The evolution of the vertebrate cerebellum: absence of a proliferative external granule layer in a non-teleost ray-finned fish

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SUMMARY The cerebellum represents one of the most morphologically variable structures in the vertebrate brain. To shed light on its evolutionary history, we have examined the molecular anatomy and proliferation of the developing cerebellum of the North American paddlefish, *Polyodon spathula*. Absence of an external proliferative cerebellar layer and the restriction of *Atonal1* expression to the rhombic lip and valvular primordium demonstrate that transit amplification in a cerebellar external germinal layer, a prominent feature of amniote cerebellum development, is absent in paddlefish. Furthermore, expression of *Sonic hedgehog*, which drives secondary proliferation in the mouse cerebellum, is absent from the paddlefish cerebellum. These data are consistent with what has been observed in zebrafish and suggest that the transit amplification seen in the amniote cerebellum was either lost very early in the ray-finned fish lineage or evolved in the lobe-finned fish lineage. We also suggest that the *Atoh1*-positive proliferative valvular primordium may represent a synapomorphy (shared derived character) of ray-finned fishes. The topology of valvular primordium development in paddlefish differs significantly from that of zebrafish and correlates with the adult cerebellar form. The distribution of proliferative granule cell precursors in different vertebrate taxa is thus the likely determining factor in cerebellar morphological diversity.

INTRODUCTION

The vertebrate cerebellum is a remarkable neural structure whose synaptic architecture is highly conserved but which varies widely in its size and gross anatomy (Voogd and Glickstein 1998). Despite the similarities in cerebellar circuit organization across vertebrates, it plays a variety of functional roles, acting principally as a coordinating centre for proprioception in anamniotes (Sherrington 1906; Wingate 2005) and having a well described function in mammalian motor learning and coordination (Manto 2008). The cerebellum also plays a significant role in primate cognition (Schmahmann 2010; Basson and Wingate 2013), and it is possible that not all cerebellar functions have as yet been described. Within the cerebellar cortex, the highly conserved geometry of the synapses between glutamatergic granule neuron axons and dendrites of GABAergic Purkinje cells indicates that a conserved processing algorithm performed by these cells is key to all these functions (Braitenberg and Atwood 1958; Meek 1992). In contrast, the wide variation in cerebellar size, even between relatively closely related species (Nieuwenhuys 1967; Nieuwenhuys et al. 1998; Sultan and Glickstein 2007; Lisney et al. 2008; Yopak and Montgomery 2008) suggests that functional specialization relies on exquisite control of relative cell number. Increasing evidence that cerebellar granule cell development, in particular, involves different migratory and proliferative strategies in different taxa (Butts et al. 2011) points to a correlation between mode of granule cell development and cerebellar morphology.

