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Citation for published version:
Joseph, NT, Morgan, K, Sellar, R, McBride, D, Millar, RP & Dunn, IC 2009, 'The chicken type III GnRH receptor homologue is predominantly expressed in the pituitary, and exhibits similar ligand selectivity to the type I receptor', *Journal of Endocrinology*, vol. 202, no. 1, pp. 179-190. https://doi.org/10.1677/JOE-08-0544

Digital Object Identifier (DOI): 10.1677/JOE-08-0544

Link: Link to publication record in Edinburgh Research Explorer

Document Version: Publisher's PDF, also known as Version of record

Published in: Journal of Endocrinology

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The chicken type III GnRH receptor homologue is predominantly expressed in the pituitary, and exhibits similar ligand selectivity to the type I receptor

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Abstract

Two GnRH isoforms (cGnRH-I and GnRH-II) and two GnRH receptor subtypes (cGnRH-R-I and cGnRH-R-III) occur in chickens. Differential roles for these molecules in regulating gonadotrophin secretion or other functions are unclear. To investigate this we cloned cGnRH-R-III from a broiler chicken and compared its structure, expression and pharmacological properties with cGnRH-R-I. The broiler cGnRH-R-III cDNA was 100% identical to the sequence reported in the red jungle fowl and white leghorn breed. Pituitary cGnRH-R-III mRNA was ~1400-fold more abundant than cGnRH-R-I mRNA. Northern analysis indicated a single cGnRH-R-III transcript. A pronounced sex and age difference existed, with higher pituitary transcript levels in sexually mature females versus juvenile females. In contrast, higher expression levels occurred in juvenile males versus sexually mature males. Functional studies in COS-7 cells indicated that cGnRH-R-III has a higher binding affinity for GnRH-II than cGnRH-I (Kd: 0.57 vs 19.8 nM) with more potent stimulation of inositol phosphate production (ED50: 0.8 vs 4.38 nM). Similar results were found for cGnRH-R-I, (Kd: 0.51 vs 10.8 nM) and (ED50: 0.7 vs 2.8 nM). The initial rate of internalisation was faster for cGnRH-R-III than cGnRH-R-I (26 vs 15.8%/min). Effects of GnRH antagonists were compared at the two receptors. Antagonist #27 distinguished between cGnRH-R-I and cGnRH-R-III (IC50: 2.3 vs 351 nM). These results suggest that cGnRH-R-III is probably the major mediator of pituitary gonadotroph function, that antagonist #27 may allow delineation of receptor subtype function in vitro and in vivo and that tissue-specific recruitment of cGnRH-R isoforms has occurred during evolution.

Journal of Endocrinology (2009) 202, 179–190

Introduction

The hypothalamic decapeptide GnRH is a central regulator of reproductive function in vertebrates (Millar 2005). Two GnRH isoforms are present in the chicken; cGnRH-I and GnRH-II (Sharp & Ciccone 2005). There is no genomic DNA evidence for a third GnRH ligand in the chicken to date. Both cGnRH-I and GnRH-II stimulate LH release from chicken pituitary in vitro (Hattori et al. 1986, Millar et al. 1986) and in vivo (Chou et al. 1985, Hattori et al. 1986, Sharp et al. 1986, Proudman et al. 2006). LH secretion in response to GnRH-II is markedly greater than that to cGnRH-I in mature laying hens. However, this is not the case in juvenile chickens of either sex, nor the cockerel (Sharp et al. 1987).

There is conflicting evidence for an effect of cGnRH-I or GnRH-II on FSH in vitro (Hattori et al. 1986, Millar et al. 1986), and in vivo (Krishnan et al. 1993, Bruggeman et al. 1998, Dunn et al. 2003, Proudman et al. 2006). Probably, cGnRH-I does not directly stimulate FSH release, but may stimulate FSH biosynthesis, while a role of GnRH-II in FSH production has not been established (Dunn et al. 2003, Sharp & Ciccone 2005).

Immunisation of laying hens against cGnRH-I, but not against GnRH-II, results in a complete regression of the reproductive system and a decrease in plasma LH (Sharp et al. 1990), suggesting that gonadotrophin secretion is controlled by cGnRH-I rather than GnRH-II. This is consistent with the presence of cGnRH-I in the median eminence (Mikami et al. 1988, Sharp et al. 1990) and the correlation between hypothalamic cGnRH-I peptide content and reproductive status (Sharp et al. 1990). In contrast, abundant levels of GnRH-II (Mikami et al. 1988, Sharp et al. 1990, Vangils et al. 1993) are not observed in the median eminence and no change in hypothalamic GnRH-II peptide content is seen in cockerels.

Journal of Endocrinology (2009) 202, 179–190

DOI: 10.1677/JOE-08-0544

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around the onset of puberty (Sharp et al. 1990). However, hypothalamic GnRH-II levels do decrease when the preovulatory release of LH is maximal or declining in the hen. Thus, GnRH-II may be involved in neuroendocrine events preceding ovulation (Wilson et al. 1990). In other avian species GnRH-II affects reproductive behaviour in ring doves and female soliciting behaviour in song sparrows (King & Millar 1995, Maney et al. 1997).

