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RESEARCH REPORT

Transit amplification in the amniote cerebellum evolved via a heterochronic shift in *NeuroD1* expression

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ABSTRACT

The cerebellum has evolved elaborate foliation in the amniote lineage as a consequence of extensive *Atoh1*-mediated transit amplification in an external germinal layer (EGL) comprising granule cell precursors. To explore the evolutionary origin of this layer, we have examined the molecular geography of cerebellar development throughout the life cycle of *Xenopus laevis*. At metamorphic stages *Xenopus* displays a superficial granule cell layer that is not proliferative and expresses both *Atoh1* and *NeuroD1*, a marker of postmitotic cerebellar granule cells. Premature misexpression of *NeuroD1* in chick partially recapitulates the amphibian condition by suppressing transit amplification. However, unlike in the amphibian, granule cells fail to enter the EGL. Furthermore, misexpression of *NeuroD1* once the EGL is established both triggers radial migration and downregulates *Atoh1*. These results show that the evolution of transit amplification in the EGL required adaptation of *NeuroD1*, both in the timing of its expression and in its regulatory function, with respect to *Atoh1*.

KEY WORDS: Cerebellum, Evolution, Atoh1, *Xenopus*, Chick

INTRODUCTION

Transit amplification is a widespread strategy in neural development that allows the fine-tuning of cell numbers in specific neuronal populations. It is mediated by transient, fate-committed progenitor cells that are spatially and molecularly distinct from precursors in the ventricular layer of the neural tube. Increasing evidence suggests that such cells can be defined by a basal cellular attachment to the pial membrane (Hansen et al., 2010) and respond to distinct mitogenic signals (Klein et al., 2005).

The impact of transit amplification on the evolution of brain structures is most clearly seen in the highly foliated, laminar structure of the mammalian cortex and cerebellum. In the cortex, variation in basal progenitor number in the subventricular zone (SVZ) is a significant determinant of cortex gyrification (Lui et al., 2011; Stahl et al., 2013). The tempo and magnitude of SVZ amplification are also likely to be responsible for variation in the relative proportions of interneuron types and their layering between mammals and between the cortical areas of a given mammal (Fietz and Huttner, 2011; Borrell and Reillo, 2012). The situation is far simpler in the cerebellum where (in both birds and mammals) a single, transit amplifying population of granule cell precursors with a distinct pial attachment (Hausmann and Sievers, 1985) forms a transient external germinal layer (EGL). Proliferation in the EGL is regulated by the morphogen Sonic hedgehog (Shh), for which underlying Purkinje cells are a prominent local source (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999; Lewis et al., 2004). Elegant genetic titration experiments have shown that Shh can precisely regulate the degree of cerebellar foliation (Corrales et al., 2006).

Despite these insights, relatively little is known about the emergence of transit amplification as a developmental strategy. In the cerebellum, there is a dramatic disjunction between developmental strategies used in birds and mammals with that in actinopterygian fish and chondrichthyes, which lack an EGL defined as a distinct basal progenitor population covering the pial surface and expressing the bHLH transcription factor *Atonal1* (*Atoh1*) (Rodriguez-Moldes et al., 2008; Kaslin et al., 2009; Chaplin et al., 2010; Butts et al., 2014). Intriguing studies by Amos Gona in the 1970s suggest that amphibians represent an evolutionarily intermediate condition. For at least part of its development, the frog displays an amniote-like EGL that is apparently non-proliferative (Gona, 1972). We investigated Gona's model in *Xenopus laevis* using modern, molecular tools and find that there is a remarkable shift from anamniote to amniote developmental mechanisms of granule cell development within a single species at metamorphosis. Furthermore, we propose that lack of proliferation in the otherwise amniote-like, postmetamorphic frog EGL is enforced by the precocious expression of another bHLH protein, NeuroD1, which in amniotes marks postmitotic granule cells. We have recapitulated this non-proliferative condition experimentally in the early chick cerebellum through the premature misexpression of *NeuroD1*. Moreover, once the EGL has formed, and in contrast to the situation in the frog, *NeuroD1* misexpression in the chick downregulates *Atoh1* and drives the radial migration of granule cells. Thus, the relative timing of *NeuroD1* expression and a change in its function with respect to *Atoh1* represent a previously unidentified regulatory mechanism for amniote cerebellum growth, providing an explanation for the origin of the proliferative EGL.

