has been induced as a direct result of D2 overexpression in the MBH. D3 is upregulated by holo-TR, therefore an increase in the local availability of T3 would be expected to produce this increase in D3 activity (Esfandiari et al., 1992). qPCR analysis of hypothalamic samples from a previous cohort did not detect a statistically significant change in the level of Dio3 transcript. However, these measurements were taken from whole hypothalami, which raises the possibility that Dio3 mRNA from other hypothalamic nuclei was reducing the impact of any detectable changes in the expression of this gene. In order to investigate this fully, Dio3 and WSB1 mRNA levels would need to be measured by qPCR in MBH punches of rAAV-D2 injected animals and compared to controls.

However, this would not explain why D3 activity was raised in the rAAV-GFP treated control group. This suggests that rAAV administration by stereotaxic surgery somehow increases the local D3 enzyme activity. This could be explained by reports that D3 activity can be stimulated by inflammation and tissue injury (Li et al., 2001, Boelen et al., 2005). Therefore, D3 expression could have been induced by the inflammatory response to stereotaxic administration of a virus to the CNS. This activation of D3 by inflammatory signals has been well characterised in leukocytes, granulocytes and muscle tissue (Boelen et al., 2005, Boelen et al., 2008), however this pathway has not been extensively characterised.
in brain parenchymal cells. In fact, reports demonstrate that bacterial LPS can decrease D3 activity and increase D2 activity in the MBH. Furthermore, the CNS lacks classical lymphatics and elicits only a transient inflammatory response to infectious particles (Lowenstein, 2002). In addition to this, rAAV has been shown to lack profound immunogenicity (Nayak and Herzog, 2009). Therefore, inflammation due to tissue damage remains becomes the likely candidate for the induction of D3 activity, given that such a response has been reported in neurones (Li et al., 2001).

The potential induction of inflammation in the MBH requires investigation. The activation of MAPK-associated factors could be analysed by ELISA as markers of tissue inflammation. In addition to new measurements of D3 enzyme activity, MAPK levels could be compared among: rAAV-GFP injected, rAAV-D3 injected, sham-injected and non-injected MBH samples. This would help ascertain whether rAAV or stereotaxic injection affects D3 expression and the MAPK signalling pathway.
Chapter 5

The Effect of rAAV-D3 Administration to the VMN of Rats Maintained on a Normal Chow Diet
5.1 Introduction
D3 plays an essential role in the regulation of T3 signalling within the CNS. Following its production by D2 in tanycytes, T3 normally enters neuronal cells in a paracrine fashion to interact with the TR (Freitas et al., 2010). D3 expression in the CNS is primarily neuronal and localises at the plasma membrane (Baqui et al., 2003, Tu et al., 1999). Therefore, intraneuronal T3 is readily inactivated by D3, which gives rise to the hypothesis that the intracellular concentration of T3 lowers when D3 is upregulated.

5.1.1 Iodothyronine Deiodinase III in the Hypothalamus
In Siberian hamsters, D3 has shown to be temporally regulated by photoperiod in the MBH. It is thought this is a mechanism for energy conservation in hibernation (Barrett et al., 2007). Deiodinase expression is essential for the responses to disease and infection whereby hypothalamic D3 is up-regulated in the hyperthyroid state and in response to the administration of bacterial lipopolysaccharide (Boelen et al., 2004b, Tu et al., 1999).

Acute administration of T3 to the VMN of male Wistar rats induced a rapid hyperphagic response without affecting locomotion (Kong et al., 2004), however, it has been suggested that a reduction in T3 signalling in hypothalamic networks could influence energy expenditure (Pelletier et al., 2008, Sjögren et al., 2007, López et al., 2010). In this respect, the affinity of TR for T3 has been targeted for investigation. Mice expressing a TRα variant with reduced affinity for T3 were reported as hypermetabolic with increased BAT sympathetic tone (Sjögren et al., 2007), an effect not explained by changes in systemic thyroid hormone status. These findings suggested that T3 could influence sympathetic innervation of BAT within the hypothalamus.

5.1.2 Sympathetic Nervous Pathways Descend from the Hypothalamus
Neurones of the SNS exist within both the CNS and the peripheral nervous system (PNS), which communicate via the sympathetic ganglia. Noradrenaline (NA) is released from post-ganglionic nerve terminals at the site of tissue innervation. The effect of NA is mediated by adrenergic receptors, of which there are three types: β, α1, and α2. β-adrenoreceptors are classified into three subtypes; β1, β2, and β3. NA binds all three β-adrenergic receptor subtypes on adipocytes. β3-adrenoreceptors are the most significant subtype in mature rodent energy homeostasis. Stimulation with a selective β3 agonist, stimulates BAT thermogenesis (Arch et al., 1984).
Although, the majority of thermogenic regulation is thought to be mediated via $\beta_3$ adrenoreceptors, deletion of these receptors in rodents does not abolish thermogenic capacity, with $\beta_1$ receptors thought to be compensatory (Susulic et al., 1995).

NA interacts with $\beta_3$-adrenergic receptors in mature brown adipocytes to activate adenylyl cyclase with subsequent intracellular elevation of cAMP production (Zhao et al., 1997). cAMP stimulates protein kinase A activity, which conveys the signal via phosphorylation of target proteins. Phosphorylation of nuclear targets, such as the transcription factor CREB, stimulates UCP-1 expression through interaction with the cAMP response element (CRE) in its promotor region (Thonberg et al., 2002).

An area within the preoptic chiasma/anterior hypothalamic nuclei (POAH) in front of the third ventricle is important in the control of body temperature (Boulant, 2000). Its destruction renders animals unable to thermoregulate (Satinoff et al., 1976). This region receives inputs from other thermosensitive areas and integrates this information to convey a message to downstream brain centres. The finding that lesioning of POAH efferent connections elicits BAT activation suggests that this pathway is an inhibitory one (Chen et al., 1998). However, a putative model consisting of a long inhibitory pathway from POAH to sympathetic neurones has often proved an inadequate explanation (Rothwell et al., 1983, Rathner and Morrison, 2006). Several areas of the hypothalamus; including the VMN, PVN, and DMN have been implicated in the central regulation of thermogenesis, which implicates these nuclei act as intermediary centres of thermogenic regulation.

Electrical and chemical stimulation of the VMN increases BAT activity (Halvorson et al., 1990). A critical role for the VMN in POAH innervation of BAT was suggested following the discovery that stimulation of BAT thermogenesis is abolished by VMN destruction (Niiijima et al., 1984). However, some doubt remains over the specific involvement of the VMN given that retrograde labelling of BAT efferent SNS neurones does not markedly identify this nucleus as a principal origin of the signal (Bamshad et al., 1999). Furthermore, the specificity of VMN lesions to VMN function has been questioned with respect to the potential damage to surrounding neurones of the ventral noradrenergic bundle (Gold, 1973), which could in fact be the true source of SNS transmission.
5.1.3 Triiodothyronine and the Sympathetic Nervous System: Brown Adipocyte Thermogenesis

T3 is essential to the stimulation of BAT thermogenesis through its stimulation of mitochondrial UCP1 expression (figure 5.1). The synergistic relationship between the SNS and T3 in BAT is demonstrated in hypothyroid rats which completely fail to elevate thermogenesis in response to NA infusion (Ribeiro et al., 2000). The NA-induced signal cascade is also directly initiated by T3, which stimulates cAMP generation due to modifications at the adrenergic receptor, its Gi protein and adenylyl cyclase (Carvalho et al., 1996, Rubio et al., 1995b, Rubio et al., 1995a). In addition, a TRE promoter has been identified in the UCP1 gene, suggesting both direct and indirect effects of T3 on its transcription and thermogenesis (Ribeiro et al., 2001).

The effect of T3 on thermogenic tissues is modulated via regulation of the deiodinases where D2 is up-regulated in BAT within a few hours of cold-exposure (Bianco and Silva, 1987). BAT adaptive thermogenesis is also stimulated by food intake and the subsequent increased production of bile acids. It has been demonstrated that binding of bile acids to their receptor, TGR5, triggers an increase in BAT intracellular cAMP which drives Dio2 expression (Watanabe et al., 2006). Furthermore, mice fed a high-fat diet supplemented with bile acids were resistant to diet-induced obesity, an effect lost in Dio2 null mice (Watanabe et al., 2006).

Sympathetic innervation of BAT descending from the hypothalamus has been directly linked with T3 signalling in the VMN. Administration of T3 to the third ventricle increased c-Fos immunoreactivity in the raphe pallidus, the inferior olive nuclei and the dorsal motor nucleus of the vagus, all of which reportedly receive neuronal projections from the VMN (López et al., 2010). This effect was associated with a dose-dependent increase in BAT sympathetic activity. Administration of an adenovirus expressing a dominant-negative TR to the hypothalamus of rats resulted in reduced expression of UCP1, UCP3 and D2 in BAT along with several enzymes of fatty acid metabolism (López et al., 2010). As already mentioned, mice expressing a TRα variant with reduced affinity for T3 were reported as being hypermetabolic with increased BAT sympathetic tone (Sjögren et al., 2007). The disparity between the findings of these two studies suggests that the exact role of T3 signalling in hypothalamic regulation of BAT activity remains to be elucidated.
In addition to BAT thermogenesis, T3 also interacts with the SNS to regulate the function of other metabolically active tissues. Thyrotoxicosis has been linked with increased hepatic gluconeogenesis and hepatic insulin resistance (Klieverik et al., 2008). Sympathetic and parasympathetic denervation of the liver attenuated the increased glucose production, suggesting that these nervous inputs are required for the T3-mediated pathophysiology. Investigations have suggested that the interactions between T3 signalling and the SNS may occur at the level of the hypothalamus. In euthyroid rats, T3 microdialysis in the PVN stimulated endogenous glucose production by 11%, which highlights hypothalamic thyroid hormone as a potential activator of sympathetic outflow to the liver (Klieverik et al., 2009).
Figure 5.1: Thermogenesis is stimulated by T3 in the Brown Adipocyte. T3 and the adrenergic nervous system act synergistically to stimulate UCP1 expression. UCP1 is inserted into the mitochondrial inner membrane to dissociate the electron transport chain from ATP synthesis, thereby promoting the dissipation of energy as heat (Celi, 2009).
5.2 Aims of Study

Until now, no investigation has been undertaken to examine the effect of reducing the local activity of T3 in hypothalamic networks. Work in this chapter was designed to chronically inactivate T3 in the VMN through overexpression of rAAV-D3 in order to investigate the physiological role of T3 in this nucleus.
5.3 Experimental Results
Male Wistar rats were bilaterally injected with either 1μl rAAV-D3 (rAAV-D3 treatment) or 1μl rAAV-GFP (controls) into the hypothalamic VMN (iVMN), as described in chapter 2.3.2. Their phenotypes were analysed using the molecular biological techniques described in chapter 2.

5.3.1 Confirmation of Vector Expression in vivo
An initial investigation was undertaken, whereby five male Wistar rats received unilateral injections of rAAV-D3 to localise the transcript. Successful expression of rAAV in the VMN was verified by radioactive WPRE in situ hybridization. The in situ hybridisation profile is shown in figure 5.2, where 5.2A represents a coronal section probed with an antisense WPRE sequence and 5.2B shows the control section probed with the sense WPRE sequence.
**Figure 5.2A:** In situ hybridisation with Wpre-anti-sense probe. Demonstrates Wpre mRNA expression in rats with unilateral iVMN injection of rAAV-D3 (white arrow).

**Figure 5.2B:** In situ hybridisation with WPRE-sense probe, to show non-specific binding in rats with unilateral iVMN injection of rAAV-D3.

