BMP signalling facilitates transit amplification in the developing cerebellum

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ABSTRACT

The external granule layer (EGL) is a transient proliferative layer that gives rise to cerebellar granule neurons and drives the foliation of amniote cerebella. The formation of and differentiation from the EGL is incompletely understood, though BMP signalling has been implicated. Here, we characterise active BMP signalling during cerebellar development in chick and human and show that while in chick BMP signalling correlates with EGL formation, humans maintain BMP signalling throughout the EGL after the onset of foliation. Using *in ovo* electroporation in chick, we show that BMP signalling is necessary for EGL formation, but not for granule neuron fate. Our data are also consistent with a second role for BMP signalling in driving differentiation of granule progenitors in the EGL. These results elucidate two key, temporally distinct roles for BMP signalling in organising first the assembly of the EGL and then the tempo of granule neuron production within it.

INTRODUCTION

Transit amplification is a significant substrate for adaptation in the development of complex neural circuitry. In both the neocortex and cerebellum, the elaboration of specialised laminae

supporting secondary proliferation is associated with increased foliation and complexity. Specialised sub-ventricular cell types which are either diminished or absent in mice, are expanded in the human cortex (Hansen *et al.,* 2010; Heide *et al.,* 2020) and, as recently shown (Haldipur *et al.,* 2019), uniquely characterise the human cerebellar rhombic lip. In the cerebellum, tetrapods display a transient, superficial layer of neural precursors, the external granule layer (EGL) (Iulianella *et al.,* 2019), which like the cortical subventricular zone is the site of transit amplification, but in this case only of a single cell type: the cerebellar granule neuron. Across different species, the degree of proliferation corresponds to the subsequent degree of elaboration of cerebellum morphology into folia. In the small, unfoliated amphibian cerebellum, the EGL is non-proliferative (Gona, 1976; Butts *et al.,* 2014b). By contrast, in the foliated cerebellum of mammals and birds, the EGL is packed full of transit amplifying granule cell precursors.

The EGL provides a superb model for understanding how transit amplification is regulated and is also clinically significant as the site of unregulated proliferation that gives rise to medulloblastoma, a devastating childhood brain tumour. The implication of Sonic Hedgehog (Shh) signalling in a large minority of medullblastoma (Pietsch *et al.,* 1997; Raffel *et al.,* 1997; Vorechovsky *et al.,* 1997) led to an examination of the role of Purkinje cell derived Shh signals in regulating EGL proliferation (Dahmane and Ruiz I Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Granule cell neurons that migrate through the Purkinje cell layer into the internal granule cell layer are the product of multiple rounds of symmetric division in the overlying EGL (Espinosa and Luo, 2008; Nakashima *et al.,* 2015; Yang *et al.,* 2015). Purkinje cell-derived Shh boosts granule cell precursor proliferation in the EGL and when Shh signalling is reduced or increased, the cerebellum is smaller or larger, respectively (Corrales *et al.,* 2004; Corrales *et al.,* 2006).

Proliferative behaviour in the EGL is intimately linked to a transition in migratory behaviour from tangential to radial (Singh and Solecki, 2015), a process driven by complex cytoskeletal rearrangements (Trivedi *et al.* 2017). This transition has recently been shown to be influenced by the level of vascularisation of the EGL and the associated oxygen tension during postnatal development (Kullmann *et al.* 2020). However, the signalling factors that modulate and terminate transit amplification and their underlying mechanisms of action are poorly understood. Recent evidence has suggested that signalling through the p75 neurotrophin receptor and Bone Morphogenetic Protein (BMP) pathways regulate cell cycle exit of granule progenitors. While removal of the p75 receptor elicits corresponding phenotypes of delayed cell cycle exit and larger

cerebella (Zanin *et al.,* 2016, 2019), the functions of the BMP pathway in cerebellar development have proved complex.

BMP signalling has a well-established function in promoting dorsal fate specification in the central nervous system (Hegarty *et al.,* 2013; Bier and De Robertis, 2015), and the EGL is derived from the most dorsal aspect of the cerebellar neuroepithelium, the rhombic lip (Alder *et al.,* 1996; Machold and Fishell, 2005; Wang *et al.,* 2005). During early development, rhombic lip specification is dependent upon BMP signalling (Chizhikov *et al.,* 2006; Machold *et al.,* 2007). However, EGL progenitors are specified following conditional deletion of BMP receptors 1a and 1b (Qin *et al.,* 2006), intracellular Smad transducers of BMP signalling (Fernandes *et al.,* 2012; Tong and Kwan, 2013), and an upstream regulator, *Meis1* (Owa *et al.,* 2018). Nevertheless, these conditional alleles all result in cerebella that are smaller and have reduced foliation, suggesting that BMP signalling is required for normal cerebellum expansion. In contrast, *in vitro*, BMP signalling antagonises Shh responses in granule cells (Rios *et al.,* 2004) and promotes differentiation (Zhao *et al.,* 2008; Ayrault *et al.,* 2010), suggesting that its role is to supress proliferation. In both cases, the significance of BMP signalling for granule precursor behaviour is unclear given evidence of relatively low levels of BMP signalling in the EGL within the mouse cerebellum at P0 (Qin *et al.,* 2006) and P4 (Rios *et al.,* 2004), around the time that folia are beginning to grow (Sudarov and Joyner, 2007).

To directly address the question of whether and how BMP signalling affects cells within the EGL we assessed BMP signalling during EGL formation and subsequent foliation in human and chick, and took advantage of the ability to experimentally manipulate BMP signalling in a targeted manner in the developing avian cerebellum. We show that granule cell precursors transduce BMP signals as the EGL forms. As the avian but not the human cerebellum form folia, responses become confined to differentiating cells. Correspondingly, blocking BMP responses in a cell autonomous fashion in chick prevents EGL formation, while upregulating a constitutively active BMP receptor drives granule cells towards precocious differentiation. Our observations suggest that BMP signalling has two distinct phases during EGL development: as well as being responsible for terminal granule differentiation, BMP signalling drives tangential migration and EGL formation, and thereby facilitates the transit amplification that characterises the amniote cerebellum.

RESULTS

Granule cells transduce BMP signals differentially across development and species

We used an antibody against phospho(p)Smad to log BMP signalling throughout cerebellar development (Figure 1a). At E5 (Figure 1b), pSmad expression is limited to cells proximal to the interface between the neurogenic neuroepithelium and the non-neurogenic roof plate, the rhombic lip. As the EGL is assembled at the pial surface up to E8 (Figure 1c), pSmad expression is uniform throughout the subpial layer. The expression of pSmad decreases in the EGL and becomes discontinuous in the granule lineage as the cerebellum begins to form folia from E10 (Figure 1d), with strong expression in the inner granule layer (IGL). By E14, expression of pSmad is seen in only a small number of EGL granule cell precursors at the crests of folia (Figure 1e, h) and is entirely absent from the EGL in the fissures (Figure 1f, h). This correlates with differences in the Purkinje cell layer, which is disorganised at the crests (Figure 1e) but in fissures comprises a uniform monolayer comparable to its mature organisation (Figure 1f). At this stage, pSmad is strongly expressed throughout the Purkinje cell and internal granule cell layer (Figure 1e, f, h). Correspondingly, *in situ* hybridisation for BMP ligands *BMP2* and *4* and receptors BmpR1a and BmpR1b reveals strong expression in the EGL at E10 but preferential expression in folia crests by E14 (Figure 1g). Likewise, BMP receptors *BmpRIa* and *BmpR1b* also show consistent expression throughout the EGL at E10 but an upregulation within the folia crests at E14.

