Literature Review

Introduction

Over 60 years ago Kennedy (1950) reported that rats with experimentally induced hypothalamic lesions developed voracious appetite and showed less discrimination in choice of diet. He proposed that this was because the hypothalamus was normally sensitive to chemical changes in the circulation. Subsequently he proposed that the lesions of the hypothalamus caused an insensitivity to a hypothetical factor in the circulation which allowed fat mass to be maintained at a relatively stable level – the lipostat model (Kennedy, 1953). Parabiotic experiments (the surgical union of two animals so that blood is exchanged between individuals) supported the lipostat model acting at the hypothalamus. Rats were parabiosed in either normal pairs or pairs where one rat had hypothalamic lesions. The normal pairs showed no significant changes, whereas the rats with hypothalamic lesions developed a voracious appetite and obesity, while the normal partner lost weight and some died, seemingly of starvation (Hervey, 1959).

The *obese* (*ob*) mutation was first described in mice in 1950, where young mice reported to have voracious appetites, were sterile and became obese, compared with their littermates (Ingalls, *et al.*, 1950). Similarly, in 1966 the *diabetes* (*db*) mutation was first described, where the mutants displayed a similar phenotype to the *obese* mutants (Hummel, *et al.*, 1966). These two mutations had near identical phenotypes, however parabiotic experiments between these mutants and wild type mice suggested that there may be a specific blood borne factor involved in the regulation of body fat that was missing in *obese* mice and that *diabetic* mice were insensitive to. This was indicated by the loss of weight in *obese* mice when joined with normal or *diabetic* mice, while normal mice lost weight and eventually died (presumably from starvation) when united with a *diabetic* mouse (Coleman, 1973). These results, when viewed with those of Kennedy (1950; 1953) were indicative of a factor missing in *obese* mice that acted at the level of the hypothalamus to regulate adiposity and energy metabolism.

In 1994 the *obese* gene product was finally identified and cloned, and it was confirmed that indeed *ob/ob* mice were not capable of producing this factor (Zhang, *et al.*, 1994). Shortly after, the *diabetes* gene was found to produce the functional receptor, and *db/db* mutants produced a truncated variant splice of the long isoform which is located in the hypothalamus (Tartaglia, *et al.*, 1995; Chen, *et al.*, 1996; Lee, *et al.*, 1996). The cloning of the obese gene protein and administration to mice showed that it was capable of reducing food intake and fat mass (Campfield, *et al.*, 1995; Pelleymounter, *et al.*, 1995; Stephens, *et al.*, 1995) and it was suggested that the protein be called leptin, derived from the Greek word 'leptós', meaning thin (Halaas, *et al.*, 1995). Following these findings there was speculation that leptin would be a panacea for obesity (Zhang, *et al.*, 1994), which is a prevalent health condition. For example over 60 % of Australians were classified as overweight or obese as of 2008 (Australian Bureau of Statistics, 2011). Unfortunately, subsequent research has shown that the mechanisms regulating body mass are far more complex. However, leptin has been found to be integral to many physiologic processes.

Structure of Leptin

Leptin is a cytokine hormone (Kline, *et al.*, 1997) 167 amino acids long, with a molecular mass of approximately 16 kDa (Zhang, *et al.*, 1994). There is a high degree of leptin structure identity in vertebrates, implying conserved function (Zhang, *et al.*, 1994), for example murine leptin shares 83.234 % identity with both human and bovine leptin (The UniProt Consortium, 2009). The structures of human and mouse leptin have been reported and there is consensus for the general structure of the protein in these species, further implying conserved function. The structure of leptin has been confirmed using nuclear magnetic resonance for murine and human proteins (with a single amino acid substitution Glu for Trp100 to induce crystal formation in human leptin). Both forms of leptin display four α-helices, two long crossover links and one short loop, all of which are consistent with a short-helix cytokine protein structure (Kline, *et al.*, 1997; Zhang, *et al.*, 1997). The dimensions of the modified human leptin were reported to be approximately 20×25×45 Å in a left hand twisted helical bundle (Zhang, *et al.*, 1997).

In the *ob/ob* mutant the amino acid sequence for leptin is truncated with a point substitution changing Arg 105 to a stop codon (Zhang, *et al.*, 1994). The implication of this is that the region downstream of Arg 105 in normal murine leptin is critical to eliciting an intracellular signal and may contain the domain which interacts with the leptin receptor binding domain and therefore truncation prevents the initiation of normal leptin signalling. In rare cases of human obesity low plasma leptin concentrations have been found in conjunction with high levels of adiposity. This has been characterised in two children with highly consanguineous parents and attributed to the deletion of a guanine nucleotide in codon 133, ultimately resulting in a premature stop codon and a non-functional leptin protein (Montague, *et al.*, 1997). It has been hypothesised that there may be 3 regions of leptin capable of interacting with leptin receptors, although two of these arise upstream of Arg 105 (Peelman, *et al.*, 2004), but it is clear that the portion of leptin downstream of Arg 105 is critical in leptin signalling in both humans and mice.

Leptin Receptors

To date, there are six known isoforms of leptin receptor, each of varying length and putative roles, designated LepR (Leptin receptor) a, b, c, d, e, and f , with LepRf only found in rats (Lee, *et al.*, 1996; Wang, *et al.*, 1996). These receptor isoforms are alternatively spliced and can be divided into three categories based on their structure: short isoforms, including LepRa, c, d, and f; the long isoform, LepRb; and a soluble receptor, LepRe (Tartaglia, *et al.*, 1995; Lee, *et al.*, 1996; Wang, *et al.*, 1996). Though there are differences in the length of the leptin receptors found between species, these receptors are homologous, with mice and humans sharing 78 % identity (Tartaglia, *et al.*, 1995; Chen, *et al.*, 1996). All receptor isoforms share a common extracellular domain to histidine 796, where LepRe diverges, resulting in a soluble receptor (LepRe), not associated with a cell. The other receptors share a transmembrane domain (34 amino acids) and all possess a Box 1 motif, involved in *Janus* kinase (JAK) signalling, with divergences at lysine 889 in the short receptor isoforms to a length characteristic of the particular isoform (Tartaglia, *et al.*, 1995; Lee, *et al.*, 1996; Wang, *et al.*,

1996) . LepRb, the long receptor isoform, is 1162 amino acids long and possesses a Box 2 motif, required for full interaction with the JAK and signal transducer and activator of transcription (STAT) pathway (Bjørbæk, *et al.*, 1997; Myers Jr., 2004; Frühbeck, 2006).

The leptin receptor has two cytokine receptor domain and a fibronectin type 3(CK-F3) binding domains in its extracellular sequence (Fong, *et al.*, 1998). A deletion and substitution study has shown that only the second of these sequences is critical in the process of leptin binding to the leptin receptor. The deletion of the first CK-F3 domain resulted in a leptin receptor still capable of binding leptin and signalling at a level comparable with the wild type receptor (LepRb), whereas substitution of the second CK-F3 domain (not deletion as this would have resulted in the movement of the first domain to the original position of the second domain) abrogated both receptor signalling and ligand binding (Fong, *et al.*, 1998). Leptin receptors are thought to exist at the cell surface in dimers, which bind leptin in a 1:1 stoichiometric ratio, forming tetrameric complexes (Devos, *et al.*, 1997; Fong, *et al.*, 1998). Upon ligand binding the receptors undergo a conformational change, allowing JAK2 to recognise the common Box 1 motif of the intracellular receptor domain and bind to the receptor, which is critical in any potential leptin signalling as leptin receptors possess no intrinsic tyrosine kinases (Ghilardi and Skoda, 1997; Sweeney, 2002). *Janus* kinase phosphorylates the intracellular tyrosine residues of the leptin receptor and the juxtaposed JAK2, which can then activate subsequent signalling cascades, depending on the receptor isoform (Bjørbæk, *et al.*, 1997; Ghilardi and Skoda, 1997; Sweeney, 2002).

Membrane bound leptin receptor isoforms (i.e. all isoforms except LepRe) appear to be internalised upon leptin binding, or independently of ligand binding, before being enveloped in clathrin coated vesicles and degradation by lysosomes (Barr, *et al.*, 1999; Lundin, *et al.*, 2000; Belouzard, *et al.*, 2004). This may seem somewhat counterintuitive, as cytokine receptors are typically recycled back to the cell surface (Sweeney, 2002). However, studies have not supported this hypothesis, indicating that new receptors are synthesised and translocated to the cell surface to replace endocytosed receptors (Barr, *et al.*, 1999; Belouzard, *et al.*, 2004). Interestingly, the large majority of

newly synthesised receptors (75-95%) are not found at the cell surface, but remain associated with organelles (Barr, *et al.*, 1999; Belouzard, *et al.*, 2004), with LepRa reaching the cell surface more quickly than LepRb (Lundin, *et al.*, 2000). This has led to the suggestion that this affects sensitivity to leptin, especially in obesity (Frühbeck, 2006), as leptin levels are typically elevated (Considine, *et al.*, 1996).

Long Receptor Isoform

The long isoform of the leptin receptor, LepRb, has been found in a number of locations including the hypothalamus (Mercer, *et al.*, 1996; Funahashi, *et al.*, 2000), the small intestine (Barrenetxe, *et al.*, 2002) and in the salivary glands (Bohlender, *et al.*, 2003), while its mRNA is found in a variety of tissues including brain, heart, lungs, spleen, digestive tract, bladder, and adipose tissue (Löllmann, *et al.*, 1997). LepRb is the only isoform of the receptor to possess a Box 2 motif in its intracellular domain, which gives it the largest signalling capacity of the leptin receptor isoforms and has led some to refer to it as the 'functional receptor' (Frühbeck, 2006). The long receptor isoform was discovered as a result of the *db/db* mouse, in which a truncated version of this receptor is found. A 106 base pair insertion results in a stop codon at amino acid 894, causing LepRb to be synthesised at the same length as LepRa and preventing translation of the Box 2 motif (Chen, *et al.*, 1996; Lee, *et al.*, 1996). These mutants have been found to be insensitive to leptin administration, obese, diabetic and infertile, whereas *db/db* mice with an artificially induced LepRb showed leptin sensitivity (Kowalski, *et al.*, 2001). These studies clearly show that LepRb (because it possesses the Box 2 motif), and therefore STAT3 are critical in normal leptin signalling and the maintenance of homeostasis.

