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Distribution and connectivity of messenger molecules in the control of energy metabolism

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Karolinska Institutet

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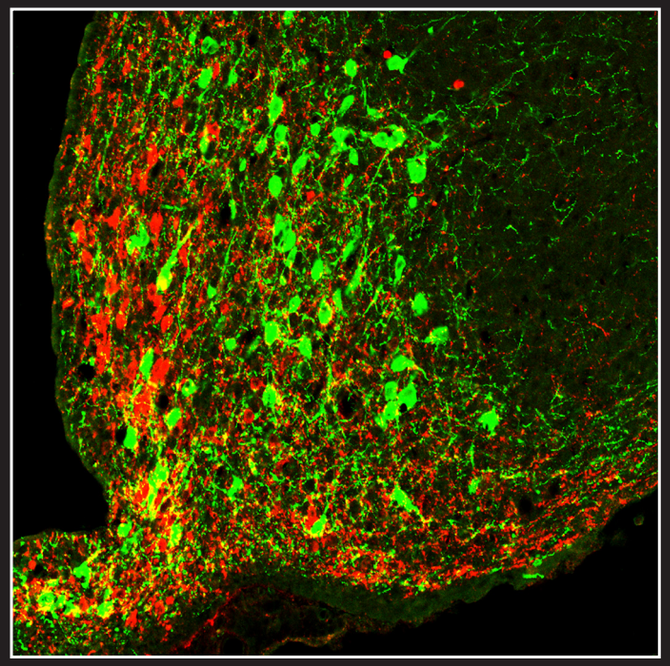
focus on neuropeptides and calcium binding proteins



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Distribution and connectivity of messenger molecules in the control of energy metabolism: *Focus on neuropeptides and calcium-binding proteins*

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Cover illustration: A confocal micrograph of a coronal section of the rat arcuate nucleus processed for immunofluorescence histochemistry for the neuropeptides agouti gene-related peptide (in red) and α melanocyte stimulating hormone (in green).

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To my parents,
for making this possible

ABSTRACT

Feeding is an essential and complex behavior which aims to provide the energy required for maintaining physiological homeostasis. The drive to feed is a powerful stimulus arising from metabolic demands, and reinforced by evolutionary pressure. The current epidemic in obesity, and associated disorders such as diabetes, makes it clinically vital to understand the mechanisms behind the control of energy metabolism. Feeding is a process governed by the central nervous system (CNS); particularly through the interplay between different hypothalamic nuclei. At the heart of the feeding neuro-circuitry lies the arcuate nucleus (ARC) which acts as a metabolic sensor, taking stock of the supply and demands of energy in the body, and coordinating food intake and energy expenditure. The work in this thesis aimed to explore the neuro-anatomical substrate of metabolic control, and the mediators involved.

The ARC contains two distinct sets of functionally antagonistic neurons. One group of neurons express the orexigenic peptides, neuropeptide Y (NPY) and agouti gene related peptide (AGRP); while the other set expresses the anorexigenic peptides, pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript. In **paper V**, we describe the histochemistry of NPY/AGRP and POMC neurons with regard to their anatomical interrelationship at the cell body and terminal level. A common experimental problem is that the ARC NPY cell bodies are difficult to distinguish and visualize in electrophysiological experiments and for immunohistochemistry. Therefore, in **paper III**, a novel transgenic mouse which expresses bright Renilla green fluorescent protein in NPY neurons was generated. Using this model, a comprehensive map of NPY-expressing cells in the CNS was generated and the effects of the satiety-inducing gut-brain bombesin peptides on ARC neurons were explored. Bombesin was found to exert powerful depolarizing actions on NPY and POMC neurons alike.

Calcium binding proteins (CaBPs) have been used extensively to delineate neuronal populations, but the ARC has not yet been subjected to such analysis. In **Paper IV** we show that three major CaBPs (calbindin D-28k, calretinin, and parvalbumin) are all expressed in the ARC, but displayed little co-localization with previously described cell groups. One exception was POMC neurons, of which distinct subpopulations stained for calbindin D-28k and calretinin, respectively. Another CaBP, nucleobindin 2 (NUCB2; also known as nesfatin), has recently been proposed as a central anorexigenic mediator. In **Paper I**, the CNS distribution of this protein was shown to include nuclei that participate in all three output channels of metabolic control, *i.e.* behavioral, endocrine and autonomic modulation. Our data also suggest that NUCB2 may not act as a cleaved and secreted messenger as proposed, but rather may play an intracellular role.

The wide distribution of NUCB2 in the neuroendocrine system prompted us to explore this protein in the pancreas (**Paper II)**. We show that NUCB2 is exclusively expressed in insulin-producing β cells, and that islet NUCB2 is dramatically decreased in the diabetic Goto-Kakizaki rat, an effect that is normalized by fasting. These data indicate that NUCB2 may play a role in metabolic control also outside of the CNS.

LIST OF PUBLICATIONS

The work in this thesis consists of the following communications. They will be referred to by their Roman numerals.

I. **Foo KS**, Brismar H, Broberger C (2008). Distribution and neuropeptide coexistence of nucleobindin2 mRNA/nesfatin-like immunoreactivity in the rat central nervous system. *Neuroscience*; 156: 563-579.

II. **Foo KS***, Brauner H*, Östenson C-G, Broberger C (2010). Nucleobindin-2/Nesfatin in the endocrine pancreas: Distribution and relationship to glycaemic state. *Journal of Endocrinology*; 204 (3): 255-63.

III. van den Pol, A., Yao Y, Fu LY*, **Foo K***, Huang H, Coppari R, Lowell BB, Broberger C (2009). Neuromedin B and gastrin-releasing peptide excite arcuate nucleus neuropeptide Y neurons in a novel transgenic mouse expressing strong Renilla green fluorescent protein in NPY neurons. *Journal of Neuroscience*; 29(14): 4622-39

IV. **Foo KS** and Broberger C (2010). Expression and co-localization patterns of calbindin-D28k, calretinin and parvalbumin in the rat hypothalamic arcuate nucleus. *Manuscript*

V. **Foo KS** and Broberger C (2010). Projections and anatomical interactions between Neuropeptide Y/Agouti gene-related peptide and Pro-Opiomelanocortin neurons in the arcuate nucleus of the rat. *Manuscript*

* Equal contribution.

CONTENTS

LIST OF ABBREVIATIONS

αMSH	α melanocyte-stimulating hormone
ACTH	Adrenocorticotropic hormone
AGRP	Agouti gene-related protein
ARC	Arcuate nucleus
BRP	Bombesin related peptide
CaBP	Calcium-binding protein
CART	Cocaine- and amphetamine-regulated transcript
CB	Calbindin D-28k
CNS	Central nervous system
CR	Calretinin
DMH	Dorsomedial hypothalamic nucleus
EIA	Enzyme Immunoassay
EW	Edinger-Westphal nucleus
GFP	Green fluorescent protein
GRP	Gastrin-releasing peptide
GI	Gastro-intestinal
GK	Goto-Kakizaki
i.c.v.	Intracerebroventricular
IF	Immunofluorescence
IML	Intermediolateral cell column of the spinal cord
i.p.GTT	Intra-peritoneal glucose tolerance test
-ir	Immunoreactive
ISH	*In situ* hybridization
LHA	Lateral hypothalamic area
LC	Locus coeruleus
-LI	like immunoreactivity
MCH	Melanin-concentrating hormone
mRNA	Messenger ribonucleic acid
NMB	Neuromedin B
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
NUCB2	Nucleobindin2
OXY	Oxytocin
POMC	Pro-opiomelanocortin
PV	Parvalbumin
PVH	Paraventricular nucleus
rGFP	Renilla GFP
SON	Supraoptic nucleus
T2DM	Type 2 diabetes mellitus
TSA	Tyramide signal amplification
VMH	Ventromedial hypothalamic nucleus

1 INTRODUCTION

1.1 BACKGROUND

In 1909, Bernhard Aschner in Vienna made the interesting observation that damage to the brain region known as the hypothalamus is sufficient to induce obesity in dogs, even when the pituitary – which is anatomically located just below the hypothalamus on the ventral side of the brain - is left intact during the procedure (Aschner, 1909). This concept was novel because although obesity was by then well-known to accompany pituitary tumors (Mohr, 1840; Babinski, 1900), it had been assumed to result from the hormonal imbalance that follows loss of this important endocrine organ (Fröhlich, 1901; Crowe, 1910; Cushing, 1912). Achner's emerging theory built on Jacob Erdheim's clinical speculation (Erdheim, 1904), that pituitary tumors might result in damage in the base of the brain which leads to obesity in patients. The hypothalamic elements, whose destruction cause such profound behavioral changes, have since attracted intense interest and are the focus of the work in this thesis. Defining these brain components, and the circuits they form, is a task that lies at the core of understanding human feeding and, in extension, may be relevant to developing new therapies for eating disorders and metabolic disease.

Overweight and obesity (defined as a body mass index over 25 and 30, respectively) are increasing concerns for modern society (WHO, 2000). Increased body weight is often associated with a variety of diseases, which ultimately increase mortality rate (Malnick and Knobler, 2006). It has been shown that 80% of obese adults have at least one or more co-morbidities, including type 2 diabetes mellitus (T2DM), hyperlipidaemia, hypertension, and cardiovascular disease (Must *et al*., 1999). The world-wide increase in T2DM is believed to be directly caused by the increase in obesity (Sullivan *et al*., 2005). Numerous elements contribute to weight gain and obesity, including genetic, metabolic, behavioral and environmental factors. In general, obesity develops when food intake exceeds energy expenditure over a period of time. To combat this, the brain has evolved an intricate system dedicated to the maintenance of energy homeostasis.

Following up on the early observations summarized above, lesion studies conducted in the 1940s laid down the foundations for studying energy metabolism in the hypothalamus. Rodent studies revealed that electrolytic ablation made in the mediobasal hypothalamus results in obesity and hyperphagia (Ranson, 1937; Hetherington, 1941) similar to the clinical description of the syndrome Fröhlich observed in tumor patients, and in line with Aschner's observation in animals. However, at that time, it was not clear why destruction of the basal hypothalamus had such dire effects on body weight. This answer emerged only when the circuitry of this brain region began to be unraveled with the revolution in histochemistry that began in the 1960's (Hökfelt, 2010), a process that continues to this day.

1.2 BASAL HYPOTHALAMUS: THE ARCUATE NUCLEUS

The basal hypothalamus is a multinucleate area located ventromedially on either side of the third ventricle. The most mediobasal aspect of this area is commonly known as the arcuate nucleus (ARC; Krieg, 1932; Chronwall, 1985). The neurons of the ARC are comprised of a heterogeneous population of which a large proportion are neuroendocrine parvocellular neurons, including the growth hormone releasing hormone and tuberoinfundibular dopamine neurons cell groups (Everitt *et al*., 1986). While these cells are to a varying extent involved in energy metabolism, the relevance of this nucleus to food intake comes primarily from its status as home to two functionally antagonistic populations of neurons that extend their projections within the brain, rather than down to the portal circulation in the median eminence like the neuroendocrine cells (Everitt *et al*., 1986). One population of neurons co-expresses mRNA for the orexigenic neuropeptides, Neuropeptide Y (NPY; Tatemoto *et al*., 1982) and agouti gene-related peptide (AGRP; Ollmann *et al*., 1997; Shutter *et al*., 1997), while the second population of neurons within the ARC expresses the polypeptide precursor pro-opiomelanocortin (POMC; Watson *et al*., 1977; Bloch *et al*., 1978; Bloom *et al*., 1978; Jacobowitz and O'Donohue, 1978) which yields anorexigenic melanocortin peptides, and cocaine-and- amphetamine related transcript (CART; Douglass *et al*., 1995; Koylu *et al*., 1997; Koylu *et al*., 1998).

