Chicken neuropeptide Y-family receptor Y4: a receptor with equal affinity for pancreatic polypeptide, neuropeptide Y and peptide YY

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Abstract

Within the neuropeptide Y (NPY) family of peptides, pancreatic polypeptide is the most divergent across species. It differs in 20 of 36 positions between human and chicken. In mammals, it binds primarily to the Y4 receptor, to which NPY and peptide YY (PYY) bind with lower affinities. Because of these large sequence differences in pancreatic polypeptide, we decided to characterise the chicken Y4 receptor. We report here that Y4 displays the least sequence conservation among the Y-family receptors, with only 57–60% overall amino acid identity between chicken and mammals, compared with 64–83% for the Y1, Y2 and Y5 receptors. After expression of the chicken Y4 receptor in COS-7 cells, ¹²⁵I-labelled porcine (p) PYY bound with a K_d of 20 pM. In competition with ¹²⁵I-pPYY, chicken pancreatic polypeptide bound with high affinity at 140 pM. Interestingly, chicken PYY bound with even greater affinity at 68 pM. The affinity of NPY, 160 pM, was similar to that of pancreatic polypeptide. Chicken Y4 is less sensitive than is mammalian Y4 to truncation of the amino terminus of the NPY molecule. RT-PCR revealed expression in several peripheral organs, including adipose tissue and oviduct. In brain, Y4 mRNA was detected in the brainstem, cerebellum and hippocampus. *In situ* hybridisation to brain sections showed expression in the dorsal motor nucleus of the vagus in the brainstem. Thus the chicken Y4 receptor is less selective and anatomically more widespread than that in mammals, probably reflecting the original properties of the Y4 receptor.

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Introduction

The neuropeptide Y (NPY) family of peptides consists, in chicken and mammals, of pancreatic polypeptide, peptide YY (PYY) and NPY itself, all of which are 36–37 amino acids long. NPY is extremely well conserved and has only one amino acid replacement between human and chicken, and four replacements between human and zebrafish (Blomqvist *et al.* 1992, Larhammar 1996, Cerda-Reverter & Larhammar 2000, Söderberg *et al.* 2000). PYY is more divergent, and pancreatic polypeptide differs greatly between species, with only 16 of 36 positions identical between human and chicken (Kimmel *et al.* 1975, Larhammar 1996, Cerda-Reverter & Larhammar 2000).

Chicken pancreatic polypeptide (chPP) was the first NPY-family peptide to be isolated (Kimmel *et al.* 1975). The physiological effects, particularly on metabolism and digestive physiology, have been a focus of intense investigation for all three peptides (Hazelwood 1993, Kalra *et al.* 1999). NPY has been implicated in the control of food intake, metabolism, blood pressure, circadian rhythms and reproduction (Kalra *et al.* 1999). NPY has also been found to stimulate feeding in birds (Kuenzel *et al.* 1987, Richardson *et al.* 1995), a reptile (Morris & Crews 1990) and fish (Lopez-Patino *et al.* 1999, Narnaware *et al.* 2000, de Pedro *et al.* 2000). Moreover, in chicken (Boswell *et al.* 1999) and fish (Silverstein *et al.* 1998, Narnaware & Peter 2001) *NPY* gene expression in the hypothalamus is

increased by food deprivation or restriction. This suggests that an involvement of NPY in the regulation of energy balance is evolutionarily very old. NPY is also expressed in neurones that send their processes toward the pituitary gland in 'lower' vertebrates, which suggests an ancient role in the regulation of neuroendocrine secretion (Vallarino *et al.* 1988, Danger *et al.* 1990). PYY and pancreatic polypeptide are expressed mainly in endocrine cells in mammals, and influence gastrointestinal secretion and gut motility. Binding sites for pancreatic polypeptide have been identified in the brain of mammals (Whitcomb *et al.* 1990, Gehlert *et al.* 1997) and chick (Adamo & Hazelwood 1984, Inui *et al.* 1992).

