RESEARCH ARTICLE

Cerebrospinal Fluid Control of Neurogenesis Induced by Retinoic Acid During Early Brain Development

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Embryonic-cerebrospinal fluid (E-CSF) plays crucial roles in early brain development including the control of neurogenesis. Although FGF2 and lipoproteins present in the E-CSF have previously been shown to be involved in neurogenesis, the main factor triggering this process remains unknown. E-CSF contains all-trans-retinol and retinol-binding protein involved in the synthesis of retinoic acid (RA), a neurogenesis inducer. In early chick embryo brain, only the mesencephalic-rombencephalic isthmus (IsO) is able to synthesize RA. Here we show that in chick embryo brain development: (1) E-CSF helps to control RA synthesis in the IsO by means of the RBP and all-trans-retinol it contains; (2) E-CSF has retinoic acid activity, which suggests it may act as a diffusion pathway for RA; and (3) the influence of E-CSF on embryonic brain neurogenesis is to a large extent due to its involvement in RA synthesis. These data help to understand neurogenesis from neural progenitor cells. *Developmental Dynamics 00:000–000, 2011.*

Key words: CSF; brain development; retinol binding protein; neural precursors; neural tube; neurogenesis

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INTRODUCTION

At early stages of embryonic development, subsequent to the closure of the anterior neuropore, the architecture of the brain primordium is formed by a pseudo-monostratified neuroepithelium enclosing the brain vesicles, which are completely filled with embryonic cerebrospinal fluid (E-CSF). Recently, it has been shown that E-CSF plays an important role in brain development at both embryonic and foetal stages and performs several crucial functions (reviewed by Gato and Desmond, 2009).

It has previously been reported, in chick and rat embryos at early stages of development, that E-CSF exerts positive pressure against the neuroepithelial walls to generate an expansive force that drives morphogenesis (Gato et al., 1993; Desmond and Jacobson, 1977; Alonso et al., 1998, 1999; Desmond et al., 2005). However, in recent years new roles for E-CSF have been demonstrated in the behaviour of neuroepithelial cell precursors, as it contributes to the regulation of the survival, proliferation, and neural differentiation of the neuroepithelial progenitor cells during early (Gato et al., 2005) and late development (Mashayekhi et al., 2002; Owen-Lynch et al., 2003; Miyan et al., 2003), as well as collaborating with the isthmic organiser in regulating mesencephalic gene expression (Parada et al., 2005a). As a result, neurogenesis is one of the most relevant processes controlled by the action of E-CSF (Gato et al., 2005; Martín et al., 2006), and although it was recently demonstrated that FGF2 contained within chick E-CSF regulates cell proliferation and neurogenesis, the existence of another

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regulatory mechanism has been suggested (Martín et al., 2006; Parada et al., 2008a).

We recently showed that, at the beginning of primary neurogenesis, both avian and mammal E-CSF proteomes include a set of molecules whose roles during development in systems other than E-CSF are related to crucial biological functions (Dziegielewska et al., 1981; Gato et al., 2004; Zapaterra et al., 2007; reviewed by Parada et al., 2007). A remarkable finding that may contribute to an understanding of E-CSF's overall effect on the neurogenesis of neuroepithelial precursors is the presence of retinol-binding protein (RBP) as well as all-trans retinol in both chick and rat E-CSF (Parada et al., 2005b, 2006, 2008b; Parvas et al., 2008). According to the literature, in systems other than E-CSF, RBP specifically binds to *all-trans* retinol, a member of the retinoid family of molecules, which is enzymatically metabolised into retinoic acid (RA), a well-known morphogen that has crucial impact on CNS development (Moro et al., 1993; McCaffery and Dräger, 2000; reviewed by Maden, 2002). Moreover, RA is known to be a neurogenic agent in both embryo and adult neural progenitor cells (Gonçalves et al., 2005; Wang et al., 2005; Jacobs et al., 2006).

RA synthesis requires the concurrence of a precursor molecule, namely all-trans retinol, a carrier molecule such as RBP whose involvement has also been described in the delivery and uptake of all-trans retinol to the cells in which it is enzymatically metabolised in RA (Kawaguchi et al. 2007), and cells expressing the particular enzymes involved in this metabolism, namely, retinaldehyde dehydrogenases (RALDHs; Reijntjes et al., 2005) and retinol dehydrogenases (RDHs; Romand et al., 2008). At the earliest stages of chick embryo brain development, RALDH3 is located in the mesencephalic-rombencephalic isthmus (IsO), which is thought to be the RA source for mesencephalic neuroepithelium in chick embryos (Blentic et al., 2003; Parada et al., 2008b).

In this context, RA diffuses from the clusters of cells expressing these enzymes, such as those forming some of the well-known organising centres, to the target cells (Duester, 2008; Blentic et al., 2003). When RA is taken up by the target cells, it binds to specific nuclear receptors (RAR), regulating a series of genes involved in neural differentiation and patterning of anterior-posterior and dorso-ventral axes (Kolm et al., 1997; Clotman et al., 1997; McCaffery and Dräger, 2000; Begemann and Meyer, 2001; Diez del corral and Storey, 2001; Kudoh et al., 2002; Maden, 2002; McCaffery et al., 2003; Duester 2008).

