Characterization of a cDNA encoding a novel avian hypothalamic neuropeptide exerting an inhibitory effect on gonadotropin release

Honoo SATAKE*[1], Miki HISADA*, Tsuyoshi KAWADA*, Hiroyuki MINAKATA*, Kazuyoshi UKENA† and Kazuyoshi TSUTSUI†[2]

*Suntory Institute for Bioorganic Research, Wakayamadai 1-1-1, Shimamoto-cho, Mishima-gun, Osaka 618-8503, Japan, and †Laboratory of Brain Science, Faculty of Integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima 739-8521, Japan

We previously isolated a novel dodecapeptide containing a C-terminal -Arg-Phe-NH$_2$ sequence, SIKPSAYLPLRF-NH$_2$ (RFamide peptide), from the quail brain. This quail RFamide peptide was shown to decrease gonadotropin release from the cultured anterior pituitary and to be located at least in the quail hypothalamo-hypophysial system. We therefore designated this RFamide peptide gonadotropin inhibitory hormone (GnIH). In the present study we characterized the GnIH cDNA from the quail brain by a combination of 3′ and 5′ rapid amplification of cDNA ends ('RACE'). The deduced GnIH precursor consisted of 173 amino acid residues, encoding one GnIH and two putative gene-related peptide (GnIH-RP-1 and GnIH-RP-2) sequences that included -LPXRF (X = L or Q) at their C-termini. All these peptide sequences were flanked by a glycine C-terminal amidation signal and a single basic amino acid on each end as an endoproteolytic site. Southern blotting analysis of reverse-transcriptase-mediated PCR products demonstrated a specific expression of the gene encoding GnIH in the diencephalon including the hypothalamus. Furthermore, mass spectrometric analyses detected the mass numbers for matured GnIH and GnIH-RP-2, revealing that both peptides are produced from the precursor in the diencephalon as an endogenous ligand. Taken together, these results lead to the conclusion that GnIH is a hypothalamic factor responsible for the negative regulation of gonadotropin secretion. Furthermore, the presence of a novel RFamide peptide family containing a C-terminal -LPXRF-NH$_2$ sequence has been revealed.

Key words: gonadotropin inhibitory hormone, mass spectrometry, quail.

INTRODUCTION

The anterior pituitary hormone gonadotropins, namely luteinizing hormone (LH) and follicle-stimulating hormone (FSH), have a major role in the control of vertebrate reproduction (reviewed in [1,2]). A hypothalamic decapeptide hormone, gonadotropin-releasing hormone (GnRH), is known to regulate and modulate the biosynthesis and release of gonadotropin from the anterior pituitary gland [3–8]. Furthermore, GnRH's gene structure [9–12] and expression [13–16], and also its physiological functions, have well been documented. However, the endogenous peptidic factors that suppress gonadotropin secretion have remained to be identified.

We have previously characterized SIKPSAYLPLRF-NH$_2$ (single-letter amino acid code), a novel 12-residue peptide containing a C-terminal Arg-Phe-NH$_2$ sequence (RFamide peptide) from the brain of the Japanese quail (*Coturnix Japonica*) [17]. The striking feature of the quail RFamide peptide is that it exerts a specific inhibitory effect on the release of gonadotropin from the cultured quail anterior pituitary in a dose-dependent manner with a threshold of 1–10 nM [17]. Furthermore, immuno-reactivities for this RFamide peptide were observed in the paraventricular nucleus and median eminence [17]. On the basis of these findings, we proposed that this quail RFamide peptide acts as a novel inhibitory factor of gonadotropin release in the hypothalamo-hypophysial system in birds and named this RFamide peptide gonadotropin inhibitory hormone (GnIH) [17]. However, the detailed physiological roles and biological significance of GnIH remained to be elucidated.

Studies on the localization of the GnIH transcript and the presence of structurally and/or functionally related peptides are expected to provide important clues to a clarification of the endogenous function of GnIH. To examine further the physiological roles and biochemical characteristics of GnIH, which possibly contributes to the establishment of a novel regulation system of gonadotropin release, a cDNA encoding the GnIH precursor polypeptide was identified. Here we describe the GnIH cDNA sequence, the localization of the mRNA in the diencephalon including the hypothalamus by Southern blotting analysis of reverse-transcriptase-mediated PCR (RT–PCR) products, and the detection of the GnIH gene-related peptide (GnIH-RP) containing the homologous C-terminal LPQRF-NH$_2$ sequence by matrix-assisted laser desorption ionization time-of-flight (MALDI–TOF) and quadrupole time-of-flight (Q-TOF) mass spectrometric analyses. These results indicated that GnIH has an essential role as a hypothalamic factor in the

inhibitory regulation of gonadotropin release and that GnIH and GnIH-RP belong to a novel RFamide peptide family carrying a C-terminal LPXRF-NH_2 sequence, where X = L or Q. To the best of our knowledge, this is the first identification of a cDNA encoding a neuropeptide that exerts an inhibitory effect on gonadotropin release and also of an avian RFamide-family peptide cDNA.

EXPERIMENTAL

Oligonucleotides

All nucleotides were obtained from Sawady Technology (Tokyo, Japan).

RNA preparation

Adult males of Japanese quail (*Coturnix japonica*) were housed in a temperature-controlled room at approx. 25 °C. Frozen brains (500 mg) were pulverized by grinding under liquid nitrogen; total RNA was extracted with Sepazol RNA II 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.

