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Pharmacological characterization of cloned chicken neuropeptide Y receptors Y_1 and Y_5

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Abstract

The neuropeptide Y (NPY) receptor subtypes Y_1 and Y_5 are involved in the regulation of feeding and several other physiological functions in mammals. To increase our understanding of the origin and mechanisms of the complex NPY system, we report here the cloning and pharmacological characterization of receptors Y_1 and Y_5 in the first non-mammal, chicken (*Gallus gallus*). The receptors display 80–83% and 64–72% amino acid sequence identity, respectively, with their mammalian orthologues. The three endogenous ligands NPY, peptide YY (PYY) and pancreatic polypeptide (PP) have similar affinities as in mammals, i.e. NPY and PYY have subnanomolar affinity for both receptors whereas chicken PP bound with nanomolar affinity to Y_5 but not to Y_1. A notable difference to mammalian receptor subtypes is that the Y_1 antagonist SR120819A does not bind chicken Y_1, whereas BIBP3226 does. The Y_5 antagonist CGP71863A binds to the chicken Y_5 receptor. Anatomically, both Y_1 and Y_5 have high mRNA expression levels in the infundibular nucleus which is the homologous structure of the hypothalamic arcuate nucleus in mammals. These results suggest that some of the selective Y_1 and Y_5 antagonists developed in mammals can be used to study appetite regulation in chicken.

Keywords: antagonists, chicken, G protein-coupled receptor, *in situ* mRNA hybridization, neuropeptide Y.

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Neuropeptide Y (NPY) is a 36 amino acid long C-terminally amidated peptide which is abundant and widespread in the central and peripheral nervous systems of all vertebrates so far studied (McDonald 1988). Physiological studies and generation of the NPY knock-out mouse have implicated NPY in feeding, blood-pressure regulation, anxiety, memory, susceptibility to seizures and regulation of reproduction (Clark *et al*. 1984; Flood *et al*. 1987; Heilig *et al*. 1989; Erickson *et al*. 1996; Kalra *et al*. 1998; Bannon *et al*. 2000), as reviewed by Kalra *et al*. (1999) and Michel *et al*. (1998). The great variety of physiological responses to NPY is mediated through G-protein-coupled receptors. So far, five different subtypes (Y_1, Y_2, Y_4, Y_5 and y_6) have been cloned in mammals (Michel *et al*. 1998; Larhammar *et al*. 2001).

The chicken (*Gallus gallus*) is an important domesticated animal which is studied for scientific as well as commercial reasons, particularly with regard to development, reproduction, growth and metabolism. NPY induces feeding when injected intracerebrally in broiler chicks (*Gallus gallus*) (Kuenzel *et al*. 1987) and NPY has been shown to be up-regulated in feed-restricted broilers (Boswell *et al*. 1999). These results imply an important role of NPY in feeding in chicken as in mammals and virtually all other vertebrates investigated.

The Y_1 receptor is the most studied NPY receptor subtype. It has mainly a postsynaptic localization and couples to adenylate cyclase in an inhibitory fashion (Larhammar *et al*. 1992) or through elevation of intracellular calcium depending on cell type (Herzog *et al*. 1992). The Y_5 receptor is the most recently cloned NPY receptor subtype. It displays less than 35% identity to other NPY receptor subtypes and it too

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The chicken Y_1 and Y_5 nucleotide sequences have been submitted to GenBank with accession numbers AY040845 (chY_1) and AY040844 (chY_5).

Abbreviations used: BAC, bacterial artificial chromosome; ch, chicken; h, human; HEK, human embryonic kidney; NPY, neuropeptide Y; p, porcine; PP, pancreatic polypeptide; PYY, peptide YY; r, rat; SDS, sodium dodecyl sulfate; SSC, standard sodium citrate; TM, transmembrane.

couples to the inhibition of adenylate cyclase (Michel *et al*. 1998). In mammals, the Y_1 and Y_5 receptor subtypes seem to be of major importance in the hypothalamic regulation of appetite (Kalra *et al*. 1999). Studies in rat and mouse show that especially Y_1, but also Y_5, plays a role in energy homeostasis (Marsh *et al*. 1998; Pedrazzini *et al*. 1998; Kanatani *et al*. 2000; Polidori *et al*. 2000). In humans, the Y_1 and Y_5 receptor genes are transcribed in opposite directions from the same region of chromosome four and have overlapping promoter regions, observations that imply a common transcriptional regulation (Herzog *et al*. 1997). Indeed, receptor mRNA distribution studies show that Y_5 has overlapping expression patterns with Y_1 in the rat central nervous system (Parker and Herzog 1999).

We have recently reported the molecular cloning of Y_2 in chicken (Salaneck *et al*. 2000). In this study, we describe the first cloning and pharmacological characterization of non-mammalian Y_1 and Y_5 receptor subtypes. [The previously cloned *Xenopus laevis* Y_1 receptor was not analysed pharmacologically (Blomqvist *et al*. 1995)]. Comparisons of sequences and pharmacological properties provide insights into the evolutionary relationships among the NPY receptors and give information on the molecular binding properties of chicken as well as mammalian receptors. To facilitate future *in vivo* studies in chicken with NPY receptor agonists and antagonists that were developed in mammals, it is first necessary to perform binding studies *in vitro* on the cloned chicken receptor subtypes. The data presented identify pharmacological tools to study the functional roles of the NPY system *in vivo* in chicken.