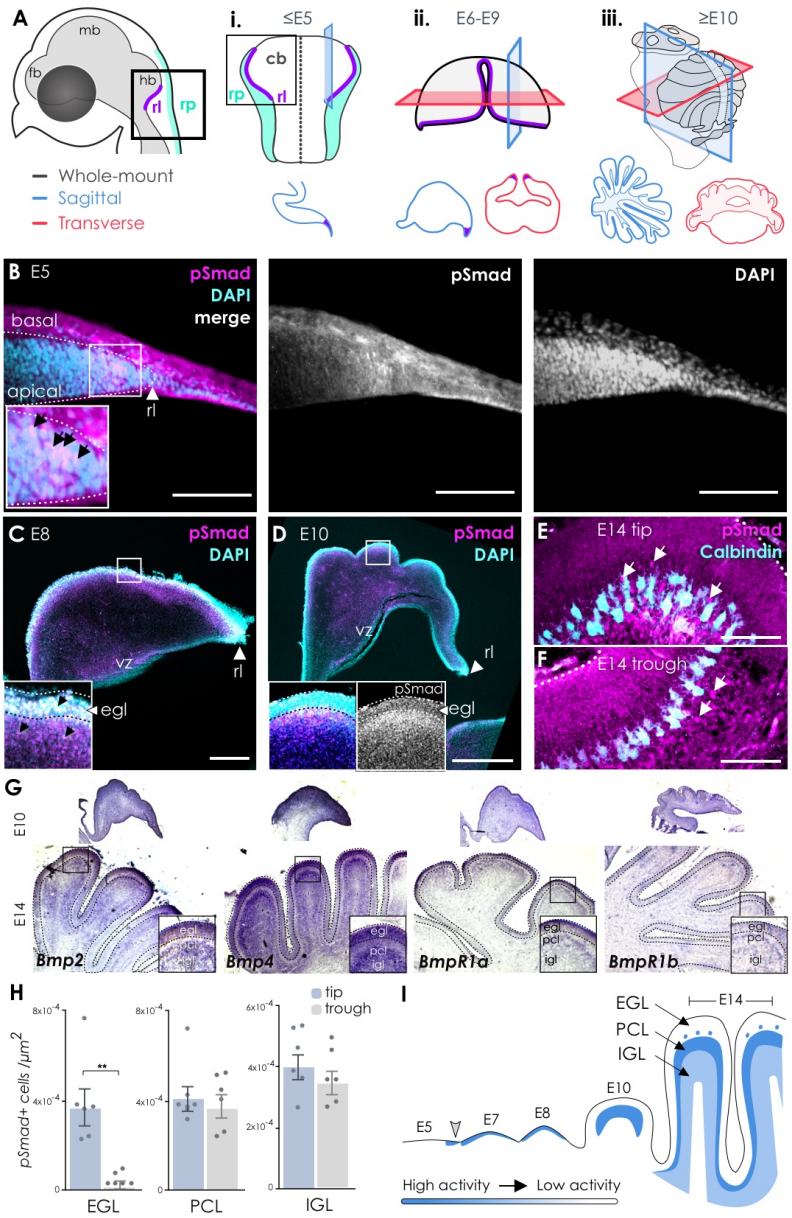

Figure 1: Characterisation of BMP activity throughout cerebellar development.

(A) A schematic showing the anatomical location of the forebrain (fb), midbrain (mb), hindbrain (hb), rhombic lip (rl) and roof plate (rp) in the chick embryo. Planes of section used throughout this study were; whole-mounted, flattened hindbrains (Ai), sagittal (blue outline) and transverse (red outline; Ai, ii) sections. An antibody against the phosphorylated 3' serine residues of Smad1/5/9 (pSmad) shows the spatial requirements of BMP activity at the E5 rhombic lip (B, n=3), during EGL establishment at E8 (C, n=3), at the onset of proliferation in the E10 EGL as folia develop (C, n=2) and at highly proliferative and foliated stages of cerebellum development (E, F; E14, n=6). (G) mRNA

expression of BMP ligands (*Bmp2, 4*) and receptors (*BmpR1a, b*) at E10 (n=3) and E14 (n=4). (H) Spatial distribution of pSmad expression in the lamina layers (EGL; external granule layer, PCL; Purkinje cell layer and IGL; inner granule layer) of the crests (blue bars, E) and fissures (grey bars, F) of sagittally sectioned E14 cerebellum (n=6 sections from 4 cerebella). Quantification of pSmad expression shows that BMP activity is significantly higher in the EGL of the folia crests compared to the fissures (G; p= 0.0015; mean ± SEM = 3.521e-005 ± 8.116e-006), however no significant differences in activity were observed between the PCL and IGL.(I) A summary of BMP activity during EGL formation and folia development in the chick (E5-E14). Scale bars B, E; 25μm, C-D; 50μm.

Taken together, our data (Figure 1g) suggest differing patterns of BMP signalling before and after the initiation of foliation. To explore whether this is conserved in the much more foliated human cerebellum, we assessed pSmad expression during early initial stages of foliation (13pcw of human development, Figure 2a-f) and later stages (19pcw, Figure 2g-l), when foliation is well underway. Formation of the human EGL is apparent by pcw10 (Figure 2a), and in common with mouse and chick, the initiation of foliation is coincident with the onset of Shh expression in developing Purkinje neurons, such that foliation and Shh expression are obvious by pcw12 (Figure 2b). As in chick, pSmad expression is present in the Purkinje cells and the internal granule layer, both at early (Figure 2c, d, e) and later foliation stages (Figure 2i, j, k). Again as in chick, at later foliation stages (pcw19) we observed a difference in the maturation of Purkinje neurons between folia crests and fissures, with a more mature monolayer being present in fissures. However, unlike the situation in chick, pSmad expression after the onset of foliation is observed in the EGL in both folia crests (Figure 2d, j) and fissures (Figure 2e, k) at both early (Figure 2c, d, e) and later (Figure 2i, j, k) stages of folia development.

