Identification of a cDNA encoding a novel amphibian growth hormone-releasing peptide and localization of its transcript

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Abstract

Recently, we identified in the bullfrog brain a novel neuropeptide with a C-terminal Leu-Pro-Leu-Arg-Phe-NH₂ sequence. This amphibian neuropeptide was shown to stimulate growth hormone (GH) release *in vitro* and *in vivo* and so was designated frog GH-releasing peptide (fGRP). In this study, we cloned a cDNA encoding fGRP from the bullfrog brain by a combination of 3' and 5' rapid amplification of cDNA ends (RACE). The deduced fGRP precursor consisted of 221 amino acid residues, encoding one fGRP and three putative fGRP-related peptides that included Leu-Pro-Xaa-Arg-Phe-NH₂ (Xaa=Leu or Gln) 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. Northern blot analysis detected a single band of approximately 1.0 kb, indicating that no alternatively spliced forms were present. Such an apparent migration was in agreement with the estimated length of the cDNA, 902 bp. *In situ* hybridization further revealed the cellular localization of fGRP mRNA in the supra-chiasmatic nucleus in the hypothalamus. In addition to fGRP, its related peptides may be hypothalamic factors involved in pituitary hormone secretion.

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Introduction

It has been established that in a variety of vertebrates hypothalamic neurons produce neuropeptides, each regulating the secretion of a particular pituitary hormone. In amphibians, growth hormone (GH) secretion from the somatotrophs is known to be under dual control: stimulatory and inhibitory. As stimulating factors, GH-releasing hormone (GHRH; for a review, see Montero *et al.* 2000), pituitary adenylate cyclase-activating polypeptide (PACAP; Martinez-Fuentes *et al.* 1994, Montero *et al.* 2000), and ghrelin (Kaiya *et al.* 2001) of amphibian origin have been nominated. As an inhibiting factor, frog somatostatin (Jeandel *et al.* 1998) has been described.

Since the molluscan neuropeptide Phe-Met-Arg-Phe-NH₂ (FMRFamide) was found in the ganglia of the venus clam (Price & Greenberg 1977), immunohistochemical studies using the antiserum against FMRFamide have provided information that the vertebrate hypothalamus possesses some unknown neuropeptide similar to FMRFamide found in invertebrates. Recently, neuropeptides having the RFamide motif at their C-termini (RFamide peptides) have been identified in the brains of several vertebrates. For instance, Leu-Pro-Leu-Arg-Phe-NH₂ (LPLRFamide), a chicken pentapeptide, was purified as

the first RFamide peptide found in the vertebrate brain (Dockray *et al.* 1983). Two pain modulatory neuropeptides FF and AF (Yang *et al.* 1985), prolactin-releasing peptide (PrRP; Hinuma *et al.* 1998), and its fish counterpart, *Carassius* RFamide (Fujimoto *et al.* 1998), are also RFamide peptides. These RFamide peptides have been shown to play some important physiological roles in neuroendocrine, behavioral, sensory and autonomic functions (Panula *et al.* 1996, 1999, Ibata *et al.* 2000).

On the other hand, we previously identified SIKPSAYLPLRF-NH₂, a novel hypothalamic RFamide peptide, which inhibited gonadotropin release in the quail and we termed this dodecapeptide gonadotropin-inhibitory hormone (GnIH; Tsutsui *et al.* 2000). Molecular cloning of cDNA encoding GnIH from the quail brain revealed that it encoded GnIH and its related peptides (GnIH-RP-1 and GnIH-RP-2), which contained a C-terminal LPXRF-NH₂ (X=L or Q) sequence (Satake *et al.* 2001). The chicken pentapeptide LPLRFamide (Dockray *et al.* 1983) may be a fragment of GnIH (Satake *et al.* 2001). In addition, cDNAs encoding novel RFamide-related peptides (RFRPs) similar to GnIH were reported to exist in mammalian brains, based on the results of a gene database search (Hinuma *et al.* 2000), and the deduced RFRPs were suggested to participate in

neuroendocrine (Hinuma *et al.* 2000) and pain mechanisms (Liu *et al.* 2001) in rodents. Two peptides have been predicted to be encoded in the cDNA of rodent RFRPs. Furthermore, we recently identified the octadecapeptide ANMEAGTMSHFPSLPQRF-NH₂ as one of the rodent RFRPs (Ukena *et al.* 2002). In addition, a pentatriacontapeptide (SLTFEEVKDWAPKIKMNKPV VNKMPPSAANLPLRF-NH₂) has been purified as one of the bovine RFRPs (Fukusumi *et al.* 2001). Collectively, these RFamide peptides identified in the brain of higher vertebrates are characterized by the LPXRFamide motif at their C-termini (LPXRFamide peptides).

