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Urotensin II from the River Lamprey (*Lampetra fluviatilis*), the Sea Lamprey (*Petromyzon marinus*), and the Paddlefish (*Polyodon spathula*)

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Urotensin II was isolated from extracts of the whole brain of the river lamprey (*Lampetra fluviatilis*) and the sea lamprey (*Petromyzon marinus*). The primary structure of the peptide from both species is the same (Asn-Asn-Phe-Ser-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val) and this amino acid sequence is identical to that of urotensin II from the dogfish and skate. Consistent with previous morphological studies indicating that the Agnatha lack a caudal neurosecretory system, urotensin II was not detected in an extract of *P. marinus* spinal cord. The data suggest that the urotensin II may have functioned in the earliest vertebrates as a neurotransmitter/neuromodulator in the central nervous system rather than as a neurohormone of the caudal neurosecretory system. Urotensin II was also isolated from an extract of the spinal cord of a chondrostean fish, the paddlefish (*Polyodon spathula*). The primary structure of the paddlefish urotensin II (Gly-Ser-Thr-Ser-Glu-Cys-Phe-Trp-Lys-Tyr-Cys-Val) is the same as that of another chondrostean, the sturgeon (*Acipenser ruthenus*). The study provides further evidence for a widespread distribution of urotensin II in vertebrate species and suggests that the primary structure of the peptide is better conserved in these phylogenetically ancient fish than in teleosts. © 1995 Academic Press, Inc.

Urotensin II (UII) has traditionally been regarded as a product of the urophyses of teleost fish (Bern *et al.*, 1985) but more recent studies have indicated that the peptide has a much wider tissue and species distribution. Immunohistochemical studies have shown that teleost fish possess an extensive extraurophyseal distribution of UII-immunoreactive neurons. In several species of marine and freshwater fish, networks of UII-containing nerve fibers were identified in the anterior spinal cord and various regions of the brain (Yulis and Lederis, 1986; 1988). UII has been purified from an extract of whole brain from the trout *Oncorhynchus mykiss* (Waugh and Conlon, 1993).

UII-immunoreactive neuronal structures have been identified in the diffuse neurosecretory system associated with the caudal spinal cord region of elasmobranch (Owada *et al.*, 1985; Onstott and Elde, 1986), holocephalan (Onstott and Elde, 1986), and chondrostean (Saenko, 1978; Belenky *et al.*, 1989; Oka *et al.*, 1989) fish. The isolation and structural characterization of UII from the dogfish *Scyliorhinus canicula* (Conlon *et al.*, 1992a), skate *Raja rhina* (Waugh and Conlon, 1993), and sturgeon *Acipenser ruthenus* (McMaster *et al.*, 1992) have shown that UII from nonteleost fishes is structurally related to the UII peptides isolated from teleost urophyses (reviewed in Waugh and Conlon, 1993). The isolation of UII from an extract of the brain of the frog *Rana ridibunda* has shown that the peptide is synthesized in the nervous tissue of an amphibian (Conlon *et al.*, 1992b).

The lampreys are extant representatives of the earliest group of jawless vertebrates (Agnatha) that diverged from the line of evolution leading to gnathostomes at least 550 million years ago. The neuroendocrine system of lampreys may display features characteristic of the

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primitive vertebrate stock from which mammals and all other vertebrates have evolved (Forey and Janvier, 1993). The paddlefish (family Polyodontidae), comprising only two extant species, the North American paddlefish, *Polyodon spathula,* and the Chinese paddlefish, *Psephurus gladius,* occupy an important position in phylogeny as one of the most primitive living members of the Actinopterygii (ray-finned fishes). The Polyodontidae and Acipenseridae (sturgeons) are closely related families that were formerly classified together as Chondrostei but the fossil record shows that in the late Cretaceous the two families were already clearly distinct from one another (Carroll, 1988).