In this report, we analyse the specific role of the RBP-all-trans retinol system contained within E-CSF in the regulation of mesencephalic neuroepithelial cell neurogenesis in chick embryos at the beginning of primary neurogenesis, by means of RA synthesis in the IsO. Using functional analysis, we demonstrate that E-CSF is a source of *all-trans* retinol for the IsO cells to synthesise RA, and that the presence of RBP in CSF is necessary for the efficient delivery of *all-trans* retinol to IsO cells for such synthesis. Finally, we show that the former mechanism is involved in mesencephalic neurogenesis during early brain development.

RESULTS

Retinoic Acid Synthesis by the IsO Is Controlled by Molecules Contained Within E-CSF

In this study, we first aimed to demonstrate whether a relationship exists between E-CSF composition and RA synthesis in the IsO, as we previously described that in chick embryos (1) this fluid contains the precursor molecule for this morphogen, i.e., all-trans-retinol, as well as the carrier protein, RBP, and (2) IsO cells are the only cells in the early brain primordium neuroepithelium expressing a recognised RA-synthesising enzyme, RALDH3, at this developmental stage. As RA is a relatively unstable compound and is present in very low concentrations in physiological conditions of biological samples, we employed a biological test to detect RA activity, based on the reporter F9-1.8 cell line culture. These cells, derived from a mouse teratocarcinoma, contain a RARE promoter coupled to the lacZ (β-galactosidase) reporter gene, and are very sensitive to RA induction. To test the responsiveness of these cells in our culture conditions, we first cultured F9-1.8 cells in a chemically defined medium supplemented with several different concentrations of commercial RA, which has been shown, by Horton and Maden (1995), to be at a physiological level in various embryonic tissues (Fig. 1A).

We then developed a co-culture technique of IsO cells and F9-1.8 reporter cells to detect the production of RA by the former (Figs. 2A and 3). In order to test basal β-galactosidase activity, we co-cultured these two cell types with a chemically defined medium; few F9-1.8 blue cells (Fig. 3A; see also Fig. 3F for a plotted comparison with other co-culture conditions) were seen, suggesting that in the absence of E-CSF, IsO cells are not self-sufficient to synthesize retinoic acid. Conversely, when F9-1.8 and IsO cells were co-cultured in a defined medium supplemented with E-CSF (Fig. 3B and F), the number of F9-1.8 blue cells significantly increased, suggesting that E-CSF contains factors that activate the synthesis of retinoic acid by the IsO cells.

Due to the presence, as previously described, of all-trans retinol and RBP in the E-CSF, we then checked the role of these two molecules in RA synthesis by the IsO cells. When F9-1.8 and IsO cells were co-cultured in a defined medium supplemented with both commercial all-trans retinol and RBP (Fig. 3C and F), the number of blue F9-1.8 cells was significantly greater than for the basal level of the chemically defined medium (Fig. 3A and F), and, what is interesting, greater also than the cells cultured in the presence of E-CSF. However, when these cells were co-cultured with a defined medium supplemented only with all-trans retinol (Fig. 3D and F), the number of blue F9-1.8 cells was similar to that for the basal condition (Fig. 3A and F), which supports the involvement of RBP in this process.

To check this point, we then co-cultured F9-1.8 and IsO cells with a defined medium supplemented with E-CSF, to which antibodies specifically recognising RPB were added to block its biological activity (Lidén and Erickson, 2005). In this experiment (Fig. 3E and F), the number of blue

F9-1.8 cells was significantly lower than when only E-CSF, or alternatively retinol + RBP, was present, but also significantly higher than for the basal level (Fig. 3A and F). This result confirms the involvement of RBP contained in CSF, in RA synthesis by the IsO. Taken together, these results suggest that (1) the retinol contained within E-CSF is the substrate for IsO cells to synthesise RA, and (2) the RPB contained within E-CSF is needed for all-trans retinol to reach the IsO cells.

We then checked whether the F9-1.8-induced activity exerted by the IsO cells in the presence of E-CSF in the culture medium, or, alternatively, by retinol + RBP, was due specifically to IsO cells, and thus to the RA they synthesise by means of RALDH3. In order to do this, we co-cultured F9-1.8 reporter cells with dorsal mesencephalic neuroepithelial cells not containing the IsO (Figs. 2B and 4). When these cells were co-cultured with the chemically defined medium (Fig. 4A; see also Fig. 4D for a plotted comparison with other co-culture conditions), the number of blue F9-1.8 cells was very low, similar to the basal condition in which F9-1.8 cells were co-cultured with IsO cells together also with the defined medium (Fig. 3A and F). Surprisingly, when these cells were co-cultured with the defined medium supplemented with E-CSF, the number of blue F9-1.8 cells significantly increased (Fig. 4B and D). In contrast, when these cells were co-cultured with the defined medium supplemented with commercial all-trans retinol + RBP (Fig. 4C and D), the number of blue cells was not significantly different from those for the basal condition. Taken together, all such findings rule out unknown RALDH activity in mesencephalic cells and strongly suggest the presence of retinoic acid within the E-CSF, directly capable of activating the reporter promoter of F9-1.8 cells, supporting the idea of CSF as a diffusion way for retinoic acid.

To further check whether E-CSF has a direct role in the reporter activity of F9-1.8 cells independent from IsO cells, i.e., if RA activity is involved, we cultured F9-1.8 cells in the absence of neuroepithelial cells with a defined medium supplemented with E-CSF at several different concentrations, thereby performing a dose-response plot. As shown in Figure 1B, the number of blue F9-1.8 cells induced by E-CSF increased in parallel to their concentration in the culture medium, which strongly supports the presence of RA activity within this embryo fluid; in addition, they were similar to values plotted in our RA dose response, where the highest dose we used was 12% of CSF in the culture medium (compare Fig. 1A with B).

