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Purification of a neuropeptide Y-related peptide from the brain of the sea lamprey and its effect on steroidogenesis

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Summary

A peptide with neuropeptide Y-like immunoreactivity was identified by radioimmunoassay in an extract of the brain of the sea lamprey, *Petromyzon marinus* using an antiserum raised against the conserved COOH-terminal region of mammalian neuropeptide Y. Purification of the peptide and determination of its primary structure showed that it was identical to peptide methionine-tyrosine (PMY), previously isolated from the intestine of the same species. Intraperitoneal injection of synthetic PMY (0.15 μg/g) into female lampreys undergoing final maturation before spawning produced a significant ($P < 0.05$) decrease in plasma concentrations of estradiol compared with control lampreys injected with vehicle only. These data suggest the hypothesis that the observed decrease in the concentration of PMY-containing cells in the intestines of lampreys during upstream migration may correlate with the increase in circulating estradiol concentrations and final maturational processes.

Introduction

Recent articles [1–6] and reviews [7,8] have discussed the molecular evolution of peptides of the neuropeptide Y (NPY) family. In tetrapods (mammals, birds, reptiles, amphibia), it is postulated that two successive duplications of a putative ancestral gene have led to separate genes encoding the homologous peptides NPY, peptide tyrosine-tyrosine (PYY) and pancreatic polypeptide (PP). In mammals, NPY is synthesized primarily in neurons of the central and peripheral nervous systems, PYY is localized to endocrine-like cells in the lower bowel and PP is produced in the pancreatic islets. In teleost and elasmobranch fish, however, NPY-related pep-

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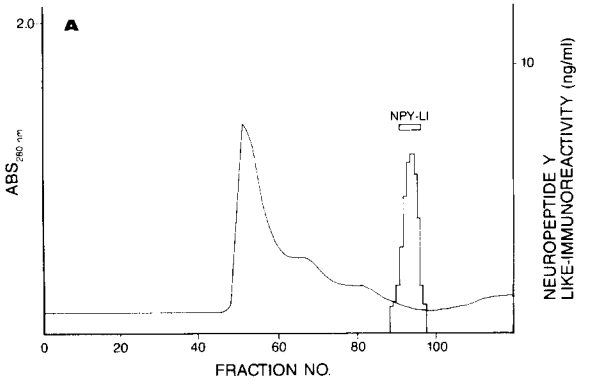
tides, whose primary structures have been strongly conserved during evolution, have been isolated from brain tissue but only PYY-related peptides have been found in both pancreatic and intestinal tissue. The lampreys are one of only two extant representatives of the vertebrate class, Agnatha or jawless fishes whose line of evolution diverged from that leading to mammals at least 550 million years ago [9]. Previous work has led to the isolation and structural characterization of a member of the NPY family from the intestine of an Agnathan, the sea lamprey *Petromyzon marinus* [10]. The localization of this peptide, termed peptide methionine-tyrosine (PMY), to endocrine-like cells in the intestinal mucosa [11] suggested that PMY was the lamprey equivalent of mammalian PYY. PMY shows 13 amino acid substitutions compared with pig NPY but exhibits the NPY/PYY-like ability to contract the guinea pig superior mesenteric artery [10]. The physiological role of PMY in the lamprey is completely unknown. In the present study, we examine an extract of sea lamprey brain for the presence of peptides of the NPY family and study the effect of synthetic PMY on steroidogenesis in adult, female sea lampreys.



