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FGF2/EGF contributes to brain neuroepithelial precursor proliferation and neurogenesis in rat embryos: the involvement of embryonic cerebrospinal fluid

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Abstract

Background: At the earliest stages of brain development, the neuroepithelium works as an interdependent functional entity together with cerebrospinal fluid, which plays a key regulatory role in neuroepithelial cell survival, replication and neurogenesis; however, the underlying mechanism remains unknown in mammals.

Results: We show the presence of fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF), in 13.5-day rat embryo cerebrospinal fluid (eCSF). Immunohistochemical detection of FGF2 expression localized this factor inside neuroepithelial precursors close to the neuroepithelial-CSF interface, suggesting that FGF2 from eCSF could originate in the neuroepithelium by apical secretion. The colocalization of FGFR1 and FGF2 in some neuroepithelial cells close to the ventricular surface suggests they are target cells for eCSF FGF2. Brain neuroepithelium EGF expression was negative. By using a neuroepithelial organotypic culture, we demonstrate that FGF2 and EGF from eCSF plays a specific role in triggering the self-renewal and are involved in neurogenetic induction of mesencephalic neuroepithelial precursor cells during rat development.

Conclusions: We propose eCSF as an intercommunication medium for neuroepithelial precursor behavior control during early rat brain development, and the neuroepithelial regulation of FGF2 and EGF presence in eCSF, as a regulative mechanism controlling precursor proliferation and neurogenesis.

KEYWORDS

EGF, FGF2, mitogens, neural precursors, neurogenesis, rat embryo

1 | INTRODUCTION

Following neurulation, the developmental brain undergoes a process termed "brain growth and morphogenesis",[1] involving two concurrent processes. First, the anterior end of the neural tube, the brain primordium, undergoes a large increase in volume, establishing a physical difference between the encephalic and spinal cord regions.[2] At the same time, because of narrowing and swelling regions in the neuroepithelial wall, the primary and secondary brain vesicles are established as an early regional patterning in the encephalon.[1]

Brain growth at this particular time has been described as "dramatic",[3] and it has been proposed that this is due to two mechanisms: firstly, positive pressure inside the brain cavity, which exerts a pushing force on the brain primordium neuroepithelial wall, inducing a rapid increase in brain volume.[1,2,4] The underlying mechanism has been attributed to apical secretion by brain neuroepithelial cells of osmotically active molecules such as chondroitin sulfate into the embryonic cerebrospinal fluid (eCSF) confined in the brain anlage cavity. This osmotic force regulates volumetric growth during the early stages of brain development.[5-7] In biological systems, it has been suggested, by means of mathematical models, that an intracavitary pressure is involved in the development of cyst structures.[8] In addition, the positive pressure exerted by eCSF on the neuroepithelial wall seems to play a role in triggering the replication of neuroepithelial cells, by pressure strength receptors known as focal adhesion kinases.[4,9]

Secondly and simultaneously, a very intense replication activity takes place in the brain neuroepithelial wall; it has been suggested that this is induced by the trophic effect exerted by eCSF on neuroepithelial cells.[10,11] There was also a previous description of cell surface receptors activated by mitogenic growth factors that were related with intense cellular replication in neuroepithelial cells.[12] Several growth factors have been proposed, such as neuroepithelial mitogens[13-16]; yet a major role has been attributed to fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF), whose mitogenic activity has been clearly established.[15,17-21] However, neither the origin of these diffusible factors nor their means of reaching the target cells are fully understood. In this regard, there have been reports of growth factors present in CSF influencing proliferation and neurogenesis in mammalian neuroepithelial precursors such as IGF2,[16] LIF2,[22] and Shh,[23,24] among others.

Despite some research[12,25] describing an autocrine-paracrine activity of FGF2 inside the neuroepithelium, other authors[16,26] propose the cerebrospinal fluid confined inside the brain anlage as a diffusible means of distributing and regulating the biological activity of trophic factors including FGF2.

In chick embryos, the presence has been reported of extra-neuroepithelial synthesized FGF2 in eCSF as a mechanism for mitotic control of neuroepithelial cells.[26] However, in mammals, the neuroepithelium itself is responsible for synthesizing and secreting FGF2 mRNA and protein, which suggests a self-regulation of mitogenic inductors in the embryonic brain by autocrine-paracrine regulation.[12,25,27] In addition, EGF brain anlage synthesis has been described in fetal stages of brain development.[28]

To date, however, the presence of these mitogenic growth factors has not been documented in mammal's eCSF at the earliest stages of brain development, despite the importance of understanding how eCSF exerts its trophic properties as a medium for neurepithelial cell intercommunication involved in brain growth and development.[11] Here, we explore the presence of FGF2 and EGF in the eCSF of rat embryos at the earliest stages of development, and report data supporting their neuroepithelial origin and involvement in the proliferative and neurogenic behavior of brain neuroepithelial cells.

2 | RESULTS

2.1 | A 13.5-day rat embryo eCSF contains FGF2 and EGF

The presence of the FGF2 growth factor in 13.5-day rat embryo eCSF was verified by western-blot. However, we used a mini-protean device from BioRad and we evaluate the molecular weight in reference to broad range standard, as a result of which the molecular weight described is approximate. Given the small amount of eCSF obtained from each embryo, we chose the 13.5-day stage instead of 12.5 days to acquire a sufficient amount of eCSF for western-blot analysis and explant cultures. A total of 20 μL of 13.5-day eCSF was electrophoresed in acrylamide gels and then transferred to a nitrocellulose membrane for immunological detection with an anti-FGF2 monoclonal antibody. As is shown in Figure 1, we detected three different bands, which were reactive to the anti-FGF2 antibody, a double band of 65.2 kDa, a more intense one with 23.4 kDa and, finally, a low molecular weight band ranging between 14 and 7.5 kDa.

We also performed a western blot analysis in the same conditions with anti-EGF antibody (Figure 1); this shows the presence of two faint bands of 62.1 and 24.1 kDa in 13.5-day rat embryo eCSF.

2.2 | FGF2 immunoexpression pattern in mesencephalic neuroepithelium

In order to clarify the origin of the FGF2 detected in rat embryo eCSF, we performed an immunohistochemical detection of this growth factor in histological sections from the mesencephalic neuroepithelium of 12.5-day rat embryos, coinciding with the age of the mesencephalic experimental explants at the beginning of the culture, as described below. Sections immunolabeled with anti-FGF2 antibody reveal (Figure 2A-C) a specific pattern of FGF2 protein expression in mesencephalic neuroepithelial cells,

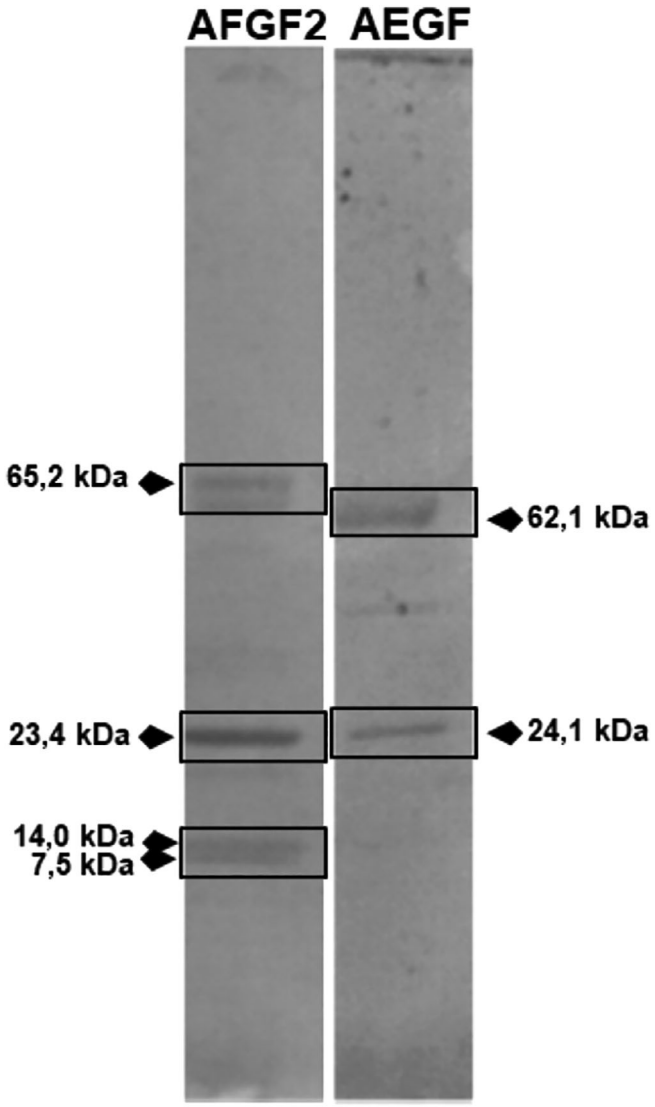

FIGURE 1 FGF2 and EGF detection in 13.5-day rat embryo eCSF by SDS-PAGE and western blot analysis. The antibody used is indicated at the top of the image. The image shows the presence of three FGF2 immunoreactive bands whose molecular weight is indicated on the left side of the image. Only two faint EGF immunoreactive bands were detected, whose molecular weights are indicated on the right side of the image. SDS, sodium dodecil sulphate

