

The Zebrafish Cerebellum

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1. Introduction

The rapid transparent *ex utero* development in zebrafish allows direct access and precise visualization of all the major events in cerebellar development. The superficial position of the cerebellar primordium and cerebellum further facilitates *in vivo* imaging of cerebellar structures and developmental events at single cell resolution. Furthermore, zebrafish is amenable to high-throughput screening techniques and forward genetics because of its fecundity and easy keeping. Forward genetics screens in zebrafish have resulted in several isolated cerebellar mutants and substantially contributed to the understanding of the genetic networks involved in hindbrain development (Bae et al., 2009, Brand et al., 1996). Recent developments in genetic tools, including the use of site specific recombinases, efficient transgenesis, inducible gene expression systems, and the targeted genome lesioning technologies TALEN and Cas9/CRISPR has opened up new avenues to manipulate and edit the genome of zebrafish (Hans et al., 2009, Scott, 2009, Suster et al., 2009, Goll et al., 2009, Hwang et al., 2013, Sander et al., 2011, Huang et al., 2011, Bedell et al., 2012). These tools enable the use of genome-wide genetic approaches, such as enhancer/exon traps and cell specific temporal control of gene expression in zebrafish. Several seminal papers have used these technologies to successfully elucidate mechanisms involved in the morphogenesis, neurogenesis and cell migration in the cerebellum (Bae et al., 2009, Chaplin et al., Hans et al., 2009, Volkmann et al., Volkmann et al., 2008). In addition, the use of genetically encoded sensors and probes that allows detection and manipulation of neuronal activity using optical methods have open up new means to study the physiology and function of the cerebellum (Simmich et al., 2012, Matsui et al., 2014). Taken together, these features have allowed zebrafish to emerge as a complete model for studies of molecular, cellular and physiological mechanisms involved in cerebellar development and function at both cell and circuit level.

2. The cerebellar anatomy and architecture

The general organization and cellular architecture of the cerebellum is highly conserved in vertebrates. The cerebellum of all jawed vertebrates consists of a major lobe, the corpus cerebelli (cerebellar corpus) and two bilateral lobes, the auricle (also known as the vestibulocerebellum, (Figure 1 A (Altman and Bayer, 1997))). The architecture of cerebellum

is highly similar to other vertebrates but there are some notable differences. The most striking differences are the lack of deep cerebellar nuclei and a well-defined white matter. Furthermore, the zebrafish fish have additional precerebellar and cerebelloid structures. The zebrafish cerebellum can be divided in three major parts, the valvula cerebelli, the corpus cerebelli and the vestibulolateral lobe (Figure 1 B-C, (Finger, 1983, Meek, 1998, Wullimann, 1997)). The eminentia granularis and the caudal lobe together form the vestibulolateral lobe that has been suggested to be homologous to the auricle of other vertebrates (Wullimann, 1997). The cerebellar corpus in zebrafish consists of a single folia and it has an anterior extension, the valvula cerebelli, which extends into the tectal ventricle below the optic tectum. The cerebellar corpus is laterally flanked by the eminentia granularis and posteriorly by the caudal lobe. In addition, zebrafish have extra structures associated to the cerebellum such as cerebelloid structures (cerebellar-like) and additional precerebellar nuclei that are not found in other vertebrates. Zebrafish has two precerebellar nuclei, the nucleus valvula lateralis and the nucleus paracommissuralis (Figure 1 C). The precerebellar nuclei are closely associated with the cerebellum through significant fiber projections. Cerebelloid structures are architecturally thought to be very similar to the cerebellum, although they are spatially well separated from the cerebellum. Cerebelloid structures are found in all vertebrate lineages except reptiles and birds (Bell, 2002, Bell et al., 2008). Two cerebelloid structures are found in zebrafish. In the hindbrain the medial octavolateral nucleus, eminentia granularis and the cerebellar crest (crista cerebellaris) form one cerebelloid structure. The torus longitudinalis together with the optic tectum forms the second cerebelloid structure in zebrafish. Like the cerebellum the cerebelloid structures process sensory signals, receive input from the periphery to the deep layers and parallel fiber input to the molecular layer.

