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Short Communication

Effects of lamprey PQRFamide peptides on brain gonadotropin-releasing hormone concentrations and pituitary gonadotropin-β mRNA expression ☆

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ABSTRACT

Within the RFamide peptide family, PQRFamide peptides that include neuropeptide FF and AF possess a C-terminal Pro–Gln–Arg–Phe–NH_2 motif. We previously identified PQRFamide peptides, lamprey PQRFa, PQRFa-related peptide (RP)-1 and -RP-2 by immunoaffinity purification in the brain of lamprey, one of the most ancient vertebrate species [13]. Lamprey PQRFamide peptide precursor mRNA was expressed in regions predicted to be involved in neuroendocrine regulation in the hypothalamus. However, the putative function(s) of lamprey PQRFamide peptides (PQRFa, PQRFa-RP-1 and PQRFa-RP-2) were not examined nor was the distribution of PQRFamide peptides examined in other tissues besides the brain. The objective of this study was to determine tissue distribution of lamprey PQRFamide peptide precursor mRNA, and to examine the effects of PQRFamide peptides on brain gonadotropin-releasing hormone (GnRH)-I, -II, and -III protein concentrations, and pituitary gonadotropin (GTH)-β mRNA expression in adult lampreys. Lamprey PQRFamide peptide precursor mRNA was expressed in the eye and the brain. Lamprey PQRFa at 100 μg/kg increased brain concentrations of lamprey GnRH-II compared with controls. PQRFa, PQRFa-RP-1 and PQRFa-RP-2 did not significantly change brain protein concentrations of either lamprey GnRH-I, -III, or lamprey GTH-β mRNA expression in the pituitary. These data suggest that one of the PQRFamide peptides may act as a neuroregulator of at least the lamprey GnRH-II system in adult female lamprey.

1. Introduction

RFamide (Arg–Phe–NH_2) peptides were first identified in invertebrates in 1977, in the ganglia of venus clams, *Marcocallista nimbosa*, as FMRFamide and it was shown to function as a cardio-excitatory neuropeptide [15,16]. FMRFamide and its related peptides were also demonstrated to be involved in other physiological processes in invertebrates including reproduction and feeding [10]. Since then, RFamide peptides have been identified in various vertebrate species, especially in species of birds and mammals. LPLRFamide, the first RFamide peptide to be discovered in a vertebrate, was isolated from the chicken brain in 1983 [1]. The first mammalian RFamide peptides were isolated from bovine brain extracts in 1985; these peptides were neuropeptide FF (NPFF) and neuropeptide AF (NPAF) [24].

The PQRFamide peptides, including NPFF and NPAF, have been shown to act as neuromodulators and neurotransmitters of the mammalian opioid system. Structurally these peptides are conserved with a C-terminal Pro–Gln–Arg–Phe–NH_2 (PQRFamide) motif [25]. The gene encoding PQRFamide peptides has been identified in mammals [14] and teleost fish [11,17]. A cDNA encoding for three mature ligands, lamprey PQRFa, PQRFa gene-related peptide (RP)-1 and PQRFa-RP-2, was also identified in the brain of lamprey, one of the most ancient vertebrate species [13]. The phylogenetic analysis from the previous study showed that the lamprey PQRFa precursor belongs to the PQRFamide peptide group. Subsequently, PQRFa, PQRFa-RP-1 and PQRFa-RP-2 were isolated from the lamprey brain [13]. Using *in situ* hybridization, PQRFa mRNA was localized in different regions of the brain suggesting possible functions in locomotor activity and neuroendocrine

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regulation of reproduction [13]. In addition, PQRFamide peptides including PQRFa, PQRFa-RP-1, PQRFa-RP-2 and LPQRFa were identified in the brain of hagfish, the other extant group of Agnathans [12]. Hagfish LPQRFa peptide significantly increased gonadotropin (GTH)-β mRNA expression in the pituitary and hagfish PQRFa and PQRFa-RP-1 and PQRFa-RP-2 also tended to increase GTH-β mRNA expression *in vitro* [12]. Hagfish PQRFamide peptides have high sequence homology to lamprey PQRFamide peptides supporting the hypothesis that one of the ancestral functions of the RFamide peptides may be as a stimulatory reproductive neuropeptide.