RBP Contained Within E-CSF Contributes to Mesencephalic Neurogenesis Regulation by Controlling RA Synthesis

Since the involvement of RA had been previously noted in the induction of neurogenesis in specific regions of the central nervous system, we then aimed to demonstrate that the capacity of E-CSF to control RA synthesis in the IsO cells affects neuroepithelial neurogenesis.

In order to show the role of the specific elements of the RBP/retinol-IsO-RA system contributing to primary neurogenic induction from the E-CSF, and so as to analyse the possible synergistic involvement of other neurogenic factors also contained within E-CSF (e.g., FGF2), an organotypic tissue culture of mesencephalic+IsO neuroepithelium was used. All the elements required to investigate neurogenesis activation by RA were included in this in vitro approach, i.e., *raldh-3*-expressing IsO cells, mesencephalic neuroepithelium capable of responding to neurogenic RA induction, and a chemically defined medium in which the different elements could be added or subtracted (see Fig. 6).

Organotypic mesencephalic+IsO neuroepithelium cultures were made under several different conditions to evaluate induced neurogenesis (Fig. 5), which was detected by beta3-tubulin immunostaining. As previously reported (Gato et al., 2005), the E-CSF present in the culture medium is capable of sustaining neuroepithelial cell survival as well as activating proliferation and neurogenesis at a level similar to that for embryos developed in ovo. Thus, as a positive control, we used explants cultured with E-CSF-supplemented medium (Fig. 5A; see also Fig. 5H for a plotted comparison between different culture conditions); this represented the maximum induction of neurogenesis mediated by E-CSF under these culture conditions. As a negative control, we used explants cultured only in a chemically defined medium (Fig. 5B and H), which represented the basal neurogenic capacity of the neuroepithelium alone, and/or due to the accumulated factors it might still contain after a 24-hr culture. This value was considered to be inherent to all culture conditions.

Since the neurogenic role of FGF2 contained within E-CSF at this developmental stage has also been previously described (Martín et al., 2006), we checked neurogenic induction of FGF2 in this experimental model system. To achieve this, we cultured mesencephalic+IsO explants with a medium supplemented with FGF2 (Fig. 5C and H) and the number of beta3-tubulin expressing cells was significantly higher than in the negative controls. Similarly, as it has been proposed that RA has neurogenic properties, we cultured explants with the medium supplemented with commercial all-trans RA (Fig. 5D and H), the number of beta3-tubulin-expressing cells being similar to that for explants cultured in the presence of FGF2. Subsequently, to verify the potential synergistic effect of RA and FGF2, mesencephalic+IsO explants were cultured in a chemically defined medium supplemented with both RA+FGF2. In these explants (Fig. 5E and H), the number of beta3-tubulin-expressing cells was higher than those cultured in the medium supplemented only with RA or, alternatively, with FGF2, and similar to the explants cultured in the E-CSF-supplemented medium. Taken together, these results suggest that both FGF2 and RA are synergistically involved in primary neurogenesis induced by E-CSF in mesencephalic progenitor cells.

Following this, and to confirm the involvement of the RBP-retinol system contained in E-CSF in inducing primary neurogenesis through the retinol uptake of IsO cells and its subsequent conversion to RA, mesencephalic+IsO explants were cultured with a medium supplemented with E-CSF, to which an antibody blocking RBP biological activity had been added (Fig. 5F and H). In these

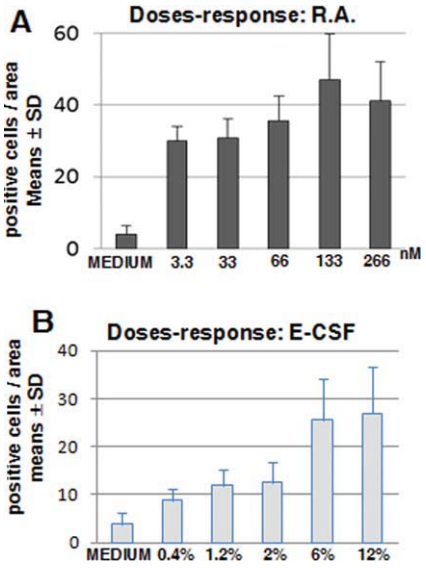

Fig. 1. Dose–response of F9-1.8 cells. **A:** Dose-response plot of F9-1.8 cells to different nM concentrations of commercial RA. **B:** Dose-response plot of F9-1.8 cells cultured with different concentrations of E-CSF.

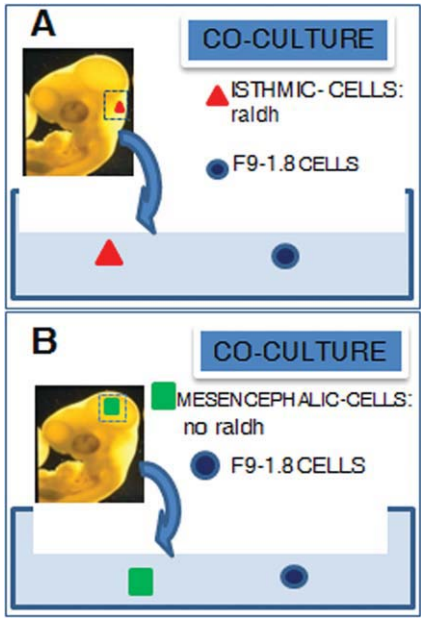

Fig. 2. Diagrams showing the co-culture of F9-1.8 cells with neuroepithelial cells. **A:** Co-culture of F9-1.8 cells with IsO cells. **B:** Co-culture of F9-1.8 cells with mesencephalic cells lacking the IsO.