**Figure 5.2C:** Schematic representation of coronal sections through the rat hypothalamus depicting the injection site. -2.76mm indicates caudal distance from bregma. Reprinted from “The Rat Brain in Stereotaxic Coordinates” (126).
5.3.2 Effect of iVMN rAAV-D3 Treatment on Food Intake and Body Weight

After bilateral iVMN injection of either 1µl rAAV-GFP (n=13) or rAAV-D3 (n=11), all animals were left to recover for 7 days. After this recovery period, consumption of normal chow and body weight gain were measured regularly.

5.3.2.1 Cumulative Body Weight Gain

Prior to surgery, there was no difference in body weight between rAAV-GFP controls (252.2 +/- 3.2g) and rAAV-D3 treated (255.7 +/- 3.0g) groups. At day 78, when the experiment was terminated, the mean body weight of the rAAV-D3 group (489.2 +/- 7.6g) was not significantly lower than the controls (511.6 +/- 13.2g) (P=0.140) (Figure 5.3A).

The difference in body weight between the two groups appears to take effect approximately 3 weeks after rAAV injection, the estimated time required for ssDNA of rAAV vectors to be converted to its dsDNA form for maximal expression.

The body weight gained by the animals from this 3 week time-point until the terminal point of the experiment was calculated. The rAAV-D3 group (129.7 +/- 4.4) gained significantly less weight than the rAAV-GFP controls (149.8 +/- 7) over this 57 day period (P=0.008) (Figure 5.3B).
Figure 5.3: Body weight following bilateral intra-VMN injection of AAV-GFP (green line) N=11, or rAAV-D3 (black line) n=13 (A) and from 21 days post-surgery (B). There was a significant reduction in body weight from day 21 in the rAAV-D3 group compared to the GFP group. Data are expressed as mean +/- SEM for both groups. * P<0.05
5.3.2.2 *Cumulative Food Intake*

After 78 days there was no difference when comparing the mass of food consumed by the rAAV-GFP controls (2146.2 +/- 50.0g) to the rAAV-D3 group (2112.8 +/- 47.6g) (figure 5.4A). These data were also matched over the 57 day period of maximal rAAV expression (figure 5.4B).
Figure 5.4: Cumulative food intake on normal chow diet following bilateral intra-VMN injection of 1µl rAAV-GFP (green line) n=11, or 1µl rAAV-D3 (black line) n=13. Data are cumulative starting from the time of rAAV injection (A) or from the 21st day after injection (B). Data are expressed as mean +/- SEM for both groups.
5.3.3 Hypothalamic Expression following rAAV-D3 Treatment
The expression of Dio3 mRNA was increased 10 fold in the rAAV-D3 group (10.2 +/- 2.1 AU) relative to the controls (1.0 +/- 0.1 AU), a difference that was statistically significant (P<0.001) by 1-way ANOVA. No significant changes were witnessed in the levels of Dio2, Mct8, Oatp1c1, Trh, Npy, Agrp, Pomc, Cart, Bdnf, Acca, Fasn, Ampk or Ucp2 mRNA (Figure 5.5).

Figure 5.5: Hypothalamic expression profile was analysed by qPCR. mRNA was quantified for Dio3, Dio2, Mct8, Oatp1c1, Trh, Npy, Agrp, Pomc, Cart, Bdnf, Acca, Fasn, Ampk, Ucp2. qPCR was performed 78 days after bilateral iVMN injection of either rAAV-GFP (green bars) n=11 or rAAV-D3 (black bars) n=13. Data are expressed as fold change relative to the mean ΔΔCt value for the rAAV-GFP controls +/- SEM. ***= P<0.001
5.3.4 Hypothalamic Deiodinase Activity following rAAV-D3 Treatment

5.3.4.1 Deiodinase Activity in Whole Hypothalamic Blocks

A batch of male Wistar rats were injected with either rAAV-D3 (n=5) or rAAV-GFP (n=4) and housed for 38 days before being decapitated when their hypothalami were dissected. Hypothalamic samples were assayed for D2 and D3 activity in collaboration with Dr Anita Boelen (Department of Endocrinology and Metabolism of the Academic Medical Centre at the University of Amsterdam).

Deiodinase activity data were expressed by correcting the level of deiodination to the protein content of the tissue sample. There was no difference in the activity of D2 in these samples. Although D3 activity was marginally higher in the rAAV-D3 treated group (12.1 +/- 1.7 fmol/min/mg) than controls (9.75 +/- 0.6 fmol/min/mg), this did not achieve statistical significance (P=0.2) (Figure 5.6).

![Figure 5.6: Dissected hypothalami were assayed for D2 and D3 activity 38 days after bilateral iVMN injection of either rAAV-GFP (green bars) (n=4) or rAAV-D3 (black bars) (n=5). Data are expressed as fmol/min/mg +/- SEM.](image-url)
5.3.4.2 Deiodinase Activity in MBH Punch Biopsies

A group of male Wistar rats were injected with either rAAV-D2 (n=8) or rAAV-GFP (n=9) and housed for 38 days before being decapitated with their brains collected. MBH punch biopsies were collected and assayed for D2 and D3 activity in collaboration with Dr Anita Boelen (Department of Endocrinology and Metabolism of the Academic Medical Centre at the University of Amsterdam).

Consistent with the results gathered from whole hypothalamic blocks, the level of D2 activity in MBH punch biopsies from rAAV-GFP treated animals (0.0092 +/- 0.001 fmol/min/mg) was not different to the level of D2 activity in rAAV-D3 treated animals (0.0076 +/- 0.002 fmol/min/mg).

However, the level of D3 activity was different in this study. The levels of D3 activity in the MBH of rAAV-GFP treated rats (23.3 +/- 3.4 fmol/min/mg) and rAAV-D3 treated rats (19.0 +/- 3.2 fmol/min/mg) were approximately double the value of D3 activity in non-injected controls (9.6 +/- 2.1 fmol/min/mg) (figure 5.7).

![Deiodinase Activity Graph](image)

**Figure 5.7**: MBH punch biopsies were assayed for D2 and D3 activity 38 days after bilateral iVMN injection of either rAAV-GFP (green bars) (n=9) or rAAV-D3 (black bars) (n=9). Non-injected MBH samples are included as a control reference value (n=2) (white bars). Data are expressed as fmol/min/mg +/- SEM.
5.3.5 Body Composition Analysis

Body composition analysis indicated a 1.2% reduction in rAAV-D3 group carcass fat mass (17.0 +/- 0.8%) compared to the rAAV-GFP group (18.21 +/- 1.0%) which was not significant (Figure 5.8) (P=0.37).

Figure 5.8: Whole body composition was analysed following carcass dissolution. Fat mass was analysed 78 days after bilateral intra-VMN injection of rAAV-GFP n=11 or rAAV-D3 n=10. Body composition data are represented by percent fat mass (black shading) and percent lean mass (grey shading). Data are expressed as mean +/- SEM.
5.3.6 Effect on Plasma HPT Axis Parameters
Radioimmunassay for plasma TSH was without a statistically significant trend between circulating TSH levels in the rAAV-D3 group (1.64 +/- 0.14mU/L) compared to controls (1.88 +/- 0.11mU/L) (Figure 5.9).

Figure 5.9: Plasma levels of TSH were measured by RIA 78 days after bilateral intra-VMN injection of rAAV-GFP (green bar) n=11 or rAAV-D3 (black bar) n=13. TSH values are shown as mU/L +/- SEM.
No change was seen in plasma fT4 levels between rAAV-D3 (20.42 +/- 1.1 pmol/L) and rAAV-GFP (20.82 +/- 1.0 pmol/L). There was no change in plasma fT3 levels between rAAV-D3 (1.24 +/- 0.1 pmol/L) and rAAV-GFP (1.26 +/- 0.1 pmol/L) (Figure 5.10).

Figure 5.10: Plasma levels of fT4 and fT3 were measured by RIA 78 days after injection of rAAV-GFP (green bars) n=11 or rAAV-D3 (black bars) n=13. fT4 and fT3 values are shown as pmol/L +/- SEM.
5.3.7 Effect on Plasma Leptin and Insulin

Plasma insulin levels were measured by RIA. These data do not show a statistically significant trend between basal insulin levels in the rAAV-D3 group (22.07 +/- 2.13pM) compared to the rAAV-GFP group (24.17 +/- 2.28pM) (Figure 5.11).

![Plasma Insulin Levels](image)

*Figure 5.11: Plasma insulin levels were measured by RIA 78 days after bilateral intra-VMN injection of rAAV-GFP (green bars) n=10 or rAAV-D3 (black bars) n=12. Values are shown as pmol/L +/- SEM.*
Plasma leptin levels were also measured by RIA. Despite the differences in body weight, the mean leptin level of the rAAV-D3 group (7.3 +/- 0.6ng/ml) was not significantly different to the rAAV-GFP group (8.64 +/- 0.9ng/ml) (Figure 5.12).

Figure 5.12: Plasma leptin levels were measured by RIA 78 days after bilateral intra-VMN injection of rAAV-GFP (green bars) n=10 or rAAV-D3 (black bars) n=12. Values are shown as ng/ml +/- SEM
5.3.8 Effect on Reproductive Axis

Plasma LH was measured by in-house RIA. There was no difference detected in the level of this hormone (5.13).

![Graph showing plasma LH levels](image)

5.13: Plasma LH levels were measured 78 days after injection of either rAAV-GFP (green bars) n=11 or rAAV-D3 (black bars) n=13. Data are expressed as mean +/- SEM for both groups.
5.3.9 Effect on Interscapular BAT

BAT tissue mass (mg) from the interscapular pad was analysed and corrected to total rat body mass (g). The mass of this BAT pad in the rAAV-D3 group (1.03 +/- 0.07 mg/g) was greater than that of the rAAV-GFP group (0.08 +/- 0.005 mg/g), which was a statistically significant difference (P=0.036) (Figure 5.14).

![Figure 5.14: Interscapular BAT pad was dissected and weighed 78 days after injection of either rAAV-GFP (green bar) N=11, or 1µl rAAV-D3 (black bar) n=13. Data are expressed as mean +/- SEM for both groups. * P<0.05](image-url)
BAT UCP-1 mRNA was up-regulated by 56% in the rAAV-D3 group (5.2 +/- 0.97) when compared to the rAAV-GFP group (2.94 +/- 0.63) (P=0.031) (Figure 5.15). However, BAT Dio2 mRNA was unchanged by rAAV-D3 treatment.

Figure 5.15: BAT Ucp1 and Dio2 mRNA levels. Ucp1 and Dio2 mRNA were quantified in 100mg dissected interscapular BAT, 72 days after injection of either rAAV-GFP (green bars) n=10 or rAAV-D3 (black bars) n=11. Data are expressed as mean +/- SEM. * P<0.05
5.4 Discussion

In this chapter, rAAV-D3 has been administered to the VMN of male Wistar rats with the intention of inducing a state of ‘local hypothyroidism’ owing to constitutive inactivation of T3. rAAV-D3 administration suppressed body weight gain without affecting systemic thyroid tone or food intake. Tissue analysis indicated a state of BAT stimulation, marked by upregulation of Ucp1 mRNA.

Differences in body mass and cumulative food intake were assessed using GEE for repeated measures. The GEE is used to analyse longitudinal data with multiple automata in order to highlight correlation amongst random fluctuations. The reduced level of body weight gained by the rAAV-D3 treated animals appeared to take effect approximately 2 to 3 weeks after vector administration. A limitation of rAAV is latency in its expression during the initial few weeks post-injection. Maximal expression may not be witnessed during this period as the conversion of ssDNA into dsDNA is rate-limiting (Ferrari et al., 1996). This conversion of rAAV dsDNA is a prerequisite for transcription and could delay the onset of expression in brain parenchyma. Additional contributors to this latency may include delays in transduction of the genetic material to the nucleus and intracellular trafficking of the necessary cofactors. Therefore, the initial absence of effect from rAAV-D3 treatment could have been due to a period of inefficient rAAV expression (Meyers et al., 1997).