Amplification of the partial GnIH cDNA fragments

All PCR amplifications were performed in a reaction mixture containing *Taq* polymerase [EX Taq polymerase (Takara Shuzo, Kyoto, Japan) or rTaq DNA polymerase (Toyobo, Osaka, Japan)] and 0.2 mM dNTP on a thermal cycler (model GeneAmp PCR System 2400; PE-Biosystems, Foster City, CA, U.S.A.). First-strand cDNA was synthesized with the oligo(dT)-anchor primer supplied in the 5′/3′ rapid amplification of cDNA ends (RACE) kit (Roche Diagnostics, Basal, Switzerland) and amplified with the anchor primer (Roche Diagnostics) and the first degenerate primers 5′-AT(A/T/C)AA(A/G)CCIAG(T/C)GCITA(T/C)(T/C)TICC-3′, corresponding to the sequence Ile^2–Pro^9. First-round PCR products were reamplified with the anchor primer and the second degenerate primers 5′-GCITA(T/C)(T/C)TICCI(T/C)TI(A/C)GITT(T/C)GG-3′, corresponding to the sequence Ala^6–Gly^{13}. Both first-round and second-round PCRs consisted of 30 cycles of 30 s at 94 °C, 30 s at 51 °C and 2.5 min at 72 °C (5 min for last cycle). The second-round PCR products were subcloned into a TA-cloning vector in accordance with the manufacturer's instructions (Invitrogen, San Diego, CA, U.S.A.). The DNA inserts of the positive clones were amplified by PCR with universal M13 primers.

Determination of the 5′-end sequence of GnIH cDNA

Template cDNA was synthesized with an oligonucleotide primer complementary to nt 684–703 (5′-ATAACATGACACAGGTT-TGC-3′), 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′-CACGGTGCATCTTTTCTGG-G-3′, complementary to nt 615–634); this was followed by further amplification of the first-round PCR products with the anchor primer (Roche Molecular Biochemicals) and gene-specific primer 2 (5′-TTCTGGGTCTTTCGGTTTCC-3′, complementary to nt 602–621). Both first-round and second-round PCRs were performed for 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 1.5 min at 72 °C. The second-round PCR products were subcloned and the inserts were amplified as described above.

DNA sequencing

All nucleotide sequences were determined with Big-Dye sequencing kits (PE-Biosystems) and a model 373A auto-mated DNA sequencer (PE-Biosystems), then analysed with GENETYX-MAC software (Software Development, Tokyo, Japan). Universal M13 primers or gene-specific primers were used to sequence both strands.

Northern blot hybridization

A digoxigenin-labelled GnIH precursor full-length cDNA was synthesized with a DNA-labelling kit (Roche Diagnostics) and used as a probe for Northern blot analysis. mRNA (1 μg) was separated on a denaturing 1 % (w/v) agarose/formaldehyde gel and fixed on a Hybond N$^+$ membrane (Amersham Pharmacia Life Science, Uppsala, Sweden) by UV irradiation. Hybridization and detection were performed in accordance with the manu-facturer's standard procedure (Roche Diagnostics). RNA size was estimated with the use of digoxigenin-labelled RNA mol-ecular markers (Roche Diagnostics).

Southern blot hybridization of RT–PCR products

The first-strand cDNA was synthesized from total RNA (1 μg) prepared from each brain region with Superscript II reverse transcriptase and an oligo(dT) primer in accordance with the manufacturer's instruction (Life Technologies, Gaithersburg, MD, U.S.A.). The oligonucleotide primer set used for the amplification of GnIH cDNA fragments was 5′-GAGCTTCC-TAACTGAGCTTC-3′ (identical with nt 10–29) and 5′-CACG-GTGCATCTTTTCTGGG-3′ (complementary to nt 615–634); primers for the amplification of β-actin cDNA fragments were 5′-TATGTGCAAGGCCGGTTTCG-3′ (identical with nt 1587–1606 in the chicken β-actin gene [18]) and 5′-ACCTCTCAT-TGCCAATGGTG-3′ (complementary to nt 3129–3148 in the chicken β-actin gene). PCR was performed for 30 cycles (30 s at 94 °C, 30 s at 55 °C and 1.5 min at 72 °C) in the PCR reaction as described above. PCR products were resolved on a 1.5 % (w/v) agarose gel followed by transfer to a Hybond N$^+$ membrane (Amersham Pharmacia Life Science). Hybridization and de-tection were performed in accordance with the DIG system protocol (Roche Diagnostics).

MS

The quail diencephalon extract was prepared as described previously [17]. After evaporation of the extract, the residue was dissolved in 1 μl of matrix solution [10 mg/ml of α-cyano-4-hydrocinnamic acid (Aldrich Chemical, Milwaukee, WI, U.S.A.) in 50 % (v/v) acetonitrile containing 0.1 % (v/v) trifluoroacetic acid] and was analysed by using MALDI–TOF-MS on a Voyager Elite mass spectrometer equipped with a delayed-extraction system and a 337 nm pulsed laser (PE-Biosystems). The accelerating voltage was 20 kV. To acquire an MS/MS spectrum, the extract was purified with a C_{18} reverse-phase column (L-column ODS; Chemicals Evolution and Research Institute, Tokyo, Japan), followed by evaporation of the resultant peptide fractions and dissolution of the residue in 50 % (v/v) methanol containing 0.1 % (v/v) formic acid. MS/MS analysis of the peptide solution was then performed with a Q-TOF tandem mass spectrometer equipped with a Z-spray nano-electrospray inter-face (Micromass, Manchester, U.K.). The needle voltage was optimized at 1000 V; the cone voltage was set at 50 V. Argon was used as the collision gas; the collision gas energy was set at 28 V.

