

Divergence of Leptin Receptor and Interleukin-6 Receptor Subunit b in Early Vertebrate Evolution and Physiological Insights from the Sea Lamprey

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Abstract

Current knowledge of class-I cytokine receptors comes primarily from studies in jawed vertebrates (gnathostomes), and their origin and evolution remain unresolved. In this study, we identified a leptin receptor-like sequence (LepRL) and three interleukin-6 receptor subunit b-like sequences (IL6RBL) from a jawless vertebrate (cyclostome), the sea lamprey (*Petromyzon marinus*). Based on structural, phylogenetic, and syntenic analyses, we deduced that these lamprey receptors are likely distinct ohnologs to gnathostome LepR and IL6RB-related receptors, respectively, that arose in the two rounds of vertebrate whole-genome duplication (1R and 2R). Notably, lamprey LepRL likely originated from a different 1R progenitor than the one giving rise to gnathostome LepR during cyclostome hexaploidization. Differential patterns in mRNA expression of LepRL and IL6RBLs were observed among adult tissues, during larval metamorphosis, and in response to juvenile feeding. Feeding stimulated hepatic expression of LepRL and IL6RBL (namely, IL6RBL1) mRNAs in correlation with upregulation of insulin-like growth factor mRNA, whereas brain LepRL and IL6RBL1 mRNA expression was correlated positively with neuropeptide Y but inversely with intestinal content in fed juveniles. Notably, these observations along with immunolocalization of LepRL in the hypothalamus suggest a role of leptin signaling in regulating energy balance that is conserved among vertebrates. Additionally, seawater exposure stimulated branchial LepRL expression coincident with increased expression of ion transporters in ionocytes, indicating a role of leptin signaling in osmoregulation. These findings provide new insight into the early evolution of class-I cytokine receptors and reveal diverse functions of the leptin signaling system in jawless vertebrate.

Keywords: lamprey, leptin receptor, IL-6 receptor, molecular evolution, energy balance, osmoregulation

Introduction

The functions of cytokines are known primarily from studies in jawed vertebrates (gnathostomes). For example, leptin is a class-I cytokine that plays a central role in regulating energy balance, and deficiency of leptin function causes hyperphagia and severe obesity in mammals (Friedman 2019; Blanco and Soengas 2021). In the central nervous system, leptin regulates appetite-regulating neuropeptides, including neuropeptide Y (NPY) and pro-opiomelanocortin (Qi et al. 2023). Leptin signals through a leptin receptor (LepR), which is primarily expressed in the hypothalamus, particularly in the arcuate nucleus (ARC) in mammals (Elmqvist et al. 1998), amphibians (Cui et al. 2014), and teleosts (Gong et al. 2016a). In addition to leptin, interleukin-6 (IL-6) family cytokines also play essential roles in energy balance and metabolic homeostasis (Cron et al. 2016; Rose-John 2018). For example, IL-6 can

suppress food intake and improve energy homeostasis, exerting beneficial metabolic effects under conditions of leptin resistance (Timper et al. 2017; Mishra et al. 2019); ciliary neurotrophic factor (CNTF) can overcome leptin resistance in diet-induced obesity through activating leptin-like pathways in the hypothalamus (Lambert et al. 2001). The IL-6 family cytokines use a common receptor subunit in their receptor complex for signaling, namely, the IL-6 receptor subunit beta (IL6RB), also known as IL-6 signal transducer (IL6ST) (Rose-John 2018).

LepR and IL6RB share structural homology in functional domains for extracellular ligand binding, transmembrane anchoring, and intracellular signaling activation and therefore are generally grouped into the superfamily of class-I cytokine receptors (Boulay et al. 2003). All the class-I cytokine receptors include one or two cytokine receptor homolog (CRH)

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domains with a common signature in the C-terminal WSXWS motif that have a function in ligand binding (Huising et al. 2006; Peelman et al. 2014). Additional extracellular domains include an immunoglobulin-like (Ig) domain immediately upstream of the CRH domain and several fibronectin type III (FN3) domains that follow the CRH domain (Peelman et al. 2014). In contrast to IL6RB that has a single CRH domain followed by three FN3 domains, LepR has duplicate CRH domains followed by two FN3 domains. However, only the second CRH domain of the LepR serves as the high-affinity ligand-binding domain (López-Hidalgo et al. 2023), and dimerized LepR can form an asymmetric architecture similar to the heterodimeric IL-6 family receptor complexes (Saxton et al. 2023). Moreover, LepR and IL6RB utilize similar molecular mechanisms in intracellular signaling transduction via the major cascades of Janus kinase (JAK) and signal transducers and activators of transcription (STAT) (Liongue and Ward 2007; Saxton et al. 2023). The structural homology and similar signaling mechanisms indicate the close relationship of LepR and IL6RB in molecular evolution, likely diverging from a common ancestor, given that a single cytokine receptor-like transmembrane protein (domeless; Dome) is identified in fruit fly (*Drosophila melanogaster*). Dome is composed of extracellular structures in the cytokine binding and FN3 domains as well as intracellular motifs essential for JAK/STAT signaling, similar to vertebrate IL6RB and LepR (Brown et al. 2001; Londraville et al. 2017; Moore et al. 2020). Other than the *Drosophila* Dome, current knowledge of the evolution of cytokine receptors and their signaling systems primarily comes from studies of jawed vertebrates (gnathostomes). The origin and early evolution of these cytokine receptors in vertebrates remain unresolved.

A group of class-I cytokine receptors are annotated in the genome of sea lamprey (*Petromyzon marinus*), a representative of jawless vertebrates (cyclostomes) (Smith et al. 2018); however, such annotation is often insufficient for assigning proper nomenclature for these lamprey receptors due to low sequence identities (generally <25%) with homologous gnathostome genes, leading to ambiguous placements in phylogenetic trees (Boulay et al. 2022). Moreover, the ligands to those receptors and their physiological functions have yet to be discovered. For example, leptin and the IL-6 family cytokines have been identified in all gnathostome taxa, but not in any other animal groups, nor in the annotation of genomes of urochordates (tunicates), cephalochordates (amphioxus), or cyclostomes (lamprey and hagfish). Earlier attempts to search for leptin and LepR in lamprey genomes were fruitless, leading to the assumption that the leptin system exists exclusively in jawed vertebrates (Hara et al. 2018; Kuraku et al. 2023). These failed attempts may have been due to the extremely low sequence identity shared between lamprey and gnathostome leptins, which made it difficult to detect in the lamprey genomes, especially given the high divergence of leptin sequences even among gnathostome taxa (Blanco and Soengas 2021). For instance, compared with human leptin, amino acid identities are 28% to 38% for birds and less than 25% for teleosts and chondrichthyans. Therefore, we made another attempt to identify LepR and related cytokine receptors in the lamprey.

In this study, we characterized four novel cytokine receptors with structural similarity to gnathostome IL6RB from the sea lamprey genome and proposed a scenario for the early evolution of LepR and IL6RB in vertebrates. To further explore

their physiological roles in sea lamprey, the patterns of mRNA expression of lamprey LepR- and IL6RB-like genes were examined in various tissues, under different nutritional states, and at different life cycle stages. We also immunolocalized sea lamprey LepR-like protein in the brain and gill and examined its response to salinity change. These studies allow us to infer the physiological roles of the leptin system in the lamprey.

Results

Identification of Lamprey LepR- and IL6RB-Related Cytokine Receptors

The sea lamprey LepR-like gene (Pma_LepRL; GenBank accession no. XM_032966187) was cloned and sequenced for its open reading frame, encoding a 988-amino acid protein (supplementary fig. S1, Supplementary Material online). Despite low amino acid identities (<28%) between Pma_LepRL and gnathostome LepRs, highly conserved domains were identified in the extracellular and intracellular regions (Fig. 1). These include an Ig domain with two conserved cysteine residues, a single CRH domain with four conserved cysteines and the signatural WSXWS motif, several FN3 domains, and a single transmembrane domain, followed by a long intracellular domain containing a JAK-binding motif (JAK box) and several C-terminal tyrosine residues, particularly a YXPQ motif for STAT activation (Fig. 1a). Unlike gnathostome LepR, such as LepRs from reedfish (*Erpetoichthys calabaricus*; Eca) and human (*Homo sapiens*; Hsa), which contain two CRH domains, Pma_LepRL consists of a single CRH domain (Fig. 1). Additionally, Pma_LepRL has conserved a third FN3 domain encoded by two additional exons, which was absent in gnathostome LepRs (supplementary fig. S1, Supplementary Material online; Fig. 1b).

Three cytokine receptors with protein architectures similar to IL6RB were identified in the sea lamprey genome using TBLASTN with Pma_LepRL as a query. A IL6RB-like gene (IL6RBL1; XM_032965904) was found on the same chromosome as LepRL. Its open reading frame was cloned and sequenced, encoding 980 amino acids with less than 28% identity to gnathostome IL6ST. The Pma_IL6RBL1 has similar structural assembly as gnathostome IL6ST, including a single CRH domain with conserved cysteine residues and WSXWS motif and three FN3 domains, followed by a transmembrane domain and a long intracellular region containing two C-terminal YXPQ motifs (Fig. 1a). Similarly, the second IL6RB-like gene (IL6RBL2; XM_032956645) has a similar receptor structure, also with two C-terminal YXPQ motifs (Fig. 1a). In contrast, IL6RBL3 (XM_032956130) shares the overall structure of IL6RB but lacks C-terminal tyrosine residues (Fig. 1a). Unlike Pma_LepRL and gnathostome LepRs with a single YXPQ motif, Pma_IL6RBL1 and IL6RBL2 contain two YXPQ motifs, like gnathostome IL6STs (Fig. 1a).

