



The long adventurous journey of rhombic lip cells in jawed vertebrates: a comparative developmental analysis

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This review summarizes vertebrate rhombic lip and early cerebellar development covering classic approaches up to modern developmental genetics which identifies the relevant differential gene expression domains and their progeny. Most of this information is derived from amniotes. However, progress in anamniotes, particularly in the zebrafish, has recently been made. The current picture suggests that rhombic lip and cerebellar development in jawed vertebrates (gnathostomes) share many characteristics. Regarding cerebellar development, these include a *ptf1a* expressing ventral cerebellar proliferation (VCP) giving rise to Purkinje cells and other inhibitory cerebellar cell types, and an *atoh1* expressing upper rhombic lip giving rise to an external granular layer (EGL, i.e., excitatory granule cells) and an early ventral migration into the anterior rhombencephalon (cholinergic nuclei). As for the lower rhombic lip (LRL), gnathostome commonalities likely include the formation of precerebellar nuclei (mossy fiber origins) and partially primary auditory nuclei (likely convergently evolved) from the *atoh1* expressing dorsal zone. The fate of the *ptf1a* expressing ventral LRL zone which gives rise to (excitatory cells of) the inferior olive (climbing fiber origin) and (inhibitory cells of) cochlear nuclei in amniotes, has not been determined in anamniotes. Special for the zebrafish in comparison to amniotes is the predominant origin of anamniote excitatory deep cerebellar nuclei homologs (i.e., eurydendroid cells) from *ptf1a* expressing VCP cells, the sequential activity of various *atoh1* paralogs and the incomplete coverage of the subpial cerebellar plate with proliferative EGL cells. Nevertheless, the conclusion that a rhombic lip and its major derivatives evolved with gnathostome vertebrates only and are thus not an ancestral craniate character complex is supported by the absence of a cerebellum (and likely absence of its afferent and efferent nuclei) in jawless fishes

Keywords: *atoh1*, cerebellum, cochlear nuclei, precerebellar systems, *ptf1a*, *wnt1*, zebrafish, rhombic lip

AMNIOTE RHOMBIC LIP AND CEREBELLAR DEVELOPMENT GENERAL

The vertebrate hindbrain (rhombencephalon) consists of an anterior metencephalon (rhombomere 1), including the cerebellum and pons, and a posterior myelencephalon (remaining rhombomeres). The embryonic anterior rhombencephalon has a dorsal rhombic groove (rhombic fossa) because the dorsal part of the neural tube wall, the alar plates, are laterally extended and form a V-shaped medulla oblongata in transverse section (Figures 1A,B). In contrast, the alar plates of the posterior rhombencephalon and the spinal cord approach each other closely in the dorsal midline and are kept together by a thin roof plate, reminiscent of a closed embryonic neural tube (Figure 1C). However, the alar plates of the anterior rhombencephalon are also interconnected by the roof plate which is greatly mediolaterally extended (Figure 1B). This epithelial tela covers the rhombic fossa and will generate (together with mesodermal vascular tissue at its peripheral side) a convoluted chorioideal plexus that is involved in cerebrospinal fluid production (Redzic et al., 2005). Interestingly, the secreted factor Sonic Hedgehog plays a specific signaling role for both the proliferation of the neuroectodermal tela itself – by acting on its germinative

domain at its lateral point of attachment at the alar plate – as well as for the mesodermal vascularization of the developing choroid plexus (Huang et al., 2009; Nielsen and Dymecki, 2010). During embryonic development, the laterally displaced alar plate edges in amniotes constitute the rhombic lip which have long been known to be highly proliferative and to give rise to various brain structures that are finally located remote from their rhombic lip origin. Here, we will first review major steps in the understanding of amniote rhombic lip (including cerebellar) development before discussing related progress in anamniotes, particularly in zebrafish. With regard to amniotes, the organization of the review aims to give a sketch of how the field progressed historically (“Lower rhombic lip,” “Cerebellum/Upper rhombic lip”) before going into modern molecular data in amniotes (“Molecular revolutions”).

LOWER RHOMBIC LIP

Both proliferation in the rhombic lip (*Rautenlippe*) and cellular migration into the pontine and inferior olivary regions are visible in normal histological material and were first described beginning in the late nineteenth century by Wilhelm His followed by various contemporaries (see Nieuwenhuys, 1998 for a review) who

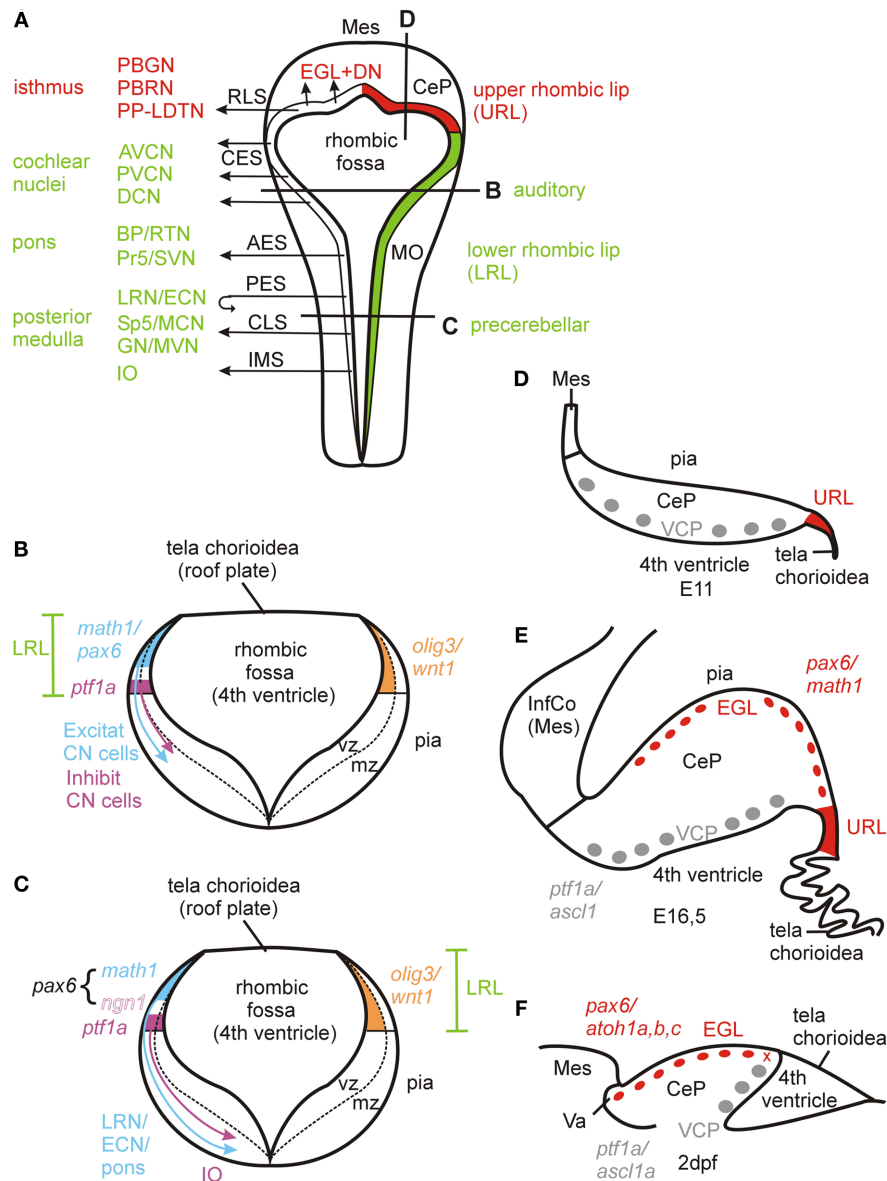


FIGURE 1 | (A–E) Amniote rhombic lip development. **(A)** Dorsal schematic view of embryonic amniote rhombencephalon showing rhombic lip and its derivatives. Many of these facts were first revealed by pre-genetic studies (see text for older citations) and later confirmed and detailed by molecular and genetic approaches. Anterior origin of primary auditory nuclei is indicated for mammals, in birds they originate along the entire lower rhombic lip (see text).

(B–C) Schematic transverse section through lower rhombic lip at two levels showing critical differential gene expression patterns. Compiled from Hoshino et al. (2005), Machold and Fishell (2005), Wang et al. (2005), Landsberg et al. (2005), Farago et al. (2006), Machold et al. (2007), Liu et al. (2008), Rose et al. (2009), Fujiyama et al. (2009), and Storm et al. (2009). Note that the *lmx1a/wnt1* positive generative zone for the choroid plexus in the most dorsal lower rhombic lip (Landsberg et al., 2005; Huang et al., 2009; Nielsen and Dymecki, 2010) is not shown. See text for some additional *math1* dependent medullary nuclei as shown in Rose et al. (2009). **(D–E)** Schematic view of sagittal sections of mouse cerebellum at E11 when EGL has not yet formed (after photo in Goldowitz and Hamre, 1998) and E16.5 when EGL has formed (after photo in Sillitoe and Joyner, 2007). Note that this difference between an anterior ventricular proliferative zone and URL emerges around E10 (DN cells formation) and that

classical descriptions of the earlier upper rhombic lip include both (see text). **(F)** Sagittal section of zebrafish cerebellar plate at 2 dpf (redrawn after Kani et al., 2010). The x symbol indicates the hypothetical site of the ventricular origin of subpial *atoh1* positive cells and, thus, the structure corresponding to the amniote URL (see text). AES, anterior extramural stream; AVCN, anteroventral cochlear nucleus; BP, basal pons; CeP, cerebellar plate; CES, cochlear extramural stream; CLS, caudal rhombic lip stream (of Rose et al., 2009); CN, cochlear nucleus; DCN, dorsal cochlear nucleus; DN, deep cerebellar nuclei; ECN, external cuneate nucleus; EGL, external granular (or germinative) layer; GN, gracile nucleus; IMS, intramural stream; InfCo, inferior colliculus; IO, inferior olive; LRL, lower rhombic lip; LRN, lateral reticular nucleus; MCN, medial cuneate nucleus; Mes, mesencephalon; MO, medulla oblongata; MVN, medial vestibular nucleus; PBGN, parabigeminal nucleus; PBRN, parabrachial nucleus; PES, posterior extramural stream; PP-LDTN, pedunculo-pontine-laterodorsal tegmental nuclei; Pr5, principal sensory trigeminal nucleus; PVCN, posteroventral cochlear nucleus; RLS, rostral rhombic lip stream; RTN, reticulotegmental pontine nucleus; Sp5, spinal trigeminal nucleus; SVN, superior vestibular nucleus; URL, upper rhombic lip; VCP, ventral cerebellar proliferation (ventricular germinal matrix).