In view of immunohistochemical findings indicating that some of the FMRFamide-like immunoreactive neurons project to the hypothalamic region close to the pituitary in amphibians (D'Aniello *et al.* 1996, Pinelli *et al.* 1999), we looked for a novel amphibian RFamide peptide existing in the bullfrog hypothalamus. As a result, a dodecapeptide, SLKPAANLPLRF-NH₂ with a C-terminal LPLRFamide motif, was isolated (Koda *et al.* 2002). Interestingly, this peptide stimulated the release of GH from the bullfrog pituitary and was designated as frog GH-releasing peptide (fGRP; Koda *et al.* 2002). With these findings as a background, in the present study we sought and identified a cDNA encoding the fGRP precursor polypeptide as the next step to investigate further the physiological roles and biochemical characteristics of fGRP, which possibly contributes to the establishment of a novel regulatory system of GH release. Here we describe the fGRP cDNA sequence and the localization of the mRNA in the hypothalamus by *in situ* hybridization.

Materials and Methods

RNA preparation

Adult bullfrogs (*Rana catesbeiana*) were used in this study. The experimental protocol was approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Hiroshima University, Japan. Total RNA of the diencephalon was extracted by the guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi 1987), and the poly(A)+RNA was then isolated with Oligotex-(dT) 30 (Daiichikagaku, Tokyo, Japan) in accordance with the manufacturer's instructions.

Amplification of the partial fGRP cDNA fragment

All PCR amplifications were carried out in a reaction mixture containing *Taq* polymerase (Ex *Taq* polymerase (Takara Shuzo, Kyoto, Japan), *PfuTurbo* polymerase (STRATAGENE, La Jolla, CA, USA) or gene *Taq* polymerase (NIPPON GENE, Tokyo, Japan)) and 0.2 mM dNTP on a thermal cycler (Program Temp Control System PC-700, ASTEC, Fukuoka, Japan). 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, Basel, Switzerland) and amplified with the anchor primer (Roche Diagnostics) and the first degenerate primers 5'-(T/C)TIAA(A/G)CCIGCIGCIAA(T/C)(T/C)TICC-3' (I represents inosine), corresponding to the fGRP sequence Leu²-Lys³-Pro⁴-Ala⁵-Ala⁶-Asn⁷-Leu⁸-Pro⁹ (Koda *et al.* 2002). First-round PCR products were reamplified with the anchor primer and the second degenerate primers 5'-GCIAA(T/C)(T/C)TICCI(T/C)TI(C/A)GITT(T/C)GG-3', corresponding to the fGRP sequence Ala⁶-Asn⁷-Leu⁸-Pro⁹-Leu¹⁰-Arg¹¹-Phe¹²-Gly¹³ (Koda *et al.* 2002). Both first-round and second-round PCRs consisted of 30 cycles for 30 s at 94 °C, 30 s at 51 °C, and 1 min at 72 °C (11 min for the 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, USA). The DNA inserts of the positive clones were amplified by PCR with universal M13 primers.

Determination of the 5'-end sequence of fGRP cDNA

Template cDNA was synthesized with an oligonucleotide primer complementary to nt 580–599 (5'-TTCAATTC TAGAGGATGGGA-3'); this synthesis was 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 and gene-specific primer 1 (5'-GACTTTAGTCTTGCCAGACA-3', complementary to nt 428–447); this was followed by further amplification of the first-round PCR products with the anchor primer and gene-specific primer 2 (5'-GGGAAT GCTTTTGGCTATTT-3', complementary to nt 380–399). Both first-round and second-round PCRs were performed for 35 cycles consisting of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C (11 min for the last cycle). The second-round PCR products were subcloned into a pPCR-Script Amp SK(+) cloning vector in accordance with the manufacturer's instructions (STRATAGENE), and the 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 City, CA, USA) and a model 373A automated DNA sequencer (PE-Biosystems), and then analyzed with DNASIS-MAC software (Hitachi Software Engineering, Kanagawa, Japan). Universal M13 primers or gene-specific primers were used to sequence both strands.