The specific receptor types through which the GnRH ligands elicit biological responses in the chicken are not known. GnRH systems exhibit species-specific recruitment of receptor subtypes and ligands within different tissues. For example, the sea bass expresses five isoforms of GnRH receptor and all but one are expressed in the pituitary (Moncaut et al. 2005). In goldfish, two subtypes of the GnRH receptor have been identified (Illing et al. 1999) and they are both expressed in the pituitary. In the bullfrog, three subtypes of GnRH receptor are expressed but only one predominates in the pituitary (Wang et al. 2001). Two subtypes of GnRH receptor occur in the African clawed frog (Xenopus laevis; Troskie et al. 2000) whereas the leopard gecko expresses three receptor subtypes in the pituitary, with one receptor subtype predominating in the anterior pituitary (Ikemoto & Park 2007). The pituitary-specific GnRH receptor subtypes expressed in the bullfrog (Wang et al. 2001), African clawed frog (Troskie et al. 2000) and leopard gecko (Ikemoto & Park 2007) differ structurally, according to the nomenclature proposed by Miller et al. and phylogenetically (Millar et al. 2004, Flanagan et al. 2007, Tello et al. 2008). The predominant pituitary GnRH receptor subtype expressed in the bullfrog is type III, the African clawed frog is type I and the leopard gecko is type III. This suggests plasticity in evolutionary recruitment of GnRH receptor subtypes for regulation of pituitary gonadotrophin production. There is also plasticity in the spatiotemporal expression patterns of GnRH receptor subtypes (Illing et al. 1999, Troskie et al. 2000, Wang et al. 2001, Moncaut et al. 2005, Ikemoto & Park 2007).

The promiscuity of receptor subtype activation by different endogenous GnRH ligands (Illing et al. 1999, Troskie et al. 2000, Wang et al. 2001, Moncaut et al. 2005, Ikemoto & Park 2007) suggests that a complex interplay between ligands and receptors may occur. The organisation of the avian GnRH system is distinct from those of teleosts, amphibians, reptiles or mammals (Ensemble database), having two receptor subtypes for two GnRH ligands (Morgan & Millar 2004). Further characterisation of the avian GnRH system may therefore provide unique information to enable further comprehension of the interplay between GnRH ligands and their receptors in the control of reproduction.

The first chicken GnRH–R isoform to be cloned, cGnRH–R–I is widely expressed (Sun et al. 2001a,b) and GnRH–II has a higher binding affinity and is more potent in stimulating inositol phosphate accumulation than cGnRH–I at this receptor (Sun et al. 2001b). This suggested that the chicken pituitary might contain an alternative GnRH receptor more selective for cGnRH–I, since this appears to be the GnRH isoform most likely to be biologically important for regulation of LH production. A novel isoform of cGnRH–R was subsequently cloned by ourselves and others (Joseph et al. 2006, Shimizu & Bedecarrats 2006; Genbank AY895154). The white Leghorn isoform of the novel receptor was designated cGNRHR2 (Shimizu & Bedecarrats 2006), but it can be classified as a type III GnRH receptor. Using PCR, this receptor showed expression of two pituitary-specific transcripts (1260 bp full length and 1065 bp splice variant; Shimizu & Bedecarrats 2006), in addition to another 501 bp splice variant that is expressed in different brain regions (Shimizu & Bedecarrats 2006). Inositol phosphate accumulation in response to GnRH-II stimulation was more potent at cGNRHR2 (Shimizu & Bedecarrats 2006) than the response to cGnRH–I. Pituitary cGNRHR2 mRNA expression levels correlated with reproductive status, suggesting that cGNRHR2 plays a role in the regulation of gonadotrophin function (Shimizu & Bedecarrats 2006).

The aims of this study were to undertake further characterisation of this second GnRH receptor isoform from a broiler chicken (commercial meat-type chicken) and compare it with the type I receptor. Using bioinformatics analysis, we confirm that cGNRHR2 is a chicken type III GnRH receptor homologue. Critically, we have established the preponderance of cGNRHR2 mRNA relative to cGnRH–R–I mRNA in the pituitary and expression was investigated during sexual development and the levels were quantified in a panel of tissues. We also performed ligand induced internalisation studies of both receptors and a detailed pharmacological analysis of the two receptors. This included analysis of ligand binding and inositol phosphate production in response to cGnRH–I and GnRH–II and an array of analogues and antagonists in order to identify a GnRH analogue that may be applied to examine receptor-specific effects on gonadotrophin production.

Materials and Methods

Animals

Birds were killed in accordance with UK Home Office guidelines. Tissues were dissected, snap frozen in liquid nitrogen and stored at −70 °C.

Peptides/primers

GnRH analogues, cGnRH–I, GnRH–II and sGnRH–III (Sigma–Aldrich) and mammalian antagonists 27, 135–25 and 135–18 were diluted in 20% propylene glycol (Sigma–Aldrich). Sequences are presented in Table 1. [His5–d–Tyr6]–GnRH peptide was synthesised with solid-phase methodology and purified by reverse–phase HPLC (Flanagan et al. 1998). Oligonucleotides (Sigma–Aldrich) diluted to 100 pM/µl were used for PCR amplification and probe synthesis.
Cloning of cGNRHR2 cDNA

Trizol (Invitrogen) was used to isolate total RNA from pooled mature male and female anterior pituitary glands from broilers. cDNA synthesis was performed with 5 μg total RNA using a first strand synthesis kit (Amersham Pharmacia Biotech Ltd). Genbank AY895154 was used as a reference for design of cDNA primers. Primers 1210f (5'-TGAGTGGGCGGTGTTTGA-3') and 3217r (5'-TAGAGGCATTGTGGAGCAGA-3') encompassing the entire coding region of the receptor were used for PCR amplification of pituitary cDNA (35 cycles of 95 °C/30 s, 61.9 °C/30 s, 72 °C/90 s). The product was cloned into Bluescript II SK (C) vector (Stratagene, The Netherlands) at the EcoR V site. The entire cDNA insert was sequenced. To make an expression construct, the fragment between the BamHI and HincII restriction sites of the Bluescript II SK(C) construct was removed and subcloned into the pCDNA3.1/myc-His-A (Invitrogen) mammalian expression vector between the BamHI and EcoR V sites. The DNA sequence of the expression construct was verified by BamHI, BsmI and PstI restriction digest and sequencing.

