1. Introduction

Neuropeptide Y receptors are G-protein coupled receptors involved in numerous physiological processes. In mammals these include appetite regulation, biological rhythms, anxiety, pain, bone formation, and release of pituitary hormones [40,50]. We have previously proposed that the ancestral jawed vertebrate had seven NPY receptors, Y1, Y2, Y4, Y5, Y6, Y7 and Y8 which can be divided into three distinct subfamilies, the Y1 subfamily (Y1, Y4, Y6 and Y8), the Y2 subfamily (Y2 and Y7) and the single gene subfamily Y5 [26,27]. All of these receptors are present in a cartilaginous fish, the elephant shark (Callorhinus millii) [27]. In mammals, five of these receptor genes are still present, one of which, Y6, is a pseudogene in several mammals including human. The Y6 gene in chicken is fully functional [5]. A few NPY receptors have been previously reported for frogs after cloning, namely Y1 in the African clawed frog Xenopus laevis [4], and Y7 in the frog Pelophylax esculentus (previously called Rana esculenta) and in the western clawed frog Silurana (Xenopus) tropicalis [16]. The decision whether to describe the western clawed frog as S. tropicalis or Xenopus tropicalis remains controversial. Cladistic analysis based upon nucleotide sequences of mitochondrial genes strongly supports the monophyly of Xenopus + Silurana which are united in the Xenopodinae but the use of two genera “underscores trenchant biological and historical differences between the two clades” [15].

The ligands are members of the NPY family of peptides, which also includes PYY (peptide YY) and PP (pancreatic polypeptide) in tetrapods. The peptide genes code for pre-propeptides that are processed by specific peptidases to generate the active peptides. All of the peptide genes code for pre-propeptides that are processed by specific peptidases to generate the active peptides. All of
the peptides examined in the NPY family consist of 36 amino acid residues, except for chicken PYY and Burmese python PP that consist of 37 and 35 amino acids, respectively [9,49]. NPY in mammals is mainly expressed in the brain and is one of the most orexigenic peptides known. It has been shown to induce feeding in different species including Siberian hamster, guinea pig, the frog X. laevis, and goldfish [11,28,29,38]. PYY and PP are mainly expressed in endocrine cells in the gastrointestinal tract in mammals. PYY in its truncated form PYY3-36 and PP have important roles as inhibitors of feeding in mammals [2,3,24,39]. All three peptides also inhibit several other roles in neuronal, cardiovascular or gastrointestinal contexts [40,50]. Endogenous truncated PYY is not known outside mammals where the peptide is cleaved by dipeptidyl peptidase IV [36]. This peptidase cannot cleave between two proline residues and all known non-mammalian PYY sequences have the amino acids Tyr-Pro-Pro, suggesting resistance to this type of cleavage.

A PYY peptide was isolated from the frog Phyllomedusa bicolor in the 1990s [37]. It was named skin PYY due to its main location, but alignment with PYY from other species did not allow functional expression. We therefore turned to the genome of S. tropicalis [21] and were able to identify three receptor genes orthologous to those of P. esculentus, i.e., Y1, Y5, and Y7, plus three additional receptor genes, Y2, Y4 and Y8. Furthermore, we have identified and synthesized the three endogenous NPY-family peptides NPY, PYY and PP. We report here an initial characterization of the NPY system of S. tropicalis.

