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Floor Plate and the Subcommissural Organ are the Source of Secretory Compounds of Related Nature: Comparative Immunocytochemical Study

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ABSTRACT

The subcommissural organ of vertebrates secretes glycoproteins into the third ventricle that condense to form Reissner's fiber (RF). Antibodies raised against the bovine RF-glycoproteins reacted with the floor plate (FP) cells of two teleost (*Oncorhynchus kisutch*, *Sparus aurata*) and two amphibian (*Xenopus laevis*, *Batrachyla taeniata*) species. At the ultrastructural level, the immunoreactivity was confined to secretory granules, mainly concentrated at the apical cell pole. In the rostro-caudal axis, a clear zonation of the FP was distinguished, with the hindbrain FP being the most, or the only (*Batrachyla taeniata*), immunoreactive region of the FP. In all the species studied, the caudal FP lacked immunoreactivity. Both the chemical nature of the immunoreactive material and the rostro-caudal zonation of the FP appear to be conservative features. Evidence was obtained that the FP secretes into the cerebrospinal fluid a material chemically related to the RF-glycoproteins secreted by the subcommissural organ. Thus, in addition to being the source of contact-mediated and diffusible signals, the FP might also secrete compounds into the cerebrospinal fluid that may act on distant targets. J. Comp. Neurol. 392:19–34, 1998.

Indexing terms: flexural organ; Reissner's fiber; teleosts; amphibians

The floor plate (FP) is a transient structure of the embryonic central nervous system (CNS). It is formed by neuroepithelial cells organized as a distinct midsagittal column, one to a few cells wide, in the ventral midline of the neural tube (Altman and Bayer, 1984; Bronner-Fraser, 1994). Together with the notochord, the FP plays a key role during early stages of CNS development, in the differentiation and spatial distribution of neurons of the ventral neural tube. This function seems to be performed through the release of several contact-mediated and diffusible factors, which act on neighboring neuroblasts to promote their differentiation into functionally distinct neuronal classes with a defined rostro-caudal and dorso-ventral pattern within the neural tube (Hirano et al., 1991; Yamada et al., 1991, 1993). It has also been postulated that FP chemotactic factors participate in the guidance of axons that cross the ventral commissure (Bovolenta and Dodd, 1990; Placzek et al., 1990).

The subcommissural organ (SCO) is a discrete region of the roof plate of the prosencephalon that differentiates

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very early in ontogenesis (Naumann, 1986; Schoebitz et al., 1986; Meiniel et al., 1990; Karoumi et al., 1990) and that remains fully active during the life span. It is formed by secretory ependymal and hypendymal cells, arranged in two cell layers, located in the roof of the third ventricle, at the entrance of the cerebral aqueduct (Oksche, 1961, 1969; Leonhardt, 1980; Rodríguez et al., 1992). It releases glycoproteins into the ventricular cerebrospinal fluid, where they condense to form a single fibrous structure known as Reissner's fiber (RF). RF extends along the aqueduct, the fourth ventricle and the central canal of the spinal cord. It ends at a terminal dilatation of the central canal located in the filum; here RF-glycoproteins undergo chemical changes before entering the blood stream (Peruzzo et al., 1987). RF grows caudally, at a constant rate, by addition of newly released glycoproteins to its rostral end (Rodríguez et al., 1992).

Polyclonal antibodies raised in rabbits against RF-glycoproteins (anti-RF) have been used for immunocytochemical surveys of the CNS of representative species of the vertebrate phylum, from cyclostomes to man (Sterba et al., 1981, 1982; Rodríguez et al., 1984). It has been demonstrated that the secretory material contained in the SCO of most species displays strong and specific immunoreactivity with the anti-bovine RF sera used. This indicates that at least some domains of the SCO glycoproteins have been conserved throughout evolution. The material recognized by these antibodies consists of N-linked high molecular weight glycoproteins (Nualart et al., 1991, 1996; Grondona et al., 1994; López-Avalos et al., 1995).

There is evidence indicating that RF-material is produced in CNS regions other than the SCO. Two structures of the ventral ependyma, the infundibular organ of cephalochordates (Olsson and Wingstrand, 1954; Sterba et al., 1983; Olsson et al., 1994) and the flexural organ, described in some anamniote embryos (Olsson, 1956, 1958; Naumann, 1986; Naumann et al., 1993; Schoebitz et al., 1993), also secrete RF-material. The infundibular organ is the exclusive RF-producing organ of cephalochordates. The material secreted by the infundibular organ cells is recognized by antibodies raised against the bovine RF (Olsson et al., 1994), thus supporting the conservative nature of the RF-glycoproteins.

The flexural organ is, in actuality, equivalent to the FP extending between the pontine and mesencephalic flexures, that is the rostralmost region of the notochordal FP (Olsson, 1993; Rodríguez et al., 1996). The flexural organ has been considered as a transient source of RF-material preceding the onset of the secretory activity of the SCO (Olsson, 1956, 1958). Studies on embryonic specimens of *pleurodeles* and rat (Naumann, 1986; Schoebitz et al., 1993; Rodríguez et al., 1996) have shown that the cells forming the flexural organ display strong immunoreactivity with anti-bovine RF sera. In the rat, the combined use of an anti-bovine RF serum and a specific marker for the rat FP cells has allowed to demonstrate that not only the hindbrain FP but also the FP of the spinal cord secrete a material recognized by the anti-bovine RF serum (Rodríguez et al., 1996).

Most FP markers available at present are antibodies against cell surface antigens (Placzek et al., 1993), usually displaying a high degree of species specificity. The anti-RF sera react with a material secreted by the FP of mammalian and non-mammalian species, thus making comparative immunocytochemical studies of the secretory activity of the FP possible. This led us to perform the present study, using four non-mammalian species, with the aim to establish in each of them: (1) the time course and spatial distribution of the FP cells reactive with the anti-RF serum; (2) the secretory nature of the material being recognized in the FP cells by the anti-RF serum; (3) the involvement of FP cells in the formation of the "first RF" in anamniotes. For this comparative analysis, the findings obtained in a similar study in the dogfish FP (López-Avalos et al., 1997) and the rat FP (Rodríguez et al., 1996) have also been considered. Important species differences were observed concerning the time course and spatial distribution of the immunoreactivity of the FP to anti-RF sera. There was, however, one finding consistent for all the investigated species, namely, the strong immunoreactivity of the FP of the hindbrain, also known as flexural organ.

MATERIALS AND METHODS

Specimens of *Oncorhynchus kisutch* (Coho salmon), *Sparus aurata* (Gilthead seabream), *Xenopus laevis* (South African clawed frog), and *Batrachyla taeniata* (Brown toad), at different embryonic and early post-hatching stages of development were utilized.

Oncorhynchus kisutch

Fourteen groups, five to 10 specimens each, ranging from 122–727 accumulated temperature units (TUs; Brown and Gratzek, 1980) were used. The average water temperature was approximately 9°C, hence nine TUs equal 1 day. Hatching occurred at about 440 TUs (approximately 49 days of development; Fig. 1). The specimens were fixed by immersion in Bouin's fluid, embedded in Paraplast, and microtome sectioned at 7 µm. Serial sections were mounted on gelatin coated glass slides and processed for immunocytochemistry and lectin histochemistry. Additionally, specimens of 220, 264, 340, and 420 TU were fixed by immersion in 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer pH 7.5, and embedded in Lowicryl K4M according to a protocol described elsewhere (Peruzzo and Rodríguez, 1989). Ultrathin sections were mounted on uncoated nickel grids and processed for immunocytochemistry or for a double immunocytochemistry-lectin histochemistry procedure.

Sparus aurata

Six groups of five specimens each, aged between 20–120 hours after fertilization, were fixed by immersion in Bouin's fluid. Hatching occurred about 50 hours after fertilization (Fig. 1). The fixed specimens were embedded in Paraplast and microtome sectioned at 8 µm. Serial sections were mounted on gelatin coated glass slides and processed for immunocytochemistry. Additionally, 80-hour-old specimens were fixed by immersion in 4% paraformaldehyde in order to perform an in toto immunocytochemistry procedure.

