Identification, localization and expression of LPXRFamide peptides, and melatonin-dependent induction of their precursor mRNA in the newt brain

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Abstract

The existence of RFamide peptides with a C-terminal LPXRFamide (X=L or Q) motif has been identified in the brain of various vertebrate species. However, the presence of LPXRFamide peptides in the urodele brain is not yet known. In this study, we cloned a cDNA encoding the precursor of LPXRFamide peptides from the newt brain by a combination of 3' and 5' rapid amplification of cDNA ends. The deduced LPXRFamide peptide precursor consisted of 233 amino acid residues, encoding four putative LPXRFamide peptides. All the peptide sequences were flanked by a glycine C-terminal amidation signal and basic amino acid on each end as an endoproteolytic site. Mass spectrometric analyses detected a nonapeptide, two decapeptides and an octapeptide produced from the precursor polypeptide in the brain as endogenous ligands. *In situ* hybridization further revealed the cellular localization of

newt LPXRFamide (nLPXRFa) precursor mRNA in the suprachiasmatic nucleus (SCN) in the newt hypothalamus. Immunocytochemistry showed a cluster of cell bodies restricted to the SCN and their terminals in the median eminence. To understand the regulatory mechanism of nLPXRFa peptide expression, we further analyzed the effect of melatonin on the expression of nLPXRFa precursor mRNA. Melatonin administration to newts increased the expression of nLPXRFa precursor mRNA in the diencephalon. These results indicate that the urodele hypothalamus possesses LPXRFamide peptides and the expression of LPXRFamide peptides is regulated by melatonin. The localization of nLPXRFa peptides further suggests that these peptides may be involved in the regulation of pituitary hormone release in newts.

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Introduction

The molluscan neuropeptide Phe-Met-Arg-Phe-NH₂ (FMRFa) was discovered in the ganglia of the venus clam (Price & Greenberg 1977), and it was suggested that the vertebrate hypothalamus possesses some unknown neuropeptides similar to FMRFa (Raffa 1988, Rastogi *et al.* 2001). In fact, over the past decade, neuropeptides that have the Arg-Phe-NH₂ (RFa) motif at their C-termini have been identified in the brains of several vertebrates. Based on the structures of vertebrate RFa peptides, to date, at least five groups of the RFa peptide family have been documented (for reviews, see Ukena & Tsutsui 2005, Tsutsui & Ukena 2006, Osugi *et al.* 2006). Among these groups of the RFa peptide family, the LPXRFamide (X=L or Q) peptide (LPXRFa peptide) group is considered to be the largest one. We recently identified several LPXRFa peptides in the brain of various vertebrates. We first identified a novel neuropeptide with a C-terminal LPLRFa motif in the quail brain (Tsutsui *et al.* 2000). It was shown

that this avian neuropeptide was located in the hypothalamo-hypophyseal system (Tsutsui *et al.* 2000, Ubuka *et al.* 2003, Ukena *et al.* 2003a) and decreased gonadotropin release *in vitro* (Tsutsui *et al.* 2000) and *in vivo* (Osugi *et al.* 2004, Ubuka *et al.* 2006, Chowdhury *et al.* 2010). We therefore designated this neuropeptide as gonadotropin-inhibitory hormone (GnIH; Tsutsui *et al.* 2000). Subsequently, several neuropeptides which were closely related to GnIH were identified in the brains of other vertebrates, such as mammals (RFamide-related peptides (RFRPs), Fukusumi *et al.* 2001, Ukena *et al.* 2002, Yoshida *et al.* 2003, Ubuka *et al.* 2009a,b), frogs (frog GH-releasing peptide (fGRP), Koda *et al.* 2002, Ukena *et al.* 2003b; Rana RFamide (R-RFa), Chartrel *et al.* 2002) and fish (goldfish (gf) LPXRFa, Sawada *et al.* 2002b, Amano *et al.* 2006). Thus, it is becoming clear that GnIH and its orthologs having a common C-terminal LPXRFa motif (LPXRFa peptides) are synthesized in the brain in a variety of vertebrates (for reviews, see Ukena & Tsutsui 2005, Tsutsui & Ukena 2006, Tsutsui 2009, 2010, Tsutsui & Osugi 2009, Tsutsui *et al.* 2010a,b).