reported the situation for the human embryo. Later, Harkmark (1954) reported rhombic lip derived migration into inferior olive and pons in the chicken embryo describing how mitotic activity in the rhombic lip is followed by the appearance of ventrally directed streams of increasingly differentiating cells into inferior olive and pons. He additionally performed lesion experiments which – while not directly demonstrating the fate of lesioned rhombic lip cells – allowed a first hypothesis on the spatiotemporal origin of migrating rhombic lip cells. An important step forward in this field emerged with autoradiography (Taber-Pierce, 1966; Altman and Bayer, 1987a,b,c,d). These studies applied tritiated thymidine to embryonic rodents and used short and long term survival times to infer birth dates, migration routes and final destination of rhombic lip derived cells within the ventral medulla oblongata (**Figure 1**). During embryonic days 13–19, the rhombic lip of the rat brain produces – in the following order – neurons destined for the inferior olive (intramural stream, IMS), the lateral reticular and external cuneate nuclei (posterior extramural stream), the pontine reticulotegmental nucleus and the basal pontine gray (anterior extramural stream). The posterior extramural stream crosses the midline floor plate and, thus, the lateral reticular and external cuneate cells become to lie on the contralateral side. All these structures are called precerebellar, since they provide axonal input to the cerebellum (mossy fibers to granule cells, except for inferior olive: climbing fibers to Purkinje cells). Most of these afferent cerebellar projections occur in a contralateral manner. Even the adult ipsilaterally projecting lateral reticular and external cuneate nuclei have a developmental origin in the contralateral rhombic lip. Horseradish peroxidase injections at various relevant developmental time points confirmed that intramural (submarginal) and extramural (marginal) streams contribute inferior olivary and lateral reticular/external cuneate neurons, respectively (Bourrat and Sotelo, 1988, 1990). The latter studies in the embryonic rat brain also showed how leading neurites pioneer the migration path of lateral reticular/external cuneate and inferior olivary neurons and provided many details of this neuronal maturation. Similar migration events of precerebellar rhombic lip neurons were also described in the mouse by SEM/TEM studies (Ono and Kawamura, 1989, 1990) and in the chick by transfecting rhombic lip cells with enhanced green fluorescent protein (EGFP) expression vectors by electroporation (Ono et al., 2004). Also, the chick–quail transplantation system was used to show inferior olive/pons origins in rhombic lip (Tan and LeDouarin, 1991). Diffusible molecules (such as Netrin1 and Slit1/2 in the floor plate) and their receptors (Netrin1 receptors DCC and Unc5, Slit receptor Robo-2), as well as Ephrins and Eph receptors are involved in neurite guided migration (incl. its termination) of precerebellar neuronal somata in a complex manner. After settlement of these neurons, these factors also regulate topographically ordered axonal pathfinding toward their cerebellar projection targets (Chédotal et al., 1997; Bloch-Gallego et al., 1999; Wingate, 2001; Sotelo, 2004).

All of these studies document rare cases of central nervous system tangential migration of neural cells, which runs perpendicular to conventional radial migration (during the latter, cells move along radial glia fibers from ventricle toward pia). Tangential migrations also exist in other parts of the brain, for example the tangentially moving cell streams from the medial ganglionic eminence into the cortex or the dorsoventral neuronal migration in the spinal

cord. Thus, rhombic lip derived migration involves a massive transgression of longitudinal zones, because these migrating alar plate cells eventually end up in the basal plate (inferior olive, pons, lateral reticular nucleus) or migrate at least through it before ending in the alar plate of the other brain side (external cuneate nucleus).

Much later – probably because of their less spectacular, shorter-distance migration – all three (dorsal, anteroventral, posteroventral) mammalian cochlear nuclei (DCN, AVCN, PVCN) were also described to derive at least partially from the rhombic lip (autoradiography: Taber-Pierce, 1967, HRP-labeling: Willard and Martin, 1986; cerebellar mutant studies: Berrebi et al., 1990, BrDU/DiI labeling: Ivanova and Yuasa, 1998). Finally, genetic studies showed in more detail that the mammalian cochlear nuclei develop from a portion of the rhombic lip that lies anterior to the region of the rhombic lip which gives rise to the extra- and intramural stream of precerebellar nuclei. For example, a sophisticated approach of intersectional and subtractive genetic mapping of *wingless* homolog *wnt1* (longitudinally expressed in the rhombic lip, see below) expressing cells in rhombomeres 3/5 (where the zinc-finger transcription factor gene *Krox20* is selectively expressed; Farago et al., 2006) or specific transgenic fate mapping of *Math5* expressing cells (*Atoh7*; Saul et al., 2008) revealed cochlear derivatives in detail. A rhombic lip origin was also reported for the chick primary auditory nuclei (Golgi stains and DiI labeling; Book and Morest, 1990). Interestingly however, the origin of chick primary auditory nuclei is not restricted to the anterior rhombic lip as in mammals, but extends throughout the medulla oblongata (Marín and Puelles, 1995; Cambronero and Puelles, 2000). This is strong evidence for an independent origin of archosaur and mammalian hearing, which is in line with the fossil record indicating an independent evolution of tympanic ears (Clack, 1997) and with parallel evolution of neuronal mechanisms, for instance for sound localization (Grothe, 2003; Grothe et al., 2004).

In summary, the longitudinally running amniote rhombic lip that flanks the rhombic fossa laterally, the so-called caudal or lower rhombic lip (LRL) gives rise to cochlear nuclei anteriorly in mammals (but all along the rhombic lip in birds) and precerebellar nuclei more posteriorly (**Figure 1**).

CEREBELLUM/UPPER RHOMBIC LIP

At their anterior pole, the longitudinally running rhombic lips fuse by forming a transverse bridge which will give rise to the primordium of the cerebellum (cerebellar plate), the deep cerebellar nuclei (DN), and the proliferative external granular layer (EGL, **Figure 1A**). To discriminate it from the caudal or lower rhombic lip, this anterior rhombic lip bridge has variedly been referred to as the rostral, cerebellar, or upper rhombic lip (URL). During embryonic development (around E10 in the mouse), it separates into two germinative zones: a fastly expanding anterior, ventricularly located ventral cerebellar proliferation (VCP, **Figure 1D**) and the so-called germinal trigone, which is the posterior rim of the initial cerebellar rhombic lip bridge (nicely depicted in Goldowitz and Hamre, 1998). While classical descriptions of the very early rhombic lip implicitly included both germinative zones, a consensus emerged meanwhile to refer only to the posterior proliferative zone (germinal trigone) as the URL and we follow this restricted definition of URL (**Figure 1A**). For our purpose here it is important

to realize that both the VCP in the developing cerebellar plate and the URL are principally ventricularly located proliferative areas, unlike the later emerging and continued proliferative EGL which is subpially located.

In amniote cerebellar development, different classes of neuronal cells were described in classical histology to be generated in subsequent waves (for an excellent review see Sotelo, 2004; for adult cerebellar organization, see: Ito, 2006). In chick cerebellar development, the first wave of postmitotic neuronal cells destined to become the deep cerebellar nuclei (DN) originates at the ventricle of the early cerebellar plate, apparently in the VCP (work of Feirabend, discussed by Dubbeldam, 1998). After migrating somewhat toward the periphery, these future DN cells move laterally down to the base of the cerebellum to their adult location. A later wave of postmitotic neuronal cells leaves the chick VCP and moves toward the periphery to eventually become Purkinje (and probably Golgi) cells. A similar early embryonic origin of DN cells was seen in autoradiographic studies in rodents, followed by Purkinje (and maybe early Golgi) cells, while late Golgi cells and molecular layer cells (basket and stellate cells) originate postnatally (mouse: Miale and Sidman, 1961; Taber-Pierce, 1975; rat: Altman and Bayer, 1985, 1987e) from interstitial proliferative cells in the ventral white matter (Sotelo, 2004).

An even later embryonic wave of amniote cerebellar neuronal cells is generated in the URL (or germinal trigone), the ventricular epithelium posterior to the now already existing cerebellar plate and has been described in normal histological and autoradiographic studies to invade the uttermost periphery of the cerebellar plate in a tangential ventrolateral to dorsomedial (e.g., mouse; Miale and Sidman, 1961) or posterior to rostral (rat: Altman and Bayer, 1987e) direction. Since these neuronal progenitors will postnatally continue to proliferate and migrate ventrally to develop into (internal) cerebellar granule cells (as first seen around 1900 by Santiago Ramon y Cajal in embryonic human brains), this peripheral cell sheet is called *external granular* or *germinal layer* (EGL). Hanaway (1967) noted in the chick that the future EGL cells are produced in the entire mediolateral extent of the ventricular cerebellar plate (presumably the URL) and move radially out into the periphery instead of tangentially invading it. The EGL of the lateral cerebellar plate is formed first, preceding that of the medial one, which possibly explains the earlier observations of an apparent latero-medial tangential invasion. Later, the entire URL was described in amniotes to give rise to cells migrating tangentially (mostly in caudorostral direction) into a subpial EGL position (retrovirally labeled clones: Ryder and Cepko, 1994; chick-quail transplants, Dil labeling: Wingate and Hatten, 1999; Wingate, 2001; Dil: Gilthorpe et al., 2002). URL cells invading the subpial cerebellar plate use clues in the overlying meninges for organized migration and the resulting adult internal granular cell masses maintain the mediolateral topology of their rhombic lip origin (reviewed in Chédotal, 2010). Thus, the founder cells of the amniote subpial EGL arise in the (ventricular) proliferative zone of the URL (posterior part of the cerebellar plate) while the rostrally expanded VCP at the ventricular side of the cerebellar plate at this developmental time point may be considered a different ventricular zone. In contrast to the DN and Purkinje neurons formed by cell waves described above, the EGL cells remain mitotic during postnatal development and eventually

give rise to an immense number of postmitotic neurons which migrate basally (e.g., Fujta, 1967; Alder et al., 1996; Zhang and Goldman, 1996; Komuro and Rakic, 1998a).

