Searching for Specific Binding Sites of the Secretory Glycoproteins of the Subcommissural Organ

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ABSTRACT The molecular organization of Reissner's fiber (RF), the structure of its proteins, and the permanent turnover of these proteins are all facts supporting the possibility that RF may perform multiple functions. There is evidence that CSF-soluble RF-glycoproteins may occur under physiological conditions. The present investigation was designed to investigate the probable existence within the CNS of specific binding sites for RF-glycoproteins. Three experimental protocols were used: (1) immunocytochemistry of the CNS of bovine fetuses using anti-idiotypic antibodies, raised against monoclonal antibodies developed against bovine RF-glycoproteins; (2) in vivo binding of the RF glycoproteins, perfusing into the rat CSF 125I-labeled RF-glycoproteins, or grafting SCO into a lateral ventricle of the rat; (3) in vitro binding of unlabeled RF-glycoproteins to rat and bovine choroid plexuses maintained in culture. One of the anti-idiotypic antibody generated by a Mab raised against RF-glycoproteins binds to choroidal cells. Furthermore, binding of RF-glycoproteins to the rat choroid plexus was obtained when: (1) the choroid plexus was cultured in the presence of unlabeled RF-glycoproteins; (2) the concentration of soluble RF-glycoproteins in the CSF was increased by isografting SCOs into a lateral ventricle; (3) radiolabeled glycoproteins were perfused into the ventricular CSF. This evidence suggests that the apical plasma membrane of the ependymal cells of the choroid plexus has specific binding sites for RF-glycoproteins, of unknown functional significance. The radiolabeled RF-glycoproteins perfused into the rat CSF also bound to the paraventricular thalamic nucleus, the floor of the Sylvian aqueduct and of the rostral half of the fourth ventricle, and the meninges of the brain and spinal cord. The labeling of the paraventricular thalamic nucleus points to a functional relationship between this nucleus and the SCO. The possibility that the SCO may be a component of the circadian timing system is discussed. *Microsc. Res. Tech. 52:541–551, 2001.*

INTRODUCTION

The subcommissural organ (SCO)-Reissner's fiber (RF) complex has been the subject of numerous investigations with the aim to clarify its functional role (see: Rodríguez and Oksche, 1993; Rodríguez et al., 1992, 1998; Severs et al., 1987, 1993). The fact that the SCO is an ancient and conserved structure of the vertebrate brain (Oksche, 1961), and that it develops very early in ontogeny (Schöbitz et al., 1986) have been taken as evidence that this gland plays an important functional role (Rodríguez et al., 1992). In all species investigated, the SCO is well developed during most part of the fetal life, thus suggesting that it may be involved in developmental mechanism(s). On the other hand, in most but not all species, the SCO is fully active during the whole life span, pointing to the possibility that during the postnatal life the SCO would play a role different from that exerted during the fetal life.

In those species in which the ontogeny of the SCO-RF complex has been investigated, it has been found that during the early stages of development the SCO secretes into the ventricle RF-glycoproteins that do not aggregate into an RF (Meiniel et al., 1990, 1991; Schöbitz et al., 1986, 1993). The first RF is formed during later periods of development, as in chick and bovine embryos (Meiniel et al., 1990, 1991; Schöbitz et al., 1986), or even after birth, as in the rat (Schöbitz et al.,1993). In the human, the SCO can be morphologically distinguished in 7- to 8-week-old embryos, and in 3- to 5-month-old fetuses is an active secretory structure. However, the SCO of human fetuses does not secrete RF-glycoproteins and does not form an RF, and there is evidence indicating that it secretes cerebrospinal fluid (CSF)-soluble glycoproteins that are different from RF-glycoproteins (Rodríguez et al., 1990, 1993; see also pages 573–590, this issue). It may be suggested that the embryonic SCO secretes CSF-soluble proteins, which may or may not be homologous to RF-glycoproteins. What is the target and fate of these proteins?

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Several in vitro studies have shown that RF proper, RF-glycoproteins, and synthetic peptides deduced from the sequence of one of the RF-glycoproteins display aggregative, anti-aggregative, and neurite outgrowing properties on cultured neurons (Meiniel, pages 484–495, this issue; Monnerie et al., 1996, 1997, 1998). All these findings support the view that during the embryonic life, the SCO is involved in neuronal development.

With the exception of the human and the bat, which do not secrete RF-glycoproteins and whose SCO undergo regression after birth, all the other species investigated do secrete RF-glycoproteins and the SCO forms an RF during the whole life span. What function does RF perform throughout the postnatal life? Recently, strong evidence has been obtained that in adult rats RF binds and transports out of the CNS monoamines (E.M. Rodríguez et al., 1999; S. Rodríguez et al., 1999; Rodríguez and Caprile, pages 564–572, this issue), confirming and extending previous findings by Hess and Sterba (1973) and Diederen et al., (1983), and substantiating the hypothesis that RF participates in the clearance of brain monoamines (Olsson, 1958; Hess and Sterba, 1973; Rodríguez et al., 1992). On the other hand, some findings suggest that RF participates in the CSF circulation (Cifuentes et al., 1994; Pérez-Fígares et al., pages 591–607, this issue), and Vío et al. (2000) have shown that the immunoneutralization of RF formation leads to a hydrocephalus.

Is it reasonable to assume that RF may participate in several different mechanisms, such as neuronal differentiation, clearance of brain monoamines, and CSF circulation? To try to answer this question the following information has to be considered. RF is a dynamic structure whose constituent proteins are continuously being assembled to its cephalic end, and disassembled at its caudal end in the filum terminale; as a consequence of this, each molecule forming RF "moves" along the fiber (Rodríguez et al., 1992, 1998). Furthermore, RF is formed by several types of proteins derived from two precursors (Nualart et al., 1991; Nualart and Hein, pages 468–483, this issue), and the major RF component, a 450-kDa glycoprotein, has several types of repeated domains (Meiniel, pages 484–495, this issue). Thus, the molecular organization of RF, the structure of its proteins, and the permanent turnover of these proteins are all facts supporting the possibility that RF may perform multiple functions.

