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Source: Zoological Science, 19(9) : 1055-1059

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.19.1055

ZOOLOGICAL SCIENCE **19**: 1055–1059 (2002)

Distribution of Growth Hormone-Like Cells in the Pituitary of Adult Sea Lampreys, *Petromyzon marinus*

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ABSTRACT—Growth hormone (GH), prolactin (PRL) and somatolactin (SL) are members of a pituitary hormone family that are believed to have evolved from a common ancestral gene by duplication and subsequent divergence. Since these hormones are found both in bony fish and cartilaginous fish, their ancestral form(s) should be present in the Agnatha. Thus, although there is no convincing evidence that the lamprey pituitary secretes GH or PRL, GH- and/or PRL-like immunoreactivity was examined in the pituitary of adult sea lampreys (*Petromyzon marinus*), using antibodies to GHs, PRLs and SL of mammalian and/or fish origins. Our initial attempt with ordinary immunohistochemical procedures failed to detect any positive reactions in the lamprey pituitary. Following the hydrated autoclave pretreatment of the sections, anti-salmon GH, anti-salmon PRL and anti-blue shark GH gave positive reactions in most cells distributed in the dorsal half of the proximal pars distalis. These results suggest that the material immunoreactive to those antibodies is related, to some extent, to GH/PRL, but enhancement of immunoreactivity to reveal this by the hydrated autoclave pretreatment of sections is needed due to low crossreactivity. The similarity of the topographic distributions within the pituitary between lampreys and teleosts suggests that lamprey GH/PRL-like cells are GH cells of the lamprey.

Key words: lamprey pituitary, growth hormone, prolactin, immunohistochemistry, hydrated autoclaving

INTRODUCTION

Lampreys and hagfish are the only two extant representatives of the oldest class of vertebrates, Agnatha (jawless fishes), whose evolutionary lineage can be traced back approximately 550 million years. Forey and Janvier (1993) have hypothesized from their phylogenetic analysis that modern lampreys are more closely related to gnathostomes than they are to hagfish. In agreement with Forey and Janvier (1993), the morphology of the pituitary gland of the lamprey is also more similar to that of gnathostome fish than it is to hagfish (Ball and Baker, 1969; Hardisty and Baker, 1982).

Among adenohypophysial hormones, only adrenocorticotropin (ACTH) and melanotropins (MSHs) have been isolated from the lamprey pituitary gland (Takahashi *et al*., 1995a). Unlike ACTH and MSH in gnathostome vertebrates, they were found to be encoded in two distinct genes in the lamprey (Heinig *et al*., 1995; Takahashi *et al*., 1995b). Nevertheless, immunoreactive (ir)-ACTH cells and ir-MSH cells were found in the rostral pars distalis and the pars intermedia, respectively (Nozaki *et al*., 1995). Furthermore, although gonadotropin (GTH) has not yet been isolated from the lamprey pituitary, ir-GTH cells were found in the ventral half of the proximal pars distalis of the sea lamprey pituitary (Nozaki *et al*., 1999a). Thus, the topographic distributions of ir-ACTH, ir-MSH, and ir-GTH cells within the pituitary of the lamprey were very similar to those of teleosts.

Growth hormone (GH), prolactin (PRL) and somatolactin (SL) are members of a pituitary hormone family that are believed to have evolved from an ancestral gene by duplication and subsequent divergence (see Rand-Weaver *et al*., 1993). GHs and PRLs have been identified in most classes of gnathostome vertebrates, while SLs are still confined to Osteichthyes (bony fishes) (Amemiya *et al*., 1999). Since GH/PRL/SL family peptides are found in three divergent lineages of gnathostome fishes, the Actinopterygii (ray-finned fishes), the Dipneustei (lung fishes), and the Chondrichthyes (cartilagenous fishes), their ancestral form(s) must be present in the Agnatha. Thus, although there is no convincing evidence that the lamprey pituitary secretes GH or PRL (Hardisty and Baker, 1982; Sower, 1998), GH and/or PRL may be present in the lamprey.