An expression construct containing cGNRHR-R-I cDNA cloned into pDNA1/Amp (Invitrogen) eukaryotic expression vector was used (Sun et al. 2001b) in parallel studies.

Oligonucleotide probes

5' end labelling using [γ-32P]-dATP and T4 polynucleotide kinase was performed with 20 pmol sense (5'-TGAGTGGGCGGTGTTTGA-3') and anti-sense (5'-TCAGGCGCGGCTAGGGGALTGGTAGTT TT TGA-3') oligonucleotides using the mirVana Probe and Marker Kit (Ambion, USA). These oligonucleotides, designed in the 5' region upstream of the translation start site in exon 1 are specific for cGNRHR2 and are not homologous to cGNRHR-R-I.

DNA probes

An actin alpha-1 skeletal muscle (ACTA1; Genbank 001030163) DNA probe was synthesised from 0.25 μg total RNA using a first strand synthesis kit (Amersham Pharmacia Biotech Ltd). Genbank AY895154 was used as a reference for design of cDNA primers. Primers 1210f (5'-TGAGTGGGCGGTGTTTGA-3') and 3217r (5'-TAGAGGCATTGTGGAGCAGA-3') encompassing the entire coding region of the receptor were used for PCR amplification of pituitary cDNA (35 cycles of 95 °C/30 s, 61.9 °C/30 s, 72 °C/90 s). The product was cloned into Bluescript II SK (C) vector (Stratagene, The Netherlands) at the EcoR V site. The entire cDNA insert was sequenced. To make an expression construct, the fragment between the BamHI and HincII restriction sites of the Bluescript II SK(C) construct was removed and subcloned into the pCDNA3.1/myc-His-A (Invitrogen) mammalian expression vector between the BamHI and EcoR V sites. The DNA sequence of the expression construct was verified by BamHI, BsmI and PstI restriction digest and sequencing.

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Table 1 Primary structure of GnRH analogues

| Peptide name          | Position of amino acid |
|-----------------------|------------------------|
| GnRH-II               | pGlu His Trp Ser His Gly Trp Tyr Pro Gly NH2 |
| cGnRH-I               | pGlu His Trp Ser Tyr Gly T
| His5-o-Tyr5 GnRH      | pGlu His Trp Ser His o-Tyr Leu Arg Pro Gly NH2 |
| cGnRH-III             | pGlu His Trp Ser Tyr Gly T
| 27                    | Ac-o-Nal(2) o-α-Me-4-Cl-Phe o-Trp Ser lpr-Lys o-Tyr Leu Arg Pro o-Ala NH2 |
| 135-18                | Ac-o-Nal(2) o-4-Cl-Phe o-Pal(3) Ser lle o-lpr-Lys Leu lpr-Lys Pro o-Ala NH2 |
| 135-25                | Ac-o-Nal(2) o-4-Cl-Phe o-Pal(3) Ser 1-MePal o-lpr-Lys Leu lpr-Lys Pro o-Ala NH2 |

*aAbbreviations for synthetic amino acids are: Nal(2), 3-(2-naphthyl) alanine; 4-Cl-Phe, 3-(4-chlorophenyl) alanine; α-Me-4-Cl-Phe, 2-methyl-3-(4-chlorophenyl) alanine; lpr-Lys, N3-isopropyllysine.

Chicken type III GnRH receptor · N T JOSEPH and others

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(sexually mature) Ross broiler breeder birds raised on 14 h light:10 h darkness. cDNA synthesis was performed on 0.5 μg of total RNA, using a 1st Strand Synthesis Kit (Amersham Pharmacia Biotech Ltd). Primers that spanned exon boundaries, 713–735f (5′-GCTCGGCTGTGCGGAGCTGT-3′; exon 2–3) and 933–952r (5′-ACGTCCCTGGAGGGAGAGAG-3′; exon 3–4) were used to amplify cGNRHR2 from various tissue cDNA samples of the pre-pubertal males and females (40 cycles of 95 °C/30 s, 62 °C/30 s, 72 °C/45 s). Real Time PCR was used to quantify the mRNA expression of cGnRH-R-I and cGnRHR2 in the pituitary gland, median eminence, small intestine and gonad using the recommended amplification conditions using SYBR-Green-I-dye (Invitrogen). Primers that spanned the exon boundaries: 713–735f and 933–952r for cGnRHR2 cDNA and primers 701–722f and 1100–1082r for cGnRH-R-I cDNA were used with pre-pubertal and sexually mature male and female tissue samples. Standard curves for amplification of each GnRH receptor homologue cDNA were made with known quantities of either chicken GnRH receptor cDNA. The cGNRHR2 cDNA in BlueScript II SK(+) vector and a cGnRH-R-I cDNA in Bluescript II SK(–) vector (containing 488 bp inserted into the EcoRV/NotI site of the vector, which corresponds to bases 1–421 of AJ304414 and 1–68 of AJ506779) were used. A linear regression analysis of known quantities of cDNA template versus cycle threshold values was plotted, allowing the calculation of the quantity of target nucleic acid from the test-sample cycle threshold values. PCR reaction with either template reached plateau phase following the same number of amplification cycles.

