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A blood–CSF barrier function controls embryonic CSF protein composition and homeostasis during early CNS development

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ABSTRACT

In vertebrates, early brain development takes place at the expanded anterior end of the neural tube, which is filled with embryonic cerebrospinal fluid (E-CSF). Most of the proteins contained within the E-CSF, which play crucial roles in CNS development, are transferred from the blood serum. Two important questions are how E-CSF is manufactured and how its homeostasis is controlled. In this respect, the timing of the blood–CSF barrier formation is controversial. Recently, the concept of a functional dynamic barrier has been introduced. This type of barrier is different from that found in adults and is adapted to the specific requirements and environment of the developing nervous system. In this study, we injected a number of proteins into the outflow of the heart and into the cephalic cavities and examined their transport rate between these two embryo compartments. The results indicated that a functional blood–CSF barrier dynamically controls E-CSF protein composition and homeostasis in chick embryos before the formation of functional choroid plexuses. We also showed that proteins are transferred through transcellular routes in a specific area of the brain stem, close to the ventral mesencephalic and prosencephalic neuroectoderm, lateral to the ventral midline, in particular blood vessels. This study contributes to our understanding of the mechanisms involved in CNS development, as this blood–CSF interface regulates the composition of E-CSF by regulating its specific composition.

Introduction

In vertebrates, early brain development takes place at the expanded anterior end of the neural tube. The development of the central nervous system (CNS) involves the simultaneous and interdependent action of several developmental mechanisms, including the establishment of positional identities, morphogenesis and histogenesis. These mechanisms are regulated by transcription factors as well as by diffusible molecules such as growth factors and morphogens acting in an autocrine/paracrine manner. Just after the closure of the anterior neuropore, the brain wall is formed by a pseudomonostratified neuroepithelium that is mainly made up of pluripotent neuroepithelial progenitor cells. This wall encloses a large cavity containing embryonic cerebrospinal fluid (E-CSF). At this developmental stage–between embryonic days E3 and E4 in chick embryos (which corresponds to developmental stages HH20 to HH23 according to Hamburger and Hamilton (1951))–the highly dynamic cellular behaviour of the neuroepithelial cells forming the brain wall includes both intense proliferation and the initiation of a period characterised by a high rate of neurogenesis. E-CSF, which is initially formed by trapped amniotic fluid, is in contact with the apical surface of all the neuroepithelial cells of the cephalic vesicles. Thus, a physiologically sealed system is formed, with a complex and dynamic protein composition that differs from that of embryonic serum (E-serum) (Gato et al., 2004). E-CSF has several crucial roles in brain anlagen development: (1) it exerts positive pressure against the neuroepithelial walls and generates an expansive force (Alonso et al, 1999, 1998; Desmond and Jacobson, 1977; Jelinek and Pexieder, 1970; Miyan et al., 2003); (2) it contributes to regulating the survival, proliferation and neurogenesis of neuroectodermal stem cells (Gato et al., 2005); and (3) it collaborates with a well-known organising centre, the mes-metencephalic boundary or isthmus (IsO), in the pattern of neuroepithelial gene expression (Parada et al., 2005a).

At E4, chick E-CSF proteome includes molecules whose role during the development of systems other than the E-CSF may account for the general functions of this fluid, as described above (Parada et al., 2006). Similar proteomes with parallel functions have been reported in mammals at equivalent developmental stages (Parada et al., 2005b; Zappaterra et al., 2007; reviewed by Parada et al., 2007). Moreover, recent studies have implicated certain proteins, lipid fractions and morphogens contained within the E-CSF (such as FGF2, apolipoproteins, retinol binding protein, retinol and low density lipoproteins) in controlling initial neurogenesis and neuroepithelial cell proliferation and survival (Martin et al., 2006; Bachy et al., 2008; Parada et al., 2008, in press).

Most of the molecules identified in chick E-CSF are not produced by the neuroectoderm itself, but by other embryonic structures. Alter-

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natively, they are stored in the yolk or the white of the egg and taken up by the chorioallantoic membrane (Parada et al., 2006). We recently demonstrated that most of the major E-CSF protein fractions are produced or stored outside of the cephalic vesicles (Parvas et al., 2008), suggesting that they are transported from the producing or storage site to the E-CSF, probably via the E-serum. Taking into account the key roles played by E-CSF during these early stages of brain anlagen development, a question arises. How is the homeostasis of this intra-cavity fluid controlled, i.e. how and to what extent is the transport of specific gene products from the E-serum to the E-CSF and *vice versa* controlled?

In adult vertebrates, CNS homeostasis is controlled by the blood-brain barrier (BBB) in vessels, which impedes transfer from the blood to the brain of virtually all molecules, except those that are small and lipophilic and, interestingly, sets of small and large hydrophilic molecules (e.g. gene products) which can enter the brain via active transport (Rowland et al., 1992). In embryos, the permeability of the BBB has usually been determined using what were considered inert tracers. For example, Wakai and Hirokawa (1978) used horseradish peroxidase (HRP) to show that capillaries in chick embryos gradually become impermeable to this molecule from as late as E13. However, the HT7 antigen, a chick-specific cell-surface glycoprotein used in experimental studies as a marker of barrier-provided vessels, has been detected as early as E6 in rhombencephalon and mesencephalon (Bertossi et al., 2002). In mouse embryos, the first capillaries that penetrate the neuroectoderm are located in the brain stem of the mesencephalon and prosencephalon at E10, which corresponds to an E3.5 chick embryo (Herken et al., 1989).

With respect to CSF composition and homeostasis, in the adult brain and in foetuses these conditions are tightly regulated by the choroid plexus, whose epithelial cells establish a blood–CSF barrier (Mollgard et al., 1979, Tauc et al., 1984). Choroid plexuses are vascular complexes related to brain ventricles that manufacture CSF by promoting the transport of some molecules from the blood plasma and by producing other molecules that are directly delivered to the brain vesicles. Developing choroid plexuses are first detected at E7 in chick and at E13 in mouse/rat embryos (Bellairs and Osmond, 2005, Emerich et al., 2005). This means that these organs cannot manufacture E-CSF or control its homeostasis at the initiation of primary brain neurogenesis, when E-CSF is known to play a crucial role in CNS development.

The permeability of blood–CSF barriers is usually expressed as a ratio of the concentration of particular molecules in the CSF with respect to blood plasma. For small molecules this ratio is much higher in the developing brain than in the adult brain (Ferguson and Woodbury, 1969 Dziegielewska et al., 1979; Habgood et al., 1993; Ek et al., 2001). This has been interpreted as evidence of greater barrier permeability (Johanson, 1989; Kniesel et al., 1996; Engelhardt, 2003; Lee et al., 2003). However, tight junctions, the morphological basis of these barriers, are present from the earliest stages of development between endothelial cells of blood vessels in BBB (Saunders and Mollgard, 1984) areas, as well as in epithelial cells of the choroid plexuses in the blood–CSF barrier (Mollgard et al., 1979; Tauc et al., 1984). Thus, it was recently suggested that different transcellular mechanisms for protein and small molecule transfer operate across the embryonic blood–CSF interface (Johansson et al., 2006).

More recently, on the basis of theoretical grounds and extensive literature, Johansson et al. (2008) argued that the developmental blood–CSF barrier restricts the passage of lipid-insoluble molecules such as gene products by the same mechanism as in the adult, i.e. by tight junctions, rendering the paracellular pathway an unlike route of entry. They suggest that proteins are transferred through transcellular routes. Thus, they introduce the concept of a functional and dynamic barrier, which is different from that of the adult as it is adapted to the specific requirements and environment of the early developing nervous system.

In this paper, we focused on protein transfer across the blood–CSF interface at the beginning of chick brain anlagen neurogenesis, shortly after the closure of the anterior neuropore. We injected several different proteins into the outflow of the heart (blood serum) and into the cephalic cavities (E-CSF) and analysed their transfer between these two embryo compartments. The results indicated a functional blood–CSF barrier that dynamically controls E-CSF protein composition and homeostasis in chick embryos from shortly after the closure of the anterior neuropore. This coincides with the initiation of maximum neurogenesis and occurs before functional embryonic choroid plexuses are formed. We also show that proteins are transferred through transcellular routes in a very specific area that is lateral to the brain stem, close to the ventral mesencephalic and prosencephalic neuroectoderm and lateral to the ventral midline. The particular blood vessels involved exhibit vascular sprouts that are in close contact with the neuroectoderm. We argue that this blood–CSF barrier function regulates the composition of E-CSF. Thus, it contributes to the crucial role of the E-CSF in CNS development.

Material and methods

Obtaining chick embryos

Fertile chicken eggs were incubated at 38 °C in a humidified atmosphere to obtain chick embryos at the desired developmental stage, i.e. at E3 (HH20), E4 (HH23) and E5 (HH26) (E is embryonic day since the beginning of incubation; HH is Hamburger and Hamilton (1951) developmental stages, as depicted in Bellairs and Osmond, 2005). For the E5 embryos, on the second day of incubation 2–3 ml of egg white were removed with a syringe to prevent the chorioallantoic membrane from sticking to the egg shell. In all cases, a further 4–5 ml of egg white was removed with a syringe prior to embryo manipulation, and a circular window was opened in the egg shell with sterile scissors. Before any manipulation, the egg shell was cleaned with a wet alcohol tissue to avoid contamination.

