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Sequential Changes in Urotensin Immunoreactivity Patterns in the Trout, *Oncorhynchus mykiss*, Caudal Neurosecretory System in Response to Seawater Challenge

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ABSTRACT—We have been investigating the possible relationship between the teleost caudal neurosecretory system and osmoregulation, by comparing immunostaining intensities of the caudal neuropeptides, urotensins I (UI) and II (UII), in fish sequentially following transfer to different water salinities. Freshwater trout (*Oncorhynchus mykiss*) were transferred from fresh water (FW) to new FW and from FW to 100% seawater (SW). After 2, 10 and 48 hr posterior spinal cords were removed, fixed and double sequentially immunostained. The 2 hr SW urophyses exhibited more UII and less UI intensity than FW ones. Perikarya anterior to the SW urophyses had less UII and more UI intensity. The 10 hr SW spinal cords showed lower intensity of UI and UII in urophyses and higher intensity of both in anterior perikarya than FW spinal cords. The 48 hr spinal cords did not show any difference in intensity for either UI or UII. We conclude that UI and UII are differentially regulated, that urophysial UI release and perikaryal synthesis are stimulated 2 and 10 hr following transfer to seawater, and that there is an initial inhibition followed within 10 hr by a stimulation of urophysial UII release and perikaryal synthesis following transfer to seawater. By 48 hr the caudal neurosecretory response to SW challenge appears to have subsided, and we hypothesize that the caudal system's role in osmoregulation may be only acute (i.e. within 48 hr following a challenge).

INTRODUCTION

In addition to the better known hypothalamo-hypophyseal system, fishes possess a second unique neuroendocrine structure at the caudal end of the spinal cord called the caudal neurosecretory system, which was first proposed by Enami (1955). In this system axons from neurosecretory neurons (Dahlgren cells) (Dahlgren, 1914; Speidel, 1919) terminate in apposition to the capillaries of a neurohemal area, the urophysis. The synthetic products of the caudal-spinal neurons are presumed to be packaged in vesicles and transported by axoplasmic flow for storage in, and release into the circulation from, the urophysis (Bern and Lederis, 1969; Lederis, 1984; Ichikawa *et al.*, 1986). There is no evidence for a caudal neurosecretory system or its hormonal products, the urotensins, as such in any higher vertebrate species. Despite considerable investigations, the precise physiological role of this neurosecretory system remains elusive. There is much evidence suggesting its involvement in ion and/or osmoregulatory functions (Maetz *et al.*, 1964; Yagi and Bern, 1965; Fridberg *et al.*, 1966; Bern and Lederis, 1969; Lederis *et al.*, 1971; Lacanilao and Bern, 1972; Chan, 1975; Chan and Bern, 1976; Chevalier, 1976, 1978; Marshall and Bern, 1979; Bern and Nishioka, 1979; Loretz *et al.*, 1981; Larson and Bern, 1987). The two major peptides that have been isolated, purified and sequenced (see Ichikawa, 1985) from this system are urotensin I (UI) and urotensin II (UII).

If the caudal neurosecretory system has a physiological role in responding to an environmental salinity stimulus and this results in a measurable change of urotensin contents in the caudal neurons and/or in the urophysis, then one way to investigate this hypothesis is by applying the double sequential immunofluorescence technique (Larson *et al.*, 1987) to look at the sequential changes in UI and UII immunoreactive intensities. Urotensin amounts could change as a result of altered synthesis, turnover, or release.

Our previous work has shown that acclimation to different salinities of water after 24 hr has an effect on urophysial staining intensities of UI and UII in the caudal neurosecretory system of *Gillichthys mirabilis* (Larson and Madani, 1988, 1989, 1991). Transfer of seawater-acclimated fish to deionized fresh water caused an increased intensity of UI- and UII-like immunoreactivity in the urophysis. Two other reports on effects of osmotic manipulation on caudal neurosecretory system immunoreactivities have appeared in recent years (Minniti *et al.*, 1989; Oka *et al.*, 1990). Oka *et al.* (1990) found increased intensities of UI-like immunoreactivity in perikarya and urophyses of freshwater-acclimated and feral charr compared with seawater-acclimated fish. Supported by the results of Minniti *et al.* (1989), we hypothesized that the caudal system response to an osmotic stimulus may be only acute. Immunoreactive differences may be more noticeable up to 24

hr following an abrupt salinity shift than after long term acclimation. The present study was undertaken to investigate if salinity of acclimation at different times after transfer affects urotensin I and II immunostaining patterns in the trout caudal neurosecretory system.

We chose commercially important rainbow trout, *Oncorhynchus mykiss*, because of their tolerance to a wide range of water salinities and their relationship to anadromous species. Some members of the same species (steelhead) naturally migrate to saline waters. We selected three time points to examine with the following rationale: 2 hr to observe effects primarily on urophysial release of stored peptides, 10 hr to look at differences in synthesis and/or release, and 48 hr to see any long term effects. Portions of this work have been previously presented (Madani and Larson, 1989; Larson *et al.*, 1991).

MATERIALS AND METHODS

Animals

Adult rainbow trout were obtained from a local supplier and kept in aerated, dechlorinated fresh tap water in 130 and 235 gallon tanks (Frigid Units, Toledo, OH) at 10-13°C under 12:12 light:dark cycles. Prior to running experiments fish were allowed approximately forty days to reestablish normal stress levels in response to their new environment and were fed a commercially prepared diet (Purina Trout Chow, St. Louis, MO) daily.

Tissue preparation

Initially, to test trout tolerance to salinity and the possible effects of osmotic stress on caudal neurosecretory immunostaining, fish were transferred to new fresh water (controls), 50% seawater, or 100% seawater, sacrificed at either 2 or 24 hr, and double immunostained. The results from 50% were indistinguishable from 100% seawater; therefore, fish transferred to 100% seawater were used exclusively in further experiments.