The clearance hypothesis (see above) implies that RF is a binding and transporting structure. Indeed, a solid body of evidence indicates that RF is a dynamic but stable structure that, while growing caudally along the aqueduct, fourth ventricle, and the whole length of the central canal of the spinal cord, remains insoluble in the CSF. However, the possibility that some of the proteins forming RF may become soluble in the CSF, has to be considered. This is a pre-requisite for the validity of the hypothesis postulating the participation of RF in neuronal differentiation (see Meiniel, pages xxx–xxx, this issue). CSF-soluble glycoproteins reacting with antibodies against bovine RF-glycoproteins have been detected in the CSF of adult rabbits by enzyme-immunoassay and immunoblotting (Rodríguez et al., 1993). If CSF-soluble RF-glycoproteins do occur under physiological connotations, as it seems to be the case for the rabbit, the question then arises about the functional significance of these proteins. The present investigation was designed to answer this question by investigating the probable existence within the CNS of specific binding sites for RF-glycoproteins. To achieve this aim, three experimental protocols were used: (1) immunocytochemistry of the CNS of bovine fetuses using anti-idiotypic antibodies, raised against three monoclonal antibodies developed against bovine RF-glycoproteins; (2) in vivo binding of the RF glycoproteins, perfusing into the rat CSF 125I-labeled RF-glycoproteins, or grafting SCO into a lateral ventricle of the rat; (3) in vitro binding of the RF-glycoproteins by rat and bovine choroid plexuses maintained in culture.

MATERIALS AND METHODS

Production of Anti-Idiotypic Antibodies

The anti-RF monoclonal antibodies (MAbs) 2A5, 3E6, and 3B1 (Pérez et al., 1995, 1996), corresponding to the idiotypes (Ab1), were purified by affinity chromatography using protein G-Sepharose (Pharmacia, LKB), coupled to hemocyanin (Schick and Kennedy, 1989), and used, separately, as antigen (Ag) to immunize four female mice (Balb/c, Charles River). Every other week, and for a period of 3.5 months, each mouse received an intraperitoneal injection of 50 μg of Ag emulsified in 50 μl of Freund's adjuvant (complete for the first injection and incomplete for the six others). Anti-idiotypic sera (Ab2) were obtained 7 days after each immunization session, pooled for each Mab, and tested in a competitive ELISA assay.

Competitive ELISA Assay for the Detection of Anti-Idiotypic Antibodies

The wells of a microtiter plate (Costar, high binding) were coated overnight, at 4°C, with 50 μl of a RF-glycoprotein extract (Nualart et al., 1991), at a concentration of 4 μg/ml, and then sequentially incubated with: (1) 300 μl blocking solution (PBS containing 0.25% bovine serum albumin and 0.05 % Tween 20), for 2 hours, and (2) 50 μl of an antibody mixture (see below), for 2.5 hours, in the dark. Antibodies were diluted in the blocking solution and incubations were performed at room temperature. After each incubation, the wells were washed twice with 0.9% NaCl containing 0.05% Tween 20. Peroxidase activity was demonstrated by adding, to each well, 50 μl 0.1 M acetate buffer, pH 6, containing 0.01 % tetramethyl benzidine (Sigma) and 0.06% perhydrol. After 10 minutes, the chromogen reaction was stopped by the addition of 50 μl 1M sulfuric acid. Absorbance at 450 nm was determined in a Microplate reader 2001 (BioWhittaker). In this ELISA assay, only Ab2 sera capable of blocking the Ab1-RF binding will be detected. The degree of inhibition caused by each Ab2 antiserum was used to estimate the titer of the competing anti-idiotype antibody.

To obtain the antibody mixture, each Ab2 serum was pre-incubated with its respective Ab1 labeled with peroxidase, at various dilutions (1:2 to 1:20), for 2 hours, in the dark.

Immunocytochemistry

The brain of two bovine embryos (approximately 75 and 100 days of pregnancy), and adult bovine choroid plexus were fixed by immersion in Bouin's fluid for

2 days. Paraffin sections (8 μm thick) were stained according to the immunoperoxidase method of Sternberger et al. (1970). Ab2 (at a dilution of 1:250 or 1:1,000), MAbs (50 μg/ml), or anti-RF antibodies (AFRU, Rodríguez et al., 1984), diluted 1:1,000, were used as primary antibodies. The sections were sequentially incubated in (1) the primary antibody for 18 hours, at room temperature; (2) the secondary antibody (rabbit anti-mouse IgG, or goat anti-rabbit IgG), diluted 1:50 for 30 minutes; (3) mouse or rabbit PAP (Sigma, St Louis, MO), diluted 1:200, for 30 minutes. DAB was used as electron donor. All antisera and PAP complex were diluted in Tris buffer, pH 7.8, containing 0.7% lambda carrageenan and 0.5% Triton X-100. Incubation in mouse pre-immune serum, as primary antibody, was used as a control test.

Perfusion of Labeled RF-Glycoproteins Into the Rat Cerebrospinal Fluid

Reissner's Fiber Glycoproteins: Extraction and Labeling. RF was obtained by perfusion of the central canal of the bovine spinal cord with 0.9% NaCl (Rodríguez et al., 1984). This procedure was performed at the Valdivia (Chile) slaughterhouse to keep postmortem time to a minimum (about 20 minutes), in order to obtain a fresh RF that will render native, non-denaturated RF-glycoproteins. The latter were solubilized in ammonium bicarbonate as previously described (Nualart et al., 1991), and labeled with 125I according to the cloramine-T method (Sambroock et al. 1989). An aliquot of the labeled RF-extract was used for SDS-PAGE-blotting and fluorography, to evaluate the integrity of the RF-proteins and their labeling with 125I.

Intracerebro-Ventricular Perfusion of 125I-RF-Glycoproteins. Twenty-two male rats of the Holtzmann (Sprague-Dawley) strain were used in the present experiment. All animals were anesthetized with ether. Subsequently, via a cannula stereotaxically implanted into the right lateral ventricle, 100 μl of the 125I-RF-glycoprotein solution (containing 3 or 6 μg RF-proteins), were perfused at a rate of 1 μl/min, by using a perfusion pump. Coordinates for the cannula placement within the lateral ventricle were: posterior from Bregma = 0.5 mm, lateral from sagittal suture = 1.8 mm; ventral from dura = 4.0 mm. After the operation, the rats were placed back into their cages and had free access to food and water. The 22 experimental rats were divided into two groups: (1) twelve rats were injected with 3 μg of labeled RF-glycoproteins and killed at the following time intervals after the end of the perfusion: 0 h (n = 2), 2 h (n = 9), 6 h (n = 1). (2) Ten rats were injected with 6 μg of labeled glycoproteins, and killed 2 h (n = 2), 4 h (n = 4), and 6 h (n = 4) after the end of the perfusion. Under ether anesthesia, the rats were vascularly perfused with a balanced washing solution and then with Bouin's fixative. The brain and the spinal cord were dissected out, frozen, and 15-μm-thick sections were obtained in a cryostat. After drying, the sections were exposed to Hiper Film B-Max for 15 days. Then, all sections were stained with hematoxylin and eosin and used to identify the radioactive brain areas.

