ELSEVIER

Developmental Brain Research 102 (1997) 69–75

DEVELOPMENTAL BRAIN RESEARCH

Research report

Rostral floor plate (flexural organ) secretes glycoproteins immunologically similar to subcommissural organ glycoproteins in dogfish (*Scyliorhinus canicula*) embryos

M.D. López-Avalos [b], M. Cifuentes [a], J.M. Grondona [a], E. Miranda [a], J. Pérez [a], P. Fernández-Llebrez [a,*]

[a] *Departamento de Biología Animal, Facultad de Ciencias, Universidad de Málaga, E-29071 Malaga, Spain*
[b] *Departamento de Biología Celular y Genética, Facultad de Ciencias, Universidad de Málaga, E-29071 Malaga, Spain*

Accepted 8 April 1997

Abstract

The subcommissural organ of vertebrates secretes glycoproteins into the cerebrospinal fluid of the third cerebral ventricle. This material polymerizes in Reissner's fiber. During ontogenetic development, besides the subcommissural organ, the ependyma lining the pontine flexure constitutes an additional Reissner's fiber-secreting gland named flexural organ. We have studied the secretion of the flexural organ and the subcommissural organ in dogfish (*Scyliorhinus canicula*) embryos using three different antisera and the lectins concanavalin A and wheat germ agglutinin. AFRU is an antiserum against the bovine Reissner's fiber; Ab-600 is an antiserum against 600 kDa dogfish subcommissural organ glycoproteins; and APSO is an antiserum against immunoaffinity purified bovine subcommissural organ secretory glycoproteins. These three antisera immunostained the flexural organ indicating that it contains epitopes similar to those present in bovine and dogfish subcommissural organ glycoproteins. It seems highly probable that the flexural organ and the subcommissural organ of dogfish embryos secrete similar compound(s). Other ependymal regions were also immunostained with Ab-600 and APSO antisera. Then, Reissner's fiber-like glycoproteins were transiently expressed by most embryonary ependymal cells. These glycoproteins might play a role in the development of the central nervous system of vertebrates. © 1997 Elsevier Science B.V.

Keywords: Floor plate; Flexural organ; Subcommissural organ; Dogfish embryo (*Scyliorhinus canicula*); Immunocytochemistry

1. Introduction

The secretory activity of neuroepithelial cells in the roof and floor plates of the vertebrate embryonic neural tube controls growing and wiring of neuronal axonal processes and neuronal differentiation. Some of these cells also release, into the lumen, large glycoproteins that polymerize to form Reissner's fiber (RF) [14] that extends along the full length of the neural tube. In the urochordate *Oikopleura dioica*, a single rostral neuroepithelial cell (fibrogen cell) [9] secretes glycoproteins that form a thin fiber. In the cephalochordate *Branchistoma lanceolatum*, there is a ventral cephalic organ (infundibular organ, IO) [10], composed of several neuroepithelial cells that also form a RF. In vertebrates, at least one RF-producing organ has been described during some phase of their life span. In embryos, the rostralmost portion of the floor plate, lining the pontine flexure, constitutes an RF-producing organ (flexural organ, FO) [8]. The secretory cells of the FO cease the production of RF glycoproteins before or after birth, depending upon the species [7,8,18]. The best-known RF-producing organ is the subcommissural organ (SCO) [11]. It is located in the roof of the diencephalic third ventricle, lining the posterior commissure at the entrance of the cerebral aqueduct. It is functionally active during embryonic life and it remains in adults, except in some species as anthropoid primates. The extended presence of RF glycoproteins in embryos and adult vertebrates suggests that they should play a role in the development and homeostasis of the central nervous system. However, in spite of the fact that RF was discovered more than a century ago, its role is still not known.

Similarities among the materials secreted by SCO, FO

* Corresponding author. Fax: +34 (5) 2132000; E-mail: LLEBREZ@ccuma.sci.uma.es

PII S0165-3806(97)00077-1

and IO have been suggested on the basis of their histochemical and immunocytochemical properties [7,9,18]. An antiserum against adult bovine RF extracts (AFRU) binds to secretory glycoproteins of SCO, FO, and IO [9,10]. This led to the asumption that materials present in all these organs could share epitopes that are also present in the RF. However, AFRU also binds to certain ependymal cells of the bovine central canal, suggesting that AFRU could contain antibodies against materials secreted by ependymal cells in addition to those secreted by the SCO [13,17]. In addition, the IO is not labeled by an anti-bovine SCO antiserum [10], suggesting that the secretions of SCO and IO are not similar. Then, a positive immunoreactivity of SCO, FO and IO to AFRU does not necessarily imply that proteins secreted by these organs are the same.