When body weight data were analysed from the 21st day post-injection, the suppressed body weight gain over the remaining 57 days was statistically significant. This suggests that AAV latency could have been responsible for the delay in phenotype presentation. During the 57 days of maximal rAAV expression, the rAAV-D3 treated group gained 23.7g less weight than the controls.

Body composition analysis was carried out to assess which constituents were accounting for this difference in body mass. Glycerol assay results indicated that rAAV-D3 treatment resulted in a 1.2% lower mean body fat percentage than rAAV-GFP control treatment. Plasma levels of leptin and insulin circulate in correlation with the level of adiposity. Both hormones demonstrated a mildly lower value in the rAAV-D3 treated group, which was consistent with the mildly reduced level body fat in this treatment group, relative to controls.

The effect on body weight was independent of food intake, given that it was consistently matched between the two groups throughout the experiment. Previous investigations of acute
T3 application to the VMN have reported an effect on food intake without alterations in locomotion (Kong et al., 2004). These differences in the mode of energy homeostasis modulation by T3 could have been due to the mode of administration. While this thesis utilises gene transfer and transient modulation of physiological T3 levels, previous investigations have involved the acute administration of supraphysiological T3 doses. Given that the increase in food intake reported by Kong et al. occurred over a 1 hour period, it is possible that the responsible mechanism was an extra-genomic effect of T3. An alternative, genomic mechanism would be that increased levels of apoTR (from D3 overexpression) or increased holoTR (from acute T3 administration) may have induced different downstream effects in VMN neurones due to their differential recruitment of transcriptional co-activators or co-repressors (Brent et al., 1989, Brent, 1994).

The expression of the deiodinases has been strongly linked to photoperiodic regulation in small mammals through interactions with melatonin signalling and effects on the reproductive axis (Barrett et al., 2007, Revel et al., 2006, Yasuo et al., 2007). Plasma luteinising hormone was measured as markers of reproductive function. However, no effect was detected in this marker, suggesting that reproductive function was unaffected.

qPCR analysis of the dissected hypothalami indicated that Dio3 mRNA was 10 fold higher in the hypothalamus of rAAV-D3 treated animals than controls. This would be expected given that the Dio3 transgene is under the control of an exogenous constitutive CMV promoter and enhanced by the downstream WPRE. There were no significant changes detected in any of the other mRNA sequences quantified, including that of Trh, which suggests that the HPT axis was unaffected. However, the increases in Mct8 and Acca mRNA were approaching statistical significance by 1-way ANOVA. MCT8 is the dominant transporter for T3 across the plasma membrane, thus its upregulation could be suggestive of a reduced intraneuronal T3 concentration. ACCA is the predominant ACC isoform targeted by T3 in the hypothalamus and its upregulation could indicate that neuronal fatty acid metabolism has been affected in response to increased D3 expression. The expression of PACAP, LEPR and MC4R was not investigated, despite their interactions with T3, therefore it would be interesting to look at these peptides in future investigations.

The activities of D2 and D3 in the hypothalamus were analysed by a deiodinase activity assay in collaboration with the University of Amsterdam. In these samples, both D2 and D3 enzyme activity were measured. D2 enzyme activity was not affected by rAAV-D3 treatment.
The activity of D3 in whole hypothalamic blocks from rAAV-D3 treated animals was not higher than the rAAV-GFP controls. It was perceived that any clear increase in D3 enzyme activity was attenuated by the detection of D3 activity in additional hypothalamic nuclei, such as the PVN. Therefore, in order to answer this hypothesis, the experiment was repeated by measuring deiodinase activity in MBH punch biopsies which would limit the detection of D3 activity in non-VMN sites.

D2 measurements in the MBH punch biopsies were ten-fold lower than those detected in whole hypothalamic blocks. This could have been due to the increased period of time that the tissue samples were exposed to ambient room temperature, during biopsy, which would have allowed D2 ubiquitination and activation to proceed. Indeed, the levels of D2 enzyme activity in whole hypothalami and MBH punches were profoundly lower than those of D3, which was consistent with data gathered in chapter 4. Given that D3 is normally expressed at a basal low level in the adult CNS, its higher level of activity than D2 in these samples suggests that D2 had been degraded to some degree.

An interesting observation was made in chapter 4 with regards to the level of D3 enzyme activity being similar in rAAV-D2 injected and rAAV-GFP injected MBH samples. This observation was again made when comparing rAAV-D3 with rAAV-GFP injected MBH samples. This reaffirms the assumption that rAAV administration to the hypothalamic parenchyma could induce D3 activity via stimulation of an inflammatory pathway. However, it is worth noting that these MBH measurements were made during the same assay batch as those presented in chapter 4. In both graphs, the non-injected control values are included, although these were processed on a separate day, potentially with different buffers and reagents.

When comparing the D3 activity results obtained from whole hypothalami to those obtained from MBH punches, it can be seen that the D3 activity in whole hypothalami is at a similar level to that of the non-injected MBH controls. Perhaps this could be explained by the fact that MBH punches demonstrate a very restricted locus of inflammation, in the site of infection and tissue damage, which is undetectable in the case of whole hypothalami. Alternatively, it could be argued that the D3 assay batch reading MBH punches was reading false positive results due to buffer instability or contamination, or even contamination of the biopsies. In any case, this experiment would need repeating with new samples alongside the
experiment outlined in chapter 4.4 to examine the effects of rAAV and stereotaxic surgery on MBH D3 activity.

A reduction of body mass despite unaltered energy consumption indicated an effect on energy expenditure. A reduced level of T3 in the hypothalamus is normally associated with stimulation of the HPT axis and BMR (Fekete and Lechan, 2007). Stimulation of the HPT axis could have accounted for the lean phenotype of rAAV-D3 treated animals had plasma fT3 and fT4 levels been affected. Plasma fT4, fT3 and TSH were not affected. These data suggest that the phenotype of rAAV-D3 treated rats was likely to be independent of HPT activity.

A prominent site of energy expenditure in rodents is BAT, with the interscapular region being the largest of the BAT pads. The mass of interscapular BAT dissected from the rAAV-D3 treated group was significantly greater than the control group. It is unknown whether this was due to hyperplasia or hypertrophy as histology was not examined in these samples. The level of UCP1 mRNA was significantly greater in the rAAV-D3 treated group by northern blot. UCP1 is often used as a marker of elevated thermogenesis and energy expenditure in rodents, thus it is possible that rAAV-D3 treatment to the VMN stimulates energy expenditure. This is supported by the cumulative body weight data which highlight that rAAV-D3 treatment causes a reduction in body weight gain without any apparent effect on food intake. The process of BAT UCP1 expression being stimulated by sympathetic activity is well established (Rubio et al., 1995a, Rubio et al., 1995b, Meyers et al., 1997, Zhao et al., 1997, Thonberg et al., 2002). The effect of NA in the brown adipocyte is markedly potentiated by the presence of T3, which is brought about by upregulation of BAT D2. No changes in the level of BAT Dio2 mRNA were picked up by northern blot. However, this enzyme is expressed at much lower levels than UCP1 in BAT, thus any changes may require a more sensitive method of mRNA quantification, such as qPCR.

These findings could be considered counterintuitive to the classical understanding of thyroid hormone physiology, in which a reduction in circulating T3 levels (hypothyroidism) is associated with increased weight gain. It is well established that D2 is upregulated during a fast, which has been highlighted as a response to plasma leptin (Diano et al., 1998, Fekete et al., 2006). However, literature exists that both disagrees (López et al., 2010) and agrees (Sjögren et al., 2007, Pelletier et al., 2008) with the idea of decreased T3 signalling in the hypothalamus increasing energy expenditure. It is noteworthy that these studies have been
primarily focused on the role of TR, but no other studies to date have investigated the effect of deiodinase overexpression in the VMN.

With T3 and adrenergic signalling co-operating at the site of BAT innervation, it is conceivable that their interaction is also relevant to sympathetic outflow descending to BAT from the VMN. Hypothalamic catecholamines have been suggested to increase the set point for TRH inhibition by T3 to promote higher T3 levels and greater thermogenesis (Fekete and Lechan, 2007). The data presented in this chapter regarding an increased BAT mass and UCP1 expression may outline an additional example of the thyroid axis and the adrenal axis interacting to regulate whole-body energy homeostasis. The potential stimulation of sympathetic drive to interscapular BAT could be investigated by pharmacological blockade of β3-adrenergic receptors following iVMN treatment with rAAV-D3. This could be performed in combination with CLAMS (Comprehensive Laboratory Animal Monitoring System) cages, which are able to detect changes in animal energy expenditure and body temperature.

The mechanism for the observed phenotype remains to be elucidated at this point. The potential products of D3 inactivation of T4 and T3 are rT3 and T2, respectively. The chronic effects of these thyroid hormone metabolites have not been fully investigated, thus it is possible that they have played a role in the observed phenotype. rT3 has been implicated in the regulation of neuronal function through extragenomic interactions (Bassett et al., 2003), therefore the role of this thyroid hormone may be significant. In order to deduce which thyroid hormones are significantly affected by rAAV-D3 treatment, assays for T3, rT3 and T2 could be performed on dissected hypothalami post-methanol extraction. The specific effects of any predominant thyroid hormone metabolites could then be investigated by their direct administration to the VMN in a similar method to that of Kong et al.

T3 modulates a number of cellular processes either by genomic interactions or extragenomic effects. The qPCR investigations failed to highlight any notable changes in targets of T3, which would suggest that the mechanism was an extragenomic one. T3 is known to affect the activity of Na⁺/K⁺ ATPases, which are present on neuronal axons and are essential to the generation of action potentials. D3 has been colocalised with Na⁺/K⁺ ATPases at the plasma membrane (Baqui et al., 2003), which implies that the two are in suitable proximity for D3 products to interact with the ATPases. There are also reports that T3 can directly regulate hypothalamic Na+/K+ ATPase activity and hypothalamic ATP content to modulate energy
homeostasis (LuGuang Luo, 2003). With this considered, it is possible that rAAV-D3 treatment has somehow affected the action potential frequency of VMN neurones to influence the activity of SNS projections to BAT. The question of whether this effect is genomic or extragenomic could be answered with the use of chromatin immunoprecipitation (ChIP), whereby interactions between holoTR and the TRE could be colocalised using a specific antibody. The potential effect on axonal Na\(^+\)/K\(^+\) ATPases could be measured using patch clamp studies to measure the action potential frequency in neurons in vitro following their transduction with rAAV-D3 in addition to the quantification of hypothalamic ATP content by ELISA.

The data of this chapter support a growing concept that thyroid hormones can manipulate sympathetic nervous activity via the hypothalamus independent of peripheral thyroid hormone status (Klieverik et al., 2009, López et al., 2010, Sjögren et al., 2007). This is potentially a significant finding in appreciating the broad means by which T3 mediates its actions on the body’s metabolic rate, which could also be characteristic of the neuroendocrine function of other hormones.
Chapter 6

The Effect of rAAV-D3 Treatment iVMN in Rats

Maintained on a High Fat Diet
6.1 Introduction
The findings reported in chapter 5 highlighted a physiological response to D3 overexpression in the VMN, where the most significant findings were an increase in BAT UCP1 expression and a reduced level of body weight gain, despite unaltered food intake. It was hypothesised that the rAAV-D3 treated animals were expending more energy than their rAAV-GFP treated counterparts by virtue of increased BAT activity, which is used as a marker for increased energy expenditure in rodents.