Microinjection of molecules

Microinjection of the several different molecules used in this study was performed in ovo with a glass microneedle (30 μm inner diameter at the tip) connected to a microinjector (Nanoject II) through a small opening made in the extraembryonic membranes with a sterilised tungsten needle. Molecules were microinjected into the mesencephalic cavity (10 pulses of 23 nl each) to monitor E-CSF/E-serum transfer, as this is the largest cavity in the avian brain at this stage of development. Alternatively, they were injected into the outflow tract of the heart (10 pulses of 23 nl each) to monitor E-serum/E-CSF transfer. Injections were always made on embryos at E4 (HH23), unless otherwise stated. The following molecules were microinjected: horseradish peroxidase (HRP; Sigma 6782, at 50 mg/ml; mw 40 kDa); bovine serum albumin (BSA; Sigma B4287, at 50 mg/ml; mw 66 kDa); immunoglobulins (IgG anti-BSA; Sigma B2901, at 34 mg/ml of total protein content, from which 5.5 mg/ml corresponds to the specific antibody; mw 180 kDa); myosin heavy chain from rabbit (MHC; Sigma, M7659, at 5 mg/ml; mw 200 kDa); human recombinant fibroblast growth factor no. 2 (FGF2), which also contains BSA as a stabiliser (Sigma, F0291; at 0.01 mg/ml; mw 17 to 34 kDa for FGF2); plasma retinol binding protein (RBP; Sigma, R9388, from human urine, at 0.01 mg/ml; 21 kDa; the recombinant protein glutathione-*S*-transferase (from *Schistosoma japonicum*) alcohol dehydrogenase (from *Drosophila lebanonensis*) (GST-Adh), purified from bacterial cultures (Martin et al., 2006; at 50 mg/ml; mw 38 kDa); ovalbumin (Sigma, A7641, at 50 mg/ml; mw 44 kDa); and biotin-dextran (BDA3000; Molecular Probes, D7135; mw 3000 Da). Prior to microinjection, FGF2, RBP and GST-Adh were labelled with FITC; ovalbumin was labelled with either FITC or Evitag® nanocrystals; and BSA was labelled with Evitag® nanocrystals or was injected unlabelled (see below for protein labelling). Proteins were dissolved in saline solution, to which fast green was added (1/5) to visualize the microinjection.

Coupling of proteins to FITC and Evitag® nanocrystals

Commercial FGF2, RBP and ovalbumin, as well as GST-Adh fusion protein, were coupled to fluorescein isothiocyanate isomer I (FITC, Sigma; FITC-1 conjugation Kit) so that they could be distinguished from the corresponding endogenous molecules during their detection (for FGF2, RBP and ovalbumin), or simply so that they could be easily detected (for GST-Adh) with an anti-FITC antibody. The coupling was made according to Sigma standard protocol, which is based on the protocol described by Harlow and Lane (1988). The unbound dye was separated by gel filtration. Molecules coupled to FITC were named FGF2-, RBP-, ovalbumin- and GST-Adh-FITC respectively.

Commercial ovalbumin and BSA were also coupled to Evitag® nanocrystals (Adirondack Green; Evident Technologies) according to the instructions of the supplier (http://www.evidenttech.com). An excess of ovalbumin and BSA (×2 with respect to standard protocols) were used so that there would be no unbound Evitags®. Molecules

coupled to Evitag® nanocrystals were named ovalbumin-Evitag and BSA-Evitag respectively.

Obtaining embryonic fluids

To detect the ratio of molecule transfer between the blood stream and the cephalic cavities and *vice versa*, as well the allantoic filtration of these molecules in some cases, E-CSF, E-serum and allantoic fluid were obtained at several different times after microinjection: 0 min, 5 min, 10 min, 20 min, 1 h and 24 h. Embryos were always microinjected at E4 (HH23), except otherwise stated. In the same way, to detect the relative concentration in the E-serum and E-CSF of some endogenous proteins (RBP, FGF2, ovalbumin and IgY) at different stages around the initiation of primary neurogenesis, E-CSF and E-serum were obtained from E3 (HH20), E4 (HH23) and E5 (HH26) control embryos. To obtain E-CSF, the embryos were dissected out of extra-embryonic membranes, rinsed twice in sterile saline solution and placed in a Petri dish. A glass microneedle (30-μm inner diameter at the tip) connected to a microinjector (Nanoject II) was carefully placed in the middle of the mesencephalic cavity under dissecting microscope control. E-CSF was slowly aspirated, avoiding contact with the neuroepithelial wall so as to obtain samples that were not contaminated by neuroepithelial cells. Enough embryos were used to obtain an adequate amount of E-CSF. To minimize protein degradation, E-CSF samples were kept at 4 °C during this procedure and immediately frozen at −20 °C until use.

Both E-serum and allantoic fluid were obtained in ovo. After opening a small window in the eggshell, the chorioallantoic membrane was dissected with a tungsten needle. The blood was obtained by microaspiration carried out in the outflow tract of the embryonic heart. The allantoic fluid was obtained by microaspiration in the allantoic vesicle, as described above for E-CSF. The embryo was held by placing a small sterile spatula underneath it. To minimize protein degradation, samples were kept at 4 °C during this procedure. Blood and allantoic fluid from different embryos were pooled and immediately centrifuged, and both samples were stored at −20 °C until use.

SDS-polyacrylamide gel electrophoresis, western-blot and slot-blot analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under denaturing conditions according to the method of Laemmli (1979), with a Miniprotean II electrophoresis system (Bio-Rad). Ovalbumin, ovalbumin–Evitag, BSA and BSA–Evitag (to monitor Evitag labelling), as well as E-CSF and E-serum of embryos microinjected with FGF2-FITC (to distinguish FGF2-FITC from the BSA-FITC generated during the coupling, as commercial FGF2 is sold stabilised with BSA) were used. Molecular mass standards of high and low range (Bio-Rad) were also used. SDS-PAGE was performed in a discontinuous buffer system for 40 min at 100 V. When needed, samples were stained with Coomassie Blue or with a Silver Stain Kit (Bio-Rad), following standard procedures.

For the western-blot analysis, proteins were electrotransferred (Trans-Blot Transfer System, Bio-Rad) from the SDS-PAGE to activated nitrocellulose membrane (Hybond-N) for 1 h at 100 V, using a basic transfer buffer following standard protocols. Detection of proteins after western-blot was performed as described below for slot-blot analysis.

For the slot-blot analysis, embryonic fluids were applied to nitrocellulose membrane (Hybond-N) using a microsample filtration manifold (Schleicher and Schuell, SRC 072/0) connected to a vacuum pump. Slot-blot was used to detect all of the microinjected molecules in the analysed embryo fluids except FGF2-FITC, i.e. HRP, BSA, immunoglobulins, MHC, GST/Adh-FITC, Ovalbumin-FITC, RBP-FITC and BDA3000 at several different dilutions. This procedure was also used to detect endogenous ovalbumin, RBP and IgY within the E-CSF and the E-serum, and the embryonic/extra-embryonic origin of the several protein fractions obtained with silver staining of E-CSF SDS-PAGE (see above). After sample application, membranes were dried to fix the proteins, and the presence of molecules was detected. The presence of HRP was detected directly by developing the slot-blot with 3-3′-diaminobenzidine tetrahydrochloride (DAB; 25 mg of DAB in 50 ml of PBS, 1 ml of $CoCl_2$, and 5 μl of H_2O_2 30% in the dark). The reaction was stopped by washing the filters with distilled water (×3).

For other microinjected proteins, membranes were blocked in 5% powdered defatted milk in PBT (MTP) for 2 h at room temperature with gentle shaking, except for samples containing BSA, which were blocked in PBT alone to avoid crossreaction. The membrane was then incubated with the corresponding primary antibody, which was properly diluted in MTP, overnight at 4 °C, except for samples containing BSA, in which the primary antibody was diluted just in PBT: i.e. mouse anti-BSA (Sigma, B2901) at 1/3000; mouse anti-MHC (Bueno et al., 1997) at 1/500; mouse anti-FITC (Sigma, F5636) at 1/2500; mouse anti-RBP (Labvision MS-428 at 1/3000; rabbit anti-ovalbumin (Calbiochem, 126705) at 1/1000; and rabbit anti-FGF2 (Sigma, F3393) at 1/2000.

After primary antibody incubation, membranes were washed with PBT (4×15 min) at room temperature, and subsequently incubated with the appropriate secondary antibody diluted in MTP for 2 h at room temperature: i.e. goat anti-mouse or alternatively goat anti-rabbit conjugated to HRP (Sigma, A0168 and A0545 respectively) at 1/3000. To detect the microinjected IgGs, membranes were incubated with the proper secondary antibody. To detect BDA3000, membranes were incubated with avidin–biotin complex kit coupled to HRP (Sigma). To detect the presence of IgY, membranes were incubated with the proper secondary antibody, anti-chicken IgY conjugated to HRP (Sigma, A-9046). Finally membranes were washed with PBT (4×15 min) at room temperature, developed with DAB, and analysed as stated above for HRP. The reaction time and sample dilution for each molecule was empirically determined to obtain the appropriate contrast with no background. The relative concentrations of these molecules were calculated using Scion Image software on the scanned slot (or western) blots, on the basis of the highest immunoreaction for each specific experiment, expressed as a ratio of the concentration.

Immunohistochemistry

To detect the protein transfer site(s), some embryos were killed 10 to 20 min after protein microinjection, dissected out of the extraembryonic membranes and fixed in paraformaldehyde 4% for confocal microscopy. Then, they were embedded in 5% agarose/10% sucrose in PBS and stored overnight at 4 °C. Subsequently, they were cut into 200 μm sections with a vibratome (Vibratome 1000 Plus). Some embryos were also immunodetected by cutting them sagittally in two halves with a scalpel. They were then washed with PBT (2×1 h, PBS+0.1% Triton X-100, from Sigma) and blocked in 10% foetal calf serum (FCS) in PBT for 1 h at room temperature. Embryos in which BSA had been microinjected were blocked with PBT alone to avoid antibody crossreaction. Then, they were incubated for 2 to 3 days at 4 °C with the corresponding primary antibody (mouse anti-FITC, Sigma F5636, at 1/2500; mouse anti-BSA, Sigma B2901, 1/1000). After being washed in PBT (3×1 h), they were incubated with the proper secondary antibody (anti-mouse conjugated to Alexa-488 at 1/500, Molecular Probes A11029). For BDA3000 microinjected embryos, the samples were washed with PBT (3×1 h) and then incubated with Streptavidin-Alexa488 at 1/500 (Molecular Probes A11029), for 90 min at room temperature. Samples from ovalbumin-Evitag and BSA-Evitag microinjected embryos were simply washed with PBT (2×20 min).

Sections and half-mount embryos were counterstained with phalloidin-TRITC at 1/2000 (Sigma, P1951) and/or with TOTO-3 at 1/1000 (Molecular Probes, T3604) in the presence of 1% Rnase (Sigma, R6513) overnight at 4 °C. After washing with PBT (3×1 h), samples were mounted on slides with Glycerol–PBS 1:1 v/v. All incubations were made whilst gently shaking. Photomicrographs were taken using a confocal microscope (Olympus) or with a dissecting microscope equipped with epifluorescence (Leica MZ16F) and were assembled with Photoshop software.