Isograft of the Subcommissural Organ Into the Lateral Ventricle of the Rat

Male rats of the Holtzmann strain (Sprague-Dawley) were used. Donor and recipient rats were 2–3 months old at the time of the operation. The host rats were anesthetized with ether and a 21-gauge cannula was stereotaxically implanted into the right lateral ventricle; the coordinates were 1.4 mm rostral from Bregma; 1.4 mm lateral to the sagittal suture; and 3.2 deep from the duramater. Donor rats were anesthetized with ether and killed by decapitation, the brain was dissected out and, under a dissecting microscope, a block of tissue containing exclusively the SCO and the posterior commissure was obtained. Each dissected SCO was sagittally divided into two halves so that they could freely be pushed down along the implanted cannula. Each recipient rat was grafted with two SCOs. Twenty minutes after transplantation, the cannula was slowly removed while injecting 10 μl saline. The rats bearing grafted SCOs were kept at a constant temperature and photoregime (LD 12:12). Water and food were provided ad libitum. Two weeks after transplantation, the rats bearing SCO grafts were anesthetized with ether and the CNS was fixed by vascular perfusion with Bouin's fluid, dissected out, and immersed in the same fixative for 2 days. Dehydration was with alcohol and embedding in Paraplast. The area of the lateral ventricles containing the grafted tissues was oriented to obtain transverse serial sections that were used for immunostaining according to the immunoperoxidase method of Sternberger et al. (1970), using an antiserum developed in rabbits against RF glycoproteins (AFRU, Rodríguez et al., 1984).

Binding of RF-Glycoproteins to Choroid Plexus

The choroid plexuses were obtained from adult rats and adult cows. The rat choroid plexus of both lateral ventricles, or an equivalent quantity of bovine choroid plexus tissue, were cultured in 250 μl of culture medium (nutrient mixture F-12, mixed with Dulbecco's modified Eagle's medium at a ratio 1:1, plus 2.5% fetal bovine serum, and 10% adult bovine serum), containing 1, 5, or 10 μg RF-glycoproteins, for 1 or 12 h, at 37°C. Then the tissue was rinsed with culture medium and transferred to 250 μl of fresh culture medium containing FITC-labeled anti-RF antibodies (AFRU, Rodríguez et al., 1984), diluted 1:250, and cultured for 1 hour, at 37°C. After several rinses in fresh culture medium, the living choroid plexus samples were analyzed under a fluorescence microscope and pictures were obtained. This procedure allowed the visualization under the microscope of the probable binding of RF-glycoproteins to the choroid plexus while the choroidal cells were still alive. Controls were carried out by following the same protocol, but incubation of the choroid plexus in the presence of RF-glycoproteins was omitted.

RESULTS

Detection of Anti-Idiotypic Antibodies by a Competitive ELISA

The six blood samples obtained from each mouse immunized with one of the Mabs (Ab1) used as antigen, namely 2A5, 3E6, and 3B1, and containing the corre-

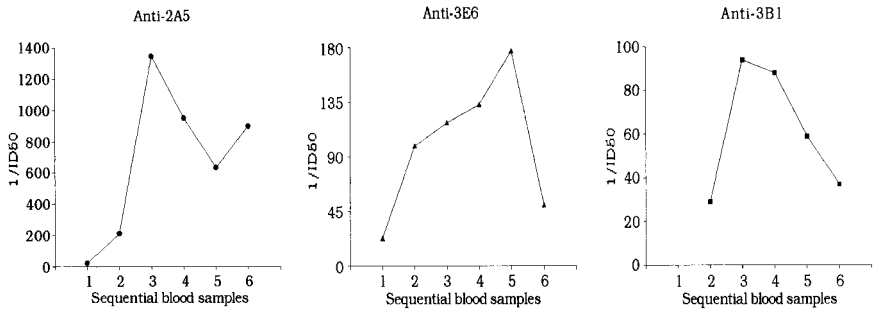

Fig. 1. Competitive ELISA. The wells were coated with Reissner's fiber glycoproteins and then incubated with an antibody mixture of the anti-idiotypic antibody (Ab2) (anti-2A5, anti-3E6 or anti-3B1) with the corresponding idiotypic antibody (Ab1) labeled with peroxidase. Previously, various mixtures in which the concentration of Ab remained constant and Ab was diluted 1:2 to 1:20480, had been prepared to estimate the ID50 parameter, that is, the dilution that causes a 50% inhibition of the specific binding of Ab1 to the RF-glycoproteins. The figure shows the evolution of 1/ID50 for each anti-idiotypic antiserum during the immunization process.

sponding anti-idiotypic antibody (Ab2), were analyzed by a competitive ELISA. All blood sample were capable of blocking the binding of the labeled Ab1 to the RF-glycoproteins, thus indicating the presence of Ab2 in all the samples. In order to estimate the titer of Ab2, the ID50 parameter was used, that is, the identification of the dilution that causes a 50% inhibition of the specific binding of Ab1 to the RF-glycoproteins. Figure 1 shows the evolution of 1/ID50 for each anti-idiotypic antiserum during the immunization process. The titer of Ab2 progressively increased in the first bleeds, decreasing again in the final bleeds.

Figure 2 shows the curves obtained for each Ab2 by increasing dilutions of that blood sample displaying the highest titer of Ab2, namely, the third bleed for anti-2A5 and anti-3B1, and the fifth bleed for anti-3E6. Anti-2A5 and anti-3E6, at a 1:20 dilution, inhibited about 90% of the Ab1-RF binding; anti-3B1 antiserum displayed the lowest titer, inhibiting about 70% of the Ab1-RF binding, at a1:20 dilution.