1.2.1 Neuropeptide Y and Agouti-Gene Related Peptide

Neuropeptide Y is a 36 amino acid peptide was first isolated in 1982 by Tatemoto and Mutt (1982) from the porcine hypothalamus. NPY is expressed in several brain regions (Chronwall, 1985; de Quidt and Emson, 1986), and accordingly has numerous roles in a variety of physiological processes (Allen *et al*., 1983). When injected into the cerebral ventricle, NPY is the most potent stimulator of food intake known (Clark *et al*., 1984; Levine and Morley, 1984; Stanley and Leibowitz, 1984). NPY signals through inhibitory G-protein-coupled receptor subtypes. Levels of NPY are indicative of the body's nutritional status with NPY mRNA expression and release of NPY increasing during fasting and decreasing after re-feeding (Sanacora *et al*., 1990; Swart *et al*., 2001; Swart *et al*., 2002). Furthermore, a rise in NPY levels precedes hyperphagia (Brady *et al*., 1990; Sahu *et al*., 1997; Tiesjema *et al*., 2007; Tiesjema *et al*., 2009).

Unlike the widely distributed and expressed NPY, AGRP is expressed exclusively in the ARC and there shown to co-localize with NPY (Broberger *et al*., 1998a; Broberger *et al*., 1998b; Hahn *et al*., 1998). Similar to NPY, the expression of AGRP is up-regulated in response to fasting (Hahn *et al*., 1998; Mizuno *et al*., 1999; Fekete *et al*., 2002; Kaelin *et al*., 2004; Fekete *et al*., 2006; Palou *et al*., 2009). Furthermore, i.c.v. injection of AGRP has been shown to induce a potent dose-dependent increase in food intake, in which a single dose can increase food intake for a week (Hagan et al., 2000; Hagan *et al*., 2001). Long term administration will ultimately lead to obesity (Rossi *et al*., 1998; Hagan *et al*., 2000). The importance of NPY/AGRP neurons in orexigenic signaling is underscored by the hypophagia and reduced body mass observed following the selective ablation of these cells (Bewick *et al*., 2005; Gropp *et al*., 2005; Luquet *et al*., 2005; Wortley *et al*., 2005; Xu *et al*., 2005).

1.2.2 Pro-opiomelanocortin

The precursor POMC is cleaved into the melanocortin family of peptides: α, β, and γ melanocyte stimulating hormone (MSH), as well as several other neuropeptides such as adrenocorticotropic hormone (ACTH) and β-endorphin (Mains *et al*., 1977; Roberts and Herbert, 1977). Among the melanocortins, αMSH may be the most prominent with regard to the regulation of energy balance in the ARC. Central administration of αMSH or agonist ligands has been shown to decrease food intake (Poggioli *et al*., 1986; Tsujii and Bray, 1989; Fan *et al*., 1997; Kask *et al*., 1998; Edwards *et al*., 2000). Hypothalamic POMC mRNA expression is also regulated by nutritional status; levels are low during fasting, and increase after re-feeding (Swart *et al*., 2002; Germano *et al*., 2007). Melanocortin anorexia is primarily mediated by the melanocortin receptors, MC3R and MC4R (Mountjoy *et al*., 1994; Mountjoy and Wong, 1997; Harrold *et al*., 1999). MC3R and MC4R are distributed throughout the CNS, in particular in areas associated with the central regulation of energy balance (Gantz *et al*., 1993; Desarnaud *et al*., 1994; Schiöth *et al*., 1996; Kishi *et al*., 2003). Mutations in the MC4R gene in humans account for as much as 5% of cases of severe obesity (Vaisse *et al*., 1998; Yeo *et al*., 1998), and MC4R sequence variants very strongly predict overweight and glucose intolerance (Chambers *et al*., 2008; Loos *et al*., 2008).The role of the MC3R in food intake remains unclear, although MC3R knockout mice have an elevated fat content and decreased lean body mass (Chen *et al*., 2000). AGRP also acts on the melanocortin receptors, as it is an endogenous antagonist of MC3R and MC4R (Lu *et al*., 1994; Ollmann *et al*., 1997; Nijenhuis *et al*., 2001; Chai *et al*., 2003; Breit *et al*., 2006; Tolle and Low, 2008).

1.2.3 Cocaine-and amphetamine-regulated transcript

Within the ARC, the majority of POMC neurons express the anorexigenic peptide precursor, CART (Elias *et al*., 1998; Kristensen *et al*., 1998). The CART gene product was initially purified from brain extracts as a peptide of unknown function (Spiess and Vale, 1980). It was first identified as a transcript in the striatum, where the mRNA expression was dramatically up-regulated after short-term exposure to cocaine and amphetamine (Douglass *et al*., 1995). CART is highly expressed in hypothalamic areas and peripherally in the pituitary and adrenal medulla (Koylu *et al*., 1997). Interestingly, CART not only co-localizes with anorexigenic peptides (POMC) but also orexigenic peptides (melanin-concentrating hormone; MCH) in the hypothalamus (Broberger, 1999; Elias *et al*., 1999; Vrang *et al*., 1999).

1.3 REGULATION OF THE ARCUATE NUCLEUS

The ARC is situated at a part of the brain endowed with a relatively permeable blood-brain barrier (BBB) and therefore constitutes an ideal site for putative brain sensors of circulating hormonal and metabolic factors coming from the periphery (Broadwell and Brightman, 1976; Broadwell *et al*., 1983; Norsted *et al*., 2008). Furthermore, due to the close proximity to the third ventricle, the ARC can sense the levels of factors found in

the cerebrospinal fluid, which is the main entrance for several peptides and hormones into the brain through the blood-CSF barrier (Elmquist *et al*., 1998a).This allows the ARC to sense blood composition and humoral messengers that reflect the metabolic state of body. This includes indices of both the supply and demand of energy in the tissues, such as leptin, insulin, ghrelin, corticosteroids, and peptide YY; for which receptors are expressed in the ARC (Margolis and Altszuler, 1967; Woods *et al*., 1979; Miyachi *et al*., 1986; Werther *et al*., 1987; Baura *et al*., 1993; Zhang *et al*., 1994; Halaas *et al*., 1995; Banks *et al*., 1996; Schwartz *et al*., 1996; Guan *et al*., 1997; Elmquist *et al*., 1998b; Kojima *et al*., 1999; Lu *et al*., 2002).

1.3.1 Peripheral inputs to arcuate nucleus

Within the ARC, the antagonistic neuronal NPY and POMC populations are sensitive to a number of the aforementioned hormones. Most prominent among these are leptin and insulin (Woods *et al*., 1979; Zhang *et al*., 1994). Insulin is produced in the pancreas, while leptin is secreted from adipocytes (Banting *et al*., 1922; Zhang *et al*., 1994). Insulin secretion is influenced by the glucose level in plasma, while leptin is secreted into the blood circulation in proportion to the body fat mass (Rezek, 1976; Maffei *et al*., 1995). Both hormones cross the BBB to access neurons in the ARC to effect energy homeostasis (Baura *et al*., 1993; Banks *et al*., 1996; Schwartz *et al*., 1996). A reduction in insulin or leptin signaling to the brain causes the body to respond as if there is a deficient level of glucose and fat, and subsequently stimulate food intake and decrease energy expenditure (Ahima *et al*., 1996; Weigle *et al*., 1997). Animal models have shown that central administration of insulin and leptin reduces feeding and body weight (Woods *et al*., 1979; Pelleymounter *et al*., 1995; Chen *et al*., 1996; Levin *et al*., 1996; Seeley *et al*., 1996; Tang-Christensen *et al*., 1999).

Both the ARC NPY/AGRP and POMC neurons contain insulin and leptin receptors and are directly regulated by these two hormones (Werther *et al*., 1987; Marks *et al*., 1990; Marks *et al*., 1992; Schwartz *et al*., 1992; Mercer *et al*., 1996; Hakansson *et al*., 1998). Selective inactivation of the leptin receptor gene in POMC neurons results in an obese phenotype (Balthasar *et al*., 2005). Leptin affects the electrical properties of NPY/AGRP and POMC neurons in an opposing manner, inhibiting NPY/AGRP neurons while stimulating POMC neurons (Cheung *et al*., 1997; Baskin *et al*., 1999; Cowley *et al*., 2001). Insulin receptors can also be found on NPY and POMC neurons and has been shown to suppress activity of NPY neurons and stimulate POMC neurons, similar to the action of leptin (Benoit *et al*., 2002; Plum *et al*., 2006). Insulin receptor expression is high in the ARC, with insulin receptor substrate-2, being the main constituent for the insulin's effect on food intake (Marks *et al*., 1990; Pardini *et al*., 2006).

In addition to leptin and insulin, one group of signal molecules that can also exert prominent metabolic effects are the bombesin-related peptides (BRPs; Martin and Gibbs, 1980; Kulkosky *et al*., 1982; Woods *et al*., 1983; Taylor and Garcia, 1985; Johnston and Merali, 1988; Flynn, 1993; Gutzwiller *et al*., 1994; Himick and Peter, 1994). Bombesin was originally isolated from skin of the frog, *Bombina bombina* (Erspamer *et al*., 1970; Anastasi *et al*., 1971), but in mammals, the dominant BRPs are gastrin-releasing peptide (GRP; McDonald *et al*., 1979), and neuromedin B (NMB;

Orloff *et al*., 1984; Minamino *et al*., 1988). These peptides are widely distributed in the gastrointestinal (GI) tract but are also found in the CNS (Panula *et al*., 1982; Wada *et al*., 1990; Mikkelsen *et al*., 1991). BRPs are released from the GI-tract following a meal, and may provide feedback inhibition from the gut to the brain to promote satiety (Banks, 1980; Gibbs, 1985; King and Hill, 1991), as they decrease meal size and duration in rodents and humans when administered peripherally or centrally (Martin and Gibbs, 1980; Kulkosky *et al*., 1982; Johnston and Merali, 1988; Flynn, 1993; Muurahainen *et al*., 1993; Gutzwiller *et al*., 1994). Intriguingly, obese humans may be less sensitive to the satiety effect of bombesin compared to those who are lean (Lieverse *et al*., 1998). Yet, the potential actions of BRPs on ARC neurons had not been addressed experimentally.