RESULTS

Characterization of cDNA encoding a GnIH precursor polypeptide

In an attempt to obtain GnIH precursor polypeptide cDNA fragments, we initially performed an RT–PCR experiment with degenerate primers corresponding to the partial GnIH sequence Ile[2]–Pro[9] and the anchor primer, followed by reamplification of the first-round PCR products with degenerate primers corresponding to the partial GnIH sequence Ala[6]–Gly[13] and the same anchor primer. Here the C-terminal amide group was thought to be derived from a C-terminal Gly residue, which is known to be a typical amidation signal. Electrophoresis of the nested PCR mixture revealed a major product of approx. 0.7 kb (results not shown). Sequencing of the subcloned inserts showed that all clones had essentially identical nucleotide sequences apart from minor differences in the 3′-terminal sequence, attributable to various lengths of the poly(A) tract. The predicted amino acid sequence included a potential RFamide peptide sequence SSIQSLLNLPQRFG 26 residues downstream of the partial GnIH sequence derived from the second-round PCR primer, implying that this cDNA clone encoded an RFamide peptide including a C-terminal sequence similar to that of GnIH (LPLRF-NH$_2$). To determine whether the cDNA clones included a GnIH sequence, we performed 5′ RACE with specific primers for the clone. A single product of approx. 0.6 kb (results not shown) was obtained and sequenced, revealing that these cDNA clones contained several putative ATG initiation codons and a single nucleotide sequence encoding the complete GnIH sequence. PCR products amplified with different polymerases had identical nucleotide sequences. This result confirmed that these cDNA clones were not generated by artifacts. The entire GnIH precursor cDNA was identified by combining nucleotide sequences determined by these RACE experiments. Figure 1 shows that the GnIH precursor cDNA was composed of 947 nt

```
                     AGAGATCCTGAGCTTCCTAACTGAGCTTCATTTGAGAGAA   40
ATGGAAATCATTTCAACCCAGAAGTTTATTCTTCTTACTTTGGCTACAGTGGCGTTTCTA  100
 M  E  I  I  S  T  Q  K  F  I  L  L  T  L  A  T  V  A  F  L   20

ACACCGCATGGTGCGTGCCTAGATGAACTAATGAAATCCAGCCTGGAAAGCAGAGAAGAT  160
 T  P  H  G  A  C  L  D  E  L  M  K  S  S  L  E  S  R  E  D   40

GATGATGATAAATATTATGAGACTAAAGACAGTATCTTGGAGGAAAAGCAGAGGAGTCTG  220
 D  D  D  K  Y  Y  E  T  K  D  S  I  L  E  E  K  Q  R  S  L   60

AATTTTGAAGAAATGAAAGACTGGGGATCAAAAAATTTCATGAAAGTGAACACCCCTACA  280
 N  F  E  E  M  K  D  W  G  S  K  N  F  M  K  V  N  T  P  T   80

GTAAACAAAGTGCCAAATTCAGTTGCTAATTTGCCTCTTAGATTTGGAAGAAGCAATCCA  340
 V  N  K  V  P  N  S  V  A  N  L  P  L  R  F  G  R  S  N  P  100

GAAGAAAGAAGCATTAAGCCAAGTGCTTATTTGCCTCTGAGATTTGGAAGAGCTTTTGGA  400
 E  E  R  S  I  K  P  S  A  Y  L  P  L  R  F  G  R  A  F  G  120

GAGAGCCTCTCTAGGCGTGCTCCAAATCTATCAAACAGGTCTGGGGATCTCCACTTGCT  460
 E  S  L  S  R  R  A  P  N  L  S  N  R  S  G  R  S  P  L  A  140

AGAAGTTCTATTCAGTCCCTTCTAAATCTGCCACAGAGATTTGGGAAGTCAGTGCCCATC  520
 R  S  S  I  Q  S  L  L  N  L  P  Q  R  F  G  K  S  V  P  I  160

AGTCTATCTCAAGGTGTCCAGGAATCTGAACCAGGGATGTGAATTCTAACAATTACATTC  580
 S  L  S  Q  G  V  Q  E  S  E  P  G  M  *                    173

AAGTTATCAAGTTATGAAGATGGAAACCGAAAGACCCAGAAAAGATGCACCGTGTATTCA  640
AAACTAAAGATCTTGAGAAGGGAATGTATATGAGAACTGAAATGCAAACCTGTGTCATGT  700
TATTATTATAATGTGTGTATTCTGCACAAACAATTGATGCATAAGGCAATGATAACTGTA  760
GTGAATGTCCAGTTTACTTCAGTGGTGGGTGTAGCACAAACTCTATTTACTTTTGTGTGA  820
CTTCCTGTCGACTGATATCTTGTTGGATGTAAAAACTAATTTAAAAACAATAGCTGTAGA  880
AATGAGAGCATTTTAAGCAAATTTAAATGAAACATCCGTAGTACAAATAAAGAAGCATGA  940
TACAAAC-poly(A)                                               947
```

Figure 1 Nucleotide sequence and deduced amino acid sequence of GnIH precursor polypeptide cDNA

The GnIH sequence is boxed and indicated in bold. GnIH-RP sequences are also boxed. Single basic amino acid cleavage sites are shown in bold. The poly(A) adenylation signal AATAAA is underlined.
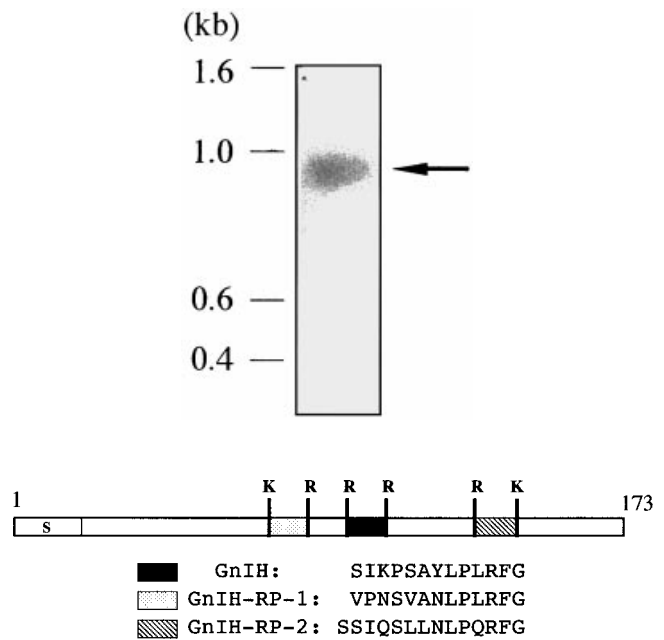