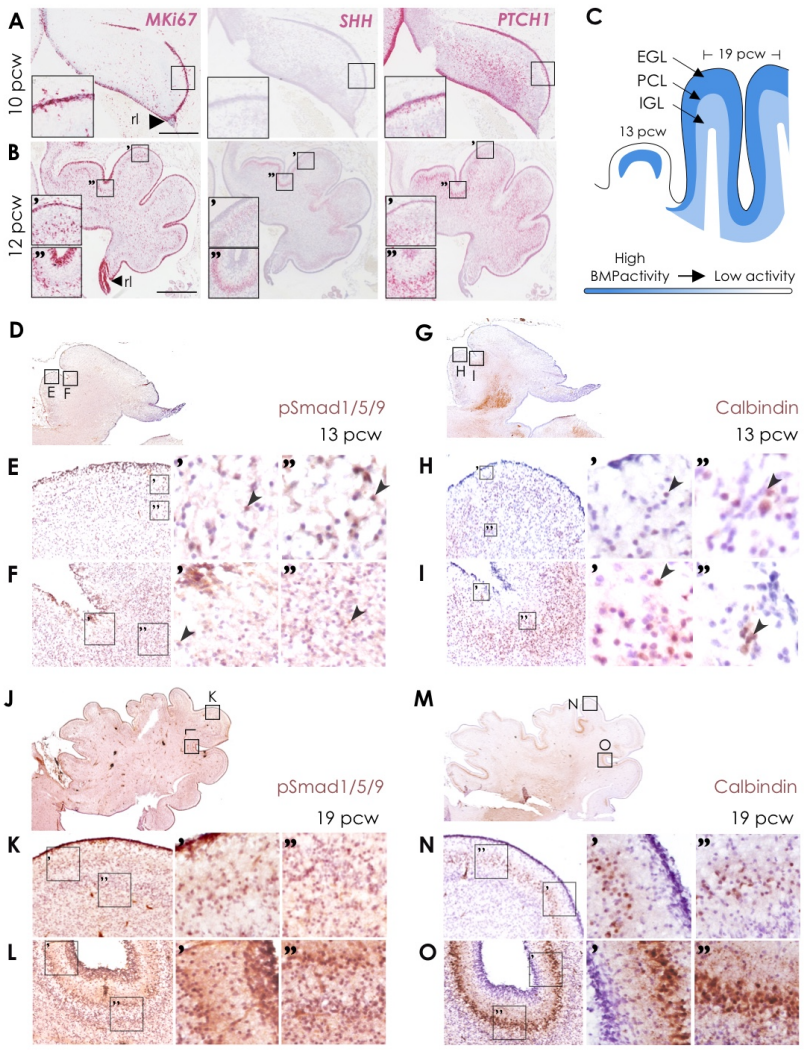

Figure 2: Shh expression and BMP activity in the embryonic human cerebellum. Expression of the proliferation marker *Ki67, SHH* and Shh receptor *PATCHED1 (PTCH1)* during EGL establishment in the 10pcw (A; n=2) and during early foliation in the 12pcw (B; n=1) human cerebellum. BMP activity, summarised in (C), is observed throughout the 13pcw human cerebellum (D, n=2) folia crests (E) and fissures (F). Calbindin expression in the 13pcw cerebellum (G) folia crests (H) and fissures (I) shows the early migration of Purkinje cell precursors. BMP activity in the 19pcw cerebellum (J; n=2) is observed in the EGL of the folia crests (K) and to a lesser extent in the EGL of the fissures (L). Calbindin expression in the 19pcw cerebellum (M) shows a less organised Purkinje cell layer in the folia crests (N) compared to the fissures (O). Scale bar A; 500µm.

BMP signalling is required for the assembly of the EGL

To determine the role of BMP signalling during the early assembly of the EGL, we used a targeted electroporation in chick at the rhombic lip at E4 (to predominantly target granule cell precursors) of a negative intracellular BMP regulator Smad6 (Xie *et al.,* 2011) or a constitutively active BMP regulator Smad1EVE, a variant of the transcription factor Smad1 in which the N-terminal SVS residue that is phosphorylated during activation is changed to EVE, (Fuentealba *et al.,* 2007; Song *et al.,* 2014).

We first confirmed that our constructs were able to affect BMP signal transduction in a predictable manner by characterising the expression of pSmad 2 days after overexpression of control, Smad1EVE, and Smad6 constructs at E3. As expected, pSmad expression was either unchanged, upregulated in a cell autonomous manner or abolished, respectively (Figure 3a).

Electroporation of a control tdTomato (tomato) construct into the cerebellar rhombic lip at E4 results in the labelling of the assembling EGL at E7 (Figure 3b). Upregulation of BMP signal transduction by overexpression of Smad1EVE at E4 resulted in tangential migration of EGL cells in a subpial pattern similar to that seen in control electroporations, albeit with a partial depletion of the EGL distal to the rhombic lip (Figure 3c). In contrast, inhibition of BMP signal transduction by overexpression of Smad6 resulted in a subpial cell-free layer in the position that a granule precursor-rich EGL would be expected to form. This cell free zone extended across the cerebellum from anterior to posterior and appears to contain axonal processes (Figure 3d).

