A novel G protein-coupled receptor for gonadotropin-inhibitory hormone in the Japanese quail (*Coturnix japonica*): identification, expression and binding activity

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Abstract

We recently identified a novel hypothalamic dodecapeptide inhibiting gonadotropin release in the Japanese quail (*Coturnix japonica*). This novel peptide was therefore named gonadotropin-inhibitory hormone (GnIH). The GnIH precursor encoded one GnIH and two GnIH-related peptides (GnIH-RP-1 and GnIH-RP-2) that shared the same C-terminal motif, Leu-Pro-Xaa-Arg-Phe-NH₂ (Xaa=Leu or Gln; LPXRF-amide peptides). Identification of the receptor for GnIH is crucial to elucidate the mode of action of GnIH. We therefore identified the receptor for GnIH in the quail diencephalon and characterized its expression and binding activity. We first cloned a cDNA encoding a putative GnIH receptor by a combination of 3' and 5' rapid amplification of cDNA ends (RACE) using PCR primers designed from the sequence for the receptor for rat RF-amide-related peptide (RFRP), an orthologous peptide of GnIH. Hydrophobic analysis revealed that the putative GnIH receptor

possessed seven transmembrane domains, indicating a new member of the G protein-coupled receptor superfamily. The crude membrane fraction of COS-7 cells transfected with the putative GnIH receptor cDNA specifically bound to GnIH and GnIH-RPs in a concentration-dependent manner. Scatchard plot analysis of the binding showed that the identified GnIH receptor possessed a single class of high-affinity binding sites ($K_d=0.752$ nM, $B_{max}=24.8$ fmol/mg protein). Southern blotting analysis of reverse transcriptase-mediated PCR products revealed the expression of GnIH receptor mRNA in the pituitary gland and several brain regions including diencephalon in the quail. These results suggest that GnIH acts directly on the pituitary via GnIH receptor to inhibit gonadotropin release. GnIH may also act on the hypothalamus to inhibit gonadotropin-releasing hormone release.

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Introduction

Recently, we identified a novel hypothalamic dodecapeptide, Ser-Ile-Lys-Pro-Ser-Ala-Tyr-Leu-Pro-Leu-Arg-Phe-NH₂, in the brain of Japanese quail (*Coturnix japonica*; Tsutsui *et al.* 2000). This avian neuropeptide was shown to inhibit gonadotropin release from the cultured quail anterior pituitary (Tsutsui *et al.* 2000). This is the first hypothalamic peptide inhibiting gonadotropin release reported in a vertebrate. We therefore termed it gonadotropin-inhibitory hormone (GnIH; Tsutsui *et al.* 2000). Because GnIH was shown to be located in neurons of the paraventricular nucleus and their terminals in the median eminence (Tsutsui *et al.* 2000, Ubuka *et al.* 2003, Ukena *et al.* 2003a), GnIH may act directly on the pituitary to inhibit gonadotropin release. In addition, GnIH-containing fibers were observed in extremely close proximity to gonadotropin-releasing hormone (GnRH) neurons in the preoptic area in birds (Bentley *et al.* 2003,

Ukena *et al.* 2003a). It is therefore plausible that GnIH may act at the level of the hypothalamus to regulate gonadotropin release as well as at the pituitary. To elucidate the mode of action of GnIH on gonadotropin release, identification of the receptor for GnIH is crucial in birds.

We also characterized a cDNA encoding the GnIH precursor in the brain of quail (Satake *et al.* 2001) and sparrow (Osugi *et al.* 2004). The GnIH precursor encoded one GnIH and two GnIH-related peptides (GnIH-RP-1 and GnIH-RP-2) that included Leu-Pro-Xaa-Arg-Phe-NH₂ (Xaa=Leu or Gln) at their C-termini (Satake *et al.* 2001, Osugi *et al.* 2004). Based on this structural feature, GnIH and GnIH-RPs are considered to be LPXRF-amide peptides as a new member of the RF-amide peptide family (Ukena & Tsutsui 2004). In addition, the chicken pentapeptide LPLRF-amide, which was previously isolated as the first RF-amide peptide found in vertebrates (Dockray *et al.* 1983), is considered to be a fragment of

GnIH (Tsutsui *et al.* 2000, Satake *et al.* 2001, Osugi *et al.* 2004).

