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Immunohistochemical Demonstration of Urotensins I and II in the Caudal Neurosecretory System of the Japanese Charr, *Salvelinus leucomaenis*, Retained in Sea Water

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Abstract This paper is concerned with part of the role and function of the caudal neurosecretory system of the charr, *Salvelinus leucomaenis*, studied by immunohistochemistry. In order to elucidate the different histologic changes, we examined the immunoreactivities of urotenisn I (UI) and urotensin II (UII) in 3 experimental groups: the feral (river) fish, the fresh-water aquarium-, and sea water aquarium-retained fish. Coexistence of UI and UII was demonstrated in most of the smaller and larger neurons distributed in and near the urophyseal system of all 3 groups. However, some of the larger neurons were immunoreactive only to a single hormone, UI or UII. Merely a few neurons indicated no reactivity for either UI or UII. No such clearcut differences were encountered immunohistochemically in the 3 groups. Neuronal and urophysial immunoreactivity to UI of feral and fresh-water-retained fish was slightly stronger than that of sea water-retained fish. Moreover, in sea water-retained fish, the intensity of immunoreactivity for UI was variable, and the number of neurons positive for UII only was somewhat larger than that in feral and fresh-water-retained fish. A series of UII-positive cerebrospinal fluid (CSF)-contacting neurons were seen in the ependymal and subependymal layers ventral to the central canal of the spinal cord in every group. These CSF-contacting neurons might constitute another neurosecretory system aside from the ordinary caudal neurosecretory system equipped with urophysis. In contrast to the hypothalamohypophysial neurosecretory system, the caudal neurosecretory system did not show any significant changes among the 3 groups. This suggests that urotensins I and II have no essential role in osmoregulation of the charr.

As was discussed in a previous paper (Oka et al., in press), the caudal neurosecretory system of fishes has not been shown conclusively to have any definitive function, though its roles in osmoregulatory, electrolyte, and vasopressor activities has been suggested (Bern, 1985; Kobayashi et al., 1986). Using a Japanese loach, Enami (1956), a pioneer researcher of this system, reported that the neurons of this system responded to osmotic manipulations. A possible correlation between secretory activity and cell diameter has been described ultrastructurally in the brook trout (charr) caudal neurosecretory system exposed to osmotic manipulations (Chevalier, 1976, 1978). After that, Sacks and Chevalier (1984) described morphometrically significant changes in the neurons of the brook charr exposed to various concentrations of saltwater and fresh-water media selectively enriched in one ion. By the detection of urotensins I and II, several workers demonstrated a possible functional significance of the urophysis. Namely, UI, as a homologue of mammalian corticotropin releasing factor (CRF), may exert an osmoregulatory function by increased cortisol secretion through its effect on the adenohypophysis (Fryer and Lederis, 1985; Lederis et al., 1985); and UII, as a partial homologue of somatostatin, may cause vasoconstriction and inhibition of prolactin secretion (Chan, 1975; Grau et al., 1982; Rivas et al., 1987). By an improved in vitro technique of urotensin binding, Woo et al. (1985) obtained data showing that the total amount of UII binding to membranes prepared from the gill and kidney of goldfish adapted to either fresh water or dilute sea water was higher than that of UI binding. In order to make comparison of different species of fishes, Owada et al. (1985) examined the immunoreactivities of UI and UII in 6 species of fresh-water teleosts and 6 species of saltwater teleosts, and found that the staining reaction in the urophysis was weaker in fresh-water fishes than in

saltwater ones.

However, no immunohistochemical investigation has been carried out on the caudal neurosecretory system of fish exposed to osmotic manipulations. Therefore, this study was designed to elucidate the changes in this system of the Japanese charr in relation to different environmental conditions.

