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Extraurophyseal distribution of urotensin II immunoreactive neuronal perikarya and their processes

(urotensins/caudal neurosecretory system/urophysis/cerebrospinal fluid-contacting neurons)

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ABSTRACT **The use of the unlabeled antibody enzyme method on serially adjacent sections permitted the demonstration of urotensin II (UII) and urotensin I (UI) immunoreactivities colocalized in most of the cells of the caudal neurosecretory system of *Catostomus commersoni*. The study of the upper regions of the central nervous system from the spinal cord anterior to the fifth preterminal vertebral region up to and including the brain stem revealed the presence of UII immunoreactivity in cerebrospinal fluid-contacting neurons, located ventral to the central canal along the entire length of the spinal cord and medulla. Beaded nerve fibers were observed projecting to the ventrolateral surface of the spinal cord and also forming a seemingly ascending immunoreactive-UII longitudinal bundle directed toward the brain. The presence of this "extraurophyseal" system of immunoreactive-UII cells and fibers suggests that the UII peptide may be released in upper regions of the central nervous system in response to stimuli conveyed via the cerebral spinal fluid. Thus, separate functions may be postulated for the urophyseal and the cerebral spinal fluid-contacting urotensin II systems.**

A caudal neurosecretory system, structurally analogous to the hypothalamic (cranial) system, exists in teleost, elasmobranch, and ganoid fishes. In teleosts, it consists of neurosecretory cells located in the caudal spinal cord that project to a highly vascularized neurohaemal region, the urophysis (1).

Two types of putative peptide hormones, urotensin I (UI) and urotensin II (UII), have been isolated from teleost urophyses; their amino acid sequences have been determined and confirmed by the synthesis of fully active peptides (2–6). These studies revealed six different forms of the naturally occurring UII peptide in the urophyses of three teleostean species (*Gillichthys* UII, *Cyprinus* UIIα, -β, and -γ, and *Catostomus* UIIA and -B). All of these UII forms are cyclic dodecapeptides sharing an identical six-residue disulfide-bridged ring in positions 6–11. The integrity of the disulfide bridge appears to be essential for UII biological activity (5, 7) and for antibody recognition in at least some cases (8).

The elucidation of the *Gillichthys mirabilis* UII sequence drew attention to a partial homology (positions 1–2 and 7–9) with somatostatin; however, analogies in the biological activities between the *Gillichthys* or other UII peptides and somatostatin are not conclusive at present (5, 7).

Previous immunocytochemical studies have shown that most or all of the caudal neurosecretory cells in several teleost species display UI or corticotropin-releasing factor (CRF)/UI immunoreactivity (7, 9–11), while UII immunocytochemistry revealed the presence of a nonreactive population of neurosecretory neurons in the caudal neurosecretory system, which is variable in percentage among different species (7, 12).

The present study was undertaken to define the localizations of UI and UII immunoreactivities in the caudal neurosecretory system and to determine if specific immunoreactive UII (IR-UII) neuronal structures are present elsewhere in the *Catostomus* central nervous system (CNS).

MATERIALS AND METHODS

Thirteen white suckers (*Catostomus commersoni*) collected from the Bow River, Calgary, Alberta, were used for this study. They were kept in aerated, dechlorinated fresh tap water at 12°C on a constant photoperiod (12L:12D) and fed *ad libitum*.

The CNS tissues were fixed in Bouin's fixative either by immersion (72 hr at 4°C) or by vascular perfusion. Perfusion was administered through the truncus arteriosus, and outflow was achieved through an incision in the right atrium. Phosphate-buffered saline (pH 7.4) was perfused for 10 min, followed by Bouin's fixative (60 min). Postfixation of perfused CNS tissues was done by immersion in Bouin's fixative (48 hr at 4°C). The fixed brains and different regions of the spinal cord, including the urophyses of nine fishes, were processed for paraffin-embedding. Serial sections (7 μm) were mounted on gelatin-coated slides and immunostained by using the unlabeled antibody–enzyme method (13), following modifications introduced by Sofroniew *et al.* (14).