Similarly, other mutants expressing abnormal LepRb isoforms further demonstrate the importance of LepRb in normal homeostatic maintenance. Other rodents with mutations of LepRb display similar phenotypes as *db/db*, i.e. hyperleptinaemia, obesity, and diabetic characteristics. A mutation in rats, *fa/fa*, results from a point mutation of Glutamine 269 changing it to Proline, which is related to the Fibronectin 3 I (F31) domain of the extracellular domain (Iida, *et al.*, 1996; Takaya, *et*

al., 1996). This F31 domain has been postulated to not be necessary for leptin signalling (Fong, *et al.*, 1998), however this mutation would indicate otherwise. In *fa/fa* rats a point mutation at Tyrosine 763 in the extracellular domain results in a premature stop codon, resulting in the rats lacking any LepR isoforms (Wu-Peng, *et al.*, 1997; Ishizuka, *et al.*, 1998). In New Zealand Obese mice three mutations on LepRb are present at residues 541 (Valine → Isoleucine), 651 (Valine → Isoleucine) and 1044 (Threonine → Isoleucine) (Igel, *et al.*, 1997). The first two mutations are found in the second and third F31 domains and may prevent leptin binding to the receptor, while the third mutation is reported to be located between the motifs required for JAK/STAT signalling (Igel, *et al.*, 1997). However, the New Zealand Black mouse has the same mutations on LepRb, but has a much thinner phenotype (Igel, *et al.*, 1997). It is not clear how this can occur, however it may be possible that there are other leptin receptors that are presently undescribed or, as the authors hypothesise, that there is receptor mediated transport present in the New Zealand Black mouse that the New Zealand Obese mouse lacks. However, as the *fa/fa* rats lack any LepR isoforms and leptin is still found in the cerebrospinal fluid (Ishizuka, *et al.*, 1998), it is also possible that leptin is produced and acts in the central nervous system (CNS). Therefore, LepRb is critical to leptin signalling and mutations to it can drastically disrupt homeostasis.

After LepRb has been tyrosine phosphorylated by JAK2 other signalling proteins can bind to it, initiating signalling cascades. Among the most important of these proteins is STAT3, which recognises the Box 2 motif and binds to the phosphorylated Tyr1138 of LepRb, in turn, becoming phosphorylated (Vaisse, *et al.*, 1996; Hekerman, *et al.*, 2005). Substitution of Ser for Tyr at amino acid position 1138 of LepRb in adult mice (*s/*s mice) has been reported to result in mice that are unable to activate STAT3 with a phenotype similar to the *db/db* mutant, with some fertility and less pronounced hyperglycaemia and obesity (Bates, *et al.*, 2003). Once phosphorylated, STAT3 then associates and dimerises with the juxtaposed STAT3 (associated with the receptor in dimer conformation) and this phosphorylated dimer translocates to the nucleus of the cell where it regulates

gene transcription and thus processes, including cell proliferation, apoptosis and protein production (Vaisse, *et al.*, 1996).

Signal transducer and activator of transcription-3 has been found to be critical in many cytokine signalling processes, notably in embryogenesis where mouse embryos developed to lack STAT3 were found to cease development after approximately 7 days (Takeda, *et al.*, 1997). Experiments using *s/s* mice have shown that LepRb induced STAT3 signalling is necessary for the regulation of both appetite and glucose homeostasis (Bates, *et al.*, 2003). This was supported by similar experiments using another STAT3 deficient mouse (RIP-Cre $STAT3^{lox/lox}$) which is reported to be STAT3 deficient at the hypothalamus and pancreatic β-cells only; these mice also exhibited hyperglycaemia and hyperphagia (Cui, *et al.*, 2004). Both studies also reported that the respective models were hyperleptinaemic, suggesting that STAT3 signalling may contribute to negative feedback regulating leptin concentrations. However, there are significant differences in the mean concentrations of circulating leptin in the models, *s/s* (7 months old) at 56.8 ng/ml, and RIP-Cre $STAT3^{lox/lox}$ (8 weeks old) at 26.0 ng/ml (compared with controls < 5.0 ng/ml). Similarly, there are differences in insulin concentrations 28 ng/ml in the *s/s* mice, 0.559 ng/ml in the RIP-Cre $STAT3^{lox/lox}$ mice, 0.21-1.4 ng/ml in controls and glucose concentrations were 2522 µg/ml in *s/s* mice, 923 µ g/ml in RIP-Cre $STAT3^{lox/lox}$ mice and 770-1532 µg/ml in controls. It must be noted that it is unclear if mice were fasted in these studies and that data must be interpreted cautiously. As the RIP-Cre $STAT3^{lox/lox}$ mice lacked any STAT3 at the hypothalamus and pancreatic cell transplant from normal donors failed to significantly rescue the phenotype, it may be concluded that the hypothalamus is critical to regulating glycaemic concentrations and that LepRb recruitment of STAT3 is critical for this, as the *s/s* mice suffer from similar pathologies. The difference in the extent of these pathologies may be due in part to the methodologies used, i.e. it is unclear when blood was sampled or how this related to the light/dark cycle, which is important as leptin concentration varies, displaying a circadian rhythm in rodents and humans when normal access to food is available (Saladin, *et al.*, 1995; Sinha, *et al.*, 1996). The differences in reported leptin concentrations may also indicate that LepRb recruited

STAT3 signalling at the hypothalamus serves to regulate peripheral leptin concentrations; the lower peripheral leptin concentration in the *s/s* mice compared with the RIP-Cre STAT3$^{lox/lox}$ may be due to partial STAT3 signalling occurring in the hypothalamus. Since hormones other than leptin, e.g. insulin, can activate STAT3 (Coffer, *et al.*, 1997), crosstalk between hormones may be partially regulating a negative feedback loop between peripheral leptin and the hypothalamus. More research is required to determine if such mechanisms and crosstalk are involved in regulating circulating leptin concentrations.

Signal transducer and activator of transcription-3 is known to activate suppressor of cytokine signalling (SOCS) 3, a negative regulator of leptin signalling (Bjørbæk, *et al.*, 1998; Sweeney, 2002; Frühbeck, 2006). Though the site of action of SOCS3 on LepRb is contentious, whether it binds at Tyr 985 (Bjørbæk, *et al.*, 2000) or Tyr 1138 (Dunn, *et al.*, 2005) to prevent further STAT3 activation, it is generally accepted that SOCS3 does inhibit leptin activation of STAT3 (Sweeney, 2002; Hegyi, *et al.*, 2004; Frühbeck, 2006). Due to this action, it has been postulated that SOCS3 prevents excessive leptin signalling by regulating a negative feedback loop, where leptin signalling would be self-limiting (Bjørbæk, *et al.*, 1999; Dunn, *et al.*, 2005). Due to this action, in models of obesity where leptin concentrations are generally elevated and there is a putative resistance to leptin (Considine, *et al.*, 1996; Friedman and Halaas, 1998), SOCS3 has been implicated as a potential mediator of leptin resistance (Bjørbæk, *et al.*, 1999; Frühbeck, 2006), where overproduction of SOCS3 may potentially prevent leptin signalling, negating the normal weight reducing effects of leptin. Therefore, understanding SOCS3 role in regulating leptin signalling may be critical in understanding the development and potential treatment of human obesity.

The range of tissues expressing LepRb throughout organisms suggests that leptin has pleiotropy, with roles in the CNS and in the periphery. Supporting this theory is the fact that LepRb can also recruit a large number of other proteins including mitogen activated protein kinase (MAPK), insulin receptor substrate (IRS), phopholipase C (PLC), phosphatidylinositol-3-kinases (PI3K), phophodiesterase (PDE) and protein kinase B (Akt) (Sweeney, 2002; Hegyi, *et al.*, 2004; Frühbeck,

2006). However, these proteins may also be activated by other hormones, e.g. other cytokines or insulin in some cases, allowing for crosstalk between leptin and these other hormones (Sweeney, 2002; Hegyi, *et al.*, 2004; Frühbeck, 2006) demonstrating the complexity of the interactions of leptin which may influence, or be influenced by, other hormones. This complexity also makes it more difficult to interpret what role leptin plays when it recruits these proteins. Thus, the pleiotropy of leptin is probably due to the myriad proteins it recruits, and understanding how these interact when activated by leptin and other hormones will need to be explored further to understand the fundamental biochemical actions of leptin.

It is clear that LepRb is critical in leptin signalling and maintenance of homeostasis and that signalling at the hypothalamus is central to this process. However, it is not clear if there is a centrally regulated negative feedback loop for leptin, and if there is, whether intracellular proteins recruited by LepRb signalling can be influenced by crosstalk from other initiating hormones to affect leptin regulation. It may also be speculated that there could be a currently unknown isoform of leptin receptor that may mediate leptin signalling, which may explain the differences seen in the New Zealand Black and *Obese* mutants, despite both having the same mutations of LepRb.

Short Receptor Isoforms

At least one short isoform of leptin receptor, LepRa, has demonstrated a limited capacity for intracellular signalling, recruiting JAK2, MAPK, and IRS-1, though at much lower levels than those seen in LepRb (Bjørbæk, *et al.*, 1997). This is thought to be due to the lack of the Box 2 motif, that only LepRb possesses, resulting in a diminished signalling capacity for the short LepR isoforms (Ghilardi, *et al.*, 1996). While LepRb is critical in leptin eliciting its effects, the fact that LepRa does display some signalling capability shows that the short isoforms of leptin receptors may be exerting some effects via signalling in the tissues where they are found. However, due to the limited signalling capacity of the short receptor isoforms it has been hypothesised that they may play major roles related to leptin transport (Tartaglia, *et al.*, 1995; Banks, *et al.*, 1996; Bjørbæk, *et al.*, 1997; Golden, *et al.*, 1997; Bjørbæk, *et al.*, 1998; Hileman, *et al.*, 2000).

