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Identification of a novel leptin receptor duplicate in Atlantic salmon: Expression analyses in different life stages and in response to feeding status

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ABSTRACT

In recent years rapidly growing research has led to identification of several fish leptin orthologs and numerous duplicated paralogs possibly arisen from the third and fourth round whole genome duplication (3R and 4R WGD) events. In this study we identify in Atlantic salmon a duplicated LepRA gene, named $LepRA_2$, that further extend possible evolutionary scenarios of the leptin and leptin receptor system. The 1121 amino acid sequence of the novel $LepRA_2$ shares 80% sequence identity with the $LepRA_1$ paralog, and contains the protein motifs typical of the functional (long form) leptin receptor in vertebrates. *In silico* predictions showed similar electrostatic properties of $LepRA_1$ and $LepRA_2$ and high sequence conservation at the leptin interaction surfaces within the CHR/leptin-binding and FNIII domains, suggesting conserved functional specificity between the two duplicates. Analysis of temporal expression profiles during pre-hatching stages indicate that both transcripts are involved in modulating leptin developmental functions, although the $LepRA_1$ paralog may play a major role as the embryo complexity increases. There is ubiquitous distribution of LepRs underlying pleiotropism of leptin in all tissues investigated. $LepRA_1$ and $LepRA_2$ are differentially expressed with $LepRA_1$ more abundant than $LepRA_2$ in most of the tissues investigated, with the only exception of liver. Analysis of constitutive $LepRA_1$ and $LepRA_2$ expression in brain and liver at parr, post-smolt and adult stages reveal striking spatial divergence between the duplicates at all stages investigated. This suggests that, beside increased metabolic requirements, leptin sensitivity in the salmon brain might be linked to important variables such as habitat, ecology and life cycle. Furthermore, leptins and LepRs mRNAs in the brain showed gene-specific variability in response to long term fasting, suggesting that leptin's roles as modulator of nutritional status in Atlantic salmon might be governed by distinct genetic evolutionary processes and distinct functions between the paralogs.

1. Introduction

In mammals the class I cytokine hormone, leptin, has long been recognized as one of the major mediators of energy balance and feed intake (Friedman, 2009; Ahima and Flier, 2000). Leptin functions are primarily exerted via the class-I helical cytokine receptor (*Ob*-R), mostly in the hypothalamus (Meister, 2000) by JAK/STAT intracellular pathways (Zabeau et al., 2004; Frühbeck, 2006), although its wide mRNA distribution in non-neuronal tissues (Tartaglia et al., 1995; Cioffi et al., 1996; Wang et al., 1996) is consistent with multiple peripheral effects (Tamashiro and Moran, 2010; Zieba et al., 2005; Fietta, 2005). Following the characterization of the first leptin gene in teleost fish (Kurokawa et al., 2005), increased access to DNA resources from non-mammalian databases have led to the identification of several teleost leptin orthologs (see review Londraville et al., 2014), including numerous duplicated paralogs possibly arisen from the third and fourth round of whole genome duplication (3R and 4R WGD) events in their ancient genome (Allendorf and Thorgaard, 1984). Several studies have indicated that leptin duplicate genes from 'diploid' and 'tetraploid' fish display distinct mRNA

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expression patterns in several organs, and suggest that the different paralogs might have already acquired distinct evolutionary pathways (Gorissen et al., 2009; Kurokawa and Murashita, 2009; Rønnestad et al., 2010; Angotzi et al., 2013; Morini et al., 2015). Few studies have targeted the teleost leptin receptors so far, but some data is available for zebrafish (*Danio rerio*, Liu et al., 2010), medaka (*Oryzas latipes*, Kurokawa and Murashita, 2009), goldfish (*Carassius auratus*, Tinoco et al., 2012), Atlantic salmon (*Salmo salar*, Rønnestad et al., 2010), orange spotted grouper (*Epinephelus coioides*, Zhang et al., 2013), fugu (*Takifugu rubripes*, Kurokawa et al., 2008), rainbow trout (*Oncorhynchus mykiss*, Gong et al., 2013), Nile tilapia (*Oreochromis niloticus*, Shpilman et al., 2014), European eel (*Anguilla anguilla*, Morini et al., 2015) and sea bass (*Dicentrarchus labrax*, Escobar et al., 2016).

We have previously identified and reported one LepR gene and two leptin paralog-pairs (LepA1/LepA2 and LepB1/B2) in Atlantic salmon (Rønnestad et al., 2010; Angotzi et al., 2013). Consistently, the tetraploid state of salmonids (Danzmann et al., 2008) suggests that two or more LepR *loci* also could exist in the DNA of this species.

With the aim to provide a broader analysis of the leptin/leptin receptor system in Atlantic salmon, we here report the identification of a novel functional (long form) leptin receptor duplicate ($LepRA_2$). We have also quantified mRNA expression of both the previously known LepR (hereafter renamed $LepRA_1$) and the novel $LepRA_2$ in four developmental stages and in selected tissues in important stages of the salmon life cycle.

Predictive models of hypothetical molecular interactions between leptin ligands and cognate receptor has been carried out in a large evolutionary comparative context (Prokop et al., 2012), and suggests that recently duplicated LepA1/LepA2 may have already acquired different binding properties in Atlantic salmon. Thus, to provide preliminary insights on $LepRA_1$ and $LepRA_2$ leptin modulatory functions, we predicted, by computational methods, their putative binding sites and analyzed the affinity properties to distinct leptins.

