Sonic hedgehog patterning during cerebellar development

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Sonic Hedgehog patterning during cerebellar development

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Abstract

Robust evidence in literature indicates that the morphogenic factor Sonic Hedgehog (Shh) actively orchestrates several aspects of cerebellar development and maturation. During embryogenesis Shh signalling is active in the ventricular germinal zone (VZ) and represents an essential signal for proliferation of VZ-derived progenitors. Later, Purkinje cell (PC)-secreted Shh sustains the amplification of neurogenic niches active during postnatal development: the external granular layer (EGL) and the prospective white matter (PWM) where excitatory granule cells and inhibitory interneurons, respectively, are produced. In addition, Shh signalling acts on Bergmann glia differentiation and during development sustains cerebellar foliation. Here we review the most relevant functions of Shh during cerebellar ontogenesis, underlying the role of this ligand in the development of different cerebellar phenotypes.

1.Introduction: the Hedgehog Pathway

Sonic Hedgehog (Shh) signalling is implicated in the regulation of key events during mammalian developmental processes [1]. The first gene of the Hedgehog family (Hh) was cloned in *Drosophila* in the early 1990s [2], and its role in controlling the proper segmental identity during fruit fly embryonic development has been identified [3, 4]. Shortly after, different Hh genes in Vertebrates were described [5, 6] and explained as a result of genome duplication. They are classified as Desert Hedgehog (Dhh), Indian Hedgehog (Ihh) and Shh [7]. In mammals, Shh is expressed starting from early embryogenesis and is one of the molecules responsible for the regulation of central nervous system (CNS) patterning [8]. Successively, during organogenesis, Shh is broadly detected in many tissues where it plays key roles as morphogen, mitogen and guidance molecule. Given its prominent role during development, alteration of its physiological functions are implicated in many human pathologies [9-11].

The spatial and temporal Shh expression pattern results from the combined action of multiple enhancer elements present in its extensive regulatory domain, controlling *Shh* transcription in different tissues [12-16]. However, only recently specific transcription factors have been discovered to directly control Shh enhancer activity. After transcription, Shh becomes an active ligand through evolutionarily conserved multistep processes [17-22] (Fig. $1A_1$). Initially, it is synthesized as a 45-kDa precursor and then it is auto-proteolytically cleaved by its own C-terminal domain into two secreted peptides: a 19-kDa amino terminus (Shh-N) with a signalling domain and a 26-kDa carboxy terminus (Shh-C), devoid of any signal transduction activity [23, 24] (Fig. $1A_1$). During auto-proteolysis, a cholesterol moiety is added to the C-terminus of Shh-N to anchor it to the plasma membrane, resulting in concentration in the lipid membrane rafts that facilitates interaction of low levels of Shh-N protein with its receptor [25-27, 18, 23]. Moreover, the cholesterol modification is also essential for a second lipophilic addition, a palmitoyl insertion at N-terminus of Shh-N [27, 18, 23] (Fig. $1A_1$). The presence of this second lipid incorporation is indispensable for Shh-N to multimerize and sequester the lipid anchor within the multimer, resulting in the detachment from the plasma membrane (Fig. $1A_1$). This soluble form of Shh-N is now able to diffuse far from its site of synthesis, resulting in a broad and long-distance effect [28-31]. The secretion of soluble multimer Shh-N can also be controlled by Dispached1 transmembrane protein (Disp1), containing a sterol sensing domain (SSD) able to displace Shh-N oligomers from lipid rafts [32, 33] (Fig. $1A_1$).

Before reaching the surface of responding cells, Shh-N moves between many cells probably under the regulation of multiple molecules and mechanisms, like megalin [34] and glypicans [35] (Fig. $1A_2$). Megalin is an endocytic receptor belonging to the low density lipoprotein receptor family, and mediates cell endocytosis of N-Shh, targeting it for lysosomal degradation or transcytosis [34]. The affinity of N-Shh for megalin is augmented when the ligand is sequestered near the cell surface by glypicans, which are heparan sulfate proteoglycans [36] (Fig. $1A_2$). Lysosomal degradation is implicated in the extracellular regulation of N-Shh concentration, whereas megalin-mediated transcytosis may facilitate long range signaling of N-Shh during early development, across neural tube epithelial cells [34, 37].