Xenopus laevis

Ten groups of five specimens each, aged between 23–146 hours after fertilization, were fixed by immersion in Bouin's fluid. Hatching occurred about 37 hours after fertilization (Fig. 1). The fixed specimens were embedded in Paraplast and microtome sectioned at 10 µm. Serial sections were mounted on gelatin coated glass slides and processed for immunocytochemistry and lectin histochemistry. Addi-

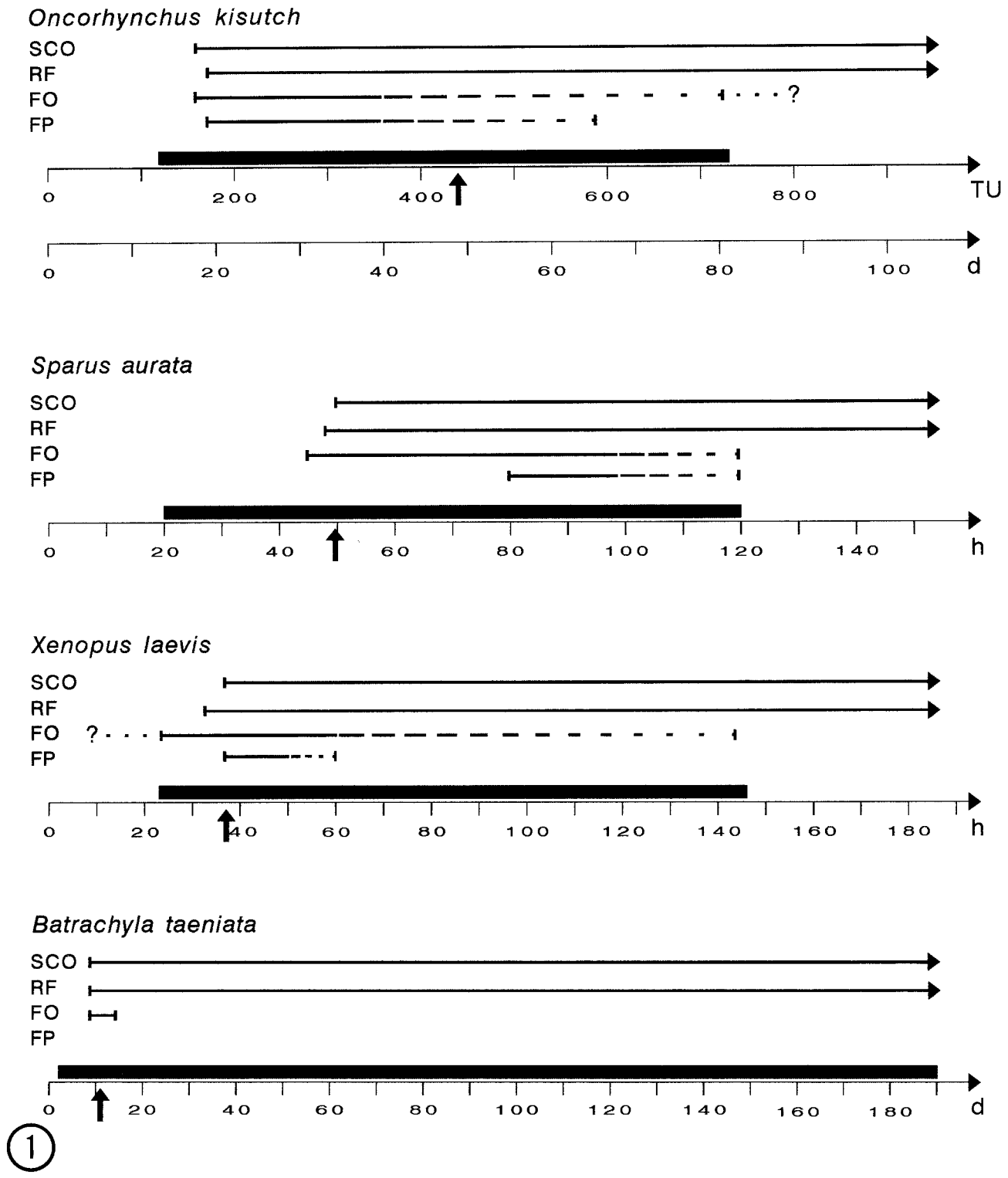

Fig. 1. Diagram representing the time course of AFRU (A = antibody, FR = fiber of Reissner, u = urea)-immunoreactivity (immunoreaction performed using AFRU sera at a 1:1,000 dilution, solid and broken lines) in the central nervous system of *Oncorhynchus kisutch*, *Sparus aurata*, *Xenopus laevis*, and *Batrachyla taeniata*, at different embryonic and early post-hatching stages of development. The length of the dashes forming the broken line indicates intensity of immunoreactivity. Arrow, hatching; Numbers; time (h, hours; d, days) or accumulated temperature units (TU) after fertilization; FO, flexural organ; FP, floor plate; RF, Reissner's fiber; SCO, subcommissural organ; Thick solid line, sampling period.

tionally, 27- and 60-hour-old specimens were fixed by immersion in a triple aldehyde mixture, containing 4% paraformaldehyde, 2% glutaraldehyde, and 1% acrolein (Rodríguez, 1969), followed by postfixation in 1% OsO4, and embedded in a mixture of Epon and Araldite. Semithin sections stained with toluidine blue were used for orientation. Ultrathin sections mounted on copper grids were stained with uranyl acetate and lead citrate.

Batrachyla taeniata

A total of 202 specimens aged between 2–190 days after fertilization were fixed by immersion in Bouin's fluid. Hatching occurred approximately on day 11 (Fig. 1). The fixed specimens were embedded in Paraplast and microtome sectioned at 8 µm. Serial sections were mounted on gelatin coated glass slides and processed for light microscopy immunocytochemistry. Additionally, specimens belonging to the 12-day-old group were fixed by immersion in a triple aldehyde mixture (Rodríguez, 1969), followed by postfixation in 1% OsO4, and embedded in a mixture of Epon and Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate.

Light microscopy immunocytochemistry

Sections of the CNS of *Oncorhynchus*, *Sparus*, *Xenopus*, and *Batrachyla*, at different stages of development, were processed according to the immunoperoxidase method of Sternberger et al. (1970) by using an antiserum raised in rabbits against bovine Reissner's fiber glycoproteins extracted in a medium containing urea (AFRU, A = antibody, FR = fiber of Reissner, U = urea; Rodríguez et al., 1984). AFRU was used at a 1:1000 dilution; incubation was at room temperature (RT) for 18 hours. Anti rabbit IgG (raised in sheep) was used as a secondary antibody (1:10 dilution, 30 minutes, RT). Rabbit peroxidase-antiperoxidase complex (PAP) was used at a 1:50 dilution (30 minutes, RT). Finally, the sections were incubated in 0.2% diaminobenzidine, 0.03% H_2O_2 in Tris buffer, pH 7.8 (15 minutes, RT). The sections were washed in Tris buffer between each step. All antibodies were diluted in Tris buffer containing 0.7% lambda carrageenan (Sigma, St. Louis, MO) and 0.5% Triton X-100.

Light microscopy lectin histochemistry

Sections of the CNS of *Oncorhynchus* and *Xenopus*, at different stages of development, were incubated with the following lectins: (1) peroxidase labeled wheat germ agglutinin (WGA; affinity = sialic acid, glucosamine) 1.25 µg/ml in Tris buffer, pH 7.8, 45 minutes, RT; (2) peroxidase labeled concanavalin A (Con A; affinity = glucose, mannose) 2.5 µg/ml in Tris buffer, pH 7.8, 45 minutes; (3) non-labeled Limax flavus agglutinin (LFA; affinity = sialic acid) 1 µg/ ml in Tris buffer, pH 7.8, 45 minutes. Incubations with WGA and Con A were followed by a developing step in 0.2% diaminobenzidine, 0.03% H_2O_2 in Tris buffer (15 minutes, RT). Incubation with LFA was followed by an immunocytochemistry procedure using an anti-LFA serum (diluted 1:5,000) raised in our laboratory (Rodríguez et al., 1990) according to the protocol described above.

Ultrastructural immunocytochemistry and lectin histochemistry

Ultrathin Lowicryl sections of *Oncorhynchus* were processed for immunocytochemistry, or for a double immunocytochemistry-lectin histochemistry procedure, using the protein A-gold (pAg) method (Roth, 1983) according to the modifications introduced by Peruzzo and Rodríguez (1989). Briefly, the immunocytochemical procedure consisted of the following steps: 1) washes in phosphate buffered saline (PBS, pH 7.2); 2) 1% bovine serum albumin (BSA) in PBS, 30 minutes, RT; 3) first antiserum (AFRU, see above) diluted 1:1,000 in PBS-BSA 1%, 18 hours, 4°C; 4) washes in PBS; 5) pAg (15 nm gold particles) diluted 1:10 in PBS from a stock solution, 45 minutes, RT; 6) washes in PBS; 7) washes with distilled water. The double labeling consisted of a complete immunostaining procedure performed on one face of the grid (using AFRU and colloidal gold particles of 15 nm as described above) followed by a lectin binding procedure on the opposite face. The lectin histochemistry method (Peruzzo and Rodríguez, 1989) involved: 1) washes in Tris buffer, pH 7.8; 2) non-labeled WGA, 1 µg/ml Tris buffer, 45 minutes, RT; 3) washes in Tris buffer; 4) anti-WGA serum, diluted 1:1,000 in Tris buffer, 1 hour, RT; 5) washes in Tris buffer; 6) pAg (5 nm gold particles) diluted 1:10 in Tris buffer from a stock solution, 45 minutes, RT; 7) washes in Tris buffer; 8) washes with distilled water. After completion of both methods, the grids were stained with uranyl acetate (2% aqueous solution, 7 minutes) and lead citrate (2 minutes), and then carbon coated.

The experimental protocols used in this investigation have been approved by animal care committees of the participating Institutions.

