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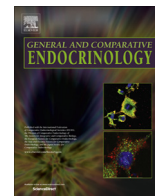
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Regulation of a putative corticosteroid, 17,21-dihydroxypregn-4-ene,3,20-one, in sea lamprey, *Petromyzon marinus*



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ABSTRACT

In higher vertebrates, in response to stress, the hypothalamus produces corticotropin-releasing hormone (CRH), which stimulates cells in the anterior pituitary to produce adrenocorticotrophic hormone (ACTH), which in turn stimulates production of either cortisol (F) or corticosterone (B) by the adrenal tissues. In lampreys, however, neither of these steroids is present. Instead, it has been proposed that the stress steroid is actually 17,21-dihydroxypregn-4-ene-3,20-dione (11-deoxycortisol; S). However, there have been no studies yet to determine its mechanism of regulation or site of production. Here we demonstrate that (1) intraperitoneal injections of lamprey-CRH increase plasma S in a dose dependent manner, (2) intraperitoneal injections of four lamprey-specific ACTH peptides at 100 µg/kg, did not induce changes in plasma S concentrations in either males or females; (3) two lamprey-specific gonadotropin-releasing hormones (GnRH I and III) and arginine-vasotocin (AVT), all at single doses, stimulated S production as well as, or to an even greater extent than CRH; (4) sea lamprey mesonephric kidneys, in vitro, converted tritiated 17 α -hydroxyprogesterone (17 α -P) into a steroid that had the same chromatographic properties (on HPLC and TLC) as S; (5) kidney tissues released significantly more immunoassayable S into the incubation medium than gill, liver or gonad tissues. One interpretation of these results is that the corticosteroid production of the sea lamprey, one of the oldest extant vertebrates, is regulated through multiple pathways rather than the classical HPI-axis. However, the responsiveness of this steroid to the GnRH peptides means that a reproductive rather than a stress role for this steroid cannot yet be ruled out.

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1. Introduction

The hypothalamus–pituitary–adrenal (HPA) axis plays a critical role in mediating stress responses in mammals, including the secretion of corticosteroid hormones that regulate metabolism, growth, reproduction, immunity, and ion homeostasis (Charmandari et al., 2005). In teleost fishes, the hypothalamus–pituitary–interrenal (HPI) axis is stimulated after exposure to a physical, chemical, or perceived stressor, which causes CRH and AVT neurosecretion to stimulate the corticotrophic cells in the pituitary (Barton, 2002; Batten et al., 1990) to release ACTH. Baker et al. (1996) were able to show that CRH and AVT could act individually or synergistically to increase ACTH secretion in rainbow trout pituitary incubations. In agnathans, the HP axis is not well defined and is

suggested to be more of a diffusional process through connective tissue (Nozaki et al., 1994; Nozaki, 2008). In teleosts, ACTH is then released into circulation and stimulates the interrenal cells of the head kidney to produce cortisol, which exert various effects on target cells. Such HPI axis response to stress, similar to the HPA system of mammals, seems to have been conserved in most vertebrates.

CRH is a 41 amino acid peptide produced in the hypothalamus and belongs to a family of neuropeptides that have been highly conserved in fish, amphibians, and mammals as summarized elsewhere (Lovejoy and Balment, 1999; Ottaviani and Franceschi, 1996; King and Nicholson, 2007). However, no CRH peptide has been identified and characterized from the basal vertebrates, lampreys and hagfish. Although the sequence information of lamprey CRH was not known at the time, Close et al. (2010) demonstrated that the human form of CRH can induce changes in concentrations of S, which was proposed to be the corticosteroid hormone in the sea lamprey. To fully understand the physiological functions of

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CRH-related peptides in lamprey, in vivo experiments with endogenous lamprey CRH are needed.

The physiological functions of ACTH in lamprey have remained elusive due to a lack of experimental data supporting its definitive functions. One of difficulties associated with understanding the physiological functions of lamprey ACTH results from its unique molecular structure. While gnathostome ACTH is composed of 39–40 amino acids, lamprey ACTH is composed of 59–60, with posttranslational phosphorylation occurring at position 35 in two of the four peptides (Takahashi et al., 2006). Such modification results in four unique forms that are significantly different from the single peptide found in most vertebrates. Since the discovery of these four peptides, no published data exist on their effect on lamprey physiology. Additionally, no form of ACTH has been shown to stimulate production of S, nor is there currently any evidence for ACTH in circulation after exposure to acute stress in lamprey. The study by Close et al. (2010), however, demonstrated that intraperitoneal injection of lamprey pituitary extract stimulated dose-dependent production of S, indicating that a biological substance in the pituitary may be involved in the stress response.

In gnathostomes, the primary glucocorticoids are cortisol and corticosterone with an exception where 1α hydroxy corticosterone is thought to be a functional corticosteroid in elasmobranchs (Anderson, 2012). However, in sea lamprey, *Petromyzon marinus*, a precursor to cortisol, S, was shown to be a putative corticosteroid hormone mediating stress responses, which prompted a debate on the evolutionary mechanisms of corticosteroid signaling in vertebrates (Close et al., 2010, 2011; Thornton and Carroll, 2011). The identification and characterization of the putative corticosteroid hormone S in lamprey may lead to a better understanding of corticosteroid hormone signaling mechanisms in early vertebrates. However, whether a classical HPI axis is regulating the stress responses in lamprey still remains unclear.

Due to lamprey's unique life history, including metamorphosis during the larval stage where the anterior part of head kidney is lost, there has been debate over which tissues are responsible for the production of corticosteroid hormone (Youson, 1970). Accordingly, to fully understand the HPI axis-mediated stress responses in lamprey, it is necessary to determine the site of corticosteroid production. Given the steroidogenic pathway, the synthesis of S from various radioactive precursor steroids such as progesterone, pregnenolone, and 17α hydroxy progesterone needs to be examined in an in vitro experiment using the tissues that are known to function as kidneys in lamprey, mesonephric tissues. Furthermore, the in vivo characterization of S production in the putative interrenal tissues will confirm involvement of the interrenal tissues in the HPI axis.

The objective of this study was to examine whether stress responses in sea lamprey are mediated by the HPI axis, by testing the effects of lamprey CRH and ACTH on plasma S concentrations, and by identifying the site of corticosteroid hormone production. In addition, this study investigated the adrenocorticotrophic activities of various hypothalamic hormones such as AVT and GnRH as potential alternative pathways that regulate corticosteroid production in sea lamprey. The significance of the evolution of the HPI axis and potential alternative pathways in the basal vertebrates is discussed in terms of steroid signaling system evolution.