In order to investigate whether the SCO and FO synthesize similar proteins we performed the present comparative immunocytochemical and lectin-histochemical investigation on dogfish embryos. We used, for the first time, antisera against secretory proteins present in the bovine and dogfish SCO to analyze the secretion of the dogfish FO.

2. Material and methods

2.1. Animals

Dogfish eggs were from females captured for commercial fishing in the harbor of Malaga (Spain). Eggs were kept in sea water at 15°C. At this temperature, *Scyliorhinus canicula* embryonary development lasts about 6 months and comprises two well-defined periods. After a first period of 3 months, the egg-case partly opens and the embryo reaches a stage named pre-hatching. The remaining 3 months are mainly a period of growing after which hatching occurs [6]. We have processed embryos from day 30 (3.5-mm-long stage G of Mellingeret al. [6]) to day 90 (40-mm-long stage Q of Mellinger et al. [6]), when pre-hatching occurs. Groups ($n = 5$) of embryos measuring 3.5, 7, 10, 16, 20, 25, 30, 35, and 40 mm were made. The neural tube of 3.5-mm-long embryos is closed and corresponds to the 3-vesicle stage. The brain of 40-mm-long pre-hatching embryos has reached about the shape of the adult brains. Dogfish hatched at day 180 with a size of 100 mm. Adult dogfish brains from our collection [2,3,5] were used as controls.

2.2. Antisera

We have used the following antisera: (i) AFRU (anti-fiber of Reissner extracted in urea) is a rabbit antiserum against an extract of bovine Reissner's fiber developed in our laboratory according to Pérez et al. and Rodríguez et al. [12,13,15]; (ii) Ab-600 is a rat antiserum developed in our laboratory against an electrophoretic gel band of mol.wt. 600 kDa from SDS-PAGE of adult dogfish SCO extracts [2,5]. The 600 kDa band was chosen because its immunoreactivity in blots using AFRU and an antiserum against dogfish SCO extracts [2,3,5], then it should contain secretory glycoproteins of adult dogfish SCO; (iii) APSO

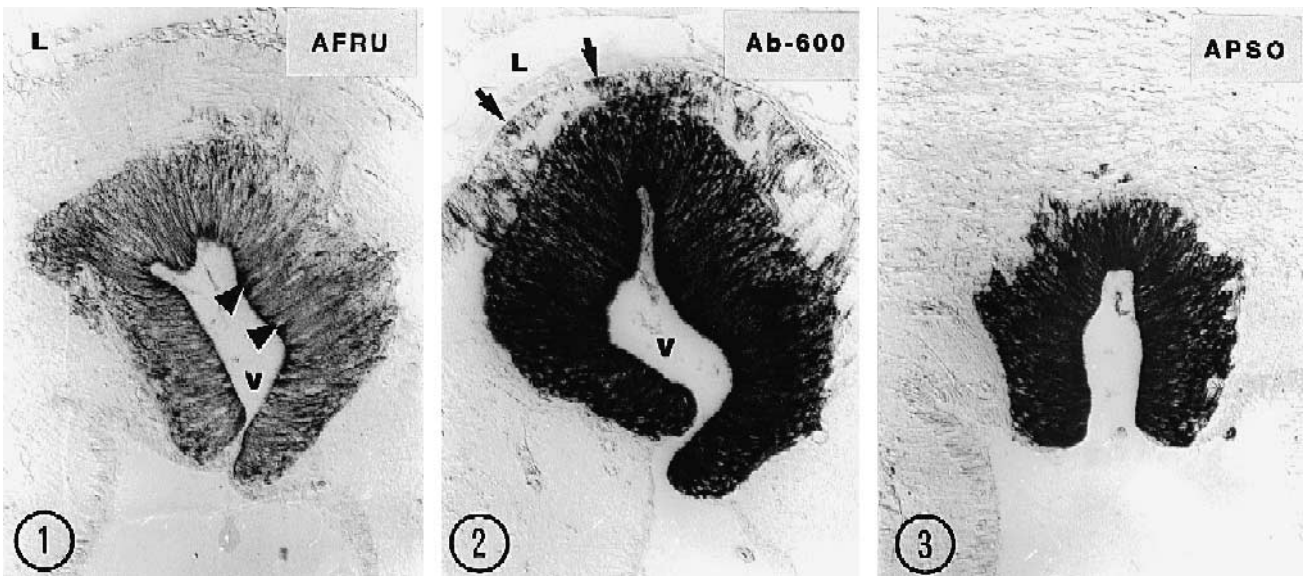

Fig. 1. Transverse sections through the subcommissural organ of adult dogfish immunostained with AFRU, Ab-600 and APSO respectively. Arrowheads, secretory materials in the apexes of the cells; arrows, basal processes; L, leptomeninge; V, third ventricle. ×69.