**Iodination of [His<sup>5</sup>-d-Tyr<sup>6</sup>]-GnRH**

[His<sup>5</sup>-d-Tyr<sup>6</sup>]-GnRH-I peptide was radioactively labelled with Iodine<sup>125</sup> using Chloramine-T (Sigma–Aldrich) and purified using Sephadex G25 chromatography (Sigma–Aldrich).

**Cell culture and gene transfection**

COS-7 cells were transiently transfected by electroporation with 10 μg expression construct. The cells were seeded in DMEM (Sigma–Aldrich) containing a final concentration of 10% FCS, 4 mM l-glutamine and 1% penicillin/streptomycin and incubated in a humidified atmosphere at 37 °C in 5% CO<sub>2</sub>.

**Whole cell radioligand binding assay**

Radioligand binding assays were performed with <sup>125</sup>I-[His<sup>5</sup>-d-Tyr<sup>6</sup>]-GnRH-I on intact cells 48 h after transfection. Cells were washed twice with 4 °C PBS + Ca<sup>2+</sup> + Mg<sup>2+</sup> (Sigma–Aldrich). The cells were then incubated for 4 h at 4 °C with <sup>125</sup>I-[His<sup>5</sup>-d-Tyr<sup>6</sup>]-GnRH (100 000 c.p.m./500 μl) and various concentrations of unlabelled GnRH analogues in 25 mM DMEM + HEPES + 0.1% BSA in triplicate. Cells were then washed twice with PBS + Ca<sup>2+</sup> + Mg<sup>2+</sup> and lysed with 0.1 M NaOH. Radioactivity was measured and non-specific binding was determined by displacement of label with 1 μM unlabelled GnRH analogue.

**Inositol phosphate assay**

Total inositol phosphate accumulation in response to GnRH stimulation was analysed as previously described (Berg et al. 1994, Pawson et al. 2003) with modifications: 24 h after transfection, cells were labelled with 1 μCi/ml per well of [<sup>3</sup>H]-myo-inositol, (Amersham Pharmacia Biotech) in 1 ml of inositol-free DMEM (Invitrogen Life Sciences) and incubated for a further 24 h. Cells were incubated at 37 °C with 0.5 ml 25 mM DMEM + HEPES (Sigma–Aldrich) containing 10 mM LiCl for two successive 5 and 30 min washes prior to stimulation with GnRH analogue in DMEM + HEPES + 10 mM LiCl for 1 h. Antagonist activity was assayed in the presence of 1 mM GnRH-II. Stimulations were terminated by replacing medium with 10 mM formic acid and incubating at 4 °C for 1 h. Water-soluble metabolites were separated as previously described (Berridge et al. 1983) with a series of washes of the Dowex resin with H<sub>2</sub>O and 60 mM ammonium formate, 5 mM sodium tetraborate prior to inositol phosphate elution in 1 M ammonium formate, 0.1 M formic acid. Assays were performed in triplicate.

**Receptor internalisation assays**

Receptor-transfected cells were incubated for 48 h prior to performing the internalisation assay as previously described (Pawson et al. 1998). Modifications to the protocol included addition of 0.5 ml acid solution (150 mM NaCl, 50 mM acetic acid, pH 2.8) to determine surface bound ligand and 0.5 ml 1 M NaOH for internalised radioligand.

**Figure 1** (A) Alignment of human, bullfrog, leopard gecko, African clawed frog and chicken GnRH receptor sequences. These are representative sequences of GnRH receptor types I, II and III. Motifs in ECL3 are depicted in bold and underlined. Ubiquitously conserved amino acid residues are shown with dark background shading. Boxed amino acids are as follows: 1 and 2, aspartic acid in the functional helix2/helix7 micro-domain of non-mammalian GnRH receptors; 3 and 4, conserved coupling arginine cage motif and alanine; 5, 6, 7 and 8, ligand binding residues, aspartic acid, asparagine, lysine and arginine respectively. Predicted transmembrane domains (TMD1–7) are indicated by a bold line. GnRH receptor subtypes are labelled according to the published terminology and presented in Roman numerals (Wang et al. 2001, Ikemoto & Park 2007). (B) A phylogenetic tree based on multiple sequence alignment of representative, reptilian, amphibian and avian GnRH receptor sequences. The tree was constructed using TreeTop phylogenetic tree prediction software (Yushmanov & Chumakov 1988).
Non-specifically bound ligand was measured at time point zero and used to normalise data. Assays were performed four times with six replicate points per experiment.

Statistical analysis

ANOVA was performed on Real time PCR data using GenStat version 9 (VSN International Ltd, UK). Other results were analysed using GraphPad Prism version 4.0 (GraphPad Software, CA, USA). Non-linear regression models were applied to the ligand binding and inositol phosphate assay data. Receptor internalisation rates were analysed as previously described (Pawson et al. 1998).

Results

Cloning of the cGNRHR2 cDNA

We cloned a PCR cDNA product from broiler (meat-type birds) pituitary samples that was identical to the sequence from Gallus gallus gallus genomic DNA (ENSGALG00000020923; Ensembl 2004) and White Leghorn strains (Genbank AY895154). An expression construct containing the entire coding sequence, 24 bp 5’ to the start codon and 100 bp 3’ to the stop codon was prepared.

Gene structure of cGNRHR2

Bioinformatic comparisons of the cDNA and genomic DNA sequences confirmed that the receptor consists of a 419 amino acid protein encoded by 4 exons (Shimizu & Bedecarrats 2006) on chicken chromosome 10 linked to the type I receptor. The amino acid sequence identity of the two cGnRH-Rs is 53% (Fig. 1A). The amino acid sequences of both cGnRH-R receptors were compared with human, bullfrog, leopard gecko and African clawed frog GnRH receptor subtypes. The highest homology to cGnRH-R-I was with bullfrog-II GnRH-R. The highest homology to cGnRH-R occurred with bullfrog-I and leopard gecko-I GnRH-Rs. Phylogenetic analysis performed by ourselves (Fig. 1B) and others (Flanagan et al. 1998, 2003) clearly classes this receptor as a type III GnRH receptor homologue. We therefore refer to it as cGnRH-R-III from here on.