For immunohistochemistry on semithin sections, embryos in which BSA had been microinjected in the cephalic vesicles were fixed in paraformaldehyde 4%–glutaraldehyde 0.1%. The area of protein transfer was dissected from the rest of the embryo, embedded in Lowicryl HM20 by freeze substitution, and then sectioned. The presence of transported BSA was detected with anti-BSA specific antibody (see above), which was subsequently detected with an anti-mouse gold antibody (10 nm gold particles; Sigma G7652). The signal was amplified using the LM/EM silver enhancing kit (British Biocell International, BBI-SEKL 15). Finally, immunostained semithin sections were counterstained with 0.1% toluidine blue.

Organotypic cultures of neuroepithelium

Organotypic cultures of both dorsal and ventral mesencephalic neuroepithelium were developed basically as described in Gato et al. (2005). Briefly, chick embryos at E4 (HH23) were dissected out of extraembryonic membranes, and the ectoderm covering the mesencephalic vesicle was removed using a tungsten needle. Subsequently, the mesencephalic roof, or the ventral mesencephalon, was cut with microscissors. After extensive washing in a serum-free medium, the explants were placed on small pieces of Millipore filters (0.8-μm pore size), which had been boiled in distilled and deionised water. After equilibrating the filters in a serum-free medium for 15 min, the explants were positioned on them so that the apical surface was in close contact with the filter. To avoid detachment of the explants, they were peripherally fixed to the filter with a tungsten needle. They were then transferred to a culture well containing a serum-free medium (DMEM F12, Sigma) supplemented with 1% ascorbic acid. Then, a 23 nl drop of RBP-FITC or the solvent was placed on top of the mesoderm covering the ectodermal explant, using a microinjector (Nanoject II). Ten minutes after the protein had been laid out, the explants were processed for immunohistochemistry, immunodetected and photomicrographed as described (see above).

Results

Permeability of the cephalic neuroectoderm

To test the functional effectiveness of the putative chick embryonic blood–CSF interface just after closure of the anterior neuropore, i.e. to check the permeability of the cephalic neuroectoderm, a small-sized tracer (BDA3000) was microinjected into the cephalic cavity or into the outflow of the heart of embryos at E3 (HH20) and E4 (HH23). First of all, we analysed whether BDA3000 was transferred from the blood to the E-CSF and *vice versa*. E-serum and E-CSF were removed 20 min after BDA3000 microinjection, and the presence of this tracer was monitored by slot-blot (Figs. 1A, B). When BDA3000 was microinjected into the outflow of the heart of either E3 or E4 embryos, it was detected in both the E-serum and the E-CSF. In the same way, 20 min after BDA3000 microinjection into the cephalic vesicles, BDA3000 was also detected within both the E-CSF and the E-serum of both E3 and E4

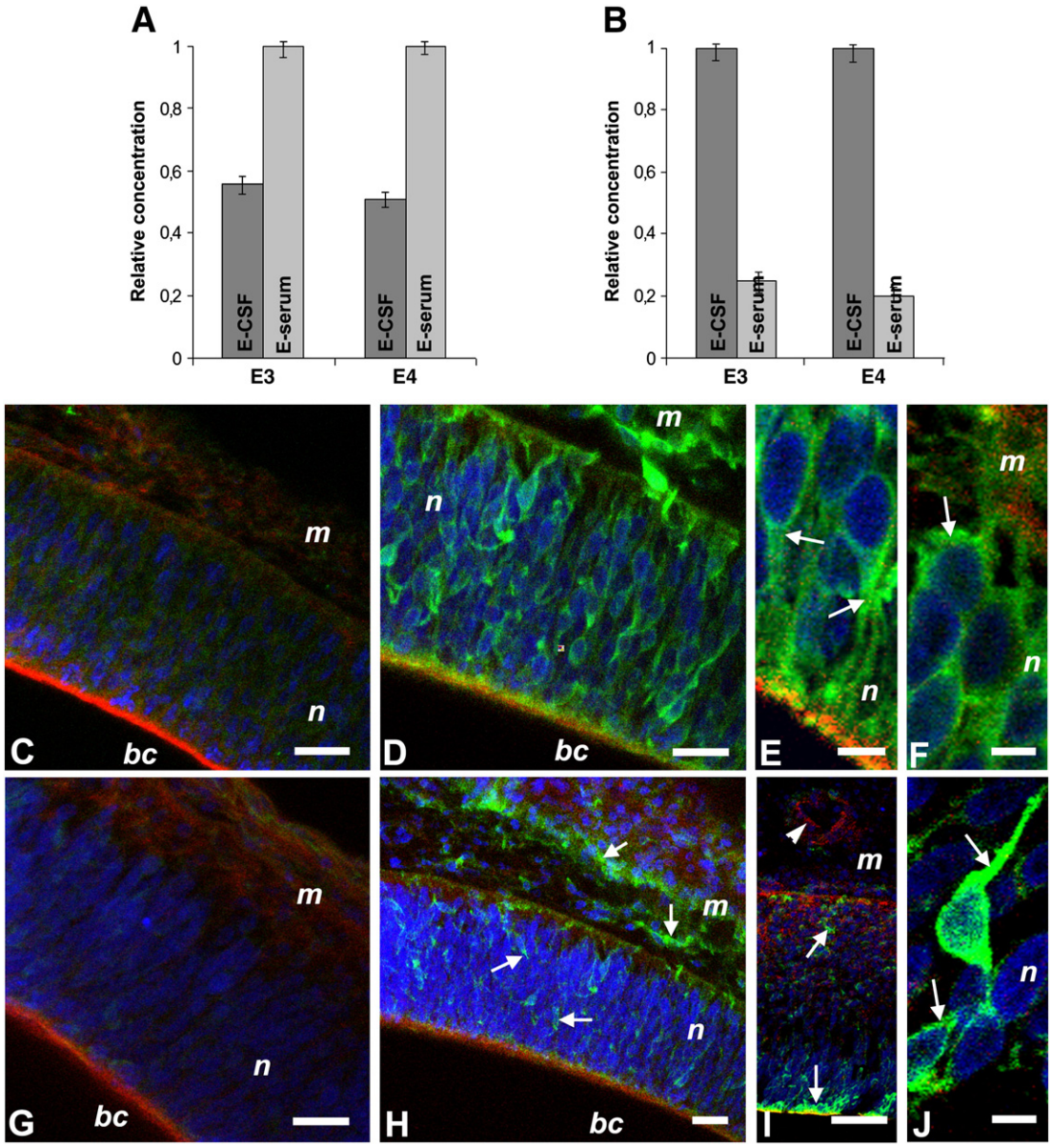


Fig. 1. Permeability of the embryonic blood–CSF interface at E3 and E4. (A) Bar chart showing BDA3000 transfer from the blood stream to the cephalic cavities 20 min after microinjection into the outflow of the heart. (B) Bar chart showing BDA3000 transfer from the brain cavities to the blood stream 20 min after microinjection into the cephalic cavities. (C) Negative control of dorsal mesencephalic neuroepithelium for an E3 embryo. (D) Dorsal mesencephalic neuroepithelium of an E3 embryo 20 min after BDA3000 microinjection into the cephalic cavities. (E) Magnification of the apical pole of the neuroepithelium shown in panel D. Note that the tracer is only present in the cytoplasm of the neuroepithelial cells (arrows). (F) Magnification of the basal pole of the neuroepithelium shown in panel D. Note that the tracer is only present in the cytoplasm of the neuroepithelial cells (arrow). (G) Dorsal mesencephalic neuroepithelium of an E4 embryo 20 min after BDA3000 microinjection into the cephalic cavities. Note the absence of the tracer within dorsal neuroepithelial cells. (H) Ventral mesencephalic neuroepithelium lateral to the ventral midline of an E4 embryo 20 min after BDA3000 microinjection into the cephalic cavities. Note the presence of the tracer within the cytoplasm of some neuroepithelial cells and in the adjacent mesenchyma (arrows). (I) Ventral neuroepithelium lateral to the ventral midline of an E4 embryo 20 min after BDA3000 microinjection into the cephalic cavities. Note the presence of the tracer within the cytoplasm of neuroepithelial cells (arrow) and in the vicinity of a blood vessel located within the mesenchyma (arrowhead). (J) Magnification of I, showing the presence of BDA3000 within neuroepithelial cells' cytoplasm including the cytoplasmic processes (arrows). All images were taken with a confocal microscope. BDA3000 is shown in green; TOTO3 nuclei counterstaining is shown in blue; and phalloidin tissue contrast is shown in red. The bar charts show the standard error. Five individual experiments were performed for each condition. Scale bars: C, D, G, H and I, 0.05 mm; E, F and J, 0.02 mm. Abbreviations: bc, brain cavity; m, mesenchyma; n, neuroectoderm.

embryos. In the first experiment, the relative concentration of the tracer was approximately 2-fold higher in the E-serum than in the E-CSF, whereas in the second experiment it was approximately 4-fold higher in the E-CSF than in the E-serum.

Then, to check whether this inert tracer was being transported across the neuroepithelial cells through a transcellular route or whether it crossed the neuroectoderm through extracellular spaces (i.e. to verify whether the brain cavity was physiologically sealed at these developmental stages), the microinjected embryos were processed for immunohistochemistry to monitor the location of BDA3000 within the neuroectodermal tissue. At E3 (Figs. 1C–F) the tracer was clearly visible in most, if not all, neuroectodermal cells of the neuroepithelium of the distinct cephalic vesicles. Interestingly, it was always detected inside cell cytoplasm and throughout cytoplasmic processes, from the apical to the basal pole of the neuroepithelium (at this stage the neuroepithelium is organised as a pseudomonostratified tissue). It was never observed within extracellular spaces. It was also observed within the mesenchymal cells adjacent to the basal pole of the neuroectoderm. Similarly, at E4 (Figs. 1G–J) when the tracer was injected either into the vesicles or into the outflow of the

heart, it was detected inside neuroepithelial cell cytoplasm, throughout the cytoplasmic processes, and never in extracellular spaces. However, unlike the results for E3 embryos, the area of transport was restricted to a very specific zone, which was located in the ventral mesencephalic neuroepithelium lateral to the floor plate, in the brain stem. No other neuroepithelial sites in the cephalic neuroectoderm showed the presence of this tracer. It was also observed within mesenchymal cells adjacent to the basal pole of the neuroectoderm. Interestingly, the areas of BDA3000 transfer across the neuroepithelium were always close to blood vessels immersed within the mesenchyma (Fig. 1I). Taken together, these results indicate that during CNS development the cephalic cavities are physiologically sealed at least from E3 onwards. In addition, in the developmental period between E3 and E4, the area for blood–CFS transfer (and *vice versa*) for this tracer becomes restricted to a very specific zone.