A group of six adult fish (350-400 g, 29-35 cm) were randomly picked from a freshwater holding tank. Three of the fish were transferred to a 25 gallon aquarium containing new fresh water (FW) as a control for transfer and environmental stress, and three fish were transferred to an identical 25 gallon tank containing water salinified to 35 ppt (100% seawater (SW)) with Instant Ocean Salts (Aquarium Systems, Mentor, OH). After 2 hr, 10 hr, and 48 hr one fish was removed from each tank and sacrificed. Anterior to the caudal fin, the skin and muscle tissue covering the vertebrae were trimmed away, and the tail was transected at the level of the 5th vertebra anterior to the last vertebral element (urostyle). The caudal spinal cords with attached urophyses were dissected out from the spinal column and fixed quickly by immersion in cold paraformaldehyde, (4% in 0.13 M phosphate buffer, pH 7.4) (Sigma Chemical Co., St. Louis, MO) overnight at 4°C. Then the tissues were transferred to three changes of 10% (w/v) sucrose in 0.01 M phosphate buffer containing 0.9% NaCl, pH 7.4 at 4°C over a three day period. The FW and SW spinal cords from the same time points were frozen, paired and positioned on top of previously-sectioned, flat blocks of frozen OCT compound (Tissue-Tek, Elkhart, IN) on chucks. Liquid OCT was layered on top of the spinal cord pairs, and the blocks were frozen by immersion in liquid Freon-12 (Ig-lo Products Corp, Hernando, MS). Longitudinal cryostat sections (16 μm) of the paired spinal cords were mounted by melting onto gelatin coated slides which had been previously layered by spraying with teflon (Fluoroglide FB, Norton, Wayne, NJ) and silicone (Slipicone 316 Release Agent, Dow Corning Corp., Midland, MI) around a 2 cm circular area that would contain the tissue section. The slides were stored frozen at -20°C prior to immunostaining. This fish salinity transfer experiment was repeated five times and approximately 30-40 slides, containing two sections each, were obtained from each set of paired spinal cords.

Antisera

Rabbit antiserum to synthetic ovine CRF (INCSTAR Corp., Stillwater, MN) was applied to identify UI-like immunoreactivity in the spinal cord sections. Evidence that the CRF-antiserum cross-reacted with UI in the fish spinal cord and urophysis but failed to cross-react significantly with the frog skin peptide sauvagine (Fisher *et al.*, 1984) was previously reported by Onstott and Elde (1984) and Larson *et al.* (1987) and further substantiated by our preabsorption controls. To localize UII, tissues were treated with a rabbit antiserum raised against synthetic *Gillichthys* UII coupled to keyhole limpet hemocyanin which was provided kindly by Dr. D. Pearson, California State University, Los Angeles. To differentiate the two rabbit primary antisera, the IgG fraction of anti-UII serum was covalently bound with biotin. Thus, avidin labeled with rhodamine could bind specifically only to biotinylated anti-UII as described by Larson *et al.* (1987). No cross-reactivity of this antiserum with somatostatin, arginine vasopressin, arginine vasotocin, oxytocin, neurotensin, substance P, vasoactive intestinal peptide and CRF was found by radioimmunoassay (see Bern *et al.*, 1985).

Double immunofluorescence-staining procedure

We employed the indirect, double sequential immunofluorescence technique as described by Larson *et al.* (1987) and Larson and Madani (1991) to immunostain both CRF/UI and UII in the spinal cord sections. Pattern and intensity differences in immunoreactivity between FW control and the SW spinal cord sections at all time points were observed using an epifluorescence-equipped microscope (Nikon, Optiphot). Single color photographic exposures of the fluorescein (indicating UI-like immunoreactivity) and rhodamine (indicating UII-like immunoreactivity) fluorescence, and double exposures of both together were taken.

Controls

a) Preabsorption controls for UI and UII

To test for specific binding of anti-CRF and anti-UII sera to the corresponding peptides in the tissues, adjacent sections were incubated with anti-CRF serum preabsorbed with 10 μM UI (10 μl of synthetic *Catostomus* UI or synthetic ovine CRF containing 1 nmol of peptide were mixed with 10 μl of anti-CRF (1:10 dilution) plus 80 μl of antisera dilution buffer) and anti-UII serum preabsorbed with 10 μM UII (10 μl of synthetic *Gillichthys* UII containing 1 nmol of peptide were mixed with 25 μl of biotinylated anti-UII (undiluted) plus 65 μl of antisera dilution buffer) for 24 hr at 4°C as a substitute for the primary antisera. Preabsorptions of the antisera with CRF, sauvagine, and somatostatin have been tested previously (Larson *et al.*, 1987).

b) Stress and circadian controls

To determine the possible effects of transfer stress or circadian rhythms on UI and UII immunostaining patterns, fish were taken directly from the large freshwater holding tank (FW-tank) at the 2 hr and 10 hr time points. Their caudal spinal cords were removed and blocked individually. The 16 μm cryostat sections of 2 hr and 10 hr FW-tank spinal cords were placed on the same microscope slide with the 2, 10, or 48 hr FW-transferred sections. The slides containing all possible combinations of transferred and controlled fish spinal cords were processed by double sequential immunofluorescence. The possible transfer stress effect was determined by comparison of the 2 and 10 hr FW-tank with the respective 2 and 10 hr and with the 48 hr FW-transferred fish spinal cords. To assess any circadian rhythm variation in immunostaining, 2 and 10 hr FW-tank spinal cords on the same slide were compared, as well as, the 2, 10 and 48 hr FW-transferred spinal cords.

c) Blind evaluation of results

The slides were evaluated for intensity and pattern differences of

UI- and UII-like immunoreactivity between FW and SW spinal cords by a blind method in which the spinal cords were positioned in the blocks and coded, and observations were made without any pre-knowledge of the investigator to rule out any bias in judgment.

RESULTS

Preabsorption controls

UI blockage of the CRF/UI antiserum eliminated specific immunoreactivity of UI in the spinal cord sections and permitted visualization of the UII immunoreactivity. The homologous blockage of UII antiserum prevented specific immunoreactivity of UII and allowed visualization of specific immunoreactive(IR)-UI in the spinal cord sections. Simultaneous blockage of both primary antisera prevented immunostaining for either UI or UII completely. The UI and UII antisera appeared specific within the parameters tested.

Distribution of UI- and UII-like immunostaining in freshwater-maintained fish

Immunohistochemical localization of CRF/UI- and UII-like IR products of the caudal neurosecretory perikarya, nerve fibers, and urophysis were found in longitudinal sections of trout spinal cord. CRF/UI-like IR products specifically displayed fluorescein fluorescence (green), UII-like immunoreactivity exhibited rhodamine fluorescence (red), and the simultaneous

