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Disruption of embryonic blood-CSF barrier in chick embryos reveals the actual importance of this barrier to control E-CSF composition and homeostasis in early brain development

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ABSTRACT

In vertebrates, early brain development takes place at the expanded anterior end of the neural tube. After closure of the anterior neuropore, the brain wall forms a physiologically sealed cavity that encloses embryonic cerebrospinal fluid (E-CSF), a complex and protein-rich fluid that is initially composed of trapped amniotic fluid. E-CSF has several crucial roles in brain anlagen development. Recently, we reported the presence of transient blood–CSF barrier located in the brain stem lateral to the ventral midline, at the mesencephalon and prosencephalon level, in chick and rat embryos by transporting proteins, water, ions and glucose in a selective manner via transcellular routes. To test the actual relevance of the control of E-CSF composition and homeostasis on early brain development by this embryonic blood-CSF barrier, we block the activity of this barrier by treating the embryos with 6-aminonicotinamide gliotoxin (6-AN). We demonstrate that 6-AN treatment in chick embryos blocks protein transport across the embryonic blood-CSF barrier, and that the disruption of the barrier properties is due to the cease transcellular caveolae transport, as detected by CAV-1 expression cease. We also show that the lack of protein transport across the embryonic blood-CSF barrier influences neuroepithelial cell survival, proliferation and neurogenesis, as monitored by neurepithelial progenitor cells survival, proliferation and neurogenesis. The blockage of embryonic blood-CSF transport also disrupts water influx to the E-CSF, as revealed by an abnormal increase in brain anlagen volume. These experiments contribute to delineate the actual extent of this blood-CSF embryonic barrier controlling E-CSF composition and homeostasis and the actual important of this control for early brain development, as well as to elucidate the mechanism by which proteins and water are transported thought transcellular routes across the neuroectoderm, reinforcing the crucial role of E-CSF for brain development.

INTRODUCTION

In vertebrates, early brain development takes place at the expanded anterior end of the neural tube. After closure of the anterior neuropore, brain walls form a large and physiologically sealed cavity enclosing embryonic cerebrospinal fluid (E-CSF), a complex and protein-rich fluid. Chick and rat E-CSF proteomes analyzed at embryological stage E4 (E is for days of incubation) or HH23 (according to Hamburger and Hamilton, 1951) [1] and at E12.7 respectively include molecules whose roles explain the general functions reported for this fluid [2-5]. Thus, E-CSF is involved in several crucial roles in brain anlagen growth and development: (1) it exerts positive pressure against the neuroepithelial walls and generates an expansive force [6-11]; (2) it contributes to regulate the survival, proliferation and neurogenesis of neuroectodermal stem cells [12]; and (3) it collaborates with a well-known organising centre, the mesencephalic-metencephalic boundary or midbrain-hindbrain isthmus (IsO), to regulate neuroepithelial gene expression patterning [13].

The microenvironment of the CNS is important for neuronal development and function, including E-CSF composition and homeostasis. The existence of a transient blood-CSF barrier located at the brain stem lateral to the ventral midline, at the mesencephalon and prosencephalon level, between embryonic days E3 and E5 in chick embryos have been identified [14]. This blood-CSF barrier, which may operate in rat embryos at en equivalent brain developmental stages, i.e. at E12.7-E13.7, as suggested by the presence of the same specific transporters [15, 16], is formed by specific blood vessels immersed in the mesoderm and the neuroectoderm close to them, and controls E-CSF composition and homeostasis during this early stages of brain development, before the formation of functional choroids plexuses –the organs fulfilling blood-CSF barrier function in later foetuses and adults.

It has been demonstrated that this embryonic blood-CSF barrier transports proteins in a selective manner through transcellular routes [14], most probably by means of caveolae as detected by the presence of CAV-1 in both blood vessels and the neuroectoderm conforming the embryonic blood-CSF barrier [16]; water and ions through specific transporters, identified by the presence of aquaporins 1 & 4 (AQP1 and AQPO4) and inwardly rectifying K+ channel (Kir4.1) in the blood vessels but not in the neuroectoderm [15]; and glucose, by means of GLUT-1 transporter, detected in both blood vessels and the neuroectoderm conforming the embryonic blood-CSF barrier [16].

However, the actual relevance of the control of E-CSF composition and homeostasis on early brain development exerted by this embryonic blood-CSF barrier has not been experimentally tested. To achieve this objective, we blocked the activity of the embryonic blood-CSF barrier by treating the embryos with a specific toxin, namely 6-aminonicotinamide gliotoxin (6-AN), a known antimetabolite of nicotinamide [17-20]. Despite 6-AN is a commonly used gliotoxic substance exerting a known inhibitory effect on the pentose phosphate shunt and a secondary effect on the direct glycolytic pathway [21, 22], several in vivo investigations including ultrastructural analysis of the endothelial cell tightness and vascular permeability monitoring have shown that it also breaks-down the integrity of the BBB [20, 22-26]. In this respect, it is important to highlight that we did use this substance only as a tool for its BBB blockage properties, irrespective of its other features.