Materials and Methods

Tissue extraction

Whole brains from approx. 2000 mature male and female sea lampreys (*Petromyzon marinus*) were col-

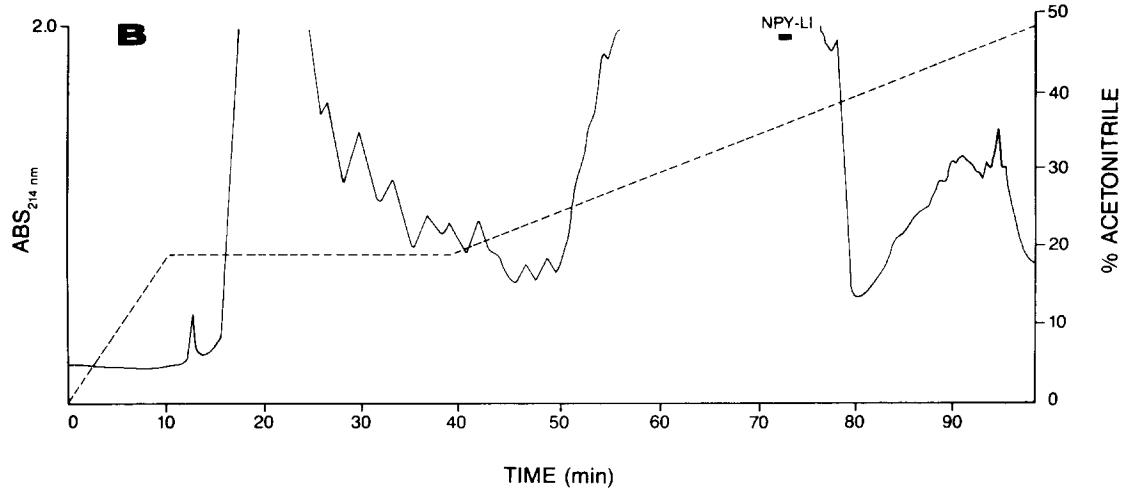
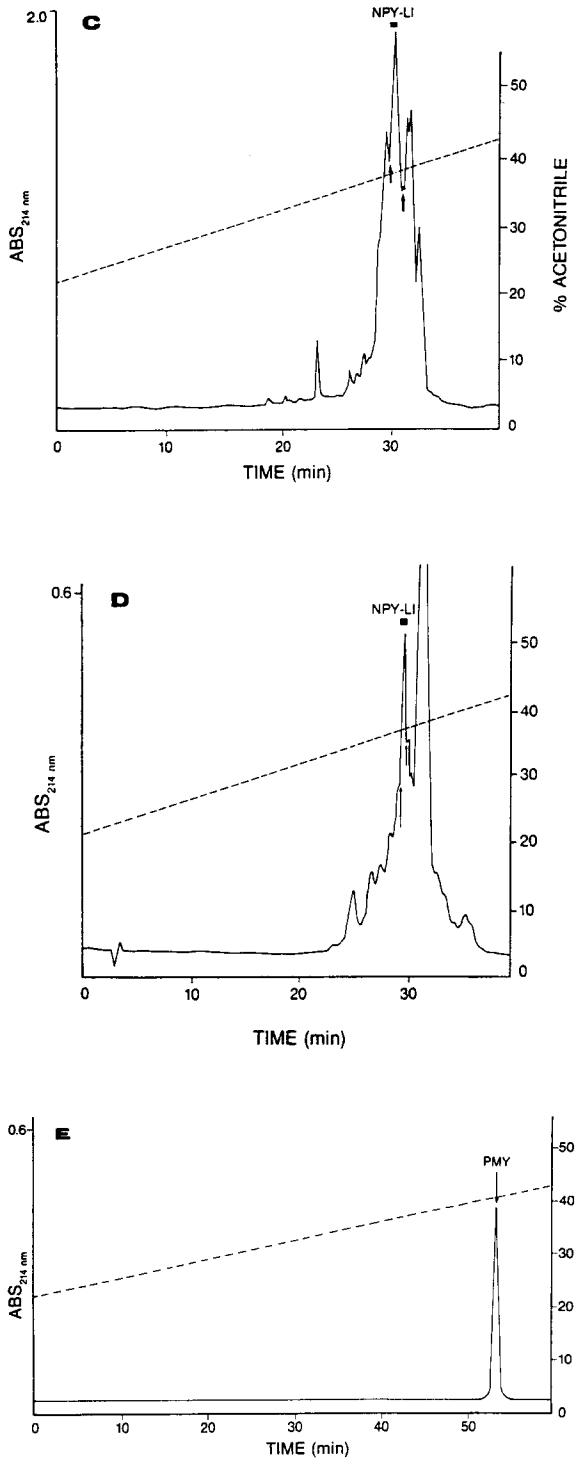