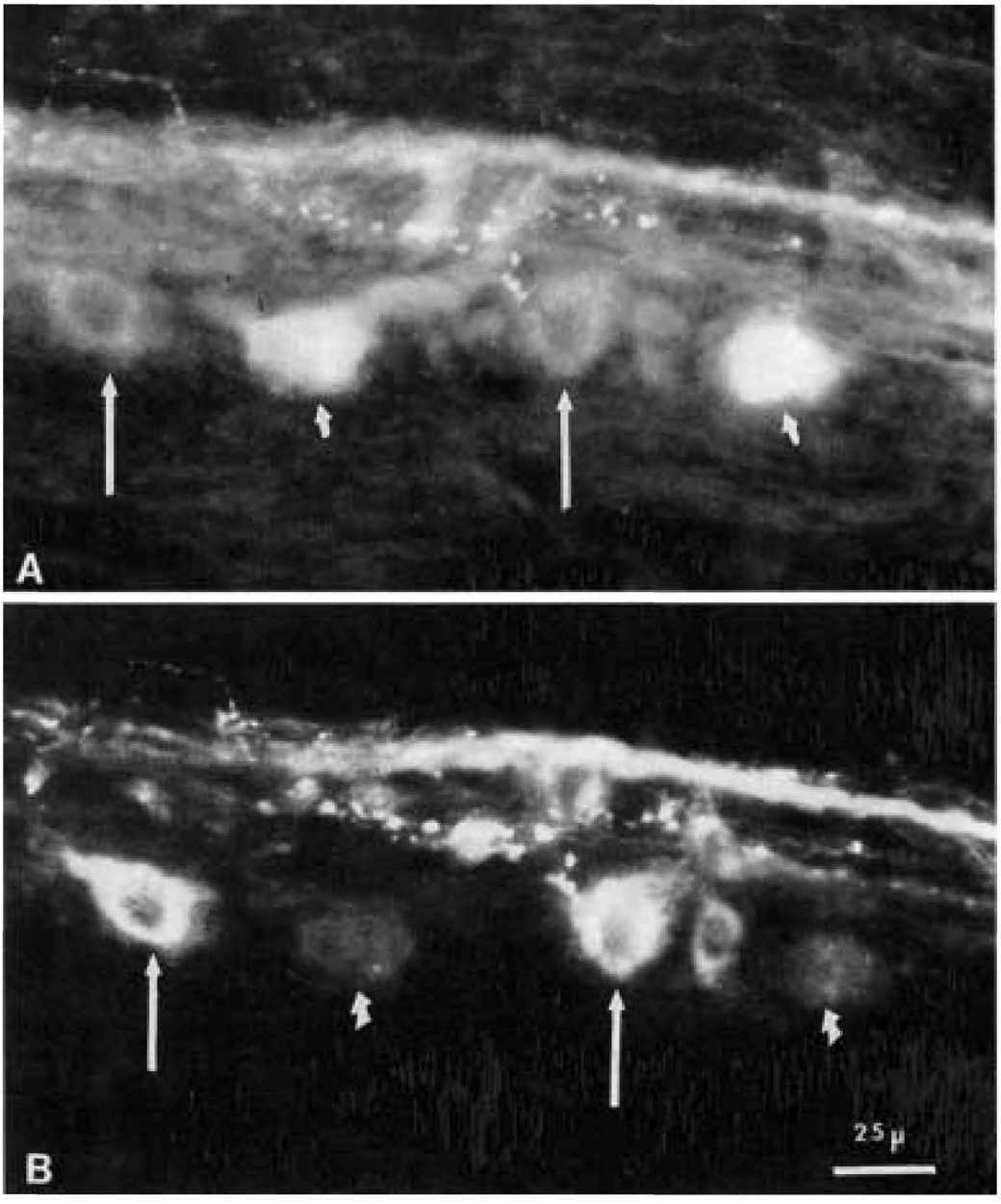

Fig. 1. Perikarya immunostained with variable intensity in the FW fish spinal cord. In the same tissue section photographed for CRF/UI (A) and UII (B), some cells (short arrows) stained more intensely for CRF/UI and less for UII. Other cells (long arrows) stained more intensely for UII and less for CRF/UI. Photographic exposure times were (A) 35 sec and (B) 6 sec. (Mag. x 615).

presence of both IR-UI and IR-UII appeared orange or yellow in doubly exposed photographs.

Perikarya IR to CRF/UI and UII antisera were identified primarily in spinal cord locations corresponding to the four preterminal vertebral segments anterior to the urophysis and in the spinal cord posterior to the urophysis (filum terminale). These perikarya did not represent uniform populations throughout the spinal cord. Perikarya IR for both CRF/UI and