The aim of the present study was to extend our knowledge of the molecular evolution of the UII family by isolating and characterizing structurally the peptide from two species of Agnatha, the river lamprey (*Lampetra fluviatilis*) and the sea lamprey (*Petromyzon marinus*), and from the North American paddlefish.

MATERIALS AND METHODS

Animals. Paddlefish (12 specimens of both sexes; age 14 months; body weight 1.2–1.8 kg) were collected at the Aquaculture Research Center (Frankfort, KY). The tissues were immediately frozen on dry ice and stored at −20° until time of extraction. River lamprey of both sexes (600 specimens; age 1–2 years; 75–250 g body weight) were collected in October during their spawning run in tributaries of the River Neva near the Gulf of Finland. Sea lamprey of both sexes (approximately 2000 mature specimens) were collected in June and July at Hammond Bay Biological Station (Millersburg, MI) as described earlier (Sower *et al.*, 1993).

Tissue extraction. Neuronal tissue from the entire spinal cord of the paddlefish (64 g) was homogenized at 0° in 10 vol ethanol:0.7 *M* HCl (3:1 v/v) as previously described (Nguyen *et al.*, 1994). The homogenate was stirred for 3 hr at 0° and centrifuged (4000*g* for 30 min). The ethanol was removed from the supernatant under reduced pressure and, after further centrifugation (4000*g* for 30 min), the extract was pumped onto eight Sep-Pak C18 cartridges (Waters Associates, Milford, MA) connected in series. Bound material was eluted with acetonitrile:water:trifluoroacetic acid (70.0:29.9:0.1) and freeze-dried.

Whole brain from the river lamprey (17 g) and sea lamprey (155 g) and neuronal tissue from the spinal cord (tail to last branchiapore) of the sea lamprey (27 g) were separately extracted by boiling for 5 min in 1 *M* acetic acid (10 vol) and homogenized using a Waring blender. After centrifugation (1600*g* for 60 min at 4°), peptide material in the supernatants was isolated using Sep-Pak C18 cartridges as described above.

Radioimmunoassay. Urotensin II-like immunoreactivity (UII-LI) was measured by a procedure previously described (Conlon *et al.*, 1992a; Waugh and Conlon, 1993) using an antiserum raised against dogfish UII. The antiserum is directed against the conserved cyclic region of the peptide and shows 0.5% cross-reactivity with somatostatin-14.

Purification of paddlefish UII. The paddlefish spinal cord extract was redissolved in 1% (v/v) trifluoroacetic acid/water (10 ml) and chromatographed on a 25 × 1-cm Vydac 218TP510 (C18) column (Separations Group, Hesperia, CA) equilibrated with 0.1% (v/v) trifluoroacetic acid/water at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min, maintained at this concentration for 30 min, and raised to 49% (v/v) over 60 min using linear gradients. Absorbance was measured at 214 and 280 nm and fractions (1 min) were collected. The fraction containing UII-LI (denoted by the bar in Fig. 1A) was rechromatographed on a 0.46 × 25-cm Vydac 214TP54 (C4) column equilibrated with acetonitrile: water:trifluoroacetic acid (21.0:78.9:0.1) at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 49% (v/v) over 50 min using a linear gradient. Paddlefish UII was purified to near homogeneity by successive chromatographies on a 0.46 × 25-cm Vydac 219TP54 phenyl column and a 0.46 × 25-cm Vydac 218TP54 (C18) column under the same conditions used for elution of the C4 column.

Purification of lamprey UII. The extracts of whole brain from the sea lamprey and river lamprey, after partial purification on Sep-Pak cartridges, were redissolved in 1% (v/v) trifluoroacetic acid/water (10 ml) and separately chromatographed on the semipreparative Vydac C18 column and the analytical Vydac C4 and C18 columns under the same conditions used for the purification of paddlefish UII.

