Neurothelin: An Inducible Cell Surface Glycoprotein of Blood–Brain Barrier–specific Endothelial Cells and Distinct Neurons

Burkhard Schlosshauer and Karl-Heinz Herzog

Max-Planck-Institut für Entwicklungsbiologie, 7400 Tübingen, Federal Republic of Germany

Abstract. The blood–brain barrier is characterized by still poorly understood barrier and transport functions performed by specialized endothelial cells. Hybridoma technology has been used to identify a protein termed neurothelin that is specific for these endothelial cells. Neurothelin is defined by the species-specific mouse mAb 1W5 raised against lentil-lectin–binding proteins of neural tissue from embryonic chick.

In the posthatch chick, neurothelin expression is found on endothelial cells within the brain but not on those of the systemic vascular system. Injection of the monoclonal antibody in vivo leads to labeling of brain capillaries, indicating that the corresponding antigen is expressed on the luminal surface of brain endothelial cells. Transplantation of embryonic mouse brain onto the chick chorioallantoic membrane results in rodent brain vascularization by the avian vascular system. Subsequently, normally mAb 1W5–negative endothelial cells, originating from blood vessels of the chick chorioallantoic membrane, are induced to express neurothelin when they are in contact with mouse neural tissue.

In contrast to differentiated brain neurons that do not express neurothelin, neurons of the nonvascularized chick retina synthesize neurothelin. However, neurothelin is not found on retinal ganglion cell axons terminating on 1W5-negative brain cells.

1W5 immunoreactivity was also found in the pigment epithelium that forms the blood–eye barrier. Putting epithelial cells into culture results in concentration of neurothelin at cell–cell contact sites, leaving other cell surface areas devoid of antigen. Therefore, the distribution of neurothelin appears to be regulated by cell–cell interactions.

In Western blot analysis, neurothelin was identified as a protein with a molecular mass of ~43 kD. The protein bears at least one intramolecular disulfide bridge and sulfated glucuronic acid as well as α-D-substituted mannose/glucose moieties.

The exclusive neurothelin expression in the posthatch chick on endothelial cells of the central nervous system but not on systemic endothelial cells makes neurothelin a marker specific for blood–brain barrier–forming endothelial cells. The spatiotemporally regulated neurothelin expression in neurons suggests an interaction between vascularization and neuronal differentiation.

NEURAL activity is essentially based on different states of membrane polarization. These states are highly dynamic and depend on minimal fluxes of distinct ions across the cytoplasmic membrane of neurons (Koester, 1985). Therefore, in the brain interstitium, a deliberate homeostasis concerning various components, including small ions such as Na^+, K^+, and Ca^{2+}, appears to be an unrenounceable prerequisite for functioning of the nervous system. On the other hand, the need for homeostasis is permanently challenged by the variable composition of the blood, which provides the crucial nutrient support for the central nervous system. This physiological situation leads to the necessity of the blood–brain barrier, which prevents the neurons from constant exposure to a continuously changing environment (Bradbury, 1979).

Most extracerebral blood vessels are lined by a permeable or fenestrated endothelium, whereas capillaries within the brain are generally not fenestrated (Rhodin, 1975). Brain capillaries are also completely ensheathed by astroglial endfeet, in contrast to systemic vessels (for review see Bradbury, 1984). The physiological relevance of this organization became evident in tracer experiments. Horseradish peroxidase, a 43-kD protein that can be visualized on the ultrastructural level in electron microscopy, does not penetrate through brain capillaries after injection into the vascular system (Reese and Karnovsky, 1967). Direct application into the brain, however, leads readily to diffusion of the enzyme even between glial endfeet surrounding brain capillaries, but not beyond the endothelial cell lining (Brightman and Reese, 1969). The hypothesis deduced from these experiments, that endothelial rather than astroglial cells form the blood–brain barrier, is supported by the observation that brain endothelial cells lack most pinocytotic vesicles and are interconnected by tight junctions (Reese and Karnovsky, 1967). This points

first of all to a reduced transcellular transport via pinocytosis and, secondly, to the lack of extracellular diffusion via interstitial spaces (paracellular route) across the endothelial lining of capillaries in the brain (Raviola, 1977).

The formation of a blood barrier, however, prevents the direct access of neurons to nutrients that are supplied via systemic circulation. Therefore, distinct transport systems bridging the blood–brain barrier have been developed (Betz and Goldstein, 1978; Betz and Goldstein, 1986; Betz et al., 1980; Dick et al., 1984). However, the molecular complexity of the blood–brain barrier, as well as the developmental sequence that leads to the formation of the barrier–carrier complex, is still ill defined.

Monoclonal antibodies have proved helpful tools in neurobiology (Reichardt, 1984; Milstein, 1986) since they allow identification and functional characterization of distinct molecules. We have taken this approach to reveal a possibly novel component of blood–brain barrier–forming endothelial cells.

Here we present data about the antigen called neurothelin that may contribute to the understanding of the blood–brain barrier–carrier complex. This glycosylated cell surface protein exhibits characteristics of a blood barrier marker in that it is expressed in endothelial cells within the brain but not in extracerebral blood capillaries. Neurothelin expression is subject to a pronounced developmental regulation and can be induced in transplantation experiments in extracerebral endothelial cells by brain cells.

For simplicity, the term blood–brain barrier marker is used in this presentation to refer to the functional complex of barrier and carrier components.

Materials and Methods

Materials

Neural retina, pigment epithelium, tectum opticum, brain capillaries, and kidney were dissected from White Leghorn chicken (embryos and hatched animals) in addition to brain material from BALB/c-Byl mice. All chemicals used were from Merck (Darmstadt, FRG) if not stated otherwise. To all homogenization and solubilization buffers, enzyme inhibitors 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid, aprotinin, leupeptin, pepstatin, and PMSF from Sigma Chemical Co. (St. Louis, MO) were added in concentrations as indicated in Schlosshauer (1989). Lentil-lectin Sepharose and CNBr-activated Sepharose were from Pharmacia Fine Chemicals (Uppsala, Sweden). Biotinylated concanavalin A (Sigma Chemical Co., 50 μg/ml), streptavidin-conjugated horseradish peroxidase (Dianova GmbH, Hamburg, FRG; 0.8 μg/ml) were applied for 16 h at 4°C. Ascites fluid with mAb D3 against the 180-kD isoform of the neural cell adhesion molecule (N-CAM)[1] (Schlosshauer, 1989), mAb C4 (Schlosshauer, 1989), mAb HNK-1 (gift from S. Chang, University of Pennsylvania, Philadelphia, PA), and mAb 1W5 were used at a dilution of 1:1,000 for 16 h at 4°C. Rhodamine- and fluorescein-conjugated $F(ab')_2$ fragment goat anti-mouse IgG (heavy and light chain) and peroxidase-conjugated $F(ab)_2$ fragment goat anti-mouse were obtained from Dianova. 4,6-diamidino-2-phenylindole (DAPI) was bought from Sigma Chemical Co.

Methods

Immunogen Processing. Cytoplasmic membranes of chick retinae at E10 were enriched by sucrose gradient centrifugation (Hoffman et al., 1982; Walter et al., 1987). Membranes were solubilized with Triton X-100 and ultracentrifuged, and the clear supernatant was applied to a lentil-lectin affinity column as described (Schlosshauer, 1989). Eluted glycoconjugates were used for five immunization cycles of BALB/c mice according to Schlosshauer (1989).

1. *Abbreviations used in this paper*: DAPI, 4,6-diamidino-2-phenylindole; N-CAM, neural cell adhesion molecule.

Generation of monoclonal antibodies was performed by fusion of mouse spleen cells with NS-1 myeloma cells (Fazekas de St. Groth and Scheidegger, 1980), and hybridoma supernatants were screened on histological sections composed of chick head tissues of three different developmental stages (Schlosshauer, 1989). 1W5 hybridoma cell lines were subcloned three times by limited dilution.

In Vivo Immunolabeling. Egg shells of E2 or E3 chick embryos were fenestrated (~2 cm^2) and incubated for another 12–17 d. Injection of 0.2–2 mg affinity-purified mAb 1W5 IgG or mAb D3 IgG in 50–100 μl F12 medium (sterile filtrated) into blood vessels of the chorioallantoic membrane was performed between E14 and E19. After circulation of the antibodies for 20–30 min, embryos were killed, perfused with PBS and 4% paraformaldehyde, and processed for cryosectioning. Tissue sections were incubated exclusively with a rhodamine-labeled secondary antibody to reveal in vivo–bound mAb 1W5 or mAb D3.

Transplantation. Based on approaches of Ausprunk et al. (1975) and Risau et al. (1986*b*), fragmented and whole E11 mouse brains were transplanted aseptically on chick chorioallantoic membranes of E9 chick embryos. Beforehand, chick eggs (~250) had been fenestrated and the windows had been sealed reversibly at the second day of incubation. After transplantation, eggs were incubated for up to 12 d. Thereafter, six viable transplants were processed for immunohistochemistry (see below). Neural structures were identified with the aid of mAb D3 against $N\text{-}CAM_{180}$ (Schlosshauer, 1989).

