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# Origin of the prolactin-releasing hormone (PRLH) receptors: Evidence of coevolution between PRLH and a redundant neuropeptide Y receptor during vertebrate evolution☆

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**Abstract**

We present seven new vertebrate homologs of the prolactin-releasing hormone receptor (PRLHR) and show that these are found as two separate subtypes, PRLHR1 and PRLHR2. Analysis of a number of vertebrate sequences using phylogeny, pharmacology, and paralogon analysis indicates that the PRLHRs are likely to share a common ancestry with the neuropeptide Y (NPY) receptors. Moreover, a micromolar level of NPY was able to bind and inhibit completely the PRLH-evoked response in PRLHR1-expressing cells. We suggest that an ancestral PRLH peptide started coevolving with a redundant NPY binding receptor, which then became PRLHR, approximately 500 million years ago. The PRLHR1 subtype was shown to have a relatively high evolutionary rate compared to receptors with fixed peptide preference, which could indicate a drastic change in binding preference, thus supporting this hypothesis. This report suggests how gene duplication events can lead to novel peptide ligand/receptor interactions and hence spur the evolution of new physiological functions.


*Keywords:* prrp; Cloning; Binding; Evolution; Pharmacology; Prolactin; Hormone; Neuropeptide Y; PRLHR; Coevolution

Gene families account for a large part of the genes in the human genome [1,2]. The molecular mechanisms by which gene families develop are, however, difficult to study and therefore relatively unknown. S. Ohno proposed that the most economical way of expanding the genome was to copy present genes instead of creating totally new ones [3], and this is thought to occur 0.01 times/gene/million years in eukaryotic genomes [4]. Segmental duplications or whole genome duplications (tetraploidizations) are mechanisms for large-scale gene expansions [3,5,6]. Gene families can also expand through local tandem gene duplications, leaving the new gene in the vicinity of the parent gene. After duplication

*Abbreviations:* PRLH, prolactin-releasing hormone; PRLHR, PRLH receptor; NPYR, neuropeptide Y receptor; GPCR, G-protein-coupled receptor; ACTH, adrenocorticotropin; TM, transmembrane region; EL, extracellular loop; HMM, Hidden Markov Model; LG, linkage group; PRH, prolactin-releasing hormone precursor; JTT, Jones–Taylor–Thornton model; MYA, million years ago; HEK, human embryonic kidney cells; Hsa, *Homo sapiens* chromosome; PYY, neuropeptide YY; GG, *Gallus gallus*; C-RF amide, *Carassius auratus* RF amide; MCR, melanocortin receptors; MSH, melanocyte-stimulating hormone.

☆ Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. AY572193–AY572195, BK005177–BK005183, BK005487, BK005488, and BK005491.

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events, there are different scenarios that may occur. Often, one of the copies will gain destructive mutations (non-functionalization), silencing the gene and bringing the organism back to the one-copy situation prior to the duplication [7]. If both genes are retained in the genome, different possibilities may occur: (1) both genes may accumulate mutations, leading to a level or tissue distribution of expression and function that summed together resemble the one-copy situation (subfunctionalization) [8]; (2) both genes may keep their original expression level and tissue distribution, leading to an increased expression level of the protein [9]; or (3) mutations may give one of the copies a new function or a different expression pattern (neofunctionalization) [8,10].

The G-protein-coupled receptor (GPCR) superfamily is one of the most abundant gene families in the human genome including more than 2% of the total number of genes [2]. The number of GPCRs has increased remarkably in higher vertebrates, with the exception of certain species-specific expansions like the chemosensory in *Caenorhabditis elegans*. The increase in numbers may be related to their role in complex and specialized functions of the immune system and central nervous system, in which the GPCRs play a crucial role. The need for many diverse and specialized functions of the GPCRs has resulted in a flexible and dynamic superfamily, binding a very broad repertoire of ligands, such as ions, catecholamines, lipids, and peptides. There are over 800 GPCRs in the human genome. These receptor proteins can be phylogenetically divided into five main families: the secretin, the adhesion, the glutamate, the frizzled, and the rhodopsin receptors of which the rhodopsin family can be subsequently divided into the α, β, γ, and δ groups [11].

The scope of this work is the coevolution of the prolactin-releasing hormone (PRLH) and its receptor, the prolactin-releasing hormone receptor (PRLHR/GPR10) [12]. This receptor is a member of the β group in the rhodopsin family of GPCRs [11]. We chose to refer to this receptor as PRLHR, in accordance with the HUGO Gene Nomenclature Committee, rather than the orphan receptor name GPR10 because the receptor has been shown to bind PRLH [13]. PRLHR is expressed in several parts of the rat brain and most notably in the reticular thalamic nucleus, the periventricular and the dorsomedial hypothalamus, the nucleus of the solitary tract, and the anterior pituitary [14]. This receptor binds two endogenous ligands, the 20- and the 31-amino-acid-long PRLH, of which the 20-amino-acid-long peptide is the truncated version of the longer one [13]. These ligands are expressed in the dorsomedial hypothalamus, the nucleus of the solitary tract, and the ventrolateral reticular nucleus of the rat brain [14]. PRLH is shown to decrease food intake in rats [15,16]. The effect on food intake is possibly mediated by altering the release of other hypothalamic neuropeptides [17]. The role of PRLH in prolactin secretion in mammals has been questioned and recent studies show that the peptide may influence the secretion of adrenocorticotropin (ACTH) from the hypothalamus [18]. Moreover, mutations of the *PRLHR* gene were recently found to be associated with lowered blood pressure in a UK Caucasian population [19].

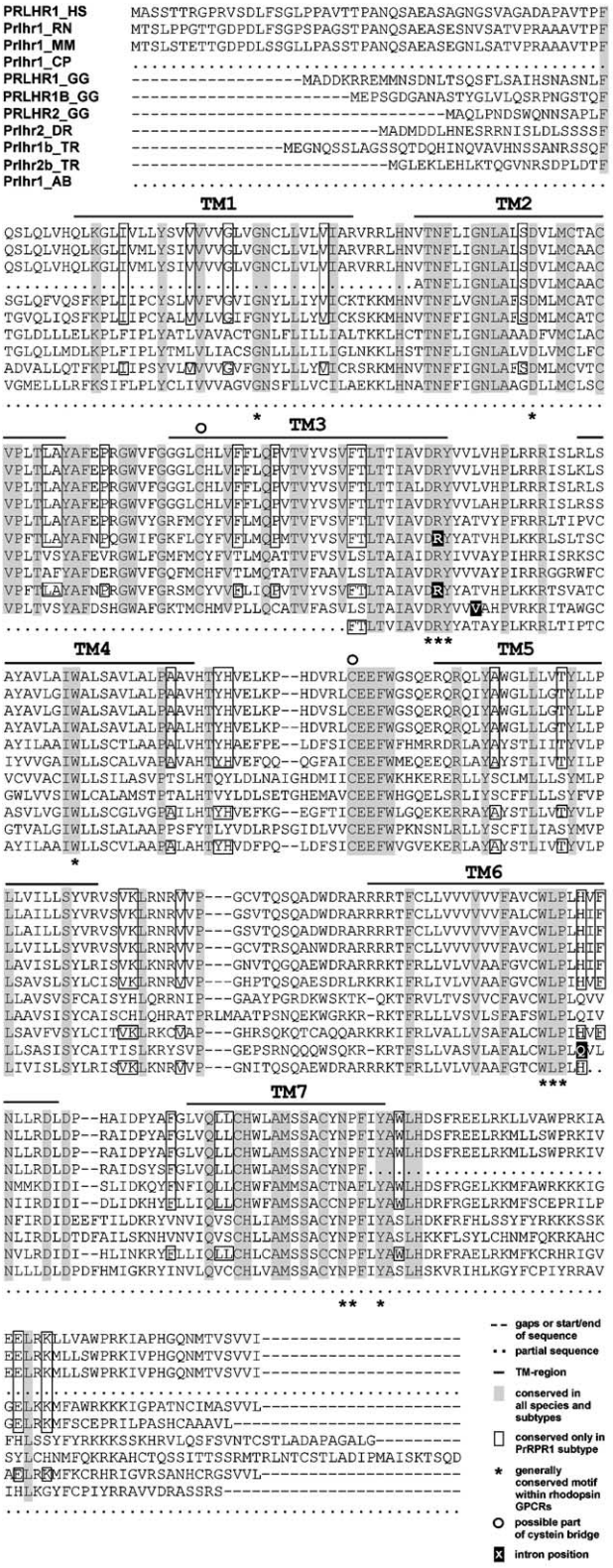
In this study we investigated the sequences of PRLH and the PRLHR and related proteins from a wide range of vertebrates, spanning human to sturgeon. We determined their phylogenetic relationships, their chromosomal positions, and the pharmacological profile of the human PRLHR. The results revealed two members of the PRLHR family, in which certain species have kept different subtypes of the receptor. We suggest that PRLH may have coevolved with an ancestral neuropeptide Y (NPY) binding receptor (NPYR), generating the PRLHR family.

## Results

### *PRLHR cloning, mining, and phylogeny*

We found six new full-length genes similar to the human *PRLHR* in the following vertebrate species: three from chicken, two from pufferfish, and one from zebrafish. Fragments ranging between transmembrane (TM) region 3 and TM6 were also obtained from guinea pig and sturgeon (Fig. 1). These protein sequences share conserved structural motifs (indicated with stars in Fig. 1), well known to be highly conserved within the rhodopsin family of GPCRs, suggesting that they are functional proteins [11,20]. Phylogenetic analysis using maximum parsimony clustered the PRLHR-like sequences into two separate subtypes, here named PRLHR1 and PRLHR2 (Fig. 2). The distance and likelihood analysis displayed similar results (these trees can be retrieved from the authors upon request). The PRLHR1 subtype (previously called GPR10/UHR1) consists of four mammalian protein sequences, two representatives from chicken, one from pufferfish, and one from sturgeon, whereas the PRLHR2 subtype consists of one chicken, one pufferfish, and one zebrafish sequence. We chose to call the human PRLHR PRLHR1 from now on, to separate the specific human PRLHR protein from the general PRLHR family. All the *PRLHR*-like genes are intronless except *PRLHR1B* from chicken and the two pufferfish sequences. The positions of the introns in chicken *PRLHRB1* and pufferfish *prlhr1b* are conserved (Fig. 1). The chicken *PRLHR1B* intron is 1421 bp long (of which the identity of approximately 90 bp is missing in the genomic sequence), whereas the pufferfish *prlhr1b* has a 191-bp-long intron. The pufferfish *prlhr2b* has two introns, 87 and 885 bp long, respectively, of which none of the intron positions are conserved within the *PRLHR* family (Fig. 1). There are clear differences in amino acid conservation between the subtypes (indicated by boxed residues in Fig. 1). The overall amino acid preservation between the subtypes is 36.8–42.9%, while within the subtypes it is 50.6–66.6% (PRLHR1, including only one of the mammalian receptor sequences in the comparison) and 49.4–59.4% (PRLHR2). The branches containing the two PRLHR subtypes were separated by a node with 99.2% bootstrap support (992 of

1000 bootstraps). Likewise, the node that separates the NPYR from the PRLHR family has a bootstrap support of 99.2%. The sturgeon and the guinea pig receptors grouped with the PRLHR1 subtypes with 905 and 633 of 1000 bootstraps, respectively.