The proliferative activity of the EGL is regulated by differentiated Purkinje neurons via the Sonic Hedgehog signaling (SHH)-ligand, which acts on SHH receptors (Patched, Smoothed) and transcription factors (Gli) expressed by EGL cells (Traiffort et al., 1998, 1999; review: Martí and Bovolenta, 2002; Sillitoe and Joyner, 2007; Chédotal, 2010). Thus, this represents a case of transit amplification (Kriegstein and Alvarez-Buylla, 2009). After entering the Vitronectin-rich premigratory zone, EGL cells become postmitotic (reviewed in Sotelo, 2004). During further cerebellar cortex development in amniotes, granule cells migrate ventrally in a radial glia guided fashion and will eventually cross the Purkinje cell layer to generate the densely populated deep adult internal granular layer (IGL; for reviews on this issue, see: Hatten, 1990, 1999; Hatten and Mason, 1990; Rakic, 1990; Hatten and Heintz, 1995; Hatten et al., 1997; Curran and D'Arcangelo, 1998; D'Arcangelo and Curran, 1998; Komuro and Rakic, 1998b; Goldowitz and Hamre, 1998; Voogd and Glickstein, 1998; Sotelo, 2004; Chédotal, 2010).

More recently, using an EGFP-expressing transgenic rat strain as source for transplants into wild type rats, the ventricular VCP was shown to produce all inhibitory cerebellar cell types (GABA-/glycinergic) in the emerging cerebellar plate (Purkinje cells/Golgi and Lugaro cells of internal granule layer/basket and stellate cells of the molecular layer; Leto et al., 2006; recent review on adult cerebellar organization: Ito, 2006), as similarly found in mice (Weisheit et al., 2006). The VCP also generates the inhibitory fraction of deep cerebellar nuclei (DN; see more details below). In complement, the URL gives rise to an early stream of excitatory (glutamatergic) deep cerebellar nuclear cells (mouse: between E10–E12), which invade tangentially the subpial developing cerebellar plate (the so-called nuclear transition zone, a forerunner of the EGL) and then descend ventrolaterally to contribute to the DN shown with *in situ* hybridization (ISH) studies of transcription factor expressing genes *tbr1*, *tbr2*, and *pax6* (Fink et al., 2006). Subsequently, the URL forms the EGL on the peripheral (subpial) side of the cerebellar plate (mouse: between E13 and E16) which will continue to proliferate during postnatal development (mouse: 2 weeks) and it will produce myriads of excitatory (internal) granule and unipolar brush cells (Englund et al., 2006; Leto et al., 2006; review: Sillitoe and Joyner, 2007).

MOLECULAR REVOLUTIONS

NEW TOOLS

At the turn of the millennium, sophisticated genetic studies extended rhombic lip and cerebellar development toward an improved understanding of the underlying genetic processes (mostly mouse, but see Wilson and Wingate, 2006 for chicken). The early active genetic network in the midbrain–hindbrain boundary (MHB) involving transcription factor-encoding genes *otx2* (midbrain), *gbx2* (hindbrain), *pax2/5* (MHB), and *fgf8* and *wnt1* (both encode for proteins that can act as secreted morphogens) eventually leads to the formation of the cerebellar anlage defined by the expression of the homeodomain encoding genes *engrailed 1/2* (Acampora et al., 2001; Liu and Joyner, 2001; Wang and Zoghbi, 2001; Wurst and Bally-Cuif, 2001; Sgaier et al., 2005). Genes with later continued expression in

rhombic lip/cerebellar plate derivatives may serve as “natural” markers to trace their fate (such as *tbr1* for glutamatergic DN cells; see above). Most importantly, site specific recombinase systems (Cre-*loxP* and Flp-*FTR*) were introduced (Branda and Dymecki, 2004) to demonstrate the fate of cells expressing developmentally relevant genes expressed in the early cerebellar plate and rhombic lip after embryonic downregulation of this informative gene expression. Also, detailed comparisons of gene expression and phenotype in wild type and mutant organisms helped to decipher developmental mechanisms. An example is the paired-box/homeobox gene *pax6*, whose diverse roles in eye, nose, and cortex development are complemented by critical functions in rhombic lip and cerebellar development (Engelkamp et al., 1999). In the mouse, *pax6* is strongly expressed between 12.5 and 13.5 days in the LRL and URL plus the latter’s derivative, the EGL, but not in the remaining cells of the cerebellar plate. The *pax6* expressing LRL progeny can be followed in anterior (AES) and posterior extramural streams (PES) toward their destination, i.e., reticulotegmental and basal pontine nuclei, lateral reticular/external cuneate nuclei, respectively, but not in the intramural stream (IMS) and its destination, the inferior olive (Figure 1A). In the *pax6* homozygous loss of function mutant *small eye (sey)*, the formation of basal pons (but not reticulotegmental nucleus and inferior olive) and LNR/ECN is impaired. Since in the *sey* mutant, proliferation in the LRL is not affected (pre-migratory cells actually accumulate there), and the Netrin1 receptor DCC fails to be expressed in AES cells (in those few that migrate to form the reticulotegmental nucleus), *pax6* may be associated with a function in migration, not proliferation. Proliferation is also not affected in the EGL of *sey* mutants: postmitotic cells accumulate in the pre-migratory zone of the cerebellar plate, but fail to migrate. In cells destined for the reticulotegmental pontine nucleus, *pax6* function must be redundantly taken over by other gene(s).

GENES INVOLVED IN INHIBITORY VS EXCITATORY NEURON DEVELOPMENT

The basic Helix-Loop-Helix (bHLH)-factor-encoding gene *ptf1a* is expressed in the ventricular ventral cerebellar proliferation (VCP) and adult animals of its corresponding mouse mutant *cerebellless* have only a rudimentary cerebellum and show associated motor deficits (Hoshino et al., 2005; Hoshino, 2006). The adult cerebellar rudiment contains large glutamatergic (DN type) cells, but no GABAergic cells. However, during embryonic cerebellar plate development, the EGL does initially develop and glutamatergic markers (Calretinin) are expressed in its descendants (while GABAergic markers such as Calbindin remain absent). The EGL eventually disappears gradually during postnatal stages. Further, lineage tracing involving a Cre-recombinase-*lacZ* strain allowing for the visualization of *ptf1a* expressing cells and their progeny revealed that these cells only develop into cerebellar GABAergic and glial cells, but not glutamatergic cells (Hoshino et al., 2005). Thus, the primary effect in *cerebellless* mutants is that no GABAergic cells are formed from the ventricular cerebellar proliferation (VCP), while EGL formation and glutamatergic cell production from it is initially unaffected (large DN cells, see above). Later, presumably because of the missing mitogenic SHH signal from Purkinje cells (see above), the EGL disappears and granule cells and a functional cerebellum are not formed as a secondary consequence.

Interestingly, adult *cerebellless* mutants also lack (glutamatergic) inferior olive and pontine nuclei, which – although embryonically formed – disappear postnatally. This likely occurs as a secondary consequence because these precerebellar nuclei lack a cerebellar innervation target (see above). In the hindbrain, *ptf1a* is also expressed in the ventral part of the LRL (apparently not affected in *cerebellless*) and cells descending from this area develop into all types of inhibitory (GABAergic, glycinergic) cells of the cochlear nuclei (Fujiyama et al., 2009), while glutamatergic cochlear nuclear cell types develop from the dorsal part of the LRL where another bHLH factor, the *atonal* homolog *atoh1* or *math1* – and also *pax6* – are expressed. Surprisingly, inferior olivary neurons (although glutamatergic) develop mostly from the *ptf1a* expressing cells in the posterior part of the ventral LRL (see below; Figures 1B,C).

In-depth analyses of mouse LRL revealed that *wnt1* is expressed throughout the entire dorsoventral extent of the developing LRL, while *math1* and the bHLH gene *neurogenin1 (ngn1)* are restricted to its dorsal and a ventrally adjacent part, respectively (Landsberg et al., 2005; see Figure 1C). Importantly, *wnt1* is expressed in a decreasing dorsoventral gradient in the LRL, likely extending ventrally beyond the *ngn1* domain (Landsberg et al., 2005; Ray and Dymecki, 2009). Using Cre and Flp based fate mapping studies, Rodriguez and Dymecki (2000) and Landsberg et al. (2005) showed that mossy fiber-type precerebellar nuclei (BP, RTN, LRN, ECN) derive from the strongly *wnt1* and *math1* expressing dorsal LRL domain, while the climbing fiber system (IO) derives from weakly *wnt1* and strongly *ngn1* expressing ventral LRL domain. This is consistent with the reported *ptf1a* expression domain origin of IO (Hoshino et al., 2005; Yamada et al., 2007), although the *ptf1a* domain may be even more ventral than that of *ngn1* in the posterodorsal LRL (Ray and Dymecki, 2009), and thus, the IO cells might be molecularly parcellated (Landsberg et al., 2005; Ray and Dymecki, 2009). In any case, the anteroventral LRL has a *ptf1a* domain, but lacks *ngn1* expression – and gives rise to inhibitory cochlear nuclei cells (Fujiyama et al., 2009; see above). In *pax6* mutant *sey* mice, a dorsal expansion of *ngn1* expression and concurrent regression of *math1* expression is seen, accompanied by the disruption of extramural streams (mossy fiber cells) and an increase of migrating cells in the intramural stream (IMS) to the IO (Landsberg et al., 2005), consistent with an earlier report on the *pax6* mutant *sey* phenotype (Engelkamp et al., 1999). This indicates that PAX6 acts in rhombic lip development and rhombic lip derived migration by limiting *ngn1* expression.

The bHLH gene *olig3* has a similar graded expression in the LRL as *wnt1* does (Figures 1B,C covering dorsoventrally *math1*, *ngn1*, and *ptf1* domains, with the gradient tapering out more slowly ventrally than that of *wnt1* (Liu et al., 2008; Ray and Dymecki, 2009; Storm et al., 2009). Accordingly, *olig3* has a role in the development of both mossy and climbing fiber precerebellar nuclei as shown both by histochemical marker analysis of *olig3* null mutant mice (Liu et al., 2008) and Cre based genetic fate mapping (Storm et al., 2009). As pointed out by Ray and Dymecki (2009), *olig3* may represent the best and most inclusive LRL marker.