Models of elevated thermogenesis can be further investigated using techniques such as indirect calorimetry and response to an adrenergic challenge. These techniques are relatively expensive and limited by available resources. In this case, the level of resistance to diet-induced obesity (DIO) has been investigated in rAAV-D3 treated animals by introducing a high fat diet (HFD) after administration of the virus.

The regulation of food intake by hypothalamic thyroid hormone has recently received much attention, with the finding that several enzymes of fatty acid metabolism are modulated by T3 administration to the VMN (López et al., 2010). The role of thyroid hormones in DIO and hypothalamic fatty acid metabolism are reviewed in this chapter introduction.

6.1.1 Diet-Induced Obesity
Obesity can be defined as an increase in adipose mass resulting from imbalance between energy intake and energy expenditure. A number of autosomal recessive mutations have been identified in rodents that give rise to the obese phenotype ob/ob and db/db mice (Halaas et al., 1995, Zhang et al., 1994). The products of the ob and db genes represent the receptor-ligand pairing of leptin and LEPR, which form a hypothalamic negative feedback pathway of adipose homeostasis, as defined by adipose deposits and dietary fat consumption.

It has been demonstrated in DIO mice that HFD increases hypothalamic expression of LEPR and NPY, whilst down-regulating POMC (Huang et al., 2004). Additionally, a direct relationship has been suggested between an ability to increase plasma leptin levels in response to a HFD and resistance to DIO (Surwit et al., 1997). However, it has been shown in humans that circulating leptin levels can be out of proportion to changing body fat stores (Havel et al., 1996). Hence, the involvement of other forms of hypothalamic fat ‘sensing’ have been explored to improve our understanding of this disease.
6.1.2 Thyroid Hormone in Diet-Induced Obesity

Plasma thyroid hormone tone positively correlates with nutritional status, particularly lipid availability, where fat stores and plasma leptin modulate the TRH set point to affect TRH, TSH and plasma thyroid hormone levels (Coppola et al., 2005, Germain, 1986). Furthermore, there have been studies demonstrating that food restriction and weight loss are accompanied by reduced plasma concentrations of thyroid hormone and decreased energy expenditure (Araujo et al., 2009, Kiortsis et al., 1999). These studies have shown that weight loss-induced reductions in plasma thyroid hormone and energy expenditure can be reversed by low dose administration of leptin in rodents and humans. Therefore, elevated plasma leptin levels in obese subjects would be expected to drive an increase in plasma thyroid hormone levels to raise the metabolic rate and combat weight gain.

It has been demonstrated that HFD induced hyperactivation of the HPT axis in rats, characterised by 2.24, 1.6 and 3.7 fold elevations in hypothalamic TRH expression, thyroid iodide uptake and serum TSH, respectively (Araujo et al., 2010). Plasma thyroid hormone levels were within the normal range, despite D1 activity being increased in the thyroid gland, liver and kidneys, indicating that localised tissue thyroid hormone metabolism had been stimulated. Interestingly, D2 activity in the pituitary was reduced, which would trigger an increase in TSH synthesis and release. In addition, BAT D2 activity was reduced, indicating suppressed sympathetic tone and energy expenditure.

6.1.3 Hypothalamic Fatty Acid Sensing

Although fatty acids are not readily oxidised for energy production in the brain, the major enzymes of fatty acid metabolism are expressed within the hypothalamus, exhibiting some key roles. The multifunctional enzymes ACC and FAS are required for the de novo synthesis of fatty acids. ACC catalyzes the synthesis of malonyl-CoA from acetyl-CoA and hydrogen carbonate ions in the presence of ATP. FAS catalyzes the synthesis of long-chain fatty acids from acetyl-CoA and malonyl-CoA in the presence of NADPH.

There is evidence to suggest that alterations in hypothalamic malonyl CoA concentration can modulate energy homeostasis in a physiological fat ‘sensing’ mechanism. In the fasted state, hypothalamic malonyl CoA levels rapidly decrease to 0.1 to 0.2 µM, whereas this value increases to 1.2 – 1.8 µM in the refed state (Wolfgang and Lane, 2008, Hu et al., 2003).
Intra-ARC injection of rAAV overexpressing malonyl CoA decarboxylase (MCD), an enzyme that catalyzes conversion of malonyl CoA into acetyl CoA, thereby reducing hypothalamic malonyl CoA levels, induces hyperphagia (He et al., 2006). Thyroid hormone is known to modulate the transcription of ACC, AMPK and FAS in hepatic tissue both in vivo and in vitro (Roncari and Murthy, 1975, Volpe and Marasa, 1975). These three enzymes are thought to be key mediators of hypothalamic fatty acid sensing.

Figure 6.1. The regulation of food intake in response to hypothalamic malonyl CoA content. In the refed state, the conversion of acetyl CoA to malonyl CoA is stimulated. The increased levels of malonyl CoA lead to the suppression of food intake. C75 inhibition of FAS potentiates this pathway by virtue of reduced malonyl CoA conversion to fatty acid.
6.1.3.1 Acetyl CoA Carboxylase

Two isoforms of ACC exist, ACCα and ACCβ, which derive from the *Acca* and *Accb* genes, respectively. It is hypothesised that ACCα, which is highly expressed in the liver, is important for lipid biosynthesis, whereas ACCβ is predominantly involved in fatty acid oxidation. T3 regulates the transcriptional activity of the *Acca* gene in a mechanism that is potentiated by SREBP1.

It has been shown that ICV leptin administration can activate ACC to increase ARC malonyl CoA levels and, consequentially, food intake (Gao et al., 2007), which is attenuated by subsequent administration of the ACC inhibitor, 5-tetradecyloxy-2-furoic acid (TOFA). This occurred despite leptin inhibition of ARC AMPK, suggesting that malonyl CoA may be a downstream mediator of AMPK signalling in hypothalamic regulation of appetite, although this is by no means conclusive evidence. Mice deficient in *Accb* have increased appetite, supporting its importance to the regulation of energy homeostasis. Genetic deficiency of *Acca* in mice is lethal to the embryo, which restricts understanding of its involvement in physiology.

6.1.3.2 AMP-Activated Protein Kinase

AMPK is a serine/threonine kinase that responds to metabolic stresses that deplete cellular adenylate charge. It is highly expressed in cells that exhibit a high level of oxidative capacity and those of a high lipogenic capacity. AMPK is a heterotrimeric protein consisting of a catalytic α subunit and regulatory β and γ subunits (Kemp et al., 2007). A number of different isoforms of each subunit exists with varying tissue distribution (Stapleton et al., 1996).

It is generally considered that AMPK acts as a ‘fuel sensor’ that increases fatty acid β-oxidation during states of energy demand and decreases β-oxidation when energy levels are sufficient. This modulation of fatty acid oxidation is due, in part, to the regulation of ACC activity, which is phosphorylated and inhibited by AMPK (Chen et al., 2000).

In the hypothalamus, increasing glucose entry into the cell raises ATP levels and inhibits AMPK activity (Cota et al., 2006). However, there are several non-nucleotide modulators of AMPK activity. The orexigenic peptide ghrelin stimulates AMPK activation independent of adenylate charge (López et al., 2008). In addition, the adipokines leptin and adiponectin alter AMPK activity to affect appetite (Minokoshi et al., 2002, Kadowaki et al., 2008).
6.1.3.3 Fatty Acid Synthase

Fatty acid synthase (FAS) is an important enzyme in de novo fatty acid biosynthesis, in which it converts malonyl CoA to malonyl ACP. This enzyme was first identified as an important player in appetite regulation when the FAS inhibitors C75 and cerulenin were shown to induce potent anorexigenic effects associated with a reduction in hypothalamic NPY expression and increased malonyl CoA concentrations. This is supported by the finding that mice with FAS deleted in the hypothalamus display a reduced appetite in addition to reduced body weight (Chakravarthy et al., 2007). Several studies have since demonstrated the involvement of FAS in appetite, such as the report that the anorexigenic effects of the oestrogen receptor antagonist, tamoxifen, were caused by its downregulation of FAS in the VMN, being completely independent of changes in AMPK or ACC activity.

The role of hypothalamic FAS in the regulation of energy homeostasis appears to be rather complex, given the range of published causes and effects. It has recently been reported that FAS expression in the VMN of Sprague-Dawley rats is downregulated in response to a fast (López et al., 2008), which could be considered counterintuitive to previous findings. The mechanism for this effect was proposed as being mediated by the gut hormone ghrelin, which is heavily implicated in the hypothalamic regulation of energy homeostasis. It was reported that FAS was downregulated in the VMN as a direct response to AMPK activation by ghrelin, thus highlighting a mechanism of hormonal regulation of food intake via its modulation of fatty acid metabolism (López et al., 2008). Considering this controversy over the role of FAS in appetite pathways, the specific nature of its involvement remains to be elucidated.

6.1.3.4 Thyroid Hormone Regulation of Hypothalamic Fatty Acid Metabolism

T3-induced hyperphagia is associated with increased hypothalamic AMPK activity and inhibition of ACC (Ishii et al., 2008). The notion of hypothalamic fatty acid metabolism being affected by T3 in the regulation of energy homeostasis has recently been supported by further evidence. In this investigation, rats rendered hyperthyroid by chronic subcutaneous administration of T4 exhibited an increase in food intake marked by upregulation of hypothalamic Fasn and increased hypothalamic malonyl CoA content. Central administration of T3 to the 3rd ventricle resulted in a rapid decrease in phosphorylated AMPK (pAMPK) and phosphorylated ACC (pACC), the active form of each molecule. Consistent with this, the hyperthyroid-associated weight loss was attenuated by 3rd ventricular administration of
AICAR (aminoimidazole carboxamide ribonucleotide) and TOFA, which are inhibitors of AMPK and ACC, respectively (López et al., 2010).

The role of AMPK in T3 signalling in the VMN was further explored in this investigation by employing an adenovirus encoding a constitutively active AMPKα, which was administered to the VMN of hyperthyroid Sprague-Dawley rats. The effect was a reduction in VMN malonyl CoA content, with body weight gained in the hyperthyroid, but not euthyroid rats. There were no reported effects on food intake despite an increase in markers of BAT activation (López et al., 2010).

6.2 Aims of Study
The aim of this study was to investigate the metabolic effects of Dio3 overexpression in the VMN of male Wistar rats, when they are maintained on a high fat diet.
6.3 Experimental Results

6.3.1 Body Weight

Male Wistar rats received either rAAV-D3 (n=10) or rAAV-GFP into the VMN (n=11). Both groups were maintained on a NCD until the 17\textsuperscript{th} day post-injection, at which time they were transferred to a HFD. After 72 days of the investigation, the rAAV-D3 group (314.8 +/- 15.6g) had gained 19.1g more body weight than rAAV-GFP controls (295.7 +/- 10.0g) (figure 6.2A).

Upon analysis of the data, it became evident that the rAAV-D3 began to gain more weight than rAAV-GFP controls following the introduction of HFD on day 17. During the 55 day period of high fat feeding, rAAV-D3 treated animals gained 27.2g more weight than their rAAV-GFP counterparts, which was approaching statistical significance (P=0.07) (figure 6.2B).
Figure 6.2: The effect of HFD on cumulative body weight gain. Animals were injected with either rAAV-GFP (green line) N=11, or rAAV-D3 (black line) n=10. Cumulative body weight gain data were plotted for the 72 day investigation, where HFD was introduced at day 17 (A). Cumulative data were also plotted for the period of HFD alone (B). Data are expressed as mean +/- SEM for both groups.
6.3.2 Body Weight Gain and Energy Consumption per Day of HFD

The body weight gain and food intake data were reanalysed to determine the mean change in body weight per day post-injection relative to energy consumption. For this, food intake data were converted from weight in grams to kilojoules of energy, where NCD contains 10.74kj/kg and HFD contains 21.914 kj/kg.