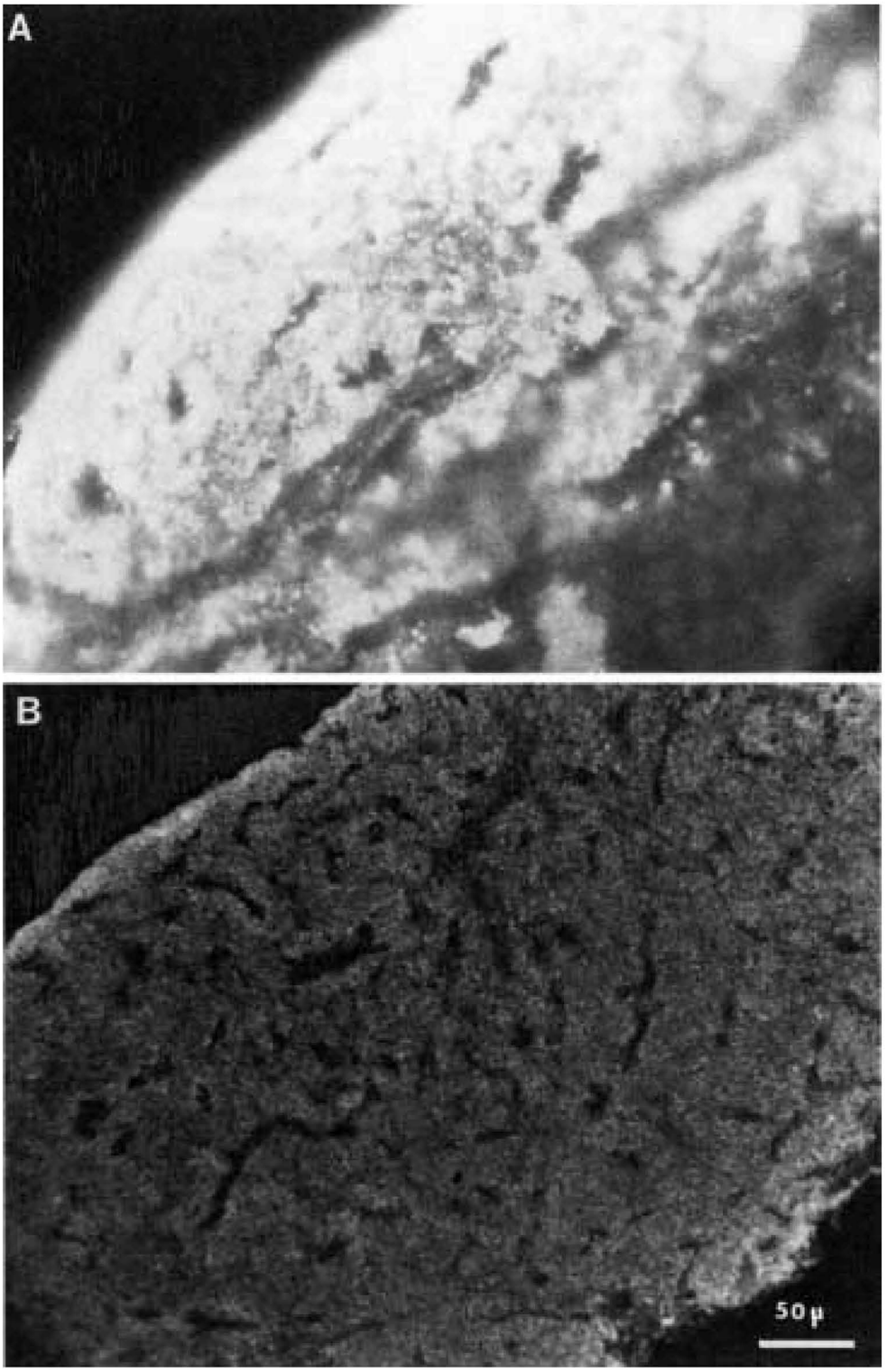

Fig. 2. Comparison of CRF/UI-like immunofluorescent intensities in the urophysis 2 hr following transfer. (A) Urophysis from control fish maintained for 2 hr in new FW. (B) Urophysis from fish 2 hr after transfer to SW. Notice the more intense UI-like immunofluorescence in the urophysis of fish maintained in FW. Photographic exposure times for both were 20 sec. (Mag. x 307).

UII located just dorsal and posterior to the urophysis were small and arranged in a compact mass. Moving progressively anterior from the urophysis the number of these small perikarya decreased as larger IR perikarya increased. The perikarya were variable in shape, size and degree of immunostaining intensity for UI and UII. A small number of perikarya intensely

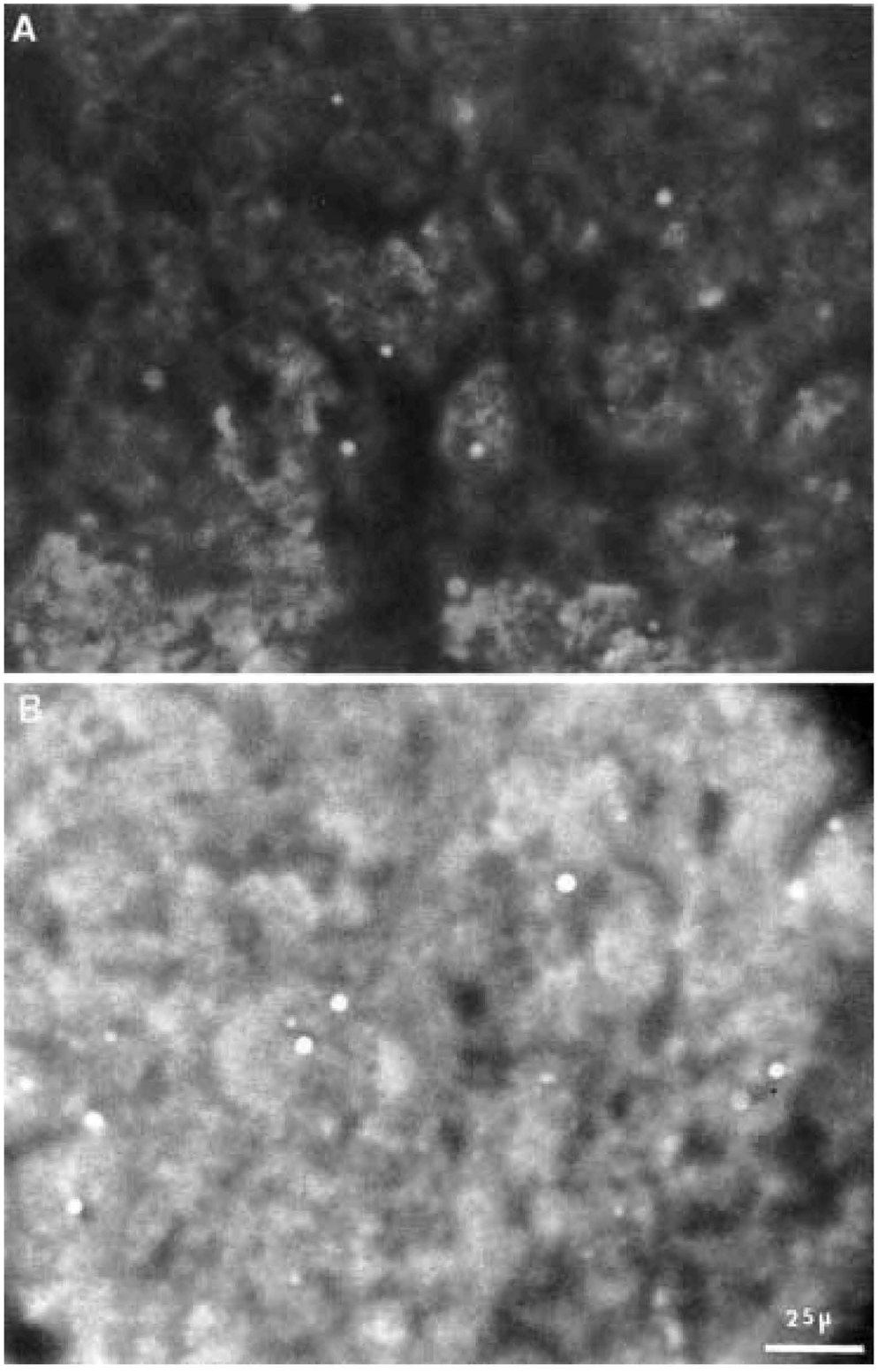

Fig. 3. Comparison of UII-like immunofluorescent intensities in the urophysis 2 hr following transfer. (A) Urophysis from control fish maintained for 2 hr in new FW. (B) Urophysis from fish 2 hr after transfer to SW. Notice more intense UII-like immunofluorescence in the urophysis of fish transferred to SW. Photographic exposure times for both were 12 sec. (Mag. x 615).

CRF/UI-IR and less IR for UII and vice-versa were observed occasionally (Fig. 1). Perikarya IR for CRF/UI only or UII only were rare. Thus, the great majority of the identifiable neurosecretory cells in the caudal spinal cord appeared to be clearly IR for both peptides.

