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Evolutionary mechanisms that generate morphology and neural-circuit diversity of the cerebellum

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Running Title: Evolution of the cerebellum

Abstract

The cerebellum is derived from the dorsal part of the anterior-most hindbrain. The vertebrate cerebellum contains glutamatergic granule cells (GCs) and gamma-aminobutyric acid (GABA)ergic Purkinje cells (PCs). These cerebellar neurons are generated from neuronal progenitors or neural stem cells by mechanisms that are conserved among vertebrates. However, vertebrate cerebella are widely diverse with respect to their gross morphology and neural circuits. The cerebellum of cyclostomes, the basal vertebrates, has a negligible structure. Cartilaginous fishes have a cerebellum containing GCs, PCs, and deep cerebellar nuclei (DCNs), which include projection neurons. Ray-finned fish lack DCNs but have projection neurons termed eurydendroid cells (ECs) in the vicinity of the PCs. Among ray-finned fishes, the cerebellum of teleost zebrafish has a simple lobular structure, whereas that of weakly electric mormyrid fish is large and foliated. Amniotes, which include mammals, independently evolved a large, foliated cerebellum, which contains massive numbers of GCs and has functional connections with the dorsal telencephalon (neocortex). Recent studies of cyclostomes and cartilaginous fish suggest that the genetic program for cerebellum

development was already encoded in the genome of ancestral vertebrates. In this review, we discuss how alterations of the genetic and cellular programs generated diversity of the cerebellum during evolution.

Key words: cerebellum, evolution, proneural gene, Fgf, Shh

Introduction

The cerebellum is part of the central nervous system (CNS) in the vertebrate brain and functions in some aspects of motor coordination and motor learning. The cerebellum is also involved in higher cognitive and emotional functions (Ito, 2005, Ito, 2006, Ito, 2008). The functions of the cerebellum rely on neural circuits that involve a relatively small number of neuronal types (Fig. 1). The simple organization of the cerebellum makes it an attractive model for studying development and functions of the CNS. Although the basic structure of the cerebellum is conserved among vertebrates, there is diversity in its gross morphology and neural circuits. For instance, cyclostomes have a

small cerebellum or display no clear cerebellum structure, whereas mammals have a large, foliated cerebellum (Bone, 1963, Kusunoki *et al.*, 1982, Larsell, 1947, Nieuwenhuys, 1967, Altman & Bayer, 1997, Butler & Hodos, 2005). During evolution, the vertebrate cerebellum acquired diversity as animals changed in response to the environment and other factors. Mammalian cerebella became large and complex, and evolved connections with the neocortex for precise motor control and cognitive functions. Notably, abnormalities in the mammalian-specific components of cerebellar neural circuits are linked to human diseases, such as psychiatric and neoplastic diseases. Some autistic spectrum disorders (ASD) are also linked to abnormalities in cerebellar neural circuits (Courchesne *et al.*, 1988, Ritvo *et al.*, 1986, Fatemi *et al.*, 2002, Tsai *et al.*, 2012). A dysregulation of GC progenitor proliferation causes medulloblastoma, a type of pediatric malignant brain tumor (Jones *et al.*, 2012, Pugh *et al.*, 2012, Robinson *et al.*, 2012). Thus, while the mammals modified their cerebellar neural circuits to elicit higher brain functions during evolution, they also became susceptible to certain brain diseases. Therefore, elucidating the mechanisms behind the evolution of the cerebellum not only increases our understanding of brain evolution, but also clarifies the etiology

and pathology of cerebellum-related human diseases.

Conserved cerebellar structure

Vertebrates have a cerebellum, but amphioxi and ascidians do not (Hudson, 2016, Lacalli *et al.*, 1994). Thus, the cerebellum is a CNS region established in vertebrates. Most, if not all, cerebella have two major types of cerebellar neurons, the glutamatergic granule cells (GCs) and the gamma-aminobutyric acid (GABA)ergic Purkinje cells (PCs) (Altman & Bayer, 1997, Butler & Hodos, 2005, Hashimoto & Hibi, 2012, Hibi & Shimizu, 2012) (Fig. 1A). The GCs and PCs receive afferent inputs through mossy fibers (MFs) and climbing fibers (CFs), respectively, from neurons outside the cerebellum (precerebellar nuclei) (Fig. 1A, B). The CFs originate from the contralateral side of inferior olive nuclei (IOs) in the caudal hindbrain and terminate on the soma and dendrites of PCs. The MFs originate from neurons in non-IO precerebellar nuclei located in various brain regions, and innervate the dendrites of GCs. The MF information is conveyed to the dendrites of PCs through GC axons that are aligned parallel to one another (called parallel fibers: PFs). The information from CFs and MFs

is integrated in the PCs. The PCs project their axon to projection neurons (excitatory neurons in the deep cerebellar nuclei [DCNs] in most vertebrate cerebella; eurydendroid cells [ECs] in teleost cerebella), which send their axon outside the cerebellum, such as to the red nuclei in the tegmentum (Fig. 1A, B). Some PCs also directly project their axon to vestibular nuclei (also known as octaval nuclei in teleosts), which are located in the hindbrain and involved in body balance control (Fig. 1B).

PCs and GCs are located in distinct areas called the Purkinje cell layer (PCL) and granule cell layer (GCL), and PC dendrites receive the GC axons (PFs) in the molecular layer (ML) (Fig. 1A). Although the cerebellum has this three-layer structure, there is variation among vertebrates in the topographic arrangement of the layers. In addition to PCs, GCs, and the projection neurons, the cerebellum contains interneurons that modify the activity of the cerebellar neural circuits. Golgi cells and stellate cells are GABAergic interneurons located in the GCL and ML, respectively, that receive GC axonal inputs and modify the GC and PC activities (Fig. 1A). The mammalian cerebellum is reported to contain other interneurons, such as GABAergic basket cells, candelabrum cells, Lugaro cells, and glutamatergic unipolar brush cells (UBCs) (Fig.

1A), although their presence in other vertebrate cerebella has not been well investigated. Glial cells such as astrocytes and Bergmann glial cells (radial glia-like cells in the cerebellum), and oligodendrocytes are also present in most of the cerebella analyzed (Altman & Bayer, 1997, Butler & Hodos, 2005).

The cerebellum can be divided into two parts: the corpus cerebelli (CCe) and the auricles (Fig. 1C). The cerebellum of ray-finned fishes but not other vertebrates has a rostral extension of the CCe termed the valvula (Va). GCs in the CCe receive a variety of MF inputs whereas those in the auricles mainly receive the vestibular MF inputs. Although the basic structure of the CCe and the auricles is conserved, their proportions and the size and complexity of the cerebellum vary by species (Fig. 2A).

Diversity in cerebellum structure

Cyclostomes

Cyclostomes, such as lamprey and hagfish, are jawless vertebrates (agnathans) that split from other vertebrates (gnathostomes) at a very early stage of vertebrate evolution. Hagfish do not have a morphologically apparent cerebellum (Bone, 1963, Kusunoki *et*

al., 1982, Larsell, 1947, Nieuwenhuys, 1967, Butler & Hodos, 2005). Lampreys have a small cerebellum (CCe only) that contains GC-like cells and neurons with complex dendrites (Nieuwenhuys, 1967). Lampreys also have cerebellar commissures that presumably contain afferent MFs from the spinal cord (Nieuwenhuys & Nicholson, 1967).

Cartilaginous fishes

Cartilaginous fishes (chondrichthyans), such as sharks, are the most basal gnathostomes (jawed vertebrates). They have a relatively large cerebellum, which includes the CCe and auricles, and contains well-differentiated GCs and PCs. Unlike other vertebrate cerebella, the GCL and PCL are not continuously aligned but are segregated into two domains; the GCs form long bilateral cylindrical columns called eminentiae granularis at the core of the CCe, and the PCs congregate on the lateral wall of the CCe (Butler & Hodos, 1996, Pose-Mendez *et al.*, 2016b) (Fig. 2A). The cartilaginous fish cerebellum contains DCNs that include projection neurons and interneurons, located in the cerebellar peduncles that connect the cerebellum with other regions of the CNS. The

DCN projection neurons receive afferents from PCs and send their efferent axons outside the cerebellum, including the red nuclei (Alvarez-Otero *et al.*, 1996, Paul & Roberts, 1984, Butler & Hodos, 2005, Ebbesson & Campbell, 1973). These features indicate that the cartilaginous fish have cerebellar neural circuits similar to those of amniotes, although there are some differences between them.