The CNS tissues of four additional fishes, fixed by immersion, were processed for Vibratome sectioning. Agar-agar (4%) solution, heated to 80°C, was used for pseudoembedding of the tissue blocks that, after hardening, were Vibratome-sectioned at 100 μm.

Primary antisera raised against synthetic *Gillichthys* UII, *Catostomus* UI, and somatostatin (Peninsula Laboratories, Belmont, CA) were used for this study. Radioimmunoassay characterization of the UII and UI antisera showed 100% crossreactivity of the UII antiserum with all of the presently known forms of the UII peptide (3, 5, 6) and no detectable crossreactivity (<0.013%) with UI and somatostatin. The UI antiserum crossreacted <0.15% with ovine or rat CRF in radioimmunoassay, and no displacement of the labeled UI could be observed with >30 ng of UI per assay tube. No immunostaining of well-documented mammalian IR-somatostatin fibers was observed when the UII antiserum was tested on rat median eminence sections.

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Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; UI, urotensin I; UII, urotensin II; CRF, corticotropin-releasing factor; IR-UI and IR-UII, immunoreactive UI and UII.

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All of the antisera were diluted in a solution containing 0.1% Triton X-100 (Sigma) and 0.7% Carrageenan (Sigma) in Tris buffer (pH 7.7).

The procedure for paraffin sections involved incubation with the first antiserum (UII antiserum diluted 1:800, UI antiserum diluted 1:800, or somatostatin antiserum diluted 1:3000) for 18 hr; with the second antiserum [goat anti-rabbit Ig (Sigma) diluted 1:25] for 30 min; and with peroxidase-antiperoxidase (Dako Accurate Chemicals, Westbury, CT; diluted 1:50) for 30 min. After the incubations with the antisera, the sections were treated with a 0.2% 3,3'-diaminobenzidine tetrahydrochloride solution containing 0.01% H_2O_2 for 15 min. Tris buffer washings (3 times for 5 min each) were performed between each step. The same concentrations were maintained for the immunostaining of Vibratome sections, but the time of antiserum incubations and buffer washings was extended (first antiserum, 48 hr; second antiserum, 90 min; peroxidase-antiperoxidase, 90 min; and Tris buffer washings, 3 times for 15 min each).

For the comparison of UI and UII localization in the urophyseal region, adjacent paraffin sections of the caudal spinal cord containing the urophysis were stained alternatively for UI and UII. Specificity of the UI immunostaining was assessed by using the UI antiserum saturated with an excess of *Catostomus* UI or ovine CRF as described (11). Homologous adsorption tests for UII and UI antisera showed an absence of immunostaining. The immunostaining of caudal neurosecretory cells by the UI antiserum adsorbed with ovine CRF was not impaired.

Controls for the UII immunostaining were done by using a somatostatin antiserum (diluted 1:3000) in sections adjacent to those immunostained for UII or by using an antiserum against UII saturated with an excess of antigen (*Gillichthys* UII).

RESULTS

Caudal Spinal Cord and Urophysis. The immunostaining of adjacent sections with antisera against UI and UII revealed that all of the identifiable neurosecretory cells in the sucker caudal spinal cord were stained with the UI antiserum. UII immunoreactivity was observed coexisting with UI immunoreactivity in most of the cells, but there was a small number of immunoreactive UI (IR-UI) perikarya and their processes that showed a weak staining or were not stained at all with the antiserum against UII (Fig. 1). These cells were generally located laterally in the rostral population of large Dahlgren cells.

The ventral bundle of fibers formed by the processes of the caudal neurosecretory cells and the accumulation of fiber terminals in the urophysis were also immunostained with both antisera. Colocalization of UI and UII in the processes of the neurosecretory cells could be confirmed repeatedly when the same thick processes were visualized in adjacent sections. Thin beaded fibers were also present and immunostained by either antiserum; however, the technique used did not permit the determination of UI–UII colocalization occurring in the thin fibers.