Materials and methods

Isolation and sequencing of the chicken Y_1 receptor

Degenerate PCR primers were designed from sequence alignments of all known Y_1 receptor sequences. Primers were Y1G2F and Y1G1R (Berglund *et al*. 1999) which are directed to trans-membrane (TM) regions 2 and 7 of the Y_1 receptor gene. The primers were used to obtain a PCR product from genomic chicken DNA (White Leghorn). Touchdown PCR was performed using DNA polymerase AmpliTaq Gold (Perkin-Elmer, Stockholm, Sweden). PCR cycles were as follows: First 20 cycles: denaturation at 94°C for 20 s, annealing at 55–45°C (lowered by 0.5°C/cycle) for 30 s, elongation at 72°C for 30 s; then 25 cycles: denaturation at 94°C for 30 s, annealing at 44°C for 30 s, elongation at 72°C for 30 s. The 800 bp long PCR product was used as probe, to screen a genomic chicken bacterial artificial chromosome (BAC) library (Zimmer and Verrinder Gibbins 1997) in order to isolate a full-length clone. Fifty thousand clones from the library have been gridded in duplicate on nylon filters in high-density format. The library was screened with ^{32}P-labelled probe (Megaprime™ DNA labelling system, Amersham, Uppsala, Sweden). Hybridizations were performed for 16 h at 60°C in 50% formamide and 6 × standard sodium citrate (SSC). Washes were done at 60°C in 0.2 × SSC and 0.1% sodium dodecyl sulfate (SDS). Positive BAC clones were sequenced by primer walking to obtain the full-length chicken (ch)Y_1 receptor sequence.

Isolation and sequencing of the chicken Y_5 receptor

All BAC clones isolated in the screening with the Y_1 probe were also positive in Southern hybridizations using a pig Y_5 receptor probe. Positive BAC DNA *Bgl*II restriction fragments were subcloned into a *Bam*HI-digested pUC18 plasmid. The clones were screened with the pig Y_5 probe at intermediate stringency at 55°C in 25% formamide, 6 × SSC, 10% dextran sulfate, 5 × Denhardt's solution and 0.1% SDS, for 16 h. Filters were washed twice for 30 min at room temperature (20°C) in 2 × SSPE/0.1% SDS (20 × SSPE: 3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA pH 7.7) twice for 30 min at 55°C in 2 × SSPE/0.1% SDS, and once for 45 min at 55°C in 0.5 × SSC/0.1% SDS. Autoradiographic film (Hyperfilm, MP, Amersham, UK) was exposed to the filter for 2.5 h. Positive clones were sequenced, and generated the full-length sequence for the chY_5 receptor.

Receptor sequence alignments and tree construction

The full-length amino acid sequences were aligned using Clustalw1.7 (Thompson *et al*. 1994) to all known Y_1, Y_5 and Y_2 sequences as well as the human (h)Y4, mouse y_6 and the *Lymnea stagnalis* NPY receptor sequences. Also, trans-membrane (TM) regions were aligned by hand in Megalign (Lasergene, DNASTAR, CA, USA). Trees were calculated for maximum parsimony using PAUP* 4.04 (Swofford 1998; Sinauer Ass. Inc., Sunderland, MA, USA) and for character-state evolution using MacClade 4.0 (Maddison and Maddison 2000; Sinauer Ass. Inc.). Parsimony analyses were conducted using heuristic searches, random addition sequence with 1000 replicates, tree-bisection-reconnection branch-swapping, and MULPARS options in effect, and branches having maximum length zero collapsed to yield polytomies. Tree lengths include only character-state changes optimized unambiguously. All amino acid substitutions, and insertions and/or deletions (gaps) were weighted equally, and characters analyzed unordered. Analyses of full-length and TM sequences yielded similar results.

Cloning into expression vector

For the chY_1 receptor PCR primers were designed, matching the 5′-and 3′-untranslated regions immediately flanking the coding region. A PCR reaction was run with proof-reading enzyme PLATINUM® *Pfx* DNA Polymerase (Life Technologies, Stockholm, Sweden) and the PCR product was gel-purified with QIAquick Gel Extraction Kit (QIAgen, Stockholm, Sweden). The PCR product was cloned using the Gateway™-system, according to manufacturer's recommendations (Life Technologies). Briefly, the PCR primers included recombination recognition sites (bold letters) for modified phage lambda recombination enzymes: cY_1GW.attB1.Fwd1: 5′-GGGG**ACAAGTTTGTACAAAAAAGCAGGCT**AACATGA-ATGCTTCGGTTCTTGAT-3′, cY_1GW.attB2.Rev1: 5′-GGGG**ACC-ACTTTGTACAAGAAAGCTGGGT**ACGCTTCAAGGTTTGG-TGTAGAT-3′. The PCR product was cloned into an expression vector, via a donor vector (Gateway™ pDONR™201 Vector, Life Technologies) using modified phage lambda enzymes (Life Technologies). The expression vector was a modified pCEP4 plasmid

(Invitrogen, Groningen, the Netherlands), with an intron from pCI-neo (Promega, Falkenberg, Sweden) located 5′ to the cloning site. This construct has previously been shown to enhance gene expression from the cytomegalovirus promoter (Marklund *et al*. 2002). Also, the vector contained a Gateway™ cloning cassette, obtained by using the Gateway™ Vector Conversion Reagent System (Life Technologies), with a ccdB-gene (confers cell-death when expressed in *Escherichia coli*) and recognition sites for modified lambda recombinases. The cloned PCR product in the expression vector was fully sequenced, and found to be identical with the genomic sequence.