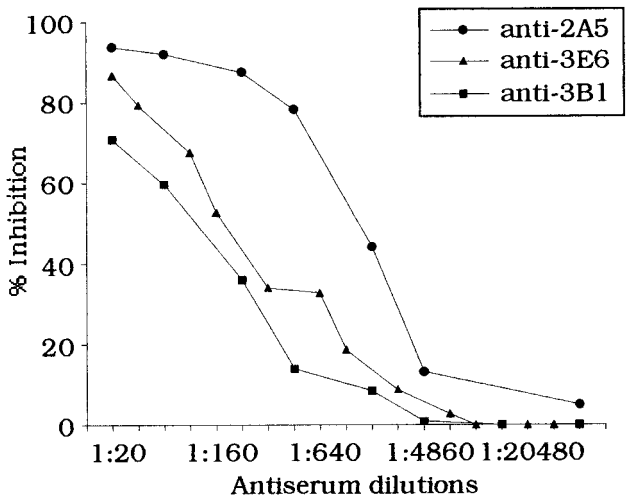

Fig. 2. Competitive ELISA. The wells were coated with Reissner's fiber glycoproteins and then incubated with antibody mixtures in which the concentration of the labeled idiotypic antibody (Ab1) remained constant and the blood sample containing the anti-idiotypic antibody (Ab2) underwent increasing dilutions (1:20 to 1:20480) (anti-2A5, anti-3E6 and anti-3B1). Curves were obtained for each Ab2 (anti-2A5, anti-3E6 and anti-3B1) by increasing dilutions of that blood sample displaying the highest titer of Ab2 deduced from Figure 1. Anti-2A5 and anti-3E6, at a 1:20 dilution, inhibited about 90% of the Ab1-RF binding; anti-3B1 antiserum displayed the lowest titer.

Anti-Idiotypic Antibody Generated by a Mab Raised Against RF-Glycoproteins Binds to Choroidal Cells

The SCO of the 75- and the 100-day-old embryos, displayed essentially the same pattern of immunoreactivity when immunostained with Mab 3B1; namely, a strong reaction of the apical secretory granules of the ependymal and hypendymal cells, and a weak reaction in other regions of the cytoplasm, most likely corresponding the cisternae of the rough endoplasmic reticulum (Fig. 3A,B). No RF was found in these embryos.

The blood sample of each immunized mouse showing the highest titer of Ab2 was used to immunostain sections of the CNS of the 75- and 100-day-old embryos. A positive immunoreaction was obtained only with the anti-3B1 serum. In both embryos, the reaction was circumscribed to the ependymal cells of the choroid plexus (Fig. 3C). Virtually all choroidal cells presented a positive reaction at the apical plasma membrane (Fig. 3D–F). A population of cells also presented a strong reaction throughout the cytoplasm (Fig. 3 D, E). The ciliated ependyma lining the ventricles did not react with this anti-idiotypic antibody (Fig. 3C).

Radiolabeled RF-Glycoproteins Perfused Into the Rat CSF Bind to Certain Brain Regions

Immunoblotting analyses of RF-extracts performed previously using anti-RF sera have shown the presence of six or more immunoreactive bands (see Nualart et al., 1991; Nualart and Rodríguez, 1996).The fluorography of the radiolabeled RF-extract carried out in the present investigation showed that all bands were labeled, with 450-kDa band being the major component.

The rats with intracerebro-ventricular perfusion of 125I-RF- glycoproteins showed radioactive labeling of the following regions: (1) Paraventricular thalamic nucleus. All experimental rats, irrespective of the amount of RF-glycoproteins perfused or the post-perfusion interval, showed a distinct labeling of this thalamic nucleus (Figs. 4A–C). The strongest labeling was found 2 and 4 hours after the perfusion. (2) Choroid plexus. The strongest labeling of the choroid plexus was found 2 and 4 h after perfusing 6 μg RF-glycoproteins (Fig. 4D,E). (3) The floor of the Sylvian aqueduct and fourth ventricle. The labeled area included the whole length of the aqueduct and the floor of the rostral half of the fourth ventricle (Fig. 5A–C). The strongest labeling