Structural characterization. Automated Edman degradation was performed using an Applied Biosystems Model 471A sequenator modified for on-line detection of phenylthiohydantoin (PTH) amino acids under gradient elution conditions. The detection limit for the PTH derivatives was 0.5 pmol. Electrospray ionization mass spectrometry was performed at the Novo/Nordisk Research Institute, Bagsvaerd, Denmark, using an API III LC/MS/MS system (Sciex, Thornhill, Ontario, Canada) as previously described (Conlon *et al.*, 1993). Approximately 20 pmol of peptide was used and the accuracy of mass measurement was 0.02%.

RESULTS

Purification of Paddlefish Urotensin II

The elution profile of the extract of paddlefish spinal cord, after partial purification on

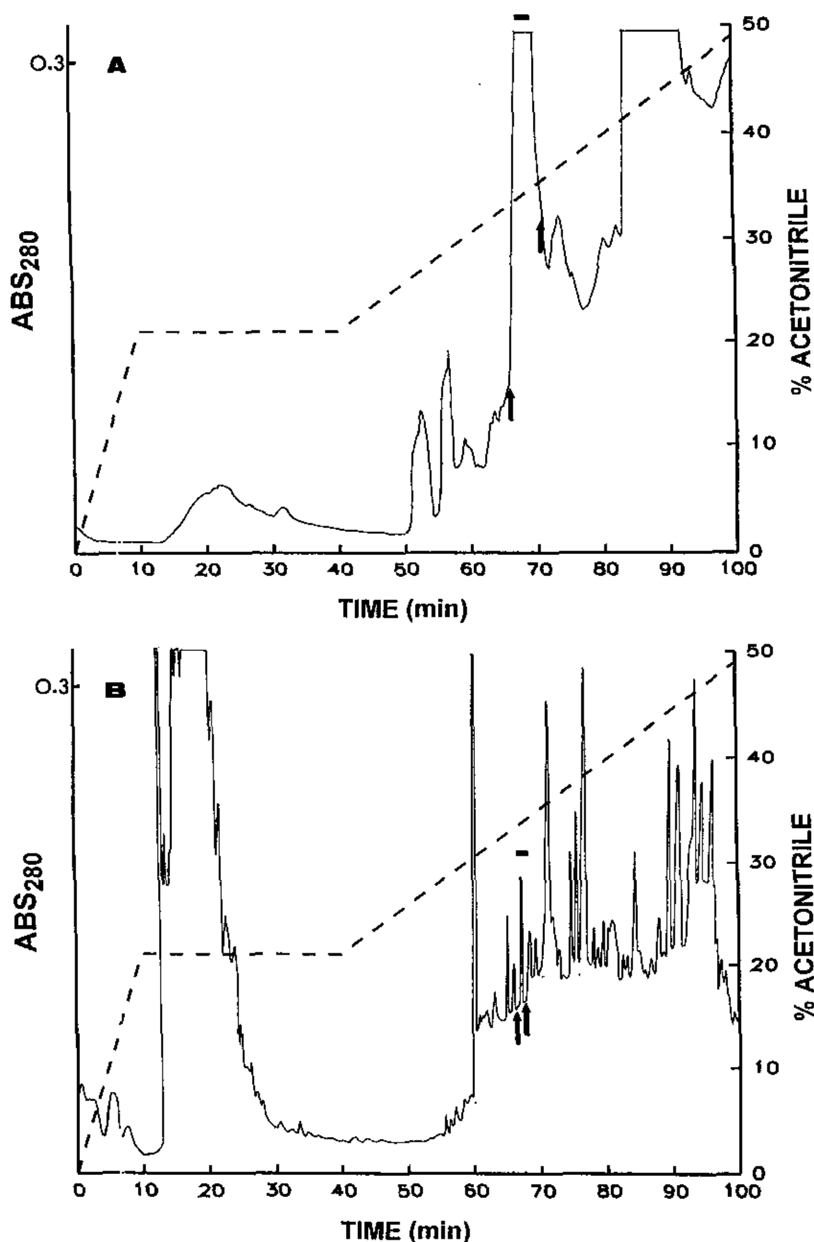

FIG. 1. Reversed-phase HPLC on a semipreparative Vydac C18 column of an extract of (A) paddlefish spinal cord and (B) river lamprey brain, after partial purification on Sep-Pak cartridges. The bar denotes the fractions containing urotensin II-like immunoreactivity. The dashed line shows the concentration of acetonitrile in the eluting solvent and the arrows show where peak collection began and ended.

