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Neuropeptide Y-family receptors Y_6 and Y_7 in chicken

Cloning, pharmacological characterization, tissue distribution and conserved synteny with human chromosome region

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The peptides of the neuropeptide Y (NPY) family exert their functions, including regulation of appetite and circadian rhythm, by binding to G-protein coupled receptors. Mammals have five subtypes, named Y_1, Y_2, Y_4, Y_5 and Y_6, and recently Y_7 has been discovered in fish and amphibians. In chicken we have previously characterized the first four subtypes and here we describe Y_6 and Y_7. The genes for Y_6 and Y_7 are located 1 megabase apart on chromosome 13, which displays conserved synteny with human chromosome 5 that harbours the Y_6 gene. The porcine PYY radioligand bound the chicken Y_6 receptor with a K_d of 0.80 ± 0.36 nM. No functional coupling was demonstrated. The Y_6 mRNA is expressed in hypothalamus, gastrointestinal tract and adipose tissue. Porcine PYY bound chicken Y_7 with a K_d of 0.14 ± 0.01 nM (mean ± SEM), whereas chicken PYY surprisingly had a much lower affinity, with a K_i of 41 nM, perhaps as a result of its additional amino acid at the N terminus. Truncated peptide fragments had greatly reduced affinity for Y_7, in agreement with its closest relative, Y_2, in chicken and fish, but in contrast to Y_2 in mammals. This suggests that in mammals Y_2 has only recently acquired the ability to bind truncated PYY. Chicken Y_7 has a much more restricted tissue distribution than other subtypes and was only detected in adrenal gland. Y_7 seems to have been lost in mammals. The physiological roles of Y_6 and Y_7 remain to be identified, but our phylogenetic and chromosomal analyses support the ancient origin of these Y receptor genes by chromosome duplications in an early (pregnathostome) vertebrate ancestor.

Neuropeptide Y (NPY) is one of the most abundantly expressed signaling peptides in the central nervous system of vertebrates. It forms a family of related peptides, usually 36 amino acids long, together with peptide YY (PYY) in vertebrates and in addition pancreatic polypeptide (PP) in tetrapods [1–4]. One of the exceptions to the 36-amino acid rule is chicken PYY (cPYY), which has an additional alanine residue at the N terminus [5]. The peptides are involved in a variety of neuronal and endocrine functions, including regulation of appetite and circadian rhythm, as well as cardiovascular, reproductive and gastrointestinal functions [6,7]. NPY is known as one of the most potent endogenous stimulators of feeding in mammals [8] and also stimulates food intake in birds [9–12]. Fasting leads to increased NPY mRNA levels in chicken

Abbreviations
CHO, Chinese hamster ovary; cNPY, chicken neuropeptide Y; cPP, chicken pancreatic polypeptide; cPYY, chicken peptide YY; Hsa, *Homo sapiens* chromosome; pNPY, porcine neuropeptide Y; PP, pancreatic polypeptide; pPYY, porcine peptide YY; PYY, peptide YY.

hypothalamus [13]. PP injected into the brain also leads to increased feeding [11,14,15], but this effect may be nonphysiological as PP has not convincingly been demonstrated to be produced within the brain. Recently, an endogenous cleavage product of PYY, fragment $PYY_{3–36}$, released from gastrointestinal endocrine cells after meals, was reported to reduce food intake in mammals [16], but this observation has been questioned in several studies and supported by only a few, as reviewed recently [17]. Moreover, PP has been reported to reduce appetite in mammals after meals [18]. These effects of endocrine $PYY_{3–36}$ and PP have not yet been investigated in chicken.

The NPY-family peptides exert their actions by binding to a family of G-protein-coupled receptors called the Y family. In mammals this family consists of subtypes named Y_1 through Y_6 [19], except that Y_3 has only been postulated from pharmacological experiments and probably does not exist as a separate gene [20,21]. The Y_1, Y_4 and Y_6 subtypes form the Y_1 subfamily, together with teleost fish Y_b [22], and they exhibit $\approx 50\%$ amino acid sequence identity to each other, while each of these is only 30% identical to the Y_2 and Y_5 subfamilies [23,24]. Subtype Y_2 forms a subfamily with the recently discovered Y_7 receptor, which has been found in zebrafish *Danio rerio* [25], rainbow trout *Oncorhynchus mykiss* [26] and two species of frogs, *Xenopus tropicalis* and the marsh frog *Rana ridibunda* [25]. These two subtypes are $\approx 50\%$ identical to each other. The Y_5 receptor, finally, is the sole member of the third subfamily. We have previously reported the cloning and pharmacological characterization of four chicken NPY (cNPY)-family receptors, namely Y_1, Y_2, Y_4 and Y_5 [27–29].

The genes for Y_1, Y_2 and Y_5 are clustered together on *Homo sapiens* chromosome 4 (Hsa4), the Y_4 gene is located on Hsa10 and the Y_6 gene is on Hsa5. These three chromosomes share members of numerous other gene families [3,23,30], supporting the idea that they all arose from a common ancestral chromosome through duplications that took place in an early gnathostome ancestor. The phylogenetic analyses show that Y_1, Y_2 and Y_5 subfamilies are very distantly related, thus the ancestral chromosome carried a representative for each of these three subfamilies before the chromosome duplications. After the duplications, some genes were lost, but interestingly the gene losses seem to differ between the vertebrate lineages. For instance, mammals have lost Y_7 and teleost fishes seem to have lost Y_1, Y_5 and Y_6 [3,23].

Appetite stimulation by NPY in mammals is mediated by receptors Y_1 and Y_5 [8,31], whereas the debated appetite reduction by $PYY_{3–36}$ has been reported to be signaled by the Y_2 receptor [16]. PP in mammals is selective for Y_4, which presumably mediates the appetite inhibition of this peptide [18], but in chicken, PYY binds to Y_4, in addition to PP [27].

The physiological role of Y_6 in mammals is unknown, and for this reason the International Union of Pharmacology (IUPHAR) receptor nomenclature committee has recommended that the mammalian receptor is written y_6 (i.e. with a small y). However, for consistency we will use the designation Y_6 for all species in this report. The Y_6 receptor seems to be functional in mouse [32,33] and rabbit [34] and the mouse receptor has been found to be functional in cAMP assays [35]. However, its pharmacological properties are uncertain because of conflicting reports [32,35]. Surprisingly, the Y_6 receptor has been found to be nonfunctional as a result of frameshift mutations in several mammals, namely human and several other primates [32,34,36], pig [37] and guinea-pig [38], and it has been lost in rat [39]. On the other hand, the gene has an intact open reading frame in a distant relative of the pig, the collared peccary [40]. As the mutations differ between the species that have an inactive Y_6 gene, it has probably been independently inactivated several times (except among primates who share the same inactivating mutations) [38]. The Y_6 gene in the shark, *Squalus acanthias*, appears to be functional [41].

Even less is known about the Y_7 gene, as it is absent in mammals. The only pharmacological information available is for the zebrafish receptor [25], which binds with subnanomolar affinity to endogenous NPY and PYY as well as to the porcine peptides. The truncated peptides $NPY_{13–36}$ and $NPY_{18–36}$ have lower affinity by orders of magnitude, which makes the zebrafish Y_7 receptor clearly different from its closest relative, Y_2, which can respond to these peptide fragments in mammals and chicken. Zebrafish Y_7 was found to be expressed in brain, eye and intestine [25].

To shed further light on receptors Y_6 and Y_7, particularly their enigmatic evolutionary histories, we report here the cloning and characterization of these receptors in chicken. This completes the initial characterization of all six NPY-family receptors identified so far in chicken.

Results

Cloning and phylogenetic analysis of chicken Y_6 and Y_7

A chicken Y_6 sequence was obtained from chicken genomic DNA by degenerate PCR and used to screen

a chicken BAC library at high stringency. Two BAC clones were isolated, one of which was sequenced with primers based on the original PCR clone and gave the remaining part of the coding region. The coding part of the Y_6 gene is contained within one exon and encodes a protein of 374 amino acids displaying the characteristics of other NPY family receptors (Fig. 1), including two well-conserved cysteines presumed to link extracellular loops 1 and 2 and two putative glycosylation sites in the N-terminal extracellular domain. The C-terminal tail contains two conserved cysteines, either or both of which may serve as palmitoylation sites to anchor the cytoplasmic tail to the inner side of the cell surface membrane. The overall identity between chicken and those mammalian Y_6 sequences that appear to be functional (mouse, rabbit and peccary) is 61–63%. These three mammalian sequences share $\approx$80% sequence identity. Nevertheless, several types of phylogenetic analyses, including the tree obtained with the Neighbor–Joining method in Fig. 2, unambiguously identify the gene as an orthologue of mammalian Y_6 (as does the conserved synteny with mammalian Y_6, see below).