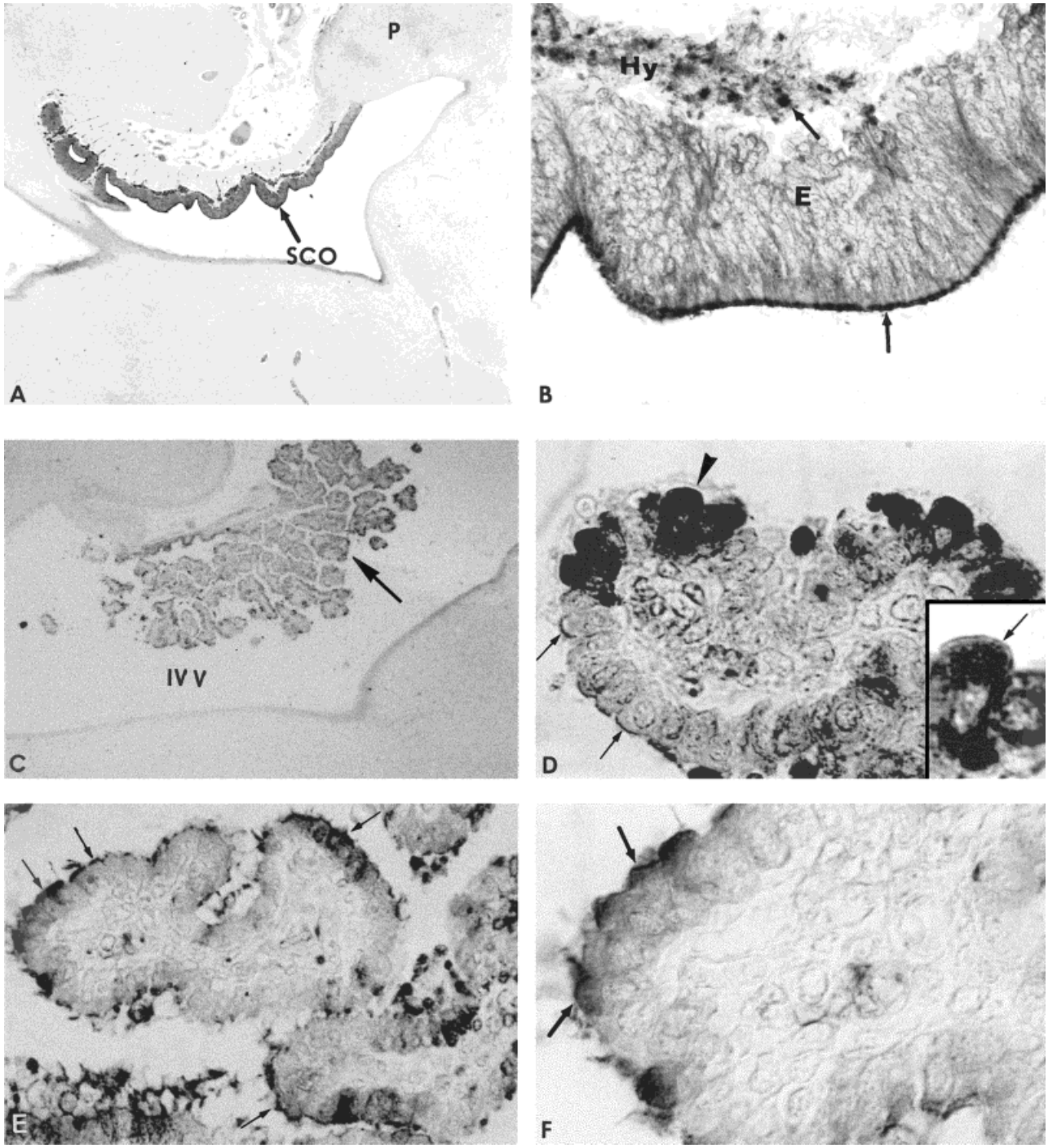

Fig. 3. Sagittal paraffin section through the brain of a 75-day-old bovine embryo. **A:** Subcommissural organ (SCO) immunostained using Mab 3B1. P, pineal gland. ×16. **B:** Detailed magnification of A to demonstrate the strong immunoreaction of the apical secretory granules (arrows) of the ependymal (E) and hypendymal (Hy) cells. ×250. **C,D:** Choroid plexus of fourth ventricle immunostained using the anti-idiotypic antibody against Mab 3B1. C: Low magnification showing that selective immunoreaction of the choroid plexus (arrow). IV V, fourth ventricle. ×16. D: Detailed magnification of previous figure revealing the immunolabelling of the apical plasma membrane of the choroidal cells (arrows) and the strong staining of the cytoplasm of a populations of choroidal cells (arrowheads). ×625. **Inset:** High magnification of the cell indicated by arrowhead in D, showing that the plasma apical plasma membrane of the strongly reactive cells is also labeled (arrow). ×1,200. **E:** High magnification of one of the choroidal villi shown in C. Virtually all choroidal cells have their apical plasma membrane labeled (arrows). ×250. **F:** Detailed magnification of E showing the strong immunoreaction of the apical plasma membrane of the choroidal cells (arrows). ×625.