Materials and methods

Specimens of the Japanese charr, *Salvelinus leucomaenis* (Pallas), were obtained by angling in several rivers in the vicinity of the Sado Marine Biological Station, Niigata University. The collection of specimens extended from June 1987 to February 1988. About 50 specimens, 146.7–211.4 mm in length and 42.0–73.0 g in body weight, of both sexes were first kept in a freshwater outdoor aquarium (tank). Half of them were then transferred into dilute sea water for acclimation. After 2 to 3 weeks, they were kept in normal sea water. As controls for aquarium-reared fish, some feral ones fixed at the spot were used. The aquarium-reared fish were sacrificed 3, 7, 11 and 13 days after acclimation. They were anesthetized with 0.1% aminobenzoate-methansulfonate (MS 222). After each tail was cut at a position about 6–7 segments of caudal vertebra from its tip, the caudal spinal cord was removed and immersed in Bouin's fixative. Serial sections cut at 6–9 μm thickness in cross and sagittal directions were made following routine paraffin embedding procedure.

Each correlative section was put on a slide glass coated with dilute gelatin. Primary rabbit antiserum raised against a synthetic *Catostomus* UI (Sues and Lederis, 1981; Sues et al., 1986) and primary UII antiserum against synthetic *Gillichthys* UII (Yulis and Lederis, 1986) were used. The specificity of both immunoreactions was checked by absorption tests after Sues and Lederis (1981), Sues et al. (1986), and Yulis and Lederis (1986).

Immunohistochemical staining was conducted by the peroxidase-antiperoxidase (PAP) method of Sternberger (1986). In addition, to discriminate the localization and distribution of caudal neurosecretory cells of charr, we carried out routine staining with azan trichrome and a combination of aldehyde fuchsin (AF)-azan.

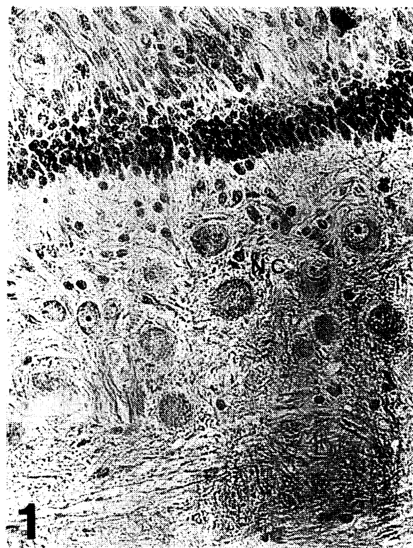

Fig. 1. Caudal neurosecretory cells (NC) of the Japanese charr, *Salvelinus leucomaenis*. Using azan trichrome, the cytoplasm is stained with the acidic dye azocarmine. ×240.

Results

As was described by Honma and Tamura (1967), the urophysis of the Japanese charr is an elongate, somewhat bow-shaped swelling of the ventral spinal cord and extends between the second- and the third-to-last vertebrae. The caudal neurosecretory cell, either monopolar and bipolar, is large, the perikaryon of which is 7–40 μm in diameter. However, the cells (neurons) located near the urophysis are relatively smaller than the others found more anteriorly. The nucleus, long ovoid to round in shape, is 5–13 μm in diameter. The cytoplasm is stained with acidic dyes (Fig. 1). However, no detectable difference was recognized between the sea water- and freshwater-reared fish. In addition, no clearcut difference was detected among the fish sacrificed at different intervals after acclimation.

Sea water-reared fish. Cells immunoreactive to UI antiserum were multimorphic, variable in size, and very variable in staining intensity, although every urophysis was stained with UI antiserum. On the contrary, every UII-reactive cell showed strong staining intensity without marked variation. Accordingly, more cells and their axons were stained with UII antiserum than with UI antiserum (Figs. 2, 3).

