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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