Eur. J. Biochem. **268,** 6146–6154 (2001) © FEBS 2001

A neuropeptide Y receptor Y1-subfamily gene from an agnathan, the European river lamprey

A potential ancestral gene

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We report here the isolation and functional expression of a neuropeptide Y (NPY) receptor from the river lamprey, *Lampetra fluviatilis*. The receptor displays ≈ 50% amino-acid sequence identity to all previously cloned Y1-subfamily receptors including Y1, Y4, and y6 and the teleost subtypes Ya, Yb and Yc. Phylogenetic analyses point to a closer relationship with Y4 and Ya/b/c suggesting that the lamprey receptor could possibly represent a pro-orthologue of some or all of those gnathostome receptors. Our results support the notion that the Y1 subfamily increased in number by genome or large-scale chromosome duplications, one of which may have taken place prior to the divergence of lampreys and gnathostomes whereas the second duplication probably occurred in the gnathostome lineage after this split. Functional expression of the lamprey receptor in a cell line facilitated specific binding of the three endogenous lamprey peptides NPY, peptide YY and peptide MY with picomolar affinities. Binding studies with a large panel of NPY analogues revealed indiscriminate binding properties similar to those of another nonselective Y1-subfamily receptor, zebrafish Ya. RT-PCR detected receptor mRNA in the central nervous system as well as in several peripheral organs suggesting diverse functions. This lamprey receptor is evolutionarily the most distant NPY receptor that clearly belongs to the Y1 subfamily as defined in mammals, which shows that subtypes Y2 and Y5 arose even earlier in evolution.

Keywords: NPY; PYY; evolution; gene duplication; G-protein coupled receptor; Lamprey.

Neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP) are closely related 36-amino-acid neuroendocrine peptides found in all tetrapods. NPY and PYY have also been found in nontetrapod gnathostomes as well as agnathans [1]. These peptides have a large number of physiological effects in the nervous system, the circulatory system and the gastrointestinal tract. NPY is most abundant in the nervous system and stimulates food intake, regulates blood pressure and influences release of pituitary peptides [2,3]. PYY and PP are found in the gastrointestinal tract and are released upon food intake. In nontetrapod vertebrates PYY is also present in the central nervous system (CNS) [4,5]. PY, a PYY-like peptide is only found in certain teleost fishes, and appears to have arisen from a fairly recent gene duplication [1,6]. In the river lamprey, *Lampetra fluviatilis*, the ancestral *PYY* gene appears to have undergone a separate gene duplication, resulting in peptide YY and peptide MY [4,7].

The effects of the NPY family of peptides are relayed by a family of G-protein coupled receptors [8–10]. Five receptors have been cloned in mammals; Y1, Y2, Y4, Y5 and y6, most of which have high affinities for NPY and PYY. Y4 is the only receptor for which PP has higher affinity than NPY or PYY. The *y6* gene still lacks a physiological correlate, hence it is designated with a lower case 'y'. In humans and other primates, the *y6* gene is a pseudogene. Sequence comparisons of the NPY receptor genes reveal different degrees of identity between the subtypes. The mammalian Y1, Y4 and y6 subtypes are ≈ 50% identical at the amino-acid level, and form the Y1 subfamily. The *Y2* and *Y5* genes are only 30% identical to each other and to each member of the Y1 subfamily. Mapping of the receptor genes in human, pig and mouse, supports the theory that the vertebrate genome has expanded by means of large-scale chromosomal or total genome duplications early in vertebrate evolution [11]. In human and pig, the *Y1*, *Y2* and *Y5* genes are localized on the same chromosome. This suggests, together with their low degree of identity to each other, that these subtypes resulted from ancient local duplications, possibly prior to vertebrate evolution [12]. Subtypes Y4 and y6 are found on other chromosomes and presumably arose later by two separate duplications of the chromosome first harboring the *Y1* gene. Duplicates of the *Y2* and *Y5* genes have not been found in any species and have possibly been lost during the course of evolution, at least in mammals, but unpublished results from a frog and zebrafish suggest a second *Y2* gene (R. Fredriksson & D. Larhammar, unpublished data).

Three NPY Y1-like receptors have been cloned from the zebrafish *Danio rerio* (zf). Despite their clear sequence similarity to the Y1, Y4 and y6 sequences, they all seemed to represent unique teleost receptors and were named Ya, Yb

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Abbreviations: NPY, neuropeptide Y; PYY, peptide YY; PMY, peptide MY; PP, pancreatic polypeptide; *Lf*, *Lampetra fluviatilis*; zf, zebrafish; CNS, central nervous system.

Note: the nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL under accession number AF340022.

(Received 29 June 2001, revised 18 September 2001, accepted 28 September 2001)

and Yc [13–15]. These three subtypes, particularly Ya, exhibit quite indiscriminate pharmacological profiles, binding NPY, PYY and PP, even though the latter has not been found in teleosts, as well various peptide analogues [16]. The receptor subtypes found in mammals have yet to be isolated in any teleost. The theory of large-scale genomic duplications may provide an explanation for the origin of the teleost genes for *Ya*, *b* and *c*. One of these could represent the expected fourth Y1-like paralogue, not yet found in mammals, and the second zebrafish receptor gene could have resulted from the genome doubling proposed for teleost fishes [17–19]. The third gene, *Yc*, is most probably due to a recent duplication in the zebrafish lineage [15,20].

Similar conclusions concerning genome duplications have been drawn from studies of other gene families, of which the *Hox* gene clusters have provided most inter-species information [21]. The cephalochordate *Amphioxus* has one *Hox* gene cluster, while four can be found on separate chromosomes in tetrapods [22]. The zebrafish has seven *Hox* gene clusters located on separate chromosomes, supporting an additional genome duplication in teleosts, giving rise to eight *Hox* clusters, one of which was lost [17]. It has been postulated that the extant members of the ancient group of jawless fishes, hagfish and lampreys, form basal groups in the craniate lineage intermediate to *Amphioxus* and gnathostomes, having undergone one large-scale genome duplication whereas the second doubling may have occurred in the gnathostome ancestor [21]. An increase in the number of developmental genes could provide an explanation for the increased body-plan complexity in gnathostomes [23].

