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Early embryonic brain development in rats requires the trophic influence of cerebrospinal fluid

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ABSTRACT

Cerebrospinal fluid has shown itself to be an essential brain component during development. This is particularly evident at the earliest stages of development where a lot of research, performed mainly in chick embryos, supports the evidence that cerebrospinal fluid is involved in different mechanisms controlling brain growth and morphogenesis, by exerting a trophic effect on neuroepithelial precursor cells (NPC) involved in controlling the behaviour of these cells. Despite it being known that cerebrospinal fluid in mammals is directly involved in corticogenesis at fetal stages, the influence of cerebrospinal fluid on the activity of NPC at the earliest stages of brain development has not been demonstrated. Here, using "in vitro" organotypic cultures of rat embryo brain neuroepithelium in order to expose NPC to or deprive them of cerebrospinal fluid, we show that the neuroepithelium needs the trophic influence of cerebrospinal fluid to undergo normal rates of cell survival, replication and neurogenesis, suggesting that NPC are not self-sufficient to induce their normal activity. This data shows that cerebrospinal fluid is an essential component in chick and rat early brain development, suggesting that its influence could be constant in higher vertebrates.

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1. Introduction

Early brain development is a complex process which is not fully understood. During this period, the brain primordium is influenced by several factors which regulate not only the behaviour of the neuroepithelial precursor cells (NPC) but also positional information, growth and morphogenesis. In the last decades, substantial evidence has helped to support the hypothesis that embryonic cerebrospinal fluid (E-CSF) plays a key role in brain development (Gato and Desmond, 2009; Miyan et al., 2003; Lowery and Sive, 2009). Also, ventricular hydrostatic pressure created by E-CSF accumulation inside the brain ventricles has been demonstrated to be directly responsible for brain expansion and morphogenesis in chick and rat embryos (Desmond and Jacobson, 1977; Desmond, 1985; Gato et al., 1993; Alonso et al., 1998, 1999). Furthermore, E-CSF is also capable of regulating particular aspects of neuroepithelial behaviour having trophic influence on NPC promoting neurogenesis, cell survival and increasing their mitotic activity (Gato et al., 2005), as well as promoting some genetic expression pattern (Parada et al., 2005b). The complex protein composition of E-CSF both in chick and rat embryos (Dziegielewska et al., 1981; Gato et al., 2004; Parada et al., 2005a, 2006) suggests that this trophic action of E-CSF may be due to the presence of molecules with high biological activity, such as growth factors and morphogens. We have shown that the presence of the fibroblast growth factor 2 (FGF2) in chick embryo E-CSF plays a key role in the mitotic activity of the mesencephalic NPC (Martin et al., 2006), whilst others have demonstrated that hydrostatic pressure in the brain cavity regulates mitosis (Desmond et al., 2005).

However, these studies have been carried out mainly in chick embryos and, despite similarities in early brain development between birds and mammals, significant differences can be appreciated not only from the morphological and histological points of view but also in the protein composition of E-CSF, as has been evaluated by proteomic analysis (Parada et al., 2005a, 2006). These phylogenetic differences support the possibility that CSF could influence NPC growth and differentiation via different mechanisms in mammals in relation to birds.

What is more, it also has been shown that rat CSF plays a key role in cortical development during fetal stages (Miyan et al., 2006), despite nothing being known about the role of E-CSF on NPC proliferation and differentiation during the earliest stages of brain development in rat embryos. In this article, we attempt to clarify whether embryonic CSF has similar trophic properties in mammals

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as in chicks whilst maintaining the ability to induce survival, replication and neurogenesis in rat NPC. Our findings have led us to propose that the trophic effect of embryonic CSF is similar for different phyla, and can perhaps be considered a general developmental mechanism.

2. Experimental procedures

2.1. Obtaining embryonic cerebrospinal fluid

Wistar strain rats were mated overnight and examined for a sperm-positive vaginal plug the following morning, which was considered the first gestational day. 13.5-day rat embryos were explanted, dissected from the extra-embryonic membranes, and the E-CSF from the mesencephalic cavity was aspirated, as previously described (Gato et al., 2004). To minimise protein degradation, E-CSF samples were kept at 4 °C, aliquoted, lyophilised and frozen at −40 °C until used.

2.2. Organotypic cultures of mesencephalic neuroectoderm

In this study we used 12.5-day rat embryos. Explanted embryos were dissected out of the extra-embryonic membranes, the dorsal region of the mesencephalon was removed with micro-scissors, and the neuroepithelial explants were cultured as described previously (Gato et al., 2005). The explants, which include neuroectoderm and surface ectoderm, were placed on small rectangles of Millipore filters (0.8 μm pore size) in a chemically defined serum-free medium (DMEM: F12, Sigma) supplemented with 1% ascorbic acid and cultured at 37 °C with 5% CO^2 for 24 h.