After the identification of GnIH in birds, we further sought to identify novel hypothalamic LPXRF-amide peptides similar to GnIH and GnIH-RPs in other vertebrates. The isolated LPLRF-amide peptide from bullfrog hypothalamus possessed growth hormone-releasing activity, and was designated as frog growth hormone-releasing peptide (fGRP) (Koda *et al.* 2002). This fGRP is structurally identical to *Rana* RF-amide (R-RFa) purified by Chartrel *et al.* (2002). The fGRP precursor also encoded one fGRP and three related peptides (fGRP-RP-1, -2, and -3; Sawada *et al.* 2002a), which were identified as mature LPXRF-amide peptides (Ukena *et al.* 2003b). fGRP-RP-2 also stimulated the release of growth hormone and prolactin (Ukena *et al.* 2003b). Furthermore, we characterized a cDNA that encoded three LPXRF-amide peptides (gfLPXRFa-1, -2 and -3) from the goldfish brain and identified gfLPXRFa-3 as a mature peptide (Sawada *et al.* 2002b). Turning to mammals, cDNAs that encode LPXRF-amide peptides have been detected in mammalian brains with a gene database search (Hinuma *et al.* 2000). Mammalian cDNAs encoded three peptides, which were termed RF-amide-related peptide-1, -2 and -3 (RFRP-1, -2 and -3). Subsequently, mammalian RFRP-1 and -3 were identified in the bovine and rat brain (Fukusumi *et al.* 2001, Ukena *et al.* 2002, Yoshida *et al.* 2003), although RFRP-2 was not an LPXRF-amide peptide. In mammals, Hinuma *et al.* (2000) have found that the deduced human LPXRF-amide peptide, human RFRP-1, increased prolactin release in the rat. Another group has also reported the stimulatory effect of RFRP-1 on prolactin release (Samson *et al.* 2003). Hinuma *et al.* (2000) have further reported the receptors for rat and human RFRP, which were G protein-coupled receptors (GPCRs).

With these findings as a background, in this study we sought to identify the receptor for GnIH (avian LPXRF-amide peptide) in the quail, based on the structure of the receptor for rat RFRP, an orthologous peptide of GnIH. Here we show a novel avian GPCR for GnIH, which specifically binds to GnIH and GnIH-RPs with high affinities. To understand the mode of action of GnIH on gonadotropin release, we further characterized the expression of GnIH receptor in the pituitary and different brain regions.

Materials and Methods

Animals and RNA preparation

Sexually mature males (3 months of age) of the Japanese quail were purchased from the Tokai Yuki Company (Toyohashi, Japan). They were housed in a temperature-controlled room ($25 \pm 2^\circ\text{C}$) under daily photoperiods of 16 h light and 8 h dark (long day; lights on at 0700 h), and

were given quail food and tap water *ad libitum*. All birds were isolated in individual cages and the experimental protocol was approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the committee of Hiroshima University (Higashi-Hiroshima, Japan).

All birds were killed by decapitation between 1000 and 1200 h. The cerebrum, diencephalon, mesencephalon and cerebellum were carefully removed using fine forceps under a dissecting microscope, snap-frozen immediately in liquid nitrogen and used for RNA isolation. Total RNA from quail diencephalons was extracted with Sepazol-RNA I Super reagent (Nacalai Tesque, Kyoto, Japan) followed by the isolation of poly(A)⁺ RNA with Oligotex-(dT) 30 (Daiichikagaku, Tokyo, Japan) in accordance with the manufacturer's instructions.

5'/3' Rapid amplification of cDNA ends (RACE) and degenerate PCR cloning

Utilizing the isolated poly(A)⁺ RNA of quail diencephalons and primers corresponding to amino acid sequences of the first extracellular loop (DNATCKM, amino acids 112–118) and second intracellular loop (IVHPFRE, amino acids 145–151) of rat RFRP receptor (accession number AB040103; Hinuma *et al.* 2000), nested degenerate PCR was performed to determine the cDNA 3'-end sequence of a putative quail GnIH receptor with the oligo(dT)-anchor primer supplied in the 5'/3' RACE kit (Roche Diagnostics). Both first- and second-round PCRs consisted of the following conditions: 94°C , hold for 3 min; five cycles of 94°C for 30 s, 47°C for 30 s, 72°C for 2 min; 30 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 2 min; hold for 3 min at 72°C (10 min in the second-round PCR). The second-round PCR products were subcloned into a TA-cloning vector, pGEM-T, in accordance with the manufacturer's instructions (Promega). The DNA inserts of the positive clones were amplified by PCR with universal M13 primers.

Cloning of the 5'-end of a putative quail GnIH receptor cDNA was carried out with the Advantage GC-2 PCR kit (Clontech). Template cDNA was synthesized with an oligonucleotide primer, 5'-CACGTAGATGTGGGAAAAGAG-3', complementary to nucleotides 748–768 (Fig. 1A), followed by dA-tailing of the cDNA with dATP and terminal transferase (Roche Diagnostics). The tailed cDNA was amplified with the oligo(dT)-anchor primer (Roche Diagnostics) and gene-specific primer 1 (5'-CTG GTGACAGTTAGGGTGAT-3', complementary to nucleotides 622–641), followed by further amplification of the first-round PCR products with the anchor primer and gene-specific primer 2 (5'-AGCACCCAGATGATGGCAAT-3', complementary to nucleotides 574–593). Both first-round and second-round PCRs were performed for 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min (10 min for last cycle). The second-round PCR