The main postulated role for short isoforms of the leptin receptor is as a transport mediator for leptin as leptin in circulation has been shown to enter the brain via a saturable process, indicating that the process is receptor mediated (Banks, *et al.*, 1996). This is supported by the finding of short leptin receptor isoforms at the choroid plexus of mice (where the LepRa was originally cloned from) (Tartaglia, *et al.*, 1995), humans (LepRa and LepRc) (Couce, *et al.*, 1997) and rats (Bjørbæk, *et al.*, 1998), and also in mouse cerebral microvessels which comprise the blood brain barrier (Bjørbæk, *et al.*, 1998; Hileman, *et al.*, 2002). More recent work has shown that cells expressing LepRa – LepRd can endocytose and subsequently exocytose intact (^{125}I- labelled) leptin, suggesting that all leptin receptor isoforms are capable of mediating leptin transport (Tu, *et al.*, 2007). Following intracerebroventricular injection of iodinated leptin in mice, high concentrations were found at the choroid plexi, further suggesting that short leptin receptor isoforms are involved in leptin transport (Maness, *et al.*, 1998), although it has also been reported that leptin leaves the brain via a non-saturable process, suggesting that this process does not involve leptin receptors (Banks, *et al.*, 1996). Additionally, lack of leptin receptors in mice has been shown to reduce the amount of exogenous leptin transported into the brain and that this is not reduced further by the administration of further leptin, suggesting another, non-saturable process of blood-to-brain transport (Hileman, *et al.*, 2002) and knockout of LepRa only reduces leptin transport into the brain by 25.6 % (Li, *et al.*, 2013).

Further support for the involvement of short isoforms of leptin receptor in leptin transport comes from the relative high densities of LepRa and LepRf in rat kidneys (Tartaglia, *et al.*, 1995; Wang, *et al.*, 1996; Fei, *et al.*, 1997), the main putative site of leptin clearance in vertebrates (Cumin, *et al.*, 1997; Meyer, *et al.*, 1997; Esler, *et al.*, 1998; McMurty, *et al.*, 2004). Leptin has been found to be excreted intact in the urine of rats (Cumin, *et al.*, 1997), where leptin binding in the kidneys has been found to be saturable (Serradeil-Le Gal, *et al.*, 1997; Hill, *et al.*, 1998), supporting the hypothesis that leptin is cleared by the kidneys and that short isoforms of leptin receptor are involved in leptin transport. Furthermore, bilateral nephrectomy in rats resulted in an increase in leptin concentration, although clearance was occurring elsewhere (Cumin, *et al.*, 1997). In chickens leptin is found to

accumulate in the lungs 4 hours after administration (McMurty, *et al.*, 2004), and in mice there is a high density of leptin receptors (both LepRa and LepRb) (Tartaglia, *et al.*, 1995; Fei, *et al.*, 1997) and LepRa mRNA (Löllmann, *et al.*, 1997), possibly indicating an alternate route of elimination, metabolism or a target tissue. This evidence demonstrating a transport role for the short isoforms of leptin receptors is supportive of the original adipostat theory, as these receptors potentially allow peripheral leptin to enter the CNS where it may exert an effect, notably at the hypothalamus.

Soluble Receptor Isoform

The soluble leptin receptor, ~116 kDa, is found in circulation where it is known to bind to free leptin, rendering it inert (Li, *et al.*, 1998; Huang, *et al.*, 2001). Although this would seem to negate leptin's ability for signalling, it seems that when leptin is released from this tetrameric complex (made up of 2 LepRe and 2 leptin molecules) that it is in an active form. This was seen in an experiment conducted in rats and mice, where adenoviruses were engineered to induce an overexpression of LepRe or β-galactosidase (control). In both species overexpression of LepRe resulted in increased leptin concentration without increased leptin expression. Moreover, when the viruses were administered to *ob/ob* mice and exogenous leptin was subsequently administered (12.5 µg/day), the mice which overexpressed LepRe demonstrated more exaggerated weight loss and decrease in appetite than the mice that overexpressed β-galactosidase over 12 days (Huang, *et al.*, 2001). There may also be other proteins which bind leptin in circulation, as in rats a protein of ~50 kDa has been found which binds leptin and subsequently releases it in a biologically active form, thus increasing the half-life of leptin (Hill, *et al.*, 1998). If these binding proteins are binding circulating leptin and increasing its half-life, this would potentially increase the duration of leptin's effects as leptin could be gradually released from these proteins during times of decreased leptin expression, e.g. fasting, effectively increasing the bioavailability and bioactivity of leptin (Huang, *et al.*, 2001).

It would appear that LepRe would be beneficial in preventing obesity by promoting and prolonging the effects of leptin. This is supported by the finding that in lean humans there is a higher proportion of circulating leptin bound to LepRe (32.9 %) than in obese people (18.2 %), with this

becoming more exaggerated during a 24hr fast (71.7 % versus 26.1 %, respectively). However, the concentrations of bound leptin are not exceptionally dissimilar at basal levels (5.2 ng/ml in lean subjects and 6.3 ng/ml in obese subjects) (Sinha, *et al.*, 1996) and as such the amount of bound leptin found in the obese subjects may represent a saturation of LepRe. It may be possible that action of LepRe contributes to leptin resistance by increasing its biological activity (Considine, *et al.*, 1996); however, it is possible that in lean subjects the leptin concentration is not high enough that LepRe has any significant effects promoting pathological change.

Physiology of Leptin

Central Effects of Leptin

The work of Kennedy (1950; 1953), Hervey (1959) and Coleman (1973; 1978) clearly indicated that appetite is regulated at the hypothalamus, at least in part, by a factor that was missing in *ob/ob* (leptin) mice and received by a mechanism missing in *db/db* mice (LepRb). The discovery and cloning of the obese gene and its product allowed the administration of leptin to animals in experiments to study its effects. The initial studies found that the leptin was capable of inducing satiety and reducing adiposity in *ob/ob* mice (Campfield, *et al.*, 1995; Halaas, *et al.*, 1995; Pelleymounter, *et al.*, 1995; Stephens, *et al.*, 1995) . Later studies also found that humans who did not produce any functional leptin, analogous to *ob/ob* mice, were hyperphagic and obese, and that leptin administration at approximately physiologic levels resulted in some correction of these conditions as well as some diabetic characteristics and increasing metabolic rate (Farooqi, *et al.*, 1999; Farooqi, *et al.*, 2002; Licinio, *et al.*, 2004). In animal models injections of leptin directly into the cerebral ventricles were found to act more quickly and at a lower dose than injections in the lateral tail vein in *ob/ob* mice indicating a role in the CNS (Campfield, *et al.*, 1995).

Further support for a hypothalamic role for leptin is found in receptor distribution of leptin receptors in the brain, notably LepRb, which has been found via several different methods (immunocytochemistry, *in-situ* hybridisation, RT-PCR) to be at particularly high density in the arcuate (ARC), dorsomedial (DMH), ventromedial (VMH), and the ventral premammillary nuclei of the hypothalamus, areas thought to be involved in the regulation of appetite and body mass (Mercer, *et al.*, 1996; Schwartz, *et al.*, 1996; Håkansson and Meister, 1998; Funahashi, *et al.*, 2000), as well as in the cerebellum (Elmquist, *et al.*, 1998). Further, an *in vitro* study utilising immunohistochemistry has shown that neurons expressing LepR in rat hypothalami activate STAT3 in response to incubation with leptin (Håkansson and Meister, 1998) and *in vivo* peripheral leptin administration at a physiologic dose (intraperitoneally or intravenously) is capable of activating STAT3 and SOCS3 in *ob/ob* and wild type mice, but not in *db/db* mice (Vaisse, *et al.*, 1996). This is consistent with the report of an increase in SOCS3 mRNA in the ARC of young (10 days) rats 30 mins after acute leptin administration (3 µg/kg body mass, given intraperitoneally), although no suppression of appetite was found, this may indicate that LepRb is critical in this signalling, but perhaps its effects cannot be elicited before a particular stage of development (Proulx, *et al.*, 2002). Although STAT3 activation occurs in response to other cytokines and growth factors (Aaronson and Horvath, 2002), these experiments support the hypothesis that leptin signals in the hypothalamus via LepRb as no STAT3 signalling was detected in *db/db* mice.

Interestingly, neurons of rat ARC, paraventricular (PVN), VMH and lateral (LH) hypothalamic nuclei have been found to stain positively for LepRb on their dendrites and somata (Funahashi, *et al.*, 2000), further implying a role for leptin to act at the hypothalamic level and that leptin is capable of directly acting on specific neurons to exert its actions. The administration of leptin intracerebroventricular suppressed appetite at much lower doses than when administered subcutaneously (0.6-40 µg/kg body mass versus 400-4000 µg/kg body mass, respectively), further implying that leptin acts in the CNS (Stephens, *et al.*, 1995). Localisation of labelled leptin administered *in vivo* shows accumulation at the ARC in mice (Banks, *et al.*, 1996), and the incubation

of mouse brain with labelled leptin results in leptin binding at the ARC, which doubles in concentration in brains from fasted (48 hours) mice (Baskin, *et al.*, 1999) and many neurons of the PVN, LH and ARC which stain positively for LepRb also stain positively for neuropepetide Y (NPY) and proopiomelanocortin (POMC) (Håkansson and Meister, 1998). In POMC neurons of the ARC in mice, leptin has shown that it can increase the number and frequency of action potentials by two mechanisms: depolarisation by activating a non-specific cation channel; and by decreasing inhibition induced by neighbouring NPY/gama-aminobutyric acid (GABA) neurons by hyperpolarisation (Cowley, *et al.*, 2001). Therefore, leptin is capable of directly and specifically stimulating, and inhibiting, neurons of the hypothalamus to elicit its effects.

The study of NPY neurons of adult rat ARCs has shown that leptin can also transiently (~ 15 mins) prevent ghrelin (which stimulates appetite) induced increase in cytosolic Ca^{2+} concentration and for the duration of the experiment (20 mins) inhibited orexin-A induced increase in cytosolic Ca^{2+} concentration (Kohno, *et al.*, 2008). Leptin is also capable of inhibiting the activation of glucose receptive ATP-sensitive K^{+} channels in the hypothalami of normal, but not *fa/fa*, rats, and it is proposed that leptin acts on these channels directly to hyperpolarise the neuron (Spanswick, *et al.*, 1997). This would provide a physiologic means for leptin to inhibit the signalling of NPY, and, if extrapolated to other orexigenic neurons expressing LepRb, may explain how leptin exerts its inhibitive effect on the orexigenic pathways. Similarly, POMC neurons, identified by green fluorescent protein expression in transgenic mice, are reported to be depolarised by non-specific cation channels (Cowley, *et al.*, 2001) and leptin fragment amino acids 22-56 causes a depolarisation in 82% and 67% of type I and II cells from rat PVN (Powis, *et al.*, 1998). These studies indicate that leptin is capable of selectively and directly influencing the neuronal signalling in hypothalamic nuclei thought to regulate appetite.