Here we demonstrate that when chick embryos are treated with 6-AN, protein transport across the embryonic blood-CSF barrier blocks for both endogenous and exogenously microinjected proteins, and that the disruption of the barrier properties is due to the cease of CAV-1 expression, thus linking protein transport to caveolae-mediated transcellular transport. We also show that the lack of protein transport across the embryonic blood-CSF barrier influences neuroepithelial cell survival, proliferation and neurogenesis. Moreover, the blockage of embryonic blood-CSF transport also disrupts water influx to the E-CSF, as revealed by an abnormal increase in brain anlagen volume, also linking water transport to caveolae-mediated transcellular transport across the neuroectoderm. Taken together, these results contribute (1) to delineate the actual extent of the blood-CSF embryonic barrier controlling E-CSF composition and homeostasis, (2) to check the importance of this control for early brain development, and (3) to elucidate the mechanism by which proteins and water are transported thought transcellular routes across the neuroectoderm, reinforcing the crucial

role of E-CSF for brain development.

MATERIAL AND METHODS

Obtaining chick embryos

Fertile chicken eggs (*Gallus gallus*; White-Leghorn strain) were incubated at 38 °C in a humidified atmosphere to obtain embryos at the desired developmental stage, i.e. at E3 (HH20), E4 (HH23), E5 (HH26) and E6 (HH29) (E stands for embryonic day from the beginning of incubation; HH stands for the Hamburger and Hamilton [1951] [1] developmental stages, as described in Bellairs and Osmond, 2005) [27]. Local and European guidelines for animal research were followed (CEEA – Comitè Ètic d'Experimentació Animal -Ethical Committee for Animal Experimentation- code number: DMAiH 3777).

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). Injections were always made on chick embryos at E4 (HH23), unless otherwise stated. The following molecules were microinjected: bovine serum albumin (BSA, Sigma B4287, at 50 mg/ml; mw 66 kDa); and ovalbumin (Sigma, A7641, at 50 mg/ml; mw 44 kDa). Ovalbumin was previously labelled with FITC to distinguish it from the endogenous one. Ovalbumin-FITC coupling was made according to Sigma standard protocol, as described in Parvas et al. (2008) [14] (FITC-1 conjugation Kit, Sigma), and the unbound dye was separated by gel filtration. 6-aminonicotinamide (6-AN, Sigma A68203, at 0.4 µg/µl) was microinjected into the brain cavity at different stages of chick development, i.e. E3 (HH20), H4 (HH23) and E5 (HH26).

Obtaining embryonic fluids

Embryonic fluids were obtained as previously described [14]. Briefly, for E-CSF, after dissecting the embryos out of extraembryonic membranes, 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. Then E-CSF was slowly aspirated, avoiding contact with the neuroepithelial wall. Conversely, E-serum was obtained in ovo. After opening a small window in the eggshell, the chorioallantoic membrane was dissected with a tungsten needle. Then the blood was microaspired thought the outflow tract of the embryonic heart, and immediately centrifuged to remove cells. A minimum of 10 embryos were used for each experiment. To minimize protein degradation, samples were kept at 4°C during these procedures and immediately frozen at -20°C until use.

Detection and quantification of molecules from embryonic fluids

Slot-blot was used to detect and quantify all of the analysed molecules in the embryo fluids as described in Parvas et al. (2008) [14]. Briefly, embryonic fluids were applied to nitrocellulose membrane (Hybond-N) using a microsample filtration manifold (Schleicher and Schuell, SRC072/0) connected to a vacuum pump. After sample application, membranes were dried to fix the proteins, and 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-FITC (Sigma, F5636) at 1/2500; and rabbit anti-ovalbumin (Calbiochem, 126705) at 1/1000.

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. Finally membranes were washed with PBT (4×15 min) at room temperature, and developed with 3-3′-diaminobenzidine tetrahydrochloride (DAB; 25 mg of DAB in 50 ml of PBS, 1 ml of CoCl2,

and 5 µl of H_2O_2 30% in the dark). The reaction was stopped by washing the filters with distilled water (×3). The reaction time and sample dilution for each molecule was empirically determined to obtain the appropriate contrast with no background.

Finally, the relative concentrations of these molecules were calculated using Scion Image software on the scanned slot blots, on the basis of the highest immunoreaction for each specific experiment, expressed as a ratio of the concentration, as described in Parvas et al. (2008) [14].