No cells or thick processes were found displaying only immunoreactivity for UII in the urophyseal region.

Rest of the Spinal Cord and Brain. The study of the spinal cord, rostral to the fifth preterminal vertebral joint, revealed the presence of IR-UII perikarya, not immunoreactive for UI, located intraependymally or in the hypendyma just below the spinal central canal (Fig. 2*a*). These neurons formed a continuous column of cells along the remaining rostral length of the spinal cord and part of the medulla and displayed a thick dendrite-like process directed toward the central canal and a varicose axon projecting ventrally. In the more rostral locations, the dendrite-like processes of these IR-UII cells showed unique bulbous dilatations that protruded into the central canal and seemed to be in direct contact with the cerebrospinal fluid (Fig. 2 *b* and *d*). These bulbous protrusions were also evident but not immunostained in the control sections used for this study (Fig. 2*c*). The most caudal cells of this midsagittal column of IR-UII neurons were observed at the level of the fourth-to-sixth preterminal vertebrae, overlapping in one instance (one caudal neurosecretory neuron located anterior to the most caudal IR-UII CSF-contacting neuron), with the most rostral IR-UI caudal neurosecretory neurons located laterally.

The beaded axons of these neurons formed a medial plexus ventral to the column of the IR-UII cells (Fig. 2*a* and Fig. 3). From this plexus, fibers left bilaterally in a ventrolateral direction and then bent to form a dense longitudinal bundle (Fig. 2 *e* and *f* and Fig. 3), which was localized at the border between the ventral and lateral funiculi. Scattered IR-UII neurons were also observed between the medial plexus and the longitudinal bundle (Fig. 2*a*).

Some beaded fibers left the longitudinal bundle and projected toward the external surface of the spinal cord (Fig. 2*e* and Fig. 3) where they joined thick smooth IR-UII fibers running longitudinally (Fig. 2 *f* and *g*). Both types of fibers contributed to the formation of complex clusters of fibers located near the ventrolateral surface of the spinal cord (Fig. 2 *f* and *g* and Fig. 3), where they displayed large dilatations that were surrounded by a diffuse halo of IR-UII "dust" (Fig. 2 *f* and *g*).

The caudal end of the longitudinal bundle was not clearly defined, but IR-UII fibers forming this structure and the bundle became progressively more evident from caudal to rostral, remaining visible through the anterior length of the spinal cord. The bundle became more diffuse at the level of the medulla, where some of the fibers spread toward the ventral surface of the brain where large dilatations were seen amidst an IR-UII halo (dust) (Fig. 4*a*), while other fibers seemed to continue rostrally.

In the medulla, below the fourth ventricle, faintly stained IR-UII perikarya and fibers were present (Fig. 4*b*). It was not

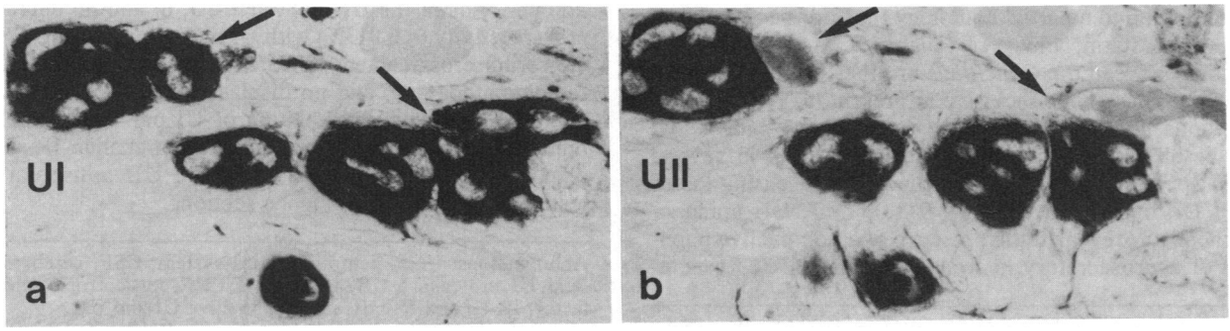

FIG. 1. Adjacent parasagittal 7-μm sections of the caudal spinal cord immunostained for UI (*a*) and UII (*b*). While most of the caudal neurosecretory cells display both immunoreactivities, some large IR-UI cells (arrows) show only a faint UII immunoreactivity. (×251.)