For the chY5 receptor, the forward primer was located in the 5′-untranslated region, just before the start codon and contained a recognition site (bold letters) for restriction enzyme *Hind*III *(*cY5HindIII.Fwd1: 5′-GCGGGC**AAGCTT**ATGGATTTAGGATTCAAAGA-3′). The reverse primer, that was located over the stop codon, had the recognition site (bold letters) for restriction enzyme *Bam*HI (cY5BamHI.Rev1: 5′-GGCG**GGATCC**CTACTAATCACAACCAAATC-3′). The PCR reaction using proofreading PLATINUM® *Pfx* DNA Polymerase (Life Technologies) was purified using a QIAquick PCR Purification Kit (Qiagen), cut with *Hind*III (Amersham, Uppsala, Sweden) and *Bam*HI (Amersham), and the 1.3 kilobase pair fragment was purified on a 1% agarose Tris Borate EDTA gel using the QIAquick Gel Extraction Kit and ligated into the expression vector pCEP4 which also contained the pCI-neo vector intron sequence 5′-to the cloning site. The vector had been cut and purified with *Hind*III/*Bam*HI. The generated receptor clone was completely sequenced and found to be identical with the genomic sequence.

Transfection protocol

For transient transfections human embryonic kidney (HEK) 293 EBNA-1 cells were transfected with FuGENE™ 6 Transfection Reagent (Boehringer Mannheim, Biberach an der Riss, Germany), diluted in Optimem medium (Gibco BRL, Stockholm, Sweden) according to the manufacturer's recommendations. After transfection, cells were grown in Dulbecco's MEM/Nut Mix F-12 without L-glutamine (Gibco BRL) containing 10% fetal calf serum (Biotech Line AS, Slangerup, Denmark), 2.4 mM L-glutamine (Gibco BRL) and 2.5 mg/mL G-418 (Gibco BRL), 100 units of penicillin/100 μg streptomycin/mL (Gibco BRL) until harvesting, after 48 h. Cell membrane pellets were frozen in aliquots at −80°C. Cells with semistable expression were selected for by growth in the presence of 100 μg/mL hygromycin, starting three days after transfection, in the same growth medium as described above. Receptor expression was confirmed by radioligand binding.

Peptides and non-peptide ligands

Porcine (p)NPY, p[Leu31,Pro34]NPY, pNPY(2–36), pNPY(3–36), pNPY(13–36), pNPY(18–36), hPP and rat (r)PP were purchased from Bachem, King of Prussia, PA, USA, p[D-Trp32]NPY from Peninsula Laboratories Inc., CA, USA. The non-peptidic Y_1 antagonist BIBP3226 (Rudolf *et al*. 1994) and the non-peptide Y_2 antagonist BIIE0246 (Doods *et al*. 1999) were provided by Boehringer-Ingenheim PharmaKG, Biberach an der Riss, Germany. The non-peptide Y_1 antagonist SR120819A (Serradeil-Le Gal *et al*. 1995) was provided by Sanofi, France. Chicken PP and chPYY were purchased from Schafer-N, Copenhagen, DK. The non-peptide Y5 antagonist CGP71863A (Criscione *et al*. 1998) was provided by Dr Jarl Wikberg, Department of Pharmaceutical Pharmacology, Uppsala University, Sweden.

Binding assays

The thawed aliquots of membranes were resuspended in 25 mM HEPES-buffer (pH 7.4) containing 2.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 2 g/L bacitracin and homogenized using an Ultra-Turrax homogenizer. Saturation experiments were performed in a final volume of 100 μL with 0.8–1.1 μg protein for chY_1, or 2.1–2.5 μg protein for chY_5, and [^{125}I]pPYY (Amersham) for 2 h at room temperature. This radioligand had iodinated tyrosines at 21 and 27 and had a specific activity of 4000 Ci/mmol. Saturation experiments were carried out with 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. Competition experiments were performed in a final volume of 100 μL. Various concentrations of the peptides pNPY, hPP, rPP, chPP, chPYY, p[Leu31,Pro34]NPY, pNPY(2–36), pNPY(3–36), pNPY(13–36), pNPY(18–36), p[D-Trp32]NPY, two non-peptide Y_1 antagonists SR120819A and BIBP3226, a non-peptide Y_2 antagonist BIIE0246, and a non-peptidic Y_5 antagonist CGP71863A were included in the incubation mixture along with [^{125}I]pPYY. Incubations were terminated by filtration through GF/C filters (Filtermat A from Wallac Oy, Turku, Finland), which had been presoaked in 0.3% polyethyleneimine, using a TOMTEC (Orange, CT, USA) cell harvester. The filters were washed with 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 Microbeta counter. The results were analyzed using the PRISM 2.0 software package (Graphpad, San Diego, CA, USA). Protein concentrations were measured using Bio-Rad Protein Assay (Bio-Rad, Solna, Sweden) with bovine serum albumin as standard.

In situ hybridization

Animal procedures were in accordance with United Kingdom Home Office regulations. Brains from four Bantam chickens (two male, two female; Roslin Institute flock, Roslin, Midlothian, UK) were processed for *in situ* hybridization. The birds were maintained on a photoperiod of 14L10D and had free access to food and water. 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, Hemel Hempstead, Hertfordshire, UK) 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 buffer (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 hybridization solution. For each of the chY_1 and chY_5 receptors, a mixture of two oligonucleotide probes complementary to the respective mRNA sequence was used for *in situ* hybridization. The oligonucleotide sequences for the Y_1 receptor were: 5′-GGAAGACACATGATGGTCACTAGAAGGTCAGAAAAGGACAGGTTGA-3′ and 5′-TGCCAAGATAAGCAAACATC-