Ray-finned fishes

The cerebellum of ray-finned fishes (actinopterygians) displays the three-layer structure (ML/PCL/GCL) (Fig. 1A, 2). The cerebella of ray-finned fishes are widely diverse in size and complexity. Bichirs (*Polypterus*) and paddlefish are the basal ray-finned fishes that did not undergo the teleost-specific whole-genome duplication (Amores *et al.*, 1998, Postlethwait *et al.*, 1998). The bichir cerebellum has a large Va, while the paddlefish cerebellum has a long mediolateral axis and a small Va (Nieuwenhuys, 1997, Butts *et al.*, 2014c) (Fig. 2A, 4A). The zebrafish cerebellum has a simple structure consisting of the Va, CCe (rostro-medial lobe), and auricle (caudolateral lobe), which consists of the lobus caudalis (LCa) and the eminentia granularis (EG, which is different from the

eminentiae granularis in the cartilaginous fish cerebellum) (Bae *et al.*, 2009, Hibi & Shimizu, 2012) (Fig. 2A). The LCa and EG contain GCs, whose axons make synapses with the PC dendrites in the cerebellum (Fig. 1A) and further extend caudally to connect with the dendrites of PC-like cells called crest cells in the dorsal hindbrain (Volkmann *et al.*, 2008, Bae *et al.*, 2009, Takeuchi *et al.*, 2015b). Similar extra-cerebellar projections from auricle GCs are also seen in cartilaginous fish (Montgomery, 1981), but not in amniotes. However, the caudal neural circuits may function similarly to those involving the caudal-most lobe (flocculus) in amniote cerebella (Fig. 1C).

In contrast to the cartilaginous fish cerebellum, that of teleosts and possibly all ray-fined fish has projection neurons called ECs that are located near the PCs and receive the axons of PCs (Fig. 1A) (Bae *et al.*, 2009, Ikenaga *et al.*, 2005, Ikenaga *et al.*, 2006). The ECs send their axon to neurons in the tegmentum (red nucleus) (Matsui *et al.*, 2014a, Matsui *et al.*, 2014b, Takeuchi *et al.*, 2015a) like the DCN projection neurons, suggesting that the ECs and the DCN projection neurons are functionally equivalent. However, unlike the DCN neurons, the ECs have dendrites that receive GC axons (Fig. 1A), indicating that the neural circuits involving the cerebellar projection neurons are

different between ray-finned fishes and other vertebrates with DCNs.

In contrast to the simple folded (lobular) structure of zebrafish and medaka cerebella, mormyrid fishes, such as *Gnathonemus petersii* (known as elephant nose fish), have a large, foliated Va and CCe; the Va is highly folded and covers the dorsal side of the rostral CNS (Fig. 2A) (Han *et al.*, 2006, Meek *et al.*, 2008, Nieuwenhuys & Nicholson, 1967). In addition to the LCa and EG, mormyrid fish have an electrosensory lateral line lobe (ELL), located posterior to the LCa. Mormyrid fish generate weak electric fields with electric organs, and perceive the electric properties of objects through epidermal electroreceptors (tuberous organs) to sense their surrounding environment. The ELL and the cerebellum (Va/CCe/LCa/EG) are involved in processing the electrosensory information (Bell *et al.*, 2008). However, although the ELL is present in all weakly electric fishes, only the mormyrid fish among them have a proportionally enlarged cerebellum (Nieuwenhuys, 1997), and it is not clear whether the enlarged cerebellum is linked to the evolution of the electrosensory system in these fish. Although the PC dendrites in the zebrafish cerebellum do not show the typical planar polarity, those in the mormyrid cerebellum have a palisading structure that can

efficiently receive inputs from PFs (Meek & Nieuwenhuys, 1991, Meek *et al.*, 2008).

Amphibians, birds, and mammals

After metamorphosis, amphibian cerebella have the three layers and the CCe/auricle structure seen in amniotes (Butler & Hodos, 2005, Butts *et al.*, 2014b) (Fig. 2A). They also have DCNs (Montgomery, 1988, ten Donkelaar *et al.*, 1991). However, the amphibian cerebella are small and not foliated. Amniotes such as aves (birds) and mammals have a foliated cerebellum that contains massive numbers of GCs and PCs (Fig. 2A). Amniote cerebella have multiple DCNs, including fastigial, interposed (emboliform and globose), and dentate nuclei (from medial to lateral), which contain glutamatergic projection neurons and GABAergic interneurons. In addition to the three continuous layers (ML/PCL/LCL), amniote cerebella have white matter (WM) that contains myelinated axons, and cerebellar peduncles that contain afferent and efferent fibers for the cerebellum (Altman & Bayer, 1997) (Fig. 2A). The middle region of the amniote CCe is morphologically distinguishable from the lateral regions of the CCe, and is termed the vermis (Altman & Bayer, 1997, Butler & Hodos, 2005) (Fig. 1C). In

mammals, the GCs in the lateral regions of the CCe, termed the cerebellar hemispheres (Fig. 1C), receive MFs from the precerebellar pontine nuclei, which receive afferent inputs from the neocortex (cortico-pontine tracts); the information from the GCs in the cerebellar hemispheres is integrated in the PCs and transmitted through the lateral DCNs (the dentate nuclei) to the thalamus, which has connections with the neocortex (cortico-cerebellar system) (Fig 1B, C). Thus, the cerebellar hemispheres have afferent and efferent connections with the neocortex and are involved in higher cognitive and emotional functions; they are also called the neocerebellum (Altman & Bayer, 1997, Butler & Hodos, 2005). The caudal-most lobe of the mammalian cerebellum, the flocculus, has connections with the vestibular nuclei and functions in body balance control; it is equivalent to the auricles in other vertebrate cerebella (Altman & Bayer, 1997, Butler & Hodos, 2005) (Fig. 1B, C).

These comparative anatomical studies indicate that evolution of the cerebellum may have involved several steps (Fig. 2B): (1) the cerebellum domain was established in the ancestor of the vertebrates; (2) differentiation of the cerebellum occurred in the ancestral gnathostomes before the cartilaginous fishes split off; (3) the

continuous layer formation was established before the ray-finned fishes split off; (4) ECs evolved in ray-finned fishes; (5) the ELL evolved in weakly electric fishes, and the cerebellum was expanded in mormyrid fishes; (6) the cerebellum was independently expanded in amniotes, and the connection between the cerebellum and neocortex was expanded, especially in mammals. In the remainder of this review, we will discuss the general mechanisms that control cerebellum development and how alterations in these mechanisms could have generated diversity of the cerebellum.