The chicken Y_7 sequence was identified in the chicken genome database by BLASTX searching with the zebrafish Y_7 sequence. The full-length sequence was cloned by PCR from White Leghorn genomic DNA. The chicken Y_7 protein sequence is encoded by a single exon and encompasses 385 amino acids with conserved cysteines, as in zebrafish Y_7 as well as various Y_2 sequences, and a presumed glycosylation site in the N-terminal extracellular region (Fig. 3). Phylogenetic analyses identify the gene as most similar to Y_7 from zebrafish (65% overall identity) and frogs [25] as well as Y_7 sequences from other teleost fishes (T. A. Larsson and D. Larhammar, unpublished), and separated with maximum bootstrap support from Y_2 in chicken and the other species (Fig. 4).

Organ distribution of Y_6 and Y_7 mRNA

RT-PCR was performed on total RNA prepared from various tissues. The PCR products were separated on agarose gels (Figs 5 and 6). Note that the assay was not designed to be quantitative. The mRNA for Y_6 was only detected in the hypothalamus among the brain regions (Fig. 5A). Among the other organs, Y_6 mRNA was detected in liver, kidney and pro-ventriculus (Fig. 5C). Weak signals were also observed from small intestine and adipose tissue. Actin was used as a positive control for the brain regions (Fig. 5B) as well as the peripheral organs (Fig. 5D). The Y_7 mRNA was exclusively observed in the adrenal gland among the organs and brain regions analyzed (Fig. 6). For comparison, the figure also shows the distribution of Y_2 mRNA, amplified from the same cDNA samples, which could be detected in all organs except liver and gizzard, and actin, which was used as a positive control.

Pharmacological characterization

The coding region of chicken Y_6 was transferred to a modified pCEP-4 expression vector [42] and expressed in human HEK-293 EBNA cells selected with hygromycin for semistable expression. The radioligand ^{125}I-porcine peptide YY (pPYY) showed specific binding to chicken Y_6 in a concentration-dependent manner with a K_d of 0.80 ± 0.36 nM (mean ± SEM of three experiments, data not shown). The low expression level, as shown by low numbers of radioligand counts, precluded reliable competition experiments. We therefore also tried to stably express the Y6 receptor in Chinese hamster ovary (CHO) cells using the pcDNA 3 vector (which worked well for chicken Y7, see below). We performed saturation binding experiments on membranes from these cells with ^{125}I-pPYY but detected no, or very low, specific binding. Instead, we investigated whether signal transduction responses could be measured after the addition of various peptides (tested after expression with the modified pCEP-4 vector in HEK-293 EBNA cells). We used the endogenous peptides cPYY and chicken pancreatic polypeptide (cPP), as well as porcine NPY (pNPY) and pPYY, in four types of signal transduction assays, namely cAMP production, intracellular calcium release, inositol phosphate formation and extracellular acidification measured in a microphysiometer (only cPYY was tested in the microphysiometer assay). However, no measurable responses were observed, although peptide concentrations exceeding 1 μM, sometimes up to 15 μM, were used. Control experiments with other NPY-family receptors run in parallel confirmed that the assays worked.

The chicken Y_7 coding region was inserted into the expression vector pcDNA 3.0. The construct was transfected into CHO cells and selected for stable expression with G-418. The radioligand, ^{125}I-pPYY, displayed specific binding to chicken Y_7 in a concentration-dependent manner with a dissociation constant (K_d) of 0.14 ± 0.01 nM (mean ± SEM, $n = 3$). Figure 7 shows a representative saturation curve. Scatchard analysis of the specific ^{125}I-pPYY binding resulted in a linear plot consistent with a noncooperative, apparently single class of binding sites (Fig. 7, inset).

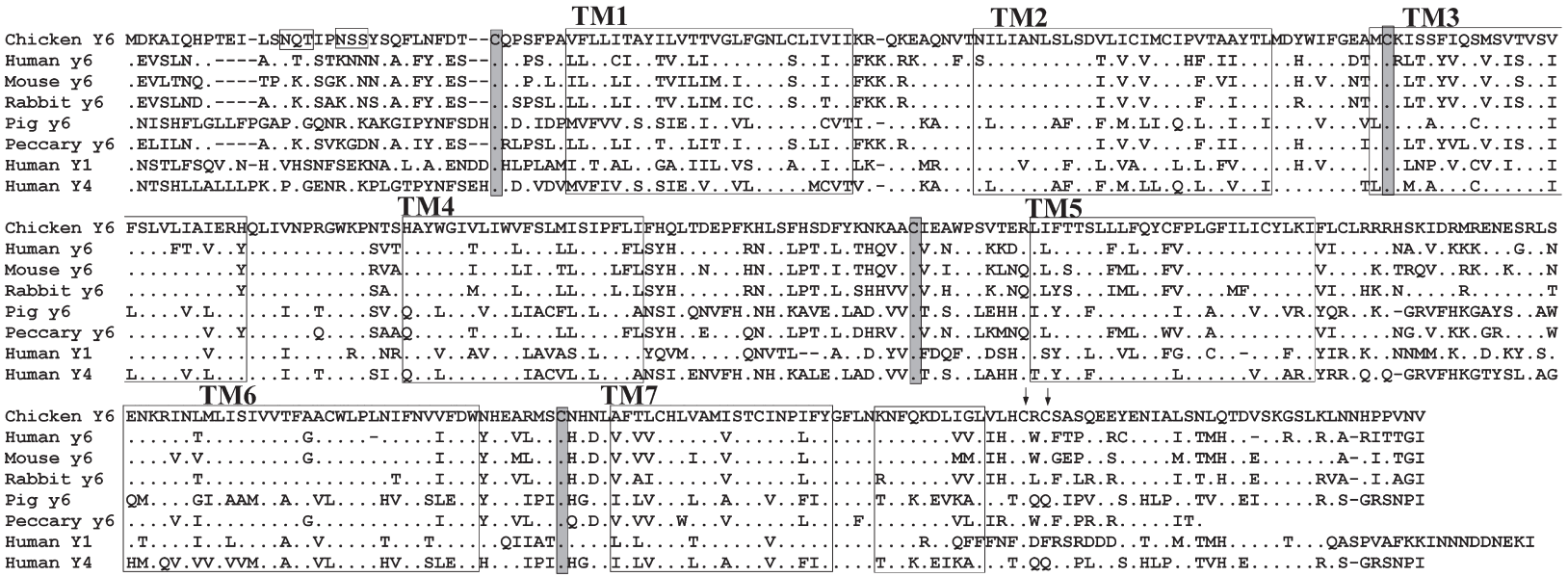


Fig. 1. Alignment of Y_6 sequences. Amino acid alignment of the Y_6 sequences from chicken, human, mouse, rabbit, pig and peccary, together with Y_1 and Y_4 (which also belong to the Y_1 subfamily) from human. Sequences were aligned using the UNIX version of CLUSTALW 1.82 [51] with default parameters. The alignment was bootstrapped 100 times using SEQBOOT from PHYLIP [52]. The chicken Y_6 sequence serves as a master. The frameshifted Y_6 pseudogenes (human and pig) were adjusted to restore the open reading frame. Boxes mark the putative transmembrane (TM) regions as predicted from comparisons with the crystal structure of bovine rhodopsin [58]. Clear boxes mark putative glycosylation sites in the N-terminal part of chicken Y_6, and shadowed boxes indicate cysteines potentially involved in disulfide bridges. Two arrows mark cysteines in the C-terminal tail, potentially serving as attachment sites for a palmitoyl moiety anchoring the tail to the cell-surface membrane. Sequence UniProt accession numbers: chicken Y6, (ABA86950); Human Y6, Q99463 (pseudogene); mouse Y6, Q61212; rabbit Y6, P79217; pig Y6, AF227955 (pseudogene); peccary Y6, Q6Y2G1; human Y1, P25929; human Y4, P50391.