Furthermore, in gnathostome fishes (i.e., salmon, sturgeon and dogfish) ir-GH cells are always found in the dorsal part of the proximal pars distalis (Nozaki *et al*., 1999b), where most cells remain uncharacterized in the lamprey

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pituitary. Thus, as an analogy to more advanced fishes, if GH cells are present in the lamprey pituitary, they may be found in the dorsal half of the proximal pars distalis. In support of this idea, earlier studies have reported in the sea lamprey that cells in the dorsal half of the proximal pars distalis were stained positively by anti-rat PRL antibody (Wright, 1984), and by one of three anti-substance P antibodies (Nozaki and Gorbman, 1985). Those PRL- and/or substance P-positive cells may correspond to lamprey GH cells.

Based on above-mentioned assumptions, this study was aimed to detect GH- and/or PRL-like immunoreactivity in the pituitary of adult sea lampreys using antibodies to GHs, PRLs and SL of various vertebrate origins.

MATERIALS AND METHODS

Animals

Twenty adult sea lampreys (*Petromyzon marinus*) of both sexes were used. They were collected in May and June 1997 during their upstream spawning migration from the sea, from traps located at the upstream end of a salmon ladder on the Cocheco River in Dover, New Hampshire, U.S.A. The animals were transported to the Fish Hatchery of University of New Hampshire, and were kept in a fresh water flow-through system supplied with ambient local reservoir water, under a natural photoperiod up to one month. They were approximately 65 cm in total length and weighed approximately 900 g at the time of sacrifice.

Tissue preparations

Lampreys were killed by decapitation after anesthesia by immersion in ethyl m-aminobenzonate methane-sulfonate (MS222). After decapitation, the brain and pituitary were rapidly removed and immersed in Bouin-Hollande sublimate solution (Romeis, 1948) for about 24 hr. The fixed tissues were put in 70% ethanol, and were supplied to us by Prof. Stacia A. Sower, University of New Hampshire. The tissues were dehydrated through a series of increasing concentrations of ethanol. Deposited mercuric chloride was removed by treatment with iodine-potassium iodine in 90% ethanol for 1–2 days. Tissues were embedded in Paraplast, and serial sagittal sections of 6 μm were mounted on glass slides which were coated with 0.01% poly-l-lysine solution (Sigma).

Antisera and immunohistochemistry

The primary antisera used for the present study are listed in Table 1, together with sources, lot numbers and working dilutions. In a preliminary study, ordinary immunohistochemical procedures were applied in the sections of the sea lamprey pituitary. However, none of antibodies to GH, PRL or SL gave positive reactions. Accordingly, the staining procedures were modified as follows. After deparaffinization and hydration, sections were autoclaved at 100°C for 5 min, by immersion in 10 mM sodium citrate buffer, pH 6.0 (hydrated autoclaving, Shin *et al*., 1991). Then, ordinary immunohistochemical procedures were performed by use of a Vectastain avidin-biotin peroxidase complex (Elite ABC) kit. In brief, the tissue sections were incubated for 30 min in methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase activities, and washed in PBS. To reduce nonspecific staining, sections were treated with normal goat serum for 30 min. Primary hormonal antisera were applied to the sections for about 12 hr, and the biotinylated secondary antibody solution and ABC reagent were each applied for 1 hr. The final reaction product was visualized with 3,3'-diaminobenzidine tetrahydrochloride in 10 mM Tris-HCl containing 0.003% hydrogen peroxide. The sections were then counterstained with hematoxylin, washed in running water, dehydrated through an increased ethanol series, and mounted in Entellan New (Merck).

To test the specificity of the immunostaining, the following control stains were done: (1) replacement of primary antisera with normal rabbit serum, and (2) preabsorption of the primary antisera with corresponding antigens (chum salmon GH, chum salmon PRL, or blue shark GH; 20 μg/ ml antibody at working dilutions).

RESULTS

Results obtained are summarized in Table 1.

A moderately intense immunoreaction to anti-salmon GH was found in the proximal pars distalis, where most GH-positive cells were concentrated in the dorsal half, with a few GH-positive cells scattered in the ventral half (Figs. 1a and b). Anti-blue shark GH and anti-salmon PRL also gave a

Table 1. Results on immunostaining in the pituitary of adult sea lampreys.

