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Glial migratory streams in the developing hindbrain: A slice culture approach

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Abstract

Compared to our knowledge of neurogenesis, relatively little is known about glial cell specification and migration during central nervous system development. We have established a novel chick hindbrain slice preparation which permits examination of gliogenesis in its native environment, providing a means to study the signaling pathways involved in glial cell specification and migration during development. Cells in the hindbrain slice preparations mature in a manner which is similar to *in vivo* developmental timing and patterning paradigms. To demonstrate the utility of this approach, we examined the effect of the retinoic acid signaling pathway on cells in these slices, showing that addition of exogenous *trans*-retinoic acid to slice cultures promotes expression of a marker of mature astrocytes, glial fibrillary acidic protein (GFAP), while the inhibition of endogenous retinoic acid synthesis reduces GFAP expression; the results suggest a role for retinoic acid in modulating glial differentiation. Using these hindbrain slice cultures, we have used two different approaches to label glial progenitors specifically at the ventricular zone and have observed for the first time the ventrally-directed migration of these cells from the ventricular zone of the hindbrain. This slice culture system is thus an innovative and robust tool for examining glial cell migration and the extracellular molecular and signaling pathways which regulate glial differentiation.

Introduction

While there have been extensive studies of neuronal differentiation and migration during brain development, relatively few studies have focused on glial cell specification and migration. In recent years, the genes and signaling pathways involved in the timing and induction of gliogenesis have begun to be elucidated by both *in vitro* and *in vivo* studies (Domowicz et al., 2008; Guillemot, 2007; Sauvageot and Stiles, 2002). However, the interactions between these pathways are difficult to ascertain using *in vitro* models of dissociated cells, and *in vivo* mouse knockout and transgenic approaches are time consuming and laborious. Therefore, we sought to develop a system to study the process of glial cell migration and specification that maintains the cells in their native environment, allows labeling of individual migrating cells, and permits the addition of agents to selectively test the functional roles of individual signaling pathways, while also being cost effective and easy to perform.

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Organotypic slice cultures are advantageous over *in vitro* culture of dissociated cells as they maintain the native environment of the cells. The importance of the native environment in neuronal and glial specification has been demonstrated previously in the mouse cortex where differentiation of neuronal progenitors co-cultured with cortical slices was influenced by the age of the tissue from which the cortical slice was prepared (Morrow et al., 2001). Additionally, slice cultures can be incubated in the presence of agents designed to interfere with or promote endogenous signaling pathways, thus allowing for the examination of molecules controlling cell differentiation and migration (Zhou et al., 2007).

The chick is an excellent model system for the study of gliogenesis; large numbers of eggs are readily available at a low cost, and the access to unlimited numbers of embryos at multiple stages permits testing of various experimental manipulations in a single set of cultures. While cortical slice cultures of mouse and rat brain have been often used for the examination of neuronal, and to a much lesser extent glial cell, differentiation and migration (Haydar et al., 1999; Kakita and Goldman, 1999; Polleux and Ghosh, 2002; Suzuki and Goldman, 2003), they require the use of timed pregnant mice, necessitating the maintenance of a large mouse colony in order to obtain the desired number of embryos for each experiment. Additionally, although methods for electroporation and viral infection have been established for both mouse and chick, the relative ease of the *in ovo* compared to the *in utero* methods renders the chick advantageous over the mouse in terms of localized gene manipulation (Ishii et al., 2004; Krull, 2004; Lipshutz et al., 1999; Saito and Nakatsuji, 2001).

Our recent identification of a subpopulation of glial cells in the chick hindbrain which appear to migrate and differentiate into astrocytes (Domowicz et al., 2008) provides the foundation for utilizing the chick hindbrain as a new system in which to study gliogenesis. Within the hindbrain at embryonic days 8–10 (E8–10) is a highly patterned population of cells which possess migratory morphology and express glial-specific genes (Domowicz et al., 2008), such as brain lipid binding protein (BLBP), glutamine synthetase (GS) and the glutamate transporter, GLAST, as well as the chondroitin sulfate proteoglycan aggrecan (Akimoto et al., 1993; Feng et al., 1994; Shibata et al., 1997). These cells are present in the ventricular zone of the hindbrain and in discrete medial- and lateral-positioned streams descending from the ventricular zone. Later in development, these aggrecan-positive cells express the mature-astrocyte marker glial fibrillary acidic protein (GFAP) (Domowicz et al., 2008; Eng, 1985). While our study (Domowicz et al., 2008) was the first to document the glial nature of this subpopulation of cells in the hindbrain, it remains unclear whether the cells within these discrete steams are indeed migratory, and importantly, what factors control the differentiation of these cells from glial progenitors to astrocytes.

To examine the differentiation and migratory potential of glial cells we developed a novel chick hindbrain slice culture assay. Chick hindbrain slices maintained in culture mature in a manner comparable with *in vivo* tissue, sustaining relatively normal developmental timing and patterning. We have demonstrated the utility of hindbrain slice cultures as a means to examine signaling pathways involved in gliogenesis by adding exogenous *trans*-retinoic acid (RA) or inhibitors of RA synthesis to the culture media; the result reveals a potential novel role for RA signaling in glial cell differentiation. Labeling of glial progenitor cells within the ventricular zone using both electroporation and transfection of the slice cultures has shown for the first time that there is a migratory population of glial cells within the chick hindbrain. We have shown this culture system to be an innovative and robust approach for examining glial cell migration and the factors which regulate glial differentiation, allowing individual migratory glial cells to be tracked and the role of exogenous and endogenous signaling molecules involved in glial differentiation to be examined.

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Materials and Methods

Chick hindbrain slice preparation and culture

Fertilized white Leghorn chicken eggs were purchased from Sharp Sales (West Chicago, IL). Brains from embryonic day (E8) chicks were dissected in cold complete HBSS (1xHBSS, 30 mM D-glucose, 2.5 mM HEPES pH 7.4, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 4 mM $NaHCO_3$). Following the removal of the meninges, the hindbrain was cut from the midbrain and embedded in 3% low melting point agarose (SeaPlaque GTG Agarose, Lonza) with the rostral end toward the bottom of the block. Beginning from the caudal end of the tissue, 250 μm sections were made with a Leica 1030S vibratome. Slices, still surrounded by agarose, were placed on poly-L-lysine (76.3 μg/ml, Sigma) and laminin (7.63 μg/ml, Sigma) coated polyethylene terephthalate (PET) track-etched membranes (6-well format, 1 μm pore size, Becton-Dickinson) which were placed in 6-well insert companion plates (Becton-Dickinson). Following transfer to the membranes, all excess solution was removed from around the slices and 1.8 ml slice medium (70% v/v Basal Eagle Medium, 25.8% v/v Complete HBSS, 20 mM D-glucose, 1 mM L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 5% heat-inactivated horse serum) was placed below each membrane insert. The slices were maintained at the air-medium interface for up to 4 days in culture at 37°C and 5% CO_2, with half-volume medium changes every second day.

For slice treatments, 100% ethanol (control), retinoic acid (0.5 mM stock solution in ethanol), citral (60 mM stock solution in ethanol) or DEAB (100 mM stock solution in DMSO) at the indicated concentrations were added to the slice medium at the start of the culture. Fresh treatment was added when the slice media was changed on the second day in culture. For BrdU incorporation, BrdU at a final concentration of 10 μM was added to slices 1 h prior to fixation.

For labeling of live cells in the slices, either electroporation or transfection of CMV-EGFP (Clontech) at a final concentration of 1 μg/μl was used. For electroporation experiments, the DNA was mixed with FastGreen dye (2.5ug/uL final concentration, Sigma), and injected into the fourth ventricle of the E8 chick brain. GenePaddle electrodes (Harvard Apparatus) were positioned such that the positive electrode sat on the ventral side of the head, just below the beak and the negative electrode rested on the dorsal brain, above the developing cerebellum and fourth ventricle. The head was then electroporated with 4 × 45 V pulses of 100 ms with a 100 ms interval using a BTX ECM830 Electro Square Porator (Harvard Apparatus) followed by dissection from the surrounding head tissue and preparation of slices as described above. For transfection experiments, the DNA was mixed in a 5% w/v glucose solution with *in vivo*-jetPEI (Polyplus Transfection) at an N/P ratio of 10. The N/P ratio describes the ionic balance of the DNA-jetPEI complexes, and refers directly to the number of cationic nitrogen residues in the *in vivo*-jetPEI per DNA phosphate (Remy et al., 1998). Complexes were allowed to form for 15 minutes at room temperature, and then Fast Green dye (2.5 μg/μl final concentration, Sigma) was added to allow visualization of the DNA solution. The mixture was applied to slice prepared from E8 brain by micropipette in the region between the dorsal ventricular zone of each slice and the surrounding agarose.

mRNA *in situ* hybridization

In situ hybridization was performed on slices as previously described for whole-mount tissue, following 96 h in culture and fixation overnight at 4°C in 4% PFA in PBS (Domowicz et al., 2008). Digoxygenin-labeled probes used in this study were the same as described previously (Domowicz et al., 2008).

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Immunohistochemistry

Slices were fixed overnight in 4% PFA at 4°C and then were washed and processed for whole mount immunofluorescence as previously described for mouse cortical slices (Polleux and Ghosh, 2002). Briefly, slices were blocked and antibodies were diluted in slice staining solution (1x PBS, 3% Normal lamb serum, 3% BSA, 0.3% Triton) and blocking was performed for 4 h at room temperature. Primary and secondary antibody incubations were done overnight at 4°C. For BrdU staining, following overnight fixation in 4% PFA at 4°C, the slices were prepared for staining as described previously (Morrow et al., 2001). Briefly, slices were post-fixed for 30 minutes in 70% ethanol, permeabilized in 0.4% Triton-X100 in 1x PBS for 1 h, and incubated in 2N HCl in water for 30 minutes followed by incubation in 1M Tris-Cl, pH 8.0 for 30 minutes. Slices were then processed as above.