Lampreys are thought to have diverged from the lineage leading to gnathostomes some 460 million years ago [24]. Because of the position within the craniate tree of the lamprey between *Amphioxus* and gnathostomes, and being an established model organism for neuroscientific studies [25], it is an attractive choice for the cloning of NPY receptors. Furthermore, we have previously cloned NPY and PYY [4] and peptide MY (PMY) has been isolated from tissue extracts [26] of a representative species in this order, the European river lamprey, *L. fluviatilis*, thereby allowing studies with endogenous ligands. The NPY system has also been partially characterized with anatomical and functional studies [4,27–29]. In prespawning female sea lamprey, *Petromyzon marinus*, PMY produces a decrease in estradiol plasma concentrations, suggesting a role for the peptide in regulating maturational processes [30].

We report here the molecular cloning and functional expression of a Y1 subfamily receptor in the European river lamprey, *L. fluviatilis*, which sheds light on the evolution of this receptor family and also has implications for the genome evolution of agnathans.

MATERIALS AND METHODS

PCR cloning and DNA sequencing

Sequences from all known Y1-like NPY receptors were retrieved from GenBank and aligned using Lasergene DNASTAR MEGALIGN software. Degenerate PCR primers were designed to match highly conserved domains of the Y1 receptor gene from human, rat, mouse, pig, dog, guinea pig, and *Xenopus*, the *Y4* gene from human, rat, mouse, guinea pig and the *y6* gene from mouse and rabbit as well as the zebrafish *Ya*, *Yb* and *Yc* genes. The following PCR conditions were used: 95 °C for 2 min, one cycle; 95 °C for 30 s, 48 °C for 45 s decreasing 0.5 °C each cycle, 72°C 1 min, for 10 cycles; 95 °C for 30 s, 42 °C for 45 s and 72 °C for 1 min, 35 cycles, 72 °C for 7 min, one cycle using Gibco BRL *Taq* polymerase. Degenerate primers designed to represent all possible codon combinations of the amino-acid sequences (Y/I)TL(M/S)(D/N)(H/N)W (nucleotide sequence 5′-TAYACXHTXATGGAYYAYTGG-3′) and (F/I)YG(F/W)LN (nucleotide sequence 5′-TTRTTXARRA AXCCRTARAA-3′) were used in the PCR reaction with genomic DNA template extracted from muscle tissue from one specimen of *L. fluviatilis* (DNA Isolation kit for Cells and Tissues, Boehringer Mannheim). The product was cloned into vector pCR II using the TOPO TA-cloning kit (Invitrogen) and transformed to TOP10 cells (Invitrogen). Plasmid DNA was prepared (Promega) from 50 bacteria colonies. The plasmid inserts were sequenced with vector specific primers using ABI PRISM Dye Terminator cycle sequencing kit according to the manufacturer's instructions (PerkinElmer) and analyzed on an automated ABI 310 fluorescent-dye sequencer (Applied Biosystems Inc.). Three plasmid inserts revealed a high degree of identity with *NPY Y4*, *Ya*, *Yb* and *Yc* receptor sequences. All three clones were identical.

Cosmid library screening

Nylon membranes from a Lamprey (*L. fluviatilis*) gridded cosmid library were supplied by RessoursenZentrum/PrimärDatabank (RZ/PD; (Max Planck Institut für Molekulare Genetik, Berlin-Charlottenburg, Germany). A ^{32}P-labeled probe was constructed with one of the NPY receptor clones using the Megaprime labeling system (Amersham). Hybridization was carried out overnight at 55 °C in 25% formamide, 6 × NaCl/Cit, 10% dextrane sulphate, 5 × Denhardt's solution and 0.1% SDS. The filters were washed twice in 2 × NaCl/Cit, 0.1% SDS at room temperature for 5 min, and twice in 0.5 × NaCl/Cit, 0.1% SDS for 30 min at 55 °C. The cosmid clone MPMGc55H1438Q3 produced a strong hybridization signal and was ordered from RZ/PD. Cosmid DNA preparations were made from the clone using FlexiPrep kit (Amersham Pharmacia Biotech) and sequenced according to the protocol above, using primers designed from the original PCR clone, and the reverse primers designed for cloning into an expression vector (see below).

Alignments and tree construction

Full-length amino-acid sequences from Y1 subfamily receptors were retrieved from GenBank [accesion numbers: human Y1 (A26481), Y2 (U36269), Y4 (XM011880); mouse Y1 (Z18281), Y4 (NM008919) y6 (U58367); rabbit y6 (D86521); chicken Y2 (AF309091), *Xenopus laevis* Y1 (L25416); zebrafish Ya (AF037400), Yb (AF030245), Yc (AF037401); cod Yb (AF073925) and *Squalus acanthias* Y4]. Chicken Y1 and Y4 and peccary y6 sequences were determined in our laboratory and will be reported separately. Sequences were aligned with Lasergene DNASTAR software using PAM 250 and BLOSSUM scoring matrices. After visual inspection of the alignments, highly divergent regions corresponding to the lamprey amino acid 1–27 (N-terminus–TM1), 237–244 (intracellular loop 3 coresponding to amino

acids GREGGGNG in the lamprey sequence, including the two gaps between amino acid 236 and 237 in the lamprey sequence) and 316–365 (TM7–C terminus) were eliminated from the alignment as these regions contain large differences in amino-acid sequence as well as length, thus making alignment impossible. The rapidly evolving termini are usually excluded from comparisons between receptor subtypes thereby facilitating phylogenetic analyses over large evolutionary distances (see for example [10,31–33]). The chicken and human Y2 receptor sequences were used as outgroups. Trees were then constructed with PAUP 4.0 software (Smithsonian Institution, Washington D.C., USA) using maximum parsimony. Robustness of the nodes was assessed by BOOTSTRAP analysis in PAUP and a consensus tree was generated. Calculations were made with 100 replicates and 10 random addition heuristic searches for each node. Trees were also constructed using the neighbor-joining method. All trees were constructed with equal weighting in all positions with all gaps were coded as a twenty-first character state.