Seasonal breeding amphibians serve as excellent animal models to understand physiological roles of GnIH and its orthologs (LPXRFa peptides) and their regulatory mechanisms. Recently, a novel neuropeptide with a C-terminal LPLRFa sequence was identified in the bullfrog (fGRP; Koda *et al.* 2002) and European green frog brain (R-RFa; Chartrel *et al.* 2002). Molecular cloning of cDNA encoding the precursor of fGRP further exhibited that it encodes fGRP and its related peptides (fGRP-RPs; Sawada *et al.* 2002a). fGRP and fGRP-RPs were invariably equipped with -LPXRF (X=L or Q) at their C-termini (Koda *et al.* 2002, Sawada *et al.* 2002a, Ukena *et al.* 2003b). In amphibians, GnIH orthologs (fGRP and its related peptides) having a common C-terminal LPXRFa motif have been identified only in frogs, anuran species. However, the presence of GnIH orthologs in the urodele brain is unclear. To generalize the presence of GnIH orthologs in amphibians, it is imperative to identify GnIH orthologs in newts, the urodele species. Therefore, the first aim of this study was to identify GnIH orthologs (LPXRFa peptide) in the newt brain. In the present study, we sought to identify a cDNA encoding the LPXRFa peptide precursor polypeptide and its mature endogenous peptides in the newt brain. The localization of its transcript in the newt brain was further investigated. To understand the physiological role of LPXRFa peptides, it is essential to know the factor that regulates the expression of LPXRFa peptides. Therefore, the second aim was to determine the regulation of the expression of the identified newt GnIH orthologs (LPXRFa peptide) by melatonin because this nocturnal hormone acts to regulate the expression of GnIH and its orthologs in birds and mammals (Ubuka *et al.* 2005, Mason *et al.* 2010). We therefore analyzed the effect of melatonin administration on the expression of LPXRFa peptide precursor mRNA in the newt brain.

Materials and Methods

Animals

Adult male Japanese red-bellied newts (*Cynops pyrrhogaster*) were collected and used in the present study. Newts were kept in ordinary water aquariums maintained at $18 \pm 2^\circ\text{C}$ under a daily photoperiod cycle of 12 h light:12 h darkness (lights on at 0700 h). The experimental protocols were approved in accordance with the Guide for the Care and Use of Laboratory Animals of Waseda University (Tokyo, Japan).

RNA preparation and amplification of the partial nLPXRFa peptide cDNA fragments

Total RNA of the brain was extracted with Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan), in accordance with the manufacturer's instructions. All PCR amplifications were carried out in a reaction mixture containing Taq polymerase (Ex Taq polymerase (Takara Shuzo, Kyoto, Japan) or gene

Taq polymerase (Nippon Gene, Tokyo, Japan)) 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) and amplified with the anchor primer (Roche Diagnostics) and the first degenerate primers 5'-(T/C)TIA-A(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 re-amplified with the first degenerate 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 of 30 s at 94°C , 30 s at 51°C and 1 min at 72°C . The second-round PCR products were subcloned into a pGEM-T Easy vector in accordance with manufacturer's instructions (Promega). The DNA inserts of the positive clones were amplified by PCR with universal M13 primers.

Determination of the 3'-end sequence of nLPXRFa peptide cDNA

First-strand cDNA was synthesized as described above and amplified with the anchor primer and gene-specific primer 1 (5'-CTGGAAGAAGCAAAAAGTAG-3', corresponding to nt 340–360). The PCR was performed for 30 cycles consisting of 1 min at 94°C , 1 min at 55°C and 1 min at 72°C . The PCR products were subcloned and the inserts were amplified as described above.

Determination of the 5'-end sequence of nLPXRFa peptide cDNA

Template cDNA was synthesized with an oligonucleotide primer complementary to nt 461–480 (5'-GGCTGGATG-GAACGTTTGCT-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 2 (5'-CGTCCAAATCTCTGTGGTAA-3', complementary to nt 431–450); this was followed by further amplification of the first-round PCR products with the anchor primer and gene-specific primer 3 (5'-TTCGGGACACTTTTAC-GAAT-3', complementary to nt 410–428). 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 . The second-round PCR products were subcloned and the inserts were amplified as described above.

DNA sequencing

All nucleotide sequences were determined with a Thermo Sequence cycle sequencing kit (Amersham Pharmacia

Biotech), IRDye 800 termination mixes version 2 (NEN Life Science Products, Boston, MA, USA), and a model 4200-1 G DNA sequencing system and analysis system (LI-COR, Lincoln, NE, USA), then analyzed with DNASIS-MAC software (Hitachi Software Engineering). Universal M13 primers or gene-specific primers were used to sequence both strands.