Primary Cultures. Single-cell cultures of retina and pigment epithelium of E6 chick embryos were obtained by trypsinization, trituration, and plating on laminin-coated (20 μg/ml for 16 h at 37°C) 8-well slides (Flow Laboratories, Inc., McLean, VA) as outlined earlier (Schlosshauer et al., 1988; Schlosshauer, 1989) or on the retinal basal lamina (Halfter et al., 1987). For explant cultures, E6 chick retinae were flat-mounted on black nitrocellulose filters, cut into 275-μm strips, and placed onto laminin-coated glass coverslips (Halfter et al., 1981). Incubation in F12 culture medium (Gibco Laboratories, Grand Island, NY) containing serum and methylcellulose (Walter et al., 1987) was performed for 24–48 h for explant cultures and 6 h to 12 d for single-cell cultures.

Immunofluorescence Assays. Cell surface antigen expression was examined by incubation of viable, nonfixed cell cultures with 1W5 hybridoma supernatant or diluted 1W5 ascites added to culture medium at a dilution of 1:1,000 for 1 h. After washing and fixation, a rhodamine-conjugated secondary antibody was applied.

Labeling of brain capillaries isolated from posthatch chicken according to Brendel et al. (1974) was performed after initial fixation. For histological investigations, chick retinae and mesencephalic regions of different developmental stages as well as mouse brain at P7 were dissected and fixed in paraformaldehyde for 16 h at 4°C. After sucrose impregnation (30% sucrose for 24 h at 4°C) 12-μm cryostat sections were produced and immunolabeled as detailed earlier (Schlosshauer et al., 1988).

Counterstaining of cell nuclei with DAPI was achieved by incubation of tissue sections with 0.5 μg/ml DAPI in PBS for 2 min.

Protein Characterization. For the purification of neurothelin, cytoplasmic membranes of chick retina at E9–10 were solubilized with 1% Triton X-100 in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl (2 mg protein/ml), ultracentrifuged, and applied to an mAb 1W5 affinity column (2 mg IgG/ml CNBr-Sepharose) at a flow rate of 5 ml/h (Schlosshauer, 1989). Beforehand, mAb 1W5 had been affinity purified on protein A–Sepharose (Pharmacia Fine Chemicals) according to the manufacturers' instructions. After thorough washing, neurothelin was eluted at pH 11.5 without detergent, dialyzed against 1:10 PBS, and lyophilized. Cell membranes from retina, brain, pigment epithelium, and kidney were partially purified according to Wallenfels (1979); brain capillaries were isolated according to Brendel et al. (1974).

Protein quantification was carried out according to Lowry et al. (1951). PAGE (Laemmli et al., 1970), applying 10 and 14% acrylamide gels, silver staining of SDS gels (Ansorge, 1985), and Western blot analysis (Towbin et al., 1979; Hawkes et al., 1982) were used for qualitative and quantitative characterization of the antigen. Reduction of samples for gel electrophoresis was achieved by addition of 0.1 M DTT to the SDS sample buffer (Cleland, 1964).

Results

Specific Neurothelin Expression in Brain Capillaries

Staining of blood vessels from the lumbar region of the body and the extraembryonic chorioallantoic membrane and com-

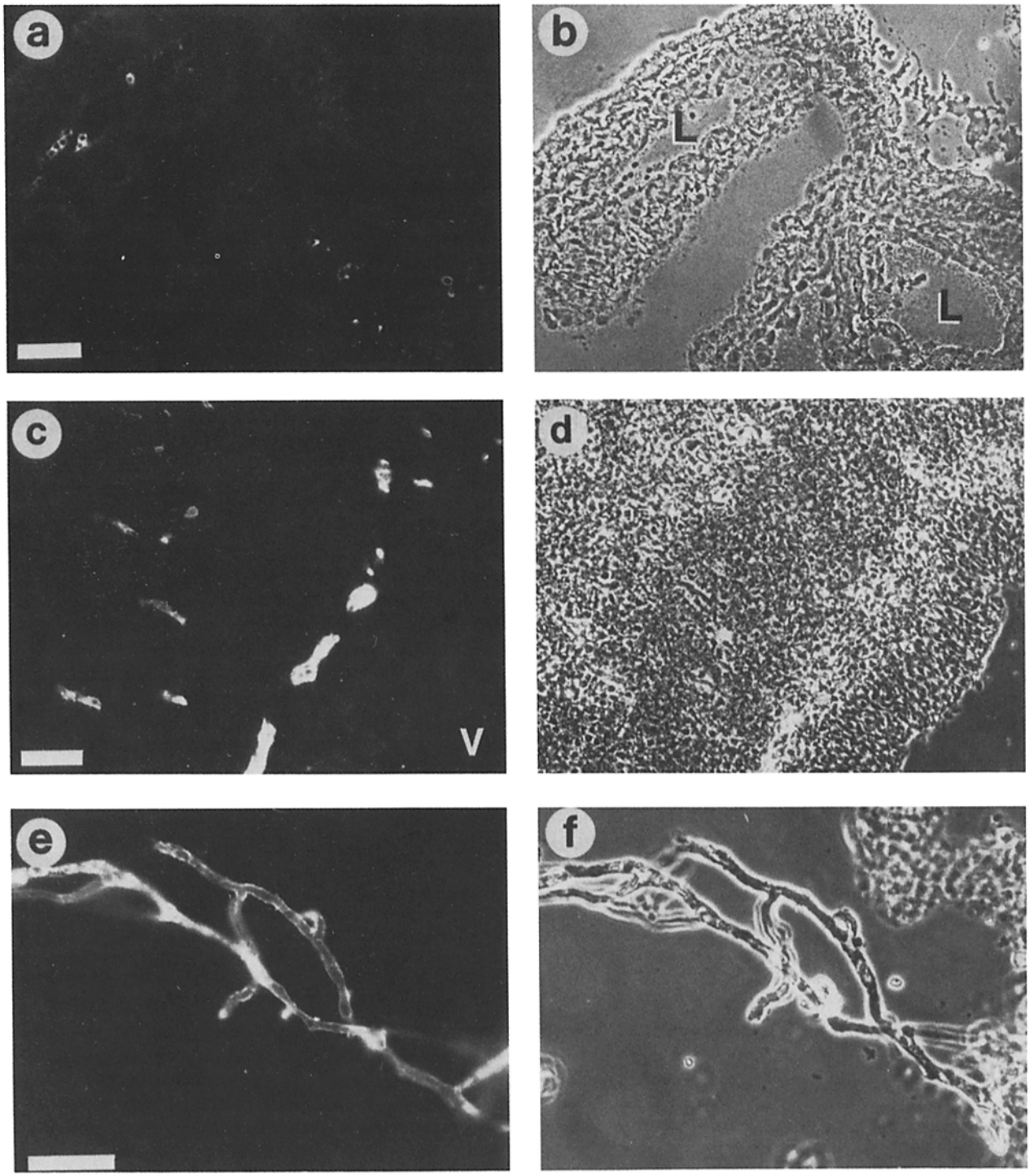

Figure 1. Neurothelin in the brain. Paraformaldehyde-fixed cryostat sections and isolated blood capillaries of embryonic chick specimens were incubated with mAb 1W5 specific for neurothelin and a rhodamine-labeled secondary antibody. (*a*, *c*, and *e*) Immunofluorescence micrographs; (*b*, *d*, and *f*) corresonding phase-contrast images. (*a* and *b*) Chorioallantoic membrane at E15; (*c* and *d*) tectum opticum at E10; (*e* and *f*) isolated brain capillaries at P7. Note that besides some marked erythrocytes in the lumen of both vessels (*a*) no staining of the vessel endothelium is observed. Neurons are neurothelin negative, whereas brain capillaries are positive (*c*). Similarly, isolated brain capillaries from posthatch chicken are stained with mAb 1W5, in contrast to copurified neuronal remnants (*e* and *f*). *L*, lumen of chorioallantoic membrane blood vessels; *V*, ventricle. Bars, 50 μm.

paring these with capillaries from the central nervous system demonstrated that capillaries outside the central nervous system are essentially devoid of neurothelin (Fig. 1 *a*).