A

1 CTC CGC AAG CAG CGG TGA CGG CGG CAC AGG AGC GAT GCT CAG CGG CGG GGA CCC CCG GCT GGA GGA GAC CTT CTT CCC CAC CTC CCG ATG CAG GCG CTG CGG 102
M Q A L R 5
103 CAC CAG GAG CCA AGC GGG GAC ACC TTC AAT GGC AGC TGG GAT AAT GCC AGT GAG AGC CAA ATC CTG AAG GAA AAC TAC ACC TTC TTG GCA TAC TAC CAG CAC 204
H Q E P S G D T F N G S W D N A S E S Q I L K E N Y T F L A Y Y Q H 39
205 TCC TCG CCC GTG GCT GTG ATG TTC ATC CTA GCC TAC ACC TTC ATC CTC ATG TGC ATG ATT GGC AAC ATC CTG GTG TGC TTC ATC GTG AAG AAC CGA 306
S S P V A V M F I L A Y T F T M I F L M C M I G N I L V C F I V V K N R 73
307 CAG ATG CGG ACG GTC ACC AAC ATG TTC ATC CTC AAC CTT GCC ATC AGC GAC CTG CTG GTG GGC ATC TTC TGC ATG CCT ACA ACT CTG GTG GAC AAC CTC ATC 408
Q M R T V T N M F I L N L A I S T M L D L L V G I F C M P T T L V D N L I 107
409 ACG GGG TGG CCT TTT GAT AAT GCC ATG TGC AAA ATG AGT GGC TTG GTA CAG GGC ATG TCT GTC TCC GCC TCT GTT TTC ACG CTG GTG GCT ATC GCA GTG GAG 510
T G W P F D N A M C K M S G L V Q G M S V S A T M L S V F T L V A I A V E 141
511 AGG TTT CGC TGC ATC GTC CAC CCG TTC CGG GAG AAG CTG ACG CTG AGG AAA GCC CTG GTG ACC ATT GCC ATC ATC TGG GTG CTG GCC CTG CTC ATC ATG TGC 612
R F R C I V H P F R E K L T L R K A L V T I A I I W V T M V L A L L I M C 175
613 CCC TCC GCC ATC ACC CTA ACT GTC ACC AGG GAG GAG CAC CAC TTC ATG CTG GAT ACC TAC AAC AAC TCC TAC CCC CTC TAC TCC TGC TGG GAGGCC TGG CCC 714
P S A I T L T V T R E E H H F M L D T Y N N S Y P L Y S C W E A W P 209
715 GAG ACA GAG ATG AGG AAG ATC TAC ACT ACC GTC CTC TTT TCC CAC ATC TAC GTG GCA CCC CTC GCC CTC ATT GTC ATC ATG TAT GCC CGC ATT GCC TTC AAG 816
E T E M R K I Y T T V L F S H I Y V T M V A P L A L I V I M Y A R I A F K 243
817 CTC TTC AAG TCA GCA GCG CCC GCC CGC AGC ACC CGG CCT GAG GAG CCCGAG GGG AGG AAG GTGTCC CGT AGG AAG GCG AAG GTC ATC AAC ATG CTC ATC ATC 918
L F K S A A P A R S T R P E E P E G R K V S R R K A K V I N M L I I 277
919 ATG GCC CTC TTC TTC ACC CTC TCC TGG CTG CCC CTC TGG ACA CTG ATG CTG CTT ACA GAC TAC GGG CAC CTC AGC GAG CAC CAG CTG CAC CTG GTC ACC GGC 1020
M A L F T M V L F T L S W L P L W T L M L L T D Y G H L S E H Q L H L V T G 311
1021 TAC ATC TTT CCC TTT GCC CAC TGG CTG GCT TTC TTC AAC AGC AGC GCC AAC CCC ATC ATC TAC GGC TAC TTC AAT GAG AAC TTC CGA CGG GGC TTC CAG GAG 1122
Y I F P F A H W L T M V L A F F N S S A N P I I Y G Y F N E N F R R G F Q E 345
1123 GCC TTCAGAGCCCTTTC TGG TTG CCA CCG TGC CGG CAG CAC CGT GGG CCC TAT GGT GCC CGC AAC CGT GTC TTC ACC CAG CCC CAG GCC AGC GAC TCT CCC 1224
A F R A P F W L P P C R Q H R G P Y G A R N R V F T Q P Q A S D S P 379
1225 CCGCAC TCA GAA TCG AGGCCA CTG GCA TCC CGA CGG GCC GGC ATC CCT GCG TGG AAT GGC TGA GCA GCT GAG GCC ACC AGC TTG CCT GAT AGA TGT GGG GAC 1326
P H S E S R P L A S R R A G I P A W N G * 399
1327 CTC GTG CAT GGC ACT GCT GTA GGG TGG ACA CCC CAT ATC ATT GTG GGT TTG GGG TCT TGA TGT GAT GAT CAG TGA GTG TCC AAC CTA AGG GCT TGT TGG GAG 1428
1429 TGGAGG GGA ATA GGGGGT TTGTAATGT CACTGC AATAAA GGCACGATG CTG-poly(A) 1479