ATGAGTTGA
 ATGCACTTGAAAATAGGCACATTGACTATAAGAACCATCATGCTTTTCACGCTTGCACC 69
 M H L K I G T L T I R T I M L F T L C T 20

 TTCCTCACTCTTTTCGCCTTTTCCACCTGCTTTGATGAGACTAAAACAATCAATCTAGAA 129
 F L T L F A F S T C F D E T K T I N L E 40

 AGTCAAGAGATATATGATGACTTGTTTGAGTCCAAAGAAGATTACAGAATGAGAGGAAT 189
 S Q E I Y D D L F E S K E D L Q N E R N 60

 GGTAATTCTGATGAGTATAGGTACATGGGATCCAACAGCATAAATGACATTAACAGATAC 249
 G N S D E Y R Y M G S N S I N D I N R Y 80

 AACAGACTGTCCATGCAGCCTGAGAACGAATTTCTACAGGAACGAAGCCTTAAACCTGCT 309
 N R L S M Q P E N E F L Q E R **S L K P A** 100

 fGRP
 GCAAATCTGCCTCTGAGATTTGGAAGAACATCCGATGACAAAATAGCCAAAAGCATTTCCC 369
A N L P L R F G R T S D D K I A K S I P 120

 AGCTTTGATAAAATAGCCAAAAGCATTCCCAATCTACCGCAACGGTTTGGGCGTTACTTG 429
 S F D K I A K **S I P N L P Q R F G** R **Y L** 140

 fGRP-RP-1
 TCTGGCAAGACTAAAGTCCAGTCGATGGCTAATTTACCGCAGAGGTTTGGAAAGAGCACAA 489
S G K T K V Q S M A N L P Q R F G R **A Q** 160

 fGRP-RP-2
 TATACCAACCATTTTGTTCACCTCTCTTGATACATTACCACTGCGATTTGGAAGAACGCCG 549
Y T N H F V H S L D T L P L R F G R T P 180

 fGRP-RP-3
 CATTCTGATAGACTTCAATATGAAATGAATTCCCATCCTCTAGAATTGAAAAACCTGAA 609
 H S D R L Q Y E M N S H P L E L K N P E 200

 GAAGACAGTGACAGGAAGAAAAGACAAGCAATGACCTTCCGAATACGAACGGATCTCCAG 669
 E D S D R K K R Q A M T F R I R T D L Q 220

 ATGTAGCAGCTAGTGAATCGCCACAAAACCCATCATCTGTCCAACACTGTATTTCAAAC 729
 M * 221

 AGCCAGATATGCACTCTTTGCACATAGTGGTGAAATCAGCTTTACGCTAATGCTTGACCC 789

 ACTTTGAAATCAAAGAAAAGTGATGAAAATGCAAAGCAAAGAAATGCATCAGAAAAC TG 849

 TGATGAAAACAATTATTTAGTTAAAGTACAACAAAAC TACATTATTATACTGT-poly(A) 902

Figure 1 Nucleotide sequence and deduced amino acid sequence of frog GH-releasing peptide (fGRP) precursor polypeptide cDNA. The sequences of putative LPXRFamide peptides including fGRP are boxed. Putative precursor convertase (PC)-recognition sites of fGRP-RP-1, -2 and -3 are underlined. The asterisk indicates the stop codon.

Figure 2 Size of the fGRP transcript. Northern blot analysis of mRNA prepared from the bullfrog brain. mRNA was purified from approximately 100 µg total RNA and subjected to Northern blot hybridization with a DIG-labeled fGRP RNA probe. The single positive band is indicated by the arrow. The positions of RNA molecular mass markers are shown on the left.

Northern blot analysis

A digoxigenin (DIG)-labeled antisense RNA probe was produced with an RNA labeling kit (Roche Diagnostics) from a part of the fGRP precursor cDNA (complementary to nt 2–855, including all open reading frames) and used for Northern blot analysis. mRNA 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 u.v. irradiation. Hybridization and detection were performed in accordance with the manufacturer's standard procedure (Roche Diagnostics). RNA size was estimated with DIG-labeled RNA molecular markers (Roche Diagnostics).