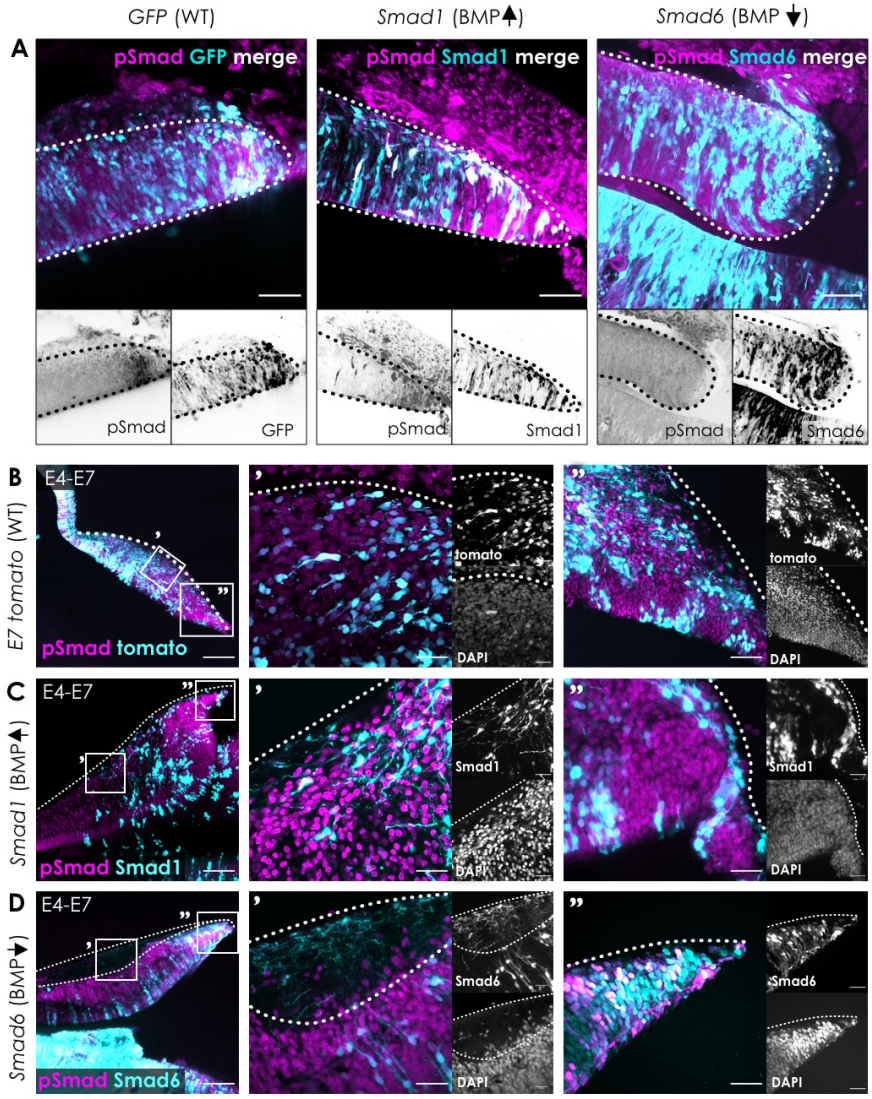

Figure 3: BMP signalling is required for establishing the external granule layer. (A) E3 hindbrains were electroporated with pCAß-GFP alone (n=3), or in combination with pCAß-Smad1EVE-IRES-GFP (Smad1; BMP↑; n=3) or pCAß-Smad6 (Smad6; BMP↓; n=3), incubated to E5, then embedded in gelatine and sectioned sagittally at 100µm. Sections were then immunolabelled using an antibody against phosphorylated Smad1/5/9 (pSmad) to confirm either no change in BMP activity (WT), or up- or down-regulation of BMP signalling at the rhombic lip in Smad1::GFP and Smad6::GFP electroporated embryos, respectively. E4 embryos were electroporated with pCAß-tdTomato alone (B, n=3), or in combination with Smad1 (BMP↑; C, n=7) or Smad6 (BMP↓; D, n=7), further incubated to E7 and then sagittally sectioned. Dotted white lines A; rhombic lip, B-D; pial surface. Scale bars A; 50µm, B-D; 100µm, B'-D"; 25µm.

BMP signalling affects tangential migration but not the specification of granule cells

In order to explore the cell autonomous effects of our manipulations upon cellular migratory behaviour, we examined the morphology of cells migrating away from the rhombic lip. We first re-examined the normal time course of granule cell differentiation from our control embryos electroporated with tomato at E4 and harvested at E7. Granule cell precursors exhibit a variety of morphologies consistent with the reported steps of proliferation and early migration within the EGL (Hanzel *et al.*, 2019), with migrating granule cell precursors being predominantly bipolar or unipolar (Figure 4a). However, no cells at this age had yet entered the internal granule cell layer and adopted the T-shape axonal morphology that characterises definitive granule cells after their exit of the EGL.

Because granule cell precursors undergo multiple rounds of division, electroporated constructs are rapidly diluted and no longer expressed in the EGL by E10. To log the acquisition of definitive morphologies by granule cells we therefore took advantage of the Tol2 transposon system (Sato *et al.,* 2007) to indelibly label granule cell progenitors and their progeny in a mosaic fashion at E2 and examined the cerebellum at E14, once granule differentiation is well under way (Figure 4b). At E14, the internal granule cell layer is occupied by granule cells with T-shaped axons.

When we experimentally manipulated BMP signalling, we first observed that following electroporation at E3, down-regulation of BMP signal transduction decreases migration away from the rhombic lip, whereas up-regulation promotes it (data not shown). When we examined the progeny at E7 of rhombic lip cells that had been electroporated with Smad6 at E4, we were surprised to find a preponderance of mature granule cell morphologies (Figure 4c). This suggests that not only are cells still produced at the rhombic lip but that these are able to develop precociously into definitive granule neurons despite the absence of a transit amplifying EGL (Figure 3d). These results indicate that BMP signalling is not required for cells to be generated at the rhombic lip and undergo differentiation into granule neurons. Rather, tangential migration leading to the formation of an EGL is inhibited when BMP signalling is perturbed.

When we examined cells expressing the constitutively active BMP receptor Smad1EVE, we were again surprised to find mature granule neuron morphologies amongst labelled cells deep to the EGL (Figure 4d). In contrast to downregulation of BMP signal transduction (Figure 4c), we also observed morphologies consistent with those of normal granule cell precursors of the EGL

(Figure 4a). Cell autonomous upregulation of BMP signal transduction thus produces a complex result where some cells retain a progenitor identity while others differentiate prematurely into neurons with T-shaped axons.

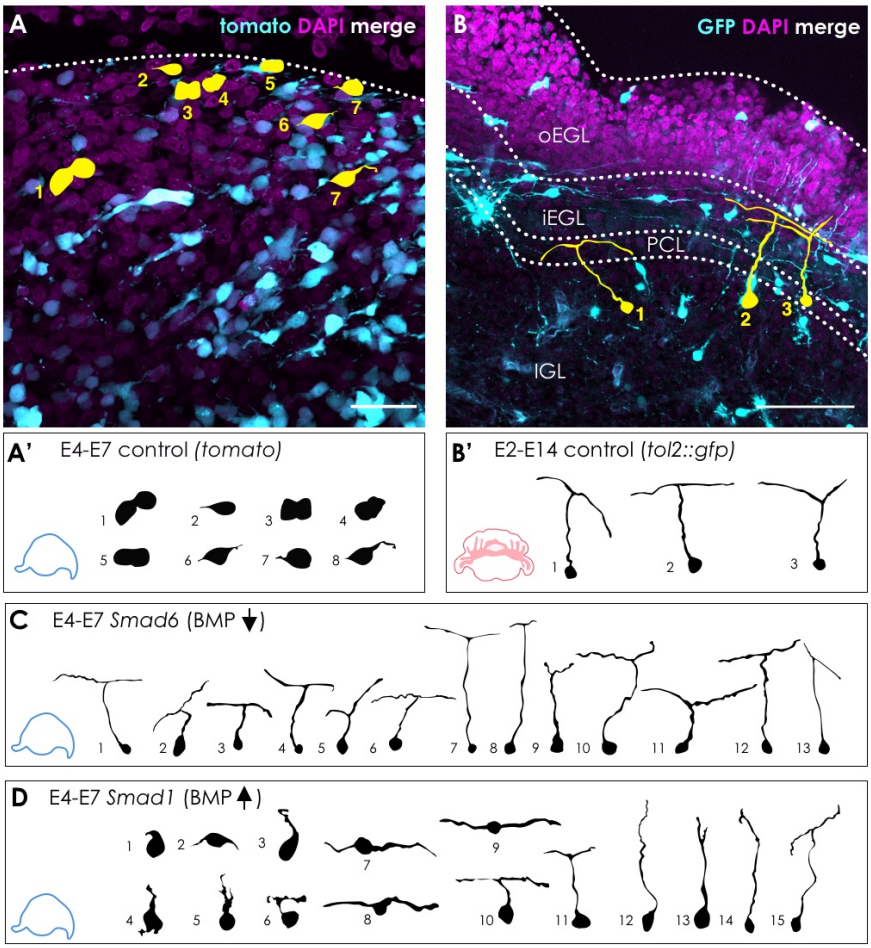

Figure 4: BMP signalling is required for the initial tangential migration of rhombic lip derivatives. (A) Sagittal sections from E7 embryos electroporated at E4 with tomato control DNA show migrating or proliferative granule precursor cells (cell traces A, A') towards the pial surface (dotted white line). (B) To visualise granule cell morphologies at a foliated stage of development, embryos were electroporated at E2 with tol2::GFP and tol2-transposase and sectioned transversely at E14 (cell traces; B'). Cell traces from the E14 cerebellum show classic T-shaped axon and parallel fibre morphologies (B'), which are also observed following knock-down of BMP signalling at E7 (C). Following upregulation of BMP signalling at E7, granule cell morphologies are varied, showing various stages of granule cell maturation (D). Scale bars A; 200µm, C, D; 25µm. C'-F cell traces not to scale.

