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Leptin in Teleost Fish

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Introduction

Subtle deregulation of energy balance can result in severe diseases such as obesity and anorexia nervosa. The prevalence of obesity has reached epidemic proportions across the globe, but mostly in Western civilization. More than 60% of US adults are overweight (Body Mass Index (BMI) > 25 kg/m²) or obese (BMI > 30 kg/m²) and the number of obese children is growing tremendously, predisposing them to a plethora of chronic conditions including cardiovascular disease and type 2 diabetes. Obesity results from a higher caloric intake than energy expenditure. Therefore food intake is considered key in the etiology of obesity. As leptin plays a crucial role in energy metabolism and food intake, research the physiology of leptin has boosted over the past decade.

Over the past decades, numerous signalling molecules that affect energy homeostasis, have been identified (for detailed reviews we refer to Schwartz et al., 2000 and Morton et al., 2006). One of the classic studies regarding body weight control comes from Coleman and Hummel (1969), using parabiosis experiments. When they surgically joined an ob/ob mouse which is obese owing to a genetic defect, to a lean congenic mouse, this abrogated food intake by the ob/ob mouse. It followed that the wild-type mouse possesses a soluble, circulating factor that is capable of inhibiting food intake in the ob/ob mouse. This soluble factor reduced food intake and weight gain of the obese parabiont and partially reverted the profoundly obese phenotype. Almost three decades later, the soluble factor predicted by Coleman and Hummel was discovered and named leptin after the Greek word λεπτος, for lean (Zhang et al., 1994). Leptin is a 16 kDa member of the class-I helical cytokine family that is produced by adipocytes. Leptin, as well as insulin, meets the criteria for a candidate adiposity signal since both are hormones originating from the periphery that signal information regarding energy stores. Both circulate in blood plasma in proportion to body fat (Bagdade et al., 1967, Considine et al., 1996) and enter the central nervous system (CNS), via a saturable transport, in proportion to their plasma level (Baura et al., 1993, Schwartz et al., 1996). Moreover, leptin and insulin receptors are present on brain neurons involved in the regulation of food intake and energy balance (Baskin et al., 1988, Tartaglia et al., 1995).

Information regarding energy status is transmitted from the periphery to the hypothalamus. The arcuate nucleus (ARC) is a region in the hypothalamus that plays a key role in the

integration of peripheral signals that monitor energy stores. It contains two distinctive and antagonistic sets of neurons. One set expresses neuropeptide Y (NPY) and agouti-related protein (AgRP) and is orexigenic (Broberger et al., 1998). The other set of neurons expresses cocaine and amphetamine regulated transcript (CART) and pro-opiomelanocortin (POMC, in these neurons processed to α -melanocyte stimulating hormone (α -MSH)) and is anorexigenic (Elias et al., 1998). Leptin activates the POMC/CART neurons and inhibits the NPY/AgRP neurons and as a result inhibits food intake.

The NPY/AgRP and POMC/CART neurons inhibit each other and signal to higher order neurons in the paraventricular nucleus (PVN), perifornical area (PFA) and the lateral hypothalamic area (LHA) (Elmquist et al., 1998; 1999). Neurons in the PVN produce several neuropeptides, involved in food intake control, such as corticotropin-releasing factor (CRF), thyrotropin-releasing hormone (TRH) and oxytocin which all reduce food intake (Kow and Pfaff, 1991, Verbalis et al., 1995). The PFA and LHA produce several orexigenic neuropeptides, such as melanin-concentrating hormone (MCH) (Qu et al., 1996) and orexins A and B (also known as hypocretin-1 and -2; de Lecea et al., 1998, Sakurai et al., 1998). Many other brain areas contribute to the central integration of cues that regulate food intake and energy metabolism including the ventromedial hypothalamus (VMH) and the paraventricular nucleus (PVN) (Hashimoto et al., 2004, Kuperman and Chen, 2008).

In mammals, the leptin receptor is present in feeding centres in the hypothalamus (Tartaglia et al., 1995) and is processed into at least six isoforms; one long receptor protein, OB-Rb (considered the 'classic' leptin receptor and responsible for leptin's biological actions) and five truncated receptor proteins OB-Ra, -Rc, -Rd, -Re and -Rf (Wang et al., 1998). The five truncated receptors are the result of alternative splicing and can bind leptin but fail to induce a second messenger pathway, as they all lack the key intracellular phosphorylation domains. OB-Ra and OB-Rc have high expression levels in the choroid plexus and brain capillaries and a role in the transport of leptin across the blood-brain-barrier (BBB) was suggested by Tartaglia and co-workers (1995). The OB-Re is soluble because it lacks the intracellular domain and the transmembrane domain. It is speculated that the OB-Re associates with leptin in circulation, and thus acts as a leptin binding protein (Li et al., 1998).