The general vertebrate cerebellum consists of a three-layered cortex and an underlying white matter (Altman and Bayer, 1997). This three layered arrangement is well recognizable in zebrafish (Figure 1 D). However, the eminentia granularis and caudal lobe consists of a single granule cell layer. In contrast, to tetrapods zebrafish lack a well-defined white matter beneath the cerebellum. In teleost fish three distinct subtypes of inhibitory neurons have been found in the cerebellar cortex: stellate, Golgi and Purkinje cells. Stellate and Golgi cells are interneurons and only project within the cerebellum. The stellate cells are scattered in the molecular cell layer of teleost fish, while Golgi cells primarily are found in the granule cell layer (Figure 1 D, (Butler A., 2005, Delgado and Schmachtenberg, 2008, Hans et al., 2009, Meek et al., 2008)). In most vertebrates the Purkinje cell is the sole cell type that projects outside the cerebellum to the deep cerebellar nuclei. The deep cerebellar nuclei are located in the white matter beneath the cerebellum. In zebrafish and other teleosts this is different. Firstly, teleost fish have an additional efferent cell type in the cerebellar cortex, the eurydendroid cell, and the teleost Purkinje cell projects to the eurydenroid cells (Figure 1 D (Nieuwenhuys et al., 1974, Alonso et al., 1992, Bae et al., 2009, Meek, 1992)). Secondly, teleost fish lack deep cerebellar nuclei. However, the eurydendroid cells directly innervate similar targets in the brain stem and spinal cord as the deep cerebellar nuclei of other vertebrates and may thus have an equivalent function (Butler A., 2005, Bae et al., 2009, Ikenaga et al., 2005, Murakami and Morita, 1987). Similar to other vertebrates abundant glutamergic granule cells are found in the granule cell layer of zebrafish (Bae et al., 2009, Kaslin et al., 2009). Other less abundant cerebellar interneuron types such as unipolar brush, Lugaro, and basket cells have not yet been identified in zebrafish.

The vertebrate cerebellum receives afferent input from two principal sources, mossy and climbing fibers (Figure 1 E,(Altman and Bayer, 1997)). In zebrafish the climbing fibers originate in the inferior olive in the caudal hindbrain and predominately terminate on the soma and the proximal dendrites of Purkinje neurons (Figure 1 E (Bae et al., 2009, Folgueira

et al., 2006, Wullimann and Northcutt, 1988, Xue et al., 2008)). Similar to other vertebrates the mossy fiber-like pathway in teleost fish originates from multiple sources such as the spinal cord, reticular formation and tegmentum (Figure 1 E (Finger, 1978, Folgueira et al., 2006, Kani et al., Volkmann et al., Wullimann and Northcutt, 1988, Wullimann and Northcutt, 1989)). Systematic retrograde tracing experiments have not yet been undertaken in zebrafish but in agreement above mentioned studies, mossy fiber-like input from several precerebellar nuclei has been reported in juvenile zebrafish (Bae et al., 2009, Kani et al., Volkmann et al.). The cerebellar output from the eurydendroid cells in zebrafish has recently been mapped by genetic methods (Matsui et al., 2014) and is in agreement with studies from other teleosts (Folgueira et al., 2006, Ikenaga et al., 2002, Ito and Yoshimoto, 1990, Wullimann and Northcutt, 1988). Targets such as the thalamus, pretectal nuclei, tegmental nuclei and motor and premotor centers are innervated by the eurydendroid cells.