The NPY-family peptides bind to rhodopsin-like G-protein-coupled receptors; five subtypes, Y1, Y2, Y4, Y5 and Y6, have been cloned in mammals (Larhammar *et al.* 2001). The Y1 and Y6 receptors have the greatest sequence identity to one another, at about 60%, whereas Y4 has a slightly lower identity of 46–50%. These form the Y1 subfamily proposed to have arisen through chromosome duplications early in vertebrate evolution (Wraith *et al.* 2000, Larhammar *et al.* 2001). The Y2 and Y5 receptors have less than 35% identity to each other and to the Y1 subfamily, and probably originated at or before the origin of vertebrates (Larhammar *et al.* 2001). The Y1 receptor differs from all other NPY receptor subtypes in that it has an intron in the coding region. In zebrafish, three distinct subtypes have been cloned: Ya, Yb and Yc (Lundell *et al.* 1997, Ringvall *et al.* 1997, Starback *et al.* 1999). Their relationships to the mammalian receptors is unclear, but as each of them shares 45–50% identity to the Y1 subfamily receptors, they clearly belong to this subfamily.

The Y4 receptor was originally named the PP1 receptor, because of its preference for pancreatic polypeptide as a ligand. In mammals, the affinity of pancreatic polypeptide for this receptor is much greater than the affinities of NPY and PYY (Bard *et al.* 1995, Lundell *et al.* 1995, 1996, Gregor *et al.* 1996). The rat receptor is even more selective for pancreatic polypeptide than are the human and guinea pig receptors (Lundell *et al.* 1996, Eriksson *et al.* 1998, Berglund *et al.* 2001). In mammals, the Y4 receptor has a modest neuronal expression in the paraventricular nucleus and in the brainstem (Dumont & Quirion 2000, Larsen & Kristensen 2000), but has a wider peripheral expression in the

gut, which agrees well with the role of pancreatic peptide in regulating gastrointestinal functions (Hazelwood 1993, Voisin *et al.* 2000). In our efforts to delineate the evolution of the NPY-family receptors and their functions, we have recently cloned chicken Y2 receptor (Salaneck *et al.* 2000) and Y1 and Y5 receptors (Holmberg *et al.* 2002). We report here the cloning of the chicken (ch) Y4 receptor and describe its less selective binding properties and its broader anatomical distribution, which may be more similar to the ancestral situation after it was duplicated from the common ancestor of the Y1, Y4 and Y6 receptors.

Materials and methods

Isolation and sequencing of chY4 receptor clones

Several combinations of degenerate primers were used for PCR on genomic DNA from White Leghorn chickens. One primer pair yielded a product of 580 bp with high sequence identity to mammalian Y4. The forward primer was based on conserved sequences at the junction of TM3 and the intracellular loop from the Y1 and zebrafish Yb receptors, and had the nucleotide sequence: 5'-ATT/C GCT/A C/TTT GAA C/AGT CAT/C CA-3'. The reverse primer was based on conserved residues from TM7 in the Y1 receptor, and had the sequence: 5'-CCA/G TAA/G AAG/A/T ATG GGG/A TTG/A/T/C ACA/G CA-3'. The following conditions were used with AmpliTaq DNA polymerase, Stoffel fragment (Perkin Elmer, Foster City, CA, USA): 2 min at 95 °C for one cycle, then 40 s at 94 °C, 40 s at 52 °C, 1 min at 72 °C for 22 cycles, followed by 30 s at 94 °C, 40 s at 42 °C, 1 min at 72 °C for 20 cycles and extension for 7 min at 72 °C. The PCR product was reamplified for 40 s at 94 °C, 40 s at 42 °C, 1 min at 72 °C for 30 cycles and 7 min at 72 °C, followed by gel purification (GFX PCR DNA and Gel Band Purification Kit, Amersham Pharmacia Biotech Inc.) and subsequently subcloned into the pCR2 1-TOPO vector using the TOPO TA cloning kit (Invitrogen). Sequence determinations were performed on clones with M13 forward and reverse primers using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase (Perkin Elmer). The sequence reactions were run on an ABI PRISM 310 Genetic

Analyzer. To obtain the full-length receptor sequence, a genomic chicken bacterial artificial chromosome (BAC) library was screened as described elsewhere (Holmberg *et al.* 2002) and a positive clone was sequenced using primer walking.

