An *In Vivo* Investigation into the Actions of the Hypothalamic Neuropeptide, QRFP

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

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<td>26 RFamide peptide</td>
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<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
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<td>ABHD5</td>
<td>abhydrolase domain containing 5</td>
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<td>AgRP</td>
<td>agouti-related peptide</td>
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<td>αMSH</td>
<td>α-melanocyte-stimulating hormone</td>
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<td>analysis of variance</td>
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<td>arcuate nucleus of the hypothalamus</td>
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<td>SCN</td>
<td>suprachiasmatic nucleus</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-acetate-EDTA buffer</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
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<tr>
<td>TMN</td>
<td>tuberomammillary nucleus</td>
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<tr>
<td>Ucp1</td>
<td>uncoupling protein 1</td>
</tr>
<tr>
<td>Vglut2</td>
<td>vesicular glutamate transporter 2</td>
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<tr>
<td>VLPO</td>
<td>ventrolateral preoptic nucleus</td>
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<td>VMN</td>
<td>ventromedial hypothalamic nucleus</td>
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<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
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<td>WAT</td>
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<td>WT</td>
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Abstract

An In Vivo Investigation into the Actions of the Hypothalamic Neuropeptide, QRFP
Submitted by Christopher Cook for the degree of Doctor of Philosophy
The University of Manchester, 2017

Pyroglutamylated RFamide peptide (QRFP) is a peptide expressed at highest levels in the mediobasal hypothalamus, with lower levels of expression found in several tissues in the periphery. QRFP acts through a G-protein coupled receptor (Gpr103) of which, uniquely in rodents, there are two isoforms. Administration of exogenous QRFP induces feeding in satiated rodents, along with acute increases in locomotor activity and energy expenditure. Furthermore, a number of effects have been observed on tissues in vitro. It is currently unclear which of these actions may translate into a physiological function of QRFP. This thesis, therefore, aimed to elucidate the physiologically relevant functions of QRFP, through the use of new transgenic mouse lines, with studies being guided by results from the administration of exogenous QRFP.

By central injection of QRFP, we confirmed a number of in vivo responses: increased food intake, increased locomotor activity, increased energy expenditure and alterations in the handling of glucose. Our observations guided further study of these endpoints in our FlEx-Qrfp mouse (a Qrfp knock-out line), and two further lines lacking either of the Gpr103 receptor isoforms. Data from these receptor knock-out lines suggest some redundancy in the system, although expression of both receptors appears to be required for full QRFP-induced responses.

Following our observations that exogenous QRFP induced locomotor activity, irrespective of time of administration, we have begun to investigate the potential role of QRFP in the sleep/wake cycle. FlEx-Qrfp knock-out mice are hypoactive, particularly during the dark phase of the light cycle, providing evidence for an important role for QRFP here, similar to that observed with another hypothalamic neuropeptide, orexin. Subsequently we have conducted neuronal mapping studies, using a Qrfp-cre model. QRFP neurons are located in the mediobasal hypothalamus, surrounding the ventromedial hypothalamic nucleus. The more lateral distributed cells intermingle with orexin-containing neurons. We show that QRFP neurons project to regions associated with the control of arousal, such as the locus coeruleus, dorsal raphé nucleus, tuberomammillary nucleus and ventral tegmental area. Retrograde tracing was used to confirm the locus coeruleus connection.

Our FlEx-Qrfp knock-out mouse had no obvious metabolic phenotype when maintained on chow diet, but gained less weight when fed a high-energy diet. This was reflected by a lower fat mass in the adipose tissue of knock-out mice, coupled with lower fat storage in the liver. This body-weight phenotype was not caused by decreased feeding, or increased locomotor activity, thermogenesis or energy expenditure. FlEx-Qrfp knock-out mice showed an increased expression of lipolytic genes in white adipose tissue and liver. We have also shown that central administration of QRFP alters glucose handling, causing a blunting of the initial peak in glucose excursion following oral glucose loading. We propose that these two actions may be linked and we hypothesise that QRFP neurons may normally inhibit sympathetic output from the brain, thereby reducing sympathetic drive to tissues such as the pancreas and adipose tissue.
Declaration

The author declares that no portion of the work referred to in the thesis has been submitted in support of an application for another degree, or qualification, of this or any other university or other institute of learning.

Christopher Cook
March 2017

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Acknowledgement

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Secondly, to all the people behind the scenes that made this work possible: the BBSRC for funding my research; the staff in the BSF, without whom none of this would have been possible (in particular Ian, Mike, Brian, Emma and Allison whose expertise has made my life that much easier); my advisor David Bechtold; and finally all the members of the SML lab: the plethora of knowledge and expertise you possess is phenomenal, and the support and advice you’ve all offered has been a massive help, I’ve genuinely loved working alongside you all. Special mentions to Nic and Amy (for quite literally teaching me everything I know, back when it was just us three), Kelly (for all your help and expertise in the lab), Claire and Tansi (for never (openly) minding the endless questions – I hope you enjoy getting your desk space back!!), and Ed (it’s always nice to have a fellow PhD around, even if just for the solitary clap at the end of lab meetings!). I’ll miss being part of the lab (and not just for the cheese and wine nights…) and I hope you’ll keep in touch.

Which just leaves Simon: quite simply the best supervisor I could ever have hoped for. Amongst the horror stories it’s been a fantastic experience working in your lab. Despite the growth of the group, you’ve always been there to offer advice, a helpful pointer or a positive spin on another negative result (!), and the fact that so many of the group want to remain in your lab is testimony to you and the fantastic lab you have assembled. As a final word, I imagine it was a bit of a gamble taking me on, considering my total lack of knowledge of the brain before my PhD. I hope I have repaid your faith in me, and that my PhD has provided at least as many answers as questions!

Well then, I suppose it’s time for the main event…
Chapter 1
INTRODUCTION
1.1 Energy homeostasis

The ‘routine’ of our lives varies greatly from one day to the next, and differences in the amount of food we eat, or exercise we undertake, result in large fluctuations of overall energy levels across the full circadian profile. Despite these short-term changes, adult humans and other mammals possess a remarkable ability, to maintain a relatively stable body weight over long periods of time. This body weight maintenance is the product of energy homeostasis: a careful balance between energy intake, in the form of food eaten, and multiple contributors to energy expenditure. Whilst body weight, when viewed over long periods of time, appears to show stability, at any one time the body is in a state of either positive or negative energy balance. Positive energy balance, caused by high energy intake relative to energy expenditure, results in excess net energy which might be stored in the body, primarily in specialised adipose tissue depots. Negative energy balance, due to low energy intake relative to energy expenditure, results in an energy deficit which is balanced by the compensatory release of this stored energy. Rarely do energy intake and expenditure balance out perfectly over a 24-hour period, but alterations over the subsequent days act to maintain homeostasis.

That said, there are limitations in the ability to maintain stable body weight, as seen by the current world-wide trend towards obesity. Superficially, obesity is a very simple disease to explain (discounting rare monogenetic errors): chronically elevated levels of energy intake, in comparison with energy expenditure, leads to a state of prolonged positive energy balance, promoting storage of excess energy as fat in the body. Theoretically, therefore, reversing a state of obesity should be a simple case of inducing a state of prolonged negative energy balance, through increasing energy expenditure and decreasing intake. Of course, the practicalities are not this simple: it is well-documented that, despite short-term weight loss being relatively easily achieved, maintaining weight loss is much more challenging. It seems likely that the reason for this is evolutionary: historically, humans evolved as hunter-gatherers with much less access to food, and a requirement to actively participate in its acquisition. Evolutionary pressures have resulted in the development of mechanisms to avoid severe weight loss, and to promote efficient assimilation of energy when food is available. In the past, the limits of food availability, the work required to acquire it, and evolutionary pressure to avoid excessive weight (to allow efficient locomotion and escape from predators), will have acted to minimise the occurrence of obesity. However, this environment has changed which may have predisposed humans towards obesity.

We are beginning to understand the broader mechanisms that influence energy homeostasis, but the finer details, and how they amalgamate into a cohesive system, are still not fully understood. It has long been appreciated that both peripheral and central factors affect energy homeostasis, with their integration occurring in the brain. Signals from peripheral organs communicate the energy status of the animal, primarily in terms of glucose and adiposity levels, through negative
feedback systems. In turn, these signals are processed in the brain, and appropriate effector responses are triggered, which leads to further feedback from the periphery. As such, communication between the brain and the peripheral tissues leads to a constant fine tuning of the factors contributing to energy homeostasis.

The targets of some of the peripheral hormones involved in energy homeostatic signalling are relatively well characterised. For example, both ghrelin and leptin induce their central effects through interactions with neurons of various hypothalamic nuclei. Thus, the identity of some of the circuits activated by these hormones, and the molecular characteristics of neurons involved, are being elucidated. One thing that is clear is the complexity of the signalling pathways involved in energy homeostasis. A variety of signals enter the brain, conveying an array of messages about the periphery, which must be correctly identified, understood and reacted to. Multiple pathways have been reported that seemingly carry out very similar functions, leading to the suggestion of a high degree of redundancy. However, as we develop ever more advanced methods to investigate these pathways, the intricacies of their functions are revealed and we develop a better understanding of the nuances of each pathway. A pertinent example is the large number of neuronal populations that have been reported to positively influence feeding, all of which are termed ‘orexigenic’. However, as more details are uncovered, it is obvious that these populations can influence different aspects of the orexigenic response, such as food seeking, meal initiation and food preference. Constant analysis is required to update our models of energy homeostasis, and a number of neuronal populations in the hypothalamus are still poorly defined. The interaction of these populations within our current models is important to understand.

To this end, my PhD has focussed on defining a neuronal population in the basal hypothalamus, characterised by their expression of pyroglutamylated RFamide peptide (QRFP). In order to understand the role these neurons may play in energy homeostasis, it is important to appreciate our current knowledge of the signalling pathways involved, and the mechanisms responsible for both detecting and responding to changes in energy balance.

1.2 The control of food intake
The essence of maintaining energy balance relies on tight regulation of the mechanisms contributing to both sides of the homeostatic equation. The sole source of energy intake comes from food consumed, meaning its regulation is of utmost importance. Aberrant feeding, both excessive and insufficient, can result in serious health issues if left unchecked over the long term. Importantly, therefore, multiple mechanisms are in place to control food intake, primarily mediated in the brain in response to both internal and external signals. As discussed earlier, mechanisms to prevent drastic weight loss seem to be more robust and effective than those in place to prevent weight gain, as evidenced by an increasing world-wide prevalence of obesity (NCD Risk Factor Collaboration 2016).
There is constant demand for energy in the body, for example to carry out basal metabolic function. However, animals do not tend to consume food at regular, defined intervals through a 24-hour period. Rather, discrete meals are consumed, taking in far more energy than is immediately required by the animal, but which will sustain the animal until the next meal is consumed. Humans are a particularly extreme example, with the majority of energy being consumed in two or three meals, whilst rodents similarly exhibit bouts of feeding. Obviously, the circadian sleep/wake cycle, whereby a large proportion of time is spent asleep, has a major influence on feeding. Laboratory rodents show notably high levels of feeding in the first few hours of waking, with another spike in the final few hours before sleep (Ho & Chin 1988). Energy consumed in the period before sleep is particularly important for ensuring the animal has sufficient energy to last through sleep, meaning the animal has to effectively predict future requirements. This large intake of food leads to a strong positive energy balance, which develops into a strong negative energy balance by the time the animal wakes, causing compensatory feeding in the morning. However, in order to maintain energy homeostasis, food intake also is influenced by the size of previous meals: if a large amount of energy was consumed earlier, the subsequent caloric intake is decreased to account for this (Ho & Chin 1988). Therefore, feeding control is a delicate balance between hunger and satiety signals to ensure that feeding is initiated and terminated appropriately.

1.2.1 Peripheral signals regulating food intake

The primary hunger signal in the body is ghrelin, a hormone produced by the stomach (Kojima et al. 1999; Date et al. 2000). Levels of circulating ghrelin are influenced heavily by the energy status of the animal, with large increases following fasting (Tschöp et al. 2000), suggesting ghrelin is responsible for feelings of hunger that result in feeding. It is unusual that ghrelin is secreted from the stomach, but causes the majority of its actions on feeding by acting in hypothalamic brain regions (discussed below). Furthermore, it seems that sensitivity to ghrelin may also be increased in states of negative energy balance (Luckman et al. 1999). Importantly, during a meal, circulating levels of ghrelin decrease, thereby reducing its orexigenic action (Tschöp et al. 2001a).

However, simply reducing the orexigenic drive is insufficient to terminate feeding, and instead, specific signals convey satiation to the brain. Satiation is the sub-conscious response that brings about meal termination, and satiating signals are generated prior to any perceived fullness. Complementing the reduction in ghrelin signalling during a meal, satiation signals increase as a meal is consumed, with both hormonal and physical responses playing important roles, leading to meal termination before physical ‘fullness’ is reached. For example, gastric distension, the physical stretch of the stomach, is an inhibitory signal for feeding (Phillips & Powley 1996), whilst hormonal signals such as cholecystokinin (CCK) or glucagon-like-peptide 1 (GLP1) are secreted in response to nutrient intake, with synergistic actions working to reduce feeding (Schwartz et al.)
1991). These signals converge predominantly in the vagal complex of the hindbrain, either via central peptidergic action, or activation of vagal afferent nerves (Smith et al. 1981; Moran et al. 1990; van Bloemendaal et al. 2014). Furthermore, the secretion of ghrelin is inhibited by elevated levels of CCK (Gomez et al. 2004), providing evidence of an interconnected system acting in synergy to control feeding behaviour.

Interestingly, these short-term signals of satiation are insufficient to maintain long-term homeostasis, as seen by an inability of repeat CCK injections to reduce long-term food intake (West et al. 1984). This is because they only act to terminate eating; thus, while meal size is reduced, animals compensate for this by eating more meals to maintain long-term energy balance. A longer-term feeling of satiety leads to decreased meal frequency, as observed during periods of positive energy balance, after a large meal for example. Therefore, other signals are responsible for long-term regulation of feeding, with one particularly important hormone being leptin. Leptin is produced in adipose tissue, with circulating levels proportional to body fat content, and elevated levels of the hormone counteract the positive energy balance (Siegrist-Kaiser et al. 1997; Elmquist et al. 1998; Friedman & Halaas 1998; Frühbeck et al. 1998). This is achieved partly by enhancing sensitivity to peripheral signals such as CCK (Matson et al. 1997; Wang et al. 2000), to reduce food intake during periods of chronic positive energy balance when fat stores are already replete. Concordantly, weight loss results in decreased leptin levels, due to decreased fat content, which supports signals to increase food intake (Ahima et al. 1996).

Acting in a complementary manner to leptin, insulin is produced by pancreatic β-cells. The archetypal action of insulin is its ability to increase glucose uptake into insulin-sensitive tissues, during times of elevated glucose levels after a meal, and subsequently reduce hepatic glucose production. However, insulin may also regulate feeding and body weight through direct actions in the brain, as demonstrated by brain-specific insulin receptor knock-out mice exhibiting obesity (Brüning et al. 2000), and centrally-administered insulin acutely inhibits food intake (Mcgowan et al. 1993; Air et al. 2002). Furthermore, fasting and weight loss lead to a similar decrease in insulin as with leptin, leading to a reduction in the anorexigenic signals provided by these hormones. Importantly, insulin has been shown to act through similar mechanisms to leptin in the brain, with direct action on both neuropeptide Y (NPY) and agouti-related peptide (AgRP), and pro-opiomelanocortin (POMC) neurons (Cowley et al. 2001; Benoit et al. 2002; van den Top et al. 2004; Könner et al. 2007). Both leptin and insulin have inhibitory effects on NPY/AgRP neurons and stimulatory effects on POMC neurons, leading to the promotion of negative energy balance: reduced food intake and long-term reductions in body weight.

Leptin in particular, therefore, provides an inhibitory ‘brake’ on the orexigenic drive when adiposity levels increase; a mechanism which should prevent excessive energy intake. However, this system also protects against drastic weight loss, when a reduction in leptin signalling releases
the inhibitory tone on the orexigenic pathways, allowing them to become more active. Hypothetically, the orexigenic effects of ghrelin should also act in a similarly self-regulating manner as leptin: chronic positive energy balance during obesity does lead to reduced circulating ghrelin (Tschöp et al. 2001b), which should reduce the drive for energy intake, and restore homeostasis. Therefore, disruption of central leptin and insulin signalling causes obesity and hyperphagia, and loss of ghrelin signalling reduces weight gain and feeding. However, the systems in place seem inherently geared towards preventing starvation rather than weight gain, as suggested by the ease with which Western society is developing obesity.

1.2.2 Central aspects of food intake regulation

It has been recognised for decades that the hypothalamus plays a critical role in food intake. In particular, seminal work, using lesions and electrical stimulation of the lateral or ventromedial hypothalamus, identified these areas as a ‘feeding centre’ and ‘satiety centre’, respectively (Hetherington & Ranson 1940; Anand & Brobeck 1951; Elmquist et al. 1999). This crude model has been revised constantly over the subsequent half century, with specific orexigenic and anorexigenic neuronal populations identified in hypothalamic nuclei such as the arcuate nucleus (Arc), dorsomedial hypothalamic nucleus (DMN), lateral hypothalamic area (LHA), paraventricular hypothalamic nucleus (PVN) and ventromedial hypothalamic nucleus (VMN). Interconnectivity between these regions leads to a highly complex signalling network capable of modifying the homeostatic responses necessary to maintain energy balance.

In particular, the Arc occupies a region at the base of the hypothalamus, adjacent to the median eminence, which lacks a complete blood brain barrier, allowing access to circulating factors (Knigge & Scott 1970). The Arc contains two of the most important neuronal populations, in terms of feeding regulation: those co-expressing NPY and AgRP, and those expressing POMC. NPY/AgRP neurons strongly promote feeding when activated (Krashes et al. 2013), through connections with different neuronal populations in the hypothalamus, whereas similarly widespread neuronal connections containing POMC-derived peptides inhibit food intake, although they appear to influence longer-term responses (Zhan et al. 2013). The prepro hormone, POMC, is enzymatically processed to produce a variety of peptides, including α-melanocyte-stimulating hormone (αMSH). Concordantly, these neurons are differentially regulated by changes in energy balance: a negative energy balance promotes NPY/AgRP signalling to increase feeding (Hahn et al. 1998), whilst POMC neuronal signalling is increased under conditions of positive energy balance (Hagan, M et al. 1999). Primarily, AgRP is an endogenous inverse agonist for the melanocortin receptors (MC3R and MC4R), at which αMSH induces its effects, whilst NPY induces feeding through activation of its own Y1 and Y5 receptors (Figure 1.1). Furthermore, NPY/AgRP neurons also contain γ-aminobutyric acid (GABA) which is an inhibitory neurotransmitter, and local projections onto POMC neurons lead to strong inhibition of their activity.
Figure 1.1: Transduction of peripheral hormonal signals by NPY/AgRP and POMC neurons

POMC neurons signal primarily through the action of α-MSH on the MC3R and MC4R GPCRs, which are coupled to G_s-proteins, producing excitatory responses. NPY/AgRP neurons signal through three mechanisms: GABA, NPY and AgRP. Both GABA and NPY produce direct inhibitory actions on target neurons by interacting with GABA_A receptors and Y1/Y5 receptors, respectively. AgRP is an endogenous inverse agonist at MC3R and MC4R and so antagonises the activity of neurons with these receptors.

Ghrelin activates NPY/AgRP neurons to mediate its central actions, leading to an increase in their inhibitory output, including direct GABAergic connections on POMC neurons. Conversely, leptin and insulin inhibit NPY/AgRP neurons, thereby disinhibiting their target neurons. POMC neurons are also activated by both leptin and insulin, to increase their output, which is enhanced by the concurrent reduction of GABAergic inhibition from NPY/AgRP neurons.
Therefore, NPY/AgRP neurons actively promote feeding, by antagonising anorexigenic signals from, and directly inhibiting, POMC neurons. POMC neurons, on the other hand, inhibit feeding primarily through the release of the biologically active neuropeptide αMSH, to act at the aforementioned MC3R and MC4R receptors.

1.2.3 Integration of peripheral and central signalling mechanisms

These two distinct populations in the Arc are of critical importance for mediating the central responses to peripheral hormones: leptin, insulin and ghrelin. Ghrelin acts through its G-protein coupled receptor (GPCR), growth hormone secretagogue receptor (GHSR) to activate the phospholipase C pathway, leading to increased intracellular Ca\(^{2+}\) (Malagón et al. 2003). NPY/AgRP neurons express ghrelin receptors and around 40% of NPY/AgRP neurons are directly activated by ghrelin, with ghrelin-induced feeding inhibited by Y1 receptor antagonism (Nakazato et al. 2001). Due to the inhibitory inputs from the NPY/AgRP neurons, onto POMC neurons, this action of ghrelin also serves to indirectly inhibit POMC neurons, thereby potentiating its orexigenic effects.

Conversely, leptin binds to the leptin receptor (LepR), leading to phosphorylation of signal transducer and activator of transcription 3 (STAT3) and activation of the phosphoinositide 3-kinase (PI3K) signalling pathway, whilst insulin, acting through its own receptor (INS1), directly activates PI3K signalling, with both hormones producing similar effects (Varela & Horvath 2012). These actions are mediated by direct interaction with both NPY/AgRP and POMC neurons, where they activate POMC neurons but inhibit NPY/AgRP signalling (Cowley et al. 2001), to reduce orexigenic drive. Leptin and ghrelin also induce rapid rewiring of the excitatory and inhibitory inputs onto NPY/AgRP or POMC neurons (Pinto et al. 2004), to further modulate their orexigenic and anorexigenic actions, whilst further evidence also shows that ghrelin and leptin antagonise each other’s actions in the Arc via these neurons (Shintani et al. 2001; Kohno et al. 2007).

1.2.4 Interconnectivity of hypothalamic nuclei in feeding regulation

These two Arc neurons extend projections throughout the hypothalamus, making important connections with a variety of second-order neurons within nuclei such as the DMN, LHA, PVN and VMN (Figure 1.2). In particular, NPY/AgRP neurons inhibit their targets via direct action of GABA or NPY, or through AgRP-mediated inhibition of melanocortin receptors, in comparison to the excitatory inputs of the POMC neurons via α-MSH. A major orexigenic output of the Arc is mediated by connections by NPY/AgRP and POMC neurons onto second-order neurons in the LHA (Stanley et al. 1993; Atasoy et al. 2012; Betley et al. 2013). Furthermore, reciprocal connections back to the Arc demonstrate the interconnectivity of these feeding circuits (Horvath et al. 1999).
The LHA is a large, heterogeneous region of the hypothalamus that not only receives functional inputs from other hypothalamic nuclei, but also sends important projections widely in the brain to influence feeding (Figure 1.3). Two of the major neuronal populations in the LHA are characterised by their expression of orexin (Sakurai et al. 1998) or melanin-concentrating hormone (MCH) (Chambers et al. 1999). Orexin and MCH are expressed in separate neurons of the LHA, although the populations intermingle with one another (Peyron et al. 1998). Both populations play important roles in promoting food intake, as discovered by the ability of central administration of either peptide to elicit robust feeding behaviour (Edwards et al. 1999).

Furthermore, these neurons both receive innervation from NPY/AgRP and POMC neurons (Elias et al. 1998), projections that are differentially regulated by leptin in the Arc (Elias et al. 1999), highlighting their importance in regulating metabolic responses. It is also important to recognise that, whilst a large proportion of the response of hypothalamic neurons to leptin and ghrelin are

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**Figure 1.2: The interconnectivity of Arc NPY/AgRP and POMC neurons**

Two main populations of neurons in the Arc play crucial roles in the control of energy homeostasis: NPY/AgRP- and POMC-expressing neurons. These neurons are differentially responsive to peripheral hormones such as ghrelin, leptin and insulin (although these hormones can also act directly on other neurons in the brain, such as those in the LHA). NPY/AgRP and POMC neurons project widely in the hypothalamus, connecting with neurons in multiple nuclei to transduce their multiple effects on energy homeostasis.

**Left:** Ghrelin promotes a state of positive energy balance by enhancing NPY/AgRP signalling, which subsequently inhibits negative energy balance-promoting POMC neurons.

**Right:** Leptin/Insulin promote a state of negative energy balance by enhancing POMC signalling, and simultaneously inhibiting NPY/AgRP neurons.

Arc: arcuate nucleus; LHA: lateral hypothalamic area; PVN: paraventricular hypothalamic nucleus; VMN: ventromedial hypothalamic nucleus; 3v: 3rd ventricle
mediated via the Arc neurons, the LHA contains neurons that are directly sensitive to both ghrelin and leptin (Guan et al. 1997; Lawrence et al. 2002a; Leinninger et al. 2009). The roles of orexin and MCH in feeding behaviour are more complex than simply inducing feeding during times of homeostatic need: both peptides play active roles in hedonic feeding and reward-seeking behaviour (discussed in detail below). However, the mechanism by which these effects are achieved is different for each peptide, primarily due to their different projections. Orexin neurons project to the ventral tegmental area (VTA), where they synapse onto dopaminergic neurons and cause a release of dopamine in the downstream nucleus accumbens (NAcc) (Korotkova et al. 2003). MCH neurons, on the other hand, directly project to the NAcc (Sears et al. 2010). Interestingly, both peptides are active in reward-based tests such as operant responding, as demonstrated by impairments in rewarding aspects of feeding in models that lack signalling of either peptide (Sharf et al. 2010; Mul et al. 2011).

**Figure 1.3: The interconnectivity of the LHA in the control of feeding**

The LHA is an area of critical importance for both homeostatic and hedonic aspects of feeding. Projections from the LHA innervate neurons in major nuclei of the hypothalamus to regulate homeostatic feeding behaviour. Neurons in the LHA innervate the mesolimbic pathway in both the VTA and the NAcc. In particular, orexin neurons innervate dopamine neurons of the VTA, whilst MCH neurons act directly on the NAcc.

Arc: arcuate nucleus; DMN: dorsomedial hypothalamic nucleus; LHA: lateral hypothalamic area; NAcc: nucleus accumbens; PVN: paraventricular hypothalamic nucleus; VMN: ventromedial hypothalamic nucleus; VTA: ventral tegmental area; 3v: 3rd ventricle
Located medially to the LHA, the VMN is also innervated by projections from both NPY/AgRP and POMC neurons (Chee et al. 2010). The VMN is an important direct target for both leptin (Irani et al. 2008) and ghrelin (Lawrence et al. 2002a), similar to the Arc, confirming its importance in the regulation of feeding. Furthermore, specific deletion of leptin receptors in the VMN leads to increased weight gain (Dhillon et al. 2006). To date there are yet to be any peptidergic neuronal sub-populations identified within the VMN, meaning the pathways involving the VMN remain unclear, although the majority of neurons are understood to be glutamatergic. Importantly, as discussed below, the VMN is also a critical part of the nutrient-sensing pathways located in the hypothalamus, a function likely to be linked to its role in energy homeostasis.

1.3 Homeostatic and hedonic control of feeding

The existence of multiple mechanisms for stimulating feeding, and countering negative energy balance, suggest a degree of redundancy in the system, supporting the idea that endogenous systems may be primarily geared towards promoting energy intake. However, the multiple mechanisms for inducing feeding are likely to exhibit less redundancy than perhaps originally thought. Whilst neuropeptides that are capable of inducing food intake are frequently grouped as orexigenic, this terminology may lead to the misconception that each mediates the same response. However, as techniques become more advanced, it is becoming clear that many neuropeptides are actually mediating slightly different aspects of the feeding response. This has led to an appreciation that feeding is not a single process, but rather a series of steps from seeking and identifying food, the actual consumption of the foodstuff, and the continued consumption until satiation is achieved. For example, orexin and MCH both cause orexigenic responses, but it is now apparent that orexin’s actions are more involved in the initiation stage of feeding, whereas MCH’s are more associated with the continued consumption of energy dense foods (Barson et al. 2013). These subtle differences show that the different aspects of feeding are differentially regulated, and potentially open avenues to target these different aspects for future treatments.

It is now accepted that there are two different aspects contributing to food intake: homeostatic and hedonic feeding. Homeostatic feeding is the better understood of the two: it is the feeding response induced by metabolic needs, to return the energy balance to equilibrium. As discussed, homeostatic feeding is induced by negative energy balance and inhibited by positive energy balance. Hedonic feeding has been seen as non-homeostatic, in that it stimulates feeding in excess of the homeostatic needs of the animal. For example, whilst rodents are particularly efficient at maintaining consistent food intake and body weight, when fed a diet high in fat they will overeat and become obese. However, to consider the two as entirely separate processes would be wrong, as there are clear interactions between the homeostatic and hedonic systems.
The initiation of the feeding response, following the detection of negative energy balance, leads to feelings of hunger which are associated with discomfort and provide a negative valence to the animal. The consumption of food removes this hunger, which fulfils the homeostatic requirements of the system, but can equally be viewed as inherently hedonic: the removal of hunger is itself rewarding. In this way, the hedonic system is critical for homeostatic feeding, by providing the necessary drive to find and consume food. This is highlighted by the involvement of the dopaminergic system, which is classically associated with reward. Activation of dopamine neurons in the VTA leads to dopamine release in the NAcc and this increases the motivation of animals to obtain a reward (Di Chiara & Imperato 1988; Corrigall et al. 1994; Kim et al. 2012).

Indeed, activation of this pathway results in increased motivation of rodents to work for palatable, sweet or fatty foods. However, the dopaminergic system is also crucial for homeostatic feeding as well: mice lacking dopamine signalling will starve to death due to a lack of motivation to eat, even when food is readily available (Zhou & Palmiter 1995). Therefore, feeding of any kind is inherently rewarding, meaning that separating homeostatic and hedonic contributions entirely will lead to an incomplete understanding of regulatory processes.

The orexigenic effects observed with administration of some neuropeptides are often exacerbated when foods high in fat are provided. This suggests a contribution of hedonic feeding in the response (Brown et al. 1998; Choi et al. 2010; Perello et al. 2010), indicating that their effects on hedonic and homeostatic feeding are intrinsically linked. Furthermore, the incentive salience of food (its rewarding value) is also regulated according to the energy status of the animal, as food-deprived animals value food higher, and are willing to work more to obtain it (Sclafani & Ackroff 2003; Sharma et al. 2012).

Hedonic feeding is likely a major contributor to obesity, with the hypothesis that the majority of people would be their ideal weight without its influence. This is because fatty and sugary foods are inherently rewarding: consumption of these leads to activation of the aforementioned brain structures in the reward pathway (Hajnal et al. 2003; Valdivia et al. 2014). Importantly, it seems that the caloric content, rather than the taste, is responsible for this effect as loss of taste receptors does not reduce the effect (de Araujo et al. 2008). This makes evolutionary sense in an uncertain environment, where the time until the next meal is not known, and consuming large amounts of energy-dense food will provide sustenance between meals. However, in an environment where food is plentiful, a drive to consume excess calories is unnecessary and can lead to obesity. As a further contributor to hedonistic regulation of feeding, certain foods are ‘liked’ more than others. This ‘liking’ is manifested by sub-conscious responses to foods, whereby sweet and fatty foods elicit signals in the brain, primarily through the opioid system, that reinforce their consumption (Peciña & Berridge 2005; Peciña et al. 2006). Therefore, the natural
reward of eating energy-dense foods is reinforced by an inherent 'liking' of these foods, leading to a state of positive reinforcement for their consumption.

1.4 Control of energy expenditure

The other side of the energy balance equation is energy expenditure. Unlike energy intake, where feeding is the only input, expenditure is a product of a number of outputs: primarily physical activity, basal metabolism and adaptive thermogenesis. Basal metabolism is by far the largest contributor to the equation, responsible for around two thirds of total energy expenditure. Whilst it is known that basal metabolism slows down, thereby using less energy, with ageing, generally basal metabolism is proportional to lean mass and so remains relatively constant. As such, there are no physiological mechanisms that modify basal metabolism in order to maintain energy homeostasis. Contrastingly, physical activity is also a major contributor to energy expenditure, but its levels vary drastically during the day, and from one day to the next. Whilst humans actively engage in voluntary exercise (sometimes in an attempt to stimulate weight loss), this is a conscious decision and weight management in this manner is not encountered in other animals. There is little, if any, evidence to suggest that natural mechanisms exist to regulate physical activity levels specifically to regulate energy balance. Instead, increased physical activity in animals is primarily goal-oriented: finding food or a mate, or escaping predation, with energy expenditure a consequence rather than a specific goal. Furthermore, voluntary exercise is also known to be a fairly poor method of weight loss due to the accompanying increases in food intake and decreases in metabolic rate, resulting in a difficulty to maintain weight loss.

Importantly, adaptive thermogenesis is primarily responsible for maintaining constant body temperature in endothermic animals, and energy expended in this way is strongly influenced by environmental factors. Mammals maintain their body temperature in two ways: shivering and non-shivering adaptive thermogenesis. The latter involves activation of brown adipose tissue (BAT), composed of specialised adipocytes with a high number of mitochondria expressing uncoupling protein-1 (Ucp1). This Ucp1 is responsible for the production of heat in BAT by uncoupling the oxidation of substrates, such as fatty acids and glucose, from the production of adenosine triphosphate (ATP) which is how energy is usually stored in the body (Golozoubova et al. 2001; Nedergaard et al. 2001). By allowing protons to cross the inner membranes of mitochondria, Ucp1 facilitates the release of energy as heat rather than storing it in chemical bonds, which contributes to thermogenesis and maintenance of body temperature.

The primary control of BAT comes from the central nervous system (Bamshad et al. 1998), via noradrenergic neurons of the sympathetic nervous system that activate β3 adrenergic receptors on BAT adipocytes (Arch & Kaumann 1993). Whilst non-shivering thermogenesis in BAT is primarily activated in response to cold, consumption of high-energy diet can also activate this process, and it can be inactivated during times of strong negative energy balance to conserve
energy (Rothwell et al. 1984). Whether diet-induced thermogenesis contributes to energy homeostasis is a hotly contested debate (Rothwell & Stock 1997; Kozak 2010). There is a lack of evidence to show that diet-induced thermogenesis evolved as a mechanism to counteract excess energy consumption: returning to the earlier point, there is no evolutionary pressure to require a system such as this, and it seems inefficient for animals to be so wasteful with vital energy, by essentially ‘burning’ excess calories. One suggestion is that the process may have evolved to protect against harmful levels of fatty acids in the circulation, due to the requirement of fatty acids for the activation of Ucp1 (Fedorenko et al. 2012), and the development of a thermogenic system could well have conveyed a strong evolutionary benefit to euthermic animals, enabling them to become the dominant lifeforms on the planet.

1.5 Central control of wakefulness/sleep

Animals are usually either diurnal or nocturnal, and behaviours such as feeding must take place during the active part of the circadian profile. Unsurprisingly, therefore, the state of arousal of the animal is an important influence on energy status, which in turn regulates the sleep/wake cycle, promoting arousal for food seeking when energy levels are low, and vice versa. Hibernating animals are a specific example: consuming sufficient energy during the summer to survive sleeping through the winter without needing to eat. However, on a shorter timescale, all animals behave similarly to ensure they can survive the inactive period of the circadian profile. Consequently, incorrect control of the sleep/wake cycle can impede homeostatic mechanisms, leading to weight gain in individuals with irregular, or disrupted, sleep patterns (Roenneberg et al. 2012). There are two main pathways in the brain that regulate the sleep/wake cycle, comprised of ascending branches originating from well-defined populations of neurons containing monoaminergic neurotransmitters (Saper et al. 2001; Saper et al. 2005). Firstly, a branch originating in the upper brainstem innervates thalamic relay neurons responsible for transmitting information to the cerebral cortex. This branch extends from two main sources in the upper brainstem, the pedunculopontine (PPT) and laterodorsal tegmental nuclei (LDTg), both of which contain acetylcholine-expressing cells. These neurons are most active during wakefulness and rapid eye movement (REM) sleep and appear to promote REM sleep (Van Dort et al. 2015). The second ascending pathway bypasses the thalamic region, and is composed of other clusters of neurons in the upper brainstem and caudal hypothalamus (Jones 2003). In particular, noradrenergic neurons in the locus coeruleus (LC) (Aston-Jones & Bloom 1981; Berridge & Foote 1991), serotonergic neurons of the raphé nuclei (the dorsal, DR, and median nuclei in particular) (Sakai & Crochet 2001), dopaminergic neurons of the ventral periaqueductal grey (PAG) (Lu et al. 2006) and VTA (Miller et al. 1983) and histaminergic neurons of the tuberomammillary nucleus (TMN) (Parmentier et al. 2002) are all important in this pathway, and are all more active in periods of wakefulness than during sleep. Interestingly, two populations of neurons in the LHA,
orexin- and MCH-containing cells, are also involved in this pathway, complementing their roles in energy homeostasis (Estabrooke et al. 2001; Hassani et al. 2009).

There are many examples in the literature of arousal-promoting effects of orexigenic peptides, but orexin and MCH are likely the most important physiologically. It is important to note that, whilst both peptides induce robust feeding behaviour, overall their signals have differing effects on energy homeostasis, primarily due to their actions on energy expenditure. Loss of orexin signalling results in hypophagia but also obesity, due to reduced activity because of a phenotype similar to human narcolepsy, and a greater tendency to enter REM sleep (Baker et al. 1982; Chemelli et al. 1999; Hara et al. 2001; Willie et al. 2003). MCH-deficient mice, on the other hand, are hypophagic but exhibit a lean phenotype, due to hyperlocomotion, secondary to being awake more and exhibiting fragmented sleep (Shimada et al. 1998; Marsh et al. 2002).

Another critical area of sleep/wake control is the ventrolateral preoptic area (VLPO), which interacts with the ascending monoaminergic neurons to form a ‘flip-flop’ sleep switch. This electrical engineering term describes a system whereby activation of one half of the circuit inhibits the second, thereby disinhibiting its own signalling. The aim of such circuits is to produce rapid transitions between one state and another, a feature observed with transitions between sleep and wakefulness. In the brain, the VLPO inhibits the arousal-promoting monoaminergic neurons to consolidate sleep, whilst reciprocal connections inhibit VLPO activity and promote wakefulness. This is a critical aspect of the regulation of sleep, as lesions in the VLPO result in aberrant transitions in both directions between sleep and wakefulness (Lu et al. 2000). Whilst orexin is now hypothesised to primarily influence arousal (with the increase in feeding caused as a direct consequence of this) it may actually be important in consolidating wakefulness. This hypothesis is supported by the identification of orexinergic action in the LC, DR, TMN and VTA (Hagan, J et al. 1999; Nakamura et al. 2000; Brown et al. 2001; Eriksson et al. 2001) but not in the VLPO, thus consolidating the arousal state. Importantly, projections from the VLPO inhibit orexin neurons, thereby inhibiting their actions on target neurons, and acting to consolidate the sleep state (Saper et al. 2005).
Nutrient sensing in the hypothalamus

The entire basis for the contribution of central signalling to the control of whole body energy balance is predicated on the ability of the brain to process inputs detailing the energy status of the animal. Earlier, the contribution of signals originating in the periphery was introduced, and the mechanisms by which the brain integrates these signals and co-ordinates relevant responses was discussed. However, the brain itself also has an inherent ability to sense levels of nutrients which provides a more direct feedback on the nutritional state of the animal. In particular, the hypothalamus contains some of the densest regions of nutrient-sensing cells in the body, capable of detecting changes in a variety of nutrients and initiating signals within the brain, to induce adaptive changes to maintain the homeostatic environment.

Within the hypothalamus, brain regions important for nutrient sensing are often similar to those regions that play crucial roles in the regulation of food intake. This is not surprising given that nutrient sensing is not a passive process, and the brain is also required to mediate effector responses, in particular to stimulate or inhibit feeding as appropriate. In particular, the VMN, LHA and Arc have all been shown to contain neuronal populations that are sensitive to nutrients such as

\[\text{Figure 1.4: Central pathways for controlling the wake/sleep cycle} \]

Monoamine-containing neurons in the LC, DR, PAG, VTA and TMN, along with specific populations in the LHA (orexin, MCH and galanin), make up one ascending pathway for controlling arousal. The second ascending pathway originates in acetylcholine-containing neurons of the PPT and LDTg, and projects into similar cortical regions via thalamic relay neurons. The VLPO and LHA orexin neurons inhibit or activate, respectively, both pathways to consolidate wake/sleep states.

DR: dorsal raphé nucleus; LC: locus coeruleus; LDTg: laterodorsal tegmental area; LHA: lateral hypothalamic area; PAG: periaqueductal grey; PPT: pedunculopontine nucleus; Thal: thalamus; TMN: tuberomammillary nucleus; VLPO: ventrolateral preoptic area; VTA: ventral tegmental area

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as glucose, fatty acids and amino acids (Blouet & Schwartz 2010), although the VMN has long been considered the most important site.

### 1.6.1 Glucose sensing

Maintaining sufficient levels of glucose is fundamental to all processes in the body. This necessitates a fine-tuned mechanism to monitor, and respond to, changes in glucose levels in a rapid manner. The pancreas is the site of secretion of insulin and glucagon, responsible for smoothing peaks and troughs in glucose levels. Elevated levels of glucose, for example after a meal, trigger insulin secretion following detection by the β-cells via the GLUT-2 receptor. It has long been believed that direct sensing of glucose by the β-cell is the most important aspect of glucose regulation, but more recently evidence has been accumulating that the hypothalamus plays an important role.

Electrophysiological studies confirmed sensitivity of neuronal populations to changes in glucose levels, with glucose sensitive neurons located in different hypothalamic nuclei (Burdakov et al. 2005). Sensing a change in glucose levels, glucose-sensing neurons regulate both the sympathetic and parasympathetic branches of the autonomic nervous system (Verberne et al. 2014). This indicates that not only do glucose sensing mechanisms exist in the brain, but that they are functional and play a role in returning glucose levels to their ideal range. Central glucose sensing directly influences pancreatic insulin and glucagon secretion, via the autonomic nervous system (Jansen et al. 1997).

Furthermore, pharmacological inhibition of glucose sensitive neurons in the hypothalamus causes a significant reduction in the first-phase insulin release following glucose loading (Osundiji et al. 2012). The critical nature of glucose to neurons has been suggested as a likely reason why the brain has evolved an ability to directly sense glucose and influence glucose homeostasis. However, insulin and glucagon secretion are not the only responses induced by changes in glucose: it is also important for the body to alter other behaviours to efficiently respond to changing energy requirements. For example, foraging or food-seeking behaviour are induced by low circulating glucose levels, in order to find food to restore energy balance (Tkacs et al. 2007). Therefore, glucose-sensitive neurons in the hypothalamus are involved in multiple physiological processes to bring about adaptive changes to respond to glucose fluctuations.

### 1.6.2 Glucose-sensitive hypothalamic populations

Whilst the hypothalamus is one of the main regions of central glucose sensing, not all neurons here are involved, even within the nuclei mentioned earlier. For example, within the LHA, orexin neurons are hyperpolarised by increasing glucose, whilst MCH neurons are excited by the same stimulus (Burdakov et al. 2005). These populations are connected to regions of the brain with known sympathetic and parasympathetic function (Bittencourt et al. 1992; Peyron et al. 1998).
Importantly, orexinergic input into the dorsal motor nucleus of the vagus (DMV) is associated with increased excitation of the parasympathetic nervous input to the pancreas, and this pathway is simulated by hypoglycaemia (Wu et al. 2004). The opposite sensitivity of orexin and MCH neurons to glucose matches their opposing roles in mediating arousal and wakefulness. Complementing the fact that orexin neurons are excited by lowering glucose, these neurons promote arousal, and consequently feeding. Conversely, MCH neurons promote rest and sleep so their excitation by increased glucose leads to a resting phase when energy conservation is more appropriate.

Studies in other nuclei have been less conclusive in determining the molecular characteristics of glucose sensitivity. In the Arc, the small number of studies conducted seem to show that NPY neurons are inhibited, whilst POMC neurons are excited, by increasing glucose levels (Burdakov et al. 2005), or at least a sub-population of these neurons act in this way. It is also unclear whether these are direct effects of glucose on these neurons or whether this is the result of glucose acting at presynaptic locations. These data make sense in terms of their physiological functions: NPY positively influences energy balance by promoting feeding, whilst POMC neurons stimulate the opposite effect.

Finally, the VMN is an area that is critically involved in glucose sensing. However, sub-populations of VMN neurons have yet to be identified phenotypically, meaning that, whilst it is clear there are both glucose-excited and -inhibited neurons present (Burdakov et al. 2005), their identities are unknown. It is also necessary to note that the vast majority of VMN neurons are glutamatergic, meaning that they exert excitatory effects (Tong et al. 2007), but exactly where these interactions occur is also unknown. It is clear, though, that the hypothalamus has an important function in glucose sensing and, through its glucose-sensitive neurons, is capable of inducing behavioural changes in animals to redress the balance of energy levels.

1.6.3 Fatty acid sensing

Recent work has also begun to elucidate a similar sensing mechanism in the hypothalamus, responsive to changes in the levels of fatty acids. When first proposed, the ability of neurons to sense changes in fatty acid levels was underappreciated compared with glucose sensing, likely due to the greater importance of glucose as a fuel for the brain. The role played by fatty acids, in terms of conveying information about energy status, is less clear cut than glucose: it is appreciated that elevated fatty acid levels are indicative of an energy surfeit in the body, and are predictive of metabolic complications such as diabetes, but it is less understood why low fatty acid levels would be an important signal. Furthermore, levels of circulating fatty acids do not appear to correlate with obesity, but instead are markedly increased during fasting. However, it has been hypothesised that triglycerides, which are increased in obesity, are metabolised to fatty acids in the brain and could be disproportionally increased compared with circulating levels (Magnan et al. 2015). Whilst fatty acids can be used as a fuel, glucose is the more important
molecule, and the brain uses fatty acids as a fuel source very rarely (Schönfeld & Reiser 2013). It is clear, however, that sensing high levels of fatty acids in the brain could provide useful feedback on energy status, with mechanisms in place to reduce their levels to avoid long-term deleterious effects. Whilst it is less clear the importance of low fatty acid levels, in vivo studies clearly show that there are neurons in the hypothalamus that are either excited or inhibited by fatty acids (Le Foll et al. 2009). Reduced fatty acids may be a signal for more long-term negative energy balance, complementing glucose sensing, by signalling when the body is running low on adipose reserves. Fatty acid-sensing neurons are likely to interact with sympathetic neurons, which are responsible for modulating lipolysis, a possible mechanism to help relieve the metabolic stresses of negative energy balance.

Regardless of its importance, clear evidence supports fatty acid sensing in the brain, with particular focus on the neurons of the VMN. Around 40% VMN neurons are excited by oleic acid, a long-chain fatty acid, whereas around 30% are inhibited (Le Foll et al. 2009). This sensing mechanism can also translate into an in vivo response to fatty acids, as intra-arterial injection of a triglyceride emulsion with heparin (a combination that results in marked increase of fatty acid levels) towards the brain inhibits feeding (Moullé et al. 2013). It is known that fatty acids can diffuse into the brain, and it is suggested that their subsequent interaction with CD36 (a fatty acid transporter) as well as their metabolism to fatty acyl coenzyme A, are primarily responsible for their actions on neurons. Down-regulation of CD36 expression in the VMN results in reduced subcutaneous adiposity in rats (Le Foll et al. 2013), confirming the importance of both CD36 and fatty acid sensing in the VMN for the regulation of peripheral adiposity. Further evidence of the importance of fatty acid sensing comes from studies showing that obesity severely blunts the oleic acid-induced feeding suppression (Morgan et al. 2004).

### 1.7 White Adipose Tissue

Quite different to early views of adipose tissue as an inert tissue solely responsible for storing triglycerides, we now appreciate that white adipose tissue (WAT) is actually a dynamic endocrine organ, responsible for secreting some of the most important hormones in the body. During times of chronic energy imbalance, signalling from WAT becomes dysregulated leading to the potentiation of obesity, insulin resistance and cardiovascular diseases. Whilst its primary function, the storage of triglycerides, is largely dependent on the prevalence of fatty acids in the body, signalling from the autonomic nervous system is important in regulating storage and release of fat from the tissue.

During times of positive energy balance, when nutrients are abundant, WAT acts as a storage depot for fatty acids, which are taken up by adipocytes and converted to triglycerides for long-term storage. Triglycerides are stored within a lipid droplet in the adipocyte, which is surrounded by a phospholipid layer. These triglycerides can be broken down, by the process of lipolysis, and
released as fatty acids when negative energy balance occurs. The WAT, therefore, acts as a buffer system for fatty acids: increasing uptake and storage during times of abundance, and increasing release during times of nutrient deficit, perhaps caused by fasting or increased exercise. The release of fatty acids is critical to bridge any gaps between energy requirements and energy availability, to avoid severe negative energy balance. Increasing WAT stores is often portrayed as a bad outcome, given the global trend towards obesity; but, under normal circumstances, fat storage by WAT is a vital function (Listenberger et al. 2003). Adequate fat stores are required to provide protection to vital organs both as insulation and a barrier against physical injury, and sufficient fat stores are required for growth and reproductive processes. Furthermore, high levels of circulating fatty acids causes lipotoxicity, with evidence showing an association with insulin resistance and other complications of obesity and diabetes (Boden et al. 1994; Boden et al. 2005).