Immunoaffinity purification and mass spectrometry

To identify endogenous mature peptides in the newt brain, we carried out affinity purification and immunoassay with the antiserum raised against fGRP. Brains ($n=200$) were boiled for 7 min and homogenized in 5% acetic acid, as described previously (Tsutsui *et al.* 2000, Koda *et al.* 2002, Ukena *et al.* 2003b). The homogenate was centrifuged at 15 000 *g* for 20 min at 4 °C and the supernatant was collected. After precipitation with 75% acetone, the supernatant was passed through a disposable C-18 cartridge column (Mega Bond-Elut; Varian, Harbor, CA, USA) and the retained material (RM) eluted with 60% methanol was loaded onto an immunoaffinity column. The affinity chromatography was carried out as described elsewhere (Sawada *et al.* 2002b, Ukena *et al.* 2002, 2003b, Osugi *et al.* 2006, Ubuka *et al.* 2008, 2009a,b). The antibodies against fGRP were conjugated to CNBr-activated Sepharose 4B as an affinity ligand. The brain extract was applied to the immunoaffinity column at 4 °C and the adsorbed materials were eluted with 0.3 M acetic acid containing 0.1% 2-mercaptoethanol. The eluted fractions were concentrated and subjected to a reversed-phase HPLC column (ODS-80TM; Tosoh, Tokyo, Japan) with two-step gradients of acetonitrile (20–27%/35 min; 27–47%/65 min) containing 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min, and fractioned every 2 min for 100 min. The immunoreactive fractions were assayed by a dot immunoblot assay, and the molecular mass of the materials were analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) (AXIMA-CFR plus; Shimadzu, Kyoto, Japan). The predicted nLPXRFa-1 (SVPNLPQRFa), nLPXRFa-2 (MPHASANLPLRFa), nLPXRFa-3 (SIQPLANLPQRFa) and nLPXRFa-4 (APSAGQFIQTLANLPQRFa) were synthesized by peptide synthesizer (PSSM-8, Shimadzu), and molecular behavior of the synthetic and native peptides were further compared using MALDI-TOF MS.

In situ hybridization of nLPXRFa peptide mRNA

The site of nLPXRFa peptide mRNA expression in the brain was localized by *in situ* hybridization. In brief, adult newts 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 at 10 µm

thickness with a cryostat at –20 °C. The sections were placed onto 3-aminopropyltriethoxysilane-coated slides. *In situ* hybridization was carried out on the whole brain sections according to our previous method (Ukena *et al.* 1999, Osugi *et al.* 2004), using the DIG-labeled antisense RNA probe. Control for specificity of the *in situ* hybridization of nLPXRFa peptide mRNA was performed using the DIG-labeled sense RNA probe, which was complementary to the antisense probe.

Immunohistochemistry

Immunohistochemical analysis was conducted using the antiserum raised against fGRP in a rabbit, as described previously (Koda *et al.* 2002). This anti-fGRP serum was confirmed to recognize specifically three nLPXRFa peptides (nLPXRFa-1, -2 and -3), by a competitive ELISA. The IC₅₀ values (concentrations yielding 50% displacement) in the competitive ELISA were estimated as follows: 9.0 nM for nLPXRFa-1 (SVPNLPQRFa), 18.9 nM for nLPXRFa-2 (MPHASANLPLRFa), 16.9 nM for nLPXRFa-3 (SIQPLANLPQRFa), 14.4 nM for fGRP (SLKPAANLPLRFa) and more than 10 000 nM for other RFamide peptides, e.g. human PrRP (TPDINPAWYASRGIRPVGRFa), human NPFF (FLFQPQRFa) and human Kiss10 (YNWNSFGLRFa). Immunohistochemical analysis was performed as described previously (Tsutsui *et al.* 2000, Koda *et al.* 2002, Ukena *et al.* 2003a, Chowdhury *et al.* 2008). In brief, adult newts were killed by decapitation, and brains were fixed as described above. The whole brain was frontally or sagittally frozen-sectioned at 10 µm thickness on a cryostat at –20 °C. Endogenous peroxidase activity was eliminated from the sections by incubation with 0.3% H₂O₂ in absolute methanol for 10 min. After blocking nonspecific binding components, the sections were immersed in the primary antiserum against fGRP at a dilution of 1:1000 (Koda *et al.* 2002). Immunoreactive products were detected with an ABC kit (Vectastain Elite Kit; Vector Laboratories, Inc., Burlingame, CA, USA), followed by diaminobenzidine reaction, according to our previous method (Tsutsui *et al.* 2000, Ukena *et al.* 2003a). The specificity of the staining was assessed by substituting the antiserum with antiserum (1:1000 dilution) that had been preadsorbed by incubating with the antigen (synthetic nLPXRFa-1, -2 and -3) in a saturating concentration (50 µg/ml) overnight before use. The localization of immunoreactive cell bodies and fibers in the brain was studied using an Olympus BH-2 microscope.