Cloning into expression vector

A fragment containing the entire coding region was generated from the BAC clone with PCR, using Pfu Turbo DNA polymerase (Stratagene, Amsterdam, Netherlands). The 5' primer was based on the upstream sequence: 5'-CTT CAT TTG GTT ATC ACC TTT TTG GAT A-3' and the 3' primer: 5'-TGC TTT TAT ACC CGT AGG TAT GAC-3'. The PCR fragment was purified and cloned into the expression vector pTEJ-8. The insert was sequenced and found to be identical to the BAC sequence.

Transfection procedure

For transient transfections, COS-7 cells were grown in 80-mm dishes to 60–80% confluency in Dulbecco's modified Eagle's medium (DMEM) (Gibco Brl) with 10% fetal calf serum (FCS; PAA Laboratories GmbH, Linz, Austria). Each dish was transfected by adding to the culture medium 600 µl Optimem (Gibco Brl) containing 12 µg of the chY4-pTEJ-8 receptor construct and 36 µl Fugene 6 reagent (Boehringer Mannheim, Biberach an der Riss, Germany). Cells were harvested after 2 days and pelleted by centrifugation and frozen in aliquots at -80°C .

Peptides

chPP and PYY were synthesised at Schafer-N, Copenhagen, Denmark. The porcine peptides were purchased from Bachem, King of Prussia, PA. BIBP 3226 was kindly provided by Dr Karl Thomae GmbH, Biberach, Germany, and the Y2 antagonist BIIE 0226 by Boehringer Ingelheim Pharma KG, Germany.

Binding assays

For binding assays, the thawed aliquots were resuspended in 25 mM HEPES buffer (pH 7.4) containing 2.5 mM CaCl_2 , 1 mM MgCl_2 and 2 g/l Bacitracin (Sigma) and homogenised using an

Ultra-Turrax homogeniser. Binding experiments were performed in a final volume of 100 µl with 11 µg protein and ^{125}I -labelled porcine (p) PYY (Amersham Pharmacia Biotech) for 2 h at room temperature. Saturation experiments were carried out with twofold serial dilutions of radioligand. Non-specific binding was defined as the amount of radioactivity remaining bound to the cell homogenate after incubation in the presence of 100 nM unlabelled pNPY. In competition studies, various concentrations of the peptides chPP, human pancreatic polypeptide (hPP), chPYY, pPYY, pNPY, and p[Leu31-Pro34]NPY (LP-NPY), and pNPY(2–36), pNPY(13–36) and pNPY(18–36) were included in the incubation mixture along with ^{125}I -pPYY. The Y1 antagonist, BIBP 3226, Y2 antagonist, BIIE 0246, and Y5 agonist, p[D-Trp³²]NPY, were also tested in a similar manner. Incubations were terminated by rapid filtration through GF/C filters, which had been presoaked in 0.3% polyethylenimine, using a TOMTEC cell harvester (Orange, CT, USA). The filters were washed with 5 ml 50 mM Tris (pH 7.4) at 4°C and dried at 60°C . The dried filters were treated with MeltiLex A (Wallac, Oy, Turku, Finland) melt-on scintillator sheets, and the radioactivity retained on the filters counted using the Wallac 1450 Betaplate counter. The results were analysed using the Prism software package (Graphpad). Protein concentrations were measured using Bio-Rad Protein Assay (Bio-Rad, Solna, Sweden) with BSA for standards.