2. Materials and methods

2.1. Identification and phylogenetic analysis of S. tropicalis peptides and receptors

The NPY-family peptide and receptor gene sequences were identified by BLAST searches [1] in the genome of S. tropicalis in the Ensembl database (www.ensembl.org) using human and chicken peptide and receptor sequences as queries. The identified receptor DNA sequences were retrieved by PCR and sequenced (see below). An alignment was constructed using the Windows version of Clustal X 1.81 [45] using standard settings (Gonnet weight matrix, gap opening penalty 10.0 and gap extension penalty 0.20). The alignment included other vertebrate NPY receptor sequences and human somatostatin receptor 1 (accession numbers are available in Supplementary Table 1). The alignment was cut in order to remove poorly aligned sequences in the N- and C-terminal regions, resulting in a final alignment spanning from the start of transmembrane region 1 (TM1) to the end of TM7. A neighbor joining tree was constructed in Clustal X 1.81 [45] with 1000 bootstrap replicas and the somatostatin receptor 1 was used as outgroup. Phylogenetic maximum likelihood (PhyML) trees were constructed using the online execution of the PhyML 3.0 algorithm available at http://www.atgc-montpellier.fr/phyml/ [20]. The analysis was made using the JTT model of amino acid substitution: ProtTest 2.4 was used to select the model. The BIONJ was selected as algorithm to estimate the random starting tree. The number of substitution rate categories was set to 8 and amino acid substitution model parameters were estimated from the dataset. Both the NNi and SPR tree improvement methods were used together with tree topology and branch length optimization.

2.2. Cloning of partial NPY receptor gene sequences in P. esculentus

Genomic DNA for the frog Pelophylax esculentus was kindly provided by Dr. Isabelle Lihrmann, Université de Rouen, France. This species was previously called R. esculenta and is considered to be a hybrid between the pool frog Pelophylax lessonae (synonyms Rana lessonae, Rana esculenta lessonae) and the marsh frog Pelophylax ridibundus (Rana ridibunda). Degenerate PCR with primers based on gene sequences for NPY-family receptors from several mammals and chicken was run on P. esculentus genomic DNA using the stuffel Taq polymerase (Applied Biosystems) and the following PCR conditions: 120 s at 95 °C for one cycle, thereafter 30 s at 95 °C, touch-down from 55 to 42 °C for 45 s, and 60 s at 72 °C for 20 cycles, followed by 20 cycles with 30 s at 95 °C, 45 s at 42 °C and 60 s at 72 °C finishing with 5 min at 72 °C. The PCR products were cloned using the TOPO-cloning kit (Invitrogen), sequenced using the BigDye V3 terminator sequencing kit (Applied Biosystems) and the extension products were analyzed on an ABI 310 automatic sequencer. The sequence was compared to the GenBank database using the On-Line BLASTX program.

2.3. Sequencing and expression constructs for S. tropicalis receptors

Sequences corresponding to the entire coding regions of six S. tropicalis NPY receptors were amplified from genomic DNA. The PCR products were directionally cloned into the pcDNA3 vector at the XhoI and HindIII sites and transformed into chemically competent DH5α E. coli cells (Invitrogen). The insert was sequenced using receptor-specific and T7 vector-specific primers and BigDye V3 terminator sequencing kit (Applied Biosystems). The product was analyzed on an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems) automatic sequencer to confirm sequence. Sequences were analyzed using the lasergene software (DNASTAR).

2.4. Transfection

The human embryonic kidney-derived cell line HEK-293 was used to express the frog receptors for binding assays. Cells were grown to 95% confluency and transfected with 12–24 μg plasmid diluted in 750 μl OPTI-MEM® mixed with 30 μl Lipofectamine 2000 according to the manufacturer’s recommendations (Invitrogen). Transfected cells were grown in DMEM/F12 growth medium (Gibco BRL/Invitrogen) at 37 °C (5% CO2, 95% air) for 24 h. The cells were harvested in 25 mM HEPES buffer containing 2.5 mM CaCl2, 1 mM MgCl2 and stored at −80 °C.

X. laevis cell-lines XTC-Z, XL-58 and A6 were also used for transfection. These were grown in amphibian strength L-15 medium at 25 °C (5% CO2, 95% air). The amphibian strength L-15 medium was made from Leibovitz L-15 medium with l-glutamine (Invitrogen) considering the frog body fluids osmolarity and added with 10% (v/v) fetal calf serum (Gibco) and penicillin–streptomycin (Gibco).