In vertebrates, the receptor and co-receptors of N-Shh are concentrated in the primary cilium, a microtubule-based membrane protrusion [38]. Emerging evidence suggests that the cell surface machinery responsive to Shh comprise is a complex interaction network [39]. Indeed, the binding of Shh-N to its specific receptor Patched 1 (Ptch1), a 12-pass transmembrane protein, is facilitated by other transmembrane proteins, like Cdo, Boc and Growth Arrest Specific 1 (Gas1; Fig. $1B_{1-3}$; [40,

41]). These co-receptors regulate Shh signalling by binding to Shh in synergic interaction with Ptch1. They are down-regulated in response to Shh signalling in a negative feedback [42-45]. This feedback simultaneously up-regulates Ptch1 and other two additional cell surface Shh-binding proteins, hedgehog interacting protein 1 (Hhip1; [46]), a membrane-anchored glycoprotein, and patched 2 (Ptch2), a structural homolog of Ptch1 that arose from a gene duplication event [47]. Ptch2 and Hhip1 binding to Shh-N ligand compete with active ligand-receptor interactions to alter the balance between bound and unbound Ptch1, resulting in cell autonomous modulation of cell signaling activity. Furthermore, the resulting transcription of *Ptch1* gene in response to Shh signalling activation leads to an auto-inhibition process where produced Shh-N is no longer sufficient to block all the available Ptch1 [40].

In absence of ligand, Ptch1 catalytically inhibits the activity of the Shh signal transducer Smoothened (Smo), a seven-transmembrane-span receptor like protein [48], modulating the concentration of its small agonist or antagonist molecules, such as Oxysterols [49], vitamin D3 derivatives [50, 48, 51-53], cyclopamine and jervine [51] (Fig. $1B_1$). Ptch1, which is structurally related to transporter and pump protein, may send endogenous sterols away from Smo, inhibiting its action [54]. After Shh interaction with Ptch1, sterols can bind to Smo and its inhibition is released [49] (Fig. $1B_2$). Thus, the seven-transmembrane receptor shuttles from an endocytic vesicle to the cilium, while the hedgehog patched complex is internalized and degraded by lysosomes [55] (Fig. $1B_2$). To activate Smo a Shh-induced Smo conformational switch is required. In particular, in mice Shh-N can regulate the accessibility of Smo to CK1αand GRK2 kinases, which bind and phosphorylate the Smo C-tail in a dose-dependent manner, shifting the closed conformation of Smo in an open shape [56] (Fig. $1B_3$). Phosphorilated Smo activates intracellular signals regulating several protein kinases, which activate a class of transcription factors known as glioblastoma (Gli) proteins, homologs of cubitus interruptus (Ci) proteins in *Drosophila* [57]. The regulation of Gli proteins activities is similar to Ci, although in mammals three Gli proteins have been discovered (Gli1–3; [58, 59]). In the absence of Shh stimulation, the nuclear localization of Gli2 and Gli3 proteins is inhibited by the binding in the cytoplasm of the Suppressor of Fused (Sufu), which is the major negative regulator of Shh signaling in mammals (Fig. $1B_1$; [60-63]). In addition, Kif7 is also present in this complex acting as a scaffolding protein for PKA, GSK3 and CK1 kinases, responsible of Gli phosphorylation (Fig. $1B_1$). After phosphorylation, GLI2 is rapidly degraded, whereas Gli3 is cleaved and the partial Gli3 acts as a repressor, blocking transcription of downstream targets [64-66, 29, 67]. On the other hand, Gli1 transcription has been demonstrated to increase in response of Shh, and its level is a widely used biomarker for activated Shh signaling. Therefore, it is implied that Gli1 is not one of the initial transducer of the Shh signal [19]. In the

presence of Hedgehog signaling, Smo induces the inhibition of Gli phosphorylation by dissociating the Sufu-Gli-kinase complex with consequent stabilization and nuclear accumulation of Gli family members (Fig. 1B$_3$; [67, 68]). In the nucleus of responding cells all Gli transcriptional factors, possessing highly similar zinc finger DNA binding domains, can bind the same DNA motif. However, Gli1 and Gli2 mainly function as transcriptional activators, while Gli3 is a transcriptional repressor (Fig. 1B$_{1,3}$; [69, 70]), suggesting that they might act on different target genes.

2. Distinct functions of Shh during cerebellar development

The secreted signaling molecule Shh plays critical roles in pattern formation of the vertebrate CNS. During neurulation, Shh is produced by the ventral midline mesoderm as well as by the ventral neural tube, and its activity is required for the determination of ventral characteristics along the anterior-posterior neuraxis [71]. At successive stages of development Shh signalling sustains the proper formation of several CNS regions, including the cerebellum. Here, Shh critically influences the initial phases of territorial determination and regulates the following steps of cerebellar progenitor maturation in primary and secondary germinal zones.

2.1 Cerebellar territory and germinal zones

The cerebellum arises from a specialized area at the midbrain/hindbrain boundary [72-74], where, at embryonic day 8.5 (E8.5), the interaction between homeobox genes *Otx2* and *Gbx2* defines the Isthmic Organizer region (IO; [75, 76]). The IO orchestrates the development of cerebellar and mesencephalic structures through the morphogenic activity of secreted factors, Fgf8 and Wnt1 [77-79]. In the midbrain and cerebellum, Shh expressed in the ventral midline regulates dorsoventral pattering and expression of the midbrain-hindbrain organizer Fgf8 during early embryogenesis (from E8.5 to E12; [14, 80]). It has been shown that retrovirus-mediated misexpression of Shh in the early chick neural tube disrupts midbrain-hindbrain boundary formation, causing the fusion of the two lateral cerebella primordia [71].