Protein modelling studies suggest high conservation of leptin protein tertiary structure from fish to mammals (Gorissen et al., 2009; Rønnestad et al., 2010; Shpilman et al., 2014). Nevertheless, accumulating data in the literature indicates that beside conservation, the two leptin classes may have different functions, for instance in regulation of energy balance and food intake.

Remarkably, responses to catabolic state are opposite to what is observed in mammals, where increased levels of leptin in plasma have been reported during starvation (Atlantic salmon, Trombley et al., 2012; Arctic charr, *Salvelinus alpinus*, Frøiland et al., 2012; rainbow trout; Gong et al., 2013). Thus finally, for a holistic evaluation, functional patterns correlated to long term changes in nutritional status, mRNA gene expression of the leptin system repertoire known to date ($LepRA_1$, $LepRA_2$, LepA1, LepA2, LepB1-2, (common transcript) were examined in two groups of Atlantic salmon fed either *ad libitum* or a restricted ration.

2. Material and methods

2.1. Fish material

Atlantic salmon fertilized eggs from AquaGen strain were collected at Marine Harvest hatchery at Tveitevågen (Norway) at 168, 215, 262 and 332 degree-days (dd) of embryonic development, as previously reported (Angotzi et al., 2013). In short, 30 embryos were dissected at each stage and pools of triplicate samples containing 10 embryos each were prepared for further processing. In larger fish, tissue distribution of relative mRNA abundance of $LepRA_1$ and $LepRA_2$ was performed in gills, ovary, spleen, stomach, hindgut, midgut, pituitary, heart, white muscle, belly flap, adipose tissue, brain and liver isolated from post-smolt immature females (mean weight 210.2 g, length 26.2 cm; n = 6) obtained from the Aquatic Laboratory of the Bergen High Technology Center.

Brain and liver samples from parr (mean weight 31.8 g, length 14.2 cm; n = 9), post-smolt (adapted to seawater for 3 months; mean weight 179.2 g, length 24.8 cm; n = 10) and 1 sea winter old adults (mean weight 1435 g, length 48 cm; n = 10) were isolated from fish fasted 1 day before being sacrificed. To evaluate the long term effects of feeding on leptins and LepRs mRNA expression, brain and liver tissues were isolated from a subset of post-smolt females (n = 47), coming from a large scale experiment conducted at the facilities of the Institute of Marine Research (IMR) in Matredal (61 °N, Norway). Salmon of mean body weight 1750 g, length 51.6 cm, were maintained in two sea cages ($5 \times 5 \times 8$ m) with temperature from 3 to 14.8 °C. The sea cages were placed close to a river outlet in the inner part of the fjord, resulting in a brackish upper layer, with mean salinity ranging from 14.0 to 29.7 ppt at 1–8 m depth. In one of two sea cages, salmon were fed *ad libitum* (100%) commercial dry pellets (Biomar ASA, Norway) calculated on basis of body weight and water temperature for 7 days a week, and defined as normal fed group (NF; n = 22), whereas fish in the other sea cage received restricted feeding 3 days per week (about 40% the amount of food given *ad libitium*), and is defined as low fed group (LF; n = 25). Fish were starved for two days prior to each sampling, caught by a dip net and anesthetised with 10 ppt metomidate (Syndel, Vitoria, BC, Canada) in 200 L seawater. The fish were then weighed, fork length measured and killed by an overdose of MS-222 (3-aminobenzoicacid ethyl ester; Sigma, St. Louis, MO). Tissues were dissected out, snap frozen on liquid nitrogen and then stored at −80 °C until assayed. Handling of animals were performed by scientists licensed by the Norwegian animal research Authority (NARA) and under due consideration of the NARA guidelines for experimental purposes.

2.2. In silico identification and cloning of salmon $LepRA_2$

Atlantic salmon $LepRA_1$ amino acid (AA) sequence (acc.No. NP001158237) was used as a query against the cod (*Gadus morhua*) genome project *_Salmo salar* database_ by using translated tblastn search algorithm (http://www.codgenome.no/blast/blast_new.php).

Full transcript coverage was obtained by using 5′ and 3′ rapid amplification of cDNA ends (RACE) libraries prepared from liver mRNA (Quick Prep Micro mRNA Purification Kit (Amersham Pharmacia, UK), according to the manufacturer's instructions (Marathon™ cDNA Amplification Kit, Clontech, CA, USA) and using gene specific-primers (GSP) for primary and nested PCRs (P5rv/N5rv; and P3fw/N3fw primers for 5′ and 3′RACE respectively, Suppl. Tab. 1).

Full-length $LepRA_2$ transcripts were then amplified from liver tissue using Long-Range PCR Kit (Qiagen, Toronto, ON, Canada), carried out as follow: 93 °C for 3 min, 15 cycles of 93 °C for 15 s, 62 °C for 30 s, 68 °C for 4 min followed by 7 min at 68 °C (primers: cdR2fw/cdR2rv; Suppl. Tab. 1).

Gel purified products by QIAquick gel extraction kit (Qiagen, Canada) were cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced at the University of Bergen Sequencing facility using Big-Dye Terminator v3.1 chemistry in ABI PRISM377 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

2.3. RNA extraction and cDNA synthesis

For total RNA extractions, brain tissues were divided into three parts and subsequently pooled together prior cDNA synthesis. All