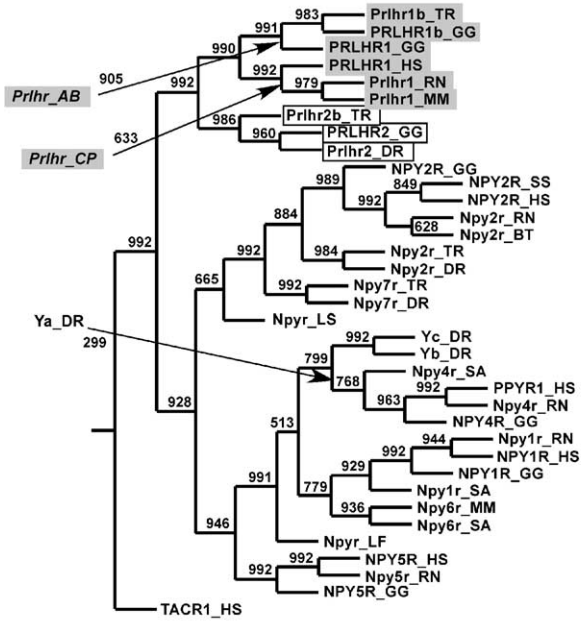




Fig. 2. Phylogenetic relationship between the PRLHR and the NPYR cluster, showing two distinct PRLHR subtypes, PRLHR1s and PRLHR2s. The tree is a maximum parsimony consensus tree originating from 1000 different trees, plotted in Tree Explorer. The phylogenetic positions of the two PRLHR-like fragments and the zebrafish Ya were calculated in a separate 1000-replica maximum parsimony analysis. AB (*Acipencer baerii baerii*), BT (*Bos taurus*), CP (*Cavia porcellus*), DR (*Danio rerio*), GG (*Gallus gallus*), HS (*Homo sapiens*), LF (*Lampetra fluviatilis*), LS (*Lymnaea stagnalis*), MM (*Mus musculus*), RN (*Rattus norvegicus*), SA (*Squalus acanthias*), SS (*Sus scrofa*), TR (*Takifugu rubripes*).

## *Chromosomal positions of the PRLHR family genes*

The positions of the *PRLHR* family of genes in human, rat, mouse, pufferfish, and zebrafish genomes were investigated using fluorescence in situ hybridization (FISH), meiotic mapping, and bioinformatic tools (Fig. 3). The human *PRLHR1* gene is found on human chromosome (Hsa) 10q26.11. This position is well conserved between the mouse and the rat genomes in which the *Prlhr1* genes map to 19D3 and 1q55, respectively. The surrounding area contains more than 24 preserved genes, suggesting a common origin. The chicken *PRLHR1* gene was positioned

Fig. 1. Amino acid sequence alignment of the cloned PRLHRs in guinea pig, chicken, zebrafish, and sturgeon; the mined receptors from pufferfish and chicken; the NCBI computer-annotated mouse sequence; and the previously published human and rat PRLHR1 sequences [12,51]. The black lines mark putative TM regions. The dashed lines indicate gaps or start/end of sequence. The dotted lines mark partial sequences and the open circles highlight interaction points for the postulated cysteine bridge. Shaded letters indicate residues conserved among all sequences in the alignment, while boxed residues show amino acids conserved only in the PRLHR1 subtype. Residues indicated with a star are generally conserved within the rhodopsin family of GPCRs. White letters on black background indicate postulated intron positions. AB (*Acipencer baerii baerii*), CP (*Cavia porcellus*), DR (*Danio rerio*), GG (*Gallus gallus*), HS (*Homo sapiens*), MM (*Mus musculus*), RN (*Rattus norvegicus*), TR (*Takifugu rubripes*).

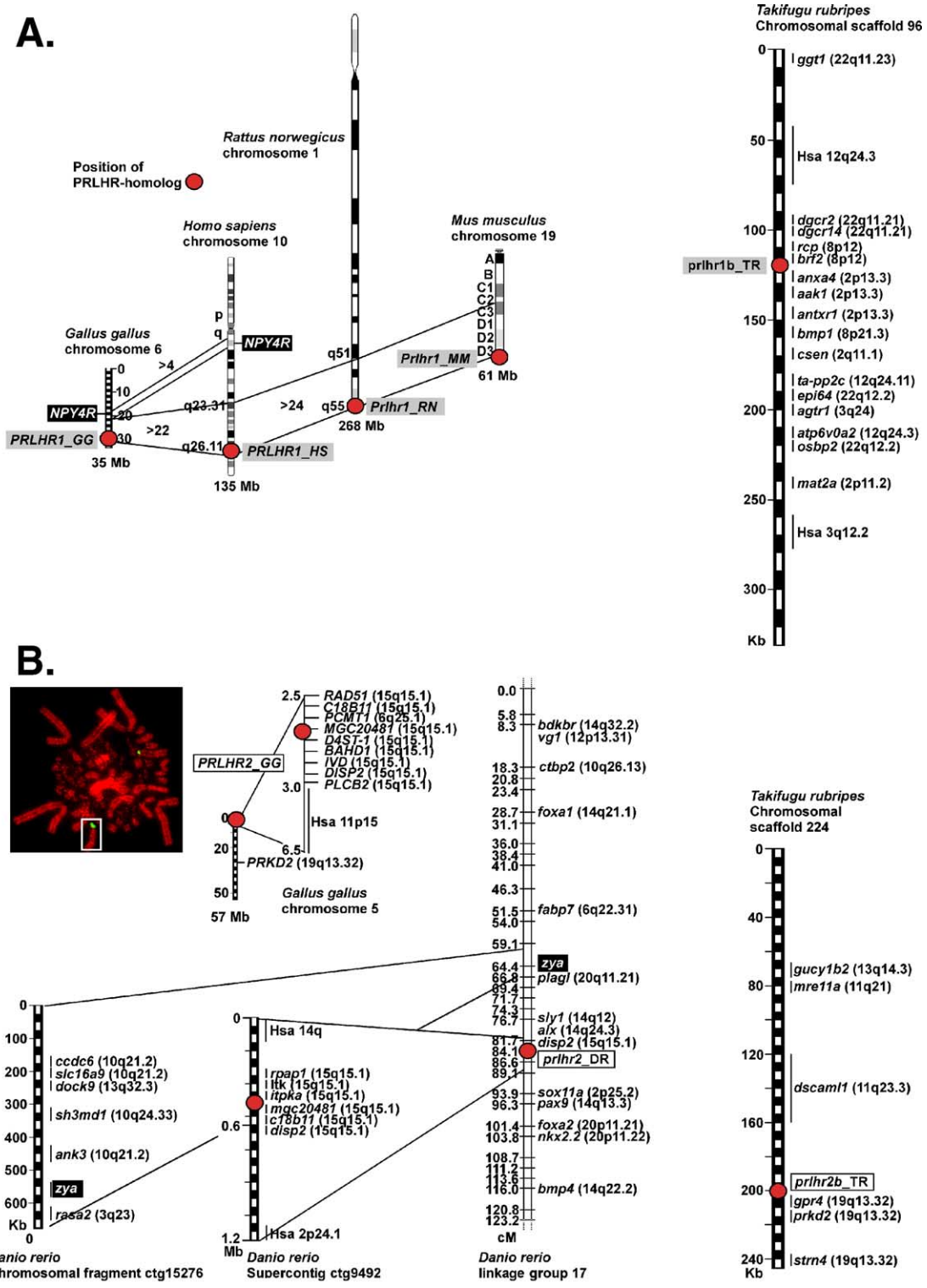



Fig. 3. The chromosomal positions for the *PRLHR* homologs in human, rat, mouse, chicken, zebrafish, and pufferfish. The positions for the *PRLHR* homologs are indicated with circles and the positions of the *NPY4R/NPY4R*-like genes are indicated with white letters on black backgrounds. (A) Chromosomal positions for the *PRLHR1* subtype. Synteny regions between the chicken, human, rat, and mouse genomes, in the area surrounding the *PRLHR1* gene, are framed. No chromosomal position is indicated for the *PRLHR1B* sequence in chicken because the sequence hits a segment that is not localized (for details regarding the synteny between human and chicken in this region, see Supplemental Information 4). (B) Chromosomal positions for the *PRLHR2* subtype. The chromosomal position of the *PRLHR2* gene in chicken can also be seen in the FISH-generated map where GG05 is surrounded by a white frame. The chicken map of GG05 was redrawn using http://www.genome.iastate.edu/chickmap/ and FISH-map-generated data, whereas the genomic positions of the two mined pufferfish receptors were investigated using http://www.ensembl.org/Fugu_rubripes/contigview. The human chromosomal backgrounds were gained from http://www.ncbi.nlm.nih.gov/mapview. The relationships among rat chromosome 1, Hsa10, and mouse chromosome 19 were put together using http://gapp.gen.gu.se/SearchRMQ.php and http://genome.ucsc.edu/. DR (*Danio rerio*), GG (*Gallus gallus*), HS (*Homo sapiens*), MM (*Mus musculus*), RN (*Rattus norvegicus*), TR (*Takifugu rubripes*). All gene names are (except zebrafish LG17, which is based on lineage analysis) according to the most similar human sequence (human chromosomal position is given within parentheses) and in accordance with the present nomenclature.

at GG06, surrounded by more than 26 genes orthologous to Hsa10q11.22–q26.12 (22 of 26 genes were also present in rat and mouse regions of conserved synteny).