explants, the number of beta3-tubulin-expressing cells was drastically reduced with respect to the positive controls, although there were more positive cells than in the negative controls; this demonstrates the involvement of RBP in the induction of primary neurogenesis and suggests that E-CSF

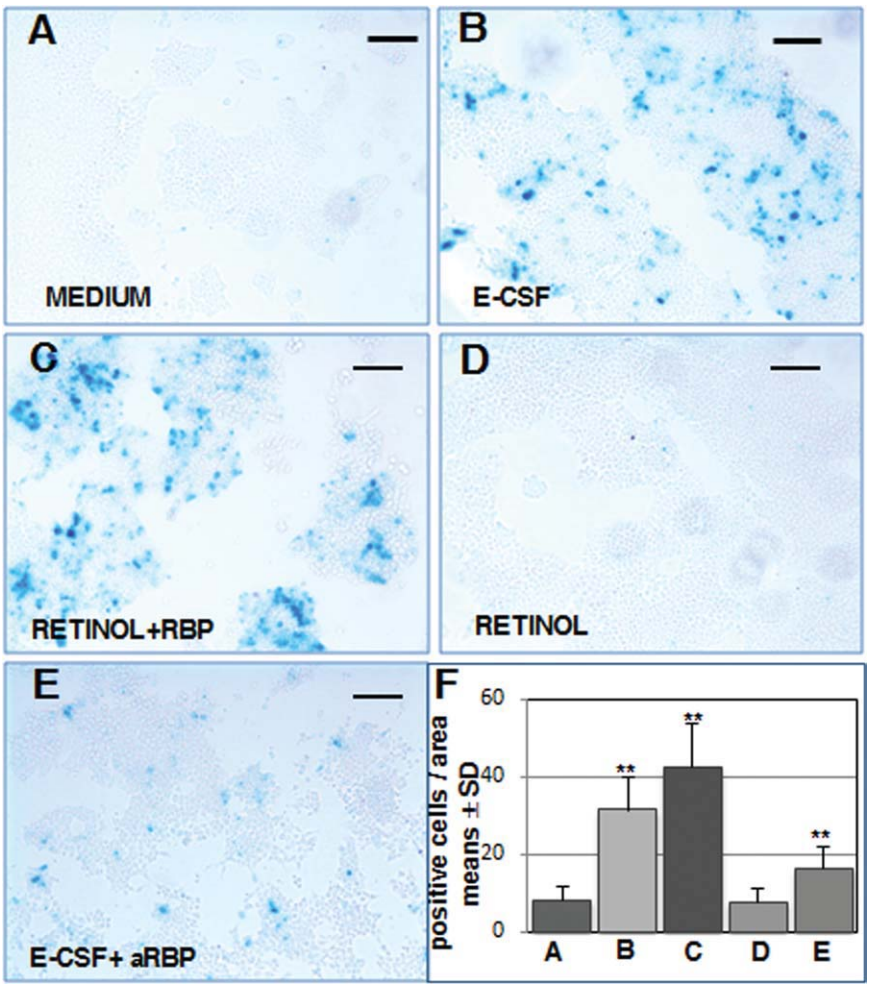

Fig. 3.

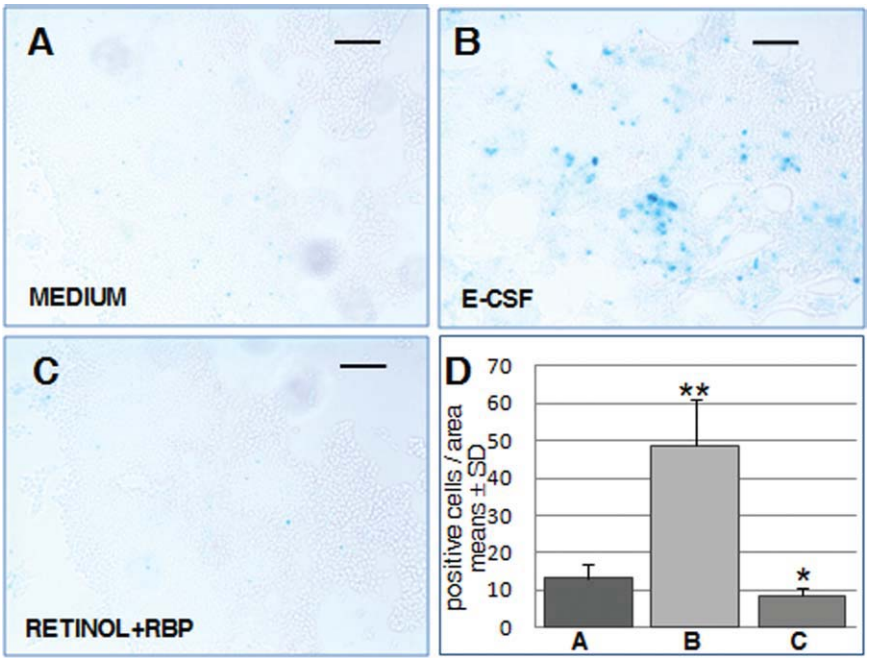

Fig. 4.

contains other factors apart from RA involved in neural differentiation, such as, for example, FGF2.

