Peptides and proteins regulating food intake: a comparative view

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Abstract

Energy homeostasis is under multiple endocrine and neural control that involves both central and peripheral hormones and neuropeptides. Disorders of energy balance (e.g. *obesitas* and *anorexia nervosa*) are caused by subtle dysregulations of these regulatory mechanisms. The hypothalamic arcuate nucleus is a main site of central regulation, where two distinct subpopulations of neurons co-express either neuropeptide Y (NPY) and agouti related protein (AgRP) or pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART); the former set of peptides increase food intake, the latter decrease food intake and obviously affect energy metabolism. Key peripheral hormones affecting energy metabolism include cholecystokinin (CCK), leptin and insulin which decrease food intake and ghrelin that increases food intake. CCK and ghrelin regulate food intake on the short term (by affecting meal size), whereas leptin and insulin regulate food intake over longer periods spanning several meals. These signals and their physiology are reasonably well-understood in mammals. On the other hand, knowledge on energy metabolism in earlier vertebrates is scant. Recently characterized central food intake regulatory mechanisms in fish suggest that they operate in a manner similar to their mammalian counterparts. Peripheral mechanisms have been poorly studied outside mammals. The recent identification of leptin in several fish species provides new insights and opportunities to enhance our understanding of the regulation of food intake. Comparative analysis of these peripheral mechanisms may shed new light on the function and evolution of the mechanisms at the basis of energy homeostasis. In this review, we summarize the recent developments in understanding of the mechanisms and signals that regulate energy balance in mammals, and compare these to what we now know about their orthologues in earlier vertebrates, with a particular focus on bony fishes.

Keywords: food intake, energy balance, peptides, hormone, leptin.

Introduction

All animals have to eat. Homeostasis of energy reserves requires a careful balance between energy intake and expenditure (Schwartz *et al.* 2000). Whereas energy intake equals (mainly) food consumption and digestion, energy expenditure is the sum of basal metabolism, physical activity and (for endotherms) thermogenesis (Spiegelman *et al.*, 2001). The daily intake of energy can vary both within and between individuals, as a result of environmental and social factors. On the short-term, mismatches in energy balance may and will occur, but in a healthy animal, energy expenditure in the long-term accurately matches the energy intake over a several meals spanning period (Edholm, 1977). A striking example of this accuracy is given by Seeley and Woods (2003): for a standard human male, a surplus of 0.5% of the consumed energy per year would result in a yearly weight gain of 0.5 kg. As the average yearly increase of weight in the USA is less than 0.5 kg per adult, this illustrates that subtle dysregulations of the balance between energy input and expenditure are at the basis of the current obesity epidemic in the western society.

In this paper, the regulation of food intake and energy metabolism in mammals is compared to that of non-mammalian vertebrates, with an emphasis on fishes. Attention will be paid to central and peripheral factors that control energy homeostasis. This paper summarizes our contribution to the 12th Benelux congress of zoology held in October 2005. We do not intend to provide an exhaustive review on the regulation of food intake, as this topic has been reviewed elsewhere in great detail; we refer for in-depth reviews to Schwartz *et al.* (2000) and Seeley and Woods (2003). For an excellent review on regulation of food intake in fish we refer to Volkoff *et al.* (2005).

Over the years, two alternative viewpoints have existed on the identity of the main peripheral indicator of nutrient status that is perceived by the brain (and the arcuate nucleus (ARC) in the hypothalamus in particular); the lipostatic theory (postulated by Kennedy, 1953) and the glucostatic theory (postulated by Mayer, 1955). The former is based on the hypothesis that the ARC monitors the storage and metabolism of fat, the latter on the hypothesis that the ARC

monitors storage and metabolism of carbohydrates. The debate has focused on which of these hypotheses provides more insight into the regulation of energy homeostasis. Each theory has its imperfections and cannot fully explain the known variety in feeding behavior or the dynamic energy balance regulation. Seeley and Woods (2003) conclude that the lipostatic and glucostatic theory are not mutually exclusive, and should be appreciated in a coordinate manner. However, in the same review, the role of glucose is downplayed since neurons are generally protected for glucose fluctuations, as it would be catastrophic for neurons to run out of metabolic energy sources. To prevent this from occurring, a decrease in circulating glucose is quickly detected by the liver and the insulin secreting β -cells in the pancreas, resulting in an immediate increase in plasma glucose and this raises the question if under physiological circumstances glucostatic regulation plays a major role in the central regulation of energy homeostasis. More likely, depletion of glucose in the central nervous system (CNS) triggers an emergency feeding response, and in most situations, fluctuations in basal glucose levels would contribute minimally to the maintenance of energy sources (Seeley and Woods, 2003).

The lipostatic theory received more attention from researchers, ultimately resulting in the discovery of leptin [from the Greek word “leptos”, meaning “lean”] (Zhang *et al.* 1994), a signal molecule produced – in mammals – in peripheral adipose tissue. Leptin signals to the central nervous system, in support of the lipostatic theory. Some fifteen years before the discovery of leptin, insulin (from the Latin word “insula”; island) produced in the pancreatic β -cells, was one of the first hormonal signals that was implicated in the control of food intake by the CNS (Woods *et al.* 1979). Leptin and insulin both meet criteria for a candidate adiposity signal as both hormones circulate in quantities proportional to body fat content (Niswender *et al.*, 2004) and enter the CNS in proportion to their plasma level (Schwartz *et al.*, 2000). Furthermore, receptors for insulin and leptin are present in brain areas involved in the regulation of food intake and energy metabolism (Schwartz *et al.*, 2000). The question that remains is how the lipostatic and glucostatic pathways are integrated.