3. Cerebellar development and neurogenesis

The initial phase of midbrain and cerebellar development in vertebrates depends on the formation and function of the isthmic organizer which lies at the midbrain-hindbrain boundary (MHB, Figure 2). The MHB organizer formation and maintenance is defined by an intricate cascade of genetic interactions that are marked by complex temporal and spatial patterns of gene expression (Figure 2). Initially the MHB is positioned along the anterior-posterior axis early within the neural plate by the opposing boundary created by mutual repression between the transcription factors *otx2* and *gbx2* (Broccoli et al., 1999, Millet et al., 1999, Rhinn and Brand, 2001, Simeone, 2000). In contrast to other vertebrates, *gbx1* and not *gbx2* positions the MHB in zebrafish (Rhinn et al., 2009, Rhinn et al., 2005). At the end of gastrulation, a complex genetic network with several region-specific transcription factors such as *Pax2/5* and *En1/2*, and the secreted molecules *Wnt1* and *Fgf8* are expressed at the *Otx/Gbx* interface (Rhinn and Brand, 2001). The secreted signals from the isthmic organizer in turn determine the development of the surrounding mid and hindbrain tissue. Studies in different vertebrates show that three parallel signaling pathways, involving *Pax2/pax2.1*, *Wnt1* and *Fgf8*, are activated independently at this interface during early embryonic stages (Figure 2 C (Canning et al., 2007, Lun and Brand, 1998, Raible and Brand, 2004, Reifers et al., 1998)). During later somitogenesis stages the three pathways become mutually dependent (Rhinn and Brand, 2001). The expression of the region-specific transcription factors is later under the control of *Fgf8*, 17, 18 and *Wnt1*, 8b 10, secreted by the isthmic organizer itself (Raible and Brand, 2004, Foucher et al., 2006, Buckles et al., 2004, Lekven et al., 2003 O'Hara, 2005 #488). These factors are involved in regulating cell proliferation and patterning of the cerebellum as well as cell differentiation and maintenance.

The cerebellar neurons and glia originate from two principal germinal zones in the hindbrain, the rhombic lip (RL) and the ventricular zone (VZ (Wingate, 2001)). Excitatory neurons are generated by the RL and the inhibitory neurons are generated from the VZ (Hoshino et al., 2005). The transcription factor *Ptf1a* marks progenitors of inhibitory neurons in the VZ, while the transcription factor *Ato1* labels progenitors for excitatory cells in the RL and subsequent EGL (Ben-Arie et al., 1997, Hoshino et al., 2005). In zebrafish the cerebellar primordium becomes morphologically distinguishable during mid-segmentation stages (Figure 2 B). The upper and lower rhombic lip parts are well recognizable one day after fertilization and *atoh1a-b* expression is detected in the whole RL (Adolf et al., 2004, Kani et al., Kim et al., 1997, Koster and Fraser, 2001), while *ptf1a* expression is confined to the lower part of the RL (Elsen et al., 2008, Kani et al., Volkmann et al., 2008, Zecchin et al., 2004). The generation of cerebellar neurons starts around two days after fertilization in zebrafish. Initiation of layer formation starts at three days after fertilization when the first differentiated Purkinje cells are

detected (Bae et al., 2009, Katsuyama et al., 2007, Volkmann et al., 2008). Granule cell production starts at two days after fertilization when granule cell precursors leave the URL (Adolf et al., 2004, Bae et al., 2009, Chaplin et al., Koster and Fraser, 2001, Toyama et al., 2004, Volkmann et al., 2008). They rapidly migrate in chain-like structures over long distance and start to differentiate (Koster and Fraser, 2006, Volkmann et al., 2008). All the three cortical layers can be distinguished five days after fertilization.

Cerebellar progenitor activity and neurogenesis continue into adulthood in zebrafish (Grandel et al., 2006, Kaslin et al., 2009, Kaslin et al., 2013). The adult cerebellar stem cell niche consists of polarized neuroepithelial-like cells that inhabit the dorsal part of the IVth ventricle (Figure 2 E). Produced cells rapidly migrate in a distinct outside-in fashion into the granule cell layer where they differentiate into granule cells. In the adult cerebellum the granule precursors migrate into the granule cell layer within 3 days (Kaslin et al., 2009). Although several subtypes of inhibitory and excitatory cells are found in the zebrafish cerebellum, mainly granule cells are produced in the adult (Kaslin et al., 2013). In amniotes granule cell precursors migrate from the URL to the cerebellar surface where they transiently form a highly proliferative second germinal zone, the external granule layer (EGL). Sonic hedgehog secreted from Purkinje neurons act as the major mitogenic signal for granule precursors in the external granule layer (Dahmane and Ruiz i Altaba, 1999, Wechsler-Reya and Scott, 1999). In contrast to amniotes, sonic hedgehog signaling and a prominent external granule cell layer is lacking in the developing and adult zebrafish cerebellum (Chaplin et al., Kaslin et al., 2009). The advent of a secondary zone of transient amplification (e.g. the external granule cell layer) seems to be an amniote specific developmental adaptation because shark, teleost fish and frogs lack an obvious external granule cell layer (Chaplin et al., Butts et al., 2014a, Butts et al., 2014b). This suggests that neurogenesis and production of granule cells in zebrafish is more likely to be controlled on the level of the primary progenitors (Kaslin et al., 2013).