The developmental genetics underlying cerebellar development are understood in some depth (Fig. 1A) following detailed study in the chick and the mouse over the last 30 years (Wang and Zoghbi 2001; Hatten and Roussel 2011). The cerebellum develops solely from two germinal zones characterized by the expression of two basic helix-loop-helix (bHLH) transcription factors: a *Ptf1a*-positive ventricular zone in the rostral-most dorsal hindbrain, which gives rise to GABAergic interneurons, including Purkinje cells (Hoshino et al. 2005); and an *Atoh1*-positive rhombic lip, which borders the roof plate, gives rise to granule cells (Ben-Arie et al. 1997), and is derived entirely from rostral hindbrain rhombomere 1 (Wingate and Hatten 1999). For the majority of development, expression of *Atoh1* is acutely maintained by interactions with the roof plate (Broom et al. 2012). However, in birds and mammals, granule precursor cells acquire the capacity to migrate away from the rhombic lip and form a secondary, transit-amplifying epithelium that is present transiently on the surface of the cerebellum. Mitogenic Sonic hedgehog (Shh)-mediated signals from underlying *Ptf1a*-derived Purkinje cells (Dahmane and Ruiz-i-Altaba 1999; Wallace 1999; Wechsler-Reya and Scott 1999) precisely regulate a large number of symmetrical divisions that eventually lead to the disappearance of the transient external germinal layer. As granule cells exit mitosis, they down-regulate *Atoh1*, switch on
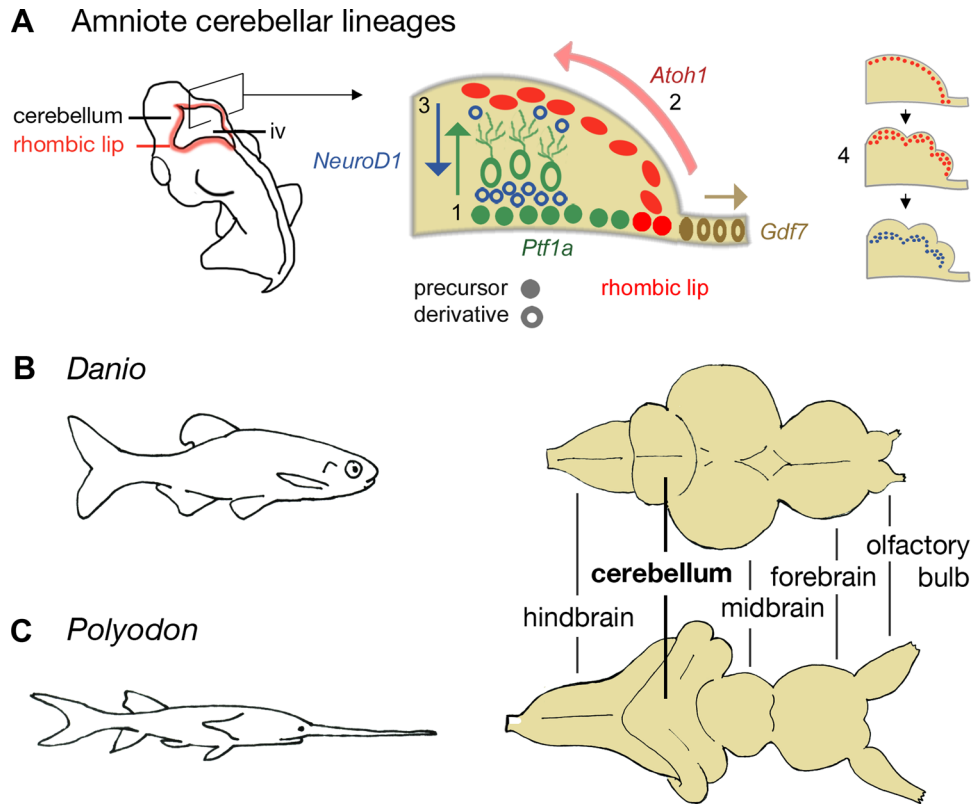
Fig. 1. Cerebellar development in amniotes and adult cerebellar form in zebrafish and paddlefish. Schematic diagram of an amniote embryo (A) showing the location of the rhombic lip and, in section, the distribution of precursors and their derivatives. Three major lineages give rise to Purkinje and other GABAergic neurons (*Ptf1a*, green), granule cells (*Atoh1*, red) and roof plate (*Gdf7*, brown). Purkinje cells migrate radially from the ventricular layer (1). Granule cells migrate tangentially to form a proliferative external germinal layer (2) and then, as *NeuroD1*-positive post-mitotic neurons, migrate radially to form an internal granule layer (3). Proliferation within the *Atoh1*-positive external germinal layer (4) leads to transverse foliation of the adult amniote cerebellum (see Fig. 6, e–g). The cerebellum of the zebrafish (*Danio rerio*) (B) differs from that of a paddlefish (*Polyodon spathula*) (C, normalized to the same scale as B): the latter displays a ribbon-like cerebellum with a convoluted and extended cerebellar rhombic lip. IV, Fourth ventricle.

the bHLH transcription factor gene *NeuroD1* (Miyata et al. 1999) and migrate into a deep, internal granule layer, below the layer of Purkinje cell bodies (Fig. 1A).

The historic description of this transient, transit-amplifying stage of granule cell development (Cajal 1894) and its obvious implications for surface area:volume ratio in the cerebellum have led to much speculation and some elegant experimental evidence (Corrales et al. 2006) for it being responsible for the highly folded structure of the cerebellum in birds and mammals (Fig. 1A4). However, the existence of this layer has been contested in the zebrafish (a ray-finned teleost fish) and the lesser-spotted dogfish (a shark) (Rodriguez-Moldes et al. 2008; Chaplin et al. 2010; Kani et al. 2010; Wullimann et al. 2011): even if present, its molecular characteristics are very different to that of amniotes. This includes, in the zebrafish, an absence of *Shh* expression in underlying Purkinje cells (Chaplin et al. 2010; Kani et al. 2010), upon which *Atoh1*-mediated transit amplification is strictly dependent in mammals (Flora et al. 2009). For teleosts, a separate solution for generating cerebellar granule cells

may have arisen in the form of the valvulus, a tongue-like rostral extension of the cerebellum that usually extends beneath the midbrain and, which, in some families such as the weakly electric tapir-fish (*Mormyrus*), becomes a highly adapted, grossly exaggerated neural structure (Nieuwenhuys and Nicholson 1969). In zebrafish, the valvulus primordium represents an enduring stem cell niche (Kaslin et al. 2009) that, in its molecular signature (Chaplin et al. 2010), resembles an extension of larval neurogenesis, rather than transit amplification, mirroring the situation observed in many regions of the zebrafish brain (Kizil et al. 2012). This distinction between extended primary neurogenesis in a ray-finned teleost fish and secondary proliferation in the cerebellum of mammals and birds (a distinction reinforced by the lack of cerebellar *Shh* in zebrafish) suggests that cerebellum development perhaps followed very different evolutionary trajectories in the actinopterygian (ray-finned) and sarcopterygian (lobe-finned) lineages of bony fishes.