Alternatively, explants were cultured with a medium supplemented with *all-trans* retinol+RBP to emulate physiological conditions (Fig. 5G and H). In these explants, the number of beta3-tubulin-positive cells was significantly higher than in the negative controls, and similar to that for explants cultured in the presence of RA, which suggests that RBP is needed to produce the maximum rate of RA synthesis in the IsO cells. Taken together, these results show that RBP from CSF indirectly contributes to neural mesencephalic differentiation, promoting the conversion of retinol into RA in the IsO.

DISCUSSION

In this report, we have shown that E-CSF plays a role in regulating RA synthesis in the mesencephalic-romboencephalic isthmus (IsO) during early chick development, and that, to some extent, this is a means by which E-CSF controls neuroepithelial neurogenesis. These results (summarized in Fig. 6) contribute to an understanding of the general role of E-CSF during early brain development.

E-CSF Contributes to Regulating RA Synthesis in the IsO

The classic view is that IsO is a source of RA, mainly due to the expression of enzymes involved in RA synthesis, i.e., RALDHs and RDHs (Reijntjes et al., 2005; Parada et al., 2008b; Romand et al., 2008). In mouse embryo models, the involvement of these enzymes in RA synthesis has been shown by using a set of knockouts (Niederreither et al., 2002; Mic et al., 2002; Ribes et al., 2006; Molotkova et al., 2007). In avians, however, no equivalent functional evidence exists, so further experimental approaches are needed. Our data, based on the co-culture of IsO cells and F9-1.8 cells engineered to monitor the presence of RA, indicate that in chick embryos, the IsO cells actively produce RA. Thus, at early stages of brain development, IsO can be considered a local source for this morphogen. Therefore, due to our knowledge that IsO is an organising centre controlling the expression of genes involved in CNS regionalisation and patterning, some of its recognised capacities as an organising centre may be the result of their capacity to produce RA. However, it should be noted that RALDH3 is not expressed in mouse Iso, but at later stages of development RALDH2 is expressed in hindbrain meninges (McCaffery et al., 2006).

Hence, one of the main contributions of this study is the demonstration that E-CSF is involved in the control of RA synthesis in the IsO. We demonstrate that E-CSF is also able to regulate the activity of one specific neuroepithelial functional area, the IsO, which indicates that E-CSF does not only have general roles for all neuroepithelial cells, but also particular roles for different populations of neuroepithelial cells.

In a previous study, we demonstrated that the presence of RBP and all-trans retinol in the E-CSF is dynamic and varies over time, and we suggested that their changes in concentration may be related to their specific roles in RA synthesis (Parada et al., 2008b). The results presented in this report demonstrate that E-CSF regulation of RA synthesis in the IsO is exerted by means of RBP and the all-trans retinol it contains. It is interesting to point out that E-CSF not only acts as a retinol reservoir for the IsO, but also facilitates its uptake by means of RBP, which therefore may act as a control mechanism for RA synthesis, suggesting a new role for E-CSF on brain development.

The traditional opinion regarding RA diffusion into cells is that it is stored as a gradient in the extracellular matrix. Our results strongly suggest the existence of another source of RA in the E-CSF. Our findings imply a previously undocumented means of possible RA supply to all brain cavity neuroepithelial cells during early stages of development, namely, via this embryonic fluid. This RA diffusion channel may act in parallel with other RA sources, such as, for example, RA synthesised by the frontonasal surface ectoderm, known to act on the prosencephalon neuroepithelium (Molotkova et al., 2007). This should be taken into account in order to understand the general role of RA on brain development. It is, therefore, interesting to note that in adult choroids plexus there is also the expression of RALDHs and RDHs, which suggests a similar diffusion method for RA in the adult brain, via CSF in the brain ventricles.

RA Is Involved in Neurogenesis Induced by E-CSF

As has been stated above, E-CSF plays a role in controlling embryonic neurogenesis. Some particular factors have also been associated with early brain neurogenesis, such as, for example, FGF2 and certain lipoproteins, although it is unclear whether they act as direct activators of neurogenesis or by indirect means. For

Fig. 3. E-CSF regulation of RA synthesis by the IsO cells (see also Fig. 6A). **A:** X-gal development of F9-1.8 cells after co-culture with IsO cells in chemically defined medium. **B:** X-gal development of F9-1.8 cells after co-culture with IsO cells in E-CSF supplemented medium. **C:** X-gal development of F9-1.8 cells after co-culture with IsO cells in defined medium supplemented with commercial all-trans retinol and RBP. **D:** X-gal development of F9-1.8 cells after co-culture with IsO cells in defined medium to which all-trans retinol has been added. **E:** X-gal development of F9-1.8 cells after co-culture with IsO cells in E-CSF supplemented medium to which antiRBP commercial antibody has been added. **F:** Quantitative analysis of F9-1.8 blue cells under several different co-culture conditions. Foot letters of each bar correspond to letters of picture in this same figure. Asterisks denote values that differ significantly ($P < 0.05$) from controls according to the two-tailed Student's *t*-test (see Table 1). Scale bar = 100 μm.

