All-trans retinol and retinol-binding protein from embryonic cerebrospinal fluid exhibit dynamic behaviour during early central nervous system development

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Embryonic cerebrospinal fluid (E-CSF) is involved in the regulation of survival, proliferation and neurogenesis of neuroectodermal progenitor cells, as well as in the control of mesencephalic gene expression in collaboration with the isthmic organizer. Recently, we showed the presence of retinol-binding protein (RBP) within the E-CSF proteome. RBP is an *all-trans* retinol carrier, a molecule that can be metabolized into retinoic acid, a morphogen involved in central nervous system (CNS) morphogenesis and patterning. Here we demonstrate the presence of *all-trans* retinol within the E-CSF and analyse the dynamics of RBP and *all-trans* retinol within this fluid, as well as the expression of retinoic acid-synthesizing enzymes during early CNS development. Our results suggest a relationship between the dynamics of these molecules and the early events of CNS patterning. *NeuroReport* 19:945–950

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Introduction

The embryonic cerebrospinal fluid (E-CSF) is a protein-containing fluid that is in contact with the apical surface of the neuroectodermal cells. In recent years, it has been shown that E-CSF has an important role in brain development at both embryonic and foetal stages [1]. It has been reported that (i) E-CSF exerts positive pressure against the neuroepithelial walls to generate an expansive force [2,3]; (ii) it contributes to the regulation of the survival, proliferation and neural differentiation of the neuroectodermal progenitor cells [4]; (iii) it collaborates with the isthmic organizer in the regulation of mesencephalic gene expression [5]; and (iv) at foetal stages, it controls the formation of the layers of neurones in the cerebral cortex as well as neuronal proliferation [6,7].

We have recently shown that both avian and mammal E-CSF proteomes include molecules, which have roles during development in systems other than E-CSF are related to the functions described above for this fluid [8,9]. The analyses of the particular roles of some of these molecules has shown that one of the growth factors contained within E-CSF, fibroblast growth factor-2, is responsible for neuroepithelial cells proliferation and also at least partially for the neurogenic properties of this fluid [10]. Moreover, we have also recently demonstrated the involvement of lipoproteins contained within the E-CSF in anterior central nervous system (CNS) neurogenesis (unpublished data) [11].

A remarkable finding that may contribute to the understanding of the overall effect of E-CSF on CNS development is the presence of plasma retinol-binding protein (RBP) and transthyretin (TTR) in both chick and rat E-CSF proteomes by proteomic analyses [8,9]. TTR forms a stable plasmatic complex with RBP that prevents the extensive loss of the latter [12]. According to the literature, RBP specifically binds to *all-trans* retinol to deliver it to the cells where it is enzymatically metabolized into the actual morphogen, retinoic acid (RA). RA has crucial roles in several developmental processes such as neural differentiation and patterning of the anterior–posterior and dorsoventral axes (reviewed in Refs [13–15]).

Despite extensive studies on the roles of RA in CNS formation, however, there are some questions remaining, for example, the route by which *all-trans* retinol reaches the cell clusters expressing the RA synthesizing enzymes. Within the embryo, only certain clusters of cells express the enzymes involved in the transformation of *all-trans* retinol into RA, that is, the alcohol-dehydrogenases and retinol-dehydrogenases (RALDHs) [16]. To contribute to the understanding of the function of E-CSF in CNS development, in this study we focus on the dynamics of retinoid system molecules contained within this fluid, that is, RBP

and retinoid derivatives, compared with the retinol-processing enzymes in the neuroepithelium, that is, RALDHs, at the moment of maximum anterior CNS neurogenesis. Our results suggest that the RBP-retinol system from E-CSF has a role in anterior CNS development and that E-CSF delivers retinol to CNS cells expressing RALDHs.