At very early stages of embryogenesis (E3–4), no elaborate system of vascularization is evident within the central nervous system. Further cytodifferentiation is accompanied by the appearance of neurothelin-positive structures that are reminiscent of blood vessels as revealed by fluorescence mi-

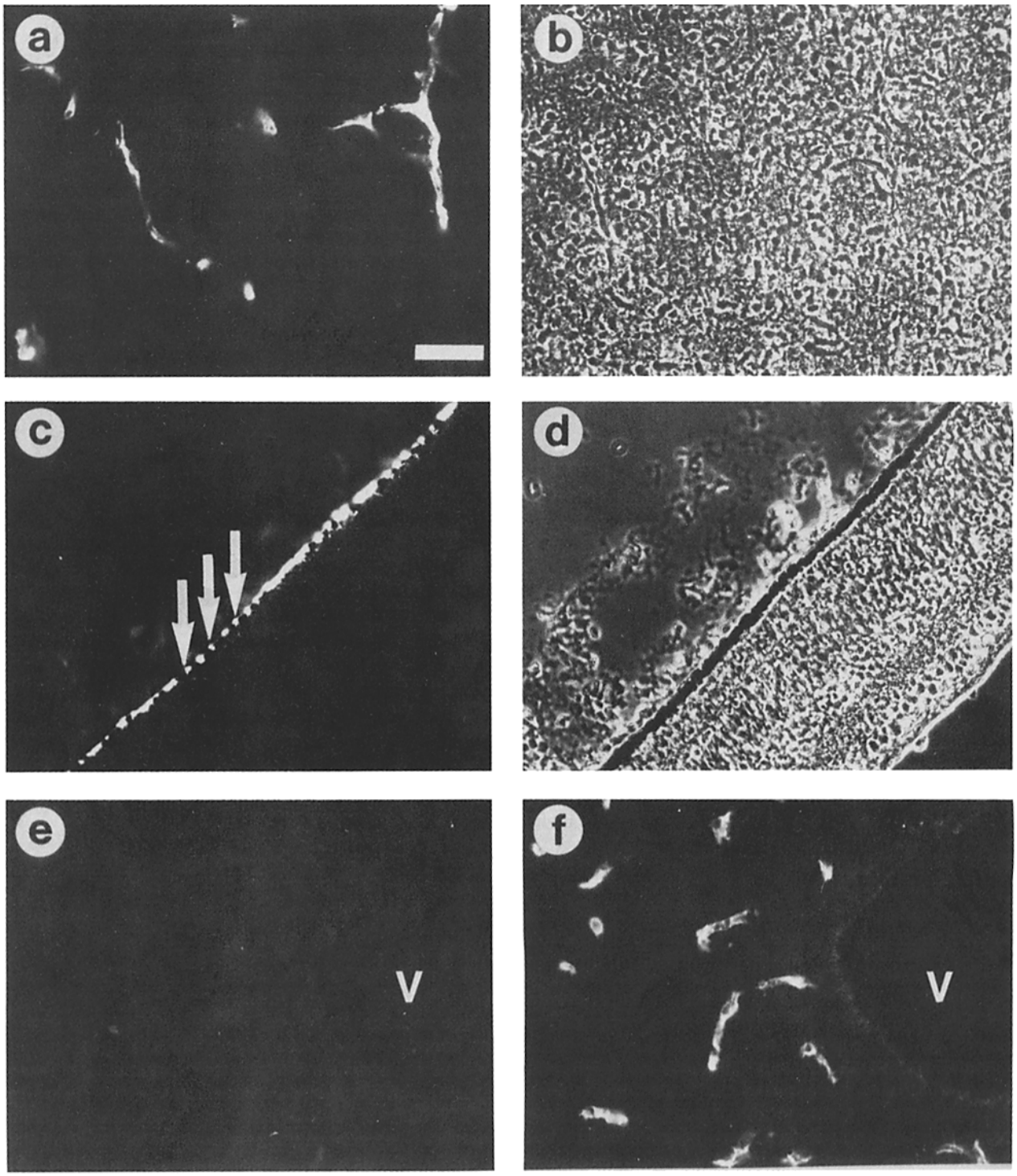

Figure 2. Neurothelin on the luminal capillary surface. Chick egg shells were fenestrated a few days after fertilization, and incubation was continued until the last week of embryogenesis (E14–19). Then affinity-purified sterile mAb 1W5 or mAb D3 against N-CAM were injected into an extraembryonic blood vessel of the chorioallantoic membrane. After 20–30 min, embryos were killed and processed for cryosectioning and immunohistochemistry using solely a secondary antibody. Alternate tissue sections of mAb D3–treated specimens were immunolabeled with mAb 1W5 after sectioning to reveal blood capillaries more clearly. (*a*, *c*, *e*, and *f*) Immunofluorescence micrographs; (*b* and *d*) corresponding phase-contrast images. (*a*, *b*, *e*, and *f*) Tectum opticum; (*c* and *d*) retina. Immunoperfusion only with mAb 1W5 results in labeling of brain capillaries, indicating antigen localization on the luminal surface of vessels. The retinal pigment epithelium with its defined gross morphology served as a control to monitor the limited diffusion of the antibody. Labeling is restricted to the pigment epithelial surface (*c*, *arrows*) facing the systemic vascular system. Note that under these conditions the neural retina remains unlabeled despite the presence of neurothelin (compare with Fig. 4, *c* and *e*). Therefore, penetration of the antibody through blood barriers does not occur. No in vivo labeling of brain capillaries with the control antibody mAb D3 is evident (*e*). Blood capillaries are visualized by mAb 1W5 on adjacent tissue sections (*f*). Bar, 50 μm.

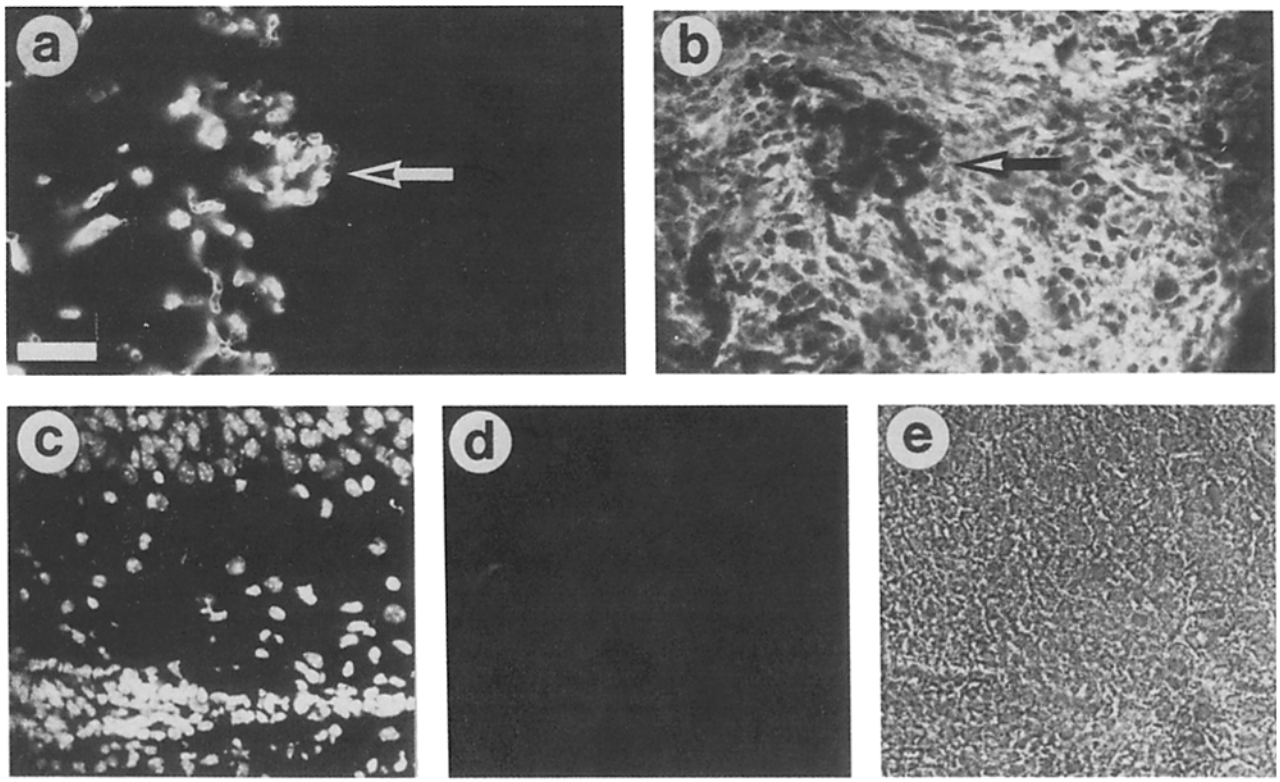

Figure 3. Neurothelin expression induced in mouse transplants. After transplantation of embryonic mouse brain at E11 onto the extraembryonic chorioallantoic membrane of chick embryos at E9, normally neurothelin-negative endothelial cells of the chorioallantoic membrane vascularized the mouse tissue and were induced to express neurothelin after 10 d. Paraformaldehyde-fixed cryostat tissue sections were immunolabeled or incubated with the nucleus marker DAPI. (*a*) mAb 1W5 staining in a transplant demonstrates chick endothelial cells in the mouse brain (*arrow*). (*b*) N-CAM$_{180}$-positive neurons revealed by mAb D3 in the same mouse brain transplant on an adjacent tissue section. (*c*, *d*, and *e*) Horizontal control section through mouse brain at P6. (*c*) Nucleus staining with DAPI. (*d*) mAb 1W5 staining. (*e*) Corresponding phase-contrast picture. Mouse brain was neurothelin negative. Bar, 50 μm.

croscopy (Fig. 1 *c*). After hatching of the chick embryo, a staining pattern similar to that at E10 is revealed in cryostat sections of differentiated optic tecta (data not shown). To directly relate mAb 1W5-positive structures in cryostat sections to distinct tissue elements, different tissue structures were isolated and incubated with the monoclonal antibody for immunostaining. Pronounced labeling could be obtained only with blood capillaries (Fig. 1 *e*), which confirms the hypothesis that within the brain, from the second week of incubation onwards, only capillaries express significant amounts of neurothelin.

Neurothelin Exposed on the Luminal Surface of Brain Capillary Endothelium

To approach the question of whether neurothelin is exposed on the luminal surface of capillaries, affinity-purified mAb 1W5 was injected into extraembryonic blood vessels of the chorioallantoic membrane in vivo. Circulation of the antibody was allowed for 20–30 min, and embryos were then killed, perfused, and processed for sectioning. Horizontally oriented head sections were incubated with a secondary rhodamine-labeled antibody. As depicted in Fig. 2 *a*, brain capillaries are clearly immunolabeled, demonstrating that the endothelial cell surface facing the lumen of the blood capillary bears neurothelin.