2. Materials and methods

2.1. Materials

Radiolabeled steroids were purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Synthetic steroids, the antibody to S, and all other chemicals and reagents were purchased

from Sigma (Sigma Aldrich Chemical Co., St. Louis, MO, USA) unless otherwise noted.

2.2. Collection and maintenance of animals

Adult sea lampreys (*P. marinus*) were obtained from the Sea Lamprey Control Program at the Department of Fisheries and Oceans in Sault Ste. Marie, Ontario in July 2010 and July 2011. Animals were transported to the University of British Columbia, Vancouver, BC, where they were held at 4–6 °C in covered, insulated tanks filled with dechlorinated tap water from the City of Vancouver. For the ACTH and AVT injection experiments, sea lamprey provided by the USFWS were transported to the Hammond Bay Biological Station and held in 1000 L flow-through tanks. The subject animals were acclimated for 7 d before the injection experiments were performed. Sea lamprey were approved for use in these experiments, which were performed according to the University of British Columbia Animal Care protocol A11-0055.

2.3. Experimental subjects

For all experiments, adult lampreys were acclimated in covered, insulated, flow-through tanks (254 L) filled with dechlorinated tap water from the City of Vancouver at 10–12 °C for at least one week before experiments.

2.4. Sampling methods

2.4.1. Blood collection

Once fish were anesthetized, fish were placed upside down in a plastic trough and blood was collected by cardiac puncture using Vacutainers coated with EDTA to prevent clotting (Becton Dickinson-Canada, Mississauga, ON, Canada). Samples were immediately placed on ice. Fish were placed in a freshwater recovery bucket and then returned to holding tanks for recovery. Total sampling time for each tank did not exceed three minutes. Blood samples were centrifuged for 12 min at 2500 rpm and 4 °C (Beckman Coulter). Plasma was frozen at –80 °C until RIA was conducted.

2.4.2. Tissue sampling

Following acclimation, fish to be euthanized were netted out and immediately immersed in an overdose of anesthetic solution (0.2–0.3 g/L of MS-222; Argent Chemical Laboratories, Inc.). At this dose, most movement stopped within one minute and death occurred within 2–3 min. Euthanized animals were placed on a surgery table, and then tissues including kidneys, gills, gonads, and livers were collected and immediately placed in L-15 incubation medium (Sigma-Aldrich) on ice. Animal remains were bagged, labeled, and disposed of according to UBC policy.

2.5. Analytical techniques

2.5.1. Radioimmunoassay

Radioimmunoassays (RIAs) were performed as in Scott et al. (1980). Briefly, RIAs were conducted in duplicate in 10 mm × 75 mm glass culture tubes (Fisher Scientific). Nine standards, also in duplicate, were made up over the range 500–1.95 pg/100 µL tube. Unknown sample tubes contained a total volume of 100 µL, made up of 20 µL plasma and 80 µL assay buffer (50 mM sodium phosphate, pH 7.4, 0.2% BSA, 137 mM NaCl, 0.40 mM EDTA, and 0.77 mM sodium azide). Binding reagent was made by adding radio-label and antibody such that when 100 µL was dispensed to all tubes, each tube would contain 5000 disintegrations per minute (DPM), and in the absence of any standard steroid, 50% of the radio-label would be bound to the antibody. Blank tubes with no antibody, and tubes necessary to determine the total and maximum DPM

counts were also included. All tubes were incubated at 4 °C overnight, separated with 500 mL of charcoal solution at 0 °C (50 mM sodium phosphate, pH 7.4, 0.1% gelatin, 1.0% dextran-coated charcoal), centrifuged at 2500 rpm, 4 °C for 12 min, decanted into 7 mL scintillation vials, and mixed with 5 mL scintillation cocktail. DPM were counted with an LS-6500 (Beckman Coulter) scintillation counter.

2.5.2. Steroid extraction

Steroid extraction was performed as in Newman et al. (2008). 100 mg of each tissue was sampled and snap frozen under liquid nitrogen. The frozen samples were ground under liquid nitrogen using a pestle and mortar. One mL of 70% ethanol was added to the ground tissue samples, followed by centrifugation. The supernatant was removed and combined before subject to solid phase extraction. For solid phase extraction, Sep-Paks (Waters, Milford, MA, USA) were activated with 5 mL methanol (MEOH) and rinsed with distilled water (ddiH₂O). The pooled supernatant was diluted and loaded onto Sep-Paks, which were rinsed with 5 mL ddiH₂O and then eluted with 5 mL MEOH. Samples were then loaded onto a CentriVap Concentrator (Labconco, Kansas City, MO, USA) to evaporate MEOH elute overnight. To each dried tube, 1 mL ETOH was added, mixed, and pipetted into microfuge tubes, which were labeled and frozen at –80 °C until needed for analysis.

2.6. Identification of CRH peptide sequence

Candidate CRH gene sequences were identified in the WGS *P. marinus* Draft Assembly (Draft v.2, 2007-02) and the NCBI Trace database. TBLASTN searches were used to identify candidate genes in sea lamprey using known vertebrate CRH amino acid sequences as queries. Sequences producing alignment hits (*E*-values <1 × 10⁻¹⁰) were added to a non-redundant list of queries and searching continued in this manner until no new contig hits were found in the assembly. Genes were predicted using GENSCAN and FGENESH and predicted amino acid sequences were tentatively identified by batch BLASTP searches against the non-redundant (nr) NCBI Protein database. Partial CRH protein sequences were found using NCBI searches for different species. Alignments of all CRH sequences shown were completed on MEGA 5 alignment.

2.7. Corticotropin-releasing hormone injections

Adult sea lampreys were injected with either corticotropin-releasing hormone or saline solution (0.90% NaCl) (control). Sea lampreys were injected intraperitoneally with corticotropin-releasing hormone (CRH₁₋₄₁), based on the sequence identified from the sea lamprey genome database, and custom synthesized by New England Peptide (Gardner, MA, USA). Following acclimation, fish were netted out of tanks, immediately immersed in anesthetic solution, and once anesthetized, placed upside down in a plastic trough. CRH dissolved in saline solution was injected intraperitoneally at doses of 0.01 µg/kg, 0.1 µg/kg, 1.0 µg/kg, 10 µg/kg, and 100 µg/kg. Once treated, fish were placed in a freshwater recovery bucket and then returned to holding tanks. Time spent for each set of injections did not exceed three minutes per tank.