RESULTS

Oncorhynchus kisutch

Immunoreactivity with AFRU appeared simultaneously in the SCO and the FP (Fig. 1) at the stage of 160 TU (18 days). The immunoreaction was most evident in the rostralmost end of the notochordal FP known as the flexural organ (FO; Olsson, 1956, 1993). As development proceeded, the FO and the SCO displayed increased immunocytochemical reactions as a result of an increased number of AFRU-immunoreactive (ir) cells and an increase in their immunoreactivity. The SCO was comprised of elongated ependymal cells arranged as a palisade below the posterior commissure (Fig. 2A). Immunoreactive hypendymal cells were never detected at the developmental stages studied. Basal processes of variable length were observed originating from the SCO cells and traversing the posterior commissure to end on the external limiting membrane of the brain (Fig. 2B,C). The RF proper was first present in the 170 TU specimens (Fig. 1). At earlier stages, aggregated AFRU-ir material not organized as a RF proper, was observed irregularly distributed inside the brain cavities

Fig. 2. Sections through the brain of *Oncorhynchus* embryos (230 TU, 26-day-old) immunostained with AFRU. **A**: Midsagittal section showing intense immunostaining of the SCO and FO. Reissner's fiber is seen in its trajectory from the SCO toward the surface of the FO, and then entering the central canal. **B,C**: Sagittal and frontal sections, respectively, of the SCO. Note the intense immunostaining in the SCO cells and in basal processes projected toward the external basal lamina of the brain (arrows). **D**: High magnification of the FO shown in a. Elongated FO cells display accumulations of AFRU-immunoreactive (ir) material in the apical cytoplasm, and in short basal processes (arrow). AFRU-ir cells extend caudad along the FP. CC, Central canal; FO, flexural organ; FP, floor plate; PC, posterior commissure; RF, Reissner's fiber; SCO, subcommissural organ. Scale bars = 50 µm in A, 20 µm in b–d.

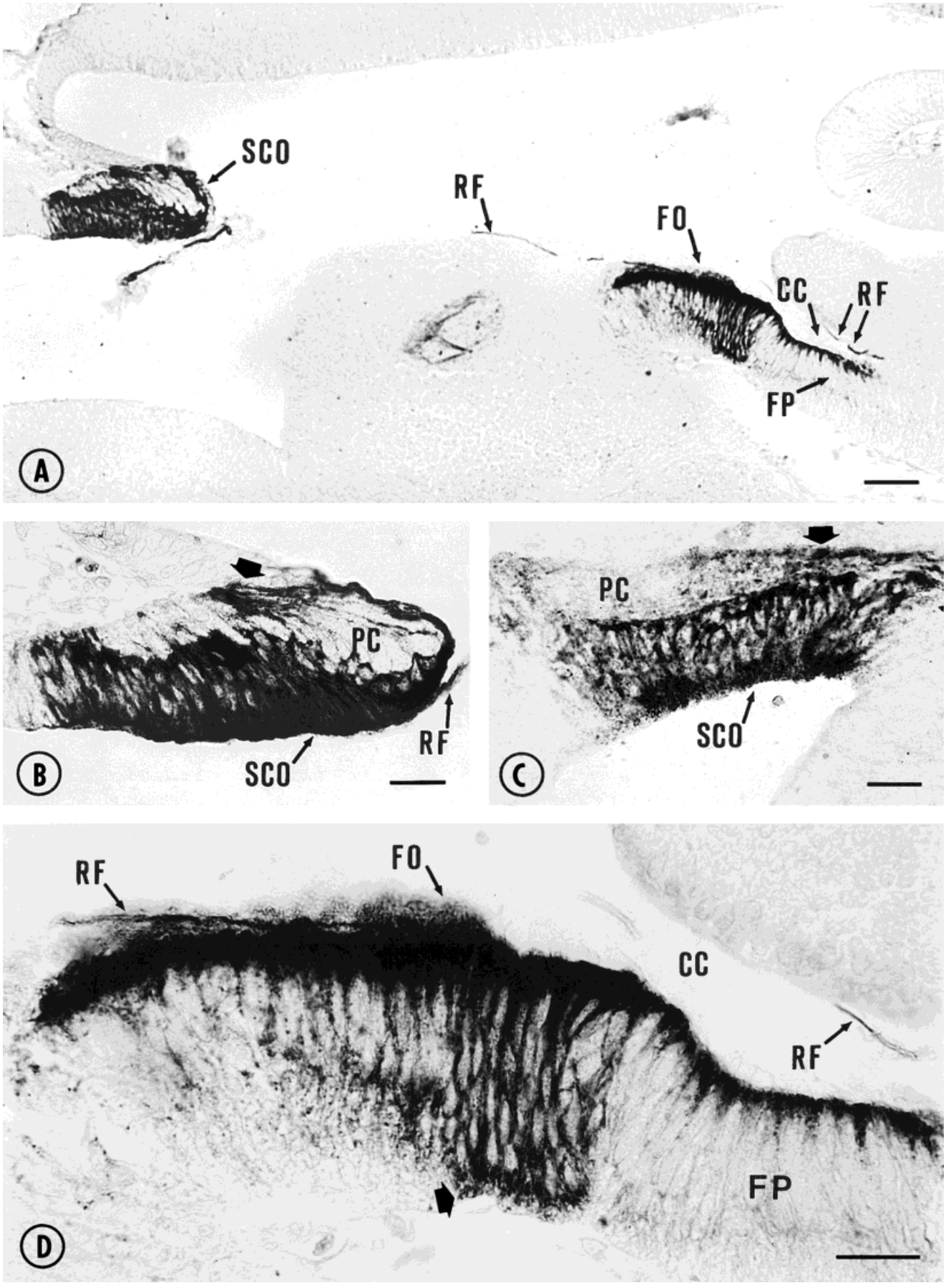

Figure 2

forming a coat on the surface of the ventricle walls. In this species, the first RF observed originated in the SCO (Fig. 2). In its trajectory to the central canal, RF established a close spatial relationship with the apical surface of the FO (Fig. 2D).

The strongest AFRU reactivity of the FO was observed in specimens between 200 and 300 TU (Figs. 2D, 3A). The FO could be clearly distinguished by the elongated shape of its cells, with the nuclei located in the intermediate and basal regions of the cytoplasm. These cells displayed intense immunoreactivity, which was more prominent in the apical and basal cell poles (Figs. 2D, 3A). AFRU-ir processes originated from the basal pole of the FO cells projected ventrally, and ended on the external limiting membrane of the brain (Figs. 2D, 3A).

The light microscopy lectin histochemistry procedure revealed that the FO and FP cells of *Oncorhynchus* bound WGA and LFA; binding of Con A rendered inconsistent results.

Ultrastructural immunocytochemistry showed that the reaction of AFRU was confined to a material contained in secretory granules distributed throughout the cytoplasm of the FO cells (Fig. 3C,D,E). Clusters of immunoreactive secretory granules were usually observed in the apical cytoplasm of the FO cells, close to the apical membrane (Fig. 3C). The combined use of AFRU-immunocytochemistry and WGA-binding on the same ultrathin sections showed a colocalization of both labels in secretory granules of the FO cells (Fig. 3D).

Specimens older than 19 days (170 TU) displayed, in addition to the reaction in the SCO and FO, AFRU-immunoreactivity in cells of the FP of the spinal cord (Fig. 1). This reaction was mainly concentrated in the apical cytoplasm and increased in intensity as development proceeded. Similarly to the FO cells, the spinal cord FP displayed the strongest reactivity in specimens between 200 and 300 TU. The immunoreactive FP cells were arranged as a midsagittal column located in the ventral wall of the central canal (Figs. 2A,D, 3B, 4A). Rostrally, the column of immunoreactive FP cells was in continuity with the FO (Fig. 2A,D). In this rostral region, fibrils of AFRU-ir material detaching from the surface of the FO and FP cells to merge with the RF proper were usually observed (Fig. 3B). The column of immunoreactive FP cells extended caudally, always in close relationship with the RF located inside the central canal (Fig. 4A). The AFRU-immunoreactivity of FP cells decreased gradually toward the caudal spinal cord; close to the ampulla caudalis the FP did not react with AFRU.

After reaching the 300 TU stage the specimens showed a decrease in the immunoreactivity of the FO and FP cells, and an increased reactivity of the SCO. The AFRU-immunoreactivity of the FP cells decreased by following a progressive pattern from caudal to rostral, the immunostaining of the FO being the latest to disappear. The last stage in which AFRU-immunoreactivity in the FO was observed was the 725 TU (Fig. 1). Specimens at this stage displayed a faint reaction in the FO contrasting with the strong reaction of the SCO (compare Fig. 4B,C).

Sparus aurata

AFRU-immunoreactivity in the FO cells first appeared, as a weak reaction, in the 45-hour-old specimens, become much stronger in older specimens and was missing in specimens sacrificed between 100 to 120 hours after fertilization (Fig. 1). The immunostaining had a homogeneous distribution throughout the cell body and basal processes of the FO cells. FP cells of the spinal cord became immunoreactive in the 80-hour-old specimens and the duration of this reactivity paralleled that of the FO (Fig. 1).

An immunoreactive RF was first observed in the lumen of the caudal neural tube of 48-hour-old specimens (insert to the right in Fig. 5A). At this stage, the FO already displayed AFRU-immunoreactivity, whereas the SCO was negative (Figs. 1, 5A). A faint immunoreaction appeared in the SCO in 50-hour-old embryos contrasting with the intense immunostaining displayed by the FO (Fig. 5B). In 80-hour-old specimens the RF was observed originating in the SCO, from where it approached to the surface of the FO cells before entering the central canal (Fig. 5C). A longitudinal layer of AFRU-ir material, probably formed by AFRU-ir material accumulated in the apical pole of the FP cells, was also evident in the in toto preparation (Fig. 5C).