Primary antibodies used in these studies are anti-BLBP (rabbit IgG, 1:400), anti-NeuN (mouse IgG, 1:100), anti-GFAP (mouse IgG, 1:2000), and anti-GFP (mouse IgG, 1:200) (Millipore), as well as anti-Transitin (EAP3, mouse IgG, 1:50, DSHB), anti-Caspase-3 (rabbit IgG, 1:500, Cell Signaling Technology), and anti-BrdU (mouse IgG, 1:500, BD). Secondary antibodies used in these studies are Rhodamine Red and AlexaFluor 488 goat anti-mouse IgG and Rhodamine Red and AlexaFluor 488 goat anti-rabbit IgG (1:500, Molecular Probes). All slides were mounted with Fluoromount-G (Electron Microscopy Sciences).

Western blotting

Lysates from slices cultured for 4 days were prepared in RIPA buffer. Total protein was normalized using BCA assay (Pierce) and equal amounts of protein were used for each sample. Samples were run on 10% SDS-PAGE gels and transferred to nitrocellulose membrane at 150 mA overnight. Primary antibodies used are anti-β-actin (mouse IgG, 1:1000, Sigma) as well as anti-BLBP and anti-NeuN as described above, and the secondary antibody was a HRP-conjungated goat anti-mouse IgG (Pierce). Signal was detected using the SuperSignal West Dura Extended Duration Substrate (Pierce) and visualized and quantified by Quantity One software (Bio-Rad).

Thymidine incorporation

Slice cultures were labeled for 24 h with 8 μCi of [^{3}H]-thymidine per well and harvested at the indicated times in culture. For each time point, duplicate sets of six slices each were collected and homogenized in a glass homogenizer. Homogenates were precipitated with an equal volume of 10% trichloroacetic acid (TCA)/4% phosphotungstic acid. Pellets were extensively washed with 5% TCA and then solubilized with 1M sodium hydroxide. Radioactivity counts were determined using a liquid scintillation counter (Tri-Carb 1900TR from Packard). Counts from different sets were normalized according to total protein content, as determined by BCA assay (Pierce) following the manufacturer's instructions.

Caspase 3/7 activity assay and quantification

Caspase 3/7 activity was measured for treated and control slice cultures after 24, 48 and 96 h in culture. For each measurement, four slices were pooled and homogenized in lysis buffer (AnaSpec, Ca), and the caspase 3/7 catalytic activity was determined by following proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AMC (AnaSpec, Ca). Production of AMC was monitored continuously with a Victor3 plate reader (PerkinElmer). Initial velocities are expressed as increase in relative fluorescent units per minute per μg of protein. Each treatment/time-in-culture condition was assayed in triplicate. Data was analyzed for statistical significance using the Student's *t*-test.

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Microscopy

Live images of migrating cells within the slices were made with a Zeiss Axiovert S100TV microscope equipped with a Retiga EXI CCD camera. *In situ* hybridization pictures were taken using a Zeiss Stemi SV11 stereomicroscope with a Zeiss AxiocamMR5 camera. Confocal images of immunostained slices and vibratome sections were obtained using a Zeiss LSM 510 laser-scanning confocal microscope through the CDIF Microscopy Facility at The University of Chicago. All images were imported into and assembled in Adobe Photoshop CS.

Results

Development of a slice culture system for the chick hindbrain

To study the differentiation and apparent migration of the glial cells in the chick hindbrain identified in our previous study (Domowicz et al., 2008), we developed an *in vitro* slice culture system of chick hindbrain. The goal was to produce a relatively rapid and reliable way to culture glial cells while maintaining the cells in their native environment. To obtain organotypic slices of chick hindbrain, the E8 brains were dissected and the hindbrain was cut from the midbrain (Fig. S1). The hindbrains minus their meninges were then embedded in low melting point agarose and slices were obtained by vibratome sectioning (Fig. S1). Following sectioning, the slices, still contained in their surrounding agarose, were transferred to poly-L-lysine- and laminin-coated PET membranes (Fig. S1). Slices were cultured at the air-media interface for up to 4 days in at 37°C and 5% CO_2.

In order to determine whether our slice culture system maintains the appropriate morphology and developmental timing of *in ovo* tissue, we immunostained vibratome sections from both *in vivo* tissue from E8–E12 embryos and slices cultured for 24–96 h (Figure 1, data not shown) for markers of radial glial and immature astrocytes (BLBP) (Feng et al., 1994), neurons (NeuN) (Mullen et al., 1992), and mature astrocytes (GFAP) (Eng, 1985). Examination of the expression of brain lipid binding protein (BLBP) in slices of chicken hindbrain at embryonic day 8 (E8) reveals the presence of a large number of BLBP-positive radial glial cells with cell bodies largely localized to the ventricular zone and processes extending from the dorsal ventricular surface to the ventral pial surface (Fig. 1A). Closer examination of the boxed region in Fig. 1A reveals many processes of radial glial cells (Fig. 1H, red arrow) and a few BLBP-positive cell bodies outside the ventricular zone (Fig. 1H, yellow arrow). Between E9–10, these radial glial cells persist (Fig. 1C, red arrow), but there are increasing numbers of BLBP-positive cells located some distance from the ventricular zone which retain a ventrally-directed leading process (Fig. 1B–C, I, yellow arrows). Higher magnification reveals the polarized morphology (a ventrally-directed leading process and the nucleus residing at the rear of the cell) and positioning of the bodies outside the ventricular zone suggesting that these cells are migratory (Figure 1I, yellow arrow). These polarized cells are primarily localized to the lateral and medial regions of the slice where we previously reported discrete streams of cells expressing glial-specific genes (Fig. 1B–C, medial stream is under red bracket) (Domowicz et al., 2008); the region in between these streams contains BLBP-positive cells which largely maintain their radial glial morphology (Fig. 1B–C). By E12, there are few radial glia or cells with migratory morphology and most of the BLBP-positive cells have astrocyte-like morphologies, with a multiple processes emanating from a centralized cell body (Fig. 1D, K, green arrowheads). The pattern of BLBP-expressing cells found in the slices was comparable with the appearance and behavior of BLBP-positive cells *in ovo* (Fig. 1E–G), with polarized cells localized outside the ventricular zone being positioned in similar streams as in the *in vivo* tissue (Fig. 1E–F, medial stream under red bracket). At higher magnification, the morphology of cells possessing the characteristics of radial glial and polarized migratory cells were similar to comparable *in vivo* stages (Fig. 1L–M, red and yellow arrows). As time in culture increased, fewer radial glial cells and greater numbers of cells with ventrally polarized processes were observed, as was

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seen with E8–10 *in vivo* tissue. By 96 h in culture, many of the BLBP-positive cells had the morphology of maturing astrocytes (Fig. 1N, green arrowheads), as was seen in E12 tissue (Fig. 1K, green arrowheads). Note that there are differences in the over all size of the cultured slices compared to the slices prepared from *in vivo* tissue, as the agarose surrounding the slice prevents its expansion from its original size (Compare Fig. 2D,G). This is also reflected in a higher cell density in the cultured slices at 96 h in culture compared to the in vivo tissue at E12 (Fig. 1K, N, data not shown).

Examination of the expression of NeuN in tissue from embryos E8–E12 reveals that with the exception of minor differences due to anterior-posterior position of the slices, the pattern of NeuN staining is refined between E8–E9, and remains constant between E9–E12 (Fig. 2A–D). In the cultured slices, the pattern of NeuN immunoreactivity at 24 h closely matches that of the *in vivo* tissue, and there were no dramatic changes in expression during the culture period from 24–96 h (Fig. 2E–G).

GFAP protein expression in E8–E12 tissue only became apparent at E12, diffusely localized to the ventricular zone, the dorsal-lateral region of the tissue and ventral boundary regions of the tissue (Fig. 2H–K, yellow arrow and arrowheads). Studies using the whole chick brain have demonstrated previously that GFAP mRNA is only weakly expressed beginning at E11 by Northern blotting (Domowicz et al., 2008) and GFAP mRNA is visible in the hindbrain by *in situ* hybridization beginning at E12 (data not shown). In slices in culture, GFAP protein was detectable at 48 h in culture (Fig. 2M, yellow arrowheads) and increased by 96 h in a pattern restricted to the ventricular zone, lateral-dorsal tissue and the medial-ventral boundary of the slice (Fig. 2N, yellow arrow and arrowheads). The presence of GFAP prior to E12 in the cultured slices suggests that either the process of cutting the slices causes low levels of injury-induced GFAP expression, or that factors in the slice culture media and/or the culture of the slices *in vitro* is causing some premature expression of GFAP and differentiation of glial cells. Although the expression of GFAP in cultured slices is slightly advanced relative to that *in ovo*, the pattern in which GFAP is expressed remains comparable. Therefore, despite this subtle difference in GFAP expression, developmental timing within the *in vitro* slice culture system is comparable to that observed *in vivo*, making the hindbrain culture system a valid tool for the study of glial differentiation and migration.

Perturbation of glial differentiation in slice cultures

It has been shown previously that dissociated glial cell cultures can express a different cohort of genes than the same cell types *in vivo* (Nakagawa and Schwartz, 2004), raising the question of whether the same signaling pathways which operate *in vivo* also function *in vitro*. Due to its maintenance of the native environment, the slice culture system permits the examination of the importance of the cellular milieu for glial cell differentiation and an assessment of the role specific molecules play in promoting or inhibiting glial cell differentiation.