The chicken *PRLHR1B* gene hit an unlocalized scaffold, chrUn:1-2,453,000. The 10 closest genes in this scaffold correspond to Hsa8p12 (for details see Supplemental Information 4). The pufferfish *prlhr1b* hits the Ensembl scaffold 96, on which several genes corresponding to Hsa2, 8, 22, and 12 are present. One of the closest hits represents the gene *brf2,* which also can be found close to the chicken *PRLHR1B* gene, which strengthens the orthologous relationship between the *PRLHR1B/prlhr1b* genes in chicken and pufferfish. Chicken *PRLHR1B* and pufferfish *prlhr1b* were thought to represent the *PRLHR1B* subtype based on shared intron positions, similar chromosomal surroundings, and a close phylogenetic relationship (Figs. 1–3).

The *PRLHR2* chicken gene mapped to a region close to the top of GG05 (Fig. 3). This position was recently confirmed by the release of the chicken assembly. The closest surrounding area contains genes corresponding to Hsa15q15.1, while the rest of the chromosome contains genes that correspond to Hsa11 (six genes) and Hsa19 (one gene). The full-length zebrafish *prlhr2* gene mapped to the zebrafish linkage group (LG) 17, where several genes corresponding to Hsa14, 15, and 20 are found (Fig. 3). The region closest to the zebrafish *prlhr2* contains several genes whose orthologs also can be found close to the chicken *PRLHR2*. Thus, clear synteny conservation can be seen between the Hsa15q15.1, the regions containing *PRLHR2* in chicken, and zebrafish LG17 (Fig. 3).

The pufferfish *prlhr2b* gene hits the Ensembl scaffold 224, containing several genes with human orthologs on Hsa11q and Hsa19q13.32. The distinction between the *PRLHR2* and the *PRLHR2b* subtypes is based on differences in chromosomal positioning, phylogenetic relationship, and intron conservation.

### *PRH and C-RF amide peptide mining*

One prolactin-releasing hormone precursor (PRH)-similar peptide and one C-RF amide-similar peptide were found in the chicken genome. The chicken *PRH* gene shares similarities in protein sequence and genomic structure with the human *PRH,* except for an insertion close to the position of the intron (Fig. 4). This insertion was confirmed by EST sequences. The chicken C-RF amide peptide aligns well with the C-rf amide sequences from goldfish (*Carassius auratus*) and zebrafish. The chicken C-RF amide peptide is identical to the teleost sequences in a 27-amino-acid-long stretch that covers the conserved intron–exon boundary (Fig. 4). The close relationship between the PRH and the C-RF amide peptides is especially evident in the region that, in the case of the human PRH, is cleaved into PRLH-20. The position of the intron–exon boundary is also conserved between the *PRH* and the *C-RF amide* peptide genes.

### *Genomic structure and chromosomal pattern of neuropeptide genes belonging to the Hox paralogon in human and in chicken*

Analysis of the chromosomal positions of 38 human neuropeptide genes binding to GPCRs showed that at least 14 ligand genes were positioned in the *Hox* paralogon, Hsa2q, 7, 12q, and 17q, a product of the two postulated perivertebrate-origin genome duplication events [3,21]. The *PRH* gene is situated on the q arm of Hsa2, together with genes from six other protein families, *FZD* (frizzled receptor homolog), *HOX* (homeobox gene), *RAMP* (receptor activity modifying protein), *NFE2* (erythroid-derived nuclear factor, family 2), *IGFBP* (insulin-like growth factor binding protein), and *ERBB* (erythroblastic leukemia viral oncogene homolog (receptor tyrosine kinase)), all present on the different *Hox*-bearing chromosomes (Fig. 5). The neuropeptide precursors for *NPY,* the RFRP peptides (*RFRP*), and preprotachykinin (*TAC1*) are situated on Hsa7, while the neuropeptide FF/SF/AF- precursor (*NPFF/SF/AF*), the tachykinin 3 precursor (*TAC3*), the neurotensin precursor (*NTS*), and the pro-melanin-concentrating hormone (*PMCH*) are positioned on Hsa12. Six other peptide precursors are localized on Hsa17: gastrin precursor (*GAS*), hypocretin (orexin) precursor (*HCRT*), pancreatic polypeptide (*PPY*) precursor, neuropeptide YY (*PYY)* precursor, preprotachykinin C (*TAC4*), and neuropeptide B precursor (*NPB*) (Fig. 5).

The position of the *PRH* precursor is preserved in the chicken genome, where the peptide is situated on GG07, a chromosome that corresponds to Hsa2 (Fig. 5). The gene for C-RF amide peptide, which is not present in the human genome, maps to GG02, the chromosome that shares synteny with Hsa7. The *NPY, TAC1,* and *RFRP* precursors are situated close to the *C-RF amide peptide* in the chicken genome. This suggests that the C-RF amide peptide and the PRH peptide share a common ancestry, originating in the two genome duplications early in vertebrate evolution. The *NTS* and *PMCH* precursors are positioned on GG01 and except for this no clear conserved synteny was observed for the regions in chicken that correspond to Hsa12 and Hsa17.

### *RF- or RY-containing precursors*

Looking at the RF- or RY-motif-containing precursors, PRH displays one 31-amino-acid-long mature peptide, and its gene is interrupted by a short intron that places the 20-amino-acid-long C-terminal peptide on one side of the intron and the rest of the N-terminal of the 31-amino-acid-long peptide on the other side. This intron–exon boundary is also conserved in the C-RF amide precursors from chicken and zebrafish (Fig. 4). The *NPY*-precursor gene codes for one mature peptide of 36 amino acids, in which the codon for the R (AGG) in the RY motif is interrupted by a long intron. The precursor contains in total two long introns. The same pattern can be seen in the *PPY* and the *PYY* precursor (except that both introns are short). It is intriguing that this pattern also

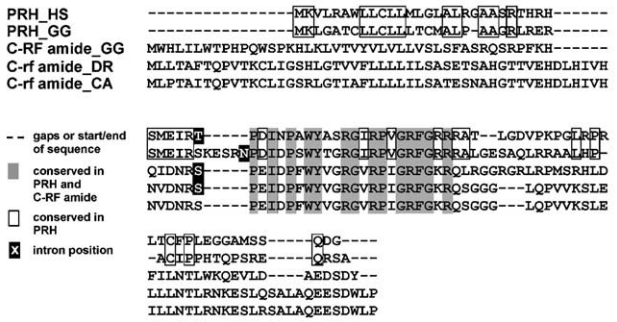



Fig. 4. Amino acid sequence alignment of: the mined PRH in chicken, the mined C-RF amide precursor in chicken, the Ensembl annotated zebrafish C-rf amide sequence, and the previously published human PRH and goldfish C-rf amide sequences [13]. The dashed lines indicate gaps or start/end of sequence. Dark-shaded letters indicate residues conserved within all sequences in the alignment, while boxed residues show amino acids conserved in the PRH precursors. White letters on black indicate proposed intron positions. There was no information available for the intron–exon boundary in goldfish. CA (*Carassius auratus*), DR (*Danio rerio*), GG (*Gallus gallus*), HS (*Homo sapiens*).

can be found, with a slight modification, in the *NPFF/SF/AF* precursor. This precursor can be processed into three mature peptides, of which NPFF and NPSF share the same C-terminal sequence. The C-terminus contains the RF motif that is interrupted by an intron in the R codon, as in the NPY/PYY/PPY precursors. The RFRP precursor can be processed into two short peptides, 12 (RFRP-1) and 8 (RFRP-3), neither of which is interrupted by introns in the genomic sequence. There seems to be a connection between the NPY/PYY/PPY and NPFF/SF/AF precursors in the processing of the mature neuropeptides. The evolutionary relationship between these neuropeptides, which are all members of the *Hox* paralogon, is complex, especially because the bovine PYY and frog PPY display the RF motif instead of the RY motif that is found in human NPY/PYY/PPY [22].

### *Comparison between the PRLHRs and the NPYRs*

Due to the similarity between PRLHR and NPY2R protein sequences (28.9% identity between full-length and 37.9% between truncated human NPY2R and PRLHR1) we included the NPYR family in this study. The degree of sequence identity between human NPY2R and PRLHR1 can be related to the full-length sequence identity of 23.6 (NPY2R–NPY1R), 25.2 (NPY2R–NPY5R), and 27.7% (NPY2R–NPY4R) between NPY2R and the other NPYRs in the family. The NPY2Rs and NPY7Rs from different species group together in the phylogenetic tree, as do the NPY4Rs, NPY1Rs, and NPY6Rs (Fig. 2). The human *NPYRs* map to Hsa5q31.2 (*NPY6R* pseudogene), Hsa4q32.1–q32.2 (*NPY2R, NPY5R, NPY1R*), and Hsa10q11.22 (*NPY4R*), the same chromosome that contains the *PRLHR1*. In zebrafish, the *zya* (*NPY4R*-like) is positioned only 20 cM from *prlhr2,* and chicken *NPY4R* is positioned only 10.6 Mb from *PRLHR1* on GG06 (Fig. 3). This repeated chromosomal pattern together with the high sequence identity prompted speculation about a common origin of the *NPYR* genes and the *PRLHR* genes.

The divergence time of the PRLHRs from the NPYRs was estimated from molecular clock calculations. The PRLHR1 subtype was shown to have a relatively high evolutionary rate (Fig. 6A). The slope of the curve for PRLHR1 is especially steep between the divergence of bird/reptile and mammal lineages. This pattern of fast evolution is shared by the NPY4R family. The NPY1Rs and the NPY2Rs display a slow rate of evolution since birds/reptiles and mammals diverged from each other. The NPY2R family shows an increased rate of evolution between the bird/reptile split and the teleost split from the line leading to mammals. The NPY1R family displays a clear and gradual increase in the steepness of the slope, from the amphibian split to the agnathan split from the main line. The approximate date of the duplication, creating the subtypes PRLHR1 and PRLHR2, was calculated to 550 ± 67 MYA, while the split between the PRLHR and the NPY2R families was calculated to 915 ± 68 MYA (Fig. 6B). The duplication generating PRLHR1 and PRLHR2 is, according to the molecular clock calculations, most likely to have occurred during the period of the tetraploidization events, while the duplication separating the NPYR family from the PRLHRs most likely happened before the rise of the vertebrates (Fig. 6B).