Fig. 1. Purification of an NPY-related peptide from the brain of the sea lamprey. (A) Gel permeation chromatography (Biogel P-10) of an extract of lamprey brain after partial purification on Sep-Pak cartridges. The fractions showed by the bar were pooled and subjected to further purification. Lamprey NPY was purified by reversed-phase HPLC on a (B) Vydac C_{18} column (C) Vydac C_4 column (D) Vydac phenyl column and (E) Vydac C_{18} column. The fractions denoted by the bar contained NPY-like immunoreactivity and the arrows show where peak collection began and ended. The broken line shows the concentration of acetonitrile in the eluting solvent. In panel E, the arrow marked PMY shows the retention time of synthetic peptide methionine-tyrosine.



lected in June and July 1990 at Hammond Bay Biological Station (Millersburg, MI) as described [12] and stored at −80°C. The tissue (155 g) was boiled for 5 min in 1 M acetic acid (2 l) and homogenized using a Waring blender. After centrifugation (1600 *g* for 60 min at 4°C), peptide material in the supernatant was isolated using Sep-Pak C-18 cartridges (Waters Associates, Milford, MA, USA) as described previously [12]. Bound material was eluted with 80% (v/v) acetonitrile/water and freeze dried.

Radioimmunoassay

NPY-related peptides were detected using antiserum 8999 which was raised against the cysteine-extended COOH-terminal hexapeptide of human NPY (Cys-Ile-Thr-Arg-Gln-Arg-Tyr·$CONH_2$) in a radioimmunoassay procedure that has been described previously [13]. Human NPY was used as standard and ^{125}I-Bolton Hunter-labelled NPY (specific activity 2200 Ci/mmol) was used as radiolabelled tracer. The antiserum cross-reacts completely with pig PYY but shows <1% reactivity with PP.

Peptide purification

The extract was redissolved in 1% (v/v) trifluoroacetic acid/water (10 ml) and chromatographed on a (5 × 90 cm) Biogel P-10 (Bio-Rad, Richmond, CA, USA) column equilibrated with 1 M acetic acid at a flow rate of 72 ml/h. Fractions (12 ml) were collected and the presence of NPY-like immunoreactivity was determined by radioimmunoassay at appropriate dilution. The fractions denoted by the bar in Fig. 1A were pooled (total volume = 72 ml) and pumped at a flow rate of 2 ml/min onto a (1 × 25 cm) Vydac 218TP510 (C-18) column (Separations Group, Hesperia, CA, USA) equilibrated with 0.1% trifluoroacetic acid/water. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min, held at this concentration for 30 min and raised to 49% over 60 min using linear gradients. Absorbance was measured at 214 and 280 nm and fractions (1 min) were collected. The fraction containing NPY-like immunoreactivity (Fig. 1B) was in-

jected onto a (0.46 × 25 cm) Vydac 214TP54 (C-4) column equilibrated with acetonitrile/water/trifluoroacetic acid (21.0:78.9:0.1, v/v) at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 42% over 40 min using a linear gradient. Lamprey PMY was purified to apparent homogeneity by successive chromatographies on a (0.46 × 25 cm) Vydac 219TP54 (phenyl) column and a (0.46 × 25 cm) Vydac 218TP54 (C-18) column with the same elution conditions used for the C-4 column.