Cultured explants and control embryos (developed “in utero” and explanted at the beginning and end of the culture time) were processed to monitor three parameters of neuroepithelial precursor cell behaviour: cell replication, apoptosis and neuronal differentiation (details below) in various sets of experiments. In all cases, the study was performed with histological sections taken from the central area of each explant to standardise results and to avoid damaged peripheral tissue during handling, as previously described (Gato et al., 2005). The aims and experiments were as follows:

(1) To establish the normal pattern of cell replication, apoptosis and neuronal differentiation at the beginning and end of the period analysed, control embryos were maintained *in vivo* until developmental stages 12.5 days (12 embryos, denominated initial controls) or 13.5 days (12 embryos, termed final controls) and then the mesencephalic neuroephithelium was explanted and processed.
(2) To test the developmental autonomy of the mesencephalic neuroepithelial precursor cells with respect to extra-neuroepithelial surrounding signals during the developmental stages analysed, a total of 20 neuroepithelial explants from 12.5-day embryos were cultured for 24 h in a defined (non-supplemented) culture medium.
(3) To test the direct influence of embryonic CSF on mesencephalic NPC behaviour, a total of 24 explants from 12.5-day embryos were cultured for 24 h in a defined culture medium supplemented with 15% embryonic CSF (13.5 days).
(4) To test the specificity of CSF on the behaviour of the explanted cells, a total of 20 explants from 12.5-day embryos were cultured for 24 h in a defined culture medium supplemented with 15% foetal calf serum (FCS).

2.3. BrdU and β3-tubulin determination

We evaluated cell replication by BrdU incorporation into cell nuclei, which was performed by adding BrdU to the culture medium at a final concentration of 5 μM for 1 h at the end of the organotypic culture. The controls (12.5 and 13.5 days) were explanted and cultured for only 1 h with the same concentration of BrdU. Subsequently, the explants were fixed in Carnoy for 20 min and embedded in paraffin. Sections from the explants were incubated with a monoclonal antibody to BrdU (Dako) at 1/100 for 30 min. As a secondary antibody we used an avidin–extravidin system conjugated to peroxidase (1/20 mouse anti-rabbit for 30 min and 1/20 extravidin for 10 min; Sigma), developed with DAB. A quantitative analysis of nuclear BrdU incorporation was performed by counting the number of BrdU-positive nuclei in 20 microscopic fields of 1400 μm^2, taken from the central region of each explant (to avoid lateral tissue damage by handling) and from five different explants. The average of each condition and the standard deviation were plotted, and the significance between values was tested by a one-way ANOVA analysis of variance with a 95% significance level ($P < 0.05$) and a Games–Howell test were performed as post-hoc multiple comparison test (Fig. 2).

To detect early neuronal differentiation, we monitored β3-tubulin (Tuj-1) expression. Histological sections taken from the explants were incubated with an anti-β3-tubulin monoclonal antibody at 1/500 (BAbCO) for 1 h and with an anti-mouse secondary antibody conjugated to FITC at 1/64 (Sigma) for 1 h. For visualisation and photographing of the preparations, we used a confocal microscope (ZEISS LSM-310). The density of β3-tubulin positive cells in the basal side of rat mesencephalic neuroepithelium at these stages of development does not permit individual cells to be distinguished; consequently, to make a quantitative analysis of neurogenesis we measured the β3-tubulin positive area in 20 images per experimental condition (taken at the same magnification from four different explants) with a GSA image analyser program. The average of each condition and the standard deviation were plotted, and the significance between values was tested by a one-way ANOVA analysis of variance with a 95% significance level ($P < 0.05$) and a Bonferroni test were performed as post-hoc multiple comparison test (Fig. 3).

2.4. TUNEL assay

Apoptotic cells were detected by the TUNEL assay on paraffin sections from formalin fixed cultured explants. Apoptotic cells were detected using the Apoptosis Detection System Fluorescein Kit (Promega) following the manufacturer's instructions. Visualisation was by means of a confocal microscope (ZEISS LSM-310). A quantitative analysis of apoptotic cells was performed by counting the number of labelled nuclei in 20 microscopic fields of 1900 μm^2, taken from the central area of each explant and from four different explants. The average of each condition and the standard deviation were plotted, and the significance between values was tested by a one-way ANOVA analysis of variance with a 95% significance level ($P < 0.05$) and a Bonferroni test were performed as post-hoc multiple comparison test (Fig. 4).

3. Results

Explanted neuroepithelium cultured 24 h “in vitro” with non-supplemented media showed a histological appearance similar to that of the final controls, as is shown in Fig. 1E and I. The histological structure of the neuroepithelium after culture was well preserved and unchanged, including apico-basal polarization of the cells. However, other cellular behaviour parameters such as replication, neurogenesis and apoptosis showed significant changes directly related to the experimental conditions. These data, together with data previously described by us (Gato et al., 2005) for chick neuroepithelium cultures, support our experimental approach with neuroepithelial organotypic cultures to test the influence of embryonic CSF.