### *Shared functional properties between the PRLHRs and the NPYRs*

The endogenous ligand for human PRLHR1 is PRLH [23]. The peptide is present both as a 31-amino-acid-long peptide and as a truncated C-terminal version of 20 amino acids [13]. These peptides have previously been included in the RF amide peptide family due to the RF-motif in the C-terminal. The $K_d$ value for the radioligand rat $^{125}$I-Prlh-31 was determined to 0.25 nM in saturation binding experiments (Table 1, Fig. 7A). This value is in agreement with published results, although the human $^{125}$I-PRLH-20 has previously been shown to display two $K_d$ values, 0.026 and 0.57 nM [23]. The competition binding experiments gave the affinities ($K_i$ values) for the human PRLH-20, rat Prlh-20, human PRLH-31, and rat Prlh-31 of 1.93, 3.49, 1.50, and 1.52 nM, respectively (Table 1, Fig. 7B). These results are also in good agreement with previous results [23]. For the evaluation of putative evolutionarily conserved binding motifs between the PRLHRs and the NPYRs, three human NPYR binding peptides were tested on the human PRLHR1. These were NPY, PYY, and a truncated version of PYY, (13-36)-PYY, which possesses a higher affinity for NPY2Rs than other NPYRs [24]. Full-length NPY and PYY bound specifically to the PRLHR1 with 3.5 and 4.5 ìM affinity, respectively, while the NPY2R-preferring (13-36)-PYY peptide displayed over sixfold higher affinity (Table 1, Fig. 7B). To test if these affinities were related only to the similarity in the structure of the C-termini, in which the NPY peptides have the RY amide

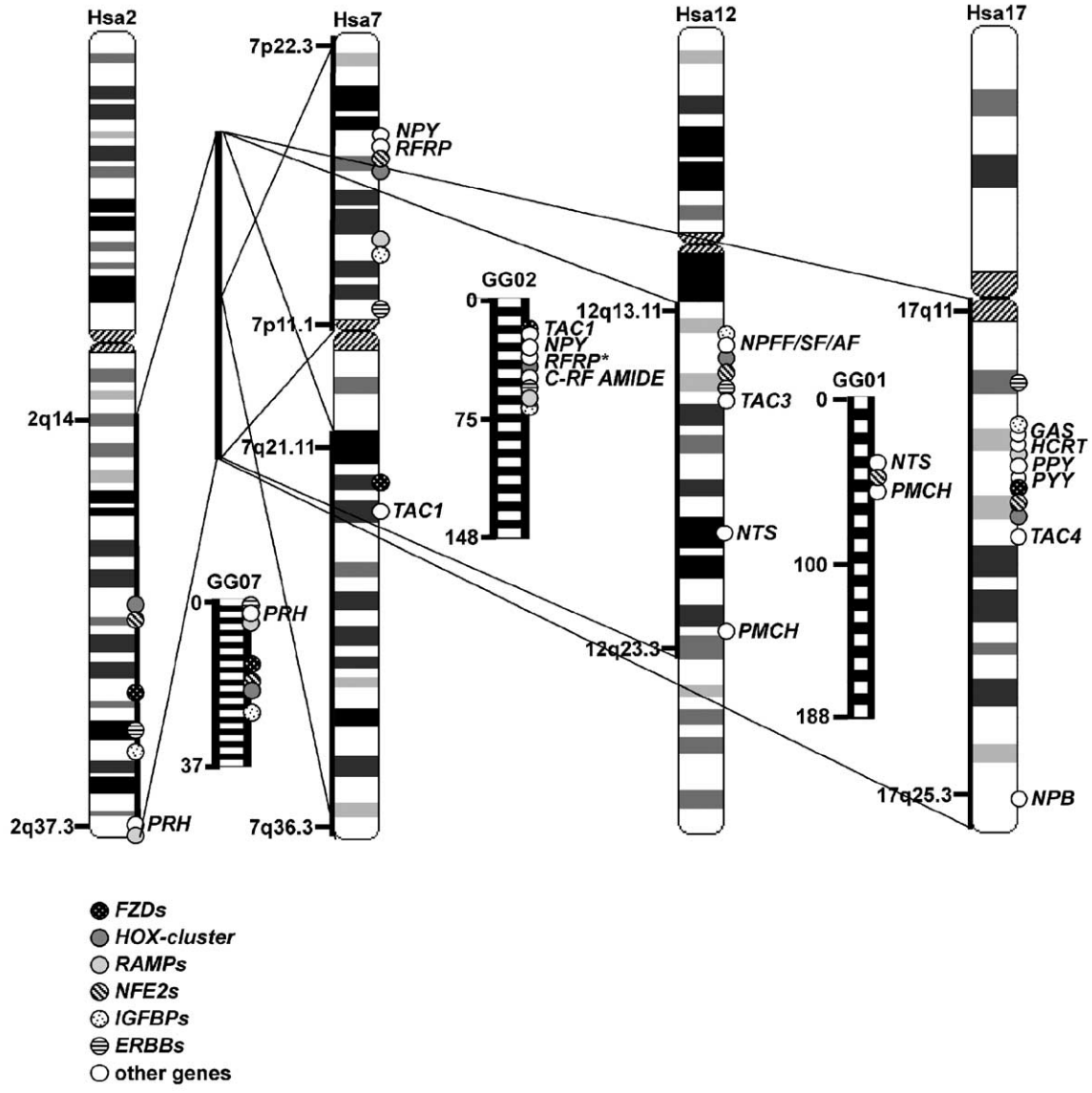



Fig. 5. Chromosomal localization of the neuropeptides belonging to the human *Hox* paralogon, 2q14–q37/7p22–p11, q21–36/12q13–q23/17q [21]. The corresponding regions of the chicken genome, GG01, GG02, and GG07, are drawn between the human chromosomes. Hsa2 corresponds to GG07, Hsa7 to GG02, and Hsa12 vaguely to GG01 (for details see Supplemental Information 5). The chicken chromosome/chromosomes corresponding to Hsa17 could not be deduced. The human paralogon is redrawn except for the neuropeptide positions [21]. *FZD* (frizzled homolog), *HOX* (homeobox gene), *RAMP* (receptor activity modifying protein), *NFE2* (erythroid-derived nuclear factor, family 2), *IGFBP* (insulin-like growth factor binding protein), *ERBB* (erythroblastic leukemia viral oncogene homolog (receptor tyrosine kinase)), *PRH* (prolactin-releasing hormone precursor), *NPY* (neuropeptide Y precursor), *RFRP* (RFRP peptide precursor (contains RPRF-1, RPRF-3)), *TAC* (tachykinin precursor), *NPFF/SF/AF* (NPFF peptide precursor (contains NPFF/NPSF/NPAF)), *NTS* (neurotensin precursor), *PMCH* (pro-melanin-concentrating hormone), *GAS* (gastrin precursor), *HCRT* (hypocretin (orexin) precursor), *PPY* (pancreatic polypeptide precursor), *PYY* (peptide YY precursor). The chromosomal positions were collected from http://genome.ucsc.edu and the chromosomal banding patterns for Hsa2, 7, 12, and 17 from http://www.ncbi.nlm.nih.gov/mapview. *RFRP** corresponds to gonadotropin-inhibitory hormone precursor (*GNIH*) in chicken (NP_989694.1). For detailed chromosomal positions see Supplemental Information 5.

motif [25], we tested several other neuropeptides with the RF amide motif. Human γ1-melanocyte-stimulating hormone (MSH), which is derived from the pro-opiomelanocortin (POMC) precursor, normally binds to the melanocortin receptors (MCRs) [26]. This peptide has been shown to interact with the MrgC11 receptor [27], plausibly due to the RF amide motif in the C-terminal. This peptide was, however, unable to bind human PRLHR1 in our assay (Table 1, Fig. 7B). Moreover, the RF-amide-containing FMRF peptide was also unable to displace the labeled PRLH-31 at the PRLHR1 (Table 1, Fig. 7B). $Zn^{2+}$ ions, which previously have been shown to bind several neuropeptide-binding GPCRs [28,29] were, however, not able to displace the binding of the radioligand to the human PRLHR1. A 10 ìM level of human PRLH-31 was not able to displace $^{125}$I-PYY from the human NPY2R (data not shown).