Structural analysis

The primary structure of the lamprey NPY-related peptide (approx. 500 pmol)) was determined by automated Edman degradation using an Applied Biosystems model 471A sequenator (Foster City, CA, USA) modified for on-line detection of phenylthiohydantoin-coupled amino acids under gradient elution conditions. Standard operating procedures were used and the detection limit was 1 pmol. Hydrolysis (24 h at 110°C in 5.7 M HCl) of approx. 500 pmol peptide was carried out and its amino composition was determined by precolumn derivatization with phenylisothiocyanate [14] using an Applied Biosystems model 420A derivatizer and 130A separation system. The detection limit for phenylthiocarbamyl-coupled amino acids was 2 pmol. The method of Schmidt et al. [15] was used to demonstrate that the COOH-terminal residue in PMY is α-amidated. The peptide (approx. 500 pmol) in 0.2 M ammonium bicarbonate (50 μl) was digested for 16 h at 37°C with 1-tosylamide 2-phenylethylchloromethylketone-treated trypsin (Sigma, St. Louis, MO) at a substrate/enzyme ratio of 50:1. The α-amidated tyrosine residue in the reaction mixture was derivatized with phenylisothiocyanate using an Applied Biosystems model 420A derivatizer and identified by reversed-phase HPLC. The retention time of synthetic L-tyrosinamide (Sigma) was determined.

Synthesis of lamprey NPY-related peptide

Lamprey PMY was synthesized using an Applied Biosystems model 430A automated synthesizer. The procedure used was a modification of that used to synthesize the salmon NPY-related peptide [16]. Briefly, p-methylbenzhydrylamine resin (0.45 mmol $-NH_2$ group) was used as solid support and all the N-α-amino acids with benzyl based side-chain protecting groups were double coupled as preformed symmetrical anhydrides (2.2 mol equivalents). Arg, Gln and Asn residues were double coupled as preformed 1-hydroxybenzotriazole esters (4.4 mol equivalents) to avoid side-reactions. At the end of the synthesis, the N-α-Boc-protecting group was removed and the peptide was detached from the resin by treatment with HF (10 ml) containing p-cresol (0.8 g) and p-thiocresol (0.2 g) for 1 h at −4°C. The peptide was purified to apparent homogeneity by chromatography on a Vydac 218TP510 (C-18) column under the conditions shown in Fig. 1C. The identity of the peptide was confirmed by Edman degradation and ^{252}Cf plasma-desorption time-of-flight mass spectrometry (observed molecular mass 4202 ± 4; calculated molecular mass 4203.8).

Biological activity

Adult female sea lampreys (body wt. 175–225 g) were maintained under natural photoperiod in flow through reservoir water at ambient temperature. In the first experiments, 20 lampreys (10 per treatment group) were injected intraperitoneally with either 0.6% NaCl (100 μl) or 0.6% NaCl (100 μl) containing synthetic lamprey PMY (0.15 μg/g body wt.). The water temperature was 13°C. After 4 h, the animals were lightly anesthetized by immersion in m-amino benzoate methanesulfonate solution (2.0 g/l) and blood samples (1 ml) were collected in heparinarized syringes by cardiac puncture. Plasma estradiol concentrations were determined by RIA as previously described [17]. The experiment was repeated 11 days later using 20 different female lampreys (10 per treatment group) and the same dose of PMY at a water temperature of 18°C. Data were analyzed by Student-Newman-Keul's test after a preliminary analysis of variance.

Results

Isolation of lamprey NPY-related peptide

The original concentration of NPY-like immunoreactivity in the extract of the lamprey brain was 25 pmol/g wet weight of tissue. The immunoreactivity, in serial dilutions of the extract diminished in parallel with the synthetic human NPY standard curve in radioimmunoassay.

As shown in Fig. 1A, NPY-like immunoreactivity in the extract, after partial purification on Sep-Pak cartridges, was eluted from a Biogel P-10 gel permeation column as a single peak with maximum immunoreactivity at the elution volume of synthetic human NPY. The fractions denoted by the bar were pooled and chromatographed on a semi-preparative Vydac C-18 column (Fig. 1B). NPY-like immunoreactivity was eluted in a single fraction, as shown. After rechromatography of this material on an analytical Vydac C-4 column (Fig. 1C), the NPY-like immunoreactivity was associated with the major peak in the chromatogram (denoted by the bar).Chromatography of this peak on an analytical Vydac phenyl column (Fig. 1D) demonstrated that the material was heterogenous. NPY-like immunoreactivity was associated with the sharp minor peak denoted by the bar. Partial amino acid sequence analysis of the major peak in the chromatogram identified this component as a fragment of glyceraldehyde-3-phosphate dehydrogenase. Lamprey NPY-related peptide was purified to apparent homogeneity by a final chromatography on an analytical Vydac C-18 column (Fig. 1E). The final yield of pure material was 2.5 nmol.