showing a cytoplasmic FGF2 presence in several of these; this seems to be restricted to their apical end in contact with the brain cavity. We can occasionally see (Figure 2C) an intense FGF2 positive material, which seems to be layered on the apical surface of the neuroepithelium. High magnification images with TOTO-3 Iodide nuclear staining (Figure 2E,F) reveals the existence of two well-defined FGF2 positive types of neuroepithelial cells close to the ventricular surface. There are cells, which express intense granular/vesicular FGF2 positive labeling with clear cytoplasmic location and others with faint FGF2 positive labeling at the nuclear level. To ensure the apical location of these FGF2 positive cells, we used correlative histological sections stained with hematoxylin-eosin (Figure 2D), detecting in the same location the neuroepithelial mitotic figures, a morphological marker of apical polarization.

In order to detect the ability of neuroepithelial cells to interact with eCSF FGF2, we conducted an immunohistochemical study with FGFR1 antibody, which has been claimed to be responsible for FGF2 mitotic activity in many biological systems. As is shown in Figure 2G, some mesencephalic neuroepithelial cells show positive immunolabeling, and these are mainly located on the apical side of the neuroepithelium in direct contact with the eCSF. Double immunostaining with FGF2 and FGFR1 antibody (Figure 2H) shows the colocalization of FGF2 and FGFR1 in specific neuroepithelial cells close to the ventricular surface together with cell which expresses only FGF2 (Figure 2I,J). These data suggest a functional relation of some neuroepithelial cells with eCSF FGF2, and are also compatible with the existence of an autocrine-paracrine mechanism previously described.[25]

As we show in Figure 2K,L), the mesencephalic neuroepithelium of 12.5-day rat embryos does not show reactivity to the EGF despite we use as positive control, adult rat skin which show EGF immunoreactivity.

2.3 | In vitro culture of neuroepithelial explants

In order to test the influence of FGF2 and EGF detected in 13.5-day rat embryo eCSF on basic cellular behavior of rat mesencephalic neuroepithelial cells, we performed the experimental approach described below; and found the following results.

2.4 | FGF2/EGF influence on neuroepithelial precursor proliferation

To evaluate cellular proliferative activity in our neuroepithelial explant culture system, we assumed that BrdU incorporation to neuroepithelial precursor cells was due to nuclear DNA synthesis.

As comparative standard values for DNA synthesis activity, we determined the number of BrdU labeled nuclei in the dorsolateral mesencephalic wall of 13.5-day rat embryos developed in utero (the age of the neuroepithelium at the end of the culture period). As is shown in Figures 3A and 4A, after 1 h of BrdU exposure of cultured neuroepithelium explants, many

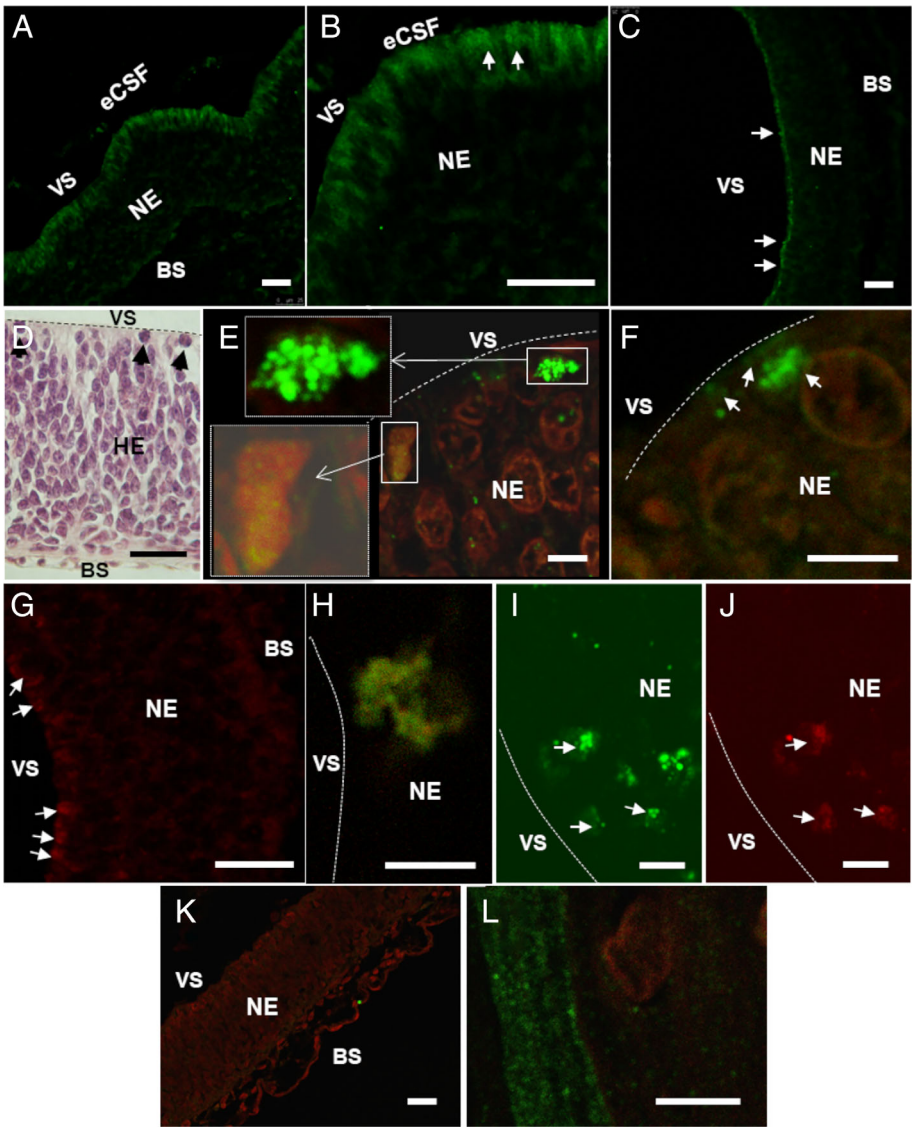

FIGURE 2 12.5-day rat embryo mesencephalic neuroepithelium sections, immunolabeled with anti-FGF2 antibody (A, B, C, E, F, and I), with anti-FGFR1 antibody (G and J) or with both (H). Images A, B, and C show intense immunolabeling in many neuroepithelial cells close to or in contact with the ventricular surface, and occasionally a thin positive layer covering the apical neuroepithelial surface (arrows in C). Image D shows hematoxylin-eosin sections correlative to immunolabeled sections, which were used to identify the apical neuroepithelial end by the presence of mitotic figures (arrows). High magnification of FGF2 positive cells with nuclear counterstain with TOTO 3 (E and F) show neuroepithelial cells with intense cytoplasmic granular or vesicular labelling (superior insert in E), and others with a faint expression of FGF2 in the nucleus (inferior insert in E). As is shown in F (arrows), some FGF2 positive cells with cytoplasmic labelling seem to be in direct contact with the ventricular surface. Immunohistochemical detection of FGFR1 in the mesencephalic neuroepithelium, showing the presence of some positive cells at the ventricular surface (arrows in G). Colocalization of both FGF2 and FGFR1 were detected in some neuroepithelial cells close to the ventricular surface (H); however, not all the FGF2 positive cells (I) expressed FGFR1 (J). These data support the responsiveness of neuroepithelial cells to the FGF2 from eCSF. Immunohistochemical detection of EGF in 12.5-day rat mesencephalic neuroepithelium was negative (K). As a positive control for antibody reactivity we used adult rat skin (L). eCSF, embryonic cerebrospinal fluid; NE, neuroepithelium; HE, hematoxylin-eosin staining; VS, ventricular surface; BS, basal surface. Scale bar in A, B, C, G, and K: 25 μm; D: 50 μm; E, F, H, I, J, and L: 5 μm

neuroepithelial cells displayed positive nuclear labeling, reflecting the high DNA synthesis activity involved in the expansion of the neuroepithelial precursor population. Figure 3A shows that in most cases the BrdU positive nucleus was located in the medial zone of the neuroepithelium, as was to be expected due to the basal-apical nuclear migration of these cells during the cell cycle. However, some labeled nuclei reach the ventricular surface and showed mitotic figures, whereas the basal side of the neuroepithelium remained free of

labeled nuclei, coinciding with βIII Tubulin positive expression (see below).