After territorial specification, cerebellar histogenesis starts at E9 in the mouse. At this age the cerebellar anlage is formed by two separated and symmetric bulges that during the following days grow and fuse together, giving rise to the unitary cerebellar plate, comprising the vermis and the two hemispheres [81]. Such developmental phase is also characterized by the formation of two germinative compartments just above the opening of the fourth ventricle: the rhombic lip (RL), located at the outer aspect of the cerebellar plate, adjacent to the roof-plate, and the ventricular zone (VZ), placed in the inner side, covering the fourth ventricle. These germinative districts are defined by the region-specific expression of two basic helix-loop-helix transcription factors: the pancreas transcription factor 1-a (Ptf1-a), expressed in the VZ [82], and the mouse homolog of Drosophila atonal (Atoh-1), present in the RL [83]. This spatially-restricted expression pattern defines the

neurochemical compartmentalization of cerebellar precursors, as all GABAergic neurons (Purkinje cells, PCs, nucleo-olivary projection neurons of deep cerebellar nuclei, DCN, and all inhibitory interneurons - basket, stellate, Golgi and Lugaro cells) originate from Ptf1-a$^+$ precursors [82, 84, 85], while glutamatergic lineages (large projection neurons of DCN, unipolar brush cells, UBCs, and granule cells) derive from Atoh-1$^+$ progenitors [85-91]. Recent experiments have shown that these transcription factors specify cerebellar progenitors within the two spatially segregated neuropithelial domains so to assure the appropriate production of GABAergic and glutamatergic neurons [85]. In particular, by means of knock-in mouse lines and by *in utero* electroporation Ptf1-a and Atoh-1 were ectopically expressed in the RL and in the VZ, respectively. Results were clear-cut, showing that ectopically Ptf1-a-expressing RL cells produced GABAergic phenotypes, whereas ectopically Atoh-1$^+$ VZ progenitors differentiate into glutamatergic populations [85]. The two primary germinative epithelia disappear at birth. Dividing VZ precursors emigrate into the cerebellar prospective white matter (PWM), whereas those of the RL move along the pial cerebellar surface, where they form the external granular layer (EGL). Postnatal neurogenesis is active in secondary PWM and EGL epithelia up to the third postnatal week to generate appropriate numbers of GABAergic and glutamatergic interneurons, respectively [81, 92].

The temporal schedule of generation of cerebellar phenotypes is also finely defined. Birthdating studies showed that projection neurons are produced first, at the onset of cerebellar neurogenesis, while both inhibitory and excitatory interneurons are generated later, during late embryonic and first postnatal life [93, 81, 94].

It is well established that Shh actively regulates the amplification of cerebellar progenitors in both embryonic and postnatal germinal zones. This morphogen, secreted by Purkinje cells from E17.5 [95-97] and by choroid plexi at earlier time points [98], controls the production of appropriate numbers of excitatory and inhibitory interneurons (Fig. 2). In addition, it modulates the correct generation and development of glial progenitors and it exerts specific functions in different phases of granule cell development, in both normal and pathological conditions, such as medulloblastoma. Finally, Shh actively orchestrates the major dynamics of cerebellar foliation, sustaining normal processes of cerebellar growth and maturation.

3.1 Shh and granule cells

Granule neurons (GNs) represent the most abundant cell type in the brain (about 10^8 granule cells in the adult cerebellum; [93, 99]. These cells derive from Atoh-1$^+$ progenitors migrating from the RL to the EGL. The entire process of granule cell production lasts from E12.5 to P14 and it is fundamental for the acquisition of regular cerebellar size and foliation [81]. Indeed it has been

observed that reduction in GN number leads to the formation of smaller cerebella [100-102] and abnormal foliation, such as the persistence of just the five cerebellar cardinal lobules at P14 in rats [103, 104]. In addition, abnormal proliferation of granule cell precursors (GCPs) is at the basis of pathological conditions such as medulloblastoma, the most common form of children malignant brain tumour (for review see [105, 106]).

Numerous studies have analysed the mechanisms underlying the initial phases of GCP proliferation and migration from the EGL mitogenic niche [97, 107, 108]. In particular, it has been demonstrated that these steps are actively controlled by mitogenic factors secreted by PCs. Indeed, the relative number of granule cells is reduced in animal models characterized by a primary PC degeneration [109-111], whereas if the loss of PCs occurs later in postnatal period (as in the *pcd* mutant mouse) the granule cell layer appears near normal [112, 111]. Shh produced by PCs is the most efficacious mitogen acting on granule cell development. Treatment of GCPs with Shh prevents the differentiation and induces a long-lasting proliferative response, while an inhibition of Shh signal dramatically reduces the mitotic activity of these precursors [97, 95, 113-115]. The pathway activated by this molecule involves the upregulation of the target genes Patched, Gli1 and Gli2, which are dynamically expressed during development by proliferating precursors [113, 116, 104]. For example, the activation of Gli2-mediated pathway is important to ensure the correct extension of GCPs in the EGL and the consequent normal patterning of cortical folia [104]. Other important mediators of Shh-induced proliferation are N-myc, cyclin D1 and cyclin D2, that directly promote the entry of precursors into cell-cycle and DNA replication [116-118]. Overexpression of these molecules is sufficient to boost GCP proliferation, but the amplitude of the response is strictly dependent on the particular molecule involved [116].