Fig. 2. For legend see Fig. 1.

Fig. 3. For legend see Fig. 1.

(anti-purified subcommissural organ glycoproteins) is a rat antiserum developed in our laboratory against immunoaffinity purified bovine SCO secretory glycoproteins. Briefly, a bicarbonate extract of 200 bovine SCOs was passed through an 1 ml column containing 10 mg of a monoclonal antibody against bovine RF named 2A5 [12,13] bound to Sepharose 4B (Pharmacia-LKB, Uppsala, Sweden). Binding buffer was 150 mM phosphate-buffered saline (PBS), pH 7.3 and elution buffer was 100 mM triethanolamine, pH 11.5. Elution fraction was neutralized with 1 M Tris buffer, pH 6.8, dialyzed against PBS, concentrated and used as immunogen.

2.3. Immunocytochemistry

Eggs were opened and embryos fixed by immersion in Bouin's fluid. After 4 h, they were dehydrated and embedded in paraffin. Sagittal and transverse, 8 μm thick, sections were immunostained according to the unlabeled PAP method of Sternberger et al. [19]. Briefly, the sections were sequentially incubated in: (1) the first antiserum (AFRU, Ab-600 or APSO) diluted 1:1000 in TCT solution, composed of 0.1 M Tris buffer, pH 7.8, containing 0.7% non-gellin seaweed lambda carrageenan (Sigma, Madrid, Spain) as the saturating agent and 0.5% Triton X-100