Cloning into an expression vector

PCR primers containing *Hin*dIII and *Xho* I sites were designed to generate a full-length clone using the cosmid clone DNA preparation as a PCR template. After digestion with *Hin*dIII and *Xho* I the PCR product was directionally ligated with T4 DNA Ligase (New England Biolabs) into a modified pCEP4 expression vector [34] to make the construct *Lf*Y-pCEP4. The construct was transformed into *Escherichia coli* cells, sequenced, and the insert found to be identical to the cosmid clone ORF.

Transfection protocol

For transient transfections 293-EBNA cells (Invitrogen) were transfected with FuGENE^{TM} Transfection Reagent (Boehringer Mannheim), diluted in Opti-MEM medium (Gibco BRL) according to the manufacturer's recommendation. After transfection, cells were grown in Dulbecco's modified Eagle's medium (Gibco BRL) containing 10% fetal bovine serum (Biotech Line AS), 24 mM L-glutamine (Gibco BRL) and 250 mg·L^{-1} G-418 (Gibco BRL), penicillin/streptomycin (100 U penicillin, 100 μg streptomycin·mL^{-1}; Gibco BRL) until harvesting by centrifugation after 48 h. Cell membrane pellets were frozen in aliquots at −80 °C.

Peptides and nonpeptidic antagonists

Porcine NPY, p[Leu31,Pro34]NPY, the series of amino terminally truncated pig NPY peptides pNPY$_{2-36}$, pNPY$_{13-36}$, pNPY$_{18-36}$, pNPY$_{25-36}$, pNPY$_{26-36}$, and bovine PP were from Bachem (King of Prussia, PA, USA); p[D-Trp32]NPY was from Peninsula Laboratories Inc. Lamprey peptides were synthesized as described below. BIBP3226 was kindly provided by K. Thomae GmBH, Biberach, Germany. SR120819A was provided by Sanofi, chicken PP and PYY were from Schafer-N, Copenhagen, Denmark. Nonpeptidic Y2 antagonist BIIE0246 was provided by Boehringer Ingelheim PharmaKG (Biberach an der Riss, Germany).

Peptide synthesis

Lampetra PMY (MPPKPDNPSSDASPEELSKYMLAVR NYINLITRQRY-NH$_2$), PYY (FPPKPDNPGDNASPEQM ARYKAAVRHYINLITRQRY-NH$_2$) and NPY (FPNKPDS PGEDAPAEDLARYLSAVRHYINLITRQRY-NH$_2$ were synthesized by solid-phase methodology on a 0.025-mmol scale using an Applied Biosystems model 432 A peptide synthesizer using a 4-(2′,4′-dimethoxy-phenyl-Fmoc-aminomethyl) phenoxyacetamido-ethyl resin (PerkinElmer). Fmoc amino-acid derivatives were activated with *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (one equivalent), 1-hydroxybenzotriazole hydrate (one equivalent) and di-isopropylethylamine (two equivalents). Deprotection of the N-terminus by piperidine was monitored by on-line measurement of the conductance of the carbamate salt of the Fmoc group and optimum coupling times were determined by the instrument in response to the deprotection times. After completion of the eighteenth cycle of synthesis, 50% of the resin was removed in order to ensure adequate solvation and the synthesis was continued using the same reagent quantities. The peptide was cleaved from the resin with trifluoroacetic acid/water/thioanisole/1,2-ethanedithiol (99.0/0.50/0.25/0.25, v/v) at 25° for 3 h.

The crude peptides were purified to near homogeneity by chromatography on a 1 × 25-cm Vydac 218TP510 C-18 reversed-phase HPLC column (Separations Group, Hesperia, CA, USA) equilibrated with 0.1% (v/v) trifluoroacetic acid/water at a flow rate of 2 mL·min^{-1}. The concentration of acetonitrile in the eluting solvent was increased from 21% to 49% over 60 min using a linear gradient. Absorbance was measured at 214 and 280 nm and the major peak in the chromatogram was collected by hand. The identity of the peptides was confirmed by automated Edman degradation and electrospray MS (PMY, observed M_r = 4194.6, calculated M_r = 4194.8; PYY, observed M_r = 4214.4, calculated M_r = 4214.8; NPY, observed M_r = 4173.4, calculated M_r = 4173.6).

Binding assays

The thawed aliquots of membranes were resuspended in 25 mM Hepes buffer (pH 7.4) containing 2.5 mM $CaCl_2$ 1 mM $MgCl_2$ and 2 g·L^{-1} Bacitracin and homogenized using an Ultra-Turrax homogenizer. Saturation experiments were performed in a final volume of 100 μL with 4–5 μg protein and [^{125}I]pPYY (Amersham) for 2 h at room temperature. This radioligand is iodinated at tyrosines 21 and 27 and has a specific activity of 4000 Ci·mmol^{-1}. Saturation experiments were carried out with serial dilutions of radioligand; nonspecific binding was defined as the amount of radioactivity remaining bound to the cell homogenate after incubation in the presence of 100 nM unlabelled pNPY. Competition experiments were performed in a final volume of 100 μL. Each concentration of each ligand was included separately in the incubation mixture along with [^{125}I]pPYY. Incubations were terminated by filtration through GF/C filters, Filtermat A (Wallac Oy, Turku, Finland), which had been presoaked in 0.3% polyethyleneimine, using a TOMTEC (Orange, CT) cell harvester. The filters were dried at 60 °C and treated with MeltiLex A (Wallac) melt-on scintillator sheets and the radioactivity

retained on the filters was counted using the Wallac 1450 Microbeta counter. The results were analyzed using the PRISM 3.0 software package (Graphpad, San Diego, CA, USA). Protein concentrations were measured using the Bio-Rad Protein Assay with BSA as standard.