Materials and methods

In-situ hybridization

Fertile chicken eggs were incubated at 38°C in a humidified atmosphere to obtain chick embryos at developmental stages HH24, HH27 and HH29 [17]. After dissecting the embryos out of extraembryonic membranes, RNA in-situ hybridization on whole-mount embryos was performed by using single-stranded digoxigenin (Boehringer Mannheim, Mannheim, Germany) antisense riboprobes as described previously[18]. *Raldh-1* riboprobe (ClaI digestion and T3 transcription), *raldh-2* riboprobe (SacII digestion and SP6 transcription) or *raldh-3* riboprobe (XhoI digestion and SP6 transcription; all from M. Maden) were synthesized according to standard protocols. Before hybridization, embryos were cut sagittally into two halves to facilitate hybridization and to avoid trapping of reagents in the cephalic cavities. For viewing and photography we used a Stemi V6 stereomicroscope (Carl Zeiss, Inc., Madrid, Spain).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis, western blot and slot-blot analyses

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed under denaturing conditions according to the method of Laemmli [19], with a Miniprotean II electrophoresis system (BioRad Laboratories, Inc., Madrid, Spain). E-CSF from embryos at developmental stage HH24 was used. The samples were obtained by microaspiration by using a Drummond microinjector in a reverse mode. To minimize protein degradation, E-CSF was aliquoted and stored at −80°C until used. Molecular mass standards of high and low range (BioRad) were also used. For western blot analysis, proteins were electrotransferred from the electrophoresis gel to nitrocellulose membrane (*Trans*-blot transfer medium, BioRad), for 1 h at 100 v, using a basic transfer buffer following standard protocols. The presence of RBP was detected with an antibody to RBP (Labvision, Astmoor, UK) mouse anti-RBP, 1/2000 in phosphate-buffered saline, and subsequently with a secondary antibody (anti-mouse IgG, Sigma-Aldrich, Madrid, Spain) conjugated to peroxidase. Anti-RBP antibody preabsorbed with commercial human RBP was used as a negative control. Briefly, human RBP (1 mg/ml, Sigma) was denatured at 94°C since according to the supplier the antibody anti-RBP recognizes an RBP epitope that becomes accessible only either on unfolded RBP or upon binding of native RBP to TTR. Denatured RBP was bound to enzyme-linked immunosorbent assay plates for 1 h at 37°C, washed in phosphate-buffered saline and afterwards incubated with the commercial antibody to RBP overnight at 4°C. The preabsorbed supernatant was used instead of primary antibody. The immunoblotting was developed using 3,2-diaminobenzidine (Sigma).

For slot-blot analysis, E-CSF from embryos at the mentioned developmental stages was loaded directly onto nitrocellulose membranes using a microsample filtration manifold device (Minifold II, Schleicher&Schuell). The presence of native RBP (i.e. RBP bound to TTR) was detected using the immunostaining protocol described above for western blot analysis. RBP concentrations were calculated by densitometry analysis using Scion Image, (Frederick, Maryland, USA) software.

High performance liquid chromatography and mass spectrometry analysis

For identification of retinoids a high performance liquid chromatography (HPLC) analysis was performed. Twenty-five micro litre of E-CSF were injected into Kromasil 100 C18 5 μm 25 × 0.46 cm (Tracer N33692) columns. HPLC was performed using the Alliance 2695 device (Waters Corporation, Milford, USA) at a flow rate of 1.8 ml/min and a detection period of 20 min. PDA 2996 (Waters) was used as a detector and all wavelengths were checked. Results were analysed using the Empower software (Waters). *All-trans* retinol and RA standards were used (Sigma). Mass spectrometry was performed from the HPLC peak using a platform II mass spectrometer and was analysed with the MassLynx (Micromass Ltd, Manchester, UK) software. Each analysis was performed in triplicate. The average of each condition and the standard deviation were plotted.

Results

All-trans retinol detection within the embryonic cerebrospinal fluid

Using two-dimensional electrophoresis, protein sequencing and database searches, we have previously reported the presence of RBP, a known *all-trans* retinol carrier, within the E-CSF proteome of chick embryos at HH24, the developmental stage that falls within the period of maximum anterior CNS neurogenesis. To further analyse whether the RBP contained within E-CSF may be involved in carrying retinoids to the neuroectoderm where they could be used as morphogenetic agents for neurogenesis and patterning, we first checked whether vitamin A and/or its derivatives were also present within E-CSF at this developmental stage (HH24). A peak with a retention time of 9,450 min at 325 nm, corresponding to the *all-trans* retinol standard, was clearly detected by HPLC (Fig. 1a). The identification of the molecular nature by mass spectrometry of the sorted HPLC peak was consistent with *all-trans* retinol (Fig. 1b). No other peaks were detected at either different retention times or different wavelengths, including the one corresponding to the RA standard.