Analysis of the effect of melatonin on nLPXRFa precursor mRNA expression

Newts were administered with melatonin through i.p. injection or loading in ordinary water aquariums. For i.p. injection, melatonin (10 µg melatonin in 50 µl saline) or vehicle (50 µl saline) was injected, and brain samples were collected after 1 h of injection. For loading in aquariums,

Table 1 PCR primers used to produce standard-DNA and competitor-DNA for competitive PCR in the newt brain

Primers used to produce standard-DNA	
nLPXRFa	
Sense	5'-CGCTTCGGCTAATTTACCAC-3'
Antisense	5'-ATAGCCAGATTCTTGGCCAC-3'
Newt β-actin	
Sense	5'-CACGGTATTGTACCAACTG-3'
Antisense	5'-AGGCAGGGCATAACCTTCAT-3'
Primers used to produce competitor-DNA	
nLPXRFa	
Sense	5'-CGCTTCGGCTAATTTACCACCCTCTGAGATACGAAAGCGC-3'
Antisense	5'-ATAGCCAGATTCTTGGCCAC-3'
Newt β-actin	
Sense	5'-CACGGTATTGTACCAACTGCCCCAGAGGAACATCCTGTA-3'
Antisense	5'-AGGCAGGGCATAACCTTCAT-3'

melatonin (2 mg melatonin in 2 l water) or vehicle (2 l water) was added into water aquariums twice in a week and newts were allowed to move freely in the aquarium. Brain samples from newts were collected after 1 week of loading with melatonin or vehicle. In both experiments, newts were kept under continuous light. To quantify the newt LPXRFa peptide precursor mRNA, competitive PCR analysis was performed as described previously (Chowdhury *et al.* 2010), with a little modification. The newt brains were removed and snap-frozen immediately in liquid nitrogen. Total RNA (including rRNA and mRNA) was isolated by the Sepasol extraction method (Sepasol-RNA I Super; Nacalai Tesque) from newt brains and reverse transcribed using oligo (deoxythymidine)_{12–18} primer (Amersham Pharmacia Biotech) and reverse transcriptase (M-MLV Reverse Transcriptase; Promega). Standard and competitor DNAs for competitive PCR analysis of the precursor nLPXRFa peptide were produced by PCR, using cDNA generated from the newt brain and primers indicated in Table 1. The PCR was conducted at 94 °C for 3 min, then 30 cycles at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, with an additional incubation at 72 °C for 3 min. PCR products were spectrometrically quantified and each aliquot was used as standard or competitor DNA.

For competitive PCR, an aliquot of the cDNA solution corresponding to 1 µg initial total RNA of each sample or standard DNA (30–3000 zmol/tube) were used as templates. Competitor DNA (300 zmol/tube) was equally applied to sample and standard tubes. PCR primers used for competitive PCR were the same as the primers which were used to produce standard DNA. The sense and antisense primers amplified a native 340 bp fragment of the nLPXRFa peptide precursor gene from sample or standard DNA, and co-amplified competitor DNA (284 bp) equally diluted in the reaction mixture. The PCR was conducted at 94 °C for 3 min, then 30 cycles at 94 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min, with an additional incubation at 72 °C for 10 min. PCR products were quantified by fluorescence of ethidium bromide on a 3UV Transilluminator (UVP, Inc., Upland, CA, USA) and subsequent two-dimensional analysis

of the gel image with an NIH Image software package (National Institutes of Health, Bethesda, MD, USA). Intensity data so derived were subjected to quantitative analysis to calculate the nLPXRFa peptide precursor mRNA concentration. Standard or competitor DNA for competitive PCR analysis of a housekeeping gene, β-actin, was also produced by PCR using primers listed in Table 1. For β-actin competitive PCR, an aliquot of the cDNA solution corresponding to 1 µg initial total RNA of each sample or standard (10–1000 amol/tube) were used as templates. Competitor DNA (100 amol/tube) was equally applied to sample and standard tubes. PCR primers used for competitive PCR were the same as the primers which were used to produce standard DNA. The sense and antisense primers amplified a native 300 bp fragment of the β-actin gene from

Figure 1 Nucleotide sequence and deduced amino acid sequence of nLPXRFa (X=L or Q) peptide precursor polypeptide cDNA. The identified nLPXRFa peptide sequences are boxed. The signal peptide is underlined. The asterisk indicates the stop codon. The poly(A) adenylation signal AATAAA is shown in bold.

standard or sample DNA, and co-amplified competitor DNA (247 bp) equally diluted in the reaction mixture. PCR products were quantified and the β -actin mRNA concentration was calculated. nLPXRFa peptide precursor mRNA level was normalized with β -actin mRNA concentration and expressed as a ratio of nLPXRFa peptide precursor mRNA concentration to β -actin mRNA concentration in the corresponding total RNA derived from each brain sample.

Statistical analysis

Results of the competitive PCR were expressed as the mean $\pm$ S.E.M. and analyzed for significance by Student's *t*-test.