While the hypothalamus is the focus for the work described in this thesis, it should be stressed that its role as a metabolic sensor is complemented by the brain stem (Berthoud and Morrison, 2008). Classically, the brainstem has been viewed as the recipient of feedback information from the alimentary tract, including not only the GI canal, but also the mouth and pharynx, where myriad sensory receptors record the presence and chemical composition of an ingested meal (Smith, 1996). Information from these receptors is relayed to the brain via cranial nerves, in particular the afferent component of the vagus nerve terminating in the brainstem nucleus of the solitary tract (NTS; Grill and Hayes, 2009). Thus, vagally mediated satiety signals, such as cholecystokinin released after a meal (Gibbs *et al*., 1973), play a major role in meal termination (Smith *et al*., 1981). When food intake is based only on brainstem feedback and the hypothalamic component is experimentally removed, as in the chronic decerebrate rat, animals are still capable of regulating meal duration in relationship to GI feedback (Grill and Norgren, 1978), but fail to adjust their intake in response to changing caloric value (Kaplan *et al*., 1993). One common interpretation of this phenomenon is that long-term control of food intake is a task exclusively carried out by forebrain (hypothalamic) structures. In recent years, however, it has become clear that many hormones such as leptin (Grill *et al*., 2002) and ghrelin (Faulconbridge *et al*., 2003) can act directly on NTS neurons to affect meal parameters, suggesting distributed actions of long- and short-term control of feeding (see also Harris *et al*., 2006).

1.4 TARGETS OF THE ARCUATE NUCLEUS

1.4.1 Hypothalamic arcuate nucleus targets

Functionally, the ARC together with its projection targets act on three output channels to maintain energy homeostasis: endocrine, autonomic and behavior (Swanson and Mogenson, 1981). Studies have demonstrated that this regulation is accomplished through the interplay between multiple distinct nuclei which form the hypothalamic circuits, rather than discrete hypothalamic feeding and satiety centers (Baskin *et al*., 1988; Unger *et al*., 1989; Baura *et al*., 1993). Four hypothalamic nuclei, in addition to the ARC, have received particular attention in this regard: the paraventricular hypothalamic nucleus (PVH), lateral hypothalamic area (LHA), dorsomedial (DMH) and ventromedial (VMH) hypothalamic nuclei.

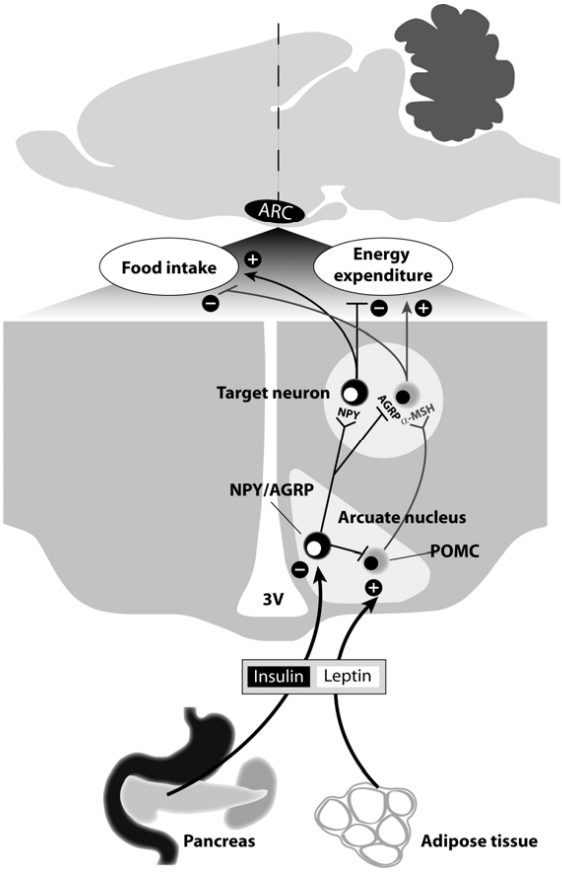

Figure 1: Schematic diagram of a rat brain in sagittal and coronal view illustrating the role of the ARC integrating peripheral and central factors regulating energy balance. Peripheral factors such as insulin and leptin enter the ARC, where they act on NPY/AGRP and POMC neurons and affect food intake and energy expenditure. 3V denotes 3rd ventricle.

1.4.1.1 Paraventricular hypothalamic nucleus

The PVH is situated adjacent to the dorsal tip of the third ventricle. It is an area where autonomic functions and endocrine system integrate (Swanson and Kuypers, 1980). Neurons in the PVH are considered to be "second order" neurons, since they receive both the anorexigenic POMC and the orexigenic NPY/AGRP signals originating from the ARC (Cowley *et al*., 1999). PVH receives input from DMH and orexigenic input from LHA (Nambu *et al*., 1999). There are two main types of neuroendocrine neurons in the PVH; the magnocellular and the parvocellular neurons (Sherlock *et al*., 1975; Swaab *et al*., 1975; Vandesande and Dierickx, 1975). Magnocellular neurons contain

oxytocin (OXY) or vasopressin, and they project directly to the posterior pituitary (Sherlock *et al*., 1975; Swanson *et al*., 1980; Wiegand and Price, 1980). Parvocellular neurons are smaller in size and express factors such as corticotrophin-releasing hormone and thyrotropin-releasing hormone (Burlet *et al*., 1979). They project to the median eminence, where they deliver releasing factors into the portal circulation which then travel to the anterior pituitary to regulate hormone secretion (Harris, 1948; Vandesande *et al*., 1977; Swanson *et al*., 1980; Wiegand and Price, 1980). Some PVH neurons also project centrally, *e.g.* both OYX and vasopressin project to the brainstem and spinal cord mediating autonomic functions (Conrad and Pfaff, 1976; Saper *et al*., 1976; Ono *et al*., 1978).

1.4.1.2 Lateral hypothalamic area

The LHA is one of the most extensively interconnected areas of the hypothalamus, due to its role in integrating an array of functions spanning from cognitive to autonomic (Bernardis and Bellinger, 1993, 1996). This ill-defined area is composed of a large and diffuse population of neurons, including those expressing the arousal-promoting hypocretin/orexin (de Lecea *et al*., 1998; Sakurai *et al*., 1998), and the sleep-associated melanin-concentrating hormone (MCH; Bittencourt *et al*., 1992; Bittencourt and Elias, 1998), although it should be noted that collectively these populations still only account for a minority of the LHA neurons (Broberger, 2005). Both of these populations receive prominent input from the orexigenic and anorexigenic populations of the ARC (Broberger *et al.,* 1998a; Elias *et al*., 1999; Horvath *et al*., 1999). The LHA engages in behavioral and autonomic output although it does not directly participate in the endocrine system.

1.4.1.3 Dorsomedial & ventromedial hypothalamic nuclei

The DMH (Bellinger and Bernardis, 2002) receives input from other hypothalamic nuclei such as the ARC, PVH, LHA and the suprachiasmatic nucleus, but it also receives information from the brainstem (Thompson and Swanson, 1998). The connection between the suprachiasmatic nucleus and the DMH neurons plays an important role in food entrainable rhythms (Chou *et al*., 2003; Gooley *et al*., 2006; Mieda *et al*., 2006). Intriguingly, there is also an induction of NPY in neurons in the DMH when the body's energy storage is depleted such as during food deprivation and lactation (Smith, 1993). Chemical lesions of VMH cells result in obesity (Marshall *et al.*, 1955), suggesting that the nucleus as a complex exerts inhibitory effects on food intake. Neurons in this region express leptin receptors (Jacob *et al*., 1997; Funahashi *et al.*, 1999). The VMH also receives input from brainstem nuclei (Fulwiler and Saper, 1985), as electrophysiological experiments revealed that VMH neurons are sensitive to stomach distention (Sun *et al*., 2006).

1.5 CALCIUM-BINDING PROTEINS IN THE ARCUATE NUCLEUS

1.5.1 Calcium-binding proteins

Since the seminal demonstration by Gibbs *et al* in (1973) that administration of cholecystokinin, a neuropeptide, resulted in abrupt meal termination, the list of neuropeptides and transmitters implicated in food intake has steadily grown. Given the complexity of signal transmission in the nervous system, it is not surprising that recent years have also revealed important roles for proteins beyond transmitters and peptides and their receptors in the central control of body weight, including pro-hormone processing enzymes, transmitter transporters, signal cascade proteins and transcription factors. A less explored class of signal molecules, though one with an established position in neuronal regulation, is calcium-binding proteins (CaBPs). The CaBPs are primarily involved in Ca^{2+} signaling and homeostasis, and have been traditionally classified as either "sensors", which facilitate signal transduction following conformational changes upon Ca^{2+} binding, or "buffers", whose function is to modulate and limit the rise in the intracellular free Ca^{2+} concentration (Dalgarno, 1984; Baimbridge et al., 1992; Heizmann, 1993; Burgoyne, 2007). It is becoming apparent, however, that several CaBPs can be involved in both functions and thus, the distinction between these groups has blurred (Schwaller, 2009).

Three particular CaBPs have been studied in the CNS due to their restricted distribution patterns, namely: calbindin D-28k (CB; Taylor and Wasserman, 1967; Jande *et al*., 1981), calretinin (CR; Rogers, 1987) and parvalbumin (PV; Henrotte, 1952; Celio and Heizmann, 1981). These proteins are members of the "EF-hand" family of CaBPs which share the structural motif of Ca^{2+} binding domain known as the EF hand (Moncrief *et al*., 1990; Lee *et al*., 1991; Nakayama *et al*., 1992). These CaBPs have mainly been used as a tool for histochemical identification of neuronal cell groups throughout the brain (Jande *et al*., 1981; Winsky *et al*., 1989; Celio, 1990; Baimbridge *et al*., 1992). In the cerebral cortex, CB, CR, and PV are expressed in largely separate populations of interneurons (Ascoli *et al*., 2008). One region of the brain which has not yet been described with regards to CaBP expression is the ARC.

1.5.2 Nucleobindin 2

One CaBP that has recently been implicated in energy homeostasis is nucleobindin2 (NUCB2). Structurally, NUCB2 is composed of 396 amino acids and contains two EF hand motifs, and a DNA binding domain (Barnikol-Watanabe *et al*., 1994). The sequence of NUCB2 is highly conserved in rodents and humans (Barnikol-Watanabe *et al*., 1994). A study in 2006 suggested a possible role for NUCB2 in the regulation of food intake (Oh-I *et al*., 2006). In this paper, it was postulated NUCB2 is a prepro-protein which is subsequently cleaved by pro-hormone convertase 1 and 3 into fragments called nesfatin-1 , -2, and -3 as shown in Fig.2 (Oh-I *et al*., 2006). This initial study suggested that only the putative fragment nesfatin-1 has an effect on suppressing food intake (Oh-I *et al*., 2006). There is much controversy, however, surrounding whether NUCB2 is in fact cleaved into nesfatin-1, -2, and -3. There is no study to date, where any of the three putative endogenous fragments are detected using western blot. Thus, whether NUCB2 is a prepro-protein or not remains elusive. NUCB2/nesfatin has

been shown in the Oh-I *et al*. (2006) study to be expressed in the hypothalamic nuclei involved in energy metabolism such as: ARC, LHA, PVH and the supraoptic nucleus (SON). However, a detailed location and distribution of the protein and its mRNA expression throughout the CNS has not yet been carried out at the onset of this thesis work. The expression of NUCB2/nesfatin with other neuropeptides involved in energy homeostasis was unknown.