Blood–CSF physiological transfer of endogenous proteins

The next step was to assess whether at the beginning of primary neurogenesis the embryonic blood–CSF interface selectively transfers proteins from the blood stream to the cephalic cavities or whether they are transferred by free diffusion (i.e. to test whether transport between the E-serum and the E-CSF is regulated). To achieve this, we checked the relative concentration in these two embryonic compartments of specific endogenous proteins that are known not to be produced by the cephalic neuroectoderm even though they have been detected within the E-CSF. These proteins were ovalbumin and RBP (Parada et al., 2006). An interval around E4, i.e. from E3 to E5, was analysed by slot-blot using specific antibodies. At all analysed developmental stages, ovalbumin, which is known to be taken from the egg reservoir, was detected at an approximately 2-fold higher concentration in the E-serum than in the E-CSF (Fig. 2A). Conversely, the relative concentration of RBP, which may be produced in the liver primordium and/or in the chorioallantoic membrane, varies with time (Fig. 2B). At E3, the relative concentration of this molecule was approximately 2-fold higher in the E-serum than in the E-CSF. However, at E4 it was almost 3-fold higher in the E-CSF than in the E-serum. Finally, at E5 its relative concentration was again slightly higher in the E-serum than in the E-CSF.

We also tested the relative concentration of endogenously produced FGF2, a molecule that is known to be produced by several different embryo tissues and organs, such as the liver primordium, the notochord, the mesonephros and the neuroectoderm, although the level of expression in neuroectoderm is much lower than in the other tissues. Moreover, FGF2 is known to be transferred from the E-serum to the E-CSF (Martin et al., 2006). The relative concentration of FGF2 was also stage-dependant, although its dynamics were different to those of RBP. In this case, the relative concentration of FGF2 was 2 to 3-fold higher in the E-CSF than in the E-serum of both E3 and E4 embryos (Fig. 2C). However, at E5 its relative concentration was 3-fold higher in the E-serum than in the E-CSF.

Finally, we checked the relative concentration of IgY, a molecule that is taken from the egg reservoir in these early developmental stages and that has not been detected within the E-CSF by proteome analysis (Parada et al., 2006). IgY was detected in the E-serum in all analysed stages, and its relative concentration was higher in E4 and E5 embryos than in E3 ones. However, it was never detected within the E-CSF (Fig. 2D). Taken together, these results indicate that the transfer across the blood–CSF interface of proteins that are normally present within the embryo fluids is developmentally regulated and that this regulation depends on each specific protein, suggesting the existence of active protein transport from E-serum to E-CSF.

Blood–CSF transfer of heterologous microinjected proteins

To further test the existence of active protein transport from E-serum to E-CSF and *vice versa* in chick embryos at E4 (HH23), we checked the transfer dynamics of a number of exogenous micro-

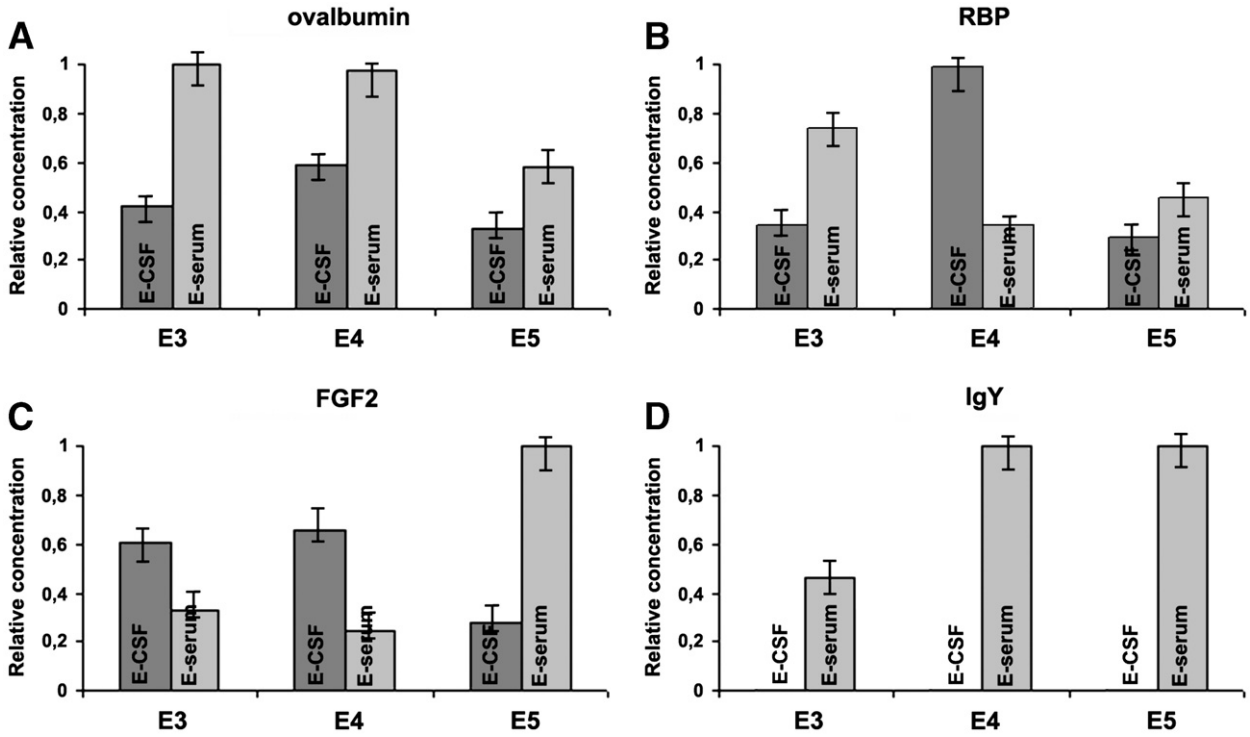


Fig. 2. Relative concentration of endogenous proteins in the E-serum and in the E-CSF at E3, E4 and E5 developmental stages. (A) Bar chart for ovalbumin. (B) Bar chart for retinol binding protein (RBP). (C) Bar chart for fibroblast growth factor no. 2 (FGF2). (D) Bar chart for IgY. Standard error is shown. Five individual experiments were performed for each condition.

injected proteins. Two different categories of proteins were used for the microinjections: (1) proteins that are not normally present in chick embryos and/or within the analyzed fluids (heterologous proteins), i.e. HRP, BSA, MHC and GST/Adh (GST/Adh was first conjugated to FITC to facilitate its detection); and (2) proteins that are normally present in the E-CSF and the E-serum of chick embryos at E4, i.e. ovalbumin, FGF2 and RBP, coupled to FITC before the microinjection, in order to distinguish them from the endogenous proteins (described in the following section). Mammalian IgG immunoglobulins, whose protein sequence differs from avian IgY immunoglobulins–although both types have a globular structure–were also used. These proteins were chosen to represent a set of different molecular weights. In all cases, E4 embryos were microinjected either into the outflow of the heart or into the cephalic cavities in ovo. E-serum and E-CSF were collected at different incubation times after microinjection, from 0 to 24 h. In some cases, the fluid contained within the allantoic vesicle was also collected. All fluids were analysed by slot-blot except for FGF2 coupled to FITC. This was analysed by western-blot, as during the FITC linkage the accompanying BSA which is used as a stabilizer by the supplier also coupled to the tracer. It is important to note that although FGF2 and RBP were of human origin, they exhibit a high degree of homology with the corresponding chick proteins. Moreover it has recently been reported that FGF2 is able to functionally substitute its endogenous counterpart in vitro (Martin et al., 2006).

With respect to the blood–CSF transfer of heterologous microinjected proteins, we first analysed HRP transfer as it was classically used to determine BBB permeability, since it has been traditionally considered an inert tracer (Figs. 3A, B). When HRP was injected into