Investigation of the retinal pigment epithelium, which forms the blood–ocular barrier equivalent to the blood–brain barrier (Cohen, 1965; Raviola, 1977), revealed that systemic mAb 1W5 application results in the exclusive labeling of the pigment epithelial surface, which is oriented towards the blood vessel–rich choroid (Fig. 2 *c*). The epithelial region facing the retina, as well as the retina itself, is devoid of label, although the retina appears to bear the highest neurothelin concentration in comparison with other brain structures (see below). These data provide additional evidence that neurothelin represents a cell surface component of blood barrier–competent cells. Using the ocular system as a control, it was clear that the monoclonal antibody, an IgG$_1$ with a molecular mass of ~150 kD, did not penetrate blood barriers, validating the use of this approach. As should be expected, penetration through extracerebral fenestrated blood capillaries of the trunk occurred readily in these experiments (data not shown). After injections of the control antibody mAb D3 against N-CAM, no staining of brain capillaries was observed (Fig. 2 *e*). However, the presence of blood capillaries in these tissue sections could be revealed by the application of mAb 1W5 after sectioning of the specimen (Fig. 2 *f*). Therefore, we conclude that the mAb 1W5 labeling in vivo is not due to unspecific IgG binding to endothelial cell surfaces.

Induction of Neurothelin Expression

Essentially no neurothelin expression could be demonstrated

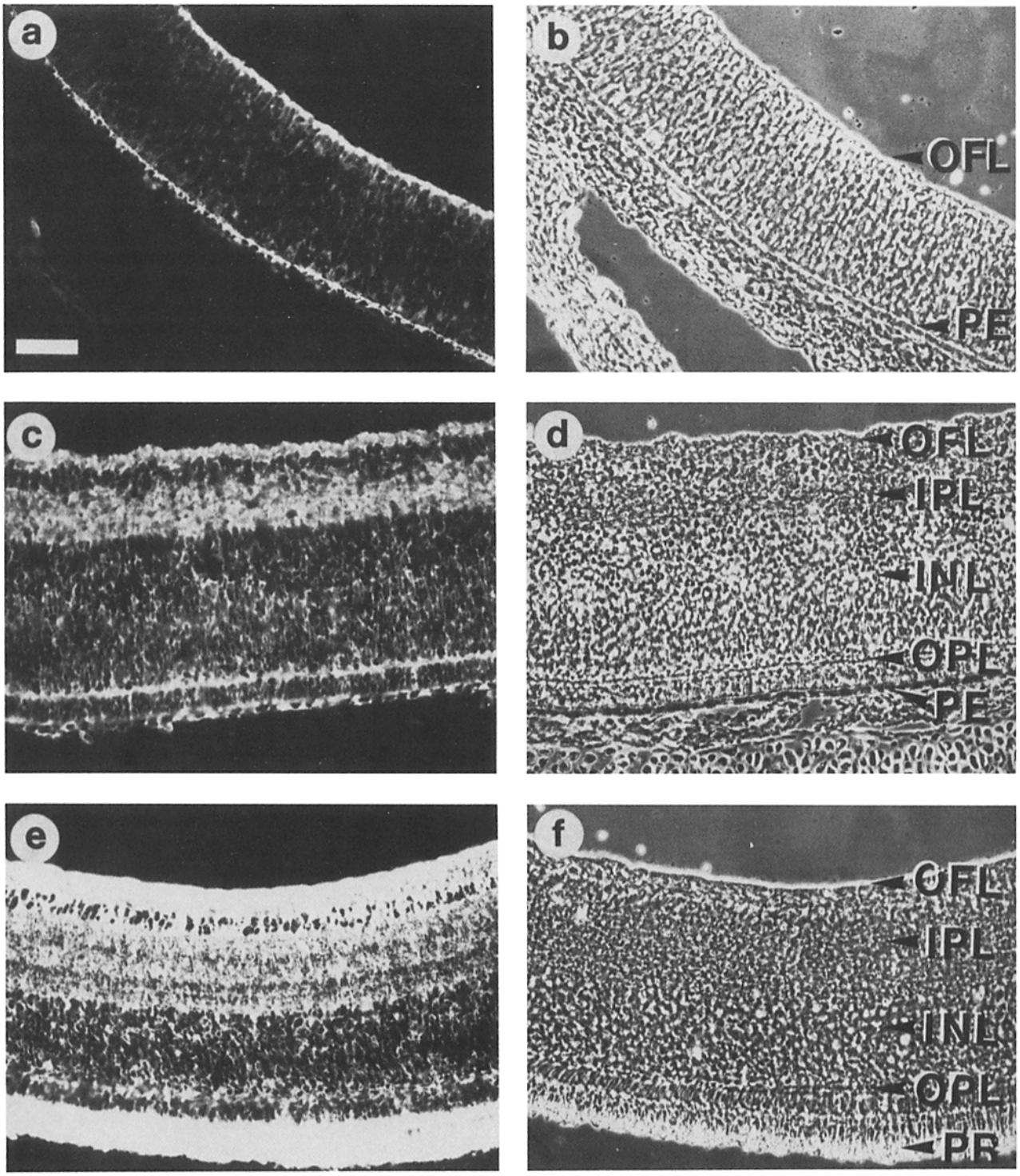

Figure 4. Neurothelin during retina development. Paraformaldehyde cryostat sections of chick retinae were immunolabeled with mAb 1W5. (*a*, *c*, and *e*) immunofluorescence micrographs; (*b*, *d*, and *f*) corresponding phase-contrast images. (*a* and *b*) Retina at E6; (*c* and *d*) retina at E10; (*e* and *f*) retina at P20. Neurothelin expression increases during histogenesis of the retina. *INL*, inner nuclear layer; *IPL*, inner plexiform layer; *OFL*, optic fiber layer; *OPL*, outer plexiform layer; *PE*, pigment epithelium; *PR*, photo receptors. Bar, 50 μm.

on blood capillaries outside the central nervous system. Therefore, neurothelin can be classified as a marker for endothelial cells that are capable of forming the blood-brain barrier. The lack of antigen expression on systemic endothelial cells raises the question of whether there is a distinct class of endothelial cells with an exceptional ability to synthesize neurothelin and whether this class populates exclusively neural tissues.

It has been shown previously that transplantation of early embryonic, not yet vascularized, brain tissue into a non-neuronal periphery leads occasionally to vascularization of neural tissue by extracerebral endothelial cells (Stewart and

Wiley, 1981), suggesting the possibility of using this system to determine whether the resulting capillaries would exhibit mAb 1W5 immunoreactivity.

For the interpretation of such experiments, it is, however, essential to determine whether endothelial cells originated from host or graft tissue. An elegant way to approach this question is heterologous transplantations between different species, if species-specific markers are available. We tested rat, mouse, and goldfish to determine whether mAb 1W5 is chick specific and could serve as an inherent species marker. We found that mAb 1W5 did not cross react with any of these species. Histological and biochemical control data from rodents are shown in Figs. 3 *d* and 8 *b*.

Subsequently, 11 d after conception, embryonic mouse brains were transplanted onto the highly vascularized chorioallantoic membrane of E9 chick embryos. To facilitate experimental manipulations, the egg shells of these embryos were fenestrated at the third day of incubation and incubated without special treatment for another 6 d. At E9 of chick embryogenesis, the brain transplantation was performed. After an additional 10–12-d incubation period, viable specimens were processed for immunohistochemistry. The survival rate of these transplantation experiments was 2.4%. Neural mouse tissue was identified by mAb D3 (Fig. 3 *b*), which binds to an intracellular domain of the N-CAM$_{180}$ (Schlosshauer, 1989). Immunoreactivity of mAb 1W5 was localized on adjacent tissue sections because both monoclonal antibodies are IgGs. Pronounced mAb 1W5 immunolabeling of capillaries that could already be identified by phase-contrast microscopy within neural mouse tissue was evident in all five transplants analyzed, as shown in Fig. 3 *a*.

The presence of mAb 1W5-positive capillaries in neural tissue of the mouse indicates that chick endothelial cells invaded the transplant and that otherwise neurothelin-negative endothelial cells of the extraembryonic chorioallantoic membrane were induced by the neural tissue to express neurothelin.

Developmental Regulation in the Retina

The retina is the only part of the chick central nervous system so far investigated that expresses neurothelin at various stages of development (Fig. 4). At E6, antigen expression is confined mainly to the vitread and sclerad surfaces of the neural retina, whereas intraretinal cells are only weakly positive for mAb 1W5. During the next 4 d of incubation, immunoreactivity becomes stronger and can be localized in all layers of the premature retina. Neurite-rich layers (optic fiber layer and inner and outer plexiform layers) show the most pronounced labeling. After hatching, neurothelin expression was found to be even higher than during embryogenesis. This is especially evident for photoreceptor segments (Fig. 4 *e*), which differentiate only at the end of retinal histogenesis.