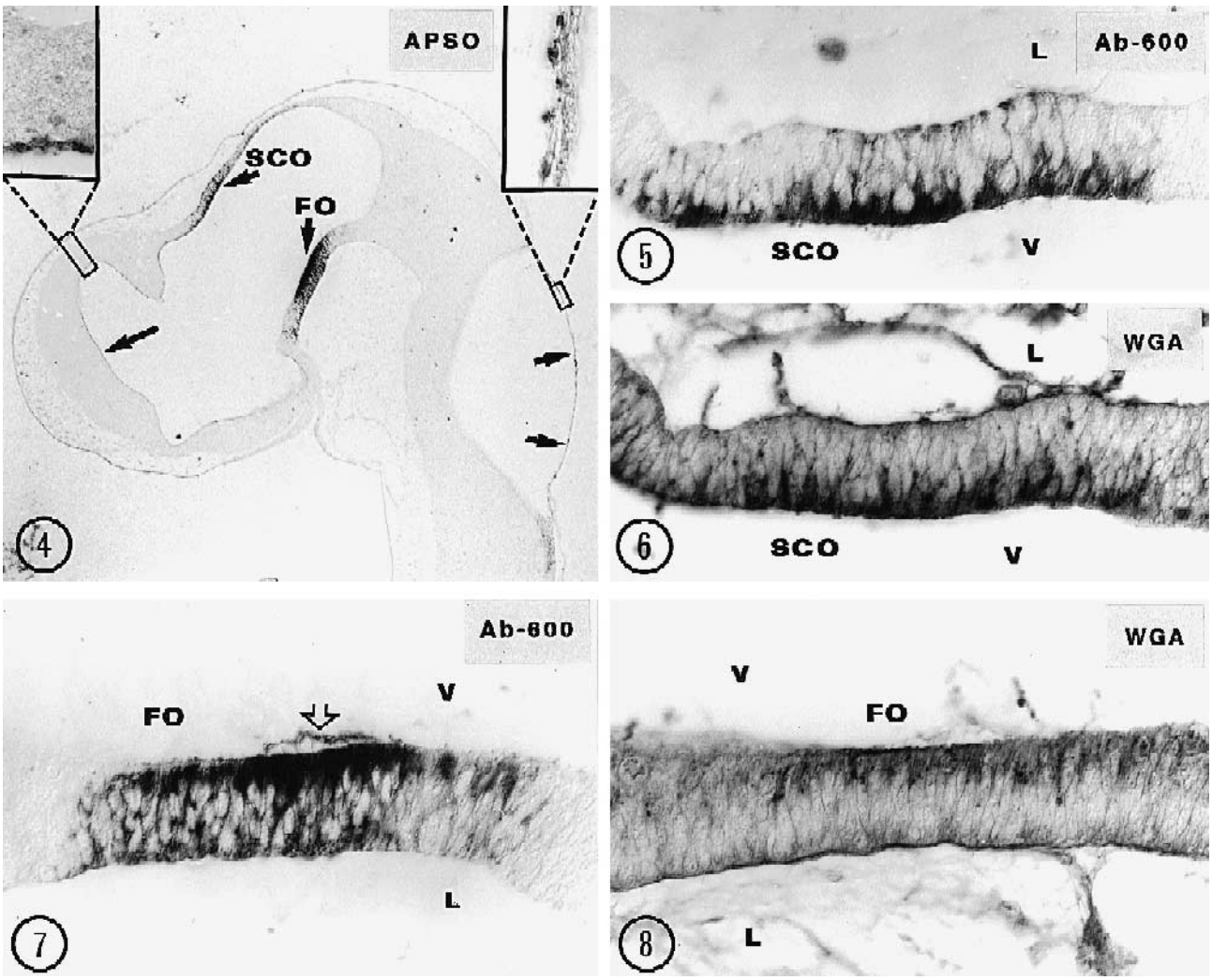

Fig. 4. Sagittal section through the head of a 10-mm dogfish embryo immunostained with APSO. Note positive subcommissural (SCO) and flexural (FO) organs. The apexes of ependymal cells in other regions were also positive (arrows, insets). ×20 (insets ×200).

Fig. 5. Sagittal section through the SCO (Fig. 5, Fig. 6) and FO (Fig. 7, Fig. 8) of a 10-mm dogfish embryo immunostained with Ab-600 (Fig. 5, Fig. 7) and stained with WGA lectin (Fig. 6, Fig. 8). Reactive materials are mostly accumulated in the apical cytoplasms. Arrow, immunoreactive fibrils; V, ventricle; L, leptomeninge. ×140.

Fig. 6. For legend see Fig. 5.

Fig. 7. For legend see Fig. 5.

Fig. 8. For legend see Fig. 5.