Sep-Pak cartridges, on a semipreparative Vydac C18 column is shown in Fig. 1A. UII-LI was eluted in the fraction denoted by the bar. After chromatography of this material on an analytical Vydac C4 column (Fig. 2A) the UII-LI (fraction denoted by the bar) was associated with the ascending limb of the major peak of uv-absorbing materials. Paddlefish UII was re-

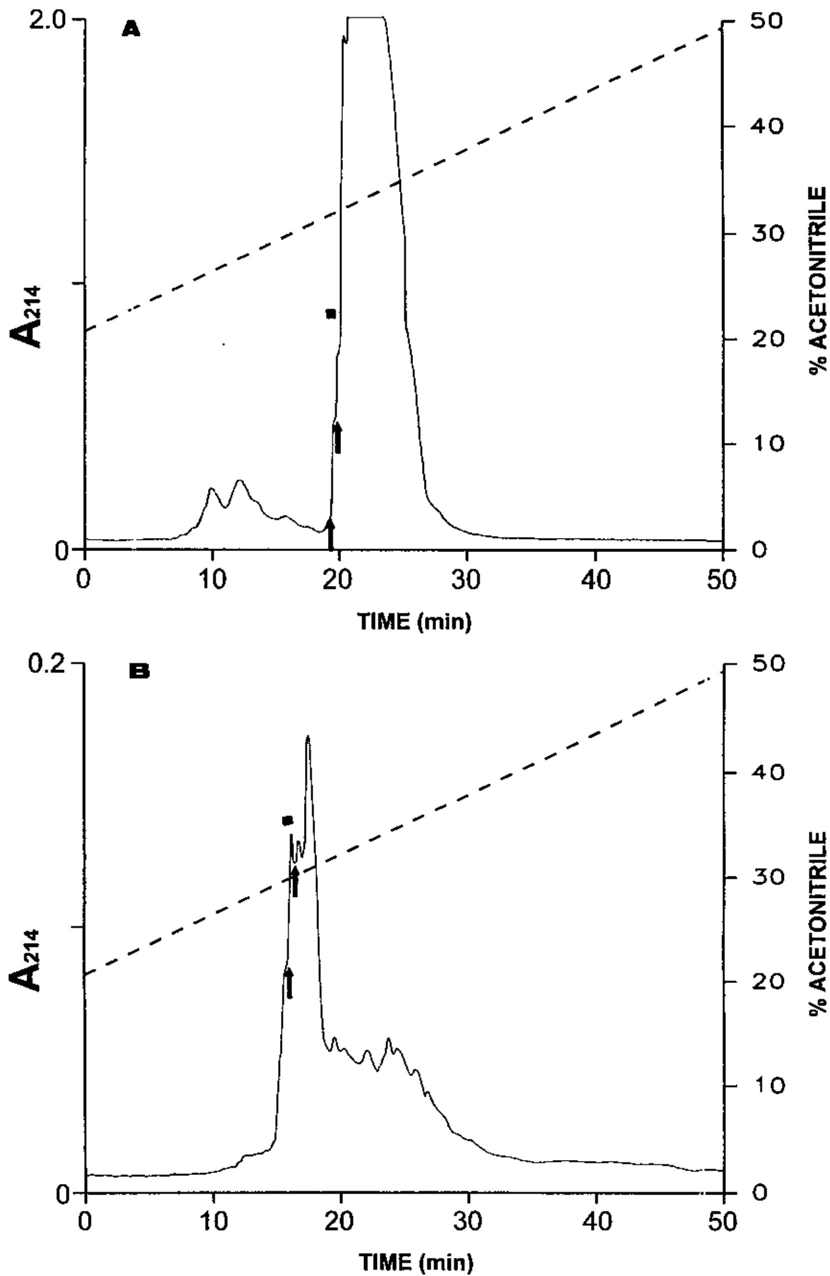

FIG. 2. Purification of urotensin II from paddlefish spinal cord by reversed-phase HPLC on (A) Vydac C4, (B) Vydac phenyl, and (C) Vydac C18 columns. The bar denotes the peak containing urotensin II-like immunoreactivity and the arrows show where peak collection began and ended.

covered from an analytical Vydac phenyl column as a discrete early eluting peak (Fig. 2B) and the peptide was purified to near homogeneity, as assessed by symmetrical peak shape, by chromatography on an analytical Vydac C18 column (Fig. 2C). The final yield of pure material, estimated from the area of the peak, was approximately 400 pmol.