Structural analysis

The amino acid sequence of the lamprey NPY-related peptide was established by automated Edman degradation as: Met-Pro-Pro-Lys-Pro-Asp-Asn-Pro-Ser - Pro^{10} - Asp - Ala - Ser - Pro - Glu - Glu - Leu - Ser-Lys-Tyr^{20}-Met-Leu-Ala-Val-Arg-Asn-Tyr-Ile-Asn - Leu^{30} - Ile - Thr - Arg - Gln - Arg - Tyr. It was possible to assign without ambiguity amino acid phenylthiohydantoin residues for 36 cycles of operation of the sequenator. The primary structure of the peptide is identical to that of PMY previously isolated from the intestine of the same species [10]. The amino acid composition of the purified PMY from lamprey brain was consistent with the proposed structure and showed that the full sequence of the peptide had been obtained (found residues/mol peptide: Asx 4.7 (5); Glx 3.4 (3); Ser 2.8 (3); Arg 3.2 (3); Thr 1.2 (1); Ala 2.3 (2); Pro 5.6 (6); Tyr 2.7 (3); Val 1.3 (1); Met 1.5 (2); Ile 1.7 (2); Leu 3.3 (3); Lys 1.7 (2)). The values in parentheses indicate the number of residues predicted from the proposed amino acid sequence. The presence of an α-amidated COOH-terminal residue in the peptide was demonstrated by chromatographic analysis of the products of digestion with trypsin, after treatment of the reaction mixture with phenylisothiocyanate. The retention time of the phenylthiocarbamyl derivative of the COOH-terminal tyrosine residue of the lamprey brain PMY was 15.19 min compared with retention times of 15.20 min and 11.67 min for the retention times of the derivatives of L-tyrosinamide and L-tyrosine, respectively.

The primary structure of the lamprey brain PMY was confirmed by chemical synthesis. A mixture of the endogenous peptide (500 pmol) and the synthetic replicate (500 pmol) were eluted from an analytical Vydac C-18 column, under the conditions shown in Fig. 1E, as a single sharp peak. Similarly, a mixture of endogenous PMY isolated from lamprey gut [10] and the NPY-related peptide isolated from lamprey brain in this study also co-eluted as a single sharp peak.

Biological activity

One injection of synthetic PMY (0.15 μg/g body weight) into female lampreys maintained in water at a temperature of 13 °C produced a significant (31%; $P < 0.05$) decrease in plasma concentrations of estradiol from 0.51 ± 0.05 ng/ml (mean ± S.E.; $n = 10$) in vehicle only treated animals to 0.35 ± 0.05 ng/ml ($n = 10$) (Fig. 2). Injection of the same dose into a different group of female lampreys maintained at a