The explants cultured solely with defined culture media (outside the influence of eCSF) displayed a significant decrease in their DNA synthesis activity (Figure 4A), which was visible in the BrdU-immunostained sections (Figure 3B).

Following the same experimental conditions but with the addition of eCSF to the culture medium, we found a statistically significant reactivation of DNA synthesis activity in mesencephalic neuroepithelial cells (Figures 3C and 4A), which attained rates slightly, albeit significantly, below those of the control ones (Figure 4A); this reveals the specific influence of eCSF on the mitotic behavior of mesencephalic neuroepithelial precursors in rat embryos. As is shown in Figure 3C, the distribution pattern of BrdU positive nuclei was similar to that observed in the controls, despite many stained nuclei displaying lower BrdU labeling intensity.

Given that, we detected two different growth factors in rat eCSF (FGF2 and EGF), we attempted to clarify the specific role of each in neuroepithelial cell DNA synthesis control. This was performed by blocking growth factor biological activity in eCSF by incorporating a specific antibody in explants cultured with defined medium supplemented with eCSF, or by the addition of the growth factor to explants cultured in defined medium. We also tested the specificity of the growth factor influence by blocking its biological activity with a specific antibody.

To corroborate the specificity of eCSF FGF2 influence in DNA synthesis, we developed explants of mesencephalic neuroepithelium cultured with eCSF-supplemented

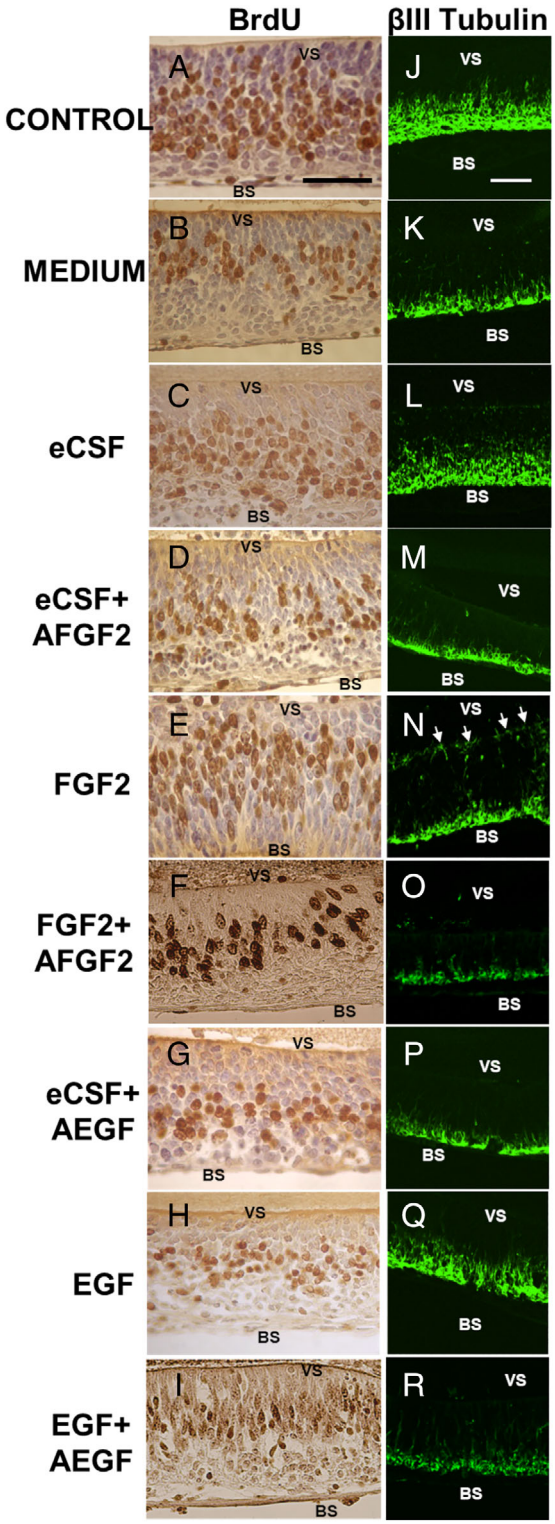

FIGURE 3 Legend on next column.

FIGURE 3 Histological sections from control 13.5-day mesencephalic neuroepithelium (A and J) or age-assimilated cultured explants (B to I and K to R), immunostained with anti-BrdU antibody (A to I), showing the brown nucleus of replicative neuroepithelial precursor cells. Images J-R correspond to anti-βIII-Tubulin-immunostained sections, showing a green cytoplasmic label, specific for newborn neurons, mainly located on the basal side of the neuroepithelial wall. Experimental conditions were as follows: control (13.5-day rat embryo mesencephalic neuroepithelium); medium (13.5-day neuroepithelial explants cultured in defined medium); eCSF (13.5-day neuroepithelial explants cultured in defined medium supplemented with 15% eCSF); eCSF+AFGF2 (13.5-day neuroepithelial explants cultured in defined medium supplemented with eCSF and with anti-FGF2 antibody); FGF2 (13.5-day neuroepithelial explants cultured in defined medium supplemented with FGF2); FGF2 + AFGF2 (13.5-day neuroepithelial explants cultured in defined medium supplemented with FGF2 and with anti-FGF2 antibody); eCSF+ AEGF (13.5-day neuroepithelial explants cultured in defined medium supplemented with eCSF and with anti-EGF antibody); EGF (13.5-day neuroepithelial explants cultured in defined medium supplemented with EGF) and EGF + AEGF (13.5-day neuroepithelial explants cultured in defined medium supplemented with EGF and with anti-EGF antibody). BS, basal side; VS, ventricular side. Scale bar in A-I: 100 μm; J-R: 100 μm