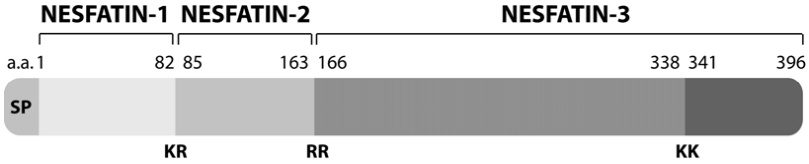

Figure 2: Schematic illustration of the 396 amino acid NUCB2. KR, RR, KK refers to pairs of proposed cleavage sites for the processing of NUCB2 into putative nesfatin fragments nesfatin-1,-2,-3. SP denotes signal peptide. a.a. denotes amino acid.

1.6 ENDOCRINE PANCREAS

The central regulation of energy balance relies on the brain's successful detection and integration of peripheral signals pertaining to metabolic state. A key component of this integrative mechanism is the ability of the hypothalamus to respond to metabolic information from the endocrine pancreas. The CNS and the pancreas not only many common signaling molecules, including neuropeptides (*e.g.* Luft *et al*., 1974), transcription factors (*e.g.* Naya *et al*., 1995), intracellular signalling mediators (see Mountjoy and Rutter, 2007), as well as proteins involved in the secretory process (*e.g.* Jacobsson *et al*., 1994), but these molecules and their receptors also interact intermittently between the two organs to collectively influence food intake and energy expenditure. The most prominent hormone is insulin (Banting *et al*., 1922), which is secreted from β-cells in the pancreatic islets of Langerhans when plasma glucose increases (typically after a meal). The interaction between insulin and its receptor allows cells to take up circulating glucose. In T2DM, the cellular sensitivity to the insulin signal is perturbed, and there may also be loss of insulin output from the β-cells, abeit not as dramatic as in T1DM (DeFronzo and Tripathy, 2009). These factors lead up to glucose intolerance, where patients have high levels of circulating glucose, with detrimental effects on the tissues (Deckert *et al*., 1978; Chase *et al*., 1989). Thus, identifying the signal repertoire of β-cells is relevant not only to understanding insulin control of appetite (as described above) but also for the pathophysiology of T2DM.

2 AIMS

In the work included in this thesis, I have studied the distribution and connectivity of peptides and proteins implicated in the homeostatic regulation of energy metabolism, with particular focus on the CNS. Specifically, the aims were to:

1. Determine the projections of neurons in the ARC that constitute the metabolic sensor

2. Determine the expression pattern of CaBPs in the ARC

3. Characterize the CNS distribution of neurons expressing NPY using a novel transgenic mouse

4. Investigate the anatomical and cellular distribution of NUCB2 in the brain

5. Examine the potential distribution of NUCB2 in the pancreas and its regulation under different metabolic conditions

3 METHODOLOGICAL CONSIDERATIONS

Detailed descriptions of the experimental procedures for the work in this thesis can be found under the *Materials and Methods* sections of the individual papers. The purpose of this section is to provide a methodological overview, considerations, and limitations to some of the techniques used.

3.1 *IN SITU* HYBRIDIZATION HISTOCHEMISTRY (ISH)

This technique (Pardue and Gall, 1969) is used to detect mRNA expression in tissues using labeled nucleotide probes complementary to the mRNA of interest (Young and Mezey, 2004). The two main concerns for *in situ* hybridization (ISH) are specificity and sensitivity of the signal. There are a few methods that can control for the specificity. The first thing to consider is the probe selection. There are four types of probe that are used for ISH: oligonucleotide, single stranded DNA, double stranded DNA and RNA probe; and each of these options have certain advantages and disadvantages (Wilcox, 1993; Jin and Lloyd, 1997). The following will focus on oligonucleotide probes, since the experiments in this thesis were performed using these reagents.

3.1.1 Probe design

Oligonucleotide probes are produced synthetically, and are commercially available unlike RNA probes. The probes are normally 40-50 base-pairs long, compared to other types of probes which are often hundreds of base-pairs long (Jin and Lloyd, 1997). An advantage of its small size is that it easily penetrates into the tissue. The short probe sequence makes it less sensitive than the long RNA/DNA probes. However, one can use a cocktail of probes against different regions of the target sequence to enhance the signal. We generated oligoprobes using the software program Oligo6, and the specificity is verified by a gene BLAST search. One of the considerations to be taken into account when generating the probes is the GC content. The G/C base-pair bonds are stronger than the A/U bond, thus the variation of GC content would require different hybridization conditions. The GC content used in the experiments is approximately 52%. Another advantage to using oligoprobes is that they are single stranded which eliminates the possibility of re-naturation in the process.

3.1.2 Tissue preparation

The treatment of tissue is also very important for the detectability of the mRNA. In general, mRNAs are synthesized and degraded at a fast rate. Therefore, it is necessary to handle the tissue as quickly as possible. Work by Dagerlind *et al* showed that freshly frozen tissue provides high sensitivity for the detection of mRNAs also without fixation (Dagerlind *et al*., 1992). Therefore, following decapitation, the tissue is rapidly dissected out and immediately frozen. It is also important to be aware that all steps should be carried out in RNase free environment to avoid degradation.

3.1.3 Probe labeling

In work presented in this thesis, oligoprobes were either radioactively (^{35}S or ^{33}P) or enzymatically (digoxigenin) labeled. The highly sensitive photo-emulsion used to reveal the signal from the radioactive probes generally gives a higher sensitivity than using the enzymatic alternative (Lewis, 1990; Feldman, 1997). However, the disadvantage of isotope-labeled probes, beyond the hazards inherent in using radioactive material, is the long exposure times required, typically several weeks (Woodruff, 1998). The radioactive isotopes emit beta particles, which cause a reduction of Ag^{+} ions to metallic silver in the photographic material. The silver grains which accumulate to form a latent image can be developed in order to visualize the expression (Buongiorno-Nardelli and Amaldi, 1970).

Digoxigenin (DIG) labeled probes utilize an indirect detection method in resemblance of immunohistochemistry, where an antibody directed against DIG and conjugated to a fluorophore or chromogenic enzyme is used to detect the presence of DIG labeled probes. One advantage of using DIG labeled probes is the higher resolution compared to radioactive probes (Panoskaltsis-Mortari and Bucy, 1995). Using the enzymatic technique, signal can easily be observed in the cytoplasm, unlike radioactive labeled probes where particles are scattered on top of the cell body. The other benefit of DIG labeling is the shorter time required to yield a signal; the staining can be revealed one day post incubation.

3.1.4 Hybridization

The main goal of hybridization is for the oligonucleotide to anneal to a complementary mRNA strand under the optimal condition. Factors that affect how well the oligoprobes will bind to the target mRNAs include: temperature, probe concentration, pH, and ion concentration (Jin and Lloyd, 1997). Changing these parameters will influence the probe's affinity for its target sequence, so that higher temperature and pH, and lower probe and ion concentration will decrease the probes' affinity to its target sequence.

Following hybridization, the slides are washed in steps with the purpose of removing unbound probes or probes loosely bound to improperly matched sequences. The washing step is done in high stringency, at a higher temperature than hybridization. This step is a delicate balance where if the condition is too strict, it will result in a loss of sensitivity; conversely, a low stringency will give rise to high background and un-specificity.

3.1.5 Specificity controls

A crucial part of any experiment is performing proper controls. It is essential to determine that the hybridization reaction is specific and that the probe binds selectively to the target mRNA sequence. One way to control for specificity of the probes is to incubate with an excess (100x) of non-labeled probe in the hybridization cocktail along

with the labeled probe. The rationale behind this method is that the non-labeled probes will compete out the labeled probes and bind up the binding sites on the tissue, resulting in an absence of signal on the tissue. Another method is to hybridize with labeled sense probes. In theory, the sense probes identify any non-specific targets it can bind to due to the purely chemical (*i.e.* sequence-independent) properties of the probe. Comparing the distribution of multiple probes targeted against different regions of the mRNA sequence can also indicate whether the observed pattern is specific or not since they should all yield the same pattern. Lastly, performing immunohistochemistry targeting the same protein of interest and comparing the distribution pattern also suggests specificity of the probes.

3.2 IMMUNOHISTOCHEMISTRY

Immunofluorescence (IF) is an antibody-based method commonly used to visualize the cellular and subcellular distribution of a protein in tissues (Coons AH, 1941; Coons and Kaplan, 1950). There are two principal methods for labeling IF; directly and indirectly. Direct IF detection entails that the primary antibody is targeted against the protein of interest and it is chemically conjugated to a fluorescent dye. The indirect method, which is the most common, consists of an unlabeled primary antibody, and the visualization requires a secondary antibody conjugated to a fluorescent dye. A benefit of using the indirect method is that it allows for amplification of signal since more than one fluorochrome-conjugated polyclonal secondary antibody can be attached to a given primary antibody; it also allows for a greater range of visualization/detection techniques. The drawback of IF, and any antibody-based technique, is the potential for cross-reactivity, especially if more than one primary antibodies is used.

3.2.1 Monoclonal vs. Polyclonal antibodies

There are two categories of antibodies, mono- and polyclonal. Monoclonal antibodies are a homogenous population of immunoglobulin directed against a single epitope (Schwaber and Cohen, 1973; Köhler and Milstein, 1975). They are generated by a single B-cell clone isolated from the spleen of an immunized mouse and fused to a myeloma cell to create a hybridoma, thus they are immunologically identical. Polyclonal antibodies are a heterogenous mixture of antibodies directed against various epitopes of the same antigen, produced by immunizing a whole animal, thus activating several different B-cell clones, and then using more or less purified serum extractions for immunochemical purposes. The animal can theoretically be from any species, though rabbits are most common. Polyclonal antibodies, which constitute a heterogeneous mix of distinct antibodies, are therefore more properly referred to as anti*serum*. In theory, polyclonal antiserum is considered to have a higher sensitivity due to its ability to recognize multiple epitopes (Ramos-Vara, 2005). However, the presence of antibodies to multiple epitopes can increase the chance for cross-reactivity and unspecificity. On the other hand, monoclonal antibodies have a high specificity because they only react with a specific epitope on a given antigen.

3.2.2 Tissue treatment

Fixation of tissues results in cross-linking of tissue proteins which preserves the antigenicity of the tissue, since there is a finite amount of antigen in the tissue and each step of tissue handling may gradually reduce the total antigen pool (Ramos-Vara, 2005). Fixation helps to prevent antigen degradation and preserve the position of the antigen to enable the easy access and binding for the antibody. The composition of fixatives and fixation time will influence staining result. For immunofluorescence applications, this is of particular importance since aldehydes can contribute inherent autofluorescence after reaction with endogenous substances (Corrodi and Jonsson, 1965). For the experiments performed in this thesis, the animals were transcardially perfused with a formalin and picric acid-based fixative (Zamboni, 1967).