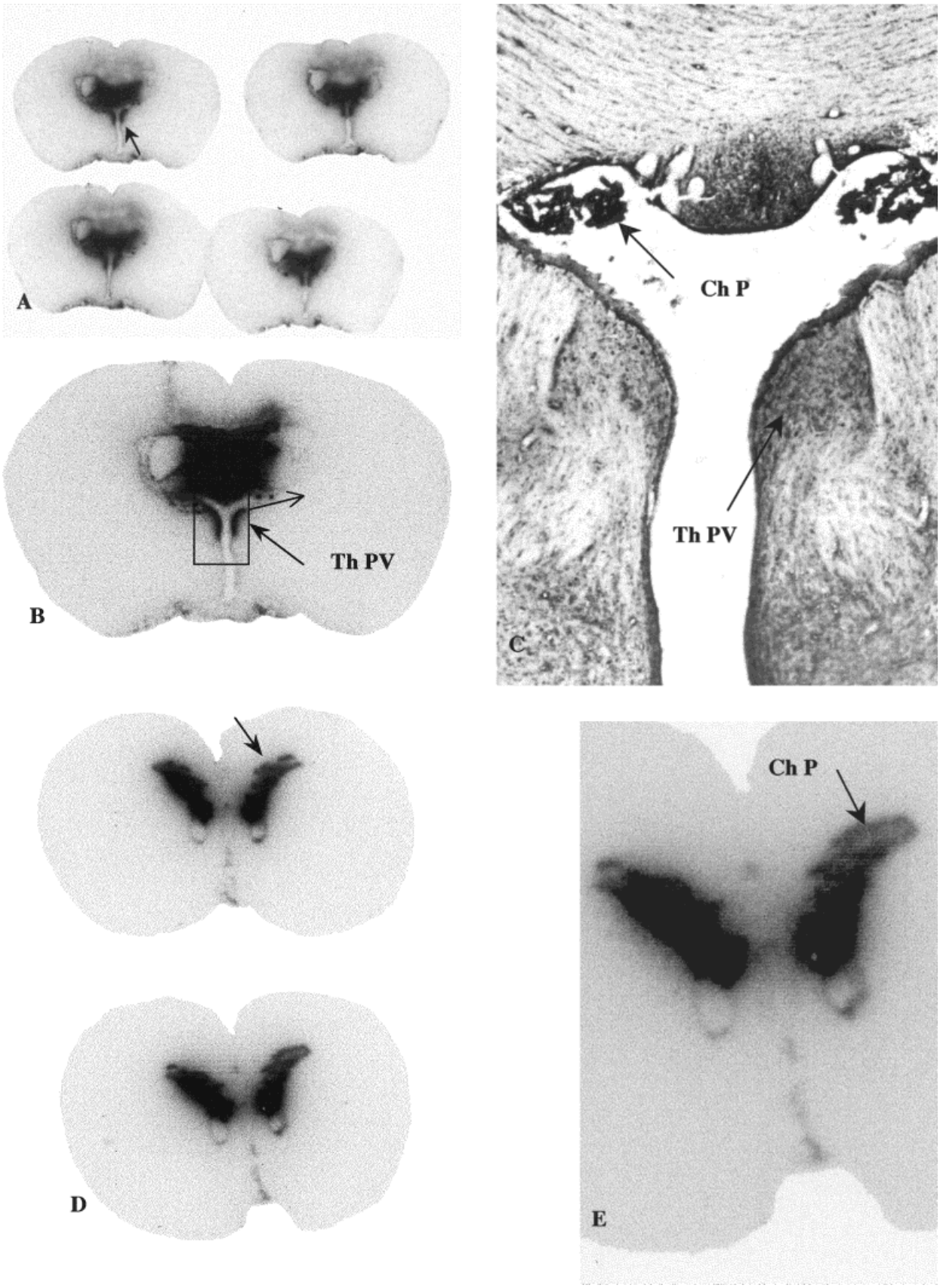

Fig. 4. Radioautography of the frontal sections through the brain of rats perfused intracerebroventricularly with 125I-RF-glycoproteins. The brain was fixed 4 hours after the end of perfusion. **A:** Four neighbor sections through the diencephalon showing the labeling of the paraventricular thalamic nucleus (arrow) and structures within the ventricle. ×3. **B:** High magnification of one of the sections shown in A showing the distinct labeling of the paraventricular thalamic nucleus (Th PV). The same section used for the radioautography was later stained with hematoxylin and eosin. The area framed by rectangle is shown in C. ×8. **C:** High magnification of the area framed in B. Hematoxylin-eosin stain. Th PV, paraventricular thalamic nucleus; Ch P, choroid plexus. ×70. **D:** Two adjacent sections through the telencephalon showing the strong labeling of the choroid plexus (arrow). ×8. **E:** Detailed magnification of one of the sections shown in D where some individual choroidal villi can be distinguished (Ch P). ×18.

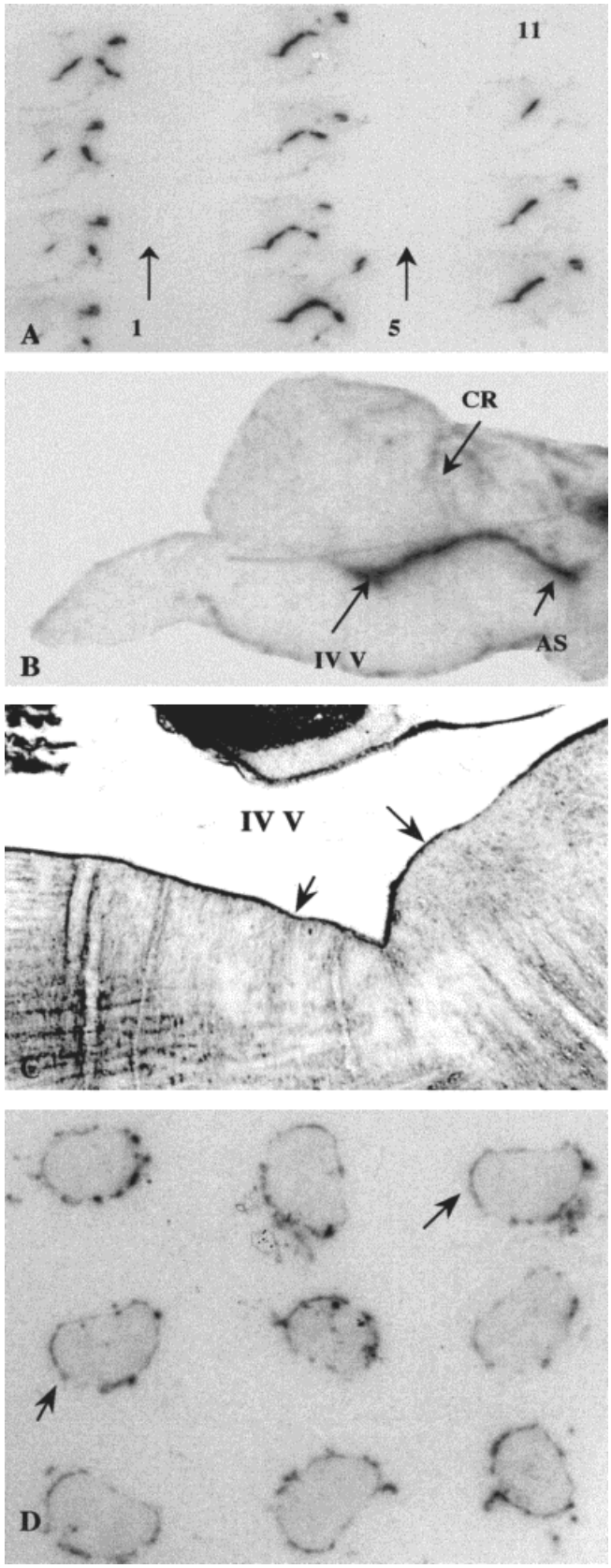

Fig. 5.

was found 2 and 4 hours after perfusing 6 μg RF-glycoproteins. (4) A distinct labeling of the surface of the brain and spinal cord (meninges?, subarachnoid space?) was found in rats killed 4 and 6 hours after perfusion (Fig. 5D).

Choroid Plexus of Rats Bearing a SCO Graft in the Lateral Ventricle Displays RF-Glycoproteins on Their Free Surface

The SCOs transplanted into the right lateral ventricle of host rats survived during the two weeks of transplantation. The ependymal and hypendymal cells showed signs of a high secretory activity (Fig. 6A; see S. Rodríguez et al., 1999). A previous study has demonstrated that the SCO grafted into a lateral ventricle releases RF-material into the CSF (S. Rodríguez et al., 1999). The present study has revealed that the choroid plexus adjacent to the graft has RF-immunoreactive material on the ventricular surface of the choroidal cells (Fig. 6A,B).

Cultured Choroid Plexuses Bind Unlabeled RF-Glycoproteins Added to the Culture Medium

The choroid plexus of both, rat and cow, cultured in the presence of RF-glycoproteins showed a positive immunoreaction on the ventricular surface of the choroidal cells when FITC-anti-RF antibodies were added to the culture (Fig. 6C,D) . A rather thick layer of immunofluorescence material was observed on the free surface of the choroidal cells; this probably corresponded to the labeling of the layer of microvilli projected by these cells (Fig. 6D). This study under a fluorescence microscope was performed while the choroidal cells were still alive. The fluorescence signal on the surface of the choroid plexus was weak when 1 μg of RF-glycoproteins was added to the culture medium, and became stronger when 5 and 10 μg proteins were used. When addition of RF-glycoproteins was omitted, no fluorescence was detected in the choroid plexus.