Xenopus laevis

In *Xenopus* a clear distinction between the FO and FP cells could not be made since they constitute a homogeneous cell population forming a continuous cell column. For descriptive purposes, those cells located in the rostral end, in the region of the ventral brain fold, will be regarded as FO. Those located in the spinal cord will be referred to as FP.

Those specimens processed 23 hours after fertilization already displayed an AFRU-reactive FO. The reaction in the FO reached its strongest intensity between the 41st and the 60th hour of development (Figs. 6A,C); then it decreased progressively to disappear in the 144-hour-old specimens (Fig. 1). The FP cells displayed AFRU-ir material in specimens collected between 37 and 60 hours after fertilization (Fig. 1). The AFRU-ir cells of the FO and FP were cylindrical, displaying a homogeneous immunostaining throughout the cytoplasm and forming a well-defined column closely associated with the underlying notochord. Midventral brain cells located rostrad to the cephalic tip of the notochord did not show AFRU-immunoreactivity. Caudally, the column of immunoreactive FP cells extended to the rostral third of the spinal cord. In the middle third, immunoreactive and non-immunoreactive cells intermingled. The FP of the caudal third of the spinal cord did not react with AFRU (Fig. 6A).

The presence of RF in the central canal and ampulla caudalis (Fig. 6E) was first observed in the 33-hour-old specimens. At this stage, FO cells were already AFRU-ir (Fig. 6D), whereas the SCO became immunoreactive only after 37 hours of development (Fig. 6B).

The light-microscopic lectin histochemistry procedure revealed that the FO and FP cells of *Xenopus* bound WGA and Con A.

The transmission electron microscopic study of the FO and FP cells of the 27-hour-old *Xenopus* specimens revealed features compatible with enhanced metabolic and secretory activities at this early stage of development. The cell nucleus occupied the mediobasal region of the cytoplasm, it was rich in euchromatin and usually displayed one or two indentations (Fig. 7A–C); abundant vitelline inclusions accumulated in the intermediate and basal cytoplasm (Fig. 7B); the apical cytoplasm was filled with mitochondria (Fig. 7B); flattened cisternae of the rough endoplasmic reticulum (RER), arranged in parallel arrays, were concentrated in the perinuclear region (Fig. 7C);

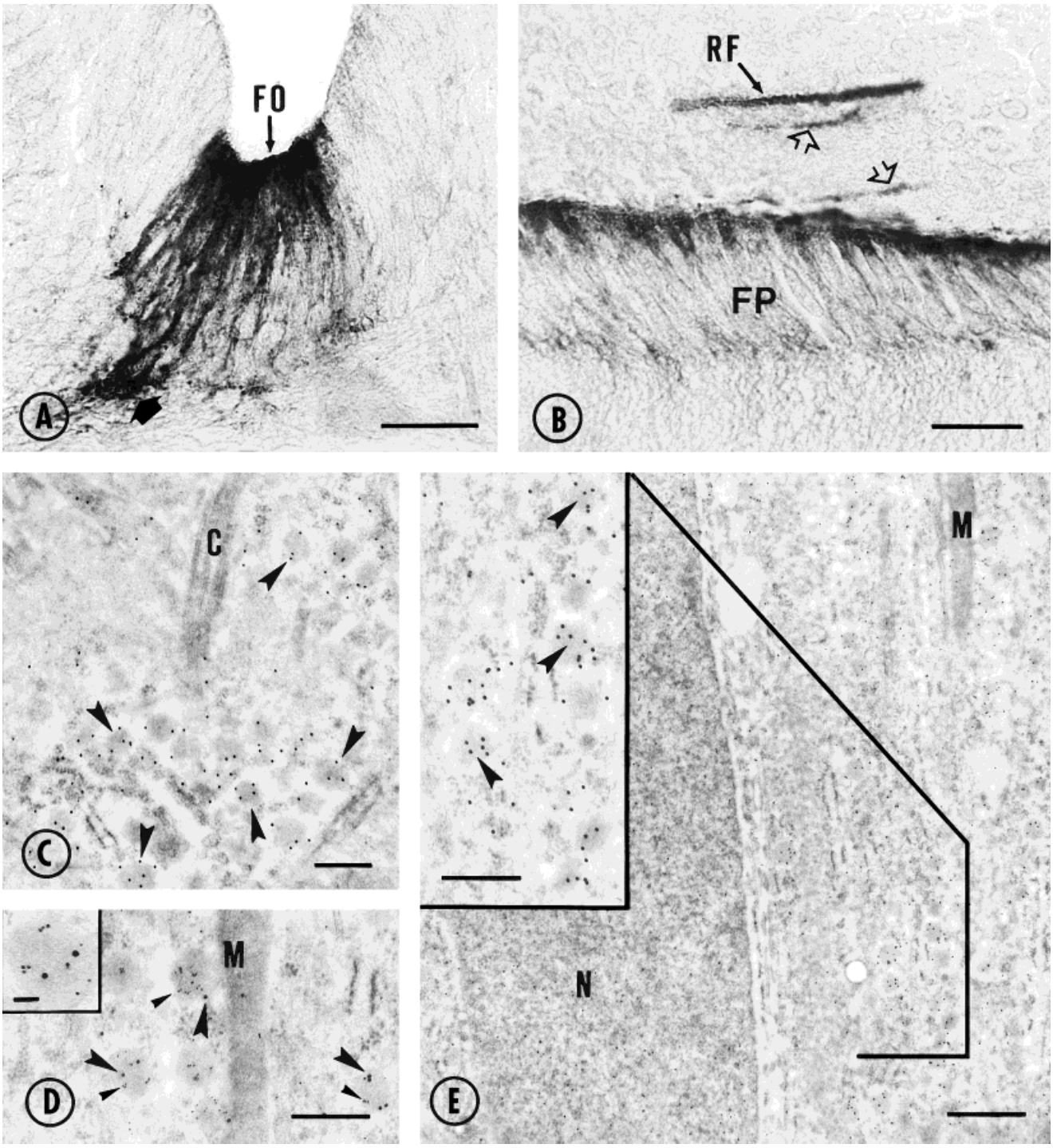

Fig. 3. Light and electron microscopic immunostaining of the FO and FP of *Oncorhynchus* embryos using AFRU. **A**: Frontal section of the FO (265 TU, 29-day-old embryo) showing basal processes ending on the external basal lamina of the brain (thick arrow). **B**: Sagittal section through the rostral end of the spinal cord showing the immunoreactive FP (250 TU, 28-day-old embryo). AFRU-ir fibrils are seen detaching from the surface of immunoreactive FP cells to merge with the RF proper (open arrows). **C**: Electron photo micrograph of the apical cytoplasm of a FO cell (340 TU, 38-day-old embryo) filled with AFRU-ir secretory granules (arrowheads) is shown. **D**: AFRU-ir material (large colloidal gold particles, large arrowheads), and wheat germ agglutinin (WGA) binding sites (small colloidal gold particles, small arrowheads) colocalize in secretory granules of the FO cells. Inset, detailed magnification of a double-labeled granule. **E**: Perinuclear region of a FO cell. The inset shows a higher magnification of an area of the cytoplasm (indicated by the angled line) containing AFRU-ir secretory granules (arrowheads). C, cilium; FO, flexural organ; FP, floor plate; M, mitochondrion; N, nucleus; RF, Reissner's fiber. Scale bars = 20 μm in A and B, 500 nm in C, 500 nm in D, 80 nm in inset, 1,000 nm in E, 500 nm in inset.