2.5. Peptide synthesis and purification

The S. tropicalis carboxyterminally amidated peptides NPY, PYY, and PP were supplied in crude form by GL Biochem. Ltd. (Shanghai, China) and were purified to near homogeneity (>98% purity) by reversed-phase HPLC on a (2.2 x 25 cm) Vydac 218TP1022 (C-18) column. The concentration of acetonitrile in the eluting solvent was raised from 21% to 56% over 60 min and the flow rate was

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6 ml/min. The structures of the peptides were confirmed by electrospray mass spectrometry.

2.6. Binding assay

Thawed aliquots of transfected cells were diluted in the binding buffer containing 2 g/l Bacitracin and thereafter homogenized. All binding experiments were performed in a total volume of 100 μl, with 125I-labeled pPYY (porcine PYY) as radioligand and with an incubation time of 3 h at room temperature. Saturation experiments were performed with 12 different concentrations of radioligand ranging from 8 to 1000 pM. Competition binding experiments were performed with the synthesized amidated native peptides NPY, PYY and PP (Fig. 1) as competing ligands which were diluted to 12 different concentrations between 10⁻⁶ and 10⁻¹¹ M. Protein concentrations were measured with the Bio-Rad Protein Assay (Bio-Rad) with bovine serum albumin as standard. The incubation was terminated by rapid filtration through GF/C filters presoaked in 0.3% polyethyleneimine using a TOMTEC cell harvester. The filters were washed with ice-cold 50 mM Tris—HCl (pH 7.4) and dried at 50 °C for 30 min and MeltiLex A (Perkin–Elmer) melt-on scintillator sheets were melted onto the dried filters. Radioactivity was measured using a Wallac 1450 Betaplate counter. Results were analyzed using Prism 4.0 software package (GraphPad). Each experiment was performed at least three times, each time with duplicate samples. The pKᵢ values were compared to each other using one-way ANOVA followed by Tukey–Kramer Multiple Comparison Test (GraphPad).

2.7. RNA isolation and cDNA synthesis

Adult *S. tropicalis* frogs were purchased from NASCO and maintained in the laboratory in well water (25–26 °C) under a 12L:12D photoperiod. Frogs were fed frog brittle (NASCO). All procedures involving animals were conducted in accordance with the guidelines of the University Committee on the Care and Use of Animals of the University of Michigan (animal care and use protocol #09860). RNA was extracted from male and female frog tissues using the Trizol Reagent (Invitrogen) following the manufacturer's instructions. cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen) and random hexamers as primers following the manufacturer's recommendations.

2.8. Primer design and quantitative real-time PCR

Primers for the quantitative PCR were designed using the Beacon Designer v4.0 (Premier Biosoft). The annealing temperature was optimized using PCR on genomic DNA. The real-time PCR reactions were performed in a MyIQ thermal cycler (Bio-Rad Laboratories, Sweden) under the following conditions: 95 °C for 4 min, followed by 50 cycles at 95 °C for 15 s, 59 °C for 30 s and 72 °C for 30 s. This was followed by 84 cycles at 55 °C for 10 s, increased by 0.5 °C per cycle. Each reaction had a total volume of 20 μl and contained cDNA synthesized from 5 ng of total RNA, 5 pM of each primer, 8 mM MgCl₂, 0.2 mM dNTP, SYBR Green (1:50,000), 0.02 μl/μl Taq DNA polymerase and buffer (without MgCl₂) (Invitrogen). Absence of genomic DNA in the cDNA was controlled for by PCR using a primer pair designed to span a small intron.

2.9. Data analysis and relative expression calculations

Gene expression data were analyzed and threshold cycle (Ct) values derived using MyIQ software v 1.04 (Bio-Rad Laboratories). Differences in the primer efficiency was corrected for using LinRegPCR [42], and Grubbs test for outliers (GraphPad) was used to calculate the mean primer efficiency for each primer pair and detection and exclusion of outliers. The Ct values were transformed into quantities using the delta Ct method [30] and the highest expression was set to 1. geNorm [47] was used to identify the most stable housekeeping gene which thereafter was used to normalize expression due to differences in cDNA concentration between samples.