RT-PCR analysis of chY4 mRNA tissue distribution

Animal procedures were in accordance with United Kingdom Home Office regulations. To determine the tissue distribution of chY4 expression, Bantam chickens (Roslin Institute flock) were killed by cervical dislocation and tissue samples were rapidly dissected and snap-frozen in liquid nitrogen before storage at -70°C . Total RNA was isolated using RNazol B (AMS Biotechnology Ltd., Abingdon, UK). A 5 µg sample of RNA was incubated with 4 U DNase I (Roche Diagnostics) at 75°C for 10 min to remove any residual genomic DNA, before being reverse transcribed using a First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech) with NotI-d(T)₁₈ as a primer. PCR was performed on a Hybaid OmniGene thermal cycler using the following primers: forward 5'-AAA CCC

AAC TTG TTG GAG G-3' and reverse: 5'-TGC TTT TAT ACC CGT AGG TAT GAC-3' in PCR buffer containing 1.5 mM MgCl₂ (Roche Diagnostics). Denaturation, annealing and extension steps were: 15 s at 94 °C, 30 s at 60 °C and 45 s at 72 °C respectively, over a total of 39 cycles. On the final cycle (the 40th) an extension time of 5 min was used. PCR amplification products were resolved by electrophoresis on a 2% agarose gel and visualised by ethidium bromide staining. Actin was used as positive control.

***In situ* hybridisation**

Brains from two male Bantam chickens (Roslin Institute flock) were processed for *in situ* hybridisation. Brains were rapidly dissected from birds killed by cervical dislocation and were immediately frozen in powdered dry ice. The tissue was stored at -70 °C before being sectioned on a cryostat (Shandon, Model OT) at 15 µm thickness. Coronal sections were thaw-mounted onto microscope slides (Superfrost Plus, Cellpath) and stored at -70 °C. Slides bearing brain sections were processed by sequential immersion at room temperature in: 4% paraformaldehyde (5 min); twice in 0.1 M sodium phosphate (5 min each); water (<5 s); 0.1 M triethanolamine, pH 8.0 (TEA, <5 s); 0.15 M acetic anhydride in 0.1 M TEA (10 min); 2 × SSC; 70%, 95% and 100% ethanol (3 min each); and blow-dried. Slides were then stored at room temperature until application of hybridisation solution. A mixture of two oligonucleotide probes complementary to chY4 receptor mRNA was used for *in situ* hybridisation. The oligonucleotide sequences were: 5'-GAT GCT AAC AAA ATA TTT ATC CTT CTT AAC TGA ACT GCT CGG TTG C-3' and 5'-GTA TTC ACT CTT CTC AAA CAT GTC CTT TCT CTT CTG TAG GCG TAA GTA-3'. The probes were labelled at the 3' end with [³⁵S]dATP, using terminal deoxynucleotidyl transferase (Amersham Pharmacia Biotech) and were purified through QIAquick spin columns (Qiagen, Crawley, UK). Specific activity of the probes was 10⁸ c.p.m./µg oligonucleotide. Hybridisation buffer consisted of 50% formamide, 4 × SSC, 10% dextran sulphate, 1% *N*-laurylsarcosine, 1 × Denhardt's solution, 200 mM dithiothreitol, and 0.1 mg/ml yeast tRNA. A 100 µl aliquot of hybridisation solution containing 3 ng of each labelled probe was applied

to each section and covered with a Parafilm coverslip. Control sections received hybridisation solution containing a 100-fold excess of each unlabelled probe. Hybridisation was performed for at least 16 h in humidified boxes at 42 °C. After hybridisation, the sections were washed (4 × 45 min) in 1 × SSC at 56 °C, and brought to room temperature while in the final wash. The tissue was then dehydrated through graded alcohols and apposed to Kodak Biomax MR film for 2–3 weeks at room temperature. Films were developed using an automatic processor (X-ograph Imaging Systems, Tetbury, UK).

(The chicken NPY-family receptor Y4 nucleotide sequence has been submitted to the GeneBank database under accession number: AF410853.)