The putative translation start codon is coded by exon 1 and the stop codon by exon 4. The amino terminal extracellular domain is coded by exon 1 and exon 2. Exon 2 also encodes transmembrane domain (TMD) 1, intracellular loop (ICL) 1, TMD2, extracellular loop (ECL) 1, TMD3, ICL2 and part of TMD4. Exon 3 encodes the rest of TMD4, ECL2, TMD5 and part of ICL3. Exon 4 encodes the rest of ICL3, TMD6, ECL3, TMD7 and the C-terminal cytoplasmic domain (Fig. 1A).

Comparisons of functionally important residues and micro-domains were performed. cGnRH-R-III like cGnRH-R-I has aspartic acid in both loci of the helix2/helix7 micro-domain, an arrangement unique to non-mammalian GnRH receptors (Zhou et al. 1994, Flanagan et al. 1999; Fig. 1A). Conservation of important coupling motifs, the arginine cage motif (Ballesteros et al. 1998) and alanine in ICL3 (Myburgh et al. 1998) is maintained in cGnRH-R-III (Fig. 1A). The two cGnRH-R-Rs exhibit conservation of the aspartic acid (Flanagan et al. 2000) and asparagine (Davidson et al. 1996) ligand-binding residues in TMD2 (Fig. 1A). The lysine in TMD3 of the cGnRH-R-III (Zhou et al. 1995) is substituted with an arginine residue. The glutamate residue in ECL3 (Flanagan et al. 1994) is not conserved in cGnRH-R-III whereas it is in cGnRH-R-I (Sun et al. 2001b). ECL3 of each receptor type has a distinctive classification motif (Millar et al. 2004), cGnRH-R-I has a PEY motif in ECL3 whereas in cGnRH-R-III this motif is PPS (Fig. 1A). The motifs known to be crucial for receptor internalisation were compared (Pawson et al. 1998, 2003), and are conserved between cGnRH-R-I and cGnRH-R-III, however, the tail of cGnRH-R-III is longer by an additional eight residues (Fig. 1A).

Northern blot analysis

Hybridisation of cGnRH-R-III antisense oligonucleotide probe was detected in the anterior pituitary but not testes or small intestine RNA (Fig. 2A). The cGnRH-R-III mRNA transcript is approximately 1600 bases long. Sense oligonucleotides did not hybridise to the RNA samples (data not shown). ACTA1 skeletal muscle 32P-dCTP labelled DNA probe hybridised to all samples (Fig. 2B), demonstrating equal RNA loading. A riboprobe also detected cGnRH-R-III mRNA transcripts of approximately 1600 bases in male and female anterior pituitary but not brain cortex or skeletal muscle RNA (Fig. 2C). There was no evidence of cGnRH-R-III mRNA transcript size variants in any tissues.

Tissue distribution of cGnRH-R-III expression and quantification relative to cGnRH-R-I

PCR amplification of GnRH receptor cDNA was ensured by designing primers spanning exon boundaries. A single 238 bp cDNA amplification product derived from exon 3 was detected in the pituitary. Amplification of cGnRH-R-III cDNA was detected in all tissues tested (anterior pituitary, hind-brain, cerebrum, cerebellum, median eminence, anterior hypothalamus, posterior hypothalamus, olfactory bulb, optic lobe, adrenal gland, kidney, small intestine, spleen, testes, ovary and liver) from pre-pubertal males and females although the signal intensity in the anterior pituitary was distinctly the highest. Relative levels of cGnRH-R-I and cGnRH-R-III mRNA measured by real-time PCR in juvenile and mature male and female tissues demonstrated that expression of cGnRH-R-III was greater than cGnRH-R-I in the pituitary, median eminence, gonads and small intestine. In the anterior pituitary cGnRH-R-III expression was 1373-fold that of cGnRH-R-I (Fig. 2F). Even though
the amplification efficiency of the two targets was similar, higher cGnRH-R-III expression was detected in the anterior pituitary compared with the median eminence, gonads and small intestine (Fig. 2D). Anterior pituitary expression of cGnRH-R-III was 92.5±3% greater than in the testes or ovary. Expression of cGnRH-R-I in the testes was 2-fold greater than in the anterior pituitary (Fig. 2E).

ANOVA demonstrated a significant relationship between age and sex on pituitary cGnRH-R-III expression (Fig. 2G). Anterior pituitary cGnRH-R-III expression was 8.4-fold lower in sexually mature males compared with juvenile males whereas the reciprocal relationship was observed in females, with 2.8-fold more anterior pituitary cGnRH-R-III expression in sexually mature females compared with juveniles (Fig. 2G).

whole cell GnRH binding assays

Cells transfected with cGnRH-R-III and cGnRH-R-I expression constructs showed binding and displacement of 125I-[His5-D-Tyr6]-GnRH with both cGnRH-I and GnRH-II in a concentration dependent fashion. The binding affinities for cGnRH-I and GnRH-II were similar at cGnRH-R-I and cGnRH-R-III (Fig. 3A). cGnRH-R-III exhibited a 35-fold higher affinity for GnRH-II than for cGnRH-I (Table 2). cGnRH-R-I exhibited a 21-fold higher affinity for GnRH-II than for cGnRH-I, similar to previous reports (Sun et al. 2001b).