Perhaps more notable, leptin alters the amount of synthesis and release of other appetite regulating hormones produced in the hypothalamus, especially in ARC. In the *ob/ob* and *db/db* mutant mice both NPY and agouti related peptide (AgRP) are upregulated, compared with normal

mice (Stephens, *et al.*, 1995; Ollmann, *et al.*, 1997; Shutter, *et al.*, 1997), possibly partially explaining their voracious appetites, suggesting that if leptin were present and able to act it would suppress the expression of these hormones. Leptin administration to the *ob/ob*, but not *db/db* mice, results in a reduction in both NPY mRNA expression (Stephens, *et al.*, 1995) and AgRP mRNA expression (Mizuno and Mobbs, 1999) showing that leptin acts via LepRb to elicit these effects. *Agouti related protein* is known to act by antagonising melanocortin receptors which are necessary for α-MSH signalling and it has been shown that blockade of MC4-R results in a blockade of leptin signalling (Seeley, *et al.*, 1997) as well as increasing concentrations of circulating leptin, as indicated by MC4-R knockout mice (Huszar, *et al.*, 1997). In contrast, in *db/db* mice there appears to be no change other than the increase in AgRP expression (Shutter, *et al.*, 1997), which may suggest that MC4-R is not dependent on LepRb signalling. Therefore, the regulation of appetite is complex and relies on the interaction of many hormones and that in the case of leptin, MC4-R must not be antagonised in order for leptin to be able to signal in ARC POMC neurons.

In 5-6 month old *ob/ob* mice chronic (daily for 30 days) subcutaneous administration of 300 µg recombinant human leptin reduces the synthesis and release of NPY, while suppressing appetite (Stephens, *et al.*, 1995). In neonatal (3-10 day old) rats chronic (postnatal days 3-10) daily administration of recombinant murine leptin (3 mg/kg body mass) intraperitoneally significantly increased SOCS3 and the anorectic POMC mRNA levels and reduced orexigenic NPY mRNA levels in the ARC, while appetite was unaffected, suggesting that leptin's anorectic traits are only attained after a certain stage of development (Proulx, *et al.*, 2002). Conversely, ghrelin administration can antagonise the action of leptin in the NPY pathway of rat hypothalami, when administered intracerebroventricular ghrelin lessens the reduction of food intake induced by leptin, while also negating and reversing leptin's effect of reducing NPY mRNA expression (Shintani, *et al.*, 2001). This suggests that leptin and ghrelin have an antagonistic relationship regulating appetite and energy balance.

Leptins' effects on appetite and metabolic rate have been postulated to mediate an adaptation to fasting, signal ling the transition between fed and fasted states. Leptin concentrations increase following a meal and decline hours after the meal (Hardie, *et al.*, 1996; Weigle, *et al.*, 1997; Elimam and Marcus, 2002), thus following a meal appetite is suppressed and metabolic rate would be expected to increase. Conversely, during periods of fasting leptin concentrations are dramatically reduced from normal levels increasing appetite and reduced thermogenesis are observed (Ahima, *et al.*, 1996; Döring, *et al.*, 1998). These adaptations would clearly be advantageous in an environment where caloric availability is limited, sporadic or seasonal, such as a famine, as lower leptin concentrations would promote food seeking behaviour while simultaneously reducing energy expenditure. Consequently in modern society with intensive agricultural systems, where a high energy diet is widely available, leptin concentrations are higher more often, notably in most cases of obesity, which is thought to contribute to leptin resistance (Considine, *et al.*, 1996). In this setting the adaptations to survive famines appear to correlate with effects that are detrimental to longevity and quality of life.

It is well established that leptin acts at the hypothalamus to regulate diet and body mass and that mechanisms for transport from the periphery to the CNS have been identified (Banks, *et al.*, 1996). However, the extent to which peripheral leptin acts in the CNS is not known and the production of leptin in the CNS has been suggested, potentially confounding the earlier hypothesis that leptin signals from the periphery to the CNS (Morash, *et al.*, 1999; Wilkinson, *et al.*, 2000). It has been known that leptin can leave the CNS and enter the peripheral circulation as cerebrospinal fluid is reabsorbed, which was thought to be a mechanism to clear leptin from the brain (Banks, *et al.*, 1996; Maness, *et al.*, 1998). Interestingly, studies in humans have shown that there is a net efflux of leptin from the brain, notably in women and obese (BMI > 28 $kg.m^{-2}$) men, when comparing afferent and efferent blood supplies of the brain (Esler, *et al.*, 1998; Wiesner, *et al.*, 1999). Whether this occurs in other vertebrates is presently unclear, though the entry of leptin into the circulation from the CNS (Banks, *et al.*, 1996; Maness, *et al.*, 1998) may support such a theory. It also appears that leptin

produced in the brain does not all act locally, as intracerebroventricular injection of iodinated leptin was found in the hypothalamus at lower concentrations than those found when administered intravenously, indicating that leptin transport from the periphery is important in directing leptin to hypothalamic targets (Maness, *et al.*, 1998). If a significant amount of leptin is produced in the brain, this may represent a negative feedback loop to regulate leptin production. Thus, leptin produced in the periphery appears to leave the circulation to enter the brain to exert an action, while leptin is simultaneously produced in the brain and enters the circulation, possibly as a central regulator of leptin's peripheral actions.

Systemic Effects of Leptin

Leptin and the Digestive Tract

Leptin has many roles outside of the CNS and has been found in the saliva of humans and rodents, and RT-PCR and RIA have indicated that the salivary glands are indeed the source of this leptin (Gröschl, *et al.*, 2001; Bohlender, *et al.*, 2003). In humans the concomitant collection of blood and saliva showed a strong positive correlation between blood and saliva leptin concentrations, suggesting that leptin in the saliva is at least in part derived from plasma leptin, although presence of mRNA indicated the production of leptin in the salivary glands is likely (Gröschl, *et al.*, 2001). LepRb has been co-localised on salivary glands in rodents, suggesting a paracrine/autocrine role for leptin in the oral cavity (Bohlender, *et al.*, 2003). However, when the functions of saliva are considered, the initial chemical breakdown and lubrication of chyme and its passage along the digestive tract, it seems plausible that leptin may play a role in more distal parts of the digestive tract, especially as leptin receptors are found in the lumen of the stomach (Gröschl, *et al.*, 2001) and in the intestine (Barrenetxe, *et al.*, 2002).

The stomach has also been identified as a source of leptin in rodents, where it is produced in epithelial cells of the fundic region comprising of chief cells (which produce digestive enzymes) from which it is released into the lumen, as well as being produced in endocrine P-cells, suggesting roles for stomach derived leptin both within and outside of the digestive tract (Bado, *et al.*, 1998; Cinti, *et al.*, 2000). One report which focussed on the corpus suggests that leptin is simply stored and released in the epithelial cells of the stomach as no leptin mRNA was found (Breidert, *et al.*, 1999). However, both Bado *et al* (1998) and Cinti *et al* (2000) reported leptin mRNA, suggesting leptin production in the stomach. While conflicting, these reports may be compatible with one another, possibly representing regional differences in leptin production within the stomachs of mammals. More recently, it has been shown that leptin is released from stomach tissues into the lumen bound to a protein, which corresponds with the external domain of LepR (produced by cleavage – not LepRe), conferring the complex with resistance to proteolysis by gastric juices (Cammisotto, *et al.*, 2006). Free leptin though is relatively stable in the lumen of the stomach (Sobhani, *et al.*, 2000), supporting the hypothesis that leptin plays a role in more distal parts of the gastrointestinal tract (GIT).

The role for leptin in the stomach remains to be completely elucidated, but evidence shows that leptin in circulation perfusing the GIT can mediate appetite regulation. Administration of leptin via the celiac artery in rats significantly reduced the size of the meal taken and co-infusion of cholecystokinin significantly further reduced meal size. Conversely, administration of leptin via the jugular vein had no significant effect on meal size and vagotomy ablated the satiety effect of leptin administration via the celiac artery, indicating that vagal afferents are involved in leptins regulation of meal size (Peters, *et al.*, 2005; Peters, *et al.*, 2006). Similarly, the administration of leptin directly into the stomachs of anaesthetised neonatal rats has been shown to stimulate brain stem neuronal activity, illustrating that vagal afferents probably are involved in satiety responses from gastric leptin (Yuan, *et al.*, 1999). This suggests that part of leptins role in regulating appetite occurs in the periphery, signalling via the vagus to the CNS.

Apart from a possible role in appetite regulation, the presence of leptin receptors in the stomach has also been postulated to provide a means for leptin to regulate protective actions for the stomach and pancreas. Experimental data show that pre-treatment with intraperitoneal leptin to rats receiving experimentally induced lesions (by intragastric administration of acidified aspirin, 75% alcohol or by water immersion restraint stress) reduced the number and size of gastric lesions (Brzozowski, *et al.*, 2000). The postulated protective action is that leptin induces gastric mucosal secretion containing prostaglandin E2 (PGE2), an increase in gastric blood flow and increased secretion of bicarbonate; it is unclear whether leptin or PGE2 is responsible for these changes, but PGE2 must be present for leptin to be able to bring on these changes. Thus, it seems plausible that leptin may cause the secretion of PGE2, which in turn, binds to prostaglandin receptors, resulting in the aforementioned physiologic changes (Takeuchi, *et al.*, 1997). Interestingly, these effects can be induced by intracerebroventricular administration of leptin, via signals from vagal efferent fibres (Brzozowski, *et al.*, 2000). This may demonstrate central regulation of gastric leptin secretion, possibly within the cephalic phase of digestion, however this would need to be investigated.