Reconstructing the ancestral organization of cerebellar development is not appreciably aided by data from chondrichthyans

(e.g., sharks and rays). Within this group, granule neurons and their proliferative, *Atoh1*-positive progenitors (Rodriguez-Moldes et al. 2008; Chaplin et al. 2010) are confined to bilateral midline ridges in the cerebellum, the prominentiae granularis (Nieuwenhuys et al. 1998), which are continuous with an extended cerebellar rhombic lip. This distinct chondrichthyan cerebellar form is radically different from both amniotes and teleosts. Therefore, to determine the ancestral condition within ray-finned fishes, we examined the molecular anatomy of cerebellar development in the North American paddlefish, *Polyodon spathula* (Fig. 1). This non-teleost ray-finned fish species is a representative of the chondrostean lineage (i.e., sturgeons and paddlefishes): a basal branch (relative to teleosts) of the ray-finned fishes (Hurley et al. 2007; Near et al. 2012). It thus represents an excellent in-group with which to reconstruct the likely ancestral ray-finned fish condition. By comparison to a teleost (Fig. 1B), the cerebellum of the paddlefish, like other chondrosteans (Nieuwenhuys et al. 1998), has a distinct ribbon-like form (Fig. 1C). Does this divergent structure represent a different mode of granule cell production? By examining gene expression and proliferation across a range of stages of paddlefish development, we demonstrate both the lack of a superficial *Atoh1*-positive, proliferative layer and the absence of *Shh* expression in the cerebellum, supporting the argument that a transient, transit amplification step in granule cell development is unique to the sarcopterygian lineage.

MATERIALS AND METHODS

Gene cloning

RNA from stage 40–46 paddlefish embryos was extracted with Trizol reagent (Life Technologies, UK). Single-strand cDNA was generated using the Superscript III First Strand Synthesis kit (Invitrogen), as per the manufacturer's instructions. The following primers were used: Atoh1 Forward AGCACA-GAGGTTATCCACAAAGTC; Atoh1 Reverse ACTTTG-TAACTCTAGATTTGCCTCC. Standard PCR conditions were used to amplify an approximately 800 bp fragment; this fragment was then cloned into the pDrive vector (Qiagen, UK). Clones were isolated and sequenced (Dept of Biochemistry DNA sequencing facility, University of Cambridge) prior to phylogenetic analysis. The GenBank accession number for *P. spathula Atoh1* is KC589703.

Phylogenetic analysis

Phylogenetic analysis of the Atonal family of proteins was performed using an alignment of the bHLH domain constructed in ClustalX and edited in SeaView (Galtier et al. 1996). Neighbor joining and maximum likelihood analysis were implemented in SeaView, with the LG model selected for the likelihood analysis following assessment of model suitability using ProtTest (Abascal et al. 2005). The robustness of the trees was assessed using 1000 bootstrap replicates. Bayesian analysis was conducted using MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003), using a mixed amino acid model combination, with two independent Markov chain Monte Carlo runs of four chains. The chains were run for 1 million generations. The burn-in phase was estimated using both likelihood plots and the average SD of split frequencies, and trees within the burn-in were removed from subsequent analysis.

Embryo collection and staining

Polyodon spathula embryos obtained from Osage Catfisheries (Osage Beach, MO, USA) over multiple spawning seasons were fixed in 4% paraformaldehyde at stages 31–45 (Bemis and Grande 1992). Embryos were processed for immunohistochemistry using a polyclonal antibody against phosphohistone H3 (αPH3, Cell Signalling Technology, Danvers, MA, USA; 1:100) and a fluorescent secondary antibody (goat α-rabbit AlexaFluor-488, Invitrogen, 1:500), or for in situ hybridization using a standard protocol (Myat et al. 1996), with riboprobes for *Atoh1*, *NeuroD1* (Modrell et al. 2011) and *Shh* (Davis et al. 2007). Selected embryos were embedded in 20% gelatin and vibratome-sectioned at 50 μm.

Whole mount embryos were photographed on a Zeiss Stemi SV6 microscope and Olympus DP camera. Sections were photographed on a Leica MZFLIII microscope using QCapture Pro 6.0, or a Nikon Eclipse 80i confocal microscope using EZ-C1 3.70 software. Images were compiled in ImageJ version 10.2 and Adobe Photoshop.

RESULTS

The ground plan of granule cell development is determined by patterns of proliferation and bHLH transcription factor expression that define the geometry and identity of proliferative cells and the presence of secondary, transit amplification. The presence of *Shh* is an additional, potential indicator of secondary proliferation in the cerebellum. We therefore examined these three parameters of cerebellar development in paddlefish embryos from stages 31 to 45, by which point the cerebellum assumes its adult form.