Lamprey PQRFamide peptides are considered to have retained ancestral characteristics compared to the PQRFamide peptides in later evolved vertebrate lineages [13]. As stated above, the functions of PQRFamide peptides within the lamprey neuroendocrine system have not been examined. Understanding the role of these PQRFamide peptides may provide insight into the evolutionary origin of RFamide peptides in vertebrates and diversity in the functions of RFamide peptides in agnathans and gnathostomes. This paper provides the first information on the effect of lamprey PQRFamide peptides on brain gonadotropin-releasing hormone (GnRH)-I, -II, and -III protein concentrations and pituitary GTH-β mRNA expression and the study of its distribution in adult lamprey tissues.

2. Materials and methods

2.1. Lampreys

Adult sea lampreys (*Petromyzon marinus*) were collected from the Cocheco River in Dover, New Hampshire during their spawning migration from the Atlantic Ocean to the freshwater spawning grounds. The lampreys were then transported to the Anadromous Fish and Invertebrate Research Laboratory in Durham, NH and were maintained from mid-May through June. Lampreys were held in an artificial spawning channel with flow-through ambient reservoir water ranging between 11 and 17 °C and natural photoperiod following the University of New Hampshire Institutional Animal Care and Use Guidelines. During the experiment, water temperature in the tanks averaged 15 °C with <1 °C variation. The adult female lampreys were in the final maturational stages of II and III, approximately 2 weeks before ovulation [19].

2.2. Tissue distribution of PQRFa transcript in the lamprey by RT-PCR

Tissue total RNAs (brain, pituitary, kidney, intestine, thyroid, liver, muscle, heart, gonad and eye) were extracted from three male and three female lampreys with 1.0 ml of QIAzol Lysis reagent (QIAGEN, Venlo, Netherlands) and digested with 5 U of RQ1 RNase-free DNase (Promega, Madison, Wisconsin, Carlsbad, California) for 1 h at 37 °C to remove genomic DNA. RNeasy lipid tissue mini kit (QIAGEN) was used for total RNA extraction of ovaries from three females according to the manufacturer's direction. Following the DNase treatment, total RNA was isolated with 0.3 ml of QIAzol Lysis reagent. For RT-PCR analysis, total RNA of each tissue from three males or three females was mixed respectively. First strand cDNA was synthesized in a 10 μl final reaction volume containing 3 μg of the total RNA, 100 U of Superscript III reverse transcriptase (Invitrogen, Carlsbad, California) and 200 ng of NotI-$(dT)_{18}$ primer according to the manufacturer's direction. The reaction mixture without SuperScript III RT was incubated for 5 min at 65 °C, then the RT was added at reaction parameters of 50 °C for 1 h and 70 °C for 15 min. PCR was performed at 95 °C for 3 min as an initial denaturation followed by 5 cycles at 95 °C for 30 s and 72 °C for 1 min, 5 cycles at 95 °C for 30 s and 70 °C for 1 min, 30 cycles at 95 °C for 30 s, 68 °C for 30 s and 72 °C for 1 min, and 72 °C for 10 min as a final extension to detect PQRFa transcript. PCR mixture was composed of 1× Advantage2 PCR buffer, 1× Advantage 2 polymerase mix (Clontech, Palo Alto, CA), 0.2 mM dNTP mix, one thirtieth of 1st strand cDNA and 200 nM forward primer (la-PQRFa F2: 5′-TCCTAGCTCTGGCCAACTGT-3′) and reverse primer (la-PQRFa R1: 5′-TAGAAG AGGCTGGGTGGA AC-3′) with a total reaction volume of 10 μl. PCR amplification of EF1α was also performed at 95 °C for 3 min as an initial denaturation followed by 35 cycles at 95 °C for 15 s and 60 °C for 1 min with 500 nM forward primer (PM EF1A ORF-F1: 5′-CCTCCATCCA TCATGGGCAAGGAAAAG-3′) and reverse primer (PM EF1A R: 5′-ACCGGCCTCAAACTCACCTA-3′) in the same composition of the PCR mixture as a positive control.