Fig. 4. E-CSF regulation of RA synthesis by the IsO cells; co-culture of F9-1.8 cells with mesencephalic cells lacking the IsO (see also Fig. 6B). **A:** X-gal development of F9-1.8 cells after co-culture with mesencephalic cells in chemically defined medium. **B:** X-gal development of F9-1.8 cells after co-culture with mesencephalic cells in E-CSF supplemented medium. **C:** X-gal development of F9-1.8 cells after co-culture with mesencephalic cells in defined medium supplemented with commercial all-trans retinol and RBP. **D:** Quantitative analysis of F9-1.8 blue cells under several different co-culture conditions. Foot letters of each bar correspond to letters of picture in this same figure. Asterisks denote values that differ significantly ($P < 0.05$) from controls according to the two-tailed Student's *t*-test (see Table 2). Scale bar = 100 μm.

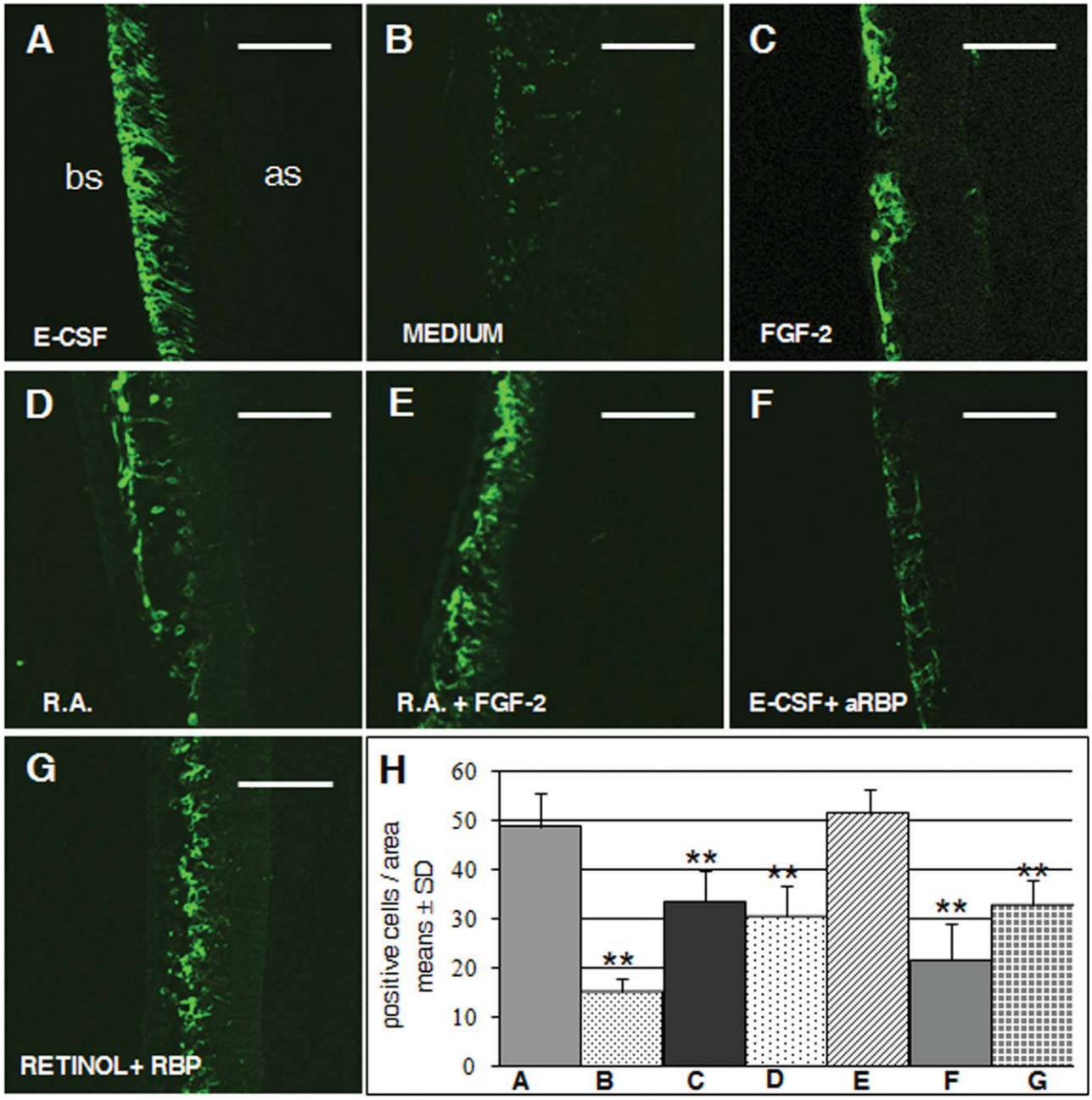

Fig. 5.

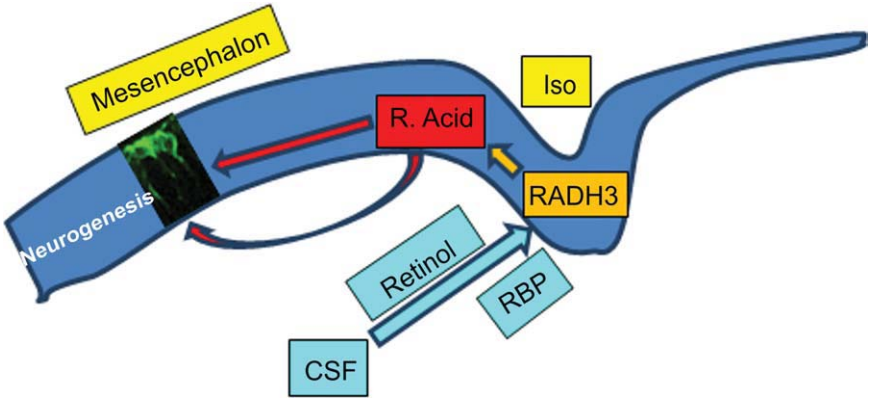

Fig. 6.