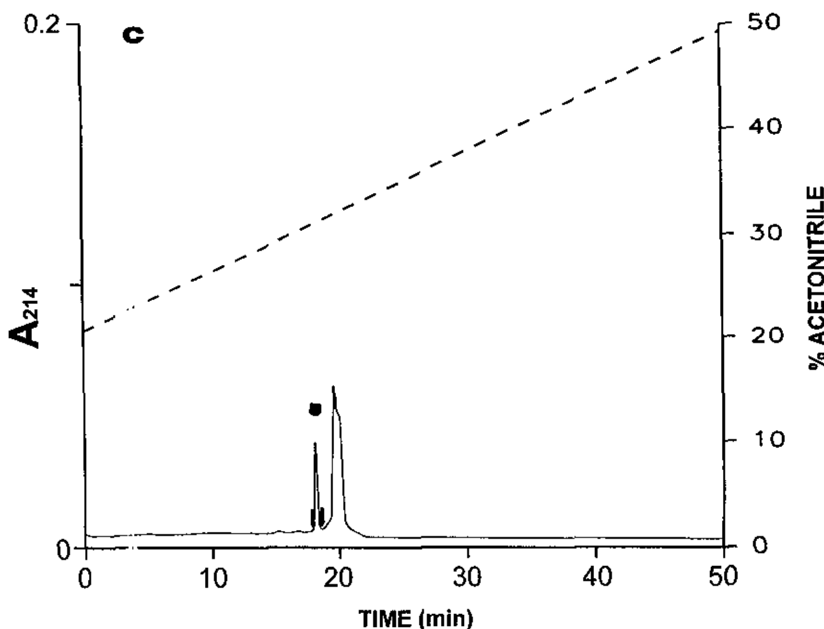

FIG. 2—*Continued*

Purification of Lamprey Urotensin II

The elution profile of an extract of river lamprey brain on a semipreparative Vydac C18 column is shown in Fig. 1B. UII-LI was associated with the well-resolved peak shown by the bar. Rechromatography of this peak on an analytical Vydac C4 column (Fig. 3A) showed that the material was heterogenous and the UII-LI was eluted in the minor peak delineated by the arrows. River lamprey UII was purified to near homogeneity by a final chromatography on an analytical Vydac C18 column (Fig. 3B). The final yield of pure peptide was approximately 300 pmol. UII from the sea lamprey was purified to near homogeneity under the same experimental conditions (chromatograms not shown) and the final yield of pure peptide was approximately 200 pmol.

UII-LI was not detected in the crude extract of sea lamprey spinal cord or in fractions of chromatographic effluent following chromatography of the extract on a Vydac C18 column under the conditions shown in Fig. 1.