2.3. Biological studies

In June 2009, 23 adult female lampreys were used for *in vivo* studies. Five lampreys in each group were intraperitoneally injected with 0.2 ml of solution individually two times 24 h apart with saline (control 0.6%), lamprey PQRFa-RP-1 at 100 μg/kg, lamprey PQRFa-RP-2 at 100 μg/kg or lamprey PQRFa at 100 μg/kg. The amino acid sequences of the lamprey PQRFamide peptides are as follows: SWGAPAEKFWMRAMPQRFa (lamprey PQRFa); AFMHFPQRFa (lamprey PQRFa-RP-1); and AGPSSLFQPQRFa (lamprey PQRFa-RP-2). These peptides were synthesized using a peptide synthesizer (PSSM-8; Shimadzu, Kyoto, Japan) and purified by a high performance liquid chromatography (HPLC) system (Prominence; Shimadzu). The purity of the synthetic peptides was over 93%. The groups consisted of four treatment groups including control, with five lampreys per treatment and three uninjected lampreys for basal sampling. After 72 h from the first injection, each lamprey was decapitated and the brain and pituitary were removed and immediately snap frozen in liquid nitrogen and stored at −80 °C.

2.4. Protein extraction and HPLC

Each lamprey brain was extracted for GnRH-I, -II, and -III as previously described [26] and modified [2,19]. One hundred and ten fractions, 1 ml each, were collected for each sample injected onto a HPLC [19]. Milli-Q water was injected onto the HPLC column after each sample. The fractions were dried using a Speed Vac system and stored at −20 °C. A total of 36 out of 110 fractions for each of 23 lamprey samples (828 samples) were assayed in duplicate to determine the brain concentration for each GnRH (GnRH-I, -II, and -III). Random water blank samples were assayed to determine if there were any contamination (no contamination was noted).

2.5. Radioimmunoassay

Duplicate 100 μl aliquots of the peak fractions were assayed to determine the concentration of lamprey GnRH-I, -II, and -III (lamprey GnRH-I: pE-HYSLEWKPG-NH_2; lamprey GnRH-II: pE-HWSHGWFPG-NH_2; lamprey GnRH-III: pE-HWSHDWKPG-NH_2; [9] using methods previously described [19,21]. I^{125} lamprey GnRH-I (lamprey GnRH-I and -III assay) and I^{125} chicken GnRH (lamprey GnRH-II assay) were iodinated respectively and appropriate synthetic lamprey GnRH was used as a standard for each of the assays. Antiserum 3952 (lamprey GnRH-III) and 135-66 (lamprey GnRH-II) were used at an initial dilution of 1:16,000 and 1:40,000, respectively. Radioimmunoassay (RIA) for lamprey GnRH-I and -III was performed on HPLC fractions 1–15, and RIA for lamprey GnRH-II was performed on HPLC fractions 35–55. For the samples in 2009, a total of 828 fractions were assayed in duplicate by RIAs. Data for hormone concentrations were analyzed using analysis of variance. Significant differences between treatment

samples and saline samples were determined using Fisher's PLSD in EXCEL. In all tests, the level of significance for different groups was $P < 0.05$.

2.6. Total RNA extraction and quantitative real time PCR assay

Total RNA from individual pituitary was extracted with 1.0 ml of QIAzol Lysis reagent (QIAGEN) and digested with 2 U of RQ1 RNase-free DNase (Promega) for 1 h at 37 °C to remove genomic DNA. After the DNase treatment, the total RNA was isolated with 0.3 ml of QIAzol Lysis reagent. First strand cDNA was synthesized in a 10 μl final reaction volume containing 500 ng of the total RNA, 100 U of SuperScript III reverse transcriptase (Invitrogen) and 200 ng of NotI-d$(T)_{18}$ primer according to the manufacturer's direction. The reaction mixture without SuperScript III RT was incubated for 5 min at 65 °C, then the RT was added at reaction parameters of 50 °C for 1 h and 70 °C for 15 min. Real time PCR assay was performed in 10 μl reaction volumes containing one tenth of 1st strand cDNA, 1× TaqMan Gene Expression Master mix (Applied Biosystems, Carlsbad, CA), 900 (GTH-β) or 500 nM (EF1α) primers and 250 nM TaqMan probe. The PCR profiles and the sequences of GTH-β primers and probe were previously reported [20]. The sequences of the primers and probe for lamprey elongation factor 1 alpha (EF1α) are as follows: PM EF1A F primer (5′-CTGGCCACAGGGACTTCATC-3′), PM EF1A R primer (5′-ACCGGCCTCAAACTCACCTA-3′), EF1A TaqMan probe (5′-FAM-ACATCGCAGGCTGACTGCGCC-TAMRA-3′).

All primers and probes for lamprey genes were designed with Primer Express software (Applied Biosystems, Carlsbad, CA). The primers spanned an intron/exon boundary and the probes were positioned on the exon/exon junction. The expression level of the GTH-β mRNA was normalized to that of EF1α (internal control). The relative amounts of mRNA were represented as mean ± SE and the statistical analysis was performed with one-way ANOVA followed by Fisher's least significant test.