3.1. E-CSF induces neuroepithelial cell replication

Neuroepithelium is a high mitotic rate tissue in which many NPC are involved in replication at the same time (Takahashi et al., 1995). BrdU incorporation into DNA has become a common tool to measure cellular replication in development. In the final control rat embryos, with 1 h BrdU pulse, many NPC incorporated this molecule into their nucleus, and the number of labelled nuclei increased dramatically (59%) between 12.5 days (initial control) and 13.5 days (final control) of development (Figs. 1B and F and 2) showing that cellular replication is a very active process in mesencephalic neuroepithelium at these stages of brain development. The BrdU labelled nuclei are located specifically in the basal 2/3 of the neuroepithelium and just starting their nuclear interkinetic migration. As shown in Fig. 2, the differences in BrdU incorporation between experimental conditions (final cont., medium, E-CSF and serum) were significant by an ANOVA analysis and a Games–Howell comparison test.

As shown in Figs. 1J and 2, the number of BrdU labelled nuclei in 24-h explants cultured in a chemically defined medium decreased 52% with respect to the final controls (Figs. 1F and 2), which showed normal BrdU incorporation into the mesencephalic neuroepithelium at the end of culture. The images reveal a restriction of the labelled nuclei to the middle third of the neuroepithelium (Fig. 1J). Both findings suggest that after a couple of hours E-CSF deprivation is influential in this large decrease in DNA synthesis. However, in a defined medium there remained in the explants, following 24 h of culture, a 48% BrdU-positive nucleus, probably the result of inertial mitotic behaviour of NPC in short time cultures (cells induced to replicate prior to the start of culture).

It is very important to note that in explants cultured with 15% E-CSF, the number of BrdU labelled nuclei (Figs. 1N and 2) attained a slightly, but significant, lower value (13%) than that of the final controls (Figs. 1F and 2). However, these same explants exhibited a (45%) increase in labelling compared with explants cultured with a defined medium (Figs. 1J and 2). In E-CSF cultured explants, no