RT-PCR and Southern blot analysis

Total RNA was prepared from CNS, liver, muscle tissue and embryonal CNS by using the acidic phenol extraction procedure. The RNA preparations were subsequently treated with DNase I (RNase free) for 20 min at room temperature (Qiagen) and purified on a RNeasy spin column (Qiagen). Approximatley 20 pg of each preparation were used as a template in Titan One Tube RT-PCR reactions (Roche Diagnostics) according to kit protocol, with specific primers (5′-AAAGTCCTCCTCCACGGGGTTGC-3′ and 5′-AGGG CCTCGCACATGAAAAAGATT-3′) coding for an ≈ 300 bp region of the receptor gene. As a negative control a reaction with total RNA from spiny dogfish, *Squalus acanthias*, liver was run with the lamprey primers. In order to exclude products from possible genomic DNA contamination in the RNA preparations an identical RT-PCR was performed without the reverse transcriptase step (data not shown). The PCR products were analyzed on a 1.5% agarose gel (Fig. 4A) and transferred to a nylon filter by blotting overnight [35]. The filter was hybridized with a random-prime-labeled 700-bp lamprey receptor probe (Megaprime kit, Amersham Pharmacia Biotech) in ExressHyb buffer (Clontech) at 65 °C for 2 h, washed twice in 2 × NaCl/Cit, 0.1% SDS for 5 min at room temperature, and twice in 0.5 × NaCl/Cit, 0.1% SDS for 30 min at 65 °C. The filter was then exposed to film (Amersham Pharmacia Biotech) at −70 °C overnight (Fig. 4B).

RESULTS

Degenerate PCR primers were constructed based upon all known *NPY Y1*, *Y4* and *y6* nucleotide sequences, as well as the zebrafish *zYa*, *Yb* and *Yc* and the cod *Yb* sequences. A 700-bp PCR product was cloned and 50 clones were sequenced of which three were found to have a high degree of identity to the Y1-like subtypes. All three clones were identical.

The clone insert was used for high stringency screening of a lamprey genomic cosmid library and one strongly hybridizing clone was isolated. Clone identity was confirmed by Southern analysis of the cosmid DNA using the original PCR product as a probe. The cosmid clone was sequenced using gene specific primers and revealed an ORF of 1100 bp (GenBank accession number AF340022) from which a putative protein of 365 amino acids could be deduced. The amino-acid sequence displayed the characteristic features of a G-protein coupled receptor, i.e. seven putative transmembrane regions, a cysteine pair linking extracellular loop 1 and loop 2, and a cysteine in the C-terminal tail where palmitoylation could serve as an anchor to the membrane and form a pseudo fourth loop (Fig. 1). Apart from these general characteristics, the amino-acid sequence of the lamprey clone exhibited a high degree of identity, ≈ 50%, to the Y1 subfamily receptors including mammalian Y1, Y4 and y6 and the teleost Ya, Yb and Yc receptor sequences. The lamprey coding sequence contains no intron (Fig. 1).

Phylogenetic trees were constructed using maximum parsimony and neighbor joining methods. Sequence from putative transmembrane region 1–7, including all intra- and extracellular loops, but excluding a highly divergent region described in materials and methods, were analyzed for all Y1-subfamily subtypes with human and chicken

