

Article

Retinoic Acid, under Cerebrospinal Fluid Control, Induces Neurogenesis during Early Brain Development

M. Isabel Alonso ^{1,2}, Estela Carnicero ^{1,2}, Raquel Carretero ¹, Aníbal De la Mano ^{1,2}, Jose Antonio Moro ^{1,2}, Francisco Lamus ¹, Cristina Martín ¹ and Angel Gato ^{1,2,*}

¹ Departamento de Anatomía y Radiología, Facultad de Medicina, Universidad de Valladolid, C/ Ramón y Cajal 7, 47005 Valladolid, Spain; E-Mails: mialonso@med.uva.es (M.I.A.); ecarnice@ah.uva.es (E.C.); raquel.carretero@uva.es (R.C.); alamano@ah.uva.es (A.D.M.); moro@med.uva.es (J.A.M.); matutautara@yahoo.fr (F.L.); mariacristina.martin.llorente@uva.es (C.M.)

² Laboratorio de Desarrollo y Teratología del Sistema Nervioso, Instituto de Neurociencias de Castilla y León (INCYL), Universidad de Valladolid, 47005 Valladolid, Spain

* Author to whom correspondence should be addressed; E-Mail: gato@med.uva.es; Tel.: +34-983-186-398.

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Abstract: One of the more intriguing subjects in neuroscience is how a precursor or stem cell is induced to differentiate into a neuron. Neurogenesis begins early in brain development and suddenly becomes a very intense process, which is related with the influence of Retinoic Acid. Here, using a biological test (F9-1.8 cells) in chick embryos, we show that “*in vivo*” embryonic cerebrospinal fluid regulates mesencephalic-rombencephalic Isthmic Retinoic Acid synthesis and this effect has a direct influence on mesencephalic neuroepithelial precursors, inducing a significant increase in neurogenesis. This effect is mediated by the Retinol Binding Protein present in the embryonic cerebrospinal fluid. The knowledge of embryonic neurogenetic stimulus could be useful in the control of adult brain neurogenesis.

Keywords: embryonic cerebrospinal fluid; retinoic acid; mesencephalic-rombencephalic isthmus; neuroepithelial precursors; neurogenesis

1. Introduction

Embryonic Cerebrospinal Fluid (E-CSF) has been shown to play key functions in brain development at both embryonic and foetal stages, [1–8]. It influences the behavior of neuroepithelial cell precursors, regulating the survival, proliferation, and neural differentiation of neuroepithelial progenitor cells [5,9,10], and also collaborates with the isthmic organizer in the regulation of mesencephalic gene expression [11]. Studies focusing on avian and mammal E-CSF proteomic composition reveal that this fluid includes a broad set of molecules, which might be responsible of their biological properties [12–15], and it has been suggested that there are specific molecules as FGF2 involved in specific cellular events, such neuroepithelial mitotic behavior [16].

Another key molecule in development, namely Retinoic Acid (RA), has been described as a powerful neurogenic agent in both embryo and adult neural progenitor cells [17–19]. RA synthesis requires the concurrence of a precursor molecule, all-trans retinol, a carrier molecule, Retinol Binding Protein (RBP), which also regulates the intake of retinol into the cells, and cells expressing the particular enzymes involved in transforming retinol to Retinoid Acid, namely Retinaldehyde Dehydrogenases (RALDHs) [20]. RA is a well-known morphogen that has a crucial impact on CNS development [21,22]. During early brain development in chick embryos the only RA metabolizing enzyme detected in the cephalic neuroepithelium, RALDH3, is located in the mesencephalic-rombencephalic isthmus (IsO), which is thought to be the RA source for brain neuroepithelium in chick embryos [23,24].

The precursor (all-trans retinol) and the carrier molecule (RBP) are present in the E-CSF from chick and rat embryos [13,14,24], which is in direct contact with the isthmic cells. At the same time, a direct involvement in neuroepithelial neurogenesis has been described for embryonic CSF both in chick [5] and rat embryos [25].

RA diffuses from the clusters of cells expressing RALDH enzymes, such as those forming some of the well-known organizing centers, to the target cells [23,26]. When RA is taken up by the target cells, it binds to specific nuclear receptors (RAR and RXR), regulating a series of genes involved in neural differentiation and patterning of anterior-posterior and dorsal-ventral axes [21,22,27–33].

Recently we showed, by means of an *in vitro* cellular culture system, that E-CSF activates neurogenesis in the mesencephalic neuroepithelial cells as a result of specific regulation of RBP-Retinol uptake in the IsO cells [34].