Immunohistochemistry

To detect CAV-1 expression, chick embryos at E3 (HH20), E4 (HH23) and E5 (HH26) were processed as described in Parvas et al. (2008). They were incubated with the corresponding primary antibody, rabbit anti-CAV1 (Beckton, C13630) at 1/500. As secondary antibody anti-rabbit conjugated to Alexa-488 at 1/500 (Molecular Probes) was used. Immunostained 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), as described in Parvas et al. (2008) [14].

Immunohistochemistry was also used to monitor neural differentiation (anti–beta3-tubulin; LabVision), apoptosis (anti–active casapse-3; Pharmingen) and cell proliferation (anti–phophohistone-H3, Millipore) in chick embryos at E3 (HH20), E4 (HH23) and E5 (HH26) previously microinjected with 6-AN, and on control embryos microinjected with saline solution. The used secondary antibodies were anti-mouse antibody conjugated to Alexa-488 or Alexa-546 at 1/500 (Molecular Probes). Quantitative analyses of beta3-tubulin– and caspase-3–expressing cells were performed by counting the number of neuroepithelial cells showing an immunostained cytoplasm in 20 microscopic fields of 1,900 μm^2, as described in Gato et al. (2005) [12]. Likewise, phosphohistone-3–expressing cell analysis was carried out by counting the number of neuroepithelial cells showing an immunostained nucleus, as described in Gato et al. (2005) [12].

Photomicrographs were taken using a confocal microscope (Olympus) or with a dissecting microscope equipped with epifluorescence (Leica MZ16F), and were assembled with Photoshop software.

Measuring cephalic cavities

Embryos previously microinjected with 6-AN as well as control embryos of at an equivalent developmental stage were examined for the width and height of the mesencephalic cavity under the dissecting microscope, as it had previously been reported that the increase in E-CSF volume parallels the volume of brain cavities [8, 28], thus reflecting water influx from the blood to the E-CSF. 6-AN microinjected embryos (n=50) were compared with controls (n=50) microinjected with saline solution, using a non-parametric statistical method (Mann-Whitney test).

RESULTS AND DISCUSSION

Disruption of protein transfer across the embryonic blood–CSF barrier

To disrupt blood-CSF barrier properties on protein transfer, 6-AN was used. 6-AN was microinjected into the mesencephalic cavities of chick embryos at E4, and relative concentration of an endogenous protein in the blood and CSF was examined, i.e. ovalbumin, at several different times after 6-AN microinjection (20 min, 2 h and 15 h). We couldn't check the relative concentration of this protein later than 15 h after 6-AN microinjection because the embryos started degenerating shortly thereafter, and died 17 h after microinjection. This protein was selected as their transport across the embryonic blood-CSF barrier had been already precisely determined [14].

20 min after 6-AN microinjection, endogenous ovalbumin, which is known to be taken from the egg reservoir and transported to the E-CSF via the blood system, was detected approximately at the same concentration in the E-CSF of both 6-AN and control embryos (Fig. 1A). 2 h after 6-AN microinjection, endogenous ovalbumin was detected at a slightly higher concentration in the E-CSF of control embryos than in the E-CSF of the 6-AN microinjected ones, although it was not statistically significant. Conversely, 15 h after 6-AN microinjection, this endogenous molecule was significantly detected at a higher concentration in the E-CSF of control embryos than in the 6-AN microinjected ones (Fig. 1A), suggesting that the

transport of proteins from the blood to the E-CSF is at least partially blocked by the use of 6-AN, and that the blockage starts to be effective between 2 to 15 h after 6-AN microinjection. A parallel dynamics was observed with respect to ovalbumin concentration in the E-serum (Fig. 1A).