Phylogeny of LepR- and IL6RB-Related Receptors

Alignment was performed by using full-length amino acid sequences of gnathostome LepRs and IL6RB-related paralogs that are present in the same paralogon, including IL6ST, interleukin-31 receptor subunit α (IL31RA), an IL31RA-like (IL31RAL; not found in human), interleukin-12 receptor β 1 and β 2 (IL12RB1 and IL12RB2), interleukin-23 receptor (IL23R), and IL-27 receptor α (IL27RA, absent in chicken), as well as an IL6RB-like that is only found in cartilaginous

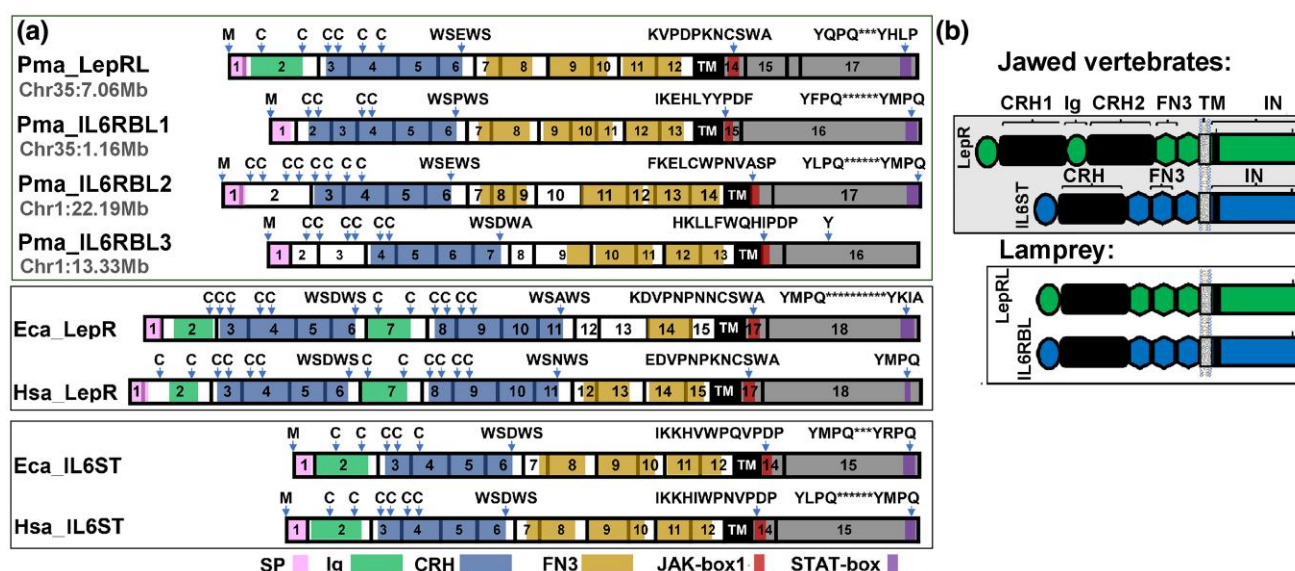


Fig. 1. Structural analyses of sea lamprey (Pma) LepR-like and IL6RB-like receptors in comparison with LepR and IL6ST from reedfish (Eca) and human (Hsa). a) All of the receptors are composed of an Ig-like domain (shaded in green), a CRH domain (in blue) and several FN3 domains (in yellow) in the extracellular region, a single transmembrane domain (TM, in black), and a long intracellular region (IN) with a JAK docking box (in red) and C-terminal Y residues for STAT activation (in purple). SP, signal peptide; other letters stand for amino acid residues; numbers represent the order of the exons. b) Schematic depicting the assemblies of functional domains of LepR and IL6RB in the lamprey and jawed vertebrates.

and ray-finned fishes (gene information listed in [supplementary table S2, Supplementary Material](#) online). Those receptors cluster into four major clades with strong support (>90%): a LepR-related and three IL6RB-related (Fig. 2a). By adding the lamprey receptors in the full-length alignment, lamprey LepRL clusters with gnathostome LepRs with good support, and all lamprey IL6RBLs cluster into a single branch as a sister group of gnathostome IL6RB-related clades ([supplementary fig. S2, Supplementary Material](#) online).

Because a common CRH domain is shared by all cytokine receptors, amino acid sequences of this domain were aligned for additional phylogenetic analysis. The phylogenetic tree of the CRH (Fig. 2b) shows the four major clades in jawed vertebrates, and lamprey LepRL clusters with the two CRH clades of gnathostome LepRs with good root support (89%). Differentially, lamprey IL6RBL2 clusters with IL23R, IL12RB2, and IL27RA with good support (78%); lamprey IL6RBL3 clusters with IL6ST, IL31RA, and IL6RBL with good support (84%); lamprey IL6RBL1 appears to be an outgroup to the two clades with good support (80%). Gnathostome IL31RAL and IL12RB1 cluster in a separate clade with good support (95%) that lacks a lamprey representative. The phylogenetic analysis of CRH domain agrees well with the phylogeny of full-length sequences and additionally reveals that lampreys IL6RBL2 and IL6RBL3 are related to distinct gnathostome clades. It also indicates that lamprey LepRL is indeed most closely related to gnathostome LepRs.

Synteny of LepR- and IL6RB-Related Genes with 13 Neighboring Gene Families

Sea lampreys LepRL and IL6RBL1 are both located on chromosome 35 (Pma_Ch35) ~5.9 million base pairs (Mb) apart, whereas the other two IL6RBLs are located on Pma_Ch1 ~8.8 Mb apart. Synteny was assessed by using six neighboring gene families in the vicinity of LepRL within

a 1 Mb region (Fig. 3) as well as six gene families in the vicinity of IL6RBL1 within a 1.5 Mb region (gene information listed in [supplementary table S3, Supplementary Material](#) online). In particular, we examine the conserved synteny of closely adjacent gene families within narrow regions, due to the extensive chromosomal rearrangements that have occurred independently in gnathostomes and cyclostomes following their split (Simakov et al. 2020; Marlétaz et al. 2024). Additionally, JAKs and LepR overlapping transcript (LEPROT) also were selected because of their loci adjacent to LepR in jawed vertebrates. In sea lamprey, paralogs in these neighboring gene families were mostly found on Pma_Ch1 and Pma_Ch44, which were determined paralogous to Pma_Ch35 associated with ancestral chordate linkage group L (CLGL) in multigenomic comparison (Marlétaz et al. 2024). The CLGs represent groups of genes, whose linkages have been preserved after the split of vertebrates and cephalochordates, and are used to assess conservation of chromosomal synteny following the ancient whole-genome duplications (WGDs) in vertebrates (Putnam et al. 2008; Simakov et al. 2020; Marlétaz et al. 2024). In this analysis, class-I cytokine receptors and the neighboring gene families were searched in the genome of amphioxus (*Branchiostoma floridae*; Bfl). Ten families were found members on Bfl_Ch15 (Fig. 3), as well as a single cytokine receptor, namely, prolactin receptor-like (PRLRL) that contains only an extracellular sequence (Ocampo Daza and Larhammar 2018).

The synteny of LepR, IL6RB paralogs, and the neighboring gene families was analyzed in a representative for Chondrichthyes, whitespotted bamboo shark (*Chiloscyllium plagiosum*; Cpl); two ray-finned fishes (Actinopterygii), spotted gar (*Lepisosteus oculatus*; Loc) and reedfish (Eca); and a representative for tetrapods, chicken (*Gallus gallus*; Gga). These species were selected due to their relatively conserved karyotypes in comparison with the deduced ancestral gnathostome karyotype (Simakov et al. 2020; Nakatani et al. 2021; Marlétaz et al. 2024). These selected gene families are mostly