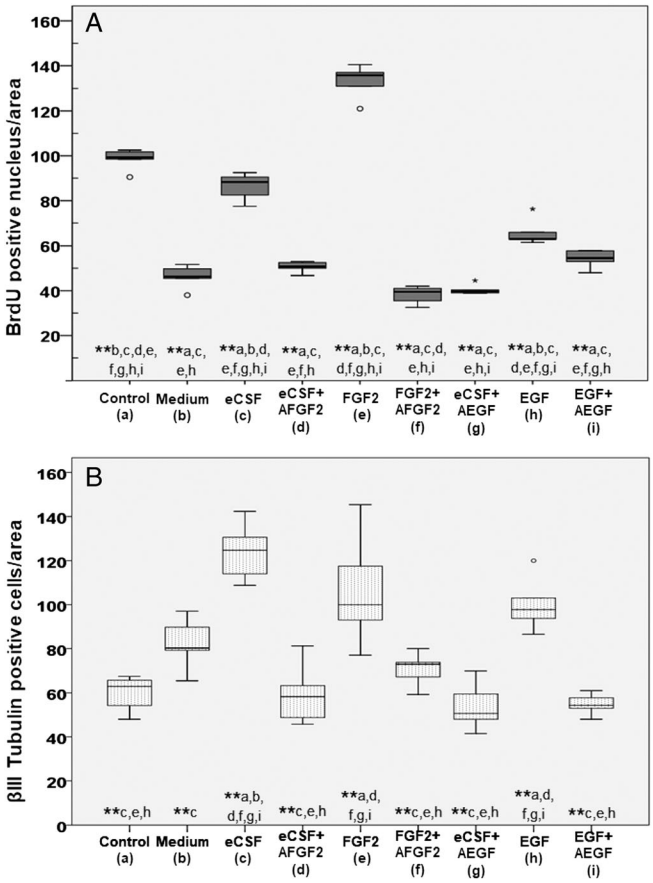

FIGURE 4 Box-plot distributions of BrdU positive nucleus/area (1400 μm^2) (A), and βIII Tubulin positive cells/area (1400 μm^2) (B) in the mesencephalic neuroepithelium. Within each box, horizontal white lines denote median values; boxes extend from the 25th to the 75th percentile of each group's distribution of values; vertical extending lines denote adjacent values (the most extreme values within 1.5 interquartile range of the 25th and 75th percentile of each group). Circles and stars represent outliers. In both cases different letters correspond to experimental conditions with statistically significant differences according to one-way ANOVA analysis of variance (n = 5): Bonferroni test post hoc comparisons test (**$P < .001$). Experimental conditions were as follows: control = 13.5-day rat embryos mesencephalic neuroepithelium; medium = 13.5-day neuroepithelial explants cultured in defined medium; eCSF = 13.5-day neuroepithelial explants cultured in defined medium supplemented with eCSF; eCSF+ AFGF2 = 13.5-day neuroepithelial explants cultured in defined medium supplemented with eCSF with anti-FGF2 antibody; FGF2 = 13.5-day neuroepithelial explants cultured in defined medium supplemented with FGF2; FGF2 + AFGF2 = 13.5-day neuroepithelial explants cultured in defined medium supplemented with FGF2 with anti-FGF2 antibody; eCSF+AEGF = 13.5-day neuroepithelial explants cultured in defined medium supplemented with eCSF with anti-EGF antibody; EGF = 13.5-day neuroepithelial explants cultured in defined medium supplemented with EGF; EGF + AEGF = 13.5-day neuroepithelial explants cultured in defined medium supplemented with EGF and with anti-EGF antibody

medium with an additional anti-FGF2 antibody to block biological activity. An analysis of these explants revealed a substantial decrease in DNA synthesis activity (Figures 3D and 4A), which was significant with respect to the eCSF-treated explants, and a significant decrease in comparison with FGF2-supplemented explants (Figures 3E and 4A). This supports our hypothesis concerning the role of FGF2 from eCSF as a key mitotic inducer for neuroepithelial precursor cells in 13.5-day rat embryos.

The addition of FGF2 to explants cultured in defined medium induced a dramatic increase in the DNA synthesis activity of neuroepithelial cells (Figure 3E), which was significant with respect to eCSF-treated explants (Figure 4A). This significant increase strongly supports the idea that FGF2 plays a key role in regulating neuroepithelial precursor DNA synthesis activity. Moreover, these data show that in physiological conditions mesencephalic neuroepithelial precursors do not develop their maximal replication potential, which seems rather to be activated by FGF2. As we show in Figure 3E, the BrdU positive nucleus distribution pattern induced by FGF2 was similar to those of the controls and the eCSF-treated explants; however, the number of positive nuclei located close to the ventricular surface seems to be increased. Figure 5A shows the quantification of BrdU positive nuclei located in the two apical nuclei rows close to the luminal surface, which was significantly greater in the FGF2 than in the eCSF-treated explants. This suggests acceleration in interkinetic nuclear migration, probably linked to the increase in DNA synthesis activity. The explants cultured with FGF2 + AFGF2 canceled out the effect of FGF2 on neuroepithelial DNA synthesis, which was statistically lower than in the case of FGF2-treated explants (Figures 3F and 4A); this showed the ability of the antibody to block FGF2 biological activity. Taken together, our results provide evidence that FGF2 from eCSF is involved in neuroepithelial precursor replication.

In addition, the explants cultured with medium supplemented with eCSF plus anti-EGF antibody (Figures 3G and 4A) showed a significantly lower number of BrdU labeled nuclei than eCSF-treated explants, which was similar to the case of eCSF plus anti-FGF2-antibody-treated explants. However, EGF-treated explants (Figure 3H) showed only a discrete but significant increase in DNA synthesis activity compared with medium or eCSF plus anti-EGF antibody-treated explants (Figure 4A). Similarly, explants supplemented with EGF and anti-EGF antibody displayed a discrete yet significant decrease in the number of Brd-labeled nuclei in comparison with EGF-treated explants (Figures 3I and 4 A).

These results provide evidence that, in these early developmental stages, EGF from eCSF was also involved in neuroepithelial precursor replication; however, EGF is

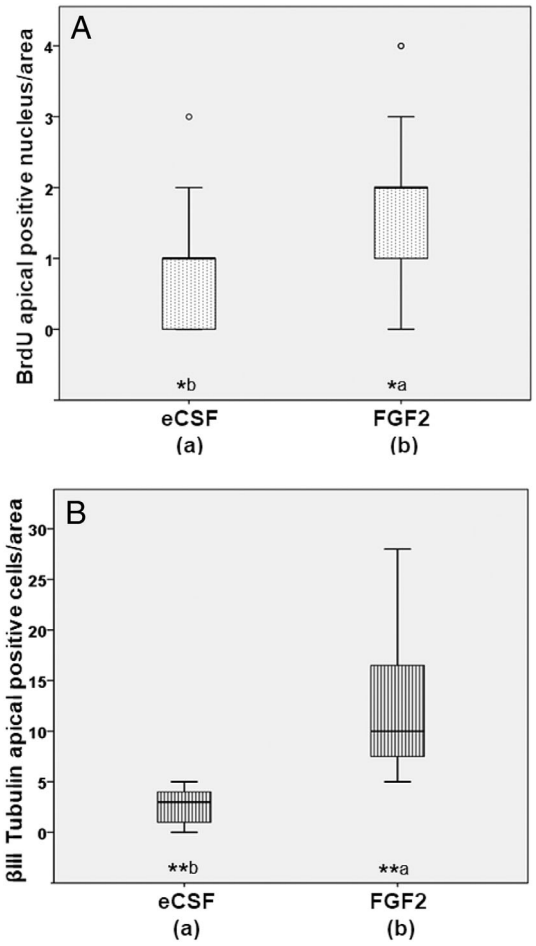

FIGURE 5 Box-plot distributions of BrdU apical positive nucleus/area (1400 μm^2) (A), and βIII Tubulin apical positive cells/area (1400 μm^2) (B) in the mesencephalic neuroepithelium upper horizontal line of box, 75th percentile; lower horizontal line of box, 25th percentile; horizontal bar within box, median; upper horizontal bar outside box, 90th percentile; lower horizontal bar outside box, 10th percentile. Circles represent outliers. In both cases statistical significance was determined by one-way ANOVA analysis of variance (n = 5) and post hoc Bonferroni test (*$P < .05$; **$P < .001$). Experimental conditions were as follows: eCSF = 13.5-day neuroepithelial explants cultured in defined medium supplemented with eCSF; FGF2 = 13.5-day neuroepithelial explants cultured in defined medium supplemented with FGF2

not able to reproduce the entire replicative activity induced by eCSF.

2.5 | FGF2/EGF influence on neuroepithelial precursor neurogenesis

Here we tried to evaluate the influence of FGF2/EGF on early neuronal differentiation from mesencephalic neuroepithelial precursors in rat embryos. As we can see in Figure 3J, in 13.5-day rat embryos (control), an intense process of neurogenesis takes place on the basal side of the mesencephalic neuroepithelium. This was detected by cytoplasmic expression of a specific marker for young neurons, βIII Tubulin, and the positive cells were evaluated as described above. Note the existence of a continuous layer of mesencephalic cells showing βIII Tubulin positive cytoplasmic expression located in the basal zone of the neuroepithelium, coinciding with the free zone of BrdU positive nuclei mentioned previously (Figure 3A).

The explants cultured in defined medium show no significant differences in the number of βIII Tubulin positive cells compared with the controls (Figures 3K and 4B) although an increase in cytoplasm labeling expression can be appreciated.

In the mesencephalic explants cultured with defined medium supplemented with eCSF (Figures 3L and 4B), we can appreciate a substantial and statistically significant increase in the number of βIII Tubulin positive cells compared with the medium treated explants (Figure 4B). These data demonstrate that eCSF induces intense neurogenic activity in neuroepithelial precursors.