cGnRH-R-III and cGnRH-R-I bound sGnRH-III and mammalian antagonists 27, 135-18 and 135-25 (Table 2). Because there was no genomic evidence for GnRH-III in the chicken, the binding affinity for sGnRH-III was tested as an alternative. cGnRH-R-III and cGnRH-R-I exhibited a 16-fold higher affinity for GnRH-II than for sGnRH-III, and both receptors exhibited a higher affinity for sGnRH-III than cGnRH-I (Table 2). Similar binding affinities were observed for all GnRH analogues and mammalian antagonists at either receptor except for mammalian antagonist 27.

cGnRH-I, GnRH-II, sGnRH-III, mammalian antagonist 135-18 and 135-25 showed respectively a 1.8, 1.1, 1.1, 3.5 and 0.6-fold higher binding affinity at cGnRH-R-I than at cGnRH-R-III. Remarkably, cGnRH-R-I has a much higher affinity for mammalian antagonist 27 (Kd: 0.38 nM) than cGnRH-R-III (Kd: 21.4 nM).

Inositol phosphate assays

cGnRH-I and GnRH-II stimulated inositol phosphate production in cGnRH-R-I and cGnRH-R-III transfected cells (Fig. 3B). The ED50 for GnRH-II was much lower than for cGnRH-I, in accordance with their binding affinities. GnRH-II was more potent than cGnRH-I at either receptor. In cGnRH-R-III transfected cells, the accumulation of inositol phosphate was six times more effective with GnRH-II stimulation than with cGnRH-I (Table 2). GnRH-II was four times more potent than cGnRH-I for production of inositol phosphate via activation of cGnRH-R-III (Table 2). The ED50 for sGnRH-III was much higher than GnRH-II and cGnRH-I at both cGnRH-Rs (Table 2). In response to cGnRH-I, GnRH-II or sGnRH-III there was a 1.6, 1.1 and 2.0-fold higher potency at cGnRH-R-I compared with cGnRH-R-III.
Mammalian antagonist 27 displayed inverse agonist properties at either receptor, decreasing inositol phosphate production below basal levels for both receptors (Fig. 4A and C). Mammalian antagonist 135-25 stimulated inositol phosphate production with very low efficacy in transfected COS-7 cells: cGnRH-R-I (ED$_{50}$: 5970 nM) and cGnRH-R-III (ED$_{50}$: 4860 nM; Table 2 and Fig. 4E and G). Mammalian antagonist 135-18 stimulated inositol phosphate in transfected COS-7 cells; cGnRH-R-III (ED$_{50}$: 1510 nM) with weak efficacy, but no stimulation of inositol phosphate was detected in cGnRH-R-I transfected cells (Fig. 4I and K).

Inhibition of 1 nM GnRH-II-stimulated inositol phosphate production by mammalian antagonist 135-18 in cGnRH-R-I transfected COS-7 cells (IC$_{50}$: 2330 nM) was more potent than for cGnRH-R-III (IC$_{50}$: 3890 nM) transfected COS-7 cells (Fig. 4J and L). Mammalian antagonist 135-25, acted as a pure agonist in cGnRH-R-III transfected cells in the presence of 1 nM GnRH-II, increasing the potency of inositol phosphate stimulation from ED$_{50}$: 4860 nM in the absence of GnRH-II to ED$_{50}$: 20.1 nM in the presence of GnRH-II (Fig. 4H). Antagonistic properties for mammalian antagonist 135-25 were observed at cGnRH-R-I (IC$_{50}$: 245 nM) transfected cells, but with little efficacy (Fig. 4F). Surprisingly, mammalian antagonist 27 exhibited large differences in antagonistic potency at cGnRH-R-I (IC$_{50}$: 2.3 nM) compared with cGnRH-R-III (IC$_{50}$: 351 nM; Fig. 4B and D).

Receptor internalisation assays

The initial rate of internalisation of $^{125}$I-[His$^5$-D-Tyr$^6$]-GnRH-I bound to cGnRH-R-III was slightly greater ($P<0.006$) than cGnRH-R-I. cGnRH-R-I internalised at an initial rate of 15.8%/min whereas the initial rate of internalisation of cGnRH-R-III was determined as 26.0%/min. The per cent maximal internalisation of cGnRH-R-I was 78.2% vs 68.9% for GnRH-R-III (Fig. 3C).

Discussion

Birds, like mammals, possess two GnRH receptor subtypes in contrast to fish, reptiles and amphibians in which three types of receptor are generally found (Morgan & Millar 2004, Ikemoto & Park 2007). The proximity of the two chicken receptor subtypes on chromosome 10 (~2 Mb apart) and their high sequence similarity, suggests they may have been derived by gene duplication.