The leptin receptor is a member of the class-I cytokine receptor family. After the binding of leptin to OB-Rb, this activated receptor pairs up with a second OB-Rb, bound by leptin, to form a functional leptin receptor complex consisting out of two leptin molecules and two Ob-Rbs. The OB-Rb contains a cytoplasmic domain that can interact with Janus kinases (JAK) and the signal transducer and activator of transcription (STAT) intracellular messengers. These are responsible for the signal transduction pathway of the leptin receptor (Zabeau et al., 2003).

Initially hopes were that leptin served a primary role as an anti-obesity hormone and that the cure for obesity resided in the exogenous administration of leptin. Indeed, treatment of ob/ob mice with exogenous leptin reduced food intake and normalized the body mass of these mice (Pellemounter et al., 1995), giving support to leptin's role as an anti-obesity hormone. Unfortunately obese humans and rodents with diet-induced obesity responded not, or weakly at best, to exogenously administered leptin (Widdowson et al., 1997, Heymsfield et al., 1999, Levin and Dunn-Meynell, 2002).

Until a couple of years ago, almost all data regarding leptin's role in regulating body weight and food intake came from rodent experiments. Direct evidence that leptin exerts effects in human physiology was lacking. However, after the identification of human leptin and its receptor (Zhang et al., 1994 and Chen et al., 1996), experiments showed that congenital leptin deficiency and a mutation to the leptin receptor gene both resulted in extreme obesity (Montague et al., 1997a and Clement et al., 1998 respectively). These results show that leptin plays a paramount role in maintaining energy homeostasis. However, natural mutations in the leptin or leptin receptor genes are very rare. Members of the few families worldwide where familial mutations in leptin have been identified as the cause of morbid obesity, do respond very well to leptin treatment (Gibson et al., 2004 and Licinio et al., 2004), but the vast majority of obese patients has high levels of circulating leptin that nonetheless fail to reduce food intake and obesity. These people have an impaired responsiveness to circulating leptin, named leptin resistance. Resistance to leptin's anti-obesity actions occurs in experimental animals (Frederich et al., 1995) as well as humans (Caro et al., 1996).

There are two mechanisms that can contribute to leptin resistance, viz. impaired leptin transport across the BBB and reduced leptin signal transduction. Evidence for impaired leptin transport across the BBB is gathered by Caro et al. (1996) and Schwartz et al. (1996). They show that leptin levels in the cerebrospinal fluid of obese humans are low in comparison to their plasma levels, indicating impaired shuttling across the BBB. Reduced leptin-receptor signal transduction can be the consequence of activation of suppressor of cytokine signalling-3 (SOCS-3) which inhibits further leptin signal transduction (Zabeau et al., 2003) or activation of Protein Inhibitors of Activated STAT (PIAS) molecules (Chung et al., 1997) that also terminate leptin's biological actions. Leptin may then fail to adequately activate or inhibit downstream POMC or NPY neurons (Scarpace et al., 2003).

The thrifty gene hypothesis offers an interesting perspective on the apparent inability of leptin signalling to prevent obesity (Neel et al., 1962). It entails that certain genes have evolved to maximize metabolic efficiency and lipid storage, and that in times of abundance promote disposition of energy (in the form of fat) to cope with future scarcity. These same pathways fail to adequately cope with the lifestyle changes in Western societies, such as ample availability of food and lack of exercise, that than lead to obesity. Following this theory Banks et al. (2006) suggest that low plasma levels of leptin signal to the brain regarding inadequate caloric reserves; leptin resistance at the BBB evolved as a defence against starvation. It may be that leptin signalling is not geared to prevent obesity but to prevent starvation and to replenish peripheral energy stores. For this reason, our brain readily reacts to low leptin levels, indicating low body energy reserves, but fails to respond adequately to high leptin levels that indicate ample energy reserves (Elmqvist et al., 1998).

Although research of leptin resistance is ongoing for years, critical questions remain unanswered, such as how chronically elevated leptin levels lead to leptin resistance? Both leptin-receptor and leptin signalling are down regulated, but the degree of down regulation of either component is insufficient to account for the largely absent responses to leptin. More information on leptin resistance will help us to understand the pathogenesis of obesity and is necessary for a successful treatment.

Evolution of Leptin

Despite the enormous attention given to leptin, no non-mammalian leptin orthologue was characterized in the decade following leptin's discovery in mice. Only recently have a number of non-mammalian leptin sequences been described. The rest of this chapter will be dedicated to an overview of these non-mammalian leptins with special emphasis on fish leptins.

Leptin-like immunoreactivity in ectotherms

The paper that reported the identification of murine leptin also featured a Southern blot experiment where a murine leptin probe was successfully hybridized to genomic DNA of chicken and eel. This led to the supposition that leptin is “evolutionarily conserved” (Zhang et al., 1994). The presence of a leptin-like molecule in ectotherms was also addressed by Johnson et al. (2000) using anti-mouse leptin antibodies to detect the presence of leptin in tissues from teleost fishes. Leptin-like immunoreactivity was detected in blood, brain, heart and liver of several teleost species. A threefold higher leptin-like reactivity was detected in plasma of fed compared to fasted fish, which is consistent with mammalian models of leptin function. Brain leptin-like immunoreactivity was also positively correlated to the percentage of body fat. In mammals, leptin is primarily produced in white adipose tissue (Zhang et al., 1994). In fish, leptin-like immunoreactivity could not be detected in intestinal adipose tissue, but a faint leptin signal was detected in the liver, which is in many fishes a main site of energy storage in the form of carbohydrates and lipid.