Immunoblocking of the biological activity of FGF2 in eCSF significantly prevents its neurogenic effects (Figures 3M and 4B).

Explants cultured with defined medium supplemented with FGF2 (Figures 3N and 4B) displayed a significant increase in the number of βIII Tubulin positive cells with respect to the explants with FGF2 immunodepletion in eCSF, which show no significant differences with eCSF-treated explants. Explants cultured with FGF2 and immunoblocked with anti-FGF2 antibody (Figures 3O and 4B) significantly canceled out the neurogenic effect of FGF2. This result supports our hypothesis that FGF2 from eCSF is partially responsible for mesencephalic neurogenesis in rat embryos.

However, in FGF2-treated explants (Figure 3N), despite the distribution pattern of βIII Tubulin positive cells at the basal side of the neuroepithelium, which gives the appearance of preservation, there are several examples of dispersed βIII Tubulin positive cells abnormally located on the luminal side of the neuroepithelium. Figure 5B shows the quantification of βIII Tubulin positive nuclei located in the two apical rows of nuclei close to the luminal surface, which was significantly greater in the case of FGF2 compared with eCSF-treated explants. These data reveal that FGF2 induces an increase in the neurogenic activity of the neuroepithelial cells, but also seems to disrupt basal-apical migratory neuroepithelial cell movements.

Our results reveal that explants cultured with defined medium supplemented with eCSF and anti-EGF antibody

displayed a significant decrease in the number of βIII Tubulin positive cells compared with the eCSF-treated samples (Figures 3P and 4B). In addition, the explants cultured with EGF added to the defined culture medium (Figures 3Q and 4B) show a neurogenic increase which does not show any statistical differences in respect of the eCSF of FGF2-treated explants (Figure 4B). Finally, the EGF neurogenic effect was statistically blocked in EGF plus anti-EGF antibody-treated explants (Figures 3R and 4B). These data support the hypothesis that EGF from eCSF plays a similar role to FGF2 in neuroepithelial neurogenesis activation.

3 | DISCUSSION

Our results offer new data concerning the composition and biological role of eCSF at the earliest stages of rat embryonic development. Here we show the presence in this fluid of growth factors, identified as FGF2 and EGF, in 13.5-day rat embryos.

We describe a 62-65 kDa band in both FGF2 and EGF western blot, a molecular weight hitherto not described for both factors. This is probably due to a nonspecific antibody joining other proteins such as Albumin or α-fetoprotein present in rat eCSF.[29] This joining can sequester some amount of FGF2 or EGF antibody in the correspondent explant culture. 1, however, reveals the presence of sufficient free antibody to be linked to the other band, suggesting that we have used enough antibodies in our neuroepithelial explant cultures. Moreover, the decrease in neuroepithelial DNA synthesis detected in eCSF plus anti-FGF2 antibody neuroepithelial explants was very similar to that induced by FGF2 plus anti-FGF2 antibody, suggesting the presence of a sufficient amount of anti-FGF2 antibody in the culture system to block eCSF FGF2 biological activity.

The 23.4 kDa band described in FGF2 western blot coincides with the high molecular weight isoform of this growth factor,[30] considered as predominantly nuclear but able to shunt to cytoplasm and to extracellular space, justifying the presence of this isoform in eCSF.[31] The 14-7.5 kDa band detected in FGF2 western blot could be coincident (given our nonprecise western blot system, see experimental procedures) with the low molecular weight isoform of FGF2, which has 18 kDa and is typically located at the cytoplasm and secreted to the extracellular space. The presence of both FGF2 isoforms, representing low and high molecular weight in eCSF, are consistent with our immunohistochemical study showing neuroepithelial cells with nuclear and cytoplasmic FGF2 labeling. Furthermore, the 24 kDa band detected in eCSF with EGF antibody was in the range of EGF linked to proteins described in the literature.[32]

Immunohistochemistry reveals the expression of FGF2 in cytoplasmic vesicles/granules of several mesencephalic neuroepithelial cells located close to the ventricular surface, and frequently in apparent contact with the eCSF; this suggests an apical secretory mechanism. However, we also detected FGF2 positive neuroepithelial cells with nuclear labeling close to, but without contact with, the ventricular surface, coinciding with that previously described by Vaccarino et al[25] or Raballo et al[12] This suggests the coexistence of both autocrine-paracrine and apical secretory mechanisms of FGF2 by neuroepithelial cells. In addition, we show the existence of mesencephalic neuroepithelial cells, which express FGFR1 close to the ventricular surface, once in contact with the eCSF. This suggests their identification as FGF2 target cells, which is supported by the presence of neuroepithelial cells with FGF2-FGFR1 colocalization at the ventricular surface. The organotypic culture of mesencephalic neuroepithelium in different experimental conditions allows us to demonstrate that FGF2 and EGF from eCSF are directly involved in triggering neuroepithelial precursor proliferation, which leads to the dramatic expansion in their population at these stages of brain development. Additionally, both factors seem to be directly involved in the triggering of neurogenesis which takes place after precursor expansion, in collaboration with other mitogens and neurogenetic inducers present in the eCSF such as IGF2,[16] LIF2,[22] and Shh,[23,24] among others (see Reference 33).

Yet there has been no description of the presence at the earliest stages of development of the two growth factors, FGF2 and EGF, in rat eCSF. Both proteins have been shown to play relevant roles both during brain development and in the adult, acting as mitogens and neurogenic inducers.[12,25,27,34] Our data contribute to a better understanding of eCSF molecular composition and its trophic effect on neural precursor behavior. In several animal species, eCSF has been proposed as a biological complex fluid, confined in the brain anlage cavity by the apical surface of neuroepithelial precursors.[1] The biochemical composition of eCSF is still to be completely clarified, although previous proteomic studies[29,35-37] have been undertaken, showing the presence of several molecules with high "biological value" such as morphogens, growth factors, and transcription factors, which justify their key role in brain development and also in adult neurogenesis.[10,11,16,38,39] eCSF is considered a key element for the diffusion of soluble signals, establishing long-distance interactions between the different neuroepithelial cell populations.[1,33,40] This brain developmental mechanism involves soluble signals such as growth factors,[16,26,41] transcription factors,[33,40,42] or morphogens such as retinoic acid.[43-46] Which can be joined to specific molecules, for instance, HSPG for

heparin-binding growth factors,[47,48] RBP for retionol,[44,46] lipoproteins,[29,40,49] or exosomes,[50,51] which protect them from degradation and regulate their biological bioavailability. In fact, we previously demonstrated the presence of RBP in eCSF, in relation with retinol transport and retinoic acid synthesis, and a certain kind of HSPG in eCSF from rat embryos, suggesting a possible interactive regulation in neural precursor behavior.[29] Here, we have also shown the presence of FGFR1 on the apical surface of some mesencephalic neuroepithelial cells in rat embryos, occasionally showing colocalization with FGF2. This supports the hypothesis that eCSF is a diffusion channel for FGF2, and suggests molecular mechanisms that regulate its bioavailability to trigger specific receptors in target cells.

We previously demonstrated the presence of FGF2 in eCSF in chick embryos; consequently, this seems to be a phylogenetically preserved mechanism for neuroepithelial stem cell mitotic replication. However, the data from this study revealed differences in the origin of eCSF FGF2 between avian and mammals. Previously, we demonstrated[26] that the chick embryo neuroepithelium does not express FGF2 mRNA, despite the fact that this growth factor is present in eCSF. We concluded that the origin of this FGF2 is extra-neuroepithelial and that it is transported by the neuroepithelium via a specific mechanism. Although the expression of FGF2 protein and mRNA in the brain neuroepithelium of rat embryos has been reported at the earliest stages of brain development,[12,25] here we show there is an accumulation of FGF2 protein close to or in contact with the apical end of mesencephalic neuroepithelial cells, strongly suggesting an apical secretory mechanism.[12,25] proposed the involvement in the regulation of the replicative activity of neuroepithelial precursors as FGF2 autocrine or paracrine mechanisms. Here we report the presence of functionally active FGF2 in the eCSF, which open the possibility of a long-distance diffusion pathway involved in regulating neuroepithelial precursor behavior during early brain development. This eCSF long-distance via for diffusible signals inside the brain cavities during development has been proposed for several molecules involved in different mechanisms during brain development and also in the adult brain.[52] Of particular interest are the studies showing the ability of adult brain stem cell niches to respond to eCSF diffusible signals, promoting an activation of neural precursor replication and neurogenesis.[53,54]

Our results contribute to an understanding of the mitogenic and neurogenic control mechanisms in the neuroepithelium, and are relevant not only in neural precursor cell population control but in macroscopic and morphogenic brain development at the earliest stages. In this regard, we previously suggested[1,9] a coordination between wall growth mechanisms (based on neuroepithelial replication) and brain cavity growth mechanisms (based on hydrostatic pressure generated by osmotic molecules) in terms of normal brain growth and morphogenesis.