Figure 2 Size of the GnIH transcript and structural organization of the GnIH precursor polypeptide

Upper panel: Northern blot analysis of mRNA prepared from the quail brain. mRNA was extracted from the brain of the quail and approx. 1 µg of RNA was subjected to Northern blot hybridization with a digoxigenin-labelled GnIH cDNA probe. The single positive band is indicated by an arrow. The positions of RNA molecular mass markers are shown on the left. Lower panel: schematic representation of the GnIH precursor. The hydrophobic leader sequence is labelled S. The predicted endoproteolytic single Arg or Lys residue is indicated in black.

containing a short 5′ untranslated sequence of 40 bp, a single open reading frame of 519 bp, and a 3′ untranslated sequence of 388 bp with the addition of various lengths of poly(A) tail. The open reading frame region began with three putative start codons at positions 41, 131 and 233 and terminated with a TGA stop codon at position 560. However, use of the ATG codon at position 131 as a start codon was highly unlikely because it did not conform to the Kozak rule [19]. A single polyadenylation signal (AATAAA) was found in the 3′ untranslated region at position 925.

Northern blot analysis of poly(A)+ RNA detected a single band of approx. 1.0 kb (Figure 2, upper panel), suggesting that no alternatively spliced forms were present. Furthermore, the apparent migration of approx. 1.0 kb was in good agreement with the estimated length of the cDNA, 947 bp. This result confirmed that the cDNA clone included a full-length nucleotide sequence encoding the GnIH precursor.

Structural organization of a GnIH precursor polypeptide

The open reading frame region encoded a 173-residue polypeptide. We predicted that the GnIH transcript would be translated with Met[1] or Met[65]. However, a hydropathy plot analysis of the GnIH precursor demonstrated that the most hydrophobic moiety, which is typical in a signal peptide region, followed Met[1] but not Met[65] (results not shown). These results suggest that Met[1] served as an initiation methionine. The cleavage site of the signal peptide was the Ala[18]-Phe[19] bond, which is supported by the −3, −1 rule [20]. The precursor included at least three RFamide peptide sequences (Figure 2, lower panel): a single copy of the GnIH sequence SIKPSAYLPLRF flanked
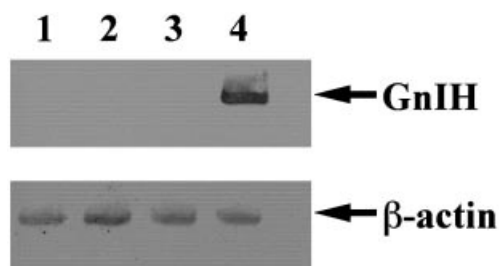
Figure 3   Localized expression of GnIH mRNA

Southern blot analysis of RT–PCR products for GnIH (upper panel) and β-actin (lower panel) transcripts isolated from the telencephalon (lane 1), mesencephalon (lane 2), cerebellum (lane 3) and diencephalon (lane 4). PCR products were resolved on 1.5% (w/v) agarose gel followed by transfer to a nylon membrane and hybridization with a digoxigenin-labelled GnIH or β-actin cDNA probe as appropriate.

by a glycine residue as a C-terminal amidation signal was located at residues 104–116, and two putative RFamide peptide sequences VPNSVANLPLRF (GnIH gene-related peptide 1, GnIH-RP-1) and SSIQSLLNLPQRF (GnIH-RP-2), both of which were also flanked by the amidation signal, were encoded at residues 84–95 and 142–154 respectively (Figures 1 and 2, lower panel). Of particular interest is the fact that GnIH-RP-1 and GnIH-RP-2 contain a C-terminal LPXRF sequence (where X represents L in GnIH-RP-1 or Q in GnIH-RP-2), which is identical with or similar to the sequence LPLRF at the C-terminus of GnIH. In addition, the GnIH-RP-1 and GnIH-RP-2 sequences display respectively 50% and 56% sequence identities to that of GnIH. Conservation of the LPXRF motif in these peptide sequences seems to indicate that the similar sequence is essential for the physiological function of these peptides. Furthermore, both the GnIH and GnIH-RPs sequences were flanked on both sides by a single Arg or Lys residue instead of a typical endoproteolytic dibasic amino acid sequence (Figures 1 and 2, lower panel).

Taken together, these findings suggest that both GnIH-RPs and GnIH are generated from the GnIH precursor, followed by participation in the regulation of some physiological functions.

Expression of the GnIH gene in brain tissues

The expression pattern of the GnIH gene in four regions of the brain was determined by Southern blotting analysis of RT–PCR products that were prepared from the telencephalon, mesencephalon, celebellum and diencephalon. Initially, we established an internal control by detecting the expression of the gene encoding quail β-actin in each region. The quail β-actin cDNA fragment was amplified with the primer set based on the chicken β-actin gene sequence [18]. A sequence analysis showed that this RT–PCR product encoded an amino acid sequence that was 96% similar to chicken β-actin, confirming the specific amplification of the quail β-actin cDNA fragment (results not shown). Southern blotting hybridization showed that the β-actin transcript was present in all tissues at a similar level (Figure 3, lower panel).

In contrast, a single hybridized band for the 625 bp GnIH RT–PCR product between nt 10 and 634 was detected exclusively in lane 4 (Figure 3, upper panel), clearly demonstrating the specific expression of the gene encoding GnIH in the diencephalon. We therefore conclude that GnIH was biosynthesized exclusively in the diencephalon, which includes the hypothalamus.