All-trans retinol and retinol-binding protein dynamics within the embryonic cerebrospinal fluid

Taking into account the rapid morphological changes that neuroepithelial cells undergo during early CNS development, and considering the reported roles for retinoids in its patterning, we then analysed the dynamics of both *all-trans* retinol and RBP within E-CSF at several different developmental stages, centred on the moment of maximum neurogenesis, from HH20 to HH29 (i.e. HH20, HH24, HH27 and HH29). HPLC analysis of *all-trans* retinol content showed that its concentration increased logarithmically during the period analysed, with its maximum increase from HH20 to HH24. Again, no other peaks were detected at any of the stages analysed.

The dynamics of RBP concentration within the E-CSF was analysed by slot-blot using a specific anti-RBP commercial

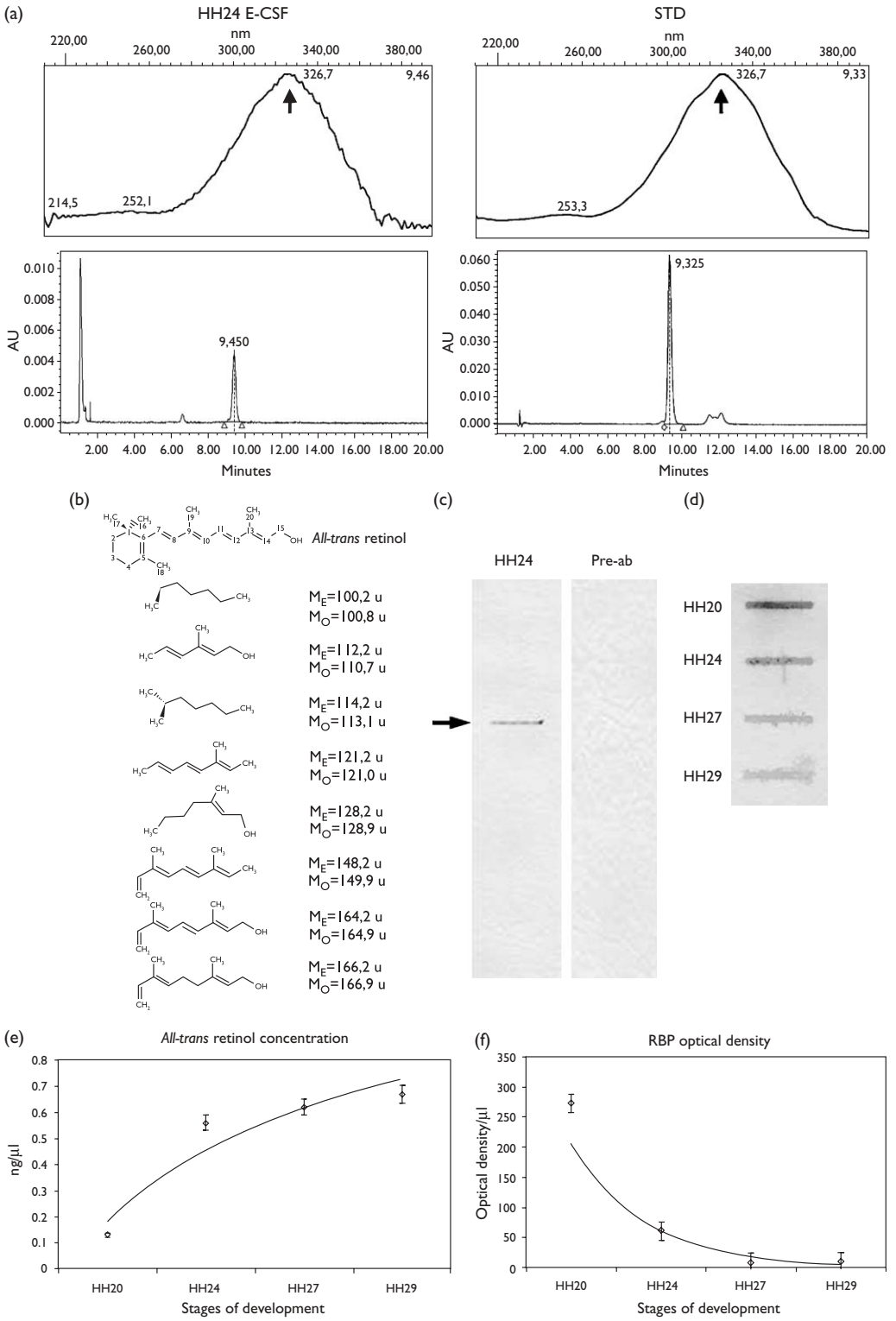

Fig. 1 *All-trans* retinol and retinol-binding protein (RBP) within embryonic cerebrospinal fluid (E-CSF). (a) High performance liquid chromatography (HPLC) chromatograms corresponding to chick E-CSF at developmental stage HH24 (left) and a standard solution containing *all-trans* retinol (right). Note a peak at the same retention time and with the same absorbance spectrum in both chromatograms. (b) Mass spectrometry analysis of the sorted HPLC peak of the E-CSF sample, corresponding to *all-trans* retinol. (c) Western blot of E-CSF using a commercial antibody to RBP and a preabsorbed anti-RBP antibody as a negative control. The arrowhead shows the band corresponding to RBP. (d) Slot-blot detection of RBP in E-CSF samples from HH20 to HH29 by using anti-RBP. (e) Plot of *all-trans* retinol concentration dynamics in the E-CSF, from HH20 to HH29, as analysed by HPLC. Note the logarithmical-like increase in *all-trans* retinol concentration. Standard deviation is plotted as a vertical bar at each analysed stage. (f) Plot of RBP dynamics by densitometry analysis of the E-CSF from the slot-blot data. Note its negative exponential-like dynamics. Standard deviation is plotted as a vertical bar at each analysed stage. aRBP, anti-RBP; M_E, expected molecular mass; M_O, observed molecular mass; Pre-ab, preabsorbed anti-RBP; *u*, atomic mass unit.