Both long and short receptor isoforms have also been found in the small intestine of rodents and humans, with long isoforms found in two distinct populations, those at the basolateral membrane and those at the brush border membrane (Lostao, *et al.*, 1998; Barrenetxe, *et al.*, 2002). This would suggest that intestinal tissues are responsive to both endocrine and exocrine (or autocrine/paracrine) leptin in some way, be it a regulatory role or simply a transport role. There is further support for leptin interacting with receptors at the basolateral membrane as intravenous administration of ^{125}I-labelled leptin results in accumulation of radioactivity in the small intestine, suggesting that leptin in circulation binds to receptors at this level (Van Heek, *et al.*, 1996; Hill, *et al.*, 1998).

Studies suggest that leptin may regulate nutrient absorption by enterocytes. In rats intravenous leptin infusion reduces the expression of apolipoprotein A4 in the small intestine (Doi, *et al.*, 2001). Apolipoprotein A4 is necessary for the transport of lipids from the GIT into the lymph system and also for the formation of chylomicrons (Barrenetxe, *et al.*, 2002). Additionally, leptin administration

over a two week period has been found to be effective in reducing cholesterol absorption in *ob/ob* and wild type mice (Igel, *et al.*, 2002). However, it is not clear whether this was due to reduced apolipoprotein A4 synthesis or by some other means. These studies would suggest that endocrine leptin (i.e. leptin in circulation) is responsible for these changes, but it is not yet clear whether leptin leaves the circulation and enters the lumen of the GIT to act on receptors in the lumen of the GIT or if it simply acts on receptors accessible via the circulation, i.e. those found at the basolateral membranes.

Within the lumen of the GIT leptin has been shown to selectively regulate sugar absorption by enterocytes. *In vtiro* studies found that D-galactose absorption is reduced in the presence of leptin. This appears to be mediated by activation of PKA and PKC, causing an inhibition of SGLT-1 (Lostao, *et al.*, 1998; Barrenetxe, *et al.*, 2004). Conversely, the same authors found that in *ob/ob* and *db/db* mice leptin increased galactose absorption, possibly due to signalling via short leptin receptor isoforms (Iñigo, *et al.*, 2004). Luminal leptin has been reported to reduce active transport of glucose via SGLT-1, with a maximal effect seen 5 min after application of leptin (Ducroc, *et al.*, 2005). However, this inhibition of SGLT-1 may indicate the change to utilisation of GLUT2 and GLUT5 transporters for sugar absorption, as concomitant administration with fructose increased both GLUT2 and GLUT5 mRNA expression in the small intestine and increased the absorption of these sugars (Sakar, *et al.*, 2009).

Leptin has also been suggested to play a role in the maturation of the mammalian digestive tract and neonatal nutrient absorption regulation. Leptin has been found in the milk and its mRNA in the mammary epithelial cells of several species including humans, mice, rats, pigs, sheep, cows and goats (Casabiell, *et al.*, 1997; Estienne, *et al.*, 2000; Bonnet, *et al.*, 2002; Smith and Sheffield, 2002). Typically in these species the maternal blood leptin concentration rises through pregnancy until 1-2 thirds term before a gradual decrease around the time of parturition, although it is not known if leptin from the circulation is found in milk or if it is synthesised in mammary tissues (Bonnet, *et al.*, 2002). As leptin is found in the milk it follows that it may play a role in neonatal physiology, a theory that is

supported by the presence of leptin receptors in the GIT. In neonatal piglets receiving a milk formula and intragastric leptin supplement, the length of the small intestine, notably jejunum, was increased as the dose of leptin increased, although this was still shorter than the small intestine of piglets on natural sow milk (Woliński, *et al.*, 2003). In human milk, leptin is typically associated with fat globules, which have been found to stain positively for leptin (Smith-Kirwin, *et al.*, 1998) and skim milk formulae have reduced leptin concentrations, presumably due to the skimming process (Resto, *et al.*, 2001). This association of leptin with fat may indicate a role for leptin in facilitating fat absorption in the gut of the neonate, which is reduced in mice with LepRb knockout in the small intestine (Tavernier, *et al.*, 2014), allowing the access of a high energy food source. This would seem to contradict the earlier report of a reduction in apolipoprotein A4 production in response to leptin in 6 week old mice, however it should be noted that these mice were given a supraphysiologic dose (5mg/kg) (Morton, *et al.*, 1998). Thus, leptin found in milk may regulate the maturation of and absorption by neonatal digestive tract.

Distribution studies have shown that the liver is a target for leptin in vertebrates (Hill, *et al.*, 1998; McMurty, *et al.*, 2004), and recent research has examined the role of leptin in the liver. In mice it has been shown that intravenous leptin infusion (0.5 μg/kg/min) increases levels of glycolysis and glycogen synthesis, but this action is reliant upon adenosine monophosphate-activated protein kinase α-2 (a specific subunit), and without it leptin does not correctly regulate the process of hepatic glucose production (Andreelli, *et al.*, 2006). Intravenous administration of leptin to rats has also been found to decrease hepatic and plasma triglyceride concentrations and increase hepatic fatty acid oxidation, however these effects are attenuated in diet induced obese rats (Huang, *et al.*, 2006), which may be analogous to most human models of obesity, suggesting that leptin resistance occurs in this model. Leptin is apparently important in both gall bladder contractility and determining bile properties as leptin resistance can reduce gall bladder responsiveness (Graewin, *et al.*, 2005), whereas administration of leptin to *ob/ob* mice caused bile salts to become less hydrophobic, a trait associated with a reduction in dietary cholesterol (Hyogo, *et al.*, 2002).

The lack of investigation for leptin in bile leads to several interesting possibilities. Following intravenous administration of ^{125}I-labelled leptin to pregnant ewes, radioactivity was detected in the gallbladder; however it is unclear whether this represented intact labelled leptin or free isotope from degraded leptin (Hunt, 2003). This means that there are two possibilities that may be implied: that leptin is degraded in the liver and is eliminated via the gall bladder and digestive tract, which would be a novel route of elimination; or that leptin is concentrated in the bile in an intact form to exert an effect in the digestive tract. The latter seems a plausible explanation as a previous study has found that more leptin arises in the duodenum of rats than could be accounted for if leptin arose from the stomach alone. While there may be other sources of leptin in the duodenum, e.g. transport from plasma across the gut wall or pancreatic secretions (Guilmeau, *et al.*, 2003), the condition of leptin in the bile needs examination to determine possible roles and is a promising avenue for investigation.

Effects on Metabolic Rate

An important feature of leptin is its reported effect of increasing metabolic rate and energy expenditure. Both *ob/ob* and *db/db* mice have lower body temperatures than wild type mice (Bray and York, 1979) and leptin administration to *ob/ob* mice normalises this by increasing metabolic rate, as indicated by indirect calorimetry (Pelleymounter, *et al.*, 1995; Breslow, *et al.*, 1999). These changes were observed with a reduction in fat mass and an increase in lean body mass, and therefore the effect of leptin on metabolic rate is thought to contribute to fat catabolism. These experiments administered leptin peripherally (intraperitoneally and via subcutaneous infusion from an osmotic pump) and both reported a reduction in food intake, indicating that leptin had access to the hypothalamus. However, the level at which leptin induces an increase in metabolic rate is unclear, as it may occur at the hypothalamus, e.g. by increasing TRH; or peripherally, e.g. by increasing oxidation of fuels in muscles.

Interestingly, leptin appears to increase energy expenditure in some animals, not by increasing active metabolic rate, but by increasing resting metabolic rate. In small placental and marsupial mammals which utilise daily torpor (a state characterised by a reduction in metabolic rate

significantly below normal resting metabolic rate, reducing energy expenditure), it has been demonstrated that leptin administration inhibits the torpor entry in fasted animals (which more readily enter torpor) and attenuated the drop in metabolic rate and body temperature normally associated with torpor bouts, but fed animals were unaffected (Döring, *et al.*, 1998; Geiser, *et al.*, 1998). It has been proposed that this adaptation represents a primitive role for leptin (Geiser, *et al.*, 1998), which may be related to the proposed role for leptin in mediating changes between the fed and fasted states (Ahima, *et al.*, 1996). It would appear that the metabolic effects of leptin may be acquired with age, as neonatal wild type and *ob/ob* mice do not respond to peripheral leptin administration with regard to food intake or metabolic rate until 28 days of age in spite of relatively high concentrations of circulating leptin (up to 20ng/ml), suggesting a neonatal resistance to leptin (Mistry, *et al.*, 1999). This neonatal resistance to leptin may be due to either leptin being bound to circulating proteins, e.g. LepRe, or receptors may not be transcribed at levels sufficient to detect any changes elicited or possibly related to weaning. Thus, leptin regulates metabolic rate in adult mammals, but this regulation may be related to age and only become detectable sometime after weaning.

It is generally accepted that leptin does not play a major role in regulating metabolic rate in humans. One report has concluded that leptin has no effect on metabolic rate, based on 30 min of indirect calorimetry, but that leptin decreases fat mass by modulating eating behaviour (Kennedy, *et al.*, 1997). Although this seems a short time for such an experiment, it is supported by the study of a nine year old girl who was unable to synthesise leptin due to a congenital mutation, analogous to the *ob/ob* mouse mutation, where treatment with exogenous leptin at a physiologic dose resulted in a modest reduction in basal metabolic rate and total energy expenditure, as indicated by the doubly labelled water technique, suggesting that again weight loss observed was due to leptin inhibiting feeding behaviour (Farooqi, *et al.*, 1999). In contrast, another study utilising the doubly labelled water technique conducted in 123 five year old children, found that there was a moderate positive correlation between serum leptin concentration and total energy expenditure and serum leptin concentration and resting metabolic rate (Salbe, *et al.*, 1997). These reports contrast with those for

research animals; however it may be that the effects on metabolic rate in humans are more subtle than those in animal models.