Results

Isolation of the chY4 receptor

In order to clone NPY-family receptors, many different combinations of degenerate primers were used for PCR on genomic DNA from several different species. One primer pair yielded a product of 580 bp in chicken that had the greatest homology to the mammalian Y4 receptor. The full-length sequence obtained from a BAC clone encodes a receptor of 377 amino acids (Fig. 1). It displays 57–60% overall amino acid sequence identity to Y4 in mammals, with 70% identity in the transmembrane regions. The identity to other members of the Y1-subfamily is lower: it has 43–46% identity to the Y1 receptors from various mammals, and similar identity to Y6 receptors from mouse and rabbit. It has respectively 48%, 52% and 51% identity to Ya, Yb and Yc receptors from zebrafish. An identity of 57–60% might be considered low for orthologous receptors between chicken and mammals, but, as the phylogenetic tree demonstrates, it clearly clusters in the Y4 branch, with a bootstrap value of 100 (Fig. 2). Furthermore, from studies in mammals, Y4 is already known to evolve more rapidly (Lundell *et al.* 1996).

The receptor protein deduced from the amino acid sequence shares many of the features of the Y4 subtype: for instance, four cysteine residues are conserved, forming two putative disulphide bridges in the extracellular domains of the receptor (Fig. 1).

Figure 1 Amino acid sequence alignment made using the Lasergene DNASTAR Megalign software. The chY4 receptor serves as master sequence in alignment with human (Lundell *et al.* 1995), guinea pig (Eriksson *et al.* 1998), pig (Wraith *et al.* 2000), rat (Lundell *et al.* 1996) and mouse (Gregor *et al.* 1996) Y4 receptors. Dashes represent gaps introduced to optimise alignment. Boxes mark putative transmembrane regions according to the crystal structure of rhodopsin (Palczewski *et al.* 2000). Consensus sequences for N-linked glycosylation in extracellular regions are underlined. Stars indicate four extracellular cysteines and one intracellular cysteine.

The chY4 sequence reveals four glycosylation sites in the amino terminus, two of which are shared with the Y4 receptors in mammals. A consensus glycosylation site in the second extracellular loop has shifted compared with that in mammals.

Pharmacological characterisation of the chY4 receptor

Membranes prepared from COS-7 cells transfected with the expression construct chY4-pTEJ-8 showed specific and concentration-dependent binding of 125 I-PYY. This radioligand identified a single class of binding sites with an affinity constant (K_d) of 21 ± 1 pM ($n=3$) and a B_{max} of 34 ± 3.6 fmol/mg protein for 125 I-pPYY (Fig. 3). As 125 I-pPYY had high affinity for the receptor, and pPYY and hPP were found to have similar affinities in competition experiments, we decided to use 125 I-pPYY for the characterisation of the receptor. In competition experiments, the endogenous peptides of pancreatic polypeptide and PYY from chicken along with hPP, were tested for their ability to inhibit 125 I-pPYY binding. Various porcine derivatives of NPY, PYY and Y-receptor antagonists were also tested. NPY, PYY and pancreatic polypeptide displayed strong inhibition of 125 I-pPYY binding. The mammalian peptides pPYY and hPP were slightly more potent than the chicken orthologues: pPYY had an inhibition constant (K_i) value of 25 ± 5.6 pM and chPYY one of 68 ± 2.8 pM

(Table 1). hPP had a slightly greater affinity than the chicken analogue: 38 ± 0.55 pM compared with 140 ± 39 pM.

NPY had an affinity similar to that of chPP, with a K_i value of 160 pM, whereas the receptor was relatively insensitive to modifications of the NPY molecule. LP-NPY, NPY(2–36) and NPY(13–36) had similar affinities: removal of 12 amino acids from the N-terminus of NPY caused only a fourfold loss of affinity, whereas removal of the next five amino acids (NPY(18–36)) caused a decrease in affinity by 1.5 orders of magnitude. The Y5 agonist p[D-Trp 32]NPY had low affinity, 43 nM, whereas the Y1- and Y2-specific antagonists displayed no affinity for the receptor ($K_i > 1000$ nM).