More studies tried to identify leptin in non-mammalian animals. Muruzábal et al. (2002) used a rabbit polyclonal antibody specific for a synthetic fragment of human leptin to study the presence of leptin in the epithelium and the enteric nervous system of several non-mammalian vertebrates (fishes, amphibians and reptiles) and reported positive signals for leptin in several tissues. Vegusdal et al. (2003) used anti-human leptin antibodies in an in-vitro culture of pre-adipocytes in Atlantic salmon (*Salmo salar*) to show that a detectable level of leptin protein was present during differentiation from pre-adipocytes to adipocytes. Several other studies reported the detection of leptin-like immunoreactivity in fish with the use of mammalian leptin-antibodies (Yaghoubian et al., 2001, Mustonen et al., 2002, Nieminen et al., 2003). However, we should interpret the immunoreactivity in fish detected with antibodies raised against heterologous, mammalian leptin with caution as these studies originated prior to the

discovery of the first fish leptin genes. It is unlikely that antibodies raised against mammalian leptin can faithfully reflect endogenous expression patterns in fish.

Leptin in fish

The first publication describing a leptin gene in a teleost fish species was by Kurokawa et al. (2005). They identified a tiger pufferfish (*Takifugu rubripes*) leptin (AB193547) by comparing genomic synteny with the human leptin. In addition, they retrieved orthologues of pufferfish leptin in the databanks for Atlantic salmon (*Salmo salar*; BI468126), Japanese medaka (*Oryzias latipes*; AB193548), Green spotted pufferfish (*Tetraodon nigroviridis*; AB193549), tiger salamander (*Ambystoma tigrinum*; CN054256) and tropical clawed frog (*Xenopus tropicalis*; scaffold 20). The pufferfish leptin protein consists of 152 amino acids; this is 15 amino acids shorter than mammalian leptins. It shows an unprecedented low amino acid identity with human leptin of merely 13%.

Shortly thereafter, we reported the identification of leptin genes in zebrafish (*Danio rerio*) and common carp (*Cyprinus carpio*) (Huisin et al., 2006b). We screened the Ensembl zebrafish genome database with mammalian leptin sequences and found a partial zebrafish leptin sequence. Aided by this partial sequence we identified two similar leptin genes in carp that enabled us to predict the complete coding sequence of zebrafish leptin (TPA database: BN000830). Carp leptin-I and leptin-II (AJ868357 and AJ868356 respectively) both consist of 171 amino acids and share 82% amino acid identity (Figure 1). This high degree of amino acid identity indicates that the two leptin paralogues probably result from a recent duplication of the carp genome, ~16 Mya (Larhammar and Risinger, 1994). Zebrafish leptin and carp leptins show approximately 60% amino acid similarity, but carp and pufferfish share merely 27% amino acid identity. This is in line with long evolutionary separation of carp and pufferfish as each represents a distinct teleost lineage that share a common ancestor that lived ~296 million years ago (Figure 2) (Hoegg and Meyer, 2005). As observed for the pufferfish leptin orthologue, amino acid identity of carp leptins compared to mammalian leptins was low, viz. 20–25%.

Despite the low amino acid similarity between fish leptins and mammalian leptin, several arguments substantiate the unambiguous orthology of fish and mammalian leptin. Both

pufferfish and carp leptins contain two cysteine residues that form a single disulphide bridge that connects the carboxyterminal ends of α -helices C and D, as in mammalian leptin (Kurokawa et al., 2005, Huising et al., 2006b). Moreover, the spacing of the cysteines is unique among class-I helical cytokines, this provides another argument that supports the orthology of fish and mammalian leptin (Huising et al., 2006a). Pufferfish and both carp leptins show a similar gene structure (Kurokawa et al., 2005, Huising et al., 2006b). They are encoded by two exons, like the human leptin gene. These two exons are separated by a short intron with consensus 5' donor (gt) and 3' acceptor (ag) splice sites. The first and second exons of the carp leptin genes differ only one and three triplets in size, respectively, from the human and mouse leptin genes. Moreover, leptin has a unique gene structure; almost all vertebrate class-I helical cytokines are encoded by at least three and in general five exons. The only other class-I helical cytokine than leptin made up out of two exons is ciliary neurotrophic factor (CNTF), but its exons differ clearly in size from those of leptin. Moreover, CNTF lacks conserved cysteine residues altogether (Huising et al., 2006a). Three-dimensional (3D) models of pufferfish, carp and human leptin show that all leptins conform to the characteristic four α -helix bundle structure (Figure 3) (Kurokawa et al., 2005, Huising et al., 2006b). These results suggest that the three-dimensional structure of leptin is conserved during vertebrate evolution.