Cell proliferation is confined to the ventricular zone and rhombic lip during cerebellar development

Cells during M-phase of mitosis were identified using an antibody against phosphohistone H3 on paddlefish embryos (Fig. 2A). Parasagittal sections through the lateral cerebellum, which encompass the hindbrain, midbrain and lateral cerebellar rhombic lip (Fig. 2, B–E), are contrasted with sagittal sections through the anterior pole of the cerebellum (Fig. 2, F–J).
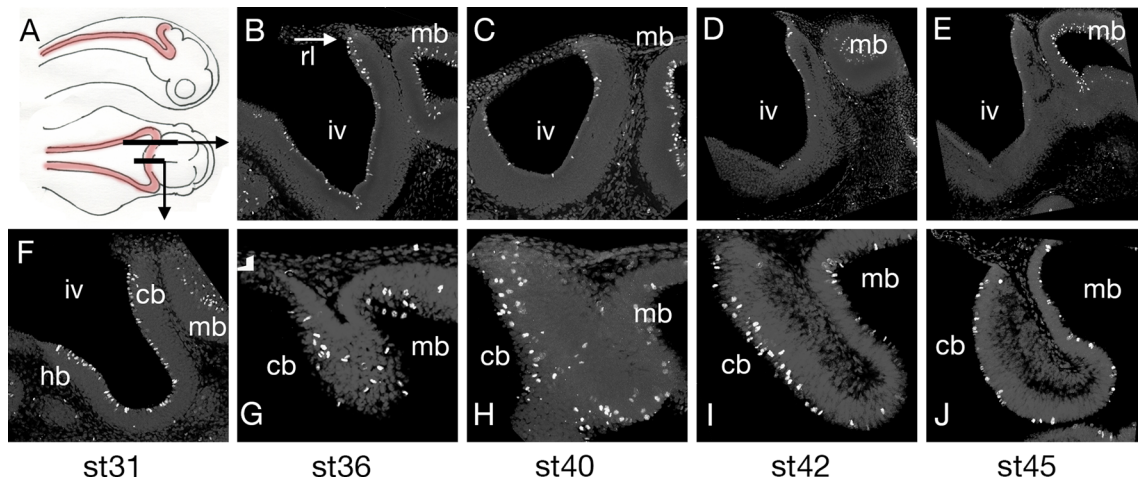
Fig. 2. Proliferation in the paddlefish hindbrain. Schematic diagram (A) of a paddlefish embryo in lateral and dorsal aspect showing the rhombic lip in red and the location of the parasagittal and sagittal sections. Immunostaining for phosphohistone H3 reveals that M-phase cells in the ventricular layer of the fourth ventricle (iv) progressively decline in number between stages 36 (B), 40 (C), 42 (D) and 45 (E), but continue to populate the midbrain (mb) ventricular surface. Sagittal sections at stages 31 (F), 36 (G), 40 (H), 42 (I), and 45 (J) showing that proliferation is selectively maintained in the midline cerebellum (CB). Anterior is to the right in all panels. Abbreviations: CB, cerebellum; HB, hindbrain; IV, fourth ventricle; MB, midbrain; RL, rhombic lip.

Between stages 36 (Fig. 2B) and 45 (Fig. 2E), there is a dramatic loss of mitotic profiles within the ventricular layer surrounding the fourth ventricle, while proliferation in the midbrain is maintained. At the earliest stage examined (Fig. 2F), there is no pronounced difference between the cerebellum, hindbrain and midbrain. However, by comparison to the subsequent decline in proliferation in the ventricular zones of the hindbrain and cerebellum, the cerebellar midline displays numerous mitotic profiles through to stage 45. This suggests that cerebellar proliferation becomes increasingly restricted to the midline anterior pole of the paddlefish cerebellum (Fig. 1C). At no point during these phases of cerebellar development is there a superficial layer of proliferative cells on the surface of the growing cerebellar anlagen that would correspond to an external germinal layer.

Expression of bHLH transcription factor genes in cerebellar granule neurons

In amniotes, granule cell proliferation and maturation are characterized by sequential expression of *Atoh1* in the superficial external germinal layer of transit amplifying precursors (Ben-Arie et al. 1997), followed by *NeuroD1* in maturing, post-mitotic cells (Miyata et al. 1999). We therefore investigated the expression of paddlefish orthologues of both *Atoh1* (Fig. S1) and *NeuroD1* (Modrell et al. 2011), in order to determine whether a transit amplification layer forms transiently during paddlefish granule cell maturation. In dorsal view, at stages 31 (Fig. 3A) and 36 (Fig. 3B), *Atoh1* is expressed throughout the rhombic lip of both the cerebellum and hindbrain with the exception of the cerebellar midline. At stage 40, the cerebellar

midline becomes *Atoh1*-positive (Fig. 3C) and subsequently becomes the exclusive site of neurogenic gene expression at stage 42, as *Atoh1* is down regulated in other parts of the rhombic lip (Fig. 3D). By stage 45, expression of *Atoh1* deep in the tissue at the cerebellar midline can only be visualized faintly in whole mount embryos (Fig. 3E). In lateral view, *Atoh1* expression outlines the lateral out-pocketing of the upper rhombic lip at stages 31 (Fig. 3F) and 36 (Fig. 3G). However, *Atoh1* is never expressed in a superficial cerebellar layer covering the pial surface that might correspond to an external germinal zone. We note that between stages 40 and 45 (Fig. 3, H–J), expression of *Atoh1*, which is essential for inner ear hair cell development (Driver and Kelley 2009; Maricich et al. 2009), is also seen in the hair cells of lateral line mechanosensory neuromasts and electrosensory ampullary organs, similar to patterns observed for other ampullary organ markers in the paddlefish (Modrell et al. 2011).