Localisation of receptor mRNA by RT-PCR and *in situ* hybridisation

The distribution of the receptor was studied by RT-PCR on mRNA isolated from peripheral tissues and brain regions. Y4 mRNA was detected in three of the eight brain regions investigated – brainstem, cerebellum and, to a much lower degree, hippocampus (Fig. 4a). In peripheral organs, Y4 mRNA was more widely expressed, most prominently in adipose tissue, spleen, oviduct and liver (Fig. 4b). Weaker expression was found in the proventriculus and other parts of the gastrointestinal tract, and the lowest expression was in heart, skeletal muscle and kidney. The integrity of

Figure 2 Distance tree of the Y1-subfamily of NPY receptors. Branch lengths correspond to sequence divergence using the neighbour-joining method of the Lasergene DNASTAR Megalign software. A bootstrap value of 100 for the chicken Y4 branch was obtained using PAUP 4.0 software. The human Y2 receptor was used as outgroup to root the tree.

the reverse-transcribed samples was confirmed by amplification, in all samples, of chicken actin mRNA. As a negative control, amplification of the same cDNA samples with primers for chY6

receptor mRNA yielded a markedly different pattern of expression (data not shown). *In situ* hybridisation of chicken brain sections with oligonucleotide probes revealed a prominent signal only in the dorsal motor nucleus of the vagus (DMV) in the brainstem (Fig. 5). No signal was observed in this area after competition with excess unlabelled probe (Fig. 5).

Figure 3 Saturation and Scatchard (inset) analyses of ^{125}I -pPY binding to membranes prepared from COS-7 cells transfected with the expression plasmid chY4-pTEJ-8. Data shown are from a representative experiment. Non-specific binding was defined by 100 nM pNPY.

Discussion

As a part of our efforts to elucidate the evolution of the NPY peptide and receptor families, we have cloned the Y4 receptor in chicken. The chicken is a highly interesting species, for both evolutionary and scientific reasons. We have recently reported the cloning of the chY2 (Salaneck *et al.* 2000) and chY1 and chY5 (Holmberg *et al.* 2002) receptors, all of which are highly conserved in chicken as compared with mammals. In contrast, the Y4 receptor reported here is much less similar to its mammalian orthologues and seems to have retained more of the original features than has Y4 in mammals.

The overall amino acid identity of chY4 is 58% to the human Y4 receptor and 57% to the rat

Table 1 Inhibition of ^{125}I -pPYY binding to membranes from COS-7 cells transfected with the expression plasmid chY4-pTEJ-8. Data represent inhibition constants (K_i) $\pm$ S.E.M. in nM concentrations for two to four experiments performed in duplicate.

Compound	K_i (nM)	<i>n</i>
chPP	0.14 $\pm$ 0.039	4
hPP	0.038 $\pm$ 0.0006	3
chPYY	0.068 $\pm$ 0.003	3
pPYY	0.025 $\pm$ 0.006	3
pNPY	0.16 $\pm$ 0.017	3
p[Leu ³¹ -Pro ³⁴]NPY	0.13 $\pm$ 0.024	3
pNPY(2-36)	0.28 $\pm$ 0.082	3
pNPY(13-36)	0.62 $\pm$ 0.36	3
pNPY(18-36)	10 $\pm$ 3	3
p[D-Trp ³²]NPY	43 $\pm$ 10	2
BIBP 3226	>1000	1
BIIE 0246	>1000	1

For substances displaying no binding, one experiment was performed. Non-specific binding was defined by 100 nM pNPY.

receptor. We have previously reported that Y4 displays remarkably low sequence identity between orders of mammals (Lundell *et al.* 1996, Eriksson *et al.* 1998) and appears to be one of the most rapidly evolving G-protein-coupled peptide receptors (Wraith *et al.* 2000): the overall identity between human and rat Y4 is only 75%, whereas Y1, Y2 and Y5 are about 94% identical between human and rat. Mammals and birds diverged from each other approximately 300 million years ago (Benton 1990) and, given the mammalian sequence divergence, it is not surprising that chY4 is only 57–60% identical to mammals. Phylogenetic analyses place the chicken receptor safely on the Y4 branch (Fig. 2). Furthermore, inclusion of receptor sequences from the coelacanth, *Latimeria chalumnae*,

and the shark, spiny dogfish, recently cloned in our laboratory – both of which seem to be Y4 receptors – makes the position of the chicken receptor even more secure (not shown).