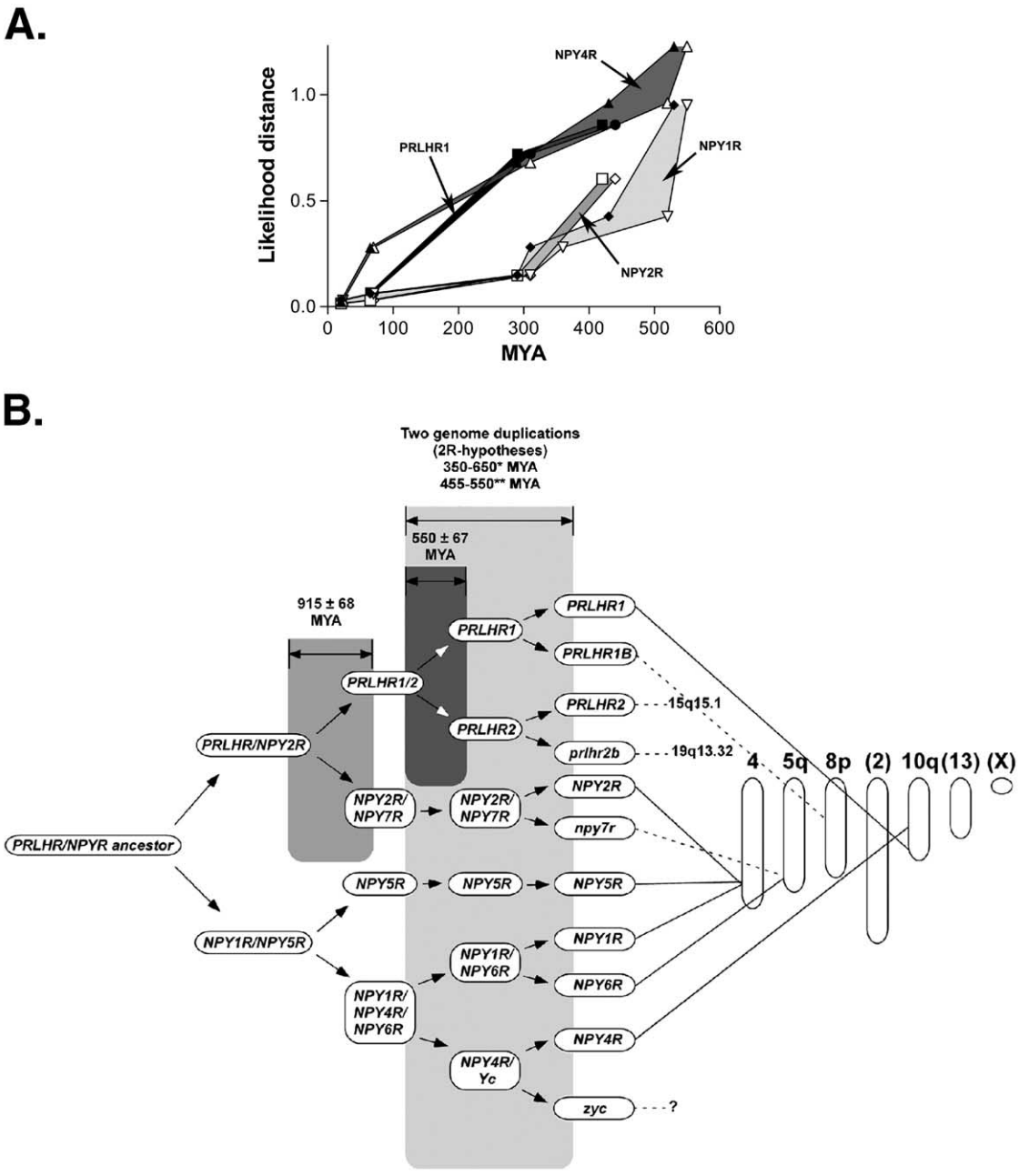



Fig. 6. Evolutionary events of the PRLHRs/NPYRs. (A) The evolutionary rate, in the vertebrate lineage, for the PRLHR1, NPY1R, NPY2R, and NPY4R subtypes. Molecular clock estimations were based on separate maximum likelihood distance calculations using TreePuzzle, plotted against the divergence time for human–rhesus monkey (20–23 MYA), human–rat (65–70 MYA), human–chicken (290–310 MYA), human–African clawed frog (310–360 MYA), human–pufferfish (420–440 MYA), human–spiny dogfish (430–520 MYA), and human–European river lamprey (530–550 MYA) [53–55]. The shaded areas represent the calculated interval between minimum divergence time and maximum divergence time evaluations (due to differences between molecular clock estimations and fossil records). The black-shaded areas represent the molecular evolution of PRLHR1s, of which the filled black squares define minimum [59] divergence time (min) according to molecular clock calculations and fossil data, while the filled black circles define maximum divergence time (max). The dark gray-shaded areas represent NPY4Rs (filled black triangles, min; white triangles, max). Medium gray displays the evolutionary rate of NPY2Rs (white squares, min; white diamonds, max) and light gray represents the NPY1Rs (filled black diamonds, min; white triangles, max). (B) A plausible scenario for the development of the PRLHRs and the NPYRs based on molecular clock estimations, chromosomal positions, and phylogenetic relationships. The light gray-shaded area denotes the time for the postulated genome duplication events based on *molecular clock estimations [60] and **fossil records [59,61,62]. The medium gray-shaded area represents the estimated time for the split between the PRLHRs and the NPYRs. The dark-shaded area shows the estimated time for the split between the two subtypes of PRLHR, the PRLHR1 and the PRLHR2. The time for the two duplication events involving the PRLHR family is based on molecular clock estimations. The chromosomal positions of the present genes are indicated with a straight line if the gene is present in the human genome. The dashed lines indicate the corresponding positions in the human genome for the pufferfish *prlhr1b* and the chicken *PRLHR1B,* the chicken *PRLHR2* and the zebrafish *prlhr2,* the pufferfish *prlhr2b* and the pufferfish *npy7r*-like. The composition of the represented paralogon, the Para*Hox* paralogon, is based on the work of Lundin et al. and Popovici et al. [5,21,30]. The portion of the picture regarding the *NPYRs* is redrawn from [24].

Table 1
Competition binding experiments with different ligands on HEK293 EBNA cells expressing the human PRLH receptor (PRLHR1)

| Ligand | $K_i \pm$ SEM |
|---|---|
| $^{125}$I-Rat Prlh-31 | 0.25 ± 0.027 nM[a] |
| Human PRLH-20 | 1.93 ± 0.14 nM |
| Human PRLH-31 | 1.50 ± 0.29 nM |
| Rat Prlh-20 | 3.49 ± 0.29 nM |
| Rat Prlh-31 | 1.52 ± 0.28 nM |
| Human NPY | 3543 ± 492 nM |
| Human PYY | 4510 ± 915 nM |
| Human 13-36 PYY | 735 ± 107 nM |
| Human γ1-MSH | $>10^{-5}$ M |
| FMRF | $>10^{-4}$ M |
| $Zn^{2+}$ | $>10^{-4}$ M |

All experiments were done in triplicate ($n = 3$).
[a] $K_d$.

Rat Prlh-31 increased intracellular $Ca^{2+}$ concentration in human embryonic kidney (HEK) 293 EBNA cells expressing human PRLHR1 (Fig. 7C). When the cells were exposed to 1 nM rat Prlh-31, a concentration close to its $K_i$ value (Table 1), a small but significant $Ca^{2+}$ elevation was observed. A 2.5-fold higher response was obtained with a 60,000-fold higher concentration, 100 μM rat Prlh-31, which we considered to give a maximum response (Fig. 7C). Thus, 40% of the maximum response was reached with 1 nM rat Prlh-31 (Fig. 7C), suggesting that the $EC_{50}$ value of the $Ca^{2+}$ elevation and the $K_i$ of rat Prlh-31 are closely related and that there thereby is no receptor reserve for $Ca^{2+}$ elevation. In contrast, NPY at a concentration of 3 μM did not evoke any $Ca^{2+}$ response (Fig. 7C), although this concentration also corresponds to this peptide's $K_i$ value. However, 3 μM NPY fully inhibited the $Ca^{2+}$ response to the subsequent application of rat Prlh-31 (Fig. 7C). Thus, NPY binds to the PRLHR1 and behaves as an antagonist.

## Discussion

This work shows that a number of basally diverging vertebrate species possess PRLHR sequences and that these receptors share their origin with the NPYRs. The phylogenetic analyses of PRLHR and NPYRs from fish, chicken, and mammals show that these receptors group together with high bootstrap support (Fig. 2). Based on the chromosomal localization and phylogenetic relationship, we suggest that the tetraploidizations generated four copies of the ancient *PRLHR* gene, PRLHR1, PRLHR1b, PRLHR2, and PRLHR2b, which were differentially retained (Figs. 3 and 6B). The PRLHR1 subtype can be found in the Para*Hox* cluster paralogon, whereas PRLHR2 belongs to the 1/11/14/15/19 paralogon [30]. Interestingly, the *PRLHR1* subtype

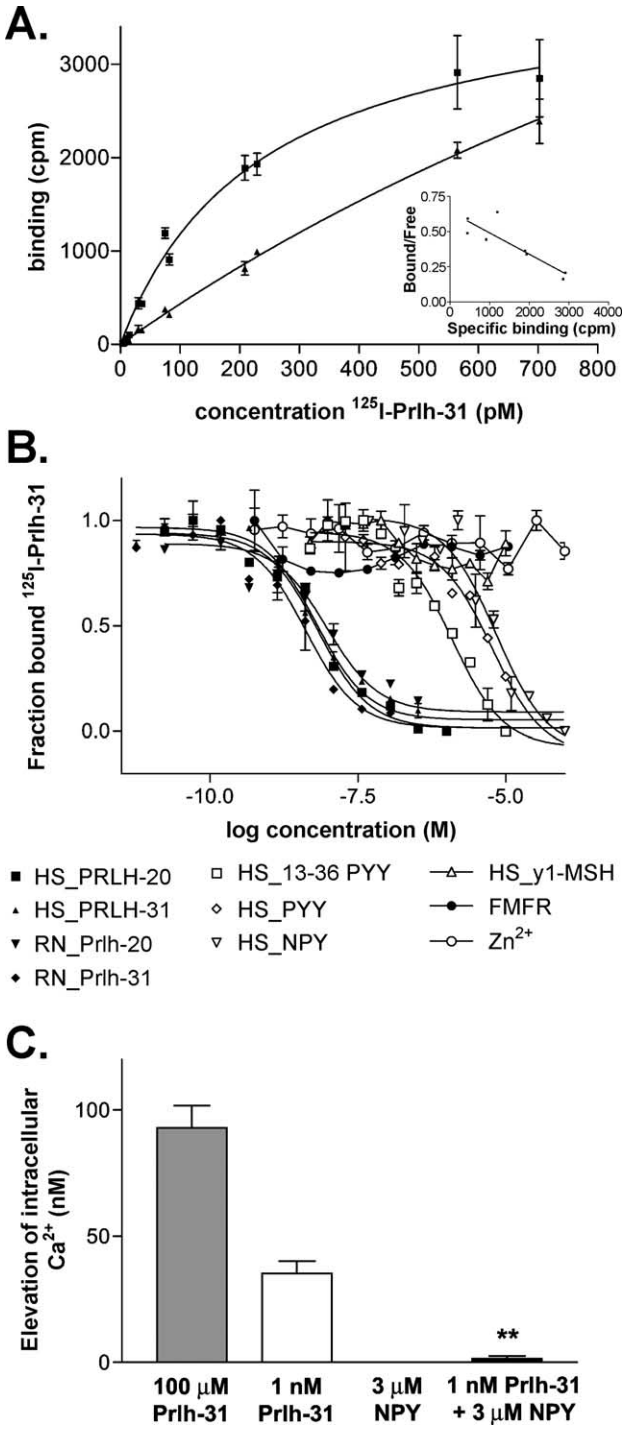



Fig. 7. Pharmacology of the human PRLHR. (A) Saturation curve for rat $^{125}$I-PRLH-31 on HEK293 EBNA membranes expressing the human PRLHR1 showing specific (squares) and nonspecific (triangles) binding. Inset: Scatchard transformation of the data shown in the saturation curve. The experiment was done in quadruplicate ($n = 4$). (B) Competition curves for both human and rat PRLH-20 and PRLH-31 and for human NPY, PYY, (13-36)-PYY, γ1-MSH, and FMRF and the metal ion $Zn^{2+}$ on HEK293 EBNA membranes, expressing the human PRLHR1, using a fixed concentration of rat $^{125}$I-PRLH-31. All experiments were done in triplicate ($n = 3$). HS (*Homo sapiens*), RN (*Rattus norvegicus*). (C) $Ca^{2+}$ responses in HEK293 EBNA cells expressing the human PRLHR1. The experiments were performed in triplicate ($n = 3$), except for maximum PRLH-evoked response (100 μM PRLH-31), which was measured in duplicate ($n = 2$). For the column labeled "1 nM PRLH + 3 μM NPY," the cells were exposed to NPY for 5 min before the application of 1 nM PRLH. $^{**}p < 0.01$ (Student's two-tailed unpaired t test) compared to the 1 nM PRLH alone.