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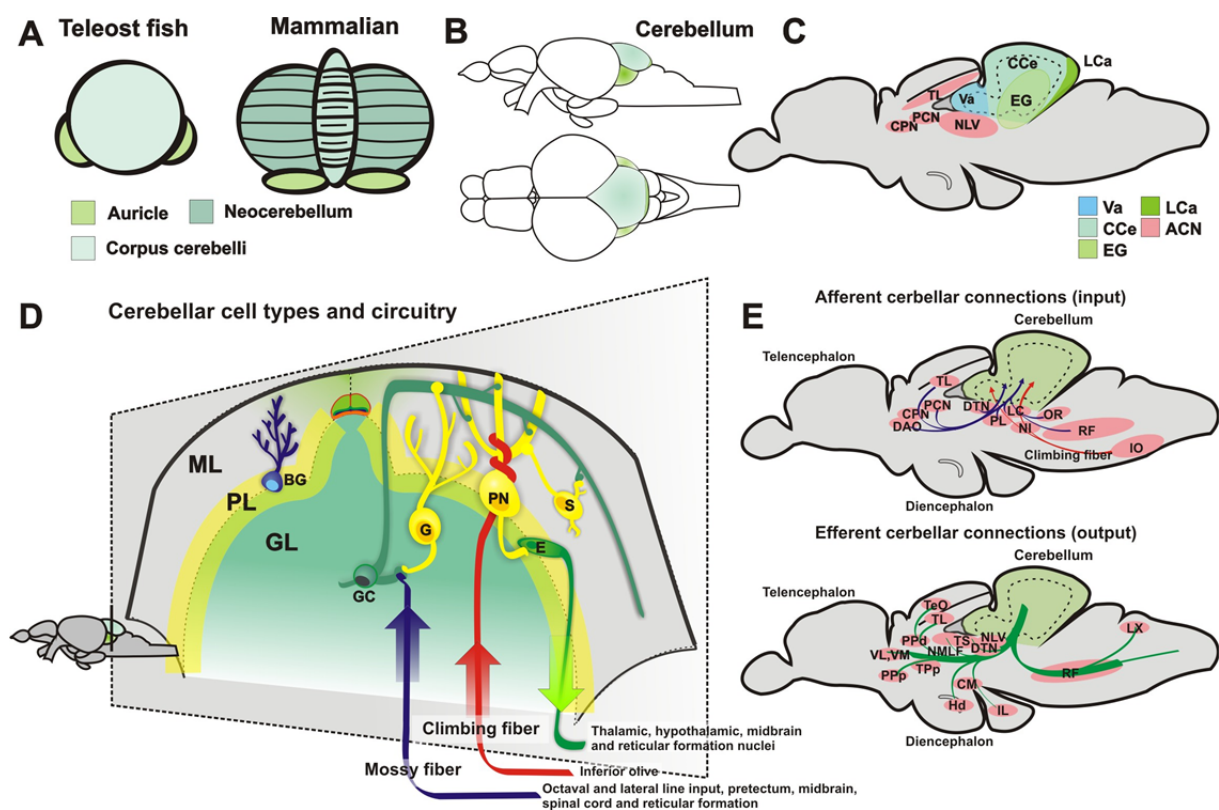