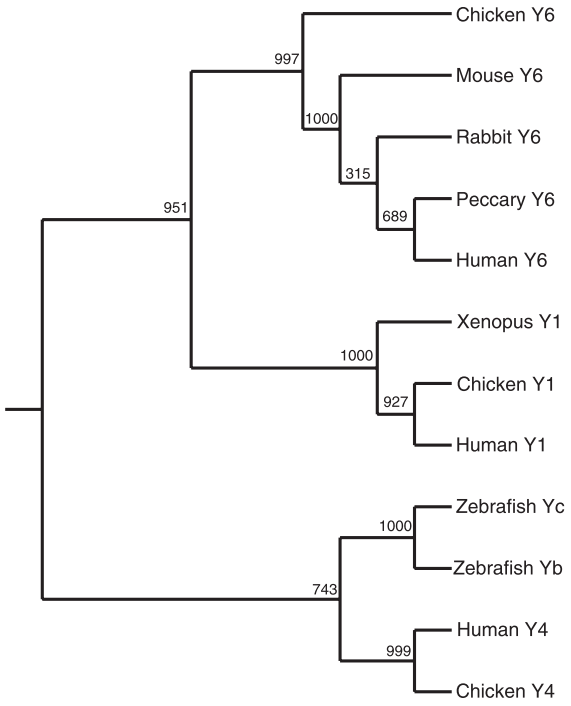


Fig. 2. Phylogenetic tree of Y_1 subfamily sequences. Phylogenetic tree of the Y_1 subfamily of receptors based on the entire coding region of the receptor genes. The consensus tree was calculated from 1000 trees using the Neighbor–Joining method of PHYLIP and plotted using TREEVIEW. The human Y_2 sequence was used as an outgroup to root the tree. Sequence UniProt accession numbers: chicken Y6, (ABA86950); mouse Y6, Q61212; rabbit Y6, P79217; peccary Y6, Q6Y2G1; human Y6, Q99463; *Xenopus laevis* Y1, P34992; chicken Y1, Q8QFM1; human Y1, P25929; zebrafish Yc, O73734; zebrafish Yb, O57463; human Y4, P50391; chicken Y4, Q8QGM3.

The affinities of peptides and nonpeptidergic ligands for chicken Y_7 were established through competition experiments with radioligand ^{125}I-pPYY (Table 1 and Fig. 8). The most potent inhibitor of ^{125}I-pPYY was pPYY, with a K_i of 0.58 nM (= pK_i of 9.24 ± 0.20, mean ± SEM). Unexpectedly, the endogenous peptide, cPYY, displayed a much lower affinity, with a K_i of 41 nM (pK_i of 7.39 ± 0.05). pNPY displayed an affinity of 10 nM (pK_i of 8.00 ± 0.15). Much lower affinities were observed for the two truncated fragments of pNPY, namely $pNPY_{3-36}$ with a K_i of 0.50 μM (pK_i of 6.28 ± 0.34) and $pNPY_{13-36}$, with a K_i of 1.1 μM (pK_i of 5.97 ± 0.02). As a result of the drastic decrease in binding of these two truncated peptides, no shorter fragments were tested. Low affinities in the micromolar range were also found for pNPY (Leu31, Pro34), the Y_2-selective (in mammals) antagonist BIIE0246 and cPP, with pK_i values of 6.56 ± 0.50, 5.68 ± 0.22 and < 6.0 (Table 1). No

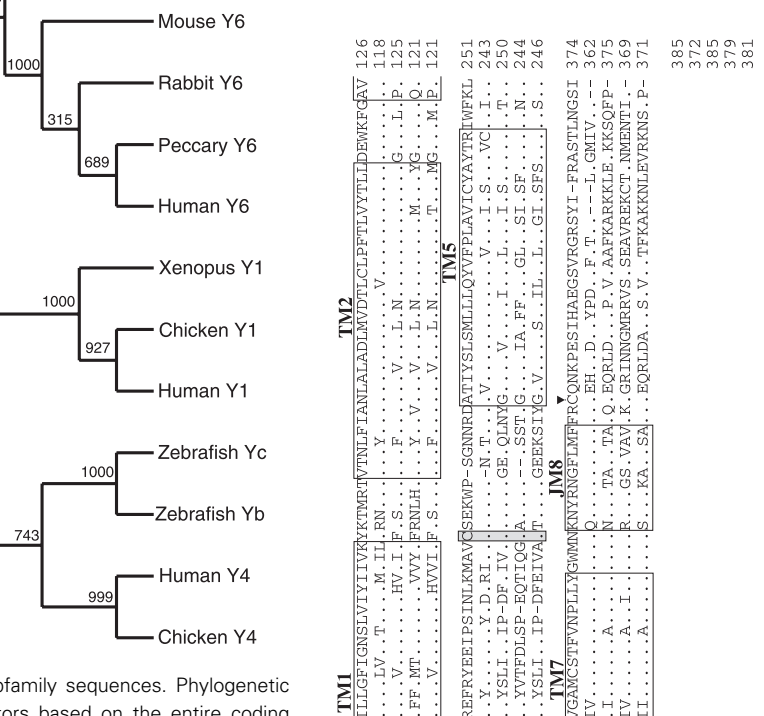


Fig. 3. Alignment of Y_7 and Y_2 sequences. Amino acid alignment of the Y_7 sequences from chicken and zebrafish with Y_2 from chicken, zebrafish and human. Sequences were aligned using the UNIX version of CLUSTALW 1.82 [51] with default parameters. The alignment was bootstrapped 100 times using SEQBOOT from PHYLIP [52]. The chicken Y_7 sequence serves as a master. Boxes mark the putative transmembrane (TM) regions as predicted from comparisons with the crystal structure of bovine rhodopsin [58]. Clear boxes mark the putative glycosylation site in the N-terminal region and shadowed boxes show cysteines potentially involved in disulfide bridges. An arrowhead marks a cysteine in the C-terminal tail that may serve as attachment sites for a palmitoyl moiety to anchor the tail to the cell-surface membrane. Sequence UniProt accession numbers: chicken Y7, Q30D05; zebrafish Y7, Q6PR57; chicken Y2, Q9DDN6; zebrafish Y2 (not yet assigned, available from the authors upon request); human Y2, P49146.

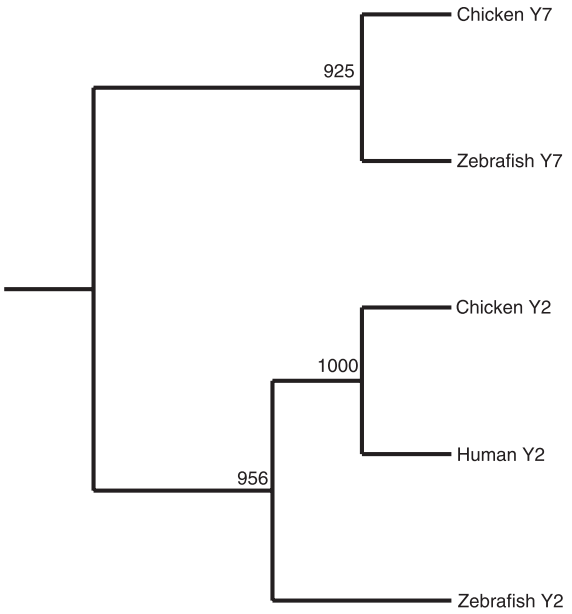


Fig. 4. Phylogenetic tree of Y_7 and Y_2 sequences. Phylogenetic tree of the Y_2 subfamily of receptors based on the entire coding region of the receptor genes. The consensus tree was calculated from 1000 trees using the Neighbor–Joining method of PHYLIP and plotted using TREEVIEW. The human Y_1 sequence was used as outgroup to root the tree. Sequence UniProt accession numbers: chicken Y7, Q30D05; zebrafish Y7, Q6PR57; chicken Y2, Q9DDN6; zebrafish Y2 (not yet assigned, available from the authors upon request); human Y2, P49146.

displacement of ^{125}I-pPYY was observed with the Y_1-selective antagonist, BIBP3226.