The expression of *NeuroD1* in the presumptive cerebellum is broadly complementary to that of *Atoh1*. Thus *NeuroD1* is absent from the cerebellum at stage 31 (Fig. 3K) and expressed in a faint transverse stripe at stage 36 (Fig. 3L). In the periphery, *NeuroD1* expression is restricted to developing cranial ganglia and the paired sense organs (Modrell et al. 2011). From stage 40 onwards, as *Atoh1* is down regulated, *NeuroD1* expression is prominent throughout the cerebellar rhombic lip and in the developing mesencephalic trigeminal nucleus in the midbrain (Fig. 3, M–O). In lateral view, the rhombic lip is clearly *NeuroD1*-negative at stages 31 (Fig. 3P) and 36 (Fig. 3Q). At stages 40–45 (Fig. 3, R–T), granule cells form a *NeuroD1*-positive domain that lies parallel to the rhombic lip, anticipating the structure of the adult paddlefish cerebellum.
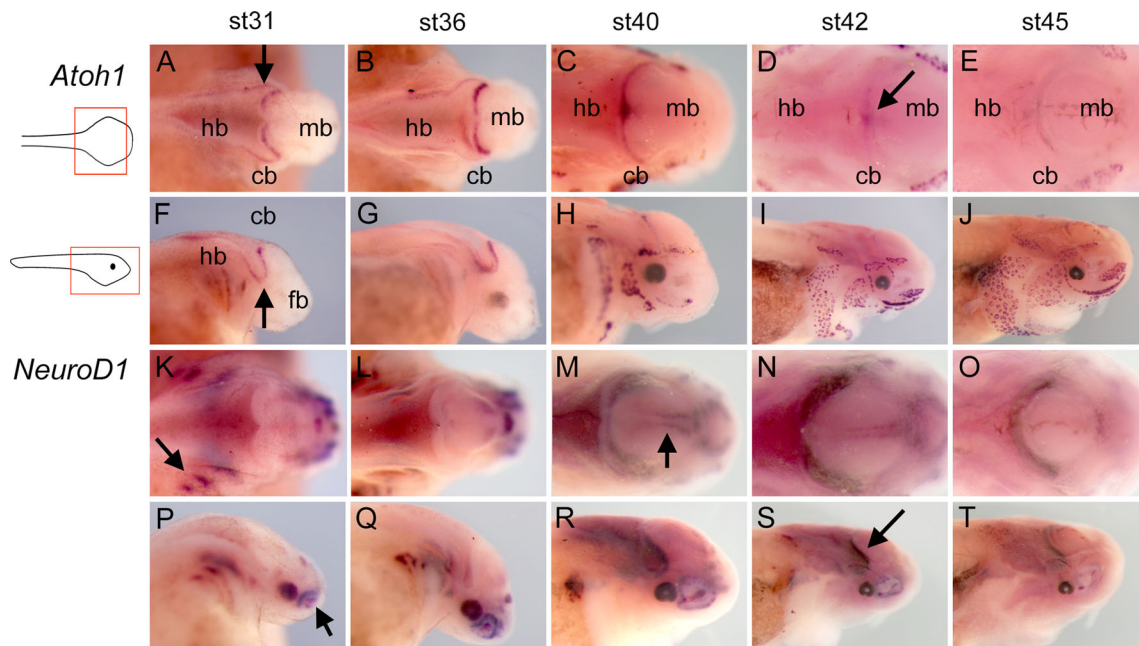
Fig. 3. *Atoh1* and *NeuroD1* expression in whole mount paddlefish embryos. Whole mount in situ hybridization for *Atoh1* (A–J) and *NeuroD1* (K–T) in paddlefish embryos between stages 31–45 in dorsal (A–E, K–O) and lateral (F–J, P–T) views. Arrows in A and F indicate the characteristic out-pocketing of the chondrostean cerebellar rhombic lip (Nieuwenhuys et al. 1998). The arrow in D indicates the refinement of *Atoh1* expression to the cerebellum midline at st42. Arrows in K and P, respectively, indicate *NeuroD1* expression in the cranial ganglia and olfactory pit. The arrow in M indicates midbrain expression of *NeuroD1*, corresponding to the position of the mesencephalic trigeminal nucleus (arrow). The arrow in S indicates the position of cerebellar *NeuroD1* expression. Abbreviations: CB, cerebellum; FB, forebrain; HB, hindbrain; IV, fourth ventricle; MB, midbrain; RL, rhombic lip.
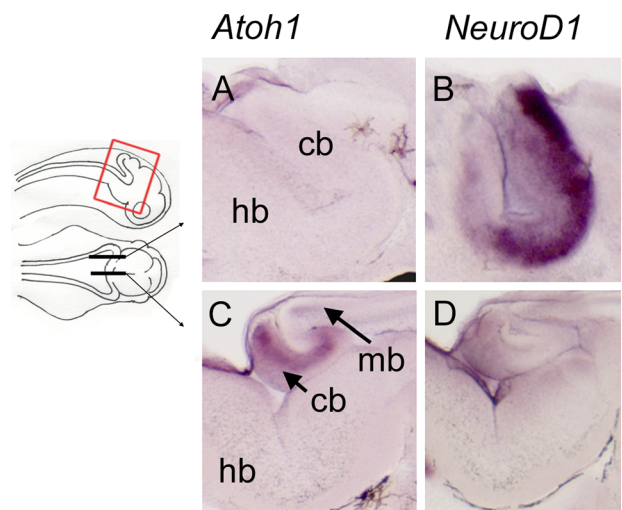
The organization of *Atoh1* and *NeuroD1* expression at stage 42 is shown in parasagittal and sagittal sections through the cerebellum in Figure 4. The lateral cerebellum is *Atoh1*-negative (Fig. 4A) but strongly expresses *NeuroD1* (Fig. 4B). In contrast, the midline of the cerebellum strongly expresses *Atoh1* (Fig. 4C), but is *NeuroD1*-negative (Fig. 4D).