Antibody to	Source	Lot No.	Working dilution	Immunoreac-tivity in PPD
Human GH	NIAMDD		× 2,200	–*
Rat GH	Wakabayashi, K.		× 10,000	–
Human PRL	NIAMDD		× 5,000	–
Rat PRL	Wakabayashi, K.		× 8,000	–
Ovine PRL	Wakabayashi, K.		× 6,000	–
Salmon GH	Kawauchi, H.	AS9-2	× 5,000	+
Salmon GH	Kawauchi, H.	8502	× 3,000	+
Salmon PRL	Kawauchi, H.	Naito	× 7,200	+
Salmon somatolactin	Kawauchi, H.	8906	× 4,000	–
Sturgeon GH	Kawauchi, H.	9201	× 3,000	–
Sturgeon PRL	Kawauchi, H.	9203	× 5,000	–
Dogfish GH	Kawauchi, H.	9601	× 2,000	+

*–, No immunoreaction, +, Moderately intense immunoreaction

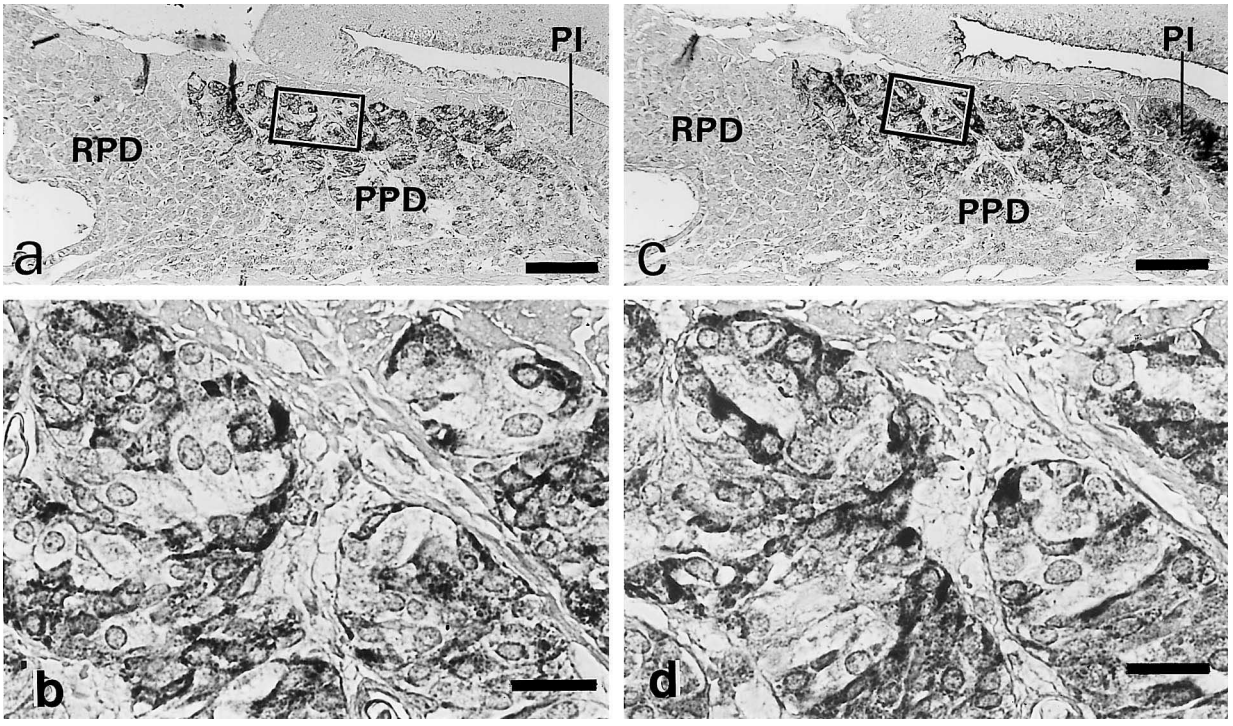

Fig. 1. (a and c) Two successive sagittal sections through the pituitary of an adult sea lamprey, stained with anti-salmon GH (a) and anti-blue shark GH (c), respectively. The areas outlined by rectangles are enlarged and shown in b and d, respectively. PI, Pars intermedia; PPD, proximal pars distalis; RPD, rostral pars distalis. Scale bars: a and c, 100 μm; b and d, 20 μm.