Shh protein is present in PC dendrites and axons during embryonic and postnatal development, and persists in the adult, suggesting multiple roles played in the developing and mature circuitry [97]. However, the decreased expression of Gli1 in the innermost part of the EGL indicates that the response to the Shh signal is progressively switched off in granule neurons [107, 104]. Cells in the deepest part of EGL, as well as in the ML and in the internal granular layer (IGL), do not express cyclin-D1, indicating their status of non-proliferating cells [119-121]. Thus, at the time when they are ready to leave the proliferative niche, GCPs undergo some cell-autonomous changes that are critical to exit the cell cycle. In addition, these cells start the expression of cyclin-dependent kinase inhibitors, such as p27. This molecule arrests their proliferation and induces the differentiation programme both *in vitro* and *in vivo* [122, 123]. However, in the p27 knockout mice GCPs are still able to leave the cell-cycle and differentiate into mature granule cells, indicating that p27 is not the unique factor responsible for this mechanism [108]. It has been suggested that extracellular matrix

(ECM) glycoproteins, such as laminin and vitronectin, can modulate GPC responses to Shh [124]. GCPs actively proliferate in the presence of Shh when cultured on laminin, which is present *in vivo* in the outer EGL, but not on vitronectin, which is normally contacted by granule cells in the deepest EGL and IGL [96, 124]. Therefore, the same molecular elements may regulate both proliferation and differentiation signalling of GCPs, depending on the pattern of ECM molecules and receptors expressed in different parts of the EGL.

3.2 Shh and GABAergic interneurons

GABAergic interneurons comprise multiple subsets of morphologically and neurochemically distinct phenotypes integrated at different levels of the cerebellar cortex and DCN. These cells are produced from late embryonic life to the second postnatal week; the peak is around P5 and the production of 75% of all inhibitory interneurons occurs prior to P7 [125]. Maricich and Herrup [126] identified the progenitors of inhibitory interneurons as a population of Pax-2^+ cells, which appear in the VZ around E12 and later emigrate into the cerebellar parenchyma. Inhibitory interneuron precursors continue to proliferate during their migration in the PWM [127-129, 125] and they generate interneuron phenotypes according to an inside-out progression. DCN interneurons are the first to be born during embryonic and early postnatal life, followed by granular layer (GL) interneurons (Golgi and Lugaro cells) and, finally, by ML ones (basket and stellate cells; [126, 128, 125, 130]. Interestingly, transplantation experiments have demonstrated that all these different interneuron subsets derive from a single population of Pax-2^+ immature interneurons that acquire mature phenotypic traits under the influence of local instructive cues provided by the PWM microenvironment [128, 129].

The cellular composition of the PWM is complex, including cells with neural stem cell (NSC)-like properties [131, 132], dividing progenitors and astrocytes, interneurons and oligodendrocytes at different maturation stages [127, 133, 134, 129, 135, 136, 121].

It has been shown that proliferative intermediate progenitors of GABAergic interneurons in the PWM are Ptf1-a^+ cells that start the expression of Pax-2 during their last S phase [126, 129, 136].

Recent findings suggest possible lineage relationships between Ptf1a^+ intermediate progenitors of GABAergic interneurons and Tnc^{low} $CD15^+$ astrocyte precursors [136]. Both populations derive from a population of Tnc^{low} and $CD133^+$ neural stem-cell-like primary progenitors of the PWM, whose production is critically maintained by PC-delivered Shh [136]. Indeed, blockade of Shh signalling in Tnc-expressing cells disrupts the PWM niche, decreasing the numbers of intermediate progenitors of both interneurons and astrocytes, causing also a dramatic reduction of their mature phenotypes [136]. Notably, such specific effect of Shh is independent of the classical role of this

mitogen in regulating GCP proliferation [97], as direct perturbation of GCP expansion does not alter GABAergic progenitor production [136].