In situ hybridization of fGRP mRNA

The site of fGRP mRNA expression in the brain was localized by *in situ* hybridization. In brief, adult bullfrogs were killed by decapitation. After dissection from the skull, the brains were fixed in 4% paraformaldehyde in PBS (pH 7.3) overnight at 4 °C and then soaked in a refrigerated sucrose solution (30% sucrose in PBS) until they sank. Whole brains were embedded in OCT compound (Miles Inc., Elkhart, IN, USA) and freeze-

sectioned frontally or sagittally at a 10 µm thickness with a cryostat at –20 °C. The sections were placed onto 3-aminopropyltriethoxysilane-coated slides. *In situ* hybridization was carried out according to our previous method (Ukena *et al.* 1999, Matsunaga *et al.* 2001) using the DIG-labeled antisense RNA probe, which was also used as a probe in the Northern blot hybridization.

Control for specificity of the *in situ* hybridization of fGRP mRNA was performed by using the DIG-labeled sense RNA probe, which was complementary to a common sequence of the antisense probe.

Immunohistochemistry of fGRP

Immunohistochemical analysis was performed as previously described (Koda *et al.* 2002). In brief, adult bullfrogs were killed by decapitation and the brains were fixed as described above. Frontal or sagittal sections (10 µm thickness) of the brain were made with a cryostat at –20 °C. After nonspecific binding components had been blocked, the sections were immersed with the antiserum raised against fGRP (Koda *et al.* 2002) at a dilution of 1:1000 overnight at 4 °C and subsequently with rhodamine-conjugated goat anti-rabbit IgG. The localization of immunoreactive cell bodies and fibers in the bullfrog brain were examined with a Nikon fluorescence microscope. The specificity of the staining was assessed by substituting the antiserum with antiserum (1:1000 dilution) that had been preabsorbed with fGRP at a saturating concentration (20 µg synthetic fGRP/ml) overnight before use (see Koda *et al.* 2002).

Results

Characterization of cDNA encoding fGRP precursor polypeptide

In an attempt to obtain fGRP precursor polypeptide cDNA fragments from the bullfrog brain, we initially performed an RT-PCR experiment with degenerate primers corresponding to the partial fGRP sequence Leu²-Lys³-Pro⁴-Ala⁵-Ala⁶-Asn⁷-Leu⁸-Pro⁹ and the anchor primer, which was followed by reamplification of the first-round PCR products with degenerate primers corresponding to the partial fGRP sequence Ala⁶-Asn⁷-Leu⁸-Pro⁹-Leu¹⁰-Arg¹¹-Phe¹²-Gly¹³ 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 (Bradbury *et al.* 1982, Suzuki *et al.* 1990, Eipper *et al.* 1991). Electrophoresis of the nested PCR mixture revealed a major product of approximately 0.65 kb (results not shown). The predicted amino acid sequence included three copies of the potential RFamide peptide sequence LPQRFG or LPLRFG downstream of the partial fGRP sequence derived

Figure 3 Cellular localization of fGRP mRNA in the brain. The expression of fGRP mRNA was localized by *in situ* hybridization. Distribution of fGRP mRNA in the suprachiasmatic nucleus (SCN) as observed in frontal (a) or sagittal (c) sections of the bullfrog brain. Lack of hybridization of fGRP mRNA by the sense probe (control) in a frontal section is evident (b). Immunohistochemical staining of a sagittal brain section (d) adjacent to that in (c) was carried out with antiserum against fGRP. Scale bars represent 100 μ m.

from the second-round PCR primer, implying that this cDNA clone encoded also an RFamide peptide including a C-terminal sequence similar to that of fGRP (LPLRF-NH₂). To determine whether the cDNA clones included also a fGRP sequence, we performed 5' RACE with specific primers for the clone. A single product of approximately 0.4 kb (results not shown) was obtained and sequenced, revealing that these cDNA clones contained a fGRP sequence. The entire fGRP precursor cDNA was identified by combining nucleotide sequences determined by these RACE experiments. Figure 1 shows that the deduced fGRP precursor encoded one fGRP and three putative gene-related peptide (fGRP-RP-1, -RP-2 and -RP-3) sequences that included LPXRF (X=L or Q) at their C-termini. The fGRP precursor cDNA was composed of 902 nt containing a short 5' untranslated sequence of 9 bp, a single open reading frame of 663 bp, and a 3' untranslated sequence of 230 bp with a poly(A) tail of various lengths (Fig. 1). The open reading frame region began with a start codon at position 10 and terminated

with a TAG stop codon at position 673. The nucleotide sequence data reported will appear in DDBJ, EMBL and Gene Bank Nucleotide Sequence Databases under the accession number AB080743.