Recent work has identified that several of the genes involved in RA synthesis and metabolism are expressed in the chick hindbrain at this critical stage of glial development (Maden, 2007; Wilson et al., 2007). Additionally, exogenous RA has been used to promote the fate of embryonic stem cells into cells with radial glial-like properties, which upon further treatment can be induced to differentiate into neurons or glia (Fraichard et al., 1995). The presence of genes involved in RA synthesis and signaling during the time of gliogenesis, as well as the effect of exogenous RA addition to neural stem cells suggests RA may be involved in the process of glial differentiation in the chick hindbrain. Therefore, to demonstrate the utility of our slice cultures for the study of gliogenesis, we examined the effect of addition of exogenous RA on the expression of glial specific genes in the chick hindbrain.

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Hindbrain slices treated with *trans*-retinoic acid (from 0.14 to 7 μM) showed a dramatic, concentration-dependent increase in the expression of the mature astrocyte marker GFAP compared to control-treated slices (1.4% ethanol) after 96 h in culture (Fig. 3A). The increase in *GFAP* mRNA (Fig. 3A) and protein (Fig. 3B) expression was localized to the ventricular zone and the discrete steams of medial and lateral glial cells at all concentrations (Figure 3A–B, red arrowheads). Although there is an increase in signal intensity in the treated slices, the overall pattern of GFAP expression is similar between control and treated slices, and therefore the effect of RA appears to be limited to a specific population of glial-committed cells. If RA treatment were to affect all cells in the slice, it would be expected that there would be an increase in GFAP expression throughout the slice, which is clearly not observed. The expression of transitin, a progenitor cell marker, although weakly expressed, did not change irrespective of treatment (Fig. 3C) (Cole and Lee, 1997;Lee and Cole, 2000). This also suggests that RA is acting specifically on a subpopulation of glial-committed cells, rather than all transitin-positive progenitors. RA has been used previously to induce neurogenesis in stem cell cultures and RA has also been shown to both promote and inhibit neurogenesis after injury (Guan et al., 2001;Jung et al., 2007;Kornyei et al., 2007). Examination of NeuN protein however revealed no changes in NeuN expression, suggesting no new neurons are generated in response to RA treatment in this system (Fig. 3D). These results suggest that RA is affecting a subpopulation of cells which at the time of culture, are restricted to the glial cell fate. Therefore, RA may serve to promote the differentiation of a subpopulation of glial progenitor cells within the chick hindbrain.

The enzymes that synthesize and metabolize RA are present in the chick hindbrain in the same regions where we observe discrete streams of glial cells extending from the ventricular zone both *in vivo* and during the duration of our slice cultures (Domowicz et al., 2008; Wilson et al., 2007), we asked whether interfering with the endogenous RA signaling pathway would inhibit differentiation of this subpopulation of glial cells. Treatment of slices with two different inhibitors of the retinaldehyde dehydrogenase enzyme required for RA synthesis, DEAB (30 μM) (Russo et al., 1988) or Citral (180 μM) (Conner and Smit, 1987; Tanaka et al., 1996), resulted in a substantial decrease in the level of GFAP expression at both the mRNA and protein levels (Fig. 4A–B). Meanwhile, inhibition of RA synthesis had no effect on the expression of transitin; subtle differences in the lateral regions of the slice are most likely due to differences in anterior-posterior position from one slice to the next (Fig. 4C). There was also no effect of RA synthesis inhibition on neurons as measured by the expression of NeuN (Fig. 4D). These results are consistent with the hypothesis that RA can promote differentiation of a subpopulation of glial cells in the chick hindbrain.

To confirm the changes in GFAP expression observed in the presence of RA or inhibitors of RA synthesis, Western blotting was performed on lysates prepared from treated and control slices using antibodies to NeuN and GFAP. These blots revealed an increase in GFAP protein with increasing RA concentration, and a decrease in GFAP levels when slices were treated with inhibitors of RA synthesis (Fig. 5A). Unlike GFAP expression, there were no changes in NeuN expression with any of the treatments (Fig. 5B). Quantification of the protein levels from the Western blots normalized to β-actin expression highlight the dramatic changes in GFAP expression and the constant levels of NeuN expression (Fig. 5C). Together, the RA and inhibitor experiments serve to illustrate the utility of the slice culture system; through the addition of extracellular factors to the slice media, the role of the various signaling pathways in glial differentiation can be assessed.

Analysis of cell division and cell death in control and treated slice cultures

In order to ensure experimental manipulations with the hindbrain slices provide accurate results, it is essential to ascertain the overall health of the tissue in culture through the

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examination of cell division and cell death in slices. Generally, in untreated tissue cells continued to divide throughout the entire slice when slices were examined at 24 h intervals between 24–96 h in culture by use of BrdU to label cells in S-phase (Gratzner et al., 1975) (data not shown). Activated caspase-3 expression, an indicator of cell death (Fernandes-Alnemri et al., 1994; Nicholson et al., 1995), appeared to be highest at 24 h and then decreased with each subsequent day in culture (data not shown).

To determine the effects of our control and experimental treatments on cell division in hindbrain slices, we examined both BrdU incorporation by immunohistochemistry and [^{3}H]-thymidine incorporation in control slices and slices treated with RA or the inhibitor DEAB at 48 and 96 h in culture. For thymidine experiments, [^{3}H]-thymidine was incorporated into the newly synthesized DNA in the slices for 24 h prior to tissue harvest. Upon scintillation counting, these experiments reveal no significant differences in the amount of thymidine incorporation between control, RA and inhibitor-treated slices (Fig. 6A). Immunohistochemistry on slices treated with BrdU for 1 h prior to harvest at either 48 or 96 h in culture demonstrated that there are no localized increases in cell division in slices with the selected treatments: control (1.4% ethanol), RA (7 μM), and DEAB (30 μM) (Fig. 6B–C). Therefore, the increase in GFAP expression seen upon RA treatment does not result from increased proliferation and subsequent differentiation of glial progenitor cells.

As indicator of cell death, the activities of caspases-3 and -7 were measured by a fluorogenic indicator assay specific for their amino-acid substrates (Materials and Methods). Activity was quantified from lysates prepared from hindbrain slices at 24, 48 and 96 h in culture. This analysis revealed that there is no significant increase in caspase-3/7 activity with treatment (control (1.4% ethanol), RA (7 μM), or DEAB (30 μM)) (Fig. 7A). Immunohistochemistry with an antibody against activated caspase-3 (Hu et al., 2000) confirmed the results from the activity assay and permitted observation of regions of localized cell death. At 48 h in culture, an equal amount of activated caspase-3 was found with all treatments, distributed throughout the slice with highest levels just proximal to the midline and the ventral boundary of the tissue (Fig. 7B). By 96 h, activated caspase-3 expression independent of treatment was largely confined to the ventral surface of the slice, where it meets the bounding agarose; this may reflect the inability of the tissue to expand with time in culture due to the restricting agarose (Fig. 7C, arrows). However, this did not affect the slice as a whole and cell division still occurred in this region (See Fig. 6C). It is worth noting that at 96 h in culture, there appears to be less caspase-3 activity in the RA treated slices as determine both through activity assays and caspase-3 immunoreactivity (Fig. 6A, C). This may indicate a neuroprotective role for RA at high concentrations and is consistent with the known neurogenic potential of RA following CNS injury (Mey, 2006). Together, the data suggest that the decrease in GFAP expression following treatment with DEAB cannot be attributed to cell death, as DEAB treated and control slices have similar levels of cell death. In sum, the *in vitro* slices undergo cell division throughout the culture period and although they experience some cell death, the slices are generally healthy. Additionally, these data show that the effects of the treatments on GFAP expression are not caused by increased cell division or cell death, thus suggesting a role for RA signaling, directly or indirectly, in regulating the expression of glial-specific genes.

Observation of migratory cells within slice cultures

As one approach to determine whether the polarized cells observed in the E8–E10 hindbrain were migratory, we utilized electroporation followed by slice culture as has been reported previously for mouse cortical tissue (Hand et al., 2005). Briefly, the head was removed from the embryo, and DNA was injected into the fourth ventricle via micropipette. The entire head was electroporated with 4 × 45 V pulses of 100 ms duration at a 100 ms interval with the electrodes oriented in the appropriate direction to drive the DNA into the ventricular zone of

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the hindbrain. Following electroporation, the hindbrain was dissected and slices prepared as described above. Slices were then fixed and immmunostained at 24, 48, 72, and 96 hours after electroporation.

GFP-positive cells are present in the dorsal ventricular zone of the slice 24 h following electroporation (Fig. 8A). As time in culture increases, GFP-positive cells increase in number and are also observed to be some distance from the ventricular zone (Fig. 8B–D). These changes in the distribution of GFP-positive cells are presumably due to the division and migration of the cells initially electroporated at the dorsal ventricular zone. Migrating cells are found in the same medial and lateral regions of the slice (Fig. 8A–D) which exhibit the morphological changes in BLBP expression between E8–E12 (Fig. 1) and which express glial specific markers in discrete medial and lateral streams (Domowicz et al., 2008), suggesting they are immature glial cells which have migrated away from the ventricular zone. Also similar to what was observed in cultures stained with BLBP (Fig. 1), the morphology of the electroporated cells changes with time in culture, from polarized with a leading process and trailing nucleus at 24–48 h to a rounded nucleus and astrocyte-like processes at 72–96 h (Fig. 8A–D, insets). In order to examine cell migration, the same slice was imaged at 16 and 72 hours following electroporation, revealing that many of the electroporated cells do migrate away from the ventricular zone over time (Fig. 8E–F). After 72 hours in culture, the slice was fixed and co-stained for GFP and BLBP. Single Z-sections obtained through confocal microscopy on this slice revealed that many of the GFP-positive cells are indeed BLBP-positive, indicating they are of the glial cell fate (Fig. 8G–I, arrows). Therefore, electroporation followed by slice culture is a valid method for examining glial cells during chick hindbrain development.