Genetic and cellular programs for cerebellum development

Establishment of the cerebellum domain

During neural development, the cerebellum domain is established in the rostral-most hindbrain as a result of signals from the isthmic organizer, which is located at the mid-hindbrain boundary (MHB) (Hidalgo-Sanchez *et al.*, 1999, Joyner *et al.*, 2000, Simeone, 2000, Wurst & Bally-Cuif, 2001). In amniotes, two homeodomain-containing transcription factors, Otx2 and Gbx2, are essential for positioning the isthmic organizer. *Otx2* and *Gbx2* are expressed in a largely complementary manner in the rostral and

caudal regions of the neuroectoderm, and the isthmic organizer forms at the boundary of *Otx2/Gbx2* expression domains (Acampora *et al.*, 1997, Broccoli *et al.*, 1999, Millet *et al.*, 1999, Simeone *et al.*, 1992, Suda *et al.*, 1997, Wassarman *et al.*, 1997) (Fig. 3A). In zebrafish, *gbx1* and *gbx2* are expressed in overlapping regions of the hindbrain (Kikuta *et al.*, 2003, Rhinn *et al.*, 2009) and function redundantly; they control the isthmic organizer formation and cerebellum development by repressing *otx* genes (Su *et al.*, 2014) (Fig. 3A). In mice, the conditional knockout of *Otx2* in the midbrain causes the ectopic induction of cerebellar neurons (Di Giovannantonio *et al.*, 2014). These data suggest that the *Gbx1/2*-mediated repression of *Otx* gene(s) is essential for the cerebellar differentiation in all vertebrates. After the cerebellar domain is established, a set of genes, fibroblast growth factor 8 (*Fgf8*), *Wnt1*, *Pax2/5*, *Lmx1b*, and *Engrailed1/2* (*Eng1/2*), are expressed in the isthmic organizer region, and comprise a genetic program that restricts and maintains the isthmic organizer, and controls the formation of the cerebellum (Harada *et al.*, 2016, Nakamura *et al.*, 2005) (Fig. 3A). Among the isthmic organizer (MHB) genes, *Fgf8* and *Wnt1* (*wnt1/3a/10b* in zebrafish) family genes play major roles in the morphogenesis of the cerebellum. The Wnt proteins regulate

development of the midbrain and cerebellum by preventing apoptosis and inducing cell proliferation (Buckles *et al.*, 2004, Dickinson *et al.*, 1994). Fgf8 (Fgf8a in zebrafish) is required isthmic organizer and cerebellum formation (Crossley *et al.*, 1996, Liu *et al.*, 1999, Martinez *et al.*, 1999, Picker *et al.*, 1999, Reifers *et al.*, 1998). Fgf8 controls the fate decision for the cerebellum by repressing *otx* genes (Foucher *et al.*, 2006). Fgf signaling also functions downstream of the *Gbx1/2*-mediated repression of *Otx* gene(s) for the isthmic organizer program and cerebellar differentiation in zebrafish (Su *et al.*, 2014), indicating that Fgf signaling is involved in both the establishment of the cerebellum domain and differentiation of the cerebellum. Fgf8 is also involved in preventing cell sorting for the MHB formation in mice (Sunmonu *et al.*, 2011) and in differentiation of the vermis (discussed below).

In amphioxus, although rostral *Otx* and caudal *Gbx* expressions are observed in the neuroectoderm, the isthmic organizer genes are not expressed (Castro *et al.*, 2006, Kozmik *et al.*, 1999). In contrast, the isthmic organizer genes *fgf8/17/18* and *wnt1*, in addition to *otx*, *gbx*, *eng*, are expressed in the MHB-like region of the hemichordate ectoderm (Pani *et al.*, 2012), suggesting that the genetic program for isthmic organizer

formation was established in ancestral deuterostomes and was subsequently lost in amphioxus. Thus, ancestral vertebrates might have inherited the genetic program for establishing the cerebellum domain during evolution.

Two proneuronal progenitor domains generate cerebellar neurons

Cerebellar neurons are generated from two neuronal progenitor domains (Fig. 3B, C): the upper rhombic lip (URL, also called the cerebellar rhombic lip) and the cerebellar ventricular zone (VZ) (Wingate, 2001, Wingate & Hatten, 1999, Zervas *et al.*, 2004) (Fig. 3B, C). The URL is located at the dorso-caudal edge of the cerebellum primordium; the neuronal progenitors in the URL express the basic helix-loop-helix (bHLH)-type proneural gene *atoh1* (the vertebrate homologue of *Drosophila atonal*), and give rise to glutamatergic excitatory neurons (Alder *et al.*, 1996, Ben-Arie *et al.*, 1997, Machold & Fishell, 2005, Wang *et al.*, 2005, Wingate, 2005, Wilson & Wingate, 2006, Kani *et al.*, 2010). The VZ is located at the rostral roof of the fourth ventricle and ventral to the URL; the neuronal progenitors in the VZ express the bHLH proneural gene *ptf1a* and give rise to GABAergic inhibitory neurons (Hoshino, 2006, Hoshino *et*

al., 2005, Kani *et al.*, 2010). Most vertebrates have one *atoh1*, whereas teleosts have three *atoh1* genes, namely *atoh1a*, *b*, and *c*, which show both overlapping and distinct expression patterns in the URL and its derivatives (Chaplin *et al.*, 2010, Kani *et al.*, 2010). There is only one *ptf1a* gene reported in vertebrates, including teleosts (Hoshino *et al.*, 2005, Kani *et al.*, 2010).

Lineage tracing in mice revealed that the *atoh1*-expressing neuronal progenitors in the URL sequentially generate neurons in the tegmental nuclei, the DCN neurons, and the GCs (Machold & Fishell, 2005, Wang *et al.*, 2005, Wilson & Wingate, 2006, Wingate, 2005) (Fig. 3B). Similarly, tracing experiments with transgenic zebrafish showed that the *atoh1a*-expressing cells give rise to the tegmental neurons and the GCs (Kani *et al.*, 2010) (Fig. 3C), suggesting that the generation of GCs from *atoh1*$^{+}$ neuronal progenitors is conserved between ray-finned fish and mammals. The roof plate, which is connected to the URL, plays an important role in specification of the *atoh1*-expressing neuronal progenitors (Chizhikov *et al.*, 2006). The roof plate cells express the LIM homeobox transcription factors Lmx1a and Lmx1b, and secrete the Bmp-family proteins, Gdf7, Bmp6, and Bmp7 (Mishima *et al.*, 2009, Chizhikov *et al.*,

2006, Alder *et al.*, 1999). In zebrafish, *gdf6a* and *gdf10a/b* are also expressed in the cerebellum adjacent to the URL (Chaplin *et al.*, 2010, Takeuchi *et al.*, 2016). These Bmp-family proteins are likely to be involved in the specification and maintenance of the *atoh1*$^{+}$ progenitors. In amniotes, the *Atoh1*$^{+}$ neuronal progenitors migrate tangentially from the URL to form the external granule (or germinal) layer (EGL), which covers the entire dorsal surface of the cerebellum (Wingate & Hatten, 1999) (Fig. 3B). In zebrafish, the *atoh1*$^{+}$ neuronal progenitor domains near the midline extend rostrally and those at the caudo-lateral edge extend laterally, forming continuous medial and caudal *atoh1*$^{+}$ domains (Kani *et al.*, 2010, Chaplin *et al.*, 2010) (Fig. 3C, 4A). The *atoh1*$^{+}$ cells in the cerebellum are proliferative, and a portion of these cells starts to express another bHLH factor Neurod1 and to differentiate into GCs in zebrafish and mice (Kani *et al.*, 2010, Miyata *et al.*, 1999). In mice, Neurod1 is expressed in postmitotic GCs that are located in the inner EGL (iEGL), and in zebrafish, it is expressed in both postmitotic and proliferating GCs in the ML (Kani *et al.*, 2010, Miyata *et al.*, 1999) (Fig. 3B, C). Immature (Neurod1^{+}) GCs migrate from the iEGL (mice) or the ML (zebrafish) inward to form the GCL in the CCe of both mammals and

zebrafish (Hashimoto & Hibi, 2012, Kani *et al.*, 2010, Mueller & Wullimann, 2002, Takeuchi *et al.*, 2015a) (Fig. 3B).

In amniote cerebella, GC progenitor proliferation in the EGL is positively regulated by Sonic hedgehog (Shh), which is secreted from PCs (Dahmane & Ruiz i Altaba, 1999, Wallace, 1999, Wechsler-Reya & Scott, 1999, Lewis *et al.*, 2004) (Fig. 3B). The Shh-dependent expansion of GC progenitors contributes to the increased size and foliation of mammalian cerebella (Corrales *et al.*, 2006, Corrales *et al.*, 2004). PC-secreted Shh is also involved in the secondary generation of astrocytes and GABAergic interneurons from neural-stem-like cells in the prospective WM (Fleming *et al.*, 2013). These data suggest that the *Shh* expression in the PCs of amniote cerebella is linked to the development of amniote-specific features of the cerebellum. In mice, the *atoh1*-expressing neuronal progenitors in the dorsal hindbrain (lower rhombic lip, LRL) also give rise to neurons in the precerebellar nuclei that send MFs to GCs, including the pontine nuclei (Landsberg *et al.*, 2005, Machold & Fishell, 2005, Wang *et al.*, 2005).