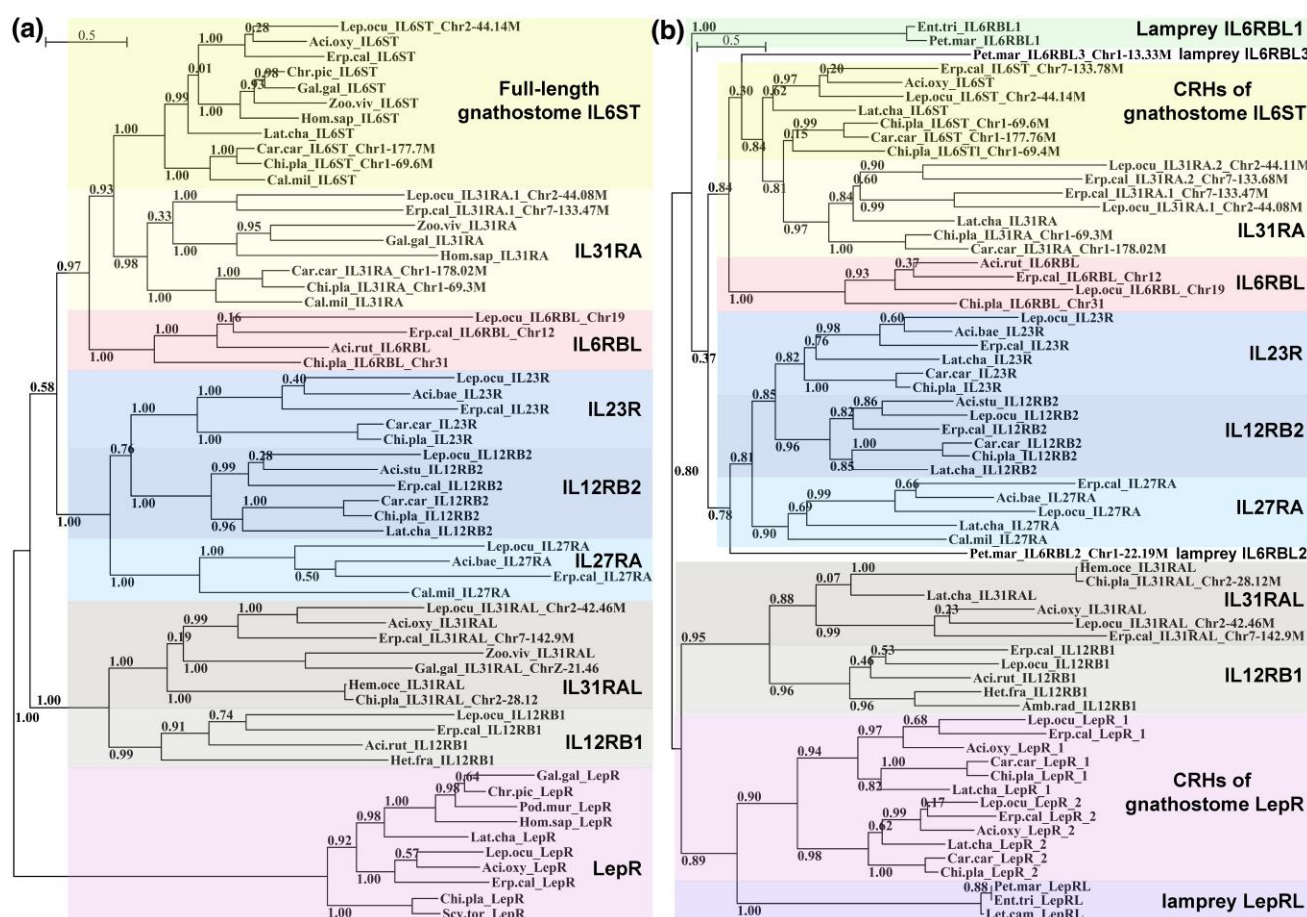


Fig. 2. Phylogenetic analyses of LepR- and IL6RB-related receptors in vertebrates. a) Full-length sequence alignment of gnathostome IL6RB-related receptors without the signal peptides and LepRs without the first CRH domain that is not essential for ligand binding. The receptors cluster into four major clades with strong node support. b) Alignment of the CRH domains of gnathostome LepR and IL6RB paralogs, as well as of lamprey LepRL and IL6RBLs, showing their relationship with the gnathostome receptors. MUSCLE algorithm and the likelihood-based phylogenetic maximum likelihood method were used in the phylogenetic analysis. Gene information is listed in [supplementary table S2, Supplementary Material](#) online.

found in the CLGL-associated paralogs that may have arisen during the two rounds of gnathostome WGD (Marlétaz et al. 2024). For instance, in spotted gar, the pair of *Loc_LG10* and *LG6* is described as the duplicates arising from the second round of WGD in jawed vertebrates (2R_{Jv}), and likewise for *Loc_LG2* and *LG19*, whereas the ancestors of the two pairs arose in the first round (1R) of vertebrate stem WGD. Similarly, these genes are located on the orthologous CLGL-associated chromosomes in bamboo shark and chicken: the pair of *Cpl_Ch11* and *Chr8* and the pair of *Gga_Ch8* and *Chr30* from 1R.1 lineage and the paralogous *Cpl_Ch1*, *Chr2*, and *Chr31* and the pair of *Gga_ChZ* and *Chr28* from 1R.2 lineage (Fig. 3). The synteny of these gene families is highly conserved among jawed vertebrates, with the most identical patterns observed between spotted gar and reedfish.

It is believed that all extant vertebrates have gone through the 1R WGD event prior to the cyclostome–gnathostome split that followed by extensive chromosomal rearrangements and cyclostome hexaploidization and gnathostome tetraploidization (Nakatani et al. 2021; Marlétaz et al. 2024; Yu et al. 2024). Thus, we deduced the 1R progenitors of the lamprey genes based on conserved synteny and phylogenetic analyses of neighboring gene families, including *JAK1/2/3/TYK2*, *MIER1/2/3*, *PLPP1/2/3/PPAD2D*, *S1PR1/2/3/4/5*, *RAB3A/B/C/D*, *PDE4A/B/C/D*, and *FOX1/2/3* (supplementary fig. S3,

Supplementary Material online). The analyses indicate that the segment including *IL6RBL2*, *PLPP3L*, and *S1PR5L* on *Pma_Ch1:22.1–23.1Mb* may have arisen from the 1R progenitors that gave rise to gnathostome *IL23R–IL12RB2* in the vicinity of *PLPP3* and *S1PR1/5*. In contrast, the segment including *IL6RBL3*, *MIER3L*, and *PDE4C/D* on *Pma_Ch1:12.6–13.3Mb* likely arose from the other 1R lineage (1R.2) that gave rise to *IL6ST–IL31RAL* in the vicinity of *MIER3* and *PDE4C/D*. Interestingly, lamprey *IL6RBL1* is adjacent to another *MIER3L* variant, *PLPP3L* variant, and *S1PR1*, and such a syntenic pattern is not present in jawed vertebrates. Different from gnathostome *LepR* adjacent to *IL23R–IL12RB2*, *RAB3B*, and *PDE4B*, lamprey *LepRL* is ~6 Mb apart from *IL6RBL1* and adjacent to *RAB3A/C* and *PDE4C/D*, which are descendant genes from the other 1R lineage (1R.2). This suggests that lamprey *LepRL* likely arose from the same 1R.2 lineage as these neighboring genes.

The mRNA Expression of LepRL and IL6RBLs among Tissues in Adult Sea Lamprey

LepRL mRNA was highly expressed in the pituitary and head kidney; moderately expressed in the brain, gill, post-kidney, muscle, visceral fat, and testes; and minimally expressed in the liver and anterior intestine (Fig. 4a). Notably, the highest

Fig. 3. Chromosomal comparison of LepR- and IL6RB-related genes and neighboring gene families in amphioxus (*B. floridae*), sea lamprey, a cartilaginous fish (the bamboo shark), two ray-finned fishes (spotted gar and reedfish), and chicken. The analysis includes six neighboring gene families (OSBP9, MIER, PLPP, ZNT7, DPH5, and S1PR) in the vicinity of IL6RBL1 on Pma_Ch35 in the range of 0.87 to 1.48 Mb and six neighboring gene families (SLC38A9, PLPP, RAB3, PDE4, FOXD, and ATG4) in the vicinity of LepRL in the range of 6.63 to 8.11 Mb, as well as JAK and LEPROT that are generally adjacent to LepR in gnathostomes. LepR and IL6RB paralogs and the neighboring gene families are found on the same paralogon in jawed vertebrates. The duplicates (α and β) of each 1R lineage are highlighted in blue and light blue (1R.1) or yellow and pink (1R.2). Based on phylogenetic analysis, the Pma_chromosomal segments originating from the same 1R lineage are marked in a similar way. In the amphioxus, ten gene families are identified on Bfl_Ch15 in the Bfl_VNyyK assembly. Notably, a single PRLRL is identified in the vicinity of LEPROT, OSBP9, ZNT7, and SLC38A9 within a 1 Mb region. It appears that lamprey LepRL arose from the 1R.2 lineage, whereas gnathostome LepR arose from the 1R.1 lineage. Gene information is listed in [supplementary table S3, Supplementary Material](#) online; loci are noted in Mb; Chr, chromosome; LG, linkage group.

mRNA levels of LepRL were detected in whole blood cells, being approximately six times higher than levels in the pituitary. The three IL6RBL mRNAs were differentially expressed among tissues, with generally low abundance in blood cells (Fig. 4b to d). In particular, IL6RBL1 mRNA was highly expressed in the brain and liver, but only slightly expressed in the pituitary and gill; IL6RBL2 mRNA was also highly expressed in the liver, but minimally expressed in the brain, pituitary, gill, and anterior intestine; IL6RBL3 mRNA was uniformly expressed in central and peripheral tissues. All IL6RBLs were moderately expressed in the kidney, muscle, and visceral fat.

The mRNA Expression of LepRL and IL6RBLs in the Liver and Gill during Metamorphosis of Sea Lamprey

Hepatic LepRL mRNA levels increased significantly in metamorphic transformers during early to midstages (stages 2 to 4 of 7) (one-way ANOVA, $P < 0.0001$; Fig. 4e), but declined at the final stage. Conversely, mRNA levels of IL6RBL1 were highest in larvae and stage-1 transformers, gradually reduced at stage 3, and rebounded at the final stage ($P = 0.0001$; Fig. 4f). In contrast, IL6RBL2 mRNA levels increased significantly at stage 3 ($P < 0.0001$; Fig. 4g). No significant changes were observed in levels of IL6RBL3 during the early stages, but levels were higher in final-stage transformers compared to pre-metamorphic larvae ($P = 0.0001$; Fig. 4h).

No significant changes were observed in branchial LepRL mRNA levels at any metamorphic stage (Fig. 4i and j). Both IL6RBL2 and IL6RBL3 were significantly upregulated at the onset of metamorphosis ($P \leq 0.001$; Fig. 4k and l). IL6RBL3 mRNA levels remained elevated in final-stage transformers compared to larvae, whereas IL6RBL2 levels at the final stage dropped to levels similar to those in larvae. IL6RBL1 mRNA was expressed at very low levels in the gill.

Effects of Feeding on LepRL and IL6RBL mRNA Expression in the Liver and Brain

During feeding trial I, feeding sea lamprey had significantly higher condition factors ($K = 100 \times M/L^3$, M = body mass, g; L = body length, cm; $K_F = 0.166 \pm 0.009$; t-test, $P < 0.0001$) than nonfeeding animals ($K_{NF} = 0.101 \pm 0.004$). Hepatic LepRL mRNA levels were significantly elevated in the feeding animals compared to nonfeeders (F vs. NF; t-test, $P = 0.0018$; Fig. 5a). Similarly, hepatic IL6RBL1 and IL6RBL2 mRNA levels were significantly elevated in feeding animals ($P = 0.01$ and $P = 0.0002$, respectively; Fig. 5b and c), but there were no significant changes in the levels of IL6RBL3 mRNA ($P = 0.1$; Fig. 5d). Feeding also significantly stimulated the mRNA expression of hepatic insulin-like growth factor (IGF) ($P = 0.0009$; Fig. 5e). Moreover, IGF expression was correlated significantly with mRNA levels of LepRL ($P = 0.02$) and IL6RBL1 ($P = 0.03$; Fig. 5f), but not with those of IL6RBL2 or IL6RBL3 ($P = 0.07$ and $P = 0.2$).