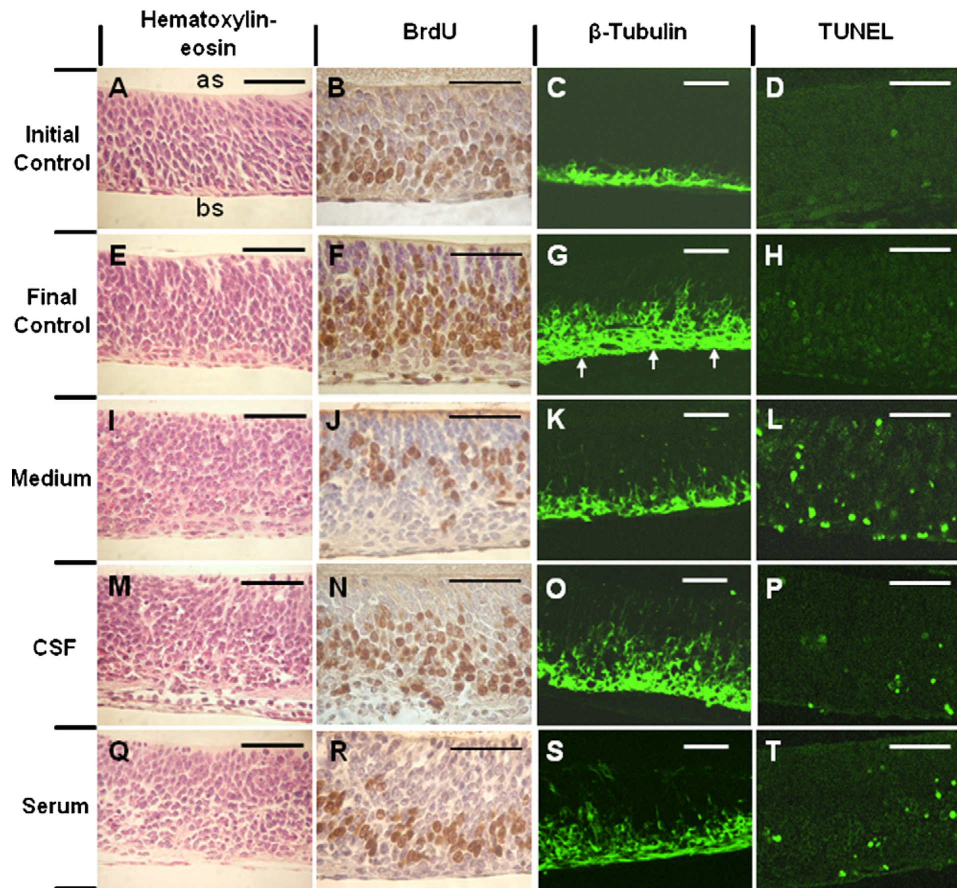


Fig. 1. (A, E, I, M and Q) Haematoxylin–eosin stained sections of mesencephalic neuroepithelium from rat embryos at 12.5-day developmental stage (initial controls) (A) and at 13.5-day developmental stage (final controls) (E). Haematoxylin–eosin stained sections of mesencephalic neuroepithelial explants after 24 h of organotypic culture (equivalent to 13.5 days of development) in various experimental conditions: (I) explants cultured with a chemically defined medium (no supplement); (M) explants cultured with a CSF-supplemented medium and (Q) explants cultured with an FCS-supplemented medium. (B, F, J, N and R) Cell proliferation analysis by BrdU incorporation into mesencephalic neuroepithelium from rat embryos at 12.5-day developmental stage (initial controls) (B) and at 13.5-day developmental stage (final controls) (F). Cell proliferation analysis, by BrdU incorporation on mesencephalic neuroepithelium, after 24 h of organotypic culture (equivalent to 13.5 days of development) in various experimental conditions: (J) explants cultured with a chemically defined medium (no supplement); (N) explants cultured with a CSF-supplemented medium and (R) explants cultured with an FCS-supplemented medium. (C, G, K, O and S) Neural differentiation monitored by β3-tubulin immunostaining of mesencephalic neuroepithelium from rat embryos at 12.5-day developmental stage (initial controls) (C) and at 13.5-day developmental stage (final controls) (G). Neural differentiation monitored by β3-tubulin immunostaining on mesencephalic neuroepithelium after 24 h of organotypic culture (equivalent to 13.5 days of development) in various experimental conditions: (K) explants cultured with a chemically defined medium (no supplement); (O) explants cultured with a CSF-supplemented medium and (S) explants cultured with an FCS-supplemented medium. Arrows in G: marginal zone. (D, H, L, P and T) Apoptosis analysis by TUNEL assay on mesencephalic neuroepithelium from rat embryos at 12.5-day developmental stage (initial controls) (D) and at 13.5-day developmental stage (final controls) (H). Apoptosis analysis by TUNEL assay of neuroepithelial explants after 24 h of organotypic culture (equivalent to 13.5 days of development) in various experimental conditions: (L) explants cultured with a chemically defined medium (no supplement); (P) explants cultured with a CSF-supplemented medium and (T) explants cultured with an FCS-supplemented medium. as: apical side, bs: basal side (all images in the same position). Scale bar in all images: 100 μm.

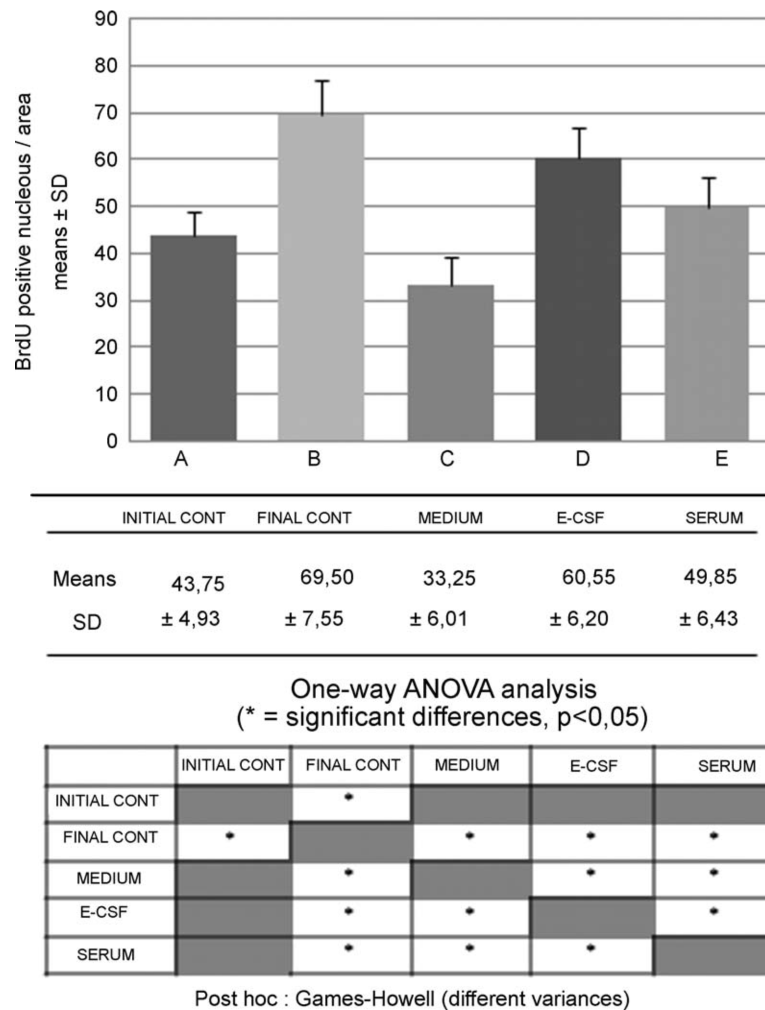
changes in the distribution of the labelled nucleus could be appreciated (compare Fig. 1N and F). Considering the mitotic level reached by the NPC cultured with only 15% E-CSF in the culture medium (in live embryos neuroepithelial cells are in contact with 100% E-CSF), these results suggest that rat E-CSF has high mitogenic properties.