Phylogenetic analysis of leptin genes of several mammalian and non-mammalian species reveal a stable clustering with a branching pattern that conforms to conventional vertebrate evolution (Figure 4). Teleostean leptins branch off before the separation of the amphibian and mammalian leptin cluster. Moreover, vertebrate leptins cluster together, apart from the other members of the class-I helical cytokine family that were included as outgroup (Huising et al., 2006a). All bootstrap values are high, corroborating the bona-fide identity of the fish leptin paralogues. The long branch lengths between mammalian and fish leptins reflect their considerable sequence dissimilarity.

Furthermore, the synteny, or gene arrangement around leptin is conserved between the human, pufferfish and zebrafish (Kurokawa et al., 2005, Huising et al., 2006b). Although information on the genomic context of the carp leptin genes is not available, the genomic context of the closely related cyprinid zebrafish is known. Both human and mouse have RNA

binding motif protein 28 (RBM28) in close proximity (<3 kb) to the leptin gene. The leptin gene of the zebrafish is positioned in the opposite orientation and in close proximity to the RBM28 gene. This conservation of synteny between zebrafish and human leptin genes further strengthens the orthology of fish and mammalian leptins.

Artefactual leptin sequences

A leptin molecule from chicken (AF012727) was reported in 1998 (Taouis et al.). At the amino acid level chicken leptin and murine leptin were 97% identical, the amino acid identity with human and rat was 83% and 96% respectively. This would represent an incredible evolutionary conservation as birds and mammals shared their last common ancestor approximately 310 million years ago (Hedges et al., 1996). In 1999, Ashwell and co-workers, independently reported the cloning of chicken and turkey leptins (AF082500 and AAC32381). Ashwell's chicken leptin shows an amino acid similarity of 90% with the murine leptin and is nearly identical to the sequence Taouis et al. (1998) found. The turkey leptin gene they identified shows 95% amino acid identity with mouse. However, several research groups failed to repeat these experiments (Friedman-Einat et al., 1999, Pitel et al., 2000). Doubts about the chicken leptin were reinforced by the results of comparative Southern blot experiments and by molecular evolutionary analysis (Friedman-Einat et al., 1999, Dunn et al., 2001). In 2000, two research groups cloned the chicken leptin receptor (Horev et al., Ohkubo et al.), and in 2003 the turkey leptin receptor was cloned (Richards and Poch., 2003). The chicken and turkey leptin receptor both have an amino acid identity of approximately 50% compared to their mammalian orthologues and this level of sequence similarity is consistent with the degree of conservation found in other class-I helical cytokine receptors in these species and with the estimated evolutionary divergence time between the avian and mammals (~310 million years). The large difference between the degree of leptin conservation and the conservation of its receptor is counterintuitive, as ligands and the ligand-binding domain of their receptors tend to co-evolve. In 2004, the draft chicken genome was published and it lacks the leptin sequence as obtained by Taouis et al., casting serious doubts on the validity of the published chicken leptin sequence. In summary, it is probable that the bird leptin sequences that are discussed above are erroneous. A bona-fide avian leptin has yet to be reported.

Once more, in 2005 and 2006, a likely contamination of experimental material with murine material occurred. Multiple fish leptin entries were submitted in the EMBL database by Dai, H., Long, L. and Dind, G. that appear to result from the contamination of experimental samples with rodent material. These sequences include: *Culter* sp. (AY497007), Common carp (*Cyprinus carpio*; AY547279), Goldfish (*Carassius auratus*; AY547322), Grass carp (*Ctenopharyngodon idella*; AY551335), Silver carp (*Hypophthalmichthys molitrix*; AY551336), Bighead carp (*Aristichthys nobilis*; AY551337), Amur catfish (*Silurus asotus*; AY551338), Wuchang bream (*Megalobrama amblycephala*; AY551339), Snakeheaded fish (*Channa argus*; AY551340), Black carp (*Mylopharyngodon piceus*; DQ784814), Japanese eel (*Anguilla japonica*; DQ784815) and Amur sturgeon (*Acipenser schrenckii*; DQ784816). Comparing these fish sequences with mouse leptin invariably reveals remarkable similarities of 97 - 99% at the nucleotide level and 97 - 100% at the amino acid level. The alleged Japanese eel leptin differs from mouse leptin at only three synonymous and one non-synonymous site. Non-synonymous (non-silent) and synonymous (silent) substitutions occur randomly but non-synonymous substitutions accumulate over time and at a more or less constant rate. Although a highly conserved leptin would in itself be conceivable, merely three synonymous substitutions over the course of 450 million years of evolution is quite unlikely. As a result, these sequences should be regarded as artefactual.