DISCUSSION

Limitations and Difficulties of the Experimental Protocols Used in the Present Study

The first approach in the present investigation was to perform binding ligand experiments using cryostat sections of the brain of rats (E-18 embryos, and adult specimens) and bovine embryos, exposed to 125I-RF glycoproteins. No binding was obtained under these conditions. This led us to standardize the procedure of

Fig. 5. Radioautography of the sagittal sections through the brain stem and transverse section through the spinal cord of rats perfused intracerebroventricularly with 125I-RF-glycoproteins. The brain was fixed 4 hours after the end of perfusion. **A:** Eleven neighbor sections through the brain stem showing the labeling of cerebral aqueduct and fourth ventricle. **B:** High magnification of one of the sections shown in A showing the distinct labeling of the floor of the Sylvian aqueduct (AS) and of the rostral halve of the fourth ventricle (IV V). The dorsal wall of the aqueduct is not labeled; this becomes evident at the collicular recess (CR). The same section used for the radioautography was later stained with hematoxylin and eosin. ×5. **C:** High magnification of the fourth ventricle (IV V) of the section shown in B. Hematoxylin-eosin stain. Arrows point the floor region displaying the label. x20. **D:** Radioautography of nine sections through different segments of the spinal cords, showing labeling of the "meningeal" region. ×7.

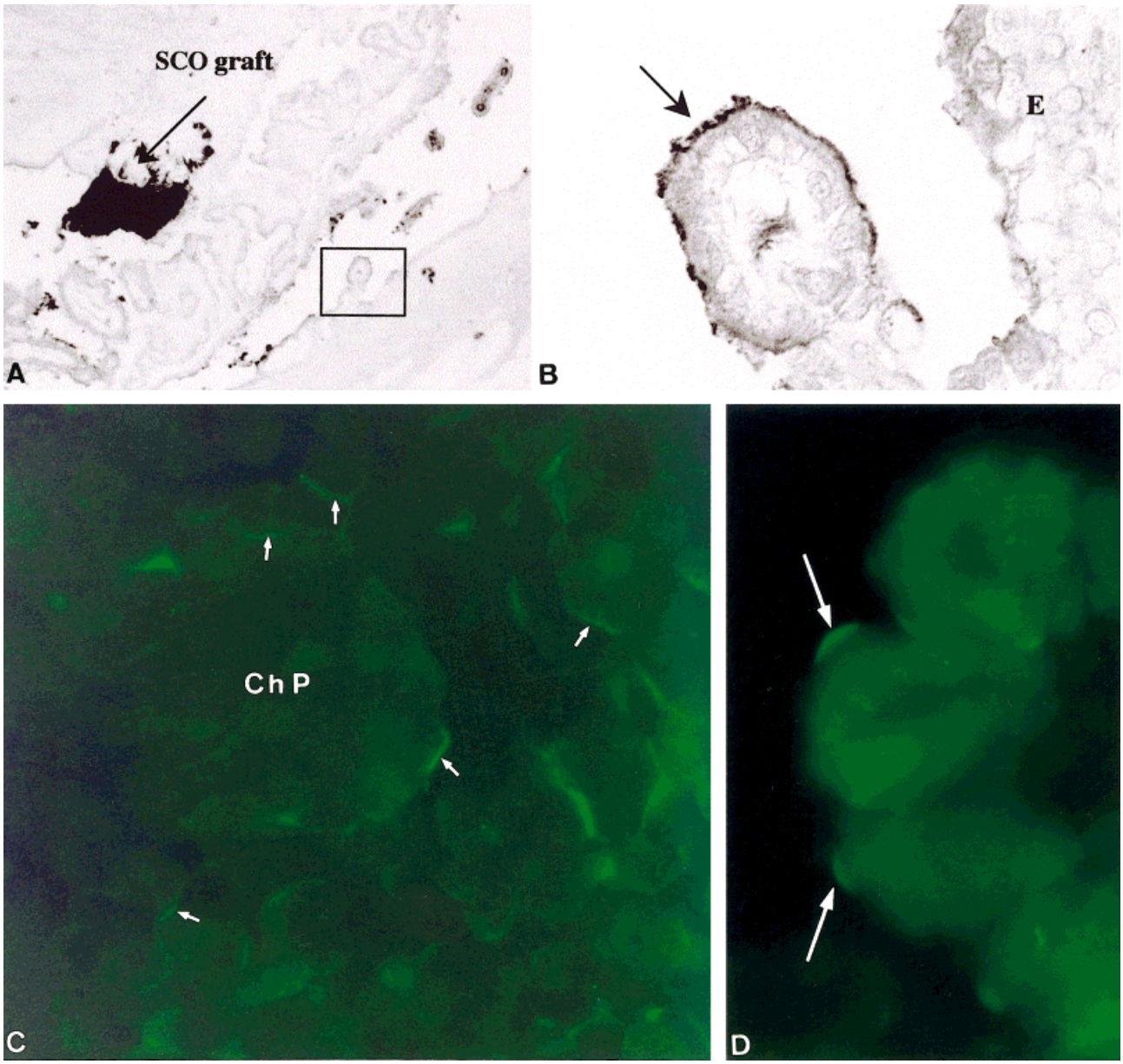

Fig. 6. **A:** Frontal section through the telencephalon of rat grafted with SCOs for 2 weeks. Immunoperoxidase staining using an anti-RF serum. The grafted SCO secretory cells appear strongly stained (SCO graft). The framed area is shown at higher magnification in B. ×60. **B:** Detailed magnification of box in A showing RF-immunoreactive material on the free surface of the choroidal cells (arrow). The adjacent ependyma (E) is devoid of immunolabeling. ×625. **C:** Rat choroid plexus cultured for 1 hour in the presence of unlabeled RF-glycoproteins (5 μg proteins/250 μl medium) and, after washing, cultured for 1 hour in the presence of FITC-labeled anti-RF antibodies. The living choroid plexus samples (CHP) were analyzed under a fluorescence microscope and pictures were obtained. The apical plasma membrane of the choroidal cells displayed varying degrees of immunofluorescence (arrows). ×250. **D:** Detailed magnification of C. There is a rather thick layer of immunofluorescence material on the free surface of the choroidal cells, probably corresponding to the layer of microvilli projected by these cells (arrows). ×625.

in vivo binding perfusing the radiolabeled RF-glycoproteins to the CSF of adult rats. The protocol used has the following limitations: (1) Since bovine RF-glycoproteins were perfused into rats, species-specificity problems may be involved. (2) A pool of various labeled RF-glycoproteins was used so that the nature of the ligand could not be established. However, as a first approach, it seemed useful to establish whether or not this pool of RF-glycoproteins displays binding sites in the CNS. (3) The protocol used does not allow carrying out displacement or competition studies, and consequently the specificity of the binding could not be established. This was partially overcome by the fact that only a few brain areas showed a consistent binding. Some of these limitations also apply to the experiments involving the in vitro binding of RF-glycoproteins to the

rat and bovine choroid plexus, and the grafting of SCO into the lateral ventricle.