3.2.3 Colchicine pre-treatment

Colchicine (Pelletier, 1820) is a natural toxin derived from the plant, *Colchicum autumnale*. It inhibits the polymerization of tubulin into microtubules (Eigsti, 1938) causing disruption of axonal transport, which results in the accumulation of peptides in the cell bodies and enhances visualization by immunohistochemistry (Hökfelt and Dahlstrom, 1971). Colchicine is injected into the lateral ventricle of the animal before sacrifice. This pre-treatment is necessary for the cellular visualization of certain neuropeptides such as ARC NPY and AGRP (de Quidt and Emson, 1986). The reservation for this technique is that it alters cell morphology, given that colchicine modifies the subcellular structure (Eigsti, 1938) and that the detectablity of dendrites and fibers are dramatically apprehended, and may in some cases affect transcription (Cortes *et al*., 1990).

3.2.4 Tyramide Signal Amplification

In contrast to standard IF procedure, tyramide signal amplification (TSA) can increase the sensitivity up to ten fold (Adams, 1992). The TSA method includes an additional step of using horseradish peroxidase conjugated with a secondary antibody which enables the peroxidase to catalyze the conversion and deposition of fluorophore onto the tissue. With this technique, two primary antibodies raised in the same species can be used for double staining if it is done sequentially (Broberger, 1999), first performing IF with TSA, and then followed by incubating the second primary antibody using conventional IF.

3.2.5 Specificity Controls

There are a few ways to evaluate the specificity of the antibody (Saper, 2009). One approach is to compare the staining patterns of several antibodies raised against the same peptide/protein of interest. Another method is to pre-absorb the antibody with the purified peptide to which the antibody has been raised against. The pool of primary antibodies will thereby be exhausted through binding to the peptide, which should abolish the staining. The expression pattern of the mRNAs of the protein of interest from ISH can also be used to verify the specificity of the antibody by comparing their distribution pattern. Western blotting can be used to identify a band

corresponding to the molecular weight of the protein of interest. One drawback to Western blotting is that not all antibodies are suitable for this method. Testing the antibody on tissue from a knock-out animal which lacks the protein of interest has been suggested as the ultimate method to demonstrate the specificity (Saper and Sawchenko, 2003).

3.2.6 Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (Egger and Petran, 1967; Carlsson *et al*., 1985) is a commonly used technique for obtaining high resolution images with depth selectivity. This technique provides the capacity to optically scan the entire specimen or part of a specimen one plane at a time with great resolution. A frequent concern in fluorescence microscopy is bleed through or overlap of the fluorescence wave lengths between different fluorochromes. Overlap in emission may result in false positive signal for one or more fluorophores. One approach to minimize the problem is to scan one laser at a time (*i.e.* sequential scanning) thus exciting one fluorophore. Another method is to ensure that the detector band-pass filter for each fluorophore is set at a strict narrow range, so that only photons within a particular wavelength are detected. However, if the potential for bleed through is a concern, it may be most prudent to perform single-staining in parallel and compare with results from double-staining.

3.3 ENZYME IMMUNOASSAY

Enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA) is a powerful technique used for quantifying and detecting the presence of an antigen in tissue homogenates or plasma and other body fluids (Engvall and Perlmann, 1971; Van Weemen and Schuurs, 1971). Different types of EIA/ELISA include, indirect, sandwich, reverse and competitive EIA. The work in **Paper II** was performed using the competitive EIA method. Briefly, the basic principle for competitive EIA is as follows, the immunoplate is pre-coated with a secondary antibody and the non-specific binding sites are blocked. The secondary antibody can bind to the Fc fragment of the primary antibody which Fab fragment will be competitively bound by both biotinylated peptide and the target peptide in the samples. Therefore, the higher the sample antigen concentration, the weaker the signal. The advantage of EIA is that the amount of antigen in a sample is quantifiable. However, the limitation is that due to the very small volume, even a small deviation of each reagent can have a compounded effect. Therefore, samples should ideally be run in triplicates. A few parameters should be taken into account when using EIA: accuracy of the measurement, detection limit and detectability range of the EIA, and the specificity of the antibodies (Porstmann and Kiessig, 1992). Accuracy is crucial because the concentration determined from the assay should be similar to the real concentration from the sample. The concentration values from the assay should be within the detection limit and range so that the values can be accurately extrapolated. Altogether, EIA is a valuable technique which complements qualitative method like immunohistochemistry.

3.4 EXPERIMENTAL ANIMAL MODELS

3.4.1 Goto-Kakizaki Wistar (GK) Rat

The Goto-Kakizaki (GK) rat provides a useful animal model in studying T2DM. The GK rat was developed by selective inbreeding of Wistar rats with the highest blood glucose over many generations (Goto *et al*., 1975). The rats are non-obese, and develop relative stable hyperglycaemia in adult life. Several colonies of GK rats, originating from breeding pairs in Japan, exist in the world, including Stockholm (Östenson, 2001). Due to the fact that this model is based on inbreeding, rather than a "pure" monogenic etiology, there are slight discrepancies between the colonies in their pancreatic islet/β-cell phenotype and morphology, and islet metabolism (Portha *et al*., 2009). In general, the GK rats at birth have a reduced number of islets (Miralles and Portha, 2001). All the experiments in **Paper II** are performed with the Stockholm GK colony, where there is a reduction in β-cell mass compared with the reduced β-cell proliferation. Stockholm colony pups are hyperglycemic at the first week after birth (Abdel-Halim *et al*., 1994). The GK model recapitulates key complications seen in human diabetics, ***e.g.*** nephropathy (Janssen *et al*., 1999), peripheral neuropathy (Murakawa *et al*., 2002), and retinopathy (Sone *et al*., 1997). The GK rat is a good non-obese model for studying T2DM, where the main defect presumably lies in the β-cell. Moreover, the hereditary component of the GK rat reflects the polygenic aspect of human diabetics. Certainly, the GK β-cell is not a blueprint for the diseased human β-cell. There are, however, valuable similarities to study and understand the aetiopathogenesis of T2DM in this rat model.

3.4.2 NPY-renilla GFP mouse

The green fluorescent protein (GFP) was first isolated from the jellyfish, *Aequorea victoria* (Shimomura *et al*., 1962). The artificial introduction of GFP as a reporter gene has enabled the visualization of specific neuronal populations in mammalian cells while conducting electrophysiological experiments, including in the hypothalamus (Cowley *et al*., 2001; van den Pol *et al*., 2004). **In Paper III**, the GFP gene from the sea pansy *Renilla reniformis* (Morin and Hastings, 1971; Ward and Cormier, 1979) was used to construct the novel NPY-GFP mouse. The NPY expression was produced from a reporter which was generated using the isolated renilla gene and adapted with human codons. Compared to the widely used jellyfish *Aequorea victoria* enhanced GFP (Morise *et al*., 1974; Chalfie *et al*., 1994; Spergel *et al*., 2001), the humanized renilla GFP (rGFP) has a lower cytotoxicity, broader pH stability, and is significantly brighter than any other known GFPs. Cellular toxicity is a cause for concern because fluorescent proteins can generate reactive oxygen species that restrict experimental time to a limited window of cell viability (Dixit and Cyr, 2003). The fluorescence of rGFP is completely stable over a wide pH range from 5.5-12.6 (Ward *et al*., 1981), enabling the study of various subcellular dynamics under different pH environment. A notable advantage of the rGFP is its remarkable brightness. The rGFP absorbs light with a five-fold higher extinction coefficient than *Aequorea* GFP, thus enhancing the brightness of the fluorescence intensity. Taken together, rGFP proved to be a powerful tool for studying the elusive ARC NPY population.

4 RESULTS AND DISCUSSION

4.1 PROJECTION OF ARCUATE POMC AND NPY NEURONS

In **Paper V,** histochemistry was used to generate a comprehensive map of the projections emanating from the NPY and POMC populations of the ARC. In this study, we took advantage of the fact that AGRP in the brain is exclusively expressed in ARC NPY neurons and thus the presence of this peptide in somata and terminals can be used as a selective marker for the relevant population *in lieu* of traditional tracing methods (Broberger *et al*., 1998b). We used αMSH as a marker for POMC neurons. It should be noted in this context, that this is not as easily interpreted, given the existence of a small population of brainstem neurons that also express POMC (Joseph *et al*., 1983; Mountjoy *et al*., 1994). While these cells are likely to supply only a minority of CNS αMSH terminals and may project to similar targets as the ARC cells (Pilcher and Joseph, 1986; Joseph and Michael, 1988), the exact origin of a given melanocortinergic axon will eventually need to be verified by conventional tracing techniques.

Our data confirm published observations but also add substantial detail to earlier literature, in particular at the subnuclear level. We show a vast, but distinct, innervation of other hypothalamic nuclei by ARC NPY/AGRP and αMSH neurons that includes the preoptic area, the periventricular nucleus, the PVH, LHA, and DMH, while largely sparing *e.g.* the suprachiasmatic and supraoptic nuclei and the core of the VMH, in agreement with other studies (Watson *et al*., 1977; Bai *et al*., 1985; Broberger *et al*., 1998b; Elias *et al*., 1998; Bagnol *et al*., 1999; Haskell-Luevano *et al*., 1999). In addition, we also show a highly targeted innervation of extrahypothalamic areas, including the bed nucleus of stria terminalis, the paraventricular thalamus (the only thalamic nucleus observed), amygdala, the periaqueductal gray area, and several autonomic regions of the brainstem, including the NTS (**Paper V**). Several major brain regions, including the cerebral and cerebellar cortices, the hippocampus, the striatum and most of the thalamus, are notably spared from innervation. The extrahypothalamic projections, though suggested by early lesion studies (Eskay *et al*., 1979) and described in immunohistochemical investigations (Watson *et al*., 1977; Dube *et al*., 1978; Jacobowitz and O'Donohue, 1978; O'Donohue *et al*., 1979; Broberger *et al*., 1998a), are often neglected, but may play important functional roles in coordinating the central regulation of energy metabolism.

With few exceptions (see below), AGRP and αMSH –immunoreactive (-ir) were found in parallel distributions in the brain (**Paper V**; Broberger *et al*., 1998b). This anatomical organization may provide a morphological correlate of the functionally antagonistic roles of these systems, allowing transport of agonist (melanocortin) and antagonist (AGRP) to the same receptors. Selective abolition of the ARC NPY/AGRP neurons leads to the termination of feeding (Bewick *et al*., 2005; Gropp *et al*., 2005; Luquet *et al*., 2005) and ultimately death (Luquet *et al*., 2005). The elimination of one cell type promotes the prevalent activity of the other, whereas the elimination of both effaces the effect of ARC in the modulation of food intake. In some areas, we also noted “braided axons” in the form of intertwined AGRP and αMSH-ir terminals. The

generality of this phenomenon remains to be determined, but may indicate a novel level of presynaptic regulation within the ARC system.