Structural Characterization

As the amounts of the pure UII peptides were low, it was decided to subject approximately 90% of the material to automated Edman degradation and the results are shown in Table 1. It was possible to establish without ambiguity the amino acid sequence of the three peptides. No phenylthiohydantoin-coupled amino acids were detected during cycles 6 and 11 of the sequence analysis, consistent with the presence of a cystine bridge in the peptides. The proposed primary structures of the UII-related peptides were confirmed by mass spectrometry. The observed molecular mass of paddlefish UII was 1406.4 ± 0.3 compared with a calculated mass (assuming the presence of a cystine bridge) of 1406.6 for the proposed structure. The observed molecular mass of *Petromyzon* UII was 1522.8 ± 0.3 and the observed molecular mass of *Lampetra* UII was 1522.6 ± 0.3 compared with a calculated mass of 1522.6 for both peptides. It is concluded that the primary structure of paddlefish UII is identical to that of sturgeon UII (McMas-

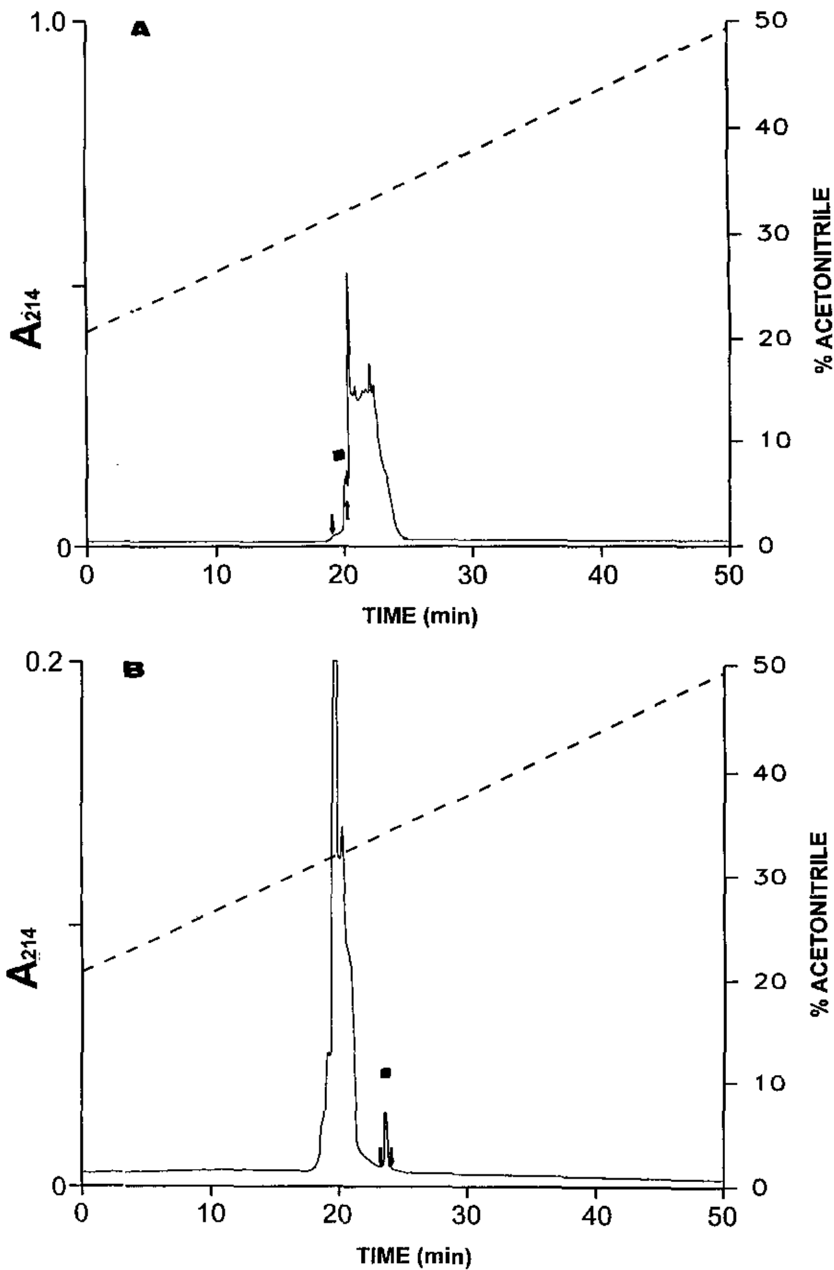

FIG. 3. Purification of urotensin II from river lamprey brain by reversed-phase HPLC on (A) Vydac C4 and (B) Vydac C18 columns. The bar denotes the peak containing urotensin II-like immunoreactivity and the arrows show where peak collection began and ended.