From our results, we can conclude that, in the early stages of rat brain development, FGF2 acts as a mitogen inducer for neuroepithelial precursors, also displaying apparently direct involvement in triggering neurogenesis. Moreover, despite the presence of EGF in eCSF but not in the mesencephalic neuroepithelium, it seems to play a role in neuroepithelial precursor proliferation (probably less intense than FGF2), also showing influence as a neurogenic inducer. These tallies with the neuroepithelial temporal gap in FGF2/EGF influence on cultured neuroepheres[15,20,21] (Kilpatrick et al, 1995). The presence of both growth factors could play a sequential key role in ongoing neurogenesis, which in rat embryos begins at 11.5 days of development, following a short period of intense replication of neuroepithelial cells as a unique activity.[55]

This study supports the hypothesis that brain neuroepithelial precursor behavior is influenced by eCSF. This influence is mediated by the activity of growth factors whose presence is regulated by the neuroepithelial cells themselves via a self-regulatory mechanism.

4 | EXPERIMENTAL PROCEDURES

4.1 | Obtaining embryonic cerebrospinal fluid

Rat embryos (wistar) of 13.5-day, were explanted and dissected from the extra-embryonic membranes. The eCSF was obtained by micro-aspiration from the mesencephalic cavity, as previously described.[35] eCSF samples were kept at 4°C during handling and then aliquoted (20 μL), lyophilized and frozen at −40°C until used. We used eCSF from 13.5-day rat embryos owing to the difficulty of obtaining a sufficient amount of eCSF at previous stages.

4.2 | eCSF western blot analysis

Denaturing sodium dodecil sulphate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli.[56] Briefly, we redissolved lyophilized aliquots of eCSF from 13.5-day rat embryos in 20 μL of distilled and deionized water to restore their original volume. eCSF samples were mixed vol/vol with the sample buffer (0.5 M Tris-HCl pH 6.8,

2% SDS, bromophenol blue, 5% β-mercaptoethanol, 10% glycerol). Molecular mass standards of broad range proteins (Biotinylated Protein Standars, BioRad) were also used. SDS-PAGE was performed in a MiniProtean device from BioRad using a 4% stacking and 12% resolving polyacrylamide gel. Following incubation of the eCSF samples and standard proteins for 5 min at 100°C, electrophoresis was conducted at 100 V until the tracking dye reached the bottom of the gel.

For western blot analysis, proteins were electrotransferred from the electrophoresis gel to nitrocellulose membrane in Trans-Blot Transfer Medium (BioRad), for 1 h at 100 V, following manufacturer protocols. Membranes were subsequently blocked in 3% nonfat dry milk in phosphate buffer saline (PBS), for 40 min at room temperature. They were then incubated with a primary antibody to FGF2 (Rabbit anti-FGF2, F3393, Sigma) at 1/2000 dilution in PBS overnight at 4°C. After extensive washes in PBS, membranes were incubated in a secondary antibody (1:2500 dilution Goat anti-rabbit IgG biotin antibody, B6648, Sigma) conjugated to peroxidase for 2 h at room temperature. Alternatively, they were incubated with an antibody to EGF (Rabbit anti-EGF, Sigma E6135) at 1/2000 dilution in PBS overnight at 4°C. After extensive washes in PBS, membranes were incubated in a secondary antibody (anti-rabbit IgG, Sigma) conjugated to peroxidase at 1/2000 dilution for 2 h at room temperature. Finally, after extensive washes in PBS, they were developed with diaminobenzidine tetrahydrochloride (DAB), following standard protocols.

4.3 | Immunohistochemical procedure

For FGF2, EGF, and FGFR1 immunostaining, 12.5-day rat embryos were fixed in Carnoy's solution for 3 h and embedded in paraffin for sectioning (8 μm) following standard procedures. The embryonic age selected was 12.5 days in order to show the presence in the mesencephalic neuroepithelium of FGF2 (or EGF) and FGFR1 at the beginning of the explant culture period and, consequently, when we incorporated the experimental conditions. Mesencephalic histological sections were treated with a heat-induced epitope retrieval procedure, with a Tris/EDTA buffer (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20, pH 9.0), at 95°C for 15 min, followed by blocking with 10% normal goat serum (Sigma) and 1% BSA in TBS for 2 h. Sections were incubated with anti-FGF2 mouse monoclonal antibody (Rabbit anti-FGF2 F-3393, Sigma-Aldrich) at 1/200 dilution. Overnight immunolabeling at 4°C was followed by a secondary antibody incubation (Alexa Fluor 488 rabbit anti-mouse IgG 1:1000 dilution for) and occasionally by a TOTO-3 Iodide (Thermo Fisher) nuclear counterstaining.

To detect FGF2 receptors in neuroepithelial cells we used a mouse IgM anti-FGFR1 antibody (clone VBS1 10 μg/mL, Millipore) at 1:100 dilution. Overnight immunolabeling at 4°C was followed by secondary antibody incubation (Alexa Fluor 568 goat anti-mouse IgM 1:1000 dilution). We occasionally performed colabeling with an anti-FGF2 antibody and an anti-FGFR1 antibody following the same procedures.

For immunodetection of EGF, sections were incubated with Rabbit anti-EGF polyclonal antibody (1 μg/mL, Abnova PAB19483). Immunolabeling was performed overnight at 4°C, followed by secondary antibody incubation (Alexa Fluor 488 goat anti-rabbit IgG (H + L) at 1:1000 dilution. As an EGF positive control, we used the same procedure in adult rat skin.

In order to clearly identify the apical end of the neuroepithelial cells, we used correlative sections to those with immunostaining, stained with hematoxylin-eosin, and identify the presence of mitotic figures as a morphological marker for the apical end.

Immunostaining without primary antibodies was used as a negative control. Immunostaining was visualized in a Leica TCS SPE confocal laser microscope.

4.4 | Organotypic culture of mesencephalic neuroepithelium

In this study, we used neuroepithelial explants from the mesencephalon of 12.5-day rat embryos cultured for 24 h up to 13.5 days of chronological age. Explants were cultured as described previously for chick embryos,[10] although in this case the area of explanted tissue was smaller given that there was less mesencephalic volume.

Briefly, following the usual protocol, the explants were placed on small pieces of filter paper (0.8 mm pore size from Millipore) in direct contact with a chemically defined serum-free medium (DMEM F12 from Sigma, supplemented with 1% ascorbic acid) and cultured at 37.8°C with 5% CO_2 for 24 h.

Mesencephalic cultured explants and 13.5-day embryos developed "in utero" (used as controls for neuroepithelial behavior at the end of the culture period) were fixed in Carnoy's solution for 3 h and embedded in paraffin for sectioning (8 μm) following standard procedures.

We evaluated three parameters in neuroepithelial tissue: histological appearance, cell replication, and neuronal differentiation, in the different experimental conditions used in the study.

First at all, we employed histological sections from the explants to develop a Hematoxylin-Eosin stain for evaluating the histological appearance of the tissue following standard procedures. We evaluate the integrity of the neuroepithelium, nuclear pseudo stratification, and apical-basal polarization by the presence of basal membrane and apical mitotic figures.

Neuroepithelial cell proliferation was evaluated by BrdU incorporation into cell nuclei, by the addition of BrdU (5 mM) to the culture medium for 1 h at the end of organotypic culture. In controls (13.5-day rat embryos) the neuroepithelium was explanted and cultured for only 1 h. With the same concentration of BrdU. Sections from the explants were incubated with a monoclonal mouse anti-BrdU antibody (clone Bu20a, from DAKO), at 1:100 dilution overnight at 4°C, after which we used an Goat anti-mouse IgG biotin antibody at 1:20 dilution for 30 min and Extravidin-Peroxidase at 1:20 dilution for 10 min, which was developed with DAB.