AGACAAAAAGAAGTATATTTCACAGAGG-3′. For the Y_5 receptor, the oligonucleotide sequences were: 5′-CACCGATCAAGGAGTACAGACGTCAGCGTGAAAGGAGAACAAA-3′ and 5′-TGCATGAGCAGTAAGGATATGGTAAAGGCAATTCTGTATGAGTCAG-3′. The probes were labelled at the 3′ end with [^{35}S]dATP using terminal deoxynucleotidyl transferase (Amersham, Little Chalfont, Buckinghamshire, UK) and were purified through QIAquick spin columns (Qiagen, Crawley, West Sussex, UK). Specific activity of the probes was 10^8 cpm/μg oligonucleotide. Hybridization buffer consisted of 50% formamide, 4 × SSC, 10% dextran sulfate, 1% *N*-laurylsarcosine, 1 × Denhardt's solution, 200 mM dithiothreitol, and 0.1 mg/mL yeast tRNA. A 100-μL aliquot of hybridization solution containing 3 ng of each labelled probe was applied to each section and covered with a parafilm coverslip. Control sections received hybridization solution containing a 100-fold excess of each unlabelled probe. Hybridization was performed for at least 16 h in humidified boxes at 42°C. After hybridization, 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, Gloucestershire, UK). The sections were not counterstained because the avian infundibular nucleus, which was the main area of this study along with other parts of the hypothalamus, is not easily defined by Nissl staining (Kuenzel and van Tienhoven 1982).

Results

The chicken (*Gallus gallus*) Y_1 and Y_5 receptor-genes were isolated from a BAC library by screening a chY_1 probe generated by PCR from genomic DNA with primers based on mammalian and *Xenopus laevis* Y_1 sequences. It is noteworthy that the chY_1 receptor gene was exceedingly difficult to clone in plasmid vectors and several cloning methods were tested before the Gateway system finally yielded a full-length clone for functional expression. The open reading frame of the chY_1 receptor is 385 amino acids and that of Y_5 is 443 amino acids. The four extracellular cysteines in the Y_1 receptor probably form two disulfide bonds and one cysteine pair presumably links extracellular loops 1 and 2 of the Y_5 receptor. Also, there is one cysteine in the carboxy terminal tail of Y_1 and two in Y_5 which presumably serve as anchors to the membrane through palmitoylation. Close to the amino terminus of each receptor there are putative glycosylation sites. In accordance with other Y_1 receptor genes, the chY_1 nucleotide sequence has an intron between the codons of

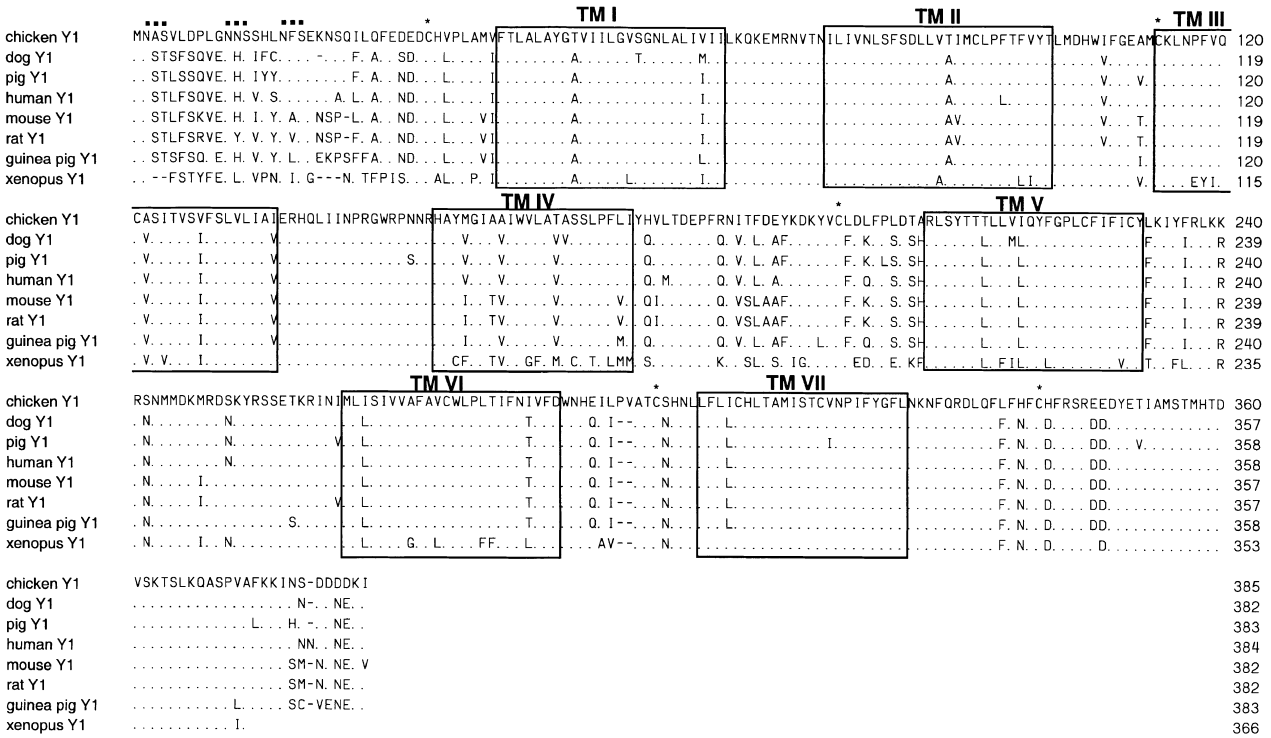

Fig. 1 Amino acid alignment. The chicken Y_1 receptor serves as master sequence in alignment with the dog, pig, human, mouse, rat, guinea pig and frog Y_1 receptors. GenBank accession numbers are dog: AF005778, pig: AF005779, human: M84755, mouse: Z18280, rat: Z11504, guinea pig: AF135061 and frog (*Xenopus laevis*): L25416. In latter sequences only positions that differ from the chicken Y_1 receptor sequence are shown while dots represent identities. Dashes indicate gaps introduced to optimize alignment. The hydrophobic segments assumed to be embedded in the cell membrane are within boxes. Tripeptides in extracellular parts marked with dotted lines conform to the consensus sequence for *N*-linked glycosylation. Stars show four extracellular cysteines and one intracellular cysteine.