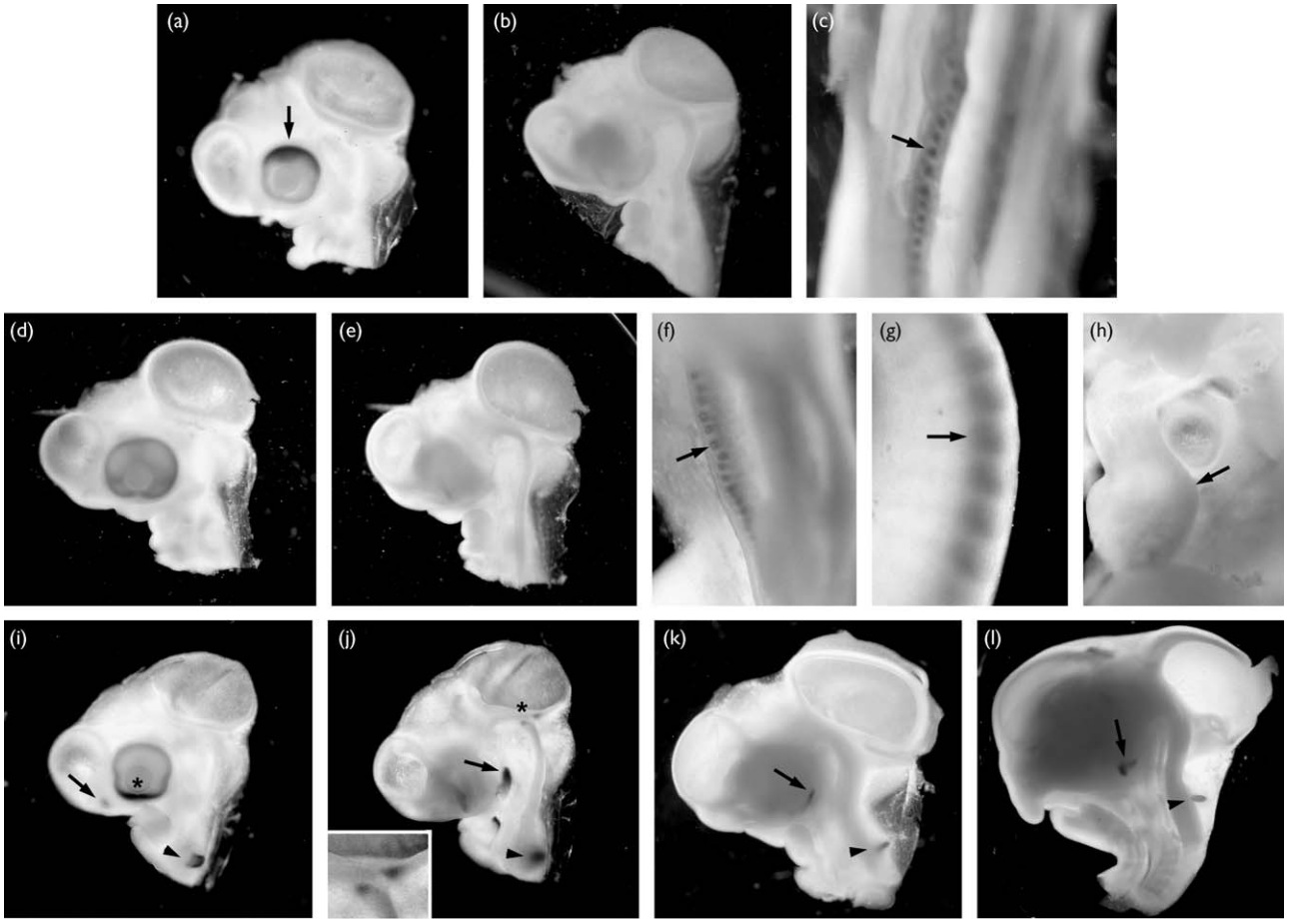

Fig. 2 Expression of retinoic acid (RA) synthesizing enzymes (*raldhs*). (a–c) Expression domains of *raldh-1* at developmental stage HH24. (a) The expression domain in the dorsal third of the eye (arrow). (b) No expression in the neuroectoderm or in its surrounding tissues. (c) Expression in the mesonephros (arrow). (d–h) Expression domains of *raldh-2* at HH24. (d and e) No expression in the neuroectoderm or in its surrounding tissues (external and inner view, respectively). (f) Expression in the mesonephros (arrow). (g) Expression in the dermamyotome of somites (arrow). (h) Expression in the mesoderm of heart inflow tracts (arrow). (i–l) Head expression domains of *raldh-3*. (i) External view of an embryonic head with typical *raldh-3* expression domains at HH24, that is, at the ventral third of the eye (asterisk), dorsal third of otic vesicle (arrowhead) and nasal placode (arrow). (j–m) Internal views of embryonic heads at HH24, HH27, HH29. In all of them *raldh-3* expression can be seen at the Rathke's pouch (arrow) and the otic vesicle (arrowhead). Note that *raldh-3* expression is detected in the IsO only at HH24 (asterisk; see inset in j).

antibody followed by densitometry analysis. First of all, the specificity of this antibody was checked by western blot (Fig. 1c). As a negative control we used preabsorbed anti-RBP. The western blot immunodetected with anti-RBP revealed a single band whose apparent molecular mass corresponded with the predicted molecular mass for chick RBP, whereas the preabsorbed anti-RBP did not bind to any protein band, thus confirming its high specificity. In contrast to *all-trans* retinol, the relative concentration of RBP decreased from HH20 to HH29 (Fig. 1d and f). Interestingly, both the maximum rise of *all-trans* retinol concentration and the maximum concentration of RBP within E-CSF coincided precisely with the moment of maximum neurogenesis within the mesencephalon (Fig. 1e and f).

Although the *all-trans* retinol concentration was also examined in the cephalic neuroectoderm, we did not detect significant variations in this molecule during the period analysed and no other retinoid derivatives were detected (data not shown). Regarding RA, the technique used in this study did not allow us to detect this molecule within the analysed tissues, a process, which is reported to be difficult [20], unless an extremely high number of embryos are used.