A quantitative analysis of BrdU incorporation was performed by counting the number of BrdU positive nuclei in 17-18 microscopic fields of 1400 μm^2, taken from the central region of each explant and from five different explants. The average of each condition with the median, minimum, maximum and quartiles were displayed in a boxplot including outlier values, and the significance between values was tested by a one-way ANOVA analysis of variance (n = 5) with a 95% significance level ($P < .05$); in addition, a Bonferroni test was performed as a post hoc multiple comparison test. Statistical analyses were performed using SPSS Statistics 24.0.

In order to evaluate changes in the spatial distribution of BrdU positive nuclei apico-basally, we counted the number of labeled nuclei in the two nuclear rows nearest to the ventricular surface, in FGF2 and eCSF explants. The data were obtained from 10 microscopic fields of 1400 μm^2 photomicrographs taken from five different explants for each experimental condition and plotted in a box-plot graph. Statistical analysis was evaluated by ANOVA (n = 5) and Bonferroni test was performed as a post hoc multiple comparison test.

To detect early neuronal differentiation, we monitored βIII Tubulin expression in histological sections from explants. These were incubated with an anti-βIII-Tubulin mouse monoclonal antibody (BAbCO MMS-435P) at 1:500 dilution for 1 h, and subsequently with an antimouse secondary antibody conjugated to FITC (Sigma Cat 1/64) for 1 h. Visualization was by means of a laser confocal microscope (Leica TCS SPE confocal laser microscope). We analyzed neurogenic activity by quantifying neuroepithelial cells with a negative nucleus surrounded by βIII Tubulin positive cytoplasm. The data were obtained from 17-18 microscopic fields of 1400 μm^2 photomicrographs taken from five different explants for each experimental condition and plotted in a box-plot graph. Statistical analysis was evaluated by ANOVA (n = 5) and Bonferroni test was performed as a post hoc multiple comparison test.

In order to evaluate changes in the spatial distribution of βIII Tubulin positive cells apico-basally, we counted the number of positive cells in the two apical neuroepithelial nuclei rows close to the luminal surface in FGF2 or eCSF explants. The data were obtained from 10 microscopic fields of 1400 μm^2 photomicrographs taken from five different explants for each experimental condition. The average of each condition was displayed in a boxplot, and value significance was tested by a one-way ANOVA analysis of variance (n = 5), with a 95% significance level ($P < .05$); in addition, a Bonferroni post hoc multiple comparison test was carried out.

4.5 | Experimental conditions in the Neuroepithelial explant culture

To establish the normal pattern of neuroepithelial cell development, cell replication and neuronal differentiation at the end of the explant culture period, control embryos were maintained in vivo until the 13.5-day developmental stage, when the mesencephalic neuroepithelium was explanted, cultured for 1 h with BrdU and processed.

To test mesencephalic neuroepithelial cell behavior avoiding extra-neuroepithelial signals (including eCSF) during the development period analyzed, a total of 10 neuroepithelial explants were cultured in a defined culture medium (DMEM F12 from Sigma, supplemented with 1% ascorbic acid).

To test the direct influence of eCSF on mesencephalic neuroepithelial cell behavior, a total of 10 explants were cultured for 24 h in a defined culture medium supplemented with 15% of 13.5-day embryonic eCSF.

In order to test the specific influence of the FGF2 (or, alternatively, EGF) detected in eCSF on the behavior of the mesencephalic neuroepithelial explants, a total of 10 mesencephalic explants for each growth factor were cultured for 24 h in a defined culture medium supplemented with 15% of 13.5-day embryonic eCSF and 2 μL of anti-FGF2 antibody diluted at 1/100 (Sigma F3393) or 1 μL of anti-EGF antibody diluted at 1/100 (Sigma-Aldrich 2520) to induce immunoblocking of biological activity.

Finally, to test the direct influence of each growth factor (FGF2 and EGF) on mesencephalic neuroepithelial cell behavior, a total of 10 mesencephalic explants for each growth factor were cultured for 24 hours in a

defined culture medium supplemented with FGF2 at a final concentration of 25 ng/mL (Sigma, F0291), or EGF at a final concentration of 10 ng/mL (Sigma, E9644) commercial growth factor. As a negative control for this condition, we performed a similar explant culture with FGF2 plus 2 μL of anti-FGF2 antibody diluted at 1/100 (FGF2 + AFGF2), and with EGF plus 1 μL of anti-EGF antibody diluted at 1/100(EGF + AEGF). Our choice of the concentration of growth factors in the culture medium was based on previous research by Kelly et al.[15]

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REFERENCES

1. Gato A, Desmond ME. Why the embryo still matters: CSF and the neuroepithelium as interdependent regulators of embryonic brain growth, morphogenesis and histiogenesis. *Dev Biol*. 2009;327:263-272.
2. Desmond ME, Jacobson AG. Embryonic brain enlargement requires cerebrospinal fluid pressure. *Dev Biol*. 1977;57:188-198.
3. Desmond ME, O'Rahilly R. The growth of the human brain during the embryonic period proper. 1. Linear axes. *Anat Embryol (Berl)*. 1981;162:137-151.
4. Desmond ME, Levitan ML, Haas AR. Internal luminal pressure during early chick embryonic brain growth: descriptive and empirical observations. *Anat Rec A Discov Mol Cell Evol Biol*. 2005;285:737-747.
5. Alonso MI, Gato A, Moro JA, Barbosa E. Disruption of proteoglycans in neural tube fluid by beta-D-xyloside alters brain enlargement in chick embryos. *Anat Rec*. 1998;252:499-508.
6. Alonso MI, Gato A, Moro JA, Martin P, Barbosa E. Involvement of sulfated proteoglycans in embryonic brain expansion at earliest stages of development in rat embryos. *Cells Tissues Organs*. 1999;165:1-9.
7. Lowery LA, Sive H. Totally tubular: the mystery behind function and origin of the brain ventricular system. *Bioessays*. 2009;31:446-458.
8. Chara O, Brusch L. Mathematical modelling of fluid transport and its regulation at multiple scales. *Biosystems*. 2015;130:1-10.
9. Desmond ME, Knepper JE, Dibenedetto AJ, et al. Focal adhesion kinase as a mechanotransducer during rapid brain growth of the chick embryo. *Int J Dev Biol*. 2014;58:35-43.
10. Gato A, Moro JA, Alonso MI, Bueno D, De La Mano A, Martín C. Embryonic cerebrospinal fluid regulates neuroepithelial survival, proliferation, and neurogenesis in chick embryos. *Anat Rec A Discov Mol Cell Evol Biol*. 2005;284:475-484.
11. Martin C, Alonso MI, Santiago C, et al. Early embryonic brain development in rats requires the trophic influence of cerebrospinal fluid. *Int J Dev Neurosci*. 2009;27:733-740.
12. Raballo R, Rhee J, Lyn-Cook R, Leckman JF, Schwartz ML, Vaccarino FM. Basic fibroblast growth factor (Fgf2) is necessary for cell proliferation and neurogenesis in the developing cerebral cortex. *J Neurosci*. 2000;20:5012-5023.
13. Dickinson ME, Krumlauf R, McMahon AP. Evidence for a mitogenic effect of Wnt-1 in the developing mammalian central nervous system. *Development*. 1994;120:1453-1471.
14. Imayoshi I, Sakamoto M, Yamaguchi M, Mori K, Kageyama R. Essential roles of notch signaling in maintenance of neural stem cells in developing and adult brains. *J Neurosci*. 2010;30:3489-3498.
15. Kelly CM, Tyers P, Borg MT, Svendsen CN, Dunnett SB, Rosser AE. EGF and FGF-2 responsiveness of rat and mouse neural precursors derived from the embryonic CNS. *Brain Res Bull*. 2005;68:83-94.
16. Lehtinen MK, Zappaterra MW, Chen X, et al. The cerebrospinal fluid provides a proliferative niche for neural progenitor cells. *Neuron*. 2011;69:893-905.
17. Kilpatrick TJ, Bartlett PF. Cloned multipotential precursors from the mouse cerebrum require FGF-2, whereas glial restricted precursors are stimulated with either FGF-2 or EGF. *J Neurosci*. 1995;15:3653-3661.
18. Martens DJ, Tropepe V, van Der Kooy D. Separate proliferation kinetics of fibroblast growth factor-responsive and epidermal growth factor-responsive neural stem cells within the embryonic forebrain germinal zone. *J Neurosci*. 2000;20:1085-1095.
19. Naruse M, Shibasaki K, Ishizaki Y. FGF-2 signal promotes proliferation of cerebellar progenitor cells and their oligodendrocytic differentiation at early postnatal stage. *Biochem Biophys Res Commun*. 2015;463:1091-1096.
20. Sanalkumar R, Vidyanand S, Lalitha Indulekha C, James J. Neuronal vs. glial fate of embryonic stem cell-derived neural progenitors (ES-NPs) is determined by FGF2/EGF during proliferation. *J Mol Neurosci*. 2010;42:17-27.
21. Tropepe V, Sibilia M, Ciruna BG, Rossant J, Wagner EF, van der Kooy D. Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. *Dev Biol*. 1999;208:166-188.
22. Chau KF, Springel MW, Broadbelt KG, et al. Progressive differentiation and instructive capacities of amniotic fluid and cerebrospinal fluid proteomes following neural tube closure. *Dev Cell*. 2015;35:789-802.
23. Huang X, Liu J, Ketova T, et al. Transventricular delivery of Sonic hedgehog is essential to cerebellar ventricular zone development. *Proceedings of the National Academy of Sciences of the United States of America*; 2010.
24. Coulter ME, Dorobantu CM, Lodewijk GA, et al. The ESCRT-III protein CHMP1A mediates secretion of Sonic Hedgehog on a distinctive subtype of extracellular vesicles. *Cell Rep*. 2018;24:973-986.e978.
25. Vaccarino FM, Schwartz ML, Raballo R, et al. Changes in cerebral cortex size are governed by fibroblast growth factor during embryogenesis. *Nat Neurosci*. 1999;2:848.
26. Martín C, Bueno D, Alonso MI, et al. FGF2 plays a key role in embryonic cerebrospinal fluid trophic properties over chick embryo neuroepithelial stem cells. *Dev Biol*. 2006;297:402-416.
27. Dono R, Texido G, Dussel R, Ehmke H, Zeller R. Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice. *EMBO J*. 1998;17:4213-4225.
28. Panchision DM, McKay RDG. The control of neural stem cells by morphogenic signals. *Curr Opin Genet Dev*. 2002;12:478-487.
29. Parada C, Gato A, Bueno D. Mammalian embryonic cerebrospinal fluid proteome has greater apolipoprotein and enzyme pattern complexity than the avian proteome. *J Proteome Res*. 2005;4:2420-2428.