RESULTS AND DISCUSSION

Different stages of the *Xenopus* life cycle exhibit different modes of cerebellar development

In amniotes, granule cell precursors migrate into the EGL as the last-born population generated from a thin strip of *Atoh1*-positive neuroepithelial precursors bordering the fourth ventricle roof plate: the rhombic lip (Gilthorpe et al., 2002; Machold and Fishell, 2005). To determine how granule cells are generated in amphibians we compared the expression of *Atoh1* (in *X. laevis*) with that of genes that characterise the rhombic lip lineage across most vertebrates: *Barhl1* [a direct downstream target of *Atoh1* (Chellappa et al., 2008)], *Lhx9* [expressed in non-granule cells, early-born rhombic lip derivatives (Rose et al., 2009)], *Zic1* [expressed in both granule cell precursors and postmitotic neurons (Aruga et al., 1998)] and *NeuroD1* [expressed in postmitotic granule neurons (Miyata et al., 1999)]. At tadpole stages (stage 48), *Atoh1* expression is confined to the rhombic lip (Fig. 1A), which is distinguished by a high density of cells in M phase of mitosis, as shown by staining for

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phosphohistone H3 (PH3; Fig. 1B). Within the cerebellum, *Atoh1* and PH3 are coextensive and confined to the rhombic lip (Fig. 1C), while sagittal sectioning reveals that the superficial layer of the cerebellum contains no proliferative cells (PCNA; Fig. 1D). In a lateral view of the mid/hindbrain region, *Barhl1*-positive rhombic lip derivatives can be seen across the dorsoventral surface of the cerebellum (Fig. 1E) and rostral hindbrain. Extra-cerebellar *Lhx9*-positive cells in ventral hindbrain (Fig. 1F) are spatially segregated from *NeuroD1*-positive postmitotic granule cells in cerebellum (Fig. 1G). At this stage of development, *Atoh1* is confined to the rhombic lip and there is no evidence of an *Atoh1*-positive EGL (Fig. 1H).