ter *et al.*, 1992) and the river lamprey and sea lamprey UII peptides are identical to UII from the spotted dogfish (Conlon *et al.*, 1992a) and the long-nosed skate (Waugh and Conlon, 1993).

DISCUSSION

Several morphological studies have concluded that the Agnatha lack a neurosecretory system in the caudal region of the spinal cord

TABLE 1
AUTOMATED EDMAN DEGRADATION OF UROTENSIN II FROM PADDLEFISH AND LAMPREY

	Paddlefish		Sea lamprey		River lamprey	
Cycle	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)
1	Gly	76	Asn	19	Asn	21
2	Ser	18	Asn	24	Asn	27
3	Thr	14	Phe	26	Phe	31
4	Ser	10	Ser	7	Ser	9
5	Glu	26	Asp	13	Asp	17
6	N.D.		N.D.		N.D.	
7	Phe	38	Phe	17	Phe	25
8	Trp	11	Trp	6	Trp	13
9	Lys	13	Lys	9	Lys	12
10	Tyr	14	Tyr	9	Tyr	14
11	N.D.		N.D.		N.D.	
12	Val	4	Val	2	Val	3

Note. N.D., not detected.

(Speidel, 1922; Sterba, 1962; Fridberg and Bern, 1968). Immunohistochemical studies have failed to demonstrate the presence of UII-containing structures in the caudal spinal cord of the hagfish, *Epatretus stouti* (Onstott and Elde, 1986), and the lampreys, *P. marinus* (Onstott and Elde, 1986) and *Lampetra japonica* (Owada *et al.,* 1985). Similarly, UII-like biological activity was not detected in extracts of the spinal cords of the hagfish, *Polisostrema stouti,* and lamprey, *Entosphenus reissneri* (Bern *et al.,* 1973). The results of the present study, showing that UII-like immunoreactivity is not detectable in an extract of the spinal cord of the sea lamprey *P. marinus,* are consistent with these earlier observations. However, convincing chemical evidence has been provided that extracts of the brains of the sea lamprey and river lamprey *L. fluviatilis* contain UII. In view of the strong phylogenetic connection between lampreys and the earliest vertebrates, this result suggests the hypothesis that UII may have functioned as a neurotransmitter and/or neuromodulator in the central nervous systems of the anscestral vertebrates before adopting the additional and/or alternative role as a neurohormone in the caudal neurosecretory system of more highly evolved species. Recent immunohistochemical studies have shown that in the frog *R. ridibunda,* which also lacks a defined caudal neurosecretory system, dense networks of UII-immunoreactive fibers are present in various regions of the brain, including the thalamus, tegmentum, tectum, and granular layer of the cerebellum (Chartrel *et al.,* 1995). This suggests that in this tetrapod, as in lampreys and some teleosts (Yulis and Lederis, 1986), the peptide is involved in regulation within the central nervous system. The observation that an antiserum to goby UII will immunostain neuronal fibers in the cerebral ganglia of the mollusc *Aplysia californica* (Gonzalez *et al.,* 1992) suggests that the gene encoding UII may have arisen even before the appearance of vertebrates.

