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Cloning and pharmacological characterization of the neuropeptide Y receptor Y5 in the sea lamprey, *Petromyzon marinus*

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ABSTRACT

The neuropeptide Y system is known to have expanded in early vertebrate evolution. Three neuropeptide Y receptors have been proposed to have existed before the two basal vertebrate tetraploidizations, namely a Y1-like, a Y2-like, and a Y5-like receptor, with their genes in the same chromosomal region. Previously we have described a Y1-subfamily and a Y2-subfamily receptor in the river lamprey, *Lampetra fluviatilis*. Here we report the identification of a Y5 receptor in the genome of the sea lamprey, *Petromyzon marinus*. In phylogenetic analyses, the Y5 receptor clusters together with gnathostome Y5 receptors with high bootstrap value and shares the long intracellular loop 3. This lamprey receptor has an even longer loop 3 than the gnathostome Y5 receptors described so far, with the expansion of amino acid repeats. Functional expression in a human cell line, co-transfected with a modified human G-protein, resulted in inositol phosphate turnover in response to the three lamprey NPY-family peptides NPY, PYY and PMY at nanomolar concentrations. Our results confirm that the Y1–Y2–Y5 receptor gene triplet arose before the cyclostome-gnathostome divergence. However, it is not clear from the NPY receptors whether cyclostomes diverged from the gnathostome lineage after the first or the second tetraploidization. Duplicates resulting from the tetraploidizations exist for both Y1 and Y2 in gnathostomes, but only a single copy of Y5 has survived in all vertebrates characterized to date, making the physiological roles of Y5 interesting to explore.

1. Introduction

The NPY (neuropeptide Y) system has attracted considerable attention due to its role in the regulation of appetite and energy balance, but also in several other biological contexts or diseases, including but not limited to blood pressure, depression, pain, cancer and bone formation [25,29,49]. The physiological functions of the NPY system are exerted by the binding of NPY-family peptides to several receptor subtypes that have different expression patterns [18,34,47]. The roles of the NPY system in appetite regulation are well characterized in mammals: NPY binds to receptors Y1 and Y5 in the hypothalamus to stimulate appetite, whereas the hormones PYY and PP have the opposite effect by binding to Y2 and Y4, respectively, in the basal hypothalamus, the vagus nerve and the brainstem [44,49]. Some functional studies of the NPY system have also been performed in other vertebrate classes like birds, reptiles, bony fish and lampreys, see for instance [3,6,30,33,35], but the information about these lineages is still limited. For detailed characterization of the biological functions in different species, evolutionary studies and identification of the individual NPY-family peptides and their receptors is required. We have therefore identified the genes, synthesized the peptides and cloned and characterized the receptors from a broad range of vertebrates [4,8–11,20–22,26,39,42,43,45,47].

The number of peptides and receptors of the NPY family has expanded through both local gene duplications and genome duplications which are two important mechanisms for the emergence of new genes and gene functions [18,24,46]. NPY and PYY have been identified in all major vertebrate lineages investigated. The tetrapod-specific pancreatic polypeptide (PP) has been confirmed to be a local duplicate of the PYY gene [12,17]. In teleost fishes, an extra gene copy for both NPY and PYY was generated [46] during the teleost-specific tetraploidization [5,13]: two copies of NPY, named NPYa and NPYb, have been identified in several species of teleost fishes, although zebrafish seems to have lost NPYb, and the so-called fish-specific peptide PY has been confirmed to be a duplicate

Abbreviations: NPY, neuropeptide Y; PYY, peptide YY; PP, pancreatic polypeptide; PMY, peptide MY; 2R, two rounds of genome doubling; 3R, third rounds of genome doubling; TM, transmembrane region; IP assay, inositol phosphate assay.

