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# Diffusion between the Neurohypophysis and the Adenohypophysis of Lampreys, *Petromyzon marinus*

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Horseradish peroxidase (HRP), a protein that can be visualized by appropriate histochemical procedures, was injected into the third ventricle of the brain of adult lampreys, *Petromyzon marinus*. Within 5 to 15 min HRP had passed through the neurohypophysis, which forms the floor of the third ventricle. It appeared at this time to have diffused throughout the connective tissue separating the adenohypophysial follicles from the neurohypophysis and from each other. This observation would indicate that it is possible for neurosecretory peptides like gonadotropin-releasing hormone to diffuse from the brain (neurohypophysis) to the adenohypophysis and thus regulate its secretory activity in lampreys. 

Of all vertebrates, only the agnatha and the teleosts lack a portal vascular system (median eminence) for transferring regulatory peptides from the brain to the adenohypophysis. The adaptive importance of such a portal system is that it makes possible central nervous regulation of such vital processes as reproduction by external (and internal) cycling environmental conditions. The teleosts have solved this structural problem by direct innervation of the pars distalis by appropriate neurosecretory neurons from the adjacent hypothalamus (Peter, 1990). The agnathans, however, have no nervous or vascular communication between the brain and neurohypophysis (Tsuneki and Gorbman, 1975). This has led to speculation that nervous regulation of the agnathan pars distalis is by diffusion of brain peptides from the adjacent neurohypophysis, across the thin connective tissue layer that separates the neural from the glandular tissues.

Proof that diffusion is an adequate basis for brain regulation of the pars distalis has rested on such experiments as those of Nozaki *et al.* (1975) and Tsukahara *et al.* (1986). They injected substances of varying molecular size into the third ventricles of hagfish. By use of staining procedures that revealed the positions of these substances a few minutes after injection, they showed that significant amounts of test materials diffused rapidly from the third ventricle, through the neurohypophysis, to the pars distalis. In several ways, however, it was felt that experiments with hagfish might not represent the diffusion hypothesis fairly. There is some question as to whether hagfish species have an environmentally regulated reproductive cycle (Gorbman and Dickhoff, 1978). Indeed, it has not been established whether the hagfish pituitary contains tropic hormones of any kind (Matty *et al.*, 1976).

Lampreys, on the other hand, are clearly seasonal and temperature responsive in the timing of their anadromous migrations and

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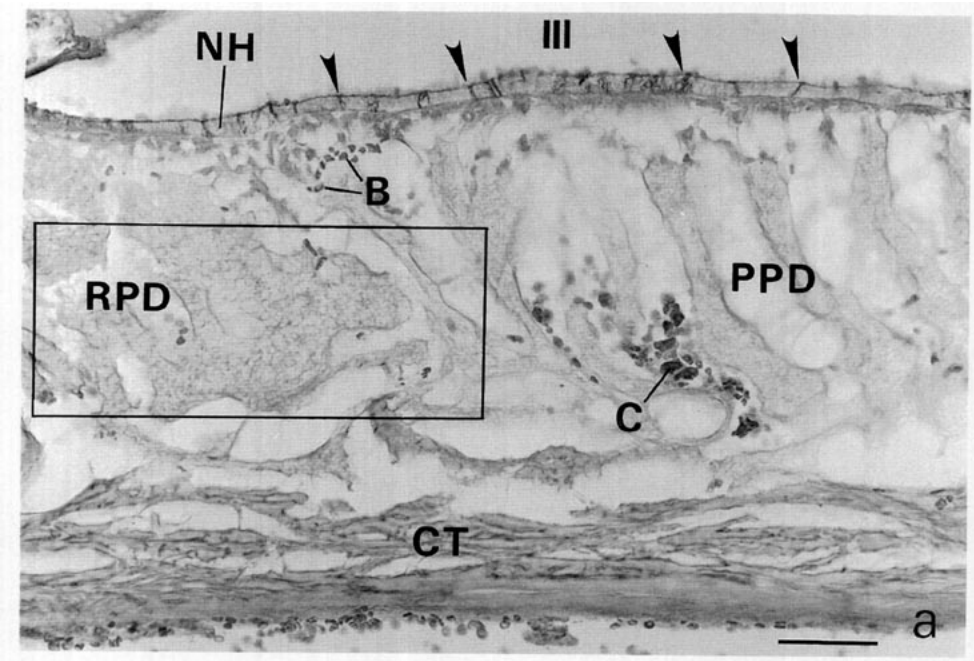
mating and breeding (Hardisty, 1979). In addition, there is anatomical evidence to support the concept of hypothalamic control of adenohypophysial function by diffusion of the neurohormones from the neurohypophysis to the pars distalis of the adenohypophysis (Nozaki *et al.*, 1984a; King *et al.*, 1988; Tsuneki, 1988). In the lamprey, GnRH-like neurons identified by immunocytochemistry project their fibers primarily into the neurohypophysis from the preoptic region (Nozaki and Kobayashi, 1979; Crim *et al.*, 1979; Nozaki *et al.*, 1984b; King *et al.*, 1988). In addition, Crim (1981) and King *et al.* (1988) using a lamprey GnRH-I antibody showed that GnRH neurons project into the third ventricle. These authors proposed an additional route of GnRH via secretion into the third ventricle and transport by tanycytes to the adenohypophysis. If indeed, as the data indicate, GnRH does diffuse across the connective tissue to the adenohypophysis, then control by the hypothalamus is probable. Accordingly, we felt it important to confirm in the adult lamprey that reasonably rapid diffusion can occur between the neuro- and adenohypophysis.