30. MacFarlane L, Murphy P. FGF2 (fibroblast growth factor 2 [basic]). *Atlas of genetics and cytogenetics in oncology and haematology*; 2010. http://AtlasGeneticsOncology.org.
31. Chlebova K, Bryja V, Dvorak P, Kozubik A, Wilcox WR, Krejci P. High molecular weight FGF2: the biology of a nuclear growth factor. *Cell Mol Life Sci*. 2009;66:225-235.
32. Taylor JM, Cohen S, Mitchell WM. Epidermal growth factor: high and low molecular weight forms. *Proc Natl Acad Sci U S A*. 1970;67:164-171.
33. Zappaterra MW, Lehtinen MK. The cerebrospinal fluid: regulator of neurogenesis, behavior, and beyond. *Cell Mol Life Sci*. 2012;69:2863-2878.
34. Zheng W, Nowakowski RS, Vaccarino FM. Fibroblast growth factor 2 is required for maintaining the neural stem cell pool in the mouse brain subventricular zone. *Dev Neurosci*. 2004;26:181-196.
35. Gato A, Martin P, Alonso MI, Martin C, Pulgar MA, Moro JA. Analysis of cerebro-spinal fluid protein composition in early developmental stages in chick embryos. *J Exp Zool A – Compar Exp Biol*. 2004;301A:280-289.
36. Parada C, Gato A, Aparicio M, Bueno D. Proteome analysis of chick embryonic cerebrospinal fluid. *Proteomics*. 2006;6:312-320.
37. Zappaterra MD, Lisgo SN, Lindsay S, Gygi SP, Walsh CA, Ballif BA. A comparative proteomic analysis of human and rat embryonic cerebrospinal fluid. *J Proteome Res*. 2007;6:3537-3548.
38. Lehtinen MK, Walsh CA. Neurogenesis at the brain-cerebrospinal fluid interface. *Annu Rev Cell Dev Biol*. 2011;27:653-679.
39. Miyan JA, Zendah M, Mashayekhi F, Owen-Lynch PJ. Cerebrospinal fluid supports viability and proliferation of cortical cells in vitro, mirroring in vivo development. *Cerebrospinal Fluid Res*. 2006;3:2.
40. Kaiser K, Gyllborg D, Procházka J, et al. WNT5A is transported via lipoprotein particles in the cerebrospinal fluid to regulate hindbrain morphogenesis. *Nat Commun*. 2019;10:1498.
41. Mashayekhi F, Azari M, Moghadam LM, Yazdankhah M, Naji M, Salehi Z. Changes in cerebrospinal fluid nerve growth factor levels during chick embryonic development. *J Clin Neurosci*. 2009;16:1334-1337.
42. Sawamoto K, Wichterle H, Gonzalez-Perez O, et al. New neurons follow the flow of cerebrospinal fluid in the adult brain. *Science*. 2006;311:629-632.
43. Alonso M, Carnicero E, Carretero R, et al. Retinoic acid, under cerebrospinal fluid control, induces neurogenesis during early brain development. *J Dev Biol*. 2014;2:72-83.
44. Alonso MI, Martín C, Carnicero E, Bueno D, Gato A. Cerebrospinal fluid control of neurogenesis induced by retinoic acid during early brain development. *Dev Dyn*. 2011;240:1650-1659.
45. Chang EH, Adorjan I, Mundim MV, Sun B, Dizon ML, Szele FG. Traumatic brain injury activation of the adult subventricular zone neurogenic niche. *Front Neurosci*. 2016;10:332.
46. Parada C, Gato A, Bueno D. All-trans retinol and retinol-binding protein from embryonic cerebrospinal fluid exhibit dynamic behaviour during early central nervous system development. *Neuroreport*. 2008b;19:945-950.
47. Kerever A, Mercier F, Nonaka R, et al. Perlecan is required for FGF-2 signaling in the neural stem cell niche. *Stem Cell Res*. 2014;12:492-505.
48. Shimokawa K, Kimura-Yoshida C, Nagai N, et al. Cell surface heparan sulfate chains regulate local reception of FGF signaling in the mouse embryo. *Dev Cell*. 2011;21:257-272.
49. Parada C, Carles Escola-Gil J, Bueno D. Low-density lipoproteins from embryonic cerebrospinal fluid are required for neural differentiation. *J Neurosci Res*. 2008a;86:2674-2684.
50. Bachy I, Kozyraki R, Wassef M. The particles of the embryonic cerebrospinal fluid: how could they influence brain development? *Brain Res Bull*. 2008;75:289-294.
51. Feliciano DM, Zhang S, Nasrallah CM, Lisgo SN, Bordey A. Embryonic cerebrospinal fluid nanovesicles carry evolutionarily conserved molecules and promote neural stem cell amplification. *PLoS One*. 2014;9:e88810.
52. Lehtinen MK, Bjornsson CS, Dymecki SM, Gilbertson RJ, Holtzman DM, Monuki ES. The choroid plexus and cerebrospinal fluid: emerging roles in development, disease, and therapy. *J Neurosci*. 2013;33:17553-17559.
53. Alonso MI, Lamus F, Carnicero E, et al. Embryonic cerebrospinal fluid increases neurogenic activity in the brain ventricular-subventricular zone of adult mice. *Front Neuroanat*. 2017;11:124.
54. Alonso MI, Gato A. Cerebrospinal fluid and neural stem cell niche control. *Neural Regen Res*. 2018;13:1546-1547.
55. Nowakowski RS, Caviness VS, Takahashi T, Hayes NL. Population dynamics during cell proliferation and neuronogenesis in the developing murine neocortex. *Results Probl Cell Differ*. 2002;39:1-25.
56. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;227:680-685.

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