Further major insights came from fate mapping of cells depending on the bHLH factor MATH1, its encoding gene has been key to investigating rhombic lip and cerebellar development because of its exclusive expression in URL (and later EGL) and dorsal LRL (apart

from anterior dorsal spinal cord, peripheral inner ear hair cells, and epidermal Merkel cells; Akazawa et al., 1995; Ben-Arie et al., 1997, 2000; Helms et al., 2000; Machold and Fishell, 2005; Wang et al., 2005). Using a *math1*-expression dependent Cre-recombinase strain in combination with a Cre-recombination mediated *lacZ* reporter line, the above discussed origin of cochlear nuclei (excitatory cells) and of mossy fiber precerebellar systems from the strongly *wnt1* expressing dorsal LRL (Landsberg et al., 2005), as well as the URL origin of the (excitatory) deep cerebellar nuclear cells and EGL of the cerebellum, was independently confirmed by determining the fate of *math1* expressing progeny (Machold and Fishell, 2005; Wang et al., 2005). These studies also demonstrated that IO formation is independent of *math1* expression. Beyond that, previously unknown *math1* expressing rhombic lip derivatives were revealed in three (auditory related) lateral lemniscal nuclei, in the superior vestibular nucleus, in the parabrachial nuclear and in an isthmic complex (the latter two related to arousal; see more below on isthmic complex). A later analysis showed that in addition excitatory neurons (mostly glutamatergic, never GABA-/glycinergic) of the (auditory) superior olive, spinal, and medial vestibular nuclei, as well as primary somatosensory nuclei (principal and spinal trigeminal, medial cuneate, and gracile nuclei) and some breathing (Kölliker-Fuse nucleus, pre-Bötzing complex, rostral ventral respiratory group) and bladder-control related (Barrington's nucleus) medullary nuclei derive from *math1* expressing LRL (Rose et al., 2009). Although the vestibular nuclei also provide mossy fiber input to the cerebellum, they are not treated usually as precerebellar nuclei like the external cuneate and lateral reticular nucleus. The temporal succession of all subpopulations in each major migratory stream was also resolved, for example in the newly described rostral rhombic lip migratory stream (RLS) which forms the early subpial nuclear transition zone (E11,5) from which the isthmic and DN cells derive first before it subsequently gives rise to the EGL (E13,5) and internal granule cells. Further, somatosensory spinal cord interneurons, which provide also mossy fiber input to cerebellar granule cells (as the precerebellar systems do), derive from the spinal continuation of the rhombic lip *math1* expression domain.

An even finer temporal and anatomical analysis of the MATH1 dependent isthmic complex was obtained by using an inducible Cre-recombinase under the control of *math1* regulatory elements (Machold and Fishell, 2005). This study showed that the isthmic complex consists of the parabigeminal nucleus and the cholinergic pedunculopontine and laterodorsal tegmental nuclei, and further detailed the already described *math1* expressing progeny. Although not reported in that study, the mammalian parabigeminal and parabrachial nuclei also contain cholinergic cells (see discussion in Volkman et al., 2010).

A normal expression study of *wnt1* and a cell fate mapping study of transgenically labeled *wnt1* expressing progeny in the mouse hindbrain (and midbrain) confirmed an LRL origin of cochlear nuclei and mossy fiber precerebellar systems, but not of the inferior olive (Nichols and Bruce, 2006). As discussed above, the IO was found to originate from the only weakly *wnt1* expressing ventral LRL (Landsberg et al., 2005). In addition, although *wnt1* expression was previously not noted prior to mouse E11,5 in URL/EGL cells (Echelard et al., 1994), Nichols and Bruce (2006) reported beginning of *wnt1* expression in URL cells from E10.5 onward and confirmed with *wnt1* expression mediated transgenic labeling the

origin of the EGL (i.e., granule cells, deep cerebellar nuclei) from the URL. Interestingly, they described an anterior cellular migration (corresponding to the RLS of *math1* expressing progeny, see above) via URL and EGL into the basal anterior rhombencephalon to form there at least part of the parabrachial and laterodorsal tegmental nuclei, indicating that the cholinergic *math1* dependent groups (Machold and Fishell, 2005; see above) are also derivatives from *wnt1* expressing precursors.

Beyond MATH1, the role of additional proneural bHLH factors has only begun to be unraveled. An ISH analysis of mouse bHLH genes *ascl1* and *neurogenin 1/2* noted their expression in the developing VCP or close to it, but an absence of expression of those genes in the URL. Thus, there is no overlap of the expression of these three genes with cerebellar *math1* domains between E10 and E13,5 and no co-expression between *ngn1* and *ngn2*, but partial overlap of expression between *ngn1/2* with *ascl1* and *ptf1a* domains (Machold et al., 2007; Zordan et al., 2008). Thus, these bHLH factor-encoding genes were interpreted to be related to the development of different inhibitory cell types. Indeed, *ascl1* was recently shown to be co-expressed in VCP cells with *ptf1a*, with ASCL1 involved in Purkinje and inhibitory DN cell development (Kim et al., 2008). In contrast, *ptf1a/ngn1* co-expressing cells develop into Purkinje, Golgi and Lugaro cells (Lundell et al., 2009). In addition, *ngn2* expression was shown to be active downstream of *ptf1a*, but not of *ascl1*. The proneural factor coding gene *neuroD* which is well established to act downstream of Neurogenins in the forebrain (reviewed in Osório et al., 2010) is required in the developing cerebellum for granule cell differentiation as shown in a comparison of wild type mice with a non-functional NeuroD mutant line (Miyata et al., 1999).

RHOMBIC LIP DEFINITIONS

Eventually, the suggestion arose to re-define and equal the rhombic lip with the extent of the *math1* expression domain bordering the rhombic fossa, including its derivatives (Wang et al., 2005). Although convenient at first sight, such a definition hinders the understanding of this complex structure at the functional systems level. The *ptf1a/ngn1* expressing ventral part of the dorsal rim of the rhombic fossa and the dorsally adjacent *math1* domain have in common that they both are the source of long distance tangential cellular migrations leading to the formation of inferior olive/inhibitory cells of cochlear nuclei and mossy fiber precerebellar nuclei/excitatory cells of cochlear nuclei, respectively. Functionally not directly cerebellar/auditory-related systems, such as the locus coeruleus, do not arise from this ventral rhombic lip territory. Instead they originate more ventrally as shown with fate mapping studies using grafting (quail-chick) and mouse brain gene expression analysis visualizing noradrenergic cell markers (dopamine- β -hydroxylase and transcription factors *Phox2a/b*; Aroca et al., 2006). This more ventral origin was confirmed in a study combining *wnt1* expression mediated transgenic labeling with tyrosine hydroxylase immunostaining (for catecholaminergic cells) where no cells in the mouse locus coeruleus were double-labeled (Nichols and Bruce, 2006). In contrast, Lin et al., 2001 claimed that the chick locus coeruleus originates from the EGL. Be that as it may, all amniote LRL derivatives share the functional context of being closely involved in cerebellar or auditory circuitry. We therefore use dorsal and ventral rhombic lip (dLRL/vLRL), respectively, for the *math1* and *ptf1a/ngn1*

expressing portions of the LRL. The URL/EGL and the ventricular VCP are comparable to at least the anterior dorsal and ventral LRL, respectively, regarding inhibitory/excitatory cell production, with the additional contribution of the URL to the cholinergic isthmic groups just discussed. However, as explained above, the early separable VCP is terminologically treated as different from the URL (germinal trigone), as the VCP does not show the long distance migratory behavior of its descendant cells as the vLRL does.

ANAMNIOTE PATTERNS

STARTING POINTS

We will now discuss existing evidence and provide new insights into similar and dissimilar patterns of rhombic lip and cerebellar development in anamniotes, particularly in the zebrafish. A natural starting point is to establish whether adult structures known to develop from rhombic lip and ventral cerebellar proliferation (VCP) in amniotes are present in zebrafish. A cerebellum with a three-layered cortex, including the major inhibitory and excitatory (glutamatergic) cell types, is present in the zebrafish (Miyamura and Nakayasu, 2001; Mikami et al., 2004; Bae et al., 2009) as in all gnathostome vertebrates (but absent in lampreys and myxinooids, Wullimann and Vernier, 2007). Deep cerebellar nuclei in teleosts are represented by eurydendroid cells, which are large excitatory cells in the Purkinje cell layer (Lannoo et al., 1991; McFarland et al., 2008; Bae et al., 2009). Thus, teleostean eurydendroid cells apparently fail to migrate into the deep of the cerebellum. They receive (inhibitory) Purkinje cell input and form the axonal output of the cerebellum (for example in the goldfish; Ikenaga et al., 2005). In the zebrafish, cholinergic isthmic nuclei exist (i.e., nucleus isthmi, secondary gustatory, and superior reticular nucleus, referred to below as the teleostean cholinergic isthmic complex; Clemente et al., 2004; Mueller et al., 2004), which are homologous to parabigeminal, parabrachial, and pedunculopontine-laterodorsal tegmental nuclei. For lack of connectional information in the zebrafish, the closely related goldfish or carp may be mentioned for the presence of an inferior olive which represents the sole cerebellar climbing fiber origin (Xu et al., 2008) and of mossy fiber-type precerebellar nuclei (lateral reticular nucleus; lateral cuneate nucleus; Wullimann and Northcutt, 1988) as well as of hearing related primary sensory nuclei (McCormick and Braford, 1994; McCormick and Hernandez, 1996). In contrast, a pons does not exist in anamniotes, including teleosts. However, do the other mentioned structures share a rhombic lip/VCP origin in teleosts? An additional immediately interesting question is whether the primary central lateral line nucleus (medial octavolateralis nucleus, MON; New et al., 1996) is a rhombic lip derivative with excitatory cells derived from *atoh1* expressing progenitors similar to the amniote cochlear nuclei. All anamniotes – except for strictly terrestrial amphibians – have this additional sensory organ and the related central processing apparatus which is entirely lost in amniotes. However, LRL derived structures have not been revealed by genetic fate mapping in teleosts so far.

