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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 adrenocorticotropic 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 17α-hydroxyprogesterone (17α-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.

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 neurones to stimulate the corticotrophe 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 steroidogenetic 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 adrenocorticotropic 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 radiolabel 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 radiolabel would be bound to the antibody. Blank tubes with no antibody, and tubes necessary to determine the total and maximum DPM