belongs to the same paralogon group as the *NPYRs,* which may indicate a common ancestry (Fig. 6B). Moreover, there are two PRLHR/NPYR-like sequences in the African malaria mosquito (*Anopheles gambiae*), which could represent genes ancestral to this group. Based on the close phylogenetic relationship between the PRLHRs and the NPYRs, we tested if peptides binding the NPYRs could bind the human PRLHR1. Quite remarkably, we found that the human NPY and two versions of the similar neuropeptide PYY bind to the human PRLHR1. NPY was also able to inhibit completely the PRLH-evoked response in PRLHR1-expressing cells, showing that NPY can act as an antagonist at high concentrations. This is even more intriguing considering that PRLH does not show any obvious sequence similarity with the NPY, PYY, or PPY peptides that bind the NPYRs. We also searched for PRLH sequences in other vertebrate and invertebrate genomes and found two sequences similar to PRH in chicken (Fig. 4). These two sequences represent proteins similar to the PRH precursor and the C-RF amide precursor (Fig. 4). C-RF amide sequences have previously been identified in goldfish and zebrafish. It is, however, notable that we could not find the PRH or the C-RF amide in any invertebrate genome. Genes coding for *NPY*-like homologs can, however, be found in several invertebrate species such as the great pond snail (*Lymnaea stagnalis*), the marine mollusc (*Aplysia californica*), and the fruit fly (*Drosophila melanogaster*) [31]. Thus, it seems clear that NPY-like peptides were present prior to the split between deuterostomes and protostomes, whereas the PRH/C-RF amide peptides most likely arose during the tetraploidizations. Considering the fact that PRLH seems to have arisen much later than the ancestral NPYR/PRLHR, we suggest that this antecedent peptide started to coevolve with a redundant NPY binding receptor, generating the PRLHR family. The results of the evolutionary rate calculations provide additional support for this hypothesis. The PRLHR1 subtype was shown to have a relatively high evolutionary rate (Fig. 6A), which could indicate a drastic change in the binding preference, from binding an NPY-related peptide to an ancestral PRLH/C-RF amide peptide. We therefore suggest that this coevolution of an NPY-binding receptor and the ancestral PRLH/C-RF amide peptide started approximately 500 MYA, in a period after the proposed tetraploidizations of the vertebrate genome but before the divergence of ray-fin and lobe-fin fish.

We aligned PRLH with numerous human peptides that bind to GPCRs to understand the origin of this peptide. There are no obvious sequence motifs common to PRLH and any other human peptide that we are aware of, except an RF motif in the C-terminus. This motif is shared by several peptides such as RFRP and the NPFF/SF/AF peptides. Several NPY/PYY/PPY peptides instead display the RY motif in the C-terminus and because both bovine PYY and frog PPY display the RF motif, this could possibly indicate a common ancestry between the RF-peptide families [22]. It has been suggested that there could be some similarities between the folding of human NPY and that of PRLH but these secondary structural properties have not been tracked from an evolutionary perspective [32]. It is, however, interesting that the *PRH,* as well as the chicken *C-RF* amide precursor, are members of the well-studied *Hox* paralogon [21] to which both the *NPY/PYY/PPY* and the *NPFF/SF/AF-RFRP* peptide genes belong (Fig. 5). This, together with the fact that the PRLH cannot be found in invertebrate genomes, suggests that the *PRH* precursor could have been generated by the two genome duplications in early vertebrate history [5,6].

In the case of neofunctionalization among the peptide binding receptors, there are two main possible scenarios, the appearance of a redundant receptor or that of a redundant ligand. The first example is represented by the MCRs. Humans have five subtypes, MC1R–MC5R, of which MC1R, MC3R, MC4R, and MC5R bind to all endogenous melanocortin ligands, α-, β-, and γ1-MSH and ACTH, whereas MC2R binds only ACTH [26]. All stimulating melanocortin ligands are processed from the same propeptide, POMC, which exists in several vertebrates and invertebrates [33–35], while the receptor family seems to have expanded during the tetraploidization events [36]. Interestingly, the nonmammalian MC1Rs, MC3Rs, MC4Rs, and MC5Rs have remarkably higher affinities for ACTH then their human orthologs have. This is evident in chicken and pufferfish [36,37]. In this case, the ligand/receptor coevolution most likely started with the appearance of redundant MSH-binding receptors, probably during the tetraploidization events. Subsequently, MC2R evolved into a specialized ACTH-binding receptor, whereas the other four receptors lost their ability to bind ACTH and instead evolved the ability to mediate their actions through the other three ligands.

A different story emerges for the NPYRs, which in human consist of four functional receptors, NPY1R, NPY2R, NPY4R, and NPY5R. The receptors in this family bind the closely related peptides NPY, PYY, and PPY but with various affinities [38]. The *PPY* precursor gene may have been formed before the appearance of the tetrapods by a local tandem duplication of the *PYY* precursor gene [22]. After this duplication, NPY4R seems to have evolved into a specialized PPY binding receptor. Evidence for this conclusion is that the human NPY4R shows a high affinity for PPY, while the affinities for the other ligands in the NPY/PYY/PPY family are decreased compared to the affinities that these ligand orthologs have for the chicken NPY4R [39]. This scenario of neofunctionalization can also be visualized by the high evolutionary rate of the NPY4Rs, relative to NPY1Rs and NPY2Rs, which have fixed peptide preferences [40,41].

The human PRLHR1, which until now was known as a member of a single-subtype receptor family, is here shown to represent a subtype of the PRLHRs, the PRLHR1s. This subtype is clearly separated from the PRLHR2s as shown by the analysis of phylogenetic relationships and chromosomal positions (Figs. 2 and 3). Both subtypes are present in chicken and pufferfish, indicating that they were present before the

divergence of ray-fin and lobe-fin fish. Because the three mammalian genomes that we analyzed contain only the *PRLHR1* gene subtype, we conclude that the gene for the PRLHR2 subtype either was lost in the mammalian lineage or has yet to be sequenced in a mammal. The human PRLHR1 protein shares high sequence identity with the human NPY2R, particularly in the upper and middle regions of TM4, TM5, and TM6. These regions have previously been shown to be important for ligand–receptor interactions in the human NPY1R [42]. A comparison with the residues in the human NPY1R, which had been reported to be crucial for the NPY binding, shows that one residue in the top of TM2 (Tyr100 (NPY1R)–Tyr120 (PRLHR1)) and two in the top of TM6 (Asn283 (NPY1R)–Asn298 (PRLHR1) and Asp287 (NPY1R)–Asp302 (PRLHR1)) are conserved in the human PRLHR1. The preservation of these residues is likely to play a role in the binding of NPY and NPY-related peptides to the human PRLHR1 (Table 1). These interactions can be the cause of the elimination of the PRLH-31 response by micromolar levels of NPY (Fig. 7C). Two important positions for binding of the NPY peptide to human NPY1R, one residue in the first extracellular loop (Asp104) and one in the top of TM6 (Trp288) [42], are different in the human PRLHR1 and this could play a role in the relatively low affinity of the NPY peptide for the human PRLHR1. These changes could perhaps explain the rapid evolution of an ancestral NPYR into a specialized PRLH-binding receptor.

In summary, we found several new full-length genes and two fragments of *PRLHR*-like genes, showing the presence of *PRLHR* in several vertebrate species. Based on phylogenetic analysis, the *PRLHR*-like genes were split into two separate subtypes, PRLHR1 and PRLHR2. Further phylogenetic and chromosomal analysis revealed a close relationship between the PRLHR and the NPYR families. Moreover, we found that three human NPYR-binding peptides bound specifically to the human PRLHR1. NPY was also capable of competitive antagonism of intracellular $Ca^{2+}$ response evoked by PRLH via the human PRLHR1. We suggest that the PRLHR family was formed when an ancestral PRLH/C-RF amide peptide started coevolving with a redundant NPY binding receptor approximately 500 MYA. These experiments show how gene duplication events can lead to novel peptide ligand–receptor interactions and hence spur the evolution of new physiological functions.

## Materials and methods

### *Receptor sequence isolation*

#### *Cloning*

Degenerate primers were based on sequences conserved between the human (P49683) and the rat PRLHR1 (O64121). The primers were used with 500 ng of guinea pig (*Cavia porcellus*), chicken (*Gallus gallus*), zebrafish (*Danio rerio*), or sturgeon (*Acipenser baerii baerii*) genomic DNA, as template, in a touchdown polymerase chain reaction (PCR) on a GeneAmp PCR System 9700 (Perkin–Elmer) using *Taq* DNA polymerase (Invitrogen) and the following conditions: 95°C for 2 min; 25 cycles of 95°C for 30 s, 55–42°C for 45 s, 72°C for 1 min 20 s; 35 cycles of 95°C for 30 s, 50°C for 45 s, 72°C for 1 min 20 s; and 72°C for 5 min. The forward primer had the sequence 5′-TTYGARCCNCGNGGNTGGGT-3′ (extracellular loop 1 (EL1)) and the reverse primers had the sequences 5′-GGNAGCCANGARAANGCRAA-3′ (TM6) and 5′-TGNARCCANGCRTADATRAA-3′ (TM7). PCR products of 540 (EL1–TM6) and 660 bp (EL1–TM7) were isolated and subsequently cloned into the pCR II-TOPO vector using the TOPO TA Cloning Kit (Invitrogen). The clones were sequenced using vector-specific primers and the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit v3.1 (Applied Biosystems, Inc., USA) and analyzed on an ABI 310 fluorescent-dye sequencer (Applied Biosystems). The sequencing results were evaluated using SeqMan from the Lasergene package (DNASTAR, Inc., USA). For identification, the sequences were compared with GenBank records using the search tool Blastx at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). Genomic DNA, used for degenerate PCR, was generously donated by Professor Dan Larhammar (Department of Neuroscience, Uppsala University, Uppsala, Sweden).