1.7.1 Endocrine function of WAT

One major function of WAT is the secretion of leptin, which plays a pivotal central role in modulating energy balance. The discovery of the obese gene (Zhang et al. 1994) led to the identification of leptin as the major hormone involved in maintenance of fat stores through a negative feedback loop: leptin acts on its receptor, LepR, to maintain energy balance if WAT is in excess. Leptin levels are positively correlated with WAT, providing feedback on the levels of adiposity in the body (Maffei et al. 1995). Importantly, short-term flux in leptin levels can drive adaptations in energy balance, without a significant fall in adiposity levels: for example, fasting induces a drop in leptin levels, independent of change in adiposity, which is itself a powerful promoter of positive energy balance (Ahima et al. 1996). The physiological roles of leptin were first investigated in studies into the phenotype of ob/ob mice (lacking the leptin gene) and db/db mice (lacking the leptin receptor), both of which develop severe obesity (Hummel et al. 1966; Friedman et al. 1991). The primary site of leptin-mediated activity is in the brain, via interaction with a wide range of neuronal targets, providing a direct mechanism for WAT tissue to modify overall energy homeostasis.

A second hormone released from WAT is adiponectin, produced by a gene very actively expressed in adipocytes. A primary role of adiponectin is to enhance the sensitivity of tissues to insulin (Berg et al. 2001), and the adiponectin receptor has been found in many insulin-sensitive tissues, such as skeletal muscle, liver and adipocytes themselves (Beylot et al. 2006). Contrasting with secretion of leptin, adiponectin levels are reduced in the obesity (Arita et al. 1999), contributing to the reduction in insulin sensitivity in the obese state. Conversely, weight loss leads to increased adiponectin secretion by adipocytes and produces improvements in insulin sensitivity (Yang et al. 2001). Adiponectin, therefore, plays an opposing role to leptin, in that it promotes positive energy balance, exemplifying the importance of WAT in overall energy homeostasis.
1.7.2 Neuronal control of WAT function

Whilst the hormones secreted from WAT can act in the brain to induce effects on whole-body energy balance, direct action of the brain on WAT was identified almost 120 years ago (Dogiel 1898). It is now well-documented that WAT receives dense neuronal innervation, in particular from sympathetic neurons of the autonomic nervous system, as identified by retrograde tracing (Bamshad et al. 1998). Despite previous assertions claiming the adrenal medulla was the primary stimulator of WAT lipolysis, studies now confirm that sympathetic input from the brain is necessary and sufficient to induce lipolysis in WAT (Bartness et al. 2014). In particular, monosynaptic retrograde tracing studies have labelled sympathetic neurons in the intermediolateral medulla (IML), the principal site of sympathetic output from the central nervous system (Bamshad et al. 1998). Building on this, modern techniques using pseudorabies virus (PRV) tracing have enabled identification of neuronal populations in the brain that control this sympathetic input. Put simply, PRV tracers behave like normal retrograde tracers, in that they infect neuron terminals and are transported back to the neuronal cell body. However, at this point they are capable of crossing back across synapses into the neuron terminals that innervate the cell body in which they have been transported. In this way they can be transported backwards through the pathway to identify higher order neurons that may be important in initiating signalling. PRV studies have, therefore, revealed that the sympathetic neurons of the IML receive input from nuclei across the hindbrain and midbrain regions of the brain (Bamshad et al. 1998). In particular, the nucleus of the tractus solitarius (NTS) plays an important role in sympathetic output, along with hypothalamic nuclei such as the DMH, PVN and LHA.

1.7.3 Intracellular signalling in the maintenance of WAT depots

There are two main mechanisms for increasing WAT stores: de novo lipogenesis, whereby triglycerides are formed from non-lipid precursors; and increased uptake of fatty acids from the circulation. De novo lipogenesis occurs more in rodents maintained on a diet high in carbohydrates but not fat (Ferramosca et al. 2014). By contrast, increased fatty acid uptake occurs when circulating levels of fat are high. Fat is transported round the body in chylomicrons or very-low-density lipoprotein particles, both of which contain large amounts of triglycerides. These are subsequently broken down by the action of lipoprotein lipase (Lpl), which converts them to fatty acids for uptake and storage in adipocytes (Mead et al. 2002). This process leads to increased WAT deposition in response to high levels of fat in the body, and is responsible for weight gain associated with overeating.

Lipolysis, on the other hand, occurs in response to negative energy balance, to provide substrate for metabolism in situations where energy intake is insufficient to cope with energy demand. It is conceivable that reduced fatty acids, associated with negative energy balance, lead to increased sympathetic output to increase lipolysis in WAT, thereby providing an alternative source of
energy. It is likely that the fatty acid sensing mechanisms discussed earlier will modulate the sympathetic output to WAT, in order to regulate levels of lipolysis.

The process of lipolysis involves the hydrolysis of triglycerides into their constituent fatty acids and glycerol, and full lipolysis requires three main sequentially-acting enzymes: adipose triglyceride lipase (Atgl), hormone-sensitive lipase (Hsl) and monoacylglycerol lipase (Mgl) (Duncan et al. 2007; Lafontan & Langin 2009; Ong et al. 2011) (Figure 1.5). The main lipolytic input into WAT is the noradrenergic sympathetic stimulation, primarily acting through β-adrenergic receptors found on adipocytes. These receptors are GPCRs coupled to a Gs protein which causes adenyl cyclase to produce cyclic adenosine monophosphate (cAMP), leading to activation of protein kinase A (PKA) (Robidoux et al. 2006). PKA phosphorylates two key proteins, perilipin A and Hsl, activating them and facilitating lipolysis (Duncan et al. 2007). Firstly, phosphorylation of perilipin A results in its structural rearrangement, facilitating lipid droplet fragmentation. However, perilipin A rearrangement also leads to the release of abhydrolase domain-containing 5 (ABHD5), which activates Atgl, causing the hydrolysis of triglyceride to diglyceride and releasing fatty acids. PKA also phosphorylates Hsl, which is located within the cytosol in its non-phosphorylated form, and causes its translocation to the lipid droplet membrane. Here it complexes with perilipin A and catalyses the breakdown of diglycerides to monoglyceride, releasing more fatty acids. The final step is the breakdown of monoglycerides, by Mgl, to release fatty acids and the glycerol backbone.
Figure 1.5: Lipolysis pathway in white adipose tissue

Noradrenergic sympathetic neurons stimulate the activation of PKA, which is responsible for the phosphorylation of perilipin A and HSL enzymes in the lipolytic pathway. Activation of ATGL, by complexing with ABHD5 released from phosphorylated perilipin A, hydrolyses TAG to DAG and releases FAs. Phosphorylated HSL translocates to the lipid droplet and complexes with phosphorylated perilipin A, leading to hydrolysis of DAG to MAG, releasing more FAs. MGL catalyses the final step of lipolysis: hydrolysis of MAG to glycerol and FAs.

ABHD5: abhydrolase domain containing 5; AC: adenyl cyclase; AMP: adenosine monophosphate; ATGL: adipose triglyceride lipase; cAMP: cyclic AMP; DAG: diglyceride; FA: fatty acid; HSL: hormone-sensitive lipase; MAG: monoglyceride; MGL: monoglyceride lipase; NA: noradrenaline; PKA: protein kinase A; SNS: sympathetic nervous system; TAG: triglyceride
1.8 RFamides

The RFamide peptides are a family of peptides found in the central nervous system of a wide range of animals, across all the major phyla. They are characterised by, and take their name from, a conserved arginine residue coupled to an amidated phenylalanine residue at their carboxy-terminal. Despite this C-terminal conservation, they show a high degree of variability at their N-terminals, which is hypothesised to be responsible for their wide range of physiological functions. The RFamide family of peptides was first identified when FMRFamide peptide was isolated from the ganglia of a clam (Price & Greenberg 1977). Currently, five main groups of RFamide peptides are recognised: kisspeptin, prolactin-releasing peptide (PrRP), neuropeptide FF (NPFF), LPXRFa and pyroglutamylated RFamide peptide (QRFP) (Bechtold & Luckman 2007). Interest in these peptides has often focussed on their potential roles in feeding regulation, following the discovery that FMRFamide peptide inhibits opioid-induced feeding in mice (Kavaliers & Hirstt 1985). The fact that feeding represents an evolutionarily conserved action of the peptides, across a wide range of species, seems to be a good indication that this may represent an important function of RFamides. However, despite multiple studies over the ensuing three decades reporting effects on feeding, it still remains to be determined whether this is a primary, physiological role of the family.

As mentioned above, the diverse N-terminal sequences of the RFamide peptides is likely to contribute to their wide-ranging actions. Whilst administration of exogenous NPFF has been shown to be capable of inhibiting feeding (Murase et al. 1996), it is unclear whether this translates to an endogenous role for the peptide. Instead, it appears that the main function of NPFF is to modulate opioid-induced analgesia (Panula et al. 1999), and it is possible that its effects on the opioid pathway may be responsible for its effects on feeding. PrRP, whose name is a misnomer given it does not stimulate prolactin release in vivo, actually does play an important role in energy homeostasis: central administration reduces feeding and body weight (Lawrence et al. 2002b), and PrRP expression is downregulated in states of negative energy balance.

Kisspeptins, on the other hand, do not appear to influence feeding, but rather play a role in the control of puberty and the secretion of gonadotrophic hormones (de Roux et al. 2003). Finally, RFamide related peptides (RFRPs), the mammalian forms of the LPXRFa group, are suggested to inhibit feeding following administration of exogenous peptide (Bechtold & Luckman 2007), similar to that observed with NPFF. Furthermore, RFRPs also seem to be capable of inhibiting opioid-mediated analgesia (Liu et al. 2001).

All of the RFamides signal via GPCRs, which show a degree of similarity between the members. In particular, the NPFF receptors appear to be highly promiscuous: high affinities for NPFF, RFRP, PrRP and QRFP (discussed below) have been reported at NPFF receptors. In fact, subsequent study has shown that RFRP actually shows higher affinity for the NPFF1 receptor than NPFF.
(Hinuma et al. 2000), resulting in the hypothesis that NPFF2 is the endogenous receptor for NPFF, whilst NPFF1 is suggested to be the endogenous receptor for RFRP. The promiscuity of the RFamides adds another layer of complexity for studies aiming to determine the functions of these peptides, an issue that will be discussed in more detail later.

Whilst they may play different roles in the body, the RFamides are almost exclusively expressed within the brain, with many (PrRP, NPFF, kisspeptin and RFRP) having key neuronal populations in hindbrain regions such as the NTS and rostral ventrolateral medulla (RVLM) (Bechtold & Luckman 2007). Interestingly, both kisspeptin and RFRP expressing neurons are also found within hypothalamic regions such as the DMN, indicating potential for a role in energy homeostasis. Neuronal populations of the RFamides frequently innervate wide ranging nuclei, with dispersed projections from these neurons reported across the brain. In summary, the RFamides represent a family of peptides linked primarily by their structure, rather than function, although it remains to be determined whether a seemingly conserved action on feeding and energy homeostasis represents a physiological link within the family.

1.8.1 Pyroglutamylated RFamide Peptide

In 2003, three groups almost simultaneously reported a novel neuropeptide member of the RFamide family (Chartrel et al. 2003; Fukusumi et al. 2003; Jiang et al. 2003), that activated an orphan receptor, SP155/AQ27, now commonly known as G-protein coupled receptor 103 (Gpr103). One group isolated a 26 amino acid peptide from the hypothalamus of frogs, leading to the name 26 RF amide peptide (26RFa) (Chartrel et al. 2003), whilst a second group identified a longer peptide of 43 amino acid length via a search of the human genome (Fukusumi et al. 2003). By determining the structure of this peptide, this group discovered a pyroglutamyl residue at the N-terminal end, hence the name pyroglutamylated RFamide peptide (QRFP). The pyroglutamyl moiety is the result of a post-translational modification commonly found in peptides with either a glutamate or glutamine residue at their N-terminal end. In this case, the N-terminal glutamate residue reconfigures to form a cyclic pyroglutamyl residue. Three years later, another group suggested that QRFP was the endogenous ligand for Gpr103 (Takayasu et al. 2006) in rodents. Subsequently, gene sequences corresponding to Qrfp/26RFa have been described in a number of animals from different taxonomic classes (mammals, birds, amphibians and fish) showing a high degree of conservation within the vertebrate phylum (Ukena et al. 2014).

26RFa represents an N-terminally truncated version of QRFP, and it has yet to be determined whether it represents an endogenous peptide in mammals. 26RFa was originally isolated from frog hypothalamus, and similar length peptides have been identified in avian (quail, chicken and zebra finch), amphibian (Xenopus) and fish (goldfish) genomes. As there have been no longer length peptides discovered in these other species, it is likely that these are the full-length peptides in these animals, and are their equivalents to mammalian QRFP: there have been no
reports of 26RFa being isolated from rodent brains unless it was specifically being sought (Takayasu et al. 2006; Prévost et al. 2015). The mature peptides (in birds, amphibians and fish) are all preceded by a lysine-arginine cleavage site, which is not present in mammalian genomes: instead a glycine-arginine cleavage site exists immediately prior to the 43 amino acid peptide sequence (Ukena et al. 2014), suggesting that mature QRFP in these animals is the major endogenous peptide. Whilst groups including ourselves have shown activity of 26RFa in rodents, it may be important to determine whether this peptide is produced normally. Different groups use either QRFP or 26RFa in their studies, so it will be essential to learn if these peptides are equally physiological.

1.8.2 Gpr103

QRFP was identified as the endogenous ligand for the orphan receptor Gpr103, which is a class A G-protein coupled receptor. Interestingly, rodents possess two isoforms of the receptor, termed Gpr103a and Gpr103b, a trait that so far appears to be unique to this group. All other animals have a single Gpr103 receptor (apart from zebrafish and coelacanth which have three), and the rodent Gpr103a most closely resembles this type. It is unclear why rodents have evolved an extra receptor isoform, but synteny analysis shows conservation of Gpr103 and its surrounding genes (Ukena et al. 2014). Interestingly, the genes either side of Gpr103 are split between chromosomes in mice, meaning it is possible that a crossover event may have been behind the development of two receptor forms in rodents.

QRFP interacts with both Gpr103 isoforms with similar affinity (Takayasu et al. 2006), and efficacy, in both rats and mice (Kampe et al. 2006; Takayasu et al. 2006). Studies have also not identified differences in the intracellular signalling of either receptor, although little work has been conducted in this area. However, in general, both QRFP and 26RFa inhibit cAMP formation in CHO cells transfected with Gpr103 (the isoform was likely to be Gpr103a due to it being conducted before Gpr103b was identified) (Fukusumi et al. 2003), whilst also increasing intracellular Ca\(_{2+}\) concentrations, in a manner that is insensitive to pertussis toxin inhibition (Jiang et al. 2003). As pertussis toxin inactivates G\(_{i/o}\) proteins, these data suggest alternative coupling, hypothesised to be G\(_q\) proteins. However, a more recent study showed QRFP reduced forskolin-induced cAMP production, but this was not replicated when QRFP was administered alone (Ramanjaneya et al. 2013). This study concluded a G\(_i\) protein coupling, meaning further investigation should aim to confirm either or both of these proposed pathways. The receptor shows strong intra-species homology, particularly in the transmembrane sequences, with over 80% homology between each of the rodent receptor isoforms and human Gpr103, whilst the rodent receptors show 77% homology to one another (Kampe et al. 2006). The potential activation of two distinct G-proteins could possibly indicate that each receptor isoform is coupled to a different one, which may lead to the discovery that each receptor is responsible for different actions of QRFP.
It is also important to consider the high homology between Gpr103 and other RFamide peptide receptors such as NPFF2, PrRP and kisspeptin, along with other peptide receptors such as NPY Y2 (Lee et al. 2001). Both QRFP and 26RFa activate the NPFF receptors, but most importantly, 26RFa appears to show particularly strong affinity for the NPFF2. A study of binding affinity profiles for RFamide peptides, at different receptors of the RFamide family, identified 26RFa binds with almost equal affinity for NPFF2 as it does for Gpr103 (Elhabazi et al. 2013). The relevance of this for normal physiology is unclear, but it is important to consider the role that cross talk may play, especially given the concerns that still exist over the endogenous expression of 26RFa compared with QRFP.

### 1.8.3 QRFP and Gpr103 expression

Early studies used quantitative reverse transcription PCR (RT-PCR) to localise expression of Qrfp and Gpr103 mRNA (Jiang et al. 2003; Fukusumi et al. 2003; Takayasu et al. 2006). All studies reported high expression of Qrfp in the brain and eye, with Gpr103 expression primarily in these tissues as well, although strong Gpr103 expression is also found within the adrenal gland. These studies showed that Qrfp is expressed fairly widely throughout the body, at low levels compared with central expression, but Gpr103 mRNA is predominantly located in the brain, with very restricted expression peripherally.

A number of other studies also have investigated the distribution of Qrfp, revealing expression within a wide range of tissues in the body, but the general consensus is that the highest level of expression is found within the brain (Chartrel et al. 2003; Jiang et al. 2003; Fukusumi et al. 2003; Takayasu et al. 2006). A lack of reliable antibody has meant the majority of the work aimed at identifying Qrfp distribution in the brain has relied on in situ hybridisation histology. Consequently, definitive localisation has been hard to determine, but it is clear that QRFP neurons are located solely within the hypothalamus, and in particular they lie within the basal regions. A number of different hypothalamic nuclei have been suggested: Arc, VMN, LHA, retrochiasmatic area, medial tuberal nucleus and the tuber cinereum area (Chartrel et al. 2003; Kampe et al. 2006; Takayasu et al. 2006). As would be expected, the distribution of Gpr103 in the brain is much more widespread than Qrfp, with both mRNA and binding found across the majority of the rostro-caudal extent of the brain (Kampe et al. 2006; Bruzzone et al. 2007). It has been reported that the Gpr103 isoforms are differentially expressed in the brain, with very little overlap observed, which could suggest a divergence in their function, although this would be a trait restricted to rodents, given that other mammals seem to possess only a single receptor (Ukena et al. 2014).

Two important studies were specifically targeted towards identifying the distribution of Gpr103 in the brain of rats (Kampe et al. 2006; Bruzzone et al. 2007). Confusingly, the latter paper reports distribution of 26RFa binding sites and Gpr103a receptor mRNA, whilst the former characterises the second receptor isoform. In general, 26RFa binding sites matched reasonably well with
Gpr103 receptor expression, when both studies are taken into account, although regions of the brain still show 26RFa binding sites with no corresponding receptor mRNA expression. It is hypothesised that these sites are likely representative of 26RFa binding to the NPFF2 receptor, confirmed by the ability of NPFF to displace radioligand binding in these sites. Both Gpr103 receptors are expressed within multiple nuclei of the hypothalamus, including the LHA, VMN, DMN, PVN and Arc, although expression of Gpr103a was reported to be higher in these hypothalamic nuclei, than Gpr103b. Furthermore, Gpr103a mRNA is strongly expressed in the paraventricular thalamic nucleus (PVT) which, despite its classical association with stress responses, is also important for GABA-mediated feeding (Stratford & Wirtshafter 2013). Given the important role of these nuclei in regulating feeding and energy balance, it seems likely that QRFP is involved in controlling certain aspects of central responses to energy balance perturbations. However, Gpr103b was found within the VTA, unlike Gpr103a, whilst neither is expressed within the NAcc. Interestingly, a third study conducted in mice reported sparse Gpr103b expression within the accumbens shell and caudate putamen (Takayasu et al. 2006), which was not observed in the rat, despite strong binding of 26RFa reported here. Both Gpr103 receptors are located in regions such as the LC, DR and PAG, with Gpr103a also found within the pontine and interpeduncular nuclei. Together, these nuclei contain neuronal sub-populations specifically involved in regulating arousal. Many of these nuclei also show Gpr103 expression in the mouse brain as well, although not all of these regions were investigated. Further complementation between mouse and rat studies can be observed by the existence of Gpr103a, but not Gpr103b, in the superior colliculus (SC), possibly linking in with the high levels of Qrfp found in the eye. Finally, evidence exists that QRFP may interact with neurons involved in autonomic signalling: both Gpr103 receptors are expressed within the spinal cord, though the precise region was not determined, whilst Gpr103a is strongly expressed in the NTS in both rats and mice, an important part of the parasympathetic vagal complex.

1.8.4 Centrally-mediated effects of QRFP

The expression of QRFP in regions of the mediobasal and lateral hypothalamus, and the widespread nature of Gpr103 expression, drew early attention to providing a role in feeding and energy homeostasis. Many studies have shown an orexigenic response to the administration of exogenous QRFP and 26RFa (Chartrel et al. 2003; Moriya et al. 2006; Takayasu et al. 2006; Primeaux et al. 2008), which is possibly amplified with the provision of highly palatable food (Primeaux et al. 2008). Furthermore, the orexigenic action of QRFP is reported to be attenuated by inhibition of NPY Y1 receptors (Takayasu et al. 2006), but is unaffected by loss of orexin signalling. Interestingly, chronically dosing QRFP centrally results in hyperphagia and obesity (Moriya et al. 2006). However, this weight gain was independent of hyperphagia, as pair-feeding QRFP-dosed mice to chow-fed controls still caused increased weight gain. This weight gain was
reported to be caused by an increase in fat mass, suggesting that QRFP may play a role in adiposity regulation, and that its actions in the brain are responsible for mediating these effects. Further evidence exists for a role of central QRFP in energy homeostasis, as levels of Qrfp mRNA are upregulated by fasting in the hypothalamus. However, only a 1.5-fold increase was induced following a very strong stimulus of a 48-hour fast, in contrast to an almost 5-fold increase in Npy mRNA (Takayasu et al. 2006).

Similar to the action of other orexigenic peptides, QRFP also influences other aspects of energy homeostasis, with strong parallels between QRFP and orexin in evidence. Like orexin, central administration of QRFP increases locomotor activity, oxygen consumption and arterial blood pressure (Takayasu et al. 2006), suggesting a broader role in energy homeostasis. As described earlier, the primary role of orexin is now considered to be stimulating arousal, with some of its secondary factors (such as feeding, locomotor activity and oxygen consumption) likely to be a direct consequence of this. The existence of Gpr103 receptors in regions associated with arousal, coupled with these in vivo responses suggests this may represent a similar part of the function of QRFP. An important avenue for future research on QRFP is to determine the primary function of the peptide.

1.8.5 Action of QRFP in peripheral tissues

Whilst the strongest expression is within the hypothalamus of the brain, QRFP is expressed in the peripheral tissues and a number of studies have begun to identify direct actions of QRFP in some of these. An initial finding of one of the original papers describing QRFP was high expression of its receptor within the adrenal gland (Fukusumi et al. 2003). This group went on to show that intravenous administration of QRFP stimulated aldosterone secretion, although, given that the highest expression of Qrfp is reported in the brain, it is unclear where the circulating QRFP would come from in order to interact with its receptor in this tissue. A second study revealed a direct effect of 26RFa on isolated perfused pancreas preparations (Egido et al. 2007), despite no previous work showing Qrfp or Gpr103 expression in the tissue. In this study, 26RFa was shown to inhibit insulin secretion, a result that was supported by a study in isolated islets (Granata et al. 2014). However, QRFP has the opposite effect: enhancing glucose-dependent insulin secretion. Interestingly, the QRFP-mediated effect was inhibited by specific inhibition of Gpr103 signalling, whereas the 26RFa-mediated action was not. It has been proposed, therefore, that 26RFa is inducing its effects in this situation via interaction at a separate receptor, most likely the aforementioned NPFF2 receptor. This further highlights the necessity to understand the physiological significance of both QRFP and 26RFa. A follow-up study by the same group has since showed that 26RFa, acting specifically through Gpr103 receptors, potentiates insulin sensitivity and insulin secretion, resulting in lower blood glucose spikes (Prévost et al. 2015), but the group did not investigate the effect of QRFP in this setting. However, interestingly this group showed
that 26RFa was secreted by the duodenum following a meal, suggesting for the first time where circulating QRFP may originate: this same group had previously shown that anorexia nervosa patients showed increased levels of circulating 26RFa compared with controls (Galusca et al. 2012). However, seemingly at odds with this discovery, their later work showed an increase in circulating 26RFa in obese people following an overnight fast, compared with normal-weight controls (Prévost et al. 2015). Furthermore, around a similar time to this study, a separate group showed that 26RFa improves glucose uptake in isolated skeletal muscle cells (Allerton & Primeaux 2015), but in this study QRFP had no effect, and no controls were included to ensure this was a Gpr103-dependent action. It is important for future studies in QRFP/26RFa to focus on this issue and ensure responses reported are not simply off-target effects, as in physiological settings these are unlikely to be primary actions.

Whilst there have yet to be any studies showing a differential role for the rodent Gpr103 receptor isoforms in the brain, one important action of QRFP in the periphery has been suggested to be mediated exclusively by one subtype. A strong antilipolytic effect of QRFP, directly on adipose cells, was shown to be a Gpr103-dependent response, with the Gpr103b receptor being solely responsible (Mulumba et al. 2010). QRFP and 26RFa increased intracellular triglyceride content and lipid uptake, and also inhibited isoproterenol-induced lipolysis. Subsequently, the same group reported that QRFP-dependent inhibition of lipolysis was associated with decreased phosphorylation of a number of key molecules in the intracellular pathway, such as perilipin A and Hsl (Mulumba et al. 2015). The precise intracellular signalling mechanisms of QRFP in this system remains to be elucidated, but the group provide strong evidence that this is a direct action of QRFP, potentially acting in a paracrine manner, given that levels of QRFP were decreased in obesity whilst Gpr103b levels were increased (Mulumba et al. 2010). The expression of QRFP in the brain as well as the periphery means that it will be important to determine in the future whether peripheral and central effects of QRFP are both equally physiological, and whether they act in synergy or independently.
Chapter 2

GENERAL METHODS
2.1 In vivo methods

2.1.1 Animals

Experiments were performed in adult, male CD1 mice (25-35g: Charles River; Sandwich, UK), adult male C57Bl/6J mice (20-30g: Harlan; Blackthorn, UK) or adult male/female transgenic mice (body weight dependent on study, bred in house, University of Manchester). Animals were housed in the animal unit in constant environmental conditions: 21±2°C and 45±10% humidity on a 12h:12h light-dark cycle (dark phase commencing at 18:00 GMT). All mice were group housed (2-5 per cage) with littermates, with ad libitum access to pelleted chow (Special Diet Services; Witham, UK) and sterile pouch water, unless otherwise stated. For some phenotyping experiments, mice were singly housed as stated in individual methods sections. Certain phenotyping experiments required mice to be fed a high-energy diet (HED) containing 5.16kcal/g, where 60% energy comes from fat (58Y1: Test Diets (IPS Ltd); London, UK).

2.1.2 Reporter/cre mouse husbandry

ROSA26-eYFP reporter mouse

The ROSA26-eYFP mouse (B6.129X1-Gt (ROSA)26Sortm1 (EYFP)Cos/J; JAX strain #006148; C57Bl/6J background) is a reporter line that can be used to express enhanced Yellow Fluorescent Protein (eYFP) in a cre-recombinase-dependent manner. The mouse expresses a STOP sequence, flanked by loxP sites, immediately upstream of the inserted eYFP gene within the Gt (ROSA)26Sor locus. When this reporter mouse is crossed with a cre-recombinase-expressing mouse, the enzymatic activity of cre-recombinase cuts at the loxP sites and removes the STOP sequence, resulting in expression of eYFP in any cre-expressing cells. The ROSA26-eYFP line was maintained as a homozygous colony.

ROSA26-ChR2/eYFP reporter mouse

The ROSA26-ChR2/eYFP mouse (Gt (ROSA)26Sortm32 (CAG-COP4*H134R/EYFP)Hze; JAX strain #012569; mixed background) is a reporter line similar to the ROSA26-eYFP line described above. In this transgenic line, a fusion gene of channel rhodopsin (ChR2) and eYFP has been inserted into the Gt (ROSA)26Sor locus. The ChR2 protein is inserted into the cell membrane, so is expressed within projection fibres as well as cell bodies, giving a benefit over the regular ROSA26 eYFP mouse in enabling neuronal connections to be visualised. The ROSA26-ChR2/eYFP line was maintained as a homozygous colony.

Gad2-cre mouse

The Gad2-cre mouse model (Gad2tm2 (cre)Zjh; Jax strain #010802; mixed background) expresses cre-recombinase under the control of the glutamic acid decarboxylase 2 (Gad2) promoter. The cre-recombinase sequence was inserted, along with an internal ribosome entry site (IRES), into the untranslated region of the Gad2 gene. The model expresses cre-recombinase in Gad2
expressing cells: the γ-aminobutyric acid (GABA)ergic population of neurons (GAD65). Breeding the Gad2-cre mouse with a floxed model would result in cre-mediated excision of the floxed region. These mice were crossed with the FiEx-Qrfp model (described fully in Chapter 5) which would result in cre-mediated rescue of Qrfp in GABAergic cells.

**Vglut2-cre mouse**

Vglut2-cre mice (Slc17a6tm2 (cre)Lowl; Jax strain #016963; mixed background) express cre-recombinase under the control of the vesicular glutamate transporter 2 (Vglut2) promoter. An IRES-linked cre-recombinase gene was inserted downstream of the stop codon for the Vglut2 gene resulting in mice that express cre-recombinase in Vglut2 cells: the glutamatergic neuronal population. Breeding this model with floxed models would produce offspring in which cre-mediated excision had removed the floxed region. These mice were crossed with the FiEx-Qrfp model (described fully in Chapter 5) which would result in cre-mediated rescue of Qrfp in glutamatergic cells.

### 2.1.3 Peptides

All peptides used were obtained from commercial suppliers and were certified pyrogen and pathogen free. Most drugs (leptin being the notable exception) were diluted in sterile saline (0.9% w/v NaCl: Braun; Melsungen, Germany) for injection by one of three routes: intracerebroventricular (ICV), intraperitoneal (ip) or subcutaneous (sc), as specified in each experiment. QRFP (between 5-10μg) (Phoenix Pharmaceuticals, Inc.; Burlingame, CA, USA), 26RFa (between 5-10μg) (Phoenix) and ghrelin (between 0.5-1μg) (Tocris Bioscience; Bristol, UK) were administered via ICV injection in a volume of 3μl using a 10μl Hamilton syringe (Hamilton Company; Reno, NV, USA) attached to a home-made injection cannula by fine bore tubing. Ghrelin and leptin (Peprotech EC Ltd; London, UK) were also administered via ip injection for certain studies, as specified, and were dosed at 2mg/kg and 4ml/kg dose volume, using 29G insulin syringes. Insulin (Sigma-Aldrich; St. Louis, MO, USA) was administered via the sc route between the shoulder blades at 1.75units/kg and 4ml/kg dose volume, using 29G insulin syringes.

### 2.1.4 Stereotaxic surgery

**ICV cannulation**

In order to enable injections to be made into the forebrain ventricles, mice were unilaterally implanted with ICV guide cannulae above the right lateral ventricle. Mice were anaesthetised with 3% isoflurane (Abbot Abbvie Ltd; Maidenhead, UK) in oxygen (1500ml/min) and the fur removed from the surgical area before iodine solution was applied to the exposed skin for at least 30 seconds. The skull was immobilised in a stereotaxic frame using ear and incisor bars. Throughout the surgical procedure, mice were maintained at the correct depth of anaesthesia using 1-2% isoflurane in oxygen (800ml/min). Depth of anaesthesia at the beginning of the procedure was
confirmed by loss of the foot-pinch reflex and depth of breathing. A small incision was made along the midline of the cranium and the meninges cleared to expose the skull which was wiped with sterile saline. A small screw (M1.4x3: Precision Technology Supplied Ltd; East Grinstead, UK) was inserted into the left parietal plate of the skull. A hole was drilled 0.4mm caudal and 1.0mm lateral to bregma through which a sterile guide cannula (made in house from a 23G needle cut to 1.2mm) was implanted. Acrylic dental cement (Simplex Rapid Powder: Kemdent; Swindon, UK / Methyl methacrylate: Metrodent; Huddersfield, UK) was used to affix the guide cannula to the screw and skull, and left for at least ten minutes to solidify. Mice were injected subcutaneously with 1ml warm, sterile, isotonic saline (for rehydration), and intramuscularly with the analgesic, buprenorphine (0.03mg/kg) (Vetergesic®: Reckitt Benckiser Healthcare; Hull, UK). The incision was sutured closed (6.0 Mersilk: Ethicon; Somerville, NJ, USA) around the guide cannula, and the wound swabbed with iodine. Mice were singly housed and allowed to recover in warm cabinets at 30°C, until moving freely, at which time they were taken back to their holding room. Experiments were carried out at least one week subsequent to the surgery, with a minimum of 48 hours in between repeat injections.

**Intracranial injections**

Stereotaxic surgery was also used for nanoinjection of substances into specific areas of the brain. The procedure for intracranial injections broadly followed that described above, although neither screws nor guide cannulae were inserted and, as such, dental cement was not required in these procedures. Holes for these injections were drilled stereotaxically in relevant positions, depending on the brain region being targeted. Rather than a guide cannula being inserted, a pulled glass pipette was inserted into the hole, attached to a nanoinjector (Drummond Scientific Company; Broomall, PA, USA) capable of delivering volumes of relevant substances in volumes in the order of nanolitres (for details see Chapter 3 Methods).

Anterograde tracing studies involved the delivery of cre-dependent, adeno-associated virus (AAV) packaged with constructs containing ChR2 (AAV-EF1a-DIO-hChR2(H134R)-mCherry: Deisseroth lab [Stanford University]; available from Vector Core, University of North Carolina) or h-synaptophysin (h-syn) tagged to mCherry (gift from Martin Myers), a fluorescent marker. Neurons within the region of injection would be infected indiscriminately by the virus, but only in those expressing cre-recombinase would the construct become expressed, resulting in selective transduction of these cells. Following surgery, mice were left to recover for two weeks to allow trafficking of the construct throughout neuronal fibres, or, in the case of h-syn, to the neuron terminals, due to it being a vesicular peptide. Injections of control AAVs with an mCherry construct that is not trafficked down neuronal projections, were made in separate mice to act as negative controls. Subsequently, mice were transcardially perfused, as described below, to enable fluorescence immunohistochemistry to be carried out. The mCherry molecule tagged to the
construct enabled visualisation of neuronal fibres or terminals, depending on the construct in question, using an anti-dsRed antibody.

Injections of retrograde tracer (Fluospheres [F8793]: Life Technologies; Carlsbad, CA, USA) were made in specific areas (described in detail in Chapter 3) to target areas containing putative terminals of neuronal projections, as identified by anterograde tracing. Retrobeads were taken up by neuronal terminals and retrogradely trafficked to the neuronal cell bodies. Mice were left to recover for two weeks following surgery before perfusion and subsequent immunohistochemistry, as described below.

2.1.5 Feeding experiments

ICV feeding experiments
Male mice used in feeding studies were singly housed for at least a week prior to study, during which time mice were acclimated to handling daily. Food was removed from cages at lights on and injections were made through indwelling cannula two hours later. Injection cannulae were designed to extend 0.5mm beyond an implanted guide cannula. Injections of peptide (concentration as specified) in 3μl saline, or saline alone, were made over 20-30 seconds with a further 15 seconds left to allow the injection to diffuse from the end of the cannula. Mice were subsequently provided ad libitum access to food, and food intake measured at one, two, four and 24 hours after injection. In experiments with a crossover design, the second injection was made a minimum of 48 hours later.

Fast-induced re-feeding studies
Mice were singly housed for at least one week prior to study and handled daily. Mice were food deprived for 20 hours from 14:00 the day before the study. Food was returned at 10:00, two hours after lights on, at the time of injection, and food intake measured at one, two, four and 24 hours later.

2.1.6 Activity testing
Specially designed cages, intersected by infrared beams, were used to measure activity levels, as beams crossed, and recorded by custom-made software (Prof. Antoni Diez-Noguera; University of Barcelona, Spain). Cages were housed within a sound attenuating cabinet running a 12:12 light:dark cycle, as experienced by mice in their regular home cages. Mice were singly housed throughout studies investigating activity levels and all mice were habituated to this set up for at least three days before measurements began.

Basal activity levels
Following habituation to the beam break cages, activity levels were measured for four 24 hours cycles. The number of beam breaks was counted in each 15 minute period and the total number
of breaks per hour was plotted over the four days. Subsequently, averages of beam breaks per hour, during both lights on and lights off, were calculated as a measure of activity, and a full 24-hour average was calculated.

**Peptide-induced acute activity changes**

The effect of peptide administration on acute activity levels was also monitored in the apparatus described above. Following ICV cannulation and recovery, mice were transferred to the beam break cages for three days acclimation. For acute studies, beam breaks were counted during five minute intervals, as this enabled activity levels to be measured as close to the actual time of injection as possible and ensured a more accurate representation of the peptide-induced profile.

### 2.1.7 Oral glucose tolerance test (OGTT)

Mice were singly housed for at least one week and handled daily. On the day of study, food was removed from cages at lights on, with the study beginning six hours later. Food was not returned until the end of the study. Blood-glucose levels were measured from the tail vein by pricking the tail, near the tip, with a 25G needle to produce a blood spot which was analysed by a hand-held glucose monitor (Accuchek: Roche Diagnostics Ltd; Burgess Hill, UK). All readings were made in duplicate unless the two readings differed by more than 10%, in which case a third reading was taken, and an average of all three used. Baseline measures were taken immediately prior to ICV injection of peptide. This was followed by oral gavage of glucose (2g/ml at 4ml/kg dose volume: Fisher Scientific; Loughborough, UK) after which the mouse was returned to its home cage. Glucose measures were made at 10, 20, 40, 60 and 120 minutes after gavage using the same tail prick as used for the baseline measure. Towards the end of the study some mice required gentle massaging of the tail, to stimulate blood flow. Results were plotted as the glucose excursion from baseline against time for each mouse, and area under the curve (AUC) calculated. Comparisons were made between control and experimental injections, at least 72 hours apart, using a crossover design.

### 2.1.8 Tissue dissection

In some studies, tissues were dissected from mice for further analysis. Mice were culled by decapitation, and relevant tissues dissected and frozen on dry ice, in either 1.5ml Eppendorf tubes or on tin foil, dependent on tissue. In order to ensure reproducibility between studies, tissues were dissected from the same areas of each mouse as follows: liver samples were taken from the base of the left lateral lobe of the liver; brown adipose tissue (BAT) was dissected in full from the interscapular region; epididymal white adipose tissue (eWAT) was dissected from the area surrounding the epididymis immediately above the testicle; and skeletal muscle samples were dissected from the gastrocnemius of the left hind leg.
2.1.9 Transcardial perfusion for tissue fixation

Animals were deeply anaesthetised using 4% isoflurane in oxygen (1500ml/min) until there was no reflex response to a toe pinch stimulus. At this point, the abdomen was opened and the diaphragm and ribcage cut, to allow access to the thoracic cavity and to expose the heart. A 23G butterfly needle was inserted into the apex of the heart and clamped in place within the left ventricle. The right atrium was cut and the body perfused with heparinised saline (10,000 units/l heparin in 0.9% NaCl: Fisher Scientific) using a peristaltic pump set to a flow rate between 10-15ml/min. Once fully exsanguinated, 4% paraformaldehyde (PFA: Sigma-Aldrich) in 0.1M phosphate buffer (PB; 95mM Na₂HPO₄ and 24mM NaH₂PO₄ in distilled water: both Sigma-Aldrich) was perfused through the animal until full contraction of limbs had occurred, from which point perfusion was carried out for a further 5 minutes. Whole brains were dissected and post-fixed overnight in 4% PFA in 0.1M PB at 4°C. PFA was then replaced with 30% sucrose (Fisher Scientific) (in PB) for cryoprotection, and brains left to equilibrate at 4°C for between 24-48 hours. Equilibrated brains were frozen on dry ice and stored at -80°C until needed.

2.1.10 Operant response

Prior to initiation of study, male CD1 mice (25-30g) were ICV cannulated, as described previously. Following a week’s recovery, mice were trained to press levers for a strawberry milk reward (Yazoo: FrieslandCampina; Amersfoot, Netherlands) as a study of motivational eating. Fully customisable, modular operant response chambers (80015NS: Campden Instruments Ltd.; Loughborough, UK) were housed in sound isolating cabinets to remove the potential for distractions during the study. Each modular chamber was equipped with two retractable levers, two stimulus lights, a house light and a reward area. The reward area, situated on the opposite wall to the levers, consisted of a hopper, into which milk could be delivered, that was covered by a clear plastic hinged door. Within the reward area was a final stimulus LED and an infrared beam capable of detecting entries into the reward hopper. External to the cage was a pump, connected to the cages by fine bore rubber tubing that was capable of delivering specific volumes of liquid directly into the reward hopper. The system was fully automated and controlled by ABET II software (Lafayette Instruments; Campden Instruments Ltd.) enabling numerous endpoints to be simultaneously and remotely measured. Exact coding for all programmes can be found in Supplementary Methods 1.

In order to avoid neophobia, mice were exposed to a small amount of milk in their home cage on two separate occasions prior to the start of study. Mice were then placed within the operant chambers in groups of four, with cage mates, in order to reduce stress levels upon first exposure to the novel environment. For the initial stages of training, mice were food restricted to around 60% of normal daily intake per mouse, and body weights measured daily. In this way mice reached, and were maintained between, 80-85% of their natural weight. Following this, on two
subsequent days, mice were placed in the chambers individually for 15 minutes, with a small amount of milk available in the reward hopper and illumination provided by the house light. During these sessions, all mice were observed to be drinking milk from the reward area.

**Fixed-Interval schedule**

The initial stage of training involved mice developing an association between a light stimulus and the availability of the reward. Cages were programmed to run for 15 minutes with the house light permanently on and both levers fully extended. Every 20 seconds the reward light (LED within reward area) was flashed five times (200ms on, 200ms off) whilst simultaneously the reward pump pulsed for 150ms, delivering 5µl milk into the reward hopper. In this way the noise of the pump was to become intrinsically linked with the flashing reward light (henceforth referred to as the ‘reward stimulus’), with which mice would be conditioned to associate the availability of milk. Mice were given two fixed-interval training sessions per day for five days, until all mice were observed to be approaching the reward area following the reward stimulus.

**Fixed-Ratio schedule**

The next progression for training was to introduce the mice to the requirement for lever pressing. A fixed-ratio schedule was set up whereby the cages were programmed to deliver milk, along with the previously described reward stimulus, following a single press on either of the extended levers (FR1), during a 20 minute training session. The house light was again permanently on and both levers extended into the cage. Mice were trained to press the levers, in the first instance, by placing a small droplet of milk on the lever which, when investigated by the mouse, led to them depressing the lever and receiving milk. Due to the association the mice had gained from fixed interval training, this reward stimulus prompted the mice to seek the milk from the reward hopper. This process was repeated with fresh milk being placed on the levers every time it had been consumed by the mouse, to help with learning, and this continued for the first few days of fixed-ratio training. Once mice had learnt the requirement to press the levers, milk was no longer provided on the levers as an incentive. After two weeks training mice were beginning to reliably press levers, although at relatively low frequency. At this stage, mice were split into two groups and rewards were restricted to a single lever. In this way, half the group were trained to press the front lever, and the other half the back lever, to obtain their milk reward, whilst presses on the incorrect lever triggered no programmed response, but were monitored throughout. Mice were trained in this FR1 schedule until they were reliably receiving 50 milk rewards in a session, over three consecutive days.

Having completed FR1, mice were then trained on an FR3 schedule, whereby each reward was only obtained after three presses on the correct lever, teaching the mice that multiple presses would be required for a reward. Within two weeks, mice were reliably obtaining 50 rewards per 15 minute session, requiring a total of 150 correct presses.
**Progressive-Ratio schedule**

Following stabilisation of responding in mice, the progressive-ratio paradigm was introduced whereby the effort required to obtain a reward progressively increases for each subsequent reward. In the present study the increase in presses required was defined by the equation: response ratio = $n^2$ (1,4,9,16,25,36,49...). As before, the house light provided permanent illumination and both levers were extended throughout the training sessions. Mice received one training session per day, lasting a maximum of 45 minutes. However, if a ten minute period elapsed with no reward obtained, the session was automatically terminated. The last completed ratio before the end of a session was defined as the breakpoint. Training continued until breakpoint stabilised for each mouse, which was identified as less than 15% variation in breakpoint over a three day period.

Once a plateau had been reached, mice were allowed *ad lib* access to regular chow and, following two days *ad lib* feeding, mice were retrained, starting at the FR1 protocol. Mice progressed quicker through each protocol second time round. Due to a lesser drive for milk in the fully fed mice, they were trained until responses plateaued at each stage, rather than to a defined end number. When fully fed mice had stabilised breakpoints in the progressive-ratio schedule, the testing phase began. Primary endpoints for operant response studies were breakpoint and correct lever pressing, whilst incorrect presses and approaches to the reward hopper were monitored throughout. In general, mice were injected, via the relevant route, 20 minutes prior to being transferred to the operant chambers where they underwent testing on the progressive-ratio schedule.

### 2.2 Ex vivo methods

**2.2.1 Sectioning of frozen tissue**

Frozen, fixed brains were cut at 30μm thickness using a freezing sledge microtome (Series 8000: Bright Instruments Ltd; Luton, UK) and coronal brain sections were divided into four sets, meaning each set contained a section every 120μm. Sections were placed into cryopreservant (30% ethylene glycol (Sigma-Aldrich), 20% glycerol (Fisher Scientific), 23mM Na$_2$HPO$_4$ and 3mM NaH$_2$PO$_4$ in distilled water) for long term storage at -20°C.

Due to the loss of structural integrity, eyes could not be sectioned using a microtome and instead a cryostat (Leica Microsystems; Wetzlar, Germany) was used to section them. Eyes were frozen in OCT (optimal cutting temperature formula) on dry ice prior to sectioning. OCT-frozen eyes were fixed to the cryostat stage using more OCT, and the stage fastened in place within the cryostat chamber. Chamber temperature was set between -18°C and -20°C whilst the stage temperature was set 2°C lower. Tissues were sectioned between 10-15μm thickness, and immediately collected onto Superfrost slides (VWR International Ltd; Lutterworth, UK) and left at room temperature for 24 hours to dry.
2.2.2 Immunohistochemistry

Fluorescence immunohistochemistry

Unless otherwise stated for specific experiments, a general protocol was used for fluorescence-based immunohistochemistry on free-floating sections. All washing and incubation steps detailed below were carried out with gentle agitation of the multi-well plates containing sections. Grouped brain sections were washed thoroughly in PB-T (0.2% v/v Triton X-100 (Sigma-Aldrich) in 0.1M PB) followed by an hour long ‘blocking’ incubation in 10% normal serum in PB-T. The serum was from the same species from which the secondary antibody was derived. Sections were incubated in the relevant primary antibody overnight at 4°C, in 1% normal serum in PB-T. Sections then were washed thoroughly with 0.1M PB, prior to incubation with appropriate secondary antibodies. Secondary antibody incubation was carried out for two hours in 5% normal serum in 0.1M PB at room temperature. Final washes were carried out in 0.1M PB followed by a single wash in distilled water prior to mounting. Slides were left overnight to dry, and covered with Prolong Gold (Invitrogen; Carlsbad, CA, USA) and a coverslip. In all studies involving visualisation of QRFP-positive cells, indirect labelling using a chicken anti-GFP antibody, that also recognises eYFP, was used (Table 2.1).

Streptavidin-based immunohistochemistry

Depending on the antibodies being used, some immunohistochemistry protocols required amplification of the signal by making use of a streptavidin-biotin complex. Most of the protocol for this was identical to that described above, with the following amendment. The secondary antibodies used in these studies were biotinylated and, following the two hour incubation in biotinylated secondary antibody, a final incubation step was performed using a streptavidin-conjugated fluorophore-linked antibody in 0.1M PB (Table 2.1), at room temperature for a further hour. These sections were then washed, as above, and mounted with Prolong Gold and a coverslip.

Peroxidase-based immunohistochemistry

An alternative method for amplifying the signal was carried out in some studies, using biotinylated secondary antibodies. For this protocol, immediately prior to the initial washing steps, sections were incubated in a peroxidase blocking solution to inhibit endogenous peroxidase activity (20% v/v methanol (Fisher Scientific) and 1.5% v/v hydrogen peroxide (Sigma-Aldrich) in 0.1M PB-T). Processing continued as the previous streptavidin-based protocol. However, rather than a streptavidin-conjugated fluorophore-linked antibody being employed, sections were incubated for one hour at room temperature in a streptavidin-conjugated horseradish peroxidase complex in 0.1M PB (GE Healthcare; Little Chalfont, UK). The antibody-peroxidase complex was visualised with a diaminobenzidine kit containing nickel (Vector Laboratories; Peterborough, UK).
The reaction was terminated by washing in distilled water and sections mounted with DPX (Fisher Scientific), a xylene-based mountant.