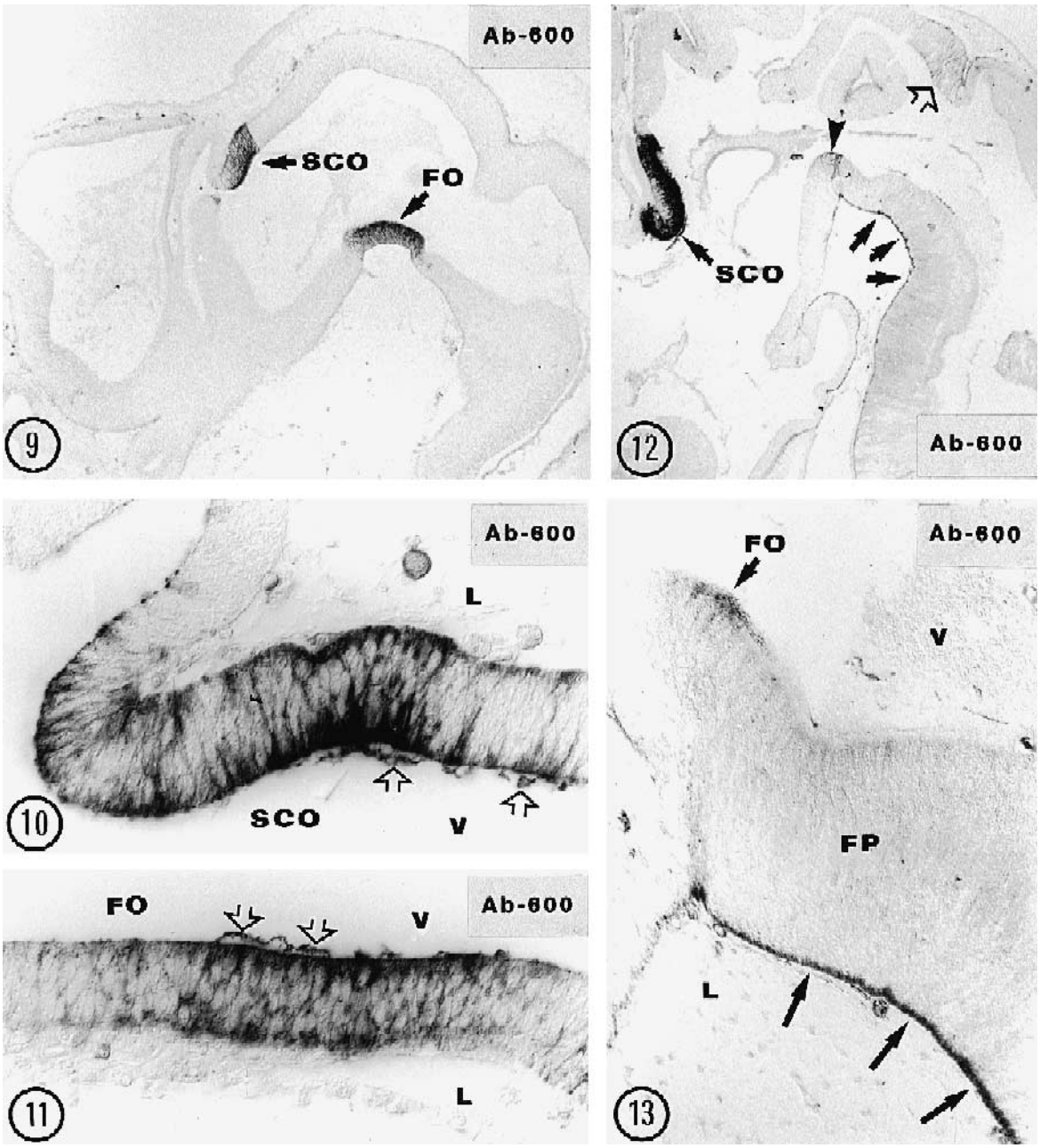

Fig. 9. Sagittal section through the head of a 20-mm-long dogfish embryo immunostained with Ab-600. Note positive subcommissural (SCO) and flexural (FO) organs. ×20.

Fig. 10. Sagittal section through the SCO (Fig. 10) and FO (Fig. 11) of a 20-mm dogfish embryo immunostained with Ab-600. Immunoreactive materials are evident in the apical and basal cytoplasms and on the ventricular surface of both organs (arrows). V, ventricle; L, leptomeninge. Nomarski optic, ×140.

Fig. 11. For legend see Fig. 10.

Fig. 12. Sagittal section through the head of a 35-mm-long dogfish embryo immunostained with Ab-600. The subcommissural organ (SCO) is almost fully developed and only some ependymal cells appeared positive in the pontine flexure (arrowheads). Cells of the mid-sagittal ventral plate appeared positive mainly at their basal endings on the leptomeninge (arrows). Also cells of the dorsal plate were immunolabeled (open arrow). ×16.

Fig. 13. Details of a sagittal section showing the remainings of the flexural organ (FO) in a 40-mm-long dogfish embryo immunostained with Ab-600. Note also reactivity of floor plate cells (arrows). V, ventricle; L, leptomeninge. Nomarski optic, ×75.

(Sigma), for 18 h at 25°C in a moist chamber; (2) the corresponding linking antibody: for AFRU, anti-rabbit IgG developed in goat diluted 1:50 in TCT, for 30 min; for Ab-600 and APSO, anti-rat IgG developed in rabbit diluted 1:500 for 1 h was used first and then anti-rabbit IgG as described above; (3) rabbit PAP (Sigma) diluted 1:100 in TCT, for 30 min. To reveal peroxidase, 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) was used as the electron donor.

2.4. Lectin histochemistry

Concanavalin-A agglutinin (Con-A) and wheat germ agglutinin (WGA) lectins were used. Sections were incubated in peroxidase-labeled lectin (Con-A, 5 μg/ml; WGA, 3 μg/ml; Sigma) dissolved in TCT buffer for 2 h at 25°C in a moist chamber; DAB was used as the electron donor.

3. Results

3.1. Adults

AFRU, Ab-600 and APSO antisera bound to secretory material of the dogfish SCO cells (Figs. 1–3). The immunostaining was particularly strong with Ab-600 and APSO. No other brain regions were stained. SCO cells displayed tall apical cytoplasms deeply immunostained at their distal portions (Fig. 1) and basal processes crossing the posterior commissure and terminating on the outer basal lamina close to the leptomeninge (Fig. 2). Con-A stained all cytoplasmatic regions whereas WGA identified secretory materials present only in the apical cytoplasms (data not shown) [5].