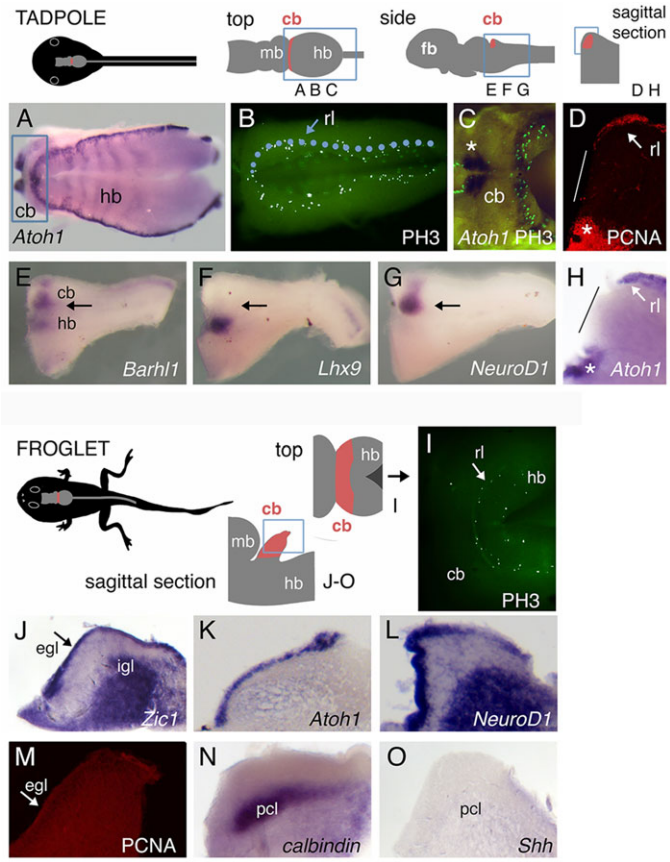

Fig. 1. *Xenopus* displays a non-proliferative EGL at metamorphosis. Schematic drawings of tadpole (stage 48: A-H) and froglet (stage 58: I-O) stages of development are shown with corresponding brain profiles [cerebellum (cb) in red] and location of whole-mount and section views (blue boxes). (A) *Atoh1* expression in whole-mount hindbrain (hb) and cerebellum. (B) Mitotic cells in the rhombic lip (rl; blue dotted line) in an equivalent embryo stained for PH3. (C) Cerebellum of embryo in A (boxed region) counterstained for PH3. The anterior extra-cerebellar *Atoh1*-postive regions (asterisk, also in D,H) correspond to the primordium of isthmic nuclei. (D) In sagittal section, PCNA staining shows that the cerebellum anlage (white line) is devoid of superficial proliferative neurons. (E) *Barhl1* is expressed in cerebellum (arrow) and hindbrain. (F) *Lhx9* is expressed in hindbrain only. (G) *NeuroD1* is expressed in cerebellum (arrow). (H) In sagittal section, *Atoh1* is not expressed on the surface of the cerebellum anlage (black line). (I) In the froglet, mitotic cells in the cerebellum are still confined to the rhombic lip. (J) *Zic1*, a marker of granule neurons at all stages of development, is expressed in both an internal granule cell layer (igl) and an external germinal layer (egl, arrow). (K) *Atoh1* is also expressed in the EGL. (L) *NeuroD1* is expressed in both layers. (M) PCNA staining in sagittal section confirms that the EGL is non-proliferative. (N) *Calbindin* is expressed in the Purkinje cell layer (pcl). (O) Purkinje cells do not express *Shh*. mb, midbrain; fb, forebrain.

After metamorphosis and the breakthrough of the arms (from stage 58), proliferation remains restricted to the rhombic lip and, as in the tadpole, there is no superficial germinal layer within the cerebellum (Fig. 1I). However, *in situ* hybridisation for *Zic1* reveals not only a large internal granule cell layer (IGL), but also labels a distinct, superficial, *Zic1*-positive cell layer (Fig. 1J), which disappears by the completion of metamorphosis (data not shown). The resemblance of this transient layer to the amniote EGL is confirmed by the specific expression of *Atoh1* (Fig. 1K). However, *NeuroD1*, which is a marker of postmitotic granule cells (Fig. 1L), is co-expressed in the EGL with both *Zic1* and *Atoh1*. Lack of proliferation within the EGL (Fig. 1M) corresponds with a Purkinje cell layer that expresses *calbindin* (Fig. 1N) but not *Shh* (Fig. 1O). The *Xenopus* EGL is thus a hybrid of progenitor and postmitotic characteristics: a pial *Atoh1*-positive transient population that nevertheless expresses *NeuroD1* and is non-proliferative, which lies adjacent to an *Shh*-negative Purkinje cell layer.

Although the presence of an EGL in anamniotes has been debated in recent years (Wullimann et al., 2011), there is little evidence for its manifestation in the cerebellum of sharks (Rodriguez-Moldes et al., 2008; Chaplin et al., 2010), basal ray-finned fish (Butts et al., 2014) and early or adult zebrafish (Kaslin et al., 2009, 2013; Chaplin et al., 2010; Kani et al., 2010). We conclude that a transit amplifying precursor layer is also absent in the frog. However, at metamorphic stages, *Xenopus* displays a transient, superficial layer of non-proliferative yet *Atoh1*-positive granule cells. Lack of proliferation in this EGL analogue might explain why the amphibian cerebellum is one of the simplest and proportionately smallest in the vertebrate radiation (Nieuwenhuys et al., 1998). These observations also suggest that an external granule cell layer might serve a function that is independent of proliferation and that, furthermore, this function is not required in either the tadpole or in the EGL-less (Rodriguez-Moldes et al., 2008; Kaslin et al., 2009, 2013; Chaplin et al., 2010; Kani et al., 2010; Butts et al., 2014) anamniote cerebellum.