Although the large number of cells transfected with a gene of interest by electroporation may be advantageous in some instances, this approach is not as well-suited for the purpose of observing and tracking the migrations of individual cells. Therefore, as an alternative approach to determine if the polarized glial cells observed in the E8–E10 hindbrain were migratory (See Fig. 1), we labeled ventricular zone cells in slice cultures with green fluorescent protein (GFP) using the *in vivo*-JetPEI lipid-based transfection reagent. Taking advantage of the boundary between the ventricular zone of the slice and the surrounding agarose, the DNA-lipid complexes were added to the slices just before the addition of the slice medium, by injecting the DNA solution with a micropipette into the space between the dorsal ventricular zone and the surrounding agarose (Fig. 9A). This method allowed the DNA-lipid complexes to remain trapped in the space between the ventricular zone and agarose, and thus reliably resulted in transfection of only cells within the ventricular zone. To ensure GFP-positive cells observed during the culture period arose from the original transfection or by mitosis of transfected cells and not from *de novo* transfection after the first time point, slices were first photographed at 40 h after transfection as the DNA-lipid complexes are stable for up to 24 h.

Analysis of GFP-positive cells at both 40 and 96 h in culture revealed that a number of these cells move ventrally away from the ventricular zone during this time frame (Fig. 9B–C, arrows). Note that GFP-positive cells are located in the ventricular zone at 40 h in culture (Fig. 9B), but by 96 h in culture these cells have a polarized morphology and have migrated away from the ventricular zone (Fig. 9C, D, arrow). Throughout this time, these cells maintain a ventrally-oriented leading process (Fig. 9D). To determine whether these migratory cells are glia, the slices were fixed and stained for GFP and BLBP. Single Z-section images of the same slice as in Fig. 9B–C obtained by confocal microscopy following immunostaining reveals that at least some of the cells which have migrated ventrally are indeed BLBP-positive glial cells (Fig. 9D–H, arrows). This is the first clear evidence that BLBP-positive glial cells within the chick hindbrain are indeed migratory and that these migrations appear to occur in discrete streams.

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Discussion

We have developed a powerful assay to examine glial cell migration and differentiation in chick hindbrain slice cultures. Chick hindbrain slices have not been previously used to study glial cell differentiation and migration, although they have been used over short culture periods to analyze neuronal connectivity and activity during development (Momose-Sato et al., 1997; Momose-Sato et al., 2001; Reyes et al., 1996). While we have only described cultures from E8 hindbrain in this paper, cultures from E6 and E7 hindbrain have also been obtained and maintained for up to 4 days using this method, and may be particularly valuable for studying the switch from neurogenesis to gliogenesis. The slice culture system accommodates numerous slices simultaneously, thereby allowing many experimental conditions to be tested in a single experiment, and should prove useful in determining the interplay between the known signaling pathways in gliogenesis.

We used this novel slice culture system to demonstrate that the addition of RA to hindbrain slice preparations increased expression of GFAP, a marker of mature astrocytes (Eng, 1985). The increase occurred specifically in the discrete streams of cells where *BLBP* mRNA is expressed at early stages (Domowicz et al., 2008) and where GFAP is normally expressed at later stages suggesting that not all the progenitor cells in the chick hindbrain are susceptible to the RA treatment. In *in vitro* culture, RA is known to induce cell differentiation and is used to prime embryonic stem cells prior to differentiation into neurons and glia (Fraichard et al., 1995). It has also been shown to increase the expression of GFAP in astrocytes while still promoting neuronal survival in dissociated cultures of rat spinal cord (Wuarin et al., 1990). The components of the RA signaling pathway from synthesizing enzymes to receptor proteins and metabolizing enzymes were recently demonstrated to be present in the chick hindbrain at the time of glial cell differentiation (Maden, 2007; Wilson et al., 2007). Three of these molecules, *retinaldehyde dehydrogenase enzyme 2 (RALDH2)*, an RA synthesizing enzyme, *cellular retinoic acid binding protein 1 (CRABP1)*, an RA binding protein, and *cytochrome P450, family 26, subfamily B, polypeptide 1 (CYP26B1)*, a metabolizing enzyme, are present in the chick hindbrain in the dorsal ventricular zone and/or in streams of cells emigrating from the ventricular zone, in a pattern reminiscent of markers (*BLBP, GLAST*) of glial progenitor cells (Domowicz et al., 2008; Wilson et al., 2007). Therefore, it is possible that endogenous RA signaling may have a role in modulating glial cell differentiation within the chick hindbrain. In the present study, the decrease in GFAP expression when endogenous RA synthesis was inhibited within slices suggests that this may indeed be the case. Additionally, a population of radially migrating cells in the E15–E17 hindbrain was also seen in a transgenic mouse expressing LacZ under the control of the RA response element (LacZ-RARE) (Zhang et al., 2003). Therefore, it is plausible that this population of glial cells within the hindbrain exists in other species, and that they may share a common mechanism of specification controlled in part by RA. While published reports indicate that the meninges may be a putative source of RA in the hindbrain (Zhang et al., 2003), the meninges are removed in our slice preparations; suggesting there may be a second source of RA in the developing hindbrain which aids in specifying glial differentiation.

In addition to their utility for the study of glial differentiation, chick hindbrain slice cultures also permit the examination of glial cell migration. This study is the first to demonstrate that the glial cells which appear in streams within the chick hindbrain are migratory, and move from the dorsal ventricular zone maintaining a ventrally-directed leading process. The discrete regions of migrating cells observed in the slice cultures and those seen in slices prepared from comparable-aged tissue are remarkably similar, indicating faithful maintenance of the overall hindbrain tissue architecture in the slice (Domowicz et al., 2008; Wilson et al., 2007; Zhang et al., 2003). Migrating GFP-positive cells were identified as glial cells by immunohistochemistry; the use of a glial-specific promoter to drive expression of EGFP would

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provide a more straightforward analysis of glial cell migration by eliminating the need for co-staining following fixation to identify the migrating cells as glial cells. Highlighting the versatility in mode of gene manipulation in these slice preparations, we utilized both electroporation and lipid-based methods of gene expression. Electroporation was quite successful at labeling large numbers of cells and will be useful in future studies which aim to examine the process of glial cell migration as a whole, or the importance of local cell signaling in glial cell differentiation. Lipid-mediated transfection by contrast, specifically and faithfully labels only a few cells at the dorsal ventricular zone and permits facile tracking of the migrations of individual cells. It is also noted that DiI-mediated labeling of cells has been performed in these slices, however we did not find this method to be as useful for examination of cell migration as electroporation and transfection, due to widespread labeling of both radial and migrating glial cells. Additionally, viral infection (Iba, 2000; Ishii et al., 2004) of the slices should be an effective means to locally label cells or alter gene expression and a gene gun delivery system (Kettunen et al., 2002) could provide a alternative method to transfect discrete regions of an individual slice. Each of these approaches has its own advantages and disadvantages, but the versatility of this slice culture system to manipulation using a wide variety of gene expression systems permits the use of whichever approach is best suited for the question at hand.

Previous clonal analysis has shown that glial cells are generated from radial glial cell detachment and migration to the appropriate position (Gray and Sanes, 1992; Voigt, 1989). Our data highlight that this process occurs in highly patterned discrete migratory streams in the hindbrain. The migration of GFP-positive glial cells is consistent with the *in vivo* expression of BLBP from E8–E12, in which BLBP-positive radial glial cells predominate at early stages, followed later in development by increasing numbers of BLBP-positive cells with cell bodies distant from the ventricular zone and ventrally directed leading processes (Domowicz et al., 2008). It is important to note however, that not all labeled cells migrate over the course of the experiment. This is consistent with the observation that while BLBP-positive cells are present all along the dorsal ventricular zone of the hindbrain, migrating cells appear to exit the ventricular zone only in discrete medial and lateral streams. Therefore is it likely that not all BLBP-positive cells in the ventricular zone migrate (Domowicz et al., 2008), supporting the notion that radial glial cells are a heterogeneous population (Pinto and Gotz, 2007). Additionally, since we utilized a ubiquitous promoter to drive the expression of GFP, it is likely that some of the cells labeled at the ventricular zone are not glial cells, or represent a different subtype of non-migratory cells.

The migration of glial cells has been demonstrated previously in the mouse cortex, where glial cells were observed to migrate radially, as well as tangentially to the surface of the brain and through the corpus callosum (Goldman et al., 1997; Kakita and Goldman, 1999; Kakita et al., 2003; Suzuki and Goldman, 2003). Migratory glial cells in the corpus callosum and cortex exhibited a migratory morphology similar to that observed for the EGFP-positive cells described here; the cells had a long leading process which was polarized in the direction of migration (Kakita et al., 2003; Suzuki and Goldman, 2003). Interestingly, these glial cells exhibited a variable rate of migration, with a marked deceleration after an initial burst of movement (Kakita et al., 2003). Similarly, the migration rates of the glial cells in the chick hindbrain are slow but variable (Fig. 8E–G, 9B–E). While the molecular mechanism of neuronal migration has been extensively characterized (Bielas et al., 2004; Kawauchi and Hoshino, 2008), the molecular machinery underlying glial migration remains much less understood. It will be of interest to use live-cell imaging in a culture chamber-equipped confocal microscope to compare rates of migration of neurons (using slices prepared from younger tissue) with those of glial cells from older tissue slices; examining the rates of glial migration coupled with determining factors which affect these rates will help to elucidate the mechanisms controlling glial cell migration.