Adaptive states such as the ingestive state that underlies control of energy balance require the parallel activation of behavioural, endocrine and autonomic controlling elements (Swanson and Mogenson, 1981). The targets of the projections from the metabolic sensor neurons in the ARC include nuclei that participate in all three functions (**Paper V**). The endocrine include parvocellular nuclei such as the periventricular nucleus, the PVH and the ARC; the innervation of the thyrotropic PVH neurons has been especially well characterized (Legradi and Lechan, 1998; Broberger, 1999; Fekete *et al*., 2000). Here, we focused on autonomic and behavioural control regions. Interestingly, the pre-autonomic rostral ventrolateral medulla and the sympathetic preganglionic cells in the intermediolateral cell column of the spinal cord (IML) appear to receive input preferentially from melanocortinergic, but not NPYergic fibers **Paper V**, see also (Saper *et al*., 1976; Elias *et al*., 1999). This biased projection of POMC neurons may indicate that sympathetic output is mainly controlled by the melanocortin system.

For the behavioral aspects, we studied regions implicated in the control of sleep and wakefulness since arousal is a necessary component for the execution of goal-oriented behavior such as feeding (Stellar, 1954; Swanson and Mogenson, 1981). The wake-promoting hypocretin/orexin neurons of the LHA (Bonnavion and de Lecea, 2010) have previously been identified as a prominent target for the ARC projection (Broberger *et al*., 1998a; Elias *et al*., 1998). Here, we also found a dense innervation of the histaminergic neurons of the tuberomammillary nucleus which play a similar physiological role (Vanni-Mercier *et al*., 1984; Haas and Panula, 2003). In contrast, innervation was sparse or absent in brainstem arousal system such as the serotonergic cells of the dorsal raphe, the cholinergic cells of the laterodorsal tegmentum and the nordadrenergic cells of the locus coeruleus (LC), suggesting that the ARC may primarily rely on hypothalamic system to recruit the arousal required to sustain food intake.

4.2 CELLULAR INTERACTION OF ARCUATE POMC AND NPY NEURONS

The competition between the anorexigenic POMC and orexigenic NPY exists not only in target nuclei, but also on a cell body level. In **Paper V**, we further examined the relationship between ARC POMC/αMSH and NPY/AGRP. In the ventromedial portion of the ARC, we observed that αMSH and AGRP-ir cell bodies are in close proximity of each other, however, no examples of double-labeled cell bodies were observed. AGRP-ir terminals were often seen in close apposition to αMSH-ir cell bodies with and without colchicine pretreatment, in line with earlier observations (Csiffary *et al*., 1990; Horvath *et al*., 1992; Broberger *et al.,* 1997; Fuxe *et al*., 1997). In contrast, no αMSH-ir terminals were observed on AGRP-ir cell bodies; though examples of putative POMC neurons auto-innervation were observed. Functionally, in **Paper III**, it was also shown that melanocortin agonists have little effect on the electrical properties of NPY neurons; similar results have been obtained by Roseberry et al. (Roseberry *et al*., 2004) who did, however, find a prominent hyperpolarization of

POMC neurons by NPY. This apparent unidirectional anatomical interaction may have a biological significance. This suggests that when NPY/AGRP neurons are active, there is a tonic inhibition of POMC cells, given that these cells also contain GABA as a transmitter (Horvath *et al*., 1997) and that the Y1 receptor expressed on POMC neurons (Fuxe *et al*., 1997; Broberger *et al*., 1997) is inhibitory (Herzog *et al*., 1992; Larhammar *et al*., 1992). Since there is no direct feedback mechanism from the POMC cells to disengage the NPY/AGRP neurons, this advocates that the feeding circuitry is wired to favor food intake.

4.3 EXPRESSION AND POTENTIAL ROLE OF CALCIUM-BINDING PROTEINS IN THE ARCUATE NUCLEUS

In **Paper IV**, we examined if CaBPs can be used as histochemical markers for specific ARC populations, similar to the way these proteins have been used to delineate populations in other brain regions, most notably cortical and striatal microcircuits (Celio and Heizmann, 1981; Celio, 1986, 1990). We focused on the distribution and co-localization pattern of three CaBPs: CB, CR, and PV. *In-situ* hybridization and IF revealed that CB, CR, and PV are all expressed in the ARC. Among these, PV was the CaBP found in the fewest number of ARC cells, in accordance with the very restricted expression of this protein in the hypothalamus reported previously (Celio and Heizmann, 1981; Celio, 1986, 1990). Notably though, these cells may represent a not previously described group of ARC neurons, as they did not co-localize with any of the markers included in the present study. With the exception of POMC neurons (see below), CR, and CB were also not found to co-localize with the neuronal markers included in the study, *i.e*. neurotensin, growth hormone releasing hormone, tyrosine hydroxylase, AGRP, galanin, dynorphin, enkephalin, and somatostatin. Although we used an extensive battery of antisera, expected to cover most of the known cell groups; it should be noted that this is a nucleus of great cellular heterogeneity (Everitt *et al*., 1986) and the complete repertoire of ARC peptides was not examined. The findings of the three CaBPs with relative little co-localization with the other ARC neuronal population was surprising, given that a lot of ARC populations have been already identified.

In other areas of the brain, such as the cerebral cortex, the CaBPs often identify distinct populations of interneurons (Celio, 1986; Kosaka *et al*., 1987; Rogers, 1987; Demeulemeester *et al*., 1988; Celio, 1990; Van Brederode *et al*., 1990). There is to date little morphological or physiological evidence for interneurons in the hypothalamus, using the classical definitions of such cells employed in "higher" brain regions. The traditional grouping of cells into projection/principal cells and interneurons may be less relevant in the hypothalamus where single cells may play both roles of "message provider" and "circuit organizer", respectively.

The functional role of CaBPs in ARC neurons is at present unclear. These proteins have been linked to the maintenance of intracellular Ca^{2+} homeostasis. For example PV is often found in fast-spiking neurons and may play a role in quickly restoring $[Ca^{2+}]_{intracellular}$ after the elevations that follow a train of action potentials (Freund *et al*.,

1992; Vreugdenhil *et al*., 2003). An interesting observation emerged from CaBP knockout mice, in which the absence of a specific CaBP is not compensated by another EF-hand family member (Schwaller, 2009). This indicates that neurons once designated to express a certain CaBP, are either incapable of turning on the expression of another EF-hand family member with similar Ca^{2+} binding properties or that the distinct properties of any other CaBP would not suffice to restore normal Ca^{2+} homeostasis (Schwaller *et al*., 2002). Future investigations will need to determine the relationship between CaBP expression and functional properties of ARC cells. It will also be of interest to see if CaBP expression changes under certain metabolic challenges.

4.4 DUAL ARCUATE POMC POPULATIONS

Interestingly in **Paper IV**, we observed that CB- and CR-like immunoreactivity is found in distinct groups of POMC populations (as shown by αMSH and CART staining). Recent accumulating evidence indicates that there exist several pools of ARC melanocortin neurons that can be differentiated based on several criteria. Thus, there is evidence for distinct GABAergic and glutamatergic melanocortin neurons (Hentges *et al*., 2009), and subgroups of POMC cells stain for the neuropeptide, pituitary adenylate cyclase-activating polypeptide (PACAP; Dürr *et al*., 2007), and cholinergic markers (Meister *et al*., 2006). Studies have shown that rostral ARC neurons project caudally to autonomic areas, whereas the more caudal ARC POMC project primarily within the hypothalamus (Swanson *et al*., 1980; Barker *et al*., 1989b; Elias *et al*., 1998; Elias *et al*., 1999). Moreover, the rostral ARC POMC neurons have been implicated in the response to insulin, while caudal ARC POMC cells display preferential sensitivity to leptin (Williams *et al*., 2010). The present findings identify another method for subdividing melanocortinergic neurons based on their expression of either CB or CR. It remains to be determined if this dichotomy correlates to the other means of differentiating POMC cells or if such divisions follow no obvious organizational principle.

4.5 VISUALIZATION OF HYPOTHALAMIC NPY NEURONS

One of the original goals– though one that was not met with success- for the study in **Paper IV** was to see if any CaBP could serve as a specific marker for NPY/AGRP neurons for *post-hoc* staining in electrophysiological experiments. Given the rapid axonal transport of the peptide messengers in these cells, these peptides themselves cannot be used for identification following intracellular or patch clamp recording, as colchicine treatment is not compatible with viability in recording. Yet, even under ideal circumstances of histochemical staining and visualization, *post-hoc* staining is very laborious and often requires recording from a large number of cells to yield a meaningful sample from the population of interest. An elegant solution to this problem is provided by mice genetically engineered to express fluorescent marker molecules such as GFP in specific molecularly defined neuronal populations (see Methodological considerations; (Spergel *et al*., 2001). A novel transgenic mouse expressing strongly fluorescent renilla GFP (rGFP) in NPY neurons was generated in **Paper III**, and this animal model was then used to map out the CNS populations expressing NPY in the

mouse and to determine the physiological characteristics and response to bombesin peptides in ARC NPY neurons.

Using immunohistochemistry, we validated that the rNPY-GFP mouse in **Paper III** faithfully express endogenous NPY. The rNPY-GFP shows similar physiological characteristics in line with other NPY-GFP lines in previous studies (Roseberry *et al*., 2004; Acuna-Goycolea *et al*., 2005). A comprehensive mapping of the rNPY-GFP illustrates that the distribution pattern mirrors that of the preproNPY mRNA expression from Allen Brain Atlas (www.brain-map.org), and agrees with previous NPY mapping studies done in rat (Chronwall, 1985; de Quidt and Emson, 1986). For example, the rNPY-GFP detected cells in the reticular thalamic nucleus, which is a population that is hard to identify with IF. Furthermore, rNPY-GFP can also distinguish the NPY population in the LHA and DMH, which is normally evident only under certain metabolic challenges and during development (Smith, 1993; Singer *et al*., 2000; Grove *et al*., 2003). Moreover, strongly fluorescent rNPY-GFP expression was found in olfactory ensheathing cells in the olfactory nerve, and in the nucleus of the solitary tract (de Quidt and Emson, 1986; Ubink *et al*., 1994).

The validity of the rNPY-GFP in the ARC was also confirmed using IHC stained with NPY, and αMSH. The rNPY-GFP co-localized with the NPY-LI, whereas it did not co-localize with the αMSH cell population in the ARC; PCR experiments corroborated this finding (**Paper III**). By comparing the distribution of NPY from IHC, and previous findings, the novel rNPY-GFP does faithfully express NPY.

4.6 EFFECT OF BOMBESIN-RELATED PEPTIDES ON NPY AND POMC NEURONS

As described in the Introduction, peripherally administered bombesin and associated mammalian peptides can provide a powerful satiety effect. In **Paper III**, the rNPY-GFP mouse was used for whole cell patch-clamp recordings in ARC slice preparations to investigate the potential response of NPY and POMC ARC neurons to BRP's. A powerful depolarization was elicited by application of bombesin, NMB and GRP, also at low doses. Through pharmacological isolation and ion substitution protocols, this response was found to involve activation on non-selective cation channels and the Na^+/Ca^{2+} exchanger. The excitatory actions of the BRPs was impressive not only for its amplitude (which was substantially larger than known depolarizing agents ghrelin and hypocretin/orexin), but also because the effect was similar on both the orexigenic ARC NPY neurons and the anorexigenic POMC neurons. The result is quite surprising given that most neuromodulators exert opposing effects on these populations. For example, ghrelin excites NPY neurons while inhibits POMC neurons (Cowley *et al*., 2003); the opposite has been reported for leptin (Cowley *et al*., 2001; Coll *et al*., 2007). This excitatory effect of BRPs on both ARC populations suggests that it might be implicated in a broad activation of the ARC homeostatic circuitry.