What, then, is the purpose of a non-proliferative EGL in metamorphic *Xenopus*? One possibility is that an amniote-like EGL may provide a means of establishing a uniform layer of late-born granule cells prior to inward radial migration into a pre-existing, definitive cerebellar neuronal scaffold. Evidence for such a scaffold comes from experiments showing that an EGL can form even when depleted of granule cells (Eddison et al., 2004). By contrast, anamniotes are characterised by a CNS that is subject to continuous growth and remodelling (Otteson and Hitchcock, 2003), negating the need for transient developmental scaffolds. We speculate that if this cytoarchitectonic role represents the ancestral condition for the EGL then transit amplification would necessarily represent a secondary evolutionary adaptation.

NeuroD1 overexpression prevents granule cell proliferation

The coincidence of a lack of transit amplification and the premature expression of *NeuroD1* in the *Xenopus* EGL prompted us to test whether *NeuroD1* is sufficient to suppress proliferation in amniotes. We misexpressed *NeuroD1* in the chick cerebellar rhombic lip at E4 and analysed the cerebellum at E8. A view of the surface of the cerebellum reveals *NeuroD1*-overexpressing GFP-positive cells coincident with regions of reduced PH3 label (Fig. 2A-C), suggesting that ectopic NeuroD1 suppresses proliferation. When viewed in parasagittal section, control GFP electroporations produce a densely labelled superficial EGL (Fig. 2D), with half of the labelled granule lineage cells residing in the EGL. By contrast, cells expressing *NeuroD1:GFP* (Fig. 2E) show a highly significant asymmetric bias in location towards the IGL ($P<0.001$). This is also reflected in PH3

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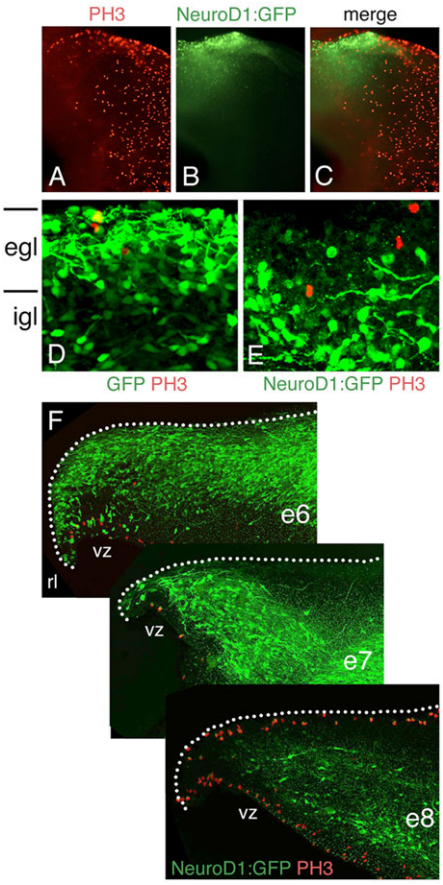