3.2. Embryos

AFRU, Ab-600 and APSO antisera strongly immunostained SCO, FO and the RF at any stage of the embryonic development. Labeling was more conspicuous with Ab-600 and APSO. Moreover, these two antisera immunostained other additional structures.

Regardless the antisera used, the first brain region appearing immunoreactive in the developing central nervous system was the flexural organ. In 3.5-mm-long embryos a few cells of the diencephalic floor showed a weak immunoreactivity (data not shown) at their apical cytoplasm. On the apical surface of these cells, immunoreactive filaments could be distinguished. In more caudal levels the lumen of the neural tube displayed a thin RF (data not shown).

In 5-mm-long embryos, immunoreactivity was evident in the FO and in the diencephalic roof (SCO region) where some ependymal cells showed labeled apical cytoplasms (data not shown).

In 10-mm-long embryos, immunoreactivity was quite evident in both SCO and FO (Fig. 4). Both organs are composed of pseudostratified epithelium of a thickness of about two or three nuclei. Their polarized secretory cells contact apically with the lumen of the embryonic brain ventricle and basally with the meninx primitiva. The apical and basal cytoplasms in both organs were immunoreactive to the three antisera. Immunoreactive fibrils were present on the surface of the secretory ependymal cells of the FO and SCO (Figs. 5 and 7). Both SCO and FO were positive to Con-A (not shown) and WGA (Figs. 6 and 8) lectins. The staining pattern with Con A was similar to that of the antisera whereas WGA bound mainly to materials located in the apical cytoplasms of both SCO and FO cells (Figs. 6 and 8).

The SCO and FO remained positive to the three antisera and the lectins while increasing in size (thickness and length) during the embryonic development. In 20-mm-long embryos (Fig. 9) an active apical secretion was quite evident in both SCO and FO (Figs. 10 and 11).

As the SCO increased in size, FO progressively regressed until, in 40-mm-long embryos, only a few cells remained positive in the pontine flexure (Figs. 12 and 13). In these embryos the SCO was well developed and secreted a strongly immunostained RF (Fig. 12).

3.3. Other regions immunostained with the antisera

While AFRU immunostained only the SCO and FO, Ab-600 and APSO antisera bound to other additional brain regions. In embryos shorther than 35 mm, APSO immunostained the apexes of the ependymal cells lining the brain ventricles and the central canal (Fig. 4, insets). The immunolabeling was particulary strong in the ependymal cells of the roof plate of 10-mm-long embryos and progressively diminished until, in 35-mm-long embryos, it could no longer be visualized.

On the other hand, in embryos larger than 35 mm in which FO is virtually absent, Ab-600 immunostained the ependymal cells of the ventral mid-sagittal plane along its entire length. Immunoreactivity was very weak in the cytoplasm of the cells and strong in the basal ending of the cells contacting the leptomeninge (Figs. 12 and 13). In addition, few ependymal cells of the dorsal mid-sagittal plane were immunostained (Fig. 12).

4. Discussion

In general, dogfish SCO and FO ontogenetic developments follow a pattern similar to those described for other vertebrate species using histochemistry and immunocytochemistry with antisera against bovine RF [7,9,18]. The FO appears first in ontogeny and, after a period of coexistence with the SCO, it gradually ceases the production of secretory materials and disappears. After that, SCO is the only

RF-producing gland. Histochemical and immunocytochemical studies have suggested similarities between the materials synthesized by both glands [7,9]. On contrary to SCO, immunoreactive secretory materials of the rat FO did not bind WGA lectin suggesting that this secretion is not glycosylated [18]. Secretory materials of the dogfish FO and SCO bound Con-A and WGA (present results), indicating that both are glycosylated. In order to study the immunochemical similarities between SCO and FO secretions we performed the present comparative immunocytochemical study.

In this work we have used three different antisera that immunostained both the SCO and FO. AFRU is an antiserum raised against the bovine RF that immunostains the SCO of all vertebrates studied [15]. However, AFRU could contain antibodies against epitopes not secreted by the SCO. In fact, immunocytochemical studies using polyclonal and monoclonal antibodies against bovine RF suggested that ependymal cells of the bovine central canal could secrete materials that aggregate in the RF [13,17]. Then, the fact that AFRU immunostains both SCO and FO in vertebrate embryos [7,18], such as in the dogfish (present results), does not prove that the materials secreted by both organs are similar. Unfortunately none of the monoclonal antibodies against the bovine RF available in our laboratory [12] immunostains the SCO or the FO in dogfish embryos (this would have been a very good evidence for the biochemical similarity of both secretions). This is the reason why we performed the present investigation using antisera against secretory glycoproteins of the proper SCO.