The longitudinal presentation of this data highlighted a spiked increase in energy intake and body weight gain, upon the switch to HFD on day 17. However, there was no statistically significant trend in these data over the duration of the investigation, as calculated by the generalised estimated equation (GEE) (figure 6.3).

![Graph showing body weight gain and energy consumption per day of HFD](image)

**Figure 6.3:** Body weight gain (triangles, right y-axis) and total energy intake (circles, left y-axis) at each time-point following bilateral intra-VMN injection of rAAV-GFP (green line) N=11, or rAAV-D3 (black line) n=10. Data are expressed as mean +/- SEM for both groups.
6.3.2.1 Body Weight and Energy Intake on HFD v NCD

Body weight and energy intake during HFD feeding were compared with NCD data from chapter 5. For this, NCD food intake data were converted from weight to kilojoules of energy.

The mean weight gained per day by the rAAV-D3 group when fed a NCD (4.6 +/- 0.2g) was not significantly lower than that of rAAV-GFP controls (6.1 +/- 0.3g) (P = 0.2). However, the mean weight gained per day on a HFD was significantly higher (P = 0.006) in the rAAV-D3 treated group (4.1 +/- 0.2g) than the rAAV-GFP controls (3.5 +/- 0.1g) (P = 0.01) (figure 6.4A).

The mean energy consumed per day during the period of normal chow feeding was not affected by rAAV treatment, which is consistent with the findings noted in Chapter 5. However, whilst the animals were maintained on a HFD, there was a significant increase in the amount of energy consumed by the rAAV-D3 group (439.42 +/- 4.8kj) compared to the rAAV-GFP group (415.69 +/- 4.0kj) (P <0.001) (figure 6.4B).
Figure 6.4: Energy parameters on HFD vs NCD. Body weight (A) and energy intake (B) during the 55 days of high fat feeding (HFD) n=21, compared to the cohorts that were maintained on 78 days of normal chow feeding (NCD – chapter 4) n=24, following administration of either rAAV- GFP (green bars) N=11, or rAAV-D3 (black bars). Data are expressed as mean +/- SEM. ** P<0.01, *** P<0.001.
6.3.3 Body Composition Analysis

Body fat percentage was calculated for each group and compared to the results obtained from chapter 5 (section 5.3.5). Body composition analysis indicated an 8.6% increase in fat mass as a percentage of total carcass mass in the rAAV-D3 group (29.2 +/- 5.0%) compared to the rAAV-GFP group (20.6 +/- 1.3%), which was a significant difference (P=0.05). Comparing these data from those obtained in chapter 5 (figure 5.7) highlighted that rAAV-D3 treatment brought about a 10.2% higher body fat percentage when animals were fed HFD rather than NCD (P=0.006) (Figure 6.5).

Figure 6.5: Body composition on HFD vs NCD. Whole body composition was analysed following carcass dissolution. Body composition data from rAAV-D3 treated and rAAV-GFP control rats maintained on a HFD, n=16, were compared to the cohorts maintained on a normal chow diet (see figure 5.7) n=24. Data are expressed as fat mass (black shading) and lean mass (grey shading) as percentages of total body mass +/- SEM. * P<0.5) ** P<0.01.
6.3.4 Effect on Plasma HPT Axis Parameters

No change was seen in plasma fT4 levels between rAAV-D3 (11.40 +/- 1.3 pmol/L) and rAAV-GFP (11.04 +/- 1.4 pmol/L). In addition, there was no change in plasma fT3 levels between rAAV-D3 (1.63 +/- 0.7 pmol/L) and rAAV-GFP (1.7 +/- 0.1 pmol/L) (Figure 6.6).

Figure 6.6: Plasma levels of fT4 and fT3 were measured by RIA 72 days after bilateral iVMN injection of either rAAV-GFP (green bars) n=10, or rAAV-D3 (black bars) n=11. fT4 and fT3 values are expressed as mean pmol/L +/- SEM.
6.3.4.1 Plasma free thyroxine on HFD v NCD

Plasma thyroid hormone measurements taken from HFD-maintained rats were compared with those from NCD-maintained rats to assess the effect of diet on systemic thyroid hormone homeostasis. Plasma fT3 measurements were not significantly affected by diet (data not shown). However, a difference was detected in plasma fT4 measurements whereby HFD appeared to reduce plasma free T4 levels, despite them remaining within the reference range for this hormone (figure 5.7).

Figure 6.7: Plasma levels of fT4 in HFD-maintained animals. rAAV-GFP – green bars (n=10), rAAV-D3 – black bars (n=11) were compared with plasma fT4 levels in NCD-maintained animals (rAAV-GFP – green bars (n=11), rAAV-D3 – black bars (n=13)). Data are expressed as mean pmol/L +/- SEM. *** P<0.001
6.3.6 Effect on Plasma Leptin

Plasma leptin levels were also measured by RIA. The rAAV-D3 group was not statistically higher plasma leptin (26.17 +/- 3.3ng/ml) than the rAAV-GFP group (23.04 +/- 3.4ng/ml) (P=0.4) (Figure 6.8).

Figure 6.8: Plasma leptin levels were measured by RIA 72 days after bilateral intra-VMN injection of rAAV-GFP (green bar) n=10 or rAAV-D3 (black bar) n=11. Values are shown as ng/ml +/- SEM.
6.3.7 Effect on Interscapular BAT
BAT tissue mass (mg) from the interscapular pad was analysed according to rat body weight (g). The mass of this BAT pad in the rAAV-D3 group (1.14 +/- 0.1 mg/g) was not significantly different to that of the rAAV-GFP group (1.23 +/- 0.09 mg/g) (P=0.38) (Figure 6.9).

![Figure 6.9: Interscapular BAT mass. Interscapular BAT pad was dissected and weighed 72 days after bilateral intra-VMN injection of 1µl rAAV-GFP (green bar) N=10, or 1µl rAAV-D3 (black bar) n=11. Data are expressed as mean +/- SEM for both groups.](image-url)
The mRNA levels of markers of adrenergic stimulation were quantified in the dissected BAT pads. UCP1 expression was 1.17 (+/- 0.09) fold higher in the rAAV-D3 treated group (P=0.12). Dio2 expression was 1.42 (+/-0.17) fold higher in the rAAV-D3 treated group (P=0.07). However, neither of these changes were statistically significant (figure 6.10).

Figure 6.10: BAT UCP1 and Dio2 mRNA levels. Ucp1 and Dio2 mRNA were quantified in 100mg dissected interscapular BAT, 72 days after injection of either rAAV-GFP (green bars) n=10 or rAAV-D3 (black bars) n=11.
6.3.8 Hypothalamic Expression Profile on HFD

Dissected hypothalami were analysed by qPCR for the expression of several genes that are important for hypothalamic function. *Fasn* mRNA was detected as being expressed higher in rAAV-D3 treated animals compared rAAV-GFP controls (5.5 fold +/- 1.8 AU), which was significant by 1-way ANOVA (P=<0.001) (figure 6.11).

![Graph showing mRNA levels for multiple genes](image)

**Figure 6.11:** Hypothalamic mRNA levels for Npy, Pomc, Agrp, Acca, Fasn, Ampkα and Ucp2 were quantified by qPCR 72 days after bilateral injection of rAAV-GFP (green bars) n=11 or rAAV-D3 (black bars) n=10. Data are expressed as fold change +/- SEM. *** P<0.001
6.3.9 Hypothalamic Fatty Acid Metabolism on HFD versus NCD

The mRNA levels of hypothalamic *Acca* and *Fasn* in rAAV-GFP and rAAV-D3-treated animals were compared between studies on HFD and NCD. *Acca* mRNA was significantly upregulated on a HFD in both rAAV groups compared to both groups on a NCD (P=<0.001), but was not significantly affected by rAAV-D3 treatment (Figure 6.11A).

*Fasn* mRNA was significantly upregulated (7.6 fold +/- 2.4 AU) in rAAV-D3 treated rats fed a HFD compared to rAAV-D3 treated rats fed a NCD (P=0.009). This upregulation of hypothalamic *Fasn* in rAAV-D3 treated rats maintained on a HFD (5.8 fold +/- 1.9 AU) was significantly greater than the upregulation of hypothalamic *Fasn* in rAAV-GFP controls maintained on a HFD (1.1 +/- 0.2 AU) (P=0.01) (Figure 6.11B).
Figure 6.12: A comparison of Acca and Fasn mRNA levels in the NCD cohorts (RNA samples from Chapter 5) n=24 versus rats fed a HFD n=21, following administration of rAAV-GFP (green bars) or rAAV-D3 (black bars). Data are expressed as mean +/- SEM for both groups. * P<0.05, ** P<0.01, *** P<0.001.
6.4 Discussion

Work in this chapter was designed to examine the response of rAAV-D3 treated rats to the challenge of a HFD. Data presented in Chapter 5 (sections 5.3.2 and 5.3.3) demonstrated that rAAV-D3 treatment on a normal chow diet causes a reduced rate of body weight gain without any noticeable difference in food intake. Assuming that the principal effect of rAAV-D3 treatment is a hypermetabolic one, the introduction of a HFD would have been expected to accentuate any significant metabolic parameters, such as cumulative body weight gain and food intake.

The initial stage of the experiment saw a similar phenotypical presentation to cohorts of chapter 5. During the initial 17 days post injection of rAAV, whilst maintained on a NCD, body weight gain was beginning to show a trend for reduction in the rAAV-D3 treated group, an effect independent of food intake. After the introduction of HFD, these animals instantaneously gained more weight than rAAV-GFP controls and consistently consumed a higher energy intake throughout the course of the investigation. This reversal of the body weight trend is clearly visible in figure 6.2, which shows a crossing-over of the body weight curves following the replacement of NCD with HFD.

When the energy intake and body weight gain data were analysed per day of high fat feeding, it was witnessed that rAAV-D3 treatment significantly increased both parameters in comparison to rAAV-GFP controls. Furthermore, both parameters were significantly increased in rAAV-D3 treated animals on a HFD compared to rAAV-D3 treated animals on a NCD, an effect not observed in the control (rAAV-GFP) group. Following termination of the experiment, body composition analysis of the rat carcasses highlighted a similar presentation to that of the body weight and food intake data when compared to the data from the NCD study in chapter 5. There was a significant increase in the body fat percentage of rAAV-D3 treated animals both when compared to controls and when compared to rAAV-D3 treated animals on a NCD. These data all suggest that rAAV-D3 treatment modulates the consumption and deposition of food, but only when maintained on a HFD.

The phenotype of BAT dissected from rats in this chapter was investigated by weighing the interscapular pads and quantifying Ucp1 and Dio2 mRNA by qPCR. The interscapular BAT mass was not significantly increased by rAAV-D3 treatment on a HFD, unlike rAAV-D3 treatment on a NCD. It is unclear why this effect was not witnessed in this experimental cohort; however, a common feature of obesity in lab rodents is white adipose infiltration of
BAT pads, which may have varied between the two treatment groups. This could be investigated histologically, whereby white and brown adipocytes could be readily distinguished, with a relative percentage estimated. The mRNA levels of *Ucp1* and *Dio2* in BAT demonstrated a trend towards upregulation following rAAV-D3 treatment, however neither was significant. This may too have been affected by differential white adipose infiltration of the interscapular BAT pad, which could have skewed the relative mRNA levels.