## MATERIALS AND METHODS

Twenty-one female adult sea lampreys approximately 65 cm in total length (*Petromyzon marinus*) were collected in May and June 1993, during their upstream spawning migration from the sea, in traps located at the upstream end of a salmon ladder on the Cocheco River in Dover, New Hampshire. They were transported to the Anadromous Fish and Aquatic Research Laboratory at the University of New Hampshire and were maintained in a freshwater flow-through system until used. The lampreys averaged 900 g in body weight.

Each lamprey was anesthetized by immersion in 1 g/liter ethyl *m*-aminobenzoate methane sulfonate (MS222) for 15 min prior to surgery in ice-cold water supplied with aeration. During the procedure, each lamprey was wrapped in a wet cloth surrounded by pieces of ice. The head was immobilized by use of pinioning screws bilaterally placed in the gill slits. The skin and dorsal fibrocranium, at a level just caudal to the pineal organ, were removed carefully, and the dorsal surface of the brain was exposed. For intraventricular injection of horseradish peroxidase (HRP), a glass microcapillary with an outer diameter of 50–100 μm, connected to a microsyringe by a polyethylene tube, was mounted on a micromanipulator. The tip under direct visual guidance under a binocular microscope was inserted in the median plane into the third ventricle at a level of the rostral end of the choroid plexus, where the central part of the neurohypophysis is located at the same vertical and transverse planes. The tip was lowered 4.5 μm from the dorsal surface of the brain to a position just above the neurohypophysis. Eighteen animals each received a single intraventricular injection of 5 to 10 μl of HRP (10 μg/μl; Sigma, Type VI-A) dissolved in 0.6% NaCl. Three animals, which were used as controls, each received 5 μl of 0.6% NaCl. Five to 15 min after the injection the animal was killed by decapitation. The brain and the attached pituitary were immediately dissected *en bloc* and immersed in ice-cold modified Karnovsky's fixative (4% glutaraldehyde and 4% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.4) for 8–12 hr. After fixation, the tissues were immersed in cold 30% sucrose in 0.1 *M* phosphate buffer (pH 7.4) for 6–24 hr. The tissues were cut sagitally at 30–40 μm thickness on a cryostat at −16°. Sections were mounted on glass slides, pretreated with gelatin–potassium dichromate solution, and were washed in 0.05 *M* Tris buffer (pH 7.6) for 10 min. Then, sections were incubated in a