Results

Characterization of a cDNA encoding the nLPXRFa peptide precursor polypeptide

To obtain LPXRFa peptide precursor cDNA fragments from the newt brain, we first performed an RT-PCR experiment with degenerate primers corresponding to the partial fGRP sequence and the anchor primer, which was followed by the re-amplification of the first-round PCR products with degenerate primers corresponding to the other partial fGRP sequence 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 ≈ 0.6 kb (not shown). The predicted amino acid sequence included three copies of the potential RFamide peptide sequence, LPQRFG, downstream of the partial fGRP sequence derived 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 (LPLRFa). To determine the 5'-end sequence, we performed 5' RACE with specific primers for the clone. A single product of ≈ 0.5 kb (results not shown) was obtained and sequenced, revealing that these cDNA clones contained a LPLRFG sequence. The entire nLPXRFa peptide precursor cDNA was identified by combining nucleotide sequences determined by RACE experiments. As shown in Fig. 1, the deduced nLPXRFa peptide precursor encoded one LPLRFa peptide and three LPQRFa peptides. The nLPXRFa peptide precursor cDNA was composed of 984 nt containing a short 5' untranslated sequence of 46 bp, a single open reading frame of 699 bp and a 3' untranslated sequence of 239 bp with the addition of various length of poly(A) tail. The open reading frame region began with a start codon at position 47 and terminated with a TGA stop codon at position 748. The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number AB537567.

Figure 2 HPLC profile of the fGRP-immunoreactive material (IM) (A) and chromatograms of mass spectrometry (MALDI-TOF MS) of native nLPXRFa-1, -2, -3 and -4 (B). The IM loaded onto the column was eluted with two-step gradients of acetonitrile (20–27%/35 min; 27–47%/65 min) containing 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min, and fractionated every 2 min for 100 min. Aliquots of each fraction were evaporated to dryness, dissolved in distilled water and spotted onto a nitrocellulose membrane. The immunoreactive fractions which were eluted with 21, 24.5, 25.5 and 38% acetonitrile are indicated by the arrows a, b, c and d, respectively (A). The immunoreactive materials in the extract of newt brain using the antiserum against fGRP showed molecular ion peaks of 1056.31 m/z $[(M+H)^+]$ (i), 1352.32 m/z $[(M+H)^+]$ (ii), 1382.33 m/z $[(M+H)^+]$ (iii) and 1958.05 m/z $[(M+H)^+]$ (iv) by MALDI-TOF MS (B).

Table 2 Behavior of native and synthetic nLPXRFa on HPLC and mass spectrometry in the newt brain

	Retention time on HPLC (min)		Observed mass (m/z $[M+H]^+$)		Calculated mass (m/z $[M+H]^+$)
	Native	Synthetic	Native	Synthetic	Synthetic
Name					
nLPXRFa-1	14–16	15	1056·31	1056·40	1056·60
nLPXRFa-2	31–33	32	1352·32	1352·37	1352·73
nLPXRFa-3	36–38	37	1382·33	1382·29	1382·79
nLPXRFa-4	71–73	71	1958·05	1958·12	1958·06

Identification of mature nLPXRFa peptides in the brain

As shown in Fig. 1, four nLPXRFa peptides (nLPXRFa-1, -2, -3 and -4) were predicted to be encoded in the cDNA. In the present study, we further investigated endogenous nLPXRFa peptides in the brain by immunoaffinity purification, combined with MS. The fGRP-immunoreactive material obtained by affinity chromatography using anti-fGRP serum was subjected to the reversed-phase HPLC purification, and the eluate was fractionated every 2 min (Fig. 2A). Each purified substance was further examined by MS. The mass values of predicted peptides were previously calculated using Protein Prospector web site (<http://prospector.ucsf.edu/prospector/mshome.htm>) on the basis of the sequence of nLPXRFa precursor polypeptide. Molecular ion peaks in the spectrum of each substance were observed at 1056·31, 1352·32, 1382·33 or 1958·05 m/z ($[M+H]^+$) on the MALDI-TOF MS (Fig. 2B and Table 2). These values were close to the mass number of 1352·73, 1056·60, 1382·79 and 1958·06 m/z ($[M+H]^+$) calculated for four deduced nLPXRFa-1, -2, -3 and -4, respectively (Table 2). The predicted nLPXRFa peptides were then synthesized and their retention time on HPLC and mass numbers were confirmed. Both native and synthetic peptides showed a similar retention time on the reversed-phase HPLC and a similar molecular mass in all four LPXRFa peptides (Table 2). These results revealed that nLPXRFa-1, -2, -3 and -4 are produced in the newt brain in mature forms.

Cellular localization of nLPXRFa precursor mRNA in the brain

Localization of nLPXRFa mRNA and the mature peptide was identified by *in situ* hybridization and immunohistochemistry. *In situ* hybridization of nLPXRFa precursor mRNA was examined in the brain, using an RNA probe with a sequence complementary to that of the nLPXRFa-1, -2, -3 and -4 precursor mRNA. Immunohistochemistry was performed using anti-fGRP serum. The binding of LPXRFa peptides to the anti-fGRP serum was completely inhibited by nLPXRFa-1, -2 and -3, demonstrating the high specificity of the antiserum (Fig. 3). An intense expression of nLPXRFa precursor mRNA was detected only in the suprachiasmatic nucleus (SCN) in the hypothalamus (Fig. 4A). The control using the sense RNA probe resulted in the complete absence of the nLPXRFa precursor mRNA expression in the SCN