Alternatively, there may be additional disparity in these mRNA levels due to modulation of UCP1 and D2 expression by the HFD, which reportedly downregulates these enzymes in BAT (Araujo et al., 2010). This may have suppressed the increases in the expression of these enzymes which were previously witnessed on a NCD. Additionally, this effect of rAAV-D3 on energy expenditure could have been affected by the change in diet at a transcriptional level, given the finding that the potency of a selective TR agonist is attenuated by HFD (Castillo et al., 2010). In this case, the effects of unliganded TR in the VMN could have been disrupted by HFD, thus abolishing the previously witnessed activation of BAT UCP1.

Plasma fT3 and fT4 measurements once again demonstrated that rAAV-D3 treatment in the VMN does not affect systemic thyroid hormone homeostasis. Plasma hormone data collected from the cohort mainatained on a HFD were compared with those from animals maintained on a NCD. There was no significant difference in plasma fT3 concentrations. fT4 levels were found to be reduced by HFD, despite operating within the normal range. This could help explain the loss of effect of rAAV-D3 on BAT UCP1 expression, given that reduced fT4 levels would decrease local T4 to T3 conversion (Bianco et al., 2002) which is important for UCP1 upregulation.

Plasma leptin was measured by RIA and, despite the significant increase in body fat composition, the plasma concentration of leptin was not significantly affected. It has been shown that circulating leptin correlates with fat mass, and the ability to increase plasma leptin allows resistance to DIO (Surwit et al., 1997). The lack of a leptin response in the rAAV-D3 treated group, despite weight gain, suggests that this treatment interferes with plasma leptin levels. An explanation could be found in a putative pathway of systemic leptin homeostasis in which sympathetic pathways are known to descend from the hypothalamus to white adipose tissue (Bartness et al., 2010, Buettner et al., 2008). At the level of the hypothalamus, LEPR is regulated by T3 in chicks (Byerly et al., 2009), whilst leptin exerts effects on hypothalamic
D2 expression (Coppola et al., 2005). Thus, rAAV-D3 treatment could have perturbed these pathways by dysregulating LEPR expression in the VMN and also by reducing the effects of leptin-induced T3 production, owing to chronic inactivation of T3. Over a long-term study, this may have influenced the sympathetic pathways descending to white adipocytes, thus rAAV-D3 could have perturbed chronic leptin secretion in these animals.

qPCR analysis of the dissected hypothalami did not detect any changes in the mRNA levels of the neuropeptides Npy, Pomp or Agrp. Given the well established effect of T3 on the regulation of enzymes of fatty acid metabolism, in addition to the pronounced effect on food intake and body weight after the introduction of HFD, the expression of some key fatty acid metabolic enzymes was analysed. There were no differences detected in the hypothalamic mRNA levels of Acca or Ampka (α-subunit), however a 5.8 fold increase in Fasn mRNA was observed in rAAV-D3 treated animals.

In order to compare this with the chapter 5 cohort, cDNA libraries from the HFD-fed animals were assayed alongside cDNA libraries from the NCD-fed animals for Acca and Fasn mRNA levels. It was found that Acca mRNA was significantly increased in the hypothalami of both experimental groups on a HFD compared to on a NCD. This could be a physiological response to the increased exposure to circulating metabolic substrates, such as triglycerides, which would require processing by fatty acid metabolic pathways in hypothalamic neurones. The systemic status of both cohorts could be compared by measuring fasting plasma triglyceride and cholesterol levels in each group, using an automated enzymatic procedure.

In order to ascertain whether FAS had been activated on a proteomic scale, the activities and protein levels of ACC and FAS would need to be measured. Following iVMN administration of rAAV-GFP or rAAV-D3, hypothalami would be dissected following cardiac perfusion with formaldehyde. In half of the cohorts, ACC and FAS protein levels could be measured by western blot, using specific antibodies for each enzyme. In the other half, ACC and FAS activity could be measured, according to cited protocols (Lopaschuk et al., 1994, Nepokroeff et al., 1975).

The involvement of FAS would need to be scrutinised for it to be considered a key mechanistic player. There are well characterised inhibitors of FAS and ACC, such as C75 and TOFA, which have been used to investigate the role of FAS in the regulation of hypothalamic malonl CoA levels (Hu et al., 2003). This model could be applied to the findings of this chapter to analyse the effect of FAS on food intake. rAAV-D3 would be administered iVMN
to rats maintained on a HFD, before injecting C75 either centrally or peripherally to pharmacologically block FAS activation and monitor how this affects acute HFD consumption. This could then be repeated using TOFA (ACC inhibitor), to confirm that the effect is restricted to FAS. Finally, C75 and TOFA would be administered simultaneously under the assumption that any effect derived from the activation of FAS could be interrupted by inactivation of ACC (see figure 6.1).

The data presented in this chapter, rather than substantiate the findings of the previous chapter, have inadvertently added weight to an emerging concept that T3 influences hypothalamic control of appetite and energy expenditure via its modulation of neuronal fatty acid metabolism. It is logical that T3, which exerts potent effects on metabolic cycles, would affect the enzymes involved in fatty acid metabolism when its activity is altered. However, the mode by which these neuronal metabolic cycles influence affect behavioural aspects such as food intake and energy expenditure are still largely unknown. It is likely that this mode involves an interaction between many players in the metabolic pathways web, rather than an isolated enzyme, although this chapter suggests that if key metabolic enzymes are important in the neuronal control of appetite, FAS should be included as one of them.
Chapter 7

Final Discussion and Conclusions
Thyroid hormones are potent stimulators of the metabolic rate, although some symptoms of hypothyroidism and hyperthyroidism are not explained by their direct effects on peripheral issues. There is growing evidence that T3 plays a prominent role in the hypothalamus in addition to its well defined effects on the HPT axis. Recent findings have identified that thyroid hormone regulates appetite, autonomic stimulation of hepatic glucose production and BAT thermogenesis, in part, via the hypothalamus without affecting systemic thyroid hormone levels (Klieverik et al., 2009, Kong et al., 2004, Sjögren et al., 2007).

In this work the key deiodinases D2 and D3 have been exploited in conjunction rAAV to generate an experimental tool for the modulation of thyroid hormone activity. Using stereotaxic techniques, these deiodinases have been overexpressed in the VMN of male Wistar rats, which has never been attempted until now. The most significant findings in this work are that D3 overexpression in the VMN increases interscapular BAT mass and reduces body weight gain on a NCD, whereas it stimulates hypothalamic FAS expression and consumption of energy on a HFD. D2 overexpression was without a pronounced physiological effect, which may have been a product of its associated regulatory pathways.

AAV was employed as a research tool in these investigations, despite the availability of a range of techniques for the manipulation of thyroid hormone levels, given its ability to provide prolonged periods of safe gene expression following a single neurosurgical session. The effects of rAAV-D3 treatment became apparent after a 21 day period, during which time the ssAAV vector was presumably being converted into a dsDNA expression cassette. This latency could have been avoided with the use of self-complementary AAV (scAAV), which is known to express more rapidly than ssAAV vectors.

However, the apparent lack of a physiological effect during this 21 day period cannot be attributed to rAAV latency with absolute certainty, as it could simply be a reflection of the kinetics of this hypothalamic system, as opposed to the rAAV design. For example, rT3 is a product of D3 metabolism, known to exert effects on neuronal plasticity. It is possible that increased levels of rT3 were taking effect much sooner, and the 21 day period of ‘latency’ was actually due to the slow development of neuronal rewiring at the site of rAAV-D3 injection.

This could be ascertained by generating and administering a scAAV-D3 vector, which should theoretically increase D3 activity more rapidly. Alternatively, D3 enzyme activity could be measured and compared in hypothalamic samples that had been dissected at various time
points from the first week up to the third week post-injection of rAAV-D3. This would help determine the potency of the effect and kinetics of the vectors used.

The rAAV vectors used in the investigations contained a CMV promoter and a downstream WPRE element to maximise mRNA accumulation. There is a range of constitutive promoters that can be used in expression cassettes, which has been extensively reviewed (Fitzsimons et al., 2002). However, the precise location of where the rAAV vectors have transduced and expressed their transgenes is not known. The use of an endogenous promoter, such as neurone-specific enolase (NSE) would have ensured that rAAV expression was confined to neuronal cells without additional expression in astrogial cells. This would have augmented the neurone-specificity of the AAV serotype 2, thus providing a useful model for characterising the role of T3 within neuronal networks of the hypothalamus.

The two plasmid transfection system utilised in production of the AAV vectors is a reliable method of producing research-grade and clinical-grade vector stocks with minimal contamination by adenovirus (Grimm et al., 1998). The dot-blot method for quantifying the viral particle number highlighted that a high yield was obtained for each vector utilised, of which the infectious titre is estimated to be greater than sixty-five percent. However, the fact that the infectious titre is unknown means that it is also impossible to know the exact number of transducing particles that have been administered to the rat VMN. Injecting more infectious particles per neurone into one rAAV treatment group than another may have experimental implications in terms of the neuronal response to the infectious load. Therefore, the effect of this disparity in infectious titre cannot be ignored when analysing the data.

The evaluation of deiodinase enzyme activity data gave some interesting conclusions as to the efficacy of each treatment. In the case of rAAV-D2, it was shown that this vector was efficient in increasing D2 activity, which was approximately ten-fold higher than that of control (rAAV-GFP) samples. This was comparable with a transgenic approach to overexpressing D2, which achieved an increase by 3 orders of magnitude (Pachucki et al., 2001). However this study also failed to produce physiologically significant effects. The ability to stimulate D2 activity by viral gene transfer is an important finding in itself, given that this could be readily used in other lines of thyroid research. However, the rAAV-D2 virus would need further characterising for efficacy, in terms of its ability to increase the local T3 pool in vivo, before confirming this. The lack of physiological effects in iVMN
rAAV-D2 treated animals could be attributed to the level of D3 activity detected in these samples, which would have had a neutralising action.

In the case of rAAV-D3, a number of analyses were carried out, each with different outcomes. Initially, the ability of the pTR-CGW-D3 packaging construct to increase D3 activity in vitro was investigated in JEG3 cells, which were transfected with the plasmid. This investigation showed that D3 activity was stimulated approximately ten-fold by pTR-CGW-D3 treatment which indicated that the construct was effective.

The ability of rAAV-D3 to increase D3 activity in vivo was investigated by administering a dose of this vector into rat VMN before analysing enzyme activity in whole hypothalamic blocks. In this case, mean D3 activity in rAAV-D3 treated samples was not significantly greater than the control treatment (rAAV-GFP), with both detected at around 10 – 12 fmol/min/mg. A subsequent investigation saw a repeat of the treatment process, although in this case MBH punch biopsies were taken to determine the level of D3 activity within a more discrete location, to rule out the influence of ‘extra-VMN’ hypothalamic structures. In this case, D3 activity was detected at the same level (around 20 fmol/min/mg) in all three treatment groups (rAAV-GFP, rAAV-D2, rAAV-D3), which was approximately double that of a non-transduced control sample. With D3 activity being much higher in MBH biopsies, it could be concluded that the source of this inflammation was within this hypothalamic region, which indicates that it was a direct response to the treatment.

It was hypothesised that the induction of D3 enzyme activity in all three groups was the result of an inflammatory pathway, stimulated by the rAAV particles and/or the stereotaxic injection process (Boelen et al., 2005, Li et al., 2001). This would require investigation before proceeding with any repeats of this study. This investigation would involve iVMN stereotaxic surgery on rats with treatment groups of saline, rAAV-GFP, rAAV-D3, sham-injection or non-injection. MBH punch biopsies would then be taken from these animals and analysed for D3 enzyme activity. A comparison could then be made to determine the cause of elevated D3 activity, or if it has been elevated at all.