2.8. Adrenocorticotrophic hormone injections

Adult sea lampreys were injected with either adrenocorticotrophic hormone or saline solution (control). Sea lampreys were injected intraperitoneally with one of four lamprey adrenocorticotrophic hormone peptides (ACTH₁₋₅₉, ACTH₁₋₅₉; 35P, ACTH₁₋₆₀, and ACTH₁₋₆₀; 35P), based on the sequences published by Takahashi et al. (2006), and custom synthesized by Bachem (Torrance, CA, USA). Following acclimation, fish were netted out of tanks, immediately immersed in

anesthetic solution, and once anesthetized, placed upside down in a plastic trough. The four ACTH peptides were dissolved in saline solution and injected at a dose of 100 µg/kg. Saline solution was used as a control. CRH injection 100 µg/kg was used as a positive control. Once treated, fish were placed in a freshwater recovery bucket and then returned to holding tanks. Time spent for each set of injections did not exceed three minutes per tank.

2.9. Adrenocorticotrophic functions of lamprey GnRHs and arginine vasotocin (AVT)

To examine whether other hypothalamic hormones exert any effects on corticosteroid production, 10 lampreys were treated with either doses of peptide hormones, including lamprey GnRH I, and GnRH III, or saline solution (control). Adult sea lampreys were injected with lamprey GnRH I and GnRH III following acclimation, and injected again with a second dose 24 h later. All of the fish treated were sampled for blood one hour after the second injection. The lamprey GnRH I and GnRH III were dissolved in saline solution and injected at doses of 100 µg/kg. In another experiment, male and female adult lampreys were treated with either 100 µg/kg dose of AVT or saline (control). The control treatment was performed on groups of four males and six females, respectively while treatment with AVT was performed on three males and three females. Blood samples were taken 1 h after the second injection.

2.10. In vitro corticosteroidogenesis (corticotropic effects of ACTH)

The objective of the first incubation experiment was to determine which, if any, of the kidney, gonads, gill, or liver were able to produce S with or without the influence of ACTH. Tissue samples were weighed, diced with a razor blade, and placed in 15 mL conical tubes containing 5 mL of L-15 incubation media on ice. The conical tubes were sealed, placed horizontally, and incubated at 10 °C for 4 h at a slow but constant shaker speed. The ACTH treatment groups were incubated with a mixture of four ACTH peptides mentioned above at a concentration of 100 ng/mL, while control groups with saline. After incubation, tubes were centrifuged at 2500 rpm, 4 °C for 12 min. The supernatant were collected and extracted using a Sep-Pak primed with methanol and deionized water. The trapped steroid was eluted with 4 mL of 100% methanol and the eluents were dried under vacuum. The dried eluents was reconstituted in RIA buffer and subject to an RIA analysis to quantify S concentrations.

After results from the first incubation experiment were obtained, the objective for the second incubation experiment was to determine which precursor steroid(s) the kidneys were able to convert to S. The procedure was the same as above, but 1–2 µCi of ³H-progesterone, ³H-pregnenolone, or ³H-17α-hydroxyprogesterone (American Radiolabeled Chemicals) were added to each incubation tube.

2.11. Thin layer chromatography

To determine whether any of the radiolabeled precursor steroids were converted to S during the second, radioactive incubations, an initial analysis of the products was performed by thin layer chromatography (TLC). Volumes corresponding to 10,000–40,000 DPM of extract were placed in 10 mm × 75 mm glass culture tubes (Fisher Scientific) containing 10 µL of standard steroids. These were dried down under nitrogen at 40 °C, resuspended in 100 µL ethyl acetate, and loaded onto separate lanes of pre-coated silica-gel TLC plates (Whatman Inc. Piscataway, NJ, USA). The plates were developed for 30 min with chloroform/ethanol/acetic acid (50/50/0.002, v/v/v) after equilibrating for 30 min. The

positions of standard steroids were noted by placing the plates under a UV source. The lanes were divided into 4 mm sections, scraped off into scintillation vials, mixed with 5 mL SafetySolve scintillation cocktail (Research Products International Corp., Mount Prospect, IL, USA) and had DPM counted in an LS-6500 (Beckman Coulter) scintillation counter.

2.12. High performance liquid chromatography

Once the initial analysis indicated which precursors were converted to S, products of the appropriate incubation media were purified by HPLC. Volumes corresponding to 15,000 DPM of extract were mixed with 20 µg of standard steroids, dried down under nitrogen at 40 °C, resuspended in 1 mL acetonitrile/water/formic acid (30/70/0.01, v/v/v), centrifuged at 14,000 rpm for 10 min, and then loaded onto a C18 reverse-phase HPLC column (Alltima, 4.6 mm × 250 mm, Alltech, Dearfield, IL, USA) fitted with a guard module. The solvents used to create the column gradient were 0.01% formic acid (solvent A) and 70% acetonitrile (solvent B) and were developed as follows: 0–10 min: 28% B; 10–60 min: 28–100% B; 60–90 min 100% B. Total development time was 90 min. Fractions were collected every 1 min between 20 min and 75 min in 16 mm × 100 mm culture tubes (Fisher Scientific). UV absorptions of eluate were monitored and recorded with a photodiode array detector (Shimadzu) to determine positions of standard steroids. Once collected, samples from each fraction were mixed with 5 mL scintillation cocktail (RPI Corp.) and DPM were counted with an LS-6500 (Beckman Coulter) scintillation counter.

2.13. Steroid acetylation and analysis by TLC

Steroid acetylation and analysis by thin-layer chromatography was performed as in Bryan et al. (2004). Briefly, volumes containing 100,000 DPM of ³H-S were fractionated on HPLC as described above. Part of the fractions (250 µL containing 50,000 DPM) corresponding to the elution position of S were placed in a 16 mm × 100 mm culture tube (Fisher Scientific) containing 10 µg of standard S. The solvents were removed, replaced by 100 µL pyridine and 100 µL acetic anhydride, covered, and left overnight at room temperature. The remaining 250 µL of the same fractions were mixed with 10 µg of standard S in a separate glass tube. The following day, the solvents in both tubes were evaporated and replaced with 100 µL of ethyl acetate. These were loaded onto separate lanes of a TLC plate, which was developed for 30 min with chloroform/ethanol/acetic acid (50/50/0.002, v/v/v) after

equilibrating for 30 min. The positions of standard steroids were noted by placing the plates under a UV source. The lanes were divided into 4 mm sections, scraped off into scintillation vials, and mixed with 5 mL scintillation cocktail (RPI Corp.). DPM were counted as above.