Lineage tracing experiments also revealed that the VZ neuronal progenitors expressing *ptf1a* (as well as *ascl1*, a homologue of *Drosophila acute-schute*)

sequentially give rise to PCs and other GABAergic interneurons ($Pax2^+$ interneurons) in the cerebellum of mice and zebrafish; *Ptf1a* is essential for the development of these neurons (Hoshino *et al.*, 2005, Kani *et al.*, 2010, Kaslin *et al.*, 2013, Leto *et al.*, 2009, Leto *et al.*, 2006, Grimaldi *et al.*, 2009, Sudarov *et al.*, 2011) (Fig. 3B, C). Within the $Ptf1a^+$ VZ domain, *Olig2* and *Gsx1* are expressed in non-overlapping caudal (dorsal) and rostral (ventral) subdomains at the beginning of neurogenesis, when PCs are generated, in the mouse cerebellum (Fig. 3B). Subsequently, in correlation with the progressive generation of the GABAergic interneurons, the *Gsx1*-expressing domains expand caudally; Olig1/2 and Gsx1 have been shown to play roles in the fate determination of PCs versus interneurons, respectively, in the mouse cerebellum (Seto *et al.*, 2014). Although *olig2* is expressed in part (caudal domain) of the *ptf1a*-expressing VZ in zebrafish, *olig2*-expressing cells do not become PCs but rather differentiate into ECs (Bae *et al.*, 2009, Kani *et al.*, 2010, McFarland *et al.*, 2008) (Fig. 3C).

The genetic deletion of *Ptf1a* in the mouse cerebellum leads to the loss of GABAergic neurons and to the ectopic formation of glutamatergic neurons from the VZ

progenitors (GCs, DCN projection neurons, and UBCs) (Millen *et al.*, 2014, Pascual *et al.*, 2007). The deletion of *Atoh1* in mice also results in the loss of glutamatergic neurons but leads to the ectopic formation of PCs from the URL (Wang *et al.*, 2005). Furthermore, expressing *Atoh1a* in the VZ suppresses *Ptf1a* expression and induces glutamatergic neurons, and expressing *Ptf1a* in the URL suppresses *Atoh1* expression and induces GABAergic neurons from the URL (Yamada *et al.*, 2014). These data indicate that *Ptf1a* and *Atoh1* mutually repress each other's expression and function, to determine cerebellar cell fates.

Extended neurogenesis in the cerebellum

In most mammals, GCs are generated from the EGL in the early postnatal period, but the EGL disappears by puberty (Sanchez-Villagra & Sultan, 2002). In zebrafish cerebellum, GCs are continuously generated from *atoh1*$^{+}$ neuronal progenitors throughout life in the ML, and they migrate into the GCL (Kaslin *et al.*, 2009, Kaslin *et al.*, 2013, Zupanc *et al.*, 2005, Kani *et al.*, 2010) (Fig. 3C). PCs are generated from *ptf1a*$^{+}$ progenitors in the zebrafish VZ at larval stages (until 1 month post fertilization),

but only a limited number of glial cells and GABAergic interneurons are generated from the VZ and the generation of PCs is scarcely detected in the adult cerebellum (Kani *et al.*, 2010, Kaslin *et al.*, 2013). Studies using *nestin:egfp* transgenic zebrafish showed that *nestin*$^{+}$ cells are neural progenitors that give rise to GCs in the adult cerebellum (Kaslin *et al.*, 2009, Kaslin *et al.*, 2013), suggesting that there are *nestin*-expressing neuronal progenitors (or neural stem cells). Some of the *nestin:egfp*$^{+}$ cells expressed *atoh1* (Kaslin *et al.*, 2013), but it was unclear whether the *nestin*-expressing cells differentiate into *atoh1*$^{+}$ progenitors or directly give rise to GCs. Actively proliferating Bergmann glial cells are found in the adult zebrafish cerebellum (Kani *et al.*, 2010), indicating that there are multiple types of neuronal progenitors or neural stem cells: *atoh1*$^{+}$ cells, *ptf1a*$^{+}$ cells, and *atoh1*$^{-}$*/ptf1a*$^{-}$ neural stem cells (e.g. *nestin*$^{+}$ neuroepithelial-like cells, glial-type stem cells), to support the extended cerebellar neurogenesis. However, it remains elusive how the neuronal progenitors (or neural stem cells) are maintained for extended periods in teleosts.

Regarding this issue, the medial GC progenitors remain in contact with the ventricle, and Fgf proteins from the ventricle have been suggested to play a role in

maintaining these cell populations in the zebrafish cerebellum (Kaslin *et al.*, 2009). *fgf8a* and *fgf12a* are expressed in GCs and PCs, respectively, and the *bmp*-family genes *gdf6a/7* and *gdf10a/b* are expressed in the vicinity of *atoh1*$^{+}$ GC progenitors at the larval stages (Chaplin *et al.*, 2010, Takeuchi *et al.*, 2016). Although it is not known whether they are also expressed in the adult zebrafish cerebellum, the continuous expression of Fgf and Bmp-family proteins may play a role in the maintenance or proliferation of neuronal progenitors or neural stem cells in the adult cerebellum.

The generation of cerebellar neurons and glial cells is also reported in the adult cerebellum of other teleost fish (Sirbulescu *et al.*, 2015, Teles *et al.*, 2012, Zupanc *et al.*, 2012), indicating that the prolonged neurogenesis of cerebellar neurons is conserved among teleosts. In some mammals, such as rabbits, a small number of GCs are generated from neuronal progenitors located at the subpial region, which is presumably an extension of the EGL, at the juvenile stage, and GABAergic interneurons and glial cells are also generated at the juvenile and adult stages (Ponti *et al.*, 2006, Ponti *et al.*, 2008). Therefore, although the timing varies according to species, there may be a common mechanism in vertebrates that controls cerebellar neurogenesis

after the initial neurogenesis phase.

Mechanisms for the development of cerebellum diversity

Establishment of the genetic program for cerebellum development

Lampreys have an MHB, which expresses cognates of the *Eng*, *Fgf8*, and *Pax2/5/8* genes, like gnathostomes (Murakami *et al.*, 2001, Murakami *et al.*, 2005). It was recently reported that hagfish and lamprey embryos express *Atoh1*, *Pax6* (a marker for GCs), and *Ptf1a* in the rhombic lip of the rostral hindbrain (Sugahara *et al.*, 2016), suggesting that neuronal progenitors for GCs and PCs are present in that region. These data also imply that the genetic program for generating the cerebellum domain was established in the ancestral vertebrates before the cyclostome clade split off (Fig. 4B).

Cartilaginous shark embryos express *Otx2*, *Gbx2*, and the MHB genes *Eng2* and *Fgf8* in a manner similar to ray-finned fish and mammals (Pose-Mendez *et al.*, 2016a). *Atoh1* is reported to be expressed in the continuous medial and caudal domains (the URL derivatives) in the shark cerebellum, as in the zebrafish cerebellum (Chaplin *et al.*, 2010) (Fig. 4A). *Ptf1a* expression in the shark cerebellum has not been reported

yet. However, since the shark cerebellum has well-differentiated PCs, GCs, and DCN neurons (projection and interneurons), and forms neural circuits similar to those of amniote cerebella (Alvarez-Otero *et al.*, 1996, Paul & Roberts, 1984, Pose-Mendez *et al.*, 2016b, Rodriguez-Moldes *et al.*, 2008, Butler & Hodos, 2005), these neurons are likely to be generated from *Atoh1*- and *Ptf1a*-expressing neuronal progenitors by mechanisms shared by other amniotes. The GCs in the shark cerebellum do not form a broad layer but instead are concentrated in the eminentiae granularis and the upper and lower auricular leafs (Pose-Mendez *et al.*, 2016b, Chaplin *et al.*, 2010) (Fig. 2A). Given the localization of the *Atoh1*$^{+}$ progenitors (Fig. 4A), the GCs generated from them do not migrate and form cylindrical structures in the shark cerebellum. It is reported that developing DCN neurons express *Lhx5*, *Lhx9*, and *Tbr1* in the shark cerebellum as in mammals (Pose-Mendez *et al.*, 2017). Therefore, the genetic programs for differentiating GCs, PCs, and DCN neurons, and for generating neural circuits were already established before the cartilaginous fishes split off (Fig. 4B).