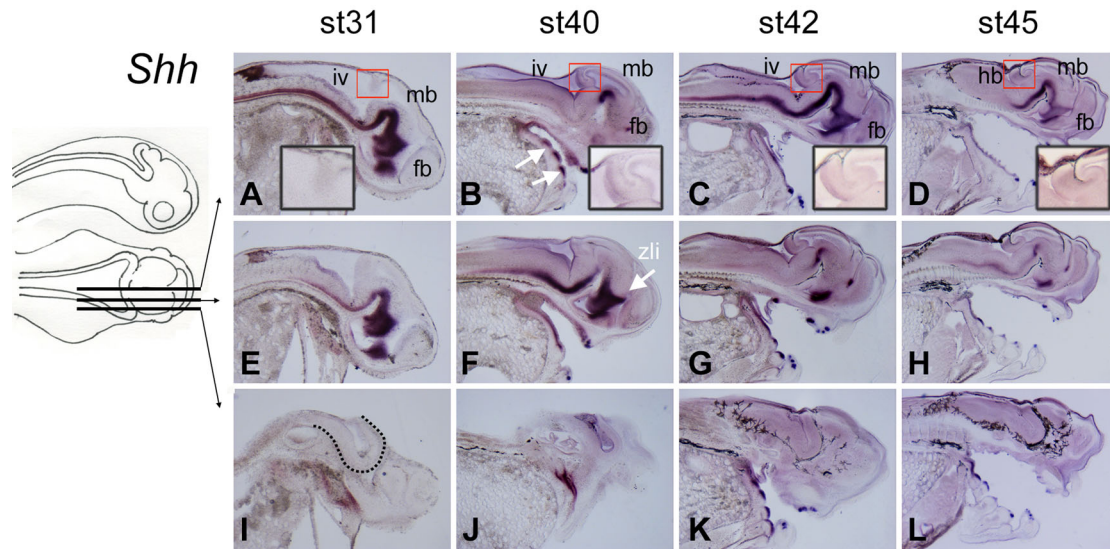## *Shh* expression in the developing paddlefish

While *Shh* is expressed in the cerebellum of sharks (Chaplin et al. 2010) and amniotes, it is absent from the cerebellum of zebrafish (Kani et al. 2010; Chaplin et al. 2010). In paddlefish, at all stages examined (stages 31 to 45), expression of *Shh* in the central nervous system (CNS) was restricted to the developing ventral midline (Fig. 5, A–D). There was no expression of *Shh* within the developing cerebellum in either medial or lateral regions (Fig. 5, E–L). From stage 40 onwards, as in other gnathostomes (Smith et al. 2009), *Shh* expression defines the site of developing tooth primordia (arrows in Fig. 5B).

DISCUSSION

We examined proliferation and bHLH transcription factor gene expression during cerebellar granule cell development in paddlefish, which together demonstrate an absence of any

Fig. 4. Complementary expression of *Atoh1* and *NeuroD1* in the medial versus lateral rhombic lip at stage 42. Sections through the rhombic lip of embryos after in situ hybridization for *Atoh1* and *NeuroD1* in parasagittal (A and B) and sagittal (C and D) planes, as indicated on the schematic. In the lateral rhombic lip, *Atoh1*-positive progenitors are absent (A) but *NeuroD1* is strongly expressed (B), consistent with previously reported distributions of granule neurons (Nieuwenhuys 1967). In the medial rhombic lip, that is, the presumptive valvulus, *Atoh1* is strongly expressed (C), while *NeuroD1* is reduced (D). Abbreviations: CB, cerebellum; HB, hindbrain; MB, midbrain.

Fig. 5. *Sonic hedgehog* is not expressed in the developing paddlefish cerebellum. Sections through embryos from stages 31 to 45 after in situ hybridization for *Shh* in sagittal (A–D) and parasagittal (E–L) planes, as indicated in the schematic. At all stages examined, *Shh* expression in the CNS is restricted to the ventral midline, with no expression in the cerebellum (red boxes, shown at higher power in insets). *Shh* expression in the CNS is lost further laterally (compare E–H with I–L). White arrows in B indicate prominent expression of *Shh* in tooth buds. The arrow in F indicates the zona limitans intrathalamica (zli), which identifies the mid-point of the diencephalon along the rostrocaudal axis. The dotted line in I demarcates the boundary of the ventral neural tube. Abbreviations: FB, forebrain; HB, hindbrain; IV, fourth ventricle; MB, midbrain; zli, zona limitans intrathalamica.

distinct external germinal layer as defined by the presence of mitotic, *Atoh1*-positive neurons in a sub-pial layer over the cerebellum (Chaplin et al. 2010).