Dynamics of retinoic acid-synthesizing enzymes within the central nervous system

As it had been reported that the enzymatic synthesis of RA, the actual morphogen for CNS development, requires the action of intracellular RALDHs, and because the expression domains of these enzymes had not been reported previously in chick embryos for developmental stages older than HH20, we verified the location of *raldh-1*, *raldh-2* and *raldh-3* expression domains in embryos at developmental stages from HH24 to HH29 (i.e. HH24, HH27 and HH29; Fig. 2). At all of these stages, *raldh-1* was expressed in dorsal neural retina and mesonephros, and *raldh-2* was detected in the mesonephros, the somites' dermamyotome and the mesodermal heart inflow tract. None of them were expressed in the cephalic neuroectoderm. *Raldh-3* expression was detected in the Rathke's pouch, the ventral third of the eye, the dorsal third of the otic vesicle and the nasal placode from HH24 to HH29. Of relevance to this study,

raldh-3 was also expressed into the IsO cells at HH24, but interestingly its expression faded afterwards and was not detected in this organizing centre or at any other cephalic neuroectoderm location at later developmental stages (HH27 and HH29). No other expression domains were detected at these late stages of development. These results suggest that the IsO might be a source of RA for neighbouring tissues such as the mesencephalic neuroepithelium.

Discussion

The analysis of the E-CSF chick proteome at HH24 has revealed several different proteins within the E-CSF, which may account for the roles exerted by the whole E-CSF on the surrounding neuroectoderm [4,5,8]. Numerous different studies in vertebrates have demonstrated that RA signalling is crucial for neural differentiation and CNS patterning, for instance, for primary neurogenesis in *Xenopus* as well as for hindbrain and spinal cord anterior–posterior patterning in mouse and chick embryos [13]. These studies, which include either increased RA supply, the analysis of the expression of RA metabolizing enzymes, or the generation of mutant mice for both RA receptors (RAR and RXR) and RA synthesizing enzymes (RALDHs) [16,21–23], have provided much relevant information on the genes and genetic networks upregulated and downregulated by RA. The source of *all-trans* retinol for the neuroepithelium, however, has not been thoroughly analysed.

To increase the knowledge of the overall functions of the E-CSF and its particular components, as has been done for FGF2 and lipoproteins [10] (and unpublished data), we analysed the presence and dynamics of *all-trans* retinol and RBP contained within E-CSF from HH20 to HH29. These stages were chosen for three main reasons: first and most important, they include the period at which the embryos show the highest rate of neural progenitor cell proliferation and differentiation [4]; second, at stages earlier than HH20 the cephalic cavities are too small to obtain enough E-CSF to evaluate the presence of these molecules; and third, at later stages the neuroectoderm of the presumptive brain begins to thicken and expand [24], making it almost impossible to obtain E-CSF samples not contaminated by neural cells.

The antibody used for RBP detection was selected so as to detect only the durable form of this protein, the one bound to TTR, as these form a stable plasmatic complex whose combined molecular mass prevents the extensive loss of RBP through glomerular filtration (reviewed in Ref. [12]).

In this study we have shown that *all-trans* retinol concentration within E-CSF increases during the period analysed, exhibiting a maximum increase from HH20 to HH24, a developmental stage which coincides with the maximum increase of neural differentiation as shown by β-3 tubulin expression [4]. In contrast, the relative concentration of functional RBP decreases continuously from HH20 to HH29, suggesting that RBP is transporting *all-trans* retinol from its storage site (i.e. the yolk of the egg) to the E-CSF where, according to the RBP decrease, it is probably released. Interestingly, both the maximum rise of *all-trans* retinol concentration and the maximum concentration of RBP within E-CSF coincides precisely with the period of maximum increase of neurogenesis, at HH24. Accordingly, *raldh3* expression, the only *all-trans* retinol metabolizing enzyme detected in the chick cephalic neuroepithelium, whose expression in the IsO have been reported previously until HH20 [16], decreases from HH24 to become undetectable at HH27. This is consistent with earlier reports in which it was demonstrated that the E-CSF as a whole collaborates with the IsO in regulating mesencephalic gene expression [5].

In mouse embryos the involvement of the retinoid system in midbrain as well as forebrain patterning and neurogenesis has not been confirmed [25], and to our knowledge it has not been analysed in chick embryos. Furthermore, phylogenetic differences are observed in RA signalling, that is, although RBP and *all-trans* retinol have been identified within the rodent and human E-CSF [9], the RA synthesizing enzymes are expressed differently in avian and rodent embryos. For instance, it is reported that *raldh-3* is not expressed in the forebrain or the midbrain in mice at E9.5-E10.5 [25] although it is expressed in the IsO in chick embryos from developmental stage HH15 to HH24 [16]. Thus, this enzyme plus *raldh-1* and *raldh-2* do not account for RA synthesis in the midbrain in mice although the presence of RA in the forebrain and midbrain in this species has been identified using the RARE-lacZ technique (reviewed in Ref. [25]), but *raldh-3* might be involved in RA synthesis in the chick IsO.