2.14. Statistical analysis

Data were expressed as mean ± SE, and analyzed by two-way analysis of variance (ANOVA), followed by Dunnett's test for multiple group comparisons using Prism 5.00 (GraphPad Software Inc, California, USA).

3. Results

3.1. Characterization of lamprey corticotropin-releasing hormone

The use of data mining identified a potential sea lamprey CRH peptide sequence with 41 amino acid residues and a C-terminal amidation. The deduced peptide sequence is SDEPPISLDLTFHLLREVLEMADEQLAQQAHTRQIMENI-NH₂. The sea lamprey CRH peptide hormone shows high sequence similarity to other known CRH peptide sequences in other species (Table 1).

3.2. Corticotropin function of corticotropin-releasing hormone

Sea lamprey injected with increasing doses of lamprey CRH between 0.01 and 100 µg/kg body weight showed increased plasma concentrations of S in a dose dependent manner after 1 h injection (Fig. 1). At a dose of 100 µg/kg body weight, the circulating S concentrations was approximately three times higher than those treated with saline.

3.3. Functional studies of adrenocorticotropin hormone

Four ACTH peptides previously identified from sea lamprey were injected along with saline as control and lamprey CRH as positive control. A two-way ANOVA was conducted that examined the effect of gender and CRH and ACTH treatments on S concentrations. There was no significant interaction between the effects of gender and treatment, $F = 1.118$, $p = 0.3556$. S concentrations significantly increased upon treatment with CRH in both males ($p < 0.001$) and females ($p < 0.01$), while S concentrations were not significantly elevated by any treatments with ACTHs (Fig. 2A and B).

Table 1

Comparison of sea lamprey CRH peptide sequence with other known CRH sequences from mammals, reptiles, and fishes.

CRH sequence	Identity (%)	Source (accession number)
S <u>DEPPISLDL</u> TFHLLREVLE MA <u>KA</u> EQLAQQ AH <u>TR</u> QIMENI IGK	100.0	SEA LAMPREY
SE <u>EP</u> PISLDL TFHLLREVLE MARAEQLAQQ AHSNRKLM <u>EI</u> IGK	86.0	RAT (AAA40965.1)
SE <u>EP</u> PISLDL TFHLLREVLE MARAEQLAQQ AHSNRKLM <u>EI</u> IGK	86.0	MOUSE (AAI19037.1)
SE <u>EP</u> PISLDL TFHLLREVLE MARAEQLAQQ AHSNRKLM <u>EI</u> IGK	86.0	HUMAN (EAW86897.1)
SE <u>EP</u> PISLDL TFHLLREVLE MARAEQMAQQ AHSNRKMM <u>EI</u> FGK	81.4	ZEBRAFISH (AAI64878.1)
SE <u>DP</u> PISLDL TFHLLREMME MSRKEQMAQQ AQNNRRMM <u>EL</u> FGK	67.4	FLOUNDER (CAD88277.1)
AE <u>EP</u> PISLDL TFHLLREVLE MARDEQLVQQ AYSNRKMM <u>DI</u> FGK	72.1	RAINBOW TROUT (CAD97421.1)
SE <u>EP</u> PISLDL TFHLLREVLE MARAEQMAQQ AHSNRKMM <u>EI</u> FGK	81.4	GOLDFISH (AAN41653.1)
SE <u>DP</u> PAISLDL TFHLLRGMME MSRAEQLA <u>EQ</u> AKNNEILMER YGK	62.8	SOLE (FR745427.1)
SE <u>EA</u> PISLDL TFHLLREVLE MARAEQMAQQ AHSNRKMM <u>EI</u> FGK	79.1	COMMON CARP (CAC84859.1)
SE <u>DP</u> PISLDL TFHLLREMME MSRAEQLAQQ AQNNRRMM <u>EL</u> FGK	72.1	TILAPIA (CAB77056.1)
SE <u>DP</u> PISLDL TFHLLREMME MSKAEQMAQQ AQNNRRMM <u>EL</u> FGK	72.1	MEDAKA (NM_001128518)
SE <u>EP</u> PISLDL TFHLLREVLE MARAEQIAQQ AHSNRKLM <u>DI</u> IGK	81.4	RANA FROG (ADJ56343.1)
AE <u>EP</u> PISLDL TFHLLREVLE MARAEQIAQQ AHSNRKLM <u>DI</u> IGK	79.1	XENOPUS FROG (NP_001165681.1)

The last amino acid residues of – GK represent an amidation site, indicating CRH peptides with a C-terminal amidation. The percentage identity of CRH sequences against lamprey CRH has been calculated to indicate similarity between the peptides. The amino acid residues that do not match with those of human CRH are underlined. Genbank accession numbers were presented in the parenthesis.

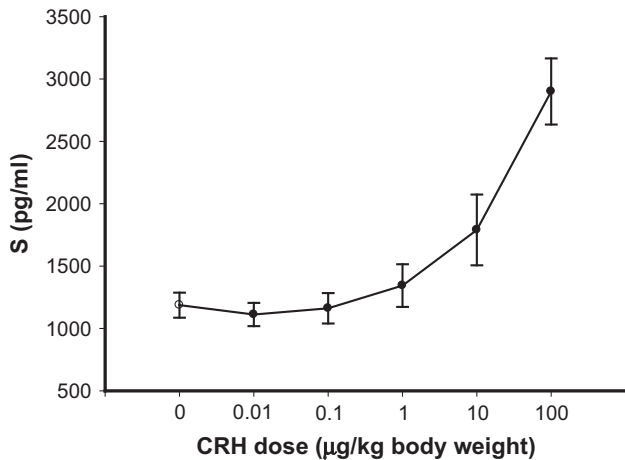


Fig. 1. Plasma 17,21-dihydroxypregn-4-ene-3,20-dione (S) concentrations of adult male ($n = 6$) and female ($n = 6$) sea lamprey after intraperitoneal injection of varying doses of lamprey corticotropin-releasing hormone (0.01–100 µg/kg body weight) or saline solution (0.90% NaCl). Data are mean \pm SE.