Here, using a similar experimental approach, we wish to ascertain if the same brain developmental mechanisms operate in live chick embryos.

2. Experimental Section

2.1. Obtaining Embryos and Cerebrospinal Fluid

Fertile chicken eggs were incubated at 38 °C in a humidified atmosphere to obtain chick embryos at the desired developmental stages according to Hamburger and Hamilton parameters [35]. Embryos were incubated to stage HH24 so as to procure E-CSF. After dissection of the embryos from the extra-embryonic membranes, E-CSF was aspirated as previously described [12]. To minimize protein degradation, E-CSF samples were kept at 4 °C, aliquoted, lyophilized, and frozen at –40 °C, until they were required for use.

2.2. Culture of F9-1.8 Cell Line

F9-1.8 cell line was maintained as previously described [36] on 1% gelatine-coated (gelatin from porcine skin Type A from SIGMA) tissue culture flasks in Advanced-DMEM (Gibco, Gaithersburg, MD) supplemented with 7.5% heat-inactivated FBS, 44 nM NaHCO₃, and 4 µg/mL G418 (from Sigma). The cells were grown to 70%, confluence. A pro-medium of 10⁷ cells/flask were re-suspended in 1 mL of medium and cultured overnight at 37 °C in a 5% CO₂ atmosphere for micro-injection. This cell line contains the Retinoic Acid Response Element (RARE) promoter coupled to the lacZ (β-galactosidase) reporter gene.

2.3. Micro-Injection of F.9-1.8 Cells “in Vivo”

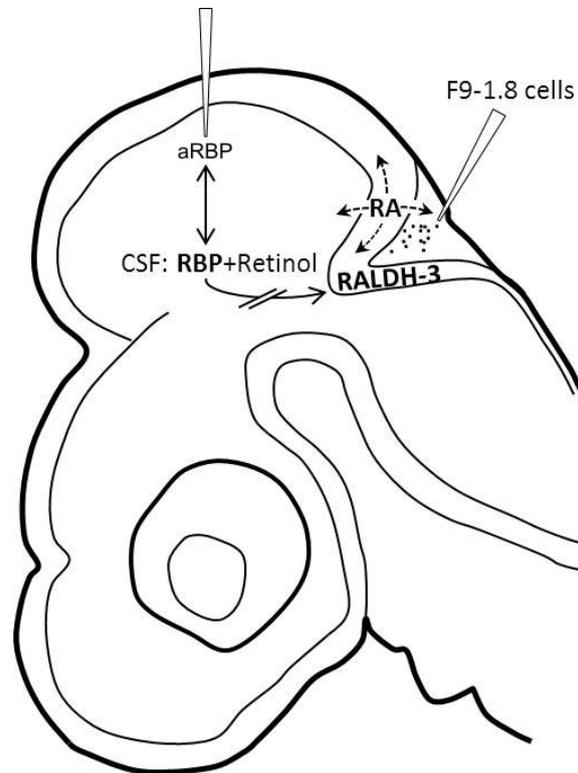
In order to test the influence of CSF on the Retinoic acid synthesis capacity of the Iso “in vivo”, we used an experimental approach, based on the use of the cell line F9 1.8, which contains the Retinoic Acid Response Element (RARE) promoter coupled to the lacZ (β galactosidase) reporter gene, which will turn blue when activate. Briefly: 21 H.H. stage chick embryos were incubated and an opening made in the shell, exposed “in ovo”. A volume of 10 µL of an F.9-1.8 cellular suspension, as described before, was microinjected in the dorsal mesenchyme, deep in the mesencephalic-rombencephalic furrow, with a 50 µm diameter Hamilton micro-syringe (Figure 1). Next, on each embryo we performed a second microinjection in the mesencephalic cavity, as we described before [16], of 40 µL of an Anti Retinoic binding protein antibody at 1/100 dilution in PBS (Lab Vision Corporation, Neomarkers), or the same amount of heat-inactivated Retinol binding protein antibody. We used eight embryos for each experimental condition. We did not perform any other type of negative control in regards to the injection of F9 1.8 cells in other embryonic structures as it is extremely difficult to find places completely free of retinoic acid influence.