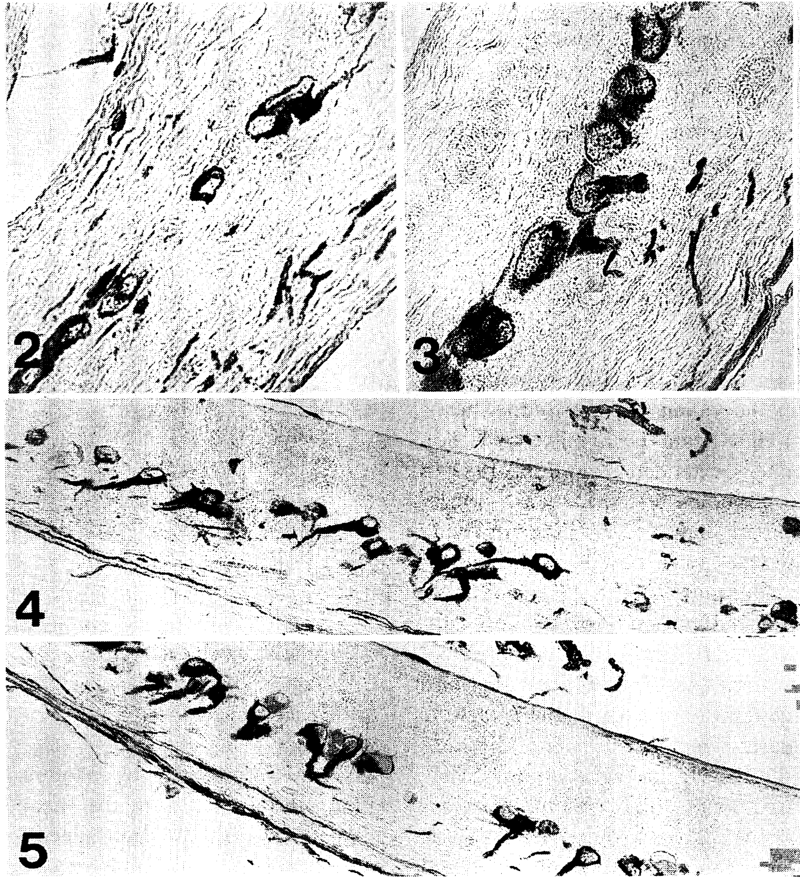

Fig. 2. UI-immunoreactive NC distributed at a far distance from the neurohemal organ (=urophysis) of a specimen reared in sea water. ×240.

Fig. 3. Correlative section adjacent to that in Fig. 2 to show the UI-immunoreactive NC. Note that the number of UII-immunoreactive NC is larger than that of UI-immunoreactive NC. ×240.

Fig. 4. UI-immunoreactive NC of a specimen reared in fresh water. ×120.

Fig. 5. Correlative section adjacent to that in Fig. 4 to show the UII-immunoreactive NC. Note that many cells reacted with both anti-UI and anti-UII sera. ×120.

Correlative sections alternatively stained with UI and UII antisera showed that the number of cells that reacted with both antisera and the number reacting only with UII antiserum were nearly the same. However, the cells immunoreactive to UI antiserum only were very scarce.

Fresh-water-reared fish. Urophyses, cells, and their axons of almost all individuals reacted strongly with UI antiserum. Similarly, the urophyses and cells of almost all individuals showed strong immunoreactivity with UII antiserum. However, the number of UII-immunoreactive cells was smaller than that of UI-immunoreactive cells.

Correlative sections alternatively stained with UI and UII antisera showed that numerous cells located in the distal portion of the urophysis reacted with both antisera (Figs. 4, 5). Nevertheless, the staining intensity of UII-immunoreactive smaller cells in the vicinity of the urophysis was stronger than that of UI-immunoreactive cells. Cells positive for UII only were very scarce, fewer in number than the cells positive for UI only. There were a few cells that did not react with either antiserum (Figs. 6, 7).

Feral fish. Cells strongly immunoreactive to UI antiserum were seen in the entire urophysial system, including both proximal smaller cells and distal larger cells. The same pattern was found