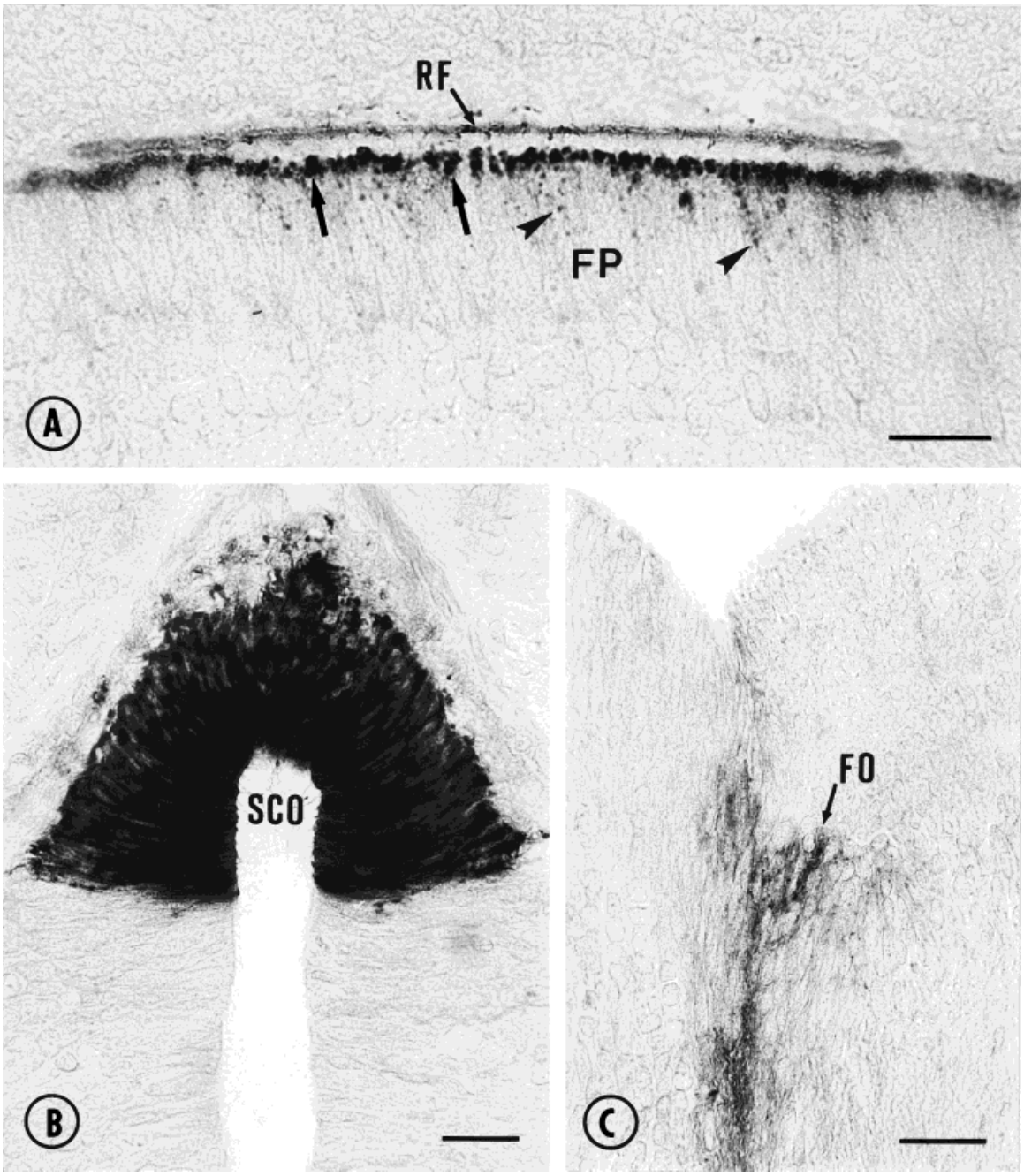

Fig. 4. Immunostaining of the central nervous system (CNS) of *Oncorhynchus* embryos using AFRU. **A**: Sagittal section of the middle third of the spinal cord (280 TU, 31-day-old embryo). The apical cytoplasm of the FP cells is filled with immunoreactive material (arrows) displaying a granular appearance (arrowheads). **B**: Frontal section of the SCO of a 725 TU (81-day-old) embryo. **C**: Sagittal section of the FO of a 725 TU (81-day-old) embryo, showing only a few immunoreactive cells. In this specimen the reaction was missing in other segments of the FP. FO, flexural organ; FP, floor plate; RF, Reissner's fiber; SCO, subcommissural organ. Scale bar = 20 µm in A and B, 30 µm in C.

apical protrusions contained electron-lucent vesicles about 130 nm in diameter and large vacuoles (Fig. 7D). A filamentous material was found on the surface of FO cells (Fig. 8A). The ultrastructure of the FO and FP cells of specimens collected 60 hours after fertilization was essentially similar to that of the 27-hour-old specimens, but a more developed RER and numerous apical protrusions were noticed in the 60-hour-old specimens. In the latter, a typical RF was found in the lumen of the central canal (Fig. 8B). RF was formed by a few bundles of longitudinal filaments ranging between 3–14 nm in thickness, bridged by short transversal filaments ranging between 4–14 nm in thickness (Fig. 8C). Masses of flocculent material were attached to RF (Fig. 8B,C).

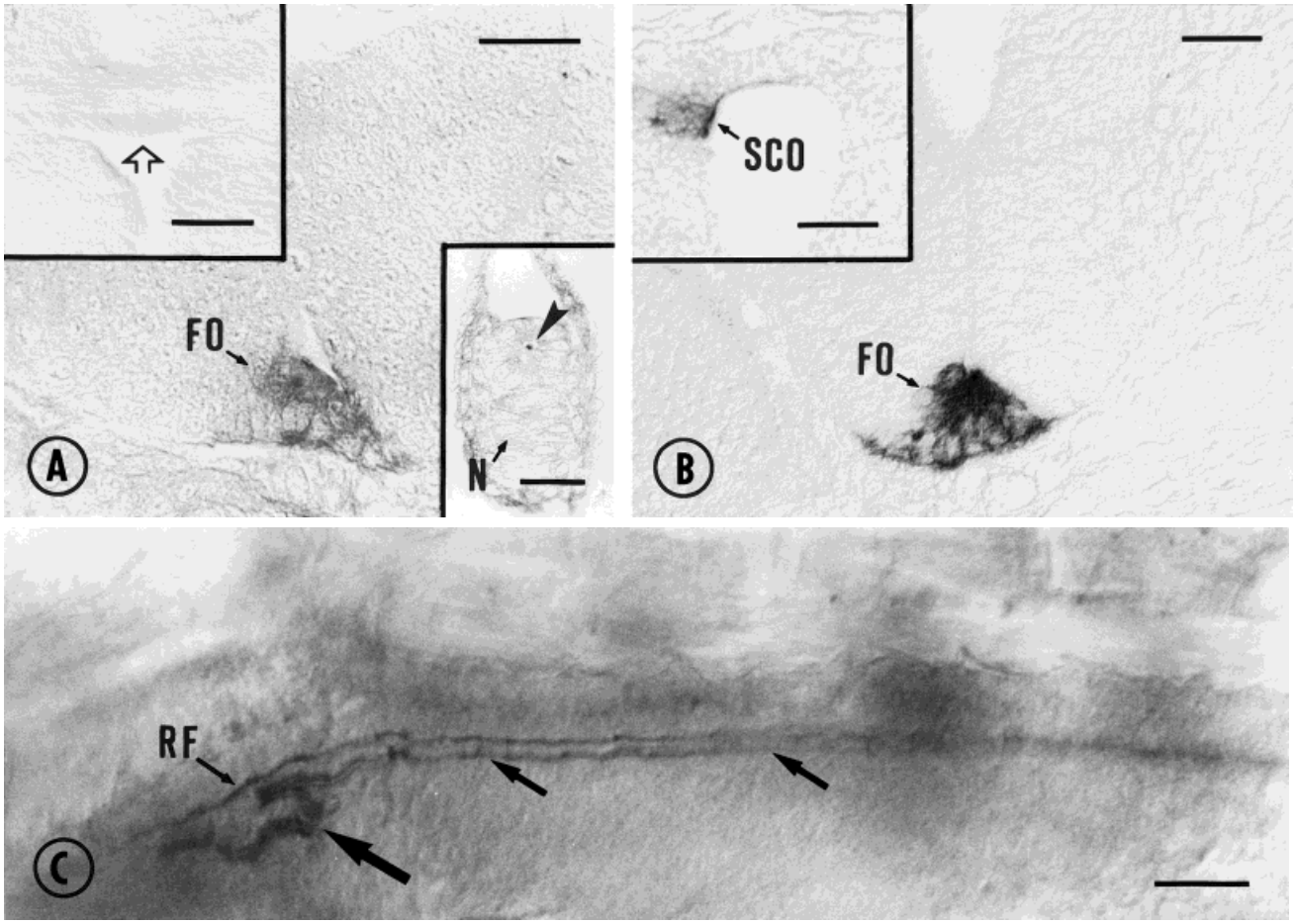

Fig. 5. Immunostaining of the CNS of *Sparus* embryos using AFRU. **A**: Frontal sections of a 48-hour-old embryo showing the presence of AFRU-ir material in the FO and an immunoreactive RF localized in the lumen of the caudal neural tube (arrowhead in inset on the right-hand side). The SCO has not yet differentiated at this stage of development (open arrow in inset on the left-hand side). **B**: Frontal sections of a 50-hour-old embryo showing increased immunostaining of the FO and the appearance of AFRU-ir cells in the SCO (inset). **C**: Whole-mount view of an 80-hour-old embryo immunostained in block with AFRU showing the course of the RF in the rhombencephalic ventricle and the central canal. The continuous AFRU-ir layer beneath the RF appears to stand for AFRU-ir material contained in the FO (large arrow) and in the FP cells of the spinal cord (small arrows). FO, flexural organ; N, notochord; RF, Reissner's fiber; SCO, subcommissural organ. Scale bars = 50 μm in A–C, 20 μm in inset of B.

Batrachyla taeniata

In this species, the AFRU-ir FP cells were circumscribed to a small patch located at the bottom of a fossa that most likely appears to be the equivalent of the isthmic fossa. The appearance of AFRU-immunoreactivity occurred simultaneously in the FO and SCO of 9-day-old embryos; at the same stage RF appeared in the central canal (Fig. 1). One day after hatching (12th embryonic day) the FO reached its maximal immunoreactivity (Fig. 9A,C). The reaction in the FO disappeared on the third day after hatching (Fig. 1), whereas the immunoreactivity of the SCO (Fig. 9B) increased progressively. The ultrastructural study of the FO cells of 12-day-old specimens revealed the presence of a well developed RER, that consisted of dilated cisternae filled with a fibrous material (Fig. 9D). Secretory granules about 115 nm in diameter occurred close to the apical membrane; aggregates of a filamentous material formed a coat on top of the cilia (Fig. 9E).