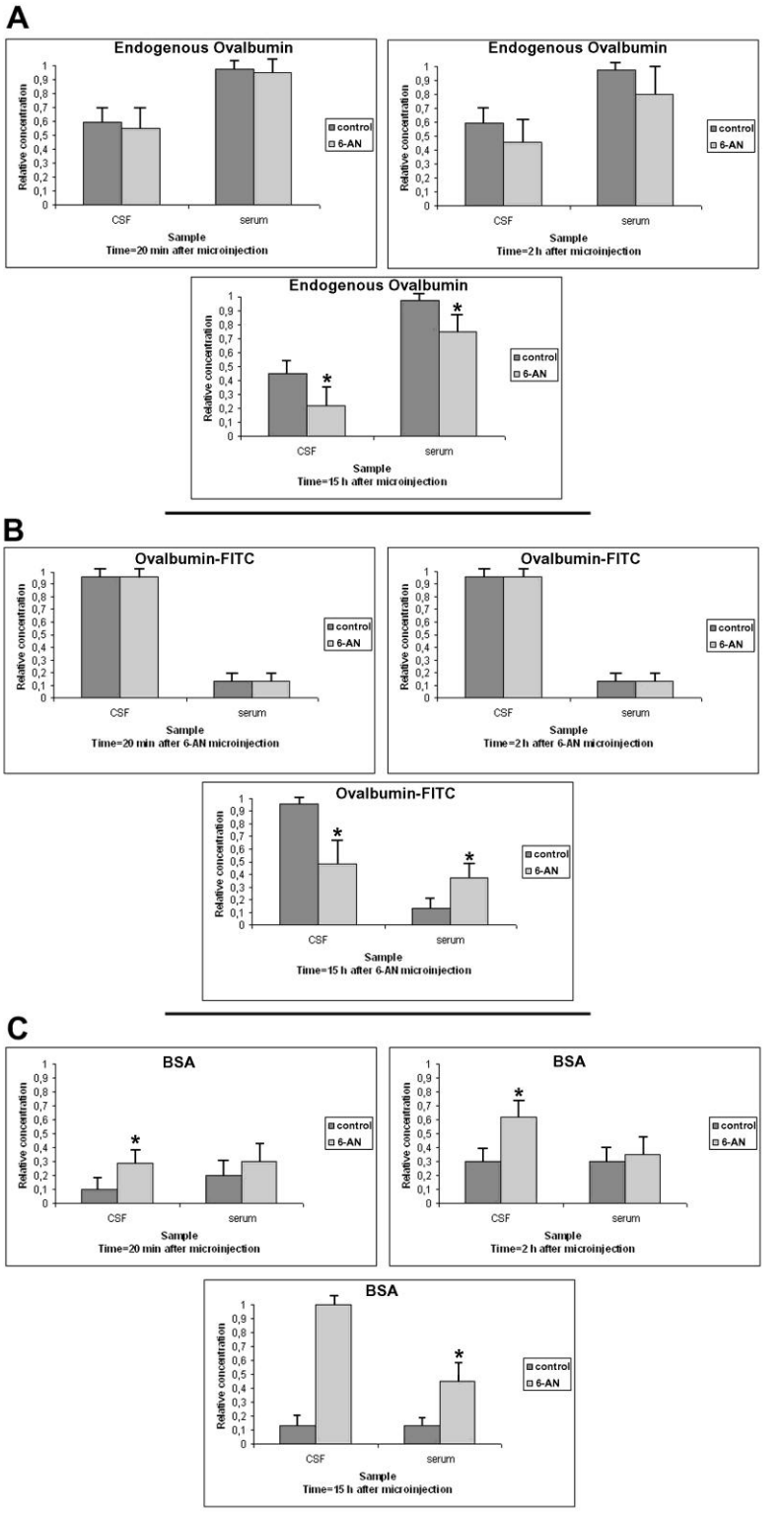

Figure 1. Bar charts showing disruption of protein transfer across the embryonic blood- CSF barrier after 6-AN microinjection into the cephalic cavities at E4. A) Endogenous ovoalbumin; B) Ovoalbumin-FITC; C) BSA. Vertical bars indicate SD, and asterisks denote values that differ significantly ($P < 0.05$) from controls according to the two-tailed Student's t-test.

To further test the extent of the disruption of protein transport across the embryonic blood-CSF barrier, we then checked the transfer dynamics of exogenous microinjected proteins after 6-AN treatment. Two different categories of proteins were used: (1) a protein that is normally present in the E-CSF and the E-serum of chick embryos at this stage of development, i.e. ovalbumin, which was previously coupled to FITC in order to distinguish it from the endogenous one; and (2) a protein that is not present in chick embryos (heterologous protein), i.e. BSA. These proteins were selected as their transport across the embryonic blood-CSF barrier had been already precisely determined [14]. In this respect, it has been reported that ovalbumin is quickly transported from the blood to the E-CSF when exogenously microinjected into the outflow of the heart, and thus it was used in this experiment as ovalbumin-FITC to monitor the blockage of protein transport from the blood to the E-CSF. Conversely, it is known BSA not to be transported from the blood to the E-CSF when microinjected into the outflow of the heart, but it is quickly remove from the E-CSF when microinjected into the brain cavity. Thus, in this experiment it was microinjected into the brain cavity to check the blockage of protein transport from the E-CSF to the E-serum.