There has been an inclination to designate GnRH receptors that are selective for GnRH-II as type II GnRH receptors. However, all non-mammalian receptors, regardless of their structural similarity are selective for GnRH-II (Pfleger et al. 2002). Other investigators have designated GnRH receptors by their tissue-specific expression. This classification is also problematic as the predominant GnRH

Figure 3 (A) Ligand binding of cGnRH-R-I and cGnRH-R-III. Competitive displacement of $^{125}$I-[His$^5$-D-Tyr$^6$]-GnRH with serial dilutions (10$^{-11}$–10$^{-6}$ M) of cGnRH-I (solid squares) and (10$^{-11}$–10$^{-6}$ M) GnRH-II (open squares) in COS-7 cells transiently transfected with cGnRH-R-I (dashed lines) and cGnRH-R-III (solid lines) expression constructs. The data presented are from four independent experiments each performed in triplicate. Non-specific binding, determined in un-transfected cells was subtracted from c.p.m. (B) Inositol phosphate production in response to (10$^{-11}$–10$^{-6}$ M) cGnRH-I (solid squares) and GnRH-II (open squares) in COS-7 cells transiently transfected with cGnRH-R-I (dashed lines) and cGnRH-R-III (solid lines) expression constructs. The data are from four independent experiments performed in triplicate. (C) cGnRH-R-I and cGnRH-R-III ligand induced internalisation rate. Per cent internalisation of $^{125}$I-[His$^5$-D-Tyr$^6$]-GnRH ligand mediated by cGnRH-R-I (solid triangle) and cGnRH-R-III (solid square). Data points are the mean ± S.E.M. of four independent experiments each performed with six replicates. Non-specific binding, determined in un-transfected cells was accounted for.
receptor expressed in bullfrog pituitary is structurally a type III receptor (Wang et al. 2001) while in African clawed frog, it is a type I receptor (Troskie et al. 2000). We therefore advocate that the receptors are grouped and designated according to their structural and phylogenetic relatedness. cGnRH-R-III was first designated as cGNRHR2 (Shimizu & Bedecarrats 2006), whereas designation as cGnRH-R-III is appropriate according to phylogenetic classification.

Delineation of ‘cognate’ ligand/receptor pairing requires the demonstration of receptor protein expression in the target cell and demonstration of ligand isoform delivery to that cell. In the chicken there is good evidence (Mikami et al. 1988, Sharp et al. 1990, Vangils et al. 1993) that cGnRH-I targets the gonadotroph and the higher expression levels of GnRH-R-III in the pituitary suggest that it is the cognate pituitary receptor. Expression of cGnRH-R-III was 1373-fold higher than cGnRH-R-I in the anterior pituitary gland. However, GnRH-R-III localisation to the gonadotroph remains to be demonstrated. Therefore, the development of analogues that differentially promote or inhibit activation of receptor subtypes may be valuable tools for deciphering the role of receptor types in terms of gonadotrophin regulation or other putative functions.

No sex differences or changes in expression were detected for cGnRH-R-I, however, a pronounced reciprocal sex difference in pituitary cGnRH-R–III expression was observed. Higher cGnRH-R–III mRNA levels occur in adult females compared with adult males. In contrast, in juvenile chickens, higher cGnRH-R–III mRNA levels occurred in males compared with females. cGnRH-R–III splice variant transcripts detected by PCR amplification (Shimizu & Bedecarrats 2006) were not detected by oligonucleotide probe or riboprobe using northern blotting, suggesting that their in vivo expression is relatively low and may be of little functional significance.

Differences in cGnRH-R–III receptor expression levels between juvenile and mature males and females were not reported previously (Shimizu & Bedecarrats 2006). This may be due to the age of animals used in different studies. The sexual dimorphism in cGnRH-R–III expression, where levels of mRNA are higher in juvenile males compared with juvenile females, is consistent with the LH response to photostimulation seen in juveniles, where there is a robust response in males but not in females (Sreekumar & Sharp 1998). However, in adult males and females, cGnRH-R–III expression levels are inversely related to sex differences in LH responsiveness to GnRH injection (Sharp et al. 1987). When chicken pituitary fragments from adult males and juveniles of both sexes are perfused, a large spike followed by a plateau in LH release in response to GnRH stimulation is observed (Liu et al. 1995). Adult laying hens do not exhibit the rapid spike of release but do show the plateau phase and the release of LH in adult hens is much reduced compared with juvenile hens (Liu et al. 1995). In general, our observations suggest that the level of GnRH-R–III mRNA may be inversely related to the release of LH by gonadotrophs in adult females. There are several possible explanations for this apparent paradox. However, cGnRH-R–III mRNA may be reflective of expression of membrane cGnRH-R–III receptor number, but that increased ovarian steroid and peptide hormones act negatively in the adult female to diminish the LH response to GnRH at the gonadotroph, through down-regulation of intercellular signalling pathways including the Ca²⁺ mobilisation mechanism (Liu et al. 1995).

As noted above, the occurrence of two or three forms of GnRH peptide hormone in different vertebrate species initially suggested the existence of different cognate receptors

| Table 2 | Receptor binding and peptide-stimulated inositol phosphate accumulation in response to cGnRH analogues in COS-7 cells expressing cGnRH-R-I and cGnRH-R-III |
|-----------------|-----------------|-----------------|-----------------|
| **Analog**      | **Ligand binding** | **IP production** | **IP production** |
|                 | **Kᵢ (nM)ᵇ**     | **cGnRH-R-I**    | **cGnRH-R-III**  |
| cGnRH-I         | 10.8 ± 1.59ᵈ     | 2.8 ± 0.20ᵈ     | 4.38 ± 0.18ᵈ    |
| GnRH-II         | 0.51 ± 0.07ᵈ     | 0.7 ± 0.14ᵈ     | 0.8 ± 0.14ᵈ     |
| cGnRH-R-III 27  | 8.40 ± 0.21ᵇ     | 10.3 ± 4.5ᵈ     | 21 ± 0.2ᵈ       |
| 135-18          | 2009 ± 156⁰   | 2.3 ± 0.3⁹     | Inverse agonist⁹ |
| 135-25          | 14 200 ± 1515⁰ | 5970 ± 2510ᵈ   | 4860 ± 807ᵈ     |

All experiments were performed on separate occasions in triplicate.
*bKi values (binding affinities) for GnRH analogues.
**ED5₀ values for agonist activity of GnRH analogues.
***IC5₀ values for antagonism of IP production stimulated by 1 nM GnRH-II.
⁴Data are mean ± S.E.M. of three to four experiments.
⁵Data are mean ± S.E.M. of two to three experiments.
⁶20 ± 8 ± 9 = agonistic effect of peptide in presence of 1 nM GnRH-II.