We originally called the Y4 receptor the PP1 receptor, because it was identified as a receptor exhibiting preference for pancreatic polypeptide (Lundell *et al.* 1995, 1996). Pancreatic polypeptide displays significantly greater affinity than NPY and PYY for the human and rat Y4 receptors: it has more than 100-fold greater affinity for the human receptor and more than 10 000-fold greater affinity for the rat receptor. Because PYY has very low affinity for rat Y4, iodinated pancreatic polypeptide was used as a radioligand in its characterisation. The Y4 receptor has been extensively studied by different groups using both iodinated PYY, pancreatic polypeptide and LP-NPY (Gehlert *et al.* 1996*b*, *c*, 1997, Gregor *et al.* 1996, Voisin *et al.* 2000, Berglund *et al.* 2001, Parker *et al.* 2001). Depending on the radioligand used, different affinities have been reported for the ligands in the various studies.

In this study, we used ^{125}I -pPYY as radioligand and found PYY to have high affinity in both saturation and competition experiments. The chicken receptor binds pancreatic polypeptide, NPY and PYY with almost equal affinity (Table 1) and, like the human and guinea pig Y5 receptors (Borowsky *et al.* 1998, Lundell *et al.* 2001), it does not discriminate between these endogenous ligands. Thus the binding profile of chY4 is quite different from that of the mammalian Y4 receptors described previously. Like the Y5 receptors, the chY4 receptor also binds NPY(2–36) and LP-NPY equally well, but chY4 differs in that it is less sensitive to N-terminal truncation of the NPY molecule. In this respect it is more similar to the Y2

Figure 4 PCR-based detection of chY4 receptor mRNA in (a) brain regions and (b) peripheral tissues. Actin served as positive control.

Figure 5 *In situ* hybridisation of ^{35}S -end-labelled oligonucleotides to chY4 receptor mRNA in a transverse section from the chicken hindbrain. A specific hybridisation signal seen in the dorsal motor nucleus of the vagus (DMV) (right) is not observed after competition with excess unlabelled oligonucleotide probes (left).

receptor (Gerald *et al.* 1995, Gehlert *et al.* 1996a): the absence of the first 12 amino acids in NPY(13–36) caused only a modest loss of affinity, whereas a further deletion of amino acids to NPY(18–36) caused a drastic loss of affinity. The broad pharmacological profile of the receptor is also reflected by the slightly greater affinities for pPYY and hPP over the corresponding endogenous peptides.

The rather promiscuous pharmacological profile of the chY4 is reminiscent of the Ya receptor that was cloned in the zebrafish (Starback *et al.* 1999). The zebrafish Ya receptor also binds truncated NPY analogues and the modified peptide LP-NPY with equal affinities. These pharmacological similarities seem to support the possibility that the Ya receptor is most closely related to Y4 among the chicken/mammal receptors Y1, Y4 and Y6. This is also supported by our recent cloning of a lamprey NPY-family receptor, which is most closely related to mammalian Y4 and zebrafish Ya and also displays a broad pharmacological profile (Salaneck *et al.* 2001). This would seem to suggest that both chicken Y4 and zebrafish Ya have retained more of the ancestral binding properties, whereas in mammals Y4 has become more selective for pancreatic polypeptide. After the appearance and rapid

divergence of pancreatic polypeptide in tetrapods, this ligand has coevolved with the Y4 receptor so that, in mammals, NPY and PYY have lost much of their affinity for Y4. Indeed, it would seem that Y4 has evolved less rapidly in chicken – it has a slightly shorter branch in Fig. 2 – but sequence information from more bird species is required to confirm this observation.