3.4. Adrenocorticotrophic functions of GnRHs and AVT

Sea lamprey injected with 100 µg/kg body weight of lamprey GnRH I, GnRH III, and S concentrations were measured. Injection of GnRH I and III resulted in plasma S concentrations increasing

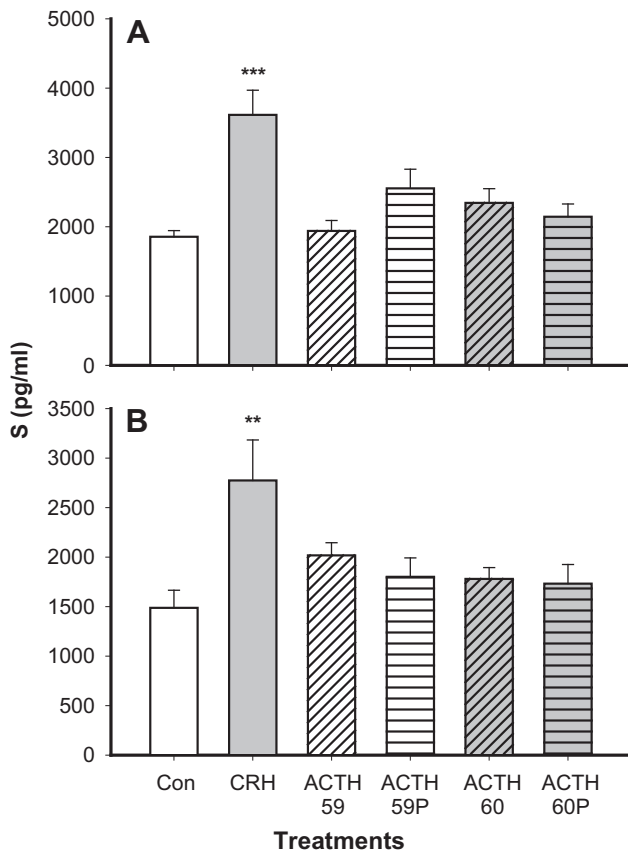


Fig. 2. Plasma 17,21-dihydroxypregn-4-ene-3,20-dione (S) concentrations of (A) adult male ($n = 5$), and (B) female sea lamprey after injection with four isoforms of lamprey adrenocorticotrophic-hormone or saline solution (0.90% NaCl) (59, 59P, 60, and 60P refer to the peptide length and phosphorylation state; C denotes saline-injected animals). Data are mean \pm SE. Asterisks indicate a significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) difference relative to control.

to 5–6 ng/mL with a slight variation between males and females (Fig. 3). A two-way ANOVA was conducted that examined the effect of gender and GnRH treatments on S concentrations. There was no significant interaction between the effects of gender and GnRH treatments, $F = 2.72$, $p = 0.07$. S concentrations significantly increased upon treatment with GnRH I (males, $p < 0.001$; females, $p < 0.001$) and GnRH III (males, $p < 0.001$; females, $p < 0.001$) (Fig. 3).

Although the stimulatory effects of the same dose of AVT were lower than those of GnRHs, plasma S concentrations increased up to nearly 4 ng/mL in both males and females. A two-way ANOVA was conducted that examined the effect of gender and AVT treatments on S concentrations. There was significant interaction between the effects of gender and AVT treatments, $F = 5.30$, $p = 0.04$. S concentrations significantly increased upon treatment with AVT both in males, $p < 0.01$ and females, $p < 0.001$ (Fig. 4).

3.5. In vitro corticosteroidogenesis

In vitro incubations of male and female sea lamprey tissues indicate that the mesonephric kidney produced higher levels of S (Fig. 5A and B). Further, incubations of the tissues with ACTH did not show any significant effects in S production. For males, mean concentration of S per mg of mesonephric tissue incubated was 1.1 ± 0.52 pg/mg. For females, mean concentration of S per mg of mesonephric tissue incubated was 4.5 ± 1.7 pg/mg. For both sexes, production by the gonads, gill, and liver was negligible.

Incubations with tritiated precursor steroids indicated that only $17\alpha P$ was biotransformed into S (Fig. 6A–F). Preliminary identification of S was obtained by running incubation media on TLC; comigration with standard S occurred only in samples incubated with tritiated $17\alpha P$ (Fig. 6A and B). Confirmation of the identification of the putative S was obtained by fractionating incubation media of samples containing $17\alpha P$ by HPLC (Fig. 6C and D), which yielded up to four peaks. In all cases, peaks corresponding to the elution point of standard S were observed, with a mean rate of conversion from $17\alpha P$ to S of $17 \pm 3.8\%$ in males and females.

Further identification was obtained by running the HPLC-purified fractions that co-eluted with standard S on TLC both before and after acetylation. In both males (Fig. 6E) females (Fig. 6F)

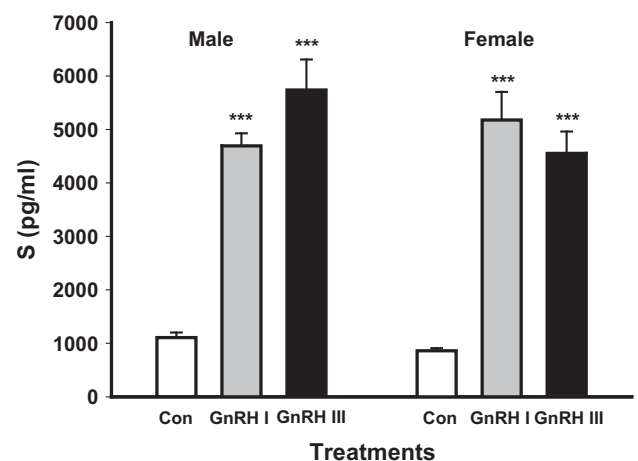


Fig. 3. Corticotrophic effects of lamprey GnRHs. Male and female lampreys ($n = 10$) were separately treated with 100 µg/kg body weight dose of lamprey GnRH I and GnRH III and the plasma levels of S were measured 1 h post injection. No stimulatory effects were observed in the lampreys ($n = 10$) treated with saline while a surge of the plasma S levels were evident in the lampreys with both GnRH I and GnRH III. Data are mean \pm SE. Asterisks indicate a significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) difference relative to control.