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able to differentially recognise the different ligands. Selectivity for GnRH-II is emerging as a general phenomenon for non-mammalian GnRH receptors due to the preconfigured β-II' turn of GnRH-II (Pfleger et al. 2002). The current study supports this concept in that both cGnRH-Rs maintain higher ligand selectivity for GnRH-II. This presents a conundrum in that cGnRH-I is the hypothalamic peptide regulating the gonadotroph (Mikami et al. 1988, Sharp et al. 1990). GnRH-II was more potent at stimulating inositol phosphate production at the cGnRH-R-III than cGnRH-I. Similarly, GnRH-II stimulates inositol phosphate production four times more potently at cGnRH-R-I than cGnRH-I, consistent with previous data (Sun et al. 2001b, Shimizu & Bedecarrats 2006). Since inositol phosphate and diacyl-glycerol are generated by the recruitment of the Gq protein and activation of PLC-β (Stojilkovic et al. 1994, Ruf et al. 2003), cGnRH-R-III can activate the protein kinase C and Ca\(^{2+}\) second messenger signalling pathways, which are the predominant mediators of LH and FSH biosynthesis and secretion (Conn & Crowley 1994, Sealfon et al. 1997). However, receptor ligand selectivity measured in vitro does not necessarily correlate with in vivo functional significance nor does it determine the designation of cognate receptors. Maybe evolutionary recruitment of ligand–receptor pairing for particular physiological processes is not determined by selection according to the highest affinity for ligand binding or efficacy in inositol phosphate production.

Considering differences between the two chicken receptors, the possibility that cGnRH-R-III may differ from cGnRH-R-I in the rate of ligand-induced internalisation was anticipated because eight additional residues occur in the carboxyl-terminal tail of cGnRH-R-III, a domain that predisposes GnRH-Rs to rapid rates of internalisation (Pawson et al. 1998). A slightly greater rate of internalisation was measured for cGnRH-R-III compared with cGnRH-R-I. The region crucial for receptor internalisation of cGnRH-R-I is distal residue 336 in cGnRH-R-I (Pawson et al. 1998). In cGnRH-R-III, this region contains all eight of the additional residues that comprise the elongated tail. The spatial arrangement of amino acid residues in the carboxyl-terminus region of cGnRH-R-III may contribute to the increase in internalisation rate, given that the known residues important for internalisation are conserved between the two receptors (Pawson et al. 1998, 2003; Fig. 1A).

Both cGnRH-R-III and cGnRH-R-I have a significantly higher binding affinity for mammalian antagonist 27 compared with mammalian antagonists 135-18 and 135-25. GnRH-R-I showed a 56-fold higher binding affinity for antagonist 27 than the binding affinity at cGnRH-R-III. The observed low binding affinities of mammalian antagonists 135-25 and 135-18 are translated into similarly low potencies in their capacity to stimulate or inhibit inositol phosphate accumulation. The sizes of these effects are too small to accurately differentiate the two receptor subtypes.

Antagonist 27 showed inverse agonistic effects at both chicken receptors. Differences in binding affinity were translated into differences in antagonistic effects at the receptors, with antagonist 27 showing a 153-fold higher antagonistic effect at cGnRH-R-I than cGnRH-R-III.
Therefore, it may be possible to utilise antagonist 27 to differentially block type I GnRH receptor function in vivo in order to study type III GnRH receptor physiology.

In summary, we directly compared pharmacological properties of cGnRH-R-I and cGnRH-R-III, and found both receptors to be similar in response to endogenous chicken GnRH ligands. Pharmacological profiling of the type I and type III GnRH receptors has established that GnRH-II is more selective and more potent in inositol phosphate accumulation at both receptors than cGnRH-I. cGnRH-R-III has a more rapid internalisation rate than cGnRH-R-I. Activation and inhibition by GnRH analogues acting on cGnRH-R-I and cGnRH-R-III has been characterised using a range ligands (sGnRH-III, mammalian antagonist 27, 135-18 and 135-25), furthering the pharmacological profile of both receptor subtypes.

The findings concerning ligand selectivity, G protein coupling and internalisation of cGnRH-R-III suggest that it could theoretically function similarly to cGnRH-R-I to regulate gonadotrophin synthesis. However, the predominant levels of cGnRH-R-III mRNA in the pituitary gland (1373-fold more than cGnRH-R-I), and the changes in expression patterns with age and sex suggest a distinct functional capacity of cGnRH-R-III compared with cGnRH-R-I.

However, further experiments are required to elucidate the role of cGnRH-R-III within the pituitary. Our studies suggest that evolutionary plasticity in the tissue-specific adoption of GnRH ligand and receptor subtypes for regulation of particular physiological functions may have occurred in birds. Birds appear to have adopted a strategy to utilise the cGnRH-R-III compared with cGnRH-R-I.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Funding**

The research was funded by a BBSRC Studentship (N J) and the Medical Research Council (K M and R P M).

**Acknowledgements**

We thank Dr RW Roeske (Indiana University, Indianapolis) for providing the mammalian antagonists. We thank Dr Adam Pawson for advising on receptor internalisation studies, Dr Zhi-Liang Lu for advice on IP assays and Nicola Miller for technical expertise. We thank Professor Peter Sharp and Dr Alan Stewart for helpful discussions.

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