Detection of both GnIH-RP-2 and GnIH in the diencephalon by MS

As mentioned above, the presence of the predicted Gly C-terminal amidation signal and a mono-Arg or Lys processing site at the N-terminus and C-terminus of the GnIH-RP sequences (very similar to the GnIH sequence) in the precursor implied that GnIH-RPs could be matured in a manner similar to that of GnIH. To examine whether GnIH-RPs also existed as mature peptides in the diencephalon, we performed a MALDI–TOF-MS analysis of the quail diencephalon extract in the mass range
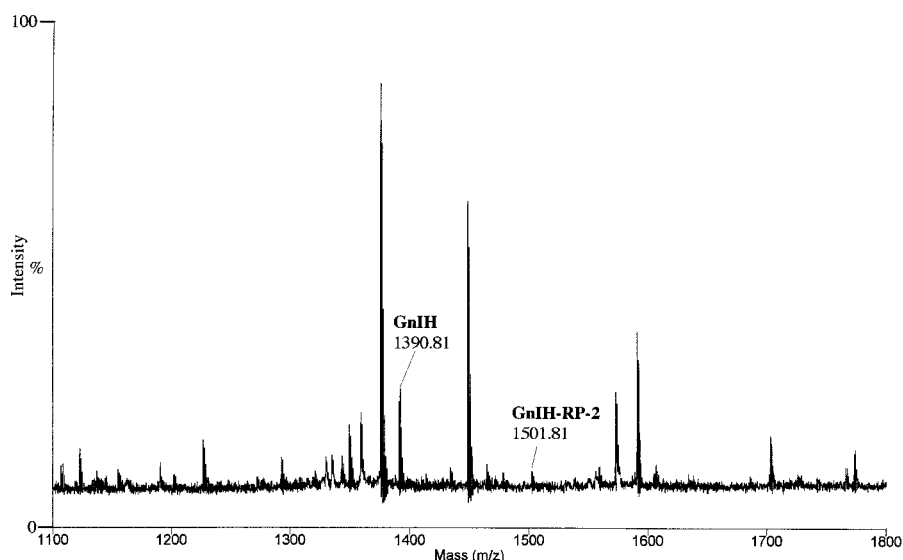

Figure 4   Detection of GnIH and GnIH-RP-2 in the diencephalon by MALDI–TOF-MS

The crude peptide was analysed on a MALDI–TOF mass spectrometer. The spectrum demonstrates the mass peaks corresponding to GnIH (1390.81) and GnIH-RP-2 (1501.81), respectively.

Table 1 Calculated and observed molecular masses of peptides

All mass values are given for the monoisotropic species. Abbreviation: n.d., not detected.

Peptide	Calculated mass, $[M+H]^+$	Observed mass, $[M+H]^+$
GnIH	1390.82	1390.81
GnIH-RP-1	1325.53	n.d.
GnIH-RP-2	1501.84	1501.81

1100–1800. As shown in Figure 4, the mass peaks corresponding not only to GnIH (1390.81; Table 1) but also to GnIH-RP-2 (1501.81; Table 1) were obtained, whereas the predicted mass of GnIH-RP-1 was not detected. These results indicated the presence of GnIH-RP-2 as well as GnIH in the diencephalon. The Q-TOF-MS/MS analysis of the purified fraction containing the predicted GnIH-RP-2 revealed that the fraction with a mass number of 1501.81 was derived from the GnIH-RP-2 amino acid sequence. Figure 5 shows fragmentation patterns of the peptide with the observed mass number of 1501.81, which agreed well

with those of synthetic GnIH-RP-2 (results not shown). Assignment of the observed typical fragment ions such as b and y″ ions confirmed that the sequence of this peptide was compatible with the GnIH-RP-2 sequence, SSIQSLLNLPQRF-NH$_2$ (Figure 5). The predicted and observed mass values of fragment ions are summarized in Table 2. These results revealed that GnIH-RP-2 is undoubtedly produced in the quail diencephalon in a mature form. Furthermore, the presence of both GnIH and GnIH-RP-2 strongly suggests that these peptides constitute a novel RFamide peptide family with a LPXRF-NH$_2$ (X = L or Q) sequence at the C-terminus.

DISCUSSION

Since the purification of FMRFamide as a cardioexcitatory molecule from the central nervous system of the bivalve mollusc *Macrocallista nimbosa* [21], numerous RFamide peptides have been identified in various species. Several mammalian and other vertebrate RFamide peptides, such as opioid modulator neuropeptide FF (NPFF) [22], prolactin-releasing peptides (PrRPs) [23] and their fish counterpart *Carassius* RFamide (C-RFa)
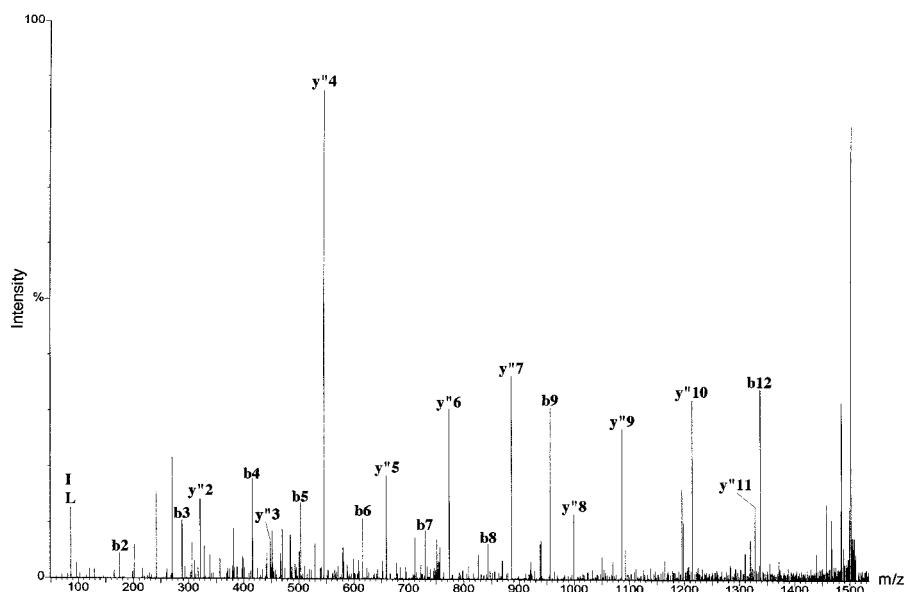
Figure 5 Sequence analysis of GnIH-RP-2 by Q-TOF-MS/MS procedures