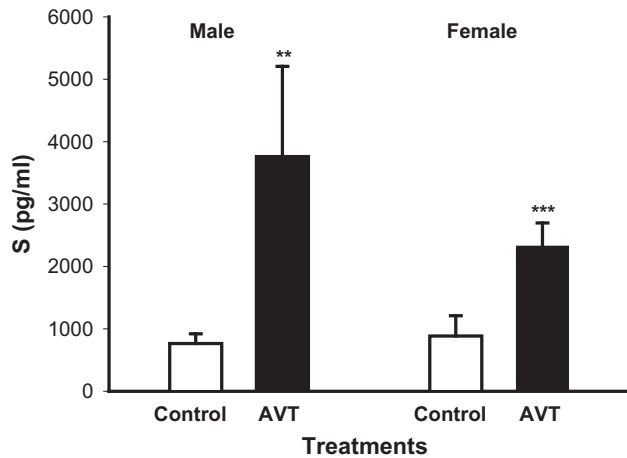


Fig. 4. Corticotropic effects of Arg-vasotocin (AVT). Male and female lampreys ($n = 3$) were separately treated with 100 $\mu\text{g}/\text{kg}$ body weight dose of AVT. The control treatment with saline was performed with groups of males ($n = 4$) and females ($n = 6$). The plasma levels of S were measured 1 h post injection. The injection of AVT strongly stimulated the production of S in both male and female lampreys. Note that the S levels changes upon the injection of AVT were statistically significant, compared to control groups with the saline injection. Data are mean \pm SE. Asterisks indicate a significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) difference relative to control.

results of radioactive co-migrations show peaks corresponding exclusively to standard S and acetylated-standard S.

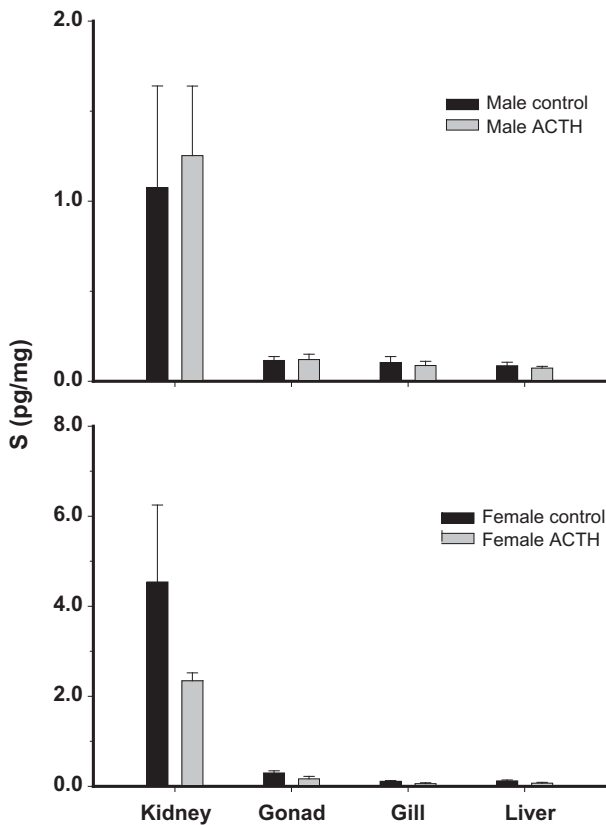


Fig. 5. Concentration of 17,21-dihydroxypregn-4-ene-3,20-dione (S) in tissues of (A) adult male ($n = 3$) and (B) female sea lamprey after incubation with or without ACTH treatment. The treatment groups were incubated with 100 ng/mL ACTH, after which S concentrations were measured and compared. There was no statistically significant difference found between the treatments. Data are mean \pm SE.

4. Discussion

Our experiments show that, in the sea lamprey, S (one of the few steroids that has been definitively identified in this species) seems to be responsive to peptides that, in higher vertebrates are involved in several different pathways: stress (CRH, AVT), reproduction (GnRH) and osmoregulation (AVT). Surprisingly, though, none of the sea lamprey ACTH peptides showed any stimulatory effects on circulatory S concentrations. The mesonephric kidneys displayed the ability to release S into the medium and to biotransform tritiated 17α -hydroxyprogesterone into a compound that behaved like S on HPLC and TLC (acetylated and unacetylated). Taken together, the data from the present study indicate that, if (as our previous studies suggested) S is a corticosteroid in the sea lamprey, its regulation is very different from that in higher vertebrates.

We have characterized a putative CRH peptide sequence by searching the lamprey genome database. In general, the amino acid sequence of lamprey CRH displayed a very high homology with other CRHs from teleost fishes, amphibians, and mammals, which is in line with the notion of high CRH conservation throughout vertebrate evolution (Lovejoy, 1996). Interestingly, the lamprey CRH showed higher homology with those of human and rats than those of other fishes, with only six amino acid residues differing from human CRH. The high homology between lamprey CRH and human CRH may explain the previous experimental data showing that injection of the human form of CRH could induce changes in S levels in the sea lamprey (Close et al., 2010). Following the injections of increasing doses of lamprey-CRH, a dose-dependent response was observed in both male and female lamprey. Concentrations of S in control groups were consistent with those previously reported in sea lamprey (Close et al., 2010), and showed very little variation. The stimulation of S production by sea lamprey CRH suggests that this peptide is exerting its corticoid steroidogenesis effects through the uncharacterized system in sea lamprey.