Effects of transfer to seawater

The urophyses from fish acclimated for 2 hr in 100%

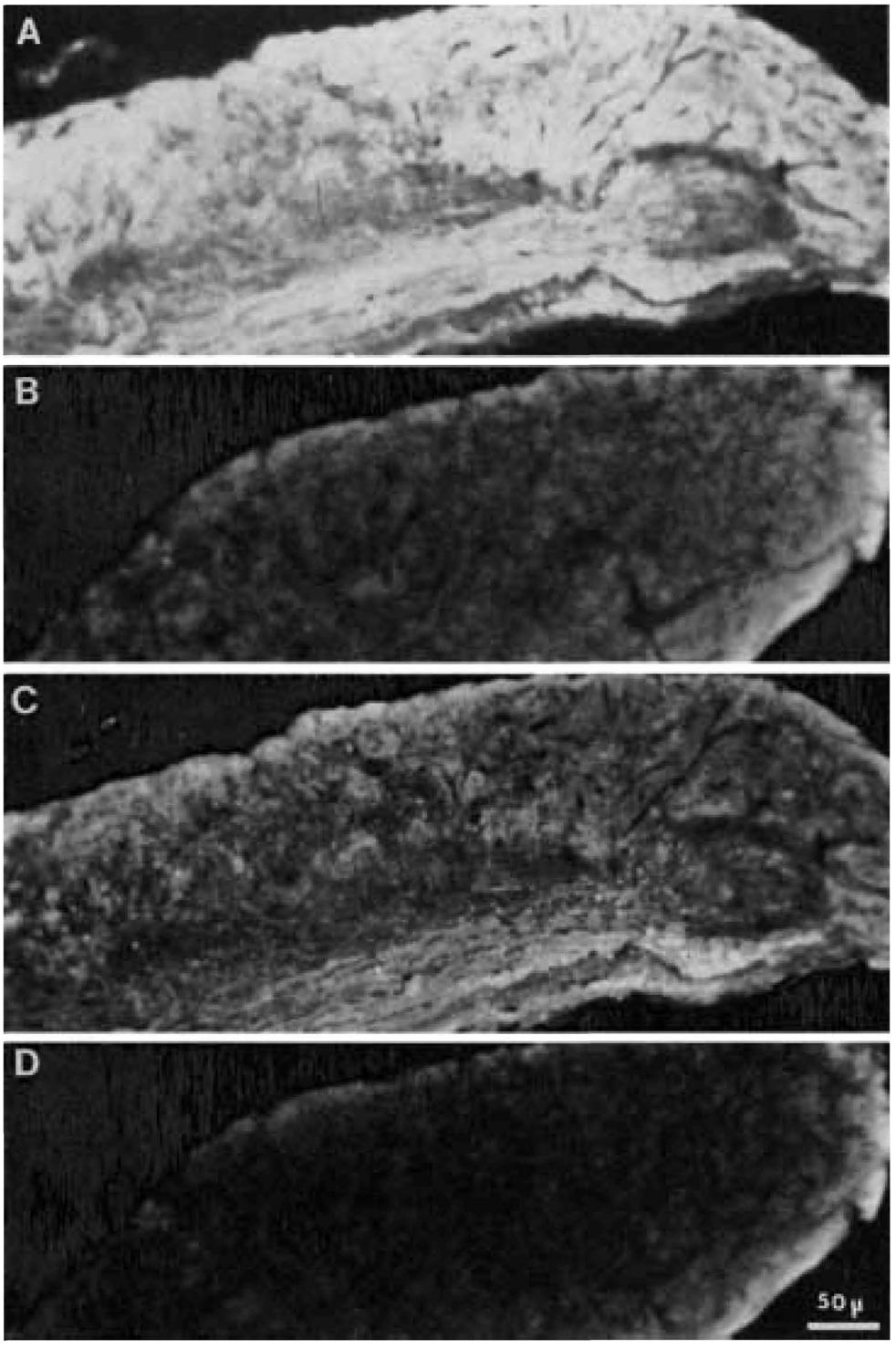

Fig. 4. Comparison of CRF/UI- and UII-like immunofluorescent intensities in the urophysis 10 hr following transfer. (A) CRF/UI-IR urophysis from control fish maintained for 10 hr in new FW. (B) CRF/UI-IR urophysis from fish 10 hr after transfer to SW. (C) UII-IR urophysis from control fish maintained for 10 hr in new FW. (D) UII-IR urophysis from fish 10 hr after transfer to SW. Notice more intense CRF/UI- and UII-like immunofluorescence in the urophysis of fish maintained in FW. Photographic exposure times were (A) and (B) 15 sec and (C) and (D) 25 sec. (Mag. x 246).

seawater exhibited less IR-UI intensity than fish maintained for 2 hr in new freshwater (Fig. 2). However, the 2 hr SW urophyses exhibited more IR-UII than 2 hr FW urophyses (Fig. 3). After 10 hr, the IR intensity was still less for UI in the SW urophyses than in the FW. The urophyses from 10 hr SW acclimated fish displayed a decreased IR intensity for UII compared with 10 hr FW control fish (Fig. 4). Analysis of the spinal cords taken 48 hr after transfer did not show any