(Fig. 4B), suggesting that the reaction was specific for nLPXRFa mRNA. However, no cell body expressing nLPXRFa mRNA was observed in other brain regions. Cellular localization of nLPXRFa peptides was further analyzed in the serial section by immunohistochemistry using the anti-fGRP serum. As shown in Fig. 4C and E, immunoreactive cell bodies were also restricted to the SCN. No nLPXRFa-immunoreactive cell body was observed in other brain regions. In addition, immunoreactive fibers were emanating from immunoreactive SCN cells to mesencephalic and rhombencephalic regions (Fig. 4C and E). In particular, immunoreactive nerve endings to the median eminence (ME; Fig. 4F) were derived from the SCN via ventral infundibular regions (Fig. 4C and E). As shown in Fig. 4D, a complete absence of the immunoreaction was observed by preincubation of the antiserum with an excess of synthetic nLPXRFa-1. Preincubation of the anti-fGRP serum with synthetic nLPXRFa-2 or nLPXRFa-3 also resulted in the disappearance of the reaction product (data not shown).

Effect of melatonin administration on the expression of nLPXRFa peptide precursor mRNA

To investigate whether melatonin is involved in the induction of nLPXRFa peptide, the expression of nLPXRFa peptide precursor mRNA in the diencephalon was measured after

Figure 3 Competition binding of nLPXRFa peptides to the antiserum raised against fGRP with various Rfa peptides as measured by competitive ELISA.

Figure 4 Cellular localization of nLPXRFa peptide mRNA and peptide in the newt brain. The expression of nLPXRFa peptide mRNA and the peptide were localized by *in situ* hybridization and immunocytochemistry, respectively. Distribution of nLPXRFa peptide mRNA in the suprachiasmatic nucleus (SCN) as observed in the frontal (A) section of the newt brain. Lack of hybridization of nLPXRFa peptide mRNA by the sense probe (control) in a frontal section is evident (B). Immunohistochemical staining of a frontal (C) or sagittal (E and F) was carried out with antiserum against fGRP. A complete absence of immunoreactions was observed by preincubation of the fGRP antiserum with synthetic nLPXRFa-1, -2 or -3 (D) (data not shown for nLPXRFa-2 and -3). Arrows and arrowheads in E and F indicate immunoreactive fibers and cells. ME, median eminence; P, pituitary. Scale bars represent 50 μ m.

melatonin administration to newts. As shown in Fig. 5A, melatonin administration through i.p. injection to newts caused a significant increase in the nLPXRFa precursor mRNA level ($P < 0.05$). Melatonin administration through loading in aquarium water also significantly increased nLPXRFa peptide precursor mRNA level (Fig. 5B; $P < 0.05$).

Discussion

In amphibians, the presence of LPXRFa peptides in the urodele brain is as yet unknown, unlike for those in the anuran brain. In the present study, we demonstrated the presence of LPXRFa peptides in the brain of the newt, which is an amphibian species of the urodele class. Thus, we can now

generalize the presence of LPXRFa peptides in amphibians. As summarized in Table 3, all the identified LPXRFa peptides in the brains of newts (this study) and other vertebrates, such as mammals, birds, frogs and fish, include a -LPXRFa sequence (X = L or Q) at their C-termini (Tsutsui *et al.* 2000, Fukusumi *et al.* 2001, Koda *et al.* 2002, Sawada *et al.* 2002b, Ukena *et al.* 2002, 2003b, Osugi *et al.* 2004, Ubuka *et al.* 2008, 2009a,b).

In the present study, we first identified a cDNA encoding nLPXRFa peptides from the newt brain by a combination of 3' and 5' RACE. We predicted that the nLPXRFa peptide transcript would be translated with Met¹, because a hydropathy plot analysis of the nLPXRFa peptide precursor demonstrated that the most hydrophobic moiety, which is typical in a signal peptide region, is followed Met¹. The cleavage site of the signal peptide was the Gly²⁵-Leu²⁶ bond. The deduced precursor polypeptide consisted of 233 amino acid residues, encoding four putative LPXRFa peptide sequences, i.e. nLPXRFa-1, -2, -3 and -4 (Fig. 1). All four peptides shared the C-terminal sequence LPXRF motif (with X representing L in nLPXRFa-2, and Q in nLPXRFa-1, -3 and -4). 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 & Chretien (1999)). Their sequences of putative nLPXRFa-2, -3 and -4 are flanked on both ends by the typical endoproteolytic sequences, i.e. RK or RFGR (Fig. 1), suggesting that mature peptides may be generated. Although, nLPXRFa-1 has the cleavage sequences of RFGR in the C-terminal sequence, N-terminal sequence did not follow typical endoproteolytic sequence (Fig. 1). Therefore, some other N-terminal processing mechanism(s) may be present in this precursor polypeptide. On the other hand, Gly preceding the

Figure 5 Effect of melatonin administration on the expression of nLPXRFa peptide precursor mRNA in the newt brain. Melatonin was administered by means of i.p. injection to newts and brains were collected after 1 h of injection (A). Melatonin was administered by means of loading into water aquarium containing newts for 2 weeks (B). Control newts received saline alone by means of i.p. injection or loading into water aquarium (A and B). Each column and vertical line represent the mean $\pm$ S.E.M. ($n = 6$ samples; one sample from one newt). * $P < 0.05$ versus vehicle by Student's *t*-test.