#### *Isolation of the chicken PRLHR2 sequence*

The degenerate chicken *PRLHR2* PCR product was labeled using the Megaprime DNA labeling system (Amersham, Uppsala, Sweden) and used as probe to screen a genomic chicken bacterial artificial chromosome (BAC) library [43]. Fifty thousand clones from the library were gridded in duplicate on nylon filters in a high-density format. Hybridizations were performed for 16 h at 60°C in 50% formamide and 6× standard sodium citrate (SSC), 5× Denhardt's, 0.1% sodium dodecyl sulfate (SDS). Washes were performed at 56°C for 1 h in 0.2× SSC and 0.1% SDS and 1 h at 65°C in 0.2× SSC and 0.1% SDS. Six positive clones were isolated and checked with the same probe by Southern hybridizations.

#### *Data mining (receptors)*

Pufferfish (*Takifugu rubripes*) PRLHR-like sequences were searched using Hidden Markov Model (HMM) building as a search strategy. The region including TM1 to TM7 for all cloned receptor sequences was aligned using ClustalW 1.82 (http://www.ebi.ac.uk/clustalw/). From the alignments, HMMs were constructed using the HMMER 2.2 package [44]. The models were constructed using HMMbuild with default settings and calibrated using HMMcalibrate. The GeneScan protein datasets for the pufferfish assembly version 14.2 were downloaded from the Ensembl ftp site, ftp://ftp.ensembl.org/pub/, and subsequently searched against the HMM, using HMMsearch. Two PRLHR-like

fragments were found, FRUP00000135820 (PRLHR2b, Scaffold_244) and FRUP00000165587 (PRLHR1b, Scaffold_96), and evaluated and reorganized using full-length homologs, knowledge of intron positions, and genomic sequences.

Two Npy2/7r-like fragments were also obtained from the pufferfish genome, FRUP00000141838 (Npy7r-like, Scaffold_2053) and FRUP00000138240 (Npy2r-like, Scaffold_22). These protein sequences were also downloaded as fragments predicted by Genscan. The full-length receptor gene predictions were then assembled in SeqMan using DNA sequence from the closest homolog and pufferfish genomic DNA from the respective scaffold.

The zebrafish *PRLHR2* sequence were obtained from working draft sequence emb|BX571712.4, searching with the cloned fragment against the NCBI zebrafish database at http://www.ncbi.nlm.nih.gov/genome/seq/DrBlast.html.

The zebrafish *npy2r*-like sequence was obtained using the pufferfish Npy2r-like as a bait on http://www.ensembl.org/Danio_rerio/. Scaffold BX255954.12 was downloaded and subsequently aligned with the bait using blast2seq at http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html to obtain the DNA sequence for the *npy2r*-like gene.

The chicken PRLHR1 sequence was obtained using tBlastx searches against the 5.3× genomic trace database downloaded from ftp://ftp.ncbi.nlm.nih.gov/pub/TraceDB/gallus_gallus using human PRLHR1 as bait. All hits with an *E* value above $10^{-15}$ were extracted into FASTA format and assembled into full-length protein using SeqMan. The assembled proteins were searched against the human PRLHR1 to obtain the chicken ortholog. The sequence overlap in the coding region was between three- and sixfold.

The accuracy of the cloned chicken *PRLHR2* sequence was investigated using the expressed sequence tag (EST) database at http://www.chick.umist.ac.uk/, finding the supporting clone 603850559F1, and the NCBI Gallus_Gallus_WGS_trace database at http://www.ncbi.nlm.nih.gov/, identifying the supporting clones gnl|ti|281513709, gnl|ti|253841912, and gnl|ti|253175448.

The *PRLHR1B* sequence from chicken was obtained by Blat searches against the chicken genome at http://genome.ucsc.edu/ using the human PRLHR1 as bait. The sequence was assembled in SeqMan using the DNA sequence from the closest homolog (pufferfish *PRLHR1b*) and the genomic sequence. Before submission to GenBank, all Ensembl-generated sequences were Blasted against the respective genome at NCBI to get NCBI accession numbers for the respective genome contigs.

The six new *PRLHR*-like full-length genes and the two fragments have been submitted to the DDBJ/EMBL/GenBank databases under Accession Nos. AY572193 (guinea pig, *PRLHR1*), AY572194 (chicken, *PRLHR2*), AY572195 (sturgeon, *PRLHR1*), BK005177 (chicken, *PRLHR1*), BK005491 (chicken, *PRLHR1B*), BK005178 (zebrafish, *PRLHR2*), BK005182 (pufferfish, *PRLHR1b*), and BK005183 (pufferfish, *PRLHR2b*) and the three Npy2/7r-like genes from pufferfish and zebrafish under Accession Nos. BK005179 (zebrafish, *npy2r*-like), BK005180 (pufferfish, *npy2r*-like), and BK005181 (pufferfish, *npy7r*-like). The accession numbers starting with BK are available in the Third Party Annotation Section of the DDBJ/EMBL/GenBank databases.

### *Chromosomal localization of receptor genes*

#### *Chicken chromosome preparation, probe labeling, and in situ hybridization*

Chicken metaphase chromosome spreads were prepared from short-term bone marrow cultures as described by Christidis [45]. The *PRLHR2*-containing BAC clone was labeled with biotin-14–dATP by nick-translation (BioNick Labeling System; Life Technologies). The hybridization mixture contained 1 μg labeled probe DNA, 10 μg chicken genomic DNA, 1 μg sonicated salmon sperm DNA, 10% dextran sulfate, 50% formamide, and 2× SSC. Overnight in situ hybridization, posthybridization washing, and signal detection with two layers of avidin–FITC (Vector) and one layer of biotinylated anti-avidin (Vector) was carried out as described elsewhere in detail [46]. The results were analyzed under a Zeiss Axioplan2 fluorescence microscope, and images were captured and processed using the CytoVision/Genus software version 2.7 (Applied Imaging). Chicken chromosomes were identified according to the recently proposed nomenclature [47].

#### *Genetic mapping in zebrafish*

From the zebrafish sequence for *PRLHR2* we used the mapping primers prrpr-f+6, 5′-CGAAAGCGAGGGTGGGTGTT-3′, and prrpr-r-231, 5′-TGTGTAAAGCGGTGGGAGTTGAC-3′. For mapping zya we used the primers zya-f, 5′-GTCTGGGGTTGCGCTCATCACTA-3′, and zya-r, 5′-AATGTGTTGTTTTGACCT-3′. These primers amplified fragments that were polymorphic by single-strand conformation polymorphism analysis on the HS meiotic mapping panel as described [48]. Maps were constructed using MapManager [49].

#### *Bioinformatic mapping and conserved synteny investigations concerning the receptor sequences*

The chicken genome was downloaded as FASTA files, one file per chromosome, from http://pre.ensembl.org/Gallus_gallus/. The files were merged into one FASTA file and formatted into a Blast database using formatdb. Regions containing the chicken *PRLHR1* and *PRLHR2* were identified using Blastn searches with the full-length chicken *PRLHR1* and *PRLHR2,* sequences that have previously been identified using degenerate PCR (*PRLHR2*) and homology searches against the chicken genome (*PRLHR1*). Regions 5 Gb upstream and downstream of the hits were extracted from the FASTA files and divided into 10,000-bp pieces using custom-made software (software can be retrieved from the authors upon request).

To identify genes in the chicken genomic regions these files were searched against a Blast database made from the NCBI RefSeq dataset [50] containing more than 18,000 inspected human protein sequences, using Blastx. A cut-off value at $E = 10^{-9}$ was used for all searches. All data were analyzed and manually inspected using Microsoft Excel. The chromosomal position for the chicken *PRLHR1B* was retrieved by Blat searches, using the full-length chicken PRLHR1B sequence, against the chicken genome at http://genome.ucsc.edu/. All hits were searched against http://genome.ucsc.edu/ to confirm the hit and retrieve the corresponding human chromosomal position. Further synteny investigations involving the chicken genome were done using the Map Manager tool at http://www.genome.iastate.edu/chickmap/. Identified gene sequences on chicken GG05 or GG06 chromosome were subsequently searched against the human genome using http://genome.ucsc.edu/ to identify the corresponding human positions.

The position of the mouse *PRLHR1* homolog was identified using http://www.ncbi.nlm.nih.gov/genome/seq/MmBlast.html and the human–mouse conserved synteny of the region was investigated using the mouse conserved synteny tool at http://genome.ucsc.edu/. The rat *PRLHR1* homolog has previously been localized to 1q55 [51]. The mouse–rat–human synteny of the region was investigated using http://gapp.gen.gu.se/SearchRMQ.php and http://www.ncbi.nlm.nih.gov/genome/seq/RnBlast.html.

The sequences of the genes belonging to LG17 in zebrafish were collected from NCBI and subsequently searched against the human genome using http://genome.ucsc.edu/ to investigate the conserved synteny of this region. The positions of the two pufferfish *PRLHR* homologs in scaffold_224 and scaffold_96 were identified using http://www.ensembl.org/Fugu_rubripes/, and neighboring genes were subsequently searched against the human genome using http://genome.ucsc.edu/.

### *Peptide sequence identification*

The chicken genome was downloaded as FASTA files, one file per chromosome, from http://pre.ensembl.org/Gallus_gallus/. The files were merged into one FASTA file and formatted into a Blast database using formatdb. Regions containing the two chicken PRH-similar sequences were identified using tBlastn searches with the full-length protein sequence of human PRH. The genomic sequences were investigated using tBlastn at http://www.ncbi.nlm.nih.gov/ in the database est_others (query limited to *Gallus gallus*) and the ChickEST database at http://www.chick.umist.ac.uk/, identifying the supporting EST clones BU445468.1 and BX933358.1 (chicken *PRH*) and BU401478.1, BU484064.1, BU481242.1, BU111815.1, CD214895.1, BU109553.1, and BU358127.1 (chicken *C-RF* amide). The sequences similar to the precursors were analyzed using SeqMan. The intron–exon boundaries for the two chicken peptide sequences were confirmed by the EST sequences. The two chicken precursor-similar sequences were aligned with the human PRH (P81277), the goldfish C-rf amide precursor (BAA76662), and the zebrafish C-rf amide precursor (ENSDART00000027397, Ensembl) using ClustalW 1.82. Before submission to GenBank, both sequences were Blasted against the chicken genome at NCBI to get NCBI accession numbers for respective genome contig. The chicken *C-RF* amide and *PRH* precursors are available in the Third Party Annotation Section of the DDBJ/EMBL/GenBank databases under Accession Nos. BK005487 and BK005488.