Conclusion

Although the results reported in this study should be interpreted with caution, the dynamics of RPB and *all-trans* retinol within E-CSF and *raldh-3* within neuroectoderm point to a possible relationship between the RBP-*all-trans* retinol system in the E-CSF and anterior CNS development, and suggest that the E-CSF delivers retinol to the RA synthesizing cells.

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References

1. Miyan JA, Nabiyouni M, Zendah M. Development of the brain: a vital role for cerebrospinal fluid. *Can J Physiol Pharmacol* 2003; **81**:317–328.
2. Desmond ME, Jacobson AG. Embryonic brain enlargement requires cerebrospinal fluid pressure. *Dev Biol* 1977; **57**:188–198.
3. 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.
4. 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; **284A**:475–484.
5. Parada C, Martín C, Alonso MI, Moro JA, Bueno D, Gato A. Embryonic cerebrospinal fluid collaborates with the isthmic organizer to regulate mesencephalic gene expression. *J Neurosci Res* 2005; **82**:333–345.
6. Miyan J, 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.

7. Owen-Lynch PJ, Draper CE, Mashayekhi F, Bannister CM, Miyan JA. Defective cell cycle control underlies abnormal cortical development in the hydrocephalic Texas rat. *Brain* 2003; **126**:623–631.
8. Parada C, Gato A, Aparicio M, Bueno D. Proteome analysis of chick embryonic cerebrospinal fluid. *Proteomics* 2006; **6**:312–320.
9. 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.
10. Martin C, Bueno D, Alonso MI, Moro JA, Callejo S, Parada C, *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.
11. Parada C, Parvas M, Bueno D. Cerebrospinal fluid proteomes: from neural development to neurodegenerative diseases. *Curr Proteomics* 2007; **4**:89–106.
12. Zanotti G, Berni R. Plasma retinol-binding protein: structure and interactions with retinol, retinoids, and transthyretin. *Vitam Horm* 2004; **69**:271–295.
13. Maden M. Retinoid signalling in the development of central nervous system. *Nat Rev Neurosci* 2002; **3**:843–853.
14. Diez del corral R, Storey K. Opposing FGF and retinoid pathways: a signalling switch that controls differentiation and patterning onset in the extending vertebrate body axis. *Bioessays* 2004; **26**: 857–869.
15. Kudoh T, Wilson SW, Dawid IB. Distinct roles for Fgf, Wnt and retinoic acid in posteriorizing the neural ectoderm. *Development* 2002; **129**: 4335–4346.
16. Blentic A, Gale E, Maden M. Retinoic acid signalling centres in the avian embryo identified by sites of expression of synthesising and catabolising enzymes. *Dev Dyn* 2003; **227**:114–127.
17. Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. *J Morphol* 1951; **88**:49–92.
18. Bueno D, Skinner J, Abud H, Heath JK. Double in situ hybridization on mouse embryos for detection of overlapping regions of gene expression. *Trends Genet* 1996; **12**:385–387.
19. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**:680–685.
20. Maden M, Sonneveld E, van der Saag P, Gale E. The distribution of endogenous retinoic acid in the chick embryo: implications for developmental mechanisms. *Development* 1998; **125**:4133–4144.
21. Marklund M, Sjodal M, Beehler BC, Jessell TM, Edlund T, Gunhaga L. Retinoic acid signalling specifies intermediate character in the developing telencephalon. *Development* 2004; **131**:4323–4332.
22. Reijntjes S, Blentic A, Gale E, Maden M. The control of morphogen signalling: regulation of the synthesis and catabolism of retinoic acid in the developing embryo. *Dev Biol* 2005; **285**:224–237.
23. Sharpe C, Goldstone KT. The control of Xenopus embryonic primary neurogenesis is mediated by retinoid signalling in the neuroectoderm. *Mech Dev* 2000; **91**:69–80.
24. Bellairs R, Osmond M. *Atlas of chick development*. London: Elsevier Academy Press; 2005.
25. Molotkova N, Molotkov A, Duester G. Role of retinoic acid during forebrain development begins late when Raldh3 generates retinoic acid in the ventral subventricular zone. *Dev Biol* 2007; **303**:601–610.