As shown in Figs. 1R and 2, the explants cultured with 15% foetal calf serum supplemented medium exhibited a decrease of 28% in BrdU labelled nuclei with respect to the final controls (whereas the difference between CSF and Serum treated explants was significant as is sowed in Fig. 2), which suggests that foetal calf serum at the concentration used is not able to induce the same mitogenic activity as the E-CSF in neuroepithelial cultured explants, and supports the theory that E-CSF has specific mitogenic properties.

3.2. E-CSF induces neurogenesis

The mesencephalic neuroepithelium of 12.5-day rat embryos (initial controls) showed the onset of neurogenesis in brain development; this is seen by the presence of a layer of cells with β3-tubulin positive cytoplasm near the basal side of the neuroepithelium. This layer had just 2–3 layers of nuclei and few cellular extensions (Figs. 1C and 3). Only 24 h later (final controls), the β3-tubulin positive cell layer increased dramatically (a 113% increase in the labelled area) in the ‘‘in vivo’’ mesencephalic neuroepithelium (Figs. 1G and 3). Now the labelled area appeared thicker, with more than 5–6 nuclear levels and many cytoplasmic extensions into the depth of the neuroepithelium. Furthermore, the immunocytochemical images reveal a differentiated layer behind the basal membrane with a small number of nuclei, suggesting the beginning of the marginal zone. These data show clearly that, at this time of development, neurogenesis is a very active process in the mesencephalic neuroepithelium, and that this period is, consequently, most appropriate for studying embryonic neuronal differentiation.

As is show in Fig. 3, the β3-tubulin differences between all experimental conditions, was significant by an ANOVA analysis



	INITIAL CONT	FINAL CONT	MEDIUM	E-CSF	SERUM
Means	43,75	69,50	33,25	60,55	49,85
SD	± 4,93	± 7,55	± 6,01	± 6,20	± 6,43

	INITIAL CONT	FINAL CONT	MEDIUM	E-CSF	SERUM
INITIAL CONT		*			
FINAL CONT	*		*	*	*
MEDIUM		*		*	*
E-CSF		*	*		*
SERUM		*	*	*	

Fig. 2. Quantitative analysis of neuroepithelial cells synthesizing DNA measured by the number of BrdU-positive nuclei. Values plotted in the chart show the mean of the BrdU-positive nucleus per area ± the standard deviation. (A) Mesencephalic neuroepithelium of 12.5-day rat embryos (initial controls). (B) Mesencephalic neuroepithelium of 13.5-day rat embryos (final controls). (C) Mesencephalic neuroepithelium explants cultured with a chemically defined medium (no supplement); (D) explants cultured with a CSF-supplemented medium, and (E) explants cultured with an FCS-supplemented medium. *Values that differ significantly ($P < 0.05$), according to the ANOVA analysis and Games–Howell post-hoc test.

and a Bonferroni comparison test. Figs. 1K and 3, show that the neuroepithelial explants cultured in a defined medium exhibited a significant decrease (45.6%) in β3-tubulin labelling with respect to the final controls, and had just 2–3 nuclear layers as well as fewer cellular extensions.

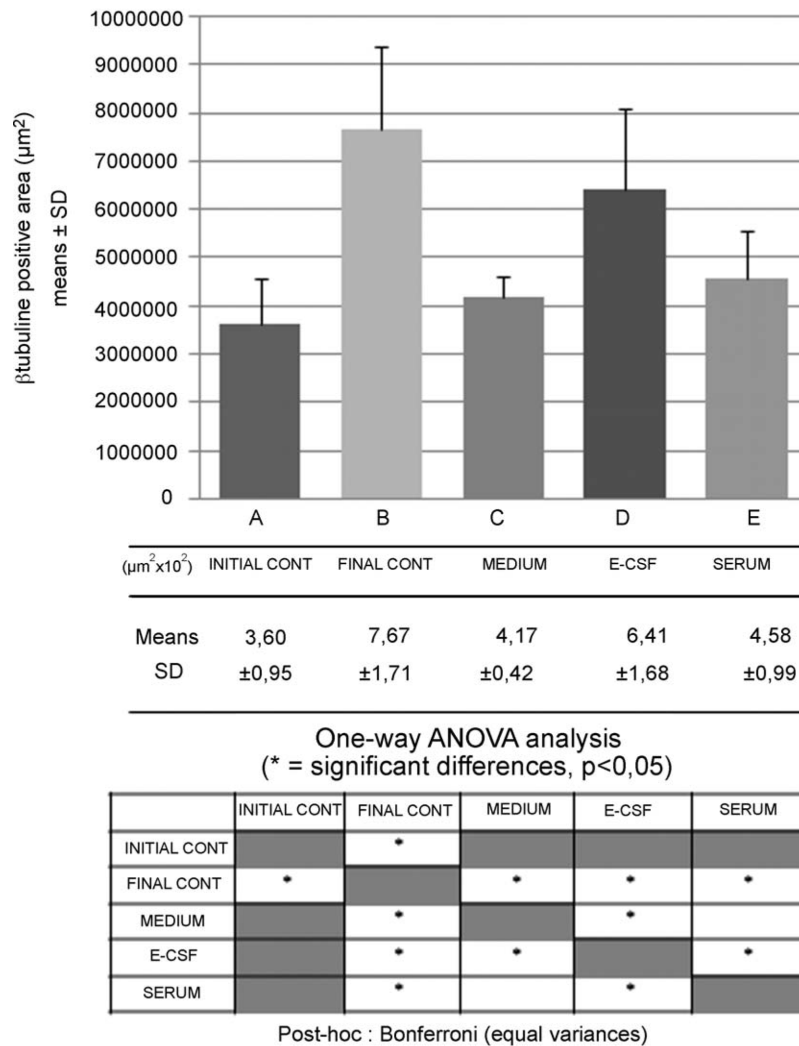
Moreover, the addition of 15% E-CSF to the culture medium (Figs. 1O and 3) induced a similar pattern of neurogenesis to that found in the final controls, with the same appearance in the number of nuclear layers and cellular extensions, although the marginal layer did not seem to be developed and there was a moderate decrease (16%) in the β3-tubulin labelled area compared with the final controls.