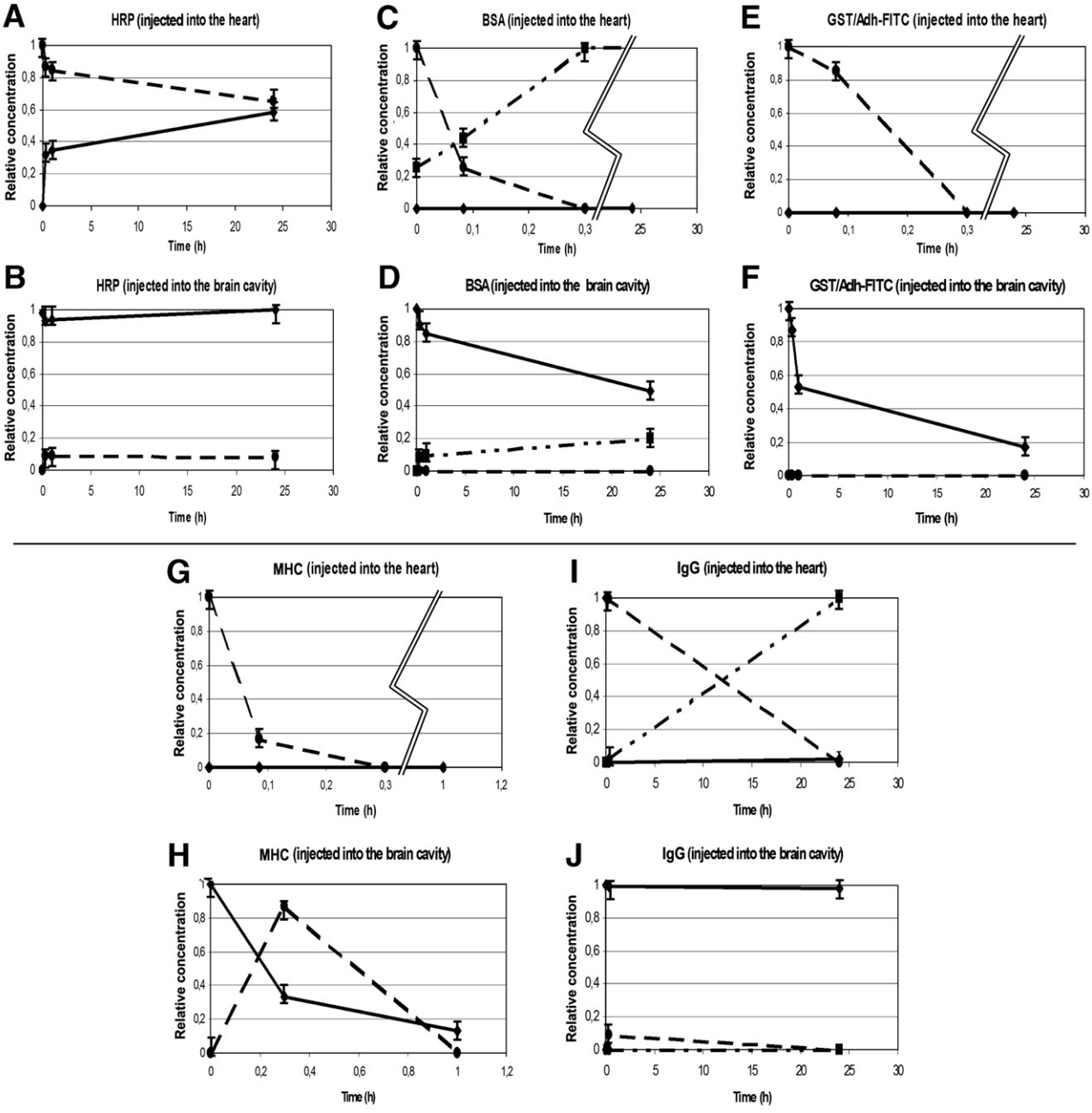


Fig. 3. Transfer dynamics of non-avian microinjected proteins. Line charts showing the transfer dynamics of proteins microinjected either into the outflow of the heart or into the cephalic cavities. Time is in hours. All embryos were microinjected at E4 (HH23). Continuous line: relative concentration of E-CSF; dashed line: relative concentration of E-serum; combined dashed and dotted line, relative concentration of allantoic fluid. Microinjected molecules and the route of microinjection are indicated in each chart. Standard error is shown. Five individual experiments were performed for each condition. Abbreviations are as in the text.

the outflow of the heart, its relative concentration in the E-serum progressively declined from 0 to 24 h, i.e. from E4 to E5. At E5 it approximately equalled HRP concentration in the E-CSF. HRP concentration within the E-CSF increased correspondingly during this period. This was the expected result for a non-barrier blood–CSF interface, and coincided with previous reports on BBB permeability at later developmental stages. However, when HRP was injected into the cephalic cavities, it remained mostly inside the cavities within the E-CSF. Very little HRP was detected within the E-serum after 24 h.

Then, we checked whether the transport dynamics of other proteins that are also not normally present in chick embryos and/or in the analysed fluids paralleled the results for HRP. We used BSA and GST/Adh, as they have a similar molecular mass to HRP, and MHC and IgG, as their molecular mass is clearly higher, although their reported 3D structure is different (i.e. globular for IgG and mostly fibrillar for MHC). Interestingly, the transport dynamics of these four proteins completely differed from that of HRP (Figs. 3C–J).

When BSA, GST/Adh or MHC was microinjected into the outflow of the heart (Figs. 3C, E and G) it was never detected within the E-CSF, and its relative concentration within the E-serum decreased very rapidly. Twenty minutes after microinjection, these proteins were no longer detected within the E-serum. Concomitantly, their relative concentrations within the allantoic fluid increased rapidly during the same period. This result indicates that, unlike HRP, these proteins are not transferred from the E-serum to the E-CSF. In addition, they are removed very rapidly from the blood stream.

In the same way, when these molecules (BSA, GST/Adh and MHC) were microinjected into the cephalic cavities (Figs. 3D, F and H), their relative concentration within the E-CSF also decreased with time, although not as rapidly. The relative concentration of MHC within the E-CSF dropped to less than a 1/4 just 1 h after microinjection. BSA and GST/Adh were still detected within the E-CSF 24 h after microinjection, although the relative concentrations had decreased by a 1/2 for BSA and a 1/4 for GST/Adh. An analysis of the E-serum and the allantoic fluid revealed that these molecules were also being removed very rapidly from the blood stream to the allantoic vesicle, as in most cases their presence within the E-serum was under the threshold of detection. Only MHC was transiently detected within the E-serum.

With respect to IgGs (Figs. 3I, J), when they were injected into the outflow of the heart their relative concentration within the E-serum decreased as reported for BSA, GST/Adh and MHC, although not as rapidly. Concentrations were just over the threshold of detection 24 h after microinjection. In this way, their relative concentration within the allantoic fluid increased proportionally during the same period, and their presence within the E-CSF was virtually undetectable. Finally, when IgGs were microinjected into the brain cavities, they were mostly detected within the E-CSF 24 h after microinjection. Very few were detected within the E-serum or within the allantoic fluid.

Blood–CSF transfer of microinjected proteins normally present in E-CSF and E-serum

Then, we analysed the transfer dynamics of some typically transferred endogenous proteins such as ovalbumin, RBP and FGF2. We microinjected them into the outflow of the heart or into the cephalic cavities (Figs. 4A–F). These gene products were previously coupled to FITC, to distinguish them from the endogenous proteins. When they were microinjected into the outflow of the heart, their concentration within the E-serum rapidly decreased. One hour after microinjection, the microinjected FGF2-FITC monitored by the conjugated FITC was not detected within the E-serum, and the concentration of RBP-FITC and ovalbumin-FITC had dropped to less than a 1/4. The relative concentration of these microinjected molecules within the E-CSF increased during the period analysed. However, this increase did not account for the total decrease in these molecules in the E-serum, as they were removed by the allantoic vesicle.

In the same way, when FGF2-FITC, RBP-FITC or ovalbumin-FITC were microinjected into the cephalic cavities, their concentration within the E-CSF rapidly decreased. One hour after microinjection, the relative concentration of all of these proteins had been reduced by approximately a 1/2, as detected by the conjugated FITC. The relative concentration of ovalbumin-FITC and RBP-FITC within the E-serum increased during the study period, although this increase did not account for the total decrease in the E-CSF, indicating that these molecules were also being rapidly removed by the allantoic vesicle. The relative concentration of FGF2-FITC within the E-serum was under the threshold of detection during the period analysed, also indicating that these molecules were being rapidly removed by the allantoids.

Microinjection of these molecules into the outflow of the heart should increase their relative overall concentration, at least transiently, in the E-serum. The transfer of these molecules to the E-CSF was detected. We then checked whether these microinjected molecules were transferred from the E-serum to the E-CSF instead of the endogenous ones (i.e. if their overall concentration within the E-CSF did not increase), or if they were transferred in addition to the endogenous ones (i.e., if their overall concentration within the E-CSF did increase or remain constant). In other words, we assessed the existence of transport regulatory mechanisms controlling the concentration of endogenous proteins within the E-CSF.

To achieve this, we microinjected FGF2, RBP and ovalbumin into the outflow of the heart, and we collected the E-CSF and the E-serum at different times thereafter, including just before microinjection (0 min), which represents the endogenous relative concentration; just after microinjection (0 min), which represents the sum of the endogenous and injected concentrations; and 5 min, 20 min, and 1 h after microinjection. Then, we analysed the relative concentration of these molecules using specific antibodies (i.e. anti-FGF2, anti-RBP and anti-ovalbumin) (Figs. 4G–I), thus detecting both the endogenous and the microinjected molecules. The relative concentration of all molecules within the E-serum increased just after microinjection. It is important to note that the immunoreaction was saturated in all cases. Thus, these values do not represent the real increase in relative concentration, which was higher. The justification for this is that if we diluted the samples more, the other values would be under the threshold of detection.

These experiments showed that the excess of proteins were rapidly removed from the E-serum, which in 5 min reached a level of relative concentration that was slightly lower than that detected just before microinjection. One hour later, all of the analysed proteins had reached their normal relative concentrations within the E-serum. In no case did the relative concentrations in the E-CSF increase above normal values at any time. Conversely, concentrations slightly decreased shortly after microinjection, paralleling the dynamics of these molecules within the E-serum. Again, like their relative concentrations in the E-serum, 1 h after microinjection all analysed proteins had reached their normal relative concentrations within the E-CSF. From these experiments it can be concluded that these microinjected molecules are transferred from the E-serum to the E-CSF "instead of" the endogenous ones, suggesting the existence of concentration regulatory mechanisms in the blood–CSF interface.

Taken together, these results also suggest that the transfer of protein molecules across the blood–CSF interface is regulated differentially, depending on each specific molecule. In addition, the allantoids contribute differentially to the elimination of protein molecules from the blood stream.

Localisation of the protein transfer area(s) in the blood–CSF interface

Finally, we investigated whether the transfer of proteins across the blood–CSF interface was restricted to a specific embryo area, as suggested by BDA3000 transfer experiments at E4. Alternatively, this transfer could be carried out by any part of the cephalic vesicles and/or