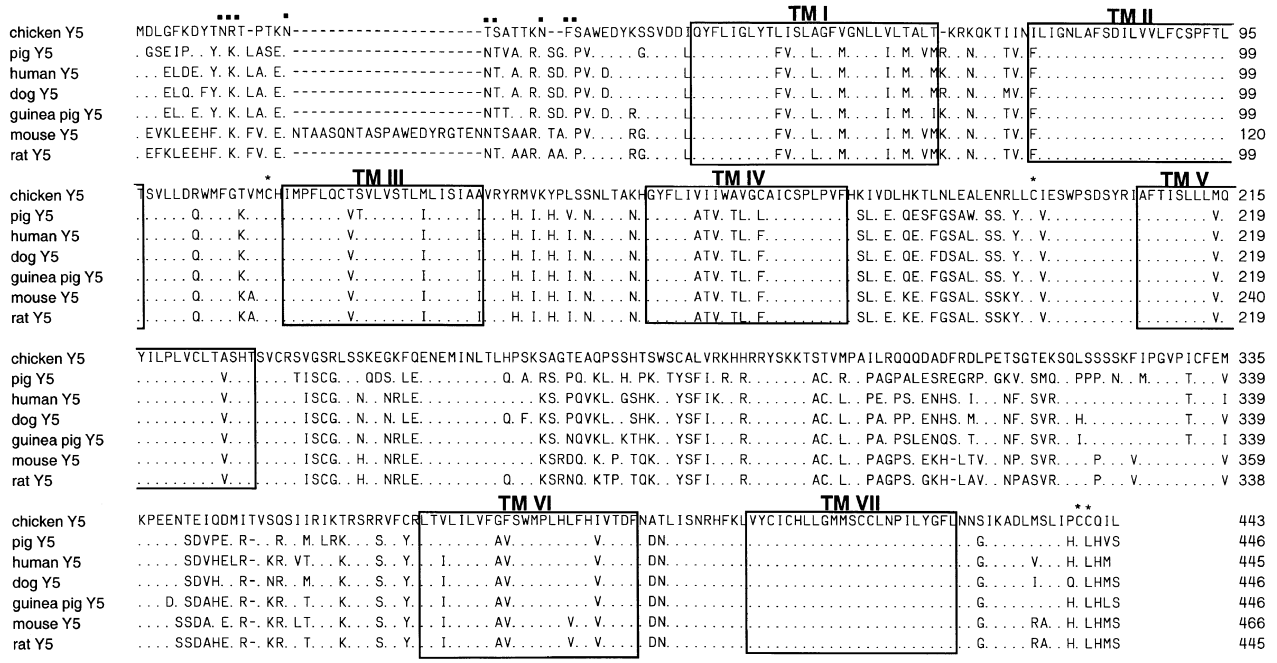

Fig. 2 Amino acid alignment. The chicken Y_5 receptor serves as master sequence in alignment with the pig, human, dog, guinea pig, rat and mouse Y_5 receptors. GenBank accession numbers are pig: AF106083, human: U56079, dog: AF049328, guinea pig: AF363240, rat: U56078 and mouse: AF022948. In latter sequences only positions that differ from the chicken Y_5 receptor sequence are shown while dots represent identities. Dashes indicate gaps introduced to optimize alignment. The hydrophobic segments assumed to be embedded in the cell membrane are within boxes. Tripeptides in extracellular parts marked with dotted lines conform to the consensus sequence for *N*-linked glycosylation. Stars show one extracellular cysteine and six intracellular cysteines.

amino acids K233 and I234. The chY_1 full-length receptor displays 80–83% amino acid identity to mammalian receptors and full-length chY_5 displays 64–72% amino acid identity to mammalian receptors. Comparing only the putative TM regions of the Y_1 receptors gives 90–92% amino acid identity to mammalian receptors. The chY_5 receptor TM regions have 85–86% amino acid identity to mammalian receptors, as calculated by the DNASTAR Software. (For alignments, see Figs 1 and 2) The phylogenetic tree shown in Fig. 3 was calculated using PAUP*4.04 and confirms that the two chicken receptors are orthologues of the mammalian Y_1 and Y_5 receptor subtypes, respectively.

The cloned chY_1 receptor, including the endogenous intron, and the Y_5 receptor were expressed separately in the mammalian cell line HEK 293 EBNA-1. Our laboratory has previously used this expression system for mammalian Y_1 and Y_5, both of which have been difficult to express in other systems, and obtained pharmacological data in good agreement with other cell lines. *In vitro* binding studies were performed using [^{125}I]pPYY as radioligand. Saturation binding assays of the Y_1 receptor gave a K_d of 0.032 nM (SEM = 0.0075 nM, $n = 3$), and a B_{max} of 608 fmol/mg protein (SEM = 201 fmol/mg protein, $n = 3$). Saturation assays of the Y_5 receptor gave a K_d of 0.113 nM (SEM = 0.0101 nM, $n = 3$), and a B_{max} of 181 fmol/mg protein (SEM = 5.508 fmol/mg protein, $n = 3$). Competition studies were done with a large panel of ligands (Table 1) including chPYY and chPP as well as several antagonists. The rank order of affinities of the ligands for the chY_1 receptor was; pNPY = p[Leu31,Pro34]NPY > chPYY > BIBP3226 = pNPY(2–36) = pNPY(3–36) = pNPY(13–36) >> SR120819A, BIIE0246, chPP, hPP, rPP, p[D-Trp32]NPY, CGP71863A. For the chY_5 receptor the rank order was; p[Leu31,Pro34]NPY, pNPY, CGP71863A = pNPY(3–36), pNPY(2–36) = hPP = chPYY = p[D-Trp32] NPY = rPP > pNPY(18–36) = chPP >> BIBP3226, SR120819A. The highest ligand affinities were in the range of 0.2–0.8 nM for both receptors ($pK_i = 9.7$–9.1). Untransfected cells gave no specific binding.