Another proof of a direct effect of Shh on inhibitory interneuron production has been also provided by recent *in vitro* data: the exogenous administration of the recombinant amino-terminal active fragment of Shh (Shh-N) on cerebellar slices obtained by PWM regions of P2 mice showed an enhanced amplification of Pax-2$^+$ cells [137]. This effect was significant both at 1 and 2 days *in vitro*, whereas it did not occur in presence of the Shh-antagonist cyclopamine [137]. In addition, Shh mitogenic effect on newborn Pax-2$^+$ cells disappears at late postnatal stages as P7, when the bulk of GABAergic interneurons have been already produced [125, 138]. Gene expression analysis of sorted GFP$^+$ cells from Pax-2-GFP transgenic mice revealed that Shh pathway is active in immature Pax-2$^+$ interneurons, which express both the Shh receptor Ptch1 and the Shh target gene Gli1 [137]. Similar results were confirmed by *in situ* hybridization: Gli1 and Ptch1 mRNAs were detected in P2 cerebella in the EGL, PCL and PWM, in line with former studies [113, 126, 96, 136]. Moreover, double Gli1$^+$/Pax-2GFP$^+$ and Ptch1$^+$/Pax-2GFP$^+$ interneurons were found, confirming the presence of an active Shh signals in Pax-2$^+$ cells [137], although its role during successive interneuron maturation remain to be clarified.

At earlier developmental stage (E16) the Gli1 mRNA was already present in the cerebellum, though less expressed compared with postnatal ages. Both Gli1$^+$ cells and double positive Gli1/Pax-2GFP cells were present in the VZ/SVZ region, or scattered in the cerebellar parenchyma, showing that during embryonic development Shh pathway is already active in Pax-2$^+$ cells located in germinal and migratory sites [137]. Exogenous administration of Shh-N on embryonic cerebellar explants increased the fraction of proliferative cells becoming Pax-2$^+$ interneurons but only at the end of the culture period, suggesting that different dynamics might regulate the production of GABAergic interneurons at embryonic and postnatal developmental stages [137]. This possibility is also supported by previous *in vivo* data showing that Shh signalling is active in the cerebellar VZ and essential to regulate the proliferation of radial glia and, consequently, the expansion of GABAergic interneurons [98]. In this case, however, endogenous PCs are not the source of Shh ligand, which is secreted by the choroid plexi and transventricularly delivered by the embryonic cerebrospinal fluid [98].

Overall these recent studies highlight the fundamental role of Shh in regulating interneuron numbers by maintaining the embryonic and postnatal niches in which these cells are produced.

3.3 Shh and cerebellar glia

Cerebellar astrocytes and oligodendrocytes comprise morphologically distinct cell types located at different sites in the cerebellar cortex and white matter [139-141].

Several evidence indicated that astrocytes (comprising Bergman Glia-BG- cells and parenchymal astrocytes) derive from the VZ [139, 142-144, 130], whereas oligodendrocyte precursors (OPCs) seem to mainly originate from exogenous sources and, at later embryonic stages, populate the cerebellar parenchyma and continue to amplify [145, 146, 134].

Some studies investigated the role of the Shh signaling pathway on both astrocytes [147-149] and oligodendrocytes [150-156] in developing and mature brain and spinal cord. In the cerebellum Shh has been described to play a direct role on both BG cells and oligodendrocytes, regulating their differentiation and proliferation, respectively. Moreover, this ligand critically maintains the VZ and PWM niches, controlling the proliferation of, respectively, radial glia cells and intermediate progenitors of parenchymal astrocytes ([98, 136]; see previous paragraph). Beside that, it is not clear whether Shh could exert some roles on mature parenchymal astrocytes.

Before Shh was shown to directly affect the BG population, Traifford et al. [157] and Wallace [113] had already observed high expression levels of Ptch and Gli1 in small cells in the PCL, presumably in BGs, suggesting that Shh signaling pathway was active in these cells. Confirmation of a direct action of Shh came shortly later, when Dahmane and Ruiz-I-Altaba [95] first demonstrated the role of this ligand in BG differentiation, but, intriguingly, not in BG progenitor proliferation [95]. These results were in accordance with previous data revealing the key role of PCs in the control of BG maturation [158, 79]. Yet, BG persist in both Gli2 mutant embryos [96] and Gli2-En1 conditional knock-out mutants, in which Gli2 deletion is restricted to cerebellar precursors [104], thus demonstrating that Shh signaling through Gli2 is dispensable for BG specification. However, the latter mutant model showed an abnormal glial morphology, characterized by the disorganization and deformity of the glial fibers. This phenotype was explained as a secondary effect of the abnormal PC morphology [104]. Similarly, Lewis et al. [97], through transgenic mice models specifically developed to prevent Shh production by PCs at different ages, observed alterations in BG only after P5, while BG morphology was normal at previous developmental stages. Also in this case the results were interpreted as a secondary effect of PC disorganization and absence of granules' parallel fibers and not as a direct consequence of Shh absence on BG differentiation. In contrast, Mecklenburg et al. [159] proposed a direct role of Shh in the regulation of BG maturation, suggesting that the altered glial morphology observed in conditional Shh mutants [97] was not subordinate to defective PCs. Indeed, Gdf10, a member of the transforming growth factor beta (TGF-ß)-superfamily strongly expressed in cerebellar BG cells from E15 (i.e. from the moment they are specified) and, thus, potentially correlated to BG specification from radial glia, was shown to be intensely reduced in conditional Shh cerebellar mutants. The rapid down-regulation of this

glial-specific gene in absence of Shh could suggests a direct role of this ligand in BG specification, despite the exact mechanisms still need to be clarified.