In fish, AVT is an important hormone that regulates many physiological and behavioural functions, including response to stress, osmoregulation, and reproduction (Balment et al., 2006). The results of our AVT injections revealed increased secretion of S into sea lamprey plasma. The involvement of neurohypophysial hormones and other neuroendocrine hormones in the regulation of corticotropic functions in fishes has been known, including the AVT and CRH/isotocin actions on ACTH secretion from pituitary of goldfish and rainbow trout (Baker et al., 1996; Fryer et al., 1985; Pierson et al., 1996), AVT and thyrotropin releasing hormone (TRH) actions on ACTH release in goldfish (Fryer et al., 1985; Lederris et al., 1994) and gilthead sea bream (Rotllant et al., 2000). The participation of the neurohypophysial hormones in direct stimulation of corticosteroid has been reported in amphibians (Larcher et al., 1992a) and mammals (Aguilera et al., 2008), indicating that the interplay between different hormonal axes or endocrine factors is widely present in vertebrates, including fishes, as summarized by Bernier and Klaren (2009). In a study using frogs, the stimulatory effect of AVT on corticosteroid secretion was found to be mediated through activation of receptors related to the mammalian V2 and/or OXT receptors in the interrenal tissues, which are positively coupled to phosphoinositide-specific phospholipase C (Larcher et al., 1992b). In a recent study, Lema (2010) identified multiple vasotocin receptors which include V1a-type, V1b-type, and V2 in fish. Expression of the receptors was wide spread in tissues including the kidney and gonads. In sea lamprey, AVT was isolated and identified from pituitary extracts which was identical to the molecule in teleosts (Lane et al., 1988). Over several decades, studies have clearly shown that AVT is involved in the HPA axis. Further studies are required to elucidate the mechanisms that AVT exerts its corticotropic function in lamprey.

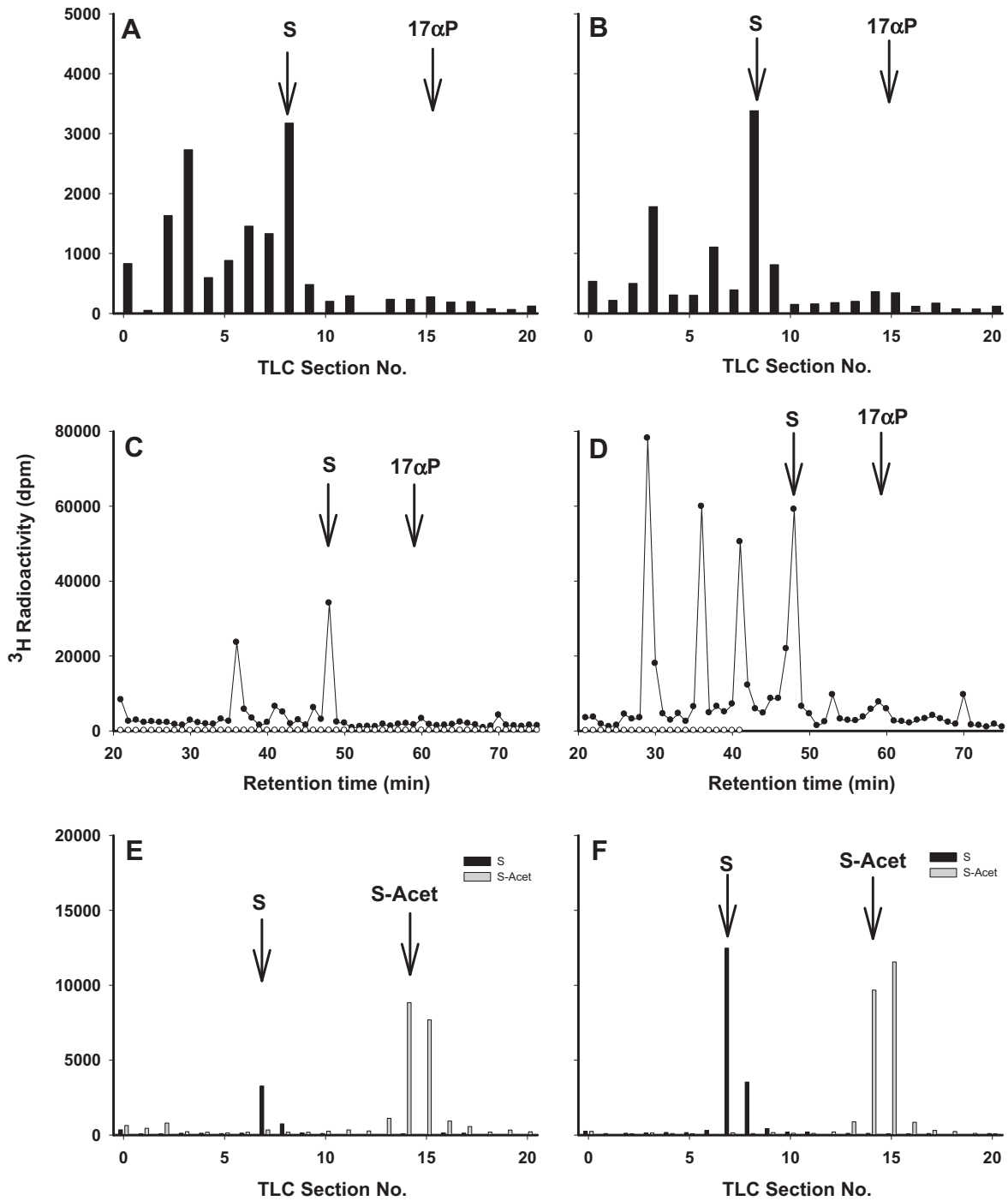


Fig. 6. Confirmation of 17,21-dihydroxypregn-4-ene-3,20-dione (S) production by mesonephric tissue. After incubation of tritiated 17 α OH-progesterone with sea lamprey mesonephric kidneys, the incubation media were extracted and analyzed by a series of analytical methods, including thin layer chromatography (TLC), HPLC, and TLC with acetylated steroids. The analytical results for identification of produced S are presented in A, C, E for males and B, D, F for females in the order of TLC, HPLC, and TLC with acetylated steroids. Note that arrows represent the elution points of standard S and 17 α P.

In most vertebrates, ACTH is a critical component of the HPA/I axis, acting on adrenal or interrenal tissues to stimulate the production of corticosteroids. In an earlier study, [Takahashi et al. \(1995\)](#) using a partial lamprey ACTH(1–43) sequence provided evidence of functionality by conversion of S in incubations to DOC and unknown products with pronephric and mesonephric tissues. However, at that time it was unknown that S was the putative corticosteroid and not a precursor steroid. Later, four lamprey ACTH peptides were identified and characterized, revealing that lamprey