Ovalbumin-FITC was microinjected into the outflow of the heart of 6-AN treated embryos at three different times after 6-AN microinjection: just immediately thereafter; 2h after 6-AN microinjection; and 15 h after 6-AN microinjection. In all experiments, E-CSF and E-serum were removed 20 minutes after ovalbumin-FITC microinjection. In this way, we were able to check the blockage of protein transfer after different times of 6-AN treatment. As a control, we used embryos that were microinjected with Ovoalbumin-FITC into the outflow of the heart but no-treated with 6-AN.

20 min and 2 h after 6-AN microinjection, ovalbumin-FITC was detected approximately at the same concentration in the E-CSF of both 6-AN and control embryos. However, at 15 h, the relative concentration of this molecule in the E-CSF of control embryos was 2-folds higher than in 6-AN treated embryos. Conversely, with respect to E-serum, the relative concentration of ovalbumin-FITC was 2- to 3-folds higher in the 6-AN treated embryos with respect to control ones (Fig. 1B). Taken together, these results suggest that the transport of proteins from the blood to the E-CSF is at least partially blocked by the use of 6-AN, and that the blockage starts to be effective between 2 to 15 h after 6-AN microinjection.

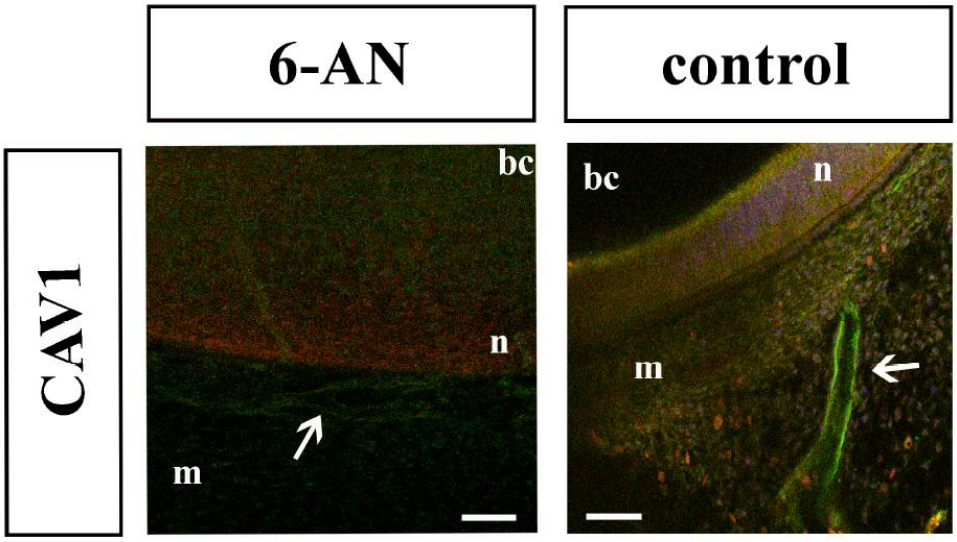

Figure 2. Immunohistochemical expression of CAV1 in microinjected chick embryos at E4 with 6-AN and control embryos (non-microinjected embryos). Merged confocal image (50 µm thick) of 200-µm vibratome sections. Note the CAV-1 immunoreactive blood vessels in control embryos (in green). Arrows point to blood vessels. Note than in 6-AN treated embryos CAV-1 expression is not detected. Cellular counterstaining is shown in red. Scale bars: 0.1 mm; Abbreviations: bc, brain cavity; m, mesechyme; n, neuroectoderm.

With respect to BSA, it was microinjected into the brain cavity of embryos to which 6-AN had been previously microinjected 20 min, 2 h and 15 h after 6-AN microinjection. As a control, we used embryos that were microinjected with BSA into the mesencephalic cavities but no-treated with 6-AN.

In this experiment, BSA removal from the cephalic cavities of 6-AN treated embryos were progressively restricted with time, from 2 to 15 h after 6-AN microinjection, as revealed by the increasingly accumulation of this molecule with respect to controls (2-folds after 20 min and 2 h, and 6- to 7-folds after 15 h). With respect to E-serum, again no significant differences between 6-AN treated embryos and controls were observed after 20 min and 2 h, but after 15 h the accumulation of BSA in the E-serum was significant (Fig. 1C). Taken together, these results also suggest that the transport of proteins from the blood to the E-CSF is at least partially blocked by the use of 6-AN, and that the blockage is increasingly effective with time.