The conclusion of Zhang et al. in 1994 that leptin is “evolutionarily conserved” appeared a plausible view, given the key role leptin plays in the regulation of energy balance in mammals. However, the discoveries of fish leptins in multiple species that share less than 25% amino acid identity with mammalian leptins prompts for leptin’s evolutionary conservation to be redeveloped. In fact, of all class I helical cytokines with orthologues identified in teleost fish to date, leptin has the lowest degree of primary sequence conservation (Huisling et al., 2006a).

Functional Aspects of Ectothermic Leptin

The low amino acid identity shared between vertebrate leptins is a reminder that we assign the name leptin solely on the basis of structural similarities. Orthologous proteins do not by default share analogous roles. This may in particular apply for molecules like leptin with its poor primary sequence conservation across the vertebrate subphylum. Information on the

possible role(s) of leptin in ectotherms is scant. Most studies on leptin in ectothermic vertebrates to date have focused on the regulation of food intake and energy metabolism. In some aspects, the physiology of energy metabolism in fish resembles mammalian physiology as most systems and hormones regarding energy metabolism in mammals are present in fish (Gorissen et al., 2006; Volkoff et al., 2003). However, the detailed interplay between those systems and hormones are still enigmatic and indeed there are some marked differences between fish and mammalian systems. For a detailed review we refer to Gorissen et al. (2006). Below we discuss the literature on the functional aspects of ectothermic leptin.

Mammalian leptin in functional studies on fish

Before the identification of teleost leptin, numerous studies used mammalian (i.e. heterologous) leptin to study functional effects in non-mammalian vertebrates. Volkoff et al. (2003) showed that both peripheral and central injections of murine leptin decreased food intake of goldfish. However, several other studies (Baker et al. (2000), Silverstein and Plisetskaya (2000) and Londraville and Duvall (2002)) reported no effect of murine leptin administration on food intake in respectively Coho salmon (*Oncorhynchus kisutch*), channel catfish (*Ictalurus punctatus*) and green sunfish (*Lepomis cyanellus*). Volkoff et al. (2003) further described that higher peripheral doses of murine leptin were required compared to centrally administered heterologous leptin, to result in an effect on food intake. This would suggest that in fish, as in mammals, murine leptin acts primarily on the brain to control energy homeostasis and is transported to the brain via a saturable transport. Furthermore, the anorexigenic effects of CART and cholecystokinin (CCK) are reinforced by central injections of murine leptin. It also inhibits NPY- and orexin A-induced food consumption. Central administration of murine leptin up regulates the mRNA expression of the anorexigens CCK and CART, while NPY mRNA expression is down regulated (Volkoff et al, 2001, 2003). This suggests that murine leptin in goldfish interacts with hypothalamic pathways to inhibit food intake, just like in mammals. However, now that we are aware of the low primary sequence conservation of leptin between fishes and mammals, we should interpret the physiological effects of mammalian leptin on fish, as well as the immunoreactivity detected with antibodies raised against mammalian leptin, with caution. At the time these experiments were conducted, no fish leptin had been cloned and the general expectation seems to have been that leptin's

role was analogous to that in mammals. Our current understanding of the poor primary sequence conservation of leptin calls for a careful re-evaluation of these experiments.

Leptin expression in fish

Pufferfish and carp leptin mRNA was expressed mostly in liver (Kurokawa et al., 2005, Huising et al. 2006b). In carp, leptin-II was more abundantly expressed in liver than leptin-I (Huising et al. 2006b). This is in line with the study of Johnson et al. (2000) that also suggested that the liver is one of the main sites of leptin immunoreactivity in fish. Kurokawa et al. (2005) performed in-situ hybridization (ISH) on liver sections of pufferfish. The hepatocytes contained abundant oil droplets and positive leptin signals were observed inside hepatocyte membranes. In pufferfish leptin was further present in the ovary. In carp both leptin genes were also abundantly expressed in thymus, kidney and to a lesser extent in other organs, such as muscle and visceral adipose tissue, but the liver by its sheer volume appears a major site for leptin production in fish. The low level of leptin in visceral adipose tissue of carp is in agreement with mammalian leptin expression, noting that the contribution of the visceral adipose tissue to circulating leptin levels in mammals is limited. Plasma leptin is mainly secreted from subcutaneous adipose tissue (Montague et al., 1997b and Woods et al., 2003) that is absent in teleost fishes. No data on leptin expression in pufferfish adipose tissue is available.

Leptin in amphibians

The cloning of leptin in *Xenopus laevis* was described by Crespi and Denver (2006). The *Xenopus* leptin orthologue is comprised of 148-amino acids (mature) protein (AY884210). The *Xenopus* leptin gene consists of two exons and displays a similarity of 35% to human and only 13% to pufferfish leptin, but the predicted tertiary structure of frog leptin was conserved compared to mammalian leptins. A tissue-distribution showed highest leptin mRNA expression in the brain and heart. However, expression was also seen in fat, liver, pituitary gland, gastrointestinal tract, lungs, kidney and gonads. *Xenopus* leptin activates *Xenopus* as well as mouse leptin receptors in vitro with approximately the same potency. Intracerebroventricular injection of recombinant *Xenopus* leptin (rxLeptin) exerted a potent anorexigenic effect. However, this response does not develop until mid-prometamorphosis. During early prometamorphosis, exogenous administered rxLeptin induces growth and