DISCUSSION

Secretory nature of the AFRU-immunoreactive material of the floor plate

In a study of the developing CNS of the rat, Rodríguez et al. (1996) found a close correlation between the immunostaining patterns obtained using AFRU and a monoclonal antibody raised against the FP-specific antigen FP4, thus confirming the FP lineage of the AFRU-ir cells of the ventral midline of the neural tube.

The secretory nature of the material recognized by AFRU in the FP cells is supported by the following findings: (1) the ultrastructural study showed that the AFRU-immunoreactivity is circumscribed to secretory granules of the FP cells; (2) these immunoreactive secretory granules are concentrated at the apical cell pole suggesting a ventricular release; (3) the presence in the central canal of aggregated AFRU-ir material at stages

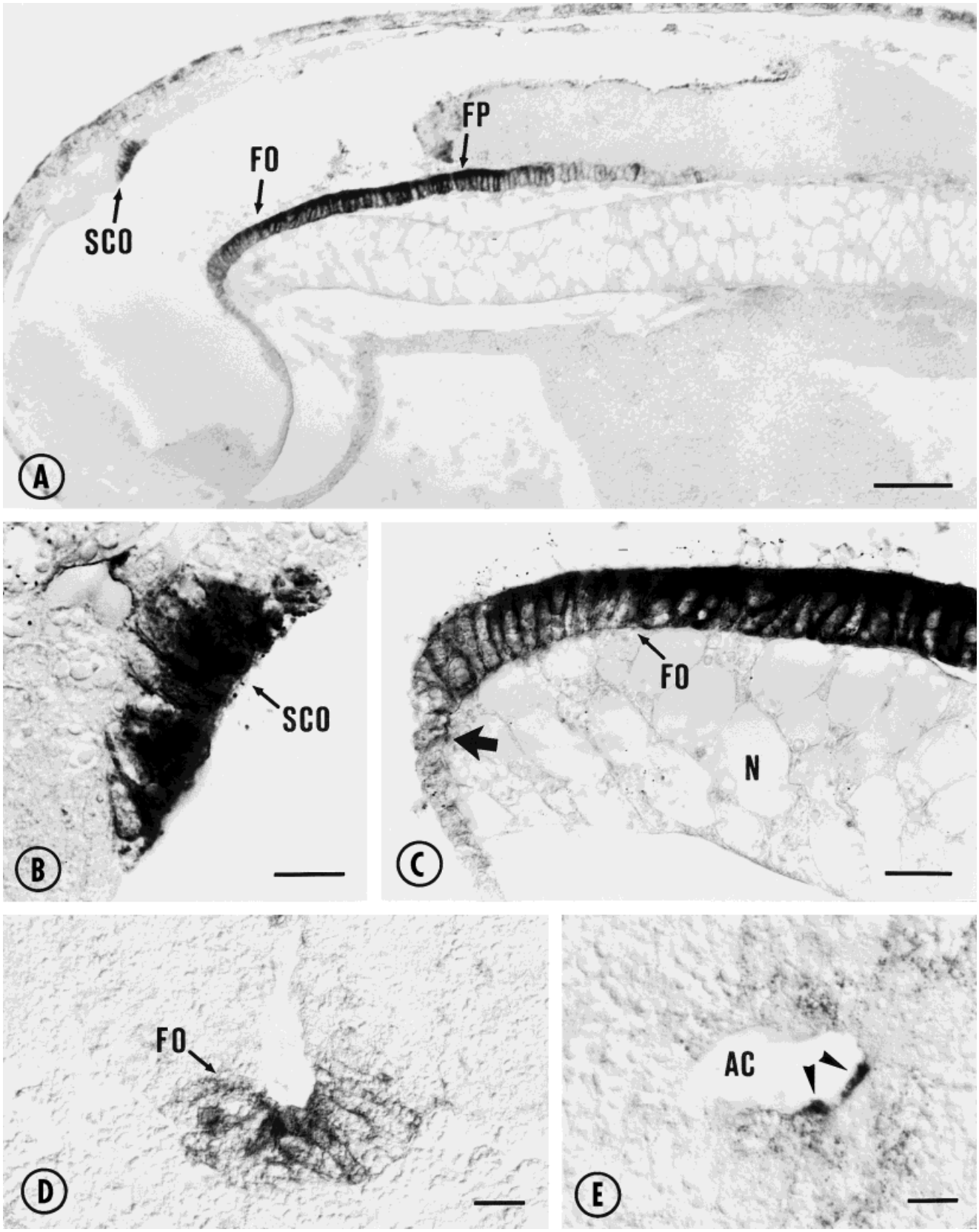

Fig. 6. Immunostaining of the CNS of *Xenopus* embryos using AFRU. **A**: Midsagittal section of a 41-hour-old embryo. The immunocytochemical reaction is confined to the SCO, FO, and FP. **B**: Higher magnification of the SCO shown in a. **C**: Higher magnification of the FO shown in a. AFRU-ir cells of the FO form a column closely apposed to the notochord. The arrow indicates cephalic end of the immunoreactive column of FP cells. **D**: Frontal section of a 33-hour-old embryo. Moderate RF-immunoreactivity is seen in cells of the FO. **E**: Frontal section of a 33-hour-old embryo. AFRU-ir material is present in the lumen of the ampulla caudalis (arrowheads). Scale bar = 30 µm. AC, Ampulla caudalis; FO, flexural organ; FP, floor plate; N, notochord; SCO, subcommissural organ. Scale bars = 200 µm in A, 30 µm in B and E, 50 µm in C and D.

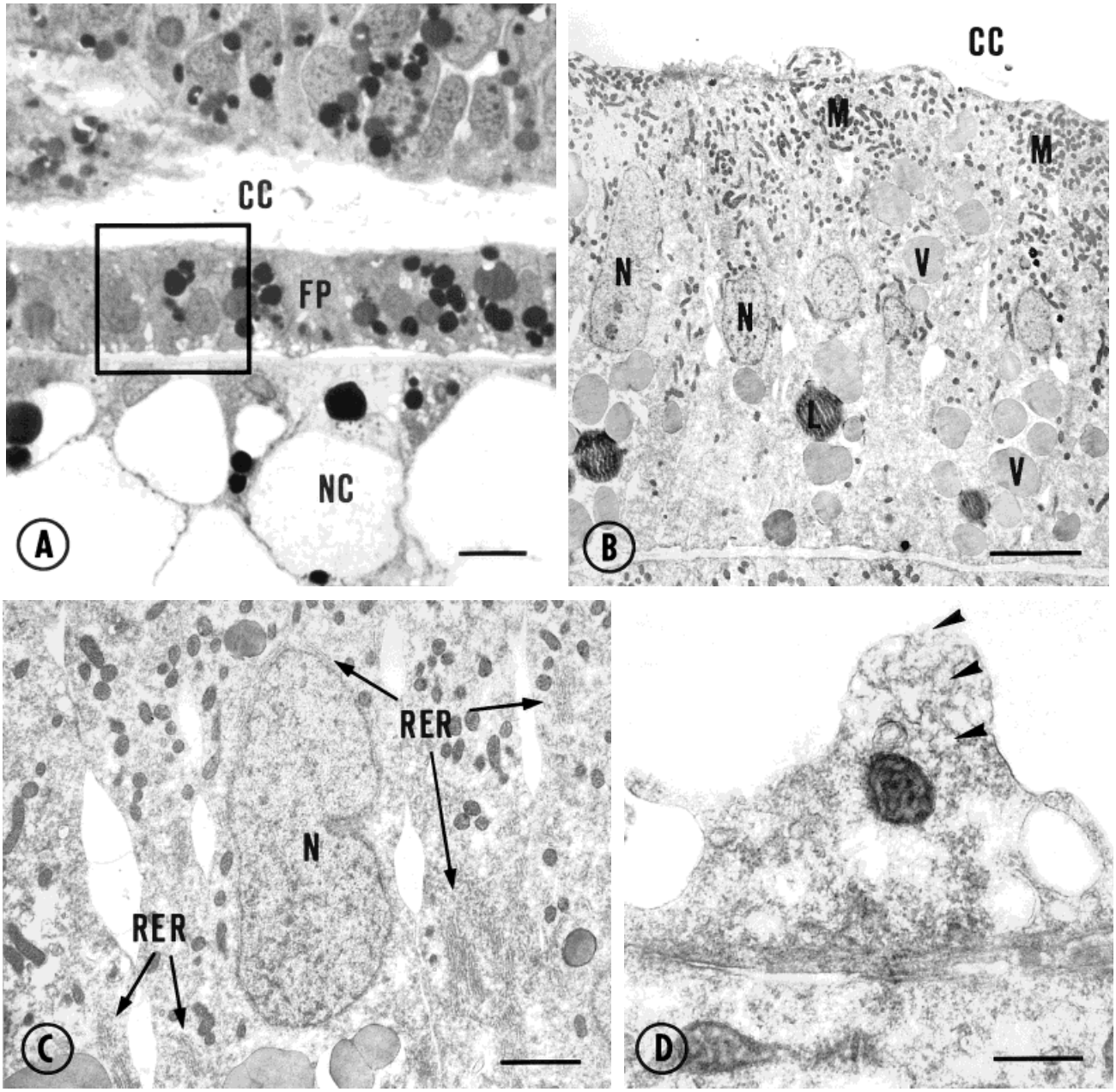

Fig. 7. Light and transmission electron microscopy (TEM) of the FP of 27-hour-old *Xenopus* embryos. **A**: Semi-thin section through the FP. The black square frames an area similar to that shown in b. **B**: Low magnification TEM of the FP showing accumulations of mitochondria in the apical cytoplasm of the FP cells and abundant vitelline inclusions and lipid droplets in the intermediate and basal cytoplasm. **C**: Perinuclear region of FP cells with parallel arrays of flattened cisternae of the rough endoplasmic reticulum. **D**: Apical protrusion of a FP cell containing electron-lucent vesicles (arrowheads) and large vacuoles. CC, Central canal; FP, floor plate; L, lipid droplets; M, mitochondria; N, nuclei; NC, notochord; RER, rough endoplasmic reticulum; V, vitelline inclusions. Scale bars = 10 μm in A, 5 μm in B, 2 μm in C, 500 μm in D.