Table 3 Amino acid sequences of LPXRFa peptides in newts and other vertebrates

Sequence	Animal	Name	References
MPHSAN LPLRFa	Human	RFRP-1	Ubuka <i>et al.</i> (2009b)
VPN LQRFa	Human	RFRP-3	Ubuka <i>et al.</i> (2009b)
SGRNMEVSLVRQVLN LQRFa	Monkey	RFRP-3	Ubuka <i>et al.</i> (2009a)
SLTFEEVKDWAPKIKMNKPVVNKMPPSAAN LPLRFa	Cow	RFRP-1	Fukusumi <i>et al.</i> (2001)
AMAHLPRLRLGKNREDSLSRWVPN LQRFa	Cow	RFRP-3	Yoshida <i>et al.</i> (2003)
SVTFQELKDWGAKKDIKMSPAPANKVPHSAAN LPLRFa	Rat	RFRP-1 ^a	Hinuma <i>et al.</i> (2000)
ANMEAGTMSHFPS LQRFa	Rat	RFRP-3	Ukena <i>et al.</i> (2002)
SPAPANKVPHSAAN LPLRFa	Hamster	RFRP-1 ^a	Kriegsfeld <i>et al.</i> (2006)
ILSRVPS LQRFa	Hamster	RFRP-3 ^a	Kriegsfeld <i>et al.</i> (2006)
SIKPSAY LPLRFa	Quail	GnIH	Tsutsui <i>et al.</i> (2000)
SLNFEEMKDWGSKNFMKVNTPTVNVKPVNSVAN LPLRFa	Quail	GnIH-RP-1 ^a	Satake <i>et al.</i> (2001)
SSIQSLLN LQRFa	Quail	GnIH-RP-2	Satake <i>et al.</i> (2001)
SIRPSAY LPLRFa	Chicken	GnIH ^a	Ikemoto & Park (2005)
SLNFEEMKDWGSKNFLKVNTPTVNVKPVNSVAN LPLRFa	Chicken	GnIH-RP-1 ^a	Ikemoto & Park (2005)
SSIQSLLN LQRFa	Chicken	GnIH-RP-2 ^a	Ikemoto & Park (2005)
SIKPFNS LPLRFa	Sparrow	GnIH ^a	Osugi <i>et al.</i> (2004)
SLNFEEMEDWGSKDIIKMNPFASMPNSVAN LPLRFa	Sparrow	GnIH-RP-1 ^a	Osugi <i>et al.</i> (2004)
SPLVKGSSQSLLN LQRFa	Sparrow	GnIH-RP-2 ^a	Osugi <i>et al.</i> (2004)
SIKPFAN LPLRFa	Starling	GnIH	Ubuka <i>et al.</i> (2008)
SLNFDEMEDWGSKDIIKMNPFVSKMPNSVAN LPLRFa	Starling	GnIH-RP-1 ^a	Ubuka <i>et al.</i> (2008)
GSSQSLLN LQRFa	Starling	GnIH-RP-2 ^a	Ubuka <i>et al.</i> (2008)
SLKPAAN LPLRFa	Frog	fGRP/R-RFa	Koda <i>et al.</i> (2002) and Chartrel <i>et al.</i> (2002)
SIPN LQRFa	Frog	fGRP-RP-1	Ukena <i>et al.</i> (2003b)
YLSGTVQSMAN LQRFa	Frog	fGRP-RP-2	Ukena <i>et al.</i> (2003b)
AQYTNHFVHSLDT LPLRFa	Frog	fGRP-RP-3	Ukena <i>et al.</i> (2003b)
PTHLHAN LPLRFa	Goldfish	gfLPXRFa-1 ^a	Sawada <i>et al.</i> (2002b)
AKSNIN LQRFa	Goldfish	gfLPXRFa-2 ^a	Sawada <i>et al.</i> (2002b)
SGTGSAT LQRFa	Goldfish	gfLPXRFa-3	Sawada <i>et al.</i> (2002b)
SVPN LQRFa	Newt	nLPXRFa-1	This study
MPHASAN LPLRFa	Newt	nLPXRFa-2	This study
SIQPLAN LQRFa	Newt	nLPXRFa-3	This study
APSAGQFIQTLAN LQRFa	Newt	nLPXRFa-4	This study

^aPutative LPXRFa peptides. C-terminal LPXRFa (X=L or Q) motifs are shown in bold.