The spectrum shows typical mass values of predicted GnIH-RP-2 fragment ions (also see Table 2). Only the observed b and y″ ions are labelled.

Table 2 Predicted and observed MS/MS fragments for GnIH-RP-2

N-terminal fragmentation ions (a, b, c″) and single amino immonium acid ions (i) are listed above the peptide sequence; C-terminal fragmentation ions (x, y″, z) are listed below. The observed mass values are indicated in bold.

a	60.0	147.1	260.2	388.2	475.3	**588.3**	701.4	815.5	**928.5**	1025.6	1153.7	**1309.8**	–
b	88.0	**175.1**	**288.2**	**416.2**	**503.2**	**616.3**	**729.4**	**843.5**	**956.5**	1053.6	**1181.7**	**1337.8**	–
c″	105.1	192.1	**305.2**	433.2	520.3	633.4	**746.4**	860.5	973.6	1070.6	1198.7	**1354.8**	–
i	60.0	60.0	**86.1**	101.1	60.0	**86.1**	**86.1**	87.1	**86.1**	70.1	101.1	129.1	120.1
	1	2	3	4	5	6	7	8	9	10	11	12	13
	Ser	Ser	Ile	Gln	Ser	Leu	Leu	Asn	Leu	Pro	Gln	Arg	Phe
	13	12	11	10	9	8	7	6	5	4	3	2	1
x	–	1440.8	1353.8	**1240.7**	1112.6	1025.6	912.5	799.4	685.4	572.3	475.2	347.2	191.1
y″	–	1414.8	**1327.8**	**1214.7**	**1086.6**	**999.6**	**886.5**	**773.4**	**659.4**	**546.3**	**449.3**	**321.2**	165.1
z	–	**1397.8**	**1310.8**	**1197.7**	**1069.6**	982.6	**869.5**	**756.4**	642.4	**529.3**	432.2	304.2	148.1

[24,25], are believed to be involved in important central regulatory functions. The quail RFamide dodecapeptide, GnIH, which was isolated from the brain, is the first example of an inhibitory peptide of gonadotropin secretion [17], in contrast with GnRH, whose structure and functions have been well identified in vertebrates. To understand further the physiological roles of GnIH and subsequently to explore the unprecedented regulatory system of gonadotropin release, we determined the GnIH cDNA sequence, the localization of the transcript and the production of the gene-related peptide in the present study. We found that the GnIH precursor polypeptide encodes two putative RFamide peptide sequences, GnIH-RP-1 and GnIH-RP-2, as well as GnIH. All three peptides share the C-terminal sequence LPXRF-NH$_2$ (with X representing L in GnIH and GnIH-RP-1, and Q in GnIH-RP-2), and their sequences are flanked on both ends by monobasic endoproteolytic residues Arg or Lys (Figures 1 and 2, lower panel) instead of a typical dibasic cleavage site. Moreover, a series of mass spectrometric analyses verified the presence of not only GnIH but also GnIH-RP-2 as a mature endogenous ligand (Figures 4 and 5 and Table 1), suggesting that cleavage occurs at the single basic amino acids present at both termini of GnIH and GnIH-RP-2. An endoproteolytic site containing a single basic amino acid is relatively unusual but is occasionally found in prepronexuropeptides and preprohormones [26–29]. Several precursor convertases (PCs) are believed to recognize and cleave a precursor polypeptide at the Arg-$(Xaa)_{2n}$-Lys/Arg sequence ($n = 1$ or 2 in many cases) rather than at basic amino acid pairs (reviewed in [29]). The C-terminal sequences of GnIH and GnIH-RPs followed by an amidation signal and a basic amino acid, the Arg-Phe-Gly-Lys/Arg sequences (Figure 1) are in complete accordance with the consensus recognition sequence. Thus we suggest that GnIH and GnIH-RPs all undergo this PC-catalysed endoproteolytic processing at their C-terminus. In contrast, the N-terminal processing mechanism is unclear, given that only a single Arg residue, instead of a common PC-recognition site, was present immediately before the N-terminus of each of the GnIH and GnIH-RP sequences (Figures 1 and 2, lower panel). Nevertheless, similar N-terminal cleavage was observed in several precursors of other RFamide peptides such as PrRP20s and C-RFa [23–25], where a single Arg residue is located immediately before active peptide sequences and the adjacent sequences contain no identified PC-recognition motif, although a dibasic amino acid site follows the C-terminus. These findings suggest that an atypical or unknown endoproteolytic mechanism, in which the single Arg residue is recognized and cleaved, is involved in the N-terminal processing of several vertebrate RFamide peptides, including GnIH and GnIH-RP-2. In addition, a related peptide, GnIH-RP-1, was not shown to be generated. A single Lys residue is located immediately before the GnIH-RP-1 sequence, in contrast with the GnIH and GnIH-RP-2 sequences, which are preceded by a single Arg residue (Figures 1 and 2, lower panel). Therefore, the single Lys residue and its surrounding sequence are unlikely to be used as a processing site during the maturation of GnIH and GnIH-RP-2, although we cannot entirely exclude the possibility that premature GnIH-RP-1 is subjected to further processing and modification or that the predicted GnIH-RP-1 is present below the detectable level for the MS performed in this study.