example, it has been suggested that FGF2 may act on neurogenesis by driving cells to terminal mitoses (Martin et al., 2006), and that the action of lipoproteins in this cellular process is the supply to the neuroepithelial cells of the necessary building materials (Parada et al., 2008b). Moreover, the presence of other neurogenic factors in the E-CSF has been suggested to account for overall neurogenesis (Martin et al., 2006; Parada at al., 2008a; Gato and Desmond, 2009). Our results show that mesencephalic neurogenesis

TABLE 1. Coculture of F9-1.8 Cells With Iso Cells[a]

	Medium	E-CSF	RETINOL+RBP	RETINOL	E-CSF +aRBP
Means	8.17	31.35*	42.52*	7.85	16.60*
SD	±3.55	±8.91	±11.29	±3.52	±5.67

[a]Values and statistical significance plotted in graph bars of Figure 3.
*$P < 0.001$.

TABLE 2. Coculture of F9-1.8 Cells With Mesencephalic Cells[a]

	Medium	E-CSF	RETINOL+RBP
Means	13.10	4.82**	8.53*
SD	±3.88	±12.26	±2.10

[a]Values and statistical significance plotted in graph bars of Figure 4.
*$P < 0.05$.
**$P < 0.001$.

in chick embryos is also driven by RA activity, which comes from the IsO, which in turn is regulated by E-CSF via the RBP and the all-trans retinol it contains. This concurs with previous reports demonstrating that RA is one of the early signalling components in the chain of molecular events that results in fully differentiated neurons in specific regions of the central nervous system (McCaffery and Dräger, 2000). Here, data presented in this report offer a better understanding of the mechanisms regulating neurogenesis from neural progenitor cells in the mesencephalon.

Furthermore, the presence of all molecular and enzymatic elements needed for RA activity taking place in adult brain has been demonstrated, especially in areas that are known to display neurogenesis from neural stem cells, like, for example, the hippocampus and the subventricular zone (McCaffery et al., 2006). In addition, a direct relationship between RA and neurogenesis in these brain areas has been demonstrated (Jacobs et al., 2006 and Bonnet et al., 2008, for hippocampus; Wang et al., 2005, and Zhang et al., 2006, for the subventricular zone). In this regard, although RA is not alone in inducing the different steps in neuronal differentiation, it may act as a master control in certain regions of the central nervous system by inducing the expression of a variety of factors that control differentiation (Joh et al., 1992; Kaplan et al., 1993; Afink et al., 1995; Xie et al., 1997; Sizemore et al., 1998; Yoshizawa et al., 1998; Maden, 2002; Duester, 2008). Consequently, the knowledge of the mechanisms involved in neurogenesis activation from neural precursors in both embryonic and adult brain, may be a hidden key to developing neuro-regenerative strategies based on the expansion and differentiation of neural precursor populations in the adult brain.

EXPERIMENTAL PROCEDURES

Obtaining Embryonic Cerebrospinal Fluid

Fertile chicken eggs were incubated at 38°C in a humidified atmosphere to obtain chick embryos at the desired developmental stages. Embryos were incubated to stage HH24 (Hamburger and Hamilton, 1951) to obtain E-CSF. After dissection of the embryos from the extra-embryonic membranes, E-CSF was aspirated as previously described (Gato et al., 2004). To minimize protein degradation, E-CSF samples were kept at 4°C, aliquoted, lyophilized, and frozen at −40°C, until they were used.

Organotypic Cultures

Organotypic cultures of both mesencephalic + IsO neuroepithelial explants were produced as described by Gato et al. (2005). Briefly, Mesencephalic + IsO neuroectodermal explants were cultured in vitro for 24 hr, between HH20 and HH23, fixed peripherally to small rectangles of Millipore (Billerica, MA) filters (0.8 μm pore size) with a tungsten needle, and then cultured with a chemically defined serum-free medium (basal medium; DMEM:F12; Sigma, St. Louis, MO) with 1% ascorbic acid, or, alternatively, with this medium supplemented with various additives, at 37°C and with 5% CO_2.

To generate different experimental conditions, the following additives were administered to 250 μl of the basal medium: 15% E-CSF; FGF2 (50 ng

Fig. 5. RBP regulation of RA neurogenic induction in vitro monitored by the presence of beta3-tubulin-positive cells. **A:** Mesencephalic+IsO neuroepithelum explants cultured in vitro with E-CSF supplemented medium. Note the presence of beta3-tubulin-positive cells. **B:** Mesencephalic+IsO neuroepithelum explants cultured in vitro with chemically defined medium. Note that the number of beta3-tubulin-positive cells is much lower than in explants cultured with E-CSF supplemented medium. **C:** Mesencephalic+IsO neuroepithelum explants cultured in vitro with defined medium to which commercial FGF2 has been added. **D:** Mesencephalic neuroepithelum+IsO explants cultured in vitro with defined medium to which commercial RA has been added. **E:** Mesencephalic+IsO neuroepithelum explants cultured in vitro with defined medium to which both commercial RA and FGF2 have been added. **F:** Mesencephalic+IsO neuroepithelum explants cultured in vitro with E-CSF supplemented medium to which antibody to RBP has been added to block its biological activity. **G:** Mesencephalic+IsO neuroepithelum explants cultured in vitro with defined medium to which commercial all-trans retinol and RBP has been added. **H:** Quantitative analysis of beta3-tubulin-positive cells under several different culture conditions. Foot letters of each bar correspond to letters of picture in this same figure. Asterisks denote values that differ significantly ($P < 0.05$) from controls according to the two-tailed Student's *t*-test (see Table 3). Scale bar = 50 μm. All images have the same orientation as A. bs, basal side; as, apical side.

