Origin, lineage and function of cerebellar glia

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Abstract: The glial cells of the cerebellum, and particularly astrocytes and oligodendrocytes, are characterised by a remarkable phenotypic variety, in which highly peculiar morphological features are associated with specific functional features, unique among the glial cells of the entire CNS. Here, we provide a critical report about the present knowledge of the development of cerebellar glia, including lineage relationships between cerebellar neurons, astrocytes and oligodendrocytes, the origins and the genesis of the repertoire of glial types, and the processes underlying their acquisition of mature morphological and functional traits. In parallel, we describe and discuss some fundamental roles played by specific categories of glial cells during cerebellar development. In particular, we propose that Bergmann glia exerts a crucial scaffolding activity that, together with the organizing function of Purkinje cells, is necessary to achieve the normal pattern of foliation and layering of the cerebellar cortex. Moreover, we discuss some of the functional tasks of cerebellar astrocytes and oligodendrocytes that are distinctive of cerebellar glia throughout the CNS. Notably, we report about the regulation of synaptic signalling in the molecular and granular layer mediated by Bergmann glia and parenchymal astrocytes, and the functional interaction between oligodendrocyte precursor cells and neurons. On the whole, this review provides an extensive overview of the available literature and some novel insights about the origin and differentiation of the variety of cerebellar glial cells and their function in the developing and mature cerebellum.

This is a review article dealing with the development of cerebellar astrocytes and oligodendrocytes and with their cerebellar-specific functions in the adult. Namely, the following points are treated:

1. Origins and differentiation of cerebellar astrocytes
2. Lineage relationships between astrocytes and other neuronal and glial types of the cerebellum
3. Role of radial glia and Bergmann glia in cerebellar corticogenesis
4. Specific role of astrocytes in function of cerebellar circuits
5. Origins and differentiation of cerebellar oligodendrocytes
6. Lineage relationships between oligodendrocytes and other neuronal and glial types of the cerebellum
7. Functional role of cerebellar oligodendrocytes and oligodendrocyte precursor cells

Origin, lineage and function of cerebellar glia

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Abstract

The glial cells of the cerebellum, and particularly astrocytes and oligodendrocytes, are characterised by a remarkable phenotypic variety, in which highly peculiar morphological features are associated with specific functional features, unique among the glial cells of the entire CNS. Here, we provide a critical report about the present knowledge of the development of cerebellar glia, including lineage relationships between cerebellar neurons, astrocytes and oligodendrocytes, the origins and the genesis of the repertoire of glial types, and the processes underlying their acquisition of mature morphological and functional traits. In parallel, we describe and discuss some fundamental roles played by specific categories of glial cells during cerebellar development. In particular, we propose that Bergmann glia exerts a crucial scaffolding activity that, together with the organizing function of Purkinje cells, is necessary to achieve the normal pattern of foliation and layering of the cerebellar cortex. Moreover, we discuss some of the functional tasks of cerebellar astrocytes and oligodendrocytes that are distinctive of cerebellar glia throughout the CNS. Notably, we report about the regulation of synaptic signalling in the molecular and granular layer mediated by Bergmann glia and parenchymal astrocytes, and the functional interaction between oligodendrocyte precursor cells and neurons. On the whole, this review provides an extensive overview of the available literature and some novel insights about the origin and differentiation of the variety of cerebellar glial cells and their function in the developing and mature cerebellum.

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1. Introduction

The anatomical and functional complexity of the cerebellum is reflected not only by the variety of its neuronal phenotypes, but also by a remarkable heterogeneity of astroglial and oligodendroglial cells. Many of these of glial types are also characterised by highly distinctive morphological features and functional properties, which are unique among the glial cells of the entire CNS. Cerebellar astrocytes and oligodendrocytes participate to crucial developmental processes and contribute to regulate physiological function in the mature cerebellum. However, while studies on cerebellar neurons and circuits have progressed during the last decades, less attention has been devoted to investigations on development, lineage, heterogeneity and functions of cerebellar glia. A comprehensive overview covering the actual knowledge on this topic, as well as the most critical issues that await clarification, is missing. Filling this gap is most important in view of the increasing relevance of glia in developmental processes and also to achieve a deeper understanding of the complexity of cerebellar structure and functioning. Here, we provide a critical review of the available data and present some personal views on the development of cerebellar astrocytes and oligodendrocytes. In addition, in order to support the concept that the peculiar features of cerebellar glia are required to carry out particular functional tasks, we discuss about the cerebellar-specific roles played by astrocytes and oligodendrocytes during development and in the operation of mature circuitries.