3. Results

3.1. Identification and phylogenetic analysis

Using BLAST searches with the human and chicken receptor amino acid sequences as queries it was possible to identify the genes encoding three peptides and six receptors in the genome database of *S. tropicalis*. The three peptide genes were identified.
as encoding NPY, PYY and PP, respectively, by comparisons with NPY-family peptide sequences previously determined in the tetraploid frog *X. laevis* [18,23,46] as shown in Fig. 1. Two distinct *X. laevis* sequences have been reported for each of the three peptides. Although it has not been formally demonstrated the published *X. laevis* sequences correspond to two gene copies (they might be allelic), it is reasonable to assume so. The *S. tropicalis* sequences conform well to the *X. laevis* sequences. The *S. tropicalis* NPY sequence is identical to one of the *X. laevis* sequences, the other differs at one position. The *S. tropicalis* PP sequence likewise is identical to one of the *X. laevis* sequences and has one difference to the other one. The *X. laevis* PYY sequences differ more from the *S. tropicalis* sequence, namely at two and four positions, respectively. For pancreatic polypeptide, we could not find the exon that encodes the last two amino acids of the mature peptide, the subsequent glycine forming the carboxyterminal amide, and the two basic amino acids for proteolytic cleavage from the precursor. The peptide was therefore synthesized based on the last two residues (plus the amide) found in pancreatic polypeptide in other amphibians, i.e., RFamide.

Two of the receptors were identified as Y1 and Y5 in our phylogenetic analyses (Fig. 2 and Supplementary Fig. 1) and are located next to each other on the same scaffold. In human the Y5 gene is located 20 kb from the Y1 gene in a head-to-head orientation, and in chicken the distance is 18 kb. The distance between Y5 and Y1 in the frog is 86 kb. It should be noted that the Y5 gene in the database, although correctly identified, has been erroneously predicted to contain several introns. Our sequence analysis shows that the frog Y5 gene lacks introns like the Y5 gene in other species. The Y1 gene has an intron of 92 nucleotides, similar in size to the corresponding intron in chicken which is 121 nucleotides and in human 97 nucleotides. One gene was identified as a Y2 receptor in the *S. tropicalis* genome database, but our phylogenetic analysis shows that it is a Y7 receptor (see Fig. 2 and Supplementary Fig. 1). The Y7 receptor is not present in mammals and this might explain the misidentification in the database. The true frog Y2 receptor ortholog is located on a different scaffold.

**3.2. Pharmacological characterization**

All six *S. tropicalis* receptor genes were inserted into expression vectors for functional expression. The receptors Y5, Y7 and Y8 gave good expression that allowed binding studies for measuring affinities. Table 1 shows the affinities for the three endogenous ligands in competition with iodinated porcine PYY. Figs. 4 and 5 show representative binding curves for each receptor. For all three receptors, PYY had significantly (*p < 0.05*) higher affinity with *K_i* values.
Fig. 3. Amino acid alignment of NPY-receptor sequences from Western clawed frog, *S. tropicalis* (Sitr); the frog *Pelophylax esculentus* (Pees); chicken, *Gallus gallus* (Gaga); and human, *Homo sapiens* (Hosa). Boxes mark the predicted transmembrane (TM) regions.
in the sub-nanomolar range, as compared to NPY and PP. NPY and PP also had high affinity for Y8, at 0.14 and 0.50 nM, respectively. For the Y5 and Y7 receptors, NPY had approximately 100-fold lower affinity than PYY, and PP more than 500-fold lower affinity. Although all six receptors were transfected, no binding to the Y1, Y2 and Y4 receptors was detected in either human HEK-293 cells or the frog cell-lines XTC-2, XL-58 and A6. Constructs with GFP-tagged receptors were made in order to allow microscopy observation of the receptors in the cells. However, fluorescence from cells transfected with the Y1, Y2 and Y4 receptors did not differ from the negative control (data not shown). The Y8 receptor was used as a positive control for GFP detection.