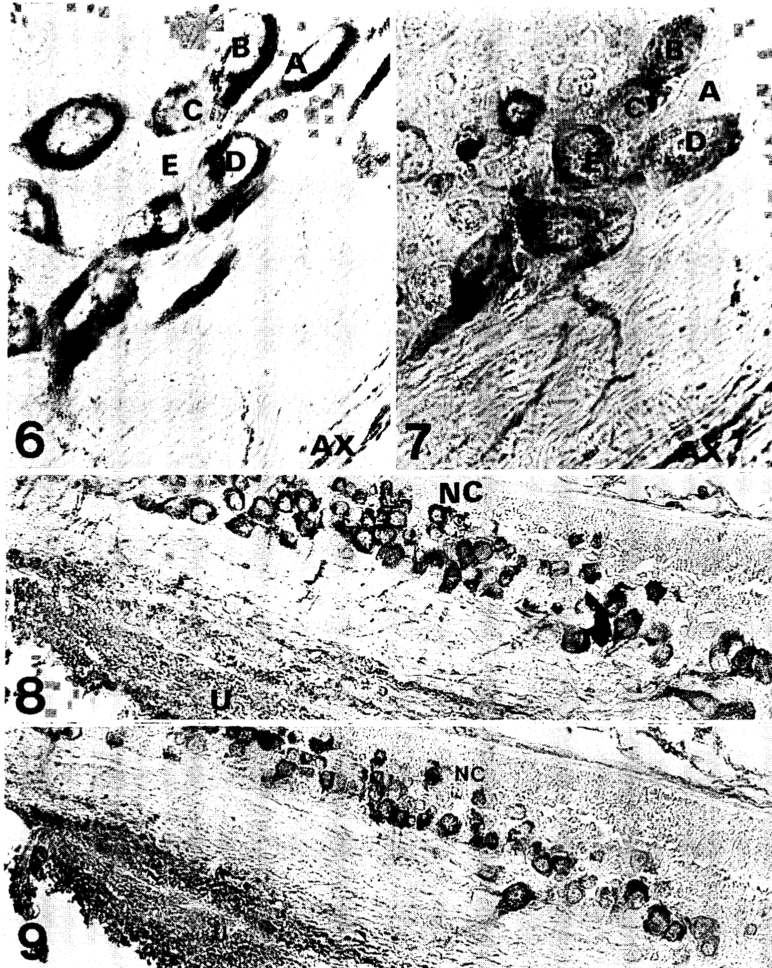

Fig. 6. UI-immunoreactive NC distributed near the urophysis of a specimen reared in fresh water. AX, immunoreactive axon. ×480.

Fig. 7. Correlative section adjacent to that in Fig. 6 to show the UII-immunoreactive NC. Note that the reactivity is weaker than that for UI and that many cells are reactive with both anti-UI and anti-UII sera. Letters A through E indicate the same cells seen in Fig. 6. ×480.

Fig. 8. UI-immunoreactive NC distributed near the urophysis of a feral specimen. U, urophysis. ×240.

Fig. 9. Correlative section adjacent to that in Fig. 8 to show the UII-immunoreactive NC. Note that many cells are reactive with both anti-UI and anti-UII sera. ×240.

for the cells immunoreactive to UII antiserum.

Observation of correlative sections alternatively stained with UI and UII antisera demonstrated that almost all the cells reacted with both antisera, although the degree of intensity was variable. Merely a few cells reacted with either UI or UII antiserum (Figs. 8, 9).

Remarkably, the neurosecretory cells, which seem to be equivalent to cerebrospinal fluid (CSF)-contacting neurons, were demonstrated to be immunoreactive exclusively with UII antiserum (Fig. 10). The bulbous processes (dendrites) that projected into the central canal, and their cell bodies were situated in the ependymal and subependymal layers ventral to the central canal. The axonal beaded fibers directed toward the ventral side of the spinal cord were seen. These UII-immunoreactive CSF-contacting neurons were distributed throughout the entire spinal cord and constituted a neurosecretory system other than the uro-

Fig. 10. Cerebrospinal fluid-contacting neurons indicating only UII immunoreactivity. They are arranged in the ependymal and subependymal layers ventral to the central canal. Some bulbous processes (dendrites) projecting into the central canal are seen. ×480.

physial one.

Anyway, we noticed that in the sea water-retained fish the intensity of immunoreactivity for UI was variable and that the number of UII-immunoreactive cells was relatively larger than that in the fresh-water-retained and in the feral fish.

Discussion

Although a considerable number of papers have been published on the primary function of the caudal neurosecretory system of fishes, the definite role of this system was heretofore not known (Bern, 1985; Kobayashi et al., 1986). Using several species of both fresh-water and saltwater teleosts, Owada et al. (1985) demonstrated that the immunoreaction of the urophysis with urotensins was stronger in saltwater fishes than in fresh-water fishes.