The opposing neurothelin profiles in the retina and the tectum opticum raise the question of how neurothelin is distributed in those cellular elements that bridge mAb 1W5-positive and -negative central nervous system regions. Ganglion cell axons are the only neural processes that extend from the retina into the brain (Rager, 1980). Identification of these axons is facilitated by mAb C4, which recognizes a fasciculation protein expressed specifically on ganglion cell axons (Schlosshauer, 1989). Labeling of consecutive cryostat sections with both antibodies demonstrated clearly that ganglion cell axons bear neurothelin inside the retina, the optic nerve and tract, as well as in the stratum opticum of the tectum opticum (Fig. 5). However, the most intriguing aspect is the absence of neurothelin in the layer beyond the stratum opticum (the stratum fribrosum et griseum superficiale), where retinal axons terminate on tectal neurites (La Vail and Cowan, 1971). This implies that the most distal segments of retinal axons, which contact neurothelin-negative tectal neurons and their neurites, are also essentially devoid of the antigen. A conclusion drawn from this observation is that a possible inhibitory signal for the neurothelin expression in brain neurons acts locally on distal retina axon segments but is not communicated to ganglion cell perikarya within the retina, allowing the expression of neurothelin in ganglion cells and their proximal axon segments.

At all developmental stages investigated (E6 until after hatching), the pigment epithelium, which surrounds the neural retina, was found to be highly mAb 1W5 positive (Fig. 4). This observation is consistent with in vitro data (see below) and the proposed role of pigment epithelial cells in blood barrier–carrier functions similar to endothelial cells of brain capillaries (Raviola 1977). Neurothelin might be one of the earliest barrier–carrier markers expressed, even at developmental stages, when the blood–ocular barrier cannot yet be demonstrated as functional.

Neurothelin Expression in Neural Cells In Vitro

Immunofluorescence data derived from tissue sections suggested that neurothelin is located in the cytoplasmic membrane of retinal neurons. To reveal whether the antigen is exposed on the cell surface, retinal cell suspensions were plated onto poly-L-lysine/laminin-coated glass surfaces and cells were immunolabeled with or without prior fixation, which leads in the case of fixation to a permeabilization of the cytoplasmic membrane. Antibody labeling was obtained in both cases, indicating that the corresponding epitope is indeed exposed on the cell surface (Fig. 6 *a*).

Immunolabeling of control cultures with mAb D3 against N-CAM indicated that nearly all retinal cells at this stage of in vitro differentiation have neural character (our unpublished observation; see also below). Therefore, it can be concluded that retinal neurons are capable of expressing neurothelin.

Retinal explant cultures were used to investigate further whether growth cones of extending axons are also mAb 1W5 positive. For this purpose, E6 chick retinae were flat mounted on adhesive nitrocellulose filters, cut into 275-μm-wide strips, and explanted upside down onto laminin-coated glass coverslips. Retrograde labeling of neurites, that extend up to 2 mm within 2 d, indicated that these processes represent ganglion cell axons (Halfter et al., 1983). Growth cones, which are easily recognized by their lamellopodial and filopodial protrusions in these cultures, also express a low amount of antigen as shown in Fig. 6 *c*. This suggests that distal axon segments with growth cones bear some neurothelin as long as axons are not in contact with tectal cells.

Histological analysis of embryonic retinae showed preferential immunolabeling of the vitread surface of the retina by mAb 1W5 (see Fig. 4 *a*). The distinct labeling in this region could be attributed to ganglion cell axons, which have been shown to bear neurothelin (see above) and to a distinct

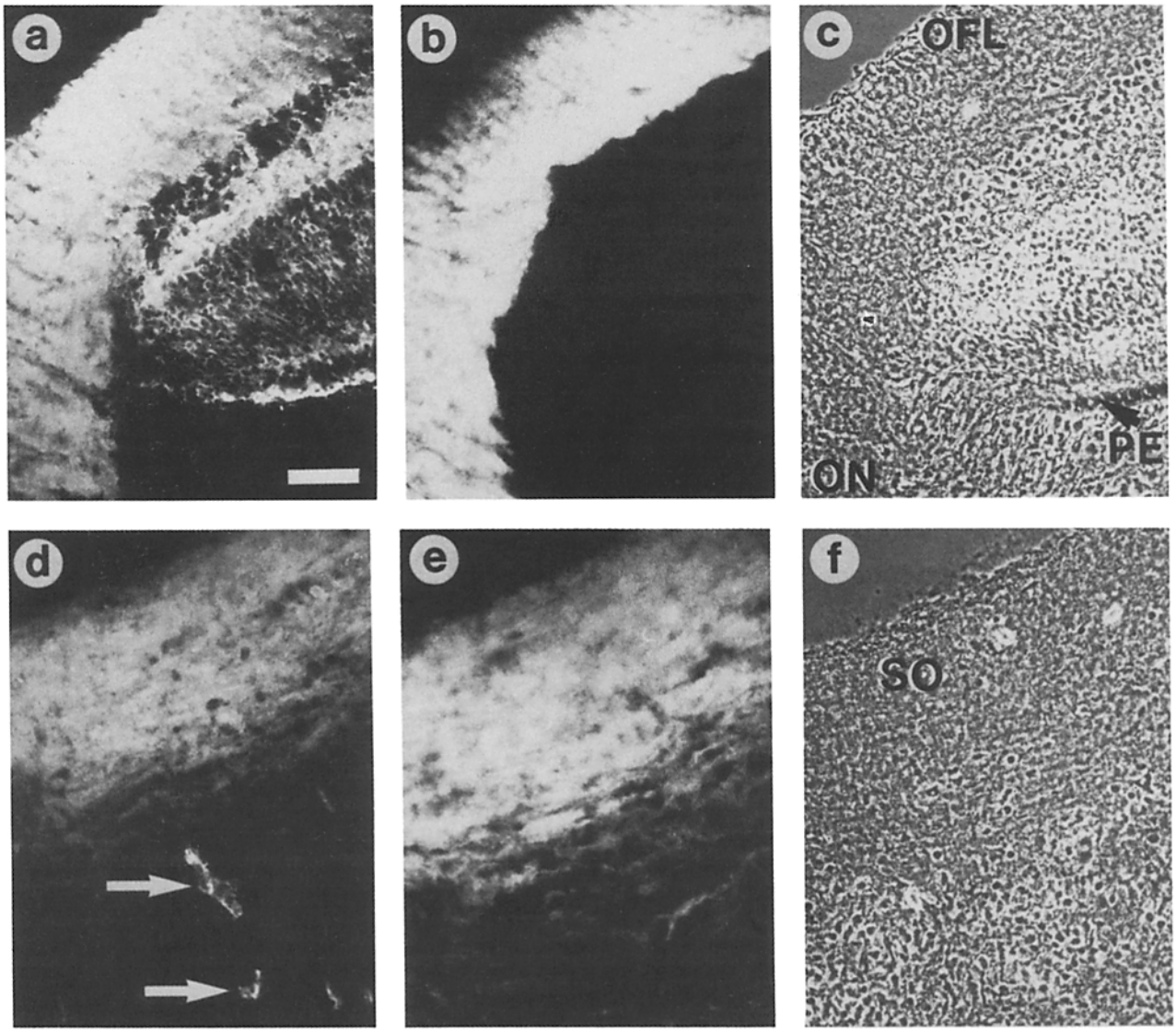

Figure 5. Neurothelin on retinal axons in situ. Immunolabeling of consecutive paraformaldehyde cryostat sections of E10 chick embryos with mAb 1W5 against neurothelin and mAb C4 against an axon-specific cell surface glycoprotein. (*a* and *d*) mAb 1W5 immunostaining; (*b* and *e*) mAb C4 immunostaining; (*c* and *f*) phase contrast. (*a*, *b*, and *c*) Retina; (*d*, *e*, and *f*) tectum opticum. Ganglion cell axons are positively identified with the aid of mAb C4; the same axons in the optic fiber layer of the retina, the optic nerve, and the stratum opticum of the tectum, as well as brain capillaries (*arrows*), bear neurothelin. However, no mAb 1W5 immunoreactivity is found in the layer beyond the stratum opticum (stratum fibrosum et griseum superficiale), where retinal axons synapse with tectal neurites. (mAb C4 immunoreactivity is not present on terminal retinal axon segments in the stratum fibrosum et griseum superficiale.) This suggests a differential distribution of neurothelin on different axon segments. *OFL*, optic fiber layer; *ON*, optic nerve head; *PE*, pigment epithelium; *SO*, stratum opticum. Bar, 50 μm.

population of neuroepithelial cells that radially span the width of retina and form specialized endfeet that separate the neural tissue from the vitreous body. These cells differentiate into Müller cells (Rodieck, 1973). The lack of a purification method for these neuroepithelial cells, however, hampers investigation of the question of whether these cells are also competent to express neurothelin. To circumvent this problem, we isolated epithelial cell endfeet rather than entire cells. A pure preparation of endfeet is obtained by isolating selectively the inner limiting membrane from embryonic retina with the aid of an adhesive poly-L-lysine–coated glass coverslip. Pressing the glass coverslip carefully against the inner limiting membrane of the flat mounted retina after removal of the vitreous body results in adhesion of neuroepithelial endfeet onto the coverslips (Halfter et al., 1987). In addition to direct histochemical analysis, this endfeet lawn was used as a substratum for cell cultures. Fig. 6 *e* reveals that neuroendothelial endfeet bear neurothelin, whereas the fibroblast shown as a control, which has engulfed the endfeet on its migration pathway, is not labeled by mAb 1W5. In summary, these data are consistent with the possibility that all neural cells derived from embryonic retina express neurothelin.