Sequence of *Atoh1* and *NeuroD1* expression in granule cell development is highly conserved but geography of expression is not

The sequential activation of first *Atoh1* and then *NeuroD1* defines the initial steps in the differentiation of granule cells in amniotes (Hatten and Roussel 2011) and zebrafish (Kani et al. 2010). Our observations of paddlefish development suggest a similar sequence in a non-teleost actinopterygian, extending this observation across all bony fish cerebellums examined thus far. In amniotes, the appearance of a transient, external germinal layer is associated with the prolonged duration of *Atoh1* expression in migratory derivatives of the rhombic lip. This pronounced *Atoh1*-positive transit amplification is absent in both zebrafish and shark (Chaplin et al. 2010). In zebrafish, this is correlated with the absence of mitogenic Shh produced by Purkinje cells, on which, in mice, *Atoh1*-mediated granule cell transit amplification depends (Flora et al. 2009). The lack of both a *Shh*-expressing domain and an *Atoh1*-positive external germinal layer in the cerebellum of paddlefish, as well as zebrafish, suggests that the transit amplification seen in amniotes is not a feature of the cerebellum in ray-finned fishes.

Our data suggest that both the origin of granule cells within the rhombic lip in shark, zebrafish (Chaplin et al. 2010; Kani

et al. 2010; Wullimann et al. 2011), birds (Broom et al. 2012) and mammals (Ben-Arie et al. 1997), and the simple progression of bHLH transcription factor gene expression that determines their fate, are conserved features of vertebrate cerebellum development. This conservation is paralleled by a synaptic arrangement between granule cells and their *Ptf1a*–derived Purkinje cell targets that is highly stereotyped and thought to be functionally essential for information processing within the cerebellum (Nieuwenhuys et al. 1998).

Divergence in proliferation zones and cerebellum structure within actinopterygians

In contrast to the conservation of embryonic origin and molecular specification of cerebellar granule cells, the morphological diversity of the cerebellum across vertebrate taxa is striking (Fig. 6). In mammals, for example, there is marked diversity in the degree of cerebellar foliation (Fig. 6, e–g). Here, structural variation appears to relate directly to proliferation within the external germinal layer in response to Shh signaling from underlying Purkinje cells (Corrales et al. 2006). However, this surface area:volume ratio explanation is unlikely to account for either the variation in chondrichthyans (Fig. 6, h–j) (Yopak et al. 2007; Yopak and Montgomery 2008) or actinopterygians (Fig. 6, a–d) (Nieuwenhuys et al. 1998), where transit amplification is, respectively, either severely attenuated or absent. How do our results explain the variation between paddlefish and zebrafish cerebellar morphology?

The developing paddlefish cerebellum both resembles and differs from that of the zebrafish cerebellum in key features. In paddlefish, as in zebrafish, the enduring site of *Atoh1* expression and granule cell proliferation into late development is the
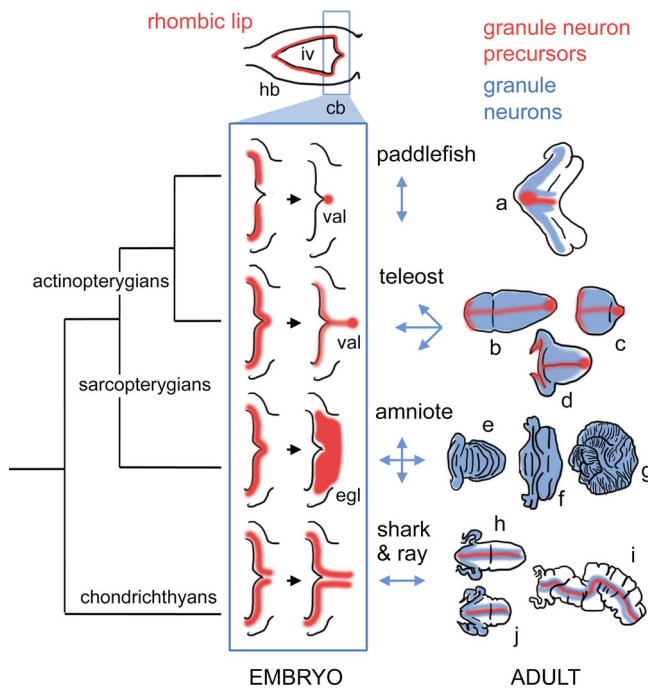
Fig. 6. Hypothetical phylogeny of granule neuron proliferative zones in the cerebellum. Hindbrain development in all vertebrate embryos can be characterized by a phase (top) in which an expanded fourth ventricle roof plate (iv) is bordered by an *Atoh1*-positive rhombic lip (red) in both the prospective cerebellum (CB) and the rest of the hindbrain (HB). Only the "upper" or cerebellar rhombic lip gives rise to granule cells in taxon-specific ways. We hypothesise that variation in the development of these proliferative zones (blue boxed schematics) and the extent of tangential migration away from these epithelia constrain the geometry of the expansion of the granule cell layer (blue arrows, right) to produce mature granule cell distributions (blue) in adult, taxon-specific cerebellar morphologies (far right). In actinopterygians, granule cell proliferation is confined to the cerebellar midline with the valvular primordium (val) at its anterior pole. In teleosts, there is a rostral developmental translocation of the valvular primordium and concurrent remodeling of the midline around a cerebellar recess (Kaslin et al. 2009), which is absent in the paddlefish. In amniotes, granule cell precursors migrate into a transient superficial external germinal layer (EGL) and no precursors are retained in the adult cerebellum. In chondrichthyans, progenitors show a similar organization, however granule neurons do not undergo tangential migration but rather condense in paramedial nuclei (Chaplin et al. 2010). Figures show hypothetical cell distributions in the cerebellar outlines (not shown to the same scale) based on model species for (a) paddlefish (a chondrostean ray-finned fish); (b–d) teleosts: b, remora (a perciform teleost), c, zebrafish (a cypriniform teleost), d, catfish (a siluriform teleost); (e–g) amniotes: e, bird, f, bat, g, giraffe; (h–j) chondrichthyans: h, dogfish (a shark), i, skate, j, stingray. Abbreviations: CB, cerebellum; EGL, external germinal layer; HB, hindbrain; IV, fourth ventricle; val, valvular primordium.