B

Figure 1 Structural analysis of a putative quail GnIH receptor. (A) Nucleotide sequence and deduced amino acid sequence of a putative quail GnIH receptor cDNA. The seven putative TMs are underlined. The N-linked glycosylation sites are indicated in bold italics. The poly(A)⁺ adenylation signal AATAAA is shown by a dotted line. (B) Hydrophobicity profile according to the analysis with DNASIS-MAC software (Hitachi, Kanagawa, Japan).

products were subcloned and the DNA inserts were amplified as described above.

DNA sequencing

All nucleotide sequences were determined with an ABI Prism Dye terminator cycle-sequencing ready reaction kit (PE-Biosystems, Foster, CA, USA) and a model 373A automated DNA sequencer (PE-Biosystems), then analysed using DNASIS-MAC software (Hitachi Software Engineering, Kanagawa, Japan). Universal M13 primers or gene-specific primers were used to sequence both strands.

Transient transfection

The full-length open reading frame of the identified putative GnIH receptor was amplified from quail diencephalon cDNA using the forward primer 5'-GCCG CCACCATGCAGCGCTGCGGCACCA-3' and the reverse primer 5'-GCTCAGCCATTCCACGCAG GGAT-3', and subcloned into TOPO-pcDNA3.1 (Invitrogen), a mammalian expression vector. 15 positive colonies were selected, subcultured, and all their plasmid DNAs purified by the Wizard *plus* SV minipreps DNA purification system (Promega) and fully sequenced. The colony that produced the plasmid construct completely identical with the receptor sequence was selected, cultured and purified by the Wizard *plus* maxipreps DNA purification system (Promega).

COS-7 cells were supplied from Riken Cell Bank (Tsukuba, Japan) and grown in 10 cm dishes as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (50 U/ml), streptomycin (50 µg/ml) and Hepes (10 mM, pH 7.4) in 5% CO₂ at 37 °C. Transfection was performed with the TransFast transfection reagent (Promega) according to the manufacturer's instructions. At 2 or 3 days after transfection, the dishes were washed twice with PBS (10 mM phosphate buffer and 140 mM NaCl, pH 7.4), and the cells were harvested. The cells were then centrifugated (1300 g for 10 min at 4 °C) and stored at -80 °C.

Radioiodination and binding assay

The synthetic C-terminal quail GnIH-RP-1 (amino acids 31–37; Satake *et al.* 2001, Osugi *et al.* 2004) with an added N-terminal tyrosine residue, YANLPLRF-amide, was radioiodinated with ¹²⁵I (Na¹²⁵I; Amersham Biosciences) by the lactoperoxidase method as described previously (Tsutsui & Ishii 1978, 1980, Tsutsui *et al.* 1985). Although GnIH contained an endogenous tyrosine residue, the binding activity of GnIH was reduced by iodination. Therefore, we used the synthetic C-terminal GnIH-RP-1 tagged with the N-terminal tyrosine residue as a ligand of iodination. Labeled YANLPLRF-amide was separated

from free ¹²⁵I on a C₁₈ reversed-phase column (Azumaya & Tsutsui 1996, Tsutsui *et al.* 1997, 1998). The specific activity of ¹²⁵I-YANLPLRF-amide was estimated at 200 µCi/nmol. For the receptor preparation, frozen COS-7 cells (1.25 × 10⁶ cells) were rapidly thawed and homogenized with a glass homogenizer with a Teflon Pestle in cold assay buffer (0.04 M Tris-HCl buffer (pH 7.4) containing 5 mM MgSO₄, 0.1% BSA and 0.1 mM phenylmethylsulfonylfluoride, a protease inhibitor; Tsutsui *et al.* 1997, 1998). The homogenates were centrifugated at 11 000 g for 20 min at 4 °C. The resulting pellets were resuspended in cold assay buffer and adjusted as the receptor preparations equivalent to 800 µg protein of crude membrane fractions of COS-7 cells/100 µl buffer.