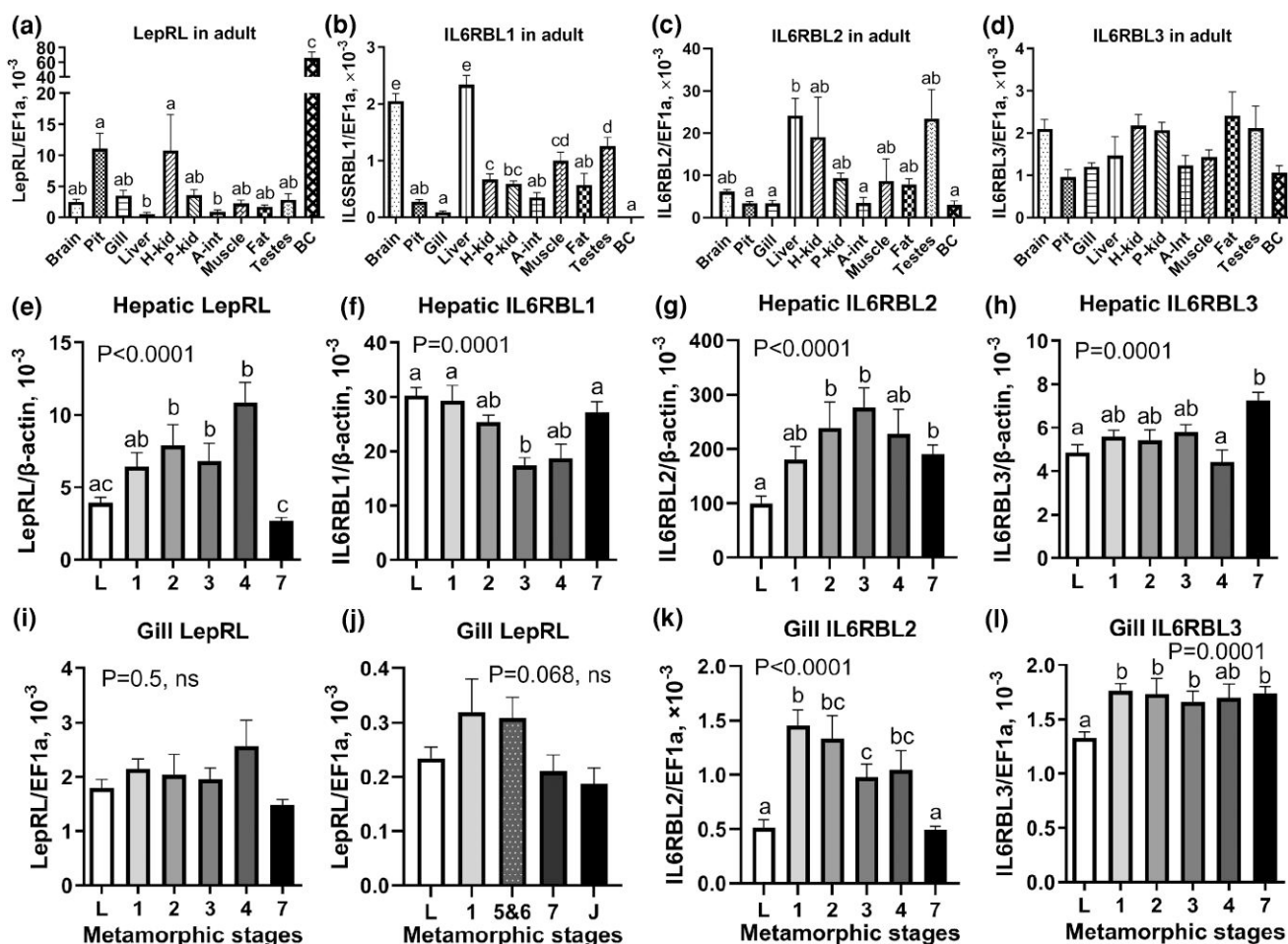


Fig. 4. The mRNA abundance of LepRL and IL6RBLs among tissues in adult sea lamprey and during metamorphosis. a to d) mRNA levels of LepRL and IL6RBLs were determined in the brain, pituitary gland (pit), gill, liver, head kidney (H-kid), posterior kidney (P-kid), anterior intestine (A-int), muscle, visceral fat, testes, and whole blood cells in adults ($N=4$). The levels were normalized to elongation factor 1a (EF1a). e to h) Hepatic LepRL and IL6RBL mRNA levels in premetamorphic larvae ($N=17$), transformers at stages 1 ($N=9$), 2 ($N=5$), 3 ($N=9$), 4 ($N=3$), and 7 ($N=17$); i, k, and l) Branchial LepRL, IL6RBL2, and IL6RBL3 mRNA levels in these animals. j) Branchial LepRL mRNA levels were examined in an additional experiment, including larvae ($N=30$); transformers at stages 1 ($N=9$), 5 and 6 ($N=8$), and 7 ($N=11$); and juveniles (J; $N=11$). Hepatic β -actin and branchial EF1a were used for normalization. Different letters denote significant differences among groups in one-way ANOVA with Turkey's post hoc test; ns, nonsignificant.

No significant changes were observed in the expression of LepRL or IL6RBL3 mRNAs in the brain (Fig. 5a and g). In contrast, brain IL6RBL1 and IL6RBL2 mRNA levels were significantly upregulated in feeding lamprey ($P=0.02$ and $P=0.01$, respectively; Fig. 5h and i). As NPY-expressing neurons are one of the main targets of leptin in the hypothalamus of mice (Lee et al. 2023), NPY was examined in the whole brain of sea lamprey, and its mRNA levels were significantly elevated in feeding animals ($P=0.03$; Fig. 5j) compared to the nonfeeding. NPY mRNA expression was significantly correlated with LepRL and IL6RBL1 in the brain ($P=0.0007$ and $P=0.0008$, respectively; Fig. 5k), but not with IL6RBL2 or IL6RBL3 ($P=0.3$ and $P=0.06$).

During feeding trial II, brain LepRL mRNA levels were downregulated in actively feeding lamprey (intestinal content scores 3 to 5) compared to postfeeders with an empty intestine (one-way ANOVA $P=0.008$; Fig. 6a). Trends toward reduced LepRL mRNA levels in the pituitary (trend $P=0.01$; Fig. 6b) and in the liver (trend $P=0.05$; Fig. 6c) during active feeding also were noted. Similarly, IL6RBL1 mRNA levels tended to decrease in feeding individuals with high intestine scores (trend $P=0.03$; Fig. 6d). Increased intestinal content was

inversely correlated with brain LepRL mRNA levels ($P=0.004$; Fig. 6e) as well as with brain IL6RBL1 mRNA levels ($P=0.03$; Fig. 6e).

Effects of Seawater Exposure on mRNA Expression of Branchial LepRL and Ion Transporters

Seawater (SW) acclimation for 14 d significantly elevated branchial LepRL mRNA levels in juvenile sea lamprey compared to freshwater (FW)-adapted individuals (two-way ANOVA, $P=0.04$; post hoc test, $P=0.001$; Fig. 7a). SW effect was also significant on branchial mRNA expression of selected ion transporters, including ATPase Na⁺/K⁺ transporting subunit beta 3 (ATP1B3; two-way ANOVA, $P=0.0003$; post hoc test, $P=0.03$ at day 3; Fig. 7b), sodium/hydrogen exchanger 3 (NHE3; two-way ANOVA, $P=0.008$; Fig. 7c), and solute carrier family 12 member 2 (SLC12A2.2, or Na⁺-K⁺-Cl cotransporter [NKCC]; $P=0.04$; Fig. 7d), but not on ATPase Na⁺/K⁺ transporting subunit alpha 3 (ATP1A3; Fig. 7e). Moreover, LepRL mRNA expression was significantly correlated with ATP1B3, NHE3, SLC12A2.2, and ATP1A3 (Fig. 7f and g).