By morphometry and bioassays of isolated rectum, Chevalier et al. (1986) demonstrated that the brook trout (charr) from acidified lakes showed markedly larger cell and nuclear sizes and a lower content of urotensin II than the charr from nonacidified lakes. This morphometric evidence has been correlated positively with ultrastructural and radioautographic indication of heightened cell synthetic activity in the cases of fresh-water- and sea water-adapted brook charr (Chevalier, 1976, 1978). Audet and Chaevalier (1981) also demonstrated that the monoaminergic nerve fibers of the urophysial system of the brook charr showed a significant decrease when the fish were transferred from demineralized water to sea water.

Examination of the present immunohistochemistry in the Japanese charr is somewhat positively consonant with the results mentioned above. However, we found that the neurosecretory cells showed a considerably variable immunoreactivity for either UI or UII. Renda et al. (1982) also observed a considerable individual (specimen's) variability in the amount of sauvagine-like immunoreactive material recognizable in the urophysial system of the fresh-water cyprinid, *Tinca tinca*. Therefore, no significant difference appears to exist between the immunoreactive neurons of fresh-water- and sea water-adapted charr in the present examination. If so, at least the caudal neurosecretory system of the Japanese charr might not be involved in the osmoregulation of fish adapted to an environment of different salinity. Further detailed study is needed to clarify this problem.

Lederis et al. (1985) proposed that UI is involved in promoting ion extrusion and water conservation in fishes living in a hyperosmotic environment. Moreover, they reported that UI-immunoreactive cells were demonstrated in the brain of *Catostomus*. In the same experimental series of the present study, one of our colleagues who wanted to examine the changes in the hypothalamo-hypophysial system of the charr found that somatostatin immunoreactivity revealed different patterns in the preoptic nucleus cells, prolactin cells, and somatotropin-producing cells (unpublished data). In this connection, because

a partial structural homology between UII and somatostatin peptides has been reported by several investigators (Pearson et al., 1980; Ichikawa et al., 1983; McMaster and Lederis, 1983), it is necessary to determine the mutual interrelationship among these cells and peptides, although the present study did not confirm the changes of UI-immunoreactive cells located in the brain.

UII-immunoreactive CSF-contacting neurons were also demonstrated along the entire length of the spinal cord and medulla of the charr. With the exception of the ratfish (*Hydrolagus*), which lacks a distinct caudal neurosecretory system (Bern and Takasugi, 1962; Onstott and Elde, 1986), and the catfish (*Ictalurus*) (Larson et al., 1987), CSF-contacting peptidergic systems have been reported not only in fish but also in mammals (Yulis and Lederis, 1986, 1988; Burnweit and Forssmann, 1979; Schroeder, 1984; Oka et al., in press). It is not yet determined whether or not the function and structural pattern of these systems are different in every different animal group.

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海水飼育したイワナの尾部神経分泌系におけるウロテンシン I および II の免疫組織化学的検出

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イワナの尾部神経分泌系の役割ないし機能の一端を明らかにするために，川で釣獲した個体を淡水槽と海水槽に飼育して，ウロテンシン I (UI) およびウロテンシン II (UII) の免疫活性を比較検討した．これらの対照として，野外で得た個体も使用した．3 群間ではことさら取り立てるほどの差異が認められなかったが，いずれの個体でも大半の小型ニューロンに UI と UII が共存することが確かめられた．一方，大型ニューロンの一部は UI か UII のどちらかの抗体のみに反応した．また，どちらにも反応しないニューロンは，ごく少数であった．一般に，野外ならびに淡水槽で飼育した個体は，海水槽の個体よりも UI に対する免疫活性が強かったが，最後者では UII 陽性ニューロンの数が他二者より多い傾向がみられた．なお，脊髄中心管の腹方に，UII のみに反応する髄液接触ニューロン系がみられ，視床下部下垂体神経分泌系や，尾部神経分泌系とは別の神経分泌系を形成していた．以上の結果は，イワナの浸透圧調整に，ウロテンシンというホルモンが，あまり重要な役割を果していないらしいことを示唆している．

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