Upregulation of BMP signalling reveals a temporal switch in GCP responsiveness at E8, coinciding with an increase of SHH-induced proliferation

Our results at E7 led us to look closely at events in the EGL between E7 and E8, which is a critical developmental step in the development of the cerebellum. By E8, proliferation has significantly increased within the EGL (Figure 5a, d) and this correlates with a sharp increase in

Ptch1 expression (a readout of Shh signalling) within the EGL (Figure 5b). EGL thickness also increases 4-fold between E7 and E8 (Figure 5c). Correspondingly, labelling of the rhombic lip by electroporation at E4 yields cellular morphologies indicative of proliferative divisions in the outer EGL (Figure 5e, f).

Upregulation of BMP signal transduction at E4 results in accelerated granule cell development and a partial loss of EGL at E7 (Figure 3c). Examining the results of the same manipulation a day later at E8, we find that the EGL is completely depleted of cells (Figure 5g). Cell tracing reveals that the majority of labelled cells bear the hallmarks of mature granule cell morphology: a cell body within the inner granule layer and a T-shaped axon (Figure 5h). The cell-free EGL can be seen to be filled with parallel fibres, reminiscent of an early-forming molecular layer (Figure 5g). Quantification of EGL cell density at E7 and E8 following electroporation shows that both upregulation and downregulation of cell autonomous BMP signal transduction results in a similar loss of EGL by E8 (Figure 5i) and premature granule cell differentiation. However, our examination of the differing patterns of cellular behaviour suggest that this reflects different developmental processes, with the EGL never forming when BMP signalling is inhibited but being prematurely depleted when BMP signalling is upregulated.

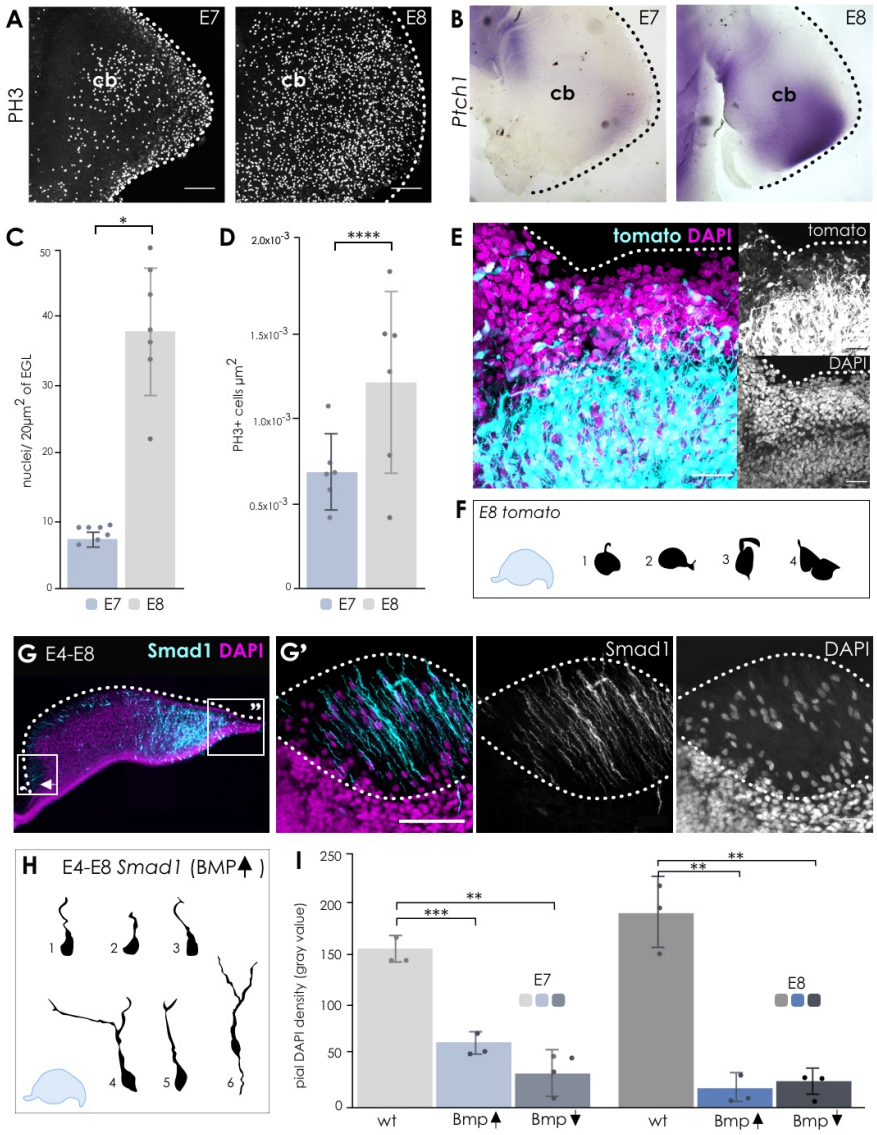

Figure 5: Upregulation of BMP signalling reveals a temporal switch in GCP responsiveness at E8, coinciding with the onset of SHH-induced proliferation. (A) Proliferation at the pial surface significantly increases between E7 and E8 ($p=0.0465$, mean ± SEM = 0.0005322 ± 0.0002344; n=6 for both E7 and E8), which coincides with the onset of Shh signalling at E8, as determined by expression of *Ptch1* mRNA (B). The nuclei density within the EGL (C) significantly increases between E7 and E8, as determined by the number of DAPI-positive nuclei per 20μm² bin of the EGL (n=6 bins per sample from n=7 for E7 and n=7 for E8; $p<0.0001$, mean ± SEM = 30.37 ± 3.556). Quantification of PH3 immunolabelling (D) of the whole-mounted cerebella (A) shows that there is a significant increase in proliferative activity between E7 and E8 ($p=0.0465$, mean ± SEM = 0.0005322 ± 0.0002344; n=6 for both E7 and E8). In a control cerebellum electroporated with tdTomato at E4 and sagittally sectioned at E8 (E; n=3), granule cell precursors still exhibit migrating or proliferative morphologies (F). In embryos electroporated at E4 with Smad1 and sectioned sagittally at E8 (G; n=4), an anterior-posterior gradient of EGL loss is observed, with a molecular layer forming in place of the EGL (G'), within which fibres can be seen extending (Smad1, cyan) and sparse DAPI labelling. (H) Granule cells in the Smad1-electroporated E8 cerebellum

exhibit more mature granule cell morphologies. (I) To show the extent of the depletion of the EGL in control (wt), Smad1 (Bmp↑) and Smad6 (Bmp↓) electroporated cerebella, the fluorescent intensity of DAPI labelling at the pial surface was quantified (n=3 samples quantified for each condition at each developmental stage). Significant losses of DAPI density was observed at both E7 and E8 for Smad1 (E7; p=0.0176, mean ± SEM = 92.33 ± 12.41, E8; p=0.0073; mean ± SEM = 173.7 ± 14.97, n=3 per condition) and Smad6 (E7; p=0.0046, mean ± SEM = -124.4 ± 8.446, E8; p=0.0248; mean ± SEM =165.8 ± 26.58, n=3 per condition). Scale bars A; 200μm, E, G'; 25μm. Cell traces in F and H not to scale.