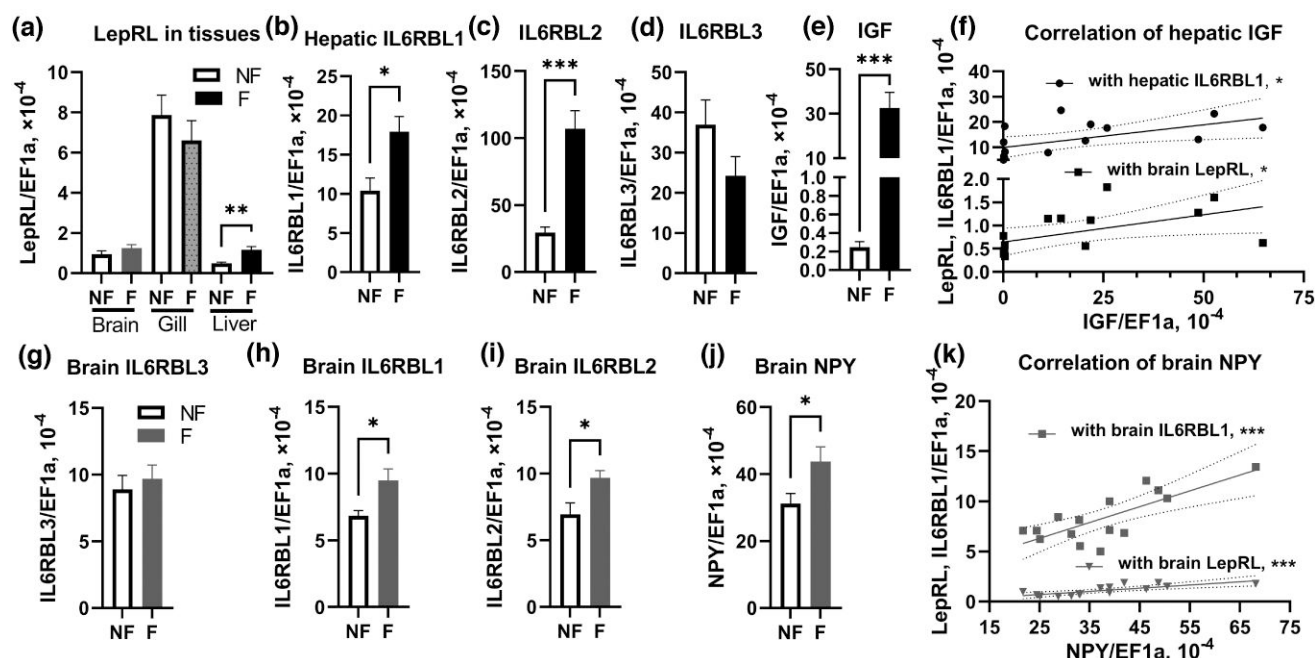


Fig. 5. The mRNA abundance of LepRL, IL6RBLs, IGF, and NPY in the liver and brain of juvenile sea lamprey during a 30-d feeding trial. a) mRNA levels of LepRL in the brain, gill, and liver were compared between nonfeeding (NF; $N=7$) and feeding (F; $N=8$; gray or black bar) juveniles. b to e) Comparison of hepatic mRNA levels of IL6RBLs and IGF; g to k) mRNA levels of IL6RBLs and NPY in the brain. f and k) Correlation of IGF and NPY mRNA expression with LepRL and IL6RBL1 in the liver and brain. Levels were normalized to the reference gene EF1a. Data are presented as mean $\pm$ SEM with significant effects indicated by * ($P<0.05$), ** ($P<0.01$), and *** ($P<0.001$).

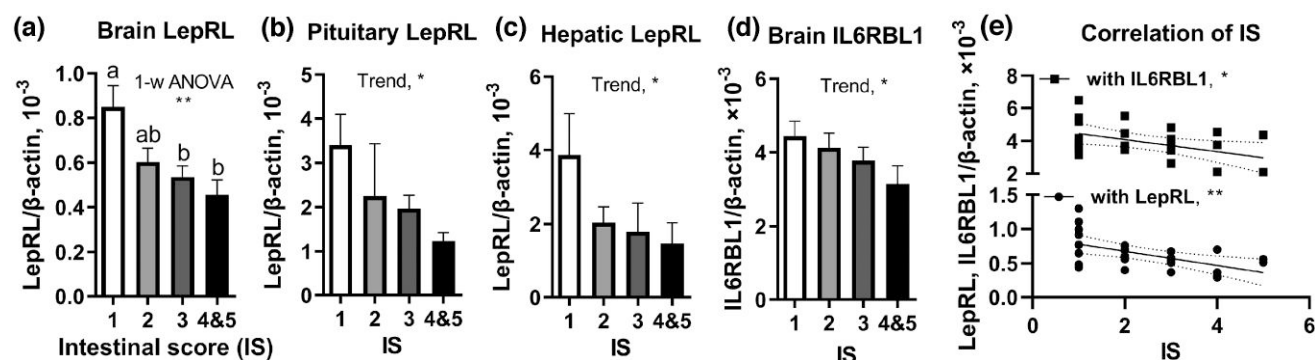


Fig. 6. The mRNA abundance of LepRL and IL6RBL1 in feeding and postfeeding sea lamprey during a 14-d feeding trial. a to d) LepRL and IL6RBL1 mRNA levels were examined in individuals scored by intestinal content and grouped into IS-1 (empty intestine; $N=9$), IS-2 ($N=5$), IS-3 ($N=5$), IS-4 ($N=4$), and IS-5 (full intestine; $N=2$). e) Correlation of intestinal scores with brain LepRL and IL6RBL1 expression. β -actin was used for normalization. Data are presented as mean $\pm$ SEM with significant effects indicated by * ($P<0.05$) and ** ($P<0.01$).

Immunolocalization of LepRL in the Brain and Gill

A recombinant protein of amino acids 710 to 766 of Pma_LepRL (a partial intracellular sequence) was produced in *Escherichia coli* and used as antigen for immunization of hens. Specificity of IgY from egg yolk in detecting Pma_LepRL was examined with cell extracts of a monoclonal HEK293 cell line stably transfected with pcDNA3.1 plasmid containing the encoding sequence of the mature protein of Pma_LepRL. A single band of the predicted size for Pma_LepRL (~ 100 kDa) was detected by immunoblotting with the IgY, indicating good antibody specificity (Fig. 8a).

The IgY was applied for immunohistochemical detection of LepRL in consecutive sections of the diencephalon of sea lamprey juveniles (Fig. 8b, region marked in gray).

Immunoreactive (ir)-staining of LepRL mainly occurred in the rostral region of the hypothalamus (Fig. 8c), primarily in the region of nucleus lateralis tuberis (nlt), but slight ir-staining was observed in the nucleus anterior tuberis (nat; Fig. 8c2). Ir-LepRL staining was barely detected in the proximal hypothalamic region connecting with hypophysis, such as the sections including rostral pars distalis (rpd) and proximal pars distalis (ppd) (Fig. 8d and e).

The distribution of LepRL also was mapped in the gill filaments of juveniles that were acclimated to FW and SW for 14 d. The ir-LepRL staining showed less density in FW-acclimated filaments (Fig. 8f) compared to SW-acclimated filaments (Fig. 8g); particularly, intense staining of LepRL was developed on ionocytes labeled by ir-staining of Na^+/K^+ ATPase (NKA) on the primary lamellae (Fig. 8h and i).

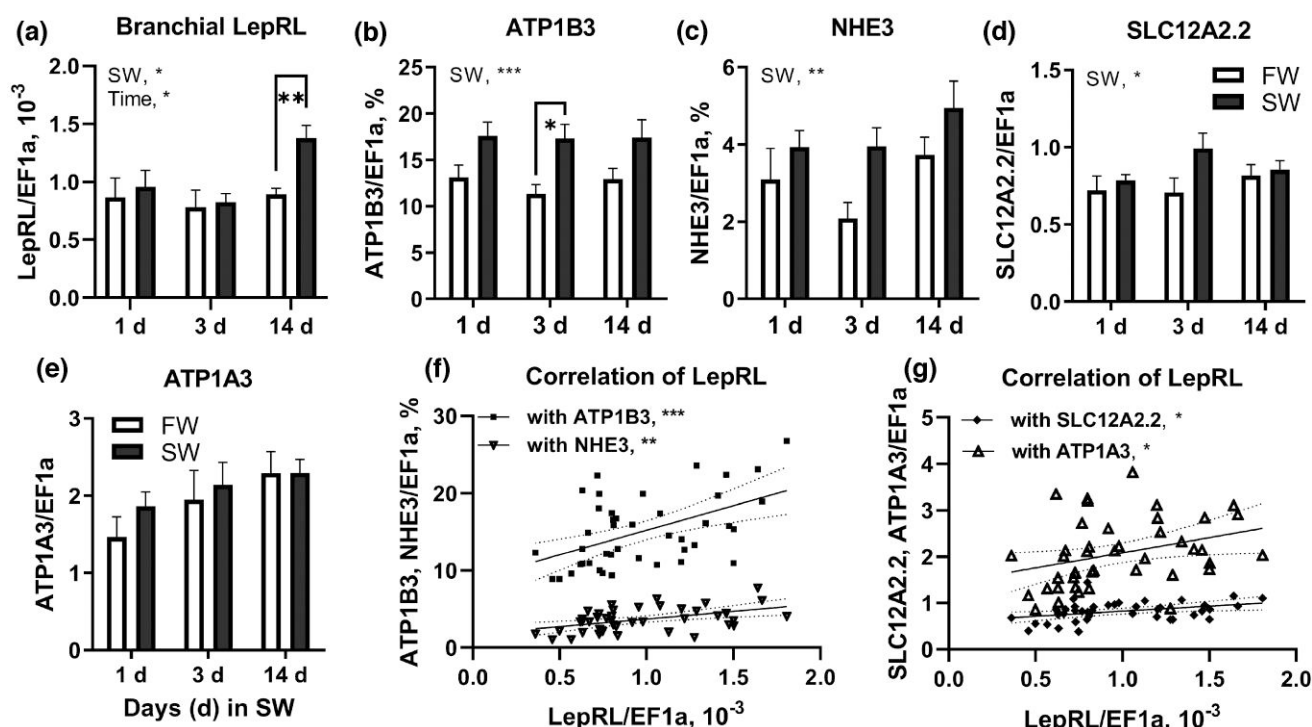


Fig. 7. The mRNA abundance of LepRL and selected ion transporters in the gill of juvenile sea lamprey during seawater acclimation. a to e) mRNA levels of branchial LepRL and ion transporters (ATP1B3, NHE3, SLC12A2.2, and ATP1A3) were compared between SW (solid bars) and FW (open bars) acclimated juveniles after 1 d ($N=6$), 3 d ($N=6$), and 14 d ($N=9$). Two-way ANOVA with Bonferroni's post hoc test showed significant SW effects at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***). f and g) Branchial LepRL expression was significantly correlated with ATP1B3, NHE3, SLC12A2.2, and ATP1A3 ($P = 0.0005$, $P = 0.001$, $P = 0.01$, and $P = 0.03$, respectively). EF1a was used for normalization. Data presented as mean $\pm$ SEM.