Cell Surface Distribution of Neurothelin

Evaluation of the cell surface distribution of an antigen provides the possibility to shed further insights into its possible

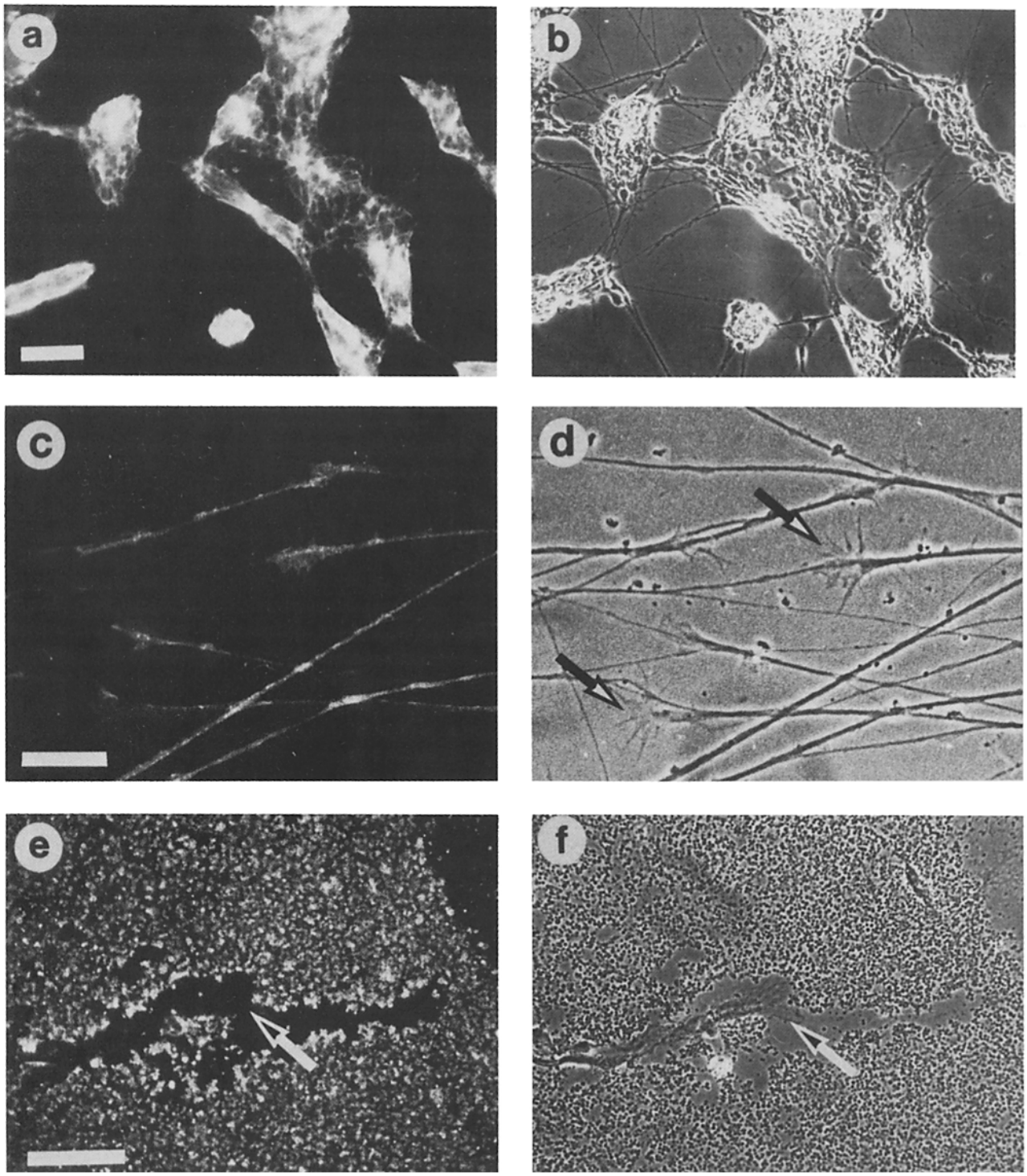

Figure 6. Neurothelin in retina cultures. Cell suspensions or tissue strips of E6 chick retinae were cultured on glass coverslips coated with laminin or retinal basal lamina, incubated for various days, and immunolabeled with mAb 1W5. (*a*, *c*, and *e*) Immunofluorescence micrographs; (*b*, *d*, and *f*) corresponding phase-contrast images. (*a* and *b*) 1-d retina cell culture on laminin; (*c* and *d*) ganglion cell axons originating from retina explants after 1 d in culture; (*e* and *f*) 2-d cell culture on basal lamina showing a fibroblast (*arrow*) that digests neuroepithelial endfeet on its pathway. Note that endfeet derived from the retinal basal lamina are neurothelin positive. Neurothelin is exposed on neural cell surfaces as indicated by immunolabeling of viable cell perikarya (*a*). Axons and growing axon tips (growth cones; *d*, *arrows*) bear also low amounts of neurothelin. Bars: (*a* and *b*) 50 μm; (*c–f*) 25 μm.

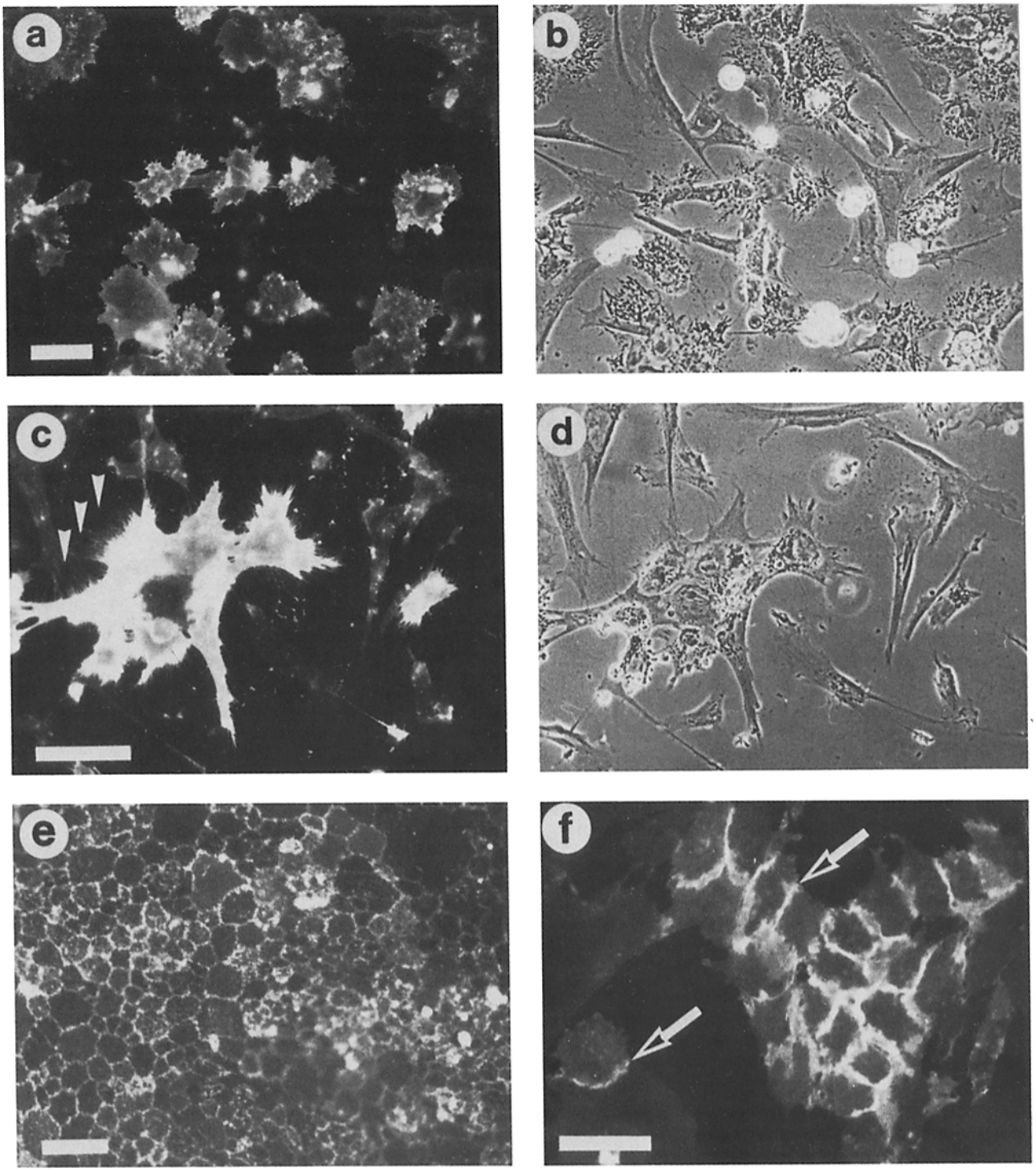

Figure 7. Neurothelin in pigment epithelium cultures. Cell suspensions of E6 chick pigment epithelium were plated on laminin, incubated for various times, and immunolabeled with mAb 1W5. (*a*, *c*, *e*, and *f*) Immunofluorescence micrographs; (*b* and *d*) corresponding phase-contrast images. (*a* and *b*) 8-h culture; (*c* and *d*) 1-d culture; (*e*) 6-d culture; (*f*) 12-d culture. Neurothelin distribution changes with time. In 8-h cultures, the antigen appears preferentially in patches; after 24 h, the antigen is homogeneously distributed on the cell surface and, after several days, predominantly at cell–cell contact sites (*f*, *arrows*). Arrowheads in *c* mark fine cell extensions, which also bear neurothelin. Pigment epithelial cells are easily identified by pigment granules in phase-contrast pictures. Bars, 50 μm.

function, such as in the case of the N-CAM$_{180}$, which was found to be concentrated at cell contact sites (Pollerberg et al., 1987).