Central regulation

The central regulation of appetite and body weight is a complex phenomenon involving interactions between the CNS and the periphery. In the hypothalamus, the ARC plays a central role in this regulation, expressing orexigenic and anorexigenic peptides. The ARC contains two distinct populations of neurons, one which co-expresses neuropeptide Y (NPY) and Agouti-related protein (AgRP) (Broberger *et al.*, 1998) and is orexigenic, and the other which expresses Cocaine and Amphetamine Regulated Transcript (CART) and pro-opiomelanocortin (POMC; the precursor of α -Melanocyte Stimulating Hormone [α -MSH]) (Elias *et al.*, 1998) and is reciprocally anorexic. The NPY⁺/AgRP⁻ and the CART⁺/POMC⁺-neurons receive multiple inputs from the periphery (Schwartz *et al.*, 2000).

Orexigenic

NPY

NPY is one of the most potent orexigenic peptides known in mammals and is expressed in key areas in the hypothalamus involved in the regulation of food intake (Halford *et al.*, 2004) including the paraventricular nucleus (PVN) and ARC (Kalra *et al.*, 1999). The orexigenic actions of NPY have been investigated thoroughly over the past decades. Injection of NPY into the third ventricle of the brain or directly into the hypothalamus increases food intake in rats (Clark *et al.*, 1985 and 1987). As is expected from a physiological signal, the rise in food intake is dose dependent, with an optimum and a decrease of efficiency at high doses (Clark *et al.*, 1987). Rats are nocturnal animals that feed predominantly in the dark. Continuous central infusion of NPY during the day light period reproduced the characteristic nocturnal feeding behavior in rats, and food intake, frequency of feeding and eating rate (g/min) were affected in a dose-dependent manner (Kalra *et al.*, 1988). Immunoneutralization of NPY blocked feeding behavior in rats (Dube *et al.*, 1994) and NPY levels increased in response to fasting and decreased upon refeeding

(Sahu *et al.*, 1988). NPY receptor (Y_1 receptor) mRNA expression was augmented in response to fasting (Xu *et al.*, 1998).

Intracerebroventricular (i.c.v.) administered NPY induces lipogenic enzymes in the liver and white adipose tissue (Billington *et al.*, 1991), thereby increasing anabolic activity. This seems a sensible approach considering that a rise in NPY-levels reflects a reduction in energy reserves, and thus the need to preserve remaining energy stores as well as increase energy consumption. Indeed, peripheral adiposity signals (leptin; insulin) decrease NPY mRNA expression (Stephens *et al.*, 1995; Schwartz *et al.*, 1996) and an absence of peripheral signals like leptin and insulin increases NPY mRNA expression and secretion (Kalra *et al.*, 1991).

Surprisingly, NPY knock-out mice show essentially normal body weight regulation (Erickson *et al.*, 1996; Ste. Marie *et al.*, 2005). One explanation for this phenomenon is that compensatory mechanisms, either cell-autonomous or circuit-based, are engaged in the absence of NPY. Since NPY producing cells in the ARC also produce AgRP (orexigenic), AgRP could mediate cell-autonomous compensatory mechanisms. However, mice lacking both NPY and AgRP also have normal body weight regulation (Qian *et al.*, 2002), although these mice fail to respond to the orexigenic effects of ghrelin (Chen *et al.*, 2004). Also, the discovery that NPY⁺/AgRP⁺-neurons produce GABA (Horvath *et al.*, 1997), leaves open the possibility of GABA-mediated, cell-autonomous compensation. In addition to cell-autonomous compensation, circuit based compensation may underlie the normal phenotype of NPY null mice. E.g., a decreased α -MSH/CART signaling may compensate for the absence of the orexigenic NPY signal (Schwartz *et al.*, 2003).

Besides direct effects on food intake and body weight regulation, NPY is known to stimulate appetitive behavior (Ammar *et al.*, 2000; van Dijk and Strubbe, 2003). I.c.v. NPY infusion stimulated the responses to obtain food, but inhibited those used to consume food (Ammar *et al.*, 2000). Direct infusion of NPY into the PVN of Wistar rats stimulated feeding behavior during the early part of the light phase (van Dijk and Strubbe, 2003).

NPY has been detected and characterized in several fish species, including goldfish (*Carassius auratus*), European sea bass (*Dicentrarchus labrax*) (Cerdá-Reverter *et al.*, 2000), rainbow trout (*Oncorhynchus mykiss*) (Doyon *et al.*, 2003) and common carp (*Cyprinus carpio*) (accession number Q9DGG7) and the actions of NPY in fish resemble those in mammalian species. Central administration of homologous and heterologous NPY in fish species increases food intake in a dose-dependent manner (Lopez-Patino *et al.*, 1999; Silverstein *et al.*, 2000) and starvation for several days induces an increase in hypothalamic NPY expression (Silverstein *et al.*, 1999; Narnaware *et al.*, 2001). Refeeding reverses the effects of starvation on NPY peptide levels and mRNA expression (Narnaware *et al.*, 2000; 2001).

Agouti and Agouti Related Protein

The agouti lethal (A^Y/a) phenotype in the mouse displays a characteristic yellowish fur compared to wild-type (black/brown) mice, in combination with hyperphagia and obesity. This phenotype is the result of ubiquitous expression of the agouti gene (designated agouti signaling protein – ASP – in species other than mouse), among others resulting in hyperphagia, hyperinsulinemia and increased linear growth (Michaud *et al.*, 1993; Miller *et al.*, 1993). The yellow fur color is the direct result of the blocking of the melanocortin 1 receptor (MC1R) by agouti, preventing α -MSH induced melanization. The obesity associated with A^Y/a -mice results from agouti antagonizing α -MSH signaling in the hypothalamus at the MC4R. A second, agouti related protein (AgRP) which is co-expressed with NPY in a population of neurons in the ARC (Hahn *et al.*, 1998) was subsequently discovered as the endogenous antagonist at the MC3R and MC4R. This AgRP inhibits the anorexigenic effects of α -MSH (MacNeil *et al.*, 2002) which are described elsewhere in this review.