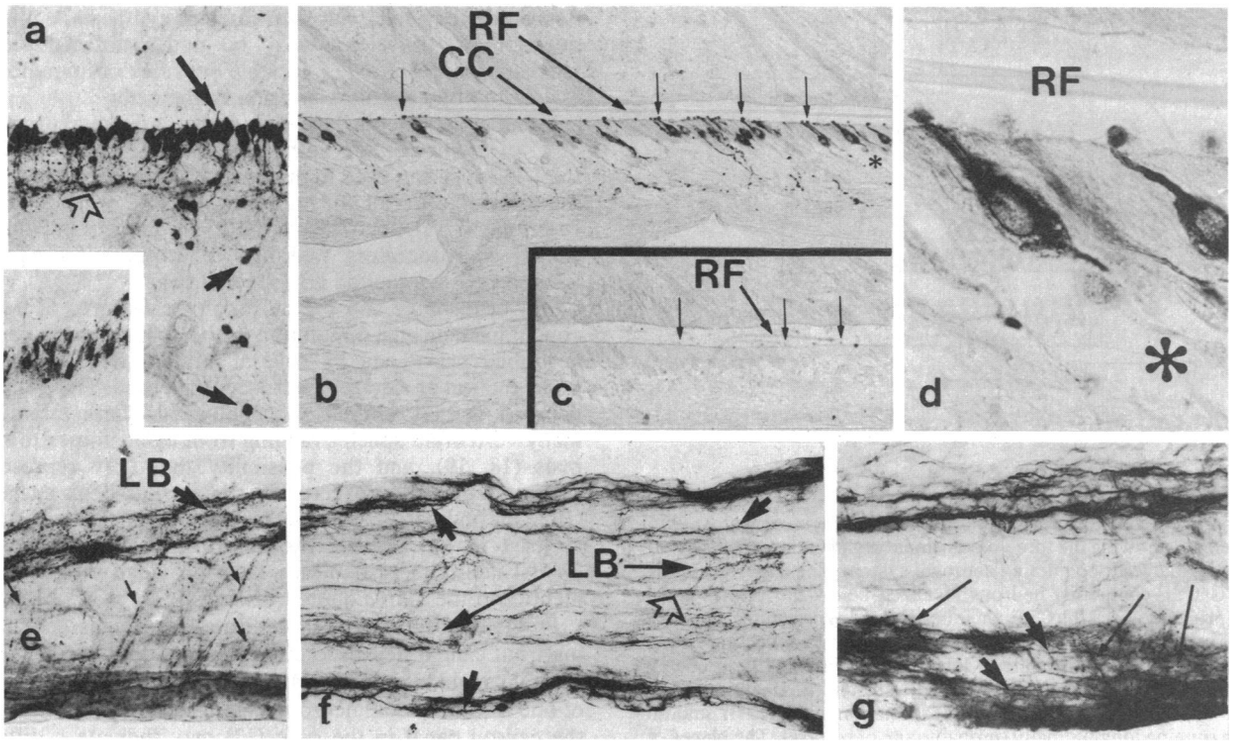

FIG. 2. Vibratome (*a*, *e*, *f*, and *g*) and paraffin (*b*, *c*, and *d*) sections of the sucker spinal cord immunostained for UII. (*a*) Midsagittal section showing the presence of intraependymal and hypendymal IR-UII neurons below the central canal (collapsed). Their dendrites are directed to the canal (large arrow), and their beaded axons project ventrally, forming a medial plexus below the cells (open arrow). Scattered IR-UII cells (small arrows) were also seen ventral to the medial plexus. (×120.) (*b*) Midsagittal section of the cervical spinal cord showing IR-UII neurons below the central canal (CC) displaying dendrites protruding into the central canal (arrows). (×123.) (*c*) Control section of the same fish and region as in *b* but immunostained with UII antiserum saturated with an excess of antigen. No immunostaining of cells or dendrite protrusions (arrows) was obtained. (×123.) (*d*) Higher magnification of the area marked by asterisk on the extreme right in *b*, showing the bulbous appearance of a dendrite terminal protruding into the central canal. (×668.) (*e*) Longitudinal oblique section revealing a longitudinal bundle (LB) of beaded IR-UII fibers and fibers in between it and the external surface of the spinal cord. (×101.) (*f*) Horizontal section showing partially the medial plexus (open arrow), the longitudinal bundle (LB), and fibers connecting both structures. Also thick IR-UII smooth fibers are seen running longitudinally (solid arrows) and approaching the external surface of the spinal cord. (×30.) (*g*) Thin beaded fibers (long arrows) and thick smooth fibers (short arrows) intermingled near the external surface of the spinal cord, displaying diffuse IR-UII halos around them. (×81.) RF, Reissner's fiber.

determined whether the fibers emanated from these cells or whether they represented innervation.