The morphology of the caudal neurosecretory system of the paddlefish *P. spathula* has been studied in detail (Brady, 1984) and it was concluded that the organization of the system is intermediate between the diffuse caudal neurosecretory system of of elasmobranchs and the more highly organized and compact system of teleosts. Although a ventral swelling or urophysis is not present, the caudal spinal region of the paddlefish exhibits a rich vascular network on its ventral aspect that is absent from the anterior spinal cord, and the capillaries of this network are in close association with the Dahlgren cell perikarya and cell processes. The terminations

of these processes are intensely basophilic and are associated with herring body-like structures indicative of high concentrations of stored neurosecretory products. Immunohistochemical studies have shown strong UII-immunoreactivity in neuronal structures throughout the paddlefish caudal spinal cord with the exception of the dorsal horns (Onstott and Elde, 1986). A similar but less intense pattern of immunostaining was observed using an antiserum raised against ovine corticotropin releasing hormone (CRH) that was reactive toward *Catostomus* urotensin I (UI). The present investigation had provided firm chemical evidence that the caudal neurosecretory system of the paddlefish synthesizes a peptide that is structurally similar to the UII peptides of the teleost fish. A peptide with CRH-LI was also isolated in low yield (approximately 100 pmol) from the paddlefish spinal cord extract (unpublished data). It was possible to determine only the amino acid sequence at the N-terminus of the peptide. This sequence (Ser-Glu-Asp-Pro-Pro-Ile-Ser-Ile-Asp-Ile. . .) is sufficiently similar to the corresponding region of *Catostomus* UI (Lederis *et al.,* 1982) (Asn-Asp-Asp-Pro-Pro-Ile-Ser-Ile-Asp-Leu. . .) to suggest that this peptide represents paddlefish UI. Further work is required to determine its complete primary structure. CRH-LI was not detected in the crude extracts of either sea lamprey spinal cord or brain in the present study.

The primary structures of the UII peptides from the lampreys and paddlefish are compared with the corresponding peptides isolated from other classes of fish in Fig. 4. The data indicate that the complete primary structure of UII has been better conserved among the phylogenetically more ancient species that among the modern teleosts examined thus far. The amino acid sequence of UII from both species of lamprey is identical to that of UII from the elasmobranch fish, the spotted dogfish *S. canicula* (Conlon *et al.,* 1992a), and the skate *Raja rhina* (Waugh and Conlon, 1993). The structure of paddlefish UII is identical to that of UII from the chondrostean fish, the sturgeon *A. ruthenus* (McMaster *et al.,* 1992). In contrast, the data in Fig. 4 show that the amino acid sequence of teleost UII peptides has been relatively poorly conserved, particularly in the N-terminal region. The observation that dogfish UII is at least 10-fold more potent than goby UII in elevating

Lamprey	Asn-	Asn-	Phe-	Ser-	Asp-	Cys-	Phe-	Trp-	Lys-	Tyr-	Cys-	Val
Dogfish/Skate	-	-	-	-	-	-	-	-	-	-	-	-
Paddlefish	Gly-	Ser-	Thr	-	Glu	-	-	-	-	-	-	-
Sturgeon	Gly-	Ser-	Thr	-	Glu	-	-	-	-	-	-	-
Goby	Ala-	Gly-	Thr-	Ala	-	-	-	-	-	-	-	-
Sucker	Gly-	Ser-	Gly-	Ala	-	-	-	-	-	-	-	-
Sucker	Gly-	Ser-	Asn-	Thr-	Glu	-	-	-	-	-	-	-
Carp	Gly-	Gly-	Gly-	Ala	-	-	-	-	-	-	-	-
Carp	Gly-	Gly-	Asn-	Thr-	Glu	-	-	-	-	-	-	-
Carp	Gly-	Ser-	Asn-	Thr-	Glu	-	-	-	-	-	-	-
Carp	Gly-	Gly-	Gly-	Ala	-	-	-	-	-	-	-	Ile
Flounder	Ala-	Gly-	Thr-	Thr-	Glu	-	-	-	-	-	-	-

FIG. 4. A comparison of the primary structures of peptides related to urotensin II from species of different vertebrate taxa. (—) denotes residue identity.

arterial blood pressure in the dogfish indicates that structural changes in the N-terminal region of the peptide affect biological activity (Hazon *et al.*, 1993).

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