3.3. Expression levels for mRNA

The expression pattern of mRNA was investigated with quantitative PCR in a panel of 19 S. tropicalis organs from a male frog, plus ovaries. In general there was a similar expression pattern for the receptors with the highest mRNA expression in skin, muscle, blood and heart (Fig. 6). The only genes with detectable neuronal mRNA expression are the Y1 and Y2 receptors as well as mRNA for the peptide NPY. Unfortunately, the assay could not be performed for Y4 in the brain regions due to shortage of material. The NPY mRNA has a similar expression level in the majority of tissues. The scale of the Y-axis also gives an indication of the small differences in expression between the tissues from this gene. For the other peptides and the receptors there is approximately a 25-fold difference between the expression in the tissues with the lowest expression compared to the tissues with the highest. The mRNA of the PYY and PP peptides display almost identical expression profiles with highest expression in skin and blood (see Fig. 7).

4. Discussion

The genome of the diploid frog S. tropicalis has been sequenced [21] and we describe here three NPY-family peptides and six NPY-family receptor genes. The sequences for the three peptides NPY, PYY and PP in S. tropicalis agree well with other amphibian and tetrapod sequences. Both NPY and PP sequences are identical to one of the sequences previously reported for X. laevis and differ from the other one at only one position (Fig. 1). Surprisingly, the two published X. tropicalis PYY sequences differ from each other at as many as six positions. The single S. tropicalis PYY sequence has two and four differences to the X. laevis PYY sequences, and the most parsimonious interpretation is that the two X. tropicalis PYY sequences have acquired two and four replacements, respectively, perhaps indicating a reduced conservative selection pressure after the duplication. We synthesized each of the three S. tropicalis peptides with a carboxyterminal amide group for binding studies of the functionally expressed receptors.

Using phylogenetic analyses we were able to identify them as the six NPY-family receptor genes encoding receptor subtypes Y1, Y2, Y4, Y5, Y7 and Y8 (Fig. 2 and Supplementary Fig. 1). It
was not possible to identify any Y6 receptor in the genome database. However, we have also cloned by PCR partial sequences for Y1, Y5, Y6 and Y7 from *P. esculentus* (only a short segment corresponding to 46 amino acids was obtained for Y1). This shows that the ancestor of the amphibian lineage had the full repertoire of receptors as that inferred for the gnathostome ancestor [26,27].
although the Y6 sequence may represent a pseudogene in *P. esculentus*. One interesting observation in this context is that this amphibian has the same three peptides as other tetrapods, but has more receptor subtypes than mammals (4–5 subtypes). In the phylogenetic tree, the Y4 receptor sequences appear in two different clusters, namely amniotes and teleost fishes together with sharks. The *S tropicalis* sequence clusters together with the amniotes, albeit with low bootstrap support. The teleost receptors were identified as Y4 based upon chromosomal location [26] and their sequences cluster with the shark sequences in the tree, thereby supporting the identity of these as Y4. The Y4 sequences have an accelerated substitution rate in all lineages compared to the other receptor subtypes, which explains why Y4 in amniotes and teleosts/sharks do not cluster together. Unfortunately, the *S tropicalis* sequence is located on a small scaffold in the database and the few gene neighbors present do not clarify its identity.

The Y4 receptor has an established partnership with PP in mammals, because PP has the highest affinity for Y4 among the three endogenous peptides, while all three peptides in chicken bind with equal affinity to the chicken Y4 receptor [31,33]. Despite testing several different cell lines, including three cell lines from the closely related frog *X. laevis*, it was not possible to functionally express the *S. tropicalis* Y4 receptor. Previous attempts to express the Y1 receptor from *X. laevis* in mammalian cells were also unsuccessful [4] and the two frog species differ at only 13 amino acid positions out of 366.