Fig. 6. Diagram showing the proposed mechanism to CSF influence in neurogenesis via RA. Schematic diagram showing how E-CSF contributes to RA synthesis. Dorsal mesencephalon and mesencephalo-rombencephalic istmo (Iso) regions are represented in dark blue. RA requires the uptake of retinol, mediated by retinol binding protein, from E-CSF (Sky blue) to Iso cells which expresses RALDH3 (orange). We propose that RA diffuses (red) from the neuroepithelium or E-CSF to target cells to induce neurogenesis (green cells).

TABLE 3. Mesencephalic + Iso Cells Explants Culture[a]

	E-CSF	Medium	FGF-2	R.A.	R.A. + FGF-2	E-CSF +aRBP	RETINOL +RBP
Means	49.80	15.25*	33.62*	30.50*	51.58	21.68*	27.80*
SD	±6.79	±2.50	±6.37	±6.45	±4.94	±7.48	±5.30

[a]Values and statistical significance plotted in graph bars of Figure 5.
*$P < 0.001$.

of human recombinant; Sigma); Retinoic Acid (RA; 33 nM of *all-trans* retinoic acid; Sigma); RA + FGF2 (at stated concentrations); 15% E-CSF + antibody to RBP (20 ng; Labvision, Fremont, CA) and *all-trans* retinol (340 nM; Fluka) + RBP (500 ng; Sigma).

Culture of F9-1.8 Cell Line and Co-Culture With Neuroepithelial Cells

F9-1.8 cell line was maintained as previously described (Schulz and Gais, 1989) on gelatine-coated tissue culture flasks in Advanced-DMEM (Gibco, Gaithersburg, MD) supplemented with 7.5% heat-inactivated FBS, 44 nM $NaHCO_3$, and 4 μg/ml G418 (Sigma). The cells were plated for experimentation at a density of 3.0×10^4 cells/cm^2 and cultured overnight at 37°C in a 5% CO_2 atmosphere. This cell line contains the RARE promoter coupled to the *lacZ* (β-*galactosidase*) reporter gene.

For the co-culture of F9-1.8 cells with neuroepithelial cells, the surface ectoderm and most of the mesenchyme were removed with tungsten needles and then 4 small pieces of IsO neuroepithelium (including the tip of the dorsolateral Iso fold, which encompassed the area of RALDH3 expression), or, alternatively, 3 small pieces of dorsolateral mesencephalic neuroepithelium (without RALDH expression), in order to obtain an equivalent amount of tissue, were micro-dissected and mechanically disaggregated in 4°C Ringer and recovered in pellet following centrifuging. After re-suspending the cells in DMEM:F12:G418, they were added to the wells containing F9-1.8 cells (Fig. 6A and B). They were then co-cultured for 24 hr at 37°C in a 5% CO_2 atmosphere. To generate the different experimental conditions, the following additives were administered to 300 μl of the basal medium: RA (3.3, 33, 66, 133, and 266 nM of *all-trans* retinoic acid; Sigma); E-CSF at 15%; E-CSF (15%) + antibody to RBP (20 ng; Labvision); *all-trans* retinol (340 nM; Fluka, St. Louis, MO) + RBP (500 ng; Sigma); and *all-trans* retinol (at the concentration stated above). In some experiments, F9-1.8 cells were cultured in the absence of neuroepitheial cells, in the basal medium supplemented with several different concentrations of E-CSF (0.4, 1.2, 2, 6, and 12%).

Colour Development of F9-1.8 Cells

After experiments with the F9-1.8 cells, these were fixed in 4% paraformaldehide in PBS, washed in PBS, and subsequently in de-ionised water, and exposed to 20 mg/ml of X-gal solution (Sigma) for 1 hr at 37°C, as described by Sonneveld et al. (1999). Culture wells have 1.9 mm^2, the F9-1.8-positive cells were counted in 20 different, randomly selected, areas of 0.55 mm^2 for each experimental condition (4 areas per each well, from 5 different wells). The results were expressed as means of positive cells/area and standard deviation, and the significant differences were evaluated by a two-tailed Student's *t*-test.

Beta3-Tubulin Determination

In order to detect early neuronal differentiation, we monitored beta 3-tubulin (Tuj-1) expression. After organotypic culture, the explants were fixed in Carnoy for 20 min, dehydrated in an alcohol series, passed through xylene, and embedded in paraffin. After the tissues had been cut transversally, they were de-paraffinized and subsequently incubated with a monoclonal antibody anti-beta3-tubulin at 1/500 (BAbCO) and an anti-mouse antibody conjugated to FITC at 1/64 (Sigma) for 1 hr at RT. For visualization and photographing of the preparations, we used a confocal microscope (Zeiss LSM-310; Zeiss, Thornwood, NY). A quantitative analysis of beta3-tubulin localization was performed by counting the number of neuroepithelial cells with immunostained cytoplasm in 20 1,900μm^2 microscopic areas, taken from the central area of each explant and from 5 different explants. The average of each condition and the standard error were plotted, and their significance was tested by an unpaired two-tailed Student's *t*-test.

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