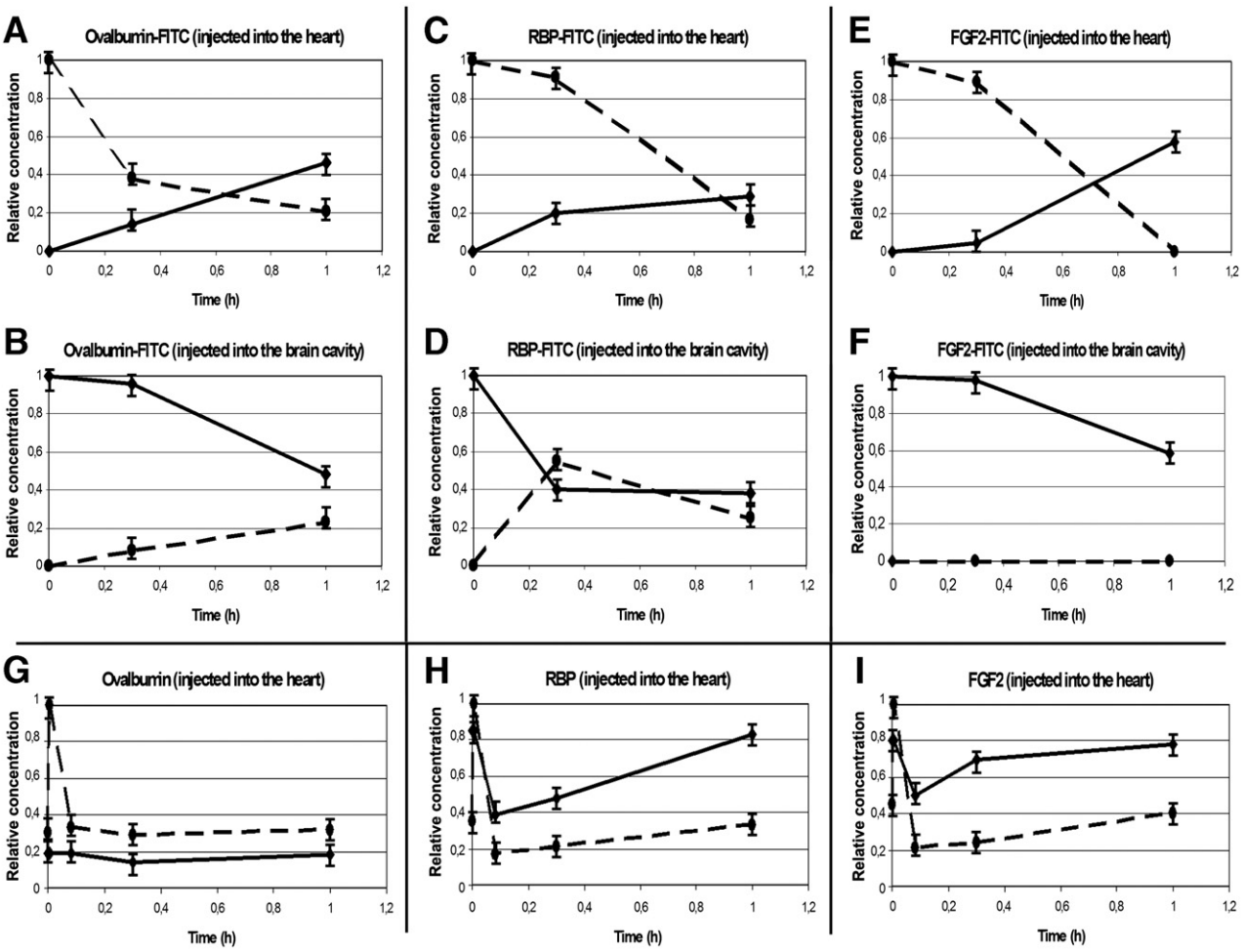


Fig. 4. Transport dynamics of microinjected proteins normally transferred across the blood–CSF interface. (A–F) Line charts showing the transfer dynamics of proteins normally present within the E-CSF and the E-serum, microinjected either into the outflow of the heart or into the cephalic cavities. These proteins were coupled to FITC to distinguish them from the endogenous ones, and thus they were detected with an antibody to this tracer. (G–I) Bar charts showing the relative concentration of proteins normally present within the E-CSF and the E-serum after microinjecting such proteins into the outflow of the heart to detect to overall increase in concentration in both the E-serum and the E-CSF. Specific antibodies were used to detect the sum of the microinjected proteins and the endogenous ones. Time is in hours. All embryos were microinjected at E4 (HH23). Continuous line: relative concentration of E-CSF; dashed line: relative concentration of E-serum. Standard error is shown. Five individual experiments were performed for each condition. Abbreviations are as in the text.

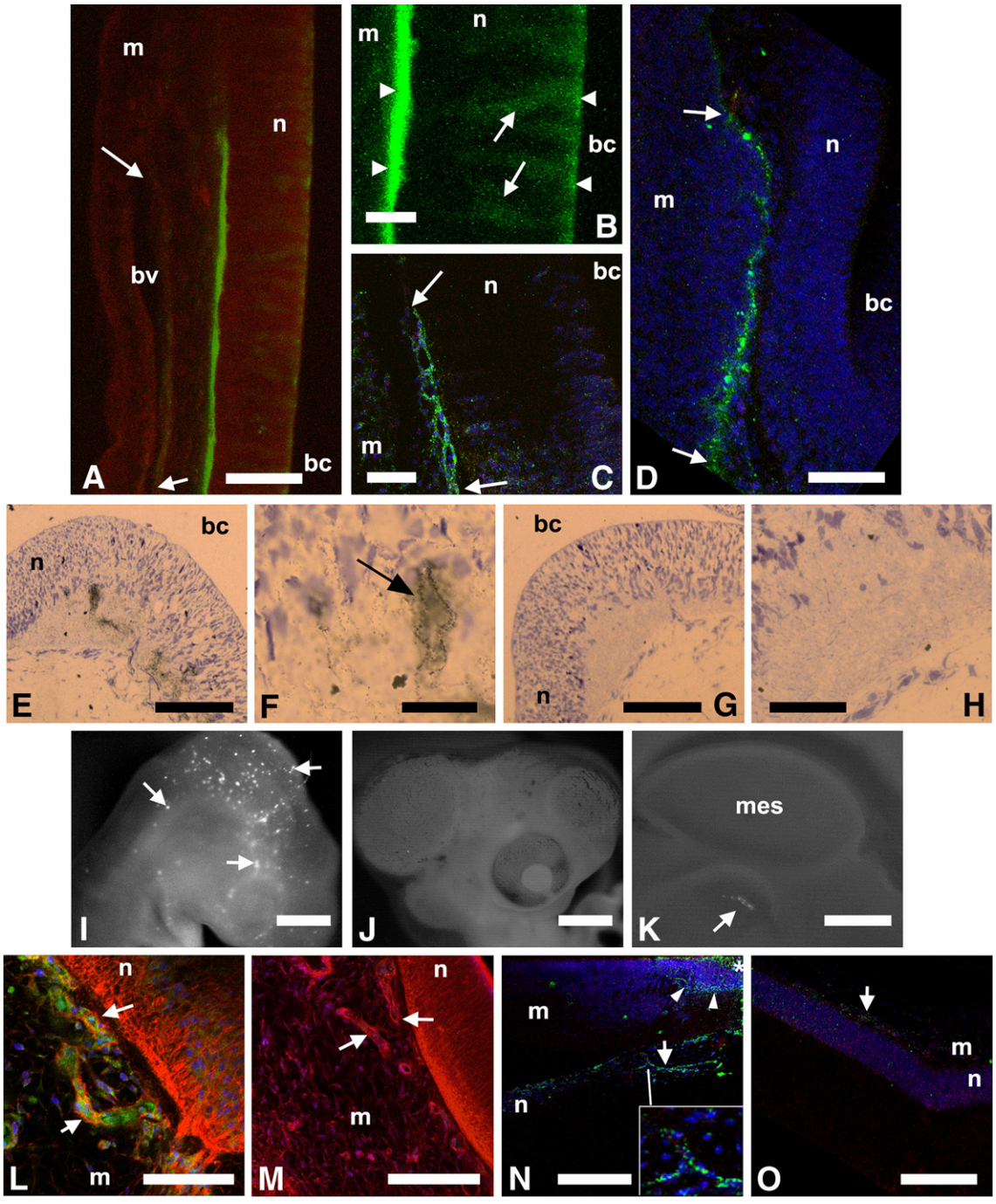
by all blood vessels, which at this developmental stage surround the cephalic cavities from within the mesenchyma. Embryos microinjected with BSA, ovalbumin-FITC or RBP-FITC were processed for immunohistochemistry 20 min after microinjection. These proteins were chosen as we had demonstrated that they were transferred from the E-CSF to the E-serum or *vice versa* within this time period. Their

Fig. 5. Protein transfer area for blood–CSF interface. Panels A–D and L–M are vibratome sections corresponding to ventral mesencephalic neuroectoderm seen under the confocal microscope. Panels E–H are semithin sections corresponding to ventral mesencephalic neuroectoderm lateral to the ventral midline seen under a light microscope. Panels I–K are whole mount heads seen under an epifluorescence-equipped dissecting microscope. Panels N–O are vibratome sections of neuroepithelial cultures seen under the confocal microscope. Unless otherwise stated, all in ovo experiments were performed by microinjecting the molecules into embryos at E4 (HH23). In vitro neuroepithelial explant experiments were performed by dissecting the neuroectoderm from embryos at E4. Nuclei TOTO3 counterstaining is shown in blue, and phalloidin tissue contrast is shown in red. (A) BSA microinjected into the brain cavities. Note the accumulation of this molecule in the basal membrane of the ventral neuroectoderm 20 min after microinjection. Also note the presence of a blood vessel within the mesenchyma close to the accumulation site (arrows). (B) Magnification of panel A showing the presence of BSA within the neuroectodermal cells (arrows). Note the accumulation of BSA at both their basal and apical poles (arrowheads). (C) Ovalbumin coupled to FITC microinjected into the outflow of the heart. Note the accumulation of ovalbumin at the basal membrane of the ventral neuroectoderm 20 min after microinjection. Also note that this accumulation is restricted to a specific area (arrows). (D) RBP coupled to FITC microinjected into the outflow of the heart. Note the accumulation of RBP at the basal membrane of the ventral neuroectoderm 20 min after microinjection. This accumulation is also restricted to a specific area (arrows). (E) Semithin sections of embryos microinjected with BSA into the cephalic cavities. Note the presence of transported BSA in vascular sprouts within the neuroectoderm, located at the ventral mesencephalon and at the most anterior part of the ventral prosencephalon, lateral to the floor plate. (F) Magnification of panel E showing the presence of BSA within the endothelium of vascular sprouts (arrow). (G) Negative control for panel E. (H) Negative control for panel F. (I) Ovalbumin coupled to Evitags® microinjected into the outflow of the heart of an embryo at E3 (HH20). Note the accumulation of ovalbumin–Evitags at different sites in the periphery of the brain cavities 20 min after microinjection (arrows). (J) Ovalbumin coupled to Evitags® microinjected into the outflow of the heart of an embryo at E4. Note that ovalbumin–Evitags do not accumulate at the periphery of the brain cavities. (K) Same embryo shown in panel F after dissecting the head in two halves. Note the accumulation of ovalbumin–Evitags at a specific location close to mesencephalic and anterior-most rhombencephalic ventral neuroectoderm (arrow). (L) Ovalbumin coupled to Evitags® microinjected into the outflow of the heart of an embryo at E4. Note the presence of ovalbumin–Evitags within some vascular sprouts located close to mesencephalic ventral neuroectoderm (arrows). (M) Negative control for panel L. Arrows points to the presence of vascular sprouts. (N) Cultured explant of mesencephalic ventral neuroectoderm lateral to the floor plate to which RBP coupled to FITC has been applied. Note the presence of RBP in the mesenchyma (asterisk), within this tissue (arrowheads) and also within neuroectodermal cells (arrows). The inset is a magnification of the neuroectoderm showing the presence of RBP within its cells. (O) Cultured explant of mesencephalic dorsal neuroectoderm to which RBP coupled to FITC has been applied. Note the presence of RBP in the mesenchyma (arrows). This molecule is completely absent from the neuroectoderm. Five individual experiments were performed for each condition. Scale bars: A–E, G, L–O, 0.05 mm; F and H, 0.02 mm; I–K, 1 mm. Abbreviations: bc, brain cavity; bv; blood vessel; m, mesenchyma; mes, mesencephalic vesicle; n, neuroectoderm.

presence within embryo tissues was analysed. BSA was used to examine CSF–blood transfer, and ovalbumin and RBP to analyse blood–CSF transfer.