Binding experiments were performed as described previously (Azumaya & Tsutsui 1996, Tsutsui *et al.* 1997, 1998). In brief, 50 µl of the non-radioactive peptide in assay buffer or the assay buffer alone, 50 µl of the ¹²⁵I-YANLPLRF-amide and 100 µl of the receptor preparation (800 µg protein of crude membrane fractions of COS-7 cells) were added to disposable plastic centrifuge tubes with a capacity of 1.5 ml. All of the reaction tubes had previously been coated with BSA to reduce the adsorption of peptides to the tube wall. The tubes were placed in a water-bath incubator with continuous shaking at 20 °C for 1 h. At the end of the incubation period, 1 ml cold assay buffer was added to each tube, and the tubes were centrifugated at 11 000 g for 3 min at 4 °C. The pellets were then washed twice with cold assay buffer, and the radioactivity of the resulting pellets was counted in an autowell γ-counter (Aloka, Tokyo, Japan). In order to examine the ligand specificity of the binding to the crude membrane fraction of COS-7 cells transfected with the putative GnIH receptor cDNA, competition-binding experiments were performed as follows: 1.25 nM ¹²⁵I-YANLPLRF-amide was incubated with different amounts of GnIH, GnIH-RP-1, GnIH-RP-2, GnIH-OH (C-terminally non-amidated GnIH) and other neuropeptides (Met-enkephalin-RF, galanin and neuropeptide Y) which lack the C-terminal LPXRF-amide motif. In the saturation-binding experiment, different amounts of ¹²⁵I-YANLPLRF-amide (0.234–3.75 nM) were incubated with or without an excess of cold YANLPLRF-amide (0.253–4.05 µM). Scatchard plots were constructed from the data obtained from the saturation-binding experiment. The dissociation constant (*K_d*) and the maximal binding sites (*B_{max}*) were then determined with Scatchard plots. Linearity of the Scatchard plots and 95% confidence intervals for the *K_d* and *B_{max}* were computed according to the method of Bliss (1967).

Southern-blot hybridization of reverse transcriptase-mediated PCR (RT-PCR) products

The first-strand cDNA was synthesized from total RNA (1 µg) prepared from the pituitary or each different brain

region with Moloney-murine-leukaemia virus reverse transcriptase (Promega) and an oligo(dT) primer in accordance with the manufacturer's instructions. The oligo-nucleotide primers used for the amplification of receptor cDNA fragments were 5'-CTCATTGTCATCATGTAT GCC-3' (identical to nucleotides 781–801) and 5'-TCA GCCATTCCACGCAGGGAT-3' (complementary to nucleotides 1267–1287). Primers for the amplification of β -actin cDNA fragments were 5'-GAGACCTTCAA CACCCCAG-3' (identical to nucleotides 310–328 in the quail β -actin gene; accession number AF199488) and 5'-GACAGAGTACTTGCGCTCAG-3' (complementary to nucleotides 935–954 in the quail β -actin gene). PCR was performed with an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min, and held at 72 °C for 3 min. PCR products were resolved on a 1.5% (w/v) agarose gel electrophoresis followed by transfer to a Hybond N⁺ membrane (Amersham Biosciences). The membrane was hybridized with digoxigenin-labeled oligonucleotide probe (5'-AATGAGAACTTCCGACG GGGC-3'; identical to nucleotides 1093–1113). Digoxigenin labeling of DNA and detection were performed in accordance with the DIG system protocol (Roche Diagnostics).

Results

Characterization of cDNA encoding GnIH receptor

We first cloned a cDNA encoding a putative GnIH receptor in the quail diencephalon by a combination of 3' and 5' RACE, based on the receptor for rat RFRP, an orthologous peptide of GnIH. As shown in Fig. 1A, the nucleotide and deduced amino acid sequences of a putative quail GnIH receptor revealed a full length of 1479 bp and an open reading frame of 1197 bp encoding 399 amino acid residues with a calculated molecular mass of 45.7 kDa. Analysis of this protein for regional hydrophobicity revealed seven putative transmembrane domains (TMs; underlined in Fig. 1A and depicted as seven hydrophobic peaks in Fig. 1B), connected by three cytosolic and three extracellular loops, extracellular N-terminal and cytosolic C-terminal domains that are characteristic of GPCRs (Ostrowski *et al.* 1992, Sun *et al.* 2001). The predicted initiator methionine codon agreed with Kozak's first-AUG rule (Kozak 1989). There were four potential Asn-linked glycosylation sites, three near the N-terminus (Asn¹⁵, ²⁰, ³⁰) and one in the second extracellular loop connecting TMIV and TMV (Asn¹⁹⁶; Fig. 1A). This putative quail GnIH receptor contained eight Cys residues, while the C-terminal Cys residue (Cys³⁵⁶) may be a site for palmitoylation (O'Dowd *et al.* 1989, Watson & Arkinstall 1994). The C-terminal region of this putative quail GnIH receptor contained five serines and one threonine which may be substrates for protein kinases

(Watson & Arkinstall 1994). The nucleotide sequence data reported will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under accession number AB183891.