DISCUSSION

In this study we have shown that BMP signalling is active in complex and spatiotemporally dynamic patterns across cerebellar development in both chick and human. In chick, the BMP signalling components are prominently expressed in the early phases of EGL formation becoming increasingly restricted to the crests of folia as development proceeds. This corresponds to the distribution of pSmad, which disappears from the EGL as folia are generated. In contrast, in humans BMP signalling throughout the EGL is maintained into foliation stages. Manipulation of this signalling in chick has revealed that BMP signalling is not directly linked to granule neuron fate, but to tangential migration and recruitment to the EGL. Phosphorylated Smad1/5/9 (pSmad) expression and experimental up- and downregulation suggest a transition in BMP signalling function to promotion of granule differentiation once the EGL has formed.

Granule cell differentiation is independent of EGL formation

Our observations add to a growing body of evidence that the intricate choreography of developmental stages of granule cell differentiation during inward migration via the EGL first described by Cajal (Cajal, 1894) is not required to produce T-shaped granule cell axons known as parallel fibres. In Cajal's scheme, after terminal migration cells exit the EGL, migrating inwards with a leading dendritic process, leaving their T-shape axons behind to extend through the molecular layer. While this sequence of events may be less stereotyped than Cajal presumed (Hanzel *et al.,* 2019), development of granule cells takes place in the absence of these steps in ray-

finned fish which do not exhibit a transient transit amplifying layer within the cerebellum (Kaslin *et al.,* 2009; Chaplin *et al.,* 2010; Butts *et al.,* 2014c).

Granule cell specification is independent of BMP signalling

Our results challenge the expectation that granule cell production would explicitly depend on a cascade of induction that initiates with BMP signalling at the rhombic lip. Classical experiments showed that BMP can induce granule cells in culture (Alder *et al.*, 1999). Correspondingly, specialised BMP-expressing cells sit adjacent to rhombic lip precursors at the rhombic lip (Chizhikov *et al.,* 2006; Campo-Paysaa *et al.*, 2019). Expression of BMP/Gdf in these cells is dependent on notch activation by adjacent neural cells (Broom *et al.*, 2012). However, conditional mouse mutants that interrupt the BMP pathway give rise to early rhombic lip phenotypes (Qin *et al.,* 2006; Fernandes *et al.,* 2012; Tong and Kwan, 2013), and a dependence of EGL cell production on BMP signalling has never been shown *in vivo*.

Our results show that manipulating BMP signalling does not result in the loss of granule progenitors but rather an alteration of either the trajectory (downregulation) or tempo (upregulation) of their development. Our observations suggest that BMP signalling is required only for the maintenance of a subpial localisation of migrating granule cell precursors adjacent to, and possibly in intimate contact with (Hausmann and Sievers, 1985), the basal lamina. Correspondingly, gain of BMP signal transduction leads to cells directly joining a subpial migration pathway but still undergoing their normal differentiation (Figure 6).

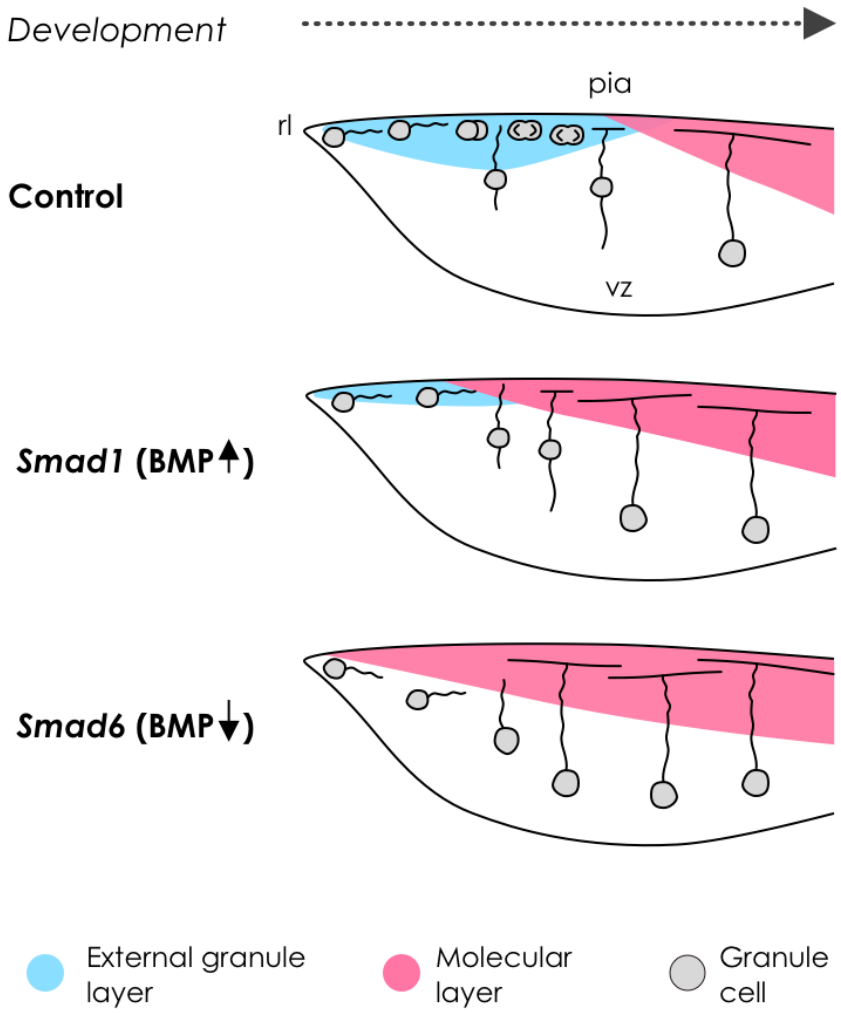

Figure 6: Tightly regulated spatiotemporal activity of the BMP signalling pathway is required for proper development of the external granule layer. In control cerebella, granule cell precursors (grey circles) migrate tangentially from the rhombic lip (rl) across the pial surface to form the external granule layer (blue), where they undergo massive transit amplification before they exit the cell cycle, differentiate and migrate radially towards the inner granule layer, eventually leaving behind a molecular layer (pink) in which their parallel fibres extend. In cerebella with increased levels of BMP signalling (Smad1, BMP↑), granule cells are initially recruited to the pial surface, however coinciding with the onset of Shh at E8 (in chick), the excess of BMP signalling forces granule cells to exit the cell cycle prematurely and migrate, leaving behind a molecular layer and a depleted cerebellum. Following inhibition of BMP signalling at the rhombic lip (Smad6, BMP↓) cells fail to be recruited to the pial surface, and instead immediately migrate ventrally and radially, before extending parallel fibres in a disorganised arrangement into the molecular layer that forms at the pial surface.