Fig. 2. *NeuroD1* expression at E4 abrogates proliferation and alters migration paths of granule cell precursors in chick. GFP:IRES:*NeuroD1* (or GFP-only control) was electroporated into the chick cerebellar rhombic lip at E4 and the cerebellum analysed at E6-8. (A) Surface view of the EGL in a whole-mount E8 cerebellum anlagen expressing GFP:IRES:*NeuroD1* and stained for PH3 (red). (B) GFP signal (green). (C) Merged PH3 and GFP images. (D) PH3 (red)-labelled section through a control GFP-electroporated cerebellum at E8. Yellow cells are proliferating granule precursors in the EGL. (E) GFP:IRES:*NeuroD1* expression drives cells from the EGL and none is co-stained for PH3. (F) Timecourse of migration of GFP:IRES:*NeuroD1*-expressing cells from the rhombic lip at E6, E7 and E8 in sagittal section counterstained for PH3 (red).

staining at E8: in GFP controls, 179 cells across 17 cerebella were co-labelled with PH3, whereas none that overexpressed *NeuroD1* was co-labelled ($P<0.001$). This suggests that *NeuroD1* expression is sufficient to terminate proliferation and suppress EGL formation by driving postmitotic granule cells into an internal layer.

We followed the timecourse at E6, E7 and E8 of rhombic lip migration following *NeuroD1* misexpression at E4 (Fig. 2F). Prior to granule cell precursor specification at E6 (Wilson and Wingate, 2006), migrating cells follow their normal subpial migration route. However, at E7 and E8, *NeuroD1*-expressing cells avoid the EGL and follow a deep migration path, presumably severing contact with the basal lamina. At no point are labelled cells seen superficially, indicating that the normal phases of accumulation within the EGL and radial migration are bypassed. This suggests that *NeuroD1* expression in granule cells has different consequences for migratory behaviour in amniote and *Xenopus* cerebellum that are manifest at the point of granule cell specification.

Together with the expression data from *Xenopus*, these observations suggest that *NeuroD1* expression suppresses proliferation and that *Atoh1* expression is not sufficient to drive transit amplification. By contrast, *Atoh1* misexpression within the mouse EGL binds *NeuroD1*-positive postmitotic granule cells to this subpial layer (Helms et al., 2001), replicating to some extent the situation within the metamorphic *Xenopus* EGL. This raises the possibility that *Atoh1* acts primarily as a determinant of cellular basal/pial attachment. Thus, although *Atoh1* expression is a necessary prerequisite for transit amplification (Flora et al., 2009), possibly by determining basal attachment (Hausmann and Sievers, 1985), whether amplification occurs is determined by the timing of the onset of *NeuroD1* expression.

Evolutionary heterochrony of *NeuroD1* expression and modification of *NeuroD1* function in amniotes

Given that differences in *NeuroD1* expression correlate with the regulation of proliferative activity and granule cell laminar distribution within the cerebellum of different species, we examined the regulatory basis of *NeuroD1* expression across tetrapods and the interaction between *NeuroD1* and *Atoh1*. Using a comparative genomic analysis of human, mouse, chick, frog and zebrafish, we identified a conserved non-coding element (CNE) upstream of the *NeuroD1* basal promoter that is 183 bp in length in mouse and conserved across osteichthyeans. We tested whether this element could reproduce species-specific *NeuroD1* expression patterns in chick. Whereas a control electroporation of GFP at the rhombic lip labels equal numbers of cells within the EGL and IGL (Fig. 3A), the orthologous proximal elements from both mouse (Fig. 3B) and frog (Fig. 3C) drive GFP expression predominantly within the IGL (Fig. 3D), when combined with the endogenous basal promoter, mirroring the endogenous expression of chick *NeuroD1*. Although it is possible that autoregulation is playing a role, we suggest that this conserved element is interchangeable between tetrapod groups. It might thus be expected to recapitulate an anamniote *NeuroD1* expression pattern if expressed in the metamorphic frog, although this remains to be tested. Which upstream factors act through this element (plausibly via epigenetic modifications) to co-ordinate the differential timing of expression of *NeuroD1* in is an important open question.