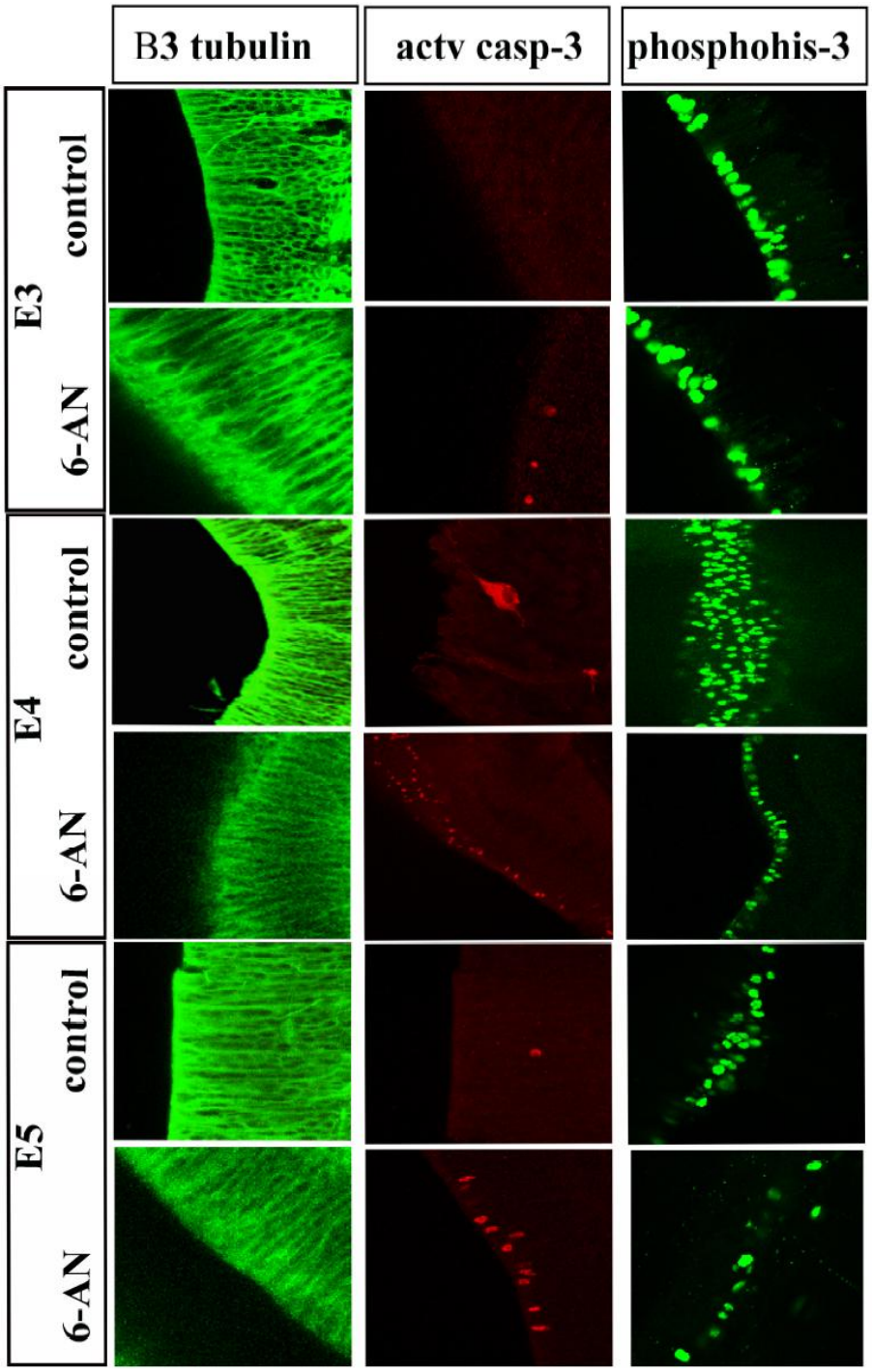

Figure 3. Neurogenesis, cell survival and cell proliferation in chick embryos at E4, E5 and E6, embryos microinjected with 6-AN and non-microinjected embryos. Beta3-tubulin immunostaining was used to evaluate neural differentiation. Active caspase-3 immunostaining was used to examine cell survival. Phosphohistone-H3 immunostaining was used to analyze cell proliferation.