Twenty minutes after BSA microinjection into the brain cavities, it was detected at a very specific location in the brain stem, within the mesenchyma basal to the neuroectoderm, close to some perineural blood vessels; specifically in the ventral mesencephalon and the most anterior part of the ventral prosencephalon, lateral to the floor plate (Fig. 5A). It was also detected within some neuroepithelial cells located exclusively adjacent to this zone (Fig. 5B), which coincided with the area of BDA3000 transfer (see Fig. 1). Concomitantly, when either ovalbumin or RBP coupled to FITC were microinjected into the outflow of the heart, their presence was detected within the mesenchyma close to the basal pole of the neuroectoderm (Figs. 5C, D). In both cases, the intensity of staining was much lower than that for BSA, and could not be visualised within the neuroectodermal cells. Interestingly, the area of transfer was also located in the same specific zone of the ventral mesencephalon and the anterior-most part of the rhombencephalon, lateral to the floor plate. This suggests that at E4 this neuroepithelial area and the perineural blood vessels close to it constitute the blood–CSF interface. The immunochemical analysis of semithin sections of embryos in which BSA had been microinjected into the cephalic cavities also showed the presence of transported BSA within the endothelium of vascular sprouts in the neuroectoderm, also located in the ventral mesencephalon and in the most anterior part of the ventral prosencephalon, lateral to the floor plate (Figs. 5E–H).

To check whether the perineural blood vessels close to these neuroepithelial area were those involved in the blood–CSF interface



for protein transfer, we microinjected ovalbumin coupled to Evitags®, as the size of these fluorescent nanocrystals may hinder or prevent the transfer of the coupled proteins, favouring their accumulation. Embryos were processed for histochemistry 20 min after microinjection into the outflow of the heart. At E3, the autofluorescence of the accumulated ovalbumin–Evitags® was detected at several different points all along the periphery of the brain cavities (Fig. 5I and data not shown), and in the blood vessels surrounding the cephalic cavities. This suggests that at this developmental stage there is no specific area for protein transfer. These results coincide with BDA3000 transfer experiments at the same developmental stage (see Fig. 1 for comparison). However, when ovalbumin coupled to Evitags® was microinjected at E4, the only area in which the nanocrystals accumulated was the ventral side of the mesencephalon and the anterior-most part of the ventral prosencephalon, within blood vessels located close to the neuroectoderm (Figs. 5J, K). These E4 embryos were also examined after vibratome sectioning and TOTO3/phalloidin counterstaining, revealing the presence of ovalbumin–Evitags® within only some blood vessels and vascular sprouts located within the mesenchyma, close to the basal pole of the neuroectoderm, in the ventral mesencephalon and the most anterior part of the prosencephalon, and lateral to the ventral midline (Figs. 5L, M). Taken together, these results indicate that these blood vessels are involved in the blood–CSF interface for protein transfer, acting concomitantly with the adjacent neuroectoderm.

Then, to check whether the capacity of the neuroectoderm to transfer proteins from the blood stream to the E-CSF at E4 was restricted to the reported areas of transport, or whether the specificity of the transfer areas was only due to the underlying blood vessels and vascular sprouts, we cultured explants of ventral and dorsal mesencephalon from E4 embryos. The apical pole of the neuroectoderm was placed in contact with the culture medium, which paralleled the E-CSF within the cephalic cavities, and the basal pole and some of the underlying mesenchyma was placed on top of the culture. RBP coupled to FITC was applied onto the mesenchyma. The explants were processed for immunohistochemistry 10 min after the protein had been laid out. When RBP was laid onto the mesenchyma adjacent to the ventral mesencephalic neuroectoderm lateral to the floor plate, it was detected within neuroepithelial cells of the neuroectoderm (Fig. 5N). This suggests that these neuroepithelial cells were enabled to transfer this protein to the cephalic cavities. However, when RBP was laid onto dorsal mesencephalic tissues, it was never detected within the neuroectoderm (Fig. 5O), suggesting that these dorsal neuroepithelial cells were not enabled for protein transfer to the brain cavities. Taken together, these results suggest that the neuroepithelial cells of the ventral mesencephalon and the most anterior part of the rhombencephalon exhibit some special features that enable them for protein transfer from the blood stream to the E-CSF.

Discussion

Recently, it has been demonstrated that at the beginning of primary neurogenesis E-CSF contains diffusible factors that contribute to the control of CNS development, including neuroepithelial progenitor cell survival, proliferation and differentiation, as well as brain anlagen growth and morphogenesis (Desmond and Jacobson, 1977; Gato et al., 2005; Parada et al., 2005a). The action on CNS development of some of the factors contained within E-CSF has been analysed, including the role of the proteoglycans, FGF2, RBP/*all-trans* retinol system, and the apolipoprotein-carried lipids (Alonso et al., 1999, 1998; Martin et al., 2006; Parada et al., 2008, in press). According to the literature, most of these molecules are produced by tissues other than the neuroectoderm or they are supplied by the mother (they cross the placenta in mammals or are taken up from the egg reservoir by the chorioallantois in avian species). Moreover, it has been demonstrated that most of the major protein fractions contained within the chick E-CSF at the beginning of brain anlagen neurogenesis are produced or stored outside of the cephalic cavities (Parvas et al, 2008). This implies that these molecules must be transferred from the blood serum to the cephalic cavities to manufacture E-CSF. However, the time of actual barrier formation is somewhat controversial. Previous studies on blood–CSF interface barrier function, based on the classical work of Wakai and Hirokawa (1978), showed that blood–CSF interface permeability to HRP in chick embryos begins to decrease at E12–E14, i.e. 8 to 10 days after the initiation of primary neurogenesis. Conversely, two recently published papers (Johansson et al., 2006, 2008) introduced the concept of a functional and dynamic barrier, which is different from that of an adult and adapted to the specific requirements and environment of the early developing nervous system. These authors also suggested that proteins are transferred through transcellular routes.

In this paper, we focused on protein transfer across the blood–CSF interface at the beginning of chick brain anlagen neurogenesis, shortly after the closure of the anterior neuropore. We have shown that (1) the cephalic cavities are physiologically sealed at least from E3 (HH20); (2) the E-CSF/E-serum ratio of chick endogenous proteins produced outside of the cephalic cavities is developmentally regulated; (3) the transfer of both chick endogenous proteins and microinjected proteins across the blood–CSF interface is tightly regulated and protein-specific; and (4) within the period E3 to E4 the transfer of proteins becomes restricted to a specific embryo area located at the brain stem lateral to the floor plate, in the ventral mesencephalon and the most anterior part of the ventral prosencephalon, taking place through specific perineural blood vessels and some vascular sprouts within the neuroectoderm at this location.

Transfer of proteins across blood–CSF interface is developmentally regulated and molecule-specific

In adult brain, the existence of an endothelial-based BBB has been recognized for more than 100 years. However, until recently, few studies applied cellular and molecular biology to this question (reviewed by Rubin and Staddon, 1999). The BBB significantly impedes entry from blood to brain of virtually all molecules, except those that are small and lipophilic. However, some sets of small and large hydrophilic molecules, such as proteins, can enter the brain by active transport. For essential nutrients such as glucose and certain amino acids, specific transmembrane transporting molecules are present in relatively high concentrations in the brain endothelial cells. Some systems also seem to be capable of shuttling macromolecules into the brain. Some of these systems are known to be receptor-mediated, e.g. by the transferrin receptor (Pardridge, 1997). There is evidence that other growth factors and cytokines have a limited ability to cross the BBB (McLay et al., 1997).

In adults and foetuses, the CSF filling brain ventricles and the subarachnoid space is manufactured by choroid plexuses, which are epithelial tissues in the brain ventricular system (for a review, see Johanson, 1995). The choroid plexus is the main component of the blood–CSF barrier, i.e. the major interface by which many hydrophilic solutes gain access to the brain from the blood, including proteins and ions. Acting like a kidney for the brain, the choroid plexus makes a major contribution to chemical homeostasis and the volume of fluids bathing neurons and glia. Choroid plexus dysfunction is responsible for a number of pathological processes, and many neurological disorders are associated with changes in the chemical composition of CSF (for review see Johanson, 1995; Johanson et al., 1999a; Miyan et al., 2003; Rubin and Staddon, 1999).

In embryos, however, nothing is known about the manufacture of E-CSF, although it has been demonstrated that this fluid plays a crucial role in early brain development, through the set of specific proteins and morphogens it contains (Gato et al., 2005; Parada et al., 2005a,

2008a,b; Martin et al., 2006). E-CSF completely occupies the cavities of the brain anlagen, whose architecture during development is highly dynamic. At E3 (HH20), just before the start of brain neurogenesis and when the neuroepithelial cells engage in a very active process of proliferation, both anterior and posterior neuropores are already closed. Thus, the E-CSF becomes completely enclosed by the neuroectoderm. It has been reported that from E3 the E-CSF has a complex protein composition that differs from that of the E-serum, and that the relative concentration of its proteins vary during development and with respect to adult CSF (Dziegielewska et al., 1980; Checiu et al., 1984; Fielitz et al., 1884; Gato et al., 2004).

However, as stated above, the time of the actual barrier formation is controversial. Despite previous works on the blood–CSF interface barrier function (Wakai and Hirokawa, 1978) showed that the permeability of the blood–CSF interface to HRP began to decrease at E12–E14, an analysis of the expression of a chick-specific cell-surface glycoprotein that is specific to barrier function provided endothelial cells (neurothelin/HT7) revealed that it is expressed from E6 in microvessels of the brain stem located in the ventral part of the rhombencephalon and mesencephalon (Bertossi et al., 2002). At later stages of development, HT7 is progressively detected in an increasing number of blood vessels, including the developing choroid plexus from E10 onwards. From E16, this antigen is uniformly distributed within the entire brain microvasculature. From these results, Bertossi et al. (2002) concluded that the HT7 antigen precedes restriction permeability to tracer HRP.