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It is likely that the processes of glial cell differentiation and migration are tightly linked. Using this *in vitro* slice culture approach, it should be possible to examine both of these processes. For example, it can be investigated whether the effect of RA on glial cell maturation increases the number of migrating cells or whether inhibition of RA signaling blocks the migration of glial cells from the ventricular zone. The maintenance of the native cellular environment, the ease of culture, the compatibility with local manipulation of gene expression using a variety of techniques, the ability to test the role of different signaling pathways by addition of agents to the culture media, the capacity to quantify enzymatic activity and perform other biochemical analyses, and the potential to follow individual migrating cells, make this system a powerful technique for probing many unanswered questions about glial cell differentiation and migration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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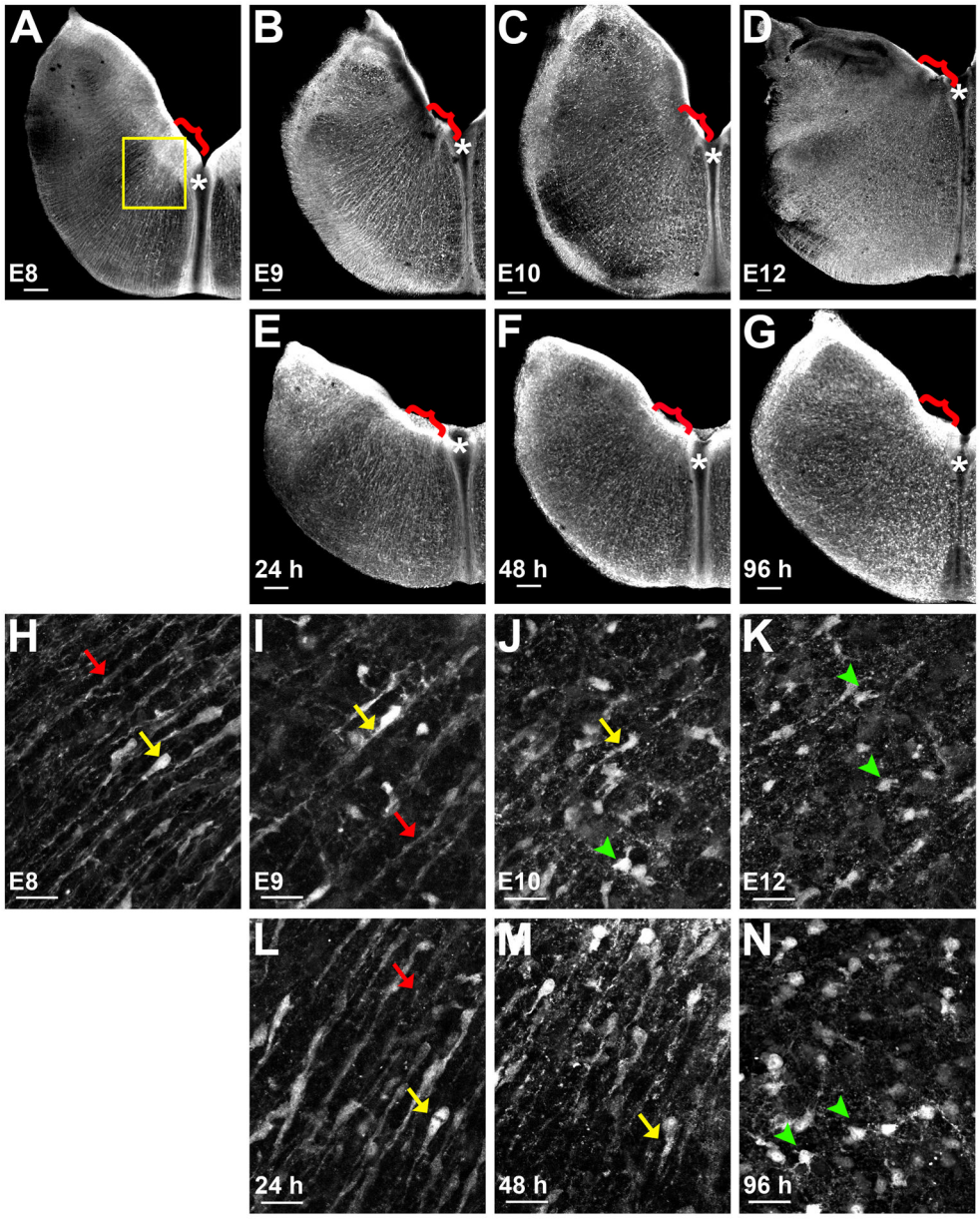

Fig. 1.

BLBP expression *in vivo* and in slice culture. All images are representative confocal single Z-sections through 250 μm thick vibratome slices. (A–D). BLBP expression *in vivo* from E8–E12. Yellow box in (A) represents the general region imaged for the higher magnification images in (H–N). (E–G). BLBP expression in slices cultured for 24 h (E), 48 h (F), and 96 h (G). The region of the slice below the red bracket in (A–G) is the location of the medial migratory stream of cells. (H–N). Higher magnification images of slices from E8–E12 tissue (H–K) and slices cultured for 24 (L), 48 (M), and 96 h (N). Red arrows denote radial glial cells, yellow arrows indicated polarized cells with a migratory morphology, and green arrowheads demarcate cells which have the morphology of maturing astrocytes. Asterisks denote the midline. Scale bars in (A–G) = 100 μm. Scale bars in (H–N) = 20 μm.

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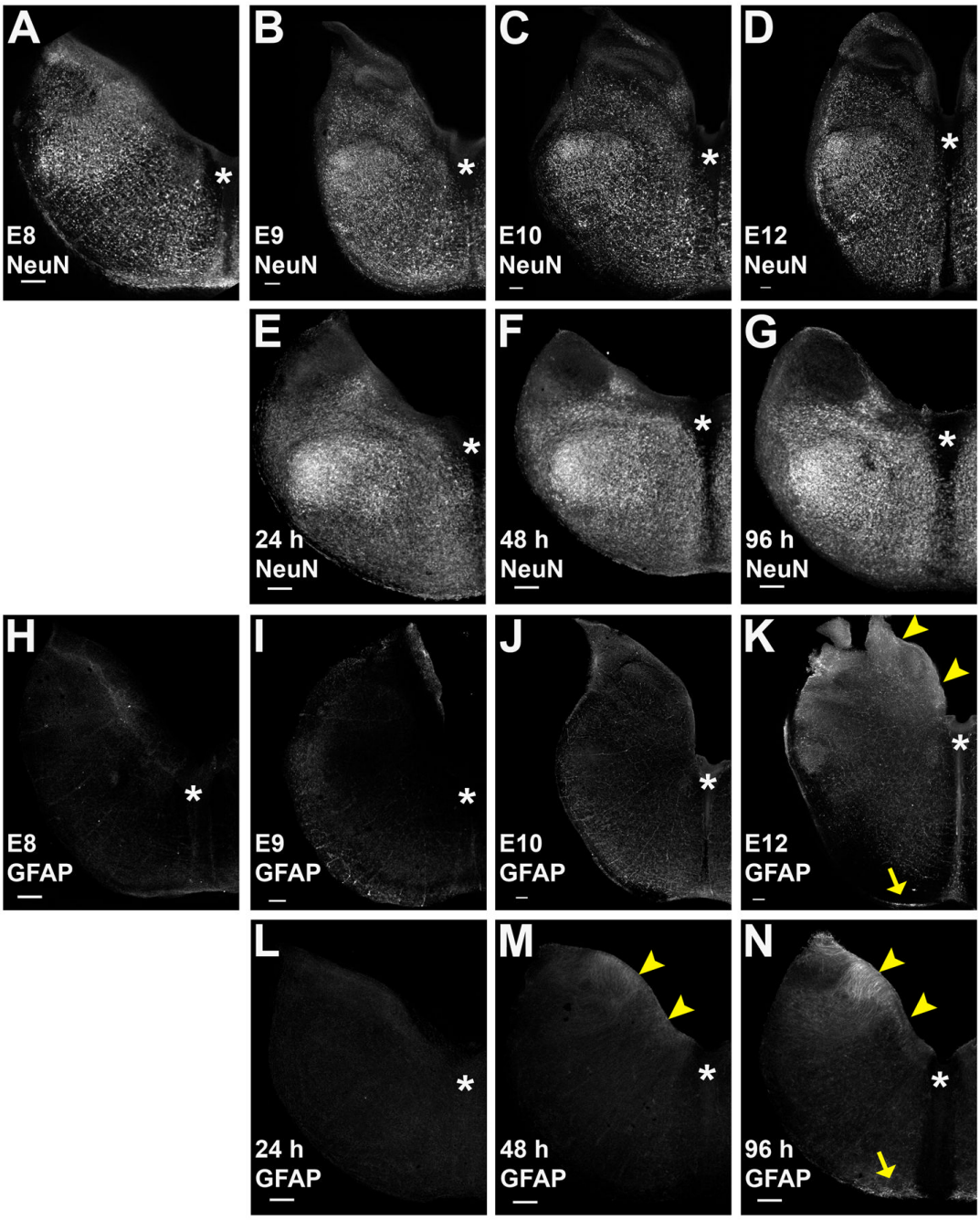

Fig. 2.