3. Results

3.1. Expression of lamprey PQRFamide peptide precursor mRNA in various tissues

Lamprey PQRFamide peptide precursor mRNA was expressed in brain and eye of adult male and female lampreys (Fig. 1). The single amplicon confirmed the specific target cDNA sequence (334 bp) was not derived from genomic DNA and all tissue cDNAs showed EF1α expression as a positive control (Fig. 1). There were not any splicing variants since the primers spanned both putative intron/exon boundaries.

3.2. Biological activities of lamprey PQRFamide peptides on GnRH concentration and GTH- expression

Female lampreys treated with lamprey PQRFa at 100 μg/kg had significant increases in lamprey brain GnRH-II ($P < 0.05$) protein concentrations compared with controls (Table 1). There were no significant changes in GnRH brain concentration in response to PQRFa-RP-1 or PQRFa-RP-2 (Table 1). There were no significant changes in GTH-β pituitary mRNA expression with any of the other treatment groups compared with the control group (Table 1).

4. Discussion

In this study, we examined the functions of three PQRFamide peptides, i.e. PQRFa, PQRFa-RP-1 and PQRFa-RP-2 on the reproductive neuroendocrine axis in the sea lamprey. Lamprey PQRFa injected at 100 μg/kg significantly increased brain concentrations of lamprey GnRH-II of mature female lamprey compared with controls. PQRFa and its related peptides (PQRFa-RP-1 and PQRFa-RP-2) did not significantly change brain protein concentrations of lamprey GnRH-I or -III or pituitary lamprey GTH-β mRNA expression. PQRFamide peptide precursor mRNA was expressed in the brain and eye of male and female lampreys. These data suggest that one of the PQRFamide peptides may act as a neuroregulator of at least the lamprey GnRH-II system in adult female lampreys. Further studies will be needed to examine the effects of these peptides in male lampreys, lampreys in different reproductive stages and testing varying doses of the PQRFamide peptides to elucidate the potential interactions between PQRFamide and GnRH systems.

Lamprey GnRH-II was identified in 2008 and shown to stimulate the hypothalamic-pituitary axis using *in vivo* and *in vitro* studies [9]. In subsequent studies, the dynamics of lamprey GnRH-I, -II and -III were examined during the final maturational stages of lampreys [19]. Sea lampreys are anadromous and semelparous, i.e., they spawn only once in their lifetime, after which they die. The final maturational stages occur after the sea lampreys return to freshwater. While the specific functions of lGnRH-II acting as a hypothalamic hormone are not known, it was shown that lGnRH-II was elevated in adult female sea lamprey at the beginning of

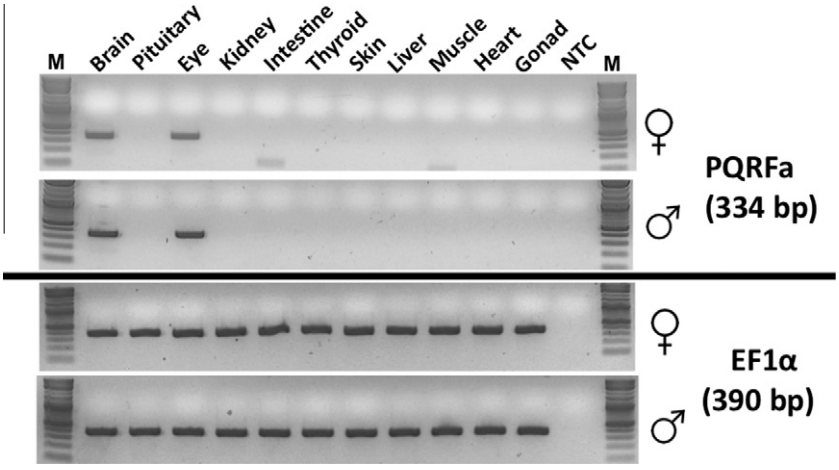

Fig. 1. Tissue distribution of the transcript of lamprey PQRFamide peptide precursor in male and female lamprey tissues by RT-PCR. Housekeeping gene EF1α used as a positive control. Lane M: DNA marker (100 bp DNA ladder); NTC: non template control.