Chromosomal location

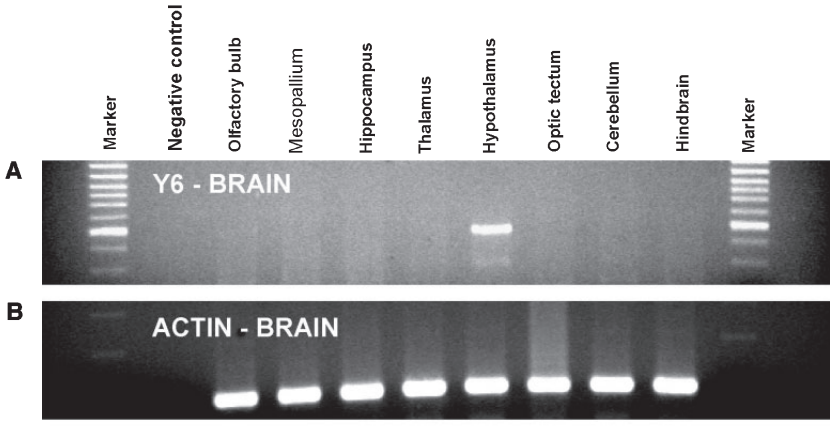
As an additional way to investigate gene orthology, we have located the chicken Y-receptor genes in the chicken genome. The two genes Y_6 and Y_7 are located approximately one megabase from each other on Gga13 (*G. gallus* chromosome 13), which shares, with Hsa5, conserved synteny for many genes (Fig. 9) including the human Y_6 gene is located as well as multiple additional genes. This supports orthology between the chicken Y_6 gene reported here and the previously identified human Y_6 gene. However, the Y_7 gene has not been found in any mammal. Adjacent to Y_6 are members of several other gene families that have representatives also on the other chicken and human chromosomes which harbor Y receptor genes. A few of these gene families are shown in Fig. 9, namely RASGEF1, SEC24, palladin and PDLIM. This observation suggests that a whole block of genes, which included all of these gene families, was duplicated early in vertebrate evolution and gave rise to the three chromosome regions that contain the Y-receptor genes [i.e. Gga4 (Hsa4), Gga6 (Hsa10) and Gga13 (Hsa5)]. For each pair of chicken–human chromosomes with conserved synteny, the sequence identity is greater between the two species (orthologues) than with the other chromosomes in the same species (paralogues), thereby confirming that the chromosome duplications took place before the separation of the lineages leading to birds and mammals.

Discussion

The discovery of the NPY-family receptors Y_6 and Y_7 came as a complete surprise, as neither had been predicted from physiological or pharmacological studies. Both were found thanks to their sequence similarity to other Y receptors, and the sequence comparisons suggested that both Y_6 and Y_7 arose before the radiation of gnathostomes in evolution [23,24,41]. Yet, Y_6 is a pseudogene in some mammals, whereas it seems to remain functional in others, and Y_7 has not been found in any mammal. Y_6 appears to be functional in the shark, *S. acanthias* [41]. To shed further light on the origin and roles of these receptors, we describe here the cloning, tissue distribution and initial pharmacological characterization, as well as the chromosomal location, of Y_6 and Y_7 in chicken.

The chicken Y_6 receptor has 61–63% amino acid identity to the functional mammalian Y_6 receptors (these are 77–82% identical among themselves), which is similar to the identity for Y_4 between chicken and mammals, but clearly lower than chicken–mammal orthologues for Y_1, Y_2 or Y_5 (disregarding the large third cytoplasmic loop of Y_5 which has diverged considerably). The phylogenetic analysis suggests that the replacement rate for Y_6 was lower earlier in evolution and that the rate has increased in the mammalian lineage (Fig. 2) [41]. This, together with the fact that the gene for Y_6 has been inactivated several times independently in mammals, indicates that the selective pressure on the gene is lower in mammals than in chicken.

Functional expression of the chicken Y_6 gene, followed by saturation-binding experiments, showed that the K_d value of radiolabeled pPYY was ≈ 0.80 nM, which is at least a twofold lower affinity than reported for other Y subtypes. The low expression level in these HEK-293 EBNA cells, as well as in CHO cells, made it virtually impossible to perform reliable competition experiments. The reason for the low affinity of the radioligand may be that pPYY differs at 12 positions from both cPYY and cNPY. We confirmed expression of the receptor in cell membranes by detection with an



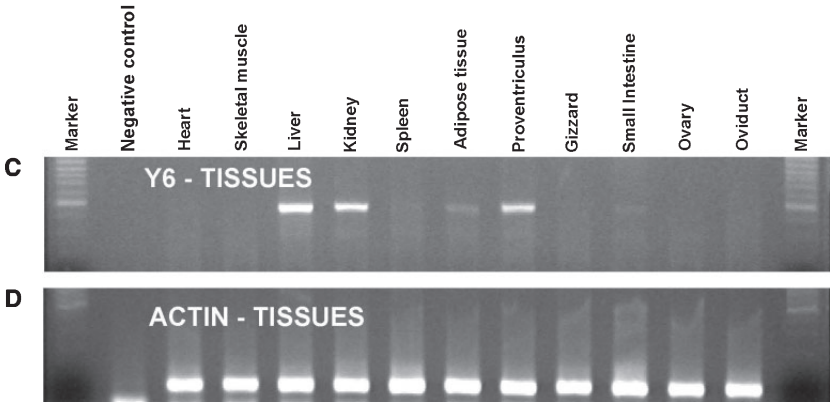


Fig. 5. RT-PCR analysis of chicken Y_6. RT-PCR analysis of Y_6 mRNA in chicken. All PCR reactions were run on cDNA made from total RNA extractions. The products were analysed on agarose gels. (A) Y_6 in brain. (B) Actin in brain. (C) Y_6 in peripheral tissues. (D) Actin in peripheral tissues. The negative control sample included water instead of cDNA. The brain regions are named in accordance with the revised nomenclature for avian telencephalon [59].

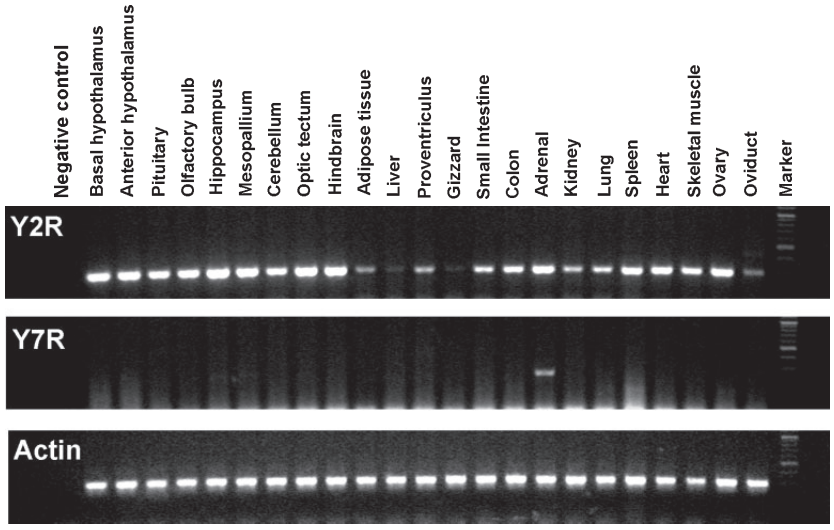


Fig. 6. RT-PCR analysis of chicken Y_7. RT-PCR analysis of Y_7 and Y_2 mRNA in chicken. All PCR reactions were run on cDNA made from total RNA extractions. The products were analyzed on agarose gels. (A) Y_2. (B) Y_7. (C) Actin. The negative control sample included water instead of cDNA. The brain regions are named in accordance with the revised nomenclature for avian telencephalon [59]. No genomic DNA contamination was detected in the mRNA samples by PCR with primers located in adjacent exons of the GnIH gene (not shown).

antibody against the epitope tag (not shown). To avoid having to rely on a high-affinity radioligand for determination of the receptor's pharmacological profile, we performed a number of functional assays to determine whether we could detect changes in signal transduction in response to various ligands. Although we tested four separate assays (cAMP, intracellular calcium release, inositol phosphate production and extracellular acidification), we found no evidence for a functional response, even at high ligand concentrations (exceeding micromolar) using pNPY, pPYY, cPYY and cPP (only cPYY for the extracellular acidification). It would seem unlikely that cNPY (unavailable) would be the sole functional agonist because it differs from the

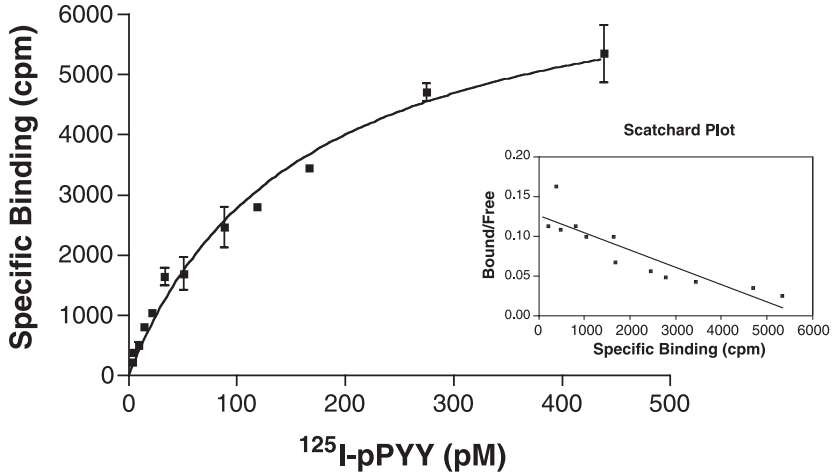


Fig. 7. Saturation binding to chicken Y_7. Saturation binding and Scatchard analysis (inset) of ^{125}I-peptide yy (pPYY) binding to cloned chicken Y_7 expressed in Chinese hamster ovary (CHO) cells. Results shown are from one representative experiment performed in duplicate. $K_d = 0.14 \pm 0.01$ nM (mean ± SEM of three experiments).