To ascertain whether the function of *NeuroD1* with respect to *Atoh1* expression has also been modified during amniote evolution, we misexpressed *NeuroD1* in the EGL of cerebellar slices prepared from E14 chick. Whereas an *Atoh1* enhancer construct robustly tags EGL cells that go on to express the *NeuroD1* reporter at 24 h *in vitro* (Fig. 3E), when *NeuroD1* is misexpressed the expression of the *Atoh1* reporter is absent (Fig. 3F). Thus, in contrast to the situation in *Xenopus*, in which both bHLH transcription factors are co-expressed in the EGL, *NeuroD1* in chick both cell-autonomously downregulates *Atoh1* expression and triggers inward radial migration.

In conclusion, whatever the extrinsic factors regulating *NeuroD1* through its functionally conserved enhancer, our study identifies that the interplay of *Atoh1* and *NeuroD1* expression establishes a temporal window for EGL proliferation that might represent a novel mechanism of growth regulation in the cerebellum. In terms of the evolution of cerebellum development, our results infer that granule progenitor transit amplification emerged through a heterochronic shift of expression of *NeuroD1* and a modification of its regulatory function with respect to *Atoh1* in an ancestral amniote.

MATERIALS AND METHODS

Electroporation of DNA constructs

The full-length chick *NeuroD1* coding sequence was cloned into pGEM-T Easy (Promega) by PCR (primers: forward, 5′-ATGACCAAGTCGTACA-GCGAGA-3′; reverse, 5′-TCACTCGTGGAAGATGGCGCTGA-3′) from cDNA prepared from E12 cerebellum with TRIzol (Invitrogen), and

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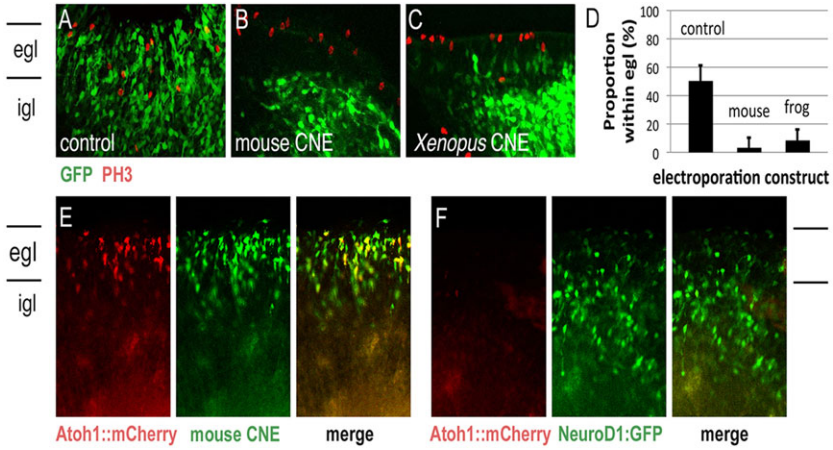

Fig. 3. *NeuroD1* regulation is conserved across tetrapods, but its activity is modified in the amniote lineage. Confocal microscopy images of sagittal sections of E8 chick cerebellum stained for GFP (green) and PH3 (red) following electroporation with: (A) a control plasmid encoding GFP, (B) the CNE upstream of the mouse basal *NeuroD1* promoter and (C) the CNE upstream of the *Xenopus* basal *NeuroD1* promoter. (D) Percentage of GFP-labelled cells in the EGL following electroporation of control (n=15), mouse (n=20) and *Xenopus* (n=12) CNE constructs. The difference between the control and either the mouse or *Xenopus* construct is highly significant (P<0.0001). Expression of the basal promoter of mouse alone is unable to drive the expression of GFP (data not shown). (E) *Atoh1* reporter driving the expression of mCherry (red), co-expressed at E14 with *NeuroD1* (mouse CNE) GFP reporter (green) labels granule cells in the EGL. After 24 h in culture, a large proportion of cells expressing *NeuroD1* retain perdurant mCherry (red), indicating that they had previously activated *Atoh1*. Most have yet to leave the EGL. (F) By contrast, when *NeuroD1* is co-expressed with the *Atoh1* reporter, no mCherry (red) can be detected and the majority of cells have exited the EGL, indicating that *NeuroD1* suppresses the activity of the *Atoh1* enhancer.

subcloned into the pCAβ-IRESeGFPm5 vector (http://www.ncbi.nlm.nih.gov/pubmed/19602272).