The divergence in Y4 receptor sequence and pharmacological properties between orders of mammals and between mammals and chicken also pertains to the expression of the receptor. Although there is a general similarity, in that the Y4 receptor seems to be expressed largely in the periphery, expression in the chicken was detected over a broader range of peripheral tissues, with the greatest expression in adipose tissue, spleen, oviduct and liver and, to a lesser extent, the proventriculus of the gastrointestinal tract. In contrast, the greatest peripheral Y4 expression in mammals is generally observed in the gut (Lundell *et al.* 1995). This indicates that Y4 may serve more varied functional roles in birds than in mammals. In general, the sites of Y4 expression relate well to what is known about the physiology of pancreatic polypeptide in the chicken. For example, the spleen, liver and proventriculus exhibited displaceable binding of

radiolabelled pancreatic polypeptide in an *in vivo* tracer study (Kimmel & Pollock 1981), consistent with our detection of Y4 receptor expression in these organs. In adipose tissue, in which strong Y4 expression was observed, pancreatic polypeptide exerts an antilipolytic effect in the chicken, but not in the rat (McCumbee & Hazelwood 1978). This may be related to the fact that, in contrast to its action in mammals, insulin is not antilipolytic in birds (Langslow & Hales 1969), and pancreatic polypeptide appears to have taken over this function. In the chicken liver, pancreatic polypeptide has been reported to mobilise glycogen (Hazelwood *et al.* 1973). The effects of pancreatic polypeptide on the spleen and oviduct have not been studied in birds, although the presence of Y4 mRNA in these organs is suggestive of a role for the receptor in mediating erythropoietic/immune and smooth muscle functions respectively.

The chicken, in common with the human, expresses Y4 mRNA in the gastrointestinal tract, suggesting that a role for the receptor in mediating gastrointestinal function has been conserved during vertebrate evolution. This view is also supported by the presence of the Y4 receptor in the DMV of the brainstem. This brain region, implicated in the central control of gastrointestinal function, is also a site of Y4 expression in mammals (Larsen & Kristensen 2000). The existence of Y4 mRNA in the gut is consistent with the reported stimulation of gastric secretion by pancreatic polypeptide from the proventriculus in the chicken (Hazelwood *et al.* 1973); this was the site in which the strongest gastrointestinal expression of Y4 was observed. Cells immunoreactive for pancreatic polypeptide and PYY (El-Salhy *et al.* 1982) have been observed in the chicken gut, consistent with a role for these peptides in mediating the effects of Y4 on gastrointestinal function. Binding of avian pancreatic polypeptide to chicken cerebellar membranes has been reported (Inui *et al.* 1992); NPY and PYY also bound here with similar affinities. Our finding of Y4 transcripts in cerebellum (Fig. 4a), along with the pharmacological data above, suggest that all three peptides may bind to Y4 in this region.

Confirmation by *in situ* hybridisation of the results obtained for Y4 mRNA expression in the central nervous system (CNS) by RT-PCR was only obtained for the brainstem, with the observation of specific hybridisation in the DMV. However, no signal was observed in the cerebellum

and hippocampus. This may reflect the sensitivity of the oligonucleotide probe methodology used. We have been unable to increase detection of chNPY receptors by the use of potentially more sensitive riboprobes. However, a more extensive comparative study to map the distribution of all NPY receptor subtypes in the chicken is under way and will involve detection of ³³P-labelled oligonucleotides, and this may reveal Y4 hybridisation in brain regions not identified in the present study. This study will also investigate whether Y4 mRNA is expressed in other regions of potential interest, such as the pituitary gland, which were not examined in the present study.

The greater affinities of the Y4 receptor for PYY and NPY in the chicken compared with that in mammals suggests that its physiological functions may be triggered by PYY and NPY, in addition to pancreatic polypeptide. However, in contrast to knowledge concerning pancreatic polypeptide, little is known about the physiological effects of NPY and PYY in peripheral organs in birds. It is likely that pancreatic polypeptide and PYY stimulate Y4 receptor function in peripheral organs and that central functions are signalled by NPY. However, it will be interesting to see if PYY and pancreatic polypeptide are present in the chicken CNS, as PYY is more abundant in the CNS in fishes (Cerdeira-Reverte & Larhammar 2000, Söderberg *et al.* 2000) and lamprey (Söderberg *et al.* 1994) than in mammals.

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