Of particular significance is that the GnIH gene was found to be expressed specifically in the diencephalon, including the hypothalamus (Figure 3), revealing that the production of GnIH is confined to this brain region. Moreover, GnIH exerted an inhibitory effect on gonadotropin release; in our previous study immunoreactivity for GnIH was located in the hypothalamus [17]. Interestingly, numerous terminals of GnIH-like immuno-

stained paraventricular nucleus neurons were observed in the median eminence [17], implying that GnIH acts as an endogenous ligand in the hypothalamo-hypophysial system. Taken together, these findings provide the evidence that GnIH is a novel hypothalamic factor involved in the negative control of gonadotropin release. We also suggest, with regard to the physiological role of GnIH as a hypothalamic gonadotropin-releasing inhibitory factor, that GnIH and/or its closely related peptides constitute a novel RFamide peptide family that is widely distributed in vertebrate species, although no vertebrate RFamide peptide family conserved between different species has ever been reported, except for PrRPs and C-RFa. We are currently attempting to characterize structurally and functionally related peptides from other vertebrate species.

In the present study we showed that GnIH and GnIH-RP-2 are produced from the GnIH precursor. Northern hybridization demonstrated that no alternatively spliced transcriptional variants occur in the brain (Figure 2, upper panel). Consequently it is most unlikely that cellularly or regionally specific differential production of GnIH and/or GnIH-RP-2 is regulated at the transcriptional level in the brain. Two post-translational processing pathways for GnIH and GnIH-RP-2 can be presumed. First, GnIH and GnIH-RP-2 are yielded simultaneously from the precursor and are packed into the same secretory vesicle in a neuron. Thus they are expected to exert some endogenous activity on a common or closely related target, leading to the co-operative or synchronous induction of physiological functions such as an inhibitory effect on gonadotropin release. Alternatively, the differential post-translational endoproteolysis of the GnIH precursor might be associated with the physiological functions of GnIH and GnIH-RP-2. Recently, some bioactive peptides originating from one precursor polypeptide have been shown to be differentially processed or sorted into distinct secretory granules [30–35], which enables peptides to undergo cell-specific or tissue-specific functional targeting. These findings support a notion that proGnIH might undergo differential proteolytic processing and/or that GnIH and GnIH-RP-2 are packed into different secretory granules for subsequent release into distinct target tissues. In this case, GnIH-RP-2 is predicted to be indirectly involved in the endogenous functions of GnIH, even if GnIH-RP-2 is devoid of the direct inhibitory activity on gonadotropin release. The biological activity and the localizations of the GnIH-RP-2 molecule are now being examined to establish both the physiological role of GnIH-RP-2 as another novel hypothalamic factor and also the functional correlation between GnIH and GnIH-RP-2.

In conclusion, we have identified for the first time the cDNA encoding the avian RFamide peptide GnIH possessing the inhibitory effect on gonadotropin release, the localization of the GnIH mRNA, and the presence of the structurally related peptide GnIH-RP-2. We believe that our findings can contribute to the further study of both the regulatory mechanism of gonadotropin release and the physiological roles of the RFamide peptide family in vertebrates.

REFERENCES

1 Vaitukaitis, J. L., Ross, G. T., Braunstein, G. D. and Rayford, P. L. (1976) Gonadotropins and their subunits: basic and clinical studies. Recent Prog. Horm. Res. **32**, 289–331

2 Wuttke, W. (1976) Neuroendocrine mechanisms in reproductive physiology. Rev. Physiol. Biochem. Pharmacol. **76**, 59–101

3 Matsuo, H., Bada, Y., Nair, R. M. G., Arimura, A. and Schally, A. V. (1971) Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. Biochem. Biophys. Res. Commun. **43**, 1334–1339

4   Burgus, R., Butcher, M., Amoss, M., Ling, N., Monahan, M., Rivier, J., Fellows, R., Blackwell, R., Vale, W. and Guillemin, R. (1972) Primary structure of the ovine hypothalamic luteinizing hormone-releasing factor (LRF) (LH-hypothalamus-LRF-gas chromatography-mass spectrometry-decapeptide-Edman degradation). Proc. Natl. Acad. Sci. U.S.A. **69**, 278–282

5   King, J. A. and Millar, R. P. (1982) Structure of chicken hypothalamic luteinizing hormone-releasing hormone. I. Structural determination on partially purified material. J. Biol. Chem. **257**, 10722–10728

6   Sherwood, N., Eiden, L., Brownstein, M., Spiess, J., Rivier, J. and Vale, W. (1983) Characterization of a teleost gonadotropin-releasing hormone. Proc. Natl. Acad. Sci. U.S.A. **80**, 2794–2798

7   Miyamoto, K., Hasegawa, Y., Nomura, M., Igarashi, M., Kangawa, K. and Matsuo, H. (1984) Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species. Proc. Natl. Acad. Sci. U.S.A. **81**, 3874–3878

8   Sherwood, N. M., Sower, S. A., Marshak, D. R., Fraser, B. A. and Brownstein, M. J. (1986) Primary structure of gonadotropin-releasing hormone from lamprey brain. J. Biol. Chem. **261**, 4812–4819

9   Seeburg, P. H. and Adelman, J. P. (1984) Characterization of cDNA for precursor of human luteinizing hormone releasing hormone. Nature (London) **311**, 666–668

10 Adelman, J. P., Manson, A. J., Hayflick, J. S. and Seeburg, P. H. (1986) Isolation of the gene and hypothalamic cDNA for the common precursor of gonadotropin-releasing hormone and prolactin release-inhibiting factor in human and rat. Proc. Natl. Acad. Sci. U.S.A. **83**, 179–183