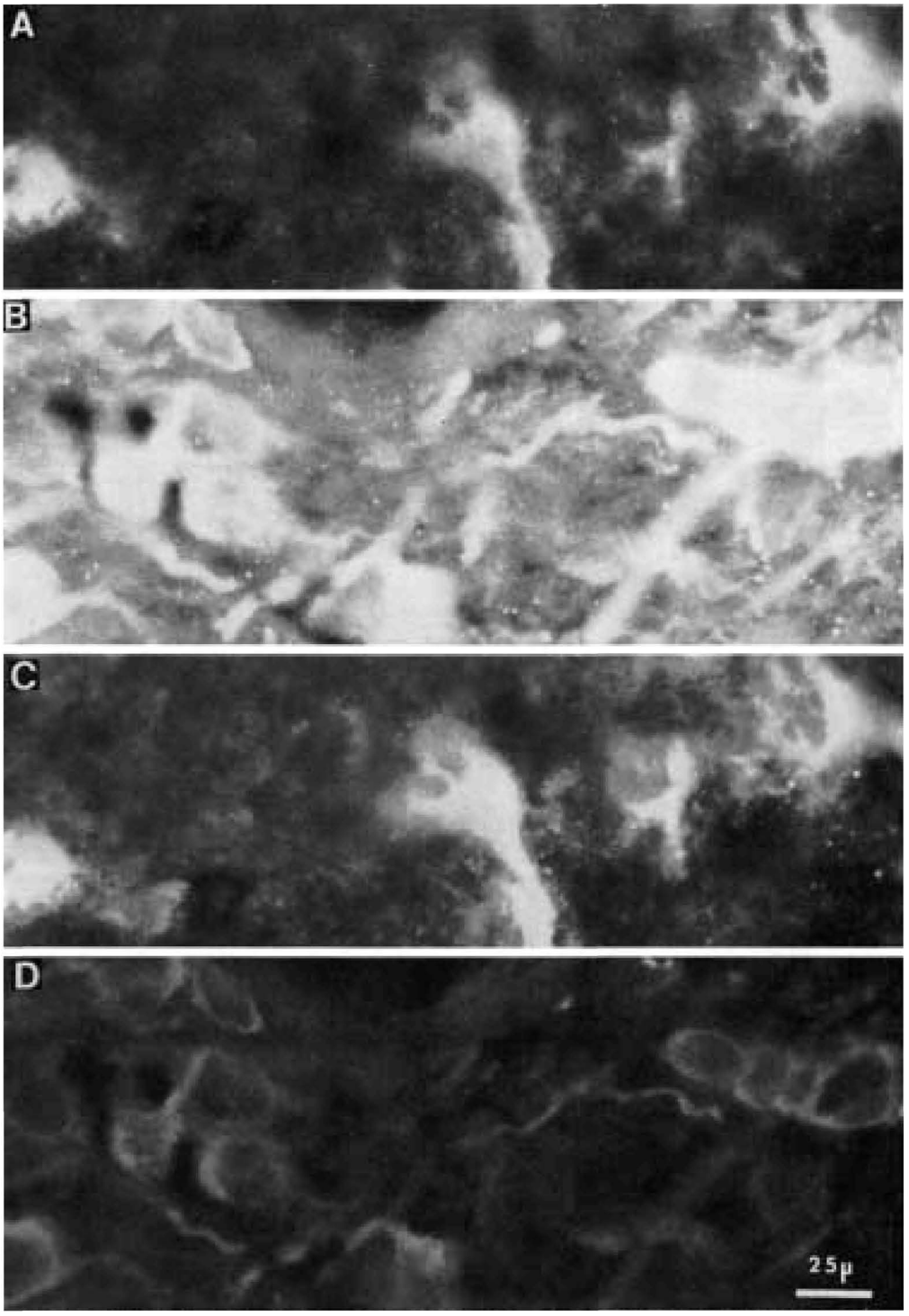

Fig. 5. Comparison of CRF/UI- and UII-like immunofluorescent intensities in perikarya anterior to the urophysis 2 hr following transfer. (A) CRF/UI-IR perikarya from control fish maintained for 2 hr in new FW. (B) CRF/UI-IR perikarya from fish 2 hr after transfer to SW. (C) UII-IR perikarya from control fish maintained 2 hr in new FW (same section as in A). (D) UII-IR perikarya from fish 2 hr after transfer to SW (same section as in B). Notice more intense CRF/UI- and less UII-IR intensities in SW perikarya. Photographic exposure times were (A) and (B) 12 sec and (C) and (D) 8 sec. (Mag. x 492).

apparent differences in intensity for either UI- or UII-like immunoreactivity in urophyses.

Two hours after transfer, the perikarya from fish in FW exhibited less IR-UI intensity than those from fish transferred to SW. Comparison of perikarya anterior to 2 hr SW urophyses showed less UII-like immunoreactivity than 2 hr FW

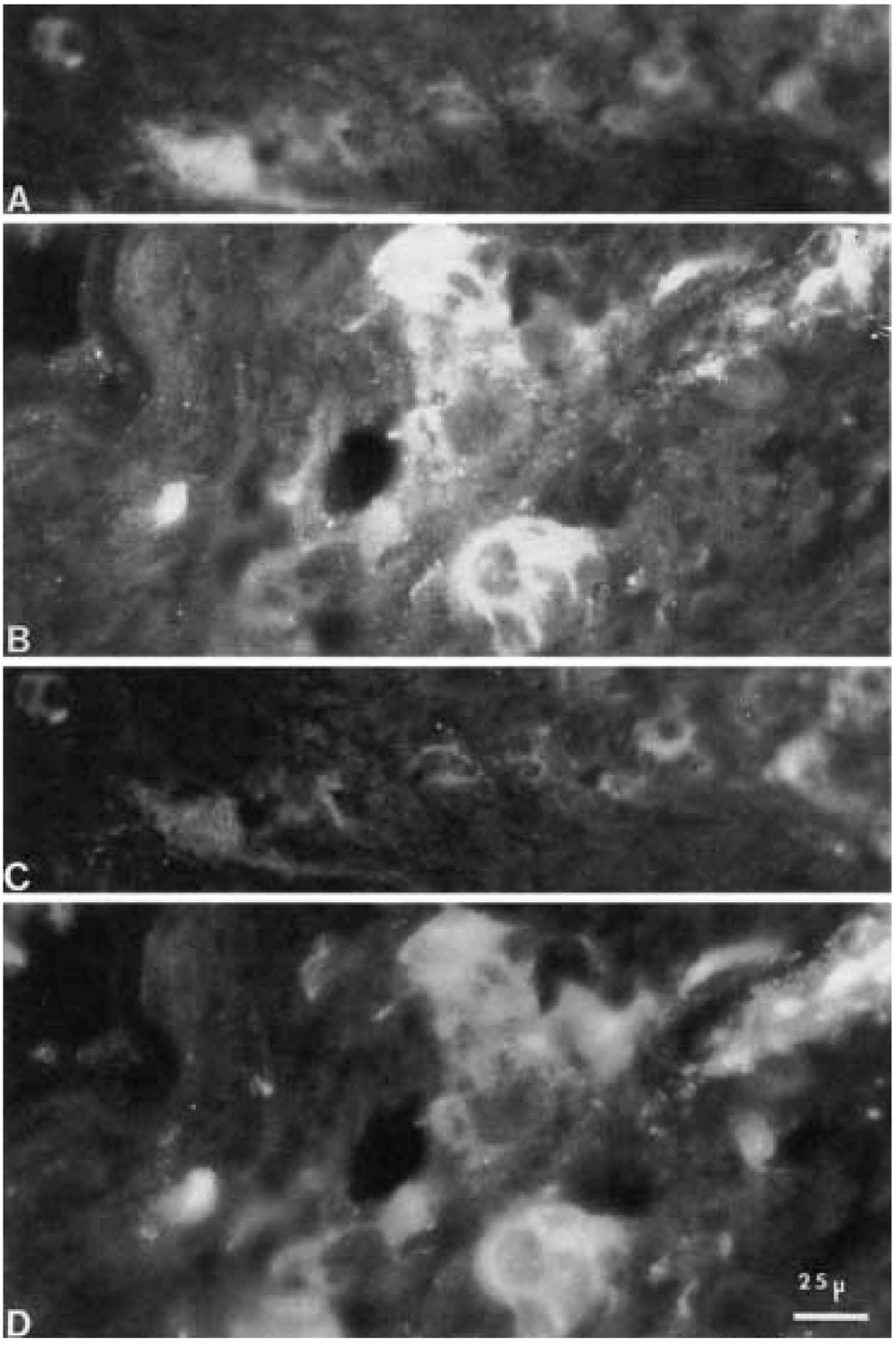

Fig. 6. Comparison of CRF/UI- and UII-like immunofluorescent intensities in perikarya anterior to the urophysis 10 hr following transfer. (A) CRF/UI-IR perikarya from control fish maintained for 10 hr in new FW. (B) CRF/UI-IR perikarya from fish 10 hr after transfer to SW. (C) UII-IR perikarya from control fish maintained 10 hr in new FW(same section as A). (D) UII-IR perikarya from fish 10 hr after transfer to SW (same section as B). Notice more intense CRF/UI- and UII-like immunofluorescence in SW perikarya. Photographic exposure times were (A) and (B) 12 sec and (C) and (D) 8 sec. (Mag. x 492).