when the SCO still has not differentiated (see below) points the FP cells as the source of this secreted material. The co-localization of WGA binding sites and AFRU-reactive material within the same granules of the FP cells would suggest that the AFRU-ir secretory material of the FP is a glycosylated peptide or a glycoprotein. In the bovine SCO, AFRU recognizes three glycoproteins of 540, 450, and 320 kDa (Nualart et al., 1991). About 20% of the molecular mass of these compounds consist of N-linked complex-type carbohydrate chains recognized by WGA (Nualart and Rodríguez, 1996). From a series of polyclonal and monoclonal antibodies raised against the glycoproteins secreted by the bovine SCO, only some of them react with the FP of the rat (Rodríguez et al., 1996) and cow (unpublished observations). Furthermore, immunoblots of extracts of the bovine FP show no reaction of AFRU with high molecular weight compounds as those present in the SCO (R.I. Muñoz, personal communication). An antiserum

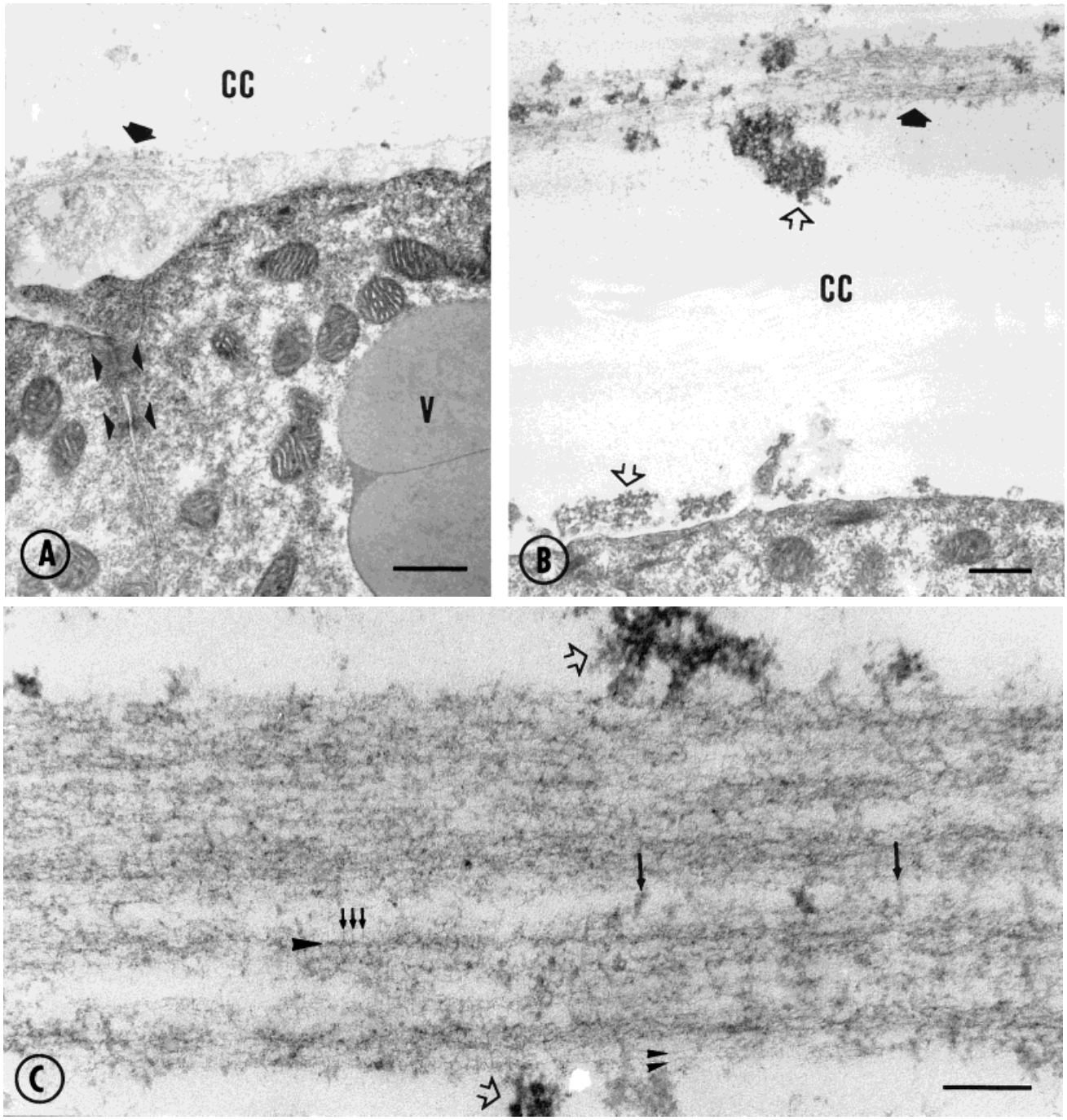

Fig. 8. TEM of the spinal cord of *Xenopus* embryos. **A**: Filamentous material (arrow) on the free surface of FP cells of a 27-hour-old embryo. Arrrowheads point to components of a junctional complex between FP cells. **B**: RF (solid arrow) inside the central canal of a 60-hour-old embryo. Electron-dense floccular material appears attached to RF and free surface of FP cells (open arrows). **C**: Detailed magnification of RF of a 60-hour-old embryo. RF is formed by parallel bundles of longitudinal filaments ranging in thickness between 3 (small arrowheads) and 14 nm (large arrowhead) bridged by short perpendicular filaments ranging in thickness between 4 (small arrows) and 14 nm (large arrows). Open arrows point to floccular material attached to RF. CC, Central canal; V, vitelline inclusion. Scale bars = 500 μm in A and B, 200 μm in C.

against the dogfish SCO glycoproteins reacted with the SCO and the FP of the dogfish, but it did not recognize the secretion of the SCO and FP of other vertebrate classes (López-Avalos et al., 1997). Therefore, it may be postulated that the AFRU-ir compound(s) expressed in the FP and the glycoproteins secreted by the SCO, although do not correspond to identical compounds, share some epitopes, and probably the carbohydrate chain too.

Partial aminoacidic sequences deducted from cDNA sequences encoding for SCO secretory glycoproteins con-