Shh was also shown to affect the proliferation of cerebellar oligodendrocytes, the glial component responsible for the myelin synthesis. Bouslama-Oueghlani et al. [160] used cerebellar organotypic cultures to investigate the influence of PCs (i.e. the only type of cerebellar neuron to be myelinated) on the timing of oligodendrocyte differentiation. In particular, using cerebellar slices cultures in which the number of PCs was significantly different, authors found that soluble factors produced by PCs were able to affect OPC population. Among these factors Shh was shown to be downregulated during PC postnatal maturation, whereas vitronectin resulted upregulated. Importantly, Shh and vitronectin administrated to postnatal organotypic slices had opposite effects on OPCs, stimulating their proliferation and differentiation, respectively [160]. These effects were reverted by Shh and vitronectin- antagonists. On the whole, these results highlight that both neuronal and glial development are highly synchronized in the cerebellum [160], as well as in other CNS regions [161-163].

3.4 Shh orchestrates normal cerebellar foliation

A prominent feature of cerebellar morphology is its folded appearance, whereby fissures separate its anterior-posterior extent by lobules [164]. By E18.5 four principal fissures are evident in midsagittal sections of the mouse vermis, leading to distinguish five cardinal lobes (anterobasal, anterodorsal, central, posterior and inferior lobes; [164]). Afterwards, additional (non principal) fissures divide the cardinal lobes into lobules, reaching the total of ten lobules identifiable in the adult murine cerebellum [164].

The mitogenic action of Shh on GPCs is the driving force for the initial formation of fissures [164], thereby affecting also cerebellar foliation process, which is the process leading to the morphologically unique brain structure of folia separated by fissures [96, 104]. In particular, it has been shown that Shh signaling spatially and temporally correlates with fissure formation and that Gli2 is the principal activator of Shh-induced GCP proliferation [96]. Indeed, Gli2-null mutants showed decreased foliation at birth and reduced numbers of GCPs, whereas Shh overexpression in wild-type cerebella leaded to normal cerebellar foliation but also to an increased cerebellar size, as consequence of prolonged GCP proliferation [96]. Other experiments clarified that the level of Shh signaling regulates the extent and complexity of cerebellar foliation, but non its typical pattern [104]. Namely, in the absence of Gli2 foliation proceeds but the process of lobulation is delayed and prematurely arrested; further reduction in foliation occurs in double Gli2 and Gli1 null mutant mice, whereas when the entire Shh signaling is removed foliation is totally inhibited because of a rapid exhaustion of GCPs after E17.5 [104]. Collectively, these findings suggest that Shh is not

necessary to initiate foliation and to determine the position of the fissures, but is more likely to be a regulator of the extent and complexity of foliation. This interpretation implies that at embryonic stages in the absence of Shh some GPCs are able to start mitosis, allowing the first indentations to form, and that at later stages before and after birth Shh becomes necessary to sustain the expansion of the EGL [97].

4. Shh and cerebellar pathology: evidence from medulloblastoma

Medulloblastoma (MB) is the most frequent form of primitive, neuroectodermal tumor during childhood, with estimated lethality of 30% and high clinical heterogeneity. It is widely accepted that MB originates from GCPs and four different subtypes of MBs have been identified and classified according to their transcriptional profiles: I) WNT MBs; II) Shh MBs; III) Group C, frequently associated with TGF1 beta pathway abnormalities; IV) Group D, often related to tandem duplication of SNCAIP [165-169].

Shh-MBs represent one third of the total number of MBs [170, 171], both in childhood and adult age, even though it has been demonstrated that infant and adult MBs exhibit different transcriptional and genetic profiles. Northcott et al. [167] identified a number of homeobox family members as the mostly up-regulated genes in adult forms of Shh-MBs; infant MBs, instead, revealed high expression of transcriptional regulators functioning in brain development, such as ZIC2 and ZIC5. These dissimilarities lead to large variability in clinical and prognostic aspects of the disease through different ages: this is the reason why targeting of signalling molecules is a fundamental step to set up therapeutic approaches.

It has been demonstrated that Shh-MBs derive from aberrations in varied components of Shh pathway, such as Ptch1 [114, 172], Sufu [173, 174], Gli transcription factors [175] and Smo [176]. Studies on mice lacking Ptch function demonstrated that abnormal activation of Shh pathway through repression of its inhibitors leads to the formation of MBs [177, 178]. Similar mutations have been also described in patients with nevoid basal cell carcinoma syndrome – also known as Gorin syndrome - often associated with the formation of MB in childhood [179]. According to Kim et al. [180], Shh pathway alteration via Ptch in heterozygous mice induces a subset of GCPs to maintain their proliferative activity with consequent deregulation of developmental gene expression, rather than globally increase GCPs proliferation during postnatal development or interrupt programmed cell death. However, the complexity of the Shh pathway does not allow attributing MBs formation to abnormalities of just one element of the network, as demonstrated by the fact that only a percentage of Ptch mutant mice develop MB. Taylor et al. [173], in fact, identified Sufu as another tumor-suppressor gene in a subset of desmoplastic medulloblastomas whose mutation predisposes to MB by modulating the Shh signaling: they created a model in which