Comparison of the deduced amino acid sequence of the putative quail GnIH receptor revealed a higher homology to the receptors for rat and human RFRP than to those for rat and human neuropeptide FF (NPFF; Fig. 2 and Table 1). The full length of the putative quail GnIH receptor displayed 68% and 48–50% amino acid identities to mammalian RFRP receptors and mammalian NPFF receptors, respectively (Table 1). Comparing only the TM regions of the putative GnIH receptor gave 81–86% and 71–73% amino acid identities to mammalian RFRP receptors and mammalian NPFF receptors, respectively (Table 1).

Binding properties of GnIH receptor

To assess the binding activity of the putative quail GnIH receptor, binding experiments were performed using ¹²⁵I-YANLPLRF-amide as a radioligand. The specific binding of ¹²⁵I-YANLPLRF-amide to crude membranes, which were harvested from COS-7 cells transfected with the putative quail GnIH receptor cDNA, increased linearly with the increase of crude membranes within a range less than 1000 µg protein per reaction tube (data not shown). In contrast, there was no specific binding to crude membranes of COS-7 cells expressing only the pcDNA3.1 vector. When the crude membrane fractions (800 µg protein) of COS-7 cells transfected with the putative quail GnIH receptor cDNA were incubated with different concentrations of ¹²⁵I-YANLPLRF-amide for 1 h at 20 °C, the specific binding increased and tended to equilibrate with respect to the concentration of the radioligand (Fig. 3, inset). Scatchard plots of the specific binding, which were constructed from the data of the saturation-binding experiment (Fig. 3, inset), showed a straight line, suggesting the presence of a single class of high-affinity binding sites, with a significant slope ($P < 0.01$; Fig. 3). The dissociation constant (K_d) and the maximal binding sites (B_{max}) were calculated from the fitted line of the plot. The K_d value was 0.752 nM with a 95% confidence interval of 0.595–1.03 nM and the B_{max} was 24.8 fmol/mg protein with a 95% confidence interval of 22.3–29.4 fmol/mg protein (Fig. 3).

To examine the ligand specificity of the binding to crude membranes of COS-7 cells expressing the putative quail GnIH receptor, competition-binding experiments were performed using GnIH, GnIH-RP-1, GnIH-RP-2 and GnIH-OH (C-terminally non-amidated GnIH) as competitors. ¹²⁵I-YANLPLRF-amide (1.25 nM) and crude membranes (800 µg protein) were incubated with various amounts of competitors for 1 h at 20 °C. The binding of ¹²⁵I-YANLPLRF-amide was inhibited as a function of the concentration of each GnIH, GnIH-RP-1

Figure 2 Amino acid sequence alignment of a putative quail GnIH (qGnIH) receptor with the sequences of mammalian (rat, r, and human, h) RFRP and NPFF receptors. Amino acid residues that are identical to those in the putative quail GnIH receptor are shown in white against a black background. Putative TMs are indicated above the aligned sequences. Gaps introduced to obtain optimal alignment are indicated by dashes. Accession numbers: quail GnIH receptor, AB183891; rat RFRP receptor, AB040103; human RFRP receptor, AB040104; rat NPFF receptor, AF268900; human NPFF receptor, AF268899.

and GnIH-RP-2 (Fig. 4). In contrast, GnIH-OH failed to inhibit the binding even at high concentrations (Fig. 4). Competition-binding experiments were further performed using mammalian (RFRP) and amphibian (fGRP) orthologous peptides of GnIH, a chicken fragment of GnIH (LPLRF-amide) and other neuropeptides (Met-enkephalin-RF, galanin and neuropeptide Y) which lack the C-terminal LPXRF-amide motif. As shown in

Table 2, orthologous peptides of GnIH (RFRP and fGRP) and chicken LPLRF-amide, a fragment of GnIH, also inhibited the binding with similar IC_{50} values of GnIH and GnIH-RPs. However, neuropeptides lacking the C-terminal LPXRF-amide motif (Met-enkephalin-RF, galanin and neuropeptide Y) did not inhibit the binding (Table 2).

Expression of GnIH receptor mRNA in the pituitary and brain

The expression of mRNA encoding for the putative quail GnIH receptor was determined by Southern blotting analysis of RT-PCR products from the pituitary, different brain regions and spinal cord. As an internal control, we also detected the expression of mRNA encoding quail β -actin in each different tissue. The quail β -actin cDNA fragment with the size of about 645 bp was amplified with the primer set based on the quail β -actin gene sequence in all tissues at a similar level (Fig. 5). In contrast, a single hybridized band for the 507 bp RT-PCR product

Table 1 Amino acid identities of a putative quail GnIH receptor to mammalian RFRP and NPFF receptors

	Amino acid identity (%)			
	rRFRP-r	hRFRP-r	rNPFF-r	hNPFF-r
Full length	68	68	48	50
Transmembrane	81	86	71	73

Abbreviations: rRFRP-r, rat RFRP receptor, (accession number AB040103); hRFRP-r, human RFRP receptor, (accession number AB040104); rNPFF-r, rat NPFF receptor, (accession number AF268900); hNPFF-r, human NPFF receptor, (accession number AF268899).