Ab-600 is an antiserum raised against 600 kDa secretory glycoproteins of the adult dogfish SCO. This antiserum selectively recognizes the SCO and the RF of adult dogfish [5]. Our results show that Ab-600 immunostains the developing dogfish SCO and FO, thus similar epitopes must be present in the adult SCO and the embryonic SCO and FO of the dogfish. Since Ab-600 immunostained the SCO of other elasmobranch species but not of other vertebrate classes (unpublished results), it can be concluded that epitopes identified by Ab-600 in dogfish adult SCO and embryonic SCO and FO are class-specific.

APSO is an antiserum against immunoaffinity purified bovine SCO glycoproteins. Purification was performed using the monoclonal antibody 2A5 that selectively recognizes an epitope present in the bovine SCO secretory material [12]. APSO recognized the SCO and the RF of the bovine and other vertebrates (unpublished results from the authors). The present results show that APSO selectively immunostained the SCO of adult dogfish and the SCO and the FO of dogfish embryos, thus similar epitopes must be present in the adult bovine SCO, the adult dogfish SCO and the embryonic dogfish SCO and FO. These epitopes can be considered conserved ''universal'' epitopes.

In conclusion, dogfish embryonic SCO and FO share universal and class-specific epitopes, thus it is very likely that similar glycoproteins are synthesized and secreted by both glands.

In addition to the SCO and the FO, the ependymal cells lining the brain ventricles and the central canal of dogfish embryos were transitorily immunostained with Ab-600 and APSO antisera. Immunostaining of embryonic ependymal cells with anti-bovine RF antiserum was reported before in early vertebrate embryos [7]. In the rat embryo, AFRU stains the floor plate of the hindbrain [16]; these authors propose that AFRU immunoreactive material would participate in the differentiation of the serotonergic neurons.

Ependymal materials immunostained with Ab-600 and APSO antisera should contain epitopes that are also present in the dogfish SCO and FO. Then, it seems that the capacity to synthesize RF glycoproteins is an extended property of embryonary ependymal cells. For unknown reasons, during development, most ependyma loss this ability and only the SCO remains in adult life of most vertebrates.

The function of glycoproteins secreted by SCO and FO is not known. Recently, a 2.6 kb cDNA insert of a 13 kb full length bovine SCO mRNA has been isolated and analyzed [1]. A part of the sequence showed a strong homology to compounds of the thrombospondin family and then the compound from bovine SCO was named SCO-spondin. The same authors reported that materials solubilized from bovine RF had anti-aggregative effect on chick embryonic neurons. F-spondin has been reported to be secreted by the floor plate and to play a role in neural cell adhesion and neurite extension [4]. The early presence of SCO and FO secretory glycoproteins in dogfish embryos suggests a role in the ontogenetical development of the central nervous system.

Acknowledgements

This work was supported by Grant PB93-0979 from DGICYT (Spain), Grant 95/1591 from FIS (Spain), and Grant from Junta de Andalucía PAI No. CVI-0217 (Spain).

References

[1] S. Gobron, H. Monnerie, R. Meiniel, I. Creveaux, W. Lehmann, D. Lamalle, B. Dastugue, A. Meiniel, SCO-spondin: a new member of the thrombospondin family secreted by the subcommissural organ is a candidate in the modulation of neuronal aggregation, J. Cell Sci. 109 (1996) 1053–1061.

[2] J.M. Grondona, P. Fernández-Llebrez, J. Pérez, M. Cifuentes, J.M. Pérez-Fígares, E.M. Rodríguez, Class-specific epitopes detected by polyclonal antibodies against the secretory products of the subcommissural organ of the dogfish *Scyliorhinus canicula*, Cell Tissue Res. 276 (1994) 515–522.

[3] J.M. Grondona, J. Pérez, M. Cifuentes, M.D. López-Avalos, F.J. Nualart, B. Peruzzo, P. Fernández-Llebrez, E.M. Rodríguez, Analysis of the secretory glycoproteins of the subcommissural organ of the dogfish (*Scyliorhinus canicula*), Mol. Brain Res. 26 (1994) 299–308.