development of the hind limb (the leptin receptor is expressed in this tissue). Furthermore, rxLeptin stimulates cell proliferation in cultured hind limbs from young tadpoles. Together, these data support the hypothesis that leptin can influence limb growth and differentiation during early development and it may represent a novel growth factor. Recently, Boswell et al. (2006) reported a 169 amino acids leptin-like protein in salamander (*Ambystoma tigrinum*). It shares an overall amino acid identity with mammalian leptins of approximately 29%. Phylogenetic analysis, 3D-structure and a conserved cysteine pair confirmed that the salamander leptin represents a true orthologue of mammalian leptin. Tissue expression of leptin-like cDNA differed between metamorphosed adults of different sizes suggesting possible developmental regulation. Leptin expression was most prominent in skin and testis but was also detected in fat, stomach and muscle. In contrast with the findings in fish and *Xenopus*, no evidence was found for hepatic leptin expression. This suggests possible taxonomic differences in leptin functions.

Physiological experiments

We performed physiological experiments in carp to see if leptin mRNA responds to different feeding regimes (Huising et al., 2006b). This was the first study on the regulation of leptin expression in any non-mammalian species. Six days of fasting and subsequent re-feeding did not affect hepatic leptin mRNA expression. Plasma glucose and non-esterified fatty acid (NEFA) values provided insight into the nutritional status of the animals because plasma glucose will be high during the postprandial phase, whereas increased NEFA values indicate energy mobilization via lipolysis in the absence of high glucose levels (Stich and Berlan, 2004). During fasting, carp predictably shifted from carbohydrate to lipid metabolism and, during re-feeding, they shifted back from lipid to carbohydrate metabolism as indicated by changes in plasma glucose and NEFA values. Neither fasting nor re-feeding affected hepatic leptin mRNA expression. Because the lack of response after six days of fasting, the carps were fasted for six weeks and then subsequently re-fed. After six weeks of fasting, NEFA levels increased and plasma glucose levels dropped to almost undetectable levels. Hypothalamic expression of the anorexigens CRF and POMC was decreased after six weeks of fasting, while the expression of the orexigen NPY was unaffected. At the end of six weeks of fasting, animals weighed less than 50% of the control fish. Despite all these differences between control and experimental animals indicating altered growth and nutritional status, no

differences were seen in hepatic leptin mRNA expression after six weeks of fasting. In a reverse approach, after six weeks of feeding to satiation, glucose and NEFA values did not differ between control and experimental animals. Also, the hypothalamic expression of CRF, POMC and NPY was not affected by overfeeding. Animals that were fed to satiation grew heavier and larger compared to control fish. The length of the experimental fish was also significantly larger. However, there was again no significant difference in leptin expression at the termination of the study.

We then focused on the short term effects of food intake on hepatic leptin expression. Following a single meal, carp showed a characteristic postprandial peak in plasma glucose and a concomitant drop in NEFA values. This postprandial peak in plasma glucose was followed by an acute and transient rise in expression of leptin-I and leptin-II at, respectively, three and six hours after feeding. Interestingly, before the postprandial peak of liver leptin-II, its expression dropped slightly, but significantly, below the preprandial expression values. Furthermore, the leptin peak follows the increase in plasma glucose, suggesting that leptin may be under direct glucose control. There are some studies on rodents that support this hypothesis. Mizuno and co-workers (1996) show that intraperitoneal glucose injection significantly increases leptin mRNA. Mueller et al. (1998) show that the increase of leptin is closely related to the amount of glucose taken up by rat adipocytes, which suggests a role for glucose metabolism in the regulation of leptin secretion. However, after six weeks of fasting glucose levels dropped to < 1 mM without affecting the hepatic leptin expression, suggesting that basal leptin expression is maintained independently of plasma glucose levels. It is also possible that the leptin peak is partly or completely the result via indirect actions of other anorexigenic hormones, such as insulin or CCK. As in carp, mice show a postprandial rise in leptin mRNA in the hours immediately following food intake (Saladin et al., 1995), suggesting that leptin will respond rapidly to short-term food intake in representatives of different vertebrate classes. However, after a short period of fasting, rodents show a more pronounced weight loss that is accompanied by a sheer drop in leptin expression (Saladin et al., 1995 and Bertile et al., 2003). Carp leptin expression was up nor down regulated after either short or long-term fasting and it takes up to five weeks of food deprivation for carp to lose a similar percentage of body weight that rats lose in six days. This difference in response between fish and mammals could be the result of the overt physiological differences that are

associated with endothermic and ectothermic vertebrates. Ectotherms are more flexible in their metabolic regulation because they do not need to thermoregulate. Some species of fish and *Xenopus* cope with starvation for periods of up to several months (Hall et al., 2006 and Calle et al., 2006). In summary, hepatic leptin mRNA expression in carp fluctuates acutely with food intake, but there is no evidence yet that leptin is involved in the long-term regulation of feeding and energy metabolism in carp.