FIG. 1. (a) Sagittal sections of the pituitary of adult lampreys, which were treated with intraventricular injection of HRP (a) and saline (c). In a, darkly stained elements shown are the tanycytes (large arrowheads) in the neurohypophysial wall (NH) and blood cells (B) in the blood capillaries (C) between follicular cell groups. The area outlined by a rectangle is enlarged and shown in b, where the penetration of the intraventricularly injected HRP is noted between the cells, outlining them (small arrowheads). Blood cells stain artifactually because of their high content of peroxidase (see c). In a, note also the relative density of the connective tissue (CT) below the adenohypophysis which presumably can impede further diffusion beyond the adenohypophysis. Hence, there is an accumulation of HRP immunoreaction in the dense connective tissue, in contrast to the clearing of HRP from the loose connective tissue between glandular follicles. In c, notice that only blood cells (arrows) are stained darkly. RPD, rostral pars distalis; PPD, proximal pars distalis; III, third (diencephalic) ventricle into which HRP was injected. Scale bars: (a) 100, (b) 50, (c) 100 μm.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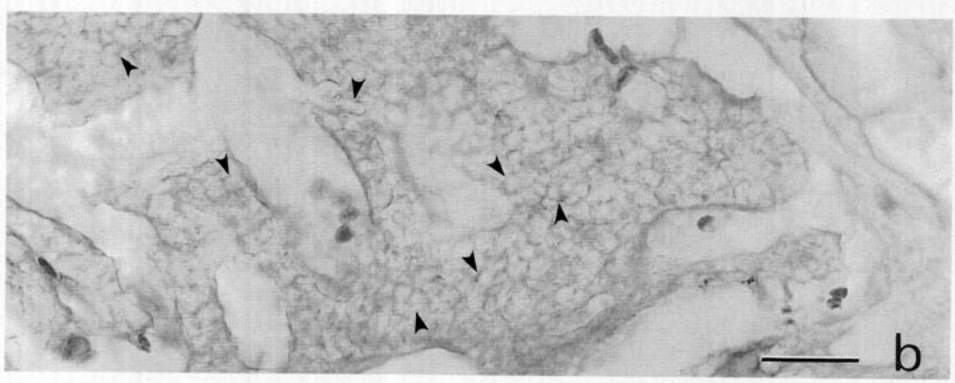
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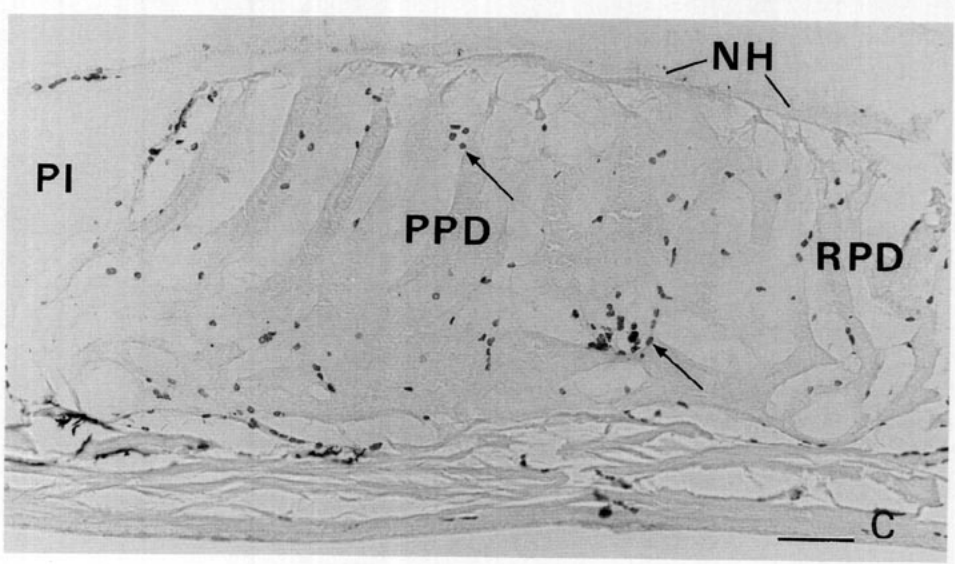
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solution consisting of 10 mg of 3,3'-diaminobenzidine tetrahydrochloride, 0.1 ml of 3% hydrogen peroxide, and 100 ml of 0.05 *M* Tris buffer (pH 7.6) for about 20 min at room temperature. Sections were then washed in distilled water, dehydrated in a graded ethanol series, cleared in Histo-clear, and mounted on slides with Permount for light microscopy.