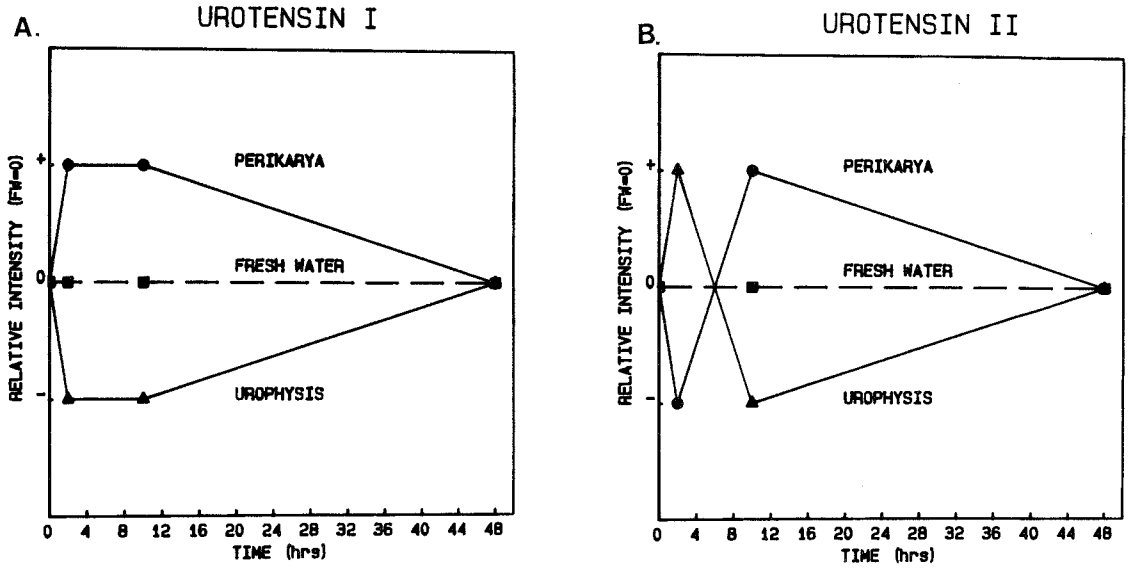

Fig. 7. Graphical representation of relative intensity differences in the perikarya and urophysis for IR-CRF/UI (A) and -UII (B). Time (abscissa) is plotted versus relative intensity (ordinate). The magnitude of intensity differences is not absolute, but rather an arbitrary, qualitative indication from the five separate experiments involving thirty fish (five each of the 2, 10, and 48 hr FW and SW transferred fish). There were no detectable differences in immunoreactive intensity levels between all FW time points and FW-transferred versus -tank fish spinal cords (---■---■---). (A) The perikarya anterior to the urophysis from SW fish showed higher intensity for UI at 2 and 10 hr and was relatively the same as FW after 48 hr (—●—●—). The urophysis from SW fish showed lower intensity for UI at 2 and 10 hr and was relatively the same as FW after 48 hr (—▲—▲—). (B) The perikarya anterior to the urophysis from SW fish showed lower intensity for UII at 2 hr, higher intensity at 10 hr, and was relatively the same as FW after 48 hr (—●—●—). The urophysis from SW fish showed higher intensity at 2 hr, lower intensity at 10 hr, and was relatively the same as FW after 48 hr (—▲—▲—).

corresponding areas (Fig. 5). By 10 hr, the perikarya anterior to SW urophyses consistently displayed an increased IR intensity for both UI and UII compared with 10 hr FW control fish (Fig. 6). There appeared to be no relative degree of differences between the treatment groups for UI and UII in perikarya anterior to the urophysis at the 48 hr time point. The relative IR intensity differences at the different time points are graphically depicted in Fig. 7. There was no difference noted in staining between male and female fish.

Stress and circadian controls

The above results indicated an effect of environmental salinity on caudal neurosecretory system immunostaining, but, to rule out any complications in the interpretation of those results from possible transfer-stress or circadian effects, fish were taken directly from the FW holding tank and compared as described in materials and methods. Comparison of the 2 hr FW-tank with the 2 hr FW-transferred fish showed no IR differences for UI or UII in their caudal spinal cords (perikarya, fibers, and urophyses). The same was true when comparing 10 hr FW-tank with 10 hr FW-transferred and 2 or 10 hr FW-tank with 48 hr FW-transferred fish spinal cords.

To rule out a possible circadian rhythm effect on UI and UII immunostaining patterns, the 2 hr FW-tank fish spinal cords were paired with the 10 hr FW-tank and a 48 hr FW-transferred fish spinal cord. There were no detectable differences in IR-UI and IR-UII between the three spinal cords, or further, between all possible combinations of transferred versus tank FW spinal cords.

DISCUSSION

Despite a persistent theme regarding the possible involvement of the caudal neurosecretory system in osmoregulation, the definite function of this system is still uncertain. The present study notes changes in the trout caudal neurosecretory system caused by a shift from a freshwater to a seawater environment. These changes began within two hours following transfer and were completed within 48 hr.

Two hr after transfer of FW-acclimated fish to SW, their urophyses exhibited decreased IR-UI intensity, while perikarya anterior to the urophysis appeared more intensely UI-IR compared with the FW-transferred controls. The UI results suggest increased urophysial release coupled with a possible stimulation of perikaryal production of the peptide in response to the hyperosmotic stimulus. An alternative explanation of inhibited UI production and release would not agree with the perikaryal observations. Increased urophysial UI degradation coupled in the perikarya with either stimulated synthesis or lessened degradation can not be ruled out from our data, but seems unlikely.

Regarding UII at 2 hr following transfer, the urophyses from the SW fish had increased IR-UII intensity and perikarya anterior to the urophysis were less intensely UII-IR than in the FW fish. The UII response to seawater challenge could be explained by an inhibition of urophysial release resulting in

accumulation of UII in the urophysis and inhibition of perikaryal synthesis followed by depletion of UII in the perikarya. Again, the alternative of stimulated UII production and release would not agree with the perikaryal results, and 2 hr would not seem to be sufficient time for significant synthesis and urophysial replenishment. However, the unlikely possibility of a decrease in urophysial UII degradation combined with increased UII degradation and/or inhibited synthesis in the perikarya would be consistent with our results.