Figure 3 Scatchard plots of the binding of ^{125}I -YANLPLRF-amide to the crude membrane fraction of COS-7 cells expressing a putative quail GnIH receptor. B, concentration of bound peptide at apparent equilibrium; F, concentration of free peptide at apparent equilibrium. Inset, saturation-binding data. ● and ○ represent specific and non-specific bindings of duplicate determinations, respectively.

between nucleotides 781 and 1287 was detected in the pituitary, cerebrum, diencephalon, mesencephalon and spinal cord, whereas no band was detected in the cerebellum (Fig. 5). The density of the band was relatively high in the diencephalon, mesencephalon and spinal cord (Fig. 5).

Discussion

We recently identified a novel hypothalamic neuropeptide inhibiting gonadotropin release in the quail and termed it

Figure 4 Competition of specific binding of ^{125}I -YANLPLRF-amide to the crude membrane fraction of COS-7 cells expressing a putative quail GnIH receptor by unlabeled GnIH, GnIH-RP-1, GnIH-RP-2 and GnIH-OH (C-terminally non-amidated GnIH). Each point depicts the mean from duplicate determinations.

GnIH (Tsutsui *et al.* 2000). We will not fully understand the action of GnIH on gonadotropin release unless the receptor for GnIH is identified. In this study we therefore sought to identify the GnIH receptor in the quail. Based on the structure of the receptor for rat RFRP, a mammalian LPXRF-amide peptide (Hinuma *et al.* 2000) which is an orthologous peptide of quail GnIH (an avian LPXRF-amide peptide), we first cloned a cDNA encoding a putative GnIH receptor. This putative GnIH receptor is considered to possess seven TMs as a new member of the GPCR superfamily by the present structural and hydrophobic analyses. Furthermore, binding experiments using the crude membrane fraction of COS-7 cells transfected with the putative GnIH receptor cDNA indicated that this membrane protein specifically binds to GnIH and GnIH-RPs and possesses high-affinity binding sites for these peptides. Taken together, the identified GnIH receptor may be a functional receptor in the quail.

Although the receptors for rat and human RFRP (mammalian LPXRF-amide peptides) have been characterized by Hinuma *et al.* (2000), the presence of the receptors for non-mammalian LPXRF-amide peptides has been unclear. Thus the identified receptor for quail GnIH is a first demonstration of the presence of the non-mammalian LPXRF-amide peptide receptor. On the other hand, several groups have identified recently the receptors for rat and human NPFF, which are also GPCRs (Bonini *et al.* 2000, Elshourbagy *et al.* 2000, Kotani *et al.* 2001). Because the C-terminal sequence of NPFF, FLFQPQRF-NH₂, is closely related to those of RFRP and GnIH, we compared the structure of the quail GnIH receptor with those of rat and human RFRP and NPFF. The deduced amino acid sequence of the quail GnIH

Table 2 Ligand specificity of specific binding to a putative quail GnIH receptor

Ligand	Animal	Sequence	IC ₅₀ (nM)
GnIH	Quail	SIKPSAYLPLRF-NH ₂	5.79
GnIH-RP-1	Quail	SLNFEEMKDWGSKNFMKVNTPTVNVKVPNSVANLPLRF-NH ₂	11.65
GnIH-RP-2	Quail	SSIQSLLNLPQRF-NH ₂	6.70
LPLRF-amide	Chicken	LPLRF-NH ₂	14.06
RFRP	Rat	ANMEAGTMSHFPSLPQRF-NH ₂	2.75
fGRP	Frog	SLKPAANLPLRF-NH ₂	3.23
GnIH-OH		SIKPSAYLPLRF-OH	>1000
Met-enkephalin-RF	Mammal, bird	YGGFMRF-OH	>1000
Galanin	Quail	GWTLNSAGYLLGPHAVDNHRSFNDKHGFT-NH ₂	>1000
Neuropeptide Y	Human, rat	YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY-NH ₂	>1000

receptor showed a higher homology to the receptors for rat and human RFRP (full length, 68%; TMs, 81–86%) than to those for rat and human NPFF (full length, 48–50%;

TMs, 71–73%). The seven transmembrane-spanning domains are suggested to be oriented to form a ligand-binding pocket (Ostrowski *et al.* 1992, Watson & Arkinstall 1994). The C-terminal LPXRF-amide motif of GnIH and RFRP may be a reason for a higher homology of the structure of TMs of these receptors. On the other hand, the presence of Cys residues in the first (Cys¹¹⁷ of quail GnIH receptor) and second (Cys²⁰⁴ of quail GnIH receptor) extracellular loops is a finding in many GPCRs. A disulfide linkage between these two Cys residues may play an important role in ligand interactions in the quail GnIH receptor, as suggested by O’Dowd *et al.* (1989), Ostrowski *et al.* (1992) and Watson & Arkinstall (1994).