## RESULTS AND DISCUSSION

Results of the injections are summarized in Fig. 1. Peroxidase reaction product could be found in the thin neurohypophysial wall and in the pars distalis even as early as 5 min after the injection into the third ventricle. The peroxidase reaction was localized in the thin intercellular spaces between the cells of the pars distalis, but not within the cells (Fig. 1b). Tanycytes in the neurohypophysis were intensely labeled (Fig. 1a). There was little labeling of material in the third ventricle, most of the free peroxidase presumably having been washed out during the processing of the tissue. In control animals, there was a peroxidase reaction only in erythrocytes (Fig. 1c). Therefore, the peroxidase reaction in the tanycytes and intercellular spaces is due to exogenous horseradish peroxidase.

It is clear, as shown in Fig. 1a, that the peroxidase injected into the third ventricle of the brain, within a period of time as brief as 5 min, had penetrated the neurohypophysis and diffused in the connective tissue trabeculae a distance equal to the entire thickness of the adenohypophysis. As might be expected, a protein as large as the HRP molecule did not cross the pituitary cell membranes, but remained in the intercellular spaces on the cell surfaces.

Within the neurohypophysis itself, the HRP reaction product was most intense at the ventricular ends of the neurohypophysial cells and in the tanycytes. The tanycytes, which extend from the ventricular surface of the neurohypophysis to its outer edge, facing the adenohypophysis, are so strongly labeled that it is reasonable to conclude that they may have actively removed the HRP from the third ventricle, accumulated it in high concentration, and possibly secreted it peripherally toward the adenohypophysis.

A similar phenomenon was observed by Nozaki *et al.* (1975) and Tsukahara *et al.* (1986), who injected HRP as well as trypan blue, a colloidal dye, into the third ventricle of the hagfish. There, also, the tanycytes became laden with HRP and deposits of the colloidal dye particles were seen in the connective tissue below the tanycytes. Accordingly, it appeared probable to these authors that the tanycytes had transported these substances across the width of the neurohypophysis. Being particulate, the trypan blue did not readily diffuse further, but remained just below the neurohypophysis.

In parallel with the above-mentioned findings in cyclostomes, previous studies in rats and Japanese quail have reported that the tanycyte of the median eminence transports intraventricularly injected HRP and GnRH to the portal vessels (Kobayashi *et al.*, 1972; Kobayashi, 1975; Knigge *et al.*, 1976). Thus it has been suggested that the tanycytes participate in the hypothalamic control over the adenohypophysial function (Kobayashi, 1975; Knigge *et al.*, 1976). However, the destruction of all tanycytes of the median eminence by use of electric cautery or intraventricular injection of saturated picric acid solution did not disturb LH secretion or estrous cycles in the rat or photostimulated testicular growth in the Japanese quail (Uemura and Kobayashi, 1977; Nozaki *et al.*, 1980). Nozaki *et al.* further observed electron microscopically that the granular axons in the palisade layer of the median eminence remain intact even after the electric cauterization of the median eminence tanycytes. Based on these observations, Nozaki *et al.* concluded that the tanycytes are not directly involved in the hypothalamic regulation of hypophysial functions of the studied vertebrates. Thus, although it is an intriguing possibility, the

involvement of the tanycytes in the hypothalamic regulation of hypophysial function is an unsettled issue in the higher vertebrates. At this time we can say little concerning the significance of absorption of HRP by tanycytes in cyclostomes.

In the present experiment in lampreys, it is not clear how the HRP moved from the third ventricle across the neurohypophysis, whether by simple diffusion, by transport through the tanycytes, or both. However, the presence of HRP reaction product throughout the adenohypophysis shows that even in the absence of a vascular portal system, a relatively efficient transport of a large peptide can take place for possible regulation of hormones of the pars distalis of Agnatha.