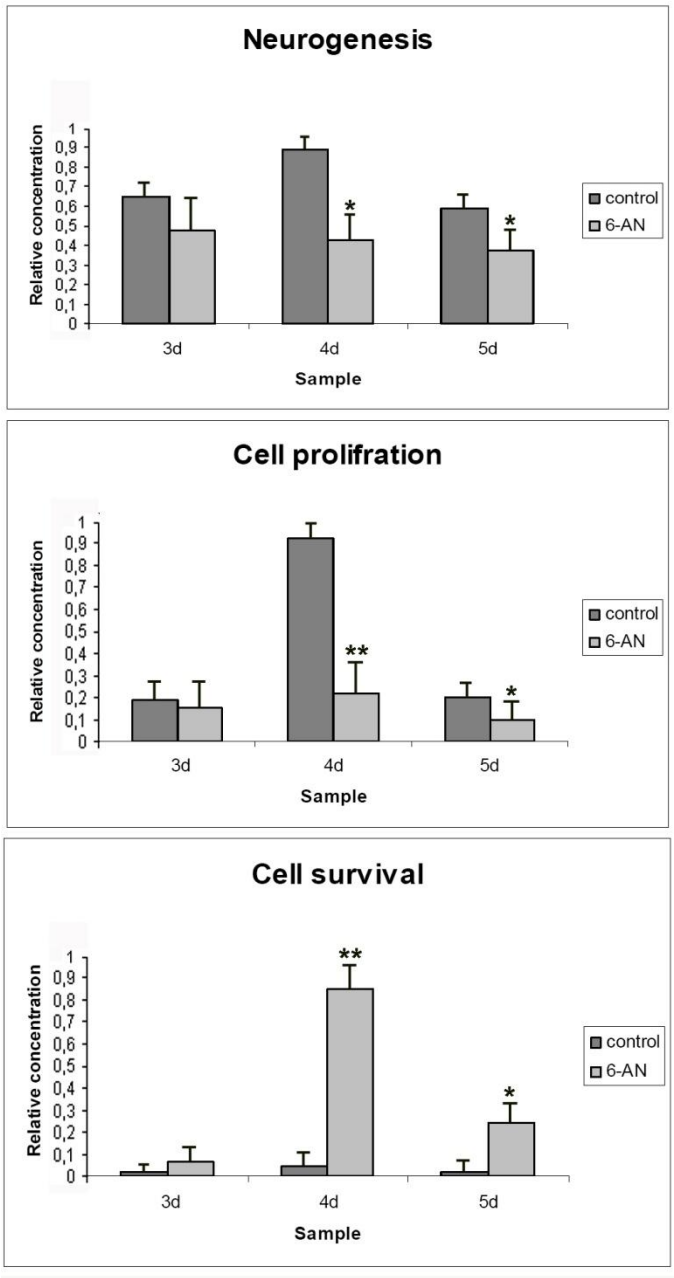

Figure 4. Plots showing quantification of the mentioned parameters of cell behavior. Results are expressed as mean 6 SD of three individual experiments per group. Vertical bars indicate SD, and asterisks denote values that differ significantly ($P < 0.05$) from controls according to the two-tailed Student's t-test.

Finally, we checked the cause of protein transport blockage. In this respect, as it had been suggested that protein transfer from the blood to E-CSF and vice versa is mediated by CAV-1 containing caveolae [16], we analysed whether protein transfer disruption induced by 6-AN treatment was due to an impairment of transcellular caveolae transport by monitoring CAV-1 expression in 6-AN treated embryos 15 h after microinjection with respect to controls. As shown in Fig. 2, CAV-1 is no longer expressed in treated embryos, as compared with controls. Taken together, these results indicate that protein transport across the blood-CSF barrier is disrupted by 6-AN treatment, and that this disruption is due, at least in part, to the interruption of transcellular caveolae transport, as detected by CAV-1 expression cease.

Disruption of protein transport affects neuroectodermal progenitor cells survival, proliferation and neurogenesis

As 6-AN can block the transport of proteins between E-serum and E-CSF and vice versa, and

molecules contained within E-CSF are involved in neuroectodermal progenitor cells survival, proliferation and neurogenesis in vitro [12], we then check whether these basic cellular parameters were also altered in 6-AN treated embryos, due to the lack of protein transport. To check this extent, chick embryos at several different developmental stages, i.e. E3 (HH20), E4 (HH23) and E5 (HH26), were microinjected with 6-AN, and examined for these basic parameters 15 h thereafter with respect to controls.

At E3, no significant differences in any of the parameters analysed were observed between 6-AN microinjected embryos and controls (Fig. 3 and 4), which is in agreement with the fact that at this developmental stage the blood-CSF barrier is still underdeveloped, as previously reported by Parvas et al. (2008) [14]. However, at E4, when this transient embryonic blood-CSF barrier is functional, cell proliferation and neurogenesis is significantly reduced in 6-AN treated embryos with respect to controls, and apoptosis is significantly increased (Figures 3 and 4). Finally, at E5 embryos, cell proliferation and neurogenesis is also significantly reduced in 6-AN treated embryos with respect to controls, and apoptosis is significantly increased, but differences are smaller than in E4 embryos.