A role for BMP signalling in the regulation of transit amplification

The conclusion of this line of evidence is that BMP signal transduction is at least permissive, if not instructive, for EGL formation. In its absence, cells fail to cling to the basal membrane as they migrate and fail to form a transit-amplifying layer. Similar results are found when NeuroD1, which marks the differentiation of granule cells, is overexpressed at the rhombic lip (Butts *et al.*, 2014b; Hanzel *et al.*, 2019) suggesting that the ability of granule cell precursors to suspend differentiation is intimately linked to the subpial location of granule cell precursors within the EGL. This germinal niche facilitates the mesenchymal-like cell behaviour that is seen in transit amplifying progenitors, including an absence of polarisation (Singh & Solecki, 2015) that is regulated by both transcriptional (Singh *et al.* 2016) and post-transcriptional (Famulski *et al.* 2010; Trivedi *et al.* 2017; Kullman *et al.* 2020) mechanisms.

When BMP signal transduction is constitutively upregulated granule cells undergo normal tangential migration and form an EGL, but their passage through this transient layer seems to be abbreviated. Under these experimental conditions, by E8 the EGL rapidly depletes. One attractive explanation is that high levels of BMP signal transduction favour terminal division of granule cell precursors as opposed to symmetrical transit amplifying divisions that produce two precursors (Nakashima *et al.,* 2015; Yang *et al.,* 2015). The balance of transit amplifying versus terminal divisions is the key regulator of EGL expansion (Espinosa and Luo, 2008). When proliferation rates are upregulated by Shh in a background of constitutively raised BMP signal transduction, granule cell precursors undergo only terminal divisions, as has been observed *in vitro* (Rios *et al.,* 2004; Zhao *et al.,* 2008). Consequently, the EGL is rapidly cleared out to make a molecular layer.

This function of BMP in driving terminal differentiation within the EGL has significant parallels with the recently described role for BMP signalling in driving terminal differentiation and radial migration of upper layer cortical progenitors (Saxena *et al.,* 2018): cortical glutamatergic neurons are derived from transit amplifying progenitors in the SVZ (Noctor *et al.,* 2004) that in mouse models proliferate in response to experimentally induced Shh signalling, mimicking the endogenous condition in humans (Wang *et al.,* 2016). Thus, BMP antagonism of Shh-driven proliferation may be a general mechanism for regulating terminal differentiation in large neuronal populations in the amniote brain.

BMP signalling in the EGL persists during initial foliation in human

Given the function of BMP signalling in driving granule differentiation, the presence of pSmad staining throughout the early human EGL (upto pcw 19) after the onset of foliation (which occurs before pcw 12) is intriguing and is consistent with the idea that the human EGL has undergone a heterochronic shift in signalling to facilitate precocious granule neuron differentiation during the early weeks of foliation. Whether this signalling persists into later embryonic and post-embryonic development is an important remaining question. In mouse, phosphoSmad expression has been detected at the rhombic lip around the time of the initiation of production of granule neurons (Qin *et al.,* 2006; Fernandes *et al.,* 2012; Ma *et al.,* 2020). Data relating to the granule lineage later in development, as with our results in chick, has exhibited variation across developmental time. While some reports have suggested that pSmad signalling is present in isolated inner EGL cells and throughout the inner granule layer (IGL) around postnatal day (P)0 (Qin *et al.,* 2006) or P4 (Rios *et al.,* 2004), more recent studies have suggested variation across the anterior-posterior extent of the EGL earlier in development at E14.5 (Ma *et al.,* 2020) and E16.5 (Fernandes *et al.,* 2012), and ubiquitous expression in both the EGL and IGL at P10 (Owa *et al.,* 2018). Thus, BMP signalling appears to exhibit considerable modulation across developmental time in mouse, as we have observed in chick.

This correlates with the behaviour of granule cells. Shh signal transduction in the EGL begins around E16.5 in mouse (Lewis *et al.,* 2004) and differentiation of granule neurons begins approximately a day later at E17.5 (Sudarov and Joyner, 2007), equating broadly with the onset of foliation. Thereafter, extensive transit amplification in the EGL and granule differentiation with radial migration happens throughout the first postnatal week. Data from clonal labelling (Espinosa and Luo, 2008; Legué *et al.,* 2015) and genetically-inducible fate mapping (Legué *et al.,* 2016) suggests that this process is largely complete by P10, at which point pSmad labelling is seen throughout the EGL and IGL (Owa *et al.,* 2018), presumably driving terminal granule differentiation of remaining progenitors.

In human, maintenance of pSmad expression throughout the EGL suggests that signalling is being transduced comparatively earlier in the life history of granule cells than in mouse and chick during foliation. This shift is reminiscent of the late postnatal mouse EGL (Owa *et al.,* 2018) and we suggest that it could represent an adaptation to enable increased production of granule neurons. The rhombic lip in humans has recently been shown to persist later in development

than is the case in mouse, and to possess a subventricular zone that presumably facilitates the huge cell production that characterises the human cerebellum, particularly the posterior lobule (Haldipur *et al.,* 2019). Our data are consistent with a model where, at least at early stages, there has also been an increase in the proliferative dynamics of the human EGL, and where the BMP signalling that we have observed drives the precocious differentiation of granule neurons beginning shortly after the initiation of foliation. Together with the persistence of the rhombic lip, BMP signalling alterations may thus contribute to the enormous numbers of granule neurons found in human (Azevado *et al.,* 2009).

BMP signalling as a regulator of the lifespan of the transient EGL

While the central importance of Shh signalling in regulating the scale of transit amplification and growth of the cerebellum is clear from a wealth of studies (Butts *et al.,* 2014a; Hibi *et al.,* 2017), our data supports a key role for BMP signalling in managing the overall programme of transit amplification. In particular, we suggest that our experiments reconcile data that BMP both inhibits Shh responsiveness (Rios *et al.,* 2004; Zhao *et al.,* 2008; Ayrault *et al.,* 2010) but is also required for a large cerebellum (Qin *et al.,* 2006; Fernandes *et al.,* 2012; Tong and Kwan, 2013). By facilitating EGL formation and then influencing the timing of differentiation, BMP signals are ideally placed to regulate the tempo of production of cells that populate the IGL. These observations are echoed by recent experiments showing that the temporal patterning of GABAergic cell production in the cerebellum is also regulated by BMP signalling (Ma *et al.,* 2020). Thus the BMP pathway has multiple roles during cerebellar development in amniotes, amongst which are two distinct roles unrelated to fate within the granule lineage: driving EGL formation initially and then promoting differentiation following the onset of foliation.

MATERIALS AND METHODS

In ovo electroporation

Fertilised hen's eggs (Henry Stewart) were incubated at 38˚C at 70% humidity. Electroporations were performed between stages HH10-25 (Hamburger and Hamilton, 1993), or between embryonic day 2 (E2) to E4. Eggs were windowed using sharp surgical scissors and the vitelline membrane covering the head removed. DNA was injected into the fourth ventricle at a final

concentration of 1-3 μg/μl in addition to trace amounts of fast-green dye (Sigma). Three 50ms square waveform electrical pulses at 5V (E2) or 10V (≥E3) were passed between electrodes that were placed on either side of the hindbrain Figure 3a). Five drops of Tyrode's solution containing penicillin and streptomycin (Sigma) was administered on top of the yolk before being resealed and further incubated for the designated number of days. Embryos were fixed in 4% PFA in PBS for 1 hour at room temperature or overnight at 4°C and then processed for histology. Table 1 summarises the DNA plasmid constructs used throughout this study.