Expression of NeuN and GFAP *in vivo* and in slice culture. All images are representative confocal single Z-sections through 250 μm thick vibratome slices. (A–D). NeuN expression *in vivo* from E8–E12. (E–G). NeuN expression in slices cultured for 24 h (L), 48 h (M), and 96 h (N). (H–K). GFAP expression *in vivo* from E8–E12. At E12 (K) there is diffuse expression of GFAP in the medial-ventral boundary of the tissue (yellow arrow) as well in the dorsal ventricular zone (yellow arrowheads). (L–N). GFAP expression in slices cultured for 24 h (S), 48 h (T), and 96 h (U). GFAP expression is evident in the dorsal ventricular zone at 48 and 96 h in culture (M–N, yellow arrowheads) and at 96 h in the medial-ventral boundary of the tissue (N, yellow arrow). Asterisks denote the midline. Scale bar = 100 μm.

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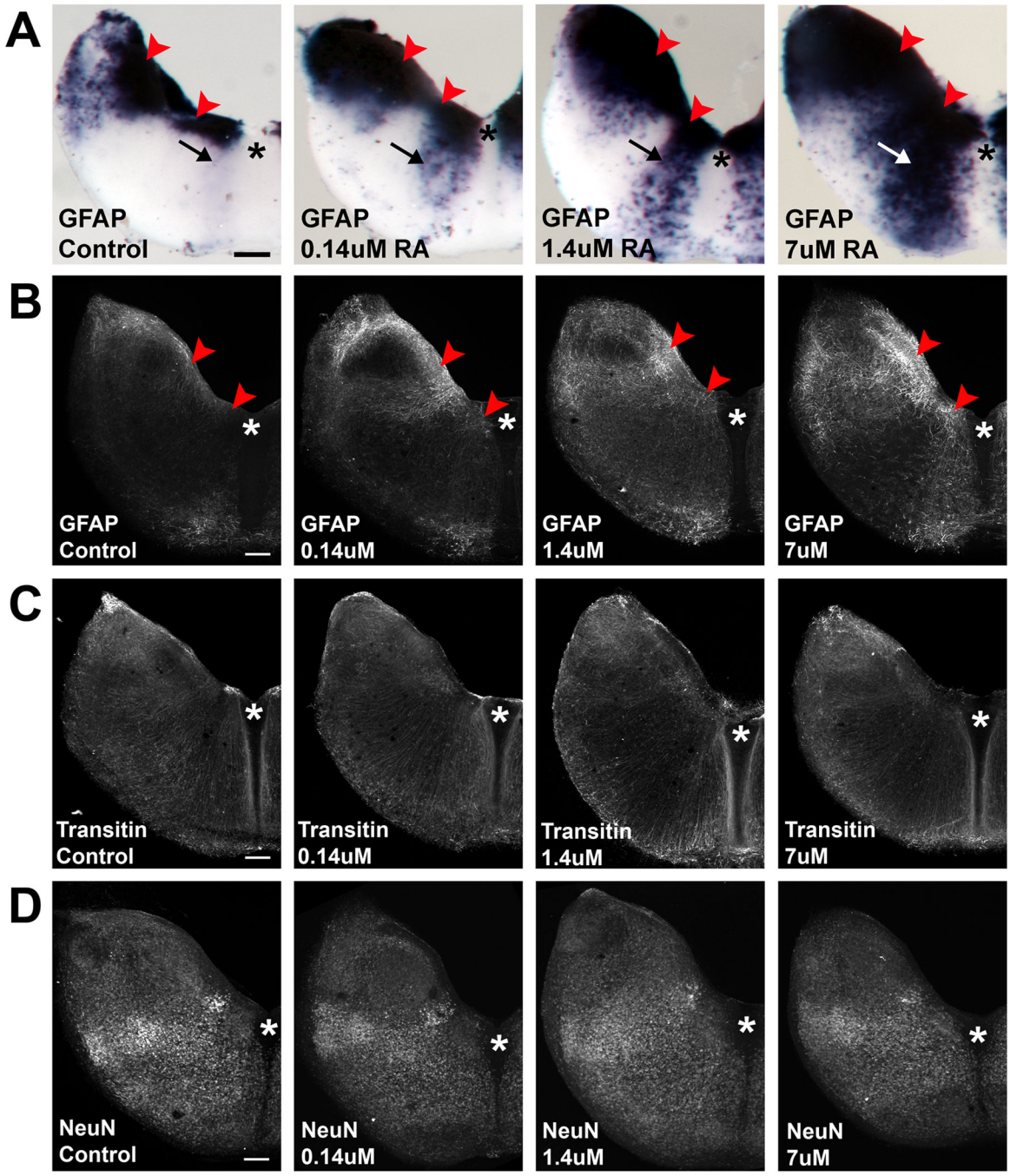

Fig. 3.

RA increases GFAP expression in hindbrain slice cultures. Slices were prepared from E8 hindbrain and cultured for 96 h with either control (1.4% ethanol) or increasing concentrations of *trans*-RA (0.14 μM, 1.4 μM or 7 μM) and then processed for whole mount *in situ* hybridization or immunohistochemistry. Panels B–D are compressed confocal Z-stacks. (A) *GFAP* mRNA expression. Note the concentration dependent increase in GFAP expression specifically in those regions which normally express GFAP: the dorsal ventricular zone (red arrowheads) and the medial migrating stream of cells (arrows). (B). GFAP protein expression, which also increases in a concentration dependent fashion in the dorsal ventricular zone and the medial and lateral discrete streams of cells (red arrowheads). Consistent levels of Transitin

(C) and NeuN (D) protein were observed with RA treatment compared to control treatment. Asterisks denote the midline. Scale bar = 100 μm.

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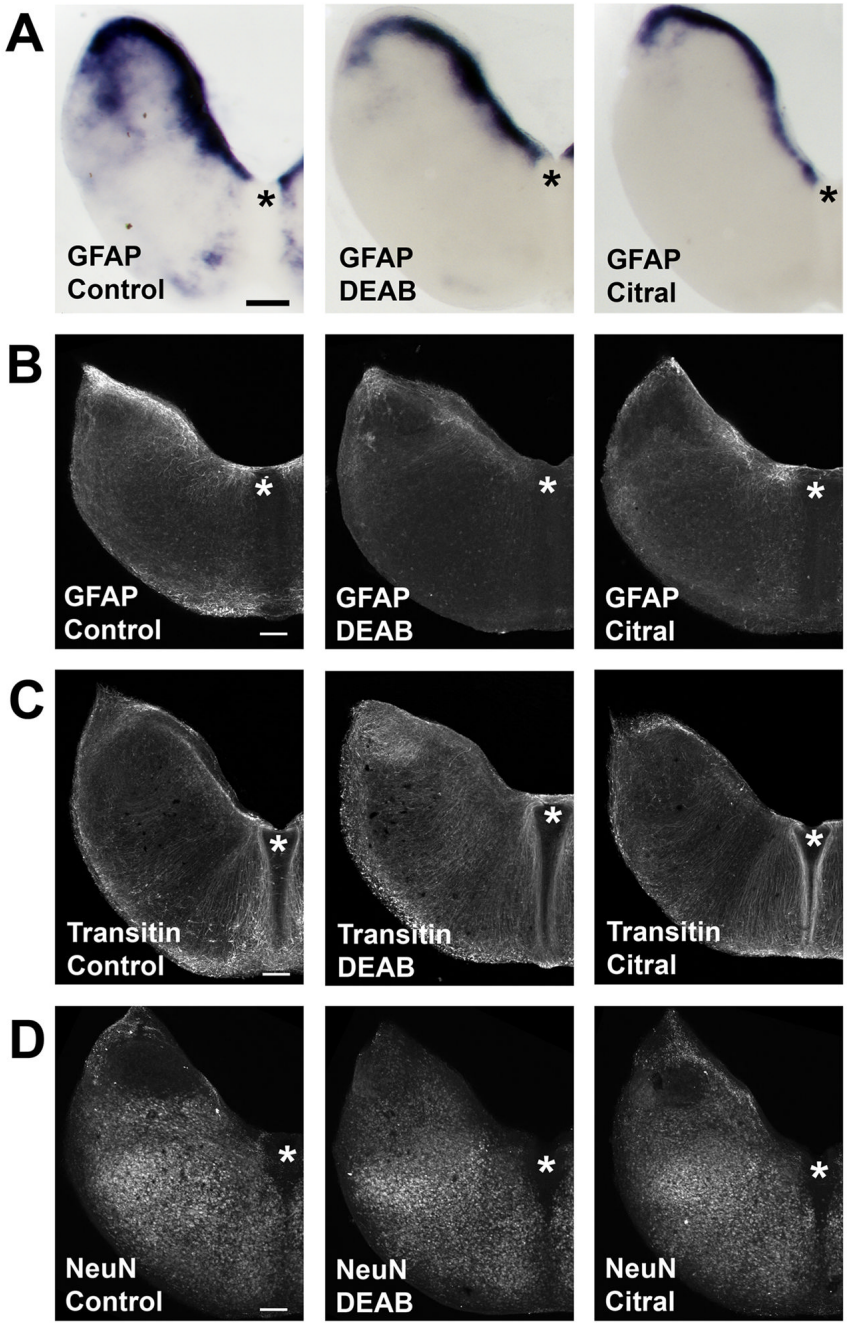

Fig. 4.