Sufu cannot bind Gli transcription factors and allocate them from the nucleus, with consequent activation of SHH target genes. More recently, it has been also demonstrated that nevoid basal cell carcinoma syndrome, traditionally associated with Ptch mutations, could be caused by heterozygous loss-of-function germline mutations in Sufu. These studies contributed to the redefinition of the risk of MB in Gorlin syndrome on the basis of the related gene: in Ptch-related forms, the risk of MB has been reduced from 5% to 2% with a probability approximately 20X higher in Sufu-related forms [181, 182].

Other studies focused on the role of Gli1 expression in MB through the identification of the subset of Gli1 transforming target genes specifically expressed in MBs [183], or through the characterization of SHH signaling pathway members Gli-3 expression in relation with prognosis, suggesting that Gli1 or Gli2 expression in pediatric medulloblastoma might confer a worse outcome [184]. However, recently, new intriguing findings partly modified this classical view of Shh-dependent proliferation of GCPs. Li et al. [185] disclosed a new population of progenitors cells in the EGL identified by the expression of the neural stem cells (NSC) marker Nestin. Surprisingly, $Nestin^+$ cells do not express Math1 and are not responsive to Shh *in vivo*, even if they express the signaling-associated machinery. Nevertheless and though they account for only 3-5% of EGL cells, they displayed enhanced tumorigenic potential and chromosomal aberrations following loss of the Shh receptor Ptch1 compared to $Math1^+$ GCPs [185], raising important questions about the cellular origin of medulloblastoma [186].

5. Concluding Remarks

The role of Shh in the development of the CNS has been extensively investigated, leading to an in-depth knowledge of extrinsic and intrinsic molecular machineries included in its signalling pathway. Moreover, beyond its role in ventral patterning, hedgehog signalling is now known to have multiple roles throughout development, favouring the processes of fate specification, oligodendrogenesis, stem cell maintenance and axon path finding. In the cerebellum, Shh pathway has been principally studied for many years in the contest of GCP proliferation. However (as described above), Shh ligand exerts additional roles on distinct cell populations, during the entire period of cerebellar development. In particular, Shh crucially sustains the expansion of neuronal and glial precursors within embryonic and postnatal niches, by means of different mechanisms, involving both cerebellar and extracerebellar strategies (Fig. 2). Moreover, Shh also induces BG maturation and oligodendrocyte amplification. As shown above, Shh signalling is a complex network involving different players that might modulate Shh function at different level, depending on the cell type. Intriguingly, the sole source of Shh during the late embryonic and postnatal development is PCs. These latter cells strategically orchestrate postnatal cerebellar morphogenesis

through the modulated secretion of Shh and vitronectin. Despite the great amount of results in these areas, a deeper knowledge of the processes regulating the timing and balance of Shh/vitronectin production by Purkinje cells will certainly reveal more surprises about cerebellar developmental mechanisms.

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Figure legends.

Figure 1. Shh signalling pathway in Vertebrates. (**A_1**) Shh protein is synthesized as 45 kDa precursor protein (**Shh**) that is palmitoylated at the N-terminus and concomitantly auto-catalytically cleaved. Successively, a cholesterol-modification occurs to obtain a 19 kDa dually lipidated N-terminal signaling protein (**SHH-N**). Shh is then trafficked to the cell surface and released from cells as a multimer (**M-Shh-N**) in a process mediated by Dispatched1 (**Disp1**). (**A_2**) Interactions with both megalin and glypicans regulate the long range Shh signalling. Binding to the transmembrane protein megalin promotes Shh internalization, resulting in either degradation or subsequent exocitosis. The Shh affinity for megalin is increased by glypicans. (**B_1**) Primary cilia are key organelles where Shh signalling takes place. On their membranes both receptors (ptch1) and co-receptors (Cdo/Boc) are exposed. These structures also contain regulatory microtubule-associated

complexes composed by Suppressor of Fused (Sufu), Kif7, PKA, GSK3, CK1 and Gli proteins. In the absence of Shh, Ptch1 prevents membrane localization and activation of Smo, retaining it on intracellular vesicles. In this context, Gli proteins are held in the microtubule-associated complex, that induces Gli protein phosphorylation (**red P**) to obtain a transcriptional repressor form (**Gli repressor**). (**B**$_2$) When Shh interacts with the Ptch1 and Cdo/Boc, Smo is shuttled from an endocytic vesicle to the cilium whereas hedgehog patched complex is internalized and degraded by lysosomes. (**B**$_3$) The de-repression of Smo induces the dissociation of Sufu-Gli-kinases complex promoting formation of the Gli activator form (**Gli activator**), that after nuclear translocation activates transcription of downstream targets.