Detection of fGRP transcript in the frog brain

Northern blot analysis of poly(A)⁺ RNA detected a single band of approximately 1.0 kb in the bullfrog brain (Fig. 2), indicating that no alternatively spliced forms were present. In addition, the apparent migration of approximately 1.0 kb was in good agreement with the estimated length of the cDNA, 902 bp. This result confirmed that the cDNA clone identified here included a full-length nucleotide sequence encoding the precursor of fGRP.

Cellular localization of fGRP mRNA in the frog brain

In situ hybridization of fGRP mRNA was examined in the bullfrog brain by using an RNA probe with a sequence

Table 1 Sequences of frog GH-releasing peptide (fGRP), putative fGRP-related peptide (-RPs) and other identified LPXRFamide peptides

Sequence	Animal	Name	Reference
SLTFEEVKDWAPKIKMNKPVVNMPPSAANLPLRF-NH ₂	Cow	RFRP-1	Fukusumi <i>et al.</i> (2001)
ANMEAGTMSHFPSLPQRF-NH ₂	Rat	RFRP-2	Ukena <i>et al.</i> (2002)
SIKPSAYLPLRF-NH ₂	Quail	GnIH	Tsutsui <i>et al.</i> (2000)
SSIQSLNLNPQRF-NH ₂	Quail	GnIH-RP-2	Satake <i>et al.</i> (2001)
SLKPAANLPLRF-NH ₂	Bullfrog	fGRP	Koda <i>et al.</i> (2002) and this study
SIPNLQRF-NH ₂	Bullfrog	fGRP-RP-1	This study
YLSGKTKVQSMANLPQRF-NH ₂	Bullfrog	fGRP-RP-2	This study
AQYTNHFVHSLDTLPLRF-NH ₂	Bullfrog	fGRP-RP-3	This study

complementary to that of the fGRP precursor mRNA. Expression was finally detected by enzyme immunohistochemistry. An intense expression of fGRP mRNA was detected only in the suprachiasmatic nucleus (SCN) in the hypothalamus (Fig. 3a and c). The control study using the sense RNA probe resulted in the complete absence of the fGRP mRNA expression in the SCN (Fig. 3b), suggesting that the reaction was specific for fGRP mRNA. Furthermore, the SCN cells expressing fGRP mRNA (Fig. 3c) were also stained by the antiserum raised against fGRP (Fig. 3d). A complete absence of such an immunoreaction in the SCN cells was observed by pre-incubation of the antiserum with synthetic fGRP (data not shown; see Koda *et al.* 2002).

Discussion

Recently, we identified a novel hypothalamic neuropeptide stimulating GH release from the bullfrog pituitary *in vitro* and *in vivo*, and therefore designated it fGRP (Koda *et al.* 2002). This peptide was characterized by a structure including the C-terminal LPLRF-NH₂ motif (Koda *et al.* 2002). In the present study, we determined the fGRP cDNA sequence and the localization of the transcript in the bullfrog brain as the next step. We first identified a cDNA encoding fGRP from the bullfrog brain by a combination of 3' and 5' RACE. We predicted that the fGRP transcript would be translated with Met¹, because a hydropathy plot analysis of the fGRP precursor demonstrated that the most hydrophobic moiety, which is typical in a signal peptide region, followed Met¹. The cleavage site of the signal peptide was the Phe²⁵-Ala²⁶ bond. The deduced precursor polypeptide consisted of 221 amino acid residues, encoding one fGRP and three putative gene-related peptide sequences, i.e. fGRP-RP-1, -2 and -3 (Fig. 1). All four peptides shared the C-terminal sequence LPXRF motif (with X representing L in fGRP and fGRP-RP-3, and Q in fGRP-RP-1 and fGRP-RP-2). In the frog, it has been demonstrated that the proprotein convertases PC1 and PC2, which are responsible for the processing of neuropeptide precursors,