Conversely, a recent study in which the transport of human and bovine albumin across the blood–CSF interface was examined in rat embryos indicated that blood-to-CSF transfer is selective from rat E13 onwards (the CNS development of a rat embryo at E13 corresponds approximately to that of an E4 to E5 chick embryo). This is in agreement with previous studies demonstrating that in rodent embryos serum proteins are excluded from the brain at a relatively early stage, indicating that a protein barrier is present early in development (Saunders et al 1991; Rubin and Staddon, 1999). In fact, it seems unlikely that all of the properties of the BBB of the blood–CSF interface are acquired simultaneously.

In this study, an analysis of the dynamics of the relative concentration of several chick endogenous proteins showed molecule-specific developmental variations in their E-CSF/E-serum ratio. The highest E-CSF/E-serum ratio for FGF2 was detected at E3 and E4, the developmental period in which this growth factor acts from the E-CSF to influence neuroepithelial progenitor cell proliferation. In this period, FGF2 also contributes to triggering brain neurogenesis (Martin et al., 2006). In the same way, the maximum E-CSF/E-serum ratio for RBP was detected at E4 but not at E3, coinciding with its suggested activity in retinol transport to the E-CSF (Parada et al., 2008). The E-CSF/E-serum ratio of ovalbumin–a protein whose role during development has not been associated with morphogenesis or neurogenesis–does not vary significantly from E3 to E5. Taken together, these results suggest that the transfer of proteins across the blood–CSF interface is developmentally regulated and molecule-specific as early as the initiation of brain neurogenesis, thus indicating that this interface has a barrier function.

The transfer ratio of microinjected endogenous proteins does not alter their concentration within the E-CSF

One of the known roles of blood–CSF interface systems with a barrier function, such as in the foetus and adult choroid plexus, is to act as a kidney-like organ that regulates the concentration of potentially harmful molecules within the CSF, including cellular and metabolic debris, excess ions and water, drugs and proteins (for reviews, see Johanson et al., 1999b; Emerich et al., 2005). Our results show that when the concentration of normal E-CSF molecules is experimentally increased, i.e. when additional molecules are microinjected into the cephalic cavities, they are rapidly removed from the cavities and subsequently from the embryo serum, in order to maintain the homeostasis of these fluids. Moreover, when the concentration of such molecules within the E-serum is experimentally increased, their overall concentration within this intracavitary fluid does not increase, although some of them (i.e. RBP, FGF2 and ovalbumin) are effectively transferred to the E-CSF to play their normal role in CNS development. The embryo rapidly eliminates excess proteins from the E-serum. Therefore, the period in which their concentration within the E-CSF may be above normal levels is very short. However, these results suggest that certain mechanisms control the concentration of molecules within the E-CSF (and also within the E-serum). Such mechanisms can detect protein concentration and eliminate any excess. Thus, these results support the existence of a barrier function in the blood–CSF interface as early as the beginning of brain neurogenesis.

The transfer of exogenous microinjected proteins is restricted and protein-specific

As mentioned above, previous works on BBB function showed that the permeability of the blood-brain interface to HRP begins to decrease at E12–E14 (Wakai and Hirokawa, 1978) in chick embryos. Thus, younger embryos may have a free diffusion system for protein transfer. This apparently contradicts the above suggestion that the transfer of proteins across the blood–CSF interface is regulated by a barrier function. To evaluate this possibility, in this study we examined the transfer of several different proteins that are not normally present in chick embryos or within the analysed fluids, including HRP. As described by Wakai and Hirokawa (1978), when HRP is supplied to the blood stream it is transferred to the E-CSF with no apparent restrictions, which supports the free diffusion hypothesis. However, this was the only exogenous molecule in the study that behaved in this way. All the other exogenous molecules, including BSA, GST/Adh, MHC and mouse IgGs, did not cross the blood–CSF interface. Apart from immunoglobulins, all of the molecules were eliminated by the allantoids shortly after microinjection. Two different explanations may account for this lack of blood–CSF interface transfer: (1) the allantoids remove these molecules very rapidly and they do not have the chance to cross the interface, irrespective of any transport control mechanisms; and (2) the specific proteins to be transferred at this interface are regulated, and these particular molecules cannot be transported. From our results, it is obvious that the allantoids remove these molecules very rapidly, but they remain in the E-serum for a certain period (more than 10 min). We demonstrated that FGF2, RBP and ovalbumin, coupled to FITC to distinguish them from the endogenous molecules, are effectively transferred across the blood–CSF interface in a very short time, much shorter than 10 min. This suggests a precisely regulated active transport mechanism across blood–CSF interface that only allows certain molecules to cross—or alternatively does not allow other molecules to cross. In this respect, it is known that in foetuses and adults the tissues involved in the barrier blood–CSF interface have numerous specific transport systems, including a broad array of receptors (Chodobski and Szmydynger-Chodobska, 2001; Emerich et al., 2005). The description of the nature of the specific transport systems acting at the embryo blood–CSF interface requires further analysis.

Likewise, when these exogenous molecules are microinjected into the cephalic cavities, they are rapidly transferred to the blood stream by means of the kidney-like function of the blood–CSF interface. However, there are two significant exceptions: HRP and mouse IgG, which are not eliminated from the E-CSF. The issue of why HRP is transferred from the E-serum to the E-CSF but not in the opposite direction still needs to be discussed. Although E-CSF contains molecules with peroxidase activity (Parada et al., 2006) that may account for the exceptional embryo transfer of HRP, its amino acid sequence is

not homologous to that of HRP, which is plant-derived. With respect to IgGs, although they are not transferred from the E-serum to the E-CSF, their removal from the E-serum is much slower than that of BSA, GST/Adh and MHC. Moreover, there is little transfer of IgGs from the E-CSF to the E-serum. This lack of IgGs transport is probably not due to a size restriction transport mechanism, as MHC–which has a similar molecular mass–is rapidly removed from the brain cavities. The differential transport dynamics of immunoglobulins with respect to BSA, GST/Adh and MHC may be due to the fact that chick embryos also contain certain types of immunoglobulins (i.e. IgY) which are taken up from the egg reservoir. Although IgY was found within the E-serum, it was not transferred to the E-CSF at the analysed developmental stages. Thus, the normal levels of immunoglobulins within the E-serum may explain the slower rate of elimination of IgGs from this fluid. In the same way, the lack of IgY transfer to the E-CSF as well as the lack of transfer of the injected IgG to the E-serum suggest that the embryo blood–CSF interface is completely impermeable to immunoglobulins.

Taken together, the results presented in this paper indicate that the transfer of proteins across the blood–CSF interface is regulated differentially depending on each specific molecule. In addition, the allantoids also contribute differentially to the elimination of proteins from the blood stream. In conclusion, the transport dynamics of all the analysed molecules indicate that the blood–CSF interface has a barrier function as early as E4 in chick embryos. This controls which molecules are transferred across the interface, as well as the rate of transport.

Blood–CSF interface for protein transfer at E4 is located in the brain stem

The different approaches used in this study to analyse the location of the blood–CSF interface reveal that during the developmental period E3 to E4 the blood–CSF transfer site for proteins is limited to a very specific area located in the brain stem, specifically in the ventral mesencephalic and rhombencephalic neuroectoderm lateral to the ventral midline. BDA3000 microinjection into the cephalic cavities shows that at E3 (HH20) protein molecules are transferred by most, if not all, neuroectodermal cells all along the cephalic neuroepithelium, which is physiologically sealed. Concomitantly, ovalbumin coupled to Evitags® microinjected into the outflow of the heart showed that several different capillaries surrounding the brain cavities are involved in such transfer. However, at E4 (HH23), an analysis of these tracers and of the transport of some other molecules (BSA, RBP and ovalbumin) by immunohistochemical procedures revealed that the area of transfer was restricted to a specific zone of the embryo located in the brain stem in the ventral mesencephalic and prosencephalic neuroectoderm, lateral to the ventral midline. Interestingly, the blood vessels in this area are the ones that show the first HT7 antigen expression from E6 on. HT7 is a chick-specific antigen for endothelial cells fulfilling barrier functions (Bertossi et al., 2002). This suggests that the barrier function reported in this paper precedes the reported detection of HT7 in these blood vessels.

Interestingly, this area does not coincide with the zones from which the different adult choroid plexuses form in a subsequent developmental stage (Bellairs and Osmond, 2005). However, in mouse embryos, it has been reported that the first capillaries which penetrate the neuroectoderm localise precisely in these areas, the brain stem of the mesencephalon and rhombencephalon, and that they do so from the ventro-lateral side, starting at mouse E10, just after the closure of the anterior neuropore (CNS-specific development of a mouse embryo at E10 approximately corresponds to that of an E3–3.5 chick embryo) (Herken et al., 1989). All these data indicate that this area is a transient place that fulfils blood–CSF barrier functions. Thus, it contributes to manufacturing E-CSF in this developmental stage.

The results using cultured explants in which RBP-FITC was laid onto the mesechyma underlying the neuroectoderm of the brain stem, or onto the mesencephalic dorsal neuroectoderm, indicate that, after the restriction of the area of protein transfer across the blood–CSF interface that occurred between E3 and E4, the neuroectodermal cells located close to the aforementioned blood vessels become the only ones capable of protein transport. This suggests that both the blood vessels and the ventral neuroectoderm of the brain stem are involved in barrier functions. In this respect, the presence of HT7 positive neuroblasts has been described at E5 in this precise location (Bertossi et al., 2002).

In conclusion, this study indicates that protein homeostasis of the E-CSF at the beginning of brain neurogenesis is controlled by the barrier function of a blood–CSF interface. This regulates the proteins that are transferred in a protein-specific manner and also controls their concentration within the E-CSF. Moreover, this study shows that after closure of the anterior neuropore and during the initiation of brain primary neurogenesis, this blood–CSF interface with barrier functions is located in the brain stem, in the ventral mesencephalon and prosencephalon lateral to the ventral midline. As mentioned above, the description of the nature of the specific transport systems acting at the embryo blood–CSF interface requires further analysis. We can also conclude that this blood–CSF interface contributes to the crucial role of E-CSF in CNS development by regulating its specific composition.

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