C-terminal cleavage site may serve as a C-terminal amidation signal (Bradbury *et al.* 1982, Suzuki *et al.* 1990, Eipper *et al.* 1991). Moreover, a series of mass spectrometric analyses verified the expression of nLPXRFa-1 (SVPNLQRFa), nLPXRFa-2 (MPHASANLPLRFa), nLPXRFa-3 (SIQPLANLQRFa) and nLPXRFa-4 (APSAGQFIQTLANLQRFa) as mature endogenous ligands. From the previous (Tsutsui *et al.* 2000, Fukusumi *et al.* 2001, Satake *et al.* 2001, Koda *et al.* 2002, Sawada *et al.* 2002a,b, Ukena *et al.* 2002, Yoshida *et al.* 2003, Ubuka *et al.* 2008, 2009a,b) and present findings (see Fig. 2 and Table 2), it may be stated that the presence of LPXRFa peptides is conserved in vertebrate brains.

Identification of the cells expressing nLPXRFa precursor 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 nLPXRFa mRNA by *in situ* hybridization. The nLPXRFa mRNA expression was localized in the SCN in the newt hypothalamus. The SCN cells expressing nLPXRFa mRNA was also stained by the antiserum that cross-reacts with nLPXRFa peptides. No cell body expressing nLPXRFa

mRNA or nLPXRFa was observed in other brain regions. In a previous study in bullfrogs (Koda *et al.* 2002), the cell bodies and terminals containing fGRP were localized immunohistochemically in the SCN and ME, respectively. It was also 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 and immunohistochemical analysis are in agreement with these previous findings. We recently identified novel hypothalamic neuropeptides containing a C-terminal LPXRFa sequence (LPXRFa peptides) in the avian and amphibian species: GnIH and its related peptides GnIH-RP-1 and GnIH-RP-2 in the quail (Tsutsui *et al.* 2000, Satake *et al.* 2001), fGRP and its related peptides fGRP-RP-1, fGRP-RP-2 and fGRP-RP-3 in the bullfrog (Koda *et al.* 2002, Sawada *et al.* 2002a, Ukena *et al.* 2003b). In addition, RFRPs, which are the orthologous peptides of GnIH, were identified in mammals: bovine RFRP-1 (Fukusumi *et al.* 2001), rat RFRP-3 (Ukena *et al.* 2002), primate RFRP-3 (Ubuka *et al.* 2009a) and human RFRP-1 and RFRP-3 (Ubuka *et al.* 2009b). The identified LPXRFa peptides are summarized in Table 3. Most of the LPXRFa peptides have

been reported as hypophysiotropic ligands. fGRP, the anuran LPXRFa peptide, stimulated the release of GH, and fGRP-RP-2 stimulated GH and PRL release (Koda *et al.* 2002, Ukena *et al.* 2003b). Therefore, it is possible that our identified nLPXRFa peptides act as endogenous ligands in the newt hypothalamo-hypophysial system to regulate pituitary hormone release. Further study is needed to determine the biological action of nLPXRFa peptides in urodele.

A regulatory mechanism(s) governing nLPXRFa peptide mRNA expression is essential to understand the physiological significance of novel newt neuropeptides. Recently, we reported that melatonin induces GnIH expression (Ubuka *et al.* 2005) and its release (Chowdhury *et al.* 2010) in the quail brain. Furthermore, we also demonstrated that the expressions of fGRP and fGRP-RPs in the anuran brain are also induced by melatonin (Chowdhury *et al.* 2008). Therefore, we hypothesized that melatonin may also be involved in the induction of nLPXRFa peptide precursor mRNA expression in urodele. Melatonin administration to newts through i.p. injection or adding in water aquarium under continuous lighting increased the expression of nLPXRFa peptide precursor mRNA. In newts, as in all vertebrates, melatonin is secreted into the blood in the dark (Chiba *et al.* 2005). In this study, we administrated melatonin under continuous light to make sure that no (or insignificant) amount of endogenous melatonin is produced. Furthermore, it was demonstrated that highest melatonin-binding activities were shown to occur in the optic tracts and in the suprachiasmatic area of the hypothalamus in newt (Tavolaro *et al.* 1995). In the current study, we demonstrated that the expression of nLPXRFa peptide precursor mRNA is restricted in the SCN. Thus, from the previous and present studies, it could be stated that the increased nLPXRFa peptide precursor mRNA is likely to be due to the direct effect of exogenous melatonin. Thus, melatonin may play a role to induce LPXRFa peptide precursor mRNA expressions both in anuran and urodele brain.

In conclusion, urodele hypothalamus expresses LPXRFa peptides like anuran, and the expression of nLPXRFa peptides is regulated by melatonin. The localization of nLPXRFa peptides indicates that these peptides may be involved in the regulation of pituitary hormone release.

Declaration of interest

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

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