**On slide fluorescence immunohistochemistry**

For immunohistochemistry on eyes sectioned by cryostat, immunohistochemistry was performed on mounted sections. The protocol for this was identical to that used for free-floating sections, described above. The main differences being that the pre-mounted sections were washed by fully submerging the slides into a bath containing relevant solution. Antibody incubations were carried out by drawing round the mounted sections with a hydrophobic pen which allowed a small puddle of antibody-containing solution to be left on the slide for the relevant time period. In order to prevent evaporation overnight, slides were left in a cool, moist environment.

**Microscopy**

Once dried, slides were observed on an upright Olympus BX51 microscope (Olympus; Southend-on-Sea, UK), using 4x/0.13 and 10x/0.30 Plan Fln objectives. Images were captured using a CoolSnap EZ Camera (Photometrics; Tucson, AZ, USA) controlled by MetaVue Software (Molecular Devices; Sunnyvale, CA, USA). Specific filter sets for DAPI, FITC and Texas Red were used to prevent bleed through from one channel to the next. All images were processed and analysed using Fiji/ImageJ (https://fiji.sc/). In order to improve contrast on images, and aide with identification of co-expression, all fluorescent images in this thesis have been false coloured: all QRFP neurons have been coloured green, with any other co-staining false coloured magenta, regardless of secondary antibody fluorophore.
### Table 2.1: Antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Origin</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-c-fos</td>
<td>SC-52 (Santa Cruz)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit anti-CART</td>
<td>H-003-62 (Phoenix)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Goat anti-ChAT</td>
<td>AB144P (Millipore)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit anti-dsRed</td>
<td>632496 (Clontech)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit anti-GAD65/67</td>
<td>AB1511 (Millipore)</td>
<td>1:500</td>
</tr>
<tr>
<td>Chicken anti-GFP</td>
<td>13970 (Cell Signalling)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Guinea pig anti-insulin</td>
<td>08-0067 (Life Technology)</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-MCH</td>
<td>H-070-47 (Phoenix)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit anti-nNOS</td>
<td>AB5380 (Millipore)</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-orexinA</td>
<td>SC-8070 (Santa Cruz)</td>
<td>1:2000</td>
</tr>
<tr>
<td>Rabbit anti-oxytocin</td>
<td>AB911 (Millipore)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit anti-pSTAT3</td>
<td>9131L (Cell Signalling)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit anti-QRFP</td>
<td>AB126388 (Millipore)</td>
<td>1:100 – 1:1000</td>
</tr>
<tr>
<td>Sheep anti-TH</td>
<td>AB1542 (Millipore)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Guinea pig anti-VGLUT2</td>
<td>AB2251 (Millipore)</td>
<td>1:500</td>
</tr>
</tbody>
</table>

**Secondary antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Origin</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donkey anti-chicken</td>
<td>703-545-155 (Jackson)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Donkey anti-rabbit</td>
<td>711-585-132 (Jackson)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Donkey anti-goat</td>
<td>705-585-147 (Jackson)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Donkey anti-sheep</td>
<td>A11016 (Invitrogen)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Goat anti-guinea pig</td>
<td>A11076 (Invitrogen)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Donkey anti-rabbit</td>
<td>711-065-152 (Jackson)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Streptavidin</td>
<td>016-580-084 (Jackson)</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

Cell Signalling Biotechnologies; Boston, MA, USA  
Clontech; Mountain View, CA, USA  
Invitrogen; Carlsbad, CA USA  
Jackson ImmunoResearch Inc; West Grove, PA, USA  
Life Technologies; Carlsbad, CA, USA  
Merck Millipore; Darmstadt, Germany  
Phoenix Pharmaceuticals, Inc.; Burlingame, CA, USA  
Santa Cruz Biotechnology; Heidelberg, Germany

### 2.2.3 Genotyping

Ear notches were collected from transgenic mice for genotyping, prior to all experimental protocols. Ear notches were heated to 95°C in lysis buffer (0.25% v/v 10M NaOH, 0.04% v/v 0.5M EDTA, in distilled H₂O) for 30-60 minutes, to extract DNA, at which point neutralising buffer (0.63% w/v Trizma hydrochloride (Sigma-Aldrich), in distilled H₂O) was added and samples vortexed briefly before transfer to ice. Polymerase chain reaction (PCR) was used to amplify specific wild-type and target alleles in order to identify genotypes, using unique wild-type and target primers (Table 2.2). Reaction mixtures differed for specific PCR reactions (Table 2.3), but all contained standard master mix components (5x buffer, MgCl₂, dNTPs, GoTaq Hot Start Polymerase (all Promega; Madison, WI, USA), as well as dimethylsulfoxide (DMSO) (Sigma-Aldrich) and/or betaine (Sigma-Aldrich) in some cases), along with unique primers. Master mixes of 22.5μl were combined with 2.5μl DNA template, and mixed in individual PCR tubes. PCR reactions were performed in a thermal cycler, using unique cycling parameters (Table 2.4).
PCR products were analysed using agarose gel electrophoresis to separate amplified DNA and bands were visualised using a UV transilluminator. Gels were made with either 1.5% or 1.8% agarose (Bioline; London, UK) and 0.005% ethidium bromide (Promega) in 1x tris-acetate-EDTA (TAE) buffer (24.2% w/v Trizma hydrochloride, 5.71% v/v glacial acetic acid, 1% v/v 0.5M EDTA, in distilled H₂O). 8μl PCR product was loaded per well and 1.5μl of Hyperladder IV marker (Bioline) loaded into separate wells. Gels were run in 1x TAE buffer at 70V for around 60 minutes.

Table 2.2: PCR primers used for genotyping

<table>
<thead>
<tr>
<th>PCR reaction</th>
<th>Primer</th>
<th>Sequence</th>
<th>Band size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cre</td>
<td>Control F</td>
<td>GGT CAG CCT AAT TAG CTC TGT</td>
<td>650 bp</td>
</tr>
<tr>
<td></td>
<td>Control R</td>
<td>GAT CTC CAG CTC CTC TGT C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Target F</td>
<td>GCC CTG GAA GGG ATT TTT GAA GCA</td>
<td>259 bp</td>
</tr>
<tr>
<td></td>
<td>Target R</td>
<td>ATG GCT AAT CGC CAT CTT CCA GCA</td>
<td></td>
</tr>
<tr>
<td>FLEX-Qrfp</td>
<td>LUS4 A</td>
<td>ACA GGG AGA AAC CAC ACT GAC ATT AGA GC</td>
<td>WT: 292 bp KO: 511 bp Rescue: 424 bp</td>
</tr>
<tr>
<td></td>
<td>LUS4 B</td>
<td>GAA AAT CAG AAC AGT CAG GCT GCT AAA GC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LUS4 C</td>
<td>AAT AAA TAC AGG GAG GGG CTA CAG ACA GC</td>
<td></td>
</tr>
<tr>
<td>Gpr103a KO</td>
<td>YS102W</td>
<td>CAT TGC CCA CTG TGT ATC G</td>
<td>WT: 566 bp KO: 794 bp</td>
</tr>
<tr>
<td></td>
<td>YS102S</td>
<td>GGA GGC AGG AAC CAC TAG CTA AAA GC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>YSNEO</td>
<td>CAT AGC CTA GAA GGG CTA CAG ACA GC</td>
<td></td>
</tr>
<tr>
<td>Gpr103b KO</td>
<td>4972-41</td>
<td>ACA TGG ATC AAC TGT TGG TAA GG</td>
<td>WT: 285 bp KO: 378 bp</td>
</tr>
<tr>
<td></td>
<td>4973-44</td>
<td>GTT GCC AAA GGC TAG GAC AAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4973-46</td>
<td>ATA CGA CCT CAT CCA TTT GAC TG</td>
<td></td>
</tr>
</tbody>
</table>

*All cre-recombinase lines were genotyped using the same protocol +RT-PCR (described below) used the same mastermix components but a final volume of 24μl and 1μl of cDNA rather than genomic DNA

Table 2.3: Genotyping PCR mastermixes

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Cre*</th>
<th>FLEX-Qrfp</th>
<th>Gpr103a KO</th>
<th>Gpr103b KO</th>
<th>RT-PCR*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x buffer</td>
<td>5μl</td>
<td>5μl</td>
<td>5μl</td>
<td>5μl</td>
<td>5μl</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2μl</td>
<td>2μl</td>
<td>2μl</td>
<td>2μl</td>
<td>2μl</td>
</tr>
<tr>
<td>dNTPs</td>
<td>0.5μl</td>
<td>0.5μl</td>
<td>0.5μl</td>
<td>0.5μl</td>
<td>0.5μl</td>
</tr>
<tr>
<td>GoTaq</td>
<td>0.125μl</td>
<td>0.2μl</td>
<td>0.2μl</td>
<td>0.2μl</td>
<td>0.2μl</td>
</tr>
<tr>
<td>H₂O</td>
<td>12.375μl</td>
<td>11.4μl</td>
<td>6.65μl</td>
<td>11.4μl</td>
<td>14.3μl</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>2.5μl</td>
<td>2.5μl</td>
<td>2.5μl</td>
<td>2.5μl</td>
<td>1μl</td>
</tr>
<tr>
<td>Primers</td>
<td>Target F &amp; R: 0.75μl each</td>
<td>LUS4 A: 1μl</td>
<td>LUS4 B: 1μl</td>
<td>LUS4 C: 1μl</td>
<td>YS102W: 0.25μl</td>
</tr>
<tr>
<td></td>
<td>Control F &amp; R: 0.5μl each</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>-</td>
<td>-</td>
<td>0.4μl</td>
<td>0.4μl</td>
<td>0.4μl</td>
</tr>
<tr>
<td>Betaine</td>
<td>-</td>
<td>-</td>
<td>6μl</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*All cre-recombinase lines were genotyped using the same protocol +RT-PCR (described below) used the same mastermix components but a final volume of 24μl and 1μl of cDNA rather than genomic DNA
Table 2.4: Genotyping PCR cycling parameters

<table>
<thead>
<tr>
<th>Cycling step</th>
<th>Cre*</th>
<th>FlEx-Orfp</th>
<th>Gpr103a/b KO</th>
<th>RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymerase activation</td>
<td>95°C, 2mins</td>
<td>94°C, 2mins</td>
<td>95°C, 2mins</td>
<td>95°C, 2mins</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C, 30s</td>
<td>94°C, 30s</td>
<td>94°C, 45s</td>
<td>95°C, 30s</td>
</tr>
<tr>
<td>Annealing</td>
<td>62°C, 60s</td>
<td>65°C, 30s</td>
<td>56°C, 60s</td>
<td>60°C, 60s</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C, 60s</td>
<td>72°C, 30s</td>
<td>72°C, 60s</td>
<td>72°C, 60s</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C, 5mins</td>
<td>72°C, 5mins</td>
<td>72°C, 5mins</td>
<td>72°C, 5 mins</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>4°C</td>
<td>4°C</td>
<td>4°C</td>
</tr>
</tbody>
</table>

*All cre-recombinase lines were genotyped using the same cycling parameters

2.2.4 Relative quantitative real time PCR (qPCR)

**RNA extraction**

Samples frozen at -80°C were used for RNA extraction using Tri-Reagent (Sigma-Aldrich) for digestion. Samples were transferred, whilst still frozen, directly into 1ml Tri-Reagent in Lysing Matrix D tubes (MP Biomedicals; Solon, OH USA). The samples were then homogenised in these tubes, in a FastPrep homogeniser (MP Biomedicals) for 40s at a speed of 6.0m/s, followed by incubation at room temperature for five minutes. 200μl chloroform (Fisher Scientific) was then added to the tubes, which were then shaken vigorously for 15 seconds and left for three minutes at room temperature for the solvent layers to begin separation. The samples were centrifuged at 12,000 rpm for 15 minutes at 4°C to form two distinct layers. 500μl was removed from the top, aqueous layer in the lysing tubes and thoroughly mixed with 500μl of pure isopropanol (Fisher Scientific). Samples were left to incubate for a further ten minutes at room temperature before being centrifuged at 12,000 rpm for ten minutes at 4°C and the supernatant discarded. The remaining RNA pellet was washed with 70% ethanol (Fisher Scientific) and centrifuged at 12,000 rpm for five minutes at 4°C and the washing step repeated to obtain a pure RNA sample. After the second wash, the supernatant was discarded and the pellet dried at 56°C to remove any remaining alcohol. Pellets were then resuspended in 150-500μl nuclease-free water, depending on pellet size, and incubated for a further ten minutes at 56°C to ensure samples were fully mixed.

RNA was extracted from WAT via a slightly modified method, to account for the potential for impurities caused by the high fat content. Following homogenisation, as above, samples were immediately centrifuged for 10 minutes. This centrifugation produced a clear fat layer at the top of the sample, with a pink layer containing RNA and DNA underneath. A known volume of the pink layer was transferred to a clean Eppendorf and 0.2x this volume of chloroform was added. Tubes were shaken vigorously for 15 seconds and left for three minutes at room temperature, to allow phase separation to begin, before being centrifuged for 15 minutes at 12,000 rpm at 4°C. A known volume of the top, aqueous layer was added to an equal volume of chloroform and mixed well followed by centrifugation at 12,000 rpm at 4°C for two minutes. A known volume of the top layer was added to an equal volume of isopropanol for RNA precipitation, followed by the usual washing steps with ethanol, as described above. The extra chloroform step served to remove
contaminating phenols from the sample, which, in WAT RNA extracted by the usual method, caused contamination and impure samples.

A NanoDrop 3300 instrument (Thermo Fisher Scientific; Wilmington, DE, USA) was used to quantify the amount of RNA within each sample as well as the quality of the RNA by analysing the absorbance ratios at 260/280nm and 260/230nm. Absorbance ratios of 1.8-2.0 (260/280nm) and 1.9-2.2 (260/230nm) were indicative of acceptable RNA quality and samples outside of these ranges were discarded from further study. These stock RNA samples were then stored at -80°C for long-term storage.

**Reverse-transcription of RNA samples**

RNA samples were converted to cDNA by employing a reverse transcription (RT) protocol. In order to reduce variation in RT efficiency, RNA stock samples were diluted to either the lowest sample concentration in the group, or to 300ng/μl, whichever was lower. In order to remove contamination by genomic DNA, 6μl of each RNA sample was incubated with an RNase-free DNase (1μl RQ1 DNase + 1μl RQ1 buffer; Promega) at 37°C for 30 minutes. The action of the DNase was inhibited by incubating samples with 1μl DNase STOP solution (Promega) at 65°C for 10 minutes. The DNase-treated RNA samples were reverse transcribed using a high capacity RNA-to-cDNA Kit (Applied Biosystems; Thermo Fisher Scientific) following manufacturers guidelines. In brief, 1μl 20x RT enzyme and 10μl 2x RT buffer were added to 9μl of the RNA sample and the mixture incubated at 37°C for 60 minutes before enzymatic activity was halted by incubating at 95°C for 5 minutes. Short-term storage of these samples was carried out at -20°C.

**Primer design**

When available, primer sequences from the literature were used for relative quantitative real-time PCR (qPCR) but, when this was not possible, bespoke primers were designed in house. Gene transcript mRNA sequences, obtained from the National Centre for Biotechnology Information (NCBI: https://www.ncbi.nlm.nih.gov/gene) database, were inputted into Primer3 (a web based design tool: http://primer3.ut.ee/). All parameters aside from those listed in Table 2.5 were kept as default settings. Primers were selected to span exon-exon boundaries, and specificity checked using the Basic Local Alignment Search Tool (BLAST: https://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure chosen primers did not show high affinity for any alternative sequences.

**Table 2.5: qPCR primer design (Primer3 parameters)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer size</td>
<td>18 – 30 base pairs</td>
</tr>
<tr>
<td>Amplicon size</td>
<td>60 – 200 base pairs</td>
</tr>
<tr>
<td>Minimum melting temp</td>
<td>58°C</td>
</tr>
<tr>
<td>Maximum melting temp</td>
<td>60°C</td>
</tr>
<tr>
<td>Optimum melting temp</td>
<td>59°C</td>
</tr>
<tr>
<td>Max Poly X (nucleotide repeats)</td>
<td>3</td>
</tr>
<tr>
<td>Limit GC content</td>
<td>40 – 60%</td>
</tr>
</tbody>
</table>
Individual forward and reverse primers arrived as oligonucleotide pellets (Sigma-Aldrich) and were resuspended in nuclease free water to a concentration of 100µM. Primer stocks were subsequently diluted to working concentrations of 10µM (mixed forward and reverse) and all primer dilutions stored at -20°C.

**Primer validation**

In order to assess the efficiency of cDNA amplification by primers, over a range of concentrations, standard curves were produced. Primers were tested in final volumes of 10µl containing 5µl QuantiFAST SYBR Green mastermix (Promega), 1.5µl nuclease free water and 0.5µl primers (0.5µM, mixed forward and reverse primers). 3µl cDNA (at 200, 100, 10, 1, 0.1, 0.01ng/µl concentrations) was added in quadruplicate on a 384-well plate. qPCR reactions were carried out in an ABI Prism 7300 Sequence Detection System (Applied Biosystems) using constant cycling parameters for all experiments (**Figure 2.1**). At the end of each protocol, the system ran standard cycling parameters to enable melt curve analysis to be performed.

![qPCR cycling parameters](image1)

**Figure 2.1:** qPCR cycling parameters

cDNA concentration was plotted against Ct value (cycle number when the fluorescent signal crossed the threshold value) to obtain standard curves. Reaction efficiencies between 90-110% were deemed acceptable for primers, as identified by slopes of between -3.6 and -3.1 (slope at 100% efficiency is -3.32) (**Figure 2.2**). Melt curve analysis was also performed to ensure primers only produced one peak, and thus only produced one amplification product.

![Standard and dissociation curves](image2)

**Figure 2.2:** Representative standard and dissociation curves from m18s primers
**qPCR**

Expression of specific genes was quantified in the cDNA samples through the use of qPCR. cDNA samples were diluted to 8.3ng/μl and 3μl loaded into each well of a 384-well clear plate. qPCR protocols were carried out using the same mastermix as used for primer validation, to which was added 3μl cDNA. All samples were run in quadruplicate and in all studies two ‘housekeeper’ genes were analysed from each sample as a positive control and to enable normalisation. The housekeeper genes used were β-actin and murine ribosomal 18s (m18s) which have previously been shown to have stable expression levels. However, during the course of this thesis, the expression of β-actin was observed to be altered between lean and overweight subjects in a number of tissues, whilst no change was observed with m18s. As a result, m18s was used as the housekeeping gene for the calculation of changes in expression level of target genes. Sequences of primers used can be found in Table 2.6.

**Table 2.6: qPCR primer sequences**

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>AGA GGG AAA TCG TGC GTG AC</td>
<td>CAA TAG TGA TGA CCT GGC CGT</td>
</tr>
<tr>
<td>Atgl</td>
<td>TGT GGC CTC ATT CCT CCT AC</td>
<td>TCG TGG TTG GTG GAG CT</td>
</tr>
<tr>
<td>Dgat2</td>
<td>AGT GGC AAT GCT ATC ATC ATC GT</td>
<td>TCT TCT GGA CCC ATC GGC CCC AGG A</td>
</tr>
<tr>
<td>Fabp4</td>
<td>GAA AAC GAG ATG GTG ACA AGC</td>
<td>TGG TGG AAG TCA CGC CTC TT</td>
</tr>
<tr>
<td>Fasn</td>
<td>GCC AAG TGG TGC TGA CT</td>
<td>CGA ATG TGG GCT TGG T</td>
</tr>
<tr>
<td>G6Pase</td>
<td>GTG GAC GCT CTG TGG ACT TT</td>
<td>GGT TCC CAG GTC TTT GAA GA</td>
</tr>
<tr>
<td>Gpr103a</td>
<td>CAG GGG AGA AAA GGC AAC TC</td>
<td>GAC CTT TTT CTT AAA ATA ACT</td>
</tr>
<tr>
<td>Gpr103b</td>
<td>TCA TAG GAT GAC CCA TGT GGC</td>
<td>AGC AAC AGT GGT AGC AGG AA</td>
</tr>
<tr>
<td>Hsl</td>
<td>GCT GGG CGT TCA CGT ACT GT</td>
<td>GTA ACT GGG TAG GCT GCC AT</td>
</tr>
<tr>
<td>Lpl</td>
<td>AGG GCT CTG CCT GAG TTG TA</td>
<td>CCA TGG CCT CAT TCC TCC TAC</td>
</tr>
<tr>
<td>m18s</td>
<td>GCC AAC GAG ATG GTG ACA AC</td>
<td>TCG TGG TGG CCT TCC GTC AAT</td>
</tr>
<tr>
<td>Pepck</td>
<td>GGG AAA TCA CCG CAT AGT CT</td>
<td>CAT GCT CAC ACA GAG ACA GG</td>
</tr>
<tr>
<td>Qrfp</td>
<td>CTG CAG TCA CAG GGA CAA GA</td>
<td>ACT GTC CCC AAG GAG GGT GG</td>
</tr>
<tr>
<td>Ucp1</td>
<td>CAC CTG GCT GGA CAC T</td>
<td>GTC CTT CCT TGG TGT ACA TG</td>
</tr>
</tbody>
</table>

Relative quantification was conducted using the ΔΔCt method (Schmittgen & Livak 2008). In brief, the Ct values, cycle number at which the gene expression reached threshold, for the target genes and the ‘housekeeper’ genes were compared between treatment groups. Ct values for each gene in each sample were averaged from the quadruplicates (with anomalies removed, calculated as values more than two standard deviations from the mean) and the average values for the target gene were subtracted from the ‘housekeeper’ gene to produce a ΔCt value. An average was taken of these ΔCt values for the control (non-treated) group and this average subtracted from each individual ΔCt value, including control samples to produce a ΔΔCt value for each sample. In order to calculate the fold difference in expression, a calculation of $2^{\Delta\Delta C_t}$ was performed. By averaging the fold differences of treated and non-treated groups, the relative expression in the non-treated group should be roughly one, and the fold difference in the treated samples is expressed relative to this control group. As such, the expression of the ‘housekeeper’ gene should remain constant.
regardless of treatment, and any changes in the fold difference of the target gene expression are attributable to the effects of treatment.

2.2.5 End point reverse transcription PCR (RT-PCR)
Following the termination of in vivo studies from which tissue was collected for further analysis, qualitative analysis of cDNA was carried out to show mice, and tissues, that expressed a specific gene, and allowed identification of those that were lacking this gene. Rather than providing quantitative data as described for qPCR, these studies were analysed using agarose gel electrophoresis as described for genotyping studies.

RT-PCR was performed in the same way as genotyping PCR, using the same mastermix components (Table 2.3), but using cDNA rather than DNA. Following amplification of the product with PCR, samples were loaded into agarose gel, as described for genotyping, and separated with electrophoresis. The subsequent imaging of these gels enabled identification of wild-type and knock-out animals by the existence, or absence, of the specific product band.

2.3 Statistical Methods
All graphs were produced using GraphPad Prism software (version 7.01: GraphPad Software, Inc; La Jolla, CA, USA), with statistical analysis performed using this same package. Unless stated otherwise, data is presented as mean ± standard error of the mean (SEM). Relevant statistical methods were applied as necessary, and tests used have been stated for each dataset, with appropriate post hoc tests applied where permitted.
Chapter 3

The Location of QRFP in the Brain and Other Tissues
3.1 Introduction

The hypothalamus is the critical centre for monitoring the metabolic status of animals by collating signals from different regions of the brain, as well as from tissues in the periphery. Neuronal populations within the hypothalamus are responsible for the maintenance of energy homeostasis, through specific actions on energy intake and expenditure. In particular, early seminal studies revealed the importance of the ventromedial and lateral hypothalamic areas in feeding regulation (Hetherington & Ranson 1940; Anand & Brobeck 1951). Electrical stimulation of the ventromedial hypothalamus produced hypophagia, whilst bilateral lesions caused voracious feeding and obesity, with the opposite effects observed with manipulations of the lateral hypothalamus. This led to the characterisation of the ventromedial hypothalamus as a satiety centre and the lateral hypothalamus as a feeding centre. Modern methodology has enabled more precise study of these large, heterogeneous regions, with, for example, distinct neuronal populations located within the lateral hypothalamic area (LHA) identified as playing a role in feeding control. Two of the best characterised LHA populations contain orexin or melanin-concentrating hormone (MCH), both of which exert orexigenic actions when centrally administered (Qu et al. 1996; Sakurai et al. 1998).

Whilst neuronal cell bodies expressing these peptides are restricted to the LHA, their projections innervate multiple regions of the brain (Bittencourt et al. 1992; Tsujino & Sakurai 2009). As a result, both of these peptides influence a number of different aspects of homeostasis, including energy intake and expenditure, and arousal/wakefulness (Barson et al. 2013), through interactions with different target populations. In fact, it is now generally appreciated that the feeding effect of orexin is secondary to a role in arousal.

In 2003, three independent groups simultaneously discovered QRFP, and its N-terminal truncated form 26RFa, within the hypothalamus (Chartrel et al. 2003; Fukusumi et al. 2003; Jiang et al. 2003), and early gene expression studies suggested a restricted distribution in the LHA and ventromedial hypothalamic nucleus (VMN) (Chartrel et al. 2003; Takayasu et al. 2006). From these initial studies, one of the most frequently reported actions of centrally administered QRFP has been an orexigenic drive in satiated animals. However, as with orexin and MCH, QRFP/26RFa administration also is reported to produce effects on arousal, energy expenditure and blood pressure (Takayasu et al. 2006). Radioligand binding studies have determined that QRFP/26RFa binding sites are also widespread in the brain, with expression of mRNA of Gpr103a/b receptors confirming location in many regions of the brain (Takayasu et al. 2006; Kampe et al. 2006; Bruzzone et al. 2007). QRFP/26RFa binding sites, and receptor expression, in the locus coeruleus (LC), tuberomammillary nucleus (TMN) and dorsal raphé nucleus (DR) provide a basis for central action on the ascending arousal system, whilst binding within the VMN, LHA and arcuate nucleus (Arc) indicate potential for a direct influence on energy balance. Furthermore, QRFP/26RFa binding sites and Gpr103 expression in the ventral tegmental area (VTA) and nucleus accumbens (NAcc) suggest QRFP could be involved in the reward pathways associated with hedonic feeding.
Finally, binding sites within the nucleus of the tractus solitarius (NTS) and the dorsal motor nucleus of the vagus (DMV), along with binding in the spinal column, provide evidence that central QRFP neurons may influence the autonomic system and peripheral organ function. To date, a lack of reliable antibody has hindered our understanding of the connectivity of QRFP neurons, something we have aimed to overcome in our present work.

More recently, studies conducted in vitro have suggested effects of QRFP/26RFa directly on tissues in the periphery. QRFP appears to exert an antilipolytic effect on white adipose tissue (WAT) or dispersed adipocytes in culture, through activation of Gpr103b (Mulumba et al. 2010), with Qrfp expression decreasing and Gpr103b increasing with obesity onset, showing an adaptive profile indicative of a paracrine role in adipogenesis. Within the pancreas, QRFP and 26RFa have been reported to cause opposing effects on glucose-stimulated insulin secretion, with QRFP expression also identified in pancreatic islets (Granata et al. 2014). It may be significant that peripheral injection of 26RFa is capable of altering glucose handling and insulin sensitivity (Prévost et al. 2015). Whilst a single human study identified changes in circulating QRFP in anorexia nervosa patients (Galusca et al. 2012), with later study indicating this originated in the gut (Prévost et al. 2015), the literature is yet to agree on whether QRFP is expressed at high enough levels in peripheral tissues to be of physiological consequence. The highest level of Qrfp mRNA expression appears to be within the hypothalamus, meaning it is possible that physiological actions of QRFP may be restricted to the brain.

Within this Chapter, we wanted to characterise both the central and peripheral expression of QRFP, using a number of novel transgenic mouse lines. We aimed to confirm definitively the expression profile of QRFP neurons and where they project to, alongside studies designed to identify the expression of Qrfp in tissues of the periphery. Overall these studies will allow us to better observe QRFP expression, and develop future experiments to investigate its role in different systems, with the view to understanding its integration into our current models of homeostatic control.

### 3.1.1 Objectives

**Identify the expression of Qrfp within a range of tissues relevant to metabolism**

Direct effects of QRFP on tissues such as WAT and pancreas indicate that QRFP may be expressed at physiologically relevant levels within tissues outside the brain. We have developed a Qrfp knock-out model (FlEx-Qrfp) which will allow us to investigate expression of Qrfp mRNA within these tissues, using knock-out animals to confirm the specificity of our results.
Visualise the QRFP neuronal population

Previous work has suggested that QRFP neurons are found within the LHA and VMN, although this work, performed using in situ hybridisation histology, only provided low power images from which cellular resolution is not possible. We have produced a Qrfp-cre mouse line which, by crossing with a cre-dependent enhanced yellow fluorescent protein (eYFP) reporter mouse, will enable us to directly label QRFP neurons for immunohistochemical visualisation. This model also permits the investigation of potential co-expression of other neuropeptides in the QRFP neurons, to ascertain whether they are a sub-population of other previously characterised neurons.

Identify regions of the brain that are innervated by QRFP neurons

Within the literature, reports of binding sites of QRFP/26RFa have suggested widespread innervation by QRFP neurons, with similarly widespread expression of Gpr103 mRNA supporting this. However, definitive identification of QRFP neuronal projections has yet to be conducted due to a lack of available tools to carry out this work. We have crossed our Qrfp-cre mouse to a cre-dependent channel rhodopsin 2 (ChR2) reporter mouse which facilitates the direct visualisation of QRFP neuronal fibres in the brain for the first time. Specific regions of interest will be identified in this model to help form hypotheses about physiological functions of QRFP. Our transgenic models will be used for tracing studies to provide further confirmation of projection sites, and to determine whether the QRFP neurons represent a homogenous population, through retrograde tracing and virally-delivered anterograde tracing.

3.2 Methods

3.2.1 Generation of transgenic Qrfp-cre mice

The Qrfp-cre mouse line was generated by GenOway (Lyon, France) through homologous recombination in embryonic stem cells from a C57Bl/6 genetic background. Through the use of a targeting vector, an IRES-cre sequence was inserted into the 3’ untranslated region of exon 2 of the Qrfp gene. The targeting vector used for the generation of this line was formed containing: long and short homology regions to the Qrfp gene (on the C57Bl/6 genetic background); an IRES-cre sequence located in the 3’ untranslated region of exon 2 of the Qrfp gene; a neomycin cassette flanked by FRT sites, for positive selection purposes; and a diphtheria toxin A cassette for negative selection purposes. Mouse genomic DNA was cloned from the C57Bl/6 background, from the regions encompassing the Qrfp exon 2, and the homology regions were then generated from these sequences. Selection of correctly targeted clones was made using the aforementioned selection sequences and these clones were injected into Balb/C blastocysts which were implanted into OF1 pseudo-pregnant females. The resulting offspring were chimeric, contributed to by the C57Bl/6 stem cells and Balb/c blastocysts, and the degree of chimerism was determined by analysis of coat colour. Highly chimeric males were bred with C57Bl/6 females to produce heterozygous offspring for the Qrfp-cre transgene. Following development of mice expressing this
transgene, the neomycin cassette was removed *in vivo* by cre-recombinase mediated excision, following breeding with a cre-recombinase expressing deleter mouse. The genotype of mice was extensively analysed by PCR and southern blot and heterozygous mice were delivered to the University of Manchester.

**Genotyping of Qrfp-cre colony**

Mice were maintained as a heterozygous, inbred colony in house, with breeding crosses set up between cre-positive and wild-type mice. All mice were identified by ear notches and the resultant biopsy used for genotyping the mice by PCR, using the protocol and primers described in General Methods.

**Generation of Qrfp-cre crosses**

Heterozygous *Qrfp*-cre mice were bred with mice from the homozygous ROSA26-eYFP line described in General Methods, to produce the *Qrfp*-cre::eYFP line. The resultant offspring were all heterozygous for eYFP and, following basic Mendelian genetics, around 50% possessed the *Qrfp*-cre transgene, as determined by PCR. The *Qrfp*-cre mice were also cross bred with mice from the homozygous ROSA26-ChR2/eYFP line, to create the *Qrfp*-cre::ChR2 model.

### 3.2.2 QRFP peripheral expression

Male FlEx-*Qrfp* mice, described fully in Chapter 5, were used for investigating the expression of QRFP in the brain as well as a number of metabolically relevant peripheral tissues. Briefly, the FlEx-*Qrfp* model is a global knock-out mouse lacking the *Qrfp* gene in the entire body. In this study, three wild-type and three knock-out mice were culled by decapitation, and brains, eye, brown adipose tissue (BAT), liver, skeletal muscle and epididymal white adipose tissue (eWAT) were all dissected, as described in General Methods. RNA was extracted from each tissue sample, and reverse transcribed to produce cDNA for reverse transcription PCR (RT-PCR) analysis. All cDNA samples were diluted to the same concentration as the least concentrated sample, or to 200ng/µl if all samples were above this concentration (hypothalamic samples were diluted to 50ng/µl due to high expression of *Qrfp* in this tissue). cDNA samples were amplified by PCR, using mastermix and primers described earlier, and agarose gel electrophoresis used to visualise the resulting product band. The presence of bands in wild-type brain samples, and absence of bands in knock-out animals was used to confirm the knock-out mice were as expected.

### 3.2.3 Immunohistochemical investigation of QRFP neurons

**Visualisation of the QRFP neurons**

Male *Qrfp*-cre::eYFP mice were used for immunohistochemistry to visualise the QRFP neuronal population. All mice were group housed with littermates and underwent no prior treatment before being anaesthetised and transcardially perfused with paraformaldehyde (PFA).
Single-label immunohistochemistry was then employed to indirectly label Qrfp::eYFP neurons, through the use of an anti-green fluorescent protein (GFP) antibody. Coronal sections through the entire brain were viewed to confirm localisation of the QRFP population.

To supplement these studies, other Qrfp-cre::eYFP brains were sectioned in the sagittal plane for investigation of the rostro-caudal extent of the QRFP population. In order to aide with this investigation, sections were dual-labelled for either tyrosine hydroxylase (TH) or orexin, using anti-TH or anti-orexin antibodies concurrently with the aforementioned anti-GFP antibody. The locations of these well-defined populations helped detail the expression profile of the QRFP population by providing known landmarks.

Eyes were dissected from PFA-perfused male Qrfp-cre::ChR2 mice for immunohistochemistry investigation of QRFP expression. Eyes were cross sectioned using a cryostat and mounted sections dried for 24 hours prior to immunohistochemistry. The expression of QRFP in the eye was investigated, by on-slide immunohistochemistry, using the anti-GFP antibody, along with the anti-TH antibody for orientation.

Single-label immunohistochemistry was performed to test direct QRFP labelling using an anti-QRFP antibody. A range of antibody concentrations, from 1:1000 to 1:100, was used in the immunohistochemistry protocol described in General Methods, with fluorescence-labelled secondary antibodies, or with biotinylated antibodies for use with the streptavidin-biotin complex. A further alteration of the protocol described in General Methods, was the addition of 3.75% acrolein (Sigma-Aldrich) to the 4% PFA solution used to perfuse the mice. Finally, Qrfp-cre::eYFP mice were injected intracranially with colchicine (Sigma-Aldrich), a neurotoxin that prevents neurotransmitter transport leading to an accumulation of transmitter in neuronal somata. 24 hours after injection, mice were transcardially perfused, with 4% PFA.

**Co-expression of QRFP with other neurotransmitters**

Additional coronal sections were used for dual-label immunohistochemistry to investigate potential co-expression of QRFP with other transmitters. Two well characterised lateral hypothalamic populations, containing orexin or MCH, were investigated due to their similar distribution and orexigenic effects *in vivo*. Immunohistochemistry techniques described in General Methods were employed using a cocktail of chicken anti-GFP and either goat anti-orexin or rabbit anti-MCH antibodies.

Most peptidergic neurons can also be classed according to their expression of the classical fast neurotransmitters γ-aminobutyric acid (GABA), glutamate, acetylcholine or catecholamines. In order to identify whether QRFP neurons co-express any of these transmitters, dual-label immunohistochemistry was carried out on Qrfp-cre::eYFP brain sections using anti-GFP, anti-glutamate decarboxylase (Gad65/67) and anti-vesicular glutamate transporter 2 (Vglut2), anti-TH
(for catecholaminergic cell identification), anti-choline acetyl transferase (ChAT) (for cholinergic cell identification), or anti-neuronal nitric oxide synthase (nNOS) antibodies.

To add clarity to these results, the aforementioned FlEx-Qrfp mice were cross-bred with Gad2-cre or Vglut2-cre mice. The full extent of the transgenic manipulation of the FlEx-Qrfp mouse, and resulting cross breeding, will be fully described in Chapter 5, but, briefly, these crosses produced mice in which QRFP was ‘rescued’ only in glutamic acid decarboxylase 2 (Gad2) or Vglut2 containing cells. Subsequently, RT-PCR and relative quantitative real-time PCR (qPCR) were carried out on hypothalami and eyes from knock-out and ‘rescue’ mice to provide evidence for whether QRFP neurons are GABAergic or glutamatergic.

3.2.4 Efferent projection of QRFP neurons: cre-dependent tracing

*Channel rhodopsin and synaptophysin tracing*

Male Qrfp-cre::ChR2 mice were used for immunohistochemistry to investigate QRFP axonal projections. Coronal sections were dual-labelled for eYFP (to visualise QRFP fibres) along with orexin, MCH, TH and ChAT. In the first instance, sections for this study were selected based on the co-ordinates of QRFP neurons previously identified, to capture the entire cross section of the hypothalamic population, and enable visualisation of how the local QRFP projections interact with orexin and MCH neurons. Further immunohistochemistry was conducted to identify QRFP axonal projections throughout the brain, using well defined populations (namely orexin and TH) to confirm nuclei of interest. These studies were complemented by sections cut in the sagittal plane to visualise the rostro-caudal extent of the QRFP axonal projections using orexin, TH, cocaine- and amphetamine-regulated transcript (CART), ChAT or oxytocin for comparison.

To supplement the above, virally targeted, cre-dependent technology was used to trace QRFP neurons. Male Qrfp-cre::eYFP mice (n=10) were used for stereotaxic injection of anterograde tracer: cre-dependent adeno-associated viruses (AAVs) packaged with ChR2 tagged to mCherry. As described in General Methods, the viruses were injected using a nanoinjector, with injections being made unilaterally. Co-ordinates for the injections were selected based on the study investigating the expression profile of the QRFP neuronal population. In order to maximise the neurons targeted, the co-ordinates used were located within one of the densest parts of the QRFP population, namely the area immediately lateral to the VMN (1.5mm posterior to bregma, 0.9mm from midline at a depth of 5.5mm, 5.7mm, 5.9mm; 3x 13.8nl). Following a two week recovery period, to allow trafficking of ChR2 down the neuron fibres, mice were transcardially perfused. Dual-label immunohistochemistry, using anti-GFP and anti-dsRed antibodies, was used on brain sections containing the hypothalamic QRFP population, to identify neurons infected with the AAV and the transport of ChR2, as shown by their expression of the tagged mCherry molecule.
A follow-up experiment used male Qrfp-cre::eYFP mice (n=12) for stereotaxic injections of a cre-dependent AAV packaged with h-synaptophysin (h-syn) tagged to mCherry. The methodology mimicked that above, with two weeks left after injection to allow trafficking of h-syn to the nerve terminals. Hypothalamic sections were dual-labelled using anti-GFP and anti-dsRed antibodies as previous.

**Retrograde tracing**

To confirm projection sites, retrograde tracers were injected into male Qrfp-cre::eYFP mice, via nanoinjection, into areas previously identified as likely candidate areas for QRFP neuron terminals: superior colliculus (SC: 0.5mm anterior to lambda, 1mm from midline at a depth of 1.2mm, 1.4mm; 2x 13.8nl) and LC (5.4mm posterior to bregma, 0.9mm from midline at a depth of 3.6mm, 3.7mm, 3.8mm; 3x 9.2nl). Following a two-week recovery period, mice were perfused and QRFP neurons visualised to identify co-localisation of retrograde tracer.
3.3 Results

3.3.1 Qrfp expression

Qrfp mRNA has been reported previously as being widespread in human and rat tissues in line with its suggested functions (Jiang et al. 2003; Fukusumi et al. 2003). Here, PCR-based methodology was used to confirm tissue distribution of Qrfp in the mouse. The validity of these results was confirmed by the comparison of Qrfp mRNA expression in a gene knock-out model. In order to investigate the expression of Qrfp, a transgenic global knock-out mouse was produced, FlEx-Qrfp (described fully in Chapter 5), which lacks Qrfp expression. RT-PCR was utilised to identify expression of Qrfp, by amplification of a cDNA product, in wild-type and null mice (n=3) in a number of metabolically relevant tissues: BAT, hypothalamus, liver, skeletal muscle, pancreas, inguinal white adipose tissue (iWAT) and eWAT. Due to previous literature reporting expression of Qrfp within the retina, the eye also was examined. Confirming previous studies, mostly on rat and human tissue, Qrfp was located in most of the tissues studied, although strongest expression was identified within the hypothalamus. As can be seen (Figure 3.1), wild-type mice showed expression of Qrfp whilst the knock-out mice did not. In a couple of tissues, gel lanes corresponding with knock-out mice also had a band, albeit a much weaker one. It was hypothesised this was likely due to over efficient primers and high levels of total RNA present in these tissues, leading to low frequency non-specific primer binding or primer dimers. To confirm this hypothesis, qPCR was employed to confirm lack of QRFP expression in a cohort of unstimulated FlEx-Qrfp mice with no Qrfp cDNA detected in knock-out animals (data not shown).

![Figure 3.1: Expression of Qrfp mRNA in peripheral tissues](image)

RT-PCR revealed Qrfp expression in FlEx-Qrfp wild-type mice. Qrfp is expressed in BAT, eye, hypothalamus, liver, skeletal muscle, eWAT and iWAT, but not pancreas, identified by white bands in wild-type (I-III) but not knock-out (IV-VI) lanes.

BAT: brown adipose tissue; Hypo: hypothalamus; Sk M: skeletal muscle; eWAT: epididymal white adipose tissue; iWAT: inguinal white adipose tissue
3.3.2 Visualisation of QRFP in the brain

To date, the only brain imaging for QRFP in the literature has been conducted using *in situ* hybridisation histology. A variety of immunohistochemistry techniques using a QRFP antibody, as described in Chapter 3 methods, were employed here, in an attempt to visualise the QRFP neuronal population. However, all attempts made so far have failed to label any neurons, and this lack of a reliable antibody for QRFP has rendered neuronal visualisation difficult.

In order to alleviate this problem, a mouse line expressing cre-recombinase under the control of the *Qrfp* promoter (*Qrfp*-cre) was engineered. This line was crossed with a reporter mouse (ROSA26-eYFP), to produce a heterozygous *Qrfp*-cre::eYFP line. Whilst this mouse model should allow visualisation of eYFP directly, the endogenous fluorescence signal is relatively weak to visualise using standard microscopy. As such, the expression profile of the QRFP-expressing cells was investigated using an antibody raised against GFP, but which recognises eYFP.

As previously reported in rat by *in situ* hybridisation histology, QRFP neurons are restricted to the hypothalamus, in particular overlapping with the LHA and periventricular hypothalamic nucleus (PeVH) (Figure 3.2). In general, in the coronal plane, the cell population surrounds the VMN with few, if any, neurons located within the nucleus itself. The appearance of eYFP in dendritic/axonal structures, provides a morphology typical of neurons. Most anteriorly, these QRFP-expressing neurons reside in the ventral part of the hypothalamus, immediately rostral to the VMN (bregma - 0.94mm) and, as the population progresses caudally, the majority of QRFP neurons are located around the lateral and dorsal limits of the VMN. A small number of QRFP-expressing neurons are localised in the PeVH, between the medial limit of the VMN and the 3rd ventricle. Neurons located dorsal to the VMN show morphology consistent with other dorsomedial hypothalamic nucleus (DMN) neuronal populations, namely a smaller size. However, it is difficult to be sure whether these few neurons are located within, or adjacent to, the DMN itself. Currently, it cannot be concluded whether the QRFP neurons represent a homogenous population, or whether there are subsets of these neurons with different functions. A peculiarity of this *Qrfp*-cre::eYFP model is the strong eYFP staining observed in the ependymal cells lining all ventricles within the brain. Previous *in situ* hybridisation studies in rats have not reported *Qrfp* mRNA expression in these cells.
Figure 3.2: QRFP neuronal population – coronal

A-F: Representative images of the expression profile of QRFP neurons in Qrfp-cre::eYFP mouse brain. Left panel: diagram adapted from (Paxinos & Franklin 2001) highlighting key hypothalamic nuclei. Right panel: representative immunohistochemical image.
A: bregma -0.94mm, B: -1.06mm, C: -1.22mm, D: -1.46mm, E: -1.58mm, F: -1.70mm

AHP: anterior hypothalamic area (posterior); Arc: Arcuate nucleus; DMN: dorsomedial hypothalamic nucleus; f: fornix; LHA: lateral hypothalamic area; opt: optic tract; Pe: periventricular hypothalamus; PVN: paraventricular hypothalamic nucleus; sox: supraoptic nucleus; VMN: ventromedial hypothalamic nucleus; 3v: 3rd ventricle
Visualisation of sagittal sections provided additional information regarding the rostro-caudal extent of this population, aided by dual-labelling for other neurons with well-defined distributions. As has been previously described, TH immunostaining is evident within a number of hypothalamic nuclei: namely the Arc, paraventricular hypothalamic nucleus (PVN) and DMN. Of primary interest to the QRFP population, TH neurons within the hypothalamus surround the VMN without entering the nucleus, with dense staining surrounding the ventral and caudal extents of the VMN. The QRFP neurons show a similar trend, in that no neurons were observed within the VMN itself, although they appear to closely surround the rostral and dorsal extents of the nucleus (Figure 3.3A). Dual-labelling for QRFP and orexin in these sagittal sections also confirmed previously described observations from the coronal sections. In particular, QRFP originate more rostrally than the orexin population, with the most rostral cells located at the ventral-most region of the hypothalamus. The QRFP population then progresses caudally through the hypothalamus whilst simultaneously extending dorsally, until QRFP and orexin neurons are intermingled (Figure 3.3B).

Figure 3.3: QRFP neuronal population – sagittal

A: Dual-label immunohistochemistry staining for TH and QRFP in the Qrfp-cre::eYFP mouse brain. TH and QRFP neurons are localised around the outside of the VMN, with the majority of the QRFP population found rostral and dorsal to the VMN (QRFP – TH)

B: Dual-label immunohistochemistry staining for orexin and QRFP in the Qrfp-cre::eYFP mouse brain. The QRFP neuronal population originates rostral to the orexin population and progresses dorso-caudally, before the two populations intermingle (QRFP – orexin)

LHA: lateral hypothalamic area; VMN: ventromedial hypothalamic nucleus
3.3.3 Visualisation of QRFP in the eye

The RT-PCR work described earlier, investigating expression of Qrfp in peripheral tissues, revealed strong expression within the eye. Furthermore, previous literature has reported expression of both Qrfp and Gpr103 in the eye as well (Takayasu et al. 2006; Fukusumi et al. 2003). It was, therefore, a logical progression to investigate the expression of QRFP within the eye using immunohistochemistry. Dual-label immunohistochemistry in Qrfp-cre::ChR2 mouse eyes confirmed strong TH expression within the internuclear layer, as a positive control. QRFP labelling also showed strong expression within the eye but this lies within the retinal ganglion cell layer (Figure 3.4). Furthermore, QRFP fibres are found within this layer, and also converging on the optic nerve, indicating the QRFP positive cells in the retina are likely to be ganglion cells projecting towards the brain.

![Figure 3.4: QRFP expression in the eye](image)

Dual-label immunohistochemistry staining for TH and QRFP in the Qrfp-cre::ChR2 mouse eye. TH is expressed within the internuclear layer whilst QRFP is labelled within the retinal ganglion cells (inner layer) of cre positive (A), but not cre negative (B), mice (QRFP – TH)
3.3.4 Investigating QRFP co-localisation with other transmitters

The distribution of the QRFP neuronal population meant it was important to investigate potential co-expression with other transmitters already characterised in this locality, particularly those which also affect feeding. To this end, immunohistochemistry was employed to identify expression of both orexin and MCH, both of which also can modify feeding behaviour. Despite some overlap in the LHA, between the regional distribution of QRFP and both the orexin and MCH populations, there is no co-expression of either of these neuropeptides within QRFP neurons, indicating that these three orexigenic neurons are discrete populations (Figure 3.5). The QRFP neuronal population originates more rostrally than either MCH or orexin, with these two populations progressing more caudally through the hypothalamus than QRFP.