The cells with semistable expression of either chY_1 or Y_5 were investigated for NPY-mediated inhibition of forskolin-induced cAMP synthesis. However, no inhibition was observed. Nor could cAMP synthesis be induced when cells were treated with pNPY only. A [^{35}S]GTPγS binding assay indicated no activation of G-protein except for the positive control run in parallel, the hY_2 receptor. Also, the chY_2 receptor has been shown to inhibit cAMP production when expressed in the same cell system (Salaneck *et al.* 2000). Perhaps the signal transduction machinery of this heterologous expression system is incompatible with the chicken Y_1 and Y_5 receptors. The HEK 293 EBNA-1 system was chosen because Y_1 and Y_5 receptors have been found to have low expression levels in less powerful systems.

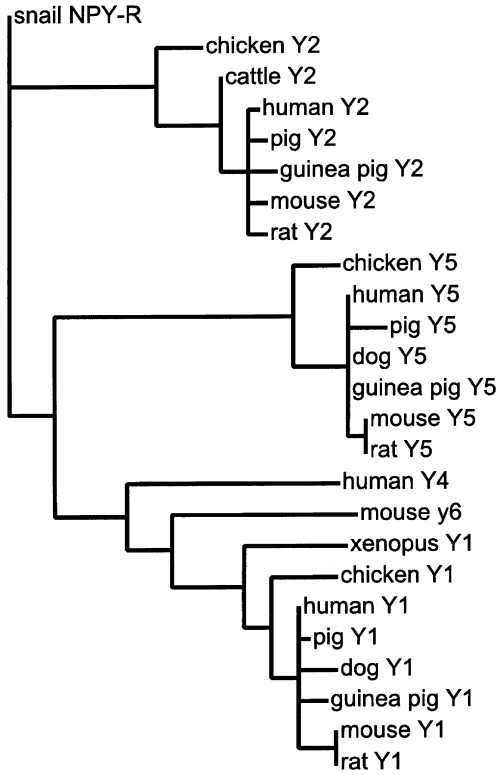

Fig. 3 Results of a maximum parsimony analysis of the TM region amino acid sequences for Y_1, Y_2, Y_5, Y_4 and y_6 receptors. Slightly shorter TM regions were defined for this analyses than for the alignments in Figs 1 and 2 because of the heterogeneity of TM regions between receptor subtypes. Phylogram: Branch-lengths are proportional to number of evolutionary changes. Tree topology is a strict consensus of 140 equally parsimonious trees (after branch-swapping on 1000 random addition sequence replicates), each of length = 466, consistency index = 0.85, rescaled consistency index = 0.79. The snail (*Lymnea stagnalis*) NPY receptor was used as an outgroup. GenBank accession numbers: Y_1: see legends to Figs 1 and 2, Y_2: human: U42766, cow: U50144, pig: AF005780, guinea pig: AF072821, rat: AY004257, mouse: D86238, chicken: AF309091, Y_4: human: Z66526, y_6: mouse: NM_010935, snail NPY receptor (*Lymnea stagnalis*): CAA57620.

In situ hybridization with antisense oligonucleotide probes to the mRNAs of the chY_1 and chY5 receptors revealed expression of both receptor mRNAs in the infundibular nucleus of the hypothalamus (Fig. 4). A stronger signal was obtained for the Y_5 receptor (Fig. 4c) than for the Y_1 receptor (Fig. 4a). For both receptors, no signal was observed in the infundibular nucleus after competition with excess unlabelled probe (Figs 4b and d).

Discussion

Both of the chicken NPY receptor subtypes Y_1 and Y_5 were found to display high sequence identity to their orthologues in mammals. The chY_1 receptor has 80–83% overall sequence identity to mammalian Y_1 with 90–92% identity in the TM regions. Chicken Y_1 has all the structural features of mammalian Y_1 such as cysteine residues and consensus sequences for glycosylation (Fig. 1). Nevertheless, there are some differences in ligand affinities as compared with Y_1 in mammals as detailed below. Saturation binding with iodinated pPYY gave a K_d of 0.032 nM. Chicken PYY has an extra alanine at the N-terminus compared with PYY in all mammals that have been investigated and also differs in several other positions (12 differences compared with pPYY) (Conlon and O'Harte 1992). It binds with a 10-fold lower affinity to the chY_1 receptor ($pK_i = 8.29$, $K_i = 5.2$ nM) than does pNPY ($pK_i = 9.67$, $K_i = 0.22$ nM). Chicken NPY differs from pNPY at only two positions (amino acid 7 and the highly variable position 17) that are unlikely to influence binding properties (Blomqvist *et al*. 1992) and was therefore not synthesized for binding studies. The Y_1 receptor in mammals is characterized by its requirement for intact NPY and a rapid loss of affinity by N-terminally truncated peptides. This is true also for the chY_1 as pNPY(2–36) binds with a pK_i of 7.61 ($K_i = 24.8$ nM) which is a 100-fold drop in affinity compared with intact pNPY. Thus, the chY_1 receptor probably interacts with the N-terminus of the peptide ligands similarly to the mammalian receptors. This would also explain why chPYY with its additional N-terminal residue has lower affinity. The extra amino acid may also account for the weaker affinity of chPYY to chY_1 than to chY_2 ($pK_i = 9.20$, $K_i = 0.6$ nM) (Salaneck *et al*. 2000) and Y_5 (see below) whereas in mammals PYY usually binds with a similar affinity as NPY to all three receptor