Orthologues of both agouti and AgRP have been identified in fish. AgRP has been characterized in several fish species, *viz.* goldfish (Cerdá-Reverter *et al.*, 2003), zebrafish (*Danio rerio*) (Song *et al.*, 2003) and pufferfish (*Takifugu rubripes*) (Klovins *et al.*, 2004). Fasting up-regulates

AgRP mRNA levels in the hypothalamus of goldfish and zebrafish (Cerdá-Reverter *et al.*, 2003; Song *et al.*, 2003).

Recently, ASP, an agouti orthologue, was characterized in the goldfish (Cerdá-Reverter *et al.*, 2005). Goldfish ASP is expressed in the skin on the ventral, but not the dorsal side of the body. Furthermore, it was demonstrated that goldfish ASP antagonized α -MSH binding and activation on the puffer MC1R and the goldfish MC4R and blocked α -MSH induced melanophore dispersion in the medaka (*Oryzias latipes*) melanophore. These data elegantly provide a visualization of the antagonistic networks which also play a major role in the regulation of energy balance. It is striking that two very distinct functions (regulation of skin color and feeding) are carried out by related signaling systems.

Anorexigenic

CART

CART peptide was first identified in the 1980s in ovine hypothalamic extracts (Spiess *et al.*, 1981). Douglass *et al.* (1995) demonstrated that CART mRNA was upregulated after administration of psychostimulant drugs such as cocaine and amphetamine (giving CART its name). Although involvement of CART in activation of the hypothalamus-pituitary-adrenal-axis was recently demonstrated (Smith *et al.*, 2004), CART is best known as an anorectic peptide. The mechanism by which CART evokes its anorectic actions is poorly understood, owing largely to the fact that no receptor has been identified for CART.

I.c.v. administration of CART antibodies increased feeding in rats (Lambert *et al.*, 1998; Kristensen *et al.*, 1998) and i.c.v. injection of recombinant CART prevented feeding (Kristensen *et al.*, 1998), and reversed feeding induced by NPY infusion. Receptors for leptin are present on CART⁺-neurons (Elias *et al.*, 1998). Larsen *et al.* (2000) showed that continuous i.c.v. administration of CART decreased feeding and body weight in a dose-dependent fashion, although the effects were limited to the first four to five days of treatment. This may imply a

short-term role in feeding behavior for CART peptides. Aja *et al.* (2001) show that i.c.v. injection of CART in the lateral ventricle decreases feeding behavior and thus food intake. I.c.v. administration of CART in the fourth ventricle (hindbrain) reduces feeding behavior (Zheng *et al.*, 2001; Aja *et al.*, 2002) and produces taste aversion conditioning (Aja *et al.*, 2002). These observations suggest that CART works at at least two sites: the hypothalamus and the hindbrain.

Goldfish have two CART peptide precursors, pro-CART I and pro-CART II (Volkoff *et al.*, 2001) and both have a widespread distribution in the brain, pituitary and several peripheral tissues including gonads and kidney. No data are yet available of i.c.v. administration of goldfish CART, but i.c.v. injection of human CART (both CART₆₂₋₇₆ and CART₅₅₋₁₀₂, which are two different mature CART peptides derived from the same protein precursor) results in decreased food intake (Volkoff *et al.*, 2000). Food deprivation and refeeding increase CART mRNA two hours after a meal for pro-CART I, whereas pro-CART II expression shows no changes in the brain, although food deprivation induces a decrease of pro-CART II in the olfactory bulbs (Volkoff *et al.*, 2000).

POMC (α -MSH)

The melanocortin peptides comprise adrenocorticotrophic hormone (ACTH) and the melanocyte-stimulating hormones (α -, β - and γ -MSH). They are derived from the hormone precursor pro-opiomelanocortin (POMC) (For a detailed review we refer to Does *et al.*, 2005) by processing by prohormone convertases 1 and 2 (PC1 and PC2). Tissue-specific expression of PC's leads to ACTH production in the corticotrope cells and α -MSH in the melanotrope cells of the pituitary (Castro *et al.*, 1997). These peptides are involved in steroidogenesis and skin-pigmentation respectively (for a review on melanocortins see: Metz *et al.*, 2006 in press). Since these first observations, many other physiological functions were discovered, including regulation of feeding and energy homeostasis (Panksepp *et al.*, 1976). Indeed, POMC⁺ neurons in the ARC produce α -MSH (Elias *et al.*, 1998) in rats. The physiological basis for these effects is attributed to

the melanocortin receptor 4 (MC4R). Targeted disruption of MC4R in mice led to increased food intake and body weight which is not reduced after administration of a synthetic MCR agonist (Huszar *et al.*, 1997; Marsh *et al.*, 1999). The MC3R is expressed in many brain areas, and MC3R deficient mice show increased fat mass, increased leptin and insulin plasma levels.

The melanocortin system is tightly linked to other systems involved in food intake and energy balance. Melanocortins and NPY have opposite effects on food intake and NPY has an inhibitory effect on POMC expression (Rahmouni *et al.*, 2001).