Though it remains unclear if leptin plays a role in energy dissipation in humans, several studies of skeletal muscle from mice and humans show that leptin does indeed regulate fat metabolism and oxidation. Skeletal muscle preparations collected from mice have shown that incubation with leptin increases fatty acid oxidation by 42% in wild type mice, 26% in *ob/ob* mice, reduces fatty acid incorporation into triacylglycerol 35% and 44%, respectively, whereas insulin directly opposes these actions, increasing fatty acid incorporation into triacylglycerol 40% and 31% respectively and decreasing fatty acid oxidation 70% and 26% respectively (Muoio, *et al.*, 1997; Muoio, *et al.*, 1999). These actions appear to be reliant upon adenosine monophosphate protein-activated kinase, as pharmacological blockade inhibited leptin's oxidative action (Solinas, *et al.*, 2004). Perhaps more interestingly, skeletal muscle from lean human donors is responsive to leptin administration, increasing fatty acid oxidation 103%, whereas skeletal muscle from obese donors was unresponsive to leptin, suggesting leptin resistance in obese donors. Obese donors' muscle also had very different basal metabolic characteristics, fatty acid uptake was 72% higher, and fatty acid incorporation to triacylglycerol was 102% higher, than values recorded in muscle from lean donors (Steinberg, *et al.*, 2002). When the role of skeletal muscles is considered, locomotion and thermogenesis, it is clear that preferential use of fuel may potentially have a considerable impact on the fat mass of an individual and illustrates that leptin does have a role in the periphery. Therefore, leptin is important in regulating fat metabolism outside of the CNS.

It has been demonstrated that leptin attenuates lipogenesis in skeletal muscle and white adipose tissue and upregulates the transcription of uncoupling protein (UCP)3 in brown adipose tissue (BAT) and skeletal muscle (Gong, *et al.*, 1997; Kawaji, *et al.*, 2001; William Jr., *et al.*, 2002). Uncoupling proteins are often found in the BAT of young and small mammals to generate heat independent of the citric acid cycle by bypassing ATP synthase (Lowell and Flier, 1997). In BAT the fat itself is oxidised as a fuel source and the action of UCPs means that heat is the only by-product of

the reaction, rather than generation of ATP (Gong, *et al.*, 1997; Lowell and Flier, 1997). This provides further evidence for leptin acting independently of the CNS, and the lack of fatty acid oxidation seen in skeletal muscle from obese donors (Steinberg, *et al.*, 2002) may also indicate that UCPs are not activated as they would normally once peripheral leptin resistance develops. It also shows that leptin is capable of inducing actions in the periphery to reduce fat mass.

Interestingly, leptin may have an effect on the thyroid, which is the main regulator of metabolic rate throughout the vertebrate body. Notably, leptin activates the PI3K pathway, which can also be activated by insulin, providing a point of cross talk in intracellular signalling (Frühbeck, 2006). This may be significant as TSH also stimulates the iodination of thyroglobulin via the PI3K pathway (Gardner and Shoback, 2007), and the implication that leptin may mediate this pathway, shows the vast potential leptin may have for affecting thyroid function and, therefore, metabolic rate. It may also be noteworthy that both leptin and T_3 upregulate UCP3, contributing to reduced fat mass (Gong, *et al.*, 1997). That leptin has some interaction with this important pathway may only be coincidental, it may indicate that leptin is closely linked to thyroid function and therefore metabolic rate regulation.

Many studies have examined the relationship between leptin and thyroid function, with conflicting results, particularly when pathologic thyroid conditions have been included or induced in the studies. Korbonits (1998) summarised many of these in her review, showing that studies in rodents have reached differing conclusions. For example, hypothyroid conditions have been associated with increased plasma leptin concentrations (Escobar-Morreale, *et al.*, 1997; Fain, *et al.*, 1997), while hyperthyroid conditions have been associated with both lower (Escobar-Morreale, *et al.*, 1997) and higher (Yoshida, *et al.*, 1997) plasma leptin concentrations. Korbonits' (1998) review also indicated that in humans hyperthyroidism has not been reported with significant changes in plasma leptin (Corbetta, *et al.*, 1997; Mantzoros, *et al.*, 1997; Valcavi, *et al.*, 1997; Leonhardt, *et al.*, 1998; Pinkney, *et al.*, 1998; Yoshida, *et al.*, 1998), but that hypothyroidism has been found in conjunction with normal plasma leptin concentrations (Corbetta, *et al.*, 1997), lower plasma leptin (Leonhardt, *et al.*,

1998; Pinkney, *et al.*, 1998) and higher leptin concentrations (Valcavi, *et al.*, 1997; Yoshida, *et al.*, 1998). This shows a lack of consensus in how, or if, leptin and the hypothalamic-pituitary-thyroid axis interact.

Further research over the last decade has not provided much clarification on the interaction between leptin and thyroid function. In rodents the administration of 8 µg/100 g body mass of leptin (a supraphysiologic dose) has been shown to increase TSH secretion *in vivo* (Ortiga-Carvalho, *et al.*, 2002; da Veiga, *et al.*, 2004) and that concomitant administration of leptin with T_3 increases O_2 consumption above what would normally be induced by T_3 alone (Wang, *et al.*, 2000). In contrast, incubation of pituitaries of rats with leptin either inhibited TSH release in normal animals (Ortiga-Carvalho, *et al.*, 2002) or had no effect in hypothyroid animals (da Veiga, *et al.*, 2004). In humans one study of normal and leptin deficient patients has shown that leptin and TSH concentrations are highly correlated in normal subjects, with similar patterns of secretion detected over diurnal cycles. In the leptin deficient patients, heterozygotes showed a weaker positive correlation between leptin and TSH concentrations, while a leptin deficient homozygote had a totally disrupted TSH secretion profile (Mantzoros, *et al.*, 2001). Several other studies have supported this hypothesis, reporting that decreased leptin concentrations are found in conjunction with decreased TSH concentrations (Hsieh, *et al.*, 2002; Rosenbaum, *et al.*, 2002; Menendez, *et al.*, 2003). However other studies have shown that there is either no relationship between leptin and TSH (Yaturu, *et al.*, 2004) or that increased thyroid function is found with decreased leptin concentrations (Isozaki, *et al.*, 2004; Oge, *et al.*, 2005).

The inconsistencies about leptin and the regulation of thyroid hormones may not be easily explained; however in several articles there is an underlying trend for leptin and thyroid hormones (i.e. TSH, T_3, and/or T_4) to have an inverse relationship (Escobar-Morreale, *et al.*, 1997; Fain, *et al.*, 1997; Pinkney, *et al.*, 1998; Isozaki, *et al.*, 2004). The inverse relationship may indicate a negative feedback loop where leptin suppresses thyroid hormone secretions and vice versa. This is suggested by the suppression of TSH secretion by rat pituitaries incubated with leptin (Ortiga-Carvalho, *et al.*, 2002; da Veiga, *et al.*, 2004) and the lack of effect on hypothyroid rat pituitary may imply that leptin

requires the presence of T_3 or T_4 to mediate some of its actions (da Veiga, *et al.*, 2004). In contrast, the positive relationship between leptin and thyroid hormones may in some cases be explained by the use of hypothyroid patients. In a hypothyroid patient TSH levels will often be relatively high as the body will attempt to increase metabolic rate, little or no T_3 or T_4 is released to act on the negative feedback loop at the hypothalamus. This may result in little inhibition of leptin release, resulting in high concentrations of both TSH and leptin, which would allow leptin to partially normalise metabolic rate. It should be noted that pathogenic thyroid conditions arise in different ways and as such any conclusions drawn from studies of these patients may result in differing hypotheses, as described. Thus, further study is required to determine the extent to which leptin and thyroid function are related.

Leptin in the Skin

Leptin and LepRb have been localised in the skin of mice where it is thought that leptin plays a role in wound healing. In the unwounded mouse skin leptin mRNA can be detected (Stallmeyer, *et al.*, 2001), and in experimentally wounded *ob/ob* and wild type mice, both intraperitoneal and topically administered leptin (0.1-10mg/kg/day) has been found to improve wound healing time (Frank, *et al.*, 2000; Ring, *et al.*, 2000). In wounded *ob/ob* mice nitric oxide dysregulation was also corrected (Kämpfer, *et al.*, 2003). Additionally, leptin has been found in the fluid of porcine wounds at up to 10 times the normal concentration found in plasma (Marikovsky, *et al.*, 2002), where it seems to regulate macrophage and neutrophil numbers (Goren, *et al.*, 2003) and may be regulated by a negative feedback loop, as indicated by the presence of SOCS-3 (Goren, *et al.*, 2006). As LepRb has been found in highly proliferative epithelial cells at wound margins in *ob/ob* mice where decreased healing time is observed (Stallmeyer, *et al.*, 2001), and *db/db* mice do not show any improvement in wound healing with the administration of leptin (Ring, *et al.*, 2000), it has been concluded that leptin mediates keratinocyte proliferation via LepRb as part of re-epithelialisation of wounds (Ring, *et al.*, 2000; Stallmeyer, *et al.*, 2001). This appears to be leptin's main role in healing, as several studies

have found that leptin does not induce angiogenesis in the tissues surrounding wound sites (Ring, *et al.*, 2000; Stallmeyer, *et al.*, 2001).

Further to a role in healing, leptin may be involved in regulating normal metabolic processes and development of skin cells. Acyl CoA:diacylglycerol acyltransferase (DGAT) is an enzyme which catalyses the final step in triglyceride synthesis in mammals (Cases, *et al.*, 1998). In mutant mice generated lacking DGAT1 (*Dgat*$^{-/-}$) post pubertal animals typically showed hair loss, poor water repulsion, poor thermoregulation (hypothermia) following water immersion, atrophic sebaceous glands, and fewer fur lipids can be detected compared with control animals (Chen, *et al.*, 2002). In contrast, *ob/ob Dgat*$^{-/-}$ mice did not present these abnormalities, and the authors proposed that leptin down regulates DGAT2, as it was found in the *ob/ob Dgat*$^{-/-}$ mice, but not *wt Dgat*$^{-/-}$ mice, therefore the *ob/ob Dgat*$^{-/-}$ mice may have had a compensatory upregulation of DGAT2 which was suppressed by leptin in the other mice. The *ob/ob Dgat*$^{-/-}$ mice did display the abnormal skin symptoms following central and peripheral leptin administration, supporting this (Chen, *et al.*, 2002). The authors concluded that the hypothalamus may control metabolism in the skin via efferent fibres; however as discussed earlier, LepRb is found in normal skin (Frank, *et al.*, 2000) and leptin may leave the brain at greater concentrations than it enters (Esler, *et al.*, 1998), suggesting that leptin may be capable of acting directly at the skin.