**Figure 3.5: Investigation of co-expression of QRFP with orexin and MCH**

**A:** Dual-label immunohistochemistry staining for MCH and QRFP in the Qrfp-cre::eYFP mouse brain. MCH and QRFP neurons intermingle within the LHA but are not co-expressed in any neurons (QRFP – MCH)

**B:** Dual-label immunohistochemistry staining for orexin and QRFP in the Qrfp-cre::eYFP mouse brain. Orexin and QRFP neurons intermingle within the LHA but no co-expression of the two exists in any neurons (QRFP – orexin)

VMN: ventromedial hypothalamic nucleus; 3v: 3rd ventricle
Neuronal populations of the lateral hypothalamus can often be separated based on their expression of one of the classical fast neurotransmitters: GABA or glutamate. To date, there have been two studies investigating which neurotransmitter QRFP neurons co-express, if either, although both studies only investigated small sub-regions of the QRFP population as part of a wider reaching study. In our own models, the co-expression of GABA and glutamate, with QRFP, was investigated in Qrfp-cre::eYFP mice through immunohistochemistry by dual-labelling with anti-Gad2 and anti-Vglut2 antibodies. Gad2 is the enzyme responsible for catalysing the production of GABA, whereas Vglut2 is involved in trafficking of glutamate through neurons. Immunohistochemistry for GABAergic or glutamatergic neuronal cell bodies did not work in the present study, perhaps due to the relatively low level of expression of the synthetic enzymes in the cell somata compared with cell terminals. This meant it was not possible to identify co-localisation of QRFP with either neurotransmitter using this technique.

In order to provide an alternative method for solving this problem, the FlEx-Qrfp model, fully described in Chapter 5, was employed. By crossing the FlEx-Qrfp mouse with Gad2-cre or Vglut2-cre mice, the expression of Qrfp could be rescued in either GABA or glutamate neurons, respectively. Qrfp expression was analysed in hypothalami and eyes from both genetic crosses. Qrfp was not expressed in eyes from Vglut2-cre mice but was partially rescued in Gad2-cre mice, indicating at least a proportion of the Qrfp-positive cells in the eye are GABAergic, but none are glutamatergic. Samples from both hypothalami showed rescue of Qrfp, which suggests that the hypothalamic QRFP population consists of both glutamatergic and GABAergic neurons (Figure 3.6).

![Figure 3.6: Identification of glutamatergic and GABAergic QRFP cells](image)

Qrfp expression was rescued in hypothalami from both Vglut2 and Gad2 crossed mice (Left), whilst expression was rescued only in eyes from Gad2 crossed mice (Right).

RT-PCR (Bottom) enabled visualisation of Qrfp rescue in a qualitative manner.
A further set of dual-labelling was carried out in the Qrfp-cre::eYFP model to ascertain whether QRFP neurons co-express other neurotransmitters, specifically in neurons containing the synthetic enzymes TH, ChAT, or nNOS. Dual-labelling in Qrfp-cre::eYFP mice, for ChAT, a marker of cholinergic neurons, revealed no co-localisation with QRFP (Figure 3.7). The ChAT neuronal population within the LHA, whilst resembling the pattern of QRFP neurons, is located much more rostrally compared with the QRFP neurons. A very small number of neurons co-express QRFP and nNOS, but there was no consistency in location between individual mice (Figure 3.7). Labelling TH, a marker of catecholaminergic neurons, identified a very small number of co-expressing neurons, generally located in the ventral region of the QRFP population (Figure 3.8). Intermingling of QRFP and TH neurons occurs within the PeVH although no co-localisation was found, and morphology is distinctly different, with QRFP neurons smaller in size. Strong expression of TH within the Arc and PVN was identified, whilst QRFP neurons border these nuclei. TH expression is also found within the DMN, with separate QRFP neurons located in close proximity to the DMN, although it is difficult to determine whether they lie within the nucleus or not.

Figure 3.7: Investigation of co-expression of QRFP with ChAT and nNOS

QRFP neurons do no express either of the neurotransmitters ChAT (A) or nNOS (B) (QRFP – ChAT/nNOS)

3v: 3rd ventricle
Figure 3.8: Investigation of co-expression of QRFP with TH

Dual-label immunohistochemistry staining for TH and QRFP in the Qrfp-cre::eYFP mouse brain. TH is expressed strongly within the ZI and periventricular area, where QRFP and TH neurons intermingle (A) without co-localising (B). TH is also strongly expressed in the ARC, with QRFP neurons lying in close proximity to both ARC and DMN nuclei (D), with no co-expression (E). A very small number of neurons co-express QRFP and TH (white arrows) (C & F). Co-localised neurons appear to be located within the ventral region of the QRFP population (QRFP – TH).

Arc: Arcuate nucleus; DMN: dorsomedial hypothalamic nucleus; Pe: periventricular hypothalamic nucleus; VMN: ventromedial hypothalamic nucleus; ZI: zona incerta; 3v: 3rd ventricle
3.3.5 Efferent projection of QRFP neurons: cre-dependent tracing

Coronal sections

Fully characterising the expression of QRFP in the brain also required investigation of areas into which axons of QRFP neurons project. The intermingling of the QRFP neuronal population with both orexin and MCH highlighted the potential for interactions between these neurons. To investigate this, the Qrfp-cre line was crossed with the ChR2 reporter mouse (ROSA26 ChR2/eYFP), to produce offspring which express a fusion protein of ChR2 and eYFP in all QRFP cells. Similar to the Qrfp-cre::eYFP mouse, the Qrfp-cre::ChR2 mouse enabled labelling of the QRFP neuronal population, with the added benefit that the ChR2 protein is a membrane-bound channel, resulting in its incorporation in the whole cell membrane, providing efficient labelling of long dendrites and axons. This means the extent of axonal and dendritic projections can be visualised, to enable further investigation of potential interactions between QRFP neurons and other populations. QRFP neuronal processes intermingle within both the orexin and MCH populations, with a few close appositions evident between QRFP axons and cell bodies of either population (Figure 3.9). One drawback of this technique, however, is that potential axon-axon connections, which contribute a major proportion of neuronal communication, are not possible to visualise.

As previously discussed in the Qrfp-cre::eYFP study, strong ependymal staining was observed in the Qrfp-cre::ChR2 mouse (Figure 3.10). It was observed that these cells, in particular those located in the third ventricular wall, towards the caudal level of the QRFP neuron population, exhibit strong staining in processes extending ventro-laterally, directly through the medial part of the VMN, appearing to end on the ventral surface of the hypothalamus. It is still unclear whether these cells, which resemble tanycytes, actively express QRFP in adults, or whether their labelling is due to developmental expression of Qrfp and, therefore, cre-recombinase.
Figure 3.9: QRFP projections through orexin and MCH neuronal populations

Dual-label immunohistochemistry staining for MCH and QRFP (left), and orexin and QRFP (right) in the Qrfp-cre::ChR2 mouse brain. QRFP projections transit through the LHA, with many in close apposition to both the MCH (A) and orexin (B) populations (QRFP – MCH/orexin)

VMN: ventromedial hypothalamic nucleus; 3v: 3rd ventricle

Figure 3.10: Projections of QRFP-expressing 3rd ventricle ependymal cells

Strong processes lead from the ependymal cells lining the 3rd ventricle, projecting ventrolaterally, through the medial VMN. These appear to terminate on the base of the hypothalamus (QRFP)

3v: 3rd ventricle
In order to aid the visualisation of QRFP neuronal projections throughout the entire brain, identification of discrete areas was aided by dual-labelling for TH (Figure 3.11, Figure 3.12). It is important to note that it is difficult to determine whether QRFP fibres originate from the hypothalamic or retinal populations.

Figure 3.11: Identification of multiple TH neuronal populations – sagittal

A: Representative sagittal brain section labelling key nuclei labelled by TH staining, used to identify specific regions containing QRFP neuronal projections.

B: Stereofluorescent microscope image of sagittal brain section labelling TH positive neuronal populations (TH)

AcbSh: nucleus accumbens shell; Arc: Arcuate nucleus; DMN: dorsomedial hypothalamic nucleus; DMV: dorsal motor nucleus of the vagus; DR: dorsal raphé nucleus; LV: lateral ventricle; NTS: nucleus of the solitary tract; PVN: paraventricular nucleus; Tu: olfactory tubercle; VMN: ventromedial hypothalamic nucleus; VTA: ventral tegmental area; 3v: 3rd ventricle; 4v: 4th ventricle
Figure 3.12: Identification of multiple TH neuronal populations – coronal

A-F: Coronal sections (corresponding to areas highlighted in Figure 3.11) identifying specific TH neuronal populations throughout the brain, to enable investigation of QRFP neuronal projections (QRFP – TH).

A: bregma +0.62mm, B: -1.46mm, C: -3.80mm, D: -4.16mm, E: -5.40mm, F: -7.08mm

AcbSh: nucleus accumbens shell; Arc: Arcuate nucleus; CPu: caudate putamen; DR: dorsal raphé nucleus; LC: locus coeruleus; NTS: nucleus of the solitary tract; PVN: paraventricular nucleus; RRF/A8: retrorubral fields (A8 dopamine cells); Tu: olfactory tubercle; VMN: ventromedial hypothalamic nucleus; VTA: ventral tegmental area; 3v: 3rd ventricle
Aside from the intermingling with the aforementioned neuronal populations, a large number of QRFP processes lie locally within the LHA, although it is unclear whether these are axons or dendrites. In the immediate vicinity of the hypothalamic QRFP cell bodies, a dense network of fibres exists, with strong projections running dorso-laterally through the LHA (Figure 3.13). Around the ventral extent of the QRFP population, multiple fibres run laterally through the supraoptic nucleus, projecting dorso-laterally through the globus pallidus, caudate putamen and corpus callosum. Fibres also lie within the paraventricular thalamic nucleus (PVT), located at similar rostro-caudal co-ordinates as the caudal extent of the QRFP population. It is noteworthy that, aside from the dense fibres within the LHA, the hypothalamus is relatively devoid of strong fibre staining, with no nuclei containing dense fibres. Of interest, however, is the identification of QRFP fibres within the TMN in sections caudal to the QRFP population.

**Figure 3.13: QRFP neuronal projections in Qrfp-cre::ChR2 mouse brain (-1.46mm)**

**A:** Coronal diagram (bregma -1.46mm) labelled with key areas of interest. Red squares represent areas in which QRFP fibres were observed

**B-D:** Immunohistochemistry in Qrfp-cre::ChR2 mouse brains identified QRFP fibres within the PVT (B). Projections are also seen passing around the ventro-lateral extent of the QRFP population (C) and in close proximity to the optic tract (D) (QRFP)

CPu: caudate putamen; f: fornix; LHA: lateral hypothalamic area; lv: lateral ventricle; mt: mammillary tract; opt: optic tract; PVT: paraventricular thalamus; VMN: ventromedial hypothalamic nucleus; 3v: 3rd ventricle
QRFP fibres were identified in rostral brain sections, within the fornix and anterior commissure. Whilst strong TH staining was observed within the caudate putamen, NAcc and olfactory tubercle, QRFP fibres were not evident within these regions (Figure 3.14). Interestingly, QRFP fibres exist within the anterior commissure but appear to be passing down the tract rather than entering the NAcc.

Figure 3.14: QRFP neuronal projections in Qrfp-cre::ChR2 mouse brain (+0.62mm)

A: Coronal diagram (bregma +0.62mm) labelled with key areas of interest. Red square represents area in which QRFP fibres were observed

B: Immunohistochemistry in Qrfp-cre::ChR2 mouse brains identified QRFP fibres within the aca, which do not enter the NAcc, highlighted by strong TH staining (QRFP – TH)

aca: anterior commissure; AcbC: nucleus accumbens core; AcbSh: nucleus accumbens shell; cc: corpus callosum; CPu: caudate putamen; lv: lateral ventricle; Tu: olfactory tubercle
Moving caudally from the hypothalamic QRFP population, dense projections in the area immediately dorsal to the mammillary body run between the medial terminal nucleus of the accessory optic tract and the rostral VTA (as identified by labelling of TH fibres) (Figure 3.15); although the direction of the projection cannot be determined with this model. Labelling of TH neurons highlights the VTA in clear detail, and labelled QRFP fibres are observed running throughout the VTA, with many in close proximity with TH neurons. Further investigation of the midbrain revealed strong staining of fibres within the interpeduncular nucleus: traversing the entire ventral part of the nucleus, from the optic tracts located laterally, through both the caudal and lateral subnuclei.

**Figure 3.15: QRFP neuronal projections in Qrfp-cre::ChR2 mouse brain (-3.80mm)**

A: Coronal diagram (bregma -3.80mm) labelled with key areas of interest. Red square represents area in which QRFP fibres were observed

B-C: Immunohistochemistry in Qrfp-cre::ChR2 mouse brains identified QRFP fibres intermingled with TH neurons in the VTA (B1, B2), whilst strong projections cross the base of the IP (C) (QRFP – TH)

Aq: aqueduct; IP: interpeduncular nucleus; PAG: periaqueductal grey; RRF: retrorubral field; SC: superior colliculus; SN: substantia nigra; VTA: ventral tegmental area
Around the same rostro-caudal co-ordinates, fibres are also identified within the periaqueductal grey (PAG) (Figure 3.16). However, as previously mentioned, all ventricles within the brain of mice derived from the Qrfp-cre line show strong staining within ependymal cells, and the aqueduct is no exception. As such, it was unclear whether the processes within the PAG were neuronal or ependymal in origin. Located ventrally to the PAG, TH neurons are labelled within the DR, with QRFP fibres passing through this nucleus. Furthermore, within this region, fibres can be traced from the very base of the brain, within the pontine nucleus, laterally through this nucleus before progressing dorsally around the lateral regions of the brain. Fibres were eventually tracked dorsally until they enter the SC. Branches of fibres also diverge from this main pathway where they enter the retrorubral field, characterised by its dopaminergic neurons (labelled with anti-TH antibody).
Figure 3.16: QRFP neuronal projections in *Qrfp*-cre::ChR2 mouse brain (-4.16mm)

A: Coronal diagram (bregma -4.16mm) labelled with key areas of interest. Red square represents area in which QRFP fibres were observed.

B-D: Immunohistochemistry in *Qrfp*-cre::ChR2 mouse brains identified QRFP fibres intermingled with TH neurons in the RRF (B) and DR (C), with projections also found within the PAG (D) (QRFP – TH).

E-H: QRFP projections were identified travelling laterally through the Pn (E) before passing around the lateral regions of the brain (F, G) to the SC (H).

Aq: aqueduct; DR: dorsal raphé nucleus; MnR: median raphé nucleus; PAG: periaqueductal grey; Pn: pontine nucleus; RRF: retrorubral field; SC: superior colliculus; ts: tectospinal tract.
Fibres of QRFP neurons also spread widely throughout the hindbrain. Using dual-label immunohistochemistry, QRFP fibres were observed within the LC, intermingling with TH neurons (Figure 3.17). These fibres traverse between the LC and the medial, and lateral, parts of the parabrachial nucleus. Ventral to the LC, at the same rostro-caudal extent, fibres are found within the raphé pallidus and raphé magnus nuclei, which project laterally to the preolivary and olivary nuclei.

![Diagram of brain regions](image)

**Figure 3.17: QRFP neuronal projections in Qrfp-cre::ChR2 mouse brain (-5.40mm)**

A: Coronal diagram (bregma -5.40mm) labelled with key areas of interest. Red square represents area in which QRFP fibres were observed

B: Immunohistochemistry in Qrfp-cre::ChR2 mouse brains identified QRFP fibres intermingled with TH neurons in the LC, with projections also found within the PBN (B). QRFP fibres were also identified projecting laterally from the RPa/RMg to the PO (C) (QRFP – TH)

LC: locus coeruleus; PBN: parabrachial nucleus; PO: preolivary nucleus; RMg: raphé magnus nucleus; RPa: raphé pallidus nucleus; 7n: facial nerve
Investigation of the dorsal vagal complex shows QRFP fibres in the NTS, identified with TH immunoreactivity, and the adjoining DMV, whilst QRFP fibres were also observed within the inferior olivary nucleus at the same rostro-caudal region (Figure 3.18).

**Figure 3.18: QRFP neuronal projections in Qrfp-cre::ChR2 mouse brain (-7.08mm)**

A: Coronal diagram (bregma -7.08mm) labelled with key areas of interest. Red square represents area in which QRFP fibres were observed

B: Immunohistochemistry in Qrfp-cre::ChR2 mouse brains identified QRFP fibres intermingled with TH neurons in the NTS, with projections travelling through the DMV (B). QRFP fibres were also found within the IO (C) (QRFP – TH)

DMV: dorsal motor nucleus of the vagus; IO: inferior olivary nucleus; NTS: nucleus of the solitary tract
**Sagittal sections**

As with the *Qrfp*-cre::eYFP mapping studies, additional *Qrfp*-cre::ChR2 mouse brains were sectioned in the sagittal plane, and immunohistochemistry employed to visualise QRFP neuronal projections throughout the brain. To aide with the identification of the projections, dual-labelling was carried out using anti-GFP and anti-TH antibodies.

As previously observed, QRFP neurons surround the VMN, along with TH neurons, and the densest projections travel dorso-laterally around the outside of the VMN, rather than through the nucleus (Figure 3.19). However, as discussed with the coronal sections, strong labelling of QRFP structures does exist within the VMN itself, although this staining is clearly from the aforementioned ependymal cells/tanycytes.

![Sagittal sections](image)

**Figure 3.19: QRFP neuronal projections in sagittal sections in Qrfp-cre::ChR2 mouse brain**

**Top:** Labelling of QRFP neuronal projections in sagittal sections identified strong projections pass around the VMN without entering the nucleus (A, B) (QRFP – TH)

**Bottom:** Sagittal sections from medial regions of the brain show fibres within the VMN (C) although careful examination reveals these are ependymal fibres rather than neuronal projections (D) (QRFP – TH)

LHA: lateral hypothalamic area; PVN: paraventricular hypothalamic nucleus; VMN: ventromedial hypothalamic nucleus
Outside the hypothalamus, QRFP fibres occur widely through the brain, although definitive identification of specific areas was more difficult in sagittal sections. However, by using the known distribution of TH neurons, some insights could be gleaned from these sections. Strong projections were identified travelling both rostral and caudal from the QRFP population, travelling alongside TH axons (Figure 3.20). Located more rostral, fibres were observed projecting towards the area surrounding the NAcc, where they appear to pass around, rather than enter, the nucleus, complementing observations from coronal sections.

![Figure 3.20: Rostral QRFP neuronal projections in Qrfp-cre::ChR2 mouse brain](image)

**Figure 3.20: Rostral QRFP neuronal projections in Qrfp-cre::ChR2 mouse brain**

**A:** QRFP fibres travel rostral to the neuronal population alongside TH fibres (QRFP – TH)

**B:** Whilst dense TH fibres are located within the NAcc, QRFP fibres appear to pass around the nucleus (QRFP – TH)

LHA: lateral hypothalamic area; NAcc: nucleus accumbens
The caudal projections, from the QRFP population, travel to the substantia nigra and VTA, where QRFP fibres are seen in close apposition with TH neurons (Figure 3.21). Further projections were observed to be travelling caudally through the hindbrain and brainstem. Neither the terminals, nor the origins, of these projections could be verified and, therefore, it was unclear which direction the projections were travelling in. These projections were observed to pass through nuclei previously highlighted in the coronal sections: namely the pontine nucleus and the preolivary and olivary nuclei, as well as the rostral ventrolateral medulla.

**Figure 3.21: Caudal QRFP neuronal projections in Qrfp-cre::ChR2 mouse brain**

**Top:** QRFP fibres travel caudal to the neuronal population alongside TH fibres (A), before travelling through the VTA where many QRFP fibres lie in close apposition with the dense TH neuronal population here (B) (QRFP – TH)

**Bottom:** QRFP fibres pass through the Pn (C) as they travel through the hindbrain before entering the spinal column (D) (QRFP – TH)

LHA: lateral hypothalamic area; Pn: pontine nucleus; VTA: ventral tegmental area
Further dual-label immunohistochemistry in sagittal sections, using anti-ChAT, anti-CART and anti-oxytocin antibodies also allowed study of QRFP projections in relation to these other neuronal populations. Labelling of the ChAT population in sagittal sections confirmed the observations in coronal sections that cholinergic neurons are located more rostrally than the QRFP population. Between ChAT-positive cells, there are sparse QRFP projections (Figure 3.22), with few close contacts evident. Further dual-labelling enabled investigation of QRFP fibres around the oxytocin population. The sagittal sections were too lateral for oxytocin PVN neurons to be seen, but oxytocin neurons within the SON were labelled, where dense QRFP projections surround the neurons, with many in close apposition. The investigation of QRFP and CART expression revealed an interesting observation in the hypothalamus. QRFP neurons surround the VMN, whilst CART neurons lie in the outer regions of the DMN (Figure 3.22). The neurons from both populations intermingle within the area immediately between the VMN and DMN, with QRFP neurons and fibres in close apposition with the CART neurons. Interestingly, a small number of QRFP neurons lie within, and QRFP fibres travel dorsally through, the DMN. In lateral regions of the DMN, dense QRFP projections pass through the CART population with many fibres seen in close apposition.
Figure 3.22: Hypothalamic QRFP neuronal projections in Qrfp-cre::ChR2 mouse brain

**Top:** Sparse QRFP fibres pass through the ChAT population, in the rostral LHA (A), whereas dense QRFP fibres surround oxytocin neurons in the SON (B) (QRFP – ChAT/oxytocin)

**Bottom:** QRFP neurons surround the VMN and intermingle with CART neurons, with QRFP fibres passing through the DMN (C) whilst laterally, QRFP fibres intermingle with CART neurons (D) (QRFP – CART)

DMN: dorsomedial hypothalamic nucleus; LHA: lateral hypothalamic area; SON: supraoptic nucleus; VMN: ventromedial hypothalamic nucleus
3.3.6 Anterograde tracing of QRFP neurons using AAVs

The use of the Qrfp-cre::ChR2 model enabled visualisation of the full extent of QRFP projection sites. However, a major drawback of this is that the direction and origin of the fibres cannot be identified. As it is currently unknown whether the hypothalamic QRFP neurons form a homogenous population, it is possible that sub-populations of QRFP neurons may project to different areas of the brain, thereby mediating alternative functions. In addition, some QRFP fibres in the brain may originate from the retinal population. In order to address these issues, the use of attenuated AAVs, packaged with specific constructs, would enable visualisation of a smaller number of neurons from the QRFP population. Initially, an AAV packaged with a ChR2/mCherry construct was injected into a region of dense QRFP neurons in the lateral extent of the population (co-ordinates: AP -1.5mm, ML +0.9mm, RV -5.9, 5.7, 5.5mm), in Qrfp-cre::eYFP mice. Following infection of neurons by the AAV, cre-dependent expression of the ChR2/mCherry construct should enable visualisation of the full extent of any transfected neurons, in much the same way as the Qrfp-cre::ChR2 model employed previously. Unfortunately, despite injection sites confirmed within the targeted region of QRFP neurons, very low levels of infection were observed rendering identification of projections of these neurons very difficult (Figure 3.23).

The two tracing techniques described previously enabled visualisation of QRFP fibres but, due to the fact that ChR2 is not a vesicular peptide, nerve terminals could not be identified. Despite the fact that many neurons communicate through axon-axon connections, identifying the regions in which neuronal fibres terminate is crucial to understanding their function. To investigate this, a second AAV was injected, this one packaged with an h-syn/mCherry construct. As a vesicular peptide, h-syn is trafficked to the neuron terminals, allowing visualisation of the target sites of infected neurons. As above, nanoinjection of this AAV into the same region of the QRFP population was made and immunohistochemistry confirmed successful sites of injection. However, rates of infection using this AAV were even lower than with the ChR2 construct, with infection seeming to occur at random in the neuronal population (Figure 3.23). As such, very few terminals would be expected to be labelled and identifying them would be almost impossible.
Figure 3.23: Nanoinjection of AAV-delivered, cre-dependent anterograde tracers

Top: Injection of AAVs packaged with a ChR2/mCherry construct resulted in sparse infection of neurons within the QRFP population (A, B) (QRFP – ChR2 tracer)

Bottom: Injection of AAVs packaged with an h-syn/mCherry construct likewise resulted in sparse infection of neurons within the QRFP population (C, D) (QRFP – h-syn tracer)
3.3.7 Retrograde tracing of QRFP projection targets

The expression of QRFP within the retinal ganglion cell layer, coupled with QRFP neuronal fibres located within the SC, suggested that these fibres originated within the eye. In order to confirm this observation, retrograde tracing was used. Stereotaxic nanoinjection of retrobeads into the SC of Qrfp-cre::eYFP mice was conducted to retrogradely trace the axons back to their origin. Immunohistochemistry labelled no QRFP cells in the hypothalamus confirming that the origin of these SC fibres was outside the brain. QRFP was labelled within the retinal ganglion cell layer as previously described, whilst retrograde tracer accumulated within this layer, overlapping with QRFP expression confirming these QRFP cells project to the SC (Figure 3.24).

Work in the Qrfp-cre::ChR2 mouse revealed fibres within the LC, leading to retrograde tracing from this nucleus to determine the extent of QRFP input here. Neurons of the hypothalamic QRFP population showed accumulation of retrobeads, verifying that these neurons project to the LC (Figure 3.24). Interestingly, neurons labelled with retrobeads were found throughout the QRFP population, suggesting no obvious spatial separation of QRFP neurons projecting here.

Figure 3.24: Nanoinjection of retrobeads in specific brain nuclei

Top: Injection of retrobeads into the SC resulted in their trafficking back to ganglion cell layer of the eye, mingling with QRFP labelling (A, B) (QRFP – retrobeads)

Bottom: Injection of retrobeads into the LC resulted in their trafficking back to QRFP neurons in the hypothalamus (C, D) (QRFP – retrobeads)
3.4 Discussion

3.4.1 Visualisation of QRFP in the brain

Numerous studies have investigated the expression of QRFP in both the rat and mouse brain. When first identified, QRFP expression was reported to be confined within the hypothalamus, by RT-PCR in mice (Jiang et al. 2003). However, more accurate descriptions have been hampered by a lack of reliable antibody for QRFP and the reported expression in the literature has been confirmed by *in situ* hybridisation histology. The development of our novel transgenic *Qrfp-cre::eYFP* model enabled us to visualise the neuronal population in greater detail.

Whilst the literature is in agreement that QRFP is confined to the hypothalamus, many different nuclei have been implicated, with expression variably reported in the Arc, LHA, medial tuberal nucleus, PeVH, retrochiasmatic area and VMN (Chartrel et al. 2003; Kampe et al. 2006; Takayasu et al. 2006; Fukusumi et al. 2003). Perhaps the most detailed, and accurate, of these studies, the distribution in rat and mouse described in the Kampe and Takayasu papers respectively, are consistent with the expression observed in our own transgenic *Qrfp-cre::eYFP* model, suggesting that the neurons themselves are similarly distributed in these rodents. More recently a GFP knock-in mouse has been reported, constitutively expressing GFP in QRFP neurons, which shows similar distribution to our own results, although the immunohistochemistry in this paper was not as detailed as ours (Okamoto et al. 2016). Our studies have confirmed that QRFP neurons primarily appear to surround the rostral half of the VMN, with dense localisation within the mediobasal, periventricular and lateral hypothalamic areas. Whilst previous studies have reported QRFP expression within the VMN and Arc, this is based on low power images with no counterstaining to confirm the boundaries of the nuclei mentioned. In contrast, co-labelling both coronal and sagittal brain sections for QRFP and TH means we can confidently state that QRFP neurons do not lie within the VMN, but rather line the outside of this nucleus. Similarly, TH counterstaining also shows that QRFP neurons, likewise, border the Arc. However, it remains unclear whether QRFP neurons lie within, or just outside, the DMN, with co-labelling for QRFP and CART suggesting a small number of QRFP neurons may lie within the ventral-most region of the nucleus.

The VMN is a major site of nutrient sensing in the brain, containing both glucose excited and inhibited neurons (Song et al. 2001) and fatty acid excited and inhibited neurons (Le Foll et al. 2009), as well as neuronal sub-populations sensitive to leptin (Elmquist et al. 1998), insulin (Cotero & Routh 2009) and ghrelin (Guan et al. 1997). The sensitivity of QRFP neurons to these endogenous stimuli is unknown (although our preliminary electrophysiology data suggests they are activated by ghrelin *ex vivo*) but the position of the neurons so close to the periphery of the VMN could potentially be an indication of a physiological function: it seems likely that connections exist between them, given their close proximity. The well-established role of the VMN in nutrient
sensing, and the ideal placement of the QRFP neurons to receive direct input from the VMN, leads
to a potential hypothesis that QRFP neurons could be involved in mediating the response to
specific changes in nutrient levels in the animal. Interestingly, the literature reports strong
expression of Gpr103 and QRFP binding sites within the VMN (Takayasu et al. 2006; Bruzzone et
al. 2007), which could suggest that QRFP neurons modulate activity of neurons within the
nucleus, or their dendrites around the periphery of the nucleus. Our present data shows that
most of the QRFP projections travel around the outside of the VMN, particularly the lateral
extent, with no dense projections observed within the nucleus itself. Whether or not QRFP acts
within the VMN will probably only be answered with electrophysiology.

It is worth noting that we have observed strong processes in the VMN, particularly the ventral
areas. However, it is clear from the relative strength of staining in these fibres that they are from
the ventricle lining ependymal cells, rather than QRFP neurons. As has already been mentioned,
the Qrfp-cre model shows very strong staining in cells lining every ventricle of the brain, which has
not been observed in any previous literature by in situ hybridisation histology. The nature of the
cre-recombinase system means that its expression will be induced in any cell that has expressed
QRFP at any point, with no differentiation between whether this is current or developmental
expression. The ubiquitous nature of the ventricle lining staining, coupled with the literature
previously having not reported this, led us to hypothesise that this is due to developmental
expression of QRFP. Therefore, we believe the ventricle lining cells no longer contain QRFP, and
this expression is not relevant to the adult role of QRFP. Further in situ hybridisation analysis of
foetal tissue may confirm this or, instead, suggest that the transgene is ‘leaky’. However, it is
interesting that the particularly strong ependymal staining at the base of the 3rd ventricle appears
to label tanycytes, the specialised cells bridging the cerebrospinal fluid to the portal capillaries.
Whilst it cannot be definitively confirmed through our present experiments, the morphology of
these labelled cells is consistent with the elongated shape and single projection terminating on
the base of the hypothalamus, as previously described (Rodríguez et al. 2005).

### 3.4.2 Investigation of co-expression of QRFP with other transmitters

Prior to our studies in the Qrfp-cre::eYFP model, no investigation of the potential for co-
expression of QRFP with other transmitters had been performed. As relatively little was known
about the QRFP population, it was important to determine whether these neurons were a sub-
population of other well-defined populations. Primarily, orexin and MCH were of major relevance
due to their high levels of expression in the perifornical LHA, similar to the lateral-most neurons of
the QRFP population, and because of the effect of these peptides on feeding. A very recent paper,
using single-cell RNA sequencing has suggested that orexin neurons contain Qrfp mRNA (Romanov
et al. 2016). However, this result was not verified by any other means of co-localisation and
appears to highlight an error in their methodology. We have shown here that there are no
neurons that co-express QRFP and orexin or MCH, definitively proving that the QRFP neurons are their own distinct population. Furthermore, if orexin neurons ever express Qrfp, then they should express cre-recombinase in our model. Our work was recently supported by the Qrfp knock-out/GFP knock-in mouse (expressing GFP in QRFP neurons rather than exon two of the Qrfp gene, see Chapter 5 for full description), which showed no co-localisation with orexin (Okamoto et al. 2016). Interestingly, so far, we have failed to identify any peptidergic transmitters co-expressed within QRFP neurons: we can conclude that QRFP neurons are not cholinergic or catecholaminergic, as they do not express ChAT or TH, nor do they contain nNOS. Due to a lack of reliable neuronal labelling, we had been unable to definitively determine whether QRFP neurons contain either of the classical fast neurotransmitters, GABA or glutamate. As a way around this, we employed two transgenic models to enable RT-PCR and qPCR analysis of Qrfp expression in Gad2-cre and Vglut2-cre mice. These models express cre-recombinase in GABA or glutamate neurons respectively which, when crossed with the FlEx-Qrfp line, cause cre-dependent rescue of Qrfp in these neurons. Recently, drop-sequencing was used to show that the QRFP neurons around the Arc are glutamatergic (Campbell et al. 2017), whilst previously the LHA neurons were suggested to be likewise (Romanov et al. 2016), although our concerns with this study were raised earlier. Our PCR data suggests that the hypothalamic QRFP population actually consists of both glutamatergic and GABAergic neurons. It is important to note that the present methodology cannot rule out the existence of neurons co-expressing neither neuropeptide. Interestingly, our study of the QRFP population in the eye reveals that these cells are definitely not glutamatergic, but our Gad2 crossed mice show rescue of at least a proportion of the Qrfp expression found in wild-type mouse eye. This result is supported by previous literature reporting that most of the retinal ganglion cells are GABAergic (Popova 2015).

Whilst we have yet to identify a co-expressed peptidergic transmitter, our results clearly show that QRFP neurons and fibres lie in very close proximity to those containing orexin and MCH, meaning that it is possible these neurons may interact with one another. It is hypothesised that there could be communication between these populations, although we were unable to verify this with our current methodology. Whilst we have doubts over the physiological relevance of QRFP in feeding (see Chapter 5), orexin and MCH also play important roles in arousal and energy expenditure, and it is possible that QRFP may interact with these neuronal populations to influence one of these other functions. The literature currently has failed to investigate whether QRFP neurons are targets of orexin or MCH, or vice versa, although one study has reported that QRFP still induces feeding in orexin null mice (Takayasu et al. 2006). Whilst this may suggest that QRFP does not signal via the orexin pathway, it is unknown whether other actions of QRFP are similarly unaffected by orexin knock-out, or, alternatively, whether loss of QRFP signalling may attenuate some aspects of orexinergic action.
3.4.3 Projections of QRFP neurons

There have been a number of studies showing wide expression of Gpr103a and Gpr103b in the brain (Takayasu et al. 2006; Kampe et al. 2006; Bruzzone et al. 2007). Using our Qrfp-cre::ChR2 mouse line, the distribution of QRFP fibres throughout the brain was investigated to identify potential regions that are targets for QRFP signalling. An important caveat to this study must be taken into consideration: ChR2 is a membrane-bound channel that is incorporated into the whole cell membrane, so does not specifically highlight axon terminals. The observations made herein, therefore, show fibres of passage but we cannot say definitively whether they mark direct targets of QRFP terminals. However, the majority of fibres observed in the Qrfp-cre::ChR2 mouse brain possess numerous axon varicosities, implying that QRFP neurons possibly communicate with other neurons along their axonal length, rather than just at classical synapses.

Sagittal sections revealed that QRFP fibres project both rostrally and caudally from the hypothalamic population, seemingly within well-defined tracts alongside fibres labelled for TH. Interestingly, QRFP fibres are found within the rostral-most limits of the fornix in the forebrain, with fibres seen passing between the fornix and corpus callosum. We cannot be certain whether these fibres are interacting with this region but they are also observed within ventral areas of the forebrain, around the level of the olfactory tubercle which complements previous work showing strong 26RFa binding within the olfactory bulbs and piriform cortex (Bruzzone et al. 2007).

Lateral hypothalamic neurons, such as orexin and MCH, play prominent roles in reward processing and projections of these populations are found accordingly within key nuclei of the mesolimbic pathway. Strikingly, dense, highly varicose QRFP fibres were observed passing through the VTA, in close apposition to TH neurons in this area. Interestingly, no QRFP fibres were observed within the NAcc, with the majority of rostral projections appearing to lie within the medial region of the prefrontal cortex, and only a few lying around the edge of the NAcc. Projections to the VTA is reminiscent of orexinergic projections (Peyron et al. 1998), in contrast to MCH projections which are found within the NAcc and not the VTA (Sears et al. 2010). Identification of strong projections within the VTA in this way potentially indicates a role for QRFP in the modulation of the mesolimbic pathways involved in reward and motivation, similar to orexin. Orexin activates the mesolimbic dopaminergic signalling pathway leading to reward-seeking behaviour and meal initiation (Choi et al. 2010), compared with MCH which seems to influence the reward pathway by promoting continued consumption of energy dense foods (Kowalski et al. 2004). Previously, it has been reported that Gpr103b is expressed within the VTA (Kampe et al. 2006) but there is also evidence of low levels of 26RFa binding sites within the NAcc, although Gpr103 expression is not confirmed here (Bruzzone et al. 2007). It is possible, therefore, that QRFP neurons interact with cells in the VTA through release of QRFP, perhaps acting via Gpr103b.
Our studies reveal a remarkable similarity between orexin and QRFP fibre distribution in key regions of the brain involved in arousal and locomotor behaviour. Orexin neurons project to parts of the ascending arousal system: the LC, DR, TMN, VTA (Peyron et al. 1998) and the PVT (Kirouac et al. 2005) all of which are involved in locomotor behaviour. Similarly, fibres in our Qrfp-cre::ChR2 mouse were also observed in these nuclei. Furthermore, QRFP fibres were observed within the ventrolateral PAG and the interpeduncular nucleus which have both also been suggested to play a role in arousal (Lu et al. 2006; Hentall & Gollapudi 1995). Our observations of fibres in these nuclei is backed up by the finding of 26RFa binding sites, and Gpr103 expression within the LC, DR, VTA, TMN, PVT, PAG and interpeduncular nucleus (Kampe et al. 2006; Bruzzone et al. 2007). Further confirmation of a likely QRFPergic effect in the LC was provided by retrograde tracing, proving QRFP neurons project to this nucleus.

These data provide a strong suggestion that QRFP plays a role in the regulation of arousal, similar to orexin, through connections in multiple regions of the brain. This supports previous literature showing acute increases in locomotion following QRFP injections (Takayasu et al. 2006) and provides evidence for a physiological role in the regulation of arousal. Interestingly, orexin has been shown to activate serotonergic neurons of the DR (Matsuzaki et al. 2002), adrenergic neurons of the LC (Hagan, J et al. 1999) and dopaminergic cells of the VTA (Nakamura et al. 2000), with the suggestion that the dopaminergic VTA neurons are responsible for mediating orexin-induced hyperlocomotion. Our studies indicate close appositions between QRFP fibres and TH neurons in the VTA which, when combined with the lack of projections within the NAcc, could potentially indicate these projections are involved with locomotor activity rather than having a direct effect on reward. For example, food seeking is a critical element of feeding behaviour and is mediated by the mesolimbic pathway, so it is possible that QRFP-induced feeding and locomotion may be linked to changes in food seeking behaviour. It would be of further interest to determine whether QRFP neurons are interacting with similar neuronal targets to orexin in these nuclei.

Another set of projections was labelled within the SC. Interestingly, these projections could be followed from the lateral regions of the pontine nucleus dorsolaterally to the SC. The primary role of the SC is the integration of sensory information to help direct goal-oriented behaviours. Previously in the literature, high levels of Qrfp and Gpr103a expression have been reported in the eye, from which the SC receives strong innervation. Accordingly, cross sectioning of the eye revealed strong eYFP localisation within the retinal ganglion cell layer of Qfpe-cre::eYFP mice, with fibres also evident in Qfpe-cre::ChR2 eyes, suggesting that QRFP is located within ganglion cells. In order to confirm the origin of the QRFP fibres within the SC, retrograde tracer was injected into the nucleus. As hypothesised, retrograde tracer labelled the ganglion cell layer, whilst no tracer was found within any of the hypothalamic QRFP neurons. It is unclear what role QRFP may be playing in the eye, but the expression of both peptide and receptor indicates an additional
paracrine role. Ganglion cells in the eye are responsible for transducing visual data from receptive cells to the brain, with many of their projections entering the SC. As the distribution of QRFP fibres in the brain strongly implicates QRFP in arousal, it is possible that these ganglion cells and their projections may also be involved in this. Importantly, the SC has been previously shown to contain QRFP binding sites and Gpr103 mRNA (Bruzzone et al. 2007).

The literature suggests a number of functions of QRFP in peripheral endocrine control: insulin secretion (Egido et al. 2007; Granata et al. 2014), lipolysis (Mulumba et al. 2010) and glucose uptake in skeletal muscle (Allerton & Primeaux 2015). These studies have all been carried out in in vitro or ex vivo set-ups but it is still uncertain whether the actions of QRFP in vivo are direct effects on peripheral tissues, or whether they are mediated centrally. Vagal signalling is a critical factor for central control of peripheral endocrine function, with the vagal complex in the brainstem acting as the key centre for centrally-mediated responses in the periphery. In particular, the NTS receives and integrates a plethora of signals: vagal inputs originating from the periphery; central input from higher brain areas such as the forebrain; and circulating factors detailing metabolic status. The effector region of the vagal complex is the adjacent DMV which is primarily responsible for vagal output to peripheral tissues, dependent on the sensory information obtained by the NTS. The detection of fibres of QRFP neurons within both the NTS and DMV provides a basis for the possibility that the actions of QRFP on peripheral endocrine responses could be mediated centrally.

The projections of QRFP neurons within the brain were investigated using both anterograde and retrograde methods. However, this did not circumvent the difficulty we have observed with infecting QRFP neurons with AAV’s. Previously it was not known whether the hypothalamic QRFP neurons represent a homogenous population, but our present data would suggest that sub-populations exist due to the finding that both glutamatergic and GABAergic cells exist in the brain. However, the retrotracing performed here did not preferentially label neurons from any specific region, meaning that further investigation is warranted to identify whether these different sub-populations are functionally discrete.

3.4.4 Summary

- *Qrfp* is expressed in a wide range of metabolically relevant tissues
- QRFP neurons are exclusively located around the VMN
  - QRFP neurons do not co-express orexin or MCH, but projections lie in close apposition
  - QRFP neurons are not cholinergic or catecholaminergic
  - Both glutamatergic and GABAergic QRFP neurons are located in the hypothalamus
- QRFP neurons project widely through the brain
  - Projections in the LC, VTA, DR and TMN suggest a role in wakefulness
Chapter 4

**METABOLIC EFFECTS OF QRFP**
4.1 Introduction

Since its discovery in 2003, the central orexigenic actions of exogenous QRFP have been reproduced robustly, but a number of other acute actions of centrally-administered QRFP have also been reported: increased locomotor activity, blood pressure and oxygen consumption (Takayasu et al. 2006). Furthermore, chronic intracerebroventricular (ICV) dosing of QRFP causes increased food intake and a body weight-independent increase in adiposity (Moriya et al. 2006).

Whilst the majority of the RFamide peptides so far studied appear to be capable of altering food intake, suggesting an evolutionarily conserved role in feeding regulation, it remains to be determined whether this is their primary, physiological role. Likewise, ICV injection of a number of the RFamide peptides influence nociception, but it is unclear if this is a true physiological effect of the peptides or due to off target effects. For example, although neuropeptide FF (NPFF) modulates opioid-induced analgesia, it is well known that the NPFF receptors are relatively promiscuous regarding other members of the RFamide family. Therefore, supra-physiological doses of peptides injected ICV could induce an effect through non-selective, non-physiological interactions. As a result, caution should be made when drawing conclusions from these types of studies, though they are invaluable in helping form hypotheses for future testing.

The previously described actions of ICV-administered QRFP indicate a number of potential functions for the peptide in the regulation of energy homeostasis. Similar work with both orexin and melanin-concentrating hormone (MCH) shows that these ‘orexigenic’ neuropeptides also play diverse, but opposing, roles on other metabolic endpoints. For example, whilst orexin injection stimulates arousal/wakefulness and increases energy expenditure, MCH increases sleep and promotes energy conservation. Even their orexigenic action differs, as orexin is suggested to be involved in meal initiation whereas MCH causes continuing consumption of energy-dense foods. It is obvious, therefore, that simply characterising a peptide as orexigenic can be misleading, especially with a lack of evidence to confirm that this is a physiological function. Rarely do peptides influence only a single endpoint, so distinguishing the primary role, and secondary responses, is a crucial step to determining how QRFP may fit into regulatory pathways.

In our studies, we set out to investigate a number of endpoints that are influenced by centrally expressed peptides. Our work was guided by previous studies involving orexin, due to the remarkable parallels that exist between the two peptides, and built upon by our results in Chapter 3. Using ICV injections we aimed to ascertain which QRFP-induced responses were robust and warranted further investigation in more physiological settings.
4.1.1 Objectives

**Determine the action of QRFP on feeding parameters**

The acute feeding response in satiated animals, following central QRFP/26RFa dosing, appears to be robust in mouse studies (Takayasu *et al.* 2006; Zagorácz *et al.* 2015; Lectez *et al.* 2009), although some groups report that the response in rats only occurs with high-fat diet (Kampe *et al.* 2006; Primeaux *et al.* 2008). We will investigate this further in our models and investigate whether there is a differential response to QRFP and 26RFa. Furthermore, access to operant response chambers will enable the investigation of QRFP in reward-based feeding, to identify whether future work should focus on QRFP in hedonic feeding. Neurons involved in the regulation of feeding are usually regulated by the nutritional status of the animal. Whilst previous literature shows an upregulation of *Qrfp* mRNA (Takayasu *et al.* 2006), we will identify whether QRFP neuronal activation is altered by fasting, employing our unique *Qrfp*-cre::eYFP model introduced in Chapter 3.

**Identify QRFP-mediated actions on energy expenditure**

Aside from promoting energy intake, ‘orexigenic’ peptides often influence energy expenditure parameters, which can be an indication of their true role. Similar to orexin, QRFP is capable of increasing arousal in mice, although it is unclear whether this is secondary to its orexigenic effects or vice versa. We will investigate the capacity for QRFP to increase wakefulness in a variety of paradigms, to try and tease these effects apart. Orexin and MCH induce opposing effects on energy expenditure, with orexin promoting energy expenditure compared with MCH-induced energy conservation. The role that QRFP may play in this system has yet to be determined, so we will investigate the acute effects of the peptide on arousal, oxygen consumption and thermogenesis to predict whether QRFP plays a complimentary role to these other lateral hypothalamic neuropeptides.

**Investigate downstream activation of neurons by centrally administered QRFP**

In Chapter 3, we identified regions of the brain that were innervated by QRFP neuronal fibres, providing a basis for the involvement of QRFP in a number of different functions. Here we will ascertain whether central QRFP activates neurons in the brain to further develop our knowledge of the signalling pathways of QRFP. We will pay particular attention to neuropeptide Y (NPY) neurons, both in the arcuate nucleus (Arc) and the dorsomedial hypothalamic nucleus (DMN), due to previous work showing attenuation of QRFP-induced feeding by NPY antagonism (Takayasu *et al.* 2006) and our own previous work showing QRFP-induced c-Fos expression in the DMN (unpublished).
4.2 Methods

4.2.1 Transgenic mouse husbandry

The Qrfp-cre::eYFP mouse model, used in this Chapter, was described in detail in Chapter 3 methods. The FlEx-Qrfp mouse model employed here will be fully described in Chapter 5.

**Npy-cre::eYFP**

The Npy-cre::eYFP model was employed in this Chapter to enable indirect visualisation of NPY neurons, through labelling for eYFP.

The Npy-cre model (Tg (Npy-cre)RH26Gsat/Mmucd; MMRRC strain #034810-UCD; mixed background) is commercially available from the MMRRC (Mutant Mouse Resource & Research Centre, www.mmrrc.org). Briefly, an intron containing a cre-recombinase cassette was inserted into the genome immediately after the ATG start codon in exon 1 of the Npy gene. The insertion was made using bacterial artificial chromosome technology, making use of the ability of a shuttle vector to insert large sequences of DNA into a target region of DNA, via homologous recombination (Yang *et al.* 1997). The resulting model expresses cre-recombinase under the control of the Npy promoter. This model was then crossed with the ROSA26-eYFP reporter mouse to produce a model expressing eYFP in NPY cells.

4.2.2 Peptides and materials

All peptides were obtained from commercial suppliers and were certified pyrogen and pathogen free. Ghrelin (Tocris) was diluted in saline for injection via two routes: ICV, 1µg per mouse, or intraperitoneal (ip), 2mg/kg. Orexin (7µg per mouse) and QRFP/26RFa (5-10µg per mouse) (both Phoenix) were dissolved in saline, to their relevant concentration for ICV injection. Leptin (Peprotech) was diluted for injection at 5mg/kg or 2mg/kg via the ip route. Leptin was diluted in 0.15mM hydrochloric acid, followed by further dilution in 0.9% sodium chloride and 0.1% bovine serum albumin. The acid was then neutralised by addition of 7.5mM sodium hydroxide to produce leptin solutions of the relevant concentration. All ICV injections were made in 3µl total volume, whilst ip injections were made in a volume of 4ml/kg.

Mice were maintained on regular RM1 chow with *ad libitum* access, unless specified, for each experiment. In some studies, in order to cause diet-induced obesity, mice were maintained on 60% high energy diet (HED) where 60% of the caloric content came from fat. Strawberry milk (Yazoo: FrieslandCampina) was used as the reward in operant conditioning studies.

4.2.3 Effect of QRFP on feeding in satiated mice

Male C57Bl/6J mice (n=20) received two doses of QRFP (5µg or 10µg per mouse, ICV) to confirm the orexigenic response of QRFP in our hands. Injections were made using Hamilton syringes, through the indwelling cannulae, two hours into the light phase and mice were given *ad libitum*
access to normal chow and intake measured. A follow up study was performed with 26RFa injections (5µg, ICV), in a separate cohort of male C57Bl/6J mice (n=13).