Leptin receptor in ectotherms

The *Xenopus tropicalis* leptin receptor consists of 1145 amino acids and is encoded by 18 exons (DQ401069) (Crespi and Denver, 2006). The *Xenopus* receptor was 37.5% identical to the human leptin receptor. In phylogenetic analysis it clusters within the vertebrate leptin receptor clade. The leptin receptor is widely distributed in the frog and is present in almost all tissues. The highest expression level of the leptin receptor was found in the pituitary gland, followed by the brain.

The first fish leptin receptor is described in 2007 (Wong et al.). Wong and co-workers characterized the leptin receptor from the marine medaka (*Oryzias melastigma*, DQ359150). In their study they also show the sequences of several leptin receptors (deduced from genomic databases) of *Tetraodon nigroviridis* (AAR25693), *Takifugu rubripes*, *Danio rerio* and *Oryzias latipes*. They describe that the putative zebrafish and medaka leptin receptor genes both consist of 18 exons and encode a protein of respectively 846 and 1055 amino acids. The *Takifugu rubripes* leptin receptor gene consists of 24 exons and encodes a protein of 1090 amino acids. The deduced *Oryzias melastigma* sequence shares highest sequence similarity with the closely related *Oryzias latipes* (81%), followed by *Takifugu rubripes* (47%) and zebrafish (36%). Multiple sequence alignment of fish leptin receptors with other vertebrate species shows that all key signature motifs and domains are conserved between mammals and fishes. A quantitative RT-PCR on several tissues of both male and female marine medakas showed that the Ob-R was abundantly expressed in gill, kidney, muscle and spleen. The GI tract shows also moderate expression and brain, eye, gonads, heart and liver show a low expression of the leptin receptor. Leptin receptor expression was studied under normoxic and hypoxic conditions. The medaka leptin receptor was significantly up regulated in gills, heart

and liver. This suggests that leptin and its receptor act pleiotropically to fulfil a range of functions in fish that we are only start to appreciate.

Perspectives

Although several leptin genes of fish species have been discovered and the first studies addressing the functions of leptin in fish have appeared, knowledge of the peripheral regulators of energy balance in fish is scarce compared to mammals. Most studies performed in ectothermic vertebrates have focused on food intake and energy metabolism. Studies in mammals suggest that leptin is not only involved in the regulation of energy metabolism but that it is a multifunctional (or pleiotropic) cytokine. Leptin potentially has a much broader significance than the regulation of food intake and energy balance solely. This is illustrated elegantly by the studies on amphibians that seem to point to a role in development and metamorphosis. It is interesting to investigate other physiological functions of leptin in ectothermic organisms, e.g. bone formation, reproduction, development and immunity. In mammals, effects of leptin on all these processes have been described (Isaia et al., 2005, Wójcik-Gładysz et al., 2006, Udagawa et al., 2007, Fantuzzi, 2006).

Regarding the function of leptin in energy balance, studies are needed to establish its precise role in fish, including the interaction of leptin with other (an)orexigenic hormones. Studies so far have gathered information on the regulation of mRNA levels of leptin under different feeding regimes in carp. However, functional data on circulating leptin protein levels are required to advance the field. Another important subject for future research is therefore the production of recombinant leptin and the leptin receptor. An initial study has recently appeared that reports the bacterial expression of pufferfish leptin, although this recombinant protein was several orders of magnitude less potent in its activation of the human leptin receptor compared to human leptin (Yacobovitz et al., 2008). Now that the first fish leptin receptor is cloned, it is possible to address binding affinity and signalling potency of teleost leptin receptors. Furthermore, we can now elucidate the intracellular signalling cascades that act downstream of leptin receptors in teleost species.

Leptin in teleost fish in particular requires our attention because it may provide us with new and interesting insights in the evolution of energy metabolism and leptin physiology. Teleost fish are among the oldest extant vertebrates and understanding of the role of leptin in fish

might reveal original or additional leptin functions. Teleost fish can offer unique and important insights in the evolution of leptin physiology that potentially offer a fresh perspective on the role of leptin in the current obesity epidemic.

Figure legends

Figure 1. Multiple amino acid alignment of teleost fish leptins. Both cysteines that form an intra-molecular disulphide bridge are indicated by asterisks, leptin's four α -helices, inferred from human leptin, are shaded. Accession numbers are listed in the legends to figure 4.

Figure 2. Overview of the evolutionary relationships between the different fish species (*Cypriniformes*: carp and zebrafish; *Beloniformes*: medaka; *Tetraodontidae*: tiger pufferfish and green spotted pufferfish) discussed in this chapter. Divergence estimates based on: Hedges (2002); Hoegg and Meyer (2005); Volff (2005); Zardoya and Doadrio (1999). Mya, millions of years ago.