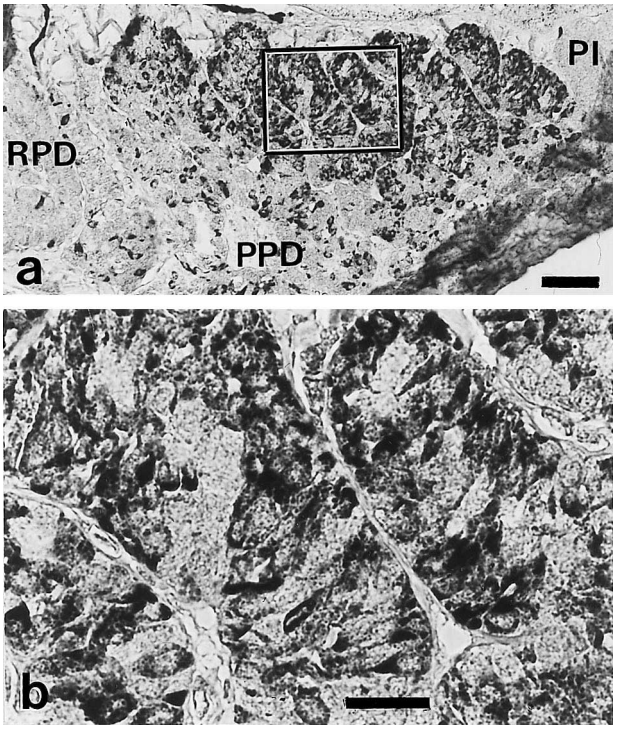

Fig. 2. (a) A sagittal section through the pituitary of an adult sea lamprey, stained with anti-salmon PRL. The area outlined by a rectangle is enlarged and shown in b. PI, Pars intermedia; PPD, proximal pars distalis; RPD, rostral pars distalis. Scale bars: a, 100 μm; b 20 μm.

similar, but slightly weaker immunoreaction in cells in the dorsal half of the proximal pars distalis (Figs. 1c and d, Fig. 2). When two successive sections were stained with one of these antibodies, respectively, it was found that the same cells were stained positively with these antibodies (Figs. 1a–d). A very few solitary cells in the rostral pars distalis also showed positive reactions to those antibodies to GH and PRL. Incidentally, although a weak positive reaction to anti-blue shark GH was also observed in most cells in the pars intermedia (Fig. 1c), such a positive reaction in the pars intermedia cells was not observed after use of other antibodies. No immunoreaction was produced in the lamprey pituitary by the remaining antibodies listed in Table 1, even after hydrated autoclaving of the sections.

DISCUSSION

The present study demonstrated that GH/PRL-like immunoreactivity in most cells are distributed in the dorsal half of the proximal pars distalis of the sea lamprey pituitary. These cells were stained positively by antibodies to salmon GH, salmon PRL and blue shark GH, after prior hydrated autoclaving of the sections. These results suggest that the material immunoreactive to those antibodies is related, to some extent, to GH/PRL, but enhancement of immunoreactivity by use of the hydrated autocrave pretreatment of sections is needed due to low crossreactivity.

Teleost PRLs lack the N-terminal disulfide loop of tetra-

pod PRL, and are more similar to vertebrate GHs than to other PRLs (Rand-Weaver *et al*., 1993). Thus, all antibodies with GH/PRL-like immunoreactivity in the lamprey pituitary were raised against GH or GH-like PRL. Accordingly, GH/PRL-like material in the sea lamprey pituitary seems to be more closely related to GH rather than to PRL or SL, as judged by immunocytochemical properties.