Table 1: DNA plasmids

Plasmid	Details	Source/ reference(s)
pCAGGS-Smad6	BMP inhibitor	Andrea Streit, KCL (Xie *et al.,* 2011)
pCAGGS-Smad1EVE-IRES-GFP	Constitutively activated Smad1	Andrea Münsterberg, UEA (Fuentealba *et al.,* 2007; Song *et al.,* 2014)
pCAGGS-GFP	GFP reporter	Richard Wingate, KCL
pCAGGS-tdTomato	Tomato reporter	
pTol2-GFP	Tol2 integrated GFP reporter	Nico Daudet, UCL (Sato *et al.*, 2007; Freeman *et al.*, 2012)
pTol2-Transposase	Tol2 transposase enzyme	

Human foetal tissue procurement

Histological analysis of the human cerebellum: Human cerebellar samples used in this study were collected in strict accordance with legal ethical guidelines and approved institutional review board protocols at Seattle Children's Research Institute, University College London and Newcastle University. Samples were collected at by the Human Developmental Biology Resource (HDBR), United Kingdom, with previous patient consent. Samples were staged using foot length with the age listed as post-conception weeks (pcw), which starts from the point at which fertilization occurred.

Samples were fixed in 4% PFA and then processed through alcohol gradients and xylene. Processed tissue was then embedded in paraffin wax prior to sectioning. Samples sectioned using the cryostat were treated with 30% sucrose following fixation. Paraffin and cryo-sections were collected at 4 and 12 □ m respectively. In situ hybridization assays were run using commercially available probes from Advanced Cell Diagnostics, Inc. Manufacturer recommended protocols were used without modification. The following probes were used in the study SHH (#600951), MKI67 (#591771) and PTCH1 (#405781). Sections were counterstained with fast green. Images

were captured at 20X magnification using a Nanozoomer Digital Pathology slide scanner (Hamamatsu; Bridgewater, New Jersey).

Tissue processing, immunohistochemistry, *in situ* hybridisation and imaging

Cerebella were dissected between E5-E14 and either whole-mounted in glycerol or embedded in 20% gelatine, 4% low-melting point (LMP) agarose or OCT and sectioned at 50μm using a vibratome (Leica) or at 15μm using a cryostat (Microm). For immunolabelling, whole-mount and gelatine sections were washed with PxDA (1x PBS, 0.1% Tween-20, 5% DMSO, 0.02% NaN_3), then 3x 30 minutes, blocked (PxDA, 10% goat serum) 2x 1 hour, and incubated in primary antibody (diluted in block) for 48 hours at 4˚C on a rocker. Tissue was washed in block for 5 mins then 3x 1 hour. Secondary Alexaflour (Thermofisher) antibodies were diluted in block (1:500) and incubated overnight at 4˚C. Samples were washed 3x 1 hour with block, 3x 3 mins with PxDA and 1 hour in 4% PFA. Sections were mounted using Fluoroshield containing DAPI (Abcam). Frozen sections were thawed at room temperature for 1 hour, washed in 1x TBS buffer (2% BSA, 1x TBS, 0.02% NaN_3, pH7.6), blocked in 1x TBS buffer for 10 minutes, and incubated in primary (diluted in 1x TBS) overnight at room temperate in a humidity chamber. Slides were washed in 500ml 1x TBS for 10 minutes (with stirring), then incubated in secondary antibody (biotinylated for DAB staining (1:300) or Alexaflour for fluorescence (1:500; diluted in 1x TBS) for 1 hour at room temperature. For DAB staining, The Strept ABC-HRP (1:100 of each A and B in 1x DAB developing buffer) was left to conjugate for 30 mins. Slides were rinsed in 1x TBS and then incubated in the conjugated Strept ABC-HRP solution for 30 minutes. Slides were rinsed in 500ml 1x TBS for 5 minutes, with stirring, and then developed for 10 minutes in DAB solution (DAKO DAB enhancer was used for pSmad1/5/9 at 1:300). Slides were washed under running water, counterstained with haematoxylin, and returned to the running water until nuclei turned blue. Antibodies, and the dilutions they were used at is summarised in Table 2.

For *in situ* hybridisation, dissected hindbrains were fixed in 4% PFA for 1 hour (and stored up to 3 months) and stained as previously described (Myat *et al.*, 1996) using a digoxygenin-labelled riboprobe (Roche) against *Patched-1 (Ptch1)*. Tissue was flat mounted in 100% glycerol and imaged from the dorsal side.

Table 2: antibodies

Target	Host species	Dilution	Source
Phosphorylated Smad1/5/9 (pSmad)	Rabbit	1:100	CST 13820
Calbindin	Mouse	1:1000-5000	Swant D28K
Phosphohistone-3 (PH3)	Rabbit	1:100	CST
DAPI	n/a	n/a	Abcam
Alexafluor-488	Goat anti-mouse	1:500	Thermofisher
Alexafluor-488	Goat anti-rabbit	1:500	Thermofisher
Alexafluor-568	Goat anti-mouse	1:500	Thermofisher
Alexafluor-568	Goat anti-rabbit	1:500	Thermofisher

Image analysis

Sections with fluorescent labelling were imaged using a Zeiss LSM 800 confocal microscope and Z-projections compiled with ImageJ (Schneider et al., 2012). Non-fluorescent samples were imaged using a Zeiss Axioscope microscope. To represent the pial migration from the rhombic lip (Figure 4a,b) the fluorescence intensity, termed *"gray value"* in ImageJ, from the rhombic lip towards the midline in an area of abundant electroporation (coloured lines; Figure 4a), was plotted as a surface histogram, obtained from the *plot profile* plugin and a curve of best fit (5th degree polynominal). ImageJ was also used to quantify the number of antigen-expressing cells per area (+ve cells/□ m^2); cells positive for pSmad labelling were manually counted using the *cell counter* plugin (Figure 1h) whereas quantification of PH3 labelling (Figure 5c,d) was done automatically by converting a compressed Z-stack to a binary image, watershed function applied and the *analyse particles* plugin applied to count positive cells in the sample. The area of the tissue being quantified was also measured in ImageJ and the number of +ve positive nuclei per μm^2 was then calculated in Excel and analysed for significance in GraphPad Prism. To measure the density of DAPI +ve nuclei at the pial surface in electroporated samples (Figure 5i), individual slices from Z-stacks of each sample were processed to binary images, and a line was drawn across the pia in ImageJ and the fluorescent density averaged across this line using the *plot profile* plugin.

Statistical analyses

All data were analysed in GraphPad Prism, and non-paired parametric t-tests were carried out to identify significance.

Data and Materials availability

The human material was provided by the Joint MRC/Wellcome (MR/R006237/1) Human Developmental Biology Resource (www.hdbr.org). Human tissue used in this study was covered by a material transfer agreement between SCRI and HDBR. Samples may be requested directly from the HDBR.

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Competing Interests

The authors have no competing interests.

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