Discussion

Molecular Evolution

The superfamily of class-I cytokine receptors consists of up to 36 members in jawed vertebrates and shares a common CRH domain, which is crucial for ligand–receptor interaction and likely originated from a common ancestral lineage (Bazan 1990; Liongue and Ward 2007). Interestingly, an additional phylogenetic tree (supplementary fig. S2, Supplementary Material online) placed the *Drosophila* cytokine receptor Dome as a sister group to the vertebrate LepR and IL6RB clades, suggesting that a common ancestral receptor existed before the divergence of protostomes and deuterostomes. A single cytokine receptor PRLRL is identified in amphioxus, whereas tandem gene duplication may have occurred in vertebrate stem lineage, giving rise to divergent ancestral genes of LepR and IL6RBs. This is suggested by the four major clades formed in the phylogeny of vertebrate receptors: a LepR-related and three IL6RB-related. Further synteny analysis indicates that the clade of IL23R, IL12RB2, and IL27RA and the clade of IL6ST, IL31RA, and IL6RBL likely arose from the 1R duplicates of the same ancestral gene during 2R_{Jv}. The twins of IL23R and IL12RB2, as well as of IL6ST and IL31RA, may have further arisen during local gene duplication. Moreover, the clade of IL31RAL and IL12RB1 lacks a lamprey representative, suggesting that the ancestral gene giving rise to these receptors may have been lost in the lamprey lineage; alternatively, descendant genes may have been eliminated due to functional redundancy with other related receptors. In contrast, novel functions may have evolved in gnathostomes after 2R_{Jv}, coinciding with the emergence of additional cytokines (IL-12 and/or IL-23), which utilize

IL12RB1 for ligand binding and signaling transduction. A differential pattern of IL31RAL loss also is observed in gnathostomes, with its absence in humans and some shark species. Additionally, lamprey LepRL clusters as a sister group to the gnathostome LepR clade, but these ohnologs likely arose from distinct 1R progenitors. Nevertheless, the divergence of these vertebrate receptors appears to have stemmed from ancestral genes related to amphioxus PRLRL, and the linkage of CLGL on the PRLRL-bearing chromosome has also been conserved in the paralogon bearing LepR and IL6RB paralogs in vertebrates.

Based on the results of the phylogenetic and syntenic analyses, we propose a scenario of the early evolution of LepR- and IL6RB-related receptors in vertebrates (Fig. 9). A triplet may have emerged on the same chromosome in the vertebrate stem lineage, leading to the emergence of the ancestral LepR/LepRL, ancestral IL31RAL, and the ancestor that gave rise to IL6ST/IL31RA and IL23R/IL12RB2. A duplicate of the ancestral IL31RAL may have been lost prior to rediploidization in jawed vertebrates, and both duplicates may have been lost in the lamprey lineage. Following 2R_{Jv}, more IL6RB paralogs, namely, IL27RA, IL6RBL, and IL12RB1, were generated and retained on the 2R copies. Notably, IL6RBL is conserved in certain cartilaginous and ray-finned fishes but has been lost in tetrapods. In contrast, only a single LepR gene was preserved in most species after rediploidization, followed by asymmetric gene loss.

In contrast to gnathostome LepR in the vicinity of PDE4B and RAB3B that are duplicates from the 1R.1 lineage, lamprey LepRL is next to PDE4C/D and RAB3C from the 1R.2 lineage. This indicates that the lamprey LepRL and gnathostome LepR are ohnologs, likely originating from distinct 1R progenitors

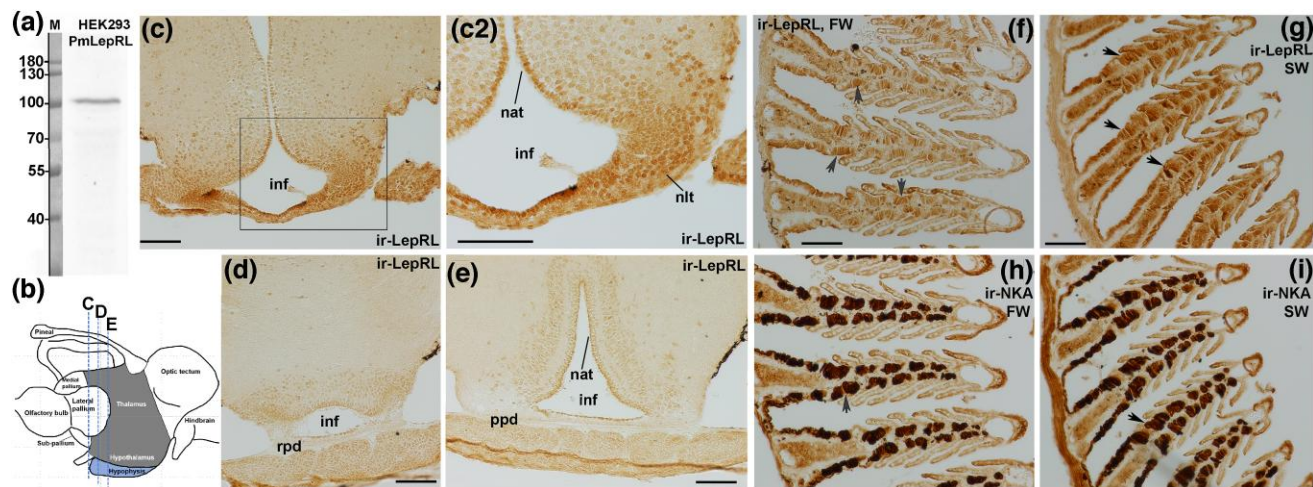


Fig. 8. Immunohistochemical labeling of LepRL in the brain and gill of juvenile sea lamprey. a) Immunoblotting detection of recombinant sea lamprey LepRL at ~100 kDa, expressed by HEK293 cells that were stably transfected with pcDNA3.1 plasmid constructed with the open reading frame of LepRL. b) Illustrations of diencephalon and hypophysis (pituitary) are shaded in gray and blue, respectively; lines C, D, and E indicate the sectioning orientations shown in c) to e) from the rostral to the caudal. c) ir-LepRL staining is found in the rostral region of the hypothalamus, primarily in the nlt and slightly in the nat; 2x magnification of the framed region in c) is shown in c2); inf, infundibulum. d and e) ir-LepRL staining was barely detected in the proximal hypothalamic regions connecting with the rpd and ppd. f and g) ir-LepRL staining in the gill filaments of juveniles acclimated in FW and SW for 14 d, respectively. h and i) ir-staining of Na⁺/K⁺ ATPase (NKA) on ionocytes in the primary lamellae. Arrows point to typical ir-staining; scale bars, 100 μm.

during cyclostome hexaploidization and gnathostome tetraploidization, respectively. Meanwhile, three lamprey IL6RBLs were retained in three separated blocks on two paralogous chromosomes after hexaploidization, followed by biased gene loss and chromosomal rearrangements (Nakatani et al. 2021; Marlétaz et al. 2024). It is possible that the two segments containing IL6RBL2 and IL6RBL3 may have been retained on Pma_Chr1 through fusion. Based on the CRH phylogeny and conserved synteny with neighboring gene families of MIER3L, PLPP3L, and S1PR1/5, we deduce that IL6RBL2 and IL6RBL3 may have arisen from the same 1R lineages as gnathostome IL23R/IL12RB2 and IL6ST/IL31RA, respectively. Lamprey IL6RBL1 may be homologous to gnathostome IL6ST, with two C-terminal tyrosine residues that can be potentially recruited for plasmatic signal transduction and STAT activation.

Notably, LEPROT gene, which is typically adjacent to LepR in jawed vertebrates (Londraville et al. 2017), is absent in the vicinity of LepRL in the lamprey. Instead, two LEPROT paralogs are found on the paralogous chromosomes to the LepRL-bearing Pma_Chr35. This suggests that the proximal loci of LEPROT and LepR may have specifically evolved in jawed vertebrates. The JAK family is also located in the paralogon, with JAK1 typically adjacent to LepR and LEPROT; however, only JAK2 is preserved in the lamprey and located in the vicinity of LEPROT on a paralogous chromosome of Pma_Chr35. It seems that a common JAK is shared by all cytokine receptors for signal transduction in the lamprey.

Structural Conservation

Gnathostome LepR may have gone through additional structural change, leading to duplicated CRH domains in the extracellular segment. Duplication of the string of exons encoding the CRH domain has been previously reported for other members of the cytokine receptor family, namely, growth hormone receptor in whale shark (*Rhincodon typus*), PRLRs in chicken and spotted anole lizard (*Anolis punctatus*), and thrombopoietin receptor (Ocampo Daza and Larhammar 2018).

Thus, CRH duplication seems to be evolutionarily feasible. Nevertheless, CRH1 has been found to be nonessential for signaling in mouse LepR isoform b (LepRb) (Peelman et al. 2014), whereas CRH2 acts as the high-affinity binding domain for leptin (Zabeau et al. 2004). In contrast, only a single CRH domain is retained in lamprey LepRL. This suggests either that lamprey LepRL did not undergo the segmental duplication observed in gnathostomes or that the nonessential CRH1 was subsequently lost. The structural similarity indicates that the lamprey LepRL possesses all essential components for ligand binding and signaling activation via the cascades involving JAK and STATs, given the conservation of JAK box and C-terminal tyrosine residues similar to mammalian LepRb (Zabeau et al. 2004). Notably, lamprey LepRL contains only a single YXPQ motif, whereas lamprey IL6RBL1 and IL6RBL2 and gnathostome IL6ST possess two C-terminal YXPQ motifs that are crucial for STAT binding and activation (Ohtani et al. 2000). The conservation of these distinct STAT-binding motifs further supports our findings that lamprey LepRL and IL6RBLs are orthologous to gnathostome LepR and IL6RB paralogs, respectively.