POMC⁺ neurons express the leptin receptor (Cheung *et al.*, 1997) and leptin administration increases POMC expression in the ARC nucleus (Thornton *et al.*, 1997) and decreases food intake, and results in weight loss (Neary *et al.*, 2004). On the other hand, white fat – which is the major tissue that produces leptin – expresses at least two MCRs, *viz.* MC2R and MC5R (Chhajlani, 1996) which play a role in lipolysis. The ligands for these receptors, ACTH and α -MSH respectively, have potent lipolytic effects (Boston, 1999; Brennan *et al.*, 2003).

Although the melanocortin system in fish is well characterized (for a detailed review of the melanocortin system in fish, we refer to Metz *et al.*, 2006 in press), little is known about the role of α -MSH in control of food intake in fish. Cerdá-Reverter *et al.* (2003a) demonstrate MC4R expression in key areas for food intake control in the goldfish brain. In contrast to mammals, the MC4R in fish is also expressed in the periphery, including spleen, gill and head kidney (Haitina *et al.*, 2004). This indicates that the MC4R may have additional functions in non-mammalian species compared to mammals. I.c.v. administration of MC4R agonists and antagonists resulted in inhibition or stimulation of food intake, respectively, in 24 hours fasted goldfish. Fasting does not modify hypothalamic POMC mRNA expression in the goldfish (Cerdá-Reverter *et al.*, 2003b). These results suggest that POMC⁺ neurons exert a tonic inhibitory effect on food intake, which can be overruled by MC4R antagonists, such as AgRP. However, as POMC is produced at several other nuclei, including the NPO, where it is processed into ACTH (Metz *et al.*, 2004), the

assessment of POMC gene expression may in this particular case be a poor indicator of the role of α -MSH on food intake.

CRH

The Corticotropin Releasing Hormone (CRH) system comprises a family of peptides (CRH, urocortins I, II and III, urotensin I (in fish) and the amphibian peptide sauvagine), two CRH-receptors and a CRH binding protein (CRH-BP). The CRH system is primarily recognized for its key role in the endocrine regulation of the stress response via the HPA-axis throughout vertebrates (Bale *et al.*, 2004; Huising *et al.*, 2004).

However, extra-HPA activities have also been reported, including regulation of energy balance and food intake in both mammals and fish (Richard *et al.*, 2000; Bernier *et al.*, 2001; Bernier, 2006). Chronic i.c.v. administration of CRH prevents further weight gain in the obese Zucker rat (Rohner-Jeanrenaud *et al.*, 1989). CRH₆₋₃₃, a CRH fragment that preferentially binds to CRH-BP and antagonizes its action reduces weight gain in the obese *fa/fa* Zucker rats (Heinrichs *et al.*, 1996) and decreases food intake in *ob/ob* (leptin deficient) mice (Heinrichs *et al.*, 2001), suggesting a role for CRH-BP in energy homeostasis, probably by modulating the anorectic actions of CRH and CRH paralogs. A link between leptin and CRH has been suggested by Arvaniti and co-workers (2001), as they demonstrate that in obese mice, leptin decreases CRH mRNA expression in the PVN.

CRH has been identified in several fish species, including common carp (Huising *et al.*, 2004), goldfish (Bernier *et al.*, 1999) and Mozambique tilapia (*Oreochromis mossambicus*) (Van Enkevort *et al.*, 2000). The CRH-like peptides urotensin I (Lederis *et al.*, 1982; Barsyte *et al.*, 1999), orthologues of urocortin II and urocortin III (Lewis *et al.*, 2001; Boorse *et al.*, 2005), the receptors CRH-R1 and CRH-R2 (Huising *et al.*, 2004; Cardoso *et al.*, 2003) and CRH-BP (Huising *et al.*, 2004; Doyon *et al.*, 2005) complete the CRH system in fish. In fish, as in other vertebrates,

CRH is the dominant hypothalamic hormone controlling the stress axis (Wendelaar Bonga, 1997; Huising *et al.*, 2004; Flik *et al.*, 2006).

In goldfish, i.c.v. injections of CRH and UI dose-dependently suppress food intake, with UI being more potent than CRH (De Pedro *et al.*, 1993; Bernier *et al.*, 2001). These effects can be reversed by pre-treatment with the CRH-R antagonist (α -helical CRH₉₋₄₁) (De Pedro *et al.*, 1997; Bernier *et al.*, 2001). These results indicate that the CRH system is involved in the regulation of food intake in fish.

Physiologically, the stress-related and the energy homeostatic effects of CRH may be linked; in socially subordinate rainbow trout, food intake is decreased compared to that in dominant individuals, possibly as a result of an increase in CRH mRNA levels in the *nucleus preopticus* (NPO) (Doyon *et al.*, 2003). This seems reasonable, as the most important objective for a stressed animal is to cope with the stressor, and energy consuming processes such as reproduction and the search for food will be temporarily arrested. So, CRH may activate the HPA axis, and simultaneously decrease feeding behavior when the organism is stressed.

Integration of hypothalamic signals

[Figure 1 in this paragraph]

The integration of all peripheral information regarding energy supplies is mediated by a highly complex hypothalamic circuit that consists of several hypothalamic nuclei including the PVN, perifornical area (PFA) and the lateral hypothalamic area (LHA), which are richly supplied by axons from ARC NPY⁺/AgRP⁺ and POMC⁺/CART⁺ neurons (Elmqvist *et al.*, 1998; 1999). Neurons in the PVN produce several neuropeptides that reduce food intake and body weight when administered centrally, such as CRH, thyrotropin-releasing hormone (TRH) and oxytocin (Kow *et al.*, 1991; Verbalis *et al.*, 1995). On the other hand, the LHA and PFA produce several food intake increasing neuropeptides, such as melanin-concentrating hormone (MCH) (Qu *et al.*, 1996) and orexins A and B respectively (de Lecea *et al.*, 1998; Sakurai *et al.*, 1998). The fact that

both ARC cell types project to the PVN, has led to the suggestion that neurons in the PVN, that express the receptors for both NPY and α -MSH, can detect and integrate orexigenic (NPY, AgRP) and anorexigenic (melanocortins) signals (Cowley *et al.*, 1999). The hypothalamic signals are integrated in the hindbrain, PVN signals stimulate, and PFA and LHA signals inhibit the termination of individual meals respectively (Schwartz *et al.*, 2000).