**4.2.4 Effect of QRFP on reward-based feeding**

A mischief of male CD1 mice (n=16) were trained in an operant responding paradigm as described in General Methods. The operant response paradigm allows investigation of the ‘wanting’ aspect of hedonic feeding and is frequently used to study the effect of peptides on motivation of animals to obtain a reward (here strawberry milk).

Throughout the initial training stages, singly housed mice were food restricted, on around 60% of normal food intake, to maintain a body weight at 85% of their pre-study weight. Lever presses and rewards earned were monitored throughout the study to identify the correct time for progression to the next step of training. When mice were stably responding in the progressive-ratio schedule, as determined by less than 15% variability between three consecutive days’ breakpoint, training was complete and the testing phase began. Mice that did not show stable breakpoints were excluded from the study, as were mice whose cannula had become blocked.

Stably responding mice were injected with either ghrelin (2mg/kg, ip) or saline and returned to their home cage for 20 minutes, with food removed. Mice were then placed in the operant chambers for a full 45 minutes, regardless of whether the schedule was terminated early due to inactivity. Upon completion, mice were returned to their home cage and food intake measured for the following 60 minutes. A crossover study was run 48 hours later. Following retraining in the progressive-ratio schedule, to re-obtain a stable baseline, mice were injected with ghrelin (0.5µg, ICV) or saline, and the same protocol as above repeated in a second crossover study, followed by a further ghrelin injection (1µg, ICV) 48 hours later.

As a further positive control, mice were retrained on the progressive-ratio schedule for a week to re-establish stable breakpoint responses. Following this, all mice were injected with orexin (7µg, ICV) or saline and the same schedule run as previous. 48 hours after the orexin crossover injections, mice were injected with QRFP (2µg or 5µg, ICV) or saline in a three way crossover study to determine the effects of QRFP on operant response.

**4.2.5 Effect of QRFP on brown adipose tissue temperature**

Brown adipose tissue (BAT) temperature is frequently used as an indirect measure of thermogenesis, with the major deposit in mice being located in the interscapular region. Infrared thermography was used to measure BAT temperature, following injections of QRFP or 26RFa. Two separate cohorts of male C57Bl/6J mice (n=13) had food removed from cages when lights came on to remove confounding effects of a recent thermogenic response to feeding. Immediately prior to the start of study, between two and three hours after lights on, interscapular BAT temperature was measured indirectly using a thermal camera (FLIR; Wilsonville, OR, USA). Mice were recorded
in their home cage for 10s, and videos were analysed using ResearchIR software (FLIR) with
correct environmental parameters incorporated into its algorithms: ambient temperature,
humidity and distance to mouse. Immediately after this, one cohort of mice was injected with
QRFP (7.5µg, ICV) or saline, whilst the other cohort was injected with 26RFa (7.5µg, ICV) or saline.
Food was withheld during measurement of BAT temperature in the subsequent two-hour period.

4.2.6 Effect of QRFP on locomotor activity
In order to investigate a potential role for QRFP in stimulating arousal and increases in activity,
males C57Bl/6J mice (n=14) were ICV cannulated and, following a week-long recovery, habituated
to beam break cages in a sound attenuating cabinet for at least four days.

Activity levels, calculated by beam breaks, were measured in five minute bins, for one hour prior
to, and for two hours after, injections with either QRFP (7.5µg, ICV) or saline. Mice were removed
from the cabinet for a maximum of five minutes to allow for injections. In addition, food intake
was also measured for the two hours following injection.

Mice were left to recover for five days before a second study was then conducted during the dark
phase of the light cycle. Based on a previous experiment investigating activity levels during the full
24-hour circadian profile, injections in the dark phase were timed to occur once the initial phase
of hyperactivity associated with lights off had finished. As such, mice were injected with QRFP
(7.5µg, ICV) or saline at least two hours into the dark phase.

The final study with this cohort of mice was a repeat of the previous night-time study with
injections made in mice with no food present. Food was removed from each mouse as lights were
switched off and activity levels measured as before. Food deprivation in the dark phase leads to
transient hyperactivity caused by increased food seeking behaviour in mice. Therefore, in order to
compensate for this, injections were made three hours after lights off and activity measured for
two further hours. Due to the fact that all mice in this study had already previously been dosed
with QRFP, at least ten days were allowed between repeat QRFP injections.

4.2.7 Effect of QRFP on handling of orally dosed glucose
Male C57Bl/6J mice (n=17) were ICV cannulated one week before an oral glucose tolerance test
(OGTT). On the day of study, mice were fasted for six hours, with food removed as lights came on,
to provide a stable baseline of blood glucose levels. Food was not returned to the mice until the
end of the study. As described in General Methods, the tail of each mouse was pricked, to
produce a spot of blood which was analysed by handheld glucose meters. Immediately after this
reading had been made, mice were injected with either QRFP (5µg, ICV) or saline. An oral gavage
of glucose was then given to each mouse (2g/ml in 4ml/kg volume) and mice placed back in their
home cage. Further glucose readings were taken at 15, 30, 60 and 120 minutes.
A second OGTT was conducted to investigate the effect of ICV administered 26RFa (5µg, ICV) on glucose handling (n=12), in a crossover design. The study closely mimicked the previously described experiment with the notable exception of time points measured. Based on results generated from the first study, time points were altered so that glucose levels were measured at 10, 20, 40, 60 and 120 minutes after gavage to provide better temporal resolution.

4.2.8 Activation of QRFP neurons by fasting
Male Qrfp-cre::eYFP mice (n=12) were fasted for 24 hours alongside fed controls before transcardial perfusion. Immunohistochemistry was employed to dual-label hypothalamic neurons for c-Fos and QRFP. The number of c-Fos expressing neurons was counted in the DMN, as well as within the lateral hypothalamic area (LHA), in the area immediately surrounding the QRFP neurons.

4.2.9 QRFP activation of neuronal populations
In order to investigate the activation of neuronal populations downstream in the QRFP signalling pathway, a study was designed to identify neurons activated by QRFP through their expression of c-Fos. Male Npy-cre::eYFP mice (n=12) were injected with either QRFP (7.5µg, ICV) or saline and returned to their home cage for two hours, after which they were transcardially perfused. Peroxidase-based immunohistochemistry was performed on sections throughout the brain, which were subsequently imaged to identify areas of QRFP-induced c-Fos expression, with particular attention paid to sections from the hypothalamus. c-Fos expression in NPY neurons was investigated by dual-label immunofluorescence, and particular attention paid to the Arc and the DMN.
4.3 Results

4.3.1 Effects of QRFP on feeding in satiated mice

It has been shown consistently that administration of QRFP, directly into the lateral ventricle of the brain of mice, results in an increase in food intake, when injections are made during the light phase. In order to verify the action of QRFP in our in-house models, a study was carried out to investigate the effect of QRFP on food intake in satiated C57Bl/6J mice (this study was led by Dr Amy Worth). ICV injection of QRFP, at 5μg and 10μg per mouse, increased food intake, compared with saline controls, one hour after injection (Figure 4.1). Food intake was increased further two hours after injection, and the increase sustained at the four-hour time point, thus indicating that the majority of QRFP-induced feeding occurred within two hours of injection. Food intake was also measured 24 hours after ICV injections, although there was no difference between QRFP- or saline-treated mice (data not shown), confirming the relatively short duration of action of QRFP and a lack of any obvious deleterious effects.

![Figure 4.1: Orexigenic effect of QRFP in C57Bl/6J mice](Image)

ICV injection of QRFP (5μg and 10μg per mouse) caused a dose-dependent increase in food intake, 1, 2 and 4 hours after injection, compared with vehicle (*p<0.05, **p<0.01, ****p<0.0001; repeated measures two-way ANOVA with Dunnett’s post hoc test)

Data shown is from experiment led by Dr Amy Worth and used here with permission
Fewer studies have been conducted using 26RFa, although those in the literature show 26RFa has a similar orexigenic effect as the full length QRFP. As confirmation of the effect of 26RFa in our in house models, ICV injections of 5μg 26RFa or saline, were made, in C57Bl/6J mice. ICV administration of 26RFa increased food intake one hour after injection, an effect that was sustained at both two and four hours after injection (Figure 4.2). As with QRFP, the majority of the 26RFa-induced feeding took place within the first two hours after injection. There was no difference between 26RFa- or saline-treated mice in their 24 hour post-injection food intake (data not shown).

Figure 4.2: Orexigenic effect of 26RFa in C57Bl/6J mice
ICV injection of 26RFa (5μg per mouse) increases food intake, 1, 2 and 4 hours after injection, compared with vehicle (**p<0.01, ****p<0.0001; repeated measures two-way ANOVA with Sidak’s post hoc test)
4.3.2 Effects of QRFP on reward-based feeding

The extent to which QRFP can influence hedonic feeding has yet to be investigated. In the present study, the effect of QRFP on operant responding was studied in mice trained to press levers to obtain strawberry milkshake in a progressive-ratio paradigm. Due to the fact that operant response is a new technique in this lab, the first study involved was set up using ghrelin as a positive control, replicating studies previously published in the literature. CD1 mice were trained to press levers reliably to obtain strawberry milkshake, progressing through fixed- and progressive-ratio training. When mice showed stable baselines in breakpoint in the progressive-ratio setting, they were injected with ghrelin (2mg/kg, ip). However, in our own model and apparatus, this injection had no effect on breakpoint or total lever presses (Figure 4.3A). As a result, the mice were implanted with indwelling cannulae to enable ICV injections to be carried out. Injections of ghrelin (0.5µg, 1µg and 2µg, ICV) or saline, were made in stably responding mice. Injections of 0.5µg and 1µg ghrelin reduced breakpoint and lever presses, with 1µg ghrelin significantly reducing both endpoints (Figure 4.3), contrary to previously reported results which have reported ghrelin to increase these parameters in both mice and rats (Perello et al. 2010; Skibicka et al. 2011). Data from the 2µg study is not included here as qualitative observations during the study showed that mice on this dose did not approach the reward receptacle or levers throughout the study. As a secondary measure, food intake during the hour following the end of the operant response program, demonstrated that the 1µg dose of ghrelin increased food intake significantly, as expected (Figure 4.3).

It has been suggested in the literature that ghrelin causes its effects on the reward system via orexin neurons, and as such a second positive control injection was made, administering 7µg orexin per mouse. The ICV injection of orexin resulted in a significant increase in both breakpoint and lever pressing (Figure 4.4), providing evidence that the model being employed in this study could be used to identify alterations in operant response. The dose of orexin was selected to induce operant responding whilst having no effect on food intake, building on previously published data (Choi et al. 2010). As predicted, no effect was observed on food intake in the half hour following operant response testing (Figure 4.4E).

The confirmation of the effect of orexin on operant responding showed that it was possible to induce increases in lever pressing using our model. As a result, the same mice were injected with an orexigenic dose and a sub-threshold dose of QRFP (5µg and 2µg, ICV, respectively) to investigate the role QRFP may play in this paradigm. Neither dose had any effect on either endpoint, although a significant increase in subsequent food intake was observed with 5µg QRFP (Figure 4.4). Presses on the inactive lever were monitored throughout each trial, to ensure increases in general arousal of the animals was not confounding the results, and no differences were induced by any treatment (data not shown).
Figure 4.3: Effects of ip and ICV ghrelin on operant behaviour

**Top:** Injection of ghrelin (2mg/kg, ip) had no effect on operant responding, as measured by breakpoint (A) and correct lever presses (B) (paired t-test)

**Middle:** Injection of ghrelin (0.5μg and 1μg, ICV), caused a dose-dependent reduction in operant responding, as measured by breakpoint (C) and correct lever presses (D) (*p<0.05; repeated measures one-way ANOVA with Dunnett’s post hoc test)

**Bottom:** 60-minute food intake, measured from termination of operant protocol, was significantly increased by ghrelin (1μg, ICV), compared with vehicle (E) (*p<0.05; paired t-test)
**Figure 4.4: Effects of orexin and QRFP on operant behaviour**

**Top:** Injection of orexin (7μg, ICV) increased operant responding, as measured by breakpoint (A) and correct lever presses (B) (*p<0.05; paired t-test)

**Middle:** ICV injection of QRFP (2μg or 5μg, ICV) had no effect on operant responding, as measured by breakpoint (C) and correct lever presses (D) (repeated measures one-way ANOVA)

**Bottom:** 60-minute food intake, measured from termination of operant protocol, was unaffected by orexin (7μg, ICV) (E) but was significantly increased by QRFP (5μg, ICV) (F), compared with vehicle (*p<0.05; paired t-test)
4.3.3 Effects of QRFP on energy expenditure

As discussed, peptides in the RFamide family play a role in a variety of different metabolic processes. One aspect of metabolism, affected by RFamides, is thermogenesis. Previous studies have shown that chronic QRFP treatment in mice leads to a reduction in rectal temperature and reduced uncoupling protein 1 (Ucp1) expression in BAT (Moriya et al. 2006), suggesting a reduction in thermogenesis. The acute effect of QRFP or 26RFa has not been fully elucidated. In two separate experiments, injections of QRFP or 26RFa (7.5μg, ICV), were made at least two hours into the light phase, and BAT temperature was measured immediately prior to, and two hours after, injection. Neither QRFP nor 26RFa induced significant changes in BAT temperature compared with saline-treated mice (Figure 4.5).

Previously, an in house study investigated the effect of ICV QRFP administration on acute energy expenditure (study conducted by Dr David Bechtold). An acute increase in VO₂ was observed following QRFP injections, when compared with saline-injected control mice, which resulted in a significant increase in total VO₂ measured over the following three-hour period (Figure 4.5C-D). Interestingly, this increased VO₂ was accompanied by a concurrent increase in the number of visits made to the food hopper following QRFP injection (Figure 4.5E-F).
Figure 4.5: Effects of QRFP on BAT temperature and energy expenditure

**Top:** BAT temperature was unaffected by injection of QRFP (A) or 26RFa (B) (both 7.5µg, ICV) compared with vehicle injection (repeated measures two-way ANOVA)

**Middle:** Injection of QRFP (10µg, ICV) increased VO₂ (C), compared with vehicle injection. VO₂ did not differ between treatment groups in the 2 hours before injection but QRFP increased total VO₂ in the 3 hours after injection (D) (***p<0.001; repeated measures two-way ANOVA with Sidak’s *post hoc* test)

**Bottom:** Food hopper visits were increased by QRFP in the same mice (E). Hopper visits did not differ between treatment groups in the 2 hours before injection whilst QRFP increased visits in the 3 hours after injection (F) (****p<0.0001; repeated measures two-way ANOVA with Sidak’s *post hoc* test)

Energy expenditure data shown (C-F) was generated by Dr David Bechtold and used here with permission
4.3.4 Effects of QRFP on locomotor behaviour

Previous studies have investigated the effect of acute QRFP dosing on activity levels in mice and have shown either an increase in locomotion, or no effect. However, none of the studies, to date, have attempted to elucidate whether the observed activity level increase is due to QRFP stimulating food-seeking behaviour, or whether the increased feeding is caused by the mice being more awake. With this aim in mind, a study was designed to investigate effects of acute ICV QRFP on activity levels, both in the presence and absence of food. ICV cannulated mice were injected with either QRFP (7.5μg) or saline during the light phase, for measurement of activity levels (Figure 4.6). In the first instance, food was left in the cages and intake was measured alongside activity levels. However, unlike previous experiments, mice administered QRFP did not show a significant increase in food intake during the study (Figure 4.6C). Activity levels were measured for one hour prior to, and two hours after, injection. As expected, no differences in activity levels were observed in the two hours before injections were made. However, despite the lack of effect on food intake in this study, QRFP administration still resulted in an increase in activity levels for 90 minutes after injection (Figure 4.6B).
Figure 4.6: Effects of QRFP on day-time locomotor activity

A: QRFP (7.5μg, ICV) increased activity levels for 60 minutes after injection (****p<0.0001; repeated measures two-way ANOVA with Sidak’s post hoc test)

B: Activity levels were no different before injections were made. QRFP (7.5μg, ICV) increased activity levels compared with vehicle controls and pre-injection activity levels (****p<0.0001 (versus pre-injection), ####p<0.0001 (versus vehicle) repeated measures two-way ANOVA with Sidak’s post hoc test)

C: Food intake, measured during the 2 hours post-injection, was not affected significantly by QRFP (7.5μg, ICV) (unpaired t-test)
The study was then replicated with the mice receiving the opposite injection to previous, around two and a half hours after lights off (Figure 4.7). It is well documented that mice do not exhibit constant levels of activity and feeding throughout the night time, with some of the highest levels occurring in the first few hours of darkness. Injections in this study were timed to take place once the mice had settled down from this initial hyperactivity. Activity levels and food intake were measured during this initial two and a half hours after lights off, prior to injection, and for another two hours after injection. In confirmation of the aforementioned circadian profile, saline-treated mice exhibited significantly higher levels of activity during the two hours pre injection compared with the subsequent two hours. Prior to injection there was no difference between groups, but QRFP-injected mice sustained the same level of activity post injection as observed pre injection, meaning that QRFP injection caused significantly increased activity levels compared with saline controls. QRFP-treated mice consumed more food than saline-treated mice following injection, showing QRFP administration increased food intake compared with saline even in sated mice. However, food intake was significantly lower in both saline- and QRFP-treated mice following their respective injections, than during the previous two hours, as expected from their circadian profiles. As a final step, mice were injected for a third time, with either QRFP or saline, during the dark phase, with food removed from the cages. As all mice had previously received a single QRFP injection, mice dosed with QRFP for this study were those that had their first QRFP injection in the first study, ensuring there were two weeks between repeat injections. Injections for this study were made slightly later than previous, around three hours after lights off, in order to account for transient hyperactivity of mice caused by increased food-seeking behaviour, due to the lack of available food. As with the previous study, no differences were observed in activity levels between either group before injection. However, with food removed, there were no significant differences between saline- and QRFP-treated mice after injection, in terms of activity levels (Figure 4.8).
Figure 4.7: Effects of QRFP on night-time locomotor activity (with food)

A: QRFP (7.5μg, ICV) increased activity levels during the dark phase (*p<0.05; repeated measures two-way ANOVA with Sidak’s *post hoc* test)

B: Activity levels were no different before injections were made. QRFP (7.5μg, ICV) increased activity compared with vehicle injections. A reduction in activity occurred following vehicle injection whilst QRFP-injected mice maintained pre-injection activity levels (*p<0.05 (versus pre-injection), #p<0.0001 (versus vehicle); repeated measures two-way ANOVA with Sidak’s *post hoc* test)

C: Food intake, measured during the 2 hours post-injection, was increased by QRFP (7.5μg, ICV), when compared with vehicle controls. A decrease in food intake occurred in both vehicle and QRFP treated mice following injections, with QRFP attenuating this decrease (**p<0.01, ****p<0.0001 (both versus pre-injection), ##p<0.01 (versus vehicle) repeated measures two-way ANOVA with Sidak’s *post hoc* test)
Figure 4.8: Effects of QRFP on night-time locomotor activity (without food)

A: QRFP (7.5μg, ICV) had no significant effect on activity levels when food was not present (*p<0.05; repeated measures two-way ANOVA with Sidak's post hoc test)

B: All mice reduced activity levels following injections, but QRFP (7.5μg, ICV) caused no effect on activity compared with vehicle. QRFP partially attenuated the reduction in activity levels in vehicle-treated mice (*p<0.05, ****p<0.0001 (versus pre-injection); repeated measures two-way ANOVA with Sidak's post hoc test)
4.3.5 Effects of QRFP on handling of orally dosed glucose

The role of signalling from the brain in glucose handling is well documented, with a critical role being played by neurons in the LHA and ventromedial hypothalamic nucleus (VMN). As such, it was hypothesised that QRFP neurons may play a role in this process. Following injection of QRFP or 26RFa, all mice received an oral gavage of glucose. QRFP significantly blunted the peak in blood glucose levels 15 minutes after gavage. However, 60 minutes after gavage, QRFP-injected mice had significantly increased blood glucose levels compared with saline injections (Figure 4.9). Area under curve (AUC) was calculated to provide a measure of glucose handling, and the AUC for the full two-hour period following injection showed no overall difference between QRFP- and saline-injected mice. However, due to the time-dependent differences observed, AUC was calculated further in two separate periods. The AUC from 0-30 minutes showed QRFP-treated mice had lower blood glucose levels during the early phase, whereas from 30-120 minutes, QRFP-treated mice had higher blood glucose. Taken together, these results indicate that QRFP is blunting the initial excursion in blood glucose levels following oral gavage, but that the increase in glucose levels is prolonged for a longer duration than saline-treated mice.

In order to confirm the effect of ICV administered QRFP on glucose handling, a second OGTT was conducted to determine the effect of ICV administered 26RFa. Sampling points of this study were slightly altered from the previous QRFP study to enable a clearer analysis of the effect of 26RFa on the initial glucose excursion. In our model, the effect of 26RFa matched up with that of QRFP, namely a blunting of the initial glucose excursion and a prolonging of elevated levels later in the study (Figure 4.10). In further corroboration, the AUC showed no differences between 26RFa- or saline-treated mice when viewed as a full two-hour profile. However, when split at the 20 minute time point, the AUC from the start of the study was decreased by 26RFa, although not quite significantly, whereas the AUC after 20 minutes was significantly increased, again indicative of a prolonged, flattened glucose profile with 26RFa treatment.
Figure 4.9: Effects of QRFP on handling of orally-dosed glucose

**Top:** QRFP (7.5μg, ICV) reduced the initial spike in blood glucose levels 15 minutes after orally dosed glucose (2g/kg), but prolonged the time during which glucose levels remained elevated (A) (*p<0.05, ****p<0.0001; repeated measures two-way ANOVA with Sidak’s post hoc test).

**Bottom:** The full glucose excursion, over 2 hours, was unaffected by QRFP, as shown by no difference in area under curve (AUC) (B). The glucose excursion in the first 30 minutes was significantly reduced by QRFP whereas, during the final 60 minutes, the glucose excursion was higher (C) (*p<0.05, **p<0.01; repeated measures two-way ANOVA with Sidak’s post hoc test).

Data shown is from experiment led by Dr Amy Worth and used with permission.
Figure 4.10: Effects of 26RFa on handling of orally-dosed glucose

**Top:** 26RFa (7.5μg, ICV) reduced the initial spike in blood glucose levels 15 minutes after orally dosed glucose (2g/kg), but prolonged the time during which glucose levels remained elevated (A) (*p<0.05, **p<0.01 ****p<0.0001; repeated measures two-way ANOVA with Sidak’s *post hoc* test)

**Bottom:** The full glucose excursion, over 2 hours, showed a trend towards being increased by 26RFa although this did not reach statistical significance (B). The glucose excursion in the first 20 minutes was reduced by QRFP, whereas during the final 100 minutes, the glucose excursion was higher (C) (****p<0.01; repeated measures two-way ANOVA with Sidak’s *post hoc* test)
4.3.6 Activation of QRFP neurons by fasting

During normal physiological situations, the activity of orexigenic neurons would be expected to be regulated by changes in energy status. For example, during times of negative energy balance, as would be seen during a fast, many orexigenic neurons are activated to stimulate feeding and food seeking behaviour. In order to determine the extent to which QFRP neurons are regulated in times of negative energy balance, Qrfp-cre::eYFP mice were fasted overnight, with ad libitum fed littermates as controls. Following this stimulus, c-Fos expression was increased in neurons of the DMN compared with fed mice (Figure 4.11). Within the LHA, c-Fos expression was significantly increased in the area immediately surrounding the QRFP population following fasting, but c-Fos did not co-localise in QRFP neurons.
Figure 4.11: Fasting-induced expression of c-Fos in the QRFP neuronal population

**Top:** An overnight fast induced c-Fos expression in the DMN (A) and in the LHA around the QRFP population (B), although QRFP and c-Fos did not co-localise in any neurons (*p<0.05, ***p<0.001; unpaired t-test)

**Middle:** Representative images of c-Fos expression in the DMN of fed (C) and fasted (D) Qrfp-cre::eYFP mice (white – c-Fos)

**Bottom:** Representative images of c-Fos expression in the LHA around the QRFP neuronal population of fed (E) and fasted (F) Qrfp-cre::eYFP mice (QRFP – c-Fos)

LHA: lateral hypothalamic area; 3v: 3rd ventricle
4.3.7 QRFP activation of neuronal populations

As discussed in the previous Chapter, very little has been observed regarding the QRFP neuronal population. This extends to a lack of knowledge regarding the pathways downstream of QRFP, and the pathways involved in mediating the effects of QRFP. Following ICV injection of QRFP, into Npy-cre::eYFP mice, very little QRFP-induced c-Fos expression was observed within the brain, and no significant increase in c-Fos was observed within any hypothalamic nucleus assessed; namely the paraventricular hypothalamic nucleus (PVN), superchiasmatic nucleus (SCN), LHA, DMN or Arc (Figure 4.12). QRFP did, however, induce a significant increase in c-Fos expression within the paraventricular thalamic nucleus (PVT), whilst a significant decrease was observed within the lateroanterior hypothalamic nucleus. An alternative indicator of neuronal activation, phosphorylated cAMP-response element binding protein (pCREB), was investigated in a second set of brain sections from the above study, but no increased pCREB expression was induced by QRFP injections compared with saline (data not shown).

Due to the small amount of c-Fos induced by QRFP, in a relatively dispersed manner within the hypothalamus, the extent to which this QRFP-induced activation was restricted to individual populations was investigated. In particular, the literature reports that inhibition of NPY receptors leads to an attenuation of QRFP-induced feeding. Therefore, NPY- and c-Fos-expressing neurons were counted in both the Arc and DMN. However, QRFP injection did not induce c-Fos expression within NPY neurons of either the Arc or the DMN (Figure 4.13).
Figure 4.12: QRFP-induced expression of c-Fos

Top: QRFP injection increased c-Fos expression in the PVT whilst reducing expression in the LA. No other hypothalamic nuclei showed any alterations in c-Fos expression (A) (*p<0.05; unpaired t-test)

Middle: Representative images of c-Fos expression in the LA nucleus following vehicle (B) or QRFP (C) injection (black – c-Fos)

Bottom: Representative images of c-Fos expression in the PVT following vehicle (D) or QRFP (E) injection (black – c-Fos)

D3v: dorsal 3rd ventricle; LA: lateroanterior hypothalamic nucleus; PVT: paraventricular thalamic nucleus; SCN: suprachiasmatic nucleus; 3v: 3rd ventricle
Figure 4.13: QRFP-induced expression of c-Fos in NPY neurons

**Top:** c-Fos was not induced in NPY neurons of the DMN after vehicle or QRFP injection (NPY – c-Fos)

**Bottom:** NPY neurons in the Arc similarly showed no c-Fos expression following vehicle or QRFP injection (NPY – c-Fos)

3v: 3rd ventricle
4.4 Discussion

4.4.1 Effect of QRFP on feeding in satiated mice

Since its discovery in 2003, QRFP has been investigated in a number of models, with a variety of influences having been described, including: aldosterone secretion (Fukusumi et al. 2003); arousal (Takayasu et al. 2006); blood pressure (Takayasu et al. 2006); bone formation (Baribault et al. 2006); thermogenesis (Moriya et al. 2006); adipogenesis (Mulumba et al. 2010); β cell survival (Granata et al. 2014); insulin secretion (Granata et al. 2014); and skeletal muscle glucose uptake (Allerton & Primeaux 2015). However, due to the localisation of QRFP neurons in the LHA, much attention has focussed on its effects on feeding. Here we have confirmed the orexigenic effects of centrally administered QRFP and 26RFa, and determined suitable doses for work with our transgenic mouse models. The orexigenic effect of both peptides in our mice may help to shed some light on an apparent discrepancy in response between rats and mice. To date, neither QRFP nor 26RFa have been reported to increase chow intake in fully satiated rats (Kampe et al. 2006; Primeaux et al. 2008). Caution must be taken with the methodology used in the Kampe paper, as they also failed to show acute ghrelin-induced feeding. Rats fed diets high in fat do seem to respond to both peptides (Primeaux et al. 2008). Two further studies have investigated the orexigenic effect of QRFP with one showing increased feeding in food-deprived rats (Lectez et al. 2009) and the other, increased consumption of milk (Zagorácz et al. 2015). Taken together, these data suggest that QRFP/26RFa-induced feeding in rats could be dependent on the rewarding value of the food: high-fat content, high-sugar content (milk), and refeeding in deprived animals, may all recruit reward-based pathways. By comparison, our results, and others previously, have shown that both peptides are capable of inducing robust feeding even in chow-fed mice (Chartrel et al. 2003; Takayasu et al. 2006; Moriya et al. 2006), highlighting the potential that rats and mice may respond differently to QRFP/26RFa. Further study would be useful to elucidate whether this is a true difference or simply a product of different methodologies.

Our results suggest QRFP/26RFa-induced feeding, in mice, resembles the actions of other orexigenic neuropeptides. In particular, both orexin (Sakurai et al. 1998) and MCH (Rossi et al. 1997), which are also produced in the LHA, stimulate robust increases in feeding over similar time frames to those described above for QRFP. Furthermore, none of the three peptides alter 24-hour food intake after a single, central injection. The stimulation of feeding by both orexin and MCH is likely due to downstream interactions with NPY signalling, as NPY antagonism partially attenuates food intake (Yamanaka et al. 2000; Chaffer & Morris 2002). Interestingly, antagonism of the NPY Y1 or Y5 receptors also reduces QRFP-induced feeding (Takayasu et al. 2006; Lectez et al. 2009; Zagorácz et al. 2015), indicating that QRFP may also interact with NPY pathways in a similar fashion. However, it could be important to note that the antagonist used in the Takayasu and
Zagoráczy studies (BIBP3226) is also an antagonist of NPFF receptors, with which QRFP has previously been shown to interact, although it unclear what relevance this may have.

Whilst in broad terms, both orexin and MCH can induce feeding, they appear to modulate different aspects of this process (Barson et al. 2013). It has been suggested that orexin may be involved in initiating feeding bouts and food seeking behaviour, whilst MCH promotes continued consumption of food high in energy, indicating that they may act in a complementary manner. It is unclear whether QRFP could play an independent role in feeding behaviour, or whether its effects are due to an interaction with either of these peptides. A previous study, showing increased arousal following QRFP injection (Takayasu et al. 2006), may point towards a similar role to orexin, where feeding is likely secondary to increased arousal. Together, these observations provide a hypothetical framework to test the similarities between QRFP and orexin function.

4.4.2 Effect of QRFP on locomotor behaviour

The ability of QRFP, orexin and MCH to stimulate feeding highlights the potential for overlap in the function of these neuropeptides. Experimentally induced orexigenic effects are also often accompanied by increased locomotor activity, and neurons within the LHA that modulate feeding behaviour often concurrently influence effects on arousal. Whilst the feeding response to orexin and MCH is of similar duration and magnitude, they play contrasting roles in the control of arousal (Barson et al. 2013). The feeding response to orexin is now generally considered to be secondary to increased arousal: central orexin injection increases arousal and reduces rapid eye movement (REM) sleep (España et al. 2001), whilst orexin neurons are most active immediately prior to waking, and during the active phase (Estabrooke et al. 2001). Furthermore, loss of orexin signalling results in a narcoleptic-like phenotype in rodents and dogs (Baker et al. 1982; Chemelli et al. 1999; Willie et al. 2003). MCH administration, on the other hand, promotes sleep (Verret et al. 2003), with MCH neurons firing maximally during REM sleep (Hassani et al. 2009) and loss of signalling resulting in increased wakefulness (Marsh et al. 2002; Zhou et al. 2005).

To date, the literature regarding the role of QRFP in arousal is controversial, although this may be confounded by the possibility of a species difference, as discussed earlier. ICV injection of 26RFa in rats produced no effect on locomotor activity, when the injection was made immediately prior to the dark phase, although activity was not measured after injections in the light phase (Kampe et al. 2006). Furthermore, microinjection of 26RFa into the medial hypothalamic area of rats also failed to induce increased locomotion in an open field test (Zagorácz et al. 2015). Studies in mice, on the other hand, confirmed that QRFP was capable of inducing a robust increase in locomotion, following central injections, in both the light and dark phase (Takayasu et al. 2006). It may be important to note that the Takayasu group also reported an intense increase in grooming behaviour following QRFP injections, although their doses of QRFP were much higher than in our
own studies. Whilst grooming behaviour is often suggested as an indirect measure of stress in animals, there were no effects on elevated plus maze scores suggesting no direct anxiogenic action (Takayasu et al. 2006). Furthermore, effects on grooming are frequently observed following injection of a variety of different peptides (Gispen et al. 1975; Sanchez et al. 1997; Ida et al. 1999; Füzesi et al. 2016), suggestive of a non-selective response to injection rather than to QRFP itself.

Our own studies confirm that doses of QRFP that are sufficient to elicit feeding behaviour, were also capable of stimulating robust, acute increases in locomotion. ICV QRFP injection during the light phase caused significant increases in activity levels for the following 90 minutes, even though, in this particular study, food intake was not significantly increased. It is unclear why feeding was not elicited in this study, in contrast to all our previous experiments, but it is possible that, in this instance, stress to the mice may have prevented the usual orexigenic effect associated with QRFP. Subsequently, we have shown that central QRFP injection also elicits increased locomotion in the dark, with concurrent increased food intake. However, when food was not available, QRFP injections did not increase activity levels above those seen in vehicle-injected mice. Close examination of the data revealed a very slight, transient effect of QRFP even in this study, but the hyperactivity of all mice, induced by increased food seeking, likely prevents this effect of QRFP from reaching statistical significance. The lack of locomotor response to QRFP in the absence of food complicates the interpretation of these data. On the one hand, this lack of response may indicate that QRFP primarily induces feeding and the increase in locomotion is secondary to this (although it is necessary to note the daytime locomotor experiment during which there was no increased feeding). Furthermore, if QRFP were involved in promoting food seeking behaviour, it would be expected that, in the absence of food, the effects of QRFP might be enhanced rather than reduced. However, it could also be argued that in a state of heightened food seeking behaviour, caused by the absence of food, it would be difficult to induce further additive effects on top of this upregulated system, due to a ceiling effect. Whilst our studies have not investigated the role of 26RFa in locomotion, given the similarities observed in the feeding studies, it is hypothesised that both peptides would induce similar effects in this parameter.

4.4.3 Effect of QRFP on reward-based feeding

It has long been appreciated that the prevalence of obesity has been steadily increasing, with particularly worrying levels evident in western society. The blame for this rise has been a more sedentary lifestyle coupled with increased availability of an obesogenic diet, and research has begun to highlight an important role for hedonic feeding (Lutter & Nestler 2009; Volkow et al. 2011). The appreciation that food could be rewarding and activate the same pathways long associated with drug addiction, has led to an abundance of studies aimed at determining the neuronal mechanisms responsible for mediating hedonic feeding. Operant responding is a well-established technique for studying the motivational aspects of hedonic feeding. One of the more
potent orexigenic peptides, ghrelin has been shown previously to enhance the motivation of both mice and rats to obtain food rewards (Perello et al. 2010; Skibicka et al. 2011; Skibicka et al. 2012). This robust effect, therefore, led to the use of ghrelin in the present study to act as a positive control, allowing confidence in protocols and techniques that are new to our laboratory.

Unexpectedly, in our experiments, ghrelin did not cause an increase in lever pressing for the milkshake reward, when injected ip or ICV, and there was actually a significant decrease in both breakpoint and lever pressing following 1µg ghrelin ICV. This conflicts with published results, where either injections of ghrelin induce a significant increase in operant responding in C57BL/6J mice (Perello et al. 2010) and rats (Skibicka et al. 2011). Although our study was conducted using the outbred CD1 strain, it seems unlikely that they would exhibit such opposing responses when our experiment confirms ghrelin similarly increases food intake. However, it is worth noting that one study has noted that C57BL/6J mice are more efficient at acquiring operant responses than CD1 mice (Baron & Meltzer 2001), although whether this observation is relevant to our current study is unclear, particularly given that our CD1 mice did acquire operant responding (see below). In our experiment, ghrelin failed to increase operant responding when 0.5µg or 1µg was injected ICV. Following 2µg ghrelin ICV, mice were observed to simply sit in a corner and not explore any part of the operant chamber. It was hypothesised that this dose may be causing an adverse behaviour; with excessive levels of hunger impacting on the mouse’s ability to perform the task.

Whilst the failure of ghrelin to elicit a previously well reported effect in this experiment is interesting, the primary purpose of using ghrelin was to act as a control and, as such, we instead tried orexin as a positive control. Indeed, 7µg orexin, injected centrally into our CD1 mice, increased both breakpoint and lever presses, without affecting the subsequent food intake. Specifically for this study, the dose of orexin was selected to complement data in rats showing that sub-threshold doses of orexin, in terms of orexigenic stimulation, are capable of inducing operant responding (Choi et al. 2010). As orexin increases activity levels generally, presses on the inactive lever were also recorded in our study, but no increase in pressing on this lever was observed, confirming that the increased operant responding was not due simply to hyperactivity of the mice.

Having demonstrated that orexin could increase operant responding in our mice, we then tested QRFP. Neither an orexigenic dose (5µg), nor a sub-threshold dose (2µg), elicited any alterations in operant responding. Our data indicate that QRFP can increase food intake without obviously modulating the reward salience of the food. This result distinguishes QRFP from both orexin and MCH, which both play modulatory roles in reward processes (Aston-Jones et al. 2009; Choi et al. 2010; Sharf et al. 2010; Nair et al. 2009; Mul et al. 2011), likely due to their interaction with the nucleus accumbens (NAcc) and ventral tegmental area (VTA) (Korotkova et al. 2003; Thorpe &
Kotz 2005; Sears et al. 2010). Potentially, the lack of effect of QRFP in this aspect is a differentiating factor between QRFP, orexin and MCH in the regulation of feeding.

### 4.4.4 Effect of QRFP on energy expenditure and thermogenesis

Central injection of orexin causes an increase in metabolic rate (Lubkin & Stricker-Krongrad 1998), whilst MCH induces a decrease in the same parameter (Glick et al. 2009), complementing their respective action on arousal. As confirmation of these effects, whilst both models are hypophagic, orexin knock-out mice are obese (Hara et al. 2001), whereas MCH knock-out mice are lean (Shimada et al. 1998). As discussed above, central QRFP administration results in increased arousal in mice, similar to orexin. The literature also reports that central QRFP injection causes an acute increase in oxygen consumption (Takayasu et al. 2006), suggesting a similar metabolic profile to orexin. Likewise, our data showed that central administration of QRFP increased oxygen consumption, although this correlates almost precisely with an increase in visits to the food hopper. These data, therefore, suggest that increased oxygen consumption may be being caused simply by feeding behaviour, rather than a direct effect of QRFP on energy expenditure. As discussed above, it is unknown whether feeding or arousal is the primary response to QRFP and, therefore, it is similarly unclear what the causal factor is in this study. Our hypothesis that oxygen consumption is caused by feeding behaviour is supported by the fact that we were unable to demonstrate an increase in BAT temperature, following central injection of either QRFP or 26RFa. Interestingly, chronic ICV QRFP administration reduces rectal temperature and Ucp1 expression in BAT (Moriya et al. 2006), which could indicate an adaptive role for QRFP in longer term energy balance, although the lack of QRFP-induced effect in our studies suggests that any effect on rectal temperature in the Moriya work is unlikely to be due to effects on thermogenesis.

### 4.4.5 Effect of QRFP on handling of orally dosed glucose

Evidence from the literature suggests a role for QRFP in the regulation of glucose handling via a direct effect on tissues in the periphery. 26RFa inhibits insulin secretion at the level of the pancreas as demonstrated in perfused rat pancreas (Egido et al. 2007), as well as isolated INS-1E β-cells and human islets (Granata et al. 2014). Conversely, QRFP actually enhances glucose-stimulated insulin secretion (Granata et al. 2014). These studies highlight a possible direct action of QRFP/26RFa on the pancreas, although it is unclear how this effect may be being mediated. The Granata paper reports expression of Gpr103 within INS-1E β-cells and human islets, and this is backed up by immunohistochemistry techniques. Our own reverse transcription PCR (RT-PCR) protocols (Chapter 3) and previous literature have reported, at best, very low level expression of either receptor type, or QRFP itself, in the pancreas (Takayasu et al. 2006; Fukusumi et al. 2003). More recently, 26RFa, but not QRFP, was shown to increase glucose uptake in skeletal myotubes in vitro (Allerton & Primeaux 2015). These results are based on in vitro models and it is currently unclear whether QRFP/26RFa are found in the circulation at sufficient levels to influence
peripheral tissues, although it is possible that the peptides may be produced locally and exert their effects in a paracrine manner.

It was our aim to determine whether central QRFP/26RFa could influence glucose handling, independent of direct actions on tissues in the periphery: it is highly unlikely that central injections of these peptides would result in them entering the circulation at a sufficient level to cause effects peripherally, enabling us to focus on centrally mediated effects. ICV injection of both QRFP and 26RFa caused markedly similar alterations to the handling of orally dosed glucose during an OGTT. In particular, both peptides lowered the initial peak of the glucose levels within the first 10-15 minutes of study. However, this reduction was counterbalanced by a subsequent prolonging of elevated glucose levels an hour after glucose loading. The overall result was that the AUC over the full two hours of the OGTT was unchanged by either peptide injection. However, if the AUC is split to investigate the early and late responses, it is clear that QRFP and 26RFa have a significant effect on the kinetics of the glucose response. Seemingly, both peptides reduce the initial glucose excursion, although whether this is due to increased insulin release, glucose uptake, or changes in glucose production, cannot be determined from the present studies.

The hypothalamus plays a major role in glucose handling through its interaction with the pancreas and insulin-sensitive tissues. It has long been known that stimulation of areas such as the VMN and LHA can cause increases in secretion of insulin and glucagon from the pancreas (Shimazu et al. 1966; Frohman et al. 1967; de Jong et al. 1977; Bereiter et al. 1981). The effects on pancreatic function are mediated by both the sympathetic and parasympathetic nervous system, primarily through projections of second order neurons onto the intermediolateral column (IML) or the dorsal motor nucleus of the vagus (DMV), respectively (Laughton & Powley 1987; Buijs et al. 2001; Mussa & Verberne 2013). A number of these second order neuronal projections originate in the LHA, from neurons containing orexin and MCH amongst others, whilst neurons of the VMN also play a role, albeit it through more indirect connections (Jansen et al. 1997; Buijs et al. 2001). The literature identified 26RFa binding sites, and Gpr103 expression, within both the DMV and the dorsal and ventral horns of the spinal column (Kampe et al. 2006; Bruzzone et al. 2007), in which the IML is located. On top of this, immunohistochemistry discussed in Chapter 3 revealed fibres of Qrfp-cre::ChR2 mice within the DMV, as well as passing into the spinal cord. It is possible, therefore, that central QRFP may act via one of these areas, leading to subsequent alterations in the function of peripheral tissues.

4.4.6 Activation of QRFP neurons by fasting

The well-documented, acute effect of QRFP on food intake, along with its expression in the LHA, is indicative of a peptide with a role in feeding regulation. If QRFP neurons were involved in physiological control of feeding, it would be expected that they would be regulated in response to the availability of food and the nutritional state of the animal. A 24-hour fast is a strong stimulus
for a mouse and causes high levels of feeding upon reintroduction of food. Fasting is reported to increase the expression of c-Fos in neurons in multiple hypothalamic nuclei (Wu et al. 2014). However, in our study, fasting did not induce c-Fos expression in QRFP neurons, potentially indicating that they are not sensitive to food deprivation. In contrast, Qrfp mRNA is upregulated in the hypothalamus of fasted mice (Takayasu et al. 2006). It is worth noting that the upregulation observed in this study was of a very small magnitude, with only a 1.5-fold increase in mRNA levels after a 48-hour fast, which is comparable to the increases in orexin mRNA following the same length fast in rats (Cai et al. 1999), but lower than a 4.5-fold increase observed in Npy mRNA levels (Takayasu et al. 2006). One interpretation of our result is that QRFP neurons are not regulated by energy status of the animal, thereby suggesting that QRFP does not play an endogenous role in feeding. Alternatively, the perceived lack of activation in this system may be because QRFP do not express c-Fos. Whilst c-Fos expression is the most commonly used marker of neuronal activation, not all neurons signal through this pathway, and it is not necessarily dependent on increased electrical activity (Luckman et al. 1994). It would be remiss to conclude that a lack of c-Fos expression directly equates to a lack of activation of a neuronal population. As discussed in the previous Chapter, ghrelin injections do not induce c-Fos expression in QRFP neurons, despite electrophysiology recordings suggesting that ghrelin does increase their firing rate. To date there have been no reports investigating whether QRFP neurons do in fact signal with c-Fos, but our results suggest that perhaps this may not be the most appropriate second messenger to evaluate the activity of the QRFP pathway.

4.4.7 QRFP-induced activation of neuronal populations

We, and others, have investigated the areas of the brain in which QRFP neuronal fibres, receptors and binding sites are located (Takayasu et al. 2006; Kampe et al. 2006; Bruzzone et al. 2007). However, previous work has not concluded the areas of the brain activated by QRFP injection. Following ICV QRFP injections, surprisingly little c-Fos was induced anywhere in the brain. Counts of c-Fos expression in specific hypothalamic nuclei also failed to identify any regions in which c-Fos expression was significantly altered compared with saline control injections. In fact, there were only two areas found where QRFP induced changes in c-Fos expression: the PVT and lateroanterior hypothalamic nucleus. Within the PVT, c-Fos expression was increased, whilst it was decreased within the lateroanterior hypothalamic nucleus. Interestingly, the PVT is classically associated with stress responses (Bubser & Deutch 1999; Otake et al. 2002), but also plays a role in arousal and locomotor activity regulation, as highlighted by high levels of orexinergic fibres (Kirouac et al. 2005). We have shown that QRFP induces locomotor activity (this Chapter) and immunohistochemistry identified QRFP fibres within the PVT (Chapter 3). Together these data may suggest that QRFP-induced arousal may be mediated by direct neuronal activation of the PVT. Recent optogenetic work confirmed that the arousal and anxiety effects induced by orexin
are mediated by activity in the PVT and locus coeruleus (LC) (Heydendael et al. 2014), and it would be interesting to investigate whether a similar model is true for QRFP. A definitive role for the lateroanterior hypothalamic nucleus is unclear but it is interesting that QRFP reduces c-Fos expression in this area, indicative of a reduced level of excitation. Immunohistochemistry in the previous Chapter did not identify QRFP fibres in this area, which suggests the c-Fos results observed here may be an indirect effect. Previous work has shown QRFP-induced feeding can be attenuated by Y1 receptor antagonism (Takayasu et al. 2006), leading to the investigation of whether QRFP caused increased c-Fos expression in NPY neurons. It was hypothesised that by viewing an individual neuronal population in this way, it may be possible to pick up QRFP-induced activation that was missed in the study of larger brain nuclei, but the low level of induced c-Fos was complemented by a lack of activation of NPY neurons in the DMN or Arc.

As discussed earlier, it is possible that the QRFP system does not readily induce c-Fos expression and that investigation of alternative markers of neuronal activation may be more appropriate methods for studying this. However, when levels of pCREB were investigated, following QRFP injections, a similar lack of increased expression was found. It is worth noting that, due to this study being optimised for c-Fos labelling, mice were culled two hours after injection whilst the optimal time for pCREB induction is considerably earlier, meaning that any effects may have been missed. On the other hand, the lack of c-Fos expression could equally be explained by QRFP neurons being inhibitory. Previous data has indicated that QRFP neurons may be glutamatergic (Campbell et al. 2017), but our studies in Chapter 3 show the QRFP population is made of a mix of both glutamatergic and GABAergic neurons.

4.4.8 Summary

- ICV QRFP and 26RFa both induce chow-feeding in satiated mice
  - Operant responding is unaltered by orexigenic, or sub-threshold doses of QRFP
- ICV QRFP causes hyperlocomotion, when administered during the day or night
- Acute increases in energy expenditure are induced by central QRFP injection
  - Likely induced by increased locomotor activity and feeding
- ICV QRFP and 26RFa injections do not alter BAT temperature
- ICV QRFP and 26RFa alter handling of orally dosed glucose
  - Prolonged, flattened glucose profile
- Neurons containing QRFP do not express c-Fos in response to fasting
- Central QRFP injections induce very few changes in c-Fos expression in the hypothalamus
Chapter 5

CONDITIONAL KNOCK-OUT OF QRFP
5.1 Introduction

Our data in Chapter 4 demonstrated a number of responses to centrally-administered QRFP, supporting previously described actions on food intake and locomotor activity (Takayasu et al. 2006), as well as identifying previously unreported effects on centrally-mediated glucose handling. These actions of exogenous QRFP provided indications of the function of QRFP, and were used to design further studies to test whether manipulation of endogenous QRFP signalling could produce complementary results. Whilst injections of exogenous peptides are an invaluable tool for providing data on the actions of peptides, these studies frequently inject supra-physiological levels of the peptide into the system, which means that their relevance to normal physiology can be called into question. In particular, off-target effects are a realistic concern with these studies, as unnaturally high levels of peptide can result in binding to receptors that ordinarily would not be activated by the levels of peptide usually encountered in the brain.