4.7 DISTRIBUTION AND EXPRESSION OF NUCB2/NESFATIN IN CNS

In **Paper I**, we studied the expression and distribution of NUCB2 mRNA and nesfatin-immunoreactive (ir) neuronal populations in the CNS using ISH and IF. The expression of NUCB2 mRNA corresponded rather well with the distribution of the nesfatin-ir cell bodies, although the IF signal was stronger and revealed more cell bodies than that of ISH. In the CNS, NUCB2/nesfatin expression was localized to distinct nuclei of the hypothalamus and to a restricted set of other brain regions. The expression of NUCB2/nesfatin was observed in neuronal populations that are traditionally implicated in energy metabolism (Fig 3). Accordingly, double IF revealed a co-localization of NUCB2/nesfatin with several neuropeptides implicated in the control of energy balance. For example, in the ARC, NUCB2/nesfatin co-localized with the POMC population, but not the NPY population. Functionally, i.c.v. injection of both melanocortins (Poggioli *et al*., 1986; Fan *et al*., 1997) and nesfatin-1 (Oh-I *et al*., 2006; Stengel et al., 2009b) leads to decreased food intake.

Outside of the hypothalamus, NUCB2/nesfatin can be observed in the thalamic parafascicular nucleus, the Edinger-Westphal nucleus (EW), LC, nuclei raphe obscurus and pallidus, NTS, and the IML. This distribution may hint at broader actions for NUCB2, but it should be noted that all stained nuclei have been implicated in the three aspects of adaptive state, *i.e.* behavior, endocrine (see below) and autonomic regulation (see Fig.3 and 4; Swanson and Mogenson, 1981). For instance, NUCB2/nesfatin-ir co-localized with MCH in neurons in the LHA and the LC, two nuclei implicated in arousal. Yosten *et al* (2009) recently showed that i.c.v. injection of nesfatin-1 fragment resulted in an increase in locomotor activity, follow by behavioral inactivity. Furthermore, the presence of NUCB2/nesfatin in various autonomic nuclei implies that it may be involved in adaptive stress response. Recent studies have shown that acute stress can trigger an increase in NUCB2/nesfatin expression the EW (Okere *et al*., 2010). Moreover, nesfatin-1 administration can increase the mean arterial pressure under stressful conditions (Yosten and Samson, 2009).

4.8 CO-LOCALIZATION OF NUCB2/NESFATIN WITH CART

One interesting observation from the double IF of NUCB2/nesfatin and neuropeptides in **Paper I** was the high degree of co-localization with CART throughout the CNS. These proteins have many shared features, such as: their anorexigenic effect; they are both cleaved from larger precursor proteins, and no receptor has been identified either. Possible functional implications underpinned by these similarities require further investigation.

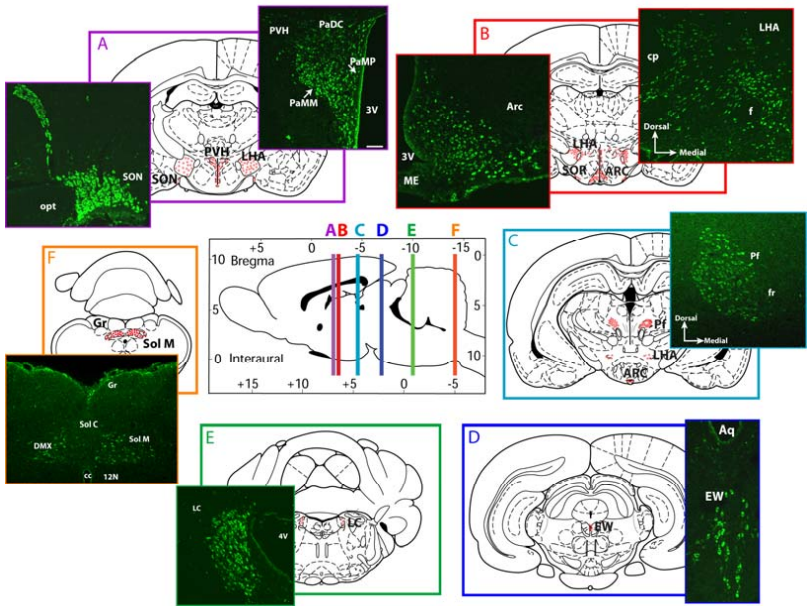

Fig 3: Schematic summary of the distribution of NUCB2/nesfatin-ir in the rat brain. Coronal templates of the rat brain taken from atlas of Paxinos and Watson, 2007. Drawings are arranged from rostral (A) to caudal (F) orientation.

4.9 ANOREXIGENIC ACTIONS OF NUCB2/NESFATIN

The mechanism by which NUCB2/nesfatin elicits satiety has been investigated in several studies. The data in **Paper I** showed the co-localization of NUCB2/nesfatin with OXY neurons in the PVH. Later studies have suggested that anorexia induced by NUCB2/nesfatin involves the PVH OXY pathway (Maejima *et al*., 2009). The study showed that NUCB2/nesfatin requires functional OXY receptors, since pre-treatment with an OXY receptor antagonist reversed the food and water intake effects of NUCB2/nesfatin, and abolish the anorexigenic effect of αMSH (Maejima *et al*., 2009; Yosten and Samson, 2010). The downstream target of the NUCB2/nesfatin OXY pathway might be the brainstem POMC neurons. Administration of nesfatin-1 has been demonstrated to activate the brainstem POMC population (Maejima *et al*., 2009; Shimizu et al., 2009). Double IF from **Paper I** showed that the NUCB2/nesfatin in the brainstem co-localized with CART; if this is also the POMC population remains to be determined. Taken together, the catabolic actions of NUCB2/nesfatin have been suggested to include activation of the melanocortin system which consequently stimulates the central OXY system resulting in the inhibition of food and water intake.

4.10 ENERGY EXPENDITURE EFFECTS OF NUCB2/NESFATIN?

The original study by Oh-I *et al*. (2006) focused on the regulation of food intake and showed that chronic infusion of nesfatin-1 decreases food intake and suppresses body weight gain over a 10 day period. However, while there was a slow desensitization of the anorexigenic effect during chronic nesfatin-1 treatment, the relative decrease in body weight gain during this period actually increased. This discrepancy may indicate that non-feeding effects, *e.g.* increased energy expenditure, may underlie the continued relative weight loss. Moreover, a study on the effect of single i.c.v. injection of nesfatin-1 revealed that nesfatin-1 does not modulate the 24hr cumulative food intake, yet still resulted in reduced body weight 24hr following the injection (Stengel *et al*., 2009a). In **Paper I**, we identified a series of novel areas involved in afferent and efferent autonomic control, including the parafascicular nucleus, the EW, caudal raphe, the NTS and the IML, in addition to the ARC, that express NUCB2. These histochemical findings may offer anatomical substrates for NUCB2 actions on fuel utilization in metabolic control.

4.11 NUCB2 SECRETED OR MESSENGER MOLECULE?

The subcellular distribution of NUCB2/nesfatin differed from that of any other feeding regulating molecules. Firstly, nesfatin-LI is absent from terminals, and primarily observed homogenously in the cytoplasm and proximal dendrites. Secondly, Oh-I *et al.* (2006) suggested that NUCB2 is cleaved into the three nesfatin fragments, based on the existence of pairs of basic amino acids that may form substrates for proteolytic processing. The molecular weight for the entire NUCB2 precursor is around that shown by Oh-I *et al* (2006). However, a western blot analysis from **Paper I** demonstrated that antiserum targeted against nesfatin-1 yields a major band at 43kDa, not dissimilar from 47.5kDa weight of the intact NUCB2 protein: a similar result was shown by Oh-I *et al* (2006) who also failed to detect an endogenous band at the predicted nesfatin-1 size of ca. 9.7 kDa. These findings suggest that NUCB2 might not be cleaved at the putative processing site into nesfatin-1, -2, and -3 as originally proposed. Moreover, antisera targeted against all three putative nesfatin fragments resulted in the same anatomical distribution pattern throughout the brain and pancreas (**Paper I**). Taken together, our data argue against further processing of NUCB2 and the secretory role of nesfatin fragments. As shown when it was first discovered, NUCB2 contains a signal peptide on the N-terminal, a DNA binding protein, putative cleavage sites, and two calcium-EF hands (Barnikol-Watanabe *et al*., 1994). It is therefore possible that NUCB2 functions as an intracellular signal molecule. This interpretation is not uncomplicated. A recent study using immunoelectron microscopy revealed that nesfatin-1-LI in PVH is localized in the secretory vesicles around the Golgi complex, and changes in electrical properties have been reported in magnocellular neurons and the ARC following application of nesfatin-1 fragment (Price *et al*., 2008a;b).

4.12 NUCB2/NESFATIN IN THE ENDOCRINE PANCREAS

In **Paper I**, we found a strong expression of NUCB2/nesfatin-1 in almost all hypothalamic neuroendocrine populations and in the anterior pituitary gland, which lead us to investigate the connection to the endocrine system in **Paper II**. Given the similarities between the signaling molecule repertoire in the brain and the pancreas, we anticipated the presence of NUCB2/nesfatin-1 in the islets of Langerhans. In pancreatic islets, NUCB2/nesfatin-LI was distributed uniformly over cell bodies and was absent in the nucleus; similar to the staining pattern observed in the CNS. Double IF in **Paper II** revealed that NUCB2/nesfatin was present exclusively in insulin expressing cells (β-cells) in both human and rat islets, but the subcellular staining pattern between the two peptides was partly non-overlapping. The slight discrepancy between the insulin and NUCB2/nesfatin-LI within the β-cell suggests that they might share the same cellular compartment. Electron microscopy may be required to determine the precise subcellular localization of NUCB2. The other islet cells did not contain NUCB2-LI. The finding of NUCB2-LI in insulin producing β-cells, lead us to investigate the relationship between the two proteins *in vivo*.

4.13 ISLET AND SERUM IMMUNOREACTIVE NUCB2/NESFATIN LEVEL

We measured the level of NUCB2-LI in isolated rat islet homogenates, islet perifusates and serum by enzyme immunoassay (EIA), in control Wistar and GK rats under different metabolic conditions. In islet homogenates, no difference was found between *ad-lib* feeding and fasting in Wistar control rats; this is in contrast to the hypothalamus where fasting decreases NUCB2 expression (Oh-I *et al*., 2006). In islets from GK rats, however, a significantly lower level of NUCB2-LI was seen compared to controls, which, intriguingly, normalized following fasting. The reason for the decreased NUCB2 is unclear at present, but a role in GK diabetogenesis cannot be ruled out. This is an all the more compelling issue, as levels were restored by fasting, considering that many metabolic parameters can be improved in diabetic patients by dietary modification.