Leptin may also be involved in glucose metabolism and have an immune function in the skin. In recent studies the skin and hair have been shown to be sources of glucocorticoids, which increase glucose metabolism and inhibit immune reactions. Local production of cortisol in the skin has been hypothesised to allow immediate delivery of cortisol to required sites in response to skin damage without having to mobilise stores from the zona fasciculata (Sharpley, *et al.*, 2010). This is supported by *in vitro* work showing that hair produces CRH, ACTH, and cortisol; all the necessary hormones for a peripheral hypothalamus-pituitary-adrenal (HPA) axis analogue. Further, hair is capable of secreting cortisol in response to ACTH and cortisol production acts in a negative feedback loop to prevent further secretion of CRH, as is seen in the actual HPA axis (Ito, *et al.*, 2005). As leptin is capable of

recruiting inflammatory mediators (Loffreda, *et al.*, 1998), it may work antagonistically with cortisol in regulating immune reaction to skin injury. POMC and α-MSH are also found in the skin, interacting with the hypothesised HPA-axis analogue. In the hypothalamus these hormones oppose the actions of leptin on appetite; however in the skin they may be complimentary. POMC and α-MSH induce melanin synthesis in the skin, conferring protection against damage caused by ultraviolet radiation (Thody and Graham, 1998; Millington, 2006), while leptin may prevent damage caused by oxidation if exposure lasts 48-72 hours, by which time leptin induces the synthesis of protective proteins (Savini, *et al.*, 2003). Interestingly, leptin concentrations in the skin are independent of plasma concentrations. An experiment examining the effects of diet and exercise found that there was no correlation between plasma leptin concentration and leptin staining found in subcutaneous adipose tissue (Xie, *et al.*, 2007). Similarly, cortisol concentrations in the skin appear to be regulated independently of location, which may suggest an independence from the central HPA-axis (Sharpley, *et al.*, 2010).

The problem of leptin resistance, seen in most human models of obesity, is prominent too when examining leptin's role in the skin. Overexpression of leptin in keratinocytes in mice leads to leptin and insulin resistance. The resistance to these two hormones results in increased glucose metabolism, weight gain, organomegaly, increased bone mass, and impaired wound healing (Rico, *et al.*, 2005). Experiments using SENCAR (sensitivity to carcinogenesis) mice demonstrated that mice on a dietary calorie restricted regimen had lower plasma leptin concentrations than mice fed *ad libitum* with or without exercise, but all mice tested had similar concentrations of leptin in their skin. The calorie restricted groups had reduced PI3K and Ras recruitment following the application of 12-O-tetradecanoylphorbol-13-acetate, a cancer promoter in these mice, possibly indicating a reduced susceptibility to tumorigenesis in mice with lower plasma concentrations (Xie, *et al.*, 2007). It is not clear whether plasma leptin concentration had any effect on carcinogenesis, but it is generally accepted that leptin resistance develops in obesity and that obese patients tend to present with more health problems, including cancers (WHO/IASO, 2000; Australian Bureau of Statistics, 2005).

Therefore, dietary calorie restriction may be an effective part of treatment in preventing obesity and leptin resistance.

Leptin and Reproduction

One of the most important roles for leptin is that it appears to be necessary for animals to undertake pubertal development. As indicated by the *ob/ob* and *db/db* mice, a lack of leptin or leptin signalling results in an adult phenotype exhibiting infertility (Ingalls, *et al.*, 1950; Hummel, *et al.*, 1966), a lack of gonadotrophin secretion, and hypogonadism, presumably caused by the lack of gonadotropins (Batt, *et al.*, 1982; Cheung, *et al.*, 1997). In *ob/ob* mice, the administration of leptin rescues fertility, gonadotrophin secretion and reverses hypogonadism (Mounzih, *et al.*, 1997). In humans who do not secrete functional leptin, the same pattern of hypogondotrophic hypogonadism is reversed in adults and allows for appropriately aged children to undergo pubertal development, while not initiating puberty in very young children (Farooqi and O'Rahilly, 2005). In fact in a child with leptin deficiency, after one year of treatment with exogeneous leptin, pulsitile nocturnal FSH and LH secretion were detected, consistent with pubertal initiation (Farooqi, *et al.*, 1999). This clearly illustrates that leptin is necessary for pubertal development to occur.

It may be that leptin signals to the CNS, and possibly directly to the gonads, when the body has sufficient fuel stores to reproduce and therefore undergo puberty. As leptin rescues fertility in *ob/ob* mice (Mounzih, *et al.*, 1997) and it acts at the hypothalamus to induce appetite control (Campfield, *et al.*, 1995; Halaas, *et al.*, 1995; Pelleymounter, *et al.*, 1995), it follows that leptin may allow for pubertal development by acting at the hypothalamus. In girls suffering from anorexia nervosa, an 11 week program increasing their BMI resulted in increased plasma leptin, LH, FSH and free T_3 concentrations (Holtkamp, *et al.*, 2003), indicating that leptin was indeed acting at the hypothalamus. Further, it was reported that FSH and LH secretion was achieved once leptin exceeded a threshold concentration of 1.2ng/ml and 1.85ng/ml respectively (Holtkamp, *et al.*, 2003). However,

most of these girls still did not begin menstruating, suggesting that hypothalamic action alone is insufficient to initiate changes in reproductive status following a prolonged energy imbalance (Holtkamp, *et al.*, 2003). Another report has found that in anorexic patients LepRe is upregulated, compared with normal subjects, which may limit leptin's effects (Misra, *et al.*, 2004). This is intuitive, as leptin is catabolic, in a state of negative energy balance the body will attempt to conserve energy, but it shows that leptin receptor expression is also altered in anorexia. This may indicate that other receptors, notably LepRb, have their expression altered, which may be critical in regulating reproductive function.

Leptin receptors (LepRb) are expressed at the ovary, but *in vitro* these are upregulated at a low concentration (0.3ng/ml) and a 'normal' concentration (10ng/ml), in comparison with an intermediate 'normal' concentration (1ng/ml) (Di Yorio, *et al.*, 2008). Similarly, fasting in mice causes an upregulation of leptin receptors at the hypothalamus (Baskin, *et al.*, 1998). However, these experiments were only conducted over 4 and 48 hours, respectively, and so the effect of long term caloric restriction on leptin receptor expression in the ovaries is not clear. In male mice leptin receptors are expressed in the testes in cells which may facilitate the maturation of sperm (Herrid, *et al.*, 2008). As the ovaries and testes are homologous, leptin may act in the ovaries to recruit follicles at ovulation, but this needs examination. Thus, leptin may act directly on the gonads, as well as at the hypothalamus to regulate reproductive function and induce pubertal changes.

In rodents leptin administration is capable of inducing puberty. Several independent studies have shown that subcutaneous or intraperitoneal injections of leptin at supraphysiologic doses result in earlier vaginal opening, vaginal oestrous, and vaginal cycling. Leptin treated rodents also mate at a younger age and reproductive tissues are relatively heavier than those observed in control animals (Ahima, *et al.*, 1997; Chehab, *et al.*, 1997). Further evidence for leptin playing a role in puberty is seen in rats where leading up to vaginal opening there is a nightly peak in plasma leptin concentrations observed, which is most pronounced around the time of vaginal opening before a decline in dioestrous (Nagatani, *et al.*, 2000). While the studies using leptin administration

successfully induce earlier puberty, the large doses do not allow the examination of normal physiologic conditions, nor for the hypothesis that leptin initiates puberty to be properly explored. In fact it has been shown that rats treated with leptin and fed at 70% there is only a partial reversal of pubertal inhibition caused by food restriction compared with control animals (Cheung, *et al.*, 1997). This indicates that leptin may only be a permissive factor for puberty to occur, rather than the initiating factor, but due to the complex interaction of leptin with many other hormones, and the unknown site(s) of action in relation for leptin (i.e. hypothalamus, gonads, both or other sites) we can only speculate as to how leptin is involved in pubertal development. These data may not be conclusive as the large doses of leptin are unlikely to be observed in normal physiologic conditions; however, in rats a nocturnal peak in leptin concentration has been reported in the nights leading up to vaginal opening, when the peak was most pronounced in the study.

Conclusion:

In spite of the vast literature generated studying leptin, to date only two studies have attempted to describe leptin distribution in whole animals, and both of these found that very little leptin actually entered the brains of the animals studied (Hill, *et al.*, 1998; McMurty, *et al.*, 2004). This suggests that leptin may have major roles in the periphery. Thus, specific actions of leptin have been defined in certain tissues; however whether leptin preferentially targets specific tissues and its distribution over time (i.e. over a time course experiment) remains to be properly determined, as only one (Hill, *et al.*, 1998) or two (McMurty, *et al.*, 2004) time points were examined in the cited studies. This potentially has serious implications for the understanding of the physiology of leptin in the periphery as target tissues may not have been examined, nor has the amount of circulating leptin entering the digestive tract been determined. These and other aspects of leptin physiology in the periphery will be explored in the proposed experimental work.

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Leptin has Minimal Effects on Metabolic Rate in Mice

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This chapter has been removed as it has been submitted for publication elsewhere.

Pharmacokinetics of Leptin in Female Mice

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Short Title

Leptin distribution in mice

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Leptin in the Reproductive Tracts of Mice: A Description of Minor Targets of Leptin

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A Pharmacokinetic Model of Leptin Distribution

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Leptin Pharmacokinetics: Links Between the Digestive Tract and Plasma

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Short Title

Pharmacokinetics of Leptin and the Gut

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General Discussion and Conclusions

Since the discovery of its production in adipose tissue (Zhang, *et al.*, 1994), leptin has been known for its effects on energy balance, notably that it ameliorates the obese phenotype expressed in *ob/ob* mice, which lack endogenous leptin production (Campfield, *et al.*, 1995; Halaas, *et al.*, 1995; Pelleymounter, *et al.*, 1995; Weigle, *et al.*, 1995). The long form of the leptin receptor, LepRb, is found at high density in the hypothalamus and animals which lack a functional LepRb are unresponsive to leptin (Ghilardi, *et al.*, 1996; Lee, *et al.*, 1996). As leptin circulates at concentrations that correlate with fat mass (Considine, *et al.*, 1996) it has been proposed that leptin produced in the periphery signals the amount of energy in the periphery in the form of fat to the hypothalamus, thereby allowing animals to maintain a stable body mass (Friedman and Halaas, 1998).