The explants cultured with 15% foetal calf serum (Figs. 1S and 3) showed a decrease of 40.3% in their β3-tubulin labelled area with respect to the final controls, with a large reduction in the number of nuclear layers whilst several cellular extensions could be appreciated. These explants also showed a significant reduction in their β3-tubulin labelled area with respect to the E-CSF treated (Fig. 3). Considering that stimulation by E-CSF in our experiments was performed with 15% E-CSF, in contrast to the ''in vivo'' controls in contact with 100% E-CSF, our results are highly suggestive of E-CSF also being a powerful inducer of neurogenesis in NPC.

3.3. The absence of E-CSF induces an apoptotic increase in neuroepithelial cells

The study of apoptotic cells detected by TUNEL reveals that both the initial and final controls exhibited a very small number of apoptotic nuclei per section (Figs. 1D and H 4), with a mean of two labelled nuclei which were not significant. As is show in Fig. 4, the TUNEL data differences between experimental conditions were significant by an ANOVA analysis and a Bonferroni comparison test, except between initial and final controls and CSF and Serum.

Compared with the final controls, the number of apoptotic nuclei in explants cultured in a defined medium increased significantly (Figs. 1L and 4), with a mean of 36.7 labelled nuclei distributed uniformly throughout the neuroepithelium. However, the histological sections stained with haematoxylin–eosin (Fig. 1Q) did not show signs of neuroepithelial degeneration. In the explants cultured with a medium supplemented with 15% E-CSF (Fig. 1P), the number of apoptotic nuclei per section did increase with respect to the final controls, reaching means of 14.4 immunoreactive nuclei per section (Fig. 4). However, this increase was significantly less than that of the number of labelled nuclei in explants in a non-supplemented medium (Figs. 1L and 4). Finally, the explants cultured with a 15% foetal calf serum supplemented



(μm²x10²)	INITIAL CONT	FINAL CONT	MEDIUM	E-CSF	SERUM
Means	3,60	7,67	4,17	6,41	4,58
SD	±0,95	±1,71	±0,42	±1,68	±0,99

One-way ANOVA analysis
(* = significant differences, p<0,05)

	INITIAL CONT	FINAL CONT	MEDIUM	E-CSF	SERUM
INITIAL CONT		*			
FINAL CONT	*		*	*	*
MEDIUM		*		*	
E-CSF		*	*		*
SERUM		*		*	

Post-hoc : Bonferroni (equal variances)

Fig. 3. Quantitative analysis of neuroepithelial cells undergoing neuronal differentiation measured by β3-tubulin-positive expression. Values plotted in the chart show the mean of the β3-tubulin-positive surface per area ± the standard deviation. (A) Mesencephalic neuroepithelium of 12.5-day rat embryos (initial controls). (B) Mesencephalic neuroepithelium of 13.5-day rat embryos (final controls). (C) Mesencephalic neuroepithelium explants cultured with a chemically defined medium (no supplement); (D) explants cultured with a CSF-supplemented medium, and (E) Explants cultured with an FCS-supplemented medium. *Values that differ significantly ($P < 0.05$), according to the ANOVA analysis and Bonferroni post-hoc test.

medium (Figs. 1T and 4), exhibited more apoptotic cells compared with the explants cultured with an E-CSF-supplemented medium, but the difference was not significant (Fig. 4).