are abundantly expressed in the brain including the hypothalamus (Vieau *et al.* 1998, Gangnon *et al.* 1999). Several proprotein convertases (PCs) are usually considered to recognize and cleave a precursor polypeptide at the Lys/Arg-(Xaa)*n*-Lys/Arg sequence (*n*=0, 2, 4 or 6; reviewed in Seidah & Chrétien 1999). Their sequences of putative fGRP-RP-1, -2 and -3 are flanked on both ends by the typical endoproteolytic sequences, i.e. KIAK or RFGK (Fig. 1), suggesting that mature peptides may be generated. Although fGRP (SLKPAANLPLRF-NH₂) purified previously has the cleavage sequences of RSLK and RFGK in the precursor polypeptide, the N-terminal cleavage does not occur between Lys and Pro (Fig. 1). Therefore, some other N-terminal processing mechanism(s) may be present in this precursor polypeptide. On the other hand, Gly preceding the C-terminal cleavage site may serve as a C-terminal amidation signal (Bradbury *et al.* 1982, Suzuki *et al.* 1990, Eipper *et al.* 1991). Future study is required to characterize mature endogenous fGRP-RPs by mass spectrometric analysis and clarify their biological functions.

Identification of the cells expressing fGRP mRNA in the brain must be taken into account when studying the action of neuropeptides. In the present study, we therefore characterized the site showing the expression of fGRP mRNA by *in situ* hybridization. The fGRP mRNA expression was localized in the SCN in the bullfrog hypothalamus. The control study using a sense RNA probe resulted in a complete absence of the fGRP mRNA expression, suggesting the validity of the *in situ* hybridization technique. In our previous study (Koda *et al.* 2002), the cell bodies and terminals containing fGRP were localized immunohistochemically in the SCN and median eminence (ME) respectively. It was demonstrated earlier that the frog ME receives innervation from SCN neurons (Carr *et al.* 1991, D'Aniello *et al.* 1996). Thus the present results obtained by *in situ* hybridization analysis are in agreement with the previous immunohistochemical findings (Koda *et al.* 2002). Taken together, it is possible that fGRP acts as an endogenous ligand in the bullfrog hypothalamo-hypophysial system. Although immunoreactive cell bodies were found not only in the SCN but

also in the anterior preoptic area (POA) and medial septal area (MSA; Koda *et al.* 2002), we detected the fGRP mRNA signal only in the SCN. It may be that the expression level of fGRP mRNA in the POA and MSA was too low to detect the signal in these brain regions with the present *in situ* hybridization or that some other undiscovered peptide(s) that cross-reacts with the antiserum against fGRP exists in the POA and MSA. Notwithstanding such discrepancy in the POA and MSA, many neuropeptides, such as thyrotropin-releasing hormone, galanin, PACAP, and somatostatin are known to be expressed in the SCN of the frog (Lamacz *et al.* 1989, Laquerrière *et al.* 1989, Lázár *et al.* 1991, Yon *et al.* 1992). We need to elucidate the co-localization of fGRP/fGRP-RPs and these neuropeptides and their interaction in the frog SCN.

We recently identified novel hypothalamic neuropeptides containing a C-terminal LPXRF-NH₂ sequence (LPXRFamide peptides) in the avian and amphibian species: GnIH (SIKPSAYLPLRF-NH₂) and its related peptide GnIH-RP-2 (SSIQSLNLPQRF-NH₂) in the quail (Tsutsui *et al.* 2000, Satake *et al.* 2001) and fGRP (SLKPAANLPLRF-NH₂) in the bullfrog (Koda *et al.* 2002). In addition, similar neuropeptides RFRPs were identified in mammals: RFRP-1 (SLTFEEVKDWAPKI KMNKPVVNKMPPSAANLPLRF-NH₂) in the bovine (Fukusumi *et al.* 2001) and RFRP-2 (ANMEAGTMS HFPSLPQRF-NH₂) in the rat (Ukena *et al.* 2002). As summarized in Table 1, all of the identified neuropeptides are LPXRFamide peptides. fGRP-RPs are also LPXRFamide peptides (Table 1). The present study suggests that fGRP-RPs as well as fGRP may be involved in the regulation of secretion of GH and other pituitary hormones in the bullfrog. The mammalian RFRP has been suggested to regulate prolactin secretion (Hinuma *et al.* 2000). Therefore, the presence of LPXRFamide peptides regulating pituitary hormone secretion may be a conserved property of the vertebrate brain. To draw a firm conclusion, we are now conducting studies on fish.

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