Equally, injecting peptides into the periphery, or direct application to isolated cells and tissues, can cause responses that may be unseen in the endogenous system. Importantly for this thesis, 26RFa shows high affinity for the NPFF2 receptor, with the suggestion that binding here occurs with similar affinity as for the Gpr103 receptors, and almost as efficaciously as neuropeptide FF (NPFF) itself (Elhabazi et al. 2013). This interaction has been subsequently proposed as being responsible for conflicting effects of QRFP and 26RFa in at least one study (Granata et al. 2014; Prévost et al. 2015).

Previously, responses invoked by administration of orexin or melanin-concentrating hormone (MCH), directly into the brain, have been validated using transgenic models. These two peptides were both originally identified for their orexigenic properties when centrally administered (Rossi et al. 1997; Sakurai et al. 1998), but genetic knock-out mice revealed other phenotypes that may be of more physiological relevance. For example, orexin knock-out mice exhibit a phenotype similar to human narcolepsy (Hara et al. 2001), and whilst they are still hypophagic, they are overweight, providing evidence that feeding effects may be a secondary role of orexin. MCH knock-out mice, on the other hand, exhibit hypophagia but are lean, caused by increased energy expenditure and locomotor activity (Shimada et al. 1998). It is important, therefore, to validate the actions of exogenous peptides using studies in which endogenous signalling has been manipulated, before drawing conclusions about physiological functions of a peptide.

With this in mind, we have developed a genetic knock-out mouse line, globally lacking QRFP through targeted disruption of the Qrfp gene: the FlEx-Qrfp mouse. The generation of this mouse provided us the opportunity to identify its phenotype, and draw more detailed conclusions about the role of QRFP. We have focussed primarily on endpoints suggested by our work in Chapter 4: feeding, activity, body weight and glucose handling, using previous literature to guide further development of our studies to investigate the cause of any phenotypic effects observed. During
the compilation of this thesis, a second group have described another transgenic model, lacking endogenous QRFP signalling, in which they have conducted many of the same experiments as described here in our model (Okamoto et al. 2016). In this Chapter, we will discuss the data generated from our own model and interpret what these results may imply for the physiological role of QRFP, drawing from this second model to support our hypothesis.

5.1.1 Objectives

Investigate the involvement of QRFP in energy homeostasis

Neuropeptide populations in the lateral hypothalamic area (LHA) are often involved in a range of metabolic functions, rather than mediating single actions. Our studies in Chapter 4 confirmed QRFP promoted acute increases in both feeding and energy expenditure. These effects indicate potential similarities between orexin, MCH and QRFP in terms of homeostatic energy regulation. Investigation of orexin or MCH knock-out mice revealed that these animals exhibited opposing alterations to feeding and energy expenditure. We will attempt to elucidate whether QRFP plays a complementary role to these other peptides in this regard, and whether the orexigenic effect of QRFP is a primary physiological function.

Investigate the role of QRFP in body-weight regulation

Chronic administration of QRFP results in increased fat mass, which is not dependent on increased food intake (Moriya et al. 2006); furthermore, disruption of QRFP signalling in mice reportedly causes a lean phenotype (Okamoto et al. 2016). Using our FlEx-Qrfp model we will investigate this obesity resistance under normal and obesogenic conditions, and specifically investigate the effects on adiposity. We will begin investigating whether the proposed lean phenotype is due to impaired fat accumulation or increased breakdown, and whether this is tissue specific or representative of changes in the whole body. To date it is unclear whether these effects of QRFP would be mediated centrally or through direct action on tissues in the periphery; we will discuss the data supporting both arguments, drawing from our own studies and those in the literature.

Determine the function of QRFP in glucose handling and insulin secretion

We reported a central effect of QRFP and 26RFa on glucose handling, in Chapter 4. Interestingly, previous work has described similar effects following peripheral administration of 26RFa (Prévost et al. 2015). It is presumed that these studies describe independent effects of QRFP/26RFa and, as such, it is unknown whether both, or either, are physiological. We will investigate the effect of both hypo- and hyperglycaemic stimuli on our FlEx-Qrfp model to determine whether a similar action can be observed in this model.
Identify the extent to which QRFP signalling is involved with the mediating the actions of peripheral endocrine regulators

Ghrelin and leptin are two of the most important regulators of feeding and body weight homeostasis. Most of their metabolic effects are mediated centrally, through interaction with numerous different brain regions, including in the hypothalamus. The sensitivity of QRFP neurons to these hormones has not been elucidated previously, so this will be investigated using in vivo studies in our FlEx-Qrfp mouse, and immunohistochemistry techniques in our Qrfp-cre models, described in Chapter 3. These data should provide further evidence on the likelihood of QRFP influencing feeding regulation as its primary function.

5.2 Methods

5.2.1 Generation of a Qrfp knock-out mouse model

The FlEx-Qrfp mouse line was generated by GenOway (Lyon, France) through homologous recombination in embryonic stem cells from a C57Bl/6J genetic background. Initially, exon 2 of the Qrfp gene was flanked by two specially modified loxP and lox511 sites before being inserted back into the genome in an inverted orientation through use of a targeting vector. The targeting vector used to generate this line contained: long and short homology regions to the Qrfp gene (on the C57Bl/6J background); the inverted exon 2 of the Qrfp gene, flanked by the two modified lox sites; a FRT-flanked neomycin cassette, for positive selection; and a diphtheria toxin A cassette to enable negative selection. The positive and negative selection sequences were used for selection of correctly targeted clones, which were injected into C57Bl/6J blastocysts for implantation into OF1 pseudo-pregnant females. Embryonic stem cell clones were originally isolated from 129Sv mice which are agouti in colour, enabling the degree of chimerism to be assessed by coat colour. Highly chimeric males were bred with C51Bl/6J females to produce heterozygous offspring, which were then bred with C57Bl/6J Flp deleter mice, causing the deletion of the FRT-flanked neomycin cassette. The resulting mouse model is a constitutive knock-out of the Qrfp gene due to the inversion of exon 2 rendering the animal incapable of transcribing functional QRFP (Figure 5.1). Breeding with a cre-recombinase mouse enables ‘rescue’ of QRFP expression in a cre-dependent manner through its interaction with both lox sites which reinverts exon 2 to its wild-type configuration. The genotype of this model was extensively analysed by PCR and southern blot before heterozygous mice were delivered to the University of Manchester.
5.2.2 Genotyping of the FlEx-Qrfp colony

Mice were maintained as a heterozygous colony with genotyping of every animal carried out following weaning. Ear notches were taken from each mouse, for identification purposes, and DNA extracted for PCR-based analysis of genotype, as described in General Methods. A trio of primers were designed by GenOway to enable identification of all three possible genes: wild-type, knock-out and cre-mediated rescue.

5.2.3 Effect of Qrfp knock out on body weight and composition

Effect of Qrfp knock out on body weight and composition

In order to evaluate the metabolic phenotype of QRFP knock out, multiple studies were conducted in both male and female mice. Male and female FlEx-Qrfp mice (n=19) were weaned onto normal chow at three weeks of age, with body weights recorded weekly from six to 20 weeks. Separate cohorts of FlEx-Qrfp mice (n=17), were switched to high-energy diet (HED) from six weeks of age and body weights again measured weekly until 20 weeks of age. Two days before the end of each study, blood glucose levels were measured in each mouse. Immediately following this, mice were shallowly anaesthetised to enable body length to be measured, from the tip of the nose to the base of the tail (an average of three measurements was taken).

A separate cohort of male FlEx-Qrfp mice (n=15) was switched onto HED at ten weeks of age to investigate the effect of HED feeding on body composition. Immediately prior to HED introduction, mice underwent quantification of total fat and lean mass through the use of quantitative magnetic resonance (QMR) (EchoMRI; Houston, TX, USA). Body weights were measured alongside fat and lean mass in order to enable these values to be normalised to body...
weight. QMR was subsequently employed every two weeks throughout the study from ten until 24 weeks of age.

Mice were culled by cervical dislocation and tissues dissected for additional ex vivo analysis. Whole livers were removed, weighed and, through QMR, fat content calculated. The right epididymal white adipose tissue (eWAT) deposit was dissected and weighed, with dissected tissues were frozen on dry ice, prior to longer term storage at -80°C and analysis by relative quantitative real-time PCR (qPCR).

RNA was extracted from samples, as described in General Methods, and the standard qPCR protocol employed to measure mRNA levels. Liver samples were analysed for phosphoenolpyruvate carboxykinase (Pepck) and glucose-6-phosphatase (G6Pase) expression; and eWAT, inguinal WAT (iWAT) and liver samples were also analysed for adipose triglyceride lipase (Atgl), hormone-sensitive lipase (Hsl), lipoprotein lipase (Lpl), fatty acid synthase (Fasn), diacylglycerol acyltransferase-2 (Dgat2) and fatty acid binding protein-4 (Fabp4).

Alongside this study, a second cohort of male FlEx-Qrfp mice (n=12) were switched to HED from ten weeks of age, with measures of adiposity recorded via QMR prior to HED introduction and then two and four weeks later. Terminal measures were made as above, to investigate the changes to the same parameters in a shorter timeframe. As before, eWAT and iWAT were dissected, with RNA extracted and qPCR analysis performed, to identify changes in relative expression of the same genes as before.

### 5.2.4 Effect of Qrfp knock out on food intake

A separate cohort of male FlEx-Qrfp mice (n=11) was used to investigate the effect of QRFP knock out on food intake. Mice were singly housed and allowed a week to acclimatise to their surroundings. Mice were allowed ad libitum access to regular chow with intake measured at the same time on three consecutive days, after which an average of the daily food intake was calculated. Following this, cumulative food intake was measured over a six-day period, during which time the food in each cage was left unchanged and the amount of food eaten was calculated after four, five and six days. Mice were then fasted overnight, for 20 hours, before food was returned two hours after lights on, and intake measured after one, two and four hours.

Following these measures, the cohort of mice was switched to HED and allowed ad libitum access for a week prior to studies starting. Three identical studies to those described above were conducted in mice fed HED, to discern any differences in feeding between the genotypes that had not been revealed in chow-fed mice.
5.2.5 Effect of Qrfp knock out on energy expenditure

Effect of Qrfp knock out on VO\textsubscript{2}/VCO\textsubscript{2} (CLAMS)

Indirect calorimetry provides a method of investigating metabolism through the exchange of respiratory gases, O\textsubscript{2} and CO\textsubscript{2}. In order to conduct this study, mice were acclimated to Comprehensive Laboratory Animal Monitoring System (CLAMS) cages (Columbus Instruments; Columbus, OH, USA), which record changes in the levels of these gases and enable calculation of O\textsubscript{2} consumption and CO\textsubscript{2} production. Male FlEx-Qrfp mice (n=8) were placed in individual CLAMS cages for three days prior to study start. Mice were maintained on chow with ad libitum access throughout the study. VO\textsubscript{2} and VCO\textsubscript{2} levels were measured for four consecutive days and averages calculated for day and night for each mouse. These mice were then switched to HED for 4 weeks, in their home cages, to induce weight gain before a second period in the CLAMS cages. Immediately prior to mice being housed in CLAMS cages, lean and fat mass were measured.

Effect of Qrfp knock out on thermogenesis

Thermogenic effects of QRFP were investigated using the FlEx-Qrfp model by measuring the temperature of brown adipose tissue (BAT) in the interscapular region. Male mice were maintained on chow diet (n=15) from weaning until they were ten weeks of age, at which time they were introduced to HED. Immediately prior to HED, the temperature of interscapular BAT was measured, on two consecutive days between two and three hours into the light phase. Two days before each thermal imaging time point, each mouse was lightly anaesthetised and the back shaved from the base of the skull to the midpoint of the back. BAT temperature was measured in conscious mice using a thermal imaging camera (FLIR) by recording a 10s video of each mouse moving freely around its home cage. Videos were analysed using ResearchIR software (FLIR) with correct environmental parameters incorporated into its algorithms: ambient temperature, humidity and distance to mouse. In this manner, BAT temperature was measured immediately prior to HED introduction and then after two, four and fourteen weeks, at which point mice were culled by cervical dislocation. BAT was dissected and weighed, before being frozen on dry ice for storage at -80°C, and qPCR analysis of uncoupling protein 1 (Ucp1) mRNA expression.

Effect of Qrfp knock out on activity levels

Ten-week old, male FlEx-Qrfp mice (n=17) were singly housed in the same specially designed beam break cages described in Chapter 4. Following four days habituation to these cages, activity levels, as measured by beam breaks, were measured over a four-day period. Numbers of beam breaks were collected into 15 minute bins and, following the end of the study, the total beam breaks per hour was calculated along with average hourly breaks during either the day or night periods.
5.2.6 Effect of Qrfp knock out on glycaemic control

Studies in the previous Chapter identified a role for exogenous QRFP in the handling of orally dosed glucose. In order to investigate the extent to which this effect is mimicked in another model, an oral glucose tolerance test (OGTT) was conducted in male FlEx-Qrfp mice (n=13). Singly-housed mice were fasted for six hours, from lights on, in order to produce a stable glucose baseline. Resting blood glucose levels were measured in knock-out and wild-type mice. Immediately following this, mice were orally gavaged with a glucose solution (2g/ml) and blood glucose levels measured at 10, 20, 40, 60 and 120 minutes after gavage. At the end of the study, glucose excursions were plotted and area under curve calculated for each mouse.

A second method for investigating glycaemic control was carried out, looking at how FlEx-Qrfp mice handled acute hypoglycaemia, as induced by a bolus injection of insulin. Male FlEx-Qrfp mice (n=17) had food removed for two hours prior to the start of the study, as lights came on. After baseline blood glucose levels were measured, mice were injected with insulin (1.75 units, sc) or saline in a crossover design. Glucose levels were measured in each mouse 30, 60, 90 and 120 minutes after insulin administration.

5.2.7 Effect of Qrfp knock out on response to endocrine regulators

Effect of absence of Qrfp on response to ghrelin

Activation of the QRFP population by metabolically important hormones would reveal a role for QRFP in mediating endocrine effects. As such, a cohort of male Qrfp-cre::eYFP mice (n=9) were intracerebroventricularly (ICV) cannulated and injected with either ghrelin (1µg, ICV) or saline, two hours into the light phase. ICV injections were made in singly-housed mice to enable food intake to be measured concurrently, as a positive control for ghrelin activity. Two hours after injection, mice were perfused transcardially. Brain sections throughout the hypothalamus were labelled for c-Fos and indirectly for QRFP using anti-c-Fos and anti-GFP antibodies (Table 2.1). Neurons labelled positively for c-Fos were counted in anterior and posterior sections of the arcuate nucleus (Arc), in representative sections from both ghrelin- and saline-injected mice, as a positive control for ghrelin-induced neuronal activation. Within the LHA, QRFP-expressing neurons were counted in identical sections in both ghrelin- and saline-treated mice, with the number of c-Fos positive neurons in both cases also counted, to enable calculations of percentage activation of QRFP neurons by ghrelin.

A cohort of male FlEx-Qrfp mice (n=17) was injected with ghrelin (2mg/kg, ip), or saline, two hours after lights on. Food intake was measured after one, two, four and 24 hours. The study was designed as a crossover, with mice receiving the other injection a week later.
**Effect of absence of Qrfp on sensitivity to leptin**

In a similar study to that described above, male Qrfp-cre::eYFP mice (n=14) were injected with leptin (5mg/kg, ip) or vehicle, two hours into the light phase. An hour after injection, mice were transcardially perfused and brains sections throughout the hypothalamus were labelled for phosphorylated signal-transducer and activator of transcription-3 (pSTAT3), a marker of leptin signalling, and QRFP/GFP (for antibodies, see Table 2.1). Neurons labelled for pSTAT3 were counted in the Arc, and within the LHA surrounding the QRFP neurons, enabling the percentage of QRFP neurons expressing pSTAT3 to be calculated.

A cohort of male FlEx-Qrfp mice (n=9) were fasted for 20 hours, overnight, and then dosed two hours into the light phase with either leptin (5mg/kg, ip) or vehicle. Food intake was measured two, four and six hours after this injection, with a crossover injection made one week later.

Subsequently, a second study measured the effect of QRFP knock out on leptin-induced suppression of overnight feeding. Male FlEx-Qrfp mice (n=10) were injected with leptin (5mg/kg, ip) or vehicle as lights began ramping down. Food intake was measured after two, four and six hours, with a crossover injection being made one week later. In a final experiment, female FlEx-Qrfp mice (n=11) were injected with a lower dose of leptin (2mg/kg, ip), or vehicle, and food intake measured.
5.3 Results

5.3.1 Validation of the FlEx-Qrfp knock-out mouse model
Reverse transcription PCR (RT-PCR) identified Qrfp expression in a variety of tissues from wild-type mice and, concordantly, no amplified product in knock-out mice (see Chapter 3). qPCR was also used on hypothalamic tissue, confirming the lack of Qrfp expression in knock-out animals, thus validating the model. Whilst further work using Western and Southern Blotting has not been conducted in house, the model was verified thoroughly by GenOway prior to its delivery, using these techniques.

5.3.2 Effect of Qrfp knock out on body weight
Peptides that exhibit an acute orexigenic action often also have longer-term effects on body weight, as has been suggested in a previous study with chronic dosing of exogenous QRFP (Moriya et al. 2006). However, it is unclear whether administration of this nature is representative of a normal physiological process. In order to investigate the role QRFP plays in body-weight homeostasis, growth curves were produced for FlEx-Qrfp mice maintained on either chow or HED (Figure 5.2, Figure 5.3). In male mice maintained on chow, there were no differences in body weight between knock-out and wild-type mice at any time point. However, when HED was provided from six weeks of age, knock-out mice were significantly lighter on HED than their wild-type counterparts, from 13 weeks through to the end of study (Figure 5.2). Similar results were achieved with female mice, with no body weight differences in chow-fed mice, whilst HED-fed mice diverged at 17 weeks of age (Figure 5.3). Previously published data identified a defect in bone development in Gpr103a knock-out mice leading to a reduction in body length (Baribault et al. 2006). In order to identify whether FlEx-Qrfp mice exhibited similar growth defects, body lengths were measured at the end of each chow growth curve. Male and female FlEx-Qrfp knock-out mice were not different in body length, measured from nose to base of tail, from wild-type mice (Figure 5.2, Figure 5.3).
Figure 5.2: Body-weight phenotype of male FlEx-Qrfp

**Top:** Male FlEx-Qrfp knock-out mice are normal weight when maintained on chow diet (A) (repeated measures two-way ANOVA)

**Middle:** Male FlEx-Qrfp mice gain less weight when maintained on HED than wild-type littermates (B) (**p<0.001; repeated measures two-way ANOVA with Sidak’s post hoc test)

**Bottom:** Body length of FlEx-Qrfp knock-out mice is no different to wild-type mice when maintained on chow (C) (unpaired t-test)
Figure 5.3: Body-weight phenotype of female FlEx-Qrfp

**Top:** Female FlEx-Qrfp knock-out mice are normal weight when maintained on chow diet (A) (repeated measures two-way ANOVA)

**Middle:** Female FlEx-Qrfp mice gain less weight when maintained on HED than wild-type littermates (B) (*p<0.05; repeated measures two-way ANOVA with Sidak’s post hoc test)

**Bottom:** Body length of FlEx-Qrfp knock-out mice is no different to wild-type mice when maintained on chow (C) (unpaired t-test)
Terminal blood glucose levels were measured immediately prior to tissue dissection, between three to five hours into the light phase (Figure 5.4). Glucose levels were not different between male mice of either genotype, on either diet. Similarly, female knock-out mice were not different from wild-type controls on either diet.

Figure 5.4: Effect of Qrfp knock out on glycaemia of FlEx-Qrfp mice

Top: Glucose levels of male FlEx-Qrfp knock-out mice are not different from wild-type mice when maintained on either chow (A) or HED (B) (unpaired t-test)

Bottom: Glucose levels of female FlEx-Qrfp knock-out mice are not changed from wild-type levels in mice maintained on chow (C) or HED (D) (unpaired t-test)
5.3.3 Effect of Qrfp knock out on food intake

Due to the orexigenic effect observed following acute dosing of QRFP, and the phenotypic effect on body weight caused by QRFP knock out, it was hypothesised that FlEx-Qrfp mice may also exhibit a feeding phenotype (Figure 5.5). However, daily food intake was not different between male mice fed regular chow diet. Furthermore, cumulative food intake over six days was also not different between knock-out and wild-type mice on chow. These studies were then repeated in the same mice following a week-long acclimation to HED, to detect any dietary differences that could be responsible for the alterations in body weight. Following acclimation to HED, it was confirmed that no difference existed in body weight between the genotypes, and food intake for each mouse had settled to a constant level (data not shown). Once again, neither daily nor cumulative food intake showed any difference between genotypes.

Finally, food intake was measured over a four-hour period following a 20-hour fast (Figure 5.5), during maintenance on either chow or HED. Fasting-induced refeeding at one, two and four hours was no different between genotypes, on either diet.
Figure 5.5: Effect of Qrfp knock out on feeding in FlEx-Qrfp mice

Top: Average 24-hour intake of chow (A) or HED (B) was no different between FlEx-Qrfp knock-out and wild-type mice (unpaired t-test)

Middle: FlEx-Qrfp knock-out and wild-type mice consumed the same amount of both chow (C) or HED (D) over 6 days (repeated measures two-way ANOVA)

Bottom: Following a 20-hour fast, FlEx-Qrfp knock-out and wild-type mice consumed equal amounts of chow (E) or HED (F) over 4 hours (repeated measures two-way ANOVA)
5.3.4 Effect of Qrfp knock out on activity levels

Another major component of energy expenditure, physical activity can have a large effect on energy balance, leading to alterations in body weight, particularly in obese mice (Figure 5.6). The activity levels of unstimulated male knock-out mice were significantly lower compared with wild-type littermates, during lights off, with a strong parallel trend during lights on. As a result, average activity levels over a full 24-hour period were significantly lower in knock-out mice.

**Figure 5.6: Effect of Qrfp knock out on locomotor activity in FlEx-Qrfp mice**

A: Circadian activity profile of FlEx-Qrfp knock-out and wild-type mice (data presented as mean)

B: Average 24-hour activity levels are significantly lower in FlEx-Qrfp knock-out mice compared with wild-type littermates (*p<0.05; unpaired t-test)

Bottom: FlEx-Qrfp mice are significantly less active during the dark phase (C), which is primarily caused by a reduction in activity in the first half of this period (D) (**p<0.01; two-way ANOVA with Sidak’s post hoc test)
5.3.5 Effect of Qrfp knock out on thermogenesis

Thermogenesis has a critical effect on energy balance in small mammals and, thus, the temperature of the interscapular BAT depot was recorded as an indirect measure of thermogenesis (Figure 5.7). This depot provides a readily accessible region to non-invasively measure BAT temperature using an infrared imaging camera in longitudinal studies. Immediately prior to mice being transferred onto HED, at ten weeks of age, BAT temperature of FlEx-Qrfp mice was measured and no difference was found between knock-out and wild-type littermates. BAT temperature was then measured after two, four and fourteen weeks of maintenance on HED. A gradual reduction in adaptive thermogenesis was observed with the onset of obesity, observed as a reduction in BAT temperature. However, no significant differences were detected between genotypes in obese, HED-fed mice.

The entire interscapular BAT was also dissected, and knock-out mice exhibited lower total BAT mass than wild-type controls (Figure 5.7), although this difference was lost when BAT weight was normalised to body weight. Relative expression levels of Ucp1 mRNA in BAT from knock-out and wild-type mice were investigated using qPCR, and found to be significantly higher in knock-out mice (Figure 5.7E).
Figure 5.7: Effect of Qrfp knock out on BAT temperature in FlEx-Qrfp mice

**Top**: BAT temperature was no different in chow- (A) or 14-week HED-fed (B) knock-out mice compared with wild-type controls. BAT temperature was reduced by obesity in both genotypes (unpaired t-test)

**Middle**: BAT weight was significantly lower in knock-out mice (C), although when normalised to body weight, both genotypes were no different (D) (**p<0.01; unpaired t-test)

**Bottom**: Expression of Ucp1 was elevated in BAT tissue from FlEx-Qrfp knock-out mice after 14 weeks HED feeding, compared with wild-type controls (E) (**p<0.01; unpaired t-test)
5.3.6 Effect of Qrfp knock out on energy expenditure

A common method for analysing energy expenditure in mice is through indirect calorimetry to measure changes in levels of respiratory gases, O$_2$ and CO$_2$ (Figure 5.8). VO$_2$ and VCO$_2$ were not different between chow-fed mice, and respiratory exchange ratio (RER) was similarly unaltered. Due to the previous observation of reduced lean mass in knock-out mice, and the importance of this for energy expenditure, the above endpoints were also normalised to lean mass rather than body weight, but there were still no differences observed (data not shown).

![Figure 5.8: Effect of Qrfp knock out on energy expenditure in FlEx-Qrfp mice (chow)](image)

Basal VO$_2$ (A & B), basal VCO$_2$ (C & D), and RER (E & F) are not different between chow-fed FlEx-Qrfp knock-out and wild-type littermates (Left: data presented as mean; Right: two-way ANOVA)
Maintaining these mice on HED for four weeks caused all mice to gain weight, but without a difference in body weight between genotypes (data not shown). This second study was conducted and analysed exactly as above, and VO\textsubscript{2}, VCO\textsubscript{2} and RER again were not different in knock-out mice, compared with wild-type mice (Figure 5.9). Interestingly, RER of HED-fed mice shows a reliance on fat metabolism at all times, compared with the normal circadian profile of chow-fed mice, which is indicative of a greater reliance of carbohydrate metabolism during waking hours (Figure 5.8E, Figure 5.9E).

**Figure 5.9:** Effect of Qrfp knock out on energy expenditure in FlEx-Qrfp mice (HED)

Basal VO\textsubscript{2} (A & B), basal VCO\textsubscript{2} (C & D), and RER (E & F) are also not different between HED-fed FlEx-Qrfp knock-out and wild-type littermates (Left: data presented as mean; Right: two-way ANOVA)
5.3.7 Effect of Qrfp knock out on body composition

Previous literature suggests a potential role for QRFP in inhibiting lipolysis and enhancing fatty acid uptake in adipocytes (Mulumba et al. 2010; Mulumba et al. 2015). Thus, measures of adiposity were recorded in male FlEx-Qrfp mice during the onset of diet-induced obesity. At ten weeks of age, mice were transferred to HED and measures of body weight and composition were conducted weekly for two weeks, then fortnightly for the remainder of the study (Figure 5.10).

Confirming the earlier growth curves, there was no difference between wild-type and knock-out mice in terms of body weight at ten weeks of age, immediately prior to being introduced to HED. As shown previously, male knock-out mice gained significantly less weight when maintained on HED (Figure 5.10). Presumably due to their older age, the difference between genotypes occurred faster than previous studies, just one week after HED feeding was initiated. Within a week of introduction to HED, both genotypes had approximately doubled their percentage fat mass. Over the following weeks a divergence in body composition emerged, with knock-out mice gaining less fat mass than wild-type mice, a result which was significant between four and twelve weeks.

When normalised to account for their lower body weight, and presented as percentage fat mass, knock-out mice have significantly lower percentage fat mass from four to eight weeks of study. Concurrently, though both genotypes significantly increased their lean mass during the study, wild-type mice gained significantly more lean mass than their knock-out counterparts. Coupled with this, wild-type mice consistently exhibited higher lean mass throughout the study compared with the knock-out mice. During maintenance on HED, wild-type mice gained significantly more lean mass between 10 and 14 weeks into the study.
Figure 5.10: Effect of Qrfp knock out on body composition of FlEx-Qrfp mice

A: FlEx-Qrfp knock-out mice gained significantly less weight on HED than wild-type controls (****p<0.0001; repeated measures two-way ANOVA with Sidak’s post hoc test)

B: Reduced body weight in FlEx-Qrfp mice was associated with a reduction in body fat as measured by quantitative magnetic resonance (***p<0.001; repeated measures two-way ANOVA with Sidak’s post hoc test)

C: When normalised to body weight, FlEx-Qrfp knock-out mice exhibited significantly reduced percentage body fat compared with wild-type controls (*p<0.05; repeated measures two-way ANOVA with Sidak’s post hoc test)

D: Lean mass increased in both FlEx-Qrfp knock-out and wild-type mice, although a greater increase occurred in wild-type mice. Throughout the study, lean mass in wild-type mice was elevated compared with knock-out mice (****p=0.0001 [versus wild-type week zero], ###p<0.001 [versus knock-out week zero], ‡‡p<0.01, ‡‡‡‡p<0.0001 [wild-type versus knock-out]; repeated measures two-way ANOVA with Sidak’s post hoc test [wild-type versus knock-out] or repeated measures two-way ANOVA with Dunnett’s post hoc test [within genotype comparisons])
At the culmination of this experiment, livers were dissected (Figure 5.11), and knock-out mice had lower total weight of liver, which corresponded with a decreased percentage fat content, compared with wild-type mice. qPCR was subsequently performed on liver samples (Figure 5.12). No differences in G6Pase expression were evident between the genotypes, but Pepck was upregulated in knock-out mouse liver. Further investigation of expression levels of Atgl, Hsl, Dgat2 and Lpl revealed upregulation of Atgl only.

Figure 5.11: Effect of Qrfp knock out on liver of FlEx-Qrfp mice

Reduced adiposity in HED-fed FlEx-Qrfp knock-out mice was accompanied by lower liver weight (A) and liver fat content (B) (**p<0.01, ***p<0.001; unpaired t-test)
qPCR analysis of livers from HED-fed FlEx-Qrfp mice revealed no alterations in expression of G6Pase (A), but Pepck was increased in knock-out mice (B). Analysis of adipogenic gene expression revealed an increase in Atgl (C) expression in knock-out mice, but no effect on Hsl (D), Dgat2 (E) or Lpl (F) (*p<0.05; unpaired t-test)
The relative expression of a number of genes involved in lipolysis and lipogenesis was investigated, using qPCR on eWAT and iWAT (Figure 5.13). Within the iWAT depot, knock-out mice had significantly elevated levels of Atgl, Hsl, Dgat2 and Lpl, with no change in levels of Fasn or Fabp4. Upon dissection, the right eWAT depot was found to be heavier in knock-out mice, despite their overall reduced adiposity (Figure 5.13G). Of note, however, was the observation that wild-type mice appeared to have greater deposits of WAT in the abdomen surrounding organs, but smaller deposits in the well-defined epididymal fat pads. The eWAT depot did, however, show corresponding trends to iWAT, in most of the genes, with significantly increased levels of Hsl and Dgat2 and a trend of increased Atgl and Lpl, and no change in Fabp4. The notable difference between iWAT and eWAT samples was a significant increase in Fasn expression in the eWAT, but not iWAT, depot of knock-out mice.
Figure 5.13: Effect of Qrfp knock out on gene expression in WAT of FIEg-Qrfp mice

Relative expression levels of Atgl (A), Hsl (B), Lpl (C) and Dgat2 (D) were increased in both inguinal and epididymal WAT from HED-fed FIEg-Qrfp knock-out mice, whilst Fasn (F) was significantly increased in epididymal WAT only. Levels of Fabp4 (E) were unchanged in either WAT depot. eWAT mass was increased in HED-fed FIEg-Qrfp knock-out mice (G) (*p<0.05, **p<0.01; unpaired t-test)
The observations of alterations in fat accumulation in FlEx-Qrfp mice fed HED, implicate QRFP in adipogenesis and/or fat storage. However, investigating gene expression at the end of the study meant that it was unclear whether these changes were causing the phenotype, or were a result of the reduced adiposity. In order to investigate the causal relationship, a second study was carried out with the same adiposity measures recorded as above (Figure 5.14). In this study, mice were culled four weeks after introduction to HED, at which point wild-type mice had gained more weight than knock-out mice, although this had yet to reach significance. Similarly, fat mass was not different between the genotypes after four weeks of HED. As seen in the longer-term body composition study, lean mass was significantly lower in knock-out mice, compared with wild-type controls, throughout the study. qPCR analysis of the previously discussed adipogenic genes was performed on both eWAT and iWAT from these mice (Figure 5.15). In contrast to the longer-term study, no differences were found between knock-out and wild-type mice in any of the genes measured.

The reduced fat mass and increased expression of lipolytic genes within WAT depots seemingly explained the reduced body weight phenotype described earlier. However, as the body weight effect was not observed in chow fed mice, a study was conducted to identify whether this lack of phenotype corresponded with unaltered lipolytic gene expression. eWAT was dissected from chow fed, male FlEx-Qrfp mice, and RNA extracted for qPCR. Relative levels of Hsl, Atgl, Dgat2, Lpl, Fabp4 and Fasn were not different between knock-out and wild-type mice, in contrast to HED-fed mice (data not shown).
Figure 5.14: Short-term effect of Qrfp knock out on body composition of FlEx-Qrfp mice

A: No difference in weight gain was observed after 4 weeks HED feeding (repeated measures two-way ANOVA)

B: Fat mass was not different between FlEx-Qrfp knock-out and wild-type mice after 4 weeks of HED, although the previously reported reduced adiposity was beginning to develop (repeated measures two-way ANOVA)

C: Fat mass normalised to body weight also showed no difference after 4 weeks (repeated measures two-way ANOVA)

D: Lean mass was lower in FlEx-Qrfp knock-out mice compared with wild-type mice, throughout the study, as previously reported (**p<0.001; repeated measures two-way ANOVA with Sidak’s post hoc test)
Figure 5.15: Effect of Qrfp knock out on gene expression in WAT of FlEx-Qrfp mice

Relative expression levels of Atgl (A), Hsl (B), Lpl (C), Dgat2 (D), Fabp4 (E) and Fasn (F) were unchanged in knock-out mice fed HED for 4 weeks. eWAT mass was unchanged by 4 weeks HED feeding (G) (unpaired t-test)
5.3.8 Effect of *Qrfp* knock out on glycaemic control

As described in Chapter 4, acute dosing of QRFP directly into the brain of mice results in alterations in glucose handling. In order to elucidate the role played by QRFP in a different physiological setting, an OGTT was undertaken in chow-fed FIEG-FlEx mice (Figure 5.16). At the initiation of the study, mice were fasted for six hours and no differences in baseline blood glucose levels were observed between knock-out and wild-type mice. This supports our previous results from the replete chow-fed mice in the body weight experiment. Following oral dosing, the fasted knock-out and wild-type mice both showed increases in blood glucose levels of around 7-8mmol/L, with levels peaking at around 20 minutes. There were no differences between knock-out or wild-type mice at any time point during the study, resulting in no difference between area under curve of glucose excursion.

As a further investigation for a potential action of QRFP in glycaemic control, the effect of insulin-induced hypoglycaemia was studied in FIEG-FlEx mice by dosing mice with a bolus injection of insulin (1.75U, sc) (Figure 5.16). All mice showed the expected decrease in glucose levels, with no difference observed between knock-out and wild-type mice.
Figure 5.16: Effect of Qrfp knock out on hyper- and hypoglycaemia in FlEx-Qrfp mice

A & B: Full blood glucose excursion profile is not different between genotypes (A), resulting in no difference in area under curve (B) (unpaired t-test)

C: Fasting blood glucose levels are the same in chow-fed FlEx-Qrfp knock-out and wild-type mice (unpaired t-test)

D & E: Insulin injections reduce blood glucose levels in both knock-out and wild-type mice (D), with no difference in response between genotypes (E) (**p<0.01, ****p<0.0001; paired t-test)
5.3.9 Effect of Qrfp knock out on response to endocrine regulators

Effect of absence of Qrfp on ghrelin-induced feeding

The orexigenic hormone, ghrelin, stimulates a number of neuronal populations in the brain to mediate its effects on feeding. Whether QRFP neurons are involved in the orexigenic effects of ghrelin had not been reported previously. Therefore, the extent to which a ghrelin injection (1μg, ICV) activated QRFP neurons was investigated, through labelling of c-Fos (Figure 5.17). As a positive control, food intake was measured for two hours after injection and, as expected, ICV ghrelin induced a significant increase in food intake compared with saline (data not shown). As a positive control for ghrelin-induced neuronal activation, c-Fos expression was increased in both anterior and posterior sections of the Arc, following ghrelin injection (Figure 5.17A-C).

Corresponding brain sections, containing QRFP neurons, were selected from both ghrelin- and saline-injected mice, and c-Fos-positive nuclei counted in identical areas in each brain. Ghrelin induced significantly greater c-Fos expression in the LHA immediately surrounding the QRFP population, and a small, though statistically significant, increase in c-Fos expression within QRFP neurons themselves. However, it is not clear whether this small increase is functionally relevant, particularly given the relatively low number of QRFP neurons in the entire LHA population.

As ghrelin administration induced this small increase in c-Fos expression in QRFP neurons, an experiment was employed to investigate the effects of ghrelin in FlEx-Qrfp mice (Figure 5.18). This study enabled the possibility of an indirect effect of ghrelin, through QRFP neurons, to also be investigated. As expected, ip-administered ghrelin increased food intake. This increase was observed in both wild-type and knock-out mice, confirming that the absence of QRFP in this model has no attenuating effect on acute, ghrelin-induced feeding, even when normalised to account for body weight differences (data not shown). Unexpectedly, 24-hour food intake was significantly increased following ghrelin injections, compared with saline, in both knock-out and wild-type mice.
Figure 5.17: Ghrelin-induced expression of c-Fos in QRFP neurons

A-C: Ghrelin injections induced c-Fos expression in the Arc (A) (**p<0.01, ***p<0.001; unpaired t-test) (representative images of Arc following vehicle (B) and ghrelin (C) injections: white – c-Fos)

D-G: Ghrelin injections induced c-Fos expression in the LHA around QRFP neurons (D) leading to a small but significant increase in percentage of activated QRFP neurons (E) (* p<0.05, ***p<0.001; unpaired t-test) (representative images of LHA following vehicle (F) and ghrelin (G) injections: QRFP – c-Fos)
Effect of absence of Qrfp on sensitivity to leptin

Leptin is a well-characterised catabolic hormone, causing its metabolic effects through a variety of neuronal populations. It had yet to be determined whether QRFP neurons are regulated by leptin and, as such, a study was instigated to address this. Qrfp-cre::eYFP mice were injected with leptin (5mg/kg, ip) and their brains processed for pSTAT3 staining (Figure 5.19). As a positive control for leptin action, the number of pSTAT3-expressing neurons was increased significantly within the Arc compared with vehicle controls (Figure 5.19A-C). Within the LHA immediately surrounding the QRFP neuronal population, relatively little pSTAT3 expression was observed with leptin treatment, although a significant increase was detected. There was no co-localisation of pSTAT3 within QRFP neurons indicating that this population may not be a direct target for leptin.

As with ghrelin, the lack of an obvious direct action on QRFP neurons by leptin, would not preclude QRFP neurons from mediating some of the leptin actions indirectly. Thus, male FlEx-Qrfp mice were fasted overnight for 20 hours, and dosed with leptin or vehicle two hours after lights on. The inhibition of fast-induced refeeding caused by intraperitoneal (ip) administration of leptin has been documented previously, but in the present study this was not evident in wild-type mice at any point (data not shown).

In a complementary experiment, the same mice were subsequently injected ip with leptin as lights began ramping down at night, in order to investigate its effect on night-time food intake (Figure 5.20A-B). Leptin caused a decrease in feeding in wild-type mice, which was significant only at the six hour time point, whilst food intake in knock-out mice was inhibited by leptin injection after four hours.

Figure 5.18: Ghrelin-induced feeding in FlEx-Qrfp mice

Ghrelin increased food intake in both FlEx-Qrfp knock-out and wild-type mice (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; repeated measures two-way ANOVA with Sidak’s post hoc test)
The earlier onset of response of FlEx-Qrfp knock-out mice to leptin encouraged a follow up overnight feeding study to be carried out, using a lower dose of leptin (2mg/kg, ip), in order to investigate whether FlEx-Qrfp mice showed increased sensitivity, or a prolonged response, to leptin (Figure 5.20C-D). As before, knock-out and wild-type mice consumed the same amount of food following vehicle injections. Leptin inhibited food intake in wild-type mice, although this effect was only significant after 12 hours of study, whilst knock-out mice also exhibited significant reductions in food intake, from four hours after injection.
Figure 5.19: Leptin-induced expression of pSTAT3 in QRFP neurons

**Top:** Leptin injections induced pSTAT3 expression in the Arc (A) (****p<0.0001; unpaired t-test) (representative images of Arc following vehicle (B) and leptin (C) injections: white – pSTAT3)

**Bottom:** Leptin injections induced pSTAT3 expression in the LHA around QRFP neurons (D) with no co-localisation (***p<0.001; unpaired t-test) (representative images of LHA following vehicle (E) and leptin (F) injections: QRFP – pSTAT3)
Figure 5.20: Leptin-inhibited feeding in FlEx-Qrfp mice

Two doses of leptin ([5mg/kg, ip; A] and [2mg/kg, ip; B]) reduced food intake in male FlEx-Qrfp mice, with leptin having similar effects in both genotypes (*p<0.05, **p<0.01, ***p<0.001; ****p<0.0001; two-way ANOVA with Sidak’s post hoc test)
5.4 Discussion

5.4.1 Investigating the physiological role of QRFP using the FlEx-\textit{Qrfp} mouse

The physiological role of \textit{Qrfp} remains uncertain, even though a number of effects have been observed following QRFP administration, as discussed in Chapter 4 (Chartrel \textit{et al.} 2003; Moriya \textit{et al.} 2006; Takayasu \textit{et al.} 2006; Kampe \textit{et al.} 2006; Egido \textit{et al.} 2007; Mulumba \textit{et al.} 2010; Granata \textit{et al.} 2014; Allerton \& Primeaux 2015). However, caution must be exerted when drawing conclusions from bolus injections of exogenous peptides into the brain, due to the relatively high doses used, and the question of how representative of normal physiological responses these effects are. Despite the potential drawbacks, studying the effects of administrating exogenous peptide on a system can lead to important insights into the function of the peptide in question and help guide further, relevant investigation. With this in mind, the generation of a knock-out mouse line, FlEx-\textit{Qrfp}, provides an excellent model with which to study the proposed role for QRFP in the regulation of body metabolism.

As highlighted in the introduction, a recent paper has described the phenotype of another \textit{Qrfp} knock-out mouse (Okamoto \textit{et al.} 2016). Whilst this model is relevant for comparison to our own FlEx-\textit{Qrfp} model, differences between how these mice have been generated make direct comparisons between the two relatively complex. As a result, we will discuss our model with regards to previous literature, and then compare the phenotypes of the two models.

5.4.2 Effect of \textit{Qrfp} knock out on body weight and composition

The only study in the literature, to date, that has studied the effects of chronic, central QRFP dosing, reported hyperphagia coupled with increased body weight, which was exacerbated under conditions of a moderately high-fat diet (Moriya \textit{et al.} 2006). Our results demonstrate that FlEx-\textit{Qrfp} knock-out mice have significantly lower weight gain in both sexes, but only when maintained on HED. Further investigation in another cohort of mice, using EchoMRI to determine total fat mass, confirmed the reduced weight gain in HED-fed knock-out mice, with concurrently reduced fat mass between four and twelve weeks on the diet. Percentage fat mass, following normalisation to body weight, was also reduced in knock-out mice, from four to eight weeks after HED introduction. Interestingly, wild-type mice plateaued in body weight between 12 and 14 weeks after being introduced to HED, whilst knock-out mice seem to continue increasing weight and percentage fat mass, albeit at a slower rate. Thus, both the body weight and percentage fat mass of the two genotypes had begun to converge at the time of culling. The fact that this phenotype takes four weeks to develop, and that the fat mass of the genotypes had begun to converge towards the end of this study, suggests that the loss of \textit{Qrfp} is not stopping weight gain, but is likely slowing it down. In order to try and identify the cause of this phenotype, we designed the following studies to investigate different aspects of energy balance.
5.4.3 Effect of Qrfp knock out on feeding and activity

As shown in Chapter 4, central administration of exogenous QRFP/26RFa induces feeding and locomotor activity in mice, regardless of the time of day. In the present studies we employed the FlEx-Qrfp model to investigate the effect of loss of endogenous QRFP signalling on these endpoints, to ascertain whether they were contributing to the lean phenotype. Interestingly, knock-out mice are normophagic, in terms of basal and fast-induced feeding, but are hypoactive, significantly so during the dark phase. The lack of feeding phenotype in these mice was unexpected, considering the well-documented orexigenic action of QRFP. In contrast, orexin and MCH knock out both result in a hypophagic phenotype (Shimada et al. 1998; Hara et al. 2001), even though the orexigenic action of orexin is now considered to be secondary to its arousal-inducing effects (Chemelli et al. 1999; Hara et al. 2001; Kotz et al. 2002). Our data questions whether the orexigenic action of exogenous QRFP is physiologically relevant. Many of the members of the RFamide family acutely influence feeding behaviour following ICV injection, but it is possible these evolutionarily conserved feeding responses are secondary to other primary functions. Caution must be taken before dismissing a role for QRFP in feeding though, as a high degree of redundancy exists in orexigenic pathways, so it is possible that alternative pathways may have compensated for the loss of Qrfp in our model. Indeed, agouti-related peptide (AgRP) and neuropeptide Y (NPY), two of the most potent feeding stimuli, play critical roles in food intake in normal physiology, but mice lacking both peptide genes show no feeding or body weight phenotype (Qian et al. 2002).

However, the observed hypoactivity in the FlEx-Qrfp knock-out mice complements the peptide-induced hyperlocomotion identified in the previous Chapter, producing a strong dataset implicating QRFP in locomotor activity and arousal. The hypophagic phenotypes in orexin and MCH knock-out mice are accompanied by altered activity profiles, with orexin knock out causing a narcoleptic phenotype and MCH knock out enhancing wakefulness (Willie et al. 2003; Willie et al. 2008). In particular, the hypoactive phenotype of our FlEx-Qrfp knock-out mice shows similarity with the phenotype of orexin knock-out mice, with activity levels only significantly decreased in the dark phase. The hypoactivity of orexin knock-out mice is characterised by cataplectic episodes: sudden ‘arrests’ in activity and consciousness. Our present studies were not sensitive enough to identify whether the FlEx-Qrfp knock-out mice suffer from similar episodes, but behavioural analysis in the dark phase could easily elucidate this. Observations in Chapter 3 revealed QRFP fibres within nuclei associated with control of arousal, that also receive orexinergetic input: namely the locus coeruleus (LC) (Hagan, J et al. 1999), dorsal raphé nucleus (DR) (Matsuzaki et al. 2002), ventral tegmental area (VTA) (Nakamura et al. 2000) and tuberomammillary nucleus (TMN) (Eriksson et al. 2001). Taken together, this data suggests an effect on activity levels could be a primary role of QRFP, potentially indicating that the orexigenic action of exogenous QRFP is secondary to the promotion of an increased arousal, similar to orexin.
5.4.4 Effect of Qrfp knock out on energy expenditure

The results in this Chapter show a clear reduction in weight gain on HED which, currently, cannot be accounted for by a reduction in food intake. With this in mind, further studies were initiated to find the cause of this lower body weight, focussing on alternative aspects of energy homeostasis, primarily energy expenditure. The reduced body weight of the FlEx-Qrfp mouse on HED diet could be caused by an increase in energy expenditure through a number of mechanisms. As mentioned earlier, both orexin and MCH, whilst capable of stimulating food intake acutely, influence energy expenditure by affecting metabolic rate. Orexin/atxin3 mice develop obesity, despite being hypophagic, due to a reduced metabolic rate (Sellayah et al. 2011), whilst MCH knock-out mice are lean due to an increased metabolic rate (Shimada et al. 1998), both determined by indirect calorimetry and the measurement of oxygen consumption. Chronic infusion of QRFP led to a decrease in rectal temperature, suggesting a possible reduction in thermogenesis (Moriya et al. 2006). This decrease was observed in ad libitum and pair-fed mice indicating that the effect on energy expenditure was independent of any orexigenic action. Similarly, Takayasu and colleagues (Takayasu et al. 2006) found that central injection of QRFP stimulated an acute increase in oxygen consumption, though our own data, in Chapter 4, suggests that this might be secondary to increased arousal. In order to investigate the effect of Qrfp knock out on energy expenditure by indirect calorimetry, FlEx-Qrfp mice were housed in CLAMS cages. In this study, there was no difference between knock-out and wild-type mice, when maintained on chow or HED. The data generated in house previously showed an acute increase in metabolic rate following QRFP injection, an effect that overlapped with visits to the food hopper. Subsequent studies, described in Chapter 4, have since shown a robust increase in activity levels following QRFP administration, and it is likely that this is a driving factor behind any acute increase in oxygen consumption, as the literature also reports increases in activity levels and oxygen consumption following QRFP injection (Takayasu et al. 2006). This group also recorded significant, and prolonged, increases in blood pressure following QRFP injection which could be linked to the previously discussed grooming behaviour (Chapter 4), as a sign of elevated stress levels.