Table 1. Competition experiments with chicken Y_7.

Ligand	pK_i ± SEM	*n*
cPYY	7.39 ± 0.05	3
pPYY	9.24 ± 0.20	4
pNPY	8.00 ± 0.15	5
$pNPY_{3-36}$	6.28 ± 0.34	2
$pNPY_{13-36}$	5.97 ± 0.02	2
cPP	< 6.0	2
pNPY(Leu31, Pro34)	6.56 ± 0.50	3
BIIE0246	5.68 ± 0.22	3
BIBP3226	n.d.	1

Inhibition by various ligands of ^{125}I-porcine peptide YY (pPYY) binding to the chicken Y_7 receptor. The results are the mean ± SEM of *n* independent experiments performed in duplicate. The saturation assay gave a K_d value of 136 ± 12.5 pM. Nonspecific binding was defined in the presence of 100 nM pPYY. The data were analyzed using nonlinear regression, GRAPHPAD PRISM 2.0 software. ND, not displaced up to 10^{-1} M.
cPYY, chicken peptide YY; cNPY, chicken neuropeptide Y; cPP, chicken pancreatic polypeptide; pNPY, porcine neuropeptide Y.

tested pNPY by only two conservative replacements, namely Ser instead of Asn at position 7 (a replacement that is common among PYY sequences) and Met instead of Leu at position 17 (Met is found some mammals including human) (Fig. 10). It is possible that the cell line used (human HEK-293 EBNA) does not allow functional coupling of the chicken Y_6 receptor, owing to species differences, or that the receptor couples via a G protein or other signal transduction proteins that are not expressed in these cells. A more remote possibility is that chicken Y_6 has found a different ligand than the three known endogenous NPY-family peptides.

The Y_6 gene is expressed in hypothalamus, liver, kidney and pro-ventriculus, and weakly also in small

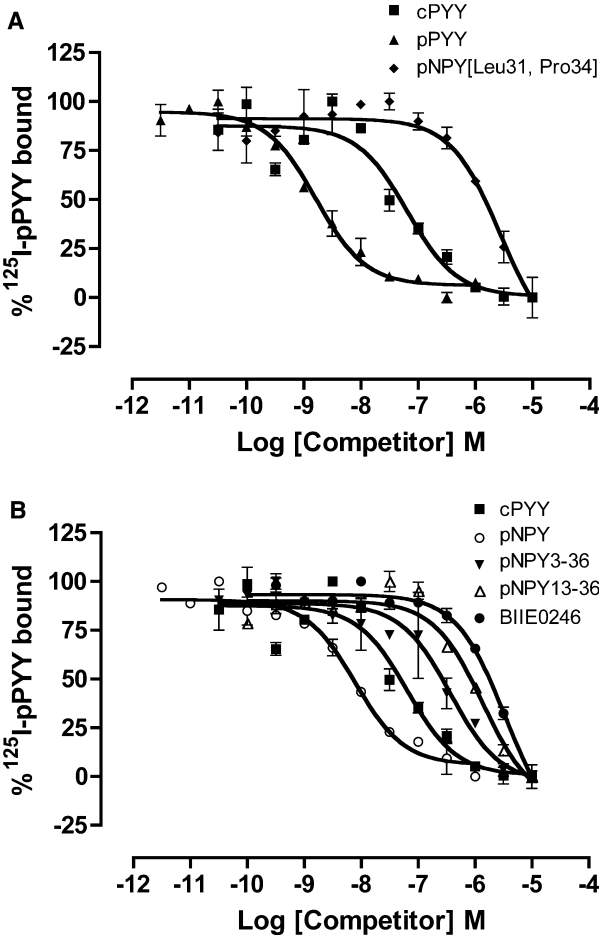


Fig. 8. (A,B) Competition binding to chicken Y_7. Inhibition of ^{125}I-peptide yy (pPYY) binding to the chicken Y_7 receptor expressed in Chinese hamster ovary (CHO) cells. Results are from one typical experiment performed in duplicate. Nonspecific binding was defined as the amount of ^{125}I-pPYY binding remaining in the presence of 100 nM unlabeled pPYY. Various concentrations of competitors were used.

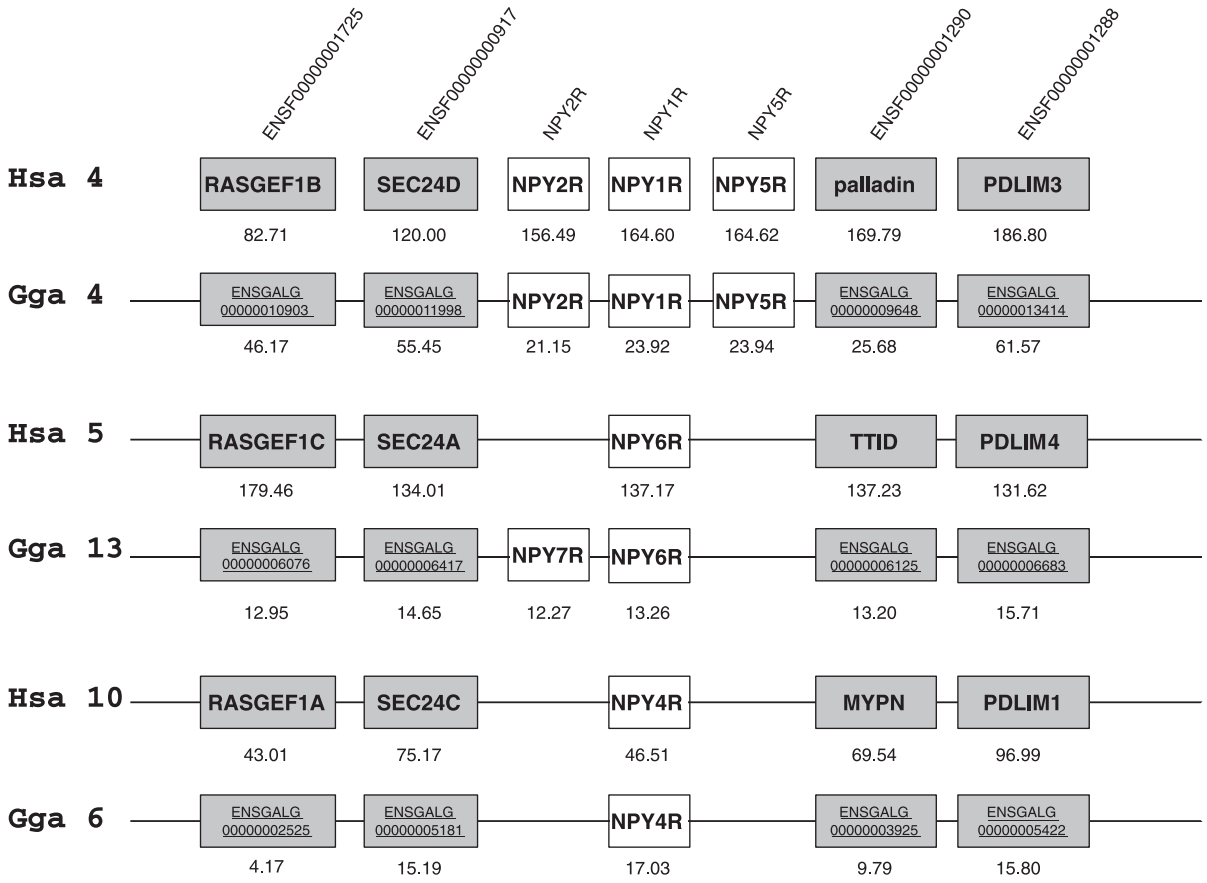


Fig. 9. Chromosome regions containing neuropeptide Y (NPY)-family receptor genes. Three chicken chromosome regions, containing NPY-family receptor genes, are shown together with their orthologous human chromosome regions. The synteny blocks also contain many other gene families with members in all three chromosome regions in both species. The map position, in megabases, is shown below each gene. Note that the gene distances are not to scale. Gene order has, in some cases, been shifted to highlight similarity with *Homo sapiens* chromosome 4 (Hsa4), because intrachromosomal rearrangements are known to occur at a higher frequency than interchromosomal rearrangements [60–62].