cerebellar midline. This territory is retained into adulthood in the zebrafish (Zupanc et al. 2005), and an intriguing remaining issue is whether the same is true of paddlefish. In zebrafish (Kaslin et al. 2013) and stickleback (Ekstrom et al. 2001), hence presumably across teleosts, this results in a linear domain of granule progenitor cell proliferation that includes the upper rhombic lip and extends along the midline of the cerebellar corpus to its anterior pole. This latter apical proliferative "niche" (Kaslin et al. 2009) has been equated to the valvular primordium (Chaplin et al. 2010; Kani et al. 2010), which gives rise, in the form of the valvulus, to the most variable structural feature of the actinopterygian cerebellum. The presumptive valvular primordium in paddlefish and zebrafish is similar in initial position, proliferative activity and gene expression and yet generates very different cerebellar morphologies (Fig. 1, B and C). Cerebellar midline remodeling around a rostrally extending ventricular protrusion in zebrafish (Kaslin et al. 2009) results in the valvular primordium becoming distanced from the rhombic lip (Fig. 6, b–d), which is the site of *Atoh1* induction via roof plate-derived Gdf7 (Alder et al. 1996; Broom et al. 2012). By contrast, in paddlefish, the valvular primordium remains close to the roof plate (Fig. 6a).

The remodeling that facilitates the developmental translocation of the valvular primordium in zebrafish may have been enabled by the teleost whole-genome duplication event that resulted in three *Atoh1* (Fig. S1) and two *Gdf7* paralogues with divergent expression patterns in zebrafish (Chaplin et al. 2010; Kani et al. 2010). The maturing cerebellar rhombic lip of zebrafish is characterized by *Gdf6* and *Atoh1c* expression. In contrast, the apical midline niche is characterized by the coordinated co-expression of *Gdf7* and *Atoh1a/Atoh1b*. Though the paddlefish lineage has undergone an independent whole-genome duplication (Crow et al. 2012), this is likely much more recent, and only a single *Atoh1* gene fragment could be isolated. In paddlefish, the cerebellar midline fails to extend such that the valvular primordium remains adjacent to the fourth ventricle roof plate, the likely exclusive source of Gdf signaling. We speculate that this simple topographic constraint on proliferation limits the paddlefish cerebellum to only lateral growth from the midline resulting in an anisomorphic, ribbon-like growth pattern reflected in its adult form (Figs. 1C and 6a).

Our results also suggest that the *Atoh1*-positive proliferative valvular primordium, which comprises a site of granule cell production that is maintained into late development, is a potential actinopterygian synapomorphy. The absence of *Shh* in the cerebellum of both paddlefish (a chondrostean actinopterygian) and zebrafish (a teleost actinopterygian), and the absence of a superficial germinal layer, argue against the valvular primordium being a direct homologue of the amniote external germinal layer. We propose that cerebellar structural diversity in actinopterygians results from the different proliferative properties of this unique valvular primordium, in terms of both rate and duration, and that this is facilitated to some extent by duplication

of the *Atoh1* gene, enabling the generation of distinct sub-domains of expression. In paddlefish, the presumptive lack of such sub-domains may "tie" the valvulus to the fourth ventricle roof plate and prevent the extension of the cerebellar midline, resulting in anisomorphic growth.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Fig. S1. Phylogenetic analysis of the Atonal homolog (Atoh) protein family in vertebrates.