In *ob/ob* animals leptin is known to normalise a low metabolic rate and body temperature (Pelleymounter, *et al.*, 1995; Breslow, *et al.*, 1999), but these results have been achieved by administering doses of leptin which range from approximate 10 times to several thousand times the circulating concentration in a normal mouse. Therefore, to examine the extent of the role of peripheral leptin in the regulation of metabolic rate a series of treatments were devised, including the administration of a supraphysiolgic dose of leptin and the administration of an anti-leptin antibody to 'normal' mice. It was found that leptin was capable of stimulating oxygen consumption, whereas the anti-leptin antibody reduced it slightly.

To examine the role of leptin in regulating metabolic rate mice were administered a high concentration of leptin or an anti-leptin antibody before mice were fed or fasted during 21 h of recording oxygen consumption. The recorded responses were very subtle and non-significant, with the anti-leptin treatment causing no apparent change in metabolic rate in either fed or fasted animals. The leptin treatment may have prevented a reduction in metabolic rate in fasted animals and caused a slight increase in metabolic rate in fed animals.

The maintenance of a normal metabolic rate in fasted mice in response to leptin administration is consistent with a previous report where leptin administration of 5 μg/g prevented the use of torpor (a

greatly reduced metabolic rate) in stripe faced dunnarts, where leptin may be signalling to the hypothalamus that there are increased energy stores available (Geiser, *et al.*, 1998). Similarly, the modest increase in metabolic rate in fed animals in response to leptin administration is consistent with previous reports (Mistry, *et al.*, 1997; Breslow, *et al.*, 1999) and with the hypothesis for leptin signalling to the central nervous system information about energy availability in the body (Friedman and Halaas, 1998).

This was the first experiment to examine the effect of removing circulating leptin on metabolic rate. It was shown that this treatment had no detectable effect on metabolic rate. The reason for this was unclear and it may be that leptin was produced in tissues, such as muscle (Wang, *et al.*, 1998), to maintain metabolic rate by means of a paracrine effect. Another possibility proposed was that leptin may have a limited role in the central nervous system and therefore its removal from circulation may have had a negligible effect on metabolic rate for this reason. Therefore, a detailed examination of leptin distribution may be justified.

To explore leptin distribution the first experiment examining its pharmacokinetics in females was performed. A physiologic dose was administered with samples taken over a 2 h time course. The experiment showed that the major roles for circulating leptin probably lies within the periphery as < 1 % of the administered dose was recovered in the central nervous system; however this does not preclude circulating leptin from having a role in the central nervous system as the hypothalamus may be very sensitive to leptin.

The major targets identified for leptin were the liver, skin, digestive tract (with contents) and kidneys, while the lungs contained a high amount of radiolabelled leptin per gram of tissue. A number of these target tissues form the interface between the body and the environment and as leptin stimulates immune responses (Loffreda, *et al.*, 1998; Farooqi, *et al.*, 2002), this may be a priming of tissues to respond to potential insult.

A large proportion of administered leptin was not recovered and is hypothesised to have entered into tissues not sampled extensively such as skeletal muscle and bones. Leptin has been reported to

stimulate oxidation of fatty acids in muscle (Muoio, *et al.*, 1999; Fabris, *et al.*, 2001; Minokoshi, *et al.*, 2002), whereas leptin is also known to stimulate haematopoiesis (Cioffi, *et al.*, 1996), which suggests that a portion of the unrecovered leptin may be in the flat bones.

Interestingly, intact leptin was recovered from the lumen of the digestive tract. Leptin is known to be secreted into the lumen of the stomach from the gastric epithelium (Bado, *et al.*, 1998) and its receptors are found in both the basolateral and mucosal surfaces of the stomach and small intestine (Sobhani, *et al.*, 2000; Barrenetxe, *et al.*, 2002) and leptin is known to regulate the expression of nutrient transporters in the small intestine, including SGLT1, GLUT2, GLUT5 and PEPT1 (Lostao, *et al.*, 1998; Buyse, *et al.*, 2001; Barrenetxe, *et al.*, 2002; Sakar, *et al.*, 2009). However, the fate of leptin in the digestive tract remains to be elucidated.

In order to determine if there may be sexual dimorphism in leptin distribution a time course experiment examining leptin pharmacokinetics in male mice was performed after a physiologic dose. As with the female mice major targets included the liver, skin, digestive tract (with contents) and kidneys. In male mice the same tissues were found to be major targets, but the accumulated dose in the digestive tract and contents was 23.2 % of the dose, almost double the proportion recovered from the same tissues in female mice. Furthermore, a lesser proportion of the administered dose was recovered in males than in females (78.7 % versus 95.3 %) and a greater proportion was estimated to have accumulated in skeletal muscle in males than in females (8.5 % versus 4.6 %). These findings seem to reflect sexual dimorphism in leptin distribution and possibly in leptin function.

After leptin administration a smaller proportion of the dose was recovered from the circulation of males than in females. This may simply have been due to the males being larger animals, while receiving the same dose, which may be implied by the similar calculated half-lives of administered leptin in both sexes. However, a greater proportion of the dose was recovered per gram of kidneys of the females (22.7 %/g versus 10.9 %/g), but the males had a much higher calculated plasma clearance rate for leptin (5.46 ml/kg/min versus 1.59 ml/kg/min). It may be that males have a higher clearance

of leptin from the plasma due to increased flow to the kidneys (Meyer, *et al.*, 1997) or due to greater access to leptin receptors (Coatmellec-Taglioni, *et al.*, 2003).

Following intravenous administration of radiolabelled leptin to male and female mice, radiolabelled leptin was recovered from the reproductive tracts of both sexes. In females < 1 % of the total administered dose was recovered from the ovaries and uterus, but in terms of dose per gram of tissue both tissues accumulated > 4 % dose/g, suggesting that leptin may play important roles in female reproduction. The pattern of recovery was irregular, which may have been due to the oestrous cycle not being accounted for as leptin receptor expression in the female reproductive tract varies over the cycle (Alfer, *et al.*, 2000; Kitawaki, *et al.*, 2000). Leptin is known to be important for embryo implantation (Antczak and Van Blerkom, 1997; Kitawaki, *et al.*, 2000; Malik, *et al.*, 2001) and inhibits the secretion of oestrogen (Zachow and Magoffin, 1997; Kendall, *et al.*, 2004), therefore it seems that these effects may rely on circulating leptin to some degree.

In the male reproductive tract a low level of leptin was recovered from the testes per gram of tissue (< 2 %/g), whereas much higher levels were found in the epididymides and seminal vesicles (> 3 %/g). In the testes leptin is known to inhibit the secretion of testosterone secretion (Tena-Sempere, *et al.*, 1999; Herrid, *et al.*, 2008), however its role in the epididymides and seminal vesicles is not clear. Leptin has been detected in the ejaculate of normal men at lower concentrations than reported for men with infertility and that concentrations are unchanged after vasectomy (Glander, *et al.*, 2002), suggesting that this leptin may be derived from the seminal vesicles and play a role in infertility. These data presented are the first to examine the distribution of circulating leptin in the reproductive tracts.

Based on the pharmacokinetic data from the study in female mice a nine pool model was developed using WINSAAM (Stefanovski, *et al.*, 2003). This is the most complex and comprehensive model describing leptin distribution to date and also predicts the size of pools of endogenous leptin and its flow in female mice. In both the fractional turnover and steady state models the major targets for

leptin from the blood were the 'other' tissues, comprising of tissues not measured in the original study including visceral adipose tissue and the musculoskeletal system.

In the fractional turnover model the tissues contributing the most leptin into the circulation were the skin and liver, both of which may represent proxies for other tissues. In the case of the skin subcutaneous adipose tissue was still attached and is a major producer of leptin (Van Harmelen, *et al.*, 1998). The model identified that the liver and digestive tract were major targets for circulating leptin, but that a negligible amount was eliminated via the faeces, which indicated that there may be reabsorption of leptin via the enterohepatic circulation, directing it back into the liver.

In the steady state model, the largest predicted pool for leptin was the digestive tract, but in both models very little leptin was predicted to be eliminated via the faeces. This seems to suggest that leptin is reabsorbed from the digestive tract, again suggesting that it will move via the enterohepatic circulation. The 'other' tissues were also found to represent a large pool for endogenous leptin, which would be consistent with visceral adipose tissue production of leptin (Van Harmelen, *et al.*, 1998).

To investigate the pharmacokinetics of leptin in the gut, mice were given an oral gavage of radiolabelled leptin at a physiologic dose and tissues were collected over a time course. Leptin was found to move aborally through the digestive tract, but in a 24 h elimination experiment < 1 % of the administered dose was eliminated via the faeces. This suggested that leptin was absorbed from the digestive tract, which was confirmed with approximately 3.5 % of the administered dose recovered from the plasma over a 2 h experiment and this was found to be 74 % intact, indicating that most of the absorbed leptin was in a form with potential signalling capacity.

The current study indicates that leptin was absorbed from the digestive tract and the previous data showing that leptin in circulation enters the lumen of the gut intact show that leptin is recycled between the gut and circulation. It is not clear what role this would play, but it may be that leptin may associate with digesta and it may regulate nutrient absorption. This has been indicated previously, as LepRb knockout from the small intestine conferred resistance to diet induced obesity on mice (Tavernier, *et al.*, 2014).

This thesis provides the most comprehensive examination of leptin pharmacokinetics to date. The novel findings provide potential new avenues for research in leptin physiology, particularly in relation to its role in the digestive tract and also in visceral adipose tissue and the musculoskeletal system, which have not been well characterised. Furthermore, the novel finding of leptin recycling between the circulation and the gut changes the paradigm for leptin pharmacokinetics in that it doesn't simply bind to a receptor and ultimately get eliminated in the urine. Further investigation may elucidate the fate of leptin in the body and whether this recycling of the hormone occurs in other tissues.

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