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                          10        20        30    36
                          |         |         |     |
NPY pig          YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY-amide
                       *         *
NPY chicken      YPSKPDSPGEDAPAEDMARYYSALRHYINLITRQRY-amide

PYY pig           YPAKPEAPGEDASPEELSRYYASLRHYLNLVTRQRY-amide
                *  *   *  **     *** ***    *
PYY chicken     AYPPKPESPGDAASPEEIAQYFSALRHYINLVTRQRY-amide

PP  pig          APLEPVYPGDDATPEQMAQYAAELRRYINMLTRPRY-amide
                 * ** *      ** ******** ** * **  *
PP  chicken      GPSQPTYPGDDAPVEDLIRFYNDLQQYLNVVTRHRY-amide
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Fig. 10. Alignments of porcine and chicken peptide sequences. Sequences comparisons between pig and chicken for each of the three peptides neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP). In each alignment, stars indicate differences between the two sequences. All of the peptides have a C-terminal amide. Sequence UniProt accession numbers: pig NPY, P01304; chicken NPY, P28673; pig PYY, P68005; chicken PYY, P29203; pig PP, P01300; chicken PP, P68248.

intestine and adipose tissue (Fig. 5). However, this does not prove functionality (e.g. even the human Y_6 pseudogene is transcribed in several tissues). Nevertheless, the fact that Y_6 has also been cloned in several ray-finned fish species (E. Salaneck and D. Larhammar, unpublished) as well as a frog (R. Fredriksson

and D. Larhammar, unpublished), and has thus existed for more than 400 million years, as corroborated by its chromosomal location in chicken as well as human (see below), supports the assumption that the gene is indeed functional, unless it has lost functionality very recently as a result of subtle mutations.

The chicken Y_7 receptor has 65% overall amino acid identity to the zebrafish Y_7 receptor (Fig. 3), and its orthology to zebrafish Y_7 is confirmed by complete bootstrap support in the phylogenetic analysis (Fig. 4). The identity between chicken Y_7 and chicken Y_2 or mammalian Y_2 is 50–55%, the same degree of identity observed between zebrafish Y_7 and Y_2. Phylogenetic analyses suggest equally strong evolutionary selection pressure for these two subtypes (data not shown).

The only other species where the Y_7 receptor has been characterized pharmacologically is the zebrafish [25]. Functional expression of the chicken Y_7 gene allows comparison of the pharmacological profile in these two species. The affinity (K_d) of ^{125}I-pPYY to chicken Y_7 was 136 ± 12.5 pM (Fig. 7), which is ≈15 times lower compared with the zebrafish Y_7 receptor for the same ligand. Moreover, several other NPY-family receptors have considerably higher affinity for this radioligand than chicken Y_7. This may be a result of the sequence differences between pPYY and endogenous cNPY. Nevertheless, the radioligand could be used for competition experiments with a panel of ligands (Table 1 and Fig. 8).

Porcine PYY competed with the radioligand for binding to chicken Y_7, with a K_i of 0.58 nM (pK_i of 9.24 ± 0.20), and displayed the highest affinity among the tested ligands. Surprisingly, cPYY showed a much lower affinity, with a K_i of 41 nM (pK_i of 7.39 ± 0.05). The concentration and amino acid composition of the peptide was analysed, and its intactness was confirmed by MALDI MS. Thus, cPYY does indeed have lower affinity than pPYY for chicken Y_7. This may be because cPYY has an additional alanine residue at the N terminus [5]. Work is in progress to determine the affinity of cPYY also to the previously cloned Y-family receptors in chicken. Among the intact peptide ligands, the rank order of potency was pPYY > pNPY > cPYY > cPP (see Table 1). Interestingly, pNPY had a lower affinity than pPYY, thereby making it unlikely that cNPY would bind with higher affinity (they differ by only two conservative replacements as mentioned above, see Fig. 10). Another observation in the same direction is that endogenous zebrafish PYY also bound with lower affinity than pPYY to zebrafish Y7 [25].

Several compounds have been developed for selectivity towards certain Y subtypes in mammals. The peptide pNPY (Leu31, Pro34) was initially claimed to be selective for Y_1, but has subsequently been found to bind also to Y_4, Y_5 and Y_6 in mammals. Thus, it can be best described as a Y_2-excluding ligand. However, we have previously reported that this peptide bound to chicken Y_2 with only 10-fold lower affinity than pNPY [28]. In the present study, we found that it bound more poorly to Y_7 with a 30-fold lower affinity than pNPY. The compound BIIE0246, which was developed as a Y_2-selective nonpeptidergic antagonist in mammals [43], bound the chicken Y_7 receptor with very low affinity, as for zebrafish Y_7 [25]. These differences in ligand affinity between Y_7 and Y_2 may prove very useful for studies of ligand–receptor interactions and 3D modeling, and we have previously been able to utilize differences between chicken and human Y_2 in antagonist binding for this purpose [44].

The two truncated peptides $NPY_{3–36}$ and $NPY_{13–36}$ had a lower affinity by 50-fold and 100-fold, respectively, compared with intact NPY. Truncated NPY fragments have also been found to lose affinity to zebrafish Y_7 and Y_2, as well as to chicken Y_2, relative to intact NPY [28], but chicken Y_7 seems to be the most extreme in this regard. Thus, the ancestral Y receptor probably required the N-terminal region of the ligands for high-affinity binding. Mammalian Y_2 receptors seem to be unique among all Y receptors in their ability to bind truncated NPY and PYY (such as $PYY_{3–36}$) with high affinity. This suggests that Y_2 in mammals acquired the ability to bind to truncated peptides recently in evolution.

In this context, it is also important to consider the possibilities of processing of the endogenous peptide ligands at the N terminus *in vivo*. Chicken PYY has the sequence AYPP, which probably makes removal of the AYP sequence to generate the equivalent of mammalian $PYY_{3–36}$ highly unlikely, as the enzyme dipeptidyl peptidase IV, which is thought to perform this cleavage, is unable to cleave a proline–proline bond, at least in mammals. An important question therefore is whether $PYY_{3–36}$ serves the postprandial appetite-reducing role in chicken as it does in mammals [16]. Perhaps this function can be performed in chicken by intact PYY (and PP).

Among all the organs investigated, chicken Y_7 mRNA could only be detected in adrenal gland. This narrow distribution is in sharp contrast to Y_2, which was almost ubiquitous (Fig. 6). The Y_7 distribution seems to be more narrow in chicken than in zebrafish, where it was found to be expressed in brain, eye and intestine [25]. Without quantification it is difficult to make comparisons of expression levels between organs and species, but the difference between Y_7 and Y_2 in the RT-PCR panel is quite striking.

To trace and date the evolutionary origin of the Y_6 and Y_7 receptors, we have also compared their chromosomal locations in the chicken genome with other species and other Y receptor subtypes. Both genes were found to be on chromosome Gga13, ≈ 1 megabase apart (Fig. 9). This chromosomal segment harbors many genes that are present on human chromosome 5, thus displaying extensive conserved synteny. Importantly, the human Y_6 gene is located on Hsa5. Thus, it seems likely that the Y_7 gene was located on this chromosome segment in a mammalian ancestor.