Figure 1. **A.** The vertebrate cerebellum consists of a major lobe, the corpus cerebelli (cerebellar corpus) and two bilateral lobes, the auricle (flocculus in tetrapods). In addition, mammals display a large lateral expansion of the corpus cerebelli, the neocerebellum. **B.** Schematic drawing of the zebrafish brain seen from the side and top. Light green illustrates the cerebellar corpus and the darker green the auricle. **C.** Schematic parasagittal overview of the zebrafish brain showing the major cerebellar parts and associated cerebellar structures. ACN=accessory cerebellar nuclei, CCe=cerebellar corpus, CPN=central pretecal nucleus, EG=eminencia granularis, LCa=caudal lobe of cerebellum, NLV=nucleus lateralis valvulae, PCN=paracommissural nucleus, TL=torus longitudinalis, Va= valvula cerebelli. **D.** The zebrafish cerebellum has a simple laminar three layered architecture consisting of a molecular layer (ML), purkinje cell layer (PL) and a granule cell layer (GL). The granule layer is consisting of small densely packed excitatory granule cells and inhibitory Golgi neurons (G). The Purkinje cell layer is inhabited with Purkinje neurons (PN), a specialized macroglia type, Bergmann glia (BG) and excitatory eurydendroid cells (E). The ML is mainly consisting of

end of gastrulation, the *otx2* and *gbx1* expression domains are sharp and complementary. Grey area= developing axial mesoderm. Light grey=yolk. Black arrows indicate the mutually repressive interactions. **B-C.** Morphogenetic events of the cerebellar primordium. At 24 hpf the midbrain-hindbrain boundary, midbrain structures and the cerebellar primordium are clearly distinguishable in the zebrafish embryo. **C.** At the end of gastrulation, a complex genetic network with several region-specific transcription factors and the secreted molecules Wnt1 and Fgf8 are expressed at the *otx/gbx* interface. The secreted signals from the isthmic organizer in turn determine the development of the surrounding mid and hindbrain tissue. **D.** Summary of the early morphogenetic events and the establishment of the progenitor domains. There is a morphogenetic rotation of the cerebellar primordium (blue arrow). From 36 hpf onwards the dorsomedial part of the IVth ventricle is shifted anteriorly creating a dorsomedial extension of the IVth ventricle (red arrow). Ventricularly located progenitors (orange line) are found in the LRL of the hindbrain and in the VZ of the cerebellum. Cerebellar progenitors adjacent to the roof plate are induced to turn in to granule cell progenitors (URL, green line). **(e).** Schematic summary of tissue growth and displacement of the progenitor niche. Displacement of the URL progenitor niche through tissue growth begins around 7 dpf. During juvenile stages there is vast generation of granule cells and massive expansion of the granule cell layer. URL progenitors (green) are maintained dorsal to the cerebellar recessus in adult zebrafish while ventricular zone derived progenitors and glia (orange) are found ventral to the recessus around the IVth ventricle. **(f)** Schematic summary of the zebrafish cerebellar progenitor niche. Neural progenitors derived from the URL are maintained in the dorsomedial part of the cerebellum around a remnant of the IVth ventricle (the cerebellar recessus). These progenitors give rise to granule neurons in a distinct outside-in fashion. **1.** Polarized neuroepithelial-like progenitors (green) are restricted to the midline of the dorsal cerebellum. The progenitors give rise to rapidly migrating granule precursors (dark green) that initially migrate dorsolaterally. During this initial phase the granule precursors still may proliferate. After reaching the meninge the granule precursors change to a unipolar morphology and start to migrate in ventrolateral direction towards the GL. The granule precursors migrate into the GL and differentiate in to granule neurons. **2.** A few glia with a radial morphology (light blue) are found close to the midline and they are used as scaffolds during the initial dorsal migration of granule precursors. **3.** Bergmann glia-like cells are interspersed in the PL (dark blue). A low amount of Bergmann glia-like cells and inhibitory neurons (yellow) are generated from VZ progenitors that are found lateral and ventral to the progenitor niche. VZ progenitors are also found ventrally around the IVth ventricle (see E). CB=cerebellar primordium, HB=hindbrain, GL=granule cell layer, IVth V= IVth ventricle, LRL=lower rhombic lip, MHB=midbrain–hindbrain boundary, ML=molecular layer, PL=Purkinje cell layer, URL=upper rhombic lip, VZ=ventricular zone