EMBRYONIC AND LARVAL GENE ACTIVITY IN ZEBRAFISH RHOMBIC LIP AND CEREBELLAR PLATE

As in amniotes, the dorsal edges of the zebrafish alar plate rhombencephalon are laterally displaced upon formation of the fourth ventricle and define in transverse view a V-shaped rhombic fossa only

covered by the roof plate (see Distel et al., 2006; their Movie 4). Furthermore, the early cerebellar plate develops between 24 and 48 h in the most anterior dorsal rhombencephalon (rhombomere 1) as bilateral, elevated flaps which eventually will cover the ventricle (Figures 2A,A'). However, the anatomical resolution does not allow identifying a structure comparable to the amniote germinal trigone/URL at the posterior margin as different from the proper cerebellar plate (Mueller and Wullimann, 2005). How about diagnostic gene expression patterns? Because of an additional whole genome duplication in early ray-finned fish (actinopterygians), modern teleosts such as the zebrafish have three paralogous *atoh1* (*math1* homolog) genes, namely *atoh1a* (*atoh1.1*), *atoh1b* (*atoh1.2*), and *atoh1c* (Kim et al., 1997; Jászai et al., 2003; Adolf et al., 2004; Wang et al., 2009; Kani et al., 2010). The *atoh1a* and *atoh1b* (not shown) gene expression patterns (Figures 2A',B' and 3B) are very similar from 1 to 2.5 days: expression domains are seen in the fused dorsal neural tube of the anterior spinal cord and most posterior hindbrain, then outline in the more rostral hindbrain the shape of the laterally displaced medullary edges (lower rhombic lip) and form in the most anterior hindbrain, where the cerebellar plate develops, a transverse bridge of expression (Köster and Fraser, 2001a), defining a URL by molecular means (see Discussion below). In addition, a strong expression is seen in the developing valvula cerebelli, an anterior part of the midline cerebellum only present in ray-finned fish (Figure 3B). After 2.5 days, *atoh1a* and *atoh1b* expression are fastly downregulated in LRL and URL, with *atoh1b* expression persisting longer in the URL, and both gene expressions are upheld in the valvula (Figure 3C). In contrast *atoh1c* expression is never seen in the LRL, but starts late (at around 2 days) to be strongly expressed in the valvula and URL (Kani et al., 2010), where it remains active in the larva beyond 9 days (Figures 2C–F and 3D).

As in amniotes, zebrafish *wnt1* expression is not restricted to the URL and LRL, but it is expressed between 16 h postfertilization (hpf) and 2 days beyond the dorsal rhombencephalon in the most dorsal mesencephalon and in the MHB, with an initial gap in the region of the cerebellar plate (Elsen et al., 2008). However, around 30 hpf, *wnt1* expression also is seen in the cerebellar plate (Lekven et al., 2003; McFarland et al., 2008).

Expression of the zebrafish *ptfla* gene starts around 32 hpf and is seen at least up to 5 days, in dorsal whole mount views in locations superficially similar to the URL and LRL expression domains of the *atoh1a* gene (Kani et al., 2010). However, in sagittal sections, it becomes clear that *ptfla* is restricted to the ventricular cerebellar proliferation (VCP) and extends later into the valvula, while *atoh1* gene expression is in a non-overlapping superficial/subpial position (Kani et al., 2010), already indicating that many *atoh1* expressing cells are in a comparable position of an amniote EGL (Figure 1F). The expression of *ptfla* in the LRL is ventral to that of *atoh1a* (A. Babaryka, R. W. Köster, personal observation).

As discussed above, the first mouse *math1* and *wnt1* dependent URL cells that invade the cerebellar plate subpially will migrate via the RLS and form the cholinergic isthmic groups (PBGN/PBRN/PP-LDTN) and deep cerebellar nuclei (DN; see Figure 1A); this is followed by the establishment of the EGL/inner granule cells (Machold and Fishell, 2005; Wang et al., 2005; Nichols and Bruce, 2006). Confocal time-lapse imaging using embryos of a zebrafish transgenic *wnt1*-Gal4-GFP strain revealed that an early migration

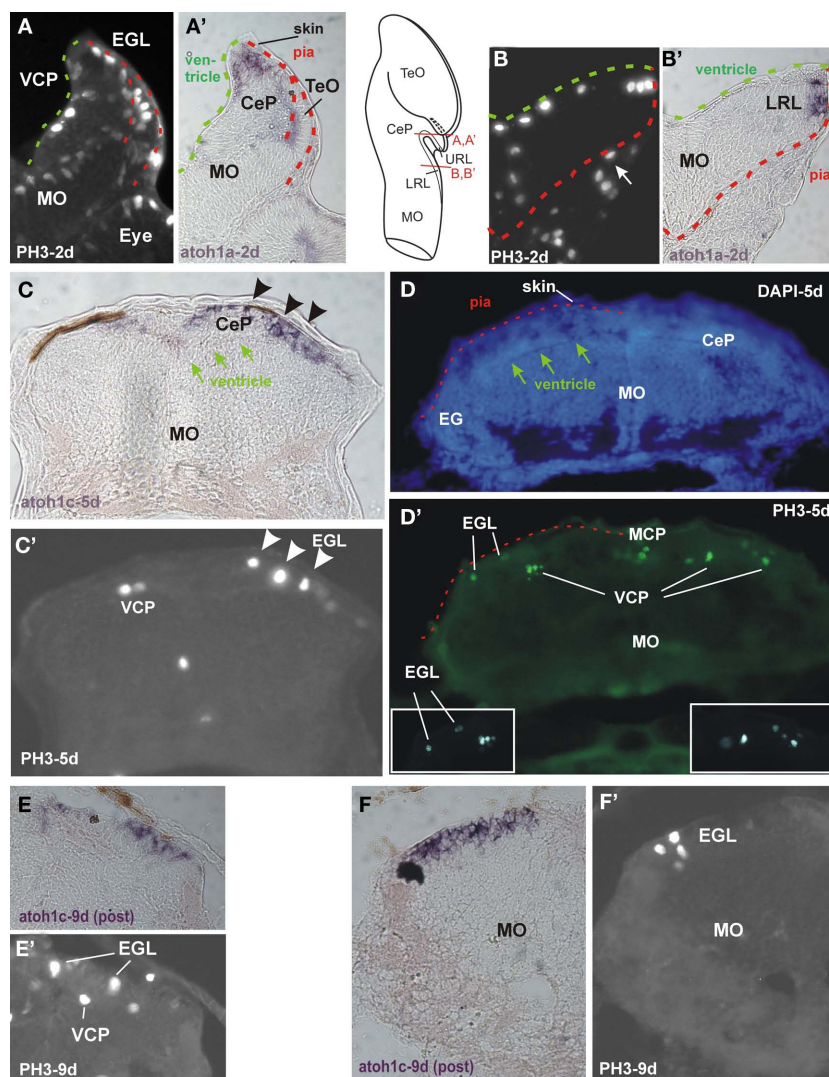
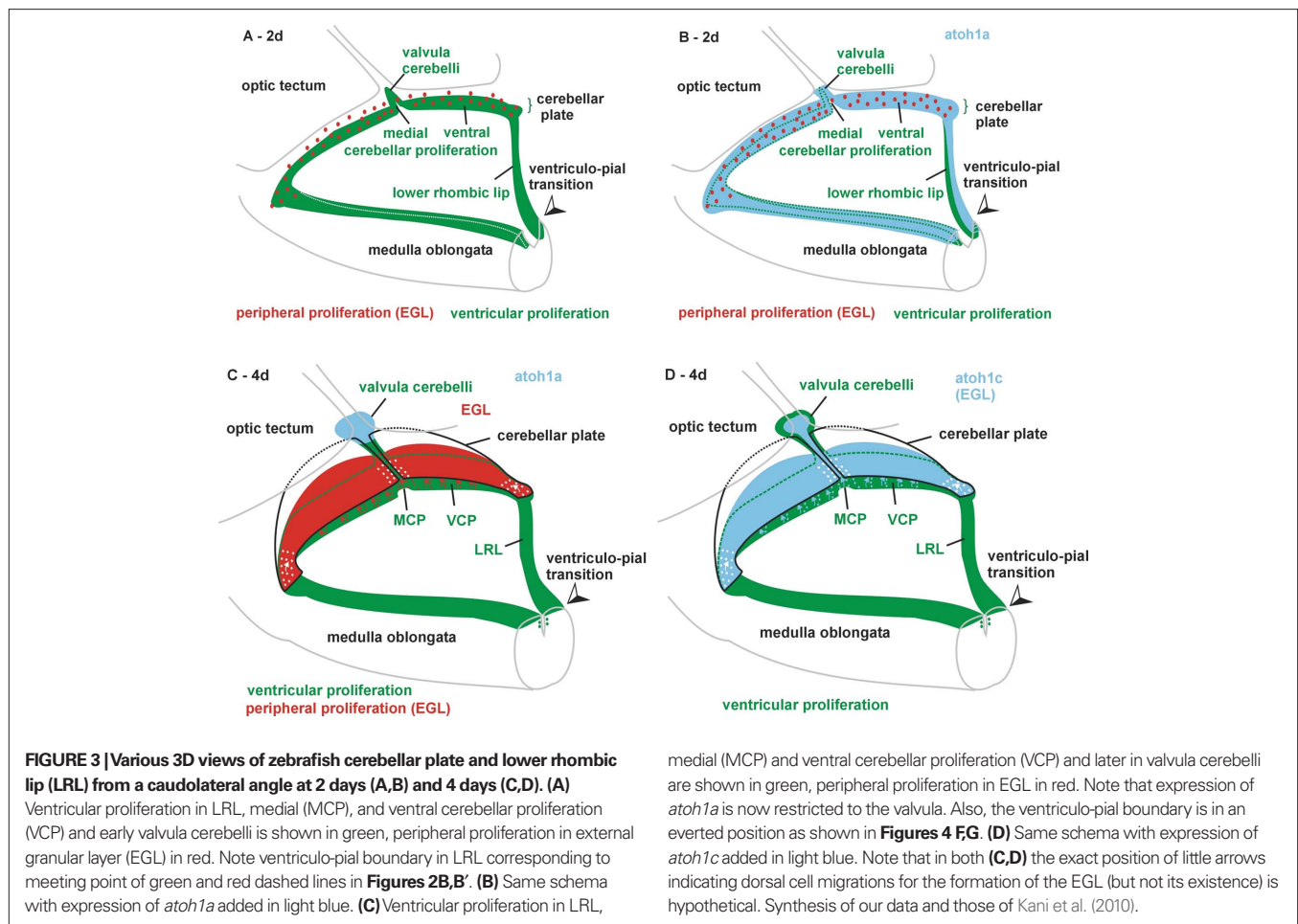