Although the lamprey cerebellum contains various types of cerebellar neurons and receives input fibers, it remains unclear whether they are primitive GCs, PCs, or

projection neurons. Many cerebellar genes are specifically expressed in the GCs and PCs of both zebrafish and mouse cerebella (Takeuchi *et al.*, 2016). Future expression analyses of these GC and PC genes in the cerebella of cyclostomes and cartilaginous fishes may reveal the extent to which the genetic program for cerebellar differentiation was established in the ancestral vertebrates (agnathans and basal gnathostomes).

Layer formation and generation of eurydendroid cells in ray-finned fishes

The *atoh1*$^{+}$ progenitors are located in the medial and caudal domains (the URL derivatives) in the zebrafish cerebellum (Chaplin *et al.*, 2010, Kani *et al.*, 2010) (Fig. 4A). However, the GCs are located in the GCL uniformly from medial to lateral in the CCe (Bae *et al.*, 2009) (Fig. 2A, 3C). Immature GCs generated from the medial *atoh1*$^{+}$ progenitors migrate into both medial and lateral regions of the GCL in the CCe in zebrafish (Kani *et al.*, 2010, Hibi & Shimizu, 2012, Hibi & Shimizu, 2014) as in mammals (Fig. 3C). GC migration is required to form the continuous GCL in the zebrafish CCe, and the program for GC migration might have been established before the splitting off of ray-finned fish. The GCs derived from the caudal *atoh1*$^{+}$ progenitors

in zebrafish exhibit little or no migration, and form the EG and LCa, like the auricles in the shark cerebellum (Kani *et al.*, 2010, Volkmann *et al.*, 2008) (Fig. 4A). In addition, the medial and caudal GC progenitors in zebrafish show distinct spatial and temporal expression patterns of the three *atoh1* genes: *atoh1a* expression is restricted to the rostral domain (Va), whereas the *atoh1b/c* expressions are maintained longer in the rostro-caudal axis at the larval and adult stages (Chaplin *et al.*, 2010, Kani *et al.*, 2010). Furthermore, GCs in the CCe and the EG/LCa show distinct expressions of some GC genes (Bae *et al.*, 2009, Takeuchi *et al.*, 2016) and form different neural circuits (Volkmann *et al.*, 2008, Bae *et al.*, 2009, Takeuchi *et al.*, 2015b). Distinct genetic programs may differentially control the development and function of the GCs in the Va/CCe and the EG/LCa in ray-finned fish. For instance, the high *atoh1a* expression in the Va may be involved in the expanded Va seen in some teleost cerebella. In such a scenario, the subfunctionalization of genes generated by the teleost-specific genome duplication might contribute to structural differences in cerebella.

ECs evolved only in the cerebellum of ray-finned fishes in place of the DCN projection neurons. There are at least two types of ECs in the zebrafish cerebellum:

olig2-expressing (*olig2*$^{+}$) and calretinin-immunoreactive (Cr-ir^{+}) ECs (Bae *et al.*, 2009, McFarland *et al.*, 2008). Although the origin of the Cr-ir^{+} ECs is not known, the *olig2*$^{+}$ ECs are mainly derived from the VZ (a minority of them are derived from the *atoh1*$^{+}$ URL) (Kani *et al.*, 2010) (Fig. 3C). *Olig2* is not essential for PC development, but in mice it is involved in suppressing the transition of PC-generating VZ progenitors to interneuron-generating VZ progenitors, biasing the system toward PC generation (Seto *et al.*, 2014). In ray-finned fish, *olig2* might function to bias the cell fate of VZ-derived cells from GABAergic to glutamatergic. ECs share some properties with PCs, including the location of their somata and their complex dendrites for receiving GC axons (Bae *et al.*, 2009, Ikenaga *et al.*, 2005, Ikenaga *et al.*, 2006). Thus, the genetic program may be the same for the development and functions of PCs and ECs that have a common VZ origin.

Formation of a large, foliated cerebellum

The expansion of GCs induced by Shh secreted from PCs in the EGL is a likely key step in the formation of the foliated cerebellum in amniotes (Corrales *et al.*, 2006, Corrales

et al., 2004). In the shark cerebellum, although *Shh* is expressed in PCs, the GC progenitors do not express the *Patched2* (*Ptc2*) gene, a receptor and target of Shh signaling (Chaplin *et al.*, 2010), suggesting that the Shh-dependent expansion of GC progenitors does not take place in cartilaginous fishes. Although *shh* is reported to be expressed in a portion of ECs (Biechl *et al.*, 2016), it is not expressed in PCs and the *ptc1* gene is not expressed in the zebrafish larval cerebellum (Chaplin *et al.*, 2010), suggesting that Shh signaling may not be involved in the expansion of GC progenitors in zebrafish and other ray-finned fishes. This possibility is consistent with the lack of a typical EGL in ray-finned fish cerebella (Chaplin *et al.*, 2010, Kani *et al.*, 2010).

In *Xenopus* cerebellum, although *Shh* is not expressed, *Atoh1*$^{+}$ (also *Neurod1*$^{+}$) cells form an EGL-like layer that does not contain proliferative cells at metamorphosis (Butts *et al.*, 2014b) (Fig. 4A). It is still under debate whether the EGL is present in anamniote cerebella (Butts *et al.*, 2014a, Wullimann *et al.*, 2011). The issue could be terminological; i.e., whether the definition of the EGL is related to its proliferation status (mitotic, postmitotic, or transit amplification; external granule layer or external germinal layer), structure (layer or column), or generation mechanism

(Shh-dependent or independent).

Atoh1^{+} proliferative GC progenitors are present in the URL derivative in the cerebellum of cartilaginous fishes and ray-finned fish. However, the proliferation of GC progenitors does not depend on Shh signaling in these species. Thus, the Shh-dependent proliferation of the GC progenitors probably evolved in the amniotes (Fig. 4A, B). Since *Shh* and *Ptc2* are expressed in the PC and the VZ progenitors, respectively, in the shark cerebellum (Chaplin *et al.*, 2010), Shh signaling may be involved in the generation of GABAergic interneurons and astrocytes in the cartilaginous fishes, as reported for mouse cerebellum (Fleming *et al.*, 2013). Thus, it may be ascertained that during evolution, amniotes kept the *Shh* expression in PCs, and GC progenitors acquired the responsiveness to Shh signaling that enabled GC expansion, whereas ray-finned fish and amphibians lost the *Shh* expression in PCs during evolution.

On the other hand, it is unlikely that mormyrid fish generated their large, foliated cerebellum by independently evolving the Shh-dependent proliferation of GC progenitors. A preliminary transcriptome analysis of *Gnathonemus petersii* indicated that the mormyrid cerebellum displays a protracted expression of genes that control the

early phase of cerebellum development at the juvenile stage (KM, TS, and MH, unpublished results), suggesting that the Shh-dependent EGL formation is not a prerequisite for the generation of foliated cerebella. Thus, mormyrid fish use a different genetic program from that of amniotes to generate a large, foliated cerebellum (Fig. 4B).

In addition to foliation, the PCs in the mormyrid cerebellum have dendrites with a palisading structure and planar polarity (Meek & Nieuwenhuys, 1991, Meek *et al.*, 2008), similar to PC dendrites in the mammalian but not zebrafish cerebellum. In mammals, the PC dendrites initially form multi-sagittal planes, which become confined to a single plane during development; this remodeling of the PC dendrites is associated with the CF innervation (Kaneko *et al.*, 2011). Sema7, a membrane-associated semaphorin, is expressed on PCs and plays an important role in eliminating the CF innervation in mouse cerebellum (Uesaka *et al.*, 2014) and possibly in the mono-polarization of the PC dendrites. The PCs in zebrafish cerebellum also express *sema7* (*sema7a*) (Takeuchi *et al.*, 2016). Although whether Sema7a has a role in eliminating the CF innervation in zebrafish is not known, it may be involved in the

mono-polarization of the PC dendrites in mormyrid fish.