Despite all these limitations, the body of evidence obtained through the different protocols permits making some propositions with respect to the probable existence within the CNS of binding sites for RF-glycoproteins.

Evidence That the Apical Membrane of the Ependymal Cells of the Choroid Plexus Has Specific Binding Sites for RF-Glycoproteins

The anti-idiotypic antibodies have been successfully used to search for receptors due to their property to mimic the antigen structure and biological activity (Knigge et al., 1989; Schick and Kennedy, 1989). Three Mab against RF-glycoproteins have been obtained previously (Pérez et al., 1995, 1996). In the present investigation, anti-idiotypic antibodies against these Mab were prepared, but only one of them (anti-3B1), when used for immunostaining of the CNS of bovine embryos, showed a positive reaction of the apical plasma membrane of the choroid plexus ependymal cells. This suggests that the epitope of Mab 3B1, present in an RF-glycoprotein, could correspond to a domain having affinity for a molecule (receptor?) present in the apical plasma membrane of the choroidal cells. The presence of anti-3B1 immunoreactive material in the cytoplasm of some choroidal cells may correspond to receptor molecules partially synthesized, or degraded, or being transported to the plasma membrane (Spengler et al., 1995).

Binding of the RF-glycoproteins to the rat choroid plexus was obtained when: (1) the choroid plexus was cultured in the presence of unlabeled RF-glycoproteins; (2) the concentration of soluble RF-glycoproteins in the CSF was increased by isografting SCOs into the lateral ventricle; (3) radiolabeled glycoproteins were perfused into the ventricular CSF. This binding appears to be specific, since no binding was detected in the walls of the lateral ventricles. In the rat, the whole volume of CSF is replaced by freshly secreted fluid approximately every 4 hours (see Pérez-Fígares et al., pages 591–607, this issue). Therefore, after the perfusion into the rat CSF of the radiolabeled RF-glycoproteins has finished, these proteins will continue to be available to their binding sites for a period of 4 hours. Since the choroid plexus still showed bound radioactive material 6 hours after the end of the perfusion, the binding of the RF-material to the choroidal cells has remained stable for at least 2 hours.

Assuming that the ependymal cells of the choroid plexus have in their apical plasma membrane specific binding sites for RF-glycoproteins, these sites would have a functional significance only if the ligand proteins are present in the CSF under physiological conditions. The only two reports dealing with this problem are contradictory. Rodríguez et al. (1993) have detected in the CSF of adult rabbits soluble glycoproteins reacting with antibodies against bovine RF-glycoproteins. On the other hand, Estivill-Torrús et al. (1998), by using a sensitive enzyme-immunoassay were unable to detect RF-glycoproteins in the bovine CSF. Thus, the problem of a probable functional relationship between the SCO-RF complex and the choroid plexus remains open to future investigations.

Is the SCO a Component of the Circadian Timing System?

Previous evidence (discussed below) and the binding of RF-glycoproteins to the thalamic paraventricular nucleus (PVT) point to a positive answer to this question.

The secretory activity of the SCO is regulated by serotoninergic innervation arising from raphe nuclei (Bouchaud and Arluison, 1977; Møllgard et al., 1978; Møllgard and Wiklund, 1979; Jiménez et al., 1993).

Performing electrolytic lesions of different raphe nuclei, Léger et al. (1983) revealed that the serotoninergic innervation of the rat SCO is mainly derived from nuclei raphe centralis superior and raphe dorsalis, each nucleus contributing about one-third of the input; the remainder is suggested to originate from the nucleus raphe pontis. The PVT is also innervated by serotoninergic fibers arising mainly from the dorsal and median raphe (Azmitia and Segal, 1978; Moore et al., 1978, Peschanski and Besson, 1984; Vertes and Martin, 1988). Thus, the nucleus raphe dorsalis innervates both the SCO and the PVT. Furthermore, serotonergic neurons of the raphe also innervate the pineal gland, the lateral habenula, and the supraquiasmatic nucleus (SCN), all of them being components of the biological timing system (Moga et al., 1995).

The components of the biological timing system, namely, the pineal gland, the lateral habenula, the PVT, and the SCN, are also connected one another. Thus, the SCN and the PVT display melatonin receptors (Lindroos et al., 1992). The PVT has reciprocal connections with the SCN, and it may function as an input component, an output component, and/or a feedback component of the circadian timing system (Moga et al., 1995).

The presence in the PVT of binding sites for RF-glycoproteins poses the interesting possibility of a functional relationship between the SCO and the PVT. In this respect, the findings by Vullings et al. (1983) and Jiménez et al. (1993) become relevant. The former found that in the frog, the SCO is more active during the night period; the latter established that numerous fibers from the frog pineal tract establish synaptic contacts with the SCO secretory cells. The PVT sends projections to the SCN, several nuclei of the medial basal hypothalamus, limbic system, the striatum, and the cortex (Moga et al., 1995). These authors concluded that the PVT is ideally situated to relay circadian timing information from the SCN to brain areas implicated in neuroendocrine, visceral, and motivational aspects of behavior and to provide feedback regulation of the circadian pacemaker, the SCN. The existence in the PVT of melatonin receptors, and probably of RF-glycoproteins receptors, puts this key nucleus under the influence of humorally mediated factors secreted by the pineal gland and the SCO. This SCO-PVT interrelationship might help to explain the disruption of the sleep-wake cycle occurring in rats with a permanent immunoneutralization of RF-formation (S. Rodríguez et al., 1999). Worth mentioning is the fact the PVT receives a dense innervation from the locus coeruleus and raphe nuclei, both nuclei implicated in the control of sleep-wake cycle (Moga et al., 1995).

Labeling of the Floor of the Cerebral Aqueduct and Fourth Ventricle by Radioactive RF-Glycoproteins

The radioautographic method utilized did not allow the microscopic identification of the labeled structures. So, it could not be established whether the binding sites were at the ventral ependyma of the aqueduct and fourth ventricle, or in subependymal structures. For the same technical limitation, the exact localization of the meningeal labeling could not be established. This latter result, however, indicates that RF-glycoproteins perfused intraventricularly can reach the subarachnoidal CSF of the brain and spinal cord.

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