Many of the genes flanking Y_6 and Y_7 on Gga13 belong to gene families that have members also on the other two chromosomes that carry Y receptor genes in chicken and human, namely Gga4/Hsa4 and Gga6/Hsa10 (Fig. 9). The observation that many gene families are represented on these three chromosomes in both species is yet another example of chromosome segments that most probably are related through common ancestry. Such a set of related chromosome regions has been termed a paralogon [45]. The three similar Y-receptor-bearing chromosomes in Fig. 9 probably arose from a common ancestral chromosome in the genome doublings (tetraploidizations) that took place in a predecessor of all gnathostomes (jawed vertebrates) or all vertebrates [46–48]. The three Y receptor subfamilies, called the Y_1, Y_2 and Y_5 subfamilies, differ more from each other than the members of each subfamilies. Therefore, it is most likely that three ancestors of these subfamilies had already arisen before the basal gnathostome tetraploidizations, meaning that a triplet of Y receptors was duplicated in the chromosome duplications. Thus, after the two rounds of tetraploidization, the ancestor could have had no less than 12 Y receptors (4×3). However, some gene losses are likely to have occurred very soon after each tetraploidization. For instance, only three of the 14 genes of the duplicated Hox clusters have retained all four copies [49], showing that gene losses are frequent after duplications. Among the Y receptors, not a single species has been found to retain any duplicates of Y_5, and in the Y_2 subfamily only Y_2 and Y_7 are known. In the Y_1 subfamily, in contrast, a full quartet probably existed after the tetraploidizations with Y_1, Y_4 (previously named Y_a in zebrafish), Y_6 and Y_b, although differential losses have occurred in different vertebrate classes (Y_b was lost in amniotes). This scenario adds further support to the hypothesis that a mammalian Y_7 gene was previously located on the equivalent of today's Hsa5 (Fig. 9).

An intriguing question is when the Y_7 gene was lost in the lineage leading to mammals. Our searches in the opossum genome database have failed do detect a Y_7 sequence, indicating that it was lost prior to the divergence of marsupial and placental mammals. Perhaps the gene was easily disposable because the mammalian ancestor had equally narrow tissue distribution as the chicken today.

In conclusion, we cloned and studied the tissue distribution and phylogeny of the chicken Y_6 and Y_7 receptors and performed the initial pharmacological characterization of the latter. It is clear, from these studies, that the Y_6 and Y_7 receptors are evolutionarily old and phylogenetically widespread, as both are present in chicken, amphibians and bony fishes. Identification of the physiological roles of these receptors in chicken and other species awaits studies using subtype-selective ligands or receptor knock-down techniques. Future studies may reveal how the Y_7 receptor was lost in mammals, how Y_6 became a pseudogene in some mammals, and what physiological functions were lost in mammals or taken over by other Y receptors.

Experimental procedures

Isolation and sequencing of the chicken Y_6 gene and cloning into an expression vector

Degenerate PCR primers, based on several mammalian and the nontetrapod Y_1 subfamily, were applied to chicken genomic DNA under the following PCR conditions: 120 s at 95 °C for one cycle; 30 s at 95 °C, touchdown from 50 °C to 42 °C for 45 s and 60 s at 72 °C for 20 cycles; 30 s at 95 °C, 45 s at 42 °C and 60 s at 72 °C for 20 cycles; then 5 min at 72 °C using *Taq* polymerase (Gibco, Gaithersburg, USA). One primer pair gave a product of the expected size. The forward primer had the sequence 5′-TAY ACX HTX ATG GAY YAY TGG-3′ and the reverse primer had the sequence 5′-AAR TAR CAX AYX AYX ARD ATR AA-3′. This product was cloned into a pCR2.1-TOPO vector (TOPO cloning kit; Invitrogen, Carlsbad, USA) and sequenced using the Big-Dye terminator sequencing kit (Applied Biosystems, Foster City, USA) and the extension products were analyzed on an ABI 310 automatic sequencer (Applied Biosystems). The sequence was compared to the GenBank database using the On-Line BLASTX program and found to be similar to the mammalian Y_6 receptors. The cloned insert was labeled using the Random Primer Labeling Kit (Amersham Bioscience, Uppsala, Sweden) and used as a probe to screen a gridded chicken genomic BAC library (RZPD, Heidelberg, Germany) at high stringency. Two BAC clones that hybridized strongly were later confirmed to be true positives by Southern hybridizations. Direct sequencing on one of the BAC clones yielded the 3′ and 5′ ends of the Y_6 gene. This sequence was annotated with the accession code DQ189216.

A fragment containing the entire coding region was generated from the BAC clone using *Pfu*-turbo DNA polymerase (Stratagene, La Jolla, USA). The 5′ primer contained a *Hin*dIII restriction site (underlined) and had the sequence 5′-gacatcaaagcttATGGATAAAGCCATT CAGCATCCT-3′, and the 3′ primer had a *Xho*I restriction site (underlined) and the sequence 5′-aagctcgagTTAGACA TTCACAGGAGGGTGGTT-3′. The PCR product was digested with *Hin*dIII and *Xho*I for 3 h, purified on a PCR purification column (Qiagen, Hilden, Germany) and thereafter ligated into a modified pCEP4 vector [42] with a FLAG epitope added to the C terminus, to facilitate detection on the cell surface. The expression construct was sequenced and found to be identical to the genomic sequence obtained from the BAC clone.

Isolation and sequencing of the chicken Y_7 gene and cloning into the expression vector

A Y_7-like sequence was identified in the Ensembl chicken genome database, version 26.1c.1 (March 2004) by BLASTX searching with the zebrafish Y_7 sequence [25]. The sequence has been annotated with the accession code DQ165551. PCR primers were designed to obtain the full-length receptor gene and included sites for ligation into the expression vector, pcDNA3 (Invitrogen, Stockholm, Sweden). Primer sequences were: primer pcDNA3cY_7.F with a *Hin*dIII restriction site (underlined; 5′-gacatcaaagcttatgctctgttgtgtccc atgc-3′) and pcDNA3cY_7.R with a *Xho*I restriction site (underlined; 5′-aagctcgagctaaacctcggtgggtccgttgcc-3′).

PCR was carried out on genomic DNA from White Leghorn kindly provided by Leif Andersson (Uppsala University, Sweden). Touchdown PCR was performed using proofreading *Pfu*Turbo®Hotstart Polymerase (Stratagene, La Jolla, CA, USA). The following PCR conditions were applied: 95 °C for 5 min, followed by 30 cycles of 45 s at 95 °C, 30 s at 55 °C and 2 min at 72 °C. In the first 30 cycles the annealing temperature was automatically decreased by 0.5 °C for each cycle. After this, another 35 cycles of 95 °C for 45 s, 50 °C for 30 s and 72 °C for 2 min, was applied. At the end, samples were held at 72 °C for 10 min. A 50 μL reaction mixture contained 1.5 U of *Pfu*Turbo®Hotstart Polymerase, 1 × cloned *Pfu* reaction buffer (Stratagene), 10 mM dNTPs (Pharmacia Biotech, Uppsala, Sweden), 5 ng of genomic chicken DNA, 20 μM forward primer and 20 μM reverse primer. The fragment containing the entire coding region of the chicken Y_7 gene was purified using a QIAquick PCR Purification Kit (Qiagen) and cut with *Hin*dIII (Amersham, Uppsala, Sweden) and *Xho*I (Amersham). The1.3 kb pair fragment was purified on a 1% agarose Tris-borate EDTA gel using the QIAquick Gel Extraction Kit and ligated into the expression vector pcDNA3 (Invitrogen). The sequence of the PCR product was determined using the BigDye™ Terminator Cycle Sequencing Ready Reaction kit (ABI PRISM™; Perkin Elmer, Foster City, CA, USA) with AmpliTaq®DNA polymerase, on an ABI PRISM 310 Genetic Analyzer, and found to be identical to the genomic sequence. The expression construct contains one upstream in-frame methionine codon (immediately after the cloning site), but this AUG codon deviates from the Kozak consensus sequence for initiation of translation [50]. Furthermore, the extension would, if translated, contain six cysteine residues, which would probably interfere with receptor processing, which is why we presume that translation was initiated at the optimal methionine shown in the alignment in Fig. 3. It is also possible that initiation occurs at the methionine at position 13, which also has an AUG context that agrees with the consensus sequence for initiation of translation.