Figure 2. Shh functions during cerebellar development.
(**A**) During the embryonic development, Shh is first secreted by choroid plexi (ChP) and is essential to radial glial cell proliferation and expansion of Ptf1a$^+$ progenitors of GABAergic neurons. Purkinje cells start the secretion of this morphogen by E17.5, modulating the correct differentiation of glial progenitors into mature BG. (**B**) Postnatally, Shh acts as a mitogen on both granule and oligodendrocyte precursor cells (GCPs and OPCs), in the EGL and PWM respectively. In the PWM, Shh also exerts a proliferative function on the neural stem-cell-like progenitors (Tnc$^+$CD133$^+$) that generate both intermediate astrocyte precursors (Tnc$^+$CD15$^+$) and GABAergic transient amplifying cells (Ptf1a$^+$). PWM, prospective white matter; IGL, internal granular layer; PCL, Purkinje cell layer; EGL, external granular layer

Figure 1
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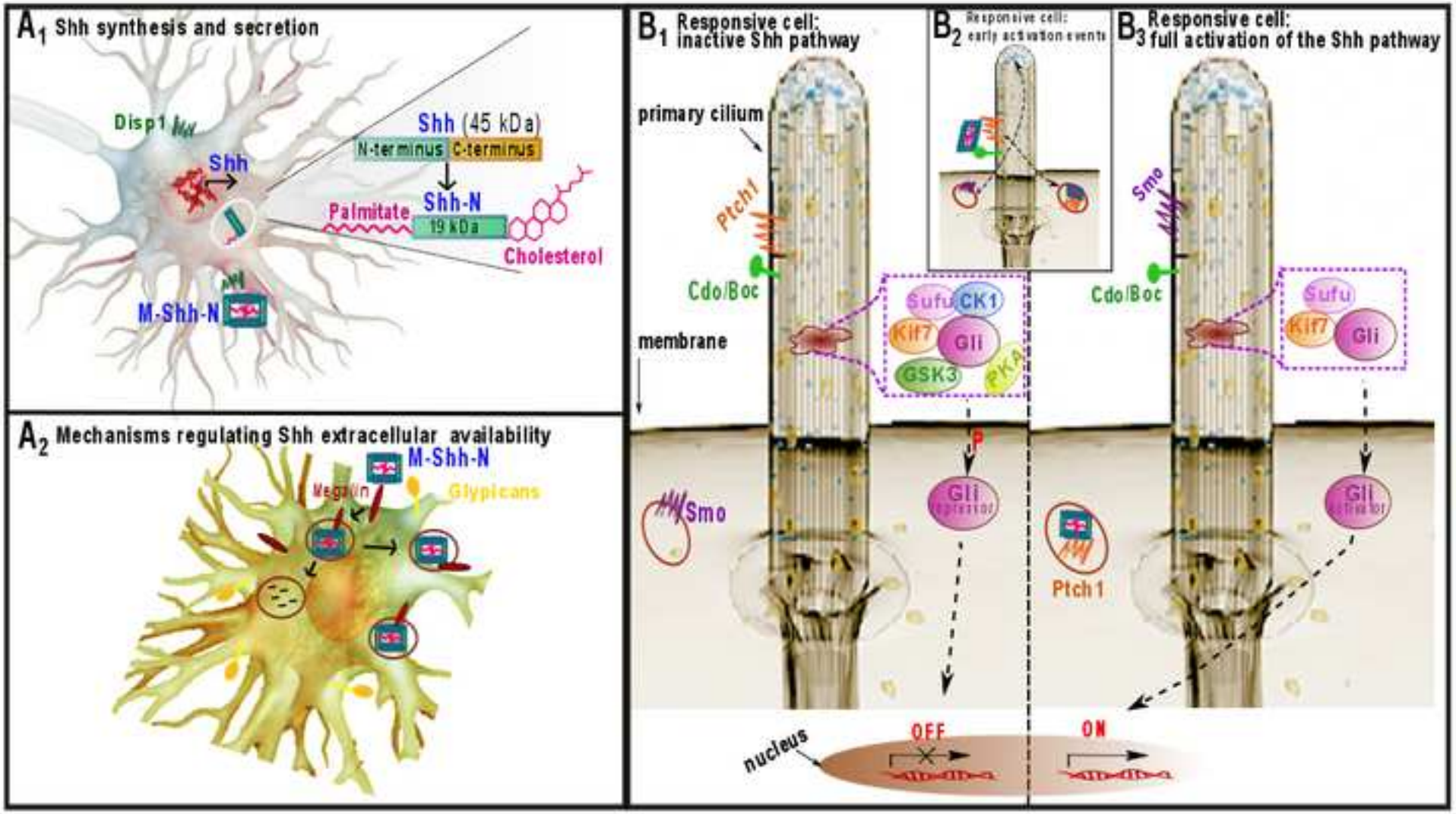

Figure 2
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