The CNS is an essential target of endocrine and humoral factors that regulate metabolic function. Hormones such as insulin, glucagon-like peptide 1 and corticosterone are known to modulate energy homeostasis through direct effects on their respective target tissues. The indirect effects of these hormones on metabolism via hypothalamic feedback pathways are well established, although their direct innervation of targets via the hypothalamus is now
receiving closer attention. This dual mechanism of hormonal regulation in energy homeostasis provides a new perspective from which we can approach neuroendocrinology and underlines the complexity of these processes.

D3 overexpression by the rAAV cassette resulted in a clear increase in Dio3 mRNA several weeks after surgery. This precipitated a reduction in body weight gained by the rAAV-D3 treatment group, despite their food intake matching that of the control group. This presentation is indicative of increased energy expenditure. A marker of sympathetic activity and energy expenditure in laboratory rodents is UCP1 expression in BAT. The interscapular BAT pads of rAAV-D3-treated animals were significantly larger complete with increased levels of Ucp1 mRNA. This suggests that D3 upregulation in the VMN stimulates sympathetic stimulation of interscapular BAT.

The mechanism of how rAAV-D3 influences rat physiology is some way from being determined and further investigations would begin at the source of the effect, in the hypothalamus. Primarily, the hypothalamic concentrations of T3 and T4 would need to be measured following iVMN rAAV-D3 treatment, in order to have an accurate profile of local thyroid hormone status. However, more important questions arise, such as; is the primary effect a genomic one and, if so, where exactly in the genome is this effect taking place? Both of these questions could be addressed with the use of ‘ChIP-seq’, which has been reported (Schmidt et al., 2008). In such an experiment, antibodies raised to TRα and TRβ would be used to immunoprecipitate genomic DNA from hypothalamic tissue. These TR-DNA precipitates would then be analysed by high-throughput sequencing to enable a genome-scale analysis of the effects of the TR system in the rAAV-D3 iVMN model. This would provide data on how rAAV-D3 treatment affects the number of TR-DNA events, in addition to a comprehensive analysis of the genomic loci involved in any effects.

As the next mediator downstream of the hypothalamus in the putative pathway, the level of SNS drive to BAT would need to be examined. This could be achieved through disruption of BAT sympathetic innervations, either surgically or chemically, using 6-OHDA (6-hydroxydopamine). The concept of impaired T3 availability leading to increased BAT activity fits in line with work employing a mutant TRα, which blocks T3 signalling (Sjögren et al., 2007, Pelletier et al., 2008). However, conclusions are complicated by recent findings that T3 administration induces the same response in Sprague Dawley rats (López et al., 2010), suggesting that some species variation may exist in the effects of T3 in this pathway.
The intriguing effect witnessed in chapter 6 asks the question of why rAAV-D3 treatment only increased energy intake and Fasn mRNA levels when animals were maintained on a HFD. There are a number of potential explanations for this which could be investigated.

It is possible that rAAV-D3 treatment in the VMN interferes with hypothalamic fatty acid sensing thus leading to the inappropriate increase in HFD consumption. This could be examined by repeating the investigation, up to the point of HFD introduction, and instead looking at how the animals respond to either peripheral injection or oral gavage of fatty acids in terms of refeeding following a fast. Alternatively, HFD could be supplied at varying percentages of cholesterol and fats, which would provide a means of developing a ‘dose-response’ profile of rAAV-D3-mediated lipo-hyperphagia.

An alternative explanation could be that rAAV-D3 treatment induces a hedonic preference for high energy foods. This would require a more simple investigation whereby rAAV-D3 treated animals are provided with a choice between NCD and HFD during the study, with the resulting profile compared to rAAV-GFP controls. However, most animal models would demonstrate an overriding preference for the HFD, which would complicate the detection and interpretation of any noticeable effects.

The pronounced upregulation of ACC on HFD is a novel and potentially important finding. Studies involving DIO mice have shown that levels ACC and FAS mRNA in adipose tissue and the liver are not significantly affected by HFD treatment (Kohsaka et al., 2007). Mice fed a 55% fat diet displayed basal activity of ACC in muscle tissue with an attenuated response of hypothalamic AMPKα to leptin administration (Martin et al., 2006). This reduced activity of AMPKα could potentially have a disinhibitory effect on hypothalamic ACC, which goes some way to supporting the effect observed in this thesis.

The upregulation of FAS on a HFD is an interesting point of discussion. The notion of FAS stimulating food intake is supported by several investigations, whereby the mechanism is thought to be based on its net reduction of hypothalamic malonyl CoA levels, which then leads to appetite stimulation (Hu et al., 2003). It was recently reported that peripheral T3 administration to Sprague Dawley rats stimulates FAS expression 1.3 fold to increase food intake on a normal chow diet (López et al., 2010). rAAV-D3 treatment would be expected to reduce local levels of T3, therefore some disparity occurs between the findings of this chapter and those of Lopez et al. However, there a number of differences between the studies, such as
the strains of Wistar rat used in the experiments, the method of hormone manipulation, the diets used in the investigations and the duration of their chronic investigations.

The role of FAS in the regulation of food intake has not been fully characterised, as demonstrated by some findings that contradict the notion that this enzyme stimulates food intake. It has been demonstrated that FAS is downregulated in the hypothalamus in response to a fast (López et al., 2008). Additionally, the orexigenic gut hormone, ghrelin, was found to increase food intake by reducing FAS mRNA levels in the VMN as a direct effect of AMPK activation. Furthermore, the regulation of FAS by T3 is generally thought to be stimulatory (Sul and Wang, 1998), although it has also been reported that it can be inhibitory (Kameda, 1995). Therefore, the role and regulation of FAS in the hypothalamus are not completely understood and further work is required to develop our understanding.

In order to bring the findings of this thesis together to a unifying mechanism, the question needs to be asked whether or not the effects observed in chapter 5 can be married with those of chapter 6. Are the effects on appetite and energy expenditure acting simultaneously on a HFD or is there a molecular pathway that is affected by HFD? However, there are at least two potential mechanisms which link the effects witnessed in these studies, which include an interference with central leptin signalling and crosstalk between the TR and other nuclear hormone receptors.

Leptin sensitivity depends on the expression of LEPR on the neurones of hypothalamic nuclei, where it is down-regulated by T3 (Byerly et al., 2009). Therefore, D3 overexpression would be expected to have a positive effect on LEPR expression which could explain the perceived stimulation of energy expenditure in chapter 5. It has been reported that HFD disrupts the nutritional regulation of LEPR expression in rodents (Sahu et al., 2002). With this considered, it is possible that the introduction of a HFD in chapter 6 was responsible for the lack of effect on BAT activity. This then raises the question of why these animals then eat more on a HFD.

It is generally accepted that, whether via direct hypothalamic stimulation or secondary to its effects on metabolism, T3 leads to appetite stimulation, although the roles of other thyroid hormone molecules may also be relevant to physiology. It has recently been reported that the central administration of the thyroid hormone derivative 3-iodothyronamine (T1AM) stimulates food intake (Dhilllo et al., 2009). It has also been shown that rT3 can induce changes in brain function by affecting neuronal plasticity (Bassett et al., 2003). The findings
of this chapter may highlight a role for D3-generated thyroid hormone metabolites whose concentrations affect HFD intake and sympathetic outflow to BAT. It could be hypothesised that D3-generated thyroid hormone derivatives have stimulated feeding whilst the transcriptional effects on LEPR have been blocked by the state of DIO. This could be investigated in further studies by iVMN administration of rT3 in diet-induced obese models.

A molecular endocrinological explanation for the observed effect could involve the phenomenon of TR crosstalk with other nuclear receptors. It is recognised that TR interacts with transcription factors which respond to nutrient signals, such as the PPARs, SREBPs, LXRαs and more (Liu and Brent, 2010). Furthermore, dysregulation of lipid metabolism by the unliganded form of TR, which would theoretically be increased by D3 overexpression, has been reported (Araki et al., 2009). The mechanisms for these interactions are diverse and involve competition for the common RXR heterodimer, response-regions on the target gene and recruitment of transcription factors.

The SREBP-1c isoform is regulated by TR and is known to direct fatty acid synthesis, owing to the presence of a SRE on the Fasn and Acca genes. SREBP1c has been directly linked to the stimulation of FAS in primary culture of hypothalamic neurones as a response to insulin signalling (Kim et al., 2007). Given that SREBP1c is less active when lipid and sterol concentrations are increased, rats on a HFD would be expected to have FAS either downregulated or expressed at a basal level. However, on a HFD, FAS was expressed at a basal level in rAAV-GFP controls and significantly increased by rAAV-D3 treatment. This could indicate that alterations in TR signalling, brought about by D3 overexpression, somehow restore SREBP activation of the Fasn gene, in an environment of increased lipid availability, through nuclear crosstalk.

In addition to SREBP signalling, PPARs have also been implicated in the regulation of genes in combination with TRs. Glucose-stimulated insulin secretion (GSIS) is affected by both PPARα signalling and TR signalling. Both PPARs and TRs compete for their common RXR heterodimer prior to their interactions with glycolytic enzymes in pancreatic islets, to regulate insulin secretion (Sugden et al., 2001, Garcia-Flores et al., 2001). It has been reported that PPARα signalling, induced by a HFD, stimulates GSIS in euthyroid rats, but inhibits GSIS in hyperthyroid rats (Holness et al., 2008). This suggests that the reversal of the rAAV-D3 phenotype by HFD could be due to a molecular interaction between PPAR and TR signalling.
There are other nuclear receptors that are affected by both HFD and TR. The regulation of energy homeostasis by photoperiod in rodents is dependent on the expression of the ‘clock’ genes, such as *Clock* and *Bmal1* in the hypothalamic suprachiasmatic nucleus (SCN). The SCN conveys signals to hypothalamic nuclei and the pineal gland to induce changes in energy homeostasis. A well characterised pathway involves photoperiodic stimulation of melatonin signalling which regulates hypothalamic *Dio2* expression (Revel et al., 2006, Yasuo et al., 2007). It has been reported that HFD disrupts circadian rhythms due to dysregulation of the ‘clock’ genes, which alters melatonin signalling and hypothalamic energy circuits (Kohsaka et al., 2007). Therefore, it is possible that the effect of rAAV-D3 is perturbed by HFD due to these interactions with rodent circadian rhythms. It has been reported that melatonin administration can attenuate body weight gain on a HFD (Puchalski et al., 2003). Therefore, if the effects of rAAV-D3 on NCD and HFD are attenuated by melatonin administration, this could highlight circadian disruption as a potential mechanism for the effects of this recombinant virus.

The findings of this thesis help further our understanding of the neuroendocrine role of thyroid hormones. For this, a tool was developed for the manipulation of local thyroid hormone homeostasis through viral gene transfer of the iodothyronine deiodinases. Furthermore, rAAV-D2 treatment produced a convincing increase in D2 activity, which has previously proved an arduous challenge. It has long been known that T3 plays an important role in the CNS, particularly in the hypothalamic pathways that influence the metabolic rate, although the specific mechanisms remain largely unclear. The key findings of this thesis are twofold: thyroid hormone influences the sympathetic nervous system through direct interactions with the hypothalamus, independent of systemic thyroid hormone tone; and stimulates the consumption of a high fat diet via transcriptional regulation of neuronal fatty acid metabolism.

With respect to the controversy surrounding the role of T3 in hypothalamic energy circuits, these key findings fall into some key points of discussion.