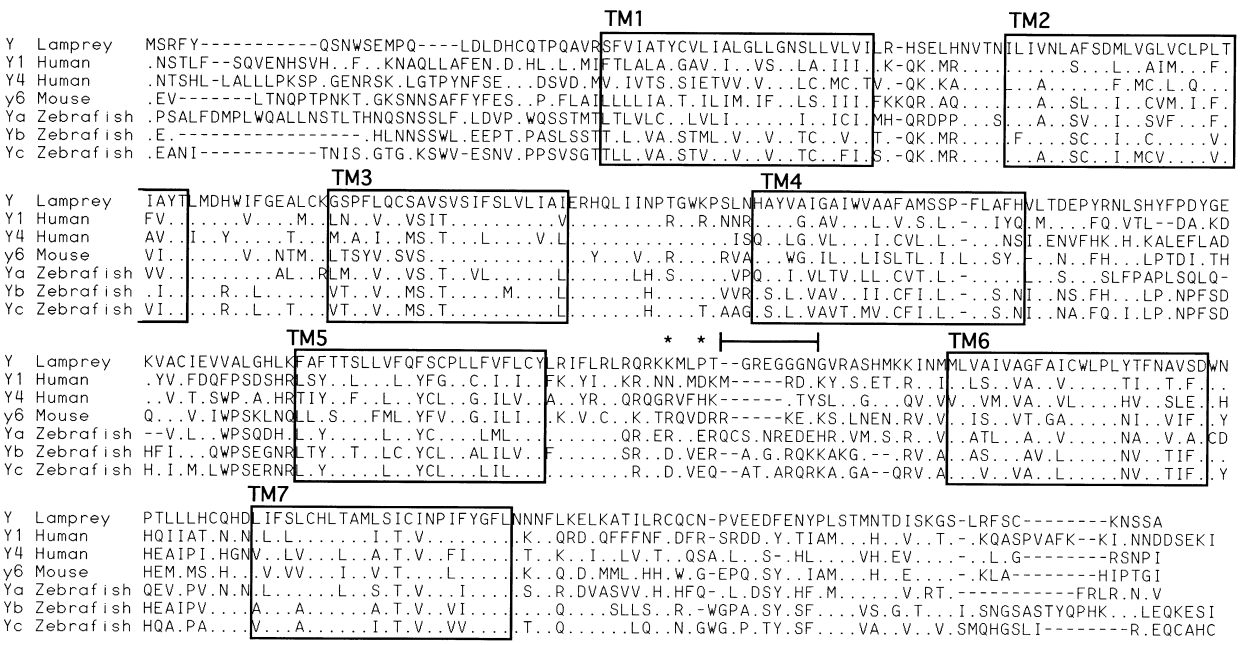

Fig. 1. Amino-acid sequence alignment. Alignments were made using Lasergene DNASTAR MEGALIGN software. The lamprey NPY receptor sequence serves as a master with human Y1, Y2 and Y4, mouse y6, and zebrafish Ya, Yb and Yc sequences. Boxes mark putative transmembrane regions. Stars indicate the two umambiguous amino-acid positions of importance for the phylogenetic analyses (see Discussion).

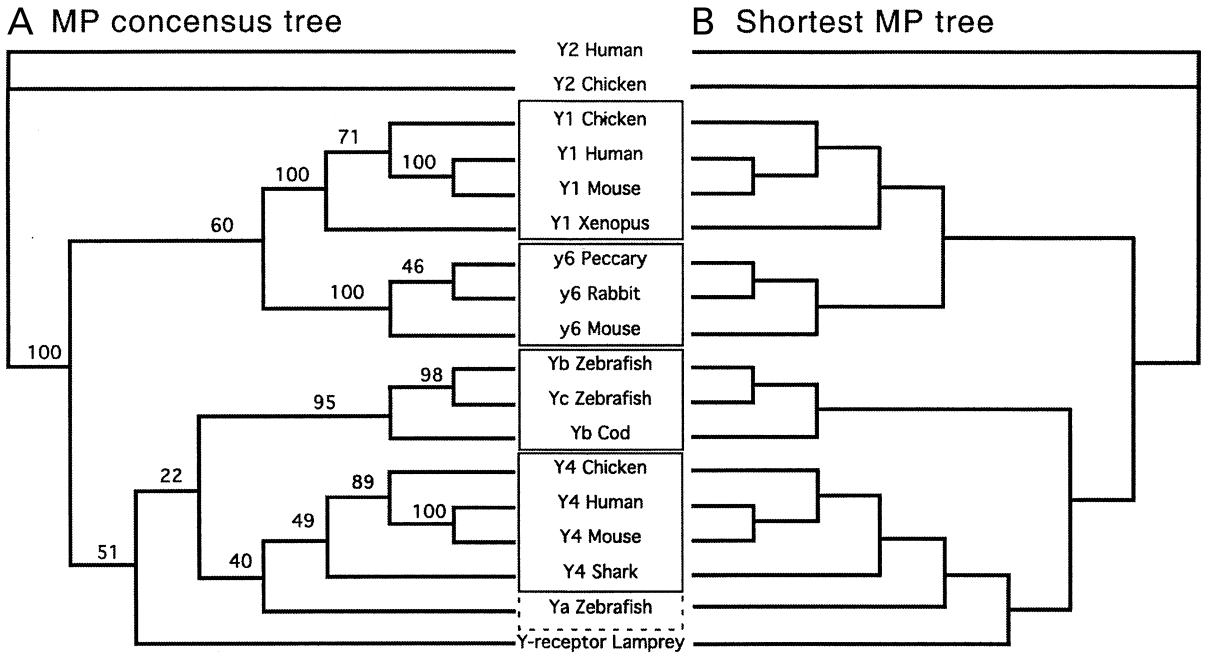

Fig. 2. Phylogenetic analyses of the Y1 subfamily amino-acid sequences. (A) Tree A is a maximum parsimony bootstrap consensus tree placing the lamprey sequence basal to all Y4, Ya, Yb and Yc sequences (PAUP 4.0b). Numbers above the nodes indicate percentage of bootstrap replicates in which the node was retained. (B) Tree B is the shortest tree generated by maximum parsimony analysis (length 1068, consistency index 0.73, homoplasy index 0.27). One of the three shortest maximum parsimony trees has topology identical to that of tree A. This tree is 0.28% longer than the shortest tree (length 1071, consistency index 0.72, homoplasy index 0.29). Also, the topology of the neighbor joining tree described in the text is identical to that of tree A. Boxes indicate four putative groups of the Y1 subfamily. The zf Ya receptor may be an orthologue of the Y4 gene and is therefore marked by a dashed box.

Y2 sequences as an outgroup. A maximum parsimony consensus tree was constructed using PAUP 4.0b software. BOOTSTRAP analysis was performed with PAUP to assess the robustness of the nodes. This tree placed the lamprey sequence basal to the Yb and Yc sequences, in addition to Y4 and Ya sequences (tree A; Fig. 2A). The shortest tree (length 1068) places the lamprey sequence basal to the Y4 and Ya subtype sequences (tree B; Fig. 2B). Another tree placing the lamprey sequence basal also to the Yb and Yc sequences, in addition to Y4 and Ya was also found among the shortest maximum parsimony trees. This tree is 0.28% longer than the shortest tree (length 1071), a difference corresponding to a single unique (or unamibiguous) amino-acid position. This tree had identical topology to tree A. Also, a neighbor-joining tree generated with PAUP using the same alignment had identical topology to tree A.