To demonstrate whether GnIH and GnIH-RPs can bind to the identified GnIH receptor, we subsequently conducted binding experiments in this study. The specific binding of the radioligand ¹²⁵I-YANLPLRF-amide (C-terminal of GnIH-RP-1 added tyrosine residue) to the crude membrane fraction of COS-7 cells transfected with the putative GnIH receptor cDNA was inhibited as a function of the concentration of GnIH, GnIH-RP-1 and GnIH-RP-2. In contrast, C-terminal non-amidated GnIH (GnIH-OH) failed to inhibit the specific binding, like other neuropeptides such as Met-enkephalin-RF, galanin and neuropeptide Y, which lack the C-terminal LPXRF-amide motif. These results suggest that the identified GnIH receptor specifically binds to GnIH and GnIH-RPs in the quail. On the other hand, competition-binding experiments indicated that RFRP and fGRP, mammalian and amphibian orthologs of GnIH, and chicken LPLRF-amide, a fragment of GnIH, also inhibited the specific binding. It is therefore considered that the identified GnIH receptor recognizes the C-terminal LPXRF-amide motif of these peptides as well as GnIH and GnIH-RPs. Scatchard plots showed the presence of high-affinity binding sites for GnIH and GnIH-RPs. The equilibrium *K*_d value calculated from the fitted line of the plots was 0.752 nM (95% confidence interval, 0.595–1.03 nM). Although physiological functions of GnIH-RPs are still uncertain in the quail, these data obtained by the

Figure 5 RT-PCR analysis together with Southern hybridization of a putative quail GnIH receptor mRNA in the pituitary, different brain regions and spinal cord. The upper panel shows the gel electrophoresis of RT-PCR products for a putative quail GnIH receptor, the middle panel shows identification of the band by Southern hybridization using digoxigenin-labeled oligonucleotide probe for a putative quail GnIH receptor. cDNA corresponding to 1 µg total RNA extracted from each tissue was used for a PCR reaction, and the PCR product was applied on one lane. The lane labeled RT (–) was left without the template as the negative control. The lower panel shows the RT-PCR for β-actin as the internal control. RT-PCR experiments were repeated five times using independently extracted RNA samples from different animals and produced similar results.

present binding experiments suggest that not only GnIH but also GnIH-RPs exert their actions after binding to the identified GnIH receptor.

To elucidate the mode of action of GnIH on gonadotropin release, the expression of GnIH receptor mRNA was further characterized by Southern blotting analysis of the RT-PCR product. Interestingly, the GnIH receptor mRNA was expressed in the pituitary as well as several brain regions and spinal cord. The expression of GnIH receptor mRNA in the pituitary suggests that GnIH acts directly on the pituitary via GnIH receptor to inhibit gonadotropin release. This mode of action of GnIH is in agreement with our previous finding that cell bodies and terminals containing GnIH were localized in the paraventricular nucleus and median eminence, respectively (Tsutsui *et al.* 2000, Ubuka *et al.* 2003, Ukena *et al.* 2003a). In addition, we could detect a higher expression of GnIH receptor mRNA in the diencephalon including the hypothalamus. From our previous studies with quails (Ukena *et al.* 2003a) and sparrows (Bentley *et al.* 2003), GnIH-containing fibers were detected in extremely close proximity to GnRH neurons in the preoptic area. Bentley *et al.* (2004) and Osugi *et al.* (2004) further reported that *in vivo* treatment with GnIH rapidly inhibits GnRH-elicited luteinizing hormone release in sparrows. Therefore, it is plausible that GnIH could be acting at the level of the hypothalamus via GnIH receptor to inhibit gonadotropin release as well as at the pituitary. On the other hand, other brain regions, i.e. the cerebrum and mesencephalon, and the spinal cord also expressed GnIH receptor mRNA. This is consistent with our previous finding that GnIH-containing fibers were distributed throughout the diencephalic and mesencephalic regions as well as in the median eminence in birds (Bentley *et al.* 2003, Ukena *et al.* 2003a). The present and previous findings suggest multiple regulatory functions of GnIH in the avian brain. GnIH may participate not only in neuroendocrine functions but also in behavioral and autonomic mechanisms, because GnIH-containing fibers were found in the ventral paleostriatum, septal area, preoptic area, hypothalamus and optic tectum (Ukena *et al.* 2003a). Behavioral and physiological studies on the basis of these distributions of GnIH and GnIH receptor are now in progress.

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