The genomic sequence of mouse covering the GENSAT BAC clone RP24-151C22 was used as the base sequence in a VISTA pairwise analysis with human, chick, *Xenopus tropicalis* and zebrafish. Conserved non-coding sequences were defined as those exhibiting at least 70% sequence homology over a sliding window of 100 bp. Using these data, reporter constructs were constructed by building non-coding sequences directly upstream of *GFP* by PCR with long primers using proof reading Fusion polymerase (NEB) and cloning into pGEM-T Easy following A-addition. The mouse construct incorporates a conserved non-coding element upstream of the endogenous mouse basal *NeuroD1* promoter and corresponds to the sequence from −401 bp to +101 bp relative to the longest 5′EST. The amphibian construct incorporates the *X. tropicalis* conserved non-coding element upstream of the *X. tropicalis* basal promoter, corresponding to the sequence from −372 bp to +96 bp relative to the longest 5′EST. As a control, we assembled a construct containing only the basal promoter from the mouse corresponding to the genomic sequence from −146 bp to +101 bp upstream of *GFP*. The Atoh1-Cre plasmid (Kohl et al., 2012) was co-electroporated with pFlox-pA-mCherry (lox-stop-lox mCherry). All constructs were confirmed by sequencing.

Constructs were expressed in fertilised brown chicken eggs (Henry Stewart) incubated at 38°C to embryonic day (E) 4. Briefly, embryos in windowed eggs were injected with DNA constructs into the fourth ventricle and an electric pulse (3×10 V/10 ms) passed through the dorsal neural tube, targeted to the rhombic lip. Eggs were sealed with tape and reincubated until E6, E7 or E8 before fixation in 4% paraformaldehyde. E14 electroporation was carried out on 300 µm slices of chick cerebellum using a modified *in vitro* protocol. Slices were cultured for 24 h at 37°C in 5% CO_2 (Green et al., 2014). Experiments involving live chick embryos were performed in accordance with UK Home Office regulatory standards.

In situ hybridisation and immunofluorescence

X. laevis probes were T/A-cloned using standard PCR from mixed larval cDNA kindly provided by Esther Bell (King's College London) into pGEM-T Easy. RNA *in situ* hybridisation was carried out on dissected whole brains or hindbrains according to standard protocols (Myat et al., 1996) using riboprobes generated for *Xenopus Atoh1*, *Barhl1*, *Zic1*, *Shh*, *calbindin* and *NeuroD1*. Immunohistochemistry was carried out using a standard protocol with rabbit anti-phosphohistone H3 (Cell Signaling Technology, 9701L; 1:100), anti-PCNA (AbCam, ab18197; 1:500) or mouse anti-GFP (Life Technologies, a6455; 1:500) and appropriate Alexa Fluor secondary antibodies (Life Technologies). Hindbrains were embedded in 20% gelatin and vibratome sectioned at 50 µm. Whole-mount *Xenopus* hindbrains were photographed on a Zeiss Stemi SV6 microscope equipped with an Olympus DP camera. Sections were photographed on a Leica MZFLIII microscope and QCapture camera. Confocal images were captured using a Nikon Eclipse 80i microscope with EZ-C1 3.70 software. Images were compiled and cell quantifications made in ImageJ (v10.2) and Adobe Photoshop (v5.5). Statistical significance was calculated using Student's t-test.

Competing interests

The authors declare no competing financial interests.

Author contributions

T.B. and R.J.T.W. designed the study. T.B. and M.H. performed experiments. T.B., M.H. and R.J.T.W. wrote the manuscript.

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