Physiological Conservation

In adult sea lamprey, LepRL mRNA expression was high in the pituitary, head kidney, and blood cells, moderate in the brain and gill, and low in the liver. In contrast, IL6RBL1 and IL6RBL2 mRNAs were highly expressed in the liver, but minimally expressed in the pituitary, gill, and blood cells. Differential expression also was observed between the two IL6RBLs in the brain. These tissue profiles indicate that these lamprey cytokine receptors likely mediate distinct physiological roles. It is conceivable that LepRL signaling in the pituitary may have roles similar to LepR in teleost fishes, in which leptin regulates pituitary hormone production (Yan et al. 2017; Ohga et al. 2021), sexual maturation, and reproduction (Tsakoumis et al. 2022). In addition, the highest levels of LepRL mRNA in peripheral blood cells (including erythrocytes and lymphocytes) of sea lamprey may be related to

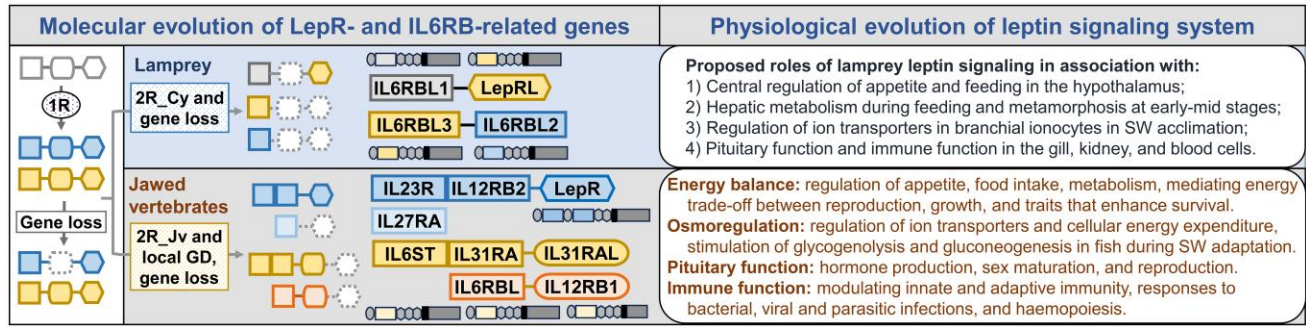


Fig. 9. Proposed scenarios of molecular evolution of LepR- and IL6RB-related genes and physiological evolution of the leptin signaling system in vertebrates. A triplet (depicted as square, stadium, and hexagon) may have emerged in the vertebrate stem lineage and duplicated during the shared 1R. A duplicate of the ancestral IL31RAL may have been lost prior to rediploidization. Gene loss is depicted using dotted-line shapes. Following gnathostome tetraploidization (2R_Jv) and local gene duplication (GD), eight IL6RB paralogs were retained in the same paralogon after asymmetrical gene loss, whereas only a single LepR was conserved. In the lamprey lineage, following hexaploidization in cyclostome (2R_Cy), three IL6RBL genes and a single LepRL were retained on the paralogon after extensive gene loss and chromosomal rearrangement. Notably, lamprey LepRL and gnathostome LepR likely arose from distinct 1R progenitors. Moreover, gnathostome LepR underwent duplication of the CRH domain and loss of an FN3 domain in the extracellular region. Despite structural modifications and distinct 1R origins, lamprey LepRL and gnathostome LepR may recruit similar mechanisms in signaling transduction and physiological functions. For instance, the leptin signaling system has fundamental roles in the regulation of energy balance and SW osmoregulation in the lamprey. The leptin system also may function in the pituitary and immune system, given high LepRL mRNA abundance in the relevant tissues of lamprey in agreement with the characterized roles in jawed vertebrates.

immune function and hemopoiesis, given that leptin supports immune function via LepRb expressed by most immune cells in mammals (Gainsford et al. 1996; Maurya et al. 2018). In mammalian immune system, leptin modulates both innate and adaptive immune responses, including (i) enhancing activation and preventing apoptosis of neutrophils, basophils, eosinophils, macrophages, and dendritic cells; (ii) stimulating proliferation of naïve T and B cells and regulating T-cell polarization; and (iii) stimulating the secretion of proinflammatory cytokines, including IL-6, IL-1b, and tumor necrosis factor (Abend Bardagi et al. 2023). Unlike gnathostomes producing immunoglobulins and T-cell receptors, lampreys have evolved an alternatively adaptive immune system, generating somatic diversification of antigen-receptor genes through recombinatorial assembly of leucine-rich-repeat modules to encode variable lymphocyte receptors (VLRs) (Cooper and Alder 2006). The VLRs are differentially expressed by T-like lymphocytes developing in the thymoid at the gill fold tips, as well as B-like lymphocytes developing in the hematopoietic typhlosole and kidneys (Boehm et al. 2018). Potentially, certain leptin functions in innate and adaptive immune systems may have evolved prior to the gnathostome–cyclostome split. The high abundance of LepRL in the head kidney and gill may be also linked to immune function and/or osmoregulation in sea lamprey. Likewise, IL6RBL2 and IL6RBL3 also were highly expressed in the kidney and gill, which may be linked to their signaling activation of IL-6 family cytokines in immunity and inflammatory responses (Heinrich et al. 2003).

Involvement in Metamorphosis

In anadromous sea lamprey, filter-feeding larvae that reach a threshold body size and accumulate high lipid reserves (4% to 14% of the body mass) undergo a nontrophic metamorphosis, marked by extensive morphological and physiological changes (Manzon et al. 2015). Metamorphosing individuals shift from lipogenesis to lipolysis by mobilizing lipid reserves from liver and nephric fold to fuel the metamorphic changes, especially during the late stages 4 to 7 (Kao et al. 1997). Concomitantly, gradual increase of hepatic LepRL mRNA expression peaks at stages 3 and 4. Similar upregulation also was

observed at IL6RBL2 mRNA levels, whereas IL6RBL1 mRNA expression was significantly downregulated. These changes may be related to the involvement of these receptors in regulating hepatic metabolism, given their well-known roles in metabolism in jawed vertebrates (Nonogaki et al. 1996; Cron et al. 2016; de Candia et al. 2021). For instance, leptin can reduce hepatic lipid content in goldfish (*Carassius auratus*) (de Pedro et al. 2006) and stimulate lipolysis in adipocytes of rainbow trout (*Oncorhynchus mykiss*) (Salmerón et al. 2015). In contrast, different transcriptional patterns were observed in the gill, with IL6RBL2 and IL6RBL3 levels significantly elevated at the onset of metamorphosis, whereas LepRL levels remained unchanged, indicating distinct functions in the gill and liver.

Responses to Nutritional Changes

In feeding sea lamprey, hepatic IGF expression was highly upregulated in correlation with the elevated mRNA levels of hepatic LepRL and IL6RBL1, but not IL6RBL2. Disparate effects of leptin on the regulation of hepatic IGF have been observed in teleost fishes. For example, leptin stimulated IGF-I expression in tilapia (*Oreochromis mossambicus*) (Douroso et al. 2017), but suppressed IGF expression in fasted rainbow trout (Gong et al. 2022a). Correlation of IGF-I and IL6ST expression also was observed in lung cancer progression, and their signaling pathways can synergistically promote cell proliferation (Tang et al. 2018). The positive responses of LepRL and IL6RBL1 expression to nutritional changes, along with their correlation with IGF expression, suggest coordinative interactions in the regulation of energy balance and growth. Although the correlation with IL6RBL2 did not reach statistical significance, this does not exclude its possible role in IGF regulation. Alternatively, IL6RBL2 upregulation may function independently of the signaling pathways involving LepRL and IL6RBL1 in IGF regulation. This independence is further supported by the correlation of NPY with LepRL and IL6RBL1, but not with IL6RBL2.

The current study shows the immunolocalization of LepRL in the nlt of the rostral hypothalamus, a piscine structure homologous to the ARC in mammals (Meek and Nieuwenhuys 1998). This distribution indicates a potential role of

hypothalamic LepRL in the central regulation of appetite and energy balance. Interestingly, brain LepRL expression was positively correlated with the upregulation of the orexigenic NPY in the feeding juveniles, compared to the individuals that never fed. Disparate effects of leptin on the regulation of NPY have been observed across gnathostome taxa. In contrast to the inhibitory effects of leptin on fasting-induced NPY expression in rat (*Rattus norvegicus*) (Morrison et al. 2005), there is no suppression of NPY expression/content in rainbow trout and goldfish after leptin administration (de Pedro et al. 2006; Gong et al. 2016a), but a transiently stimulatory effect of leptin on NPY was observed in clawed frog (*Xenopus laevis*) (Cui et al. 2014). In the current study, we were unable to test the effects of LepR signaling on neuropeptides due to the unidentified ligand in the lamprey, but the observed correlation of NPY with both LepRL and IL6RBL1 expression may indicate the conservation of hormonal coordination in appetite and neuropeptide regulation across vertebrates, potentially through molecular mechanisms similar to those in signaling of CNTF and leptin in mammals via the cascades involving JAK and STATs (Bjørnbæk et al. 1999; Lambert et al. 2001; Severi et al. 2015).

In fed sea lamprey, LepRL mRNA expression in the brain was negatively correlated with intestinal content. Such a negative correlation of the leptin signaling system with intestinal food content also was observed in rainbow trout, in which plasma leptin levels decreased soon after initiation of feeding and were negatively correlated with an increased amount of feed reaching the gastrointestinal tract (Johansson and Björnsson 2015). Conversely, plasma leptin levels are elevated in salmonids during fasting (Johansson and Björnsson 2015), concomitant with changes of hypothalamic LepR expression (Gong et al. 2016b). The activation of leptin signaling during food deprivation may represent an energy-sparing adaptation in which peripheral leptin signals an energy deficit to the central nervous system, and thus fasting-induced changes of plasma leptin levels initiate a feedback loop to limit energy deficiency (Flier and Maratos-Flier 2017), by regulating appetite and neuropeptides, suppressing somatic growth and reproduction, and mediating energy allocation to traits that enhance survival (such as immunity) (Ahima et al. 1996; Demas and Sakaria 2005; Wang et al. 2019; Gong et al. 2022a). Such a survival strategy for coping with energy deficiency by utilizing the leptin system may already be in place in lamprey, evidenced by the elevated levels of LepRL in the sea lamprey postfeeders with an empty intestine.