The increase in IR intensity levels of one urotensin while the other decreases in the same tissue compared with the controls indicates independent regulation of the two urotensins. Whether the differential regulation is occurring at the level of synthesis, degradation or release can not be answered definitively with the present methods. Because UI and UII are co-localized in the great majority of neurosecretory cells, the opposite shifts in IR intensity levels, especially in the urophysis within 2 hr, also suggest that the two urotensins are packaged in separate secretory vesicles to at least some extent. This suggestion is consistent with the findings of Yamada *et al.* (1990) using the carp, *Cyprinus carpio*. They describe neurosecretory granules in urophysial nerve endings that are IR for either UI, UII, or both peptides. Even in single nerve endings displaying both immunoreactivities all three varieties of IR vesicles were found.

By 10 hr following transfer to SW the urophyses displayed lower IR intensities for both urotensins and higher IR intensities of both in the anterior perikarya than the FW controls. This could indicate continued urophysial release and perikaryal synthesis of UI in SW. However, the UII results represent a relative IR intensity reversal between 2 and 10 hr. Possibly after longer exposure to the hyperosmotic stimulus there is a shift from the initial decreased release and synthesis of UII to a later activation of UII release and synthesis relative to the FW controls. The reversal could have occurred anytime during the 10 hr period. Immunocytochemical analysis of additional time points before and after 2 hr would be needed to pinpoint the timing of the switch. The independent change in IR-UII further argues for differential regulation and separate secretory vesicles of the two urotensins.

After 48 hr in SW we could detect no UI-or UII-IR intensity differences relative to the FW controls in either the urophyses or perikarya. Thus, to the limits of our detection, the caudal neurosecretory response to SW challenge as indicated by IR-urotensin levels appears to have subsided between 10 and 48 hr. This lack of any difference within 48 hr supports our previous hypothesis (Larson and Madani, 1991) and the results of Minniti *et al.* (1989) that the caudal system response to osmotic stimuli is relatively acute and could explain at least some of the earlier, seemingly conflicting results of others (see Larson and Madani, 1991).

The tank-transfer stress and circadian rhythm controls revealed no IR intensity differences in the caudal neurosecretory systems between any of these FW fish. Thus, the observed intensity increases and decreases in SW fish represent absolute changes and are clearly the result of the difference in water salinity. The immunochemical detection sensitivity for UII may have been different from that for UI; however, no comparisons were made or intended between intensities of IR-UI relative to those of IR-UII. All immunoreactive intensity comparisons were made qualitatively between IR-UI in SW relative to those in the FW controls or between IR-UII in SW relative to those in the FW controls.

In *Gillichthys mirabilis* we found that 24 hr after transfer of seawater-acclimated fish to fresh water their urophyses had increased intensities of IR-UI and -UII (Larson and Madani, 1991). Although the comparisons are reversed our results from trout at 10 hr after transfer of FW-acclimated fish to SW agree with those from *Gillichthys*. In both cases the fish in seawater appeared to have decreased urophysial quantities of UI and UII relative to the fish in fresh water which could reflect stimulated release of both urotensins in response to seawater challenge and release inhibition in response to freshwater challenge.

Others have reported less caudal neurosecretory contents in fish transferred from fresh water to seawater (Takasugi and Bern, 1962; Kriebel, 1980) and increased caudal perikaryal activity in response to a hyperosmotic stimulus (Enami, 1956; Yagi and Bern, 1965; Chevalier, 1976). Sacks and Chevalier (1984) also reported enlargement of the caudal neurons when brook trout, *Salvelinus fontinalis*, were transferred to seawater as we noted at 2 hr in *Oncorhynchus mykiss*. However, other studies suggesting caudal neurosecretory activation in response to a hypo-osmotic stimulus (Berlind *et al.*, 1972; Chevalier, 1978; Gauthier *et al.*, 1983; Owada *et al.*, 1985) would appear to conflict somewhat with our data. Besides the differences in species used, parameters measured, and stimuli imposed, most of these earlier studies analyzed salinity effects after days or longer which could explain some of the apparent contradictions and makes them difficult to compare with ours.

Investigations by Minniti *et al.* (1989) and Oka *et al.* (1990) have been conducted on urotensin immunoreactivities in response to environmental salinity changes. Minniti and coworkers examined changes in sauvagine/UI-like immunoreactivities in the caudal neurosecretory system of a seawater teleost, *Diplodus sargus* L., after 15, 30, 45 and 90 min of exposure to a hypo-osmotic milieu. They found a significant, acute increase of immunoreactivity mainly in the urophysis that peaked by 60 min and began to decline at 90 min. Their conclusion that urophysial UI release is inhibited by a hypo-osmotic stimulus and stimulated by a hyperosmotic stimulus agrees completely with ours and the 2 hr results for urophysial IR-UI. Using another trout species, *Salvelinus leucomaenis*, Oka *et al.* (1990) could find no consistent change in either IR-UI or -UII after several weeks in seawater and concluded that the caudal neurosecretory system had no essential role in osmoregulation of the charr. Their results are completely consistent with ours 48 hr after transfer. If the caudal neurosecretory response to an environmental salinity shift is acute and finished by 48 hr they would have missed the time period to observe changes. Their description of the immunostaining patterns for freshwater trout is very similar to

that described for freshwater-maintained *Oncorhynchus mykiss* except that we did not note cerebrospinal fluid contacting neurons immunoreactive for UII only.

Our results support a functional role for the caudal neurosecretory system in trout osmoregulation and independent regulation of its coexisting neuropeptides, UI and UII. In response to increased environmental salinity there is a relatively acute change in urotensin IR intensities that has subsided by 48 hr. We suggest that these changes may reflect an increase in UI release from the urophysis and an initial (within 2 hr) inhibition followed by (within 10 hr) increased UII release in response to a hyperosmotic stimulus. It is tempting to speculate that in addition to the possible direct effects of urotensins on osmoregulatory organs or blood flow to those organs by means of their vasoactivities, the urotensins might indirectly affect osmoregulation by controlling release of the two major fish osmoregulatory hormones, cortisol (seawater-adapting) and prolactin (fresh water-adapting) (see Bern *et al.*, 1985; Larson and Bern, 1987). Elevated circulation levels of UI could stimulate cortisol release through pituitary adrenocorticotropin stimulation and increased levels of UII could inhibit pituitary prolactin release in fish exposed to seawater (see Grau *et al.*, 1982; Lederis *et al.*, 1985; Rivas *et al.*, 1986). The role of the urotensins might be to stimulate these changes in cortisol and prolactin levels that are later maintained by other means. Radioimmunoassay of circulating levels of UI, UII, cortisol and prolactin are needed to address these questions.

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