2. Origins and differentiation of cerebellar astrocytes

2.1. The phenotypic repertoire of cerebellar astrocytes

The glia of the cerebellum was first described and classified by Ramón y Cajal (1911) in three main categories, distinguished by their morphology and position in the cerebellar tissue: i)

the glial cells of the white matter (also including oligodendrocytes), characterized by processes oriented along the direction of axonal tracts, ii) the astrocytes of the granular layer, with star-shaped bushy processes, and iii) the "neuroepithelial cells with Bergmann fibres", featuring cell bodies aligned to Purkinje cell somata and several ascending processes spanning radially the molecular layer, up to their endfeet in contact with the subpial basement membrane. The latter cells were later called "Golgi epithelial cells" (Palay and Chan-Palay, 1974), but they are commonly known as Bergmann glia (BG), and here we shall use this term. This classification of cerebellar astrocytes was essentially confirmed by more recent essays (e.g. Palay and Chan-Palay, 1974; Altman and Bayer, 1997). Nonetheless, by the analysis of Golgi preparations with high-voltage electron microscopy, Chan-Palay and Palay (1972) discovered the veil-like appendages emanating from processes of granular layer astrocytes and BG and described their relationship with the local neuropil, suggesting a role in the compartmentation of the cortical circuitries. Accordingly, the Palays distinguished "velate" astrocytes, including the bushy cells of the granular layer and BG; "protoplasmic" astrocytes, a less represented type with slender processes devoid of lamellar appendages that is encountered in the cortex; and "fibrous" astrocytes, typical of the white matter (Palay and Chan-Palay, 1974). In spite of the morphological variety of the astrocytes that populate the different subdivisions of the mature cerebellum, in the following sections devoted to the ontogenesis of cerebellar astroglia we will exclusively refer to BG and parenchymal astrocytes (the latter comprising all the other categories).

2.2. Origin of cerebellar astrocytes from the ventricular neuroepithelium

Ramón y Cajal (1911) described the origin of cerebellar astrocytes through the comparative investigation of cerebellar development in different species. He concluded that all cerebellar glia derive from the ventricular neuroepithelium (VN) and proposed that BG results from the

retraction of ependymal processes of radial glia of the cerebellar primordium. By the analysis of ^{3}H-thymidine-labelled material, Altman and Bayer (1997) defined the time course of generation of cerebellar glia from the VN of the embryonic cerebellar primordium. These authors assumed that after the completion of Purkinje cell genesis, at E16 in the rat, this germinal layer exclusively gives rise to non-neuronal elements. Therefore, at subsequent developmental stages (from E17 to birth) they identified two main sites of cell proliferation within the VN: a posterior one, located in front of the rhombic lip (RL), and a more anterior one, close to the isthmus and the nascent superior cerebellar peduncle. Altman and Bayer (1997) also noted that the cells that delaminate from the VN continue to divide in the overlying tissue and, hence, first proposed that cerebellar glia may be also generated by progenitors that proliferate within the cerebellar parenchyma. While it is now known that GABAergic interneurons also derive from parenchymal progenitors (Zhang and Goldman, 1996a; Marichich and Herrup, 1999; Leto et al., 2006), anatomical investigations of neurochemically identified glia (Yuasa, 1996, Yamada and Watanabe, 2002) and fate mapping analyses using inducible reporter genes expressed in radial glia essentially confirmed the VN origin of cerebellar astrocytes (Mori et al., 2006; Sudarov et al., 2011).

2.3. Origin of cerebellar astrocytes from the rhombic lip and the external granular layer?

While the VN generates GABAergic neurons and glia, other cerebellar types, and notably all glutamatergic neurons, derive from another germinal site, the RL (Ramón y Cajal 1911; Altman and Bayer, 1997; Machold and Fishell, 2005; Carletti and Rossi, 2008; Hoshino, 2012). The possibility that cerebellar astrocytes may also originate from the RL, and particularly from the RL-derived progenitors that populate the external granular layer (EGL), has been suggested following ^{3}H-thymidine labelling (Altman, 1972) and immunocytochemical visualization of astrocytic markers (Sievers et al., 1994a). These observations, however, have