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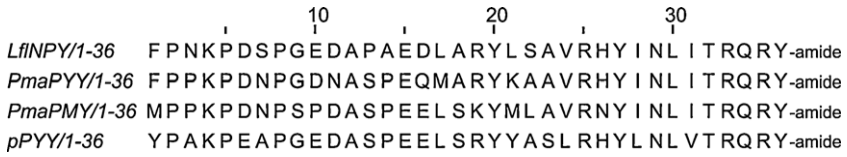

Fig. 1. Peptides used for functional assay. Four peptides were used for IP functional assay: *L. fluviatilis* NPY (LflNPY), *P. marinus* PYY (PmaPYY) and PMY (PmaPMY) and pig PYY (pPYY). LflNPY was used instead of PmaNPY as they differ by a only single conservative replacement: D16 is an E in PmaNPY.

of PYY, hence renamed to PYYb [46]. In the river lamprey *Lampetra fluviatilis*, three NPY-family peptides, NPY, PYY and PMY have been identified [40,48]. The sea lamprey *Petromyzon marinus* peptides have been isolated and PYY has the same sequence as in the river lamprey whereas NPY and PMY differ at a single position between the two species [7,28]. The evolutionary relationship between PYY and PMY in the lamprey lineage has not yet been clarified [46].

It has been proposed that three ancestral neuropeptide Y receptor genes [18] existed before the two basal vertebrate tetraploidizations, called 2R for two rounds of genome doubling [32,38]. This ancestral triplet was generated by local duplications of a single ancestral NPY receptor gene, and resulted in a Y1-like, a Y2-like and a Y5-like gene. A repertoire of seven receptor genes was present in the gnathostome ancestor (after 2R), belonging to three subfamilies based on phylogenetic analyses, Y1-like (Y1, Y4, Y6, Y8), Y2-like (Y2, Y7) and Y5-like (only Y5) [18,23,24]. Through lineage-specific deletions, local duplication and the teleost fish-specific third round genome doubling (3R), different numbers of receptors, from 4 to 7, are now maintained in mammalian, bird, amphibian and teleost fish lineages [4,8–11,20–22,26,39,42,43,45,47]. Except for the euteleost fish lineage, the Y5 gene has been cloned or identified in all these lineages, including basal ray-finned fishes as well as in the lobe-finned fish *Latimeria chalumnae* and the cartilaginous fish *Callorhinchus milii* [22,24,43]. Interestingly, the number of receptors in the Y1 and Y2 subfamilies increased during vertebrate evolution, but Y5 is the only member belonging to the Y5 subfamily. A Y1-subfamily [41] and a Y2/Y7-subfamily [24] receptor have been identified in *L. fluviatilis*, and partial sequences for a Y5-like [24] and another Y1-subfamily gene (unpublished) have also been found in this species. Lampreys constitute a highly interesting and important vertebrate lineage because they diverged from the lineage leading to gnathostomes around the time for the second vertebrate tetraploidization. Whether lampreys diverged after the first or the second tetraploidization is still not clear [16].

Here we report the complete Y5 sequence of *P. marinus* and functional studies *in vitro* with the three lamprey peptides, thereby confirming the previously proposed evolutionary scenario for the vertebrate NPY-family receptors.

2. Materials and methods

2.1. P. marinus Y5 sequence identification and analysis

The nucleotide and amino acid sequences of the putative *P. marinus* Y5 (PmaY5) was identified on the scaffold25143 in the genome assembly PMAR3 (http://genome.wustl.edu/pub/organism/Other_Vertebrates/Petromyzon_marinus/assembly/Petromyzon_marinus-3.0/output/) by TBLASTN search using human NPY Y5 as a template.