The Y5 receptor in *S. tropicalis* was found to have preference for PYY whose affinity was much higher than the affinities of NPY or PP (see Table 1). This differs from the situation in both mammals [32] and chicken [22] where the three peptides have almost equal affinities. It has been found that NPY stimulates food intake partially via the Y5 receptor in the paraventricular nucleus in mammals [28,29], but the much higher affinity of PYY for Y5 in a frog may indicate a somewhat different mechanism or physiological role. The Y5 receptor arose already in the ancestor of gnathostomes and cyclostomes, as it has been identified in a lamprey [27], and therefore must originally have been a receptor for the NPY/PYY ancestral peptide. In this perspective, it is interesting to note that in *S. tropicalis*, NPY has a much reduced affinity for Y5 whereas PYY has high affinity. It will be interesting to explore if this reflects a different function for Y5 in the frog lineage as compared to the mammalian lineage.

The Y7 receptor in *S. tropicalis* has a ligand profile which is almost identical to that of the Y5 receptor. This differs from chicken receptor Y7 where NPY had a higher affinity than chicken PYY [5]. Compared to Y7 in zebrafish or rainbow trout [16,25] the most striking observation for the chicken Y7 receptor was its overall low affinity.

The *S. tropicalis* Y8 receptor stands out from Y5 and Y7 in that all three peptides have higher affinity for this receptor than for the other two. PYY had the highest affinity at 0.042 nM. Interestingly, PP has an affinity of 0.50 nM making Y8 the only receptor among these three where PP seems to have physiologically relevant affinity. It is interesting to note that Y8 is the only receptor among these that belongs to the Y1 subfamily, as does the PP-binding receptor Y4 in amniotes. The Y8 receptor is missing in all amniote genomes.
in the database and seems to have been lost in this lineage. On the other hand, the Y8 receptor is present in duplicate (Y8a and Y8b) in the teleost fishes *Tetraodon nigroviridis* and *Takifugu rubripes* [26]. However, PP is a tetrapod-specific duplicate of PYY.

The six *S. tropicalis* NPY-family receptors have a fairly similar tissue distribution as measured by quantitative PCR of mRNA (Fig. 6). The most prominent expression was observed in skin, muscle, blood and heart. The stomach always has lower expression than the small and large intestine. There is a higher expression of the Y7 receptor in the small intestine than in the large intestine, while the other receptors have a similar expression in both parts of the gastrointestinal tract. The Y5 receptor appears to have a similar expression pattern as the Y7 receptor in the small and large intestine, however the error bars are too large to draw any firm conclusions. In the nervous system, the only receptor genes with detectable mRNA expression are Y1 and Y2, in addition to the mRNA for NPY. The Y1 receptor shows expression in both telencephalon and diencephalon while only minor expression in diencephalon is detectable for the Y2 receptor. The broad expression of the NPY-family receptors in this frog is similar to that previously reported for the two cartilaginous fishes spiny dogfish [43] and elephant shark [27].

The distribution of the NPY-family peptides in a few organs from various frogs has previously been described using immunohistochemistry methods [12,13,19,35]. In mammals NPY is mainly expressed by neurons while PYY and PP are released from endocrine cells. Our quantitative PCR analyses of the three peptides in a large organ panel show that all three peptides have surprisingly broad distribution (Fig. 7). The three peptides display a similar expression pattern in non-neuronal organs with high expression in skin. Both NPY and PYY have been isolated from frog skin and it has been demonstrated that they display antimicrobial properties [14,48]. In the nervous system the expression level of NPY is highest in diencephalon, followed by telencephalon. Expression in hindbrain and the spinal cord is also detectable. Neither PYY nor PP has any expression in these tissues. High expression for all three peptides was found in small and large intestine. More detailed studies will be necessary to see whether NPY is expressed in intestinal neurons and PYY and PP in endocrine cells.

5. Conclusions

We report here that the NPY system in the frog *S. tropicalis* is comprised of three peptides and six receptors and thereby displays greater complexity than in mammals. Binding properties and anatomical distribution overall show similarities to mammals but there are also some interesting differences that warrant further studies. An intriguing question for the future is why the NPY system in mammals has lost two of the receptors still present in frogs, Y7 and Y8, and what functions those receptors perform in frogs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2012.04.027.

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