Table 1 pK_i values determined in competition assays for various ligands, for the chicken Y_1 and Y_5 receptor

	pK_i (– log *M*) ± SEM	
Ligand	chY_1	chY_5
chPYY	8.29 ± 0.047	9.21 ± 0.079
pNPY	9.67 ± 0.112	9.64 ± 0.054
pNPY(2–36)	7.61 ± 0.035	9.46 ± 0.117
pNPY(3–36)	7.41 ± 0.083	9.51 ± 0.048
pNPY(13–36)	7.05 ± 0.017	N.D.
pNPY(18–36)	N.D.	8.78 ± 0.114
p[Leu31,Pro34]NPY	9.38 ± 0.082	9.67 ± 0.070
rPP	< 5.0	9.09 ± 0.045
hPP	< 5.0	9.32 ± 0.089
chPP	< 5.0	8.33 ± 0.035
BIBP3226	7.80 ± 0.093	< 5.0
SR120819A	< 5.0	< 5.0
p[D-Trp32]NPY	< 5.0	9.11 ± 0.039
BIIE0246	< 5.0	N.D.
CGP71863	< 5.0	9.62 ± 0.123

Radiolabelled competitor is [^{125}I]pPYY. Non-binders were determined as $pK_i < 5.0$. For all competitor assays $n = 3$, except for non-binders where $n = 2$. N.D. = not determined.

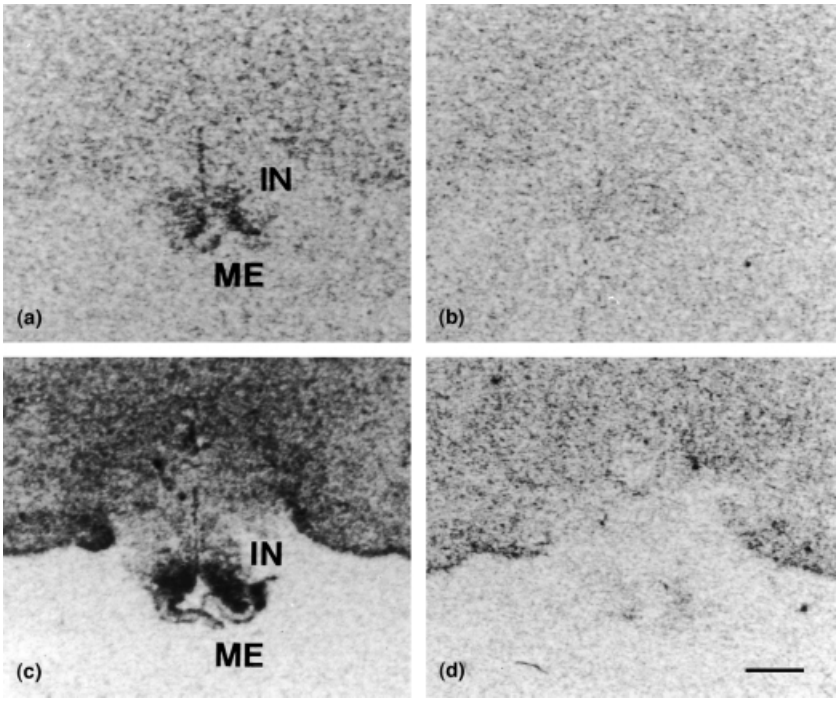

Fig. 4 *In situ* hybridization of ^{35}S-labelled oligonucleotides to chicken Y_1 (a,b) and Y_5 (c,d) receptor mRNA in coronal chicken brain sections. For both receptors, a specific hybridization signal (a,c) was observed in the hypothalamic infundibular nucleus (IN), lying above the median eminence (ME) in the medio-basal region of the hypothalamus. No signal was present in this nucleus in an adjacent section (b,d) after competition with the respective unlabelled nucleotide probes. Scale bar = 1 mm.

subtypes. This might be of importance in situations where PYY may compete with NPY. However, *in vivo* NPY predominates in the brain while PYY is presumably more abundant than NPY in the gastrointestinal tract, at least in mammals. The higher chPYY affinity to chY_2 than to chY_1 may suggest that chY_2 is the main mediator of PYY actions in the gut, at least at low concentrations. For the other tested modified and/or truncated NPY fragments, and for the Y_2 antagonist BIIE0246, the order of ligand affinities is similar to those of mammalian receptors.

Two established Y_1 antagonists that bind to Y_1 in all mammals tested to date (Michel *et al*. 1998), SR120819A and BIBP3226, exhibited great differences in affinity to chY_1. SR120819A does not bind to the chY_1 receptor in the concentration range tested, while BIBP3226 binds chY_1 with a pK_i of 7.80 (K_i = 16.8 nM). A previous mutagenesis study (Sautel *et al*. 1996) identified amino acids important for BIBP3226 binding to the hY_1 receptor. All of these amino acids are also conserved in chicken (Y211, W163, F173, Q219, N283, D287, and F286). Considering the amino acid divergence between the chicken and mammalian receptors, the loss of affinity to SR120819A is not completely surprising. Interestingly, this implies that the sites of interaction with the receptor are at least partially distinct for the two Y_1 antagonists although both were developed by mimicking the C-terminus of NPY. The chicken receptor sequence may prove helpful for structural modelling of ligand–receptor interaction, particularly since no structural binding model has yet been published for SR12089A. We are currently investigating the different binding properties of the two Y_1 antagonists by ligand-receptor computer-aided modelling and mutagenesis studies.