Inhibition of RA biosynthesis reduces GFAP expression in hindbrain slice cultures. Slices were prepared from E8 hindbrain and cultured for 96 h with either control (1.4% ethanol), or with one of the retinaldehyde dehydrogenase inhibitors, DEAB (30 μM) or Citral (180 μM), and then processed for whole mount *in situ* hybridization or immunohistochemistry. Panels B–D are compressed confocal Z-stacks. (A) Expression of *GFAP* mRNA. (B) Expression of GFAP protein. Note the decrease in GFAP expression in slices treated with Citral and DEAB at both the mRNA and protein levels. Consistent levels of Transitin (C) and NeuN (D) protein were observed with DEAB or Citral treatment compared to control treatment. Asterisks indicate the midline. Scale bar = 100 μm.

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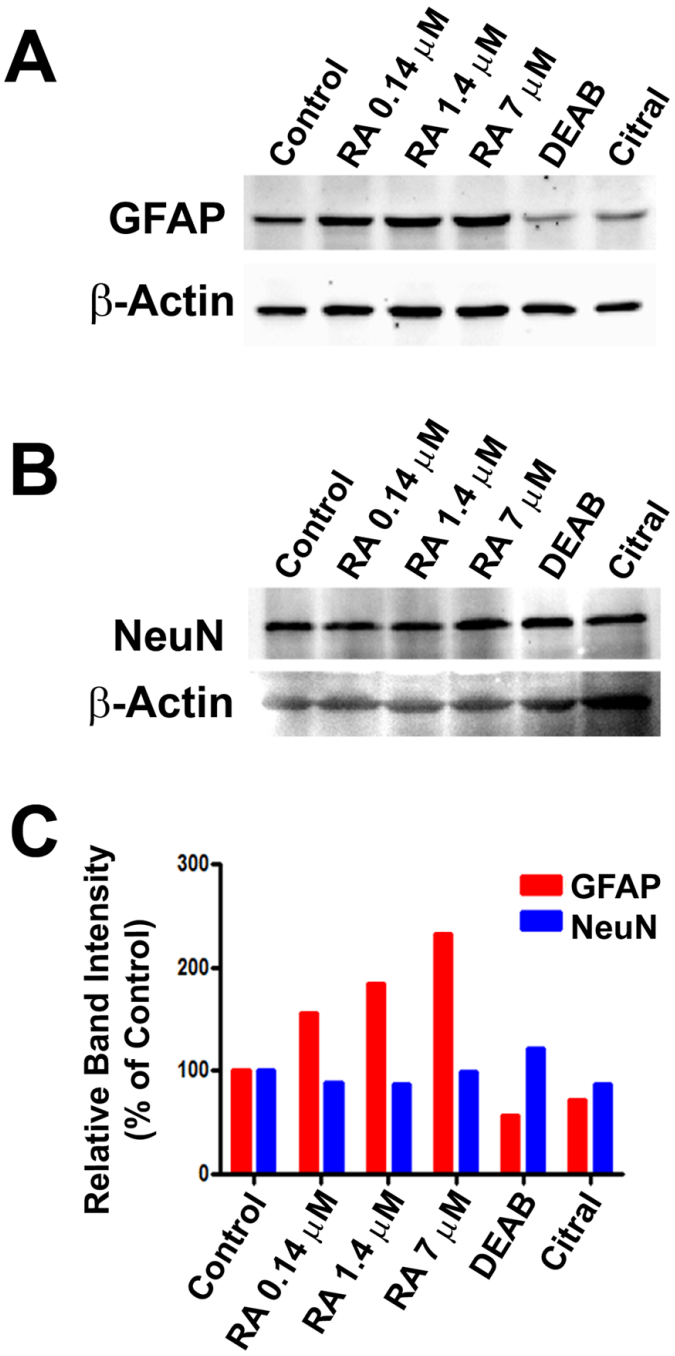

Fig. 5.

Western blotting on treated slice lysates. Lysates from control and treated slices were prepared after 96 h in culture. Equal amounts of total protein for each sample as determined by BCA protein assay were loaded on the gel. (A). Representative Western blot showing an concentration dependent increase in GFAP expression with RA addition and a decrease in GFAP expression in slices treated with inhibitors of RA synthesis. β-actin blot of the same gel serves as a loading control. (B). Representative Western blot showing no changes in the level of NeuN expression over control with any of the treatments. β-actin blot of the same gel serves as a loading control. (C). Quantification of the Western blots. The intensity of the bands in the

GFAP and NeuN blots are plotted as a percent of the β-actin control. Data is representative of three independent experiments.

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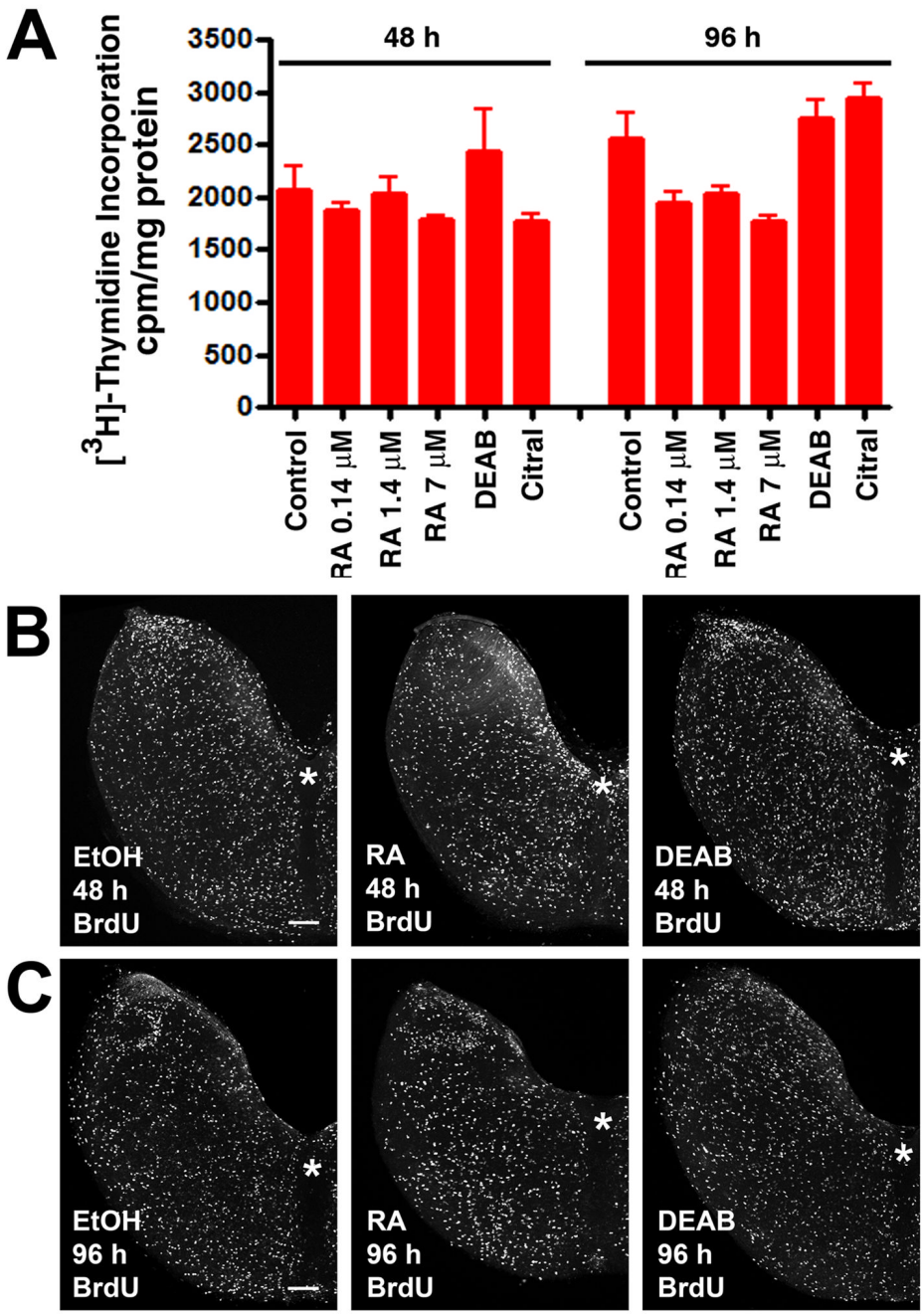

Fig. 6.

Addition of RA or an inhibitor or RA biosynthesis does not affect cell division in slice cultures. Slices were incubated for 48 or 96 h in culture with control (1.4% ethanol), 7 μM RA or 30 μM DEAB. (A) None of the treatments have an overall effect on [^{3}H]-thymidine incorporation in the slice over the 24 h period prior to harvest. Examination of BrdU incorporation 1 h prior to harvest of tissue at 48h (B) or 96 h (C) in culture reveals no significant differences in amount or pattern of BrdU incorporation between treatments. Images are compressed confocal Z-stacks. Asterisks indicate the midline. Scale bars = 100 μm