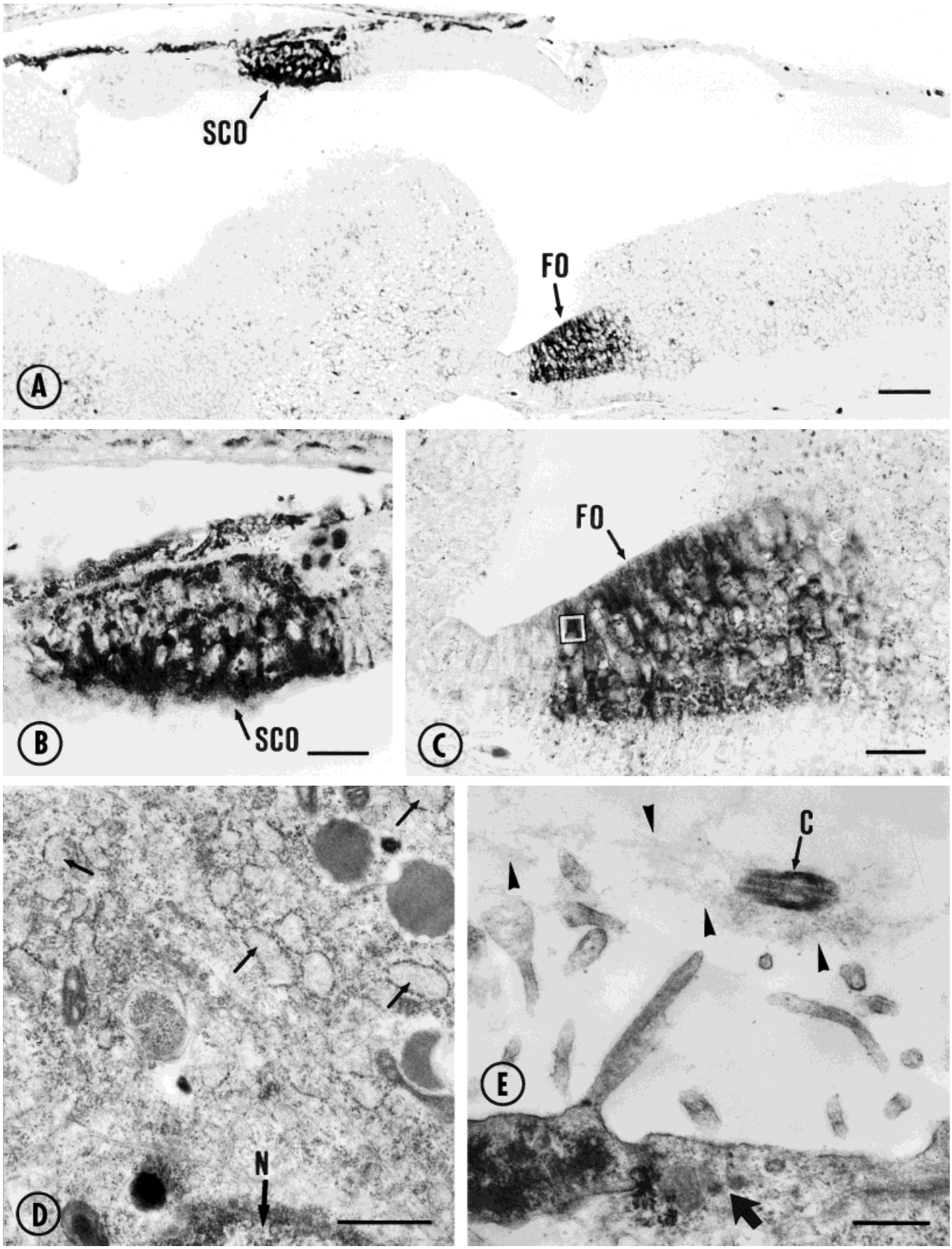

Fig. 9. Immunostaining with AFRU and TEM of the CNS of 12-day-old *Batrachyla* embryos. **A**: Midsagittal section showing intense immunostaining of the SCO and FO. **B**: Detailed magnification of the SCO shown in previous figure. **C**: Detailed magnification of FO shown in a. Rectangle frames area similar to that shown in d. **D**: TEM of a FO cell (for orientation see rectangle in c) showing accumulations of dilated RER cisternae (arrows) filled with a fibrous material. **E**: Free surface of FO. A few electron dense granules (arrow) are seen in the apical cytoplasm of a FO cell. Note the filamentous material accumulated around a cilium (arrowheads). C, Cilium; FO, flexural organ; N, nucleus; SCO, subcommissural organ. Scale bars = 50 µm in A, 20 µm in B and C, 1 µm in D, 500 µm in E.

tain some repeated domains found in the spondin family (Gobron et al., 1996; Nualart et al., 1998), including the F-spondin present in the FP and other structures of the embryonic CNS of the rat (Klar et al. 1992). The spatial and temporal distribution within the rat developing nervous system of the AFRU-ir material (Rodríguez et al., 1996) and that of the mRNA encoding for F-spondin (Klar et al., 1992) do not match, suggesting that the compound revealed by AFRU in the FP differs from F-spondin. Neither a correlation concerning time and place of expression of the Sonic hedgehog protein (Echelard et al., 1993; Krauss et al., 1993; Ekker et al., 1995; Martí et al., 1995) and the proteins revealed by AFRU in the FP was observed. It remains open for future investigations to elucidate the molecular structure of the AFRU-ir material secreted by the FP.

Conservative nature of the AFRU-ir material secreted by the floor plate

The fact that the hindbrain FP (FO) of embryos of an elasmobranch species (*Scyliorhinus canicula*, dogfish, López-Avalos et al., 1997), two teleost species (present report), three amphibian species (Naumann, 1986; and present report), the chicken (Del Brio León, personal communication), the rat (Rodríguez et al., 1996), and the cow (unpublished observation) all react with anti-bovine RF sera indicate that at least certain domains of the FP secretory compound(s) recognized by AFRU may be highly conserved in the vertebrate phylum.

The secretion of AFRU-immunoreactive material by the rostral-most end of the floor plate (flexural organ), and the zonation of the floor plate in its rostro-caudal axis, are conservative features

The term "flexural organ" was introduced by Olsson (1956) to designate a secretory differentiation of the ependyma located in the floor of the rhombencephalon of the embryonic brain, at the tip of the plica rhombomesencephalica. Using histochemical techniques, Olsson (1956, 1958) described the FO as a transient source of RF-material in embryonic specimens of *Esox lucius*, *Xenopus laevis*, and *Salmo salar*.

Our results, together with observations made by other researchers (Olsson, 1993; Fernández-Llebrez et al., 1996; Rodríguez et al., 1996), show that the FO may be regarded as a secretory specialization of the cephalic end of the notochordal FP, in which the secretion of RF-like material appears as a relevant feature (for a detailed discussion on the rostral end of the FP see Rodríguez et al., 1996). However, instead of being restricted to the region of the FO, as indicated from the early studies by Olsson (1956, 1958), in three of the four species investigated in the present study, as well as in the rat (Rodríguez et al., 1996), this secretory activity extends caudad into the FP of the spinal cord. The caudal extension of the column of AFRU-ir FP cells varies throughout the developmental stages, and among the species studied. However, consistently, the cells of the caudal third of the FP lack or have negligible amounts of AFRU-ir material. These results obtained in non-mammalian species and those obtained in the rat (Rodríguez et al., 1996) support the view that there is a zonation of the notochordal FP in the rostro-caudal axis, with its cephalic end (FO) secreting a material recognized by AFRU, and the caudal end secreting materials not recognized by AFRU. Such a zonation, which appears as a conserved evolutionary feature, must be kept in mind for future investigations concerning the FP, as well as for the interpretation of results already obtained, as those reported by Placzek et al. (1993).

The floor plate as a source of Reissner's fiber (RF)-material

An AFRU-ir FO was present at initial stages of CNS development in all the species used in the present study. In two of the species investigated (i.e., *Sparus aurata* and *Xenopus laevis*) the AFRU-immunoreactivity appeared earlier in the FO than in the SCO. Of more importance, in these two species an immunoreactive structure resembling RF was seen in the central canal of embryos displaying an AFRU-ir FO but lacking a differentiated SCO. This agrees with the situation in *Pleurodeles waltlii* (Naumann, 1986). Thus, in some species there is a developmental period in which the only source of RF-like material is the FP of the hindbrain (FO), therefore confirming early histochemical findings by Olsson (1956, 1958). This indicates that the RF-like structures found in the central canal of these species might result from the aggregation of the material secreted into the CSF by the AFRU-ir FP cells. Since the AFRU-ir material secreted by the FP is different from the AFRU-ir material secreted by the SCO (see above), it seems obvious that the RF produced by the SCO and the RF-like structure resulting from the secretion of the FP, are constituted by proteins of different nature, although sharing some epitopes recognized by AFRU.

During a long period of the CNS development of the species investigated (Fig. 1) in which the SCO and FP secrete AFRU-ir material, the RF running along the central canal would result from the aggregation of molecules secreted by both structures. This is most evident in *Oncorhynchus kisutch*; in this species the first RF originates in the SCO, and a fibrous secretory material secreted by the AFRU-ir FP cells seems to be added to the RF (fibrils, in Fig. 3B).

This situation in anamniotes differs from that of the rat; in this species the formation of RF is a postnatal event, despite the fact that the rat embryos have well developed SCO and FP displaying strong AFRU immunoreactivity (Rodríguez et al., 1996). The possibility that cells of the FP secrete two kinds of AFRU-ir compounds, namely, molecules that form RF-like aggregates in the CSF, and CSF soluble molecules, should be considered. There is strong evidence pointing to a similar situation taking place in the SCO (Rodríguez et al., 1993).

Fate of the AFRU-ir material secreted by the floor plate

The FP is considered as one of the main sources of inductive signals involved in the differentiation of the CNS (Placzek et al., 1993). Most of the efforts directed to elucidate this matter have been concentrated in the search of contact-mediated and diffusible signals derived from the FP cells (Placzek et al., 1990; Griffith and Wiley, 1991; Klar et al., 1992; Yamada et al., 1993). Several authors have obtained evidence indicating a direct contact between FP cells and the putative target (contact mediated factors), and the release of a diffusible compound into the intercellular space where, through a concentration gradient, it would trigger the differentiation of distant cells. In addi-

tion to the above mentioned mechanisms, it has been postulated that long-range acting signals, such as the peptides derived from the cleavage of the polypeptide Sonic hedgehog (Shh), might perform inductive functions acting at the luminal surface of target cells, through their release into the CSF (Lai et al., 1995; Martí et al., 1995). The spatial and temporal distribution of the AFRU-ir material in the FP of several species (Rodríguez et al., 1996; present report) differs from that of Shh peptides. The evidence presented here supports the hypothesis advanced by Rodríguez et al. (1996) that the AFRU-ir secretion of the FP also utilizes the CSF as a pathway to perform as yet unknown functions in the developing CNS. This hypothesis is further supported by the fact that in the FP cells of the spinal cord of *Oncorhynchus kisutch* the AFRU-ir secretory granules are exclusively concentrated at the apical cell pole.

At variance, the FP cells forming the FO of *Oncorhynchus kisutch* display AFRU-ir secretory granules throughout the cytoplasm, including their basal processes reaching the brain surface. The possibility that the FO cells transport AFRU-ir material along their processes and release it locally, must be considered. It has been postulated that in the rat FO, the AFRU-ir material transported along the basal cell processes participate in the differentiation of serotonergic neurons (Rodríguez et al., 1996).

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