In any case, many genes that control the formation and function of cerebellar neural circuits are conserved between zebrafish and mammals (Takeuchi *et al.*, 2016), suggesting that the genetic programs for cerebellar neural circuit formation and remodeling may have existed in the genome of the ancestral vertebrates, and were modified to generate species-specific features of the cerebellum.

Evolutionary changes in neuronal connectivity

The hemispheres in the mammalian cerebella have strong connections with the neocortex: efferent fibers (MFs) from the pontine nuclei and afferent fibers through the lateral DCNs (dentate nuclei). In teleosts, the lateral valvular nuclei (NLVs) located ventro-lateral to the Va receive projections from the telencephalon to relay information to the cerebellum (Va, CCe) (Yang *et al.*, 2004), like the pontine nuclei in mammals. Although it is not known whether the NLVs are derived from *atoh1*$^{+}$ neuronal progenitors in the LRL like the pontine nuclei, the position and structure of the NLV are different from those of the pontine nuclei, implying that they evolved independently in

teleosts and mammals. The descendant cells from *atoh1*$^{+}$ neuronal progenitors in the LRL migrate ventrally during early neurogenesis in the zebrafish hindbrain (Kani *et al.*, 2010), like those in the mouse hindbrain (Landsberg *et al.*, 2005, Machold & Fishell, 2005, Wang *et al.*, 2005).

The rostral *Atoh1*$^{+}$ LRL progenitors that give rise to the pontine nuclei might have expanded in mammals during evolution. The generation of the pontine nuclei co-evolved with the cerebellar hemispheres. In mice, high *Fgf8* expression is required to form the vermis (Fig. 1C) (Basson *et al.*, 2008, Sato & Joyner, 2009). A hyper-activation of Fgf signaling with deficiencies in *Sprouty* genes, which encode negative feedback regulators of Fgf signaling, results in an expansion of the vermis and a reduction in the hemispheres, which is correlated with a reduction in Shh (Yu *et al.*, 2011). These reports suggest that *Fgf8* plays a role in regional determination of the vermis versus the cerebellar hemispheres. Since Fgf8 positively regulates *Atoh1* expression but suppresses cerebellar differentiation (Green *et al.*, 2014), the downregulation of *Fgf8* expression and subsequent shift to *Shh* expression might have been involved in the generation and expansion of the cerebellar hemispheres in

mammals.

Prospective

We are learning more about the diversity in the structures and neural circuits of the vertebrate cerebella, and we are beginning to understand the mechanisms that control the formation of cerebellar neural circuits in model animals, including zebrafish, chickens, and mice. Transcriptome and genome analyses are revealing when and where developmental genes are expressed in the cerebellum and its primordium in different vertebrate species, including non-model animals. Accumulating information enables us to speculate about which changes in gene expression contributed to differences in the structure and/or neural circuits of the cerebellum in different species. However, there is no compelling evidence yet for a change in gene expression that is responsible for changing the structure and/or the neural connections of the cerebellum during evolution. Furthermore, there is no evidence for a genomic alteration that led to a change in gene expression responsible for evolution of the cerebellum. For instance, the expression of *Shh* in PCs is a potential key for generating the foliated cerebella in amniotes. However,

it is not known whether the expression of *Shh* in PCs is sufficient for the generation of foliated cerebella, i.e., whether the expression of *Shh* in the PCs of an anamniote cerebellum would induce an amniote-type foliated cerebellum. Other events, such as the acquisition by GC progenitors of the ability to respond to Shh, may also be required. Furthermore, it remains elusive what genomic changes in the amniote *Shh* genes caused *Shh* to be expressed in PCs, and whether such changes are conserved in the cartilaginous fish genome.

As the genomic sequence information of more vertebrate species is becoming available, comparisons of the genomic sequence of multiple species, including phylogenetic analyses of genes' regulatory and coding regions, are becoming feasible. These analyses will identify the mechanisms that control the species-specific expression of genes that are linked to evolution of the cerebellum. Recent techniques for genome editing using the TALEN and CRISPR/Cas9 systems enable us to introduce desired changes into the genome of any vertebrate species whose embryo can be manipulated. These methods will enable us to test whether identified genomic changes are responsible for the evolution of the cerebellum in various animals.

Multiple related events occurred during the evolution of the cerebellum in different species. The expansion of the cerebellar hemispheres was linked to the generation or expansion of afferent and efferent systems in the mammalian cerebella. The expansion and foliation of the cerebellum might also have been linked to the formation of electric organs, teleost-type electroreceptors (tuberous organs), and the ELL in mormyrid fish (Baker *et al.*, 2013). It is not known whether these events occurred sequentially or if they were coordinately established during evolution, and if so, what kind of genetic and cellular mechanisms were responsible for them. Thus, many questions remain to be addressed. The development of innovative experimental techniques for genome analysis, genome editing, transgenesis, and embryo manipulation, will help researchers shed light on the mechanisms behind the evolution of the cerebellum.

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Figure legends

Fig. 1. Structure of the cerebellum and cerebellar neural circuits.

(A) Cerebellar neural circuits in ray-finned fish (left panel) and mammals (right panel). (B) Afferent (left panel) and efferent (right panel) connections with the mammalian cerebellum. (C) Gross morphology of vertebrate cerebella. Anterior-posterior (A-P) direction is indicated. Ba, basket cell; CCe, corpus cerebelli; CF, climbing fiber; DCN, deep cerebellar nuclei; EC, eurydendroid cells; GCL, granule cell layer; Go, Golgi cell; Lu, Lugaro cell; MF, mossy fiber; ML, molecular layer; PC, Purkinje cell; PCL, Purkinje cell layer; St, stellate cell; UBC, unipolar brush cell. (C) is modified from (Butler & Hodos, 2005).

Fig. 2. Diversity of cerebellum structures.

(A) Structures of vertebrate cerebella. Cerebellum structures are classified into several categories according to their morphological features. Each drawing represents a sagittal section image except for the right panel of the shark cerebellum that represents a cross section image. The sagittal section images with rostral to the left. The cross section image with dorsal to the top. The GCLs are marked by shading. PCs are marked by magenta dots. (B) Changes in the cerebellum structure during evolution. Parts of (A) are modified from (Pose-Mendez *et al.*, 2016b) (shark), (Llinas *et al.*, 1968) (alligator), and (Butler & Hodos, 2005) (frog, lizard, aves/mammals). ELL, electrosensory lateral line lobe; UAL, upper auricular leaf; LAL, lower auricular leaf; WM, white matter. The other abbreviations are described in the legend for Figure 1.

Fig. 3. Cerebellum development.

(A) Establishment of the cerebellar domain. Schematic representation of the early rostro-caudal patterning of the neuroectoderm that determines the cerebellum domain (left panel), and the genetic program that maintains and restricts the isthmic organizer (right panel). (B) Mouse cerebellum development on embryonic day 15 (E15, left panel) and postnatal day 7 (P7, middle panel). Sagittal views. Right panel is a detailed illustration of the box in the middle panel. *Atoh1*$^{+}$ GC progenitors are located in the outer layer of the EGL and actively proliferate in response to Shh secreted from PCs. *Neurod1*$^{+}$ GCs are located in the inner layer of the EGL, and subsequently migrate inward to the GCL. (C) Zebrafish cerebellum development at larval (5 days post fertilization, left panel) and adult (middle and right panels) stages. Sagittal views (left and middle panels). Cross-section view (right panel). EGL, external granule (germinal) layer; TNs, tegmental nuclei; URL, upper rhombic lip; VZ, ventricular zone. Other abbreviations are described in the legend for Figure 1. (A) is modified from (Harada *et al.*, 2016, Martinez *et al.*, 2013). (B) and (C) are modified from (Hashimoto & Hibi, 2012).