Non-shivering adaptive thermogenesis is a major component of energy expenditure in small mammals, and it is well documented that alterations in environmental conditions, such as housing mice at either cold or thermoneutral temperatures, result in profound changes in energy expenditure (Cannon & Nedergaard 2011). There is also strong evidence suggesting that obesogenic diets can cause alterations in adaptive thermogenesis (Rothwell & Stock 1997; Cannon & Nedergaard 2004), although the topic of diet-induced thermogenesis remains a hotly contested phenomenon (Kozak 2010). It is possible that the body composition phenotype observed in FlEx-Qrfp mice could be as a result of altered thermogenic capacity in these mice. On chow diet, no obvious difference was observed in BAT temperature between the FlEx-Qrfp knock-out mice and wild-type littermates. However, BAT temperature was slightly elevated in FlEx-Qrfp mice.
throughout the HED experiment, but this effect was not significant. All mice exhibited a gradual decrease in BAT temperature as HED-induced obesity set in and, at the end of the study, mice from both genotypes had significantly lower BAT temperature than before they were introduced to HED. This result indicated a reduction in thermogenesis in both genotypes with the development of obesity, but no interaction between obesity and genotype was evident. Upon completion of the study, BAT tissue was dissected and absolute mass was significantly smaller in FlEx-Qrfp mice (although when normalised to total body weight, there was no difference). Relative qPCR suggested that FlEx-Qrfp mice had elevated Ucp1 mRNA levels when compared with wild-type mice. However, caution should be exercised as this is not a definitive measure of thermogenic capacity: the qPCR data represents a surrogate estimate per gram of tissue. Since the mass of tissue is decreased in knock-out mice, an increase in the thermogenic capacity of BAT is unlikely to be of any great significance, though it is possible that a modest increase might explain the minor elevation in BAT temperature (approximately 0.3°C across the study). In order to determine whether there was any browning of WAT, relative expression levels of Ucp1 in iWAT was performed, with no difference identified between wild-type and knock-out mice (data not shown). It appears unlikely, therefore, that increased adaptive thermogenesis is contributing to the body-weight phenotype.

5.4.5 Effect of Qrfp knock out on adiposity

Due to the fact that, as yet, we have been unable to identify a causal factor for the reduced weight gain in the FlEx-Qrfp mice, we set about investigating this phenotype in more detail. Previously, the role of QRFP in adipogenesis in vitro has been investigated (Mulumba et al. 2010), with the overall conclusion that QRFP exerts an antilipolytic effect, at the level of the adipocyte. Both QRFP and 26RFa, acting via the Gpr103b receptor, increased triglyceride accumulation and fatty acid uptake (with concurrent increased expression of genes associated with uptake), whilst inhibiting isoproterenol-induced lipolysis. In a second paper by the same group, QRFP inhibited phosphorylation of Hsl, a key enzyme in the breakdown of triglycerides, in 3T3-L1 adipocytes, by inhibiting protein kinase A (PKA) activity (Mulumba et al. 2015). Taken together, these data suggest a local role in adipogenesis, with QRFP functioning as an antilipolytic peptide. The fact that the body weight phenotype in our FlEx-Qrfp mice was observable only under the challenge of HED may complement the literature, suggesting a role for QRFP in the regulation of adiposity, due to the fact that in mice on chow, fat depots are maintained at low levels.

In order to investigate the possible biochemical processes being influenced by Qrfp knock out, qPCR was conducted in WAT of epididymal and inguinal origin. The relative expression levels of a number of genes was investigated in both depots: Atgl and Hsl, two key enzymes in the lipolysis pathway; Lpl, an enzyme responsible for release of fatty acids from circulating lipoproteins, and thus uptake into tissues; Fasn, the enzyme responsible for catalysing the final stage of de novo
fatty acid synthesis; \textit{Dgat2}, an enzyme catalysing the conversion of diglycerides to triglycerides; and \textit{Fabp4}, a transporter of fatty acids. In support of a suggested antilipolytic effect of QRFP, FlEx-\textit{Qrfp} knock-out mice showed elevated levels of \textit{Atgl} and \textit{Hsl}, compared with wild-type mice. In general, basal lipolysis is increased in obesity (Duncan \textit{et al.} 2007), and the increase in fatty acids is associated with the development of insulin resistance. The observation that our FlEx-\textit{Qrfp} mice have elevated levels of lipolytic genes may suggest that this is a change that is contributing to the leanness, as a reduced fat mass should lead to reduced lipolysis in theory. \textit{Lpl} and \textit{Dgat2} mRNAs were also elevated in both WAT depots in knock-out mice, despite the reduced adiposity in these animals. The development of diet-induced obesity is normally associated with upregulated expression of these enzymes (Casaschi \textit{et al.} 2005; Suzuki \textit{et al.} 2005; Goldberg \textit{et al.} 2009), making the interpretation of these results difficult. It is interesting that our study shows increased \textit{Lpl} in knock-out mice in contrast to previous work, which showed an increase of \textit{Lpl} and increased fatty acid uptake following QRFP treatment (Mulumba \textit{et al.} 2010). The increased expression of these two genes may be explained by fatty acid recycling mechanisms: increased lipolysis releases fatty acids from WAT, and between 30-60% of these can be recycled and converted back to triglycerides for storage (Reshef \textit{et al.} 2003). Therefore, it is likely that an increase in lipolysis in this model would have the same effect, with the increased expression of \textit{Dgat2} and \textit{Lpl} playing a part in this. Unexpectedly, expression of \textit{Fasn} was increased in eWAT, but not iWAT, from FlEx-\textit{Qrfp} mice, which is difficult to reconcile in mice on HED, as \textit{de novo} fatty acid synthesis would be expected to be downregulated in obesity. However, the increased eWAT mass in knock-out mice, despite reduced total body fat mass, could be linked to this increased \textit{Fasn} expression, potentially highlighting a differential regulation of this tissue.

It is important to understand that qPCR on WAT from chow-fed, and short-term HED-fed mice culled before body weight divergence, showed no difference in mRNA levels of any of the investigated genes. This suggests that the changes observed are possibly being caused by an adaptive mechanism that is only manifested as an observable phenotype as fat mass is increased in these animals. This mechanism could be being triggered by the increase in fat mass in HED-fed mice, the response to which is altered in the FlEx-\textit{Qrfp} knock-out mouse, leading to an inability to reduce lipolytic rate.

Of further interest, FlEx-\textit{Qrfp} knock-out mice had significantly lower total liver weight, which was accompanied by a reduction in fat content. Obese mice tend to develop fatty livers due to accumulation of triglycerides in the tissue, so the reduced liver fat content in the knock-out mice may support a general state of increased lipolytic activity in these mice. There was also no effect observed with \textit{G6Pase} expression, but \textit{Pepck} levels were increased in knock-out mice. These genes catalyse different stages of the gluconeogenesis pathway, with \textit{Pepck} catalysing oxaloacetate conversion to phosphoenolpyruvate (Hanson & Garber 1972; Granner & Pilkis 1990)
and G6Pase catalysing the final step of converting glucose-6-phosphate to free glucose (Barzilai & Rossetti 1993; Granner & Pilkis 1990). However, as FlEx-Qrfp mice were not hyperglycaemic, it may be more relevant that Pepck also plays a role in the glyceroneogenesis pathway, which has been identified as the primary source of glycerol in vivo (Nye et al. 2008). In turn this glycerol would likely be utilised to produce triglycerides, from the released fatty acids, which is responsible for a large proportion of fatty acid recycling. Importantly, levels of Atgl were also upregulated in livers of knock-out mice, implying that increased lipolysis may be responsible for the reduced fat content. As the expression of Qrfp and its receptors in the liver is understood to be low (Fukusumi et al. 2003; Jiang et al. 2003), it is possible that this may suggest a more centrally-mediated control of lipolysis by QRFP.

### 5.4.6 Evidence for central and peripheral actions of QRFP on adiposity

It is currently unknown whether the effects we observe on adiposity reflect an effect of QRFP in the periphery, or if they are mediated centrally. The literature suggests a direct effect on WAT, with an antilipolytic action mediated by QRFP via Gpr103b (Mulumba et al. 2010; Mulumba et al. 2015), but it remains to be determined whether this translates to a physiologically relevant effect in vivo. Importantly, the expression of Qrfp mRNA was decreased, whilst that of Gpr103b was increased, during the development of diet-induced obesity in mice, indicating a potential paracrine mechanism of action. We report here that FliEx-Qrfp knock-out mice show changes in WAT gene expression only when maintained on HED long term, which suggests that this is an adaptive change in the mice, rather than a direct effect of the gene knock out. In speculation, both leptin and insulin play important roles in regulating adiposity: leptin stimulates lipolysis (Frühbeck et al. 1998) including expression of Atgl, Hsl and Lpl; whilst insulin upregulates Lpl, Dgat2 and Fasn at the transcriptional level (Ranganathan et al. 2006). It would be interesting to ascertain whether WAT from knock-out mice shows altered sensitivity to either leptin or insulin. As non-fasted glucose levels were not different between wild-type and knock-out mice, we have no evidence of higher total-body insulin sensitivity, but this does not rule out a tissue specific effect.

Alternatively, our identification of QRFP neuronal projections passing through the brainstem and towards the spinal column (see Chapter 3), provides a possible anatomical basis for central QRFP neurons mediating peripheral effects. Furthermore, previous literature reported that chronic centrally-administered QRFP increased fat mass in HED-fed mice (Moriya et al. 2006). Many data exist detailing central control of lipolysis in WAT, specifically through the sympathetic nervous system. In particular, retrograde tracing has revealed innervation of WAT originates predominantly from sympathetic ganglia in the spinal column (Youngstrom & Bartness 1995; Foster & Bartness 2006), whilst pseudorabies virus (PRV) tracing identified neurons within the
ventromedial hypothalamic nucleus (VMN) that influence lipolysis via these sympathetic ganglia (Bartness et al. 2010; Bartness et al. 2014).

5.4.7 Effect of Qrfp knock out on lean growth
Alongside the measures of fat mass discussed above, lean mass was concurrently measured in this cohort of mice, and found to be significantly lower in FlEx-Qrfp knock-out mice compared with wild-type littermates. This reduced lean mass was evident at all stages of the study, including prior to mice being transferred to HED, although the difference was not as large as that observed between fat mass. For example, after eight weeks of HED, fat mass was 40% lower, whereas lean mass was around 15% lower. It is worth noting that both wild-type and knock-out mice increased lean mass during the study, although knock-out mice only gained around half as much lean mass as their wild-type littermates. In both male and female mice of both genotypes, when maintained on chow, body length at 20 weeks of age was similar. Due to this lack of effect on linear growth in the knock-out mouse, the difference in lean mass is likely due to alterations in muscle mass, although we have no absolute measures to definitively confirm this. A lower lean mass may be expected to result in a reduction in energy expenditure, but this is not supported by our data. Currently, we have no strong hypothesis as to why this difference may exist, although it may be of relevance that 26RFa, but not QRFP, enhanced insulin-induced glucose uptake and glycogen formation in cultured myotubes (Allerton & Primeaux 2015), although it is also possible that this action of 26RFa may be mediated by off-target effects (see Chapter 7).

A previous study suggested a role for Qrfp, acting through the Gpr103a receptor, in bone formation (Baribault et al. 2006). In this previous study, QRFP receptor knock-out mice were found to exhibit kyphosis, an increased backward curvature of the spine, leading to a pronounced ‘hump’, and a reduced body length. This effect was more common in female mice, and was caused by a reduction in bone density, coupled with a reduced osteoclast number. Taken together this data indicated that Gpr103 may play a role in skeletal formation and remodelling. Whilst the receptor isoform that had been disrupted in the production of this model was not defined, it is likely to have been Gpr103a, due to the fact that Gpr103b was not described until around the same time (Takayasu et al. 2006). Furthermore, in this model, relatively strong expression of Gpr103 was identified within the eye which, as discussed in Chapter 6, suggests the Gpr103a isoform is being targeted. Our mice were measured from nose-to-base-of-tail which, whilst not at the technical level required to diagnose kyphosis, would be sufficient to identify the deformity. Nose-to-base-of-tail length was the same between genotypes which suggests that, at least in our model, loss of Qrfp has no obvious deleterious effects on bone formation. Whilst contradictory to the aforementioned study, it is possible that compensatory mechanisms in the FlEx-Qrfp mouse may have prevented a phenotype being observed in this model or, alternatively, the kyphosis in the Gpr103 knock-out mouse may have been independent of QRFP signalling.
5.4.8 Effect of Qrfp knock out on glycaemic control

Results from Chapter 4 demonstrated the ability of both QRFP and 26RFa to influence glucose handling following ICV injection. As previously described, QRFP and 26RFa have been shown also to influence insulin secretion in both isolated islets (Granata et al. 2014) and a perfused pancreas preparation (Egido et al. 2007). In both these studies, 26RFa inhibited glucose-stimulated insulin secretion whilst QRFP acted in the opposite manner, enhancing glucose-stimulated insulin secretion (Granata et al. 2014). However, the effect of 26RFa has since been attributed to effects mediated by the NPFF2 receptor, for which 26RFa shows high affinity, due to the inability of Gpr103 inhibition to attenuate this effect. Recent work in MIN6 cells, which express Gpr103 but not NPFF2, confirms this by showing that in this set-up 26RFa induces insulin secretion akin to QRFP (Prévost et al. 2015).

Firstly, no difference was seen in unfasted glucose levels between FlEx-Qrfp genotypes. The close relationship between obesity and insulin resistance is well documented, if not completely understood, and as a result, obesity is commonly associated with elevated blood glucose levels. Since body weights of FlEx-Qrfp knock-out mice were low compared with their wild-type littermates, it was hypothesised that this might result in an improvement in insulin sensitivity and glycaemic control. However, unfasted, diet-induced obese FlEx-Qrfp knock-out mice had glucose levels no different to wild-type controls. This result was observed in both male and female mice, and chow-fed mice similarly exhibited no differences in glucose levels. Similarly, both genotypes exhibited the same correlation between body weight and glucose levels (data not shown).

In order to further investigate the effects of QRFP on glucose handling, an OGTT was conducted in FlEx-Qrfp mice to identify the physiological role of QRFP in glucose handling. No differences were observed between knock-out and wild-type mice, following orally dosed glucose. However, glycaemic control requires the body to respond to periods of hyperglycaemia, such as following a meal, as well as times of hypoglycaemia, as found during strenuous exercise. Bolus insulin injections led to hypoglycaemia followed by a recovery to baseline glucose levels over the following two hours, during which time knock-out mice showed no adverse effects on their ability to recover.

The lack of phenotype in the FlEx-Qrfp model, despite an effect of exogenous QRFP on glucose handling, has similarities with the orexigenic effects of QRFP. As before, the reason behind this lack of phenotype in the FlEx-Qrfp model is unclear but it is possible that compensatory mechanisms, in the knock-out mice, may have prevented a phenotype from being observed.

In a similar manner to the adiposity phenotype observed in the FlEx-Qrfp knock-out mice, it does not mean that the direct action of QRFP on insulin secretion at the level of the pancreas (Granata et al. 2014; Egido et al. 2007), precludes an effect of hypothalamic QRFP on glucose via neuronal control. It remains to be determined whether QRFP and its receptors are expressed at high
enough levels in the pancreas to be physiologically relevant. Two groups attempted to identify QRFP expression in a range of peripheral tissues when QRFP was first described (Jiang et al. 2003; Fukusumi et al. 2003) and both showed, at best, very low levels of QRFP in the pancreas. In contrast, there are reports in the literature showing strong expression of both QRFP and Gpr103 in the pancreas (Granata et al. 2014; Prévost et al. 2015), with two groups suggesting QRFP is expressed within the islets. It is concerning that, in order to label their target, antibodies had to be used at low dilutions, which could easily lead to off-target binding. Our results, using RT-PCR support the consensus that there is no expression of QRFP in the pancreas, but it is worth remembering that RNA extraction from pancreatic tissue can be problematic due to the high levels of endogenous RNAse. As such, it is possible that our protocol was not sensitive enough to detect QRFP expression here.

### 5.4.9 Effect of Qrfp knock out on response to endocrine regulators

During normal physiological conditions, energy balance fluctuates throughout the day but is generally kept within limits by acute changes to energy intake or expenditure. One of the main drivers of energy intake is ghrelin, which acts in the brain to promote feeding. Systemic injections of ghrelin increase food intake, primarily through the activation of NPY neurons of the Arc (Dickson & Luckman 1997; Wang et al. 2002). However, our own data, as well as previous work in the literature, shows central ghrelin induces neuronal activation in multiple hypothalamic nuclei (Lawrence et al. 2002a; Toshinai et al. 2003; Olszewski et al. 2003) complementing the distributed expression of its receptor (Zigman et al. 2006). Ghrelin induced a significant increase in c-Fos expression in both the anterior and posterior regions of the Arc, as well as within the LHA between the QRFP neurons. It was observed that ghrelin induced a small yet significant increase in c-Fos expression in QRFP neurons, but it is unlikely that activating less than 3% of QRFP neurons would result in a physiological response. However, electrophysiology recordings suggest that QRFP neurons are activated by ghrelin, as seen by an increase in firing following ghrelin administration (our laboratory, unpublished). These data suggest that QRFP neurons are sensitive to ghrelin in an ex vivo setting. The lack of ghrelin-induced c-Fos expression in QRFP neurons might indicate that they do not respond to ghrelin in vivo. Alternatively, it has been documented previously, that activation of neurons without a concurrent increase in c-Fos expression is possible (Luckman et al. 1994). Firstly, the stimulus may have been sub-threshold to induce c-Fos expression; or the stimulus may have failed to activate key secondary messenger signals that are necessary for c-Fos expression. Whilst c-Fos is the most commonly reported marker of neuronal activation, alternative markers have been investigated such as pERK in the spinal cord (Ji et al. 1999), phosphorylated cAMP responsive element binding (pCREB) in the paraventricular hypothalamic nucleus (PVN) or supraoptic nucleus (Shiromani et al. 1995; Légrádi et al. 2008) and c-Jun (Wisden et al. 1990; Herdegen et al. 1991). To date, there are no known reports in the
literature indicating whether activation of QRFP neurons induces c-Fos expression. It would be interesting to determine whether the neuronal population does signal through c-Fos, or whether an alternative pathway may be more representative of activation in QRFP neurons.

To attempt to clear up the ambiguity of the neuronal response to ghrelin, FlEx-Qrfp knock-out mice were injected with ghrelin to identify whether Qrfp knock out had an effect on the response to ghrelin-induced feeding. Ghrelin increased food intake one, two and four hours after injection in both genotypes, with no statistically significant interaction between genotype and time identified. This likely indicates that QRFP neurons are not involved in mediating the feeding response to ghrelin in vivo. Whilst it is tempting to hypothesise that this indicates a lack of involvement of QRFP in physiological feeding, it is of interest to note that exogenous ghrelin induces c-Fos expression in orexin neurons in the LHA, but has no effect on MCH neurons (Toshinai et al. 2003). As MCH is known to play an important role in the regulation of feeding, it is still possible that QRFP may also be involved but be mediating an alternative aspect of the process to that induced by ghrelin. In this case, a compensatory mechanism may be acting in FlEx-Qrfp knock-out mice leading to a lack of effect in this study.

Similar to ghrelin, leptin is a major regulator of energy balance, although generally over a longer timeframe. Leptin provides an anorectic signal to promote negative energy balance through increased energy expenditure and reduced feeding (Hwa et al. 1997; Doring et al. 1998; Friedman & Halaas 1998; Elmquist et al. 1999; Klok et al. 2007). The current study was designed to investigate whether leptin’s effect to reduce food intake is mediated by QRFP signalling, due to the fact that, similar to ghrelin, the majority of leptin’s effects are mediated through neurons of the hypothalamus (Håkansson et al. 1998; Leshan et al. 2006). Leptin did not induce pSTAT3 expression within QRFP neurons implying that QRFP neurons are unlikely to be targets of leptin signalling.

Injections of a two doses of leptin induced a significant decrease in overnight food intake in both FlEx-Qrfp wild-type and knock-out mice, confirming that loss of QRFP signalling has no attenuating effect on leptin-induced anorexia. Whilst this leptin-induced anorexia occurred quicker in knock-out mice, leptin caused no greater inhibition of feeding in either genotype, suggesting that FlEx-Qrfp knock-out mice are no differently sensitive to leptin than wild-type mice.
5.4.10 Comparison of two Qrfp knock-out mouse models

During the composition of this thesis, Sakurai and colleagues published work on another Qrfp knock-out model (Okamoto et al. 2016). Interestingly, both models involved manipulation of exon 2 of the Qrfp gene. The entire exon is inverted in our FlEx-Qrfp model, whilst in the other model, the coding region of exon 2 was replaced with a GFP sequence and a pgk-Neo cassette (consisting of a phosphoglycerine kinase promoter and a neomycin resistance gene), immediately upstream of the untranslated region of the gene. Importantly, both models have been shown to express no QRFP. For clarity, the knock-out mice from our model will be referred to as FlEx-Qrfp, whilst mice from the other model will be Qrfp/GFP.

The most evident phenotype of our FlEx-Qrfp mouse was its lean phenotype on HED, a result that is complemented in Qrfp/GFP mice, and replicated in both male and female mice in both models. Interestingly, our mice show a more pronounced attenuation of weight gain on HED, likely due to our use of a diet with 60% fat compared with the 32% fat used by the other group. Whilst FlEx-Qrfp mice show no difference in body weight on chow diet, Qrfp/GFP mice are also lean under these conditions as well. It may be important to note that body weight of Qrfp/GFP mice was recorded until 30 weeks of age, with no difference observed until 18 weeks, potentially indicating that this effect is a delayed response, and as our study terminated at 20 weeks it is possible this had not yet occurred in our mice. Importantly, they also showed that the body weight difference even in chow-fed mice was caused by reduced fat mass, although it is unclear at what age this was measured. This work ties in nicely with our hypothesis that loss of QRFP signalling results in defects in fat accumulation, and show that this is not simply an effect of diet, but that Qrfp knock-out also affects fat accumulation often seen in older mice.

It is important to discuss that the Qrfp/GFP mouse was reported to be no different to wild-type controls, in terms of lean mass, in contrast to our own model. Interestingly, in all body weight graphs, the Qrfp/GFP mice are around 5% lighter at six weeks of age, compared to wild-type controls, although no explanation is offered for what may be causing this. It is possible that this is indicative of a small reduction in lean mass, similar with our FlEx-Qrfp mouse, or the differences between the studies may be an indication of a divergence in the models. Alternatively, it is worth noting that the methodology used in each study was different: we employed QMR whilst Sakurai et al used computed tomography, and whilst we cannot comment on the accuracy of either technique for the quantification of lean mass, it is possible that differences may exist.

We described earlier that the lean phenotype of the FlEx-Qrfp mouse may be caused by an increase in lipolytic drive in the animal, and these mice exhibit no deleterious effects on feeding. However, the lean phenotype of the Qrfp/GFP model (Okamoto et al. 2016) was accompanied by a hypophagic phenotype, with knock-out mice consuming 5-6g less than wild-type controls, which
was hypothesised to be the underlying cause of leanness. We can offer no definitive reason why the two knock-out models should exhibit such different phenotypes in regards to feeding.

FlEx-Qrfp mice exhibit reduced activity levels during the dark phase of the light cycle, a similar effect to that observed in orexin knock-out mice (Willie et al. 2003). Whilst the Qrfp/GFP mouse was not less active (in terms of distance moved) during its normal circadian profile, the Sakurai paper did report impairments in the regulation of sleep and wakefulness. In particular, time spent awake was significantly decreased during the first three hours of darkness, with a concomitant increase in non-rapid eye movement (non-REM) sleep. Closer inspection of our dataset reveals that the FlEx-Qrfp mouse hypoactivity in the dark phase is predominantly caused by reduced activity in the first few hours of darkness. It is likely, therefore, that the two Qrfp knock-out models are both exhibiting the same phenotype in terms of arousal and activity, thus supporting our assertion that a primary role of QRFP is in the regulation of wakefulness. In particular, the initial hyperactive phase at the start of darkness is associated with high levels of feeding in mice, and the discovery that mice are less active, due to being less awake during this time, indicates that any feeding phenotype may be as a result of this, rather than the other way around.

In conclusion, these two models both confirm an important role for QRFP in arousal. There is also potential direct effects on body weight, which we hypothesise may be due to an increased lipolytic drive, as supported by previous literature (Mulumba et al. 2015). In contrast, Sakurai and colleagues suggest a lean phenotype is a direct result of hypophagia in their Qrfp/GFP model (Okamoto et al. 2016). It remains to be proven if the orexigenic effects of QRFP are secondary to increased arousal, in a similar manner to orexin, or not. Our work in Chapter 3 highlighted the close proximity of QRFP and orexin neurons, so it is possible that communication between these populations may be a medium for this effect.

**5.4.11 Summary**

- **Qrfp** knock-out mice gain less weight on HED than wild-type littermates
  - Reduced weight gain caused by reduced adiposity
  - No alterations in feeding or basal metabolism in knock-out mice
- Knock-out mice exhibit hypoactivity during the dark phase of the circadian profile
- Responses to hypo- and hyperglycaemia are unaffected in FlEx-Qrfp knock-out mice
- **Qrfp** knock-out mice show no alterations in responses to acute ghrelin or leptin
Chapter 6

Gpr103 Receptor Knock-Out
6.1 Introduction

In a similar manner to orexergic signalling, QRFP acts via two distinct receptor types in rodents: named Gpr103a and Gpr103b. The two orexin receptors (OxR1 and OxR2) are differentially expressed in the brain (Marcus et al. 2001), with the widespread overall expression suggested to contribute to the multifunctional action of orexin. Furthermore, it has been suggested that each receptor type mediates different aspects of orexergic action: OxR2 knock-out mice exhibit a strong narcoleptic phenotype (Willie et al. 2003), akin to that observed in orexin knock-out mice (Chemelli et al. 1999), whilst OxR1 inhibition results in impairments in motivational behaviours such as operant responding (Sharf et al. 2010). The discovery that canine narcolepsy is caused by a mutation in OxR2 seemingly confirmed this difference in function (Lin et al. 1999), but subsequent work has shown that both OxR1 and OxR2 knock-out mice show attenuation of orexin-induced arousal and wakefulness (Mieda et al. 2011).

The two QRFP receptors are also widespread in the rodent brain, with reports in the literature that they also show fairly distinct distribution (Takayasu et al. 2006; Kampe et al. 2006; Bruzzone et al. 2007). However, work by our collaborators suggests that the receptors do overlap in certain brain regions (Eli Lilly, unpublished). Whilst there have been no attempts to elucidate the function of each Gpr103 receptor type in mediating QRFP-induced actions in the brain, a number of studies have investigated their roles in mediating QRFP action directly on peripheral tissues. In particular, Gpr103b is reportedly expressed in white adipose tissue (WAT) and is reported as responsible for mediating QRFP-induced antilipolytic effects in the tissue (Mulumba et al. 2010). On the other hand, Gpr103a expression has been reported in myotubes, with no concurrent expression of Gpr103b, and is suggested to be involved in 26RFa-mediated increases in insulin sensitivity (Allerton & Primeaux 2015).

Our results in Chapter 5 highlighted a number of phenotypic traits of our FlEx-Qrfp mouse, caused by the loss of QRFP signalling. In order to investigate these effects further, we have developed two transgenic models lacking Gpr103a or Gpr103b globally, which will enable us to identify any phenotypic differences in these models, providing evidence on whether they mediate different endogenous QRFP actions. Furthermore, these models will be used to determine whether exogenous QRFP-induced actions in the brain are transduced preferentially by either of the receptor types, focusing on the orexigenic and locomotor responses observed in Chapter 4. Finally, we will cross breed our receptor knock-out models to enable us to identify whether a dual receptor knock-out model exhibits the same phenotype as our FlEx-Qrfp mouse.
6.1.1 Objectives

Investigate the contribution of Gpr103 receptors to body weight and activity phenotypes

In Chapter 5 we identified a lean phenotype in our FlEx-Qrfp knock-out mice, which was accompanied by hypoactivity of the mice during the dark phase. To determine the relative contribution of each receptor to these phenotypes, we will conduct similar experiments to Chapter 5 in each receptor knock-out model, as well as within the dual receptor knock-out line. We will also confirm whether receptor knock-out mice show a similar lack of phenotype with regards to glucose handling and feeding, as described in our FlEx-Qrfp mice.

Determine whether QRFP preferentially acts through either receptor type in the brain

Previously differential attenuation of specific aspects of the response to orexin administration was shown in knock-out mice of either receptor isoform. With this in mind, we aimed to investigate whether QRFP injections act via both receptors equally, by repeating feeding and activity studies from Chapter 4 in our knock-out models. This will provide us with an alternative avenue of study to ascertain the possibility of redundancy existing in the QRFP system.

6.2 Methods

6.2.1 Generation of individual Gpr103 receptor knock-out mice

The Gpr103a and Gpr103b mouse lines were generated by Taconic (Taconic Biosciences, Inc; Köln, Germany) through homologous recombination in embryonic stem cells from a C57Bl/6N genetic background. To create the Gpr103a knock-out model, a targeting vector was generated in which exon 1 of the Gpr103a gene and 400 base pairs of upstream proximal promoter sequence were removed. The Gpr103b knock-out model was created using a targeting vector in which exon 3 of the Gpr103b gene was flanked by modified loxP sites. These targeting vectors contained: long and short homology regions to either the Gpr103a or Gpr103b gene (on the C57Bl/6N background) (including the relevant Gpr103a or Gpr103b modifications respectively); a FRT-flanked puromycin cassette, for positive selection; and a thymidine kinase cassette, for negative selection. Correctly targeted clones, as identified by the aforementioned positive and negative selection pressures, were injected into C57Bl/6N blastocysts for implantation into OF1 pseudo-pregnant females. Offspring expressing the correct gene were selected in a similar manner to the FlEx-Qrfp mouse described in Chapter 5. The resulting Gpr103a model is a constitutive knock-out for the gene, due to the loss of both promotor sequences and exon 1 of the gene. The Gpr103b model produced is a conditional knock-out of the gene, in which Gpr103b can be deleted in a cre-recombinase-dependent manner. Breeding of this mouse line with a global cre-recombinase-expressing mouse caused the deletion of exon 3 of the Gpr103b gene, resulting in the loss of the fourth transmembrane domain, and producing a frame shift in the downstream exons and a premature stop codon in exon 4. It is highly unlikely that any transcribed mRNA would possess a
physiologically relevant function. The *Gpr103a* and *Gpr103b* models described in this Chapter are, therefore, both global knock-outs of either gene. Comprehensive analysis of these models was conducted, prior to the delivery of heterozygous mice to the University of Manchester.

### 6.2.2 Tissue expression of *Gpr103a* and *Gpr103b*

The expression of the *Gpr103a* and *Gpr103b* receptors in peripheral tissues has not previously been fully elucidated. In a similar study to that conducted in Chapter 3 for peripheral *Qfp* expression, *Gpr103a* and *Gpr103b* mutant mice were culled by cervical dislocation and the following tissues dissected: hypothalamus, eye, brown adipose tissue (BAT), liver, muscle and epididymal (eWAT) and inguinal WAT (iWAT). Tissue samples were immediately frozen on dry ice and stored long term at -80°C. As *Gpr103a* knock-out mice still possess the *Gpr103b* gene, the *Gpr103a* knock-out samples were used as wild-type samples for *Gpr103b* expression, and vice versa. Reverse transcription PCR (RT-PCR) was carried out on each tissue to identify expression of each receptor isoform. Agarose gel electrophoresis enabled visualisation of amplified cDNA product bands, and expression of each receptor was confirmed through the presence of a band in the wild-type samples, and concordant absence of a band in the knock-out samples.

### 6.2.3 Effect of *Gpr103a* or *Gpr103b* knock out on body weight

A cohort of male *Gpr103a* knock-out and wild-type littermates (n=15) were maintained on chow diet, from weaning, and body weights measured weekly from six weeks of age, for 14 weeks. Alongside this study, a cohort of male *Gp103b* knock-out and wild-type littermates (n=16) were maintained in identical conditions, and body weights again measured as above.

In a second study, a cohort of male *Gpr103a* (n=12) and *Gpr103b* mice (n=15) were maintained on high-energy diet (HED) from six weeks of age, for 16 weeks. Body weights were again measured weekly throughout the study. One week before culling, when mice had been on HED for 15 weeks, blood glucose levels were measured in all mice. Food was removed from the cages as lights came on, with glucose levels being measured four hours later to avoid effects of recent feeding. A week later, mice were fasted for 24 hours and the study repeated, with glucose readings being made at the same time of day as the above study. At the end of the full period, eWAT and liver were weighed and frozen for relative quantitative real-time PCR (qPCR) analysis.

### 6.2.4 Effect of *Gpr103a* or *Gpr103b* knock out on feeding

The effect of receptor knock-out was measured using separate cohorts of mice from both the *Gpr103a* (n=11) and *Gpr103b* (n=14) lines. Mice were singly housed for four days prior to start of study, and two measures of food intake recorded with mice maintained on regular chow. 24-hour food intake was measured and an average calculated for three consecutive days. Subsequently, cumulative food intake was measured over a week for each mouse.
The same mice were then fasted overnight, for 20 hours to investigate fast-induced refeeding. Food was returned to each mouse, two hours into the light phase, and intake measured after one, two and four hours.

It is currently unknown whether the orexigenic effect of QRFP is mediated by one or other of its receptors or whether both are involved. As a result, a study was instigated to investigate the effect of QRFP on feeding in mice lacking either of the receptor isoforms (Gpr103a n=11, Gpr103b n=11). All mice were injected with QRFP (7.5µg, ICV), or saline, in a crossover study, with mice receiving the other injection a week later. Mice were immediately returned to their home cage following injection and food intake measured for the following one, two and four hours.

6.2.5 Effect of Gpr103a or Gpr103b knock out on activity levels

Due to the previously described effects of exogenous QRFP on activity levels, and a concordant lower activity observed in mice lacking the Qrfp gene, activity levels of both Gpr103a and Gpr103b knock-out mice were measured (Gpr103a n=10, Gpr103b n=13), following three-days acclimation to individual beam-break cages. On three consecutive days, beam breaks were measured in 15 minute time bins and average breaks per hour calculated throughout full 24-hour profiles, to produce a circadian pattern. Subsequently, beam breaks were averaged during the day and night phases, enabling comparisons between genotypes during these different periods.

Two further cohorts of Gpr103a and Gpr103b knock-out mice (Gpr103a n=9, Gpr03b n=9) were used for analysis of activity levels, in order to identify which receptor mediates QRFP-induced hyperlocomotion. Once acclimated to individual beam-break cages, mice were injected with QRFP (7.5µg, ICV) or saline, two hours after lights on. Activity levels were measured for one hour prior to injection and two hours after injection. Beam breaks were recorded, as a measure of activity levels, in five minute bins and total beam crosses calculated in 30 minute intervals.

6.2.6 Generation of a dual receptor knock-out line

In order to investigate the extent to which the function of each of the Gpr103 receptors overlap, the two individual receptor lines were crossed to develop a Gpr103 dual receptor null mouse. Initially this was performed by crossing homozygous knock-out mice from each colony, breeding which resulted in offspring that were heterozygous for each receptor isoform which were then further interbred. However, the infrequency with which mice were born possessing the relevant genotype (namely dual knock-out or dual wild-type), meant that studies were impossible to conduct with this breeding program. Thus, separate homozygous knock-out and wild-type lines were created using heterozygous offspring from the initial crosses. These mice were maintained separately and, as a result, wild-type controls in these experiments were not littermates of the knock-out mice. However, care was taken to ensure that no more than three subsequent generations of each line were allowed to breed before the separate lines were cross-bred and the
dual knock-out and wild-type lines rederived, from the original individual receptor knock-out lines, to minimise genetic drift.

**F₀ cross:** \( Gpr103a \) knock-out \( \times \) \( Gpr103b \) knock-out

**F₀ offspring:** \( Gpr103a^{+/+}::Gpr103b^{+/+} \) dual heterozygous mice

**F₁ cross:** \( Gpr103a^{+/+}::Gpr103b^{+/+} \) offspring crossed together

**F₁ offspring:** \( Gpr103a \) knock-out::\( Gpr103b \) knock-out (inbred as \( Gpr103 \) dual knock-out line)

\( Gpr103a \) wild-type::\( Gpr103b \) wild-type (inbred as \( Gpr103 \) dual wild-type line)

Other genotype combinations (knock-out, wild-type and heterozygous) discarded

### 6.2.7 Effect of \( Gpr103 \) dual knock out on body weight and feeding

Following on from the earlier studies investigating the effect of \( Gpr103a \) and \( Gpr103b \) knock-out on body weight, similar studies were initiated in \( Gpr103 \) dual knock-out mice. Cohorts of male \( Gpr103 \) dual knock-out mice and wild-type controls, were maintained on HED from six weeks of age (n=20), with body weights measured weekly for 14 weeks. Concurrently, female mice from these two lines were maintained on chow and weighed weekly for 14 weeks from six weeks of age (n=22). At the end of both studies, blood glucose levels were measured in \textit{ad libitum}-fed mice, two hours into the light phase in order to reduce the likelihood of recent feeding affecting the readings. Body length was also measured in these mice at the same time point as glucose was measured.

Using a cohort of \( Gpr103 \) dual knock-out mice (n=13), alongside wild-type controls, the effect of dual receptor knock-out on food intake was measured, in mice maintained on chow. Mice were singly housed for four days prior to start of study for acclimation, after which 24-hour food intake was measured over three consecutive days to determine whether knock-out and wild-type mice differed in any way. Food intake was measured at six weeks of age to avoid significant body weight differences from complicating the results.
6.3 Results

6.3.1 Generation of receptor knock-out models
The individual Gpr103 receptor knock-out mice were maintained as heterozygous colonies, with mating of heterozygous siblings providing the full complement of genotypes. Following weaning, all mice were genotyped using the fast DNA extraction protocol detailed in General Methods. A specific trio of primers was designed for each line, enabling the identification of knock-out and wild-type alleles using PCR. Visualisation of the amplification products was performed using agarose gel electrophoresis to produce product bands. The genotype of these mice was verified using qPCR in hypothalamic samples, confirming lack of gene expression in knock-out mice.

6.3.2 Peripheral expression of Gpr103a and Gpr103b
Whilst the expression of mRNAs for the two receptor isoforms, Gpr103a and Gpr103b, has been reported in different parts of the brain, there have been few studies investigating their expression in peripheral tissues. With this in mind, a variety of metabolically relevant tissues were dissected from knock-out mice from both the Gpr103a and Gpr103b knock-out lines. Gpr103a knock-out samples were used as control samples for Gpr103b expression and vice versa. RT-PCR analysis showed strong expression of both Gpr103a and Gpr103b in the hypothalamus, and confirmed the genotype of knock-out mice (Figure 6.1). Peripherally, Gpr103a is expressed in the eye, but was not amplified in any other tissue tested. Gpr103b was amplified solely in the hypothalamic samples, although faint bands in WAT samples may indicate very low level expression in these tissues. RT-PCR, carried out on eWAT from obese mice, however, failed to amplify Gpr103b (data not shown). Finally, RT-PCR conducted using the primers employed by in a previous paper (Mulumba et al. 2010), in which Gpr103b expression was confirmed in WAT, showed amplification in hypothalamic samples from Gpr103b knock-out mice.
Figure 6.1: Expression of Gpr103a and Gpr103b mRNA in peripheral tissues

A: RT-PCR revealed expression of Gpr103a in peripheral tissues in Gpr103a wild-type (I-III) and knock-out (IV-VI) mice. Gpr103a was detected in the eye and hypothalamus as identified by white bands in wild-type lanes and lack of amplification product in knock-out lanes. Gpr103a mRNA was not identified in BAT, liver, skeletal muscle, pancreas, epididymal WAT or inguinal WAT.

B: A second study was completed as above, investigating Gpr103b expression in Gpr103b wild-type (I-III) and knock-out (IV-VI) mice. Gpr103b was amplified in the hypothalamus only.

BAT: Brown adipose tissue; Hypo: hypothalamus; Sk M: skeletal muscle; eWAT: epididymal white adipose tissue; iWAT: inguinal white adipose tissue
6.3.3 Effect of Gpr103a or Gpr103b knock out on body weight

Work in the previous Chapter revealed a phenotype in FlEx-Qrfp mice of lower body weight on HED caused by reduced adiposity. In order to determine whether these effects are mediated primarily by either of the receptor isoforms, mice from both receptor knock-out models were maintained on regular chow, or HED (Error! Reference source not found.). Knock-out mice from both Gpr103a and Gpr103b lines were not different in whole-body mass from their respective wild-type littermates, when maintained on either chow or HED. Mice from the Gpr103b line, maintained on chow, had to be culled at 16 weeks of age, due to fighting. This occurred with both knock-out and wild-type mice and, as such, was not a reflection of the genotype.

Figure 6.2: Effect of Gpr103 knock out on body weight

Top: Body weights of chow-fed Gpr103a (A) and Gpr103b (B) knock-out mice are no different from wild-type littermates (repeated measures two-way ANOVA)

Bottom: Body weights of HED-fed Gpr103a (C) and Gpr103b (D) knock-out mice are no different from wild-type littermates (repeated measures two-way ANOVA)
Immediately prior to the end of the HED growth curves, blood glucose levels were measured four hours into the light phase. Glucose levels of Gpr103a knock-out mice were significantly lower than wild-type littermates, whilst Gpr103b knock-out mice were not different from wild-type mice (Figure 6.3A). Subsequently, following an overnight fast, glucose levels of Gpr103a and Gpr103b knock-out mice were found to be no different from their relevant wild-type littermates (Figure 6.3B).

**Figure 6.3: Effect of Gpr103 knock out on resting glycaemia**

A: Fed glucose levels of Gpr103a knock-out mice were lower than wild-type littermates, but Gpr103b knock-out mice were no different (A) (*p<0.05; unpaired t-test)

B: Fasted blood glucose levels were no different between knock-out and wild-type mice from either the Gpr103a or Gpr103b models (B) (unpaired t-test)

At the completion of the HED study, mice were culled and eWAT and livers dissected. Despite no obvious body weight effect being observed in these mice, eWAT from both Gpr103a and Gpr103b knock-out mice was larger than wild-type controls (Figure 6.4A). Livers from both lines were no different in weight between genotypes (Figure 6.4B).

**Figure 6.4: Effect of Gpr103 knock out on WAT from HED-fed mice**

A: Epididymal WAT was larger in both Gpr103a and Gpr103b knock-out mice compared with wild-type littermates, following HED-induced obesity (*p<0.05, ***p<0.001; unpaired t-test)

B: Liver weight of Gpr103a or Gpr103b knock-out mice was no different to wild-type mice, following HED feeding (unpaired t-test)
6.3.4 Effect of Gpr103a or Gpr103b knock out on feeding

Knock out of Gpr103a or Gpr103b had no effect on food intake, compared with wild-type littermates, when measured as average 24-hour intake (Figure 6.5A-B) or cumulative food intake over six days (Figure 6.5C-D). Furthermore, following a 20-hour overnight fast, neither knock-out model showed any difference in refeeding at any time point measured (Figure 6.5E-F).

The orexigenic effect induced by exogenous QRFP is well documented, but no studies have investigated the receptor isoform responsible for mediating this effect, or whether some level of redundancy exists in the system. In order to investigate this, Gpr103a and Gpr103b knock-out mice were injected with QRFP (7.5µg, ICV) (Figure 6.6). As before, food intake was increased by QRFP injection in wild-type mice. However, Gpr103a and Gpr103b knock-out mice did not respond to QRFP injections, and no increase in feeding was observed at any point after injection.
Figure 6.5: Effect of Gpr103 knock out on feeding

**Top:** Average 24-hour chow intake was no different between knock-out and wild-type mice from either the Gpr103a (A) or Gpr103b (B) line (unpaired t-test)

**Middle:** Cumulative chow intake, over 6 days, was no different in Gpr103a (C) or Gpr103b (D) knock-out mice compared with wild-type littermates (repeated measures two-way ANOVA)

**Bottom:** No differences were evident in chow refeeding following a 20-hour fast, between knock-out and wild-type mice from the Gpr103a (E) or Gpr103b (F) colonies (repeated measures two-way ANOVA)
QRFP injections (7.5µg, ICV) induced feeding in wild-type, but not knock-out mice from the Gpr103a (A) and Gpr103b (B) lines (*p<0.05, **p<0.01, ****p<0.0001; repeated measures two-way ANOVA with Sidak’s post hoc test).

Figure 6.6: Effect of Gpr103 knock out on QRFP-induced food intake
6.3.5 Effect of Gpr103a or Gpr103b knock out on activity levels

To investigate the effect of knock out of either receptor, two separate cohorts of mice, from the Gpr103a and Gpr103b colonies, were habituated to beam break cages (Figure 6.7). Following three days acclimation, average activity levels were measured over four consecutive days. Activity levels were no different between knock-out and wild-type mice from either model.

The effect of knock out of Gpr103a and Gpr103b on acute QRFP-induced activity was measured in separate cohorts (Figure 6.8). As in the experiment above, no differences were observed in activity in the hour prior to injections. Following QRFP injection, an increase in activity levels was observed in wild-type mice in both cohorts. However, QRFP failed to induce significant increases in activity levels in knock-out mice from either cohort.
Figure 6.7: Effect of Gpr103 knock out on locomotor activity

**Top:** Full circadian locomotor profiles, over three days, of Gpr103a (A) and Gpr103b (B) knock-out and wild-type mice (data presented as mean)

**Middle:** Average activity levels were not significantly different between genotypes from either the Gpr103a (C) or Gpr103b (D) lines (repeated measures two-way ANOVA)

**Bottom:** Average activity levels in the first and second halves of the dark phase did not significantly differ between genotypes from either the Gpr103a (E) or Gpr103b (F) lines (repeated measures two-way ANOVA)
Figure 6.8: Effect of Gpr103 knock out on QRFP-induced locomotor activity

Top: Average traces of activity levels before and after QRFP (7.5µg, ICV) or saline injections, made at time 0, in Gpr103a (A) and Gpr103b (B) knock-out and wild-type mice (data presented as mean)

Bottom: Activity levels were no different between genotypes before injections were made. QRFP injections increased activity levels in wild-type, but not knock-out mice from both the Gpr103a (C) or Gpr103b (D) models (**p<0.01; repeated measures two-way ANOVA with Sidak’s post hoc test)
6.3.6 Effect of Gpr103 dual receptor knock out on body weight

The previously reported reduced body weight gain in FlEx-Qrfp mice maintained on HED was not mimicked in either receptor knock-out model. As such, a study was instigated to investigate the effect of loss of both receptors on body weight. As discussed in Chapter 6 methods, male knock-out and wild-type mice used in this study were not littermates, but were age matched. Mice were maintained on HED from six weeks of age and, unexpectedly, Gpr103 dual knock-out mice gained more weight compared with wild-type controls (Figure 6.9). Knock-out mice were significantly heavier than wild-type controls within six weeks of being on HED (12 weeks age). Immediately prior to dissection, blood glucose levels were measured, with no difference identified between knock-out and wild-type, HED-fed mice despite their being overweight. No difference was observed between knock-out and wild-type mice in terms of body length in this study.

Due to the unexpected results of the Gpr103 dual knock-out study above, the effect of dual receptor knock-out on body weight in chow-fed mice was investigated (Figure 6.9), to identify whether this effect was also only observable under metabolic challenge. Due to the aforementioned difficulty obtaining mice of the correct genotype, and the fact that no gender differences had been observed in the FlEx-Qrfp model, female mice were used for this study. Supporting the above results, knock-out mice became significantly overweight from 11 weeks of age, and this difference increased throughout the study. As above, blood glucose levels were measured, along with body length, immediately prior to culling the mice. No differences were observed between wild-type and knock-out mice in terms of blood glucose levels, even though knock-out mice were significantly heavier. A subsequent study revealed no difference in food intake between knock-out and wild-type mice (data not shown), indicating the body weight phenotype was not caused by hyperphagia.
Figure 6.9: Effect of dual Gpr103 knock out on body weight and glycaemia

**Top:** Male Gpr103 dual knock-out mice were overweight compared with wild-type mice on HED (A) whilst female, chow-fed knock-out mice were also significantly overweight (B) (*p<0.05, ***p<0.001, ****p<0.0001; repeated measures two-way ANOVA with Sidak’s post hoc test)

**Bottom:** Blood glucose levels of male, HED-fed or female, chow-fed Gpr103 dual knock-out mice are not different to wild-type littermates (C) (unpaired t-test)
6.4 Discussion

6.4.1 Peripheral expression of *Gpr103a* and *Gpr103b*

Work with the FlEx-Qrfp mouse in the previous Chapter confirmed that the expression of Qrfp mRNA is relatively widespread throughout the body. In contrast, the expression of the two receptors appears to be highly restricted, at least within the tissues investigated here. *Gpr103a* mRNA was only amplified in the hypothalamus and the eye, whilst *Gpr103b* mRNA was only found within the hypothalamus. The results of *Gpr103a* expression in our knock-out models confirmed PCR results in rat where it was described at significant levels only in the brain, eye, adrenal gland and testis (Fukusumi et al. 2003). It is worth noting that, whilst the receptor isoform investigated in this study was not identified, it is presumed to be *Gpr103a* due to the fact that the second receptor isoform was not identified until after this paper was published. Thus, we can have confidence in our current results, that there is significant *Gpr103a* expression in the hypothalamus and eye. *Gpr103a* has also been identified in cultured, differentiated myotubes (Allerton & Primeaux 2015) although our present study, along with the work in the Fukusumi paper, suggest there is no *Gpr103a* mRNA expression in mature skeletal muscle.