CART influences metabolic rate via thyroid hormone; CART releases TRH from hypothalamic explants (Stanley *et al.*, 2001) and inhibits suppression of TRH in the PVN of fasted rats (Fekete *et al.*, 2000). Conversely, hyperthyroidism decreases expression of CART in the PVN (Lopez *et al.*, 2002), establishing CART as a mediator of metabolic regulation.

α -MSH modulates thyroid hormone levels and fat metabolism. The MC4R is expressed on TRH-producing cells in the PVN (Fekete *et al.*, 2000) and α -MSH has a stimulatory effect on preproTRH gene transcription and thus α -MSH increases levels of thyroid hormone via the hypothalamus-pituitary-thyroid (HPT) axis. Thereby, α -MSH increases metabolic activity when the animal is fed and decreases metabolic activity when the animal is fasted, which is a crucial survival mechanism.

Peripheral regulation of food intake and body weight

To achieve energy homeostasis, meal size and meal frequency are regulated. Both the initiation of a meal, and the termination of a meal are biologically controlled processes. Whereas the endocrine initiation of a meal has only recently been established and is controlled by ghrelin (Tshöp *et al.*, 2000; Lawrence *et al.*, 2002; Chen *et al.*, 2004), termination of a meal (through satiation/satiety) is a process known for several decades and is controlled by CCK (Gibbs *et al.*, 1973). Ghrelin is released upon the absence of nutrients in the stomach and activates the NPY⁺/AgRP⁺ neurons in the ARC (Chen *et al.*, 2004) and thus food intake. CCK is released upon stimulation by nutrients in the stomach and is transmitted by vagal efferents to the

hindbrain (Moran and Kinzig, 2004; Little *et al.*, 2005). Ghrelin and CCK will be discussed in the next paragraphs.

Regulation of body weight is achieved through regulation of food intake (and the regulation of energy expenditure). Information regarding prevailing energy reserves is transmitted to the CNS by at least two signals, *viz.* leptin and insulin. These hormones both circulate in proportion to body fat, cross the blood-brain-barrier, and activate the POMC⁺/CART⁺ neurons and inactivate the NPY⁺/AgRP⁺ neurons. Since leptin and insulin signal information regarding body fat (*i.e.* energy stores) these hormones may be considered as peripheral regulators of body weight and energy reserves. Both hormones will be discussed below in addition to CCK and ghrelin, which are important hormones for the regulation of food intake.

Food intake regulation

CCK

Cholecystokinin (CCK) is found in both the brain and the gastrointestinal tract (Moran and Kinzig, 2004). In the brain, CCK functions as a neurotransmitter and has effects on diverse behaviors including satiety (Crawley and Corwin, 1994). Within the gastrointestinal tract, CCK is found in neural and endocrine cells. Endocrine cells containing CCK (I-cells) are stimulated by intestinal nutrients and secrete their product in the blood and/or surrounding tissue (Moran and Kinzig, 2004). CCK is also present in enteric vagal afferent neurons where it acts as a neurotransmitter (Larson and Rehfeld, 1979).

CCK comes in multiple molecular forms (Rehfeld, 1981; Eberlein *et al.*, 1992; Rehfeld, 1998), classified according to the number of amino acids they contain, which are all derived from a 95 amino acid proCCK (Rehfeld, 1998).

Two types of CCK receptors have been identified to date, originally named CCK-A (alimentary type) and CCK-B (brain type), according to their anatomical distribution (Moran *et al.*, 1986; Wank *et al.*, 1992; Little *et al.*, 2005). More recently, it has been established that there is

overlap in their anatomical distribution and the CCK-A and CCK-B receptors are renamed CCK₁ and CCK₂ receptors respectively (Moran and Kinzig, 2004; Little *et al.*, 2005). The CCK₁ receptor is expressed in several brain areas involved in the regulation of food intake, including the nucleus tractus solitarius (NTS) (Moran *et al.*, 1986; Hill *et al.*, 1987), and has high (adapted to respond to low concentrations of CCK; pM range) and low (adapted to respond to high concentrations of CCK; nM range) affinity binding sites (Pandya *et al.*, 1994; Little *et al.*, 2005). In rats, the inhibitory effect of CCK on food intake has been shown to be mediated by activation of the low affinity binding sites of the CCK₁ receptor (Weatherfort *et al.*, 1993). This has a major implication, as it is unlikely that postprandial concentrations of CCK occur in the range of nM. This suggests that the satiety effects of CCK are not endocrine, but must reflect higher CCK concentrations at local sites of action (Moran and Kinzig, 2004).

In their landmark study, Gibbs and co-workers (1973) demonstrated that intraperitoneal administration of the sulfated octapeptide of CCK (CCK-8) dose-dependently decreased intake of solid and liquid food in rats. CCK-8 reduced both the size, and the duration of a meal. CCK affects food intake rapidly, and the duration of the inhibition is relatively short. Therefore, CCK reduces single meal size, but does not affect total daily intake because reduced meal size is compensated by an increase in the number of meals (West *et al.*, 1984).