Fig. 4. Mechanisms that generate diversity in the cerebellum.

(A) Variations in cerebellum morphology during development. Dorsal views of the

cerebellum. *Atoh1*$^{+}$ GC progenitor domains are marked by blue shading. The proliferating GC progenitors are located in the medial and caudal regions, which are derived from the URL, in shark, paddlefish, and zebrafish. In the frog cerebellum, non-proliferating *Atoh1*$^{+}$ GCs expand to transiently form an EGL-like layer (green shading) at metamorphosis. In the mouse cerebellum, proliferating *Atoh1*$^{+}$ GC progenitors expand to form the EGL. (B) Changes in cellular (gray text) and genetic (blue text) programs that generated diversity in the cerebellum during evolution. (A) is modified from (Butts *et al.*, 2014a).

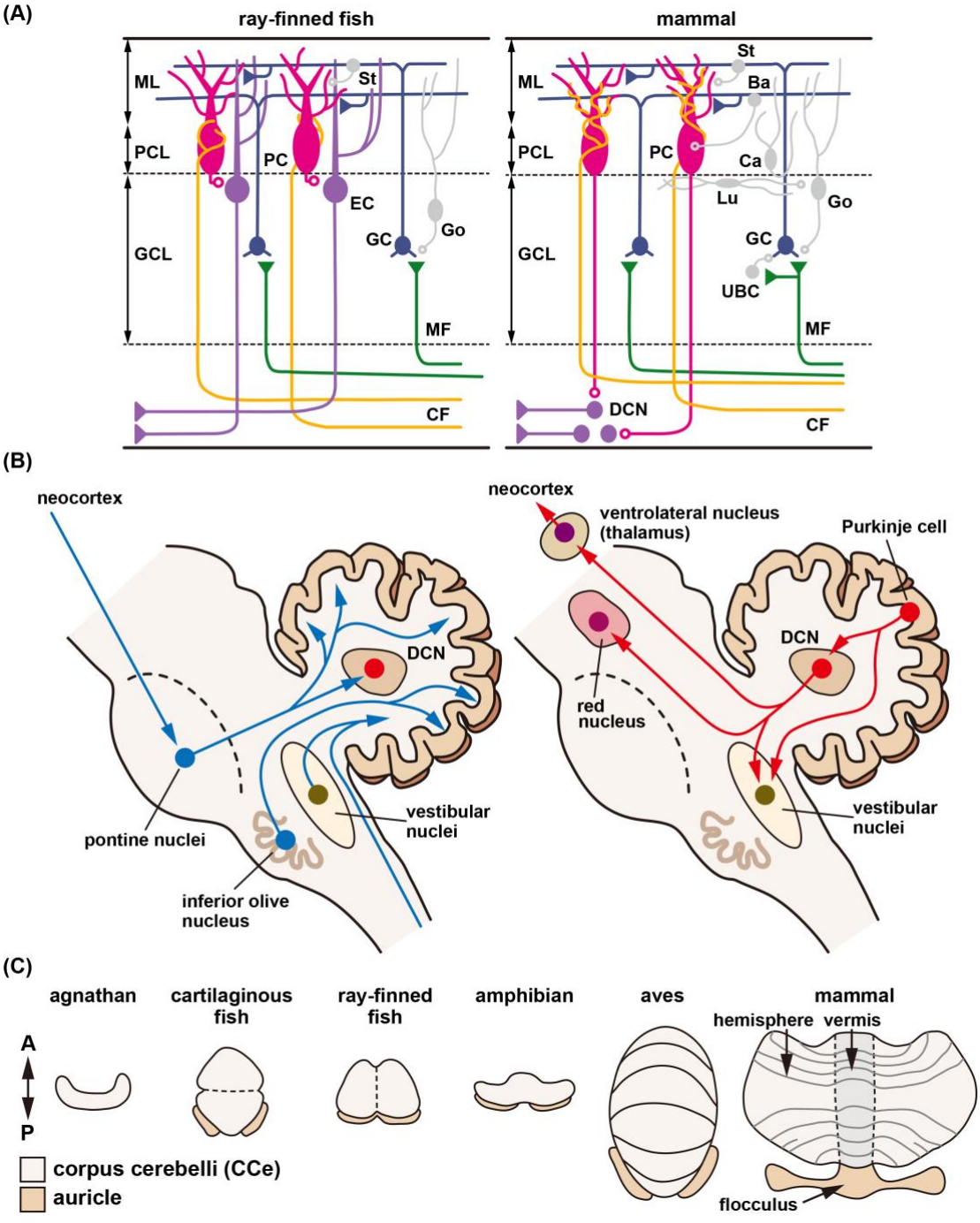

Fig. 1. Structure of the cerebellum and cerebellar neural circuits.

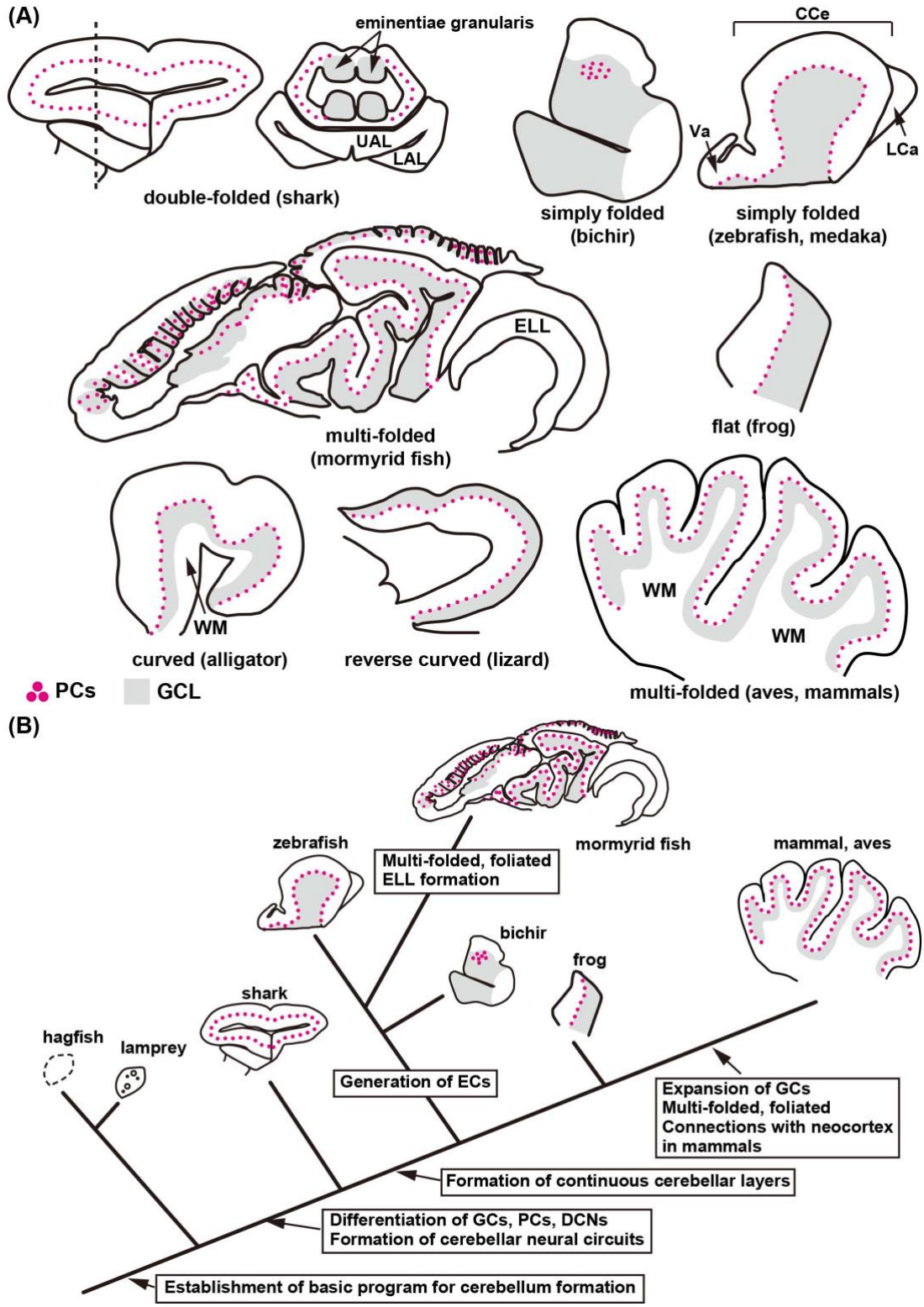

Fig. 2. Diversity of cerebellum structures.