We next investigated whether NUCB2 could be released from the endocrine pancreas by glucose challenge, since glycaemia is the main factor controlling the secretory activity of β-cells. Isolated islets from Wistar control and GK rats were incubated in two concentrations of ambient glucose, 3.3 and 16.7 mM. The experiment revealed that NUCB2-LI levels were modestly, but significantly, increased following glucose stimulation in control Wistar rats; no differences were observed in GK rats. This change (+23%) was, however, over a magnitude less than glucose-induced increase in release of the prototypical hormone produce of β-cells, insulin (+717%). This experiment thus suggests that pancreatic release of NUCB2, at least under hyperglycaemic conditions, is minor at most.

Finally, we performed an intraperitoneal glucose tolerance test (ipGTT) to measure the plasma NUCB2-LI concentration in control Wistar and GK rats. Both groups of animals showed statistically similar baseline levels of plasma NUCB2-LI, and a similar

NUCB2 response to glucose fluctuations. This finding argues against β-cells as a major contributor of plasma NUCB2, given that GK rats had decreased islet levels of this protein (see above). For both rat strains, the NUCB2-LI level decreased half an hour after i.p. glucose injection, and returned to basal level after two hours. Interestingly, the NUCB2-LI level was inversely correlated to blood glucose level. These data indicate that though plasma NUCB2-LI level may be unresponsive to fasting, it can be influenced by acute hyperglycaemia. The drop in NUCB2-LI that accompanied glucose injection is somewhat paradoxical in comparison to the anorexigenic role ascribed to the protein in the CNS; it may well be that NUCB2 has functionally distinct roles across organs. Given the current uncertainties summarized above regarding mechanism-of-action, the "what?" and "how?" of the physiological contribution of NUCB2 in metabolic control remain rather speculative.

Figure 4: The distribution of NUCB2/nesfatin is in shown to include nuclei which participate in all three output channels of metabolic control, *i.e.* behavioral, endocrine and autonomic modulation.

5 CONCLUDING REMARKS

In the work presented here, we show that the two antagonistic ARC populations, NPY/AGRP and POMC, have a very close neuranatomical interrelationship, in which they project widely throughout the CNS in a similar pattern. Noted exceptions are the melanocortin projections to the caudal autonomic control regions. The projection patterns also suggest that the ARC primarily contacts hypothalamic arousal centers to recruit the wakefulness necessary to sustain feeding behavior. On the cell body level, there appears to be a biased relationship, in the form of a unidirectional innervation from NPYergic neurons to the melanocortinergic neurons. Given that the ARC NPY neurons are difficult to visualize, a novel transgenic mouse expressing fluorescence Renilla GFP under the NPY promoter was generated. The validity of the rNPY-GFP mouse was confirmed by immunohistochemistry, and an extensive mapping of the NPY population in the CNS was performed. Using the rNPY-GFP mouse, electrophysiology experiments were conducted to examine the effect of appetite regulating peptide (bombesin) on the ARC NPY and POMC populations. Surprisingly, bombesin have a stimulatory effect on both ARC NPY and POMC neurons. The presence of several CaBPs was demonstrated in the ARC, and while these proteins showed relatively little coexistence with other known markers of ARC populations, differential expression of calretinin and calbindin-D28K could be used to differentiate two separate populations of POMC neurons. NUCB2/nesfatin is another CaBP, which has been implicated in the regulation of food intake. Based on the neuroanatomical distribution we describe, the protein may be involved in behavioral, autonomic, and endocrine regulation of energy balance, broadening the role from food intake alone. Moreover, our data suggests that NUCB2 may play an intracellular role, as opposed to acting as a secreted messenger. The distribution and expression of NUCB2 is not limited to the CNS, but it is also found in the insulin producing β-cell of the pancreas. Our data shows that the level of NUCB2 is lower in the β-cell of a T2DM animal model. While NUCB2 is released from β-cell under glucose stimulation, the level is significantly lower than the level of insulin release. Finally, NUCB2 can be detected in the plasma where it is influenced by glycaemic state. These data suggest a role in endocrine regulation which merits further investigation.

6 THERAPEUTIC IMPLICATIONS

Obesity poses a major threat to global health (WHO, 2000). While it is encouraging to note that the prevalence of adult obesity appears to have reached a plateau (Flegal *et al*., 2010), and may even be decreasing in children (Ogden *et al*., 2008), being overweight and its associated disorders presents a clinical challenge of staggering proportions. There are in fact fewer anti-obesity drugs on the market than there were five years ago, while more money has been invested into research for drug development. It is unclear whether any of the drugs currently on the market are clinically or economically cost-effective as a strategy for weight loss and long-term weight management. Diet regimes for the overweight have a very high relapse and failure rate.

The discovery of leptin a decade and a half ago (Zhang *et al*., 1994) raised hopes that this hormone could be used to treat obesity. Initially discouraging results (Heymsfield *et al*., 1999), coupled with the demonstration that obesity may represent a state of resistance to the anorexigenic effects of leptin (Maffei *et al*., 1995), however, dampened expectations. Yet, recent years have seen several promising applications of leptin substitution in leptin-deficient conditions such as lipodystrophy and hypothalamic amenorrhea (see Friedman, 2009). It may also be too early to abandon hope for its application in obesity; a sub-population of obese patients respond with significant weight loss to leptin injections (Heymsfield *et al*., 1999). Intriguingly, administration of a low dose of leptin during dieting may increase the chance to reach the weight goal and prevent relapse in obese patients by reversing some of the metabolic changes that occur during weight loss (Rosenbaum *et al*., 2002). The decrease in leptin level acts as a negative feedback signal which increase food intake and decrease energy expenditure, ultimately gaining back the lost weight (Rosenbaum *et al*., 2002).

Currently, bariatric surgical treatment is the most effective method for sustainable weight loss (Bueter *et al*., 2009) and has dramatic and immediate effects on improving hyperglycaemia and insulin sensitivity in obese diabetics (Pories *et al*., 1995). However, the inherent complications of operative procedures and anaesthesia, especially in the severely obese, limit the broad applicability of this procedure in weight management. The precise mechanism of the success behind the surgical treatment remains unknown, but it has been proposed that the procedure modulates the endogenous signals from the GI tract by elevating the level of satiety-inducing gut hormones (Bueter *et al*., 2009). Such hormonal changes have so far been difficult to pin down, but if successful may inform new pharmacological obesity therapies.

Specific in- and out-patient behavioral intervention therapies for obesity are showing early promise (Ford *et al*., 2010; McCrady-Spitzer and Levine, 2010). Behavioral intervention include dietary modification but also combating a sedentary lifestyle with a focus not just on increased exercise but also on thermogenesis associated with everyday activities, such as the fuel burned to maintain posture (Levine, 2004). However, long-term weight-loss through lifestyle modification is not easily accomplished, as anyone who has tried can attest to. Pharmacotherapy would thus be a very welcome adjuvant to the above-mentioned treatment strategies. What considerations are relevant for the development of such therapies?

The regulation of body weight is often thought of as a homeostatic system. This is likely only partly correct. A true homeostatic system maintains a controlled variable at a fixed value (Cannon, 1932). The objective of the body energy system, however, appears to be to conserve energy; forage for food in times of need; and accumulate energy in times of plenty. There has been little evolutionary pressure to reduce food intake once energy stores are filled up, or to burn off excess calories as heat due to the fact that there was a general shortage of food and that the lifespan was shorter. Therefore, this system is biased towards weight gain and storage of fat, with few mechanisms that encourage weight loss. Given the large number of potential signals involved in the regulation of energy balance, a complex integrating circuitry has evolved, with the hypothalamus playing a central role. In the heart of this circuitry lies the primary energy sensors in the ARC, which co-ordinate the metabolic needs to the demands of the internal milieu. However, the feeding circuit does not regulate within a narrow range, but it is rather an adaptable controller that adjusts to ever-changing environmental conditions.

The CNS contains multiple potential targets for the treatment of obesity. Although there are numerous peptides and neurotransmitters implicated in energy balance; they proved to be difficult to translate into viable drugs. One explanation is the substantial redundancy and compensation in the feeding circuitry, which may explain the normal body weight of the NPY knockout mouse model (Erickson *et al*., 1996). The biased nature of the homeostatic systems will restrict the efficacy of some of these approaches. There is also the challenge of developing orally bioavailable molecules that are agonists or antagonists acting on peptide receptors. Given that feeding is such an essential behavior, tampering with one component of this network might impinge on other homeostatic systems such as reproduction and sleep wake cycle, as well as the autonomic nervous system. Various anti-obesity drugs (*i.e.* Fen-phen, Rimonabant, Sibutramine) over the years have been taken off the market due to adverse cardiovascular and psychiatric side effects. Pharmacologically, a prospective drug target should take advantage of the body's own network and reinforce or manipulate the existing internal feedback signals to short circuit over feeding. It can be expected that more potential drug targets will be identified in the years to come, but pharmacological intervention alone will not suffice in the fight against obesity.

Any obesity therapy with aspirations on success will, however, need to take into account the fact that food intake in humans (and likely in most higher animals) is not a purely homeostatically driven process. In fact, it has been argued that under normal circumstances, with ready availability of high-calorie foods as is typical of modern society, the influence of homeostatic feedback is, at best, minor (see de Castro and Plunkett, 2002). While this observation may be true, it does not invalidate the importance of understanding the basic mechanisms of deficit-driven adaptive behaviour in metabolic control. Such mechanisms may also be relevant for anorexia, a common and deleterious condition that accompanies many inflammatory and neoplastic diseases and is often seen in the elderly. But knowledge of hypothalamic circuits will now need to be synthesized with greater knowledge of the non-homeostatic factors that drive human eating. Such factors include emotions, previous experience and the incentive stimulus value of various foods (Berthoud and Morrison, 2008). The feelings of satisfaction and pleasure generated by eating will in turn reinforce the compelling drive

to engage in this behavior again. Therefore, the reward value and aspect of certain enticing food for an individual should not be under estimated. Consequently, it is not surprising that the cortico-limbic systems which are responsible for generating that reward feeling can hijack the behavioral and metabolic effector mechanisms to dictate our food intake. The candidates which link the homeostatic system with the limbic system include leptin and insulin. Not only do they act on the ARC NPY and POMC neurons, they can also act directly on the mesolimbic dopamine neurons to modulate the "wanting" aspect of food (Figlewicz, 2003; Fulton *et al*., 2006; Hommel *et al*., 2006).Taken together, it is essential to understand how the metabolic need is converted into behavior and highlight the importance of crosstalk between homeostatic and reward systems involved in regulating food intake. An effective anti-obesity treatment should consist of combining pharmacological therapies with behavioral interventions.

A comprehensive strategy to combat obesity will also need to take into account the developmental aspects of this condition. It was first proposed by Barker (Barker *et al*., 1989a) that intrauterine conditions could have severe consequences on adult disease incidence. This appears to be particularly true in the metabolic realm; offspring to both obese or diabetic mothers have increased risk of inheriting these conditions later in life (Levin and Govek, 1998; Dabelea *et al*., 2000). Recent data have revealed that this is accompanied – and very possible caused – by changes in hypothalamic wiring (Bouret *et al*., 2004; Grayson *et al*., 2006; Glavas *et al*., 2010). Preventive measures at the prenatal and early postnatal stage may thus offer a low cost-high benefit strategy for combating metabolic disorders.

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