11 Reinhart, J., Mertz, L. M. and Catt, K. J. (1992) Molecular cloning and expression of cDNA encoding the murine gonadotropin-releasing hormone receptor. J. Biol. Chem. **267**, 21281–21284

12 White, S. A., Bond, C. T., Francis, R. C., Fernald, R. D. and Adelman, J. P. (1994) A second gene for gonadotropin-releasing hormone: cDNA and expression pattern in the brain. Proc. Natl. Acad. Sci. U.S.A. **91**, 1423–1427

13 Richards, J. S. (1994) Hormonal control of gene expression in the ovary. Endocr. Rev. **15**, 179–189

14 Kakar, S. S. and Jennes, L. (1995) Expression of gonadotropin-releasing hormone and gonadotropin-releasing hormone receptor mRNAs in various non-reproductive human tissues. Cancer Lett. **83**, 57–62

15 von Schalburg, K. R. and Sherwood, N. M. (1999) Regulation and expression of gonadotropin-releasing hormone gene differs in brain and gonads in rainbow trout. Endocrinology **140**, 3012–3024

16 Fernald, R. D. and White, R. B. (1999) Gonadotropin-releasing hormone genes: phylogeny, structure, and functions. Front. Neuroendocrinol. **20**, 224–240

17 Tsutsui, K., Saigoh, E., Ukena, K., Teranishi, H., Fujisawa, Y., Kikuchi, M., Ishii, S. and Sharp, P. J. (2000) A novel avian hypothalamic peptide inhibiting gonadotropin release. Biochem. Biophys. Res. Commun. **275**, 661–667

18 Kost, T. A., Theodorakis, N. and Hughes, S. H. (1983) The nucleotide sequence of the chick cytoplasmic beta-actin gene. Nucleic Acids Res. **11**, 8287–8301

19 Kozak, M. (1987) An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. **15**, 8125–8148

20 von Heijne, G. (1986) A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14**, 4683–4690

21 Price, D. A. and Greenberg, M. J. (1977) Structure of a molluscan cardioexcitatory neuropeptide. Science **197**, 670–671

22 Yang, H.-Y. T., Fratta, W., Majane, E. A. and Costa, E. (1985) Isolation, sequencing, synthesis, and pharmacological characterization of two brain neuropeptides that modulate the action of morphine. Proc. Natl. Acad. Sci. U.S.A. **82**, 7757–7761

23 Hinuma, S., Habata, Y., Fujii, R., Kawamata, Y., Hosoya, M., Fukusumi, S., Kitada, C., Masuo, Y., Asano, T., Matsumoto, H. et al. (1998) A prolactin-releasing peptide in the brain. Nature (London) **393**, 272–276

24 Fujimoto, M., Takeshita, K., Wang, X., Takabatake, I., Fujisawa, Y., Teranishi, H., Ohtani, M., Muneoka, Y. and Ohta, S. (1998) Isolation and characterization of a novel bioactive peptide, *Carassius* RFamide (C-RFa), from the brain of the Japanese crucian carp. Biochem. Biophys. Res. Commun. **242**, 436–440

25 Satake, H., Minakata, H., Wang, X. and Fujimoto, M. (1999) Characterization of a cDNA encoding a precursor of *Carassius* RFamide, structurally related to a mammalian prolactin-releasing peptide. FEBS Lett. **446**, 247–250

26 Hosaka, M., Nagahama, M., Kim, W. S., Watanabe, T., Hatsuzawa, K., Ikemizu, J., Murakami, K. and Nakayama, K. (1991) Arg-X-Lys/Arg-Arg motif as a signal for precursor cleavage catalyzed by furin within the constitutive secretory pathway. J. Biol. Chem. **266**, 12127–12130

27 Nakayama, K. (1997) Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins. Biochem. J. **327**, 625–635

28 Day, R., Lazure, C., Bazak, A., Boudreault, A., Limperis, P., Dong, W. and Linberg, I. (1998) Prodynorphin processing by proprotein convertase 2. Cleavage at single basic residues and enhanced processing in the presence of carboxypeptidase activity. J. Biol. Chem. **273**, 829–836

29 Seidah, N. G. and Chrétien, M. (1999) Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. Brain Res. **848**, 45–62

30 Fisher, J. M., Sossin, W., Newcomb, R. and Scheller, R. H. (1988) Multiple neuropeptides derived from a common precursor are differentially packaged and transported. Cell **54**, 813–822

31 Danoff, A., Cutler, D. F. and Shields, D. (1991) Heterologous expression of preprosomatostatin. Intracellular degradation of prosomatostatin-II. J. Biol. Chem. **266**, 10004–10010

32 Blomquist, J. F. and Baenziger, J. U. (1992) Differential sorting of lutropin and the free α-subunit in cultured bovine pituitary cells. J. Biol. Chem. **267**, 20798–20803

33 Klumperman, J., Spijker, S., van Minnen, J., Sharp-Baker, H., Smit, A. B. and Geraerts, W. P. M. (1996) Cell type-specific sorting of neuropeptides: a mechanism to modulate peptide composition of large dense-core vesicles. J. Neurosci. **16**, 7930–7940

34 Perone, M. J., Murray, C. A., Brown, O. A., Gibson, S., White, A., Linton, E. A., Perkins, A. V., Lowenstein, P. R. and Castro, M. G. (1998) Procorticotrophin-releasing hormone: endoproteolytic processing and differential release of its derived peptides within AtT20 cells. Mol. Cell. Endocrinol. **25**, 191–202

35 Nillni, E. A. (1999) Neuroregulation of ProTRH biosynthesis and processing. Endocrine **10**, 185–199