2.1.1. Phylogenetic analyses

The identified PmaY5 amino acid sequence was aligned with other vertebrate NPY receptor sequences and human somatostatin receptor 1 using ClustalW 2.012 with standard settings (Gonnet weight matrix, gap opening penalty 10.0 and gap extension penalty 0.20). The alignment included sequences from following species; Pma, *Petromyzon marinus*, Hsa *Homo sapiens*, Ssc *Sus scrofa*, Rno *Rattus norvegicus*, Gga *Gallus gallus*, Cmi *Callorhinchus milii*, Sac *Squalus acanthias*, Dre *Danio rerio*, Ocu *Oryctolagus cuniculus*, Lch *Latimeria chalumnae*, Tru *Takifugu rubripes* and Lfl *Lampetra fluviatilis*. Accession numbers; HsaY1 – NM_000909, SscY1 – AF106081, RnoY1 – NM_001013032, GgaY1 – NM_001031535, CmiY1 – EU637847, SacY1 – AH012614, DreY1 – EU046342, OcuY6 – D86521, GgaY6 – NM_001044687, LchY6 – ABI94073, SacY6 – AY177271, CmiY6 – EU637851, TruY8a – EU104004, DreY8a – NM_131437, DreY8b – AF030245, TruY8b – EU104005, CmiY8 – EU637853, HsaY4 – NM_005972, SscY4 – AB021678, RnoY4 – U84245, GgaY4 – AF410853, SacY4 – AY177270, CmiY4 – EU637849, DreY4 – AF037400, TruY4 – EU104002, LflY1-like – AAL66410, PmaY1-like – ENSPMAG00000009963, HsaY5 – NM_006174, RnoY5 – NM_012869, SscY5 – AF106083, GgaY5 – NM_001031130, LchY5 – ABI94072, CmiY5 – EU637850, DreY2 – XP_001343301, TruY2 – EU104001, SscY2 – AF106082, HsaY2 – NM_000910, RnoY2 – NM_023968, GgaY2 – NM_001031128, CmiY2 – EU637848, LflY2-Y7-like – EU743622, GgaY7 – NP_001032913, CmiY7 – EU637852, TruY7 – EU104003, DreY7 – AY585098, HsaSSTR1 – NP_001040. The alignment was cut to remove N-terminal and C-terminal parts, resulting in a final alignment spanning from the start of TM1 (Transmembrane region 1) to the end of TM7. A phylogenetic tree was constructed using the neighbor-joining (NJ) method with 1000 bootstrap replicates in ClustalX 2.012 [19].

2.1.2. Alignment of Y5 receptors

The identified sea lamprey amino acid sequence was aligned with other Y5 sequences using ClustalW 2.012 with standard settings (Gonnet weight matrix, gap opening penalty 10.0 and gap extension penalty 0.20). Amino acid sequences with these accession numbers were retrieved using the NCBI database. HsaY5 – NM_006174, RnoY5 – NM_012869, SscY5 – AF106083, GgaY5 – NM_001031130, StrY5 – NP_001072244, LchY5 – ABI94072, CmiY5 – EU637850, ObiY5 – EU46356, AbaY5 – EU046345, PseY5 – EU046360, SacY5 – EU046362. Abbreviations as above but the alignment also includes also these species; Str, *Silurana tropicalis*, Obi, *Osteoglossum bichirossum*, Aba, *Acipenser baerii*, Pse, *Polypterus senegalus*.

2.2. Primer design and coding region amplification

Primers for PCR amplification were designed based on PmaY5 sequence, a Kozak consensus sequence for initiation of translation was added to the 5' end of forward primer: 5'-CCT ACC ATG GCC CTC TCC ACG-3'. A reverse primer was designed where the Stop codon was replaced to give a continuous open reading frame with GFP: 5'-CCG CCC GTG ACC CAG GCA G-3'. The GC-rich PCR system, DNTPPack reagent (Roche) was used to amplify the genomic DNA using the program: 95 °C for 5 min and following 30 cycles of 30 s at 95 °C, 30 s at 62.5 °C, and 90 s at 72 °C, followed by 7 min at 72 °C. The PCR product was purified using the MinElute Gel Extraction Kit (Qiagen) according to product instructions.