CCK immunoreactivity has been detected in several fish species, including Atlantic cod (Jonsson *et al.*, 1987) and goldfish (Himick and Peter, 1994). CCK mRNA have been shown in several fish species, including goldfish, rainbow trout (Jensen *et al.*, 2001), pufferfish and flounder (Kurokawa *et al.*, 2003).

Fish possess different CCK-8 peptides that differ in amino acid number six, counting from the C-terminus. Although CCK-8 is the major product of posttranslational processing of CCK, other CCK forms (e.g. CCK-7, CCK-21) have been detected in rainbow trout, which are all fully sulfated (Johnsen *et al.*, 1998; Jensen *et al.*, 2001).

In fish, CCK is released when food is present in the gastrointestinal tract (Aldman and Holmgren, 1995). CCK has been shown to suppress food intake in fish; i.c.v. and i.p. injections of sulfated CCK-8 in goldfish decrease food intake (Himick and Peter, 1994; Volkoff *et al.*, 2003) and administration of CCK antagonists increase food intake in rainbow trout (Gélineau and Boujard, 2001). CCK mRNA increases 2 hours postprandially in the goldfish brain (Peyon *et al.*, 1999).

Ghrelin

Ghrelin is a 28 amino acid peptide that is predominantly produced in the stomach and was identified as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R) in 1999 by Kojima and co-workers (Kojima *et al.*, 1999) (“ghre” is the Proto-Indo-European root for “grow”). It contains a unique fatty acid side-chain on the third amino acid residue which is necessary for most of its biological activity. In human, this modification is an octanoyl (C8) side-chain (Kojima *et al.*, 1999).

Ghrelin-immunoreactive cells are found predominantly in the stomach, but also in the duodenum, ileum, colon, hypothalamus and pancreas (in the newly identified epsilon (ε) cells). In the intestine, ghrelin-immunoreactivity decreases from the duodenum to the colon (Kojima *et al.*, 2005).

Ghrelin controls energy homeostasis by increasing food intake (Tschöp *et al.*, 2000; Nakazato *et al.*, 2001; Lawrence *et al.*, 2002) and is a potent peripheral orexigenic hormone.

Although ghrelin is widely regarded as a peripherally expressed peptide, in a recent paper by Cowley *et al.* (2003), ghrelin expression is detected in previously uncharacterized hypothalamic neurons that are adjacent to the third ventricle between the dorsal, ventral PVN and ARC. In the ARC, these ghrelin-expressing neurons project to the NPY⁺/AgRP⁺-neurons to stimulate the release of these orexigenic peptides and to the POMC⁺/CART⁺-neurons to suppress the release

of these anorexigenic peptides. Although identified as a peripheral orexigenic signal, ghrelin in the hypothalamus is positioned such that it can regulate food intake directly.

Ghrelin has been identified in several teleost fish species, including goldfish (Unniappan *et al.*, 2002), Mozambique tilapia (Kaiya *et al.*, 2003a), Japanese eel (*Anquilla japonica*) (Kaiya *et al.*, 2003b), and rainbow trout (Kaiya *et al.*, 2003c) with high expression levels in the stomach(/gut) and moderate levels in the brain. Fish ghrelins come in multiple forms that vary in length, in the specific fatty acid modifications on the third amino acid (octanoyl; C8 or decanoyl; C10) and in the presence of an amide structure at the C-terminal end (Kaiya *et al.*, 2003a; 2003b; 2003c).

Recent studies demonstrate that similar to mammals, ghrelin in fish is involved in feeding and energy homeostasis (Unniappan *et al.*, 2002; Riley *et al.*, 2005). Both central and peripheral injections of homologous ghrelin in goldfish stimulate food intake (Unniappan *et al.*, 2002). A postprandial decrease in preproghrelin mRNA expression in the hypothalamus and gut and a subsequent decrease in serum levels of ghrelin further support the orexigenic actions of ghrelin in goldfish (Unniappan *et al.*, 2004). In tilapia, long-term treatment by osmotic mini pump (21 days) of homologous ghrelin-C8 and ghrelin-C10 result in different effects (Riley *et al.*, 2005). Whereas food consumption, fat deposition, liver weight and total fat content in the liver increased, and plasma IGF-I decreased in animals that received ghrelin-C10, ghrelin-C8 did not affect any of these parameters. Ghrelin-C8 did increase GH mRNA expression in the pituitary (although no longitudinal growth was observed between the ghrelin treated groups and the controls), suggesting differential functions for ghrelin-C8 and ghrelin-C10, perhaps via different ghrelin receptor isoforms. Furthermore, these data suggest that the orexigenic actions of ghrelin are independent of its ability to increase plasma GH levels (Riley *et al.*, 2005). More work is needed to characterize the ghrelin receptor isoforms in the tilapia. The fact that ghrelin-C10 is the major circulating form of ghrelin in tilapia (Kaiya *et al.*, 2003a) may reflect a more important role in this species for ghrelin in energy homeostasis than its GH releasing role, although ghrelin-C8 expression may be restricted to hypothalamic nuclei; indeed a low ghrelin mRNA expression is

detected by RT-PCR in the brain (Kaiya *et al.*, 2003a, b, c). Contrasting mammalian studies, peripheral ghrelin-C10 in tilapia decreases plasma levels of IGF-I (Riley *et al.*, 2005). This finding suggests that ghrelin may possess distinct physiological functions in fish (ectotherms) that differ from those seen in mammals (endotherms) where the allocation of energy might be different.