An ideal model system to study dynamics of the cell surface distribution of neurothelin was found in pigment epithelial cell cultures. Pigment epithelial cells of the eye express neurothelin at all developmental stages investigated. Epithelial cells in culture form true monolayers (Newsome and Kenyon, 1973), in which individual cells are not obscured in cell aggregates as in the case of neurons (compare Fig. 6 *a* with Fig. 7 *e*).

Pigment epithelial cells kept in culture for 8 h express neu-

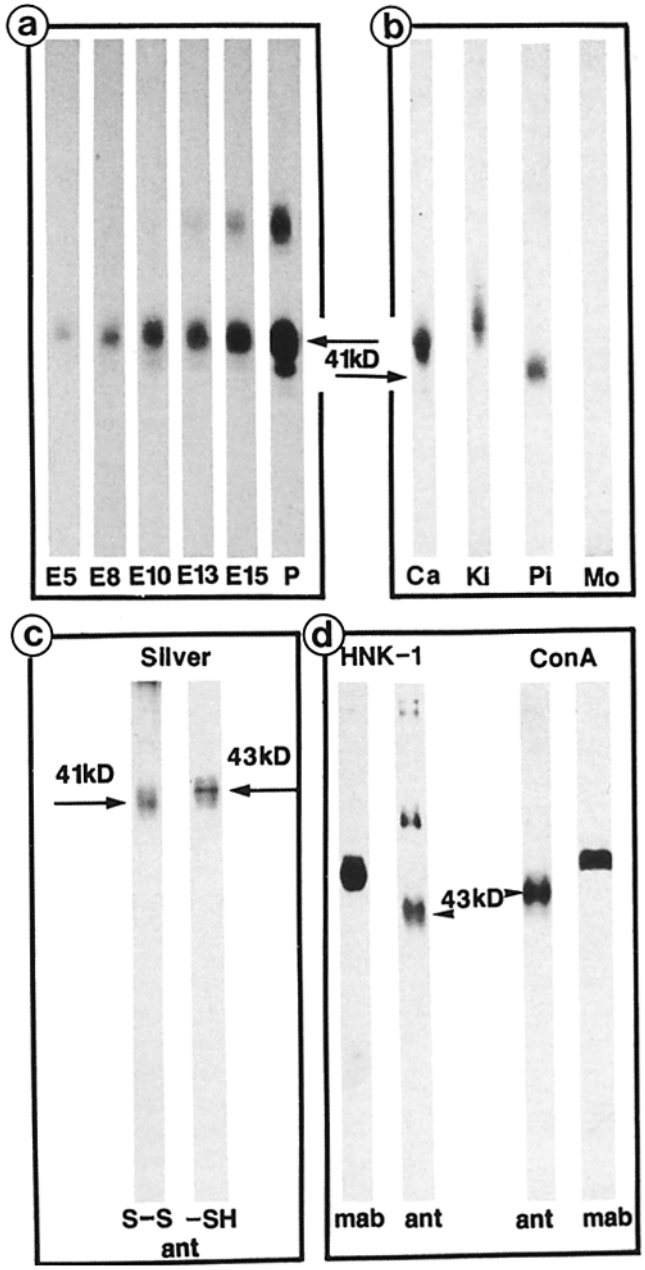

Figure 8. Biochemical characterization. The detection method used is indicated above the lanes (in *c* and *d*), and the sample analyzed is given below the lanes (*a–d*). (*a*) Chick retina samples of different embryonic (*E*) and posthatch (*P*) days were subjected to Western blot analysis using mAb 1W5 and a horseradish peroxidase–conjugated secondary antibody. Antigen expression increases during development, revealing a major band at 41 kD. At late stages, additional bands at 38 and 58 kD become more prominent. (*b*) Western blot analysis reveals neurothelin-positive bands in brain capillaries (*Ca*), kidney (*Ki*), and pigment epithelium (*Pi*), all from posthatch chicken, but not in P6 mouse brain (*Mo*). (*c*) Affinity-purified neurothelin antigen (*ant*) in nonreduced (*S-S*) and reduced (*-SH*) forms visualized by silver staining (*Silver*) of SDS gels. Breakage of disulfide bridges by reduction induces a molecular mass shift. (*d*) Western blot analysis of affinity-purified neurothelin monoclonal antibody (*mab*) and antigen (*ant*) using mAb HNK-1 (*HNK-1*) specific for sulfated glucuronic acid and the lectin concanavalin A (*ConA*) specific for α-D-substituted-glucose and -mannose. Glycosylated antigen bands are evident at 43, 58, and 160/190 kD. The glycosylated heavy chain (~50 kD) and nonglycosylated light chain (~25 kD; not visible) of the immunoglobulin serve as positive and negative controls.

rothelin at multiple sites as well as in patches preferentially at the peripheral cell rim that contacts the substratum. After 24 h in culture, pigment epithelial cells have extended an elaborate system of fine processes. These extensions, as well as the cell bodies, appear to be homogeneously labeled by mAb 1W5 in most cases. However, cultivation of cells for 6 d results in a confluent monolayer and a reorganization of the localization of the antigen because at this stage neurothelin is confined mainly to cell contact sites, whereas other cell surface areas are relatively depleted of the antigen. This is even more obvious in cells at the periphery of monolayers because neurothelin is restricted to cell contact sites. Sides of the somewhat cubic cells that do not contact neighboring cells are depleted of the antigen (Fig. 7 *f*). A possible explanation of these phenomena is that direct cell contact induces clustering of the antigen at contact sites, which may have implications for the function of neurothelin (see Discussion).

Neurothelin: A Glycoprotein

Western blot analysis of nonreduced E10 chick retina samples reveals that mAb 1W5 recognizes a major band at 41 kD, indicating that the 1W5 antigen is a protein.

The developmental sequence in the retina suggests a continuous increase of neurothelin expression, especially in plexiform layers. Therefore, antigen expression was investigated biochemically, which permits direct relation to defined protein concentration of different samples. In E5 retinae, a low but significant amount of antigen can be detected (Fig. 8 *a*). The antigen concentration increases during the next days and reaches a plateau at E10. Only during the last week of embryogenesis, starting at E15, does neurothelin concentration in the retina increase further, with the highest value reached 3 d after hatching. Particularly in posthatch stages, two additional bands at ~38 and at 58 kD become evident in Western blots.

Investigation of different tissue structures demonstrates that the major neurothelin protein band with a similar molecular mass as in retina is also expressed in brain capillaries, kidney, and pigment epithelium of posthatch chick (Fig. 8 *b*). The 58-kD protein is essentially absent from nonneuronal structures. The fact that protein bands are relatively broad and the presence of HNK-1 and concanavalin A binding sites on the antigen (see below) suggest that the slight differences in molecular mass between different tissues may be attributed to variable degrees of glycosylation. No immunoreactivity was found in neural mouse tissue.

An initial observation that mAb 1W5 bound only weakly to reduced samples in Western blot analysis raised the question of whether antibody binding could be influenced by the redox state of the antigen. Using nonreduced antigen samples gave indeed a substantially stronger signal (data not shown). Subsequently, the monoclonal antibody was affinity purified via protein A and coupled to CNBr-activated Sepharose, and, finally, the 1W5 antigen was immunoaffinity purified from solubilized retinal membranes. Application of purified neurothelin in nonreduced and reduced forms to 14% polyacrylamide–SDS gels, which provide more compressed bands than 10% gels, results in two different bands after silver staining (Fig. 8 *c*). The nonreduced and reduced forms appear at 41 and 43 kD, which confirms the initial interpretation of differences in antibody binding and indicates that neurothelin bears intramolecular disulfide bridges. The molecular mass shift is explained by the fact that breakage of covalent

S-S bridges leads to a complete unfolding of proteins in the presence of SDS. Consequently, the retardation within the gel is increased and the protein appears to have a higher molecular mass.

Further biochemical analysis was focused on possible glycosylation sites of neurothelin. Affinity-purified neurothelin was applied to SDS gels, and different saccharide probes were used in the subsequent Western blot analysis. Monoclonal antibody HNK-1, which binds specifically to sulfated glucuronic acid (Schwarting et al., 1987) as well as the lectin concanavalin A, that recognizes α-D-substituted glucose and mannose (Lis and Sharon, 1973) identifies the major 43-kD band of the reduced neurothelin protein (Fig. 8 *d*). Interestingly, a strong signal at 58 kD with HNK-1 is evident even in retina E10 samples in addition to two bands at 160 and 190 kD (Fig. 8 *d*), which could be demonstrated also with affinity-purified neurothelin applied to Western blots in conjunction with mAb 1W5 (data not shown). These data possibly imply that with increasing polypeptide length the rate of glycosylation increases and that also high molecular mass components in the retina bear the mAb 1W5 epitope.