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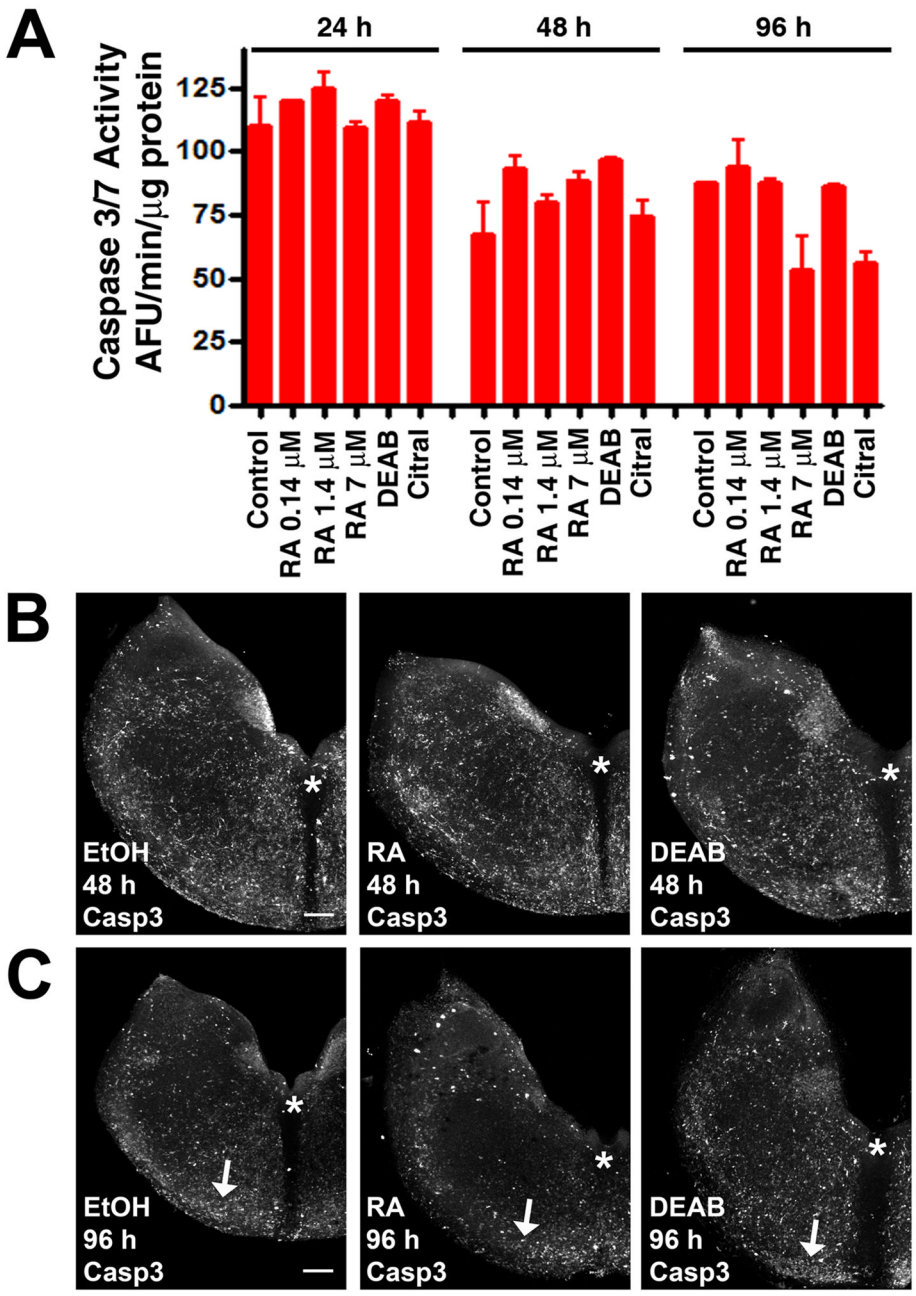

Fig. 7.

Addition of RA or an inhibitor or RA biosynthesis does not affect cell death in slice cultures. Slices were incubated for 48 or 96 h in culture with control (1.4% ethanol), 7 μM RA or 30 μM DEAB. (A) Caspase 3/7 activity as measured by a fluorgenic indicator assay from cultured slices reveals no significant differences between treatments at any time point. Note the highest activity was observed at 24 h with all treatments. (B–C) Immunohistochemistry for activated cleaved caspase-3 in slices harvested at 48h (B) and 96h (C) in culture. At 96 h, slices treated with RA had slightly lower levels of caspase-3 staining than control and DEAB treated slices. This is also observed in the caspase3/7 activity assay (A). At 96 h there was consistent staining observed with all slices near the ventral portion of the slice (arrows). Images are compressed

confocal Z-stacks and are oriented as in Figure 1. Asterisks indicate the midline. Scale bars = 100 μm.

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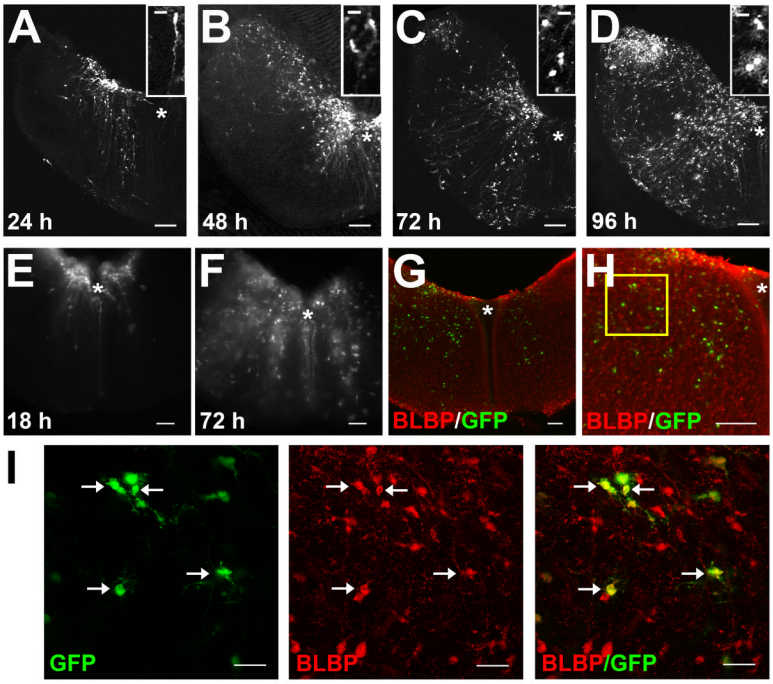

Fig. 8.

Analysis of cell migration in chick hindbrain slices by electroporation. All slices were electroporated with a CMV-EGFP plasmid and cultured for 24–96 h. (A–D). Compressed confocal Z-stacks of slices electroporated and then cultured for 24 (A), 48 (B), 72 (C) or 96 h (D). Note the increase in the number of cells away from the ventricular zone from 24–96 hours. Also note that the migration of cells occurs in the same medial stream where BLBP expression shifts between E8–E12 in Fig. 1. Insets in (A–D) show higher magnification views of representative cells from each time point, highlighting the changing morphology of these cells which parallel *in vivo* development, seen in Fig. 1. (E–H). Same slice live-imaged at 18 h (E) or 72 h (F) or in as seen in a compressed confocal Z-stack after fixation and immunostaining (G–H). Note the increase in the number of cells which have left the ventricular zone between 18 and 72 h. Boxed region in (H) is shown in (I). (I). Single confocal Z-sections through the same slice reveal many of the cells which have migrated from the ventricular zone are also positive for the immature glial marker, BLBP (I, arrows). Asterisks denote the midline. Scale bar (A–H) = 100 μm. Scale bar (Insets in A–H, I) = 20 μm.

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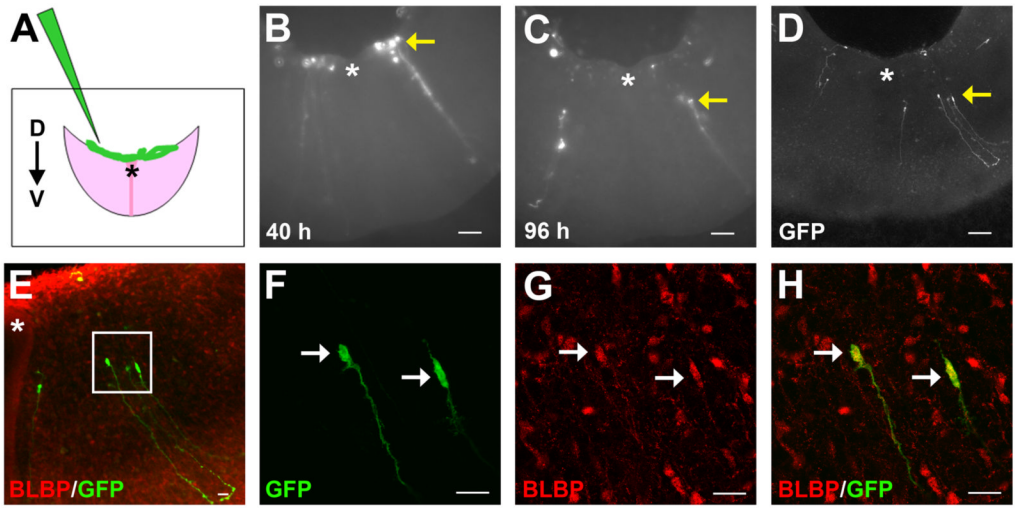

Fig. 9.

Analysis of cell migration in chick hindbrain slices by transfection. (A) Schematic of DNA-lipid complex addition to slices via micropipette. DNA-lipid complexes are injected between the dorsal ventricular zone and the surrounding agarose, essentially trapping the DNA in this region. (B–I) Same slice transfected with a CMV-EGFP plasmid imaged live at 40 h (B), and 96 h (C), and then by confocal microscopy following fixation and immunohistochemistry (D–H). At 40 h, note the GFP-positive cells near the ventricular zone (yellow arrow) (B); by 96 h, these GFP positive cells have a ventrally-oriented leading process and have moved from the ventricular zone (yellow arrow) (C–D). White box in (E) highlights two GFP-positive (green) cells which also express BLBP (red). (F–H). These same two cells shown at higher magnification, showing expression of GFP (F), BLBP (G) and the merged images of the two signals (H). Asterisks indicate the midline. Scale bars (B–D) = 100 μm. Scale bar (G–H) = 20 μm.