The pituitary gland of lampreys is organized anatomically in a pattern which resembles that of more advanced gnathostome fishes. Moreover, the topographic distributions of ACTH-like, MSH-like, and GTH-like cells within the pituitary of the lamprey are also very similar to those of teleosts (Nozaki *et al*., 1995; Nozaki *et al*., 1999). These facts suggest that other adenohypophysial hormones of the lamprey than those mentioned above, if they exist, may also exhibit topographic distributions similar to those of teleosts. In the present study, GH/PRL-like cells were detected in the dorsal half of the proximal pars distalis of the sea lamprey pituitary, where GH cells are always found in teleosts (Nozaki *et al*., 1990). The topographic similarity of immunolocations between lampreys and teleosts suggests that lamprey GH/PRL-like cells are actually GH cells of the lamprey.

Very recently, a cDNA coding for a protein with four half-cystine residues at positions homologous to those of GHs and teleost PRLs was obtained from the pituitary of sea lampreys (see Sower and Kawauchi, 2001). Using an antiserum raised against a synthetic peptide corresponding to a partial sequence of lamprey GH/PRL, cells immunoreactive to anti-salmon GH, anti-salmon PRL and anti-blue shark GH were also stained positively (data, not shown). Subsequently, a mature protein was isolated from the sea lamprey pituitary, and was identified to be GH by demonstrating an increase of expression of the lamprey IGF gene in the liver (see Sower and Kawauchi, 2001). The lamprey GH shows approximately 22% sequence identity to GHs of gnathostomes, 16–20% identity with SLs and 15–17% with PRLs (see Sower and Kawauchi, 2001).

The hydrated autoclave heating of tissues is commonly used to enhance immunohistochemical reactions for certain epitopes (Shin *et al*., 1991; Igarashi *et al*., 1994). In the present study, although none of antibodies give positive reactions in ordinary immunohistochemical procedures, the hydrated autoclave pretreatment of sections was successful to visualize GH/PRL-like immunoreactivity in cells distributed in the proximal pars distalis of the sea lamprey pituitary. In histological tissue fixation, the potential antigenic sites of antigens may be masked by the aldehyde group of formaldehyde that cross-links to amine groups in the polypeptide backbone and amino acid side chains. This may result in a decrease or loss of antigenicity. Since heating of tissues leads the denaturation and fragmentation of tissue protein, the hydrated autoclave heating may act to disconnect the cross-linkages between antigenic sites and the aldehyde groups of formaldehyde. Through this kind of mechanism, the hydrated autoclave heating probably retrieves antigenic sites damaged by the fixation.

In the present study, GH/PRL-like immunoreactivity was found in a few cells in the rostral pars distalis as well as in most cells in the proximal pars distalis. Since such GH/PRL-like cells in the rostral pars distalis exhibited the same responses to antibodies as those in the proximal pars distalis, they appeared to be a part of GH/PRL-like cells. Incidentally, GH/PRL-like cells found in the present study appeared to correspond to anti-rat PRL-positive cells reported by Wright (1984) and to anti-substance P-positive cells reported by Nozaki and Gorbman (1985) in the same species. Although Wright (1984) also reported anti-rat GH-positive cells the proximal pars distalis of the sea lamprey pituitary, those rat GH-positive cells appeared to correspond to ACTH cells in the proximal pars distalis (Nozaki *et al*., 1995).

In conclusion, the present study has demonstrated GH/PRL-like cells in the dorsal half of the proximal pars distalis of the sea lamprey pituitary. These cells were stained positively by antibodies to salmon GH, salmon PRL and blue shark GH, after hydrated autoclaving of the sections. The similarity of the topographic cellular distributions within the pituitary between lampreys and teleosts suggests that lamprey GH/PRL-like cells are the GH cells of the lamprey.

ACKNOWLEDGEMENTS

We are grateful to Professor Stacia A. Sower, University of New Hampshire, USA, who supplied the fixed lamprey pituitaries used in the present study. We are also grateful to Professors Katsumi Wakabayashi, Hormone Assay Center, Institute for Molecular and Cellular Regulation, Gunma University, and Hiroshi Kawauchi, Laboratory of Molecular Endocrinology, School of Fisheries Sciences, Kitasato University, for providing antisera and antigens used in the present study. In addition, we would like to express our appreciation to Professor A. Gorbman for his kindness in editing the manuscript.

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(Received February 10, 2002 / Accepted July 7, 2002)