Primers containing restriction enzyme sites were used to produce a PCR product that was subsequently cloned into a modified pCEP4 expression vector [34], and transiently transfected into human EBNA cells. Membranes prepared from the cells were used to perform binding experiments with porcine [^{125}I]PYY. The radioligand exhibited concentration-dependent binding to the membrane fraction with an affinity constant (K_d) of 35 ± 4 pM, (mean ± SEM, $n = 3$, each experiment run in duplicate) and a B_{max} of 234 ± 50 fmol·mg protein^{-1} (mean ± SEM, $n = 3$, each experiment run in duplicate) (Fig. 3). Competition experiments were performed using the three endogenous lamprey (*Lf*) peptides *Lf*NPY, *Lf*PYY and *Lf*PMY and an array of mammalian intact and N-terminally truncated (pNPY$_{2\text{-}36}$ and shorter) peptides. Two modified peptides with amino-acid substitutions [p(Leu31, Pro34)NPY, p(D-Trp32)NPY] as well as four nonpeptidic antagonists were also tested, belonging to the standard battery for NPY receptor profiling [9]. The highest affinities were exhibited by the endogenous lamprey peptides, followed by mammalian and truncated peptides. No binding was exhibited by the nonpeptidergic ligands over the concentration range tested (Table 1).

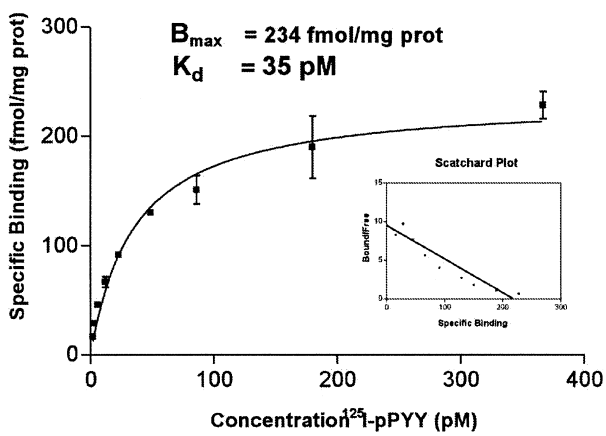

Fig. 3. Saturation binding isotherm and Scatchard plot (inset). Analyses of [^{125}I]pPYY binding to membranes prepared from HEK293 (EBNA) cells transfected with the *Lf*Y-pCEP 4 construct. Results shown are from a representative experiment ($n = 2$ for each concentration).

Table 1. Inhibition of [^{125}I]pPYY binding to membranes from EBNA cells transfected with the expression plasmid laflYrec-pCEP4. Inhibition constants $K_i \pm$ SEM expressed as pK_i (−log M) for three experiments performed in duplicate.

Ligand	pK_I (−log *M*)	SEM
Lf-NPY	10.6	0.03
Lf-PYY	11.1	0.36
Lf-PMY	10.8	0.11
pPYY	10.3	0.13
pNPY	10.3	0.14
pNPY 2–36	10.3	0.23
pNPY 13–36	10.4	0.06
pNPY 18–38	9.1	0.42
pNPY 25–36	8.5	0.03
pNPY 26–36	8.3	0.16
p[Leu31, Pro34] NPY	10.1	0.05
p[D-Trp32] NPY	7.3	0.41
bPP	9.5	0.22
BIIE0246	< 6.0	
BIBP3226	< 6.0	
CGP 71863 A	< 6.0	
SR120819A	< 6.0	

RT-PCR with primers flanking a 300-bp portion of the receptor gene detected transcripts in CNS, liver and gonads as well as in larval tissue, but not in muscle tissue (Fig. 4A). Total RNA from a shark, *Squalus acanthias*, liver was run in parallel as a negative control. Controls were performed as described in Materials and methods; no contamination was detected. A Southern blot of the same gel to a nylon filter probed with a lamprey receptor probe confirmed these results (Fig. 4B).

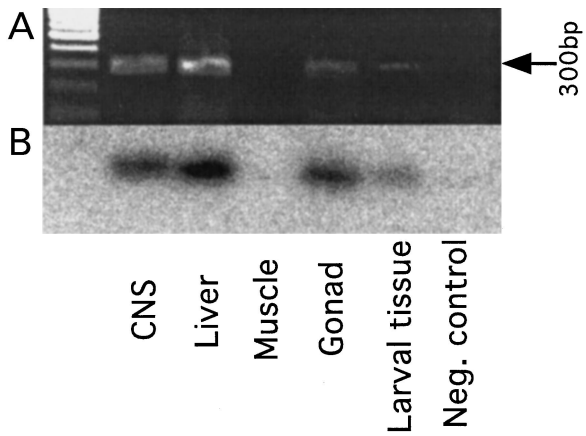

Fig. 4. RT-PCR analysis of gene expression in lamprey tissues. (A) RT-PCR using total RNA from lamprey CNS, liver, muscle, gonad and whole larval tissues. The primer pair generates a 300-bp product. The PCR reactions were performed three times with the same results. The negative control is shark liver RNA. Additional negative controls are described in Materials and methods. (B) Southern blot of the agarose gel to a nylon filter subsequently probed at high stringency with a radioactivley labeled lamprey NPY receptor probe. No additional bands were visible in comparison to the agarose gel in Fig. 4A.

DISCUSSION

The NPY receptors were first identified in mammals and for this reason the mammalian receptors have been used to define the subtypes. The lamprey receptor described here is from the class of chordates most distantly related to the mammals of which NPY receptors have been studied. We chose to clone NPY receptors in the lamprey for several reasons. First of all we wished to elucidate the evolution of the NPY receptor family as information on the time points for the gene duplications will hopefully provide a clearer understanding of the functions of the NPY system. Furthermore, sequence comparisons will help to elucidate evolutionary changes in the peptide–receptor interactions. The lampreys are a crucial class to study as they diverged from other vertebrates prior to a proposed large genome duplication event in the gnathostome lineage [36–38]. Secondly, the European river lamprey, *L. fluviatilis*, has already been well established as a model organism for neurobiological purposes [25].