Subsequently, the egg shells were sealed and the eggs were reincubated for 24 h until reaching stage 24 H.H. The embryos were dissected form extra-embryonic membranes and fixed in 4% paraformaldehyde at room temperature for 3 h. Finally, the X-GAL was developed following a standard protocol: the embryos were washed in PBS, followed by de-ionized water, and exposed to 20 mg/mL of X-gal solution diluted 1/50 in ferric solution (from Sigma) for 1 h at 37 °C, as described by Sonneveld *et al.* [37]. The X-GAL positive cells inside the embryo were photographed with a binocular microscope.

2.4. Neuroepithelial Cell Behavior Test

In order to test the effect of the different experimental conditions on the behavior of the neuroepithelial cells, we selected the main parameters, cell survival, cell replication, and neurogenesis.

Figure 1. Diagram of a saggital section of an early embryo cephalic end, showing the experimental approach used in this study. On the left, microinjection into the embryonic brain cavity of Retinol Binding Protein Antibody (aRBP) to block the Retinol uptake in mesencephalic-rombencephalic isthmus (IsO) RALDH positive cells. On the right, simultaneous microinjection F9-1.8 cells into the mesenchyme of the mesencephalic-rombencephalic furrow, as a sensitive test for Retinoic Acid activity.



2.4.1. Determination of BrdU

Incorporation of BrdU into cell nuclei was performed by microinjecting 190 nL of a 5 μ M solution of BrdU in the outflow of the heart, exactly one hour before the end of the culture period; Immediately afterwards, the embryos were fixed in Carnoy for 20 min, dehydrated, and embedded in paraffin. After transverse sectioning of the embryos, BrdU was detected with a monoclonal antibody to BrdU (Dako) at 1/100 dilution for 30 min and a secondary antibody with avidin–extravidin system conjugated to peroxidase (mouse anti-rabbit and extravidin from Sigma) was developed with DAB. We visualized and photographed the preparations using a Nikon microphot-FXA photomicroscope.

A quantitative analysis of nuclear BrdU incorporation was performed by counting the number of BrdU-positive nuclei in twenty microscopic fields of 1,400 μ m² taken from four different embryos for each experimental condition. Both, the average of each condition, and the standard error, were plotted, and their significance was tested by a two-tailed Student's *t* test.

2.4.2. Neurogenesis

We detected neuronal differentiation by β 3-tubulin expression. Embryonic sections from the embryos were blocked in 1% BSA in PBS, then incubated with a monoclonal anti-tubulin antibody at

1/500 (BAbCO), and, following extensive PBS washing, were incubated with an anti-mouse antibody conjugated to FITC at 1/64 (Sigma) for 1 h at room temperature. In order to facilitate identification of the β 3-tubulin-positive cells, we used single plane images obtained by confocal laser microscopy. A quantitative analysis of β 3-tubulin expressing cells was performed by counting the number of neuroepithelial cells with immunostained cytoplasm in 20 microscopic fields of $1,900 \mu\text{m}^2$, from at least 4 different embryos. The average of each condition and the standard error were plotted, and their significance was tested by a two-tailed Student's *t* test.

2.4.3. TUNEL Assay

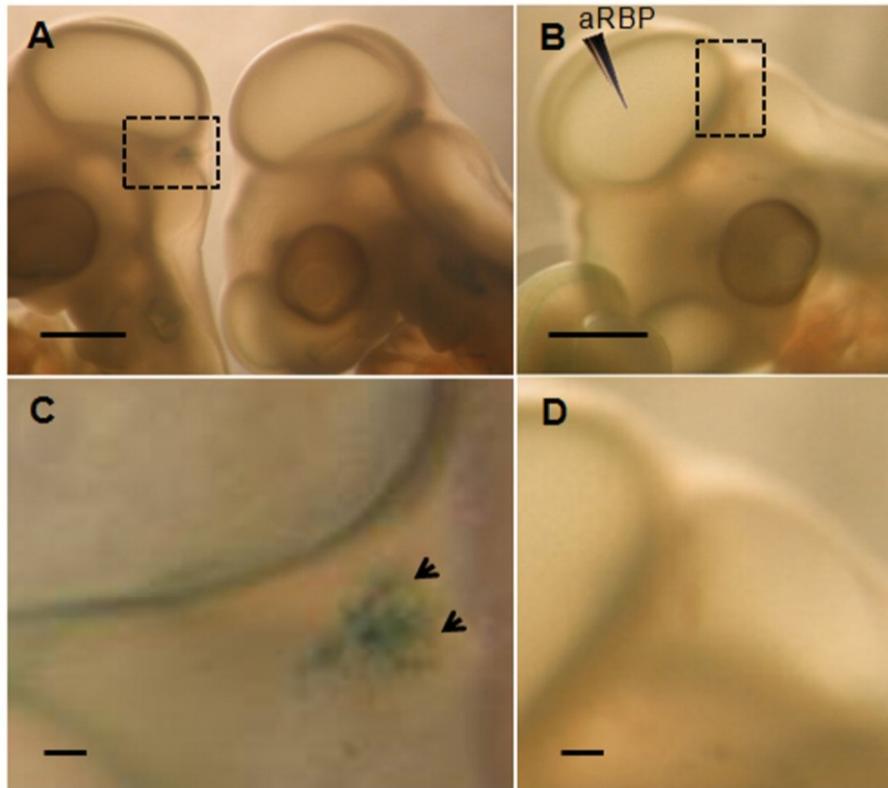
In order to ascertain a potential influence of RA on cell survival, we assessed apoptosis by means of the TUNEL technique on paraffin sections from each experimental condition. Apoptotic cells were detected using the Apoptosis Detection System Fluorescein Kit (Promega), following the manufacturer's instructions.