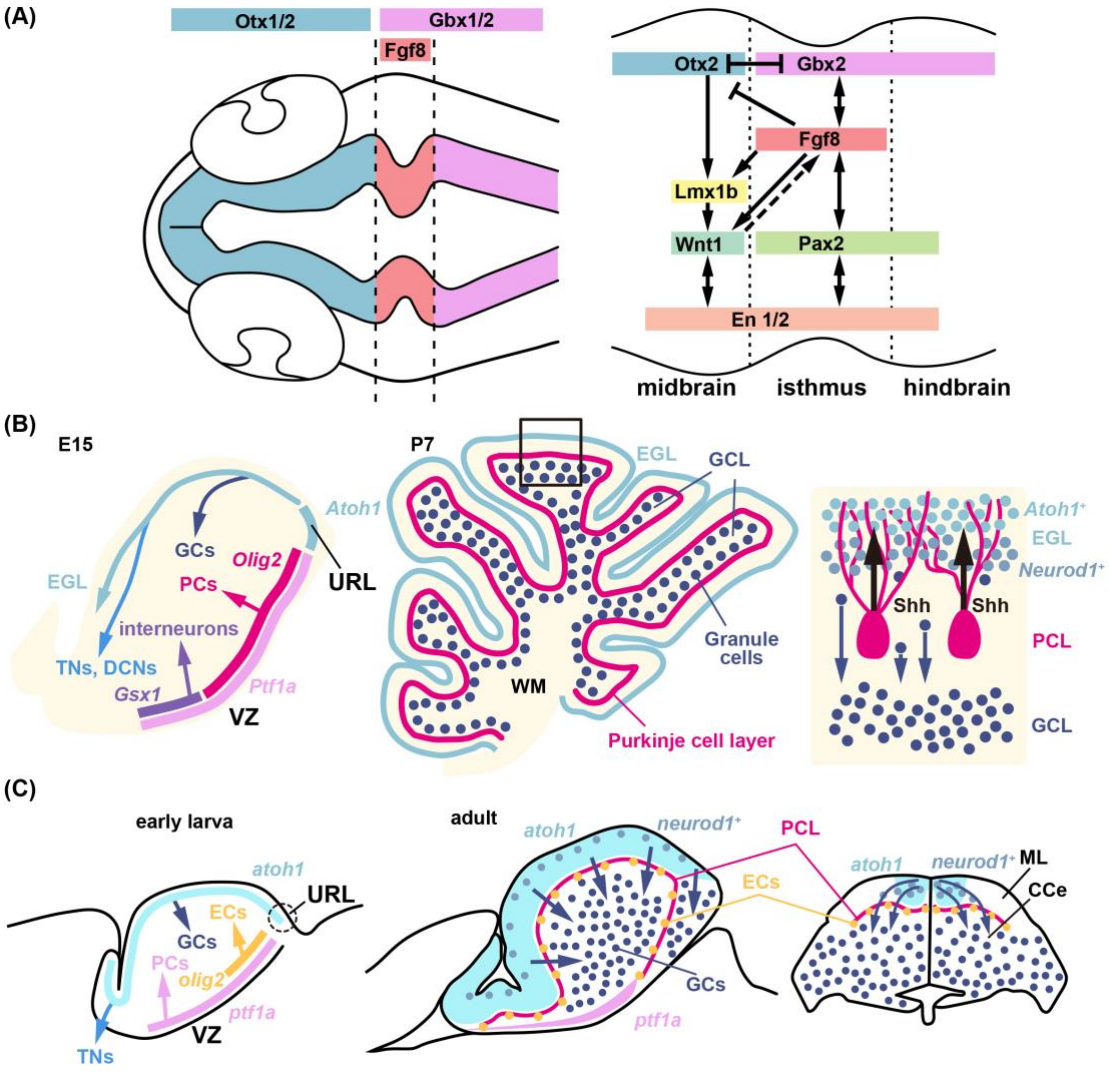

Fig. 3. Cerebellum development.

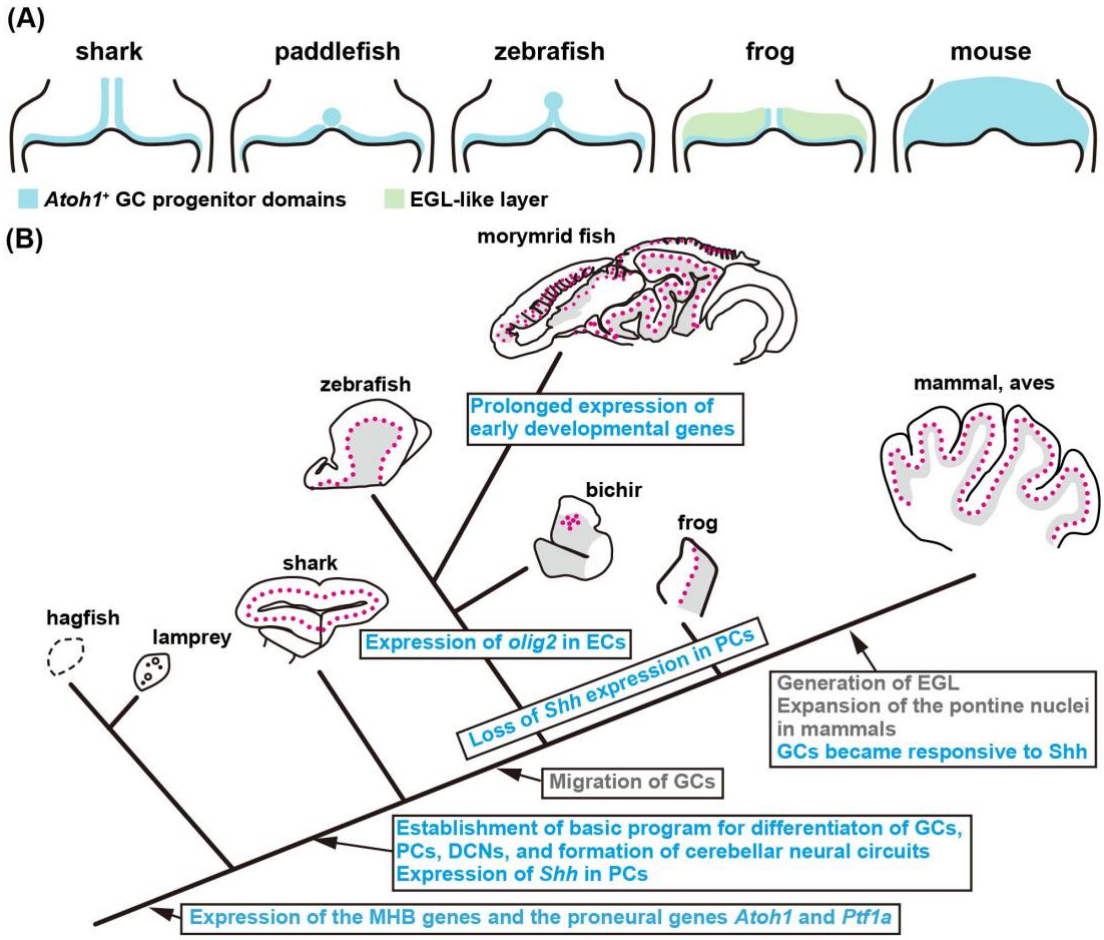

Fig. 4. Mechanisms that generate diversity in the cerebellum.