Most of the IR-UII fibers observed in the brainstem course longitudinally and spread out in the diencephalon, reaching several hypothalamic and most thalamic nuclei. Numerous fibers displaying dust-like terminal distribution patterns were observed in the mammillary bodies (Fig. 4*c*), nucleus anterioris hypothalami, nucleus pretectalis, and nucleus preglomerulosus pars lateralis. Moderate numbers of fibers were seen in the nucleus difusus lobi inferioris (Fig. 4*d*), nucleus posterioris tuberis, and most of the optic tectum layers. In the rostral regions of the thalamus, the fibers formed a distinct part of the fasciculus medialis telencephali, entering into the telencephalon where they distributed mainly in the area of the ventral telencephalic nuclei (Fig. 4*e*). From this region, some fibers projected to the dorsal telencephalon (Fig. 4*e*), and others entered into the olfactory tract, apparently ending in the olfactory bulb (Fig. 4*f*).

Although thick IR-UII smooth fibers were practically absent in the brain, numerous IR-UII fibers displaying large dilatations (up to 8-μm diameter) were seen in different locations, such as the ventral surface of the medulla (Fig. 4*a*), the nucleus difusus lobi inferioris (Fig. 4*d*), and the dorsal telencephalon. These fibers usually were found in close proximity to the external basal lamina of the CNS, displaying branches, and in some cases could be found amidst a dust-like IR-UII halo (Fig. 4*a*).

When adjacent sections of the upper spinal cord and brain were immunostained with UI, UII, and somatostatin antisera, no overlapping of the respective immunostaining patterns was observed. Central-canal CSF-contacting neurons were revealed only by the UII antiserum.

DISCUSSION

Our findings show that UI and UII immunoreactivities are colocalized in most of the neurosecretory cells and in their thick processes in the caudal spinal cord of *Catostomus commersoni*. This phenomenon had been described previously in other species of fishes (7, 15), showing different degrees of colocalization of CRF/UI and UII immunoreactivities. The absence of caudal neurosecretory neurons exclusively immunostained for UII, observed in our study, does not support the findings of Yamada *et al.* (15), who reported that UII-specific cells in the carp outnumber CRF/UI and CRF/UI–UII cells. Additional studies will be necessary to elucidate if such differences represent species variations or different physiological stages of some neurons in the caudal neurosecretory system.