Visualization was made with a confocal microscope (Zeiss LSM-310). We performed quantitative analysis by counting the number of stained nuclei of neuroepithelial cells in 10 microscopic fields of $1,900 \mu\text{m}^2$, from at least four different samples. The average of each condition and the standard error were plotted and their significance was tested by a two-tailed Student's *t* test.

3. Results and Discussion

First of all, we show the capacity of IsO to act as a physiological centre for synthesis and diffusion of RA during early brain development. We used F9-1.8 cells as a biological test for the presence of RA in live tissues, and in order to avoid the dispersion of these cells inside the brain cavity, we microinjected them deep in the cephalic mesenchyme in the dorsal region of the IsO, which has been described as the main location in the early chick embryonic brain that expresses RALDH and which consequently might produce RA. In all cases (eight embryos), after 24 h, the F9-1.8 cells microinjected into the mesenchyme, close to the IsO, became blue (Figure 2A,C), showing the presence of retinoic acid activity in the extracellular space in this area. We previously showed the ability of the IsO cells to secrete Retinoic Acid to the extracellular space "*in vitro*", and our previous results strongly suggest that the final destination of this Retinoic Acid is the CSF, acting as a restricted diffusion channel to reach the neuroepithelial cells [34]. However, here we show that "*in vivo*" F9-1.8 cells microinjected close to but outside the neuroepithelium are able to respond to RA secretion from the IsO cells, suggesting that, *in vivo*, the IsO cells produce an active form of Retinoic Acid which seems diffuse both peripherally and radially outside the IsO neuroepithelium, establishing an "area of influence" from an "organizing center". Consequently, RA could be included as a diffusible signal of IsO involved in early brain development [38]. Giving the local expression of RALDHs in cephalic developing structures [24,39] we cannot rule out the possibility that each area of RALDH expression (including those close to but outside the neuroepithelium such as retina and nasal placode) could exert its own influence on a part of the developing brain, and that this system evolves phylo and ontogenically [40], however this hypothesis needs experimental support.