The lamprey Y receptor (Fig. 1) exhibits a higher degree of identity to all of the Y1 subfamily receptors than to Y2 or Y5. Both maximum parsimony and neighbor-joining methods of tree construction place the lamprey sequence within the Y1 subfamily together with the Y4 and teleost sequences. This suggests that the lamprey receptor is neither a pro-orthologue of the entire Y1 subfamily, nor a pro-orthologue of the Y1 and y6 genes. The analysis suggests instead that the lamprey Y receptor belongs in the clade containing Y4, Ya, Yb and Yc.

It is difficult to define exactly which of these subtypes the lamprey Y receptor represents. This is not surprising given the large evolutionary distance between the lamprey and the gnathostomes, and the assumed short time interval between the divergence of these animal groups and the postulated gene duplication events. A maximum parsimony bootstrap consensus tree (Fig. 2A) places the lamprey sequence basal to all Y4/a, Yb and Yc sequences. Also, a neighbor-joining tree (data not shown) generated with the same alignment as above exhibits topology identical to that of tree A. The shortest single tree obtained with maximum parsimony analysis suggests instead that the lamprey Y receptor may be an orthologue of the Ya and Y4 ancestor (Fig. 2B). One of the three shortest trees generated by maximum parsimony does place the lamprey sequence at the base of both the Y4/Ya and Yb/Yc branches, just as the maximum parsimony bootstrap consensus and the neighbor joining trees, and is only 0.28% longer than the shortest tree. A comparison of these two trees reveals that only two unambiguous amino-acid replacements supports tree B as the shorter (Lys232 and Pro235 in the lamprey sequence). One unambiguous replacement is unique to the 'next shortest' tree (with the same topology as tree A) compared tree B. In other words, one of two possible unambiguous amino-acid replacements makes the tree B shorter than the next shortest tree. Both of these residues are at highly variable positions with three different residues represented among the other Y1 subfamily members (Fig. 1). Also, most available molecular data indicate an additional large-scale duplication in the gnathostome ancestor [37–41] as compared to the lamprey, which supports the topology of tree A in Fig. 2A. A majority of our phylogentic analyses as well as data from other gene

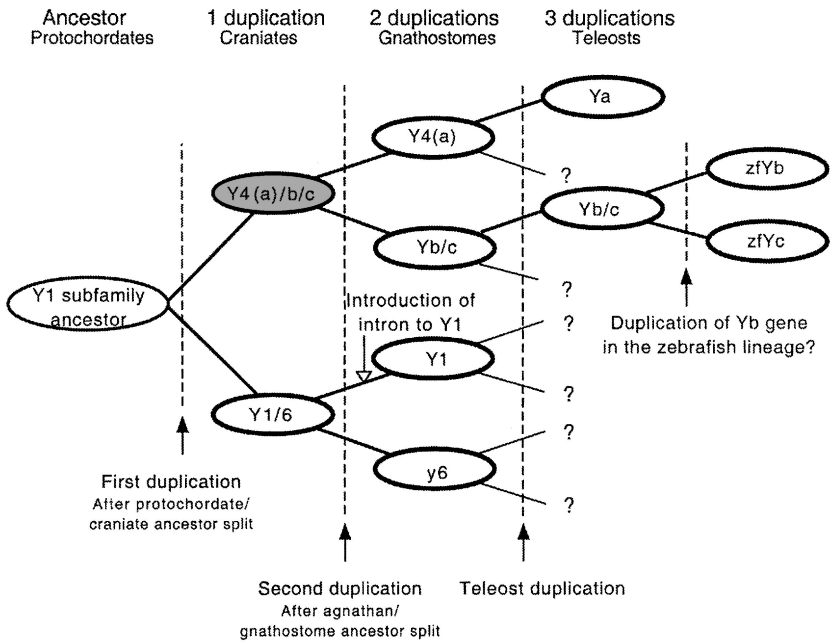

Fig. 5. Hypothetical scheme for the evolution of the NPY Y1 receptor subfamily. The possible position of the described lamprey receptor is marked by the shaded oval. Although the cloning of further NPY receptors will be necessary to confirm this, the scheme is the most parsimonious explanation consistent with current data from the Y1 subfamily phylogenetic trees as well as chromosomal duplications inferred from the linkage of several other gene families [11].

families therefore suggest that the lamprey receptor represents an ancestral orthologue to both Y4/a and Yb/c branches. Hopefully the isolation of more lamprey as well as protochordate NPY receptor genes will assist in more definitive resolution of the position of the lamprey Y receptor in the tree. While this work provides no direct evidence for the preagnathan duplication, previous work, primarily on HOX gene clusters, supports a one-to-four duplication scheme [21,42,43]. Such a scheme is proposed for the NPY receptor family in Fig. 5 and takes into consideration both the sequence analyses shown in Fig. 2 and the chromosome duplication data [11,21,22].

Although the definite position of the lamprey sequence remains unresolved, the inclusion of the sequence in phylogenetic analyses appears to have assisted in defining the evolutionary positions of other sequences. The trees indicate that the Ya receptor could have a common origin with the tetrapod Y4 subtype, or possibly even be the teleost orthologue of Y4. A recently cloned receptor gene in the shark, *Squalus acanthias* (E. Salaneck, E. T. Larson, D. Ardell and D. Carhammar, unpublished data) appears to be a Y4 receptor gene when included in the phylogenetic analysis (Fig. 2). Also, considering that Y4 in tetrapods functions as the PP receptor and that PP does not appear to exist in nontetrapods, large differences in receptor function and sequence could result when comparing fish and mammal orthologues, which could further explain the difficulties in resolving the relationship of the Ya and Y4 subtypes.