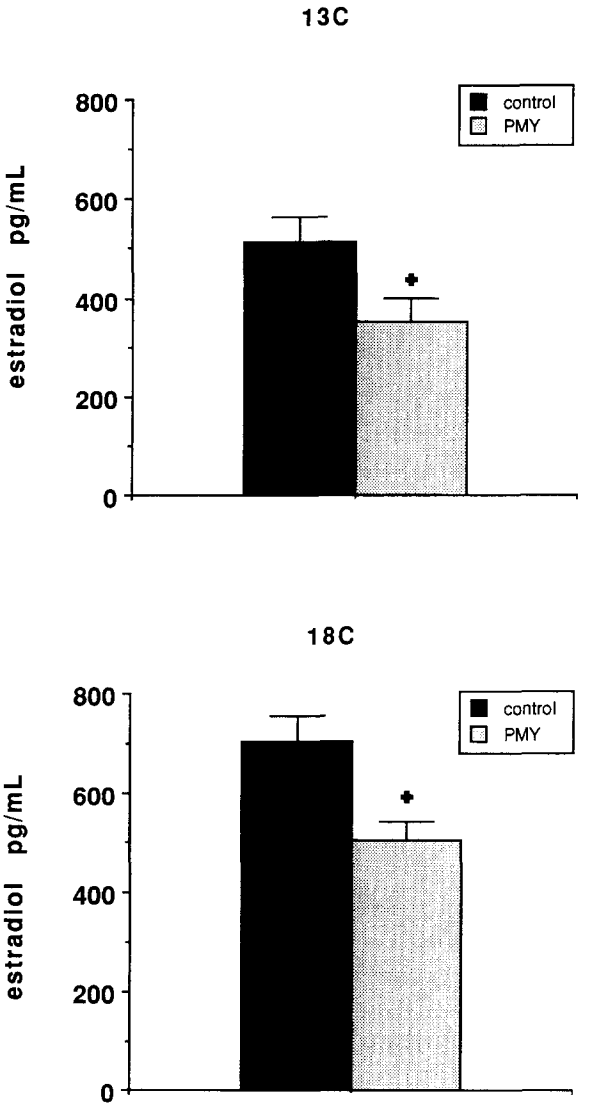


Fig. 2. Plasma estradiol concentrations in female sea lampreys in samples taken 4 h after an ip injection of saline only (control) or peptide methionine-tyrosine (PMY) (0.15 μg/g body weight). Lampreys (10 animals per treatment group) were maintained at a water temperature of 13 °C (upper panel) or 18 °C (lower panel). (+) $P<0.05$ vs control.

water temperature of 18 °C also produced a significant (29%; $P<0.05$) decrease in plasma estradiol from 0.71 ± 0.05 ng/ml ($n = 10$) in vehicle only treated animals to 0.50 ± 0.04 ng/ml ($n = 10$) (Fig. 2).

Discussion

The concentration of NPY-like immunoreactivity measured in an extract of the brain of the sea lamprey (25 pmol/g) was appreciably lower than comparable concentrations in whole brain extracts from higher vertebrates prepared and assayed under the same conditions (dogfish (elasmobranch) 95 pmol/g [5]; Atlantic cod (teleost) 121 pmol/g) [4]; European green frog (amphibian) 175 pmol/g [1] and American alligator (reptile) 643 pmol/g [18]). Using an antiserum raised against the COOH-terminal of human NPY in RIA, only one molecular form of NPY-like immmunoreactivity was detected in the brain extract. Amino acid sequence analysis established that this component was identical to peptide methionine-tyrosine (PMY) previously isolated from an extract of lamprey intestine [10]. Our study, however, cannot exclude the possibility that the sea lamprey synthesizes other member of the NPY family that either were not detected in the RIA or were not extracted from brain tissue by boiling 1 M acetic acid. In fact, multiple NPY-related peptides were detected by immuno-histochemistry and HPLC in the brain stem and spinal cord of the river lamprey, *Lampetra fluviatilis* [19] suggesting there may be considerable inter-species variation amongst the Agnathans. In the elasmobranch fish, *Scyliorhinus canicula* (European common dogfish), a peptide with strong structural similarity to human NPY was isolated from brain [5] and a different peptide with greater similarity to human PYY was isolated from the pancreas [2] and intestine (unpublished data). In the brain of the teleost fish, *Oncorhynchus mykiss* (rainbow trout), however, peptides with structural similarity to mammalian NPY and PYY co-exist [4].

Injection of a synthetic replicate of PMY (0.15 μg/g) into pre-ovulating female lampreys produced a significant decrease ($P<0.05$) in plasma estradiol concentrations. This dose was chosen as ip injection of a similar amount of lamprey GnRH-III into female lampreys produced a significant increase (244% over basal) in plasma estradiol levels [10]. Similarly, blood

samples were taken 4 h after injection as a previous study [22] has shown that estradiol levels have reached a maximum value at this time following i.p. injection of lamprey GnRH-I. Basal plasma estradiol levels show a strong positive correlation with water temperature [20] and so the effect of PMY was tested at two different temperatures. As expected, concentrations of estradiol in the vehicle-treated lampreys were higher in animals maintained at 18°C compared with 13°C but a quantitatively similar inhibitory effect of PMY on steroidogenesis was observed at both water temperatures. At the time of this investigation, no further lampreys were available to study the effect of other doses of PMY on steroidogenesis but future work will address of question of the concentration-dependence of the observed effect.