FIGURE 2 | Proliferation shown with phosphohistone 3-immunohistochemistry (PH3-IHC) and expression of bHLH genes *atoh1a* and *atoh1c* in zebrafish cerebellar plate (CeP; A,A', C,C', D,D', E,E', F,F') and lower rhombic lip (LRL; B,B') transverse sections. (A) PH3-IHC in CeP at 2 days. Note that proliferation in CeP is both on the ventricular (green dashed line) and pial (red dashed line) side. **(A')** Identical section shows *atoh1a* ISH and overlap with both proliferative zones. **(B)** PH3-IHC in LRL at 2 days. Note that proliferation is only on ventricular side (green dashed line), not on pial side (red dashed line). More lateral PH3-positive cells are in connective tissue outside of brain (arrow). **(B')** Identical section shows *atoh1a* ISH to overlap with PH3 cells at ventricle, and to extend more peripherally. **(C)** Expression of *atoh1c* at 5 days is mostly peripheral (note arrows indicating ventricle) forming an EGL (arrowheads) and colocalizes with PH3 positive cells (shown in identical section in **(C')**; arrowheads; left side

shows PH3 positive cells in *atoh1c* negative ventral cerebellar proliferation). **(D)** DAPI stain and **(D')** PH3-IHC stain (identical section) demonstrate that proliferative cells are present in ventricular cerebellar proliferation (VCP) and in external granular layer (EGL). Insets show PH3 positive cells in EGL and VCP at a different focus. Dashed red line indicates pial surface of brain with skin above it. Arrows denote ventricle. **(E,E')** and **(F,F')** demonstrate that an EGL persists into later larval development, at least 9 days. Note in particular in **(F')** how *atoh1c* expression is at the periphery of the cerebellar plate, that proliferative PH3 cells are restricted to most peripheral EGL and that proliferation is absent in the rest of the hindbrain at that stage. Methodological details will be provided elsewhere. CeP, cerebellar plate; EGL, external granular (or germinative) layer; IO, inferior olive; LRL, lower rhombic lip; MO, medulla oblongata; TeO, tectum opticum; VCP, ventral (or ventricular) cerebellar proliferation (ventricular germinal matrix).

between 24 and 48 hpf (Volkman et al., 2010) precedes granule cell migration from the EGL, which only starts around 48 hpf (Volkman et al., 2008). In particular, zygotes of this transgenic *wnt1*-reporter strain were injected with an UAS:mCherry construct to allow for precise visualization of this early migration. These *wnt1*-expressing cells co-express *atoh1a* and were shown to arise from proliferative cells (in an area indicated in **Figure 1F** with an X), move out into the subpial periphery of the cerebellar plate, before they

migrate ventrally into the tegmentum of the most anterior rhombencephalon. Furthermore, this tegmental cluster exhibits expression of cholinergic and glutamatergic markers (Volkman et al., 2010) and has differential and characteristic axonal projections to the optic tectum and the diencephalon indicating that this teleostean cholinergic isthmus complex includes the homologs of the cholinergic isthmus groups of mammals (Volkman et al., 2010; summarized in **Figure 7**). It is also interesting that no *tbr1/tbr2* expressing cells



are seen in the migrating isthmic population (Volkman et al., 2010), consistent with the fact that the eurydendroid cells, which are homologous to amniote DN, remain within the cerebellar cortex (Lannoo et al., 1991; McFarland et al., 2008; Bae et al., 2009).

THE ZEBRAFISH UPPER RHOMBIC LIP

Which region in the zebrafish brain corresponds to the amniote *atoh* positive cell producing URL (germinal trigone)? Köster and Fraser (2001a) have shown that *atoh1a* expression in the zebrafish is initiated at about 18 hpf in the cerebellar plate along the most dorsal ventricle (indicated by an X in **Figure 1F**) and then spreads into the subpial cerebellar plate periphery covering the entire cerebellar primordium up to the MHB by 20 hpf. This precedes by far the expression of *ptf1a*, which only starts around 32 hpf more ventrally (in the VCP), but directly adjacent to the *atoh1a* expression domain and this non-overlapping expression is maintained throughout development (A. Babaryka, R. W. Köster, personal observations; Kani et al., 2010). At the same time, *atoh1b* expressing cells and, in addition, from 2 days on into later larval life *atoh1c* expressing cells, are in the subpial periphery of the cerebellar plate with the *ptf1a* gene non-overlappingly expressed in the VCP (Kani et al., 2010, their **Figure 1**). In comparison to amniotes, these subpial *atoh1* cells correspond to an EGL and their ventricular point of origin as described above is in the caudal meeting point of ventral *ptf1a*

positive zone and *atoh1*-positive subpial zone (the X in **Figure 1F**). This has been shown by *in vivo* time-lapse studies that observed *atoh1a* expressing cells proliferating at the dorsal cerebellar ventricle (Distel et al., 2010). Subsequently, *atoh1/wnt1* expressing URL cells move rostrally and migrate ventrally. Early URL emigrating cells give rise to neurons of tegmental hindbrain nuclei while later arising neurons will form the inner granular layer (Köster and Fraser, 2001b, 2006; Volkman et al., 2008; Rieger et al., 2008; Kani et al., 2010; Volkman et al., 2010).

THE ZEBRAFISH EXTERNAL GRANULAR LAYER

Thus, the subpial *atoh1* expressing cells in the zebrafish from 2 days on are in a position comparable to the amniote EGL and they originate from the most dorsal cerebellar plate ventricle (which corresponds to the amniote germinal trigone). For the recognition of an EGL, a critical issue is whether proliferation takes place during these developmental events not only at ventricular, but also on the subpial side of the zebrafish cerebellar plate. Phosphohistone 3 (PH3) immunohistology clearly shows that LRL and URL behave differently in this respect. Whereas the zebrafish LRL at 2 days shows PH3 positive cells only at the ventricular side (**Figure 2B**; note that pial and ventricular surface are indicated by stippled green and red lines, respectively, in all panels of this figure), with *atoh1a* positive cells extending radially out into the pial periphery (**Figure 2B'**,

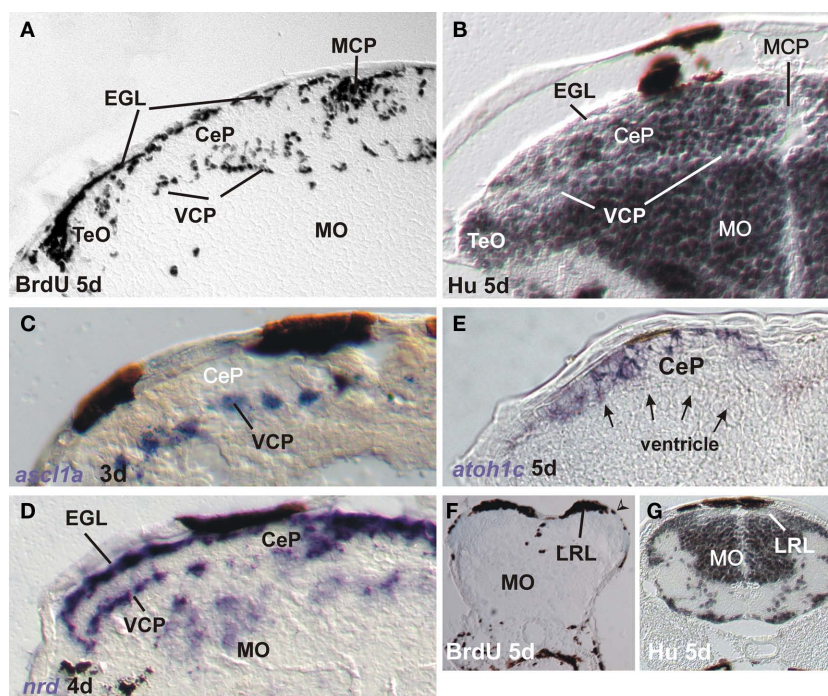


FIGURE 4 | Markers for proliferation and neurogenesis in zebrafish cerebellar plate (A–E) and [lower rhombic lip, LRL (G,F)] shown in transverse sections.

(A) Proliferation in ventral and medial cerebellar proliferation and external granular layer shown with BrdU saturation labeling (see text). (B) Differentiation in cerebellar plate shown with Hu immunohistochemistry. Note complementarity of label to BrdU pattern. (C) Ventral cerebellar expression of proneural gene *ascl1a*. (D) Ventral and dorsal cerebellar expression domains of bHLH *neuroD*. Modified from Mueller and Wullimann (2002) and Wullimann and Mueller (2002). (E) Dorsal

cerebellar expression of bHLH *atoh1c*. Note position of ventricle (arrows). (F) Proliferation at LRL level shown with BrdU saturation label. Note position of ventriculo-pial transition (arrowhead). (G) Differentiation at LRL level shown with Hu immunohistochemistry. Note Hu-free LRL. Both modified from Mueller and Wullimann (2005). CeP, cerebellar plate; EGL, external granular (or germinative) layer; IO, inferior olive; LRL, lower rhombic lip; MCP, middle cerebellar proliferation; MO, medulla oblongata; TeO, tectum opticum; VCP, ventral (or ventricular) cerebellar proliferation (ventricular germinal matrix).

the situation is different in the cerebellar plate. Here, proliferation is located both at the periventricular as well as at the subpial side (Figure 2A) and *atoh1a* extends from ventricularly located cells out into the pial periphery (Figure 2A'). In later larvae between 5 and 9 days *atoh1c* expression is seen to reach the periphery of the cerebellar plate and that some *atoh1c* positive cells at the pial periphery still do co-express PH3 (Figures 2C,C', E,E', F,F'). Of course, proliferation (PH3 reactivity) is also seen in the VCP (Figures 2D,D', E,E'). This shows that in the zebrafish cerebellar plate subpially migrated *atoh1* cells continue to proliferate into larval life, meeting one of the critical characteristics of an EGL. The LRL in later larval zebrafish brain development is more everted than at 2 days, but the ventriculo-pial transition point is still clearly identifiable (Figures 3 and 4G,F).