Taken together, these results indicate that protein transport blockage is affecting the basic cellular parameters of neuroepithelial progenitor cell from the developmental stage at which the embryonic blood-CSF barrier is active, i.e. E4, but not before that, which in turn reflects the importance of this barrier for normal early brain development. In this respect, the alteration of the normal cellular basic parameters can be due to the lack of certain factors that should normally be in the E-CSF, and/or to the presence of cellular metabolites within the E-CSF, that can not be removed from this fluid and that can exert some toxic effects.

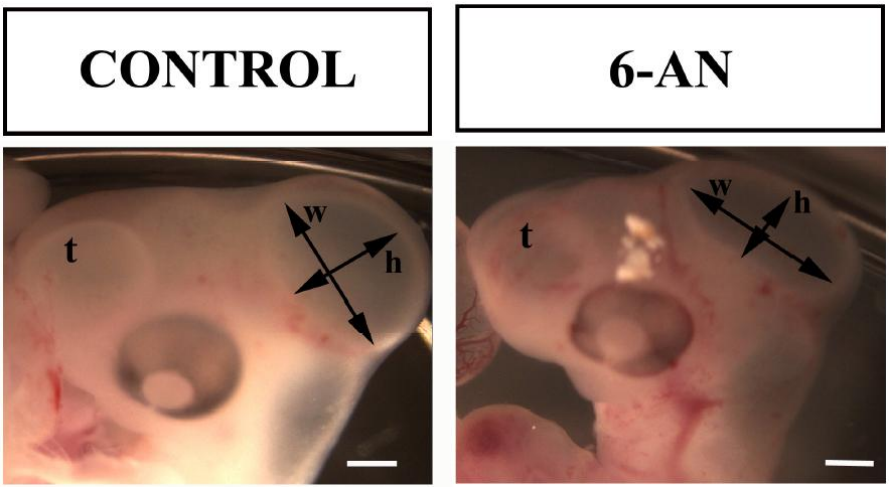

Figure 5. Comparison between an embryo treated with 6-AN to block transport across the blood–CSF barrier and a control embryo. Lines show the width and height of the mesencephalic vesicles. Also note that the telencephalic vesicles appear smaller in the treated embryo with respect to the control. Scale bar: 0.5 mm. Abbreviations: h, height of the mesencephalon; t, telencephalon; w, width of the mesencephalon.

Disruption of water transport across the embryonic blood–CSF barrier

Finally, we also checked whether the blockage of transcellular caveolae transport induced by 6-AN also affects water transport from the blood to the E-CSF, as it has been previously suggested that water uses caveolae to cross the neuroectodermal cells fulfilling blood-CSF barrier function [16]. Fifteen hours after 6-AN microinjection to E4 embryos, they were examined for the width and height of the mesencephalic cavity under the dissecting microscope (Fig. 5), as it had been reported that the increase in E-CSF volume parallels that of the brain cavities. Interestingly, mesencephalic cavities of 6-AN treated embryos were clearly smaller than those of controls. Other cavities, for example telencephalic cavities, were also smaller in treated embryos, although they were not quantified (Fig. 5).

We then studied the normal distribution of

the values. We first used the chi-square test of normality. The values obtained for the width variable were as follows: 6-AN treated embryos, chi-square = 84.64 and p-value = 9.8046 x 10-12; control embryos, chi-square = 166.72 and p-value = 0.0. The values obtained for the height variable were as follows: 6-AN treated embryos, chi-square = 83.92 and p-value = 1.33136 x 10-11; control embryos, chi-square = 137.92 and p-value = 0.0. Thus, the samples did not follow a normal distribution.

For this reason, we then used a non-parametric test to compare our variables (Mann-Whitney test; void hypothesis: both 6-AN treated embryos and controls follow the same distribution and thus they have the same mean and variance). The values obtained for the width variable were as follows: U = 603.5; p-value = 0.000008. The values obtained for the height variable were as follows: U = 211.0; p-value = 0.00. Thus, in both cases there were significant differences between 6-AN treated embryos and controls, indicating that mesencephalic expansion in 6-AN treated embryos is significantly lower than in controls.

So the disruption of the embryonic barrier produces a significant reduction in the volume of the mesencephalic vesicle, which, according to the literature, may be the consequence of a decrease in brain cavity hydrostatic pressure [11], and thus to a decrease in the water imbalance from the blood to the E-CSF. Taken together, these results indicate that the barrier function of the blood vessels and the neuroectoderm adjacent to them is not restricted to proteins, but that it also includes other molecules such as water and most possible also ions. These observations reinforce the hypothesis that these embryonic blood vessels and the neuroectoderm adjacent to them have a transient blood–CSF barrier function during early stages of brain development, before the formation of the functional choroid plexus, which appears later in development. Consequently, they are crucial for brain anlagen growth and differentiation as they modulate E-CSF composition and homeostasis.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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