The mechanism by which PMY inhibits steroidogenesis in the lamprey remains to be investigated. In the absence of experimental data, the site of action of the peptide may be at the hypothalamus, pituitary or ovary. Lamprey brain contains multiple forms of GnRH [10,21] and previous studies have shown that ip injections of lamprey GnRH-I [22] and lamprey GnRH-III [10] in spawning lampreys produce significant increases in plasma estradiol concentrations and stimulate ovulation. Similarly, in in vitro studies, lamprey GnRH-I potentiated estradiol release in cocultures of lamprey testes and pituitary but had no direct effect on estradiol release from the testes alone (Sower SA, unpublished data). PMY may thus act by inhibiting release of GnRH. Attempts to measure GnRH-like immunoreactivity in lamprey blood have been unsuccessful and so the hypothesis cannot be tested at this time. Similarly, gonadotropin(s) (GTH) have not yet been isolated from lamprey pituitary gland so that it is not possible to study the direct effects of PMY on GTH release. However, the possibility that PMY inhibits GTH release by a direct interaction with gonadotrophs deserves consideration. The ovary of the sea lamprey has been established as a site of synthesis of estradiol [23] and further studies are required to determine whether PMY exercises a direct effect on ovarian steroidogenesis that may be independent of pituitary function.

The presence of neuropeptide Y in the brains of teleost fish and in nerve terminals in close association with the gonadotropin secreting cells of the pituitary has been demonstrated by radioimmunoassay and immunohistochemistry [24]. The effects of NPY on gonadotropin release in bony fish are complex and dependent on the steroidogenic environment and reproductive status. Studies in vitro using perifused pituitary pieces from the trout [25] have shown that mammalian NPY inhibited release of gonadotropins in tissue from vitellogenic females but strongly stimulated gonadotropin release in ovulated females and in vitellogenic females previously treated with an aromatase inhibitor. It was also shown that NPY potentiated the action of GnRH on gonadotropin release from trout pituitary fragments suggesting that NPY sensitized the gonadotrophs to the action of GnRH [26]. Experiments with perifused pituitaries from goldfish at different gonadal stages did not show any inhibitory effects of NPY on gonadotropin release but the stimulatory effect of the peptide was lower in sexually regressed fish than in fish undergoing gonadal recrudescence [27]. An in vivo study has shown that intraperitoneal injections of NPY in mature trout at the end of vitellogenesis resulted in a 2-fold increase in plasma gonadotropin levels that peaked 2 to 4 h after administration [28]. Thus, the evidence that NPY plays a physiological role in reproduction in teleost fish is strong.

Analagous to the localization of PYY in the distal intestine of mammals [8], PMY is localized to endocrine-like cells in the anterior intestine of the sea lamprey, suggesting a possible hormonal role for the peptide [11]. The sea lamprey is anadromous and returns to fresh water from the ocean for its final reproduction and spawning. During its upstream migration, a decrease in the concentration of PMY-containing cells in the intestine has been shown by immunohistochemistry [11]. Several studies (reviewed in Ref. 29) have shown that increases in concentrations of circulating estradiol are associated

with reproductive activity in both male and female lampreys. Consequently, the data in the present study suggest that circulating PMY may play a physiological role in reproduction in lampreys by exerting an inhibitory action on the production of estradiol during the parasitic phase that is diminished in the period during final maturation before ovulation. Further studies are required to whether upsteam migration is associated with changes in PMY concentrations in specific areas of the brain, particularly in the preoptico-neurohypophysial region of the hypothalamus in which the majority GnRH-containing neurons are located [30].

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