Also the positions of the *Yb* and *Yc* genes become more clear after inclusion of the lamprey sequence. Their positions would suggest that they represent the fourth *Y1* duplicate that would be expected to exist after two rounds of genome doubling, although this fourth gene has not yet been found in tetrapods (Figs 2 and 5). A subsequent duplication in the zebrafish lineage after the split from the cod lineage could then explain the existence of the two closely related zebrafish genes *Yb* and *Yc*. This event is probably more recent than the tetraploidization proposed to have occurred early in the telost lineage [8]. Recent comparison of zebrafish and tetrapod gene maps revealed that ≈ 20% of the duplicated gene pairs seem to be retained in the zebrafish after the additional tetraploidization [18]. This could explain why no additional *NPY*, *PYY* or *NPY* receptor genes have been found in the zebrafish that can be directly associated with this tetraploidization event, although additional receptor genes should probably be expected (Fig. 5). Considering the extent to which we now understand the relationships of the teleost receptors, a revision of the nomenclature would seem appropriate, where the lowercase letters for at least Yb and Yc are replaced by numbers. The Ya receptor may require further studies before deciding whether it should be called Y4 or given a separate number.

Despite the large evolutionary distance, porcine (p) PYY was found to work well as a radioligand after functional expression of the lamprey Y receptor in a human cell line. Indeed, pPYY bound with an affinity in the picomolar range. All three of the endogenous lamprey peptides bound with even higher affinities with binding constants in the low picomolar range. Thus, all three peptides exhibit sufficiently high affinities to be physiological ligands *in vivo*.

Several other NPY family peptides, truncated peptides and modified peptides were tested in competition experiments and exhibited binding to the lamprey Y receptor. Remarkably, several peptide variants commonly used to differentiate between mammalian subtypes bound to the lamprey receptor. The truncated analogs NPY13-36 and shorter variants, which have until now shown binding only

to the Y2 subtype and zebrafish Ya [44], unexpectedly bound to the lamprey Y receptor. Porcine [Leu31, Pro34]NPY, a modified NPY peptide with greater structural similarity to PP [45], that binds to all Y1 subfamily receptors as well as Y5, also bound to the lamprey Y receptor. Also p[D-Trp32]NPY, a modified NPY peptide found to be a Y5-selective ligand [46], bound to the lamprey Y receptor, albeit with rather low affinity. Despite this indiscriminate binding with regard to modified and truncated peptides, none of the nonpeptidergic NPY receptor ligands showed binding to the lamprey receptor. Considering the evolutionary distance between the mammalian receptors to which these antagonists were developed and the lamprey receptor, this cannot be considered surprising. Even the chicken Y1 and Y2 receptors do not exhibit binding to specific antagonists developed to the orthologous subtypes in mammals [47] (S.K.S. Holmberg & D. Larhammar, unpublished data). In the light of these findings it would be expected that these nonpeptidergic ligands are unlikely to bind Y receptors that are evolutionarily more distant than the avian lineage.

The broad pharmacological profile of the lamprey receptor is reminiscent of the zebrafish Ya receptor, but is distinct from Yb and Yc, supporting a closer relationship with the Y4/Ya lineage as in tree B (Fig. 2). Mammalian Y4 has a more narrow profile favoring PP among the native ligands, but its preference for PP is clearly a secondary event as Y4 is evolutionarily older than PP and must originally have bound NPY and/or PYY [10].

The analysis of mRNA expression performed by RT-PCR indicated the presence of transcripts in several of the tissues examined. The CNS expression of the lamprey receptor, along with earlier studies revealing high expression levels of NPY and PYY in the lamprey CNS [4], clearly motivates further investigation of the possible neuronal functions of the lamprey receptor. The previous description of NPY effects in the lamprey spinal cord [29] encourages studies of the receptor in this context. The expression pattern also suggests that the lamprey receptor could have a role in peripheral functions of the NPY-family peptides, such as regulation of gastrointestinal tract endocrine functions and blood pressure.

In summary, we have isolated an NPY receptor from the agnathan *L. fluviatilis*. This is the first NPY receptor to be cloned from an agnathan, contributing information from a key lineage in evolution and an interesting model organism in neuroscience, and further clarifying the origin of the large family of NPY receptors. Although the exact evolutionary position of the lamprey receptor cannot be definitely assigned with the presently available sequences, it appears likely that the lamprey gene could be a pro-orthologue of Y4, Ya and Yb/c. Inclusion of the lamprey sequence in phylogenetic trees has clarified the position of other Y1 subfamily genes. Pharmacological analysis demonstrates that the receptor is functional and all three known endogenous lamprey peptides bind with picomolar affinities. The indiscriminate binding profile, resembling the zebrafish Ya receptor, adds further support for a close relationship with the Y4/Ya lineage. The widespread expression pattern suggests important and/or diverse functions for the receptor. It will be of great interest to isolate a possible additional Y1 subfamily receptor in the lamprey, expected to be more similar to Y1/y6, hopefully further clarifying the evolution of the Y1 subfamily.

ACKNOWLEDGEMENTS

The authors thank C. Bergqvist for expert technical assistance, F. Hallböök, Uppsala University, Sweden, for supplying lamprey RNA preparations, J.S. Albert, University of Florida, Gainsville, USA for valuable advice on the phylogenetic analyses, C. Burgtorf, RZ/PD Berlin, Germany for supplying the lamprey cosmid library, K. Rudolf and H. Wieland, Boeringer Ingelheim KG, Biberach, Germany, for providing BIBP3226 and BIIE0246, J. Wikberg, Uppsala University, Sweden for providing CGP 71863A and C. Serradeil-Le Gal, Sanofi, Toulouse, France for providing SR120819A. This work was supported by the Swedish Natural Science Research Council and the National Science Foundation.

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