Previous studies of the early zebrafish cerebellar plate (3–5 days) development using proliferating cell nuclear antigen (PCNA) immunohistochemistry and BrdU had already provided evidence for distinct proliferative layers in the zebrafish VCP and in a subpial position (suggestive of an EGL) with postmitotic cells in between (Mueller and Wullimann, 2002; Figures 4A,B and 5). The latter were demonstrated with immunohistochemistry for Hu proteins (Figures 4B and 5) which mark early differentiated neuronal cells (Marusich et al., 1994). Further, expression of proneural bHLH genes *ascl1a* (Wullimann and Mueller, 2002) and *neuroD* (*nrd*; Mueller and Wullimann, 2002) supported this early cerebellar plate organization. Expression of *ascl1a*

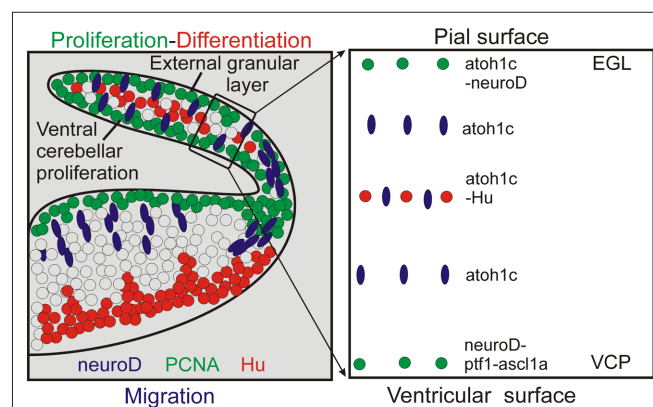
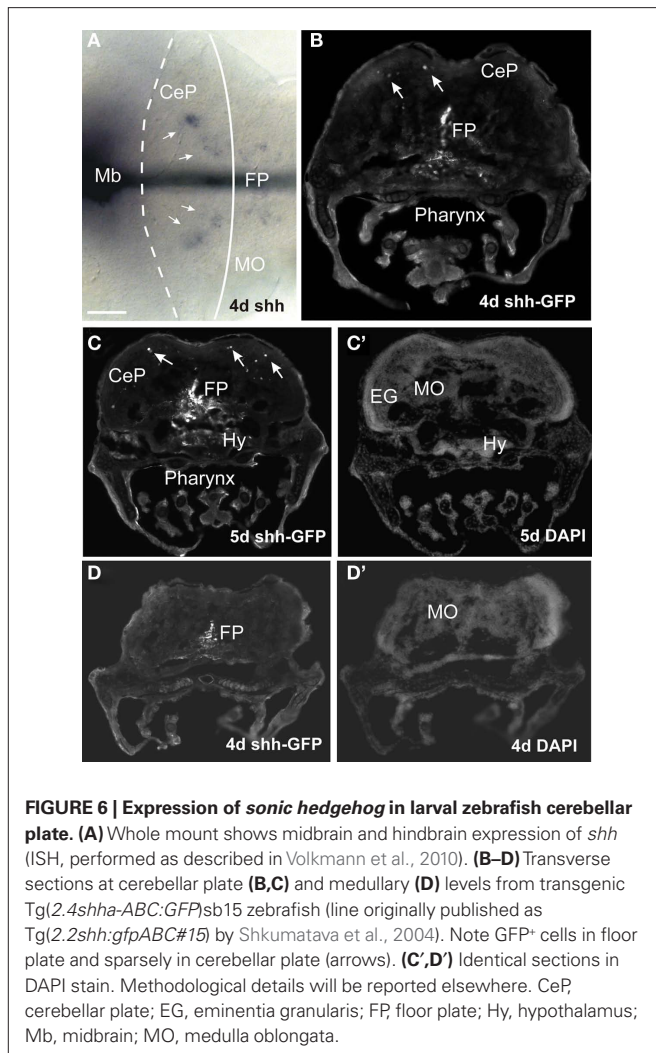
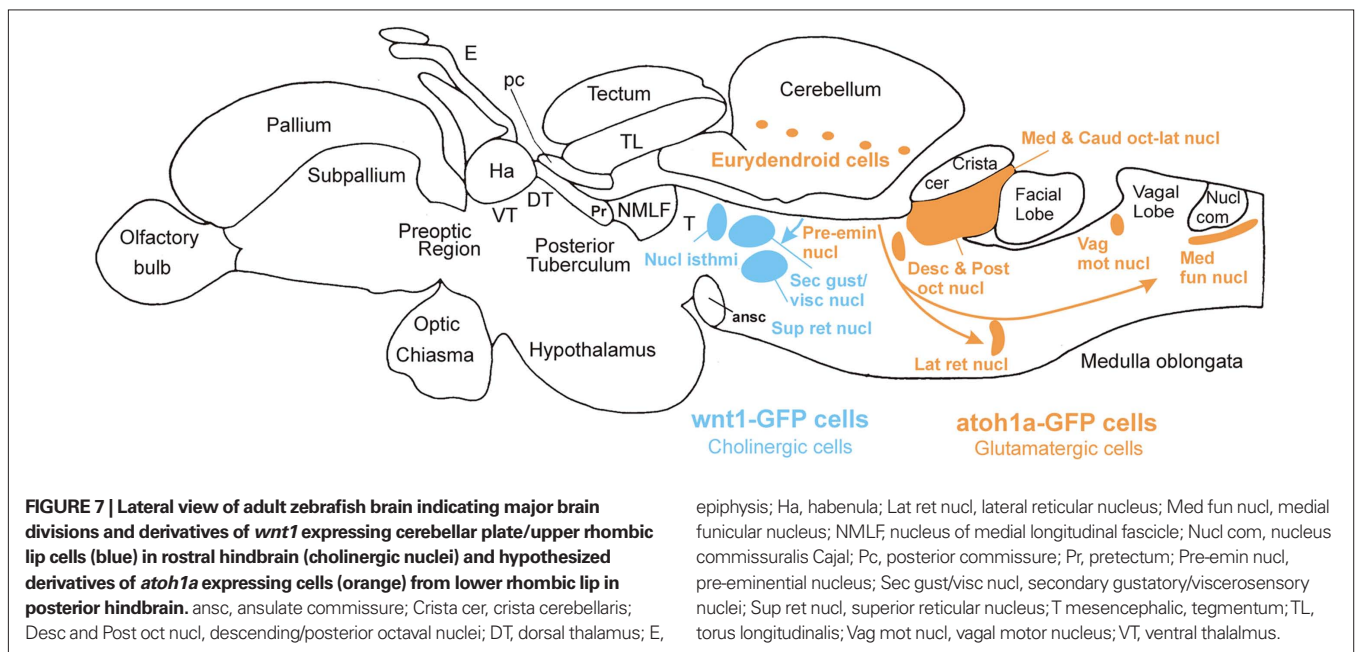


FIGURE 5 | Schema summarizes neurogenesis in zebrafish cerebellar plate. Left panel: Interpretative sketch based on localization of cellular marker in zebrafish cerebellar plate as shown in Figure 4. GABA as shown in Mueller and Wullimann (2006). Right panel: summary of markers at various dorsoventral levels of zebrafish cerebellar plate. Data as shown in left panel and compiled from Kani et al., 2010 (*ptf1*).

is only seen in the zebrafish VCP (Figures 4C and 5), whereas *nrd* is present in both the VCP and EGL (or close to them, Figures 4D and 5), leaving the sandwiched Hu-positive cerebellar area in between



free of expression. In contrast, *atoh1c* at that developmental time is expressed at the subplial side only (EGL plus additional cell layers ventrally, **Figures 4E and 5**). Since in the vertebrate forebrain *ascl1a* is involved with GABAergic and *nrd* with glutamatergic cell development, respectively (see discussion in Osório et al., 2010), these early studies already indicated the possibility that GABAergic cells are generated in the zebrafish VCP and glutamatergic cells in the EGL (Mueller and Wullimann, 2002; Wullimann and Mueller, 2002). GABA immunopositive cells are observed in the zebrafish cerebellar plate beginning with 3 days (Mueller et al., 2006). As discussed above, mouse *ascl* is indeed expressed downstream of *ptf1a* in VCP cells and involved in Purkinje cell development whereas mouse *neuroD* expression is involved in granule cell development. Intriguingly, zebrafish neurogenins (its two homologs *ngn1* and *ngn2* are apparently involved in GABAergic cell development in the mouse cerebellum, see above) were not observed to be expressed during zebrafish URL and LRL development so far (Mueller and Wullimann, 2003). Recent studies used transgenic zebrafish lines expressing reporter fluorescent proteins under the control of regulatory elements of proneural genes *ptf1a*, *wnt1*, *atoh1a*, and *olig2* (McFarland et al., 2008; Volkman et al., 2008, 2010; Distel et al., 2010; Kani et al., 2010). This work established the fate of VCP and EGL zebrafish cerebellar plate cells showing essentially correspondence to the situation seen in the mammals (see above). During 2–5 days of zebrafish cerebellar plate development, *atoh1a*:EGFP cells give first rise to the tegmental cholinergic complex, followed by the formation of inner granule cells. These studies clearly demonstrate that the *atoh1a* expressing cells also begin to express the proneural gene *nrd* already close to the EGL and *nrd* positive cells later accumulate in the cerebellar inner granule layer, thus showing their subplial origin from *atoh1a* expressing progenitors (Volkman et al., 2008). It was furthermore shown in a BrdU assay that proliferation does occur in the EGL during this developmental process (Kani et al., 2010). In contrast, *ptf1a*:EGFP cells are shown to originate in the VCP and migrate dorsally to form Purkinje cells