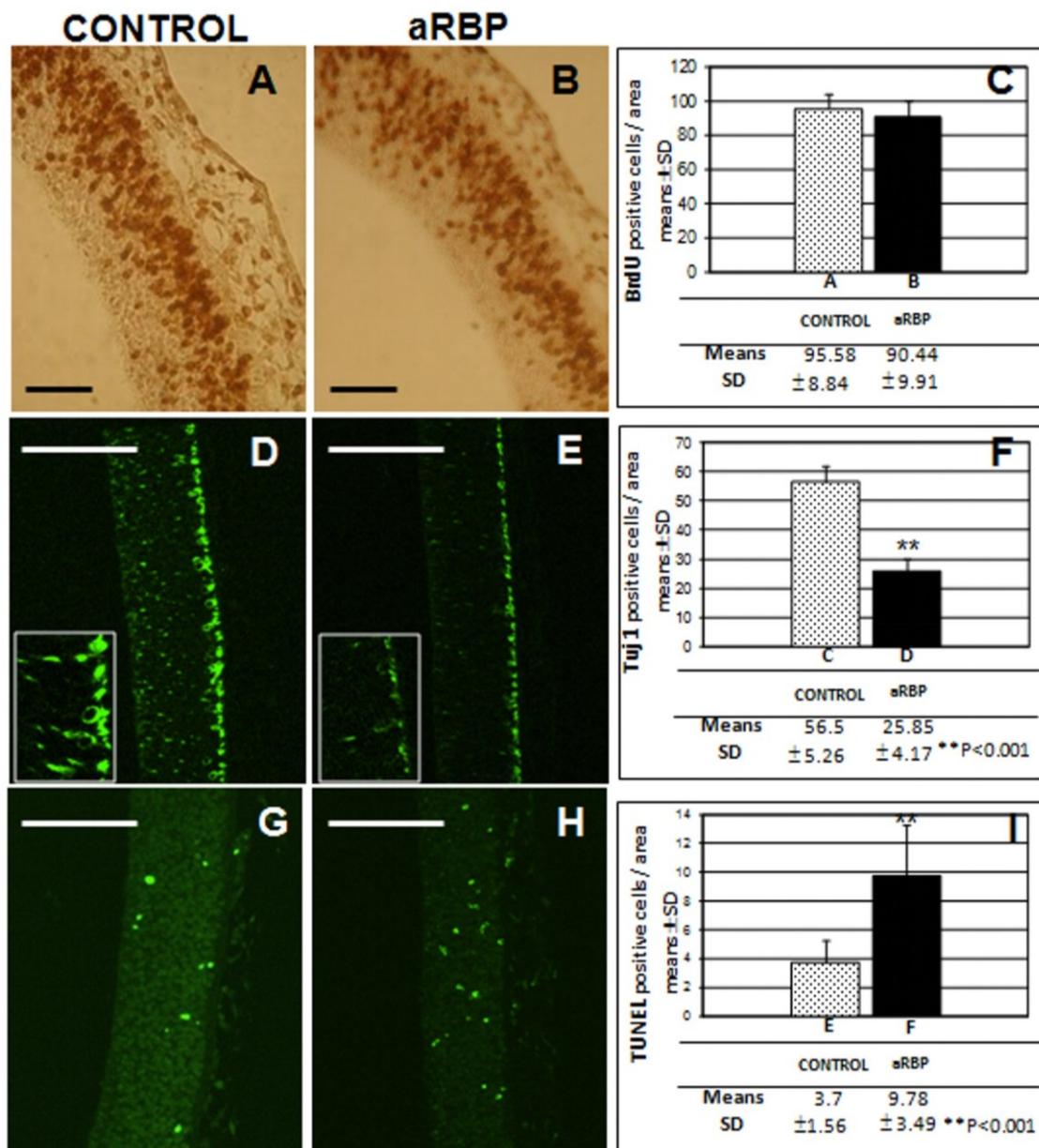
Figure 2. Photomicrographs of 23 H.H. stage chick embryos after microinjection of F9-1.8 cells in the mesencephalic-rombencephalic furrow delimited by a discontinuous line box in (A) and (B). After 24 h close to the IsO, the F9-1.8 microinjected cells develop a blue color (arrow heads in (A) and (C)) showing Retinoic Acid activity in this tissue. (C) and (D) show embryos with a similar experimental approach but with a simultaneous microinjection of Retinol Binding Protein antibody into the brain cavity. Note that the F9-1.8 cells do not develop a blue color, suggesting the blocking of retinoic acid binding activity in the area (scale bar in (A) and (B): 1 mm; scale bar in (C) and (D): 100 μ m).



Secondly, we try to corroborate our hypothesis that, *in vivo*, the embryonic CSF was able to influence Retinoic Acid activity in the IsO, regulating the retinol uptake from RALDH positive IsO cells by the activity of the Retinol Binding Protein (RBP), both components present in embryonic CSF [24,34]. As before, we used an experimental approach based on the immunoblocking of RBP activity in the brain cavity of chick embryos, with a monoclonal antibody microinjected “*in vivo*”, whilst at the same time performing a second microinjection of the F9-1.8 cells in the IsO mesenchyme to detect RA activity. It is relevant to note that in a previous study we showed that Immunoglobulins such as FGF2 monoclonal antibody microinjected inside the cavity of chick embryo neural tube, are not able to cross the neuroepithelial barrier and remains inside the cavity [25], Consequently, the effect of antibody microinjection is attributable to specific immunoblocking of CSF components. As we show in Figure 2B,D, the immunoblocking of RBP activity in CSF was able to prevent the F9-1.8 cells microinjected in the IsO mesenchyme from becoming blue in color, which signifies no RA activity in the area. This effect was complete in six embryos, and in two the number of blue cells was greatly reduced compared to controls (no antiRBP antibody microinjected in brain cavity), probably due to a

partial blocking of RBP activity. These results strongly suggest that “*in vivo*” control of Retinoic Acid synthesis by IsO cells depends on the CSF, which seems to be the main supply of the retinol for RALDH IsO cells, and also depends on the CSF RBP, which seems to be responsible for the intake of retinol inside these cells.