Involvement in SW Adaptation

Studies in teleosts revealed leptin functions in the gill, an organ that plays critical roles in gas exchange and osmoregulation. For example, hypoxia treatment stimulated LepR expression in crucian carp (*Carassius carassius*) (Cao et al. 2011). Acute SW transfer of tilapia stimulated hepatic leptin and LepR mRNA expression and appeared linked to stimulation of glycogenolysis and gluconeogenesis (Baltzegar et al. 2014). SW osmoregulation can be an energetically demanding process for euryhaline fishes to maintain the function of ion transporters for osmotic and ionic homeostasis (Norstog et al. 2022). In the current study, our results indicate that LepRL signaling likely plays a role in SW adaptation in juvenile sea lamprey by targeting branchial ionocytes, particularly through the regulation of ion transporters and cellular energy expenditure. Evidence for this comes from the observed SW effects on

stimulation of branchial LepRL expression and correlated ion transporter genes encoding an NKA- β subunit, NHE3, and NKCC. Additionally, SW exposure induced denser ir-staining of LepRL on NKA-expressing ionocytes. A similar stimulative effect of leptin signaling on NKA activity has been observed in rat kidney, where this signaling is mediated by JAK2 and extracellular signal-regulated kinase (ERK) cascades (Marciniak et al. 2005). Furthermore, NKCC and NHE3 in rat kidney proximal tubules are known to be regulated by cAMP levels and activation of protein kinase C (Honegger et al. 2006; Jaggi et al. 2015). Correspondingly, leptin can modulate cAMP levels and has varied effects on protein kinase C across physiological conditions (El-Zein et al. 2015). Thus, the potential regulation of LepRL signaling in the ion transporters and cellular energy expenditure may be mediated by mutual signaling cascades involving JAK, ERK, and cAMP.

Conclusions

This comprehensive study demonstrates that the divergence of ancestral LepR and IL6RB genes traces back to the vertebrate stem lineage. Two rounds of ancient WGD (1R and 2R), followed by extensive gene loss/retention, led to the emergence of multiple IL6RB paralogs in jawed vertebrates, while a single functional LepR was retained in most taxa after these events. In the sea lamprey, we identified three IL6RB-related genes that arose during hexaploidization, each potentially serving distinct roles in tissues. The sea lamprey LepRL contains all essential domains and motifs for ligand binding and intracellular signaling, closely resembling the structure of gnathostome LepR, but lacking a duplicated CRH domain that is not essential for signaling transduction. Despite these structural modifications, as well as a distinct evolutionary origin from 1R, the sea lamprey LepRL may utilize mechanisms of signal transduction to regulate physiological processes similar to that of LepR in jawed vertebrates. Analyses of LepRL mRNA expression in sea lamprey revealed the pleiotropic roles in central and peripheral tissues, involving metamorphosis, feeding, and SW adaptation. These findings provide new insight into the early evolution of cytokine receptors in vertebrates as well as the physiological evolution of the leptin signaling system, supporting the conservation of its fundamental roles in regulating energy balance, feeding, and osmoregulation.

Materials and Methods

Animal Experiments

Sea lampreys, including larvae and metamorphic transformers at various stages (1 to 4), were collected between June and October 2016, by electrofishing from the Sawmill River (Montague, MA, USA). The metamorphic transformers at late stages (5 and 6) were caught from a drained hydropower station canal in mid-September, reared in 1 m diameter tanks supplied with sand for burrowing and 4 L min⁻¹ flow-through Connecticut River water, where they completed metamorphosis later in November. FW postmetamorphic juveniles (body mass of 3 to 6 g and body length 13.5 to 16 cm) were used for SW exposure. The juveniles acclimated to SW (35 ppt) and FW were held at 12 °C and sampled on days 1, 3, and 14 (Gong et al. 2022b). Prespawning adults, with body mass of 600 to 800 g and body length of 65 to 75 cm, were caught during upmigration in the Connecticut River and sampled for various tissues in the lab.

Feeding Trial I: Comparing Nonfeeding and Feeding Juveniles after 30 d

Juvenile sea lamprey were first acclimated in brackish water (15 ppt) for 10 d and provided Atlantic salmon (*Salmo salar*, ~300 g body weight) for feeding. Host-attachment behavior was checked daily. Feeding lampreys with expanded intestine and residual blood-like intestinal content, as well as nonfeeding individuals that never fed and had unexpanded intestine, were euthanized using a lethal dose of MS-222 (400 mg L⁻¹ buffered with NaHCO₃, pH 7.0; Argent Chemical Laboratories, WA, USA) and sampled for blood and various tissues 30 d after being reared with host fish.

Feeding Trial II: Comparing Feeding Juveniles and Postfeeders with Various Intestinal Contents

Juvenile sea lamprey were lightly anesthetized using a nonlethal dose of MS-222 (100 mg L⁻¹ buffered by NaHCO₃, pH 7.0) and individually marked by injecting colored acrylic paints into the posterior dorsal fin. The animals were placed into 1.5 m diameter tanks with brook trout (*Salvelinus fontinalis*; ~100 g body weight) for feeding for 14 d. Behavior was recorded daily by observing the attachment on host fish. At sampling, intestinal content was determined and scored on a five-grade scale: 1, intestine was empty and lacked any solid content; 2, intestine was slightly expanded, with bile in the posterior, but not in the anterior; 3, intestine was expanded, with food content in the posterior; 4, blood-like content in both sections of intestine; and 5, intestine was full of blood-like content. Individuals that had an empty intestine, weight loss, and no history of attachment were taken as nonfeeders and excluded from the analysis. Animals were euthanized using MS-222 (400 mg L⁻¹) and sampled for blood and various tissues, which were immediately frozen and stored at -80 °C.

All procedures involving animals were conducted in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council, Washington, DC) and protocols approved by the Internal Animal Care and Use Committee at the University of Massachusetts and the U.S. Geological Survey (protocol number: 2019-0009). The study also was conducted in accordance with ARRIVE guidelines.

Database Searches and Gene Cloning

The LepR sequences from ray-finned fishes were queried for the genomes of sea lamprey, Arctic lamprey (*Lethenteron camtschaticum*), and Pacific lamprey (*Entosphenus tridentatus*) with TBLASTN (ncbi.nlm.nih.gov/assembly). Gene sequences were obtained from genome annotation and used to design primers for cloning the open reading frames of sea lamprey LepRL and IL6RBL1 (information listed in [supplementary table S1, Supplementary Material](#) online). RT-PCR products were cloned to pGEM-T plasmid (Promega Corp, Madison, WI, USA), and at least three colonies were sequenced by MacroGen USA (Rockville, MD, USA).

Real-time Quantitative PCR Analysis

Frozen tissue samples were homogenized in TRI Reagent (MilliporeSigma, St. Louis, MO, USA) by TissueLyser LT (Qiagen, Hilden, Germany) or Precellys 24 (Bertin Technologies, Montigny-le Bretonneux, France). The first strand cDNAs were synthesized from 2 µg total RNA by using high-capacity cDNA reverse transcription kit (Applied

Biosystems, Waltham, MA, USA). Quantitative PCR (qPCR) reactions included SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 500 nM primers, and cDNA samples or standard DNA samples that were linearized pGEM-T plasmids with DNA inserts of EF1a, β-actin, LepRL, or IL6RBL1. Standards for other tested genes were serial dilutions of pooled cDNA samples. qPCR was performed in a CFX96 Real-Time System (Bio-Rad Laboratories). A melting curve analysis was conducted in each qPCR run with a single peak, indicating PCR specificity. Quantification cycle (Cq) values and log (copy numbers of standard DNA) were plotted for linear regression. Copy numbers in cDNA samples were calculated from the standard curves and normalized to the reference genes, including elongation factor 1a (EF1a; Ensembl no. ENSPMAG00000010399) and β-actin (GenBank no. AJG41731). Other tested genes include LepRL (XP_032822078.1), IL6RBL1 (XP_032821795), IL6RBL2 (XP_032812536), IL6RBL3 (XP_032812021), IGF (AB081462), NPY (AY823514), ATP1B3 (XP_032827835), ATP1A3 (XP_032820581), NHE3 (XP_032830379), SLC12A2.1 (XP_032800811), and SLC12A2.2 (XP_032824630). Information on primers and qPCR efficiencies (in the range of 99% to 110%) is listed in [supplementary table S1, Supplementary Material](#) online. Primers were designed from a junction of two exons or separated exons and tested by RT-PCR and electrophoresis, showing a single band, indicating primer specificity.

Statistical Analyses

Kolmogorov–Smirnov tests were used to determine normal distribution of treatment groups, and Levene's median test or F test was used to assess equality of variances. One-way ANOVA followed by a Tukey post hoc test was applied for comparisons at various stages of metamorphosis. One-way ANOVA followed by a post hoc test for linear trend was used to compare feeding sea lampreys with various intestinal contents. Two-way ANOVA followed by Bonferroni's multiple comparisons test was applied for analysis of the SW acclimation experiment. Unpaired *t*-test with two-tailed *P* values was used to compare feeding and nonfeeding juveniles. Pearson correlation coefficients were computed in the analysis of correlation between genes or conditions. Analyses were performed with GraphPad Prism 9 (Boston, MA, USA). Differences were considered significant at *P* < 0.05 (*), *P* < 0.01 (**), and *P* < 0.001 (***).

Other Experimental Procedures

All procedures followed general protocols for sequence alignment, phylogenetic and syntenic analyses, production of recombinant proteins, antibody preparation, cell transfection and generation of stable cell lines, immunoblotting, and immunohistochemistry. The details are described in the [Supplementary Material](#).

Supplementary Material

Supplementary material is available at [Molecular Biology and Evolution](#) online.

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Author Contributions

All authors participated in research design; N.G., A.B., J.L.N., A.M.R., and S.D.M. performed the experiments; N.G. analyzed the data and drafted the paper; all coauthors commented and reviewed the final draft.

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Conflict of Interest

The authors declare no competing interest.

Data Availability

The data underlying this article are available in the article and in its online [supplementary material](#).

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