In relation to the effect highlighted in chapter 5, in which D3 stimulated BAT activity, this supports the findings which suggest that negative T3 signalling increases the BMR (Sjögren et al., 2007, Pelletier et al., 2008). However, as highlighted in chapter 1.7.5, this contradicts findings which state that positive T3 signalling stimulate the BMR (López et al., 2010).
Regarding the effect of T3 on appetite, it has previously been demonstrated that T3 promotes feeding (Kong et al., 2004, López et al., 2010, Ishii et al., 2008) although a uniform view on how this comes about could not be made. The findings of chapter 6 indicate that T3 inactivation promotes consumption of a HFD which, along with other reports (Klieverik et al., 2009, López et al., 2010), contradicts those findings that suggest that T3 stimulates appetite.

The findings in chapter 6, which suggest that FAS expression is increased by D3 in the VMN, further contradict the findings of Lopez et al who report that T3 stimulates FAS expression. Furthermore, Lopez et al reported that T3-induced FAS mediate effects on energy homeostasis by modulating the BMR, without effects on appetite, which is the exact opposite phenotype to that presented in chapter 6.

This controversy of findings in the area further increases the opacity of our understanding of these processes and necessitates further investigations. The common finding is that T3 influences the function of the hypothalamus, both in terms of appetite regulation and the setting of the body’s metabolic rate, and this thesis supports new evidence that neuronal fatty acid metabolism is involved. The disparities between publications in how T3 achieves this could be products of the various species of animal models used for these investigations. Alternatively, it could suggest that the regulation of energy homeostasis by T3 is an extremely complex process, affected significantly a number of temporal and environmental factors, such as husbandry techniques, dietary sources, housing conditions etc which may differ significantly across research institutions. Given the range of physiological pathways that T3 influences and is influenced by, it is unlikely that this hormone transmits a single, uniform effect in a system as complex as the hypothalamic energy circuits, let alone systemic metabolism.
Reference List


selenodeiodinase is located in the plasma membrane and undergoes rapid internalization to endosomes. *Journal of Biological Chemistry*, 278, 1206.


measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nature medicine*, 1, 1155.


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Appendix I: List of Solutions

3M Alcoholic KOH solution: Add 39 g KOH pastels to 200 ml 65 % ethanol.

Amasino wash buffer: Mix 250 ml 1M Na phosphate buffer, 2 ml 500 mM EDTA, and 100 ml 20 % SDS with 650 ml GDW.

Denaturing solution (RNA) (Denat): Add 300μl of 40% formaldehyde and 100μl of 20XMOPS to 1ml of formamide.

0.5M ethylenediaminetetra-acetic acid (EDTA): In 800ml autoclaved GDW, dissolve 186.1g C10H14H2O8Na2.2H2O and adjust to pH 8.0 with 1M NaOH. Make up to 1L with GDW.

4% Formaldehyde solution: Dissolve 100ml 40% formaldehyde in 1L GDW.

Gel loading buffer: mix 3.125 ml 80 % glycerol, 50 ul 0.5 M C10H14H2O8Na2.2H2O and 6.075 ml GDW, add 10 mg orange G.

10% glycerol in GDW: Add 1ml glycerol to 9ml of GDW

GTE: Mix 2.5ml 1M Tris-HCl, pH 8.0, 2ml 0.5M C10H14H2O8Na2.2H2O and 5ml 18% glucose and top up to 100ml. Sterilise by passing through a 0.2μm filter.

Hybridisation buffer: dissolve 0.5 g dried milk powder in 48 ml GDW and 0.5 ml 500 mM EDTA. Add 25 ml 1 M Na phosphate, 25 ml 20 % SDS, 1 ml 2.5 mM ATA.

Hybridisation buffer for oligo dT probing: dissolve 0.2 g milk powder in 75 ml GDW and 25 ml 20X SSPE at 37 ºC. Add 200 ul nonidet and put on ice to allow Nonidet to dissolve.

HBS Buffer: 1.36M NaCl, 0.05M KCl, 0.007M Na2HPO4.2H2O, 0.2M HEPES, 0.125M glucose passed through a sterile filter unit.

1x HEBS buffer: 1.36M NaCl, 0.05M KCl, 0.007M Na2HPO4.2H2O, 0.2M HEPES, 0.125M glucose

Kemteck Buffer: 22.4g Na2HPO4, 9.96g NaH2PO4, EDTA 14.8g, Thimersol 1g, 30% BSA 26.6ml. Made up to 4L.
**LB (amp):** Dissolve; 10 g Bacto-tryptone, 5 g Bacto-Yeast Extract, 5 g NaCl in 800ml GDW. Add 200μL 5N NaOH to achieve pH 7.5 and make up to 1L before autoclaving. When LB has cooled to room temperature, add 2ml of 50mg/ml sterile ampicillin solution per litre of LB.

**LB(amp) plates:** Take 1L of prepared LB, pH 7.5, and add 30g bacto-agar per litre of LB. Mix the contents and autoclave. When LB has cooled to ~40°C, add 2ml of 50mg/ml sterile ampicillin solution per litre of LB and mix. Pour ~20ml LB-agar into each petri dish before flaming each one to remove bubbles.

**SOB:** Dissolve; 20g Bacto-tryptone, 5g Bacto-yeast extract, 0.5g NaCl in 800ml GDW. Adjust to pH 7.0 with 5M NaOH. Before use, add 5ml of 2M MgCl₂.

**20x MOPS:** Dissolve 83.6g 3-(N-Morpholino)propanesulphonic acid (MOPS), 8.1g sodium acetate, 7.4g C10H14H2O8Na2.2H2O and 2.5ml formaldehyde to 800ml GDW. Add 833μL 3M KC. Adjust to pH 7.0 with 10M sodium hydroxide and make up to 1L before autoclaving.

**1x PBS:** In 800ml deionised water, dissolve: 8g of NaCl, 0.2g of KCl, 1.44g of Na₂HPO₄, 0.24g of KH₂PO₄. Adjust pH to 7.4 before making up to 1L volume.

**PE Buffer:** 100 mM phosphate buffer containing 2 mM EDTA and 50mM DTT. Adjust to pH 8.0.

**Phosphate Buffer (RIA buffer):** 48g of Na₂HPO₄.2H₂O, 4.13g KH₂PO₄, 18.61g C10H14H2O8Na2.2H2O, 2.5g NaN₃ were dissolved in 5L GDW, that had been boiled and allowed to cool, the pH was measured to confirm it was 7.6 ± 0.1 and the buffer was stored at 4°C.

**3M potassium acetate:** Dissolve 294.4g CH₃COOK in 500 ml GDW, add 115 ml glacial acetic acid and GDW up to 1 L.

**20mg/ml proteinase K:** Dissolve 20mg for every 1ml GDW and store at -20°C.

**Sephadex G50:** Add 8g fine grade Sephadex G-50 beads (diameter: 20-80μm) (Sigma), 2ml 100x TE and 0.1ml 20% SDS to 200ml GDW and autoclave to expand beads.

**3M sodium acetate, pH 5.2:** Dissolve 246.1g CH₃COONa in 800ml a/c GDW, adjust to pH 5.2 with glacial acetic acid and make up to 1L with GDW
5M sodium chloride: dissolve 292.2 g NaCl in 1 L GDW.

20% sodium dodecyl sulphate (SDS): Add 200g SDS to 800ml a/c GDW, heat to 60°C while stirring. Allow to cool and make up to 1L with GDW.

Solution A: 10mM Tris/HCl pH8.0, 10mM MgCl₂, 10µg/ml DNaseI

Solution B: 10mM Tris/HCl pH8.0 100mM NaCl 10mM EDTA 0.5% SDS

20x SSC: Dissolve 1.753g NaCl, 14.2g Na₂HPO₄ and 7.4g C₁₀H₁₄H₂O₆Na₂.2H₂O in 700ml GDW, adjust to pH 7.7 with 10M NaOH and make up to 1L with GDW.

20x SSPE: Dissolve 210.4g NaCl, 14.2g Na₂HPO₄ and 7.4g C₁₀H₁₄H₂O₆Na₂.2H₂O in 700mls GDW, adjust to pH 7.7 with 10M NaOH and make up to 1L with GDW.

30% sucrose solution: Dissolve 300g sucrose in 1L GDW.

50x TAE: Dissolve 242g Trizma base in 843ml GDW and mix in 57ml glacial acetic acid and 100ml 0.5M C₁₀H₁₄H₂O₈Na₂.2H₂O.

100x TE: Dissolve 121.1g Trizma base and 3.7g C₁₀H₁₄H₂O₈Na₂.2H₂O in 800ml and adjust pH 7.5 with hydrochloric acid. Make up to 1L.

TES: 50mM Tris-HCl, pH 8.0, 50mM sodium chloride, 5 mM EDTA

Universal wash buffer: mix 10 ml 20 % SDS and 10 ml 20X SSPE with 980 ml GDW.

Buffer R: Contains; 40mM Pipes buffer pH 7.6, 1.5mM DCHBS, 17.5mM magnesium-ions.

Reagent R: Contains; 0.4mM aminophenazonel, 1mM ATP, glycerol-kinase > 0.4 U/ml, glycerol-3-phosphate oxidase > 1.5 U/ml, peroxidase > 0.5 U/ml. ascorbic acid oxidase > 7.0 KU/l.

Dio2 Oligos: NCBI Ref: NM_172119.2; Location: 5’ - 106 – 1060 - 3’
5’AGCTACCGGTAGGCGAATGGGATTCAGTTG
3’AGCTGGATCCTGACTGGGAAGCAGAGTGC

Dio3 Oligos: NCBI Ref: NM_017210.3; Location: 5’ - 28 – 1518 - 3’
5’GCTGGATCCTGCTGGTGCTGGAGAAGGTGAA
3’GCTTGTACATCATTAGGCGCTGTTTCGA

SECIS Oligos: NCBI Ref: NM_172119.2; Location: 5’ - 5051 - 5651 - 3’
5’AGCTACCGGTAAAGACCTGCACGCAAG
3’AGCTGTACATGGCACACACGGTGTCTCA
Appendix II: List of Suppliers

Advanced Biotechnology Centre, Imperial College, London, UK

Animalcare Limited, Dunnington, York, UK

Applied Biosystems, Warrington, UK

ATCC, Middlesex, UK

Bright Instrument Company, Huntingdon, Cambridgeshire, UK

Campden Instruments, Lougborough, UK

Charles River, Bicester, UK

Clark electromedical instruments, Pangbourne, UK

Crystal Chem Inc, Illinois, USA

Eppendorf, Hamburg, Germany

Fisher Scientific UK, Loughborough, Leicestershire, UK

GE Healthcare, Little Chalfont, Buckinghamshire, UK

Harvard Apparatus, Kent, UK

Helena Biosciences, Sunderland, Tyne and Wear, UK

Invitrogen Life Technologies, Paisley, UK

Jet X-Ray, London, UK

Kodak, Hemel Hempstead, Hertfordshire, UK

Life Sciences Technology, Eggenstein, Germany

MP Biomedicals LLC, Orangeburg, NY

Millipore, Watford, UK

New England Biolabs (UK) Ltd, Hitchin, Hertfordshire, UK
Novagen, Nottingham, UK
Oswel DNA service, Southampton, UK
Plastics One, Roanoke Inc., Virginia, USA
Promega, Madison, WI, USA
Randox Laboratories Ltd, Crumlin, Co. Antrim
Reckitt-Benckiser Healthcare, Hull, UK
Research Diets Inc, New Brunswick, NJ, USA
Schering-Plough Ltd., UK
Seton Healthcare, Oldham, UK
Shimadzu, Kyoto, Japan
Siemens Medical Solutions, Sudbury, UK
Sigma-Aldrich, Poole, Dorset, UK
Vet-Tech Solutions Ltd., UK
VWR International Ltd, Poole, UK
Wallac, Waltham, MA