ACTHs are 20–21 amino acids longer than those of more derived vertebrates, to which they show very little similarity ([Takahashi and Kawachi, 2006](#); [Kawachi and Sower, 2006](#); [Takahashi et al., 2006](#)). Due to post-translational modifications, lamprey produce four unique ACTH peptides, whereas all other vertebrates have one ([Kawachi and Sower, 2006](#)). Unfortunately, in lamprey, it remains unknown which sequences of the ACTH peptides are necessary to stimulate biological activity, although it is known only the first 24 amino acids including the NH₂ terminal are required for

stimulation of biological activity in mammals. (Li, 1963; Evans et al., 1966; Hadley, 1992). In the present in vivo study the synthetic copies of the four ACTH peptides, including free (ACTH₅₉, and ACTH₆₀) and phosphorylated (ACTH_{59P} and ACTH_{60P}) forms at a dose of 100 µg/kg failed to induce any changes in circulating concentrations of S. These findings can be attributed to five possible reasons: (1) the ACTH forms used may not be the functional pituitary adrenocorticotrophic hormone (i.e., there might be other ACTH forms or different peptides that exert adrenocorticotrophic effects on interrenal tissues) (2) the full HPI axis involving the pathway CRH – ACTH – corticosteroid may have evolved in vertebrates after they split from the cyclostomes (i.e., the proposed lamprey ACTHs may represent peptides that have not yet gained adrenal-stimulating properties. (3) The ACTH molecules (which were made synthetically) may not have formed an appropriate structural conformation for receptor binding. (4) The injected ACTH peptides may have been rapidly degraded when injected; (5) despite our previous results and its ability to respond to CRH, S may not be the right steroid to be measuring (i.e., there may be another as yet unknown steroid that functions as a corticosteroid and is responsive to ACTH). Only further research will reveal the correct answer.

Our study showed that lamprey GnRH I and GnRH III can stimulate the plasma concentrations of S in sea lamprey. It is well established that pituitary gonadotropins (GTHs) under the control of hypothalamic GnRH(s), play an important role in gametogenesis. GTHs act by stimulating gonadal synthesis of steroid hormones. In teleosts, spermatogenesis and oocyte growth are controlled by 11-ketotestosterone and estradiol-17β respectively (Nagahama, 1994). During final maturation, there is a steroidogenic shift in the gonads to produce maturation-inducing hormone (MIH) (Nagahama, 1994). The MIHs control sperm maturation and oocyte maturation in fish. The three known MIHs in fish are 17α,20β-dihydroxy-4-pregnane-3-one (17α,20β-DP), 17α,20β,21 trihydroxy-4-pregnen-3-one (20β-S) (Senthilkumaran et al., 2004), and 17,21-dihydroxypregn-4-ene-3,20-dione (S) (Webb et al., 2002). These findings may suggest that S can be an MIH in lamprey, since GnRHs can stimulate S production. However, we have observed that in addition to S, 17α OH-P, 17α,20β-DP, and 20β-S are stimulated after lamprey GnRH I and III injections (unpublished data). It will be interesting to examine whether those three steroids act as an MIH in lampreys, given that no MIH has been identified and characterized in the lamprey.

Previous studies suggest that stress responses can be manifested not only by conventional HPI axis but also other pathways. For example, acute stress has been shown to sharply elevate plasma GTH and ACTH concentrations that can remain elevated up to four hours in brown trout (*Salmo trutta* L.) (Pickering et al., 1987; Sumpter et al., 1987). Furthermore, GTHs have been shown to stimulate adrenal/interrenal steroidogenesis. Guinea pig adrenal cells exposed to human chorionic gonadotropin were shown to stimulate cortisol and androstenedione secretion in vitro (O'Connell et al., 1994). ACTH, luteinizing hormone (LH) and partially purified salmon gonadotropin (SG-G100) injected into gonadectomized catfish (*Heteropneustes fossilis*) increased plasma cortisol and androgen concentrations (Truscott et al., 1978). Goswami et al. (1985) injected ovine LH and SG-G100 in hyposectomized and intact gravid catfish which also increased plasma cortisol concentration in both treatments. In coho salmon (*Oncorhynchus kisutch*), interrenal incubations with SG-100 and a highly purified chum salmon GTH (DE-46) demonstrated that both preparations were extremely effective in stimulating the secretion of cortisol and androstenedione equivalent to ACTH (Schreck et al., 1989). We caution making inferences from the partially purified salmon gonadotropin (SG-100) due to the possibility of cross contamination with ACTH. However, it is clear from these studies that GTHs,

specifically LH, can stimulate production of corticosteroids in vertebrates. The presence of a single gonadotropin (GTHβ) identified in sea lamprey (Sower et al., 2006) and the stimulation of S production by GnRH in the present study warrant further studies to examine the regulation of stress responses by non-conventional pathways.

The in vitro corticosteroidogenesis experiments performed in the present study demonstrated that the anterior mesonephric kidneys of the sea lamprey are possibly an endocrine organ. The measurement of S concentrations in various tissues, followed by in vitro incubation of the mesonephric kidney with ³H precursor, 17αP which was able to convert to S. The probable identity of S in the mesonephric tissue was based on TLC, HPLC and acetylation of radioactive product and RIA of non-radioactive product. In agnathan fishes, lampreys and hagfish, the identity of interrenal tissue still remains elusive, even though there have been many studies describing steroidogenic tissues called presumptive adrenalcortical tissue (PAT). As summarized in a recent review paper by Youson (2007), the pronephric and opisthonephric tissues of lampreys seem to display some anatomical, biochemical, and histochemical evidence to suggest they might be involved in pituitary-PAT axis (Seiler et al., 1981; Youson, 1972; Weisbart and Youson, 1975). However, the consensus on the identity of the PAT still awaits further scientific assertion. Therefore, further work is needed to examine the controlling factors and production of S in presumed adrenal tissues and gonads.

In conclusion, the present study demonstrates that not just CRH, but also AVT and GnRH, are able to stimulate S production, and that lamprey mesonephric tissues appear to be the main site of production of S. The lack of activity of ACTH indicates either a methodological problem (e.g., instability of the peptides) or that, if S is a corticosteroid, then its regulation in the lamprey is very different from that in higher vertebrates. To comprehensively understand mechanisms underlying stress physiology in lamprey, further research is required, including: (1) identification and characterization of a functional ACTH-like molecule; (2) regulatory mechanisms of lamprey CRH on lamprey ACTH; (3) elucidation of corticotrophic mechanisms of endocrine factors such as AVT and GnRHs; (4) establishment of a network of endocrine systems that regulate stress responses; (5) feedback regulation of noreurohypophysial and pituitary hormones by S. The results from such proposed research may provide clues to answer questions regarding the evolution of corticosteroid signaling pathways in the early vertebrate lineage.

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