Table 1
GnRH-I, -II, and -III brain hormone concentrations (ng/brain) and lGTH-β RNA expression of each treatment group of control, lamprey PQRFa, -RP-1, or -RP-2 at 100 μg/kg. * denotes significance at $P < 0.05$.

Treatment	lGnRH-I ng/brain mean ± SE	lGnRH-II ng/brain mean ± SE	lGnRH-III ng/brain mean ± SE	lGTH-β Relative Qty GTH/EFIA mean ± SE
Control	2.30 ± 2.17	0.13 ± 0.11	23.60 ± 14.31	0.57 ± 0.38
lPQRFa-RP-1 100 μg/kg	3.66 ± 3.41	0.19 ± 0.15	21.27 ± 18.72	0.60 ± 0.09
lPQRFa-RP-2 100 μg/kg	1.38 ± 2.15	0.69 ± 0.63	33.39 ± 16.12	0.374 ± 0.25
lPQRFa 100 μg/kg	13.07 ± 19.86	1.51 ± 0.75*	16.91 ± 21.15	0.49 ± 0.11

the final reproductive period with a sharp decline and remained low during the rest of this period [19]. In the current study, lamprey PQRFa significantly increased brain concentrations of lGnRH-II but not lGnRH-I or -III. Both lamprey PQRFamide peptides and lamprey GnRH-II in separate studies have been shown in close proximity to one another in the brain and hypothalamus. Using both *in situ* hybridization and immunohistochemistry, lGnRH-II was shown to be expressed in the preoptic area and hypothalamic regions [9]. In another study, PQRFamide peptides were shown to be expressed in hypothalamic regions in close proximity to GnRH neurons in the hypothalamus, specifically the nucleus commissure postopticae and smaller numbers were also expressed in the mesencephalon and the rostral part of the medulla oblongata [13]. These data lend support to the hypothesis that lamprey PQRFamide peptides may have a neuroregulatory role on hypothalamic lGnRH-II. However, until the synthesis, storage and release rates of each brain GnRH are known, elevated or decreased concentrations of GnRH-I, -II, or -III in the brains of lampreys can only be correlated with function.

In addition to the hypothalamus, PQRFamide peptide precursor mRNA expression was seen in the tegmentum of the mesencephalon and fasciculus longitudinalis medialis suggesting their role in locomotor activity and other physiological functions [13]. In the current study, PQRFamide peptide precursor mRNA expression was shown not only in the brain but also the eye, which supports other possible functions in lampreys. GnRH-II mRNA has been shown to be expressed in a variety of different tissues by RT-PCR including the brain and eye [9] suggesting a co-localization of the precursor mRNAs of PQRFamide peptides and GnRH-II within these tissues. While the specific functions are unknown, these studies suggest that there may be regulatory interactions between PQRFamide peptides and GnRH-II in the CNS including the reproductive and the visual systems. To the best of our knowledge, the expression of PQRFamide peptide precursor has not been observed in the eye of gnathostomes. As for other RFamide peptides, LPXRFamide peptide (X = L or Q) and its receptor GPR147 mRNAs are expressed in the eye of human [5] and grass puffer [18]. 26RFa and its receptor GPR103 mRNAs are also expressed in the eye of human [8] and rodents [4,22]. *Carassius*–RFamide (homolog of PrRP) immunoreactive neurons were also observed in the eye of Japanese crucian carp [23]. The function(s) of these RFamide peptides in the eye remains to be studied.

Lamprey PQRFamide peptides did not significantly alter pituitary mRNA expression of GTH-β *in vivo*. In addition, lamprey PQRFamide peptide precursor mRNA was not expressed in the pituitary. These data suggest that PQRFamide peptides may not directly be involved in pituitary regulation in lampreys. Further studies measuring protein expression of GTH and release rates will need to be done to determine whether these actions are direct or indirect.

Based on differences in functional studies in agnathans and gnathostomes, the functions of these PQRFamide peptides may have diverged following gene duplication before the agnathan–gnathostome split. As previously proposed, many features of the agnathan neuroendocrine system have retained ancestral characteristics and diverged from gnathostomes following one or two large-scale genome duplication events [3,6,7]. This theory may also support the functional divergence of RFamide peptides between the agnathans and gnathostomes. The lamprey PQRFamide peptides have been considered to be the most conserved evolutionarily. Further studies will be needed, including the identification and localization of the receptor of PQRFamide peptides in lampreys in order to understand and elucidate the complexities, interactions and functions of PQRFamide peptides with other physiological systems.

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