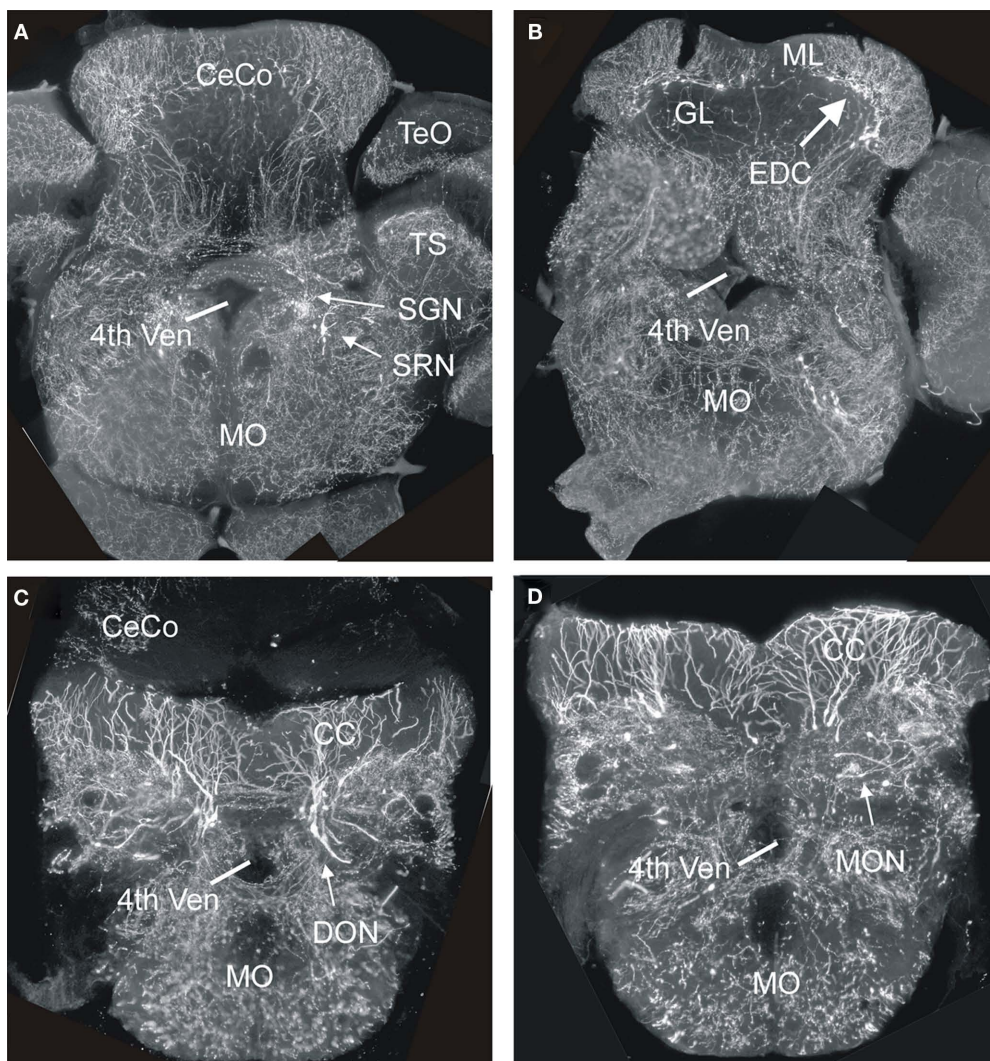


FIGURE 8 | Four transverse sections of the hindbrain from adult transgenic *Tg(atoh1a:Gal4TA4)^{hzm2} × Tg(4xUAS:GFP)^{hzm3}* zebrafish from rostral (A) to caudal (D) showing various GFP expressing cells in nuclei known in amniotes (as far as present) to derive from lower rhombic lip. Note that most fluorescent label shows fibers, not somata. The generation of these transgenic strains has

been described in detail in Distel et al., 2009, 2010). 4th ven, fourth ventricle; CC, crista cerebellaris; CeCo, cerebellar corpus; DON, descending octaval nucleus; EDC, eurydendroid cells; GL, granular layer; ML, molecular layer; MO, medulla oblongata; MON, medial octavolateralis nucleus; SGN, secondary gustatory nucleus; SRN, superior reticular nucleus; TS, torus semicircularis.

(and likely other inhibitory cerebellar cell types; Kani et al., 2010). A curious difference to the situation in the mouse was seen with respect to the origin of zebrafish eurydendroid cells, the homolog of the mammalian deep cerebellar nuclei. Early zebrafish cerebellar cells expressing the proneural factor-encoding *olig2* gene will give rise to eurydendroid cells (McFarland et al., 2008; Kani et al., 2010). Unlike in mammals, the vast majority of these (excitatory) cells does not derive from the *atoh1* expressing URL (although some do, Kani et al., 2010), but instead from the dorsal part of the *ptfla* expressing VCP, immediately ventrally adjacent to the ventricular *atoh1a* expression domain. This fact may serve to explain why teleost eurydendroid cells behave like VCP derived Purkinje neurons. Future eurydendroid cells migrate only over short distances to populate the Purkinje cell layer rather than migrating over long distances into a deep cerebellar

nuclear position like the *math1* expressing mammalian DN. A slight shift in *atoh1* or *ptfla* expression could have modulated the migration behavior and final position of these DN homologous cells in teleosts. Correlated with this is an additional difference regarding the neurotransmitter type, as descendants of *ptfla* expressing cells only give rise to GABAergic cells in mammals (see above), while in zebrafish *ptfla* expressing cells also contribute to glutamatergic eurydendroid cells. Possibly the *nrd* expression in the zebrafish VCP (Mueller and Wullimann, 2002), which is not seen in the mouse, may be related to the production of these excitatory cells from the zebrafish VCP.

In contrast to the above reports, Chaplin et al. (2010) have argued that the zebrafish has no EGL comparable to amniotes because of absence of (1) subpial proliferation, (2) ventral migration of *atoh1* expressing cells, (3) complete subpial coverage of the cerebellar

plate with *atoh1* cells in later larval stages, and most importantly, (4) absence of markers indicating the presence of transit amplification of the EGL proliferative activity via a SHH signal from Purkinje cells. The first claim is clearly refuted by data discussed above (Kani et al., 2010; data shown in **Figure 2**, summarized in **Figure 3**). In fact, Chaplin et al.'s (2010) own data (their **Figure 2G**) shows that both a VCP and EGL layer of PH3 positive cells is seen in the cerebellar plate. Similarly, the ventral migration of *atoh1a* cells has been amply documented (Köster and Fraser, 2001a, 2006; Volkmann et al., 2008; Kani et al., 2010). However, it is true that in later larval stages, the cerebellar plate is only covered caudally by proliferative *atoh1c* positive cells (only this paralog is relevant), which would be different from the ubiquitous subpial presence of an EGL in the postnatal mouse. However, a SHH mediated transit amplification of EGL proliferation in the zebrafish as seen in mammals during cerebellar development gains support by our finding of *shh* expression in the zebrafish cerebellar plate at 4–5 days using ISH or transgenic fish in which GFP expression is mediated by regulatory elements of *shh* (**Figure 6**). Thus, *atoh1* positive cells generated in the ventricular zone of the caudal URL invade the caudal cerebellar plate subpially, continue to proliferate there and give rise to cell migrations to the anteroventral tegmentum (cholinergic complex) and later inner granule cells, as seen in mammals. In conclusion, there is an EGL also in zebrafish.

THE ZEBRAFISH LOWER RHOMBIC LIP

Finally, we consider the zebrafish LRL and its potential derivatives. An immunohistochemical study on the distribution of PAX6 proteins in the zebrafish brain indicated that cerebellar and LRL domains are similar to the situation in the mouse (Wullimann and Rink, 2001). Importantly, a stream of PAX6 positive ventrally migrating cells was observed, indicative of a rhombic lip contribution to ventral medullary precerebellar nuclei. Detailed studies using transgenic zebrafish to show the fate of *atoh* or *wnt1* dependent cells are missing so far and no zebrafish hindbrain structure has been clearly shown to be derived from the LRL by fate mapping analysis. However, the use of a transgenic zebrafish Tg(*atoh1a:Gal4TA4*)^{hzm2} line to identify GFP expressing cells in the adult hindbrain produced some potentially exciting results. We found single GFP expressing cells in these brains in cerebellar eurydendroid cells (**Figures 8B and 7**), as well as in brain nuclei representing the cholinergic complex (**Figures 8A and 7**), in primary (descending and posterior) auditory nuclei (**Figures 8C and 7**), in the lateral reticular and in cells lateral to the medial funicular nucleus, the latter possibly corresponding to the lateral cuneate nucleus, which both project to the adult teleost cerebellum (Wullimann and Northcutt, 1988), in the lateral line primary mechanosensory (medial and caudal) octavolateralis nuclei (**Figures 8D and 7**), in the lateral line related pre-eminential nucleus and also in the vagal motor area (**Figure 7**). Since the location of most of these GFP labeled cells in the adult zebrafish brain is con-

sistent with an origin from *atoh1a* expressing progenitors similar to the situation in amniotes, it is tempting to conclude that the label reflects their origin in embryonic/larval *atoh1a* expressing URL and LRL cells. In addition, the data indicate that structures of the lateral line system, which are functionally similar to the auditory system, do also derive from the LRL in anamniotes. However, these data have to be treated with caution, since it is highly likely that the GFP cells might alternatively represent descendants of adult *atoh1* expressing progenitors which still persist in the zebrafish cerebellum (Kani et al., 2010), likely due to ongoing hindbrain neurogenesis in the zebrafish (Zupanc et al., 2005; Adolf et al., 2006; Kaslin et al., 2009).

CONCLUSION

The morphogenesis of cerebellar cortex development is largely conserved between ray-finned fishes and amniotes. However, the eurydendroid cells, which represent the amniote deep cerebellar nuclei, are special because they are positioned within the Purkinje cell layer in adult ray-finned fishes and are not deep to the cerebellar cortex. Thus, the migratory behavior of eurydendroid cells appears to be evolutionarily truncated. Furthermore, the molecular regulation partially differs between these vertebrate groups (number of *atoh1* genes, origin of deep nuclear cells). The data reviewed and presented here speak for the presence of an EGL in early larval zebrafish, although it seems not ubiquitously present over the entire pial cerebellar surface as in amniotes. Furthermore, evidence for the existence of a simple EGL during cerebellar development has been provided for amphibians (Urey et al., 1987) and cartilaginous fishes (Rodríguez-Moldes et al., 2008). In contrast, a functional cerebellum is absent in jawless lampreys and hagfishes (Lannoo and Hawkes, 1997). Thus, since a lower and upper rhombic lip and ventral cerebellar proliferation with their major derivatives (deep cerebellar nuclei, external/internal granular layer, inhibitory cerebellar cells, precerebellar nuclei, cholinergic tegmental nuclei) occur both in anamniotes and amniotes, they likely are shared characters of gnathostomes. The situation in jawless fishes regarding the presence of a rhombic lip and the nature of its derivatives awaits future research.

ACKNOWLEDGMENTS

We thank Bea Stiening for preparing the *in situ* hybridized and immunohistological sections (unless otherwise stated) shown in the present paper and Enrico Kühn for various technical support. Furthermore, we thank Alena Shkumatava, Sabine Fischer, Ferenc Müller, Uwe Strähle, and Carl J. Neumann for sharing their *shh-GFP* line. The Munich Graduate School of Systemic Neurosciences (GSN) and the Deutsche Forschungsgemeinschaft (DFG, project Wu 211/2-1; Mario F. Wullimann), the Studienstiftung des deutschen Volkes (Martin Distel) and the Ministry of Education and Research (BMBF, BioFuture 0311889), and the Helmholtz Association (Reinhard W. Köster) are gratefully acknowledged for support.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 07 December 2010; paper pending published: 11 January 2011; accepted: 06 April 2011; published online: 21 April 2011.

Citation: Wullimann MF, Mueller T, Distel M, Babaryka A, Grothe B and Köster RW (2011) The long adventurous journey of rhombic lip cells in jawed vertebrates: a comparative developmental analysis. *Front. Neuroanat.* 5:27. doi: 10.3389/fnana.2011.00027

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