In our study, some of the neurons displaying mainly UI immunoreactivity also showed a faint UII immunoreaction, suggesting that the lack of complete colocalization of both peptides may be due to differences in cytoplasmatic content of UII in the caudal neurosecretory cells. It is tempting to suggest that both peptides are present in all of the caudal

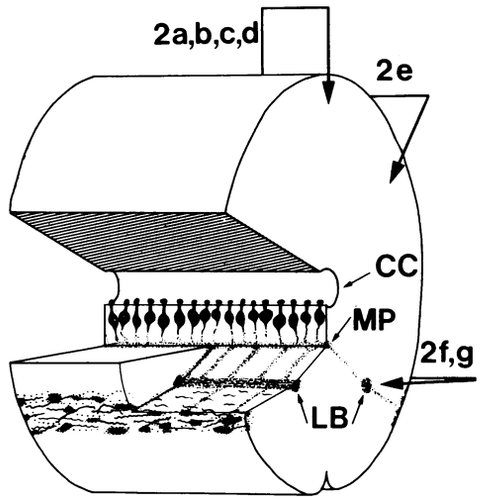

FIG. 3. Schematic representation of a segment of *Catostomus* spinal cord showing the three-dimensional organization of the IR-UII central-canal CSF-contacting neuronal system. The dendrites of the IR-UII CSF-contacting neurons localized below the central canal (CC) project toward the CSF, their beaded axons projecting ventrally to form: (*i*) a medial plexus (MP; probably interconnecting neighboring neurons), (*ii*) a bilateral longitudinal bundle (LB; probably ascending towards the brain), and (*iii*) clusters of fibers near the lateroventral surface of the cord in association with IR-UII smooth fibers running longitudinally (probable site of release). The planes of the sections relating to those shown in Fig. 2 are labeled thus 2a, b, c, d, e, f, and g (see Fig. 2).

neurosecretory cells, but their rates of synthesis, enzymatic degradation, or release may be under different control mechanisms, resulting in variable densities of immunostaining for the two peptides in some of the cells.

HPLC experiments have shown that individual urophyses of *Catostomus* contain both identified UII forms (5). Since the UII antiserum used in the present study recognizes all of the known forms of the molecule (8), we were unable to determine if UIIA or UIIB, or both, are present in either the caudal neurosecretory neurons, or in the more rostrally located CSF-contacting UII-specific cells.

The occurrence and the morphologic features of a CSF-contacting neuronal population in the spinal cord have been described previously by several authors who used silver impregnation or electron microscopy methods (16–18). Such neurons display similar morphological characteristics in many vertebrate species ranging from cyclostomes to mammals (18, 19), and the possibility that they represent a separate spinal neurosecretory system has been suggested (19). Our findings support this view and strongly suggest that, at least in the sucker, the central-canal CSF-contacting IR-UII neuronal system may have provided a biosynthetic identity to the above quoted neurons.

Previous observations of dendrite-like processes emanating from caudal neurosecretory neurons that project to the central canal in the teleost *Albula vulpes* (20) and the finding of one IR-UII caudal neurosecretory neuron in contact with the central canal in the carp (12) may indicate a different CSF-contacting system from that described here. Alternatively, those previous findings may indicate a variability in the form of one and the same CSF-contacting system.