Figure 3. Evaluation of basic cellular behavior of mesencephalic precursor cells in control chick embryos (A,D,G) or after Retinol Binding Protein immunoblocking in CSF (B,E,H). (A) and (B) show BrdU labeled nucleus used to evaluate cellular replication, of which values were plotted in (C), with no significant differences between both groups. (D) and (E) show cytoplasm expression of β 3-Tubulin (Tuj1) used to evaluate early neurogenesis, of which values were plotted in (F), and show a significant decrease in neurogenesis after RBP immunoblocking. (G) and (H) show cellular apoptosis measured by the TUNEL technique and the results were plotted in (I) and a small but significant increase of RBP immunoblocking (scale bar in (A) and (B): 50 μ m; scale bar in (D-E) and (G-H): 100 μ m).



RA activity has been involved in the induction of neural differentiation during brain development [40,41] especially in the hindbrain derivatives; however, we previously described this relation in the mesencephalic neuroepithelium in an *in vitro* model. Thus, here we tested *in vivo* the neurogenic influence of the IsO as an RA source and mesencephalic neuroepithelial precursors as target cells.

In order to ascertain whether RA synthesis controlled by E-CSF is involved in mesencephalic neurogenesis *in vivo*, we studied the basic neuroepithelial precursor behavior in the mesencephalon of the chick embryos micro-injected with RBP antibody in the mesencephalic cavity. To test the replicative activity of the mesencephalic neuroepithelial cells, we previously incorporated BrdU into the cells for 1 h and evaluated the number of BrdU positive nucleus in the mesencephalic roof. As is shown in Figure 3A,B,C, the number of positive nuclei does not differ substantially in the RBP immunoblocked embryos with respect to the control ones, which suggests that RBP activity (and subsequently RA) is not related with cellular division in neuroepithelial precursors at this developmental stage. However, when we analyzed the number of β 3-Tubulin young neurons located in the basal side of the mesencephalic neuroepithelium (Figure 3D,E), we identified a clear decrease both in number and in the labeling intensity of β 3-tubulin (Tuj1) positive cells, which, however, maintain their basal location in the neuroepithelium. The data plotted in Figure 3F show a statistically significant decrease of more than 50% of β 3-Tubulin positive cells in RBP immunoblocked embryos compared with the control specimens.

In terms of the presence of apoptotic cells in the mesencephalic neuroepithelium, made manifest by the TUNEL technique, a small number of positive cells are seen in the mesencephalic neuroepithelium of the control embryos; however, in RBP treated embryos there is a statistically significant increase in the number of apoptotic cells, more than double (Figure 3G,H,I). This effect could reflect the apoptosis of precursors unable to differentiate in neurons; however, we cannot rule out a direct influence of RA in neuronal survival.

4. Conclusions

Our results reinforce the hypothesis that, at the earliest stages of development, there is a narrow interrelation between neuroepithelial precursor cells and the CSF, which becomes an inner interneuroepithelial communication path. We also show that specific cellular events, such as neurogenesis, could be regulated by CSF in a non-direct way, such as the regulation of RA synthesis in the IsO. In the adult forebrain a relation has been described between neural precursor cells and Retinoic Acid expression [42], suggesting an ontogenically preserved role of Retinoic Acid in neurogenesis. Finally, we contribute to the knowledge of neural precursor regulatory mechanisms during development, which could be useful in the control of adult brain stem cell behavior taken in account that both (embryonic and adult) precursors seems to be the same cellular lineage [43].

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Author Contributions

J.A. Moro and A. de la Mano were responsible for microaspiration technique of CSF and for microinjection of F9 1.8 cells and RBP antibody in embryonic brain cavity. Both collaborate in the manuscript writing.

E. Carnicero and C. Martín were responsible for F9 1.8 cells culture and identification in embryonic tissues. Both work in manuscript writing.

M.I. Alonso, F. Lamus and R. Carretero were responsible for immunolabelling and TUNEL techniques, confocal images achievement and cellular counting. All three were involved in the results and discussion writing.

A. Gato was responsible for the experimental approach design, control and integration of the results and final version writing of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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