Phylogenetic analyses

Sequences were aligned using the UNIX version of CLUSTALW 1.82 [51]. The default alignment parameters were applied. The alignment was bootstrapped 1000 times using SEQBOOT from the Win32 version of the PHYLIP 3.6 package [52]. Protein distances were calculated on the bootstrapped alignments using PROTDIST from the Win32 version of the PHYLIP 3.6 with the Jones-Taylor-Thornton matrix. Trees were calculated on the distance matrixes using NEIGHBOR from the WIN32 version of the PHYLIP 3.6 package, resulting in 1000 trees. These trees were analyzed using CONSENSE from the WIN32 version of the PHYLIP 3.5 package to obtain a bootstrapped consensus tree. Trees were plotted using TREEVIEW (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

RT-PCR

To determine the tissue distribution of Y_6 gene expression, three adult laying Bantam hens (Roslin Institute flock) were killed by cervical dislocation, in accordance with United Kingdom Home Office animal experimentation regulations. For analysis of Y_2 and Y_7 gene expression, three hens of the Lohmann Brown laying strain (Roslin Institute flock) were used. Tissue samples were rapidly dissected and snap-frozen in liquid nitrogen before storage at −70 °C. Total RNA was isolated using RNA-Bee (AMS Biotechnology, Abingdon, UK) according to the manufacturer's instructions. Individual tissue blocks were homogenized using a Ribolyser (Thermo Life Sciences, Basingstoke, Hampshire, UK). A 5 μg sample of RNA was incubated with 4 U of DNase I (Roche Diagnostics, Lewes, East Sussex, UK) at 37 °C for 30 min to remove any residual genomic DNA, before being reverse transcribed using a First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech, Little Chalfont, Bucks., UK) with *Not*I-d$(T)_{18}$ as a primer. For Y_7, these were: forward primer 5′-GAGGAAATCCCATCTAT CAACC and reverse primer 5′-AGACCACGACTACCAT CACC. For amplification of Y_2, the following primers were

used: forward primer 5′- CAATTGGGAAGAAAACCAG ACA and reverse primer 5′- GCACAATGTATTCACCAG CAGA. Actin, used as a positive control to monitor the efficacy of reverse transcription, was amplified as part of the analysis of Y_6 expression using forward primer 5′-TGGGTATGGAGTCCTGTGGT and reverse primer 5′-AGACAGCACTGTGTTGGCATA. In the analysis of Y_2 and Y_7 gene expression, actin was amplified using forward primer 5′-AATCAAGATCATTGCCCCAC and reverse primer 5′-TAAGACTGCTGCTGACACC. PCR was performed using Roche *Taq* polymerase in PCR buffer containing 1.5 mM $MgCl_2$ on a Hybaid MBS system thermocycler block with an annealing temperature of 60 °C and denaturing and extension steps of 94 °C and 72 °C, respectively. Times used were 15 s denaturation, 30 s annealing and 30 s extension, with an extension time for the final cycle of 5 min. PCR was carried out for 30 cycles for actin and 35 cycles for Y_2 and Y_6 and Y_7. PCR amplification products were resolved by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. No genomic DNA contamination was present in the mRNA samples used for Y_2 and Y_7, as demonstrated by PCR with primers located in adjacent exons of the GnIH gene; no product containing the intervening small intron (874 bp) was detected (data not shown). The mRNA panel used for the Y_6 experiment was prepared using the same mRNA isolation kit, which had previously been carefully tested and selected because it did not produce genomic DNA contamination. We have used this reagent routinely with many types of tissue and have never experienced a problem with contamination. Nevertheless, as an extra safeguard, an additional incubation step with DNase was included.

Transfection protocol and membrane harvesting

For studies of Y_6, HEK 293-EBNA (Invitrogen) cells were seeded onto 90 mm dishes, grown to 50% confluence and transfected with 10 μg of the expression construct in the modified pCEP4 vector using FuGene (Roche, Basel, Switzerland) according to the manufacturer's recommendations. The construct contained a C-terminal FLAG-epitope to facilitate detection of the protein product. The cells were grown for 48 h after transfection before harvesting. For semistable transfection, HEK 293-EBNA cells were transfected as described above and grown for 24 h. The cells carrying the expression vector were thereafter selected for by growing in the presence of 500 $\mu g \cdot mL^{-1}$ hygromycin (Gibco) for 10 days. After the harvest, the cells were homogenized using an Ultra-Turrax (Janke & Kunkel, Staufen, Germany). The cell suspension was centrifuged for 3 min at 600 ***g*** and the supernatant was recentrifuged for 15 min at 31 000 ***g***. The cell pellet was resuspended in binding buffer containing 50 mM Tris/HCl, pH 7.4, 2.5 mM $MgCl_2$ and 1 mM $CaCl_2$, aliquoted and stored at −80 °C.

For studies of Y_7, CHO cells grown to 70% confluence on 90 mm dishes were transfected with 12 μg of the expression construct pcDNA3-cY_7 using FuGENE^{TM}6 Transfection Reagent (Roche), diluted in Opti-MEM medium (Gibco BRL, Stockholm, Sweden) according to the manufacturer's recommendations. Cells were grown in DMEM/Nut Mix F-12 without L-glutamine (Gibco BRL) containing 10% fetal bovine serum (Biotech Line A/S, Slagerup, Denmark), 2.4 mM L-glutamine (Gibco BRL) and 100 U of penicillin/100 μg streptomycin per mL (Gibco BRL). One day after transfection, 0.25 $mg \cdot mL^{-1}$ G-418 (= geneticin) (Gibco BRL) was added to the growth medium to select for cells with stable expression. The cells were harvested, washed and collected by centrifugation. The cell pellet was resuspended in binding buffer containing 50 mM Tris/HCl, pH 7.4, 2.5 mM $MgCl_2$ and 1 mM $CaCl_2$, aliquoted and stored at −80 °C.

Peptides and nonpeptide ligands

Chicken PYY and PP were ordered from Schafer-N (Copenhagen, Denmark). For the studies of Y_6, pNPY and pPYY peptides were purchased from Bachem (King of Prussia, PA, USA). For the studies of Y_7, pNPY, pPYY, $pNPY_{3–36}$, $pNPY_{13–36}$ and pNPY(Leu31,Pro34) were purchased from Neosystem Groupe SNPE (Strasbourg, France). Alignments of porcine and chicken peptide sequences are shown in Fig. 10. The radioligand ^{125}I-pPYY was purchased from Amersham. The nonpeptidergic antagonists for Y_1, BIBP3226 [53], and for Y_2, BIIE0246 [43], were kindly provided by Boehringer-Ingelheim PharmaKG (Biberach an der Riss, Germany).

Binding assays

Thawed aliquots of membrane were resuspended in 25 mM Hepes buffer (pH 7.4) containing 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$ and 2 $g \cdot L^{-1}$ (Y_6) or 0.2 $g \cdot L^{-1}$ (Y_7) Bacitracin and homogenized using an Ultra-Turrax homogenizer. Saturation experiments were performed in a volume of 100 μL. The reactions were incubated for 2 h at room temperature with ^{125}I-pPYY (Amersham Bioscience) as radioligand. This radioligand had iodinated tyrosines at positions 21 and 27 and a specific activity of 4000 $Ci \cdot mmol^{-1}$. Saturation experiments were carried out with serial dilutions of radioligand, and nonspecific binding was defined as the amount of radioactivity binding to the cell homogenate with 100 nM nonlabeled pPYY included in the reactions. The incubations were terminated by rapid filtration through GF/C filters (Filtermat A; Wallac Oy, Turku, Finland) that had been presoaked in 0.3% polyethyleneimine, using a TOMTEC (Orange, CT, USA) cell harvester. The filters were washed with 5 mL of 50 mM Tris/HCl, pH 7.4, at 4 °C and dried at 60 °C. The dried filters were treated with MeltiLex A (Perkin Elmer) melt-on scintillator sheets, and

the radioactivity retained on the filters was counted using the Wallac 1450 Betaplate counter (Wallac). The results were analyzed with a nonlinear regression curve fitting using the PRISM 2.0 software package (GraphPad, San Diego, CA, USA). For Y_7, competition experiments were performed in a final volume of 100 μL. Various concentrations of the competitor [i.e. cPYY, pPYY, pNPY, $pNPY_{3–36}$, $pNPY_{13–36}$, cPP, pNPY(Leu31,Pro34), BIIE0246, or BIBP3226] were included in the incubation mixture along with ^{125}I-pPYY. Saturation experiments were also analyzed with linear regression using Scatchard transformation. Hill coefficients were calculated for each individual competition experiment.

Signal transduction assays

As the Y_6 receptor did not bind the radioligand with sufficient affinity for competition assays, it was tested for functional response to the four peptides (pNPY, pPYY, cPYY, and cPP) up to a concentration of 1 μM or higher in four signal transduction assays. These assays were performed as described previously for cAMP [54], intracellular calcium release [55], inositol phosphate formation [56] and microphysiometer extracellular acidification assay [57]. Only cPYY was used in the microphysiometer assay. However, none of these four assays gave a measurable response for the chicken Y_6 receptor, although positive controls with other NPY-family receptors that were run in parallel gave robust responses (data not shown).

Synteny comparisons

The chromosomal locations of all of the chicken Y receptor genes were retrieved from the Ensembl database, version 32.1h, and compared with the corresponding human genes in the genome database, version 32.35e. The chromosomal locations were also retrieved for a few adjacent genes belonging to families with representatives on the other chromosomes of the three that harbour Y receptor genes.

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