It should be remembered that the pituitary structure we see in the modern Agnatha has been preceded by about 500 million years of evolution from the primitive stem vertebrates. Present structure of the lamprey pars distalis is approximately what can be expected to evolve in adaptation for relatively efficient diffusional control. The pars distalis is thin, mostly less than thirty cells deep, so that no cell is too distant from the neurohypophysis. The pars distalis is not a continuous layer of cells, but is broken up into follicles and cords, making it possible for diffusion to occur in the channels or trabeculae between them (Figs. 1 and 2). The connective tissue and sinusoids that separate the adenohypophysial follicles are of a loose spongy type, in contrast to the dense collagenous connective tissue that lies below. In fact, the density of the subadenohypophysial connective tissue would tend to block, or at least to slow,

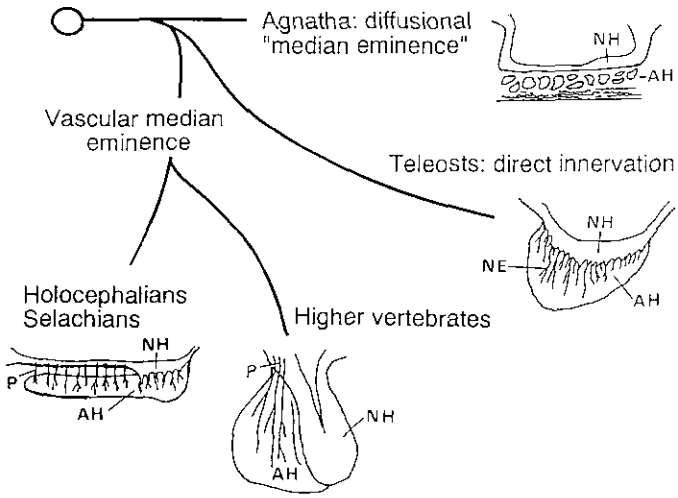


FIG. 2. Vertebrate brain–pituitary relations. Diagrammatic representation of the three types of relationships between the brain (hypothalamus) and the adenohypophysis in vertebrates. In agnathans, the thin neurohypophysis is coextensive with a thin layer of epithelial glandular cells, making diffusion between these two structures a relatively efficient means for neurosecretory regulation of the adenohypophysis. In teleosts the many neurohypophysial axonal extensions penetrate the adenohypophysis and either directly innervate synaptically the adenohypophysial cells or end near these cells for diffusional (paracrine) action of neurosecretions. In lower vertebrates (represented at the lower left) an intermediate form of vascular transport via many short vessels occurs between a thin flat area of the neurohypophysis and the coextensive relatively thin adenohypophysis. The most highly evolved median eminence is that of other vertebrates (amphibians, reptiles, birds, and mammals) usually with larger pituitaries, in which portal vessels collect neurohypophysial blood and conduct it to and within the adenohypophysis. AH, Adenohypophysis; NH, neurohypophysis; P, portal blood vessel from vascular median eminence.

further diffusion beyond the adenohypophysis.

For reasons such as we mention here, we can say that the structural features of the lamprey (and hagfish) pituitary and surrounding tissues appear to represent adaptive evolution in this group to make diffusion as efficient as possible for pituitary regulation by brain peptides. The evolution of the agnathan "diffusional median eminence" may be considered as characteristic of this group as is the anatomical system of the hypothalamic neural penetration of the pars distalis of teleosts.

We can conclude that in the evolutionary sense there have been three types of regulation of the adenohypophysis developed in the vertebrates: the agnathan diffusional type, the teleostean direct innervational type, and the vascular type seen in all other vertebrates (Fig. 2). Whether we should speak of the agnathan diffusional type as "primitive" is difficult to say. Perhaps the principal advantage of the vascular median eminence type of control of the pars distalis by the brain is that it permitted development of larger and thicker glands as vertebrates became larger and more complicated in form and the distance between the hypothalamus and pituitary increased significantly.

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