### *Chromosomal mapping of the peptide genes in the human and the chicken genome*

The human chromosomal positions of GPCR-binding peptides belonging to the *Hox* paralogon, 2q14–q37/7p22–p11, q21–36/12q13–q23/17q [21], were determined using http://genome.ucsc.edu and http://www.ncbi.nlm.nih.gov/mapview. The corresponding chromosomal positions in the chicken genome were investigated using the human sequences as queries in Blat searches against the chicken genome sequence at http://genome.ucsc.edu and in Blast searches at http://www.ensembl.org/Gallus_gallus/.

### *Phylogeny of the receptor protein sequences*

To avoid phylogenetic problems associated with differences in length of the carboxyl and the amino termini of the receptor-like sequences, only regions TM1–TM7 were analyzed. The TM regions were determined after structural comparisons with bovine rhodopsin and using the Conserved Domain Database search tool v1.6 (RPS-BLAST) at NCBI. The previously published human (P49683) and rat PRLHR (Q64121) were downloaded from NCBI, as were the sequences for the NPYRs and the human tachykinin receptor 1 (TACR1) (for accession numbers see Supplemental Information 1). The fragments from guinea pig and sturgeon were not included in the first round of calculations as they would reduce the phylogenetic information in the alignment due to the short length of these fragmentary sequences. The truncated receptors were aligned using the Win32 version of ClustalW 1.82 with default alignment parameters. The alignment was visually inspected and the NPY5Rs' extra amino acids in the third intracellular loop were removed. The alignment was subsequently bootstrapped 1000 times using Seqboot from the α3.6 version of the Phylip package to obtain a total of 1000 different alignments. This Seqboot file was then analyzed with three different methods: maximum parsimony, neighbor joining, and maximum likelihood. Maximum parsimony trees were calculated using Protpars from the α3.6 version of the Phylip package. Trees were unrooted and calculated using ordinary parsimony, and topologies were obtained using the built-in tree search procedure. Neighbor-joining trees were calculated using Protdist (Jones–Taylor–Thornton model (JTT)) and Neigh-

bor from the α3.6 version of the Phylip package with default settings. Maximum likelihood analyses were carried out using Proml from the α3.6 version of the Phylip package with default settings (JTT) and times to jumble set to 1. Consensus trees were calculated for each method using Consense from the 3.5 version of the Phylip package and plotted using TreeExplorer (http://evolgen.biol.metro-u.ac.jp/TE/TE_man.html). The phylogenetic positions of the partial sequences from guinea pig and sturgeon and Ya (NPY4R-like) from zebrafish were determined using Protpars under the same conditions as above except that the starting input file was cut, using only the portion from the end of TM3 to the end of TM6, to have equally long protein sequences. Their positions in the final parsimonious tree are indicated with arrows (Fig. 2). Sequence similarity within the PRLHR family and the NPYR family was determined using full-length protein sequences and MegAlign from the Lasergene package (DNASTAR). The sequence similarity between the analyzed neuropeptides (see Supplemental Information 2) was evaluated both using MegAlign and by manual inspection.

*Molecular clock calculations*

To establish the evolutionary rate of the different receptor subtypes, available protein sequences from human, rhesus monkey (*Macaca mulatta*), rat, chicken, African clawed frog (*Xenopus laevis*), pufferfish, spiny dogfish (*Squalus acanthias*), and European river lamprey (*Lampetra fluviatilis*) were collected for the PRLHR1, NPY1R, NPY2R, and NPY4R subtypes. Each sequence was truncated, using only TM1–TM7 for this study. One alignment was made for each subtype using the Phylip output format of ClustalW 1.82. Each separate alignment was put into TreePuzzle [52] (see Supplemental Information 3 for the input sets and accession numbers) for estimated molecular clock calculations. The distances were calculated using tree reconstruction, quartet puzzling with 1000 steps with exact parameter estimate, gamma parameter estimations from the dataset, and the JTT model of substitution. The published distances in million years ago between human and the other species [53–55] were plotted against the likelihood distances calculated by TreePuzzle using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA). A minimum and a maximum value for each divergence time was given based on differences between molecular clock estimations and fossil findings: human–rhesus monkey (20–23 MYA), human–rat (65–70 MYA), human–chicken (290–310 MYA), human–African clawed frog (310–360 MYA), human–pufferfish (420–440 MYA), human–spiny dogfish (430–520 MYA), and human–European river lamprey (530–550 MYA).

The approximate dates of divergence between the PRLHR1 and the PRLHR2 subtypes and between the PRLHRs and the NPYRs were subsequently based on distance calculations using TreePuzzle. The distances were calculated using the truncated alignment described under the phylogeny section under the following conditions: tree reconstruction, quartet puzzling with 1000 steps with exact parameter estimate, gamma parameter estimations from the dataset, and the JTT model of substitution. The calculated distance between chicken PRLHR1 and PRLHR2 was used together with the distance between pufferfish PRLHR1b and PRLHR2b for estimations of the date of the split between the subtypes. These distances were inserted into the regression formulas $y = 433.9 - 1 \times x + (-0.04631 \pm 0.1441)$ [56] and $y = 451.9 - 1 \times x + (-0.05573 \pm 0.1339)$ (max), derived from the PRLHR1 calculations, to get an approximate interval for the split. The 9 derived values (4 for each species) were inserted into GraphPad Prism 3.0 for calculation of mean ± standard deviation. The distance between the human PRLHR1 and the human NPY2R was used together with the distance between chicken PRLHR1 and chicken NPY2R and pufferfish PRLHR1b and Npy2r in the same way as described for PRLHR1 and PRLHR2, for the calculation of the split between the PRLHRs and the NPYRs. The 12 derived values (4 for each species) were put into GraphPad Prism 3.0 for calculation of mean ± standard deviation.

*Binding studies*

*Expression of the human PRLHR1 in HEK293 EBNA cells*

A semistable cell line (the plasmid is kept as a cytosolic episome under selection with hygromycin) expressing the human PRLHR1 was constructed by transfection of 12 μg of plasmid DNA using FuGENE Transfection Reagent (Boehringer Mannheim, Germany), diluted in OptiMEM medium (Gibco BRL, Sweden) according to the manufacturer's recommendation. After transfection, cells were grown in Dulbecco's modified Eagle's medium/Nut Mix F-12 w/o L-glutamine (Gibco BRL) supplemented with 10% fetal calf serum (Gibco BRL/Life Technologies), 2.4 mM L-glutamine (Gibco BRL), 250 μg/ml gentamicin (Gibco BRL), and penicillin–streptomycin (100 U penicillin, 100 μg streptomycin/ml) (Gibco BRL) for 48 h before addition of 200 μg/ml hygromycin (Gibco BRL). The cells were harvested in PBS after 3 weeks of selection.

*Membrane preparation*

After harvest, the cells were homogenized using an UltraTurrax disperser for 30 s in binding buffer containing 20 mM Tris–HCl, 5 mM $MgCl_2$, and 2 mM EDTA, pH 7.4 [23]. The cell suspension was centrifuged for 3 min at 170 relative centrifugal force (rcf), and the supernatant was recentrifuged for 15 min at 31,000 rcf. The cell pellet was resuspended in binding buffer and homogenized again for 15 s. The cell suspension was then aliquoted and stored at −20°C.

*Receptor binding assays*

Before the pharmacological studies were conducted, the amount of protein/μl was determined using the Bio-Rad

Protein Assay (Bio-Rad, Solna, Sweden). Binding studies were performed using a buffer containing 20 mM Tris–HCl, 5 mM $MgCl_2$, and 2 mM EDTA, pH 7.4 [23], in a final volume of 200 μl, and incubation for 90 min at room temperature using 0.6–0.8 μg of membrane preparation per well. Saturation studies were performed with various concentrations of rat $^{125}$I-Prlh-31. The radioligand was labeled with $^{125}$I using the chloramine-T method [57] and purified by high-performance liquid chromatography (Euro-Diagnostica AB, Malmö, Sweden). Nonspecific binding was defined as the amount of radioactivity remaining bound to the cell homogenate after incubation in the presence of 1 μM unlabeled human PRLH-20 (Neosystem, France). Competition studies were performed with various concentrations of unlabeled displacers included in the incubation mixture along with 0.2–0.6 nM rat $^{125}$I-Prlh-31. Binding affinities were calculated using GraphPad Prism 3.0. Peptides were purchased from Neosystems (France) or Sigma and $ZnCl_2$ was purchased from Sigma.

*Functional studies*

For intracellular $Ca^{2+}$ measurements, the HEK293 EBNA cells expressing the human PRLHR1 were harvested and loaded with fura-2 acetoxymethyl ester (Molecular Probes Inc., Eugene, OR, USA) as previously described [58]. Briefly, the cells were detached using PBS supplemented with 0.2 g/L EDTA, loaded with 4 μM fura-2 in Tes-buffered medium [137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.44 mM $KH_2PO_4$, 4.2 mM $NaHCO_3$, 10 mM glucose, 0.5 mM probenecid (*p*-[dipropylsulfamoyl]benzoic acid) (Sigma), and 20 mM 2-[(2-hydroxy-1,1-bis[hydroxymethyl]ethyl)amino] ethane sulfonic acid (Tes) adjusted to pH 7.4 with NaOH), washed, and stored on ice as pellets. For the measurement of intracellular free calcium, one cell pellet was resuspended in Tes-buffered medium at 37°C. The fluorescence was monitored in a stirred quartz microcuvette in the thermostatted cell holder of a Hitachi F-2000 or F-4000 fluorescence spectrophotometer at the wavelengths 340 (excitation) and 505 nm (emission). The experimental conditions were calibrated by adding 60 μg/ml digitonin (for the maximum fluorescence) and 10 mM EGTA (for the minimum fluorescence). All the measurements, except maximum PRLH-evoked $Ca^{2+}$ response (100 μM rat Prlh-31), which were made in duplicate, were performed at least three times with similar results. For competition experiments, 3 μM NPY was incubated with the human PRLHR1-expressing cells for 5 min before the addition of 1 nM rat Prlh-31.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2005.02.007.

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