The chY_5 receptor displays 64–72% identity to mammalian Y_5 receptors and shows the same basic features (Fig. 2). The lower degree of overall identity than Y_1 is primarily due to greater variability in the large third intracellular loop. The TM regions of Y_5 are almost as highly conserved as those of Y_1 with 85–86% identity. Chicken PYY as well as pNPY, pNPY(2–36) and pNPY(3–36) all bind to chY_5 with affinities in the range of 0.1–0.6 nM (Table 1). The modified peptide p[Leu31,Pro34]NPY has high affinity to chY_5 as well as to chY_1. Rat PP binds to the chY5 receptor (pK_i = 9.09, K_i = 0.8 nM), although it does not bind to the rat Y_5 receptor (Borowsky *et al*. 1998). Chicken PP binds with a lower affinity to the chY_5 receptor (pK_i = 8.33, K_i = 4.7 nM), than does both hPP (pK_i = 9.32, K_i = 0.5 nM) and rPP (pK_i = 9.09, K_i = 0.8 nM) but probably within the physiological range, provided that PP reaches the anatomical sites of Y_5 expression. The rat Y_5 receptor is expressed mainly in the central nervous system with a very restricted expression in the periphery (Gerald *et al*. 1996; Goumain *et al*. 1998), while PP is most abundant in the periphery (Pieribone *et al*. 1992). In analogy with the rat, endogenous chPP seems to have a somewhat lower affinity for the chicken Y_5 receptor (Gerald *et al*. 1996). Taken together this suggests that PP is not an important ligand of the Y_5 receptor subtype in rat and chicken. The Y_5 antagonist CGP71863A bound with a pK_i of 9.62 (K_i = 0.2 nM), which is similar to the affinity to the human receptor of 1.4 nM or pK_i 8.85 (Criscione *et al*. 1998; Duhault *et al*. 2000). Thus,

this compound may be used in studies *in vivo* to explore the role of Y_5 in appetite regulation in chicken. However, cross-reactivity with non-NPY receptors must be investigated since this compound binds to a muscarinic receptor in rat (Zuana *et al*. 2001). Also, the affinity of p[D-Trp32]NPY is higher than that reported in mammals (Borowsky *et al*. 1998; Lundell *et al*. 2001) with a pK_i of 9.11 (K_i = 0.8 nM).

The avian infundibular nucleus is the structural equivalent of the mammalian hypothalamic arcuate nucleus, a site in which Y_1 and Y_5 expression has been reported in rat, mouse, sheep (Y_1), and human (Y_1) (Mikkelsen and Larsen 1992; Jacques *et al*. 1996; Dyer *et al*. 1997; Naveilhan *et al*. 1998; Broberger *et al*. 1999; Nichol *et al*. 1999; Parker and Herzog 1999). In mammals, the arcuate nucleus is the main site of synthesis of NPY in the hypothalamus (Morris 1989). This also appears to be the case in chicken as NPY-like-immunoreactive cell bodies (Kuenzel and Fraley 1995) and NPY mRNA (T. Boswell, unpublished observations) are present in the infundibular nucleus. We detected mRNA for both Y_1 and Y_5 in this structure, thus indicating overall conservation of the anatomical distribution (Fig. 4). More detailed studies in parallel with other appetite-regulating peptides and receptors will be necessary to see if the neuronal circuitry is the same as in mammals. A more extensive mapping of chicken Y_1, Y_2, Y_4, Y_5 and y_6 mRNA distribution is underway, all of which have recently been cloned in our laboratory (Salaneck *et al*. 2000; Lundell *et al.* 2002; R. Fredriksson, E. Salaneck and D. Larhammar, unpublished results).

The general conservation of binding properties and neuro-anatomical localization of the chicken Y_1 and Y_5 receptors with their mammalian counterparts suggest that the functions of these receptors are likely to have been conserved during amniote evolution. However, physiological responses to endogenous ligands could be somewhat different due to the lower affinity of chPYY for Y_1, but not for Y_2 or Y_5. Also, the exact roles of each receptor subtype in the regulation of food intake will require detailed physiological and pharmacological experiments, and these mechanisms have still not been completely resolved even in mouse or rat despite extensive studies. The effects of NPY on the reproductive axis in the rat may be mediated through Y_1 and Y_5 receptors (Kalra *et al*. 1998; Jain *et al*. 1999; Raposinho *et al*. 1999) and these receptors could perform a similar function in the chicken because NPY-like-immunoreactive neurons in the infundibular nucleus have been implicated in the control of sexual maturation (Walsh and Kuenzel 1997).

In conclusion, the chicken NPY receptors Y_1 and Y_5 display a high degree of sequence identity with their mammalian orthologues despite divergence from mammals some 300 million years ago. The binding properties of endogenous ligands in chicken are similar to those in mammals and both receptors are expressed in the hypothalamus, suggesting that the effects on feeding, metabolism and reproduction by NPY and possibly PYY may be similar in birds and mammals. PP seems to be a less important endogenous ligand for chicken Y_5 than NPY and PYY. The Y_1 antagonist SR12089A cannot be used in chicken, but the subtype-selective antagonists BIBP3226 (Y_1) and CGP71863A (Y_5) can possibly be used for *in vivo* studies in chicken.

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