Another study identified *Gpr103a* expression in the pancreatic islets (Granata et al. 2014) in contrast to our own work and that of Fukusumi *et al.* It is well known that isolation of RNA from pancreas is difficult due to the high level of RNAse in this tissue, which could be a factor in our inability to amplify mRNA in this tissue, rather than there being a genuine lack of expression here. However, as previously discussed, the paper suggested *Gpr103a* is expressed within islets as observed by immunohistochemistry (Granata *et al.* 2014), although we have failed to amplify receptor mRNA here. Furthermore, it has previously been suggested that the direct effects of QRFP on pancreas must be mediated by a different receptor, possibly NPFF2, due to lack of expression of *Gpr103* in the pancreas (Egido *et al.* 2007).

There is little in the literature regarding the expression of *Gpr103b* in peripheral tissues. One important study identified *Gpr103b* mRNA expression in WAT (Mulumba *et al.* 2010), with no expression of *Gpr103a* detected. Our own studies, however, failed to amplify *Gpr103b* mRNA in two WAT depots. Interestingly, Mulumba and colleagues showed the expression of *Gpr103b* increased in HED-fed, overweight mice. Perhaps, in lean mice as used in our studies, the expression may have been at too low a level to be detectable using our protocol. To address this, our PCR was replicated in diet-induced obese mouse tissue, but this again failed to amplify *Gpr103b* from epididymal WAT. Finally, RT-PCR was conducted on hypothalamic tissue using the primers from the Mulumba paper and, worryingly, amplification was observed from both *Gpr103b* wild-type and knock-out tissue, suggesting their primers may not be specific.

Experiments carried out in collaboration between ourselves and Eli Lilly (not included in this thesis), suggest extensive binding for both *Gpr103a* and *Gpr103b* in different parts of the brain
(unpublished results). Therefore, in summary, our current knowledge suggests that in rodents, Gpr103a is expressed in the brain, eye, adrenal gland and testis, whereas Gpr103b is expressed solely within the brain, with the caveat that low level expression may exist in WAT.

### 6.4.2 Effect of Gpr103a or Gpr103b knock out on body weight

It is unclear whether the two Gpr103 receptor isoforms in rodents exhibit redundancy, or whether they mediate different aspects of QRFP’s physiological function. The discovery that the FlEx-Qrfp knock-out mouse exhibits reduced adiposity, leading to a lean phenotype when fed HED, implies that a major function of QRFP is in fat storage. In order to investigate the role each of the receptor isoforms on this phenotype, growth curves were produced from both genotypic knock-out models. Neither Gpr103a nor Gpr103b knock-out mice showed any changes in body weight compared to wild-type littermates, when maintained on either chow or HED.

It is unclear whether the effects of QRFP on adiposity and body weight are centrally mediated, or a direct effect on WAT. Evidence in the literature implicates Gpr103b, but not Gpr103a, in the QRFP-mediated antilipolytic effect directly on WAT (Mulumba et al. 2010). We failed to detect either Gpr103 type in two WAT depots. However, both receptor knock-out mice showed increased epididymal WAT mass compared with wild-type controls, again suggesting a degree of redundancy in the receptor function. It is interesting that Gpr103b expression is upregulated by obesity only in the eWAT depot, whilst its expression remains unchanged in inguinal and perirenal depots (Mulumba et al. 2010), bearing in mind our concerns over the specificity of these PCRs. It is certainly interesting that eWAT mass is higher in both peptide and receptor knock-out mice, in apparent contrast with whole-body fat mass.

At the culmination of the HED growth curves, blood glucose levels were measured in all mice, under both fasted and non-fasted conditions. Gpr103b knock-out mice were not different from wild-type mice in either condition whilst Gpr103a knock-out mice exhibited reduced glucose levels, but only when non-fasted. The nature of the non-fasted condition means it is possible that compounding factors, such as feeding or activity, may have played a part in this dichotomy of responses, particularly given the small numbers of animals involved in this study. Previous work in our collaboration with Eli Lilly shows that neither receptor knock-out model shows signs of impaired glucose handling (unpublished), suggesting that it is likely the alteration in non-fasted blood glucose levels in Gpr103a knock-out mice is due to confounding factors rather than the genetic manipulation itself.

The lack of an obvious phenotype in either model points to a redundancy between the two receptors, with either capable of mediating the effects of QRFP on fat storage, or, alternatively, indicates that neither receptor is responsible for the adiposity phenotype. It was hoped that the generation of a dual receptor knock-out model may allow us to answer this directly. However, it was impossible to produce sufficient numbers of dual homozygote mice from heterozygote
crossings and, therefore, it was necessary to derive two separate homozygous lines (wild-type and knock-out), which were maintained separately, to produce sufficient experimental animals. The resultant offspring showed a particularly surprising phenotype: dual knock-out mice, both chow-fed females and HED-fed males, were significantly heavier than wild-type controls. We do not wish to draw immediate conclusions from these data, not least due to our concerns over the validity of comparing between divergent homozygous lines from mixed genetic backgrounds, but this unexpected phenotype warrants further investigation. A primary focus should be on activity levels of these mice, given the phenotype observed in FlEx-Qrfp mice.

6.4.3 Effect of Gpr103a or Gpr103b knock out on QRFP-induced behaviour

We have previously discussed the similarities between the QRFP and orexin systems, including the fact that both peptides can signal via two distinct receptors. It is important to identify whether the receptor isoforms regulate different aspects of peptide action, or whether redundancy exists in the system. Previous work in the literature has identified a certain level of difference in the functions of the two orexin receptors. Similar to published reports of Gpr103 expression, orexin receptors are differentially distributed in the brain (Marcus et al. 2001; Sakurai 2007), with the suggestion that this is responsible for the slightly different roles played by each receptor isoform. In this system, OxR2 is strongly associated with the narcoleptic phenotype associated with orexin knock-out (Willie et al. 2003), although OxR1 knock-out mice also exhibit fragmented sleep (Willie et al. 2001). Interestingly, other reports show OxR1 is crucial for orexin-mediated effects on operant responding (Sharf et al. 2010), showing that whilst each receptor may predominantly mediate certain actions, a degree of redundancy exists.

To date, it has not been reported whether central Gpr103 receptors show redundancy in their function. Here we show that neither receptor knock-out model exhibited any alterations in normal or fast-induced food intake: nor was there any statistical difference in spontaneous activity. As seen in the previous Chapter, the FlEx-Qrfp knock-out mice showed no feeding phenotype, but they did show a significant reduction in night-time activity. This data confirms a certain degree of redundancy in QRFP signalling as expression of either receptor isoform is sufficient to maintain normal activity levels. As a further development of this study, ICV-injected QRFP failed to induce feeding or hyperactivity in either receptor knock-out mouse. The implications of these studies appear somewhat confusing: expression of either receptor is sufficient to maintain normal physiology, but both receptors are required to transduce the actions of exogenous QRFP.

It is difficult to reconcile the apparent redundancy identified here, with the differential receptor expression reported in the literature, in both mouse (Takayasu et al. 2006) and rat brain (Kampe et al. 2006; Fukusumi et al. 2003). Conversely, work by our collaborators, using the receptor knock-out models described herein, has shown that, at least in certain areas like the ventromedial
(VMN) and dorsomedial hypothalamic nuclei (DMN), there are relatively high levels of crossover in receptor expression (Eli Lilly, unpublished). The fact that this work was carried out in receptor knock-out models removes the possibility of cross-reactivity, potentially indicating that these results may be more reliable. Interestingly, both receptor isoforms are expressed in the raphé nuclei and the locus coeruleus (LC) in rats (Kampe et al. 2006; Fukusumi et al. 2003), both regions of which are involved in locomotor behaviour. No work has been published regarding the potential for functional dimerisation between the two Gpr103 receptor isoforms, but it is generally appreciated that dimerisation is an important aspect of signal transduction in some GPCRs. If the two Gpr103 receptor isoforms do form functional heterodimers in vivo, this could provide an explanation for the present results, although this would not be supported by the literature indicating differential expression. However, evidence has been presented in the literature that human Gpr103 can form functional heterodimers with both orexin receptors (Davies et al. 2015), proving that Gpr103 can undergo dimerisation, and raising the possibility that this may occur between the two Gpr103 types.

On the other hand, if the literature is correct, and the receptor isoforms are differentially expressed in the brain, this raises the potential for them to mediate different aspects of the response to QRFP. Returning to orexin signalling, similar to our QRFP studies, orexin administration in OxR1 or OxR2 reveals that both models show attenuated response (Mieda et al. 2011), indicating that both are required for normal signalling. As discussed, orexin receptors are differentially expressed in the brain and it has been demonstrated that two populations in particular are crucial for orexin-mediated activity: OxR1-expressing noradrenergic neurons of the LC and OxR2-expressing histaminergic neurons of the tuberomammillary nucleus (TMN) (Mieda et al. 2011). It is, therefore, possible that the Gpr103 receptors may similarly be expressed differentially and mediate different aspects of QRFP action. Furthermore, this could explain the redundancy, identified by the lack of activity phenotype in receptor knock-out mice, as each receptor could mediate a different part of the response which may not be strong enough alone to produce a phenotype. The use of electroencephalography could permit the investigation of sleep and wake states in receptor knock-out mice to elucidate whether they do, in fact, show differences in different aspects of wakefulness.

6.4.4 Summary

- Gpr103 receptors exhibit a high degree of redundancy
  - Knock-out mice show normal body weight, food intake and locomotor activity
- Both chow- and HED-fed dual receptor knock-out mice are obese
- QRFP-induced feeding and locomotor activity are lost in both receptor knock-out models
Chapter 7

GENERAL DISCUSSION
7.1 Overview of thesis

The data presented in this thesis have provided new insights into the function of QRFP in normal physiology, and we have successfully built upon the literature to help guide future work in this area. Firstly, the development of novel transgenic models has enabled us to provide the most in depth characterisation of QRFP neurons to date. We have described the distribution of these neurons and investigated their projections, with work beginning to identify specific target nuclei of QRFP neurons. This work, coupled with insights from the literature, has facilitated the development of hypotheses regarding the physiological functions of QRFP.

A major part of this thesis has involved conducting experiments to elucidate the phenotype of our novel FlEx-Qrfp mouse. We have uncovered a lean phenotype in this model during maintenance on HED, which, according to our data, is not associated with alterations in feeding, thermogenesis or energy expenditure, but may be due to an increase in lipolysis in white adipose tissue (WAT). Furthermore, we have produced data that may question the physiological role of QRFP in feeding regulation. Coupled with the lean phenotype, we have also uncovered a hypoactive phenotype in the FlEx-Qrfp mouse, drawing parallels with the orexin system. Linking this phenotype with our acute studies of exogenous peptide-induced hyperlocomotion, similar work in the literature, and our immunohistochemistry work, has provided us with strong evidence for an important role for QRFP in the control of wakefulness and arousal.

Finally, our work in receptor knock-out mice has concluded that a high degree of redundancy exists in the QRFP system, with the detection of no phenotype in either model, and no differences in their responses to exogenous peptide. Counterintuitively, the development of a dual receptor knock-out model produced a mouse exhibiting obesity, a result which contradicts the lean phenotype of our FlEx-Qrfp mice.

The following general discussion looks to combine these various results into cogent hypotheses regarding the role(s) of QRFP. Ideas are also developed regarding the possible discrepancies between central and peripheral effects of QRFP and whether our data, combined with the literature, can shed light on which of these is of most physiological relevance.

7.2 QRFP expression

The discovery of QRFP/26RFa in 2003 introduced a new member of the RFamide peptide family (Chartrel et al. 2003; Fukusumi et al. 2003; Jiang et al. 2003). An important aspect of these papers was the identification of QRFP distribution, using banks of tissues to amplify Qrfp mRNA, and in situ hybridisation histology to investigate distribution within the brain. Whilst the reported expression in peripheral tissues differed between studies, high expression in the brain was a common feature, and was shown to be restricted to the hypothalamus. These early studies pronounced QRFP as being localised solely within the ventromedial hypothalamic nucleus (VMN)
and lateral hypothalamic area (LHA) (Chartrel et al. 2003), or the arcuate nucleus (Arc) (Fukusumi et al. 2003). However, three years later, a more in-depth analysis showed similar distribution of QRFP neurons to that reported in this thesis (Takayasu et al. 2006). The generation of our transgenic Qrfp-cre model has allowed us to image the QRFP population in much greater detail than has previously been reported, and our immunohistochemistry mapping revealed interesting results: the QRFP neurons seem to surround the VMN, without entering this nucleus. Thus, neurons are found within the LHA, periventricular hypothalamus (PeVH), the tuber cinereum area and dorsomedial hypothalamic nucleus (DMN), with most lying outside the classically defined nuclei. We suggest that, due to the close proximity of the QRFP population to the VMN, previous studies may have mistakenly identified QRFP within the nucleus, which our counter-staining with tyrosine hydroxylase (TH) disproves. It is difficult, therefore, to define their location anatomically based on classic hypothalamic nuclear structure, but perhaps most illustratively, these neurons are best described as occupying the peri-VMN area.

The significance of this expression profile is currently unclear, although two possible conclusions present themselves. On the one hand, it is possible that the QRFP neurons are so placed to facilitate an interaction with the neurons of the VMN, as they are ideally situated for reciprocal connections. The importance of the VMN in sensing the nutritional status of an animal could implicate QRFP in this process as well. On the other hand, the critical nature of the VMN may mean that, as it increased in size during the evolution of its function, the QRFP population was simply dispersed around the outside of this nucleus, so as not to interfere with VMN activity. Previous literature has suggested high levels of 26RFa binding in the VMN, along with expression of Gpr103 mRNA (Bruzzone et al. 2007), which could point to the former explanation: that QRFP neurons innervate and modulate VMN function. However, our data presented here has failed to identify QRFP fibres within the VMN. Whilst our study could not conclusively identify axon terminals, it was clear that the majority of QRFP fibres project around the ventral and lateral extents of the VMN, rather than into its midst.

### 7.3 How does QRFP distribution contribute to functionality

We are unsure as to whether the QRFP population can be divided into smaller sub-populations with different functions. In the present work we have investigated the possibility of sub-populations existing in terms of their co-expression of other neurotransmitters; their differential localisation; or their projection sites. We have been unable to immunohistochemically identify co-expressed neurotransmitters in QRFP neurons, confirming that they are not a sub-population of other previously characterised neurons, but PCR-based work reveals that both glutamatergic and GABAergic QRFP neurons exist. This methodology could not separate these sub-populations anatomically, but two studies in the literature, using RNA-based methodology, have recently investigated this (Romanov et al. 2016; Campbell et al. 2017). Firstly, Romanov et al. reported
that the PeVH QRFP neurons are glutamatergic, although this is based on very low numbers of neurons. We also have questions over their methodology, due to their suggestion that QRFP and orexin co-localise in some neurons, which is not backed up by our data or the literature (Okamoto et al. 2016). Furthermore, orexin neurons are not found within the PeVH as investigated in the Romanov work. More recently, Campbell and colleagues demonstrated that QRFP neurons around the Arc are also glutamatergic. It is possible that further analysis could reveal an anatomical divergence in glutamatergic and GABAergic QRFP neurons, warranting further investigation.

It can be seen that QRFP neurons in different areas do exhibit different morphological characteristics: the more laterally located neurons appear to be larger than those ventral to the DMN, with more prominent fibres, although this could simply be a factor of their location rather than an indication of different functionality. Previous attempts have been made to distinguish two sub-populations of orexin neurons, based on their location relative to the fornix (Harris & Aston-Jones 2006), although this has largely fallen out of favour. Finally, our retrograde tracing studies also failed to distinguish sub-populations, with no obvious distinction in the location of neurons projecting to the LC, although investigation of other target regions is warranted to confirm this.

Perhaps, therefore, the expression of the Gpr103 receptor types may provide a better avenue to identify how the different roles of QRFP may be mediated. As discussed in Chapter 6, Gpr103 receptor distribution in the brain is reported to be without much overlap, which could mean that the different actions of QRFP could be mediated by the different receptor types. This system was proposed for orexin, wherein OxR2 receptors are suggested to primarily be responsible for the narcoleptic phenotype (Lin et al. 1999; Willie et al. 2003) and OxR1 receptors are important for motivated behaviours (Sharf et al. 2010), possibly leading to them contributing more to the orexigenic action. Our data from receptor knock-out models, however, does not seem to support a similar theory for QRFP signalling, due to the lack of a phenotype in either model. Neither Gpr103a nor Gpr103b knock-out mice exhibit a body weight or arousal phenotype, suggesting a degree of redundancy, whilst mice lacking either of the receptor types show attenuated orexigenic and locomotor responses to QRFP. This is difficult to reconcile with our lack of phenotype in these models but could point towards dimerisation with other receptors.

### 7.4 Comparison of QRFP and orexin

The similarities between the QRFP and orexin signalling systems were highlighted earlier in this thesis. This similarity was used to help guide our own experiments with QRFP, to determine whether the similarities extended to the function of the two peptides. Importantly, we have definitively proven that QRFP and orexin neurons are discrete populations, although the observation of dense QRFPergic fibres passing between the orexin neurons means that investigation of potential cross-talk by these neurons is warranted. It is of interest that QRFP-
induced feeding is not attenuated in orexin knock-out mice (Takayasu et al. 2006), suggesting that this action does not involve interaction with orexin neurons. However, as discussed below, the relevance of this QRFP-induced feeding to normal physiology is unclear and no one has yet determined whether other aspects of exogenous QRFP action are inhibited in orexin knock-out mice, or whether loss of QRFP signalling attenuates orexin-induced actions.

One of the more interesting results from our work has been the discovery that, whilst administration of exogenous QRFP causes an orexigenic response, it seems that QRFP neurons themselves are not obviously orexigenic, as evidenced by a lack of feeding phenotype in our FlEx-Qrfp knock-out mice. Whilst we remain cautious that compensatory mechanisms within these mice may have prevented this phenotype from being observed, the overwhelming lack of effect on this endpoint is a key difference between QRFP and orexin. Whilst, orexin’s feeding action is postulated to be secondary to changes in the arousal of animals, orexin knock-out mice are still hypophagic (Hara et al. 2001). It seems possible, therefore, that QRFP neurons may not play a physiologically relevant role in the regulation of food intake; the fact that our FlEx-Qrfp knock-out mice exhibit other phenotypes without a feeding phenotype suggests that, even if QRFP is involved in feeding regulation, it is unlikely to represent the primary function for this peptide. However, the recent identification of a hypophagic phenotype in a Qrfp/GFP model complicates matters (Okamoto et al. 2016), as discussed in Chapter 5. Despite the literature showing an increase in Qrfp mRNA in fasted mouse hypothalamus (Takayasu et al. 2006), suggesting an upregulation of QRFP signalling in this state, this upregulation was much less pronounced than that observed with Npy in the same state. An alternative explanation is that fasted mice also show increased arousal due to increased food seeking behaviour, which might explain the increase in Qrfp mRNA. To date, we have shown that QRFP neurons do not express c-Fos, the frequently used marker of neuronal activation, following an overnight fast or ghrelin injection, nor do they express pSTAT3 following leptin administration. We tentatively suggest, therefore, that QRFP neuronal activity is not regulated by acute satiety or hunger signals. However, a major caveat with functional activity experiments must be addressed: not all neurons express c-Fos upon activation (Luckman et al. 1994), meaning that our neurons could be responding but we are not using the correct method of detection. It may be important that recent unpublished data from our lab shows that ghrelin increases QRFP neuron firing rate in in vitro electrophysiology experiments, but the translatability of this to an in vivo function is currently unknown, particularly given the lack of effect on ghrelin responsiveness in FlEx-Qrfp knock-out mice.

Published work has shown that QRFP is capable of increasing arousal (Takayasu et al. 2006), similar to orexin. We have built on this literature, in our own studies presented here, to show that intracerebroventricular (ICV) QRFP can promote arousal with both day- and night-time injections. The primary function of orexin was the topic of debate for many years, chiefly due to the inherent
difficulty of separating arousal and feeding. However, the detection of a narcoleptic/cataplectic phenotype in mice with deficient orexin/orexin receptor-2 signalling seems to have solved this conundrum (Lin et al. 1999; Willie et al. 2003). We have been guided by this work to try and tackle this problem in regards to QRFP-dependent actions, but have been similarly unable to differentiate categorically between exogenous peptide-induced feeding and locomotor behaviour. However, whilst both locomotion and feeding are stimulated by ICV QRFP, our FlEx-Qrfp knock-out model exhibits hypolocomotion without a concurrent feeding phenotype. Furthermore, the Qrfp/GFP model showed reduced wakefulness and increased non-rapid eye movement (non-REM) sleep (Okamoto et al. 2016), in much the same manner as the orexin models. It is our hypothesis, therefore, that a primary role of QRFP is to modulate arousal in a similar fashion to orexin, and feeding is induced as a consequence of this. This hypothesis is backed up by the detection of QRFP neuronal fibres in regions of the brain commonly associated with arousal, that are also innervated by orexin fibres: in particular, fibres within the locus coeruleus (LC), tuberomammillary nucleus (TMN), dorsal raphé nucleus (DR) and ventral tegmental area (VTA) could be important in promoting wakefulness. Furthermore, previous literature confirms the existence of 26RFa binding sites, and Gpr103 expression, in these nuclei (Kampe et al. 2006; Bruzzone et al. 2007). Our retrograde tracing in the LC confirmed that QRFP neurons do project to, and innervate, this nucleus. Interestingly, orexin neurons activate noradrenergic neurons within the LC (Hagan et al. 1999a), histaminergic neurons of the TMN (Eriksson et al. 2001), serotonergic neurons of the DR (Brown et al. 2001) and dopaminergic neurons of the VTA (Korotkova et al. 2003), so it seems likely that, if QRFP is modulating arousal, they may similarly innervate these neurons. Further tracing work should be directed to address these regions and attempt to determine whether QRFP neurons do interact with similar targets.

Orexin specifically promotes wakefulness and decreases both REM and non-REM sleep (Willie et al. 2003). Whilst our studies demonstrate a decrease in night time activity in FlEx-Qrfp knock-out mice, they were not refined enough to provide more detailed analysis of the role of QRFP in the sleep-wake cycle. Subsequently, increased non-REM sleep and decreased wakefulness were reported in the Qrfp/GFP mouse (Okamoto et al. 2016), notably during the first few hours of the dark period. As mentioned already, QRFP neurons do not appear to be activated in response to homeostatic signals of hunger, but, if we are correct in our hypothesis that QRFP neurons are actually involved in arousal, this may not be surprising.

7.5 Endocrine functions of QRFP

In this thesis, a number of potentially separate functions of QRFP have been discussed: the promotion of arousal (Takayasu et al. 2006) (Chapter 4); an antilipolytic effect altering whole-body adiposity (Mulumba et al. 2010; Mulumba et al. 2015) (Chapter 5); and an influence on insulin secretion (Granata et al. 2014) (Chapter 4). The data presented herein strongly supports
the hypothesis that QRFP neurons can affect arousal, whilst it is still open to debate whether other functions of QRFP are centrally mediated or caused by direct action on peripheral tissues.

7.5.1 QRFP-mediated effects on adiposity

Alongside QRFP’s newly suggested role in regulating arousal, our FlEx-Qrfp knock-out mice also exhibit another striking phenotype: a reduction in diet-induced weight gain, which we suggest may be being caused by an increased lipolytic drive. These mice are leaner than wild-type littermates when fed HED, but not chow, which is hypothesised to be a result of increased hydrolysis of triglyceride. Higher triglyceride breakdown is supported by an increase in expression of Hsl and Atgl in both WAT and liver which, whilst not proof of increased enzymatic activity, does indicates enhanced activity in the lipolysis pathway. The Qrfp/GFP model also exhibited reduced body weight and fat mass when maintained on chow diet (Okamoto et al. 2016), although the longer duration of that study potentially explains this difference: C57Bl/6J mice exhibit late-onset obesity even on chow diet. This means it is possible that the reduced body weight in this model was due to reduced fat accumulation, in a similar manner to HED-induced obesity. Currently, strong arguments could be made for either a central effect of QRFP, or a direct action in peripheral tissues.

On the one side of the argument is data from a group working on in vitro and ex vivo samples that show a direct effect of QRFP in these preparations, through activation of Gpr103b receptors (Mulumba et al. 2010). This group have shown that QRFP inhibits lipolysis in WAT, specifically by inhibiting PKA phosphorylation of Hsl and the translocation of caveolin 1 to the lipid droplet (Mulumba et al. 2015). Importantly, they also reported that Qrfp mRNA in WAT is reduced in obesity, whilst Gpr103b expression is elevated, which suggests a paracrine role for QRFP.

However, end-point RT-PCR in our own mouse receptor models has failed to amplify Gpr103b in WAT of lean or obese mice. Crucially, our studies were designed to enable comparisons between wild-type and receptor knock-out mice in order to confirm the specificity of our primers. When we tested the primers used by the Mulumba group, in our knock-out model, we still obtained an amplification product, suggesting that their primers may not be specific. It may be important that Gpr103 and Npfl receptors share around 40% homology. Furthermore, whilst we, and others previously (Takayasu et al. 2006; Fukusumi et al. 2003), have detected low level Qrfp mRNA in WAT, it is unclear whether this is translated to protein at levels high enough to be of physiological relevance.

A further complication to this direct action of QRFP is the discovery that our HED-fed FlEx-Qrfp knock-out mice also have increased Atgl expression in the liver. Our RT-PCR study revealed low level Qrfp expression in the liver, but no expression of either Gpr103 receptor type. This Qrfp expression is backed up in some literature (Fukusumi et al. 2003; Jiang et al. 2003; Takayasu et al. 2006), though recently this has been contradicted (Prévost et al. 2015). Studies investigating
direct QRFP effects on the liver or isolated hepatocytes are lacking, but the observation that lipolysis appears to be up regulated in other tissues aside from the WAT confirms this is not simply a tissue-specific effect. It is likely, therefore, that if QRFP is causing an antilipolytic effect through direct action on peripheral tissues, there must be a co-ordinated peripheral response, potentially caused by circulating QRFP, which, incidentally has been reported by one group (Galusca et al. 2012; Prévost et al. 2015). Interestingly, that group also reported that 26RFa, produced in the gut, increases secretion of insulin, which exerts a strong antilipolytic action. It is possible that the increase in lipolytic gene expression in our FlEx-Qrfp knock-out mice could be caused by a reduction in insulin’s antilipolytic action, due to either reduced secretion or sensitivity caused by loss of QRFP signalling. However, this would not explain the results observed in in vitro studies (Mulumba et al. 2010). The potential implications of QRFP-induced insulin secretion will be discussed later.

Alternatively, an antilipolytic action suggested by our Flex-Qrfp model, could be mediated through central signalling pathways, leading to a general increase in peripheral lipolytic drive. Of particular note, projections of the sympathetic nervous system can stimulate lipolysis in WAT via adrenergic signalling (Peirce et al. 2014; Bartness et al. 2010), and stimulation of this neuronal input has been shown to be sufficient to induce lipolysis in WAT (Zeng et al. 2015). We do not know whether QRFP neurons activate sympathetic signalling, but fibres are present in regions such as the nucleus of the solitary tract (NTS), parabrachial nucleus (PBN), raphé pallidus (RPa) and periaqueductal grey (PAG), all of which are important in sympathetic signalling (Bamshad et al. 1998). Furthermore, 26RFa binding sites and Gpr103 expression are also found in these areas (Kampe et al. 2006; Bruzzone et al. 2007), providing anatomical basis that functional connections could exist. It may also be important that projections from the VMN and LHA play key roles in regulating both adipose and liver metabolism (Shimazu 1981); with QRFP neurons ideally placed to be involved in these pathways. In particular, pseudorabies virus (PRV) tracing from WAT depots in rats revealed that only a few neurons in the ventromedial hypothalamus were connected to the sympathetic control of WAT, and these were located around the periphery of the VMN, much as QRFP neurons are (Bamshad et al. 1998). A final observation of potential significance is that dense QRFP fibres can be observed passing through the hindbrain into the spinal column. Their destination cannot be identified from our present studies, but 26RFa binding sites and strong Gpr103 expression is reported in the dorsal and ventral horns of the spinal column (Bruzzone et al. 2007), leading to speculation that QRFP may directly innervate the intermediolateral column (IML), from which the majority of preganglionic sympathetic neurons originate.

An important study investigating the effects of chronic QRFP administration may also lend support to the idea of a centrally mediated effect of QRFP on lipolytic signalling (Moriya et al. 2006). This study administered QRFP chronically via the ICV route, over two weeks, and showed
that QRFP induced hyperphagia and obesity in both chow- and HED-fed mice. Subsequently, they confirmed that this obesity was coupled to elevated fat content in the mice. Interestingly, when QRFP-dosed mice fed HED were pair fed to vehicle controls, no difference in body weight was observed but mice still exhibited increased body fat, suggesting that central administration of QRFP is sufficient to cause fat accumulation independent of feeding. It may also be important that the pair-fed mice were hyperinsulinaemic, despite exhibiting normoglycaemia, which will be further discussed below. In conclusion, central QRFP may be involved in the regulation of peripheral lipolysis, speculatively through interaction with the sympathetic nervous system.

7.5.2 QRFP-mediated effects on insulin secretion

As with the lipolysis results discussed above, the action of QRFP on glucose handling could be mediated centrally or directly in the periphery. A strong argument can be made for a peripherally mediated role for QRFP in glucose handling, with \textit{in vitro} (Granata \textit{et al.} 2014), \textit{ex vivo} (Egido \textit{et al.} 2007) and \textit{in vivo} (Prévost \textit{et al.} 2015) protocols seeming to confirm this. Despite reporting opposing actions of QRFP and 26RFa on insulin secretion, the group responsible for these studies have subsequently suggested that this is likely due to 26RFa acting through NPFF2 receptors (Egido \textit{et al.} 2007; Granata \textit{et al.} 2014; Prévost \textit{et al.} 2015). In particular, the Prévost group discovered that intraperitoneal (ip) injection of 26RFa increased insulin secretion and caused a reduction in the glucose excursion during an ip glucose tolerance test in mice. Importantly, they also demonstrated expression of \textit{Gpr103} mRNA in liver, WAT, muscle and pancreas, although they failed to distinguish which receptor isoform they were investigating. Furthermore, these data are not supported by previous literature, or our own results (Fukusumi \textit{et al.} 2003; Jiang \textit{et al.} 2003; Takayasu \textit{et al.} 2006). The Prévost paper also revealed an increase in 26RFa in the circulation following oral glucose loading, with the suggestion that this is released from the intestines. Consequently, the response was not observed if glucose was administered intravenously, which the investigators took to mean that 26RFa was playing an incretin role (Prévost \textit{et al.} 2015). As mentioned earlier, only two papers have reported 26RFa in circulation, both of which have been conducted by the same group (Galusca \textit{et al.} 2012; Prévost \textit{et al.} 2015), using radioimmunoassay and a 26RFa antibody. It also seems contradictory that the Prévost paper reported increased 26RFa in the circulation in obesity, whilst previously \textit{Qrfp} was shown to be reduced in obese WAT (Mulumba \textit{et al.} 2010) and \textit{anorexia nervosa} patients also exhibit increased circulating 26RFa (Galusca \textit{et al.} 2012). It is of potential importance that there are still questions over the relevance of 26RFa in rodent physiology (see Chapter 1).

Our data (Chapter 4) clearly shows that centrally injected QRFP and 26RFa can both reduce the initial peak in glucose levels during an oral glucose tolerance test (OGTT). It may be important to note that we observed a subsequent increase in glucose levels in the latter half of our OGTT, although the effects of QRFP are relatively short-lived, so care should be taken in the
interpretation of this. Whilst the initial effect in our work complements the effect observed in the Prévost work, our route of dosing is unlikely to raise blood concentrations of QRFP/26RFa, presumably ruling out a direct peripheral effect. As discussed earlier, the actions of the LHA and VMN on pancreatic function are well-documented (Shimazu et al. 1966; Frohman et al. 1967; de Jong et al. 1977; Bereiter et al. 1981). Neurons of these hypothalamic nuclei affect pancreatic secretion through interaction with both the sympathetic and parasympathetic nervous systems, via connections in the IML and dorsal motor nucleus of the vagus (DMV), respectively (Laughton & Powley 1987; Buijs et al. 2001; Mussa & Verberne 2013). The detection of QRFP fibres in these areas (Chapter 3) provides a basis for central effects of QRFP on pancreatic secretion, which is supported by our in vivo studies, but how QRFP may function centrally is unclear. Currently it is unknown whether the centrally-induced effects reported here, or the peripheral effects in the literature are most physiologically relevant, or whether a combination of both exists. To summarise, we are unable to conclude whether QRFP-dependent effects on glucose are centrally and/or peripherally mediated, with data showing that both routes are capable of stimulating changes in glucose handling.

Complicating matters are our results showing a lack of impact of Qrfp knock-out on glucose levels in an OGTT, or following bolus insulin injection, suggesting that either compensatory mechanisms have prevented a phenotype being observed, or that the effects of exogenous peptide administration do not translate to an action of endogenous QRFP. Furthermore, QRFP is unable to increase glucose uptake in skeletal muscle in vitro, which is the largest insulin-sensitive tissue (Allerton & Primeaux 2015). The fact that 26RFa caused effects in this study, whilst QRFP did not, is suggestive of off target effects of 26RFa (possibly on NPFF2 receptors) similar to previous work (Granata et al. 2014).

**7.6 Mechanism for centrally mediated effects of QRFP**

Whilst speculative, the following is a potential working hypothesis for future study to test (Figure 7.1). Since they surround the anterior half of the VMN, QRFP neurons are ideally placed to modulate, or be modulated by, neurons within the VMN. It is well established that the VMN is critical for gathering information about the nutritional status of the animal, with neurons sensitive to insulin (Oomura & Kita 1981), leptin (Irani et al. 2008), glucose (Burdakov et al. 2005) and fatty acids (Le Foll et al. 2013). My hypothesis is that the QRFP neurons receive input from these nutrient-sensitive neurons, or possibly are nutrient sensing in their own right. The detection of QRFP neuron fibres in regions classically associated with sympathetic output allows us to speculate that QRFP neurons may interact with the sympathetic nervous system. The sympathetic nervous system is known to promote lipolysis in WAT, and inhibit insulin secretion in the pancreas, amongst other effects. Therefore, if activation of QRFP signalling caused inhibition of sympathetic output, this could have a major influence on peripheral metabolic processes.
If QRFP neuronal activity ordinarily inhibits sympathetic output to WAT, thereby reducing lipolysis, the loss of this signal in our FlEx-Qrfp mouse could result in increased lipolysis, causing the lean phenotype observed. The upregulation of a number of genes involved in adiposity regulation, in multiple tissues, may support this type of centrally-mediated control, which is likely only achievable through central regulation or physiologically relevant levels of QRFP in circulation. Whilst there is evidence of QRFP in circulation (Prévost et al. 2015), further work needs to be carried out to verify this, and there is no reason why peripheral actions of QRFP would preclude central regulation. Furthermore, chronic central administration of QRFP results in obesity, and more specifically increased fat mass (Moriya et al. 2006), which could be being caused by a QRFP-induced inhibition of sympathetic output inhibiting lipolysis.

Building on this model, central action of QRFP on the autonomic nervous system may also be responsible for the glucose handling actions reported herein. The sympathetic nervous system is known to inhibit the secretion of insulin, to enhance the “fight-or-flight” response. The hypothesised inhibitory actions of QRFP neurons on sympathetic output could, therefore, result in increased insulin secretion due to disinhibition of sympathetic action on beta cells (Yajima et al. 2001). In our OGTT studies, this may have manifested itself as a blunting in the initial peak of glucose excursion (Chapter 4). The fact that the FlEx-Qrfp mice do not concurrently exhibit altered glucose handling could implicate compensatory mechanisms, or that supra-physiological doses of QRFP may have induced a non-physiological inhibition of the sympathetic nervous system.
Limitations and future directions

The maintenance of energy homeostasis requires co-ordinated actions of multiple tissues throughout the body, along with input from the brain, in order to regulate a multitude of processes. There are two primary methods used to study the roles of specific peptides in the maintenance of this delicate balance: investigating the effects of administration of exogenous peptide, or the effects of genetic knock-out of the peptide on normal metabolic processes. Unfortunately, both techniques have their drawbacks, meaning that neither, in isolation, can provide definitive proof of the function of a particular peptide. In this thesis, we have tried to combine both approaches to support our conclusions, providing a basis for the hypotheses we
have formed regarding the function of QRFP. However, there still remain unanswered questions which our studies were unable to address, providing ample avenues for future study.

One of the main questions we have attempted to address in this thesis, is the primary role of QRFP in normal physiological processes. Whilst is clear that administration of exogenous QRFP results in a strong orexigenic response, a number of our results have demonstrated that feeding may be a secondary response to QRFP. Our FlEx-Qrfp model shows no sign of reduced feeding, and we have similarly failed to show that QRFP neuronal activation is regulated by hunger. In fact, our results demonstrate that loss of QRFP signalling has no effect on normal or hunger-induced feeding, and no effect on the response to leptin or ghrelin. However, it has not been possible to conduct an exhaustive investigation of all aspects of feeding behaviour. In particular, it is accepted that there are a number of steps in the feeding response, and neuropeptides such as orexin and MCH actually influence different aspects of this behaviour. It is possible, therefore, that QRFP may have an effect on an aspect of feeding that we have not been able to investigate here. Given that our knock-out model has provided evidence that QRFP may play a role in wakefulness, akin to that of orexin, food seeking and the initiation of feeding may represent a logical avenue for future study. Whilst our studies have attempted to shed some light on this, we cannot conclude for certain that QRFP-induced activity is not intrinsically linked to food-seeking behaviour. Furthermore, there appears to be a high degree of redundancy in orexigenic signals in the body, and by disrupting the Qrfp gene, it is possible that other pathways in the FlEx-Qrfp mice may have adapted to compensate for the lack of QRFP signalling.

Nevertheless, the evident disturbance of activity levels in our FlEx-Qrfp mice, suggests that any role in wakefulness may be more important than a possible feeding effect; increases in activity following QRFP injections are supported by hypoactivity in the knock-out mouse. Both orexin and MCH are also critically active in the sleep/wake cycle, alongside their effects on feeding. Orexin neurons, in particular, show highest levels of activation during the early waking period, and previous literature reports that Qrfp/GFP mice exhibit fragmented wakefulness in the early active phase (Okamoto et al. 2016). Whilst not at this technical level, our data support a reduction in activity in the early waking phase, indicating a primary role for QRFP may be consolidating wakefulness.

However, this highlights a further limitation of our current knowledge of the QRFP system, in that we have, as yet, been unable to identify any stimulus capable of activating the QRFP neurons. Primarily, we have used c-Fos as our marker of neuronal activation, which is the most frequently used method. However, it is known that not all neurons signal via this pathway, and not all stimuli induce c-Fos expression (Luckman et al. 1994), and it is possible, therefore, that investigating alternative pathways may resolve this issue.
Finally, very little is currently known about the signalling pathways downstream of the Gpr103 receptors. A number of studies have highlighted changes in intracellular signalling molecules but no one has yet described the whole pathway, or defined the G proteins these receptors couple to. QRFP induces an influx of Ca^{2+} into cells (Takayasu et al. 2006; Ramanjaneya et al. 2013) but at least two pathways have been implicated in the transduction of QRFP signalling. The effects of QRFP on insulin secretion are suggested to be dependent on G_{\alpha} signalling (Granata et al. 2014), whilst a possible effect on aldosterone secretion in the adrenal gland is dependent on G_{\alpha} proteins (Ramanjaneya et al. 2013), and the lipolytic effect involves the PI3K pathway (Mulumba et al. 2015). Given the ability of 26RFa, in particular, to activate non-Gpr103 receptors such as NPFF2, it is important that we understand the signalling pathway downstream of Gpr103. This would further help ensure that effects being investigated are due to on-target actions.
Supplementary Methods I

Operant response studies conducted in Chapter 4 were carried out using operant chambers controlled by ABETII software. This software requires manual inputting of code to run specific protocols in these cages. The operant studies described in this thesis involved 4 distinct protocols: fixed-interval, fixed-ratio 1 (FR1), fixed-ratio 3 (FR3) and progressive-ratio. The codes for each of these can be found below (all times are in seconds unless otherwise stated).

Each chamber in our set-up has 8 “inputs” and 7 “outputs” (defined below) which remained identical in each protocol

<table>
<thead>
<tr>
<th>Coding names for operant inputs/outputs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inputs</strong></td>
</tr>
<tr>
<td>Line</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
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<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
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<tr>
<td>7</td>
</tr>
<tr>
<td>8</td>
</tr>
</tbody>
</table>

Fixed-Interval schedule

Dispenses 5µl milkshake every X s (user-defined time) throughout schedule

<table>
<thead>
<tr>
<th>Group</th>
<th>Name</th>
<th>Go To</th>
<th>Condition</th>
<th>Action</th>
</tr>
</thead>
</table>
| 1     | Start| 2     | When _Schedule_Timer ≥ 1 | Turn on House_Light #1
|       |      |       |           | Turn on Left_Lever_Operate #1
|       |      |       |           | Turn on Right_Lever_Operate #1
|       |      |       |           | Start IOS_Timer

*Initialise and prepare chamber for testing*

<table>
<thead>
<tr>
<th>Group</th>
<th>Name</th>
<th>Go To</th>
<th>Condition</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Timer</td>
<td>1</td>
<td>When IOS_Timer ≥ IOS_Value</td>
<td>Start Flash RA_Light #1, total time = 1200 ms, on time = 200 ms, off time = 200 ms Pulse Feeder_Pump #1 for 200 ms Reset IOS_Timer</td>
</tr>
</tbody>
</table>

*Dispenses milkshake drop & flashes reward light every X s*

<table>
<thead>
<tr>
<th>Group</th>
<th>Name</th>
<th>Go To</th>
<th>Condition</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Finish</td>
<td>3</td>
<td>When _Schedule_Timer ≥ 900</td>
<td>No Action</td>
</tr>
</tbody>
</table>

*Ends schedule after 15 minutes*

_Schedule_Timer & IOS_Timer are simple timers

IOS_Value is a user defined variable – value altered by user depending on required gap between rewards
**Fixed-Ratio 1 (FR1) schedule**

Dispenses 5µl milkshake with every lever press (can be modified to only provide milkshake following “correct” lever presses, viz. left or right, via modification of group 2 and 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Name</th>
<th>Go To</th>
<th>Condition</th>
<th>Action</th>
</tr>
</thead>
</table>
| 1     | Start    | 2     | _Schedule_Timer ≥ 1 | Turn on House__Light #1  
|       |          |       |                 | Turn on Left_Lever_Operate #1  
|       |          |       |                 | Turn on Right_Lever_Operate #1  
|       |          |       | *Initialise and prepare chamber for testing* | |
| 2     | Lever press | 3 | When Left_Lever_Press #1 = Activated Or Right_Lever_Press #1 = Activated | Start Flash RA_Light #1, total time = 1200 ms, on time = 200 ms, off time = 200 ms  
|       |          |       |                 | Pulse Feeder_Pump #1 for 200 ms  
|       |          |       |                 | Increment _Trial_Counter  
|       |          |       | *Lever press triggers reward and flashes reward light* | |
| 3     | Lever release | 2 | When Left_Lever_Press #1 = Not Activated And Right_Lever_Press #1 = Not Activated | No Action  
|       |          |       | *Resets schedule to enable next lever press to release milk* | |
| 4     | End of schedule | - | _Trial_Counter ≥ 50 Or _Schedule_Timer ≥ 900 | Turn off Left_Lever_Operate #1  
|       |          |       |                 | Turn off Right_Lever_Operate #1  
|       |          |       | *Ends schedule when mouse achieves 50 lever presses or after 15 minutes (whichever comes first)* |  

* _Schedule_Timer is a simple timer  
  _Trial_Counter is a simple counter – increases with each lever press

**Fixed-Ratio X schedule**

Modified version of the above FR1 schedule, where X “correct” presses (user-defined value) are required for reward delivery, example here is for FR3 using right lever only. “Correct” lever can be changed via modification of group 2 and 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Name</th>
<th>Go To</th>
<th>Condition</th>
<th>Action</th>
</tr>
</thead>
</table>
| 1     | Start    | 2     | _Schedule_Timer ≥ 1 | Turn on House__Light #1  
|       |          |       |                 | Turn on Left_Lever_Operate #1  
|       |          |       |                 | Turn on Right_Lever_Operate #1  
|       |          |       | *Initialise and prepare chamber for testing* | |
| 2     | Lever count | 3 | When Right_Lever_Press #1 = Activated | Increment FR_Count  
|       |          |       | *Counts correct lever presses* | |
| 3     | Lever press | 2 | When FR_Count ≥ FR_Value_3 | Start Flash RA_Light #1, total time = 1200 ms, on time = 200 ms, off time = 200 ms  
|       |          |       |                 | Pulse Feeder_Pump #1 for 200 ms  
|       |          |       | *Every 3 lever presses triggers reward & flashes reward light* |  
| 3     | Lever release | 2 | When FR_Count < FR_Value_3 And Right_Lever_Press #1 = Not Activated | No Action  
|       |          |       | *Resets schedule to enable next lever press to release milk* | |
| 3     | Finish   | 4     | _Trial_Counter ≥ 50 Or _Schedule_Timer ≥ 900 | Turn off Left_Lever_Operate #1  
|       |          |       |                 | Turn off Right_Lever_Operate #1  
|       |          |       | *Ends schedule when mouse achieves 50 rewards or after 15 minutes (whichever comes first)* |  
| 4     | End of schedule | - | | |

* _Schedule_Timer is a simple timer  
  FR_Count is a simple counter – increases with each lever press  
  FR_Value_3 is a user defined value – value defines how many presses required for each reward  
  _Trial_Counter is a simple counter – increases with each reward released
**Progressive-Ratio schedule**

Final schedule, requiring increasing numbers of presses to obtain the next reward, with a user-defined equation defining the numbers of “correct” presses to pass each level. “Correct” lever can be changed via modification of group 2 and 3.

<table>
<thead>
<tr>
<th>Group</th>
<th>Name</th>
<th>Go To</th>
<th>Condition</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Start</td>
<td>2</td>
<td>When _Schedule_Timer ≥ 1</td>
<td>Turn on House__Light #1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Turn on Left_Lever_Operate #1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Turn on Right_Lever_Operate #1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Start Time_Out_Timer</td>
</tr>
<tr>
<td>2</td>
<td>PR Evaluation</td>
<td>3</td>
<td>When PR_Count ≥ PR_Value.value</td>
<td>Start Flash RA_Light #1, total time = 1200 ms., on time = 200 ms., off time = 200 ms</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pulse Feeder_Pump #1 for 200 ms</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Decrement Rewards_per_Step</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Start Time_Out_Timer</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Start _Trial_Timer</td>
</tr>
<tr>
<td>2</td>
<td>PR Count</td>
<td>3</td>
<td>When Right_Lever_Press #1 = Activated</td>
<td>Increment PR_Count</td>
</tr>
<tr>
<td>2</td>
<td>PR Rewards per Step</td>
<td>2</td>
<td>When Rewards_per_Step ≤ 0</td>
<td>GetNextValue PR_Value</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reset Rewards_per_Step</td>
</tr>
<tr>
<td>2</td>
<td>Time Press Out</td>
<td>4</td>
<td>When PR_Count ≥ 169 Or Time_Out_Timer ≥ 600</td>
<td>Turn off Left_Lever_Operate #1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Turn off Right_Lever_Operate #1</td>
</tr>
<tr>
<td>3</td>
<td>Lever release</td>
<td>2</td>
<td>When Right_Lever_Press #1 = Not Activated</td>
<td>No action</td>
</tr>
<tr>
<td>4</td>
<td>End of schedule</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Initialise and prepare chamber for testing*

*Counts presses and compares to PR ratio to determine when reward is due*

*Counts number of presses*

*Selects next PR ratio value when previous level completed*

*Ends schedule when mouse achieves max rewards or after 10 minutes of no rewards (whichever comes first)*

*Resets schedule to enable next lever press to release milk*

_Schedule_Timer is a simple timer_

_Time_Out_Timer is a simple timer – counts time from reward delivery for a maximum of ten minutes (if timer reaches ten minutes, schedule times out due to inactivity)_

_Trial_Timer is a simple timer – counts time taken to complete each level_

_PR_Count is a simple counter – increases with each lever press_

_PR_Value is a user-defined list – sequential numbers are selected to define lever presses required for each level (values on the list are defined by the formula n^2)_

_Rewards_per_Step is a set value (fixed at 1) – required to provide reference point to ensure one reward delivered per level, and trigger the schedule to draw the next value from the PR-Value list_
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