The specificity of HNK-1 and concanavalin A is shown by the use of reduced mAb 1W5 as a positive and negative control. Only the heavy immunoglobulin chain at ~50 kD bears glycomoieties, whereas essentially no staining is evident at ~25 kD, where the light chain is located (Fig. 8 *d*). The purity of the antigen preparation is demonstrated by the absence of immunoglobulin bands as a possible contamination from affinity chromatography.

Discussion

Endothelium and Blood-Brain Barrier

The exclusive expression of neurothelin on endothelial cells of the central nervous system but not on endothelia of the systemic vascular system renders it a likely candidate as a blood-brain barrier marker. This notion is further supported by the absence of the antigen in choroid plexus endothelium (our unpublished observation) where endothelial cells lack tight junctions and, consequently, blood-brain barrier characteristics (Rhodin, 1975). The classification as a marker protein that can be induced in vivo is also in line with data from induction experiments in vivo. Stewart and Wiley (1981) have shown that transplantation of embryonic quail brain into the coelomic cavity of chick embryos results in brain transplant vascularization by systemic vessels. The distinct nuclear morphology of quail cells allowed distinction between intermixed cells of both species. Abdominal capillaries vascularizing neural tissue clearly developed features of the blood-brain barrier. This approach first showed that embryonic endothelial cells are not predetermined to form one type of vessel or the other and, secondly, that brain tissue influences endothelial cell differentiation. Similarly, transplantation of embryonic mouse tissue onto the chick chorioallantoic membrane results in the induction of neurothelin expression in otherwise neurothelin-negative endothelial cells of extraembryonic capillaries. This is also in conjunction with equivalent transplantation data obtained for the HT-7 antigen, which has also been identified as a marker for blood-brain barrier specific endothelial cells (Risau et al., 1986*b*). Which cell type is responsible for this effect remains to be elucidated. Future investigations will be focused on glial cells as inductive elements since it has been observed that C6 glioma cells are capable of inducing gamma-glutamyl transpeptidase, as another blood-brain barrier marker in ME-2 endothelial cells via direct cell-cell contact (De Bault and Cancilla, 1980). The close association of astroglia with brain endothelial cells also suggests that an interaction between both cell types may be essential for blood-brain barrier function (Betz et al., 1989). This hypothesis is supported by the finding that the loss of cell contact between these cell types under pathological conditions as in brain tumors is accompanied by a loss of the blood-brain barrier (Bradbury, 1979).

Developmental Regulation

Vascularization, which starts around E3.5, proceeds from leptomeningeal blood vessels to ventricular brain regions (Peterson, 1968). These vessels become neurothelin positive before E9. The functional manifestation of the blood-brain barrier, however, as judged from horseradish peroxidase penetration assays, is not observed before E12-13 (Wakai and Hirokawa, 1978). Therefore, neurothelin appears to be one of the earliest molecular markers for endothelial cells that will form the blood-brain barrier.

It is of special interest that in the chick retina, which does not become populated by either endothelial cells or by astrocytes unlike other parts of the central nervous system, neurothelin expression in neurons increases. These data raise the question of whether vascularization could provide an inhibitory signal for the neurothelin expression in neurons. Neurothelin expression appears to be an event that is both reversible and possibly subject to constant regulation in neurons as well as in endothelial cells. A model to be tested comprises a triangle interrelationship: neurons influence astrocyte differentiation as suggested by Sobue and Pleasure (1984) and Hatten and Mason (1986); astrocytes affect endothelial cell differentiation (Bradbury, 1984) and induce neurothelin expression in endothelial cells; and, finally, endothelial cells suppress antigen expression in neurons. Induction and suppression experiments may be readily designed to test this model.

Antigen Function

Neurothelin is shown to be specific for those endothelial cells that form the blood-brain barrier in addition to pigment epithelial cells of the eye that form the blood-eye barrier. The barrier is due partly to tight junctions between these cells, which prevents diffusion through the interstitium (Reese and Karnovsky, 1967). Neurothelin may be associated with tight junctions, which would be consistent with the specific distribution of neurothelin at contact sites of pigment epithelial cells in vitro. This interpretation, however, contradicts the appearance of neurothelin on neurons and erythrocytes (see Figs. 1 *a* and 6 *a*) that are devoid of tight junctions.

Essential components of the blood-barrier complex are transport systems discussed above. The idea that neurothelin may serve a carrier rather than barrier function is supported by several lines of evidence.

In contrast to many higher vertebrates, the chick neural retina lacks vascularization. Instead, blood-borne nutrients

reach the sclerad retinal surface from the blood vessel–rich choroidea via the pigment epithelium, and the vitread surface possibly from the pecten via the vitreous humor. The pecten is a pigmented lamina of connective tissue that projects folded upon itself from the ventral retina (optic fissure) into the vitreous body of birds (Romanoff, 1960). Both intermediate transport structures, pigment epithelium and pecten (our unpublished observation) were found to be neurothelin positive.

Antigen expression in the retina is closely related to tissue differentiation. At E6, when the retina thickness does not span >250 μm at the fundus (Coulombre, 1955), neurothelin is located preferentially at inner and outer retina layers neighboring the pigment epithelium and vitreous humor. With subsequent increase of tissue width, internal layers express more antigen. However, at the end of the second week of incubation (~E13), the retina thickness reaches a transient minimum due to pronounced neuronal cell death (Coulombre, 1955; Hughes and McLoon, 1979). As clearly indicated by Western blot analysis, neurothelin expression also reaches an intermediate plateau during this period. Thereafter, the retina expands in thickness (Coulombre, 1955), and antigen expression rises as well.

The spatiotemporal regulation of neurothelin and, in particular, the fact that both the absolute and the relative amount of the antigen increase in the retina during embryogenesis fit well with the increasing requirement for transport mechanisms during retinal growth. According to this hypothesis, retinal neurons themselves would perform the transport functions which are mediated by endothelial cells and astrocytes in vascularized brain regions. The proposed transport function of neurothelin is also supported by the presence of neurothelin in transport interfaces in kidney tubuli (our unpublished observation).

The presence of neurothelin at cell contact sites of epithelial cells in vitro suggests a regulation of neurothelin distribution on the cell surface mediated by direct cell–cell contact. This is consistent with the observation that individual retinal axons in explant cultures show only a weak mAb 1W5 immunoreactivity, whereas fasciculated axons in vitro as well as in situ exhibit stronger immunofluorescence. Intriguing in this respect is the result that neurothelin could be observed on retinal axons only in that region of the brain (stratum opticum) where these axons remain separated from neurothelin-negative tectal neurons. This is also in line with neurothelin expression on distal axon segments and growth cones that do not synapse with tectal cells in vitro. Once retinal axons leave the stratum opticum and penetrate as individual fibers into the stratum fibrosum et griseum superficiale to synapse finally on tectal neurites (Rager, 1980), these retinal axons also appear to be neurothelin negative. Therefore, the antigen distribution on the cell surface is likely to be subject to a fine-tuned regulation on retinal neurons as well as on epithelial cells. The distinct distribution of cell surface proteins on different axon segments has also been reported for a distinct class of cell surface glycoproteins called fasciclins in *Drosophila* (Bastiani et al., 1987; Patel et al., 1987) as well as for TAG1 and the cell adhesion molecule L1 on mammalian spinal cord neurites (Dodd et al., 1988) and different N-CAM isoforms on ganglion cell axons (Schlosshauer et al., 1984). The discovery that the subcellular distribution of neurothelin is tightly regulated in neurons, as well as in epithelial cells, promises to reveal more readily the causal network behind these phenomena by elucidating analogies between both systems.

Molecular Identity

Comparison of neurothelin with other markers of the blood–brain barrier suggest that it represents a novel glycoprotein. Gamma-glutamyl transpeptidase and cholinesterase, regarded as endothelial cell markers in the mammalian central nervous system, have not been detected in the chick brain (Risau et al., 1986*a*). Alkaline phosphatase has been localized in the luminal and antiluminal cytoplasmic membrane of brain capillary endothelial cells (Betz et al., 1980) but appears only at later stages of chick embryogenesis (Risau et al., 1986*a*). The same holds true for the transferrin receptor (Risau et al., 1986*a*). The 43-kD neurothelin is also distinct from a glucose transporter of the blood–brain barrier, which has been identified as a 53-kD protein in different species (Dick et al., 1984). Whether the HT7 antigen (Risau et al., 1986*b*) or the 46-kD protein from bovine brain capillaries (Partridge et al., 1986) share homologies with neurothelin awaits further investigations.

mAb 1W5 promises to be a helpful tool for the elucidation of molecular structures involved in blood–brain barrier and carrier functions. The discovery that the antigen is subject to a stringent regulation during development in neurons as well will certainly facilitate understanding of functional implications of neurothelin, which are likely to be related in endothelial and neuronal cells. In addition, the abundant expression of neurothelin in the retina renders it an ideal model system for further biochemical and functional characterization since the retina is easily accessible and provides a homogeneous neural cell population with respect to antigen expression. Therefore, combining experimental approaches in neuroembryogenesis and angiogenesis is likely to provide further insight into the regulation of the blood barrier–carrier complex.

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