Recently, Iqbal *et al.* (2006) demonstrate that injection of ovine ghrelin into the third ventricle is unable to stimulate voluntary food intake in sheep, in contrast to the other mammalian species investigated so far. This finding is even more puzzling, since a rise in ghrelin plasma levels is seen in sheep before feeding (Sugino *et al.*, 2002; 2004), as in other mammalian species (Tschöp *et al.*, 2000; Cummings *et al.*, 2003). The different response to ghrelin between sheep and other mammals may lie in the fact that sheep are ruminants, whereas the other mammals studied (rat, mice and human) are monogastric. In sheep, ghrelin is produced in the abomasum (Hayashida *et al.*, 2001) (the fourth stomach of ruminants) in contrast with monogastric species, where ghrelin is produced in the proximal part of the stomach. The rumen (first stomach) is not emptied between meals and these ruminant characteristics may underline the differences observed in ghrelin effects in these species. Iqbal *et al.* (2006) observe a postprandial rise in GH levels, and it is likely that ghrelin (that was initially discovered as an inducer of GH secretion) regulates central GH secretion.

Body weight regulation

Leptin

Three decades ago, parabiosis experiments (where two mice or rats are surgically joined to allow the passage of circulating signals) with *ob/ob* mice (a mutant genotype marked by intense hyperphagia and obesity) and wild-type littermates suggested the absence of a circulating factor in the *ob/ob* mice responsible for the hyperphagia and obesity (Coleman *et al.* 1969; 1973).

Indeed, twenty years later, Zhang and co-workers (1994) demonstrated that the hyperphagia and obesity observed in *ob/ob* mice resulted from a recessive mutation of the *obese*

gene. This gene encodes a unique member of the class-I α -helical cytokine family, a 16 kDa protein which was named leptin and is secreted (mainly) by white adipose tissue. This makes leptin a perfect candidate to inform the brain about nutritional status and reserves. Indeed, leptin is shuttled across the blood brain barrier by a truncated form of the leptin receptor (Kastin *et al.*, 1999) and leptin receptors are present on the NPY⁺/AgRP⁺-neurons and the POMC⁺/CART⁺-neurons (Schwartz *et al.*, 2000; Leibowitz *et al.*, 2004) and this suggests that leptin affects food intake by simultaneously stimulating anorexigenic factors and inhibiting orexigenic factors. In-vivo experiments confirm that this is indeed true as administration of leptin, both peripherally and centrally, reduces food intake in mammals. Even more importantly, leptin is secreted into the bloodstream in proportion to the total amount of body fat (Schwartz *et al.*, 2000; Niswender *et al.*, 2004), and is therefore a reliable indicator of available energy reserves.

Until the recent identification of a cDNA encoding leptin in pufferfish (Kurokawa *et al.*, 2005) and duplicate leptin genes in common carp (Huisling *et al.*, 2006), leptin had only been isolated in mammals. Kurokawa *et al.* also performed a database search and found leptin in several other fish species (Medaka, Atlantic salmon (*Salmo salar*) and freshwater puffer; *Tetraodon nigroviridis*) and amphibians (*Xenopus tropicalis* and *Ambystoma tigrinum*). The deduced amino acid sequence of puffer leptin is only 13.2% identical to human leptin. In fact, leptin is one of the class-I helical cytokines with the poorest primary sequence conservation throughout vertebrates (Huisling *et al.*, 2006). However, the conservation of a pair of cysteine residues that forms a disulphide bond, conservation of gene structure, phylogenetic analyses and a predicted conservation of tertiary structure (four α -helices) between fish and mammalian leptin confirms that fish leptins are true orthologues to leptin of mammalian species (Kurokawa *et al.*, 2005; Huisling *et al.* 2006 in press; Huisling, pers. observation.). Puffer leptin is expressed mainly in the liver, whereas the main site of mammalian leptin expression is fat tissue. Interestingly, the hepatocytes of puffer contain abundant oil droplets reminiscent of adipocytes.

Leptin-like immunoreactivity was detected in several fish species in the blood, liver and adipocytes, including burbot (*Lota lota*) and sea lamprey (*Petromyzon marinus*) (Johnson *et al.*, 2000; Yaghoubian *et al.*, 2001; Mustonen *et al.*, 2002; Vegusdal *et al.*, 2003). In goldfish, both peripheral and central injections of murine leptin decrease food intake (Volkoff *et al.*, 2003). In the same study, it was found that higher doses of peripheral injection are required to exert the same effects as central injections, which suggests that in fish, as in mammals, leptin exerts at least part of its actions in the brain. Injections of murine leptin decrease NPY mRNA expression in the goldfish brain whereas CART and cholecystokinin (CCK) mRNA is upregulated (Volkoff *et al.*, 2001; 2003). These data suggest that in fish, leptin also interacts with hypothalamic nuclei to inhibit food intake. However, now that we are aware of the poor primary sequence conservation between fish and mammalian leptins, we should interpret these effects of mammalian leptin on food intake in fish, as well as the immunoreactivities detected with antibodies against human or mouse leptin, with caution. Therefore, the recent identification of leptin in fish will provide new insights and possibilities for research considering the regulation of food intake in fish, and new understandings of the evolution of leptin and the role(s) it plays in energy metabolism throughout vertebrates.

The first experiments assessing the function of carp leptin suggest that (at least in this species) leptin is important for the regulation of food intake, but not for body weight (Huising *et al.*, in preparation). We interpret these findings in light of the ectothermic metabolism of fish which allows many species of fish to respond to fluctuations in food availability by arrested or accelerated growth.

Insulin

Although leptin has lately received the lion's share of the attention of researchers, insulin, produced by the pancreatic β -cells, was one of the first peripheral signals described to be involved in the regulation of food intake. Woods and co-workers demonstrated in 1979 that chronic i.c.v.

administration of insulin reduces food intake and body weight in baboons. Entry of insulin in the brain has been demonstrated in several species, including dog, rodents and human (Baura, *et al.*, 1993; Schwartz *et al.*, 2000). Insulin receptors have been characterized in the brain, also in key areas for the regulation of food intake, including the ARC (Wilcox *et al.*, 1989).