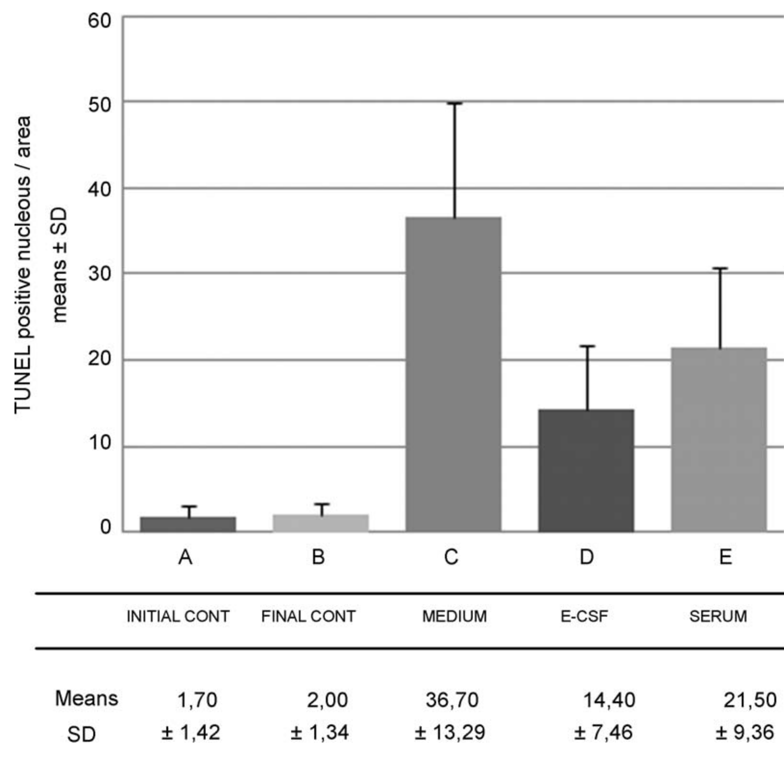
The fact that all the explants cultured *in vitro* revealed significantly more apoptosis than the *in vivo* controls, may be due to an effect of the culture technique, however, the difference between E-CSF treated explants with respect to the non-supplemented medium ones, suggests that factors within the E-CSF prevent cell death within the neuroepithelium *in vivo*. These results suggest that E-CSF induces neuroepithelial cell survival, lending weight to our hypothesis that the E-CSF trophic effect is necessary for different aspects of neuroepithelial cell behaviour.

4. Discussion

4.1. CSF exerts a trophic influence on the behaviour of NPC

The results reported here, together with our previous research on chick embryos (Gato et al., 2005; Parada et al., 2005b), strongly support our theory that the neuroepithelium is not self-sufficient in activating its normal cellular behaviour. The neuroepithelium works in an interdependent and co-operative way with the CSF as a functional entity during early brain development (Gato and Desmond, 2009).

Our results clearly show that E-CSF exerts a trophic influence on the behaviour of NPC in rat embryos, contributing to support the hypothesis that the behaviour of NPC, during embryogenesis as well as in adulthood, has a direct dependence on the content of the brain cavities, i.e., cerebrospinal fluid. The influence of CSF on neural precursor cells in the adult brain (which could be analogous to NPC during development), has been described in rodents in the subventricular zone, where a direct contact has been shown between NPC and the CSF via cellular projections through the ependymal layer. These projections could act as receptors of biological signals. The presence of cilia on certain subependymal cells has also been described in adult rodents, and this could serve as an area for signals for replication or differentiation from the CSF (Götz and Stricker, 2006; Ninkovic and Götz, 2007). Other examples of the influence of the CSF upon the behaviour of adult brain neural precursor cells have been established by Sawamoto et al., 2006, in which they demonstrated in adult mice that the oriented migration of neuronal precursor cells from the subventricular zone towards the rostral migratory stream depends on a



One-way ANOVA analysis
(* = significant differences, p<0,05)

	INITIAL CONT	FINAL CONT	MEDIUM	E-CSF	SERUM
INITIAL CONT					
FINAL CONT			*	*	*
MEDIUM		*		*	*
E-CSF		*	*		
SERUM		*	*		

Post-hoc : Bonferroni (equal variances)

Fig. 4. Quantitative analysis of neuroepithelial cells undergoing apoptosis measured by TUNEL technique. Values plotted in the chart show the mean of the apoptotic nucleus per area $\pm$ the standard deviation. (A) Mesencephalic neuroepithelium of 12.5-day rat embryos (initial controls). (B) Mesencephalic neuroepithelium of 13.5-day rat embryos (final controls). (C) Mesencephalic neuroepithelium explants cultured with a chemically defined medium (no supplement); (D) explants cultured with a CSF-supplemented medium, and (E) explants cultured with an FCS-supplemented medium. *Values that differ significantly ($P < 0.05$), according to the ANOVA analysis and Bonferroni post-hoc test.

gradient of chemorepulsive molecules in the CSF, created by the beating of cilia in lateral brain ventricles. Furthermore, changes in CSF composition have been related with certain neurodegenerative diseases, suggesting that the CSF is involved with normal and abnormal brain behaviour (Parada et al., 2007).

A very interesting theory has been proposed by Miyan et al. (2006), namely, that the relation between the CSF and neuronal precursor cells might be different in each ventricle and also in the subarachnoid space, especially in corticogenesis. Their theory is based on experiments that analysed the behaviour of neuronal precursor cells during foetal brain development in rat embryos.