Figure 3. Protein model of human leptin (A), carp leptin-I (B), and carp leptin-II (C). Typical is the four-helix bundle conformation, conserved in human and carp. Reproduced with permission from (Huising et al., 2006b). Copyright 2006, The Endocrine Society.

Figure 4. Phylogenetic tree of vertebrate leptin amino acid sequences. Phylogeny was reconstructed on the basis of amino acid differences (p-distance) using the Neighbour-Joining algorithm in MEGA 3.0. The size of the dots at the branch nodes represent the confidence level of a 1000 bootstrap replications. Clusters of mammalian, amphibian, and fish leptins are indicated in different shades of grey. The vertebrate leptin cluster is delineated by a dashed line. Included as an outgroup are growth hormone (GH) and ciliary neurotrophic factor (CNTF). Accession numbers are as follows: human leptin, P41159; macaque leptin, Q28504; dog leptin, O02720; cat leptin, Q9N2C1; pig leptin, Q29406; cow leptin, P50595; mouse leptin, P41160; Norway rat leptin, P50596; fat-tailed dunnart leptin, AF159713; South African clawed frog leptin, AY884210; *Tetraodon nigroviridis* leptin, AB193549; tiger pufferfish leptin, AB193547; zebrafish leptin, AM920658; common carp leptin-I, AJ836745; common carp leptin-II, AJ836744; human GH, P01241; mouse GH, P06880; human CNTF, P26441; mouse CNTF, P51642.

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Helix A

Helix B

Human MHWGTLCGFLWLWPYLYFYVQAVP-----IQK--VQDDTKTLIKTIIVTRINDISHTQSVSSKQKVTG---LDFIPGLHPILTLKMDQTLAVYQQILTSMP
 Mouse MCWRPILCRFLWLWSYLSYVQAVP-----IQK--VQDDTKTLIKTIIVTRINDISHTQSVSAKQRVTG---LDFIPGLHPILSLKMDQTLAVYQQVLTSLP
 Common carp leptin-I MYFSALL-YPCILAMLSLVHGIP-----IHSDSLKNLVKLQADTIIIRIKDHNAELKLYPKLLIGDPELYPEVPADKPIQGLGSIMDTITTFQKVLQRLP
 Common carp leptin-II MYFSVLL-YPCILGMLSLVHAIP-----VHPDSLKNLVKLQADTIIILRIKDHNEKLLKSPKLLIGDPELYPEVPANKPIQGLGSIVETLSTFFHKVLQRLP
 Zebrafish MRFPALR-STCILSMLSLIHCIP-----VHQHDRKN-VKLQAKTIIIVRIREHIDGQNLPTLIIGDPGHYPEIPADKPIQGLGSIMETINTFFHKVLQKLP
 Tiger pufferfish --MDHILALVLAALLPLSLCVALPGALDAMDVEKMKSKVTWKAQGLVARI-DKHFP--DRGLRFDTD-----KVEGSTSVVASLESYNLISDR-
 Green spotted pufferfish --MDYTLALALSLLQLSMCTPVP---MMQDSGRMKTAKAWMVQQLLVRLKDNVWPHFDMPTFSAD-----DLEGSASIVARLENFNLSLISDN-
 Medaka -MDSALVLFALFLHCLNVATAAPVN---PELQEMKSNVIDIAKEPSLRLENI IQTSIGPKFSPSPD-----ELNGLSSIMAVLECTNQISDN-

Helix C

Helix D

Human SRNVIQISNDLENLRDLLHLVAFSKSCHLPWA-SGLETLDLGGVLEASGYSTEVVALSRLQGSLODMLWQLDLSPGC 167
 Mouse SQNVLQIANDLENLRDLLHLVAFSKSCHLPQT-SGLQKPESLDGVLEASLYSTEVVALSRLQGSLODILQQLDVSPEC 167
 Common carp leptin-I KGRVSIHIDLSTLLGHLKERMTSMHCTSKEP-ANGRALDAFLEDNATHHITVRYLALDRLKQFMOKLLVNLQDLKSC 171
 Common carp leptin-II KGHVSQIRNDLFTLLGYLKDRMTSMRCTLKEP-ANERSLDAFLENNATHHITFGFLALDRLKQFMOKLIVNLQDLKSC 171
 Zebrafish NKHVDQIRRDLSLLGYLEG---MDCTLKES-TNGKALDAFLEDSASYPFTLEYMTLNRLKQFMOKLIDNLQDLKIC 166
 Tiger pufferfish FGGVSIKTEISSLAGYLNHWREGN-CQEQQPK-VWP-----RRNIFNHTVSLEALMRVREFLKLQKNDLLERC 152
 Green spotted pufferfish LGDVLQIKAEISSLTGYLNNWRHNN-CKEQRPRTAVPGLPQEPQRRKDFIQSVTIDALMSMKEFLNLLQNLQDLHEIC 160
 Medaka FDEAKKIKVDISSLMDSMSEWSDKH-CGEQPSTQAEEN-----QTSRRFSITESMQAVTRLKHFLLLQNSDQLEIC 155

A



B



C