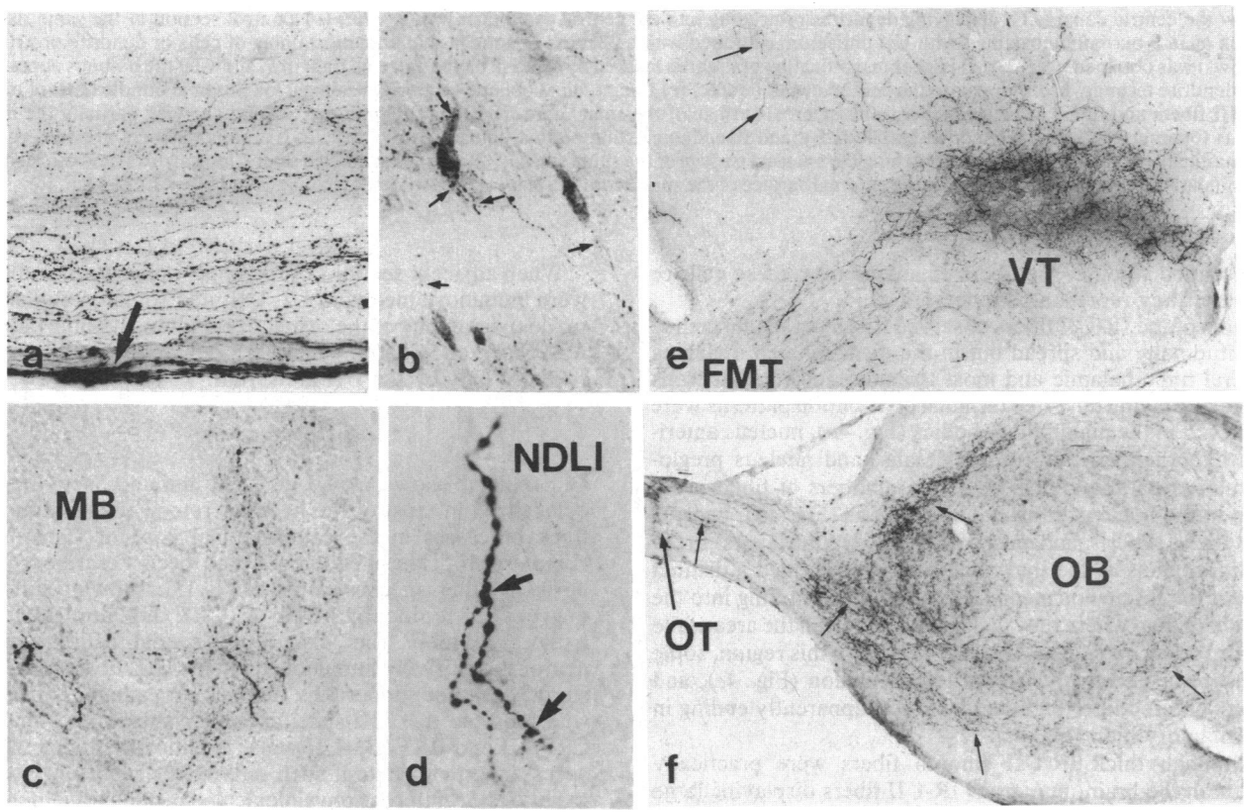

FIG. 4. Sagittal Vibratome sections of the sucker brain immunostained for UII. (*a*) IR-UII fibers located close to the ventral surface of the medulla. Some show large dilatations and diffuse halos of IR-UII material (arrow). (×105.) (*b*) IR-UII neurons located below the fourth ventricle; IR-UII beaded fibers appear to be making axodendritic and axosomatic contacts (arrows). (×152.) (*c*) Distribution of IR-UII beaded fibers in the mammillary bodies (MB). (×108.) (*d*) IR-UII fiber displaying collateral branches and large beads near the ventral surface of the brain in the region of the nucleus diffusus lobi inferioris (NDLI). (×259.) (*e*) IR-UII beaded fibers forming part of the fasciculus medialis telencephali (FMT) and distributed in the ventral telencephalon (VT). Some fibers project dorsally (arrows), reaching the dorsal surface of the telencephalon. (×23.) (*f*) IR-UII fibers in the olfactory tract (OT) and distributed in the olfactory bulb (OB) (arrows). (×55.)

The shape of the apical dendrites of the CSF-contacting IR-UII neurons located more distally in the sucker spinal cord is comparable to that described in other species of fishes (18, 19, 21); however, the CSF-containing IR-UII neurons located in the most anterior spinal cord and medulla display dendrites with bulbous protrusions into the central canal similar to those described in mammals (18, 21).