The experiments we report here using explants of rat neuroepithelium, together with our similar findings using neuroepithelial explants from chick embryos, clearly show the dependence of the neuroepithelium on trophic factors in the CSF (Gato et al., 2005; Parada et al., 2005b). Others have also found that the neuroepithelium requires factors from the CSF for proliferation and differentiation (Vaccarino et al., 1999a,b; Panchision and McKay, 2002; Dono, 2003). All of these results support the theory that, at the earliest stages of development, the brain neuroepithelium is not self-sufficient, but rather that it is totally dependent on the CSF to both differentiate and attain normal cellular replication rates. Consequently, we conclude that CSF is a major regulator of neural precursor cells behaviour throughout the entire lifetime of the brain, and that its role needs to be taken into account for a real understanding of the biology of these cells.

4.2. CSF might be involved in the regulation of neural replication, neurogenesis and cell survival by different mechanisms

Our results help to demonstrate that CSF plays relevant roles in early brain development in different species of higher vertebrates. Today we have sufficient experimental evidence to support the idea that the CSF influences early brain development on the basis of its complex biological composition (Gato et al., 2004; Parada et al., 2005b, 2006, 2007; Gato and Desmond, 2009). The presence of osmotically active components in CSF such as proteoglycans and ions, appears to generate the expansive force involved in early brain growth and morphogenesis (Desmond and Jacobson, 1977; Gato et al., 1993; Alonso et al., 1998, 1999; Lowery and Sive, 2005).

This hydrostatic pressure exerted by CSF has also been shown to control the replication of NPC (Desmond et al., 2005). Both of these mechanisms highlight the influence of physical mechanisms on early brain growth.

Additionally, there is much research data about how specific components of the CSF are able to control different aspects of the behaviour of NPC. Our results here show that the CSF is involved in controlling the mitotic activity of NPC during embryonic brain development in rats. Others have shown that the CSF exerts a clear influence on neural precursor replication during rat corticogenesis during foetal development (Mashayekhi et al., 2002; Owen-Lynch et al., 2003; Miyan et al., 2006; Salehi and Mashayekhi, 2006). The mechanism by which CSF exerts this control in the early brain development of rodents is still unexplained. However, a considerable amount of research suggests that the control of neuronal replication is via FGF2 (Vaccarino et al., 1999a,b; Ford-Perriss et al., 2001; Raballo et al., 2000; Panchision and McKay, 2002; Dono, 2003; Maric et al., 2007). At the earliest stages of brain development in chicks, the neuroepithelium develops a selective transport mechanism of CSF molecules through the neuroepithelial wall (Marzesco et al., 2005; Bachy et al., 2008; Parvas et al., 2008; Johansson et al., 2008) including FGF2, and these are selectively accumulated in CSF and are actively involved in regulating the mitotic behaviour of brain neuroepithelial cells in chicks (Martin et al., 2006). Moreover, it has been demonstrated that FGF2 receptors (FGFR1 and FGFR3) are present on the NPC in chicks (Walshe and Mason, 2000).

Many have demonstrated the expression in rodents of FGF2 and EGF mRNA and their receptors in the brain neuroepithelium during development, suggesting an autocrine–paracrine autoinduction of mitotic behaviour (Ozawa et al., 1996; Panchision and McKay, 2002; Dono, 2003; Trokovic et al., 2003, 2005; Blak et al., 2005; Plata-Salamán, 1991; Wong and Guillaud, 2004). This may well be true, although our results show that the NPC mitotic behaviour is not self-sufficient but requires stimulation from some molecule/s in the CSF. Consequently, work is in progress in our laboratory to try to detect mitogenic growth factors in the CSF during brain development in rats.

Our experiments also show that CSF promotes neurogenesis from NPC. We cannot rule out the possibility that some neurogenesis might result from the mitotic effect of CSF, which induces NPC to attain their "terminal mitosis". However, we have previously suggested that specific neurogenic factors are present in CSF, such as retinoic acid (Martin et al., 2006). Retinoic acid has been shown to be a powerful neurogenic inducer in adult brain NPC (McCaffery et al., 2006; Jacobs et al., 2006; Molotkova et al., 2007; Rawson and LaMantia, 2007; Romand et al., 2008; Bonnet et al., 2008). The presence of retinol (the precursor of retinoic acid) and that of the retinol binding protein (the transporter) as well as the RALDHs (the converter enzymes), have been detected in chick and rat CSF and neuroepithelium, respectively (Parada et al., 2005a, 2006, 2008a,b). These findings suggest the involvement of retinoic acid synthesis in the CSF and its subsequent role as a regulator of neuroepithelial neurogenesis. Work is in progress in our laboratory to further elucidate this hypothesis.

Finally, we show that CSF brings about the survival induction of NPC during early brain development in rats. A similar effect has been described in chicks by us (Gato et al., 2005) at early embryonic stages, and by Salehi and Mashayekhi (2006) in rat foetal corticogenesis. In chick brain development, it has been suggested that certain survival factors are present which remain unknown and require further research (Martin et al., 2006); however, we cannot rule out the possibility of an indirect effect by the influence of CSF on mitotic behaviour or differentiation of NPC. The results reported here support the theory that, in higher vertebrates including mammals, CSF exerts a critical trophic influence on brain NPC during development.

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