Demonstration that insulin signaling in the CNS affects food intake and energy homeostasis is more complicated than doing so for leptin, since insulin fluctuations directly modulate plasma glucose (by mediating glucose uptake in muscle and liver) and low glucose itself is a signal that will increase food intake; the reverse occurs at high glucose concentrations (Lovett *et al.*, 1970). This complicates the assessment of the direct actions of insulin. Furthermore, animals without insulin or with an insulin receptor deficiency die before reaching adulthood.

However, as i.c.v. insulin administration decreases NPY mRNA and protein levels in the ARC (Schwartz *et al.*, 1992) this suggests a direct anorexic role for insulin. Moreover, direct administration of insulin in the CNS as well as local deletion of insulin receptors in the brain demonstrate a function for insulin in energy homeostasis. When insulin is administered i.c.v., a dose-dependent decrease of food intake and body weight is seen in baboons and rats (Woods *et al.*, 1979; Chavez *et al.*, 1995). The disruption of insulin receptors in neurons in mice results in higher food intake, more body fat and increased weight gain (Brüning *et al.*, 2000).

In fish, especially compared to mammals, insulin seems to play important roles in lipid and protein metabolism (Ince, 1983; Plisetskaya, 1990) and free amino acids are the most potent secretagogues for insulin release in fish species (Mommensen *et al.*, 1991). Possibly because insulin appears to play a less important role in glucose metabolism in fish, the role of insulin in the control of food intake in fish has not been extensively studied. Moreover, as fasting of rainbow trout for six weeks resulted in a decrease of plasma insulin (Moon *et al.*, 1989) and feeding this species with a high carbohydrate diet increased plasma insulin levels (Hilton *et al.*, 1987), insulin seems to be involved in energy homeostasis indeed. More recently, Soengas and Aldegunde (2004) demonstrated that i.c.v. injection of insulin in rainbow trout inhibited food intake after 26

hours and this effect was still apparent after 74 hours. So, insulin elicits effects on the long term; between hours and days.

Perspectives

The central mechanisms involved in the regulation of food intake are relatively well characterized in mammals as well as in non-mammals and generally appear to function similarly throughout all vertebrates classes studied to date. On the other hand, our understanding of peripheral regulators of food intake in earlier vertebrates is scarce compared to mammals. Only very recently, with the identification of leptin in fish and the first reports on the food intake regulating actions of ghrelin in fish, new insight and understanding in these mechanisms came into focus. These results suggest that peripheral signals indicating nutrient status differ in their function between endotherm and ectotherm species. These differences may stem from the different management of energy reserves between ectotherms and endotherms, as the latter require much tighter control of food intake to ensure continued thermoregulation (Somero *et al.*, 2005).

Also, the very different actions of ghrelin in ruminants (sheep) and birds (chickens) may give us more understanding in the evolution and function of ghrelin in humans. Now that we know that leptin is poorly conserved from fish to human, studies with homologous leptin are needed to verify previous results obtained with murine leptin administration in fish and to identify new effects of leptin in ectotherm species.

Teleost fish in particular, with an estimated number of as many as 35,000 extant species that have successfully adapted to a large and diverse array of different habitats provide many useful and interesting model systems for comparative studies on energy homeostasis. Peripheral signals such as leptin, ghrelin, and insulin now require our attention, as knowledge on these factors in earlier vertebrates may provide new insights in the evolution of energy metabolism and might reveal clues that assist in our understanding of the ongoing obesity epidemic.

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Figure captions

Figure 1: Overview of the regulation of food intake and body weight. In mammals (A), the effects of peripheral indicators of nutrient status with brain centers that regulate food intake are indicated. The ARC contains two distinct subsets of neurons that co-express either NPY and AgRP or CART and POMC, the former peptides increase, while the latter set decreases food intake. The ARC receives multiple endocrine input from the periphery, where leptin, insulin (that both circulate in proportion to body fat), ghrelin (expressed by the stomach wall in response to low carbohydrate levels in the stomach), and CCK (expressed by stomach endocrine cells in response to nutrient stimulation) relay information about nutrient status and energy reserves to the hypothalamus. The NPY⁺/AgRP⁺ and CART⁺/POMC⁺ neurons inhibit each other and signal to higher order neurons in the PVN, including CRH neurons. These CRH neurons project to the hindbrain, where (in combination with CCK) food intake is terminated. The differences in the regulation of feeding and energy metabolism in fish (B) compared to mammals are shown in italics and possible interactions that remain to be established are indicated by question marks. These differences include the nomenclature of the hypothalamic nuclei involved: the NLT and NPO instead of the ARC and PVN. Furthermore, in contrast to mammals where the adrenal gland produces glucocorticoids, the head kidney fulfils this function in fish. Fish leptin is expressed and produced mainly in the liver. Note that some fish species (including common carp) do not possess a classical stomach, the main site of ghrelin production in these species remains to be established. Abbreviations: ACTH: adrenocorticotrophic hormone, AgRP: agouti related protein, ARC: arcuate nucleus, CART: cocaine and amphetamine regulated transcript, CCK: cholecystokinin, CRH: corticotropin releasing hormone, GC's: glucocorticoids, HPA-axis: hypothalamus-pituitary-adrenal axis, MSH: α -melanocyte stimulating hormone, NLT: *nucleus lateralis tuberosus*, NPO: *nucleus preopticus*, NPY: neuropeptide Y, PVN: paraventricular nucleus.

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1B