Vigh *et al.* (19) described a ventrally directed tract of varicose fibers originating in CSF-contacting neurons and ending close to the ventrolateral surface of the spinal cord by means of terminal enlargements. Similar features were observed in the extraurophyseal IR-UII system of the sucker, especially in the UII-immunostained thick Vibratome sections, which allowed a better visualization of fiber projections. Additionally, our results showed also a longitudinal IR-UII bundle running ventrolaterally that was not described in the previous studies (19).

The observation of a diffuse halo of IR-UII dust around some IR-UII fibers may be artifactual. However, the selectivity of this phenomenon to some fibers only (usually close to the external surface of the CNS) supports the possibility of a visualization of extracellular immunoreactive peptidergic material, which might be located in or may have been released from "synaptoid" terminals. Therefore, this dust may be an immunocytochemical corroboration of previous electron microscopic findings by Vigh *et al.* (19), who describe the existence of neurosecretory terminals making close contact with the external basal lamina of the spinal cord.

In several brain regions, such as the mammillary bodies, nucleus anterior hypothalami, nucleus pretectalis, and nucleus preglomerulosus pars lateralis, IR-UII fibers displaying large dilatations around cell bodies and coursing along dendrite-like processes of unstained neurons were seen. Such images were considered suggestive of terminal endings on the basis of previous descriptions relating light and electron microscopy findings (22). Some IR-UII fibers displaying similar features were observed surrounding weakly stained IR-UII neurons in the brain stem, suggesting an innervation of IR-UII cells by fibers from other IR-UII cells. The origin of these fibers has not been determined.

The staining pattern obtained with an antiserum against somatostatin was different from that of UII, suggesting that both somatostatin and UII peptides are present in the fish CNS, forming separate peptidergic systems. Interestingly, the CSF-contacting somatostatin-reacting neurons in the teleostean brain (23) show close similarities with the UII central-canal CSF-contacting neurons described here.

Our studies showed that the "extraurophyseal" distribution of IR-UII and IR-UI (11) is different, suggesting the possibility that the colocalization of the two urophyseal peptides may occur only in the caudal neurosecretory system.

It is tempting to speculate, on the basis of the morphologic design of this extraurophyseal UII system, that central-canal CSF-contacting IR-UII neurons of *Catostomus* may respond to events originating in the CSF, releasing their content into the subarachnoid space and/or the meningeal vasculature [a pathway suggested by Vigh *et al.* (19)]. Moreover, functional connections may be anticipated between the spinal-cord CSF and some brain areas, where IR-UII fibers and their terminals occur.

Previous light or electron microscopic studies have reported the presence of numerous stereovili and kinocilia present on spinal CSF-contacting terminals, which approach the Reisner's fiber by passing through the lumen of the central canal (18, 21). Such morphological features appear to apply to the CSF-contacting IR-UII neurons and the Reisner's fiber in the present studies (Fig. 2 *b* and *d*). It has been proposed that such an interrelationship may indicate the perception by mechanoreceptors of gravitational vibration or flow stimuli (e.g., lateral line organ, labyrinthine receptors, organ of Herring; for a review, see ref. 18). Application of appropriate experimental approaches (e.g., immunization of animals against UII or passive immunization with UII antisera) may help to establish if the above function is likely to apply, envisaging the perception of stimuli from the Reisner's fiber by the bulbous processes of the IR-UII cells and transmitting the information in the form of altered UII secretion within the brain (or blood stream).

The apparent subdivision of UII-containing neurons into two morphologically recognizable systems—the caudal neurosecretory or urophyseal and the anterior spinal or CSF-contacting—may be indicative of separate functions. Only physiologically oriented experiments, perhaps considering osmoregulatory–reproductive functions on the one hand and mobility, equilibrium, vibrational phenomena on the other, may provide long-awaited clues to the physiological roles of the urophyseal peptides and the caudal system against the anterior (spinal CSF-contacting; pituitary; cerebral) systems of both types of urotensins.

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