[4] A. Klar, M. Baldassare, T.M. Jessell, F-spondin: a gene expressed at high levels in the floor plate encodes a secreted protein that promotes neural cell adhesion and neurite extension, Cell 69 (1992) 95–110.
[5] M.D. López-Avalos, J. Pérez, J.M. Pérez Fígares, B. Peruzzo, J.M. Grondona, E.M. Rodríguez, Secretory glycoproteins of the subcommissural organ of the dogfish (*Scyliorhinus canicula*): evidence for the existence of precursor and processed forms, Cell Tissue Res. 283 (1996) 75–84.
[6] J. Mellinger, F. Wrisez, M.J. Alluchon-Gerard, Developmental biology of an oviparous shark, *Scyliorhinus canicula*, in: T. Uyeno, R. Arai, T. Taniuchi, K. Matsuura (Eds.), Proceedings of the 2nd Int. Conference on Indo-Pacific Fishes, Ichthyological Society of Japan, Tokyo, 1986, pp. 310–332.
[7] W.W. Naumann, W. Lehmann, P. Debbage, The subcommissural organ and ontogenetic development of the brain, in: A. Oksche, E.M. Rodríguez, P. Fernández-Llebrez (Eds.), The Subcommissural Organ. An Ependymal Brain Gland, Springer, Berlin, 1993, pp. 61–69.
[8] R. Olsson, The development of Reissner's fiber in the brain of the salmon, Acta Zool. (Stockh.) 37 (1956) 235–250.
[9] R. Olsson, Reissner's fiber mechanisms: some common denominators, in: A. Oksche, E.M. Rodríguez, P. Fernández-Llebrez (Eds.), The Subcommissural Organ. An Ependymal Brain Gland, Springer, Berlin, 1993, pp. 33–39.
[10] R. Olsson, R. Yulis, E.M. Rodríguez, The infundibular organ of the lancelet (*Branchiostoma lanceolatum*, Acrania): an immunocytochemical study, Cell Tissue Res. 277 (1994) 107–114.
[11] A. Oksche, E.M. Rodríguez, P. Fernández-Llebrez, The Subcommissural Organ. An Ependymal Brain Gland, Springer, Berlin, 1993.
[12] J. Pérez, B. Peruzzo, G. Estivill-Torrús, M. Cifuentes, K. Schoebitz, E. Rodríguez, P. Fernández-Llebrez, Light- and electron-microscopic immunocytochemical investigation of the subcommissural organ using a set of monoclonal antibodies against the bovine Reissner's fiber, Histochem. Cell Biol. 104 (1995) 221–232.
[13] J. Pérez, O. Garrido, M. Cifuentes, F.J. Alonso, G. Estivill-Torrús, G. Eller, F. Nualart, M.D. López-Avalos, P. Fernández-Llebrez, E.M. Rodríguez, Bovine Reissner's fiber (RF) and the central canal of the spinal cord: an immunocytochemical study using a set of monoclonal antibodies against the RF-glycoproteins, Cell Tissue Res. 286 (1996) 33–42.
[14] E. Reissner, Beiträge zur Kenntnis vom Bau des Rückenmarks von *Petromyzon fluviatilis* L., Arch. Anat. Physiol. 77 (1860) 545–588.
[15] E.M. Rodríguez, A. Oksche, S. Hein, S. Rodríguez, R. Yulis, Comparative immunocytochemical study of the subcommissural organ, Cell Tissue Res. 237 (1984) 427–441.
[16] E.M. Rodríguez, M.A. Del Brío León, P. Riera, J. Menendez, K. Schoebitz, The floor plate of the hindbrain is a highly specialized gland. Immunocytochemical and ultrastructural characteristics, Dev. Brain Res. 97 (1996) 153-168.
[17] S. Rodríguez, S. Hein, R. Yulis, L. Delannoy, I. Siegmund, E.M. Rodríguez, Reissner's fiber and the wall of the central canal in the lumbo-sacral region of the bovine spinal cord. Comparative immunocytochemical and ultrastructural study, Cell Tissue Res. 240 (1985) 649–662.
[18] K. Schoebitz, E.M. Rodríguez, O. Garrido, M.A. Del Brío-León, Ontogenetic development of the subcommissural organ with reference to the flexural organ, in: A. Oksche, E.M. Rodríguez, P. Fernández-Llebrez (Eds.), The Subcommissural Organ. An Ependymal Brain Gland, Springer, Berlin, 1993, pp. 41–49.
[19] L.A. Sternberger, P.H. Hardy, J.J. Cuculis, H.G. Meyer, The unlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish-peroxidase-antiperoxidase) and its use in identification of spirochetes, J. Histochem. Cytochem. 18 (1970) 315–333.