not been confirmed by several studies using different approaches, including ultrastructural investigation (Swarz and Del Cerro, 1977), quail-chick chimeras (Hallonet et al., 1990, 1993), transplantation of purified EGL or RL cells (Gao and Hatten, 1994; Alder et al., 1996) and retroviral labelling of progenitor cells in the postnatal cerebellum (Zhang and Goldman, 1996a, 1996b). Accordingly, it is now generally accepted that, *in vivo*, EGL progenitors are strictly committed to the granule cell fate and do not contribute to the genesis of cerebellar glia (Gao and Hatten, 1994; Alder et al., 1996; Ben Arie et al., 1997; Machold and Fishell, 2005). Nevertheless, analysis of Math1/Math1-null chimeras, a distinctive marker of the RL-derived glutamatergic neuronal lineages (Hoshino, 2012), suggested that at least some BG cells may originate from RL precursors (Jensen et al., 2004), and the gliogenic potential of RL cells has been also corroborated by experimental manipulation of Notch signalling (Machold et al., 2007). Another report indicated that cells isolated from the outermost layers of the EGL and exposed *in vitro* to Sonic Hedgehog (Shh) and Bone Morphogenetic Protein-2 (BMP-2) acquire astrocytic identities (Okano-Uchida et al., 2004). Finally, it has been recently shown that the EGL contains a population of GFAP-positive precursors that eventually become Math1-positive progenitors and differentiate into granule cells (Sibereis et al., 2010).

On the whole, these findings indicate that RL precursors, together with their EGL derivatives, are not homogeneous, but comprise cells of neuronal and glial lineages (Jensen et al., 2004; Machold and Fishell, 2005). Hence, it is plausible that a minor contingent of cerebellar astrocytes actually derives from RL precursors. On the other hand, the available evidence indicates that, during physiological development, EGL progenitors exclusively generate granule neurons and the possible origin of astrocytes from this germinal layer remains to be unequivocally demonstrated. Indeed, it is not clear whether the GFAP-positive EGL precursors (Silbereis et al., 2010), which may be multipotent *in vitro* (Okano-Uchida et al., 2004), actually differentiate into mature astrocytes *in vivo*.

2.4. Ontogenetic relationships between astrocyte and neuronal lineages

Cerebellar neurons and astrocytes are generated from the same germinal neuroepithelia (Carletti and Rossi, 2008; Hoshino, 2012), indicating that the two lineages might be related. While a common origin of cerebellar neurons and astrocytes has been suggested by some fate mapping studies (Mori et al., 2006; Sudarov et al., 2011), the available clonal analyses are not conclusive. Two reports based on somatic recombination were carried out by examining a neuron-specific reporter (Mathis et al., 1997; Mathis and Nicolas, 2003), which prevented the identification of a shared ancestor with glial types. On the other hand, another study performed using retroviral labelling of E13.5 VN cells exclusively yielded glial clones (Miyake et al., 1995), whose identification and cellular composition were not fully certain.

Further indication that both cerebellar neurons and glia may stem from common ancestors comes from studies of Notch activity in the cerebellar primordium. Notch is expressed in the VN, but not in the RL, starting from E10.5 in the mouse (Machold et al., 2007). Selective Notch ablation in the cerebellar territory results in precocious neuronal differentiation (Lütolf et al., 2002; Machold et al., 2007), leading to rapid exhaustion of the progenitor pool and hypomorphic cerebella (Lütolf et al., 2002). Conversely, constitutive activation of Notch, by retroviral expression of the Notch Intracellular Domain in E9.5 cerebellar progenitors, promotes the generation of astrocytes at the expense of neurons (Machold et al., 2007). In this context, Notch would repress Math1 upregulation in RL progenitors by antagonizing the proneural activity exerted by BMPs released by the roof plate. It is still unknown whether a similar mechanism also applies for VN progenitors. However, the ablation of Bmi1, a component of the polycomb repressor complex that reduces gliogenesis through inhibition of BMPs (Zhang et al., 2011), results in reduced numbers of interneurons (and oligodendrocytes) and concomitant increased numbers of astrocytes (Van

der Lugt, 1994; Jacobs et al., 1999; Zhang et al., 2011). Taken together, these findings suggest that Notch acts on the BMP pathway to regulate a switch between neuronal and astroglial fates in multipotent progenitors of the cerebellar primordium.

The germinal neuroepithelia of the cerebellar primordium can be subdivided in a number of discrete domains, identified by distinctive distribution patterns of transcription factors (Chizhikov et al., 2006; Zordan et al., 2008; Hoshino, 2012). Notably, while Math1-expression defines the RL (also called C1 domain), Ptf1-a corresponds to the VN (the C2 domain; two others domains, C3 and C4, have been identified, whose descendants are still uncertain; Chizhikov et al., 2006). The latter region is further characterized by the discrete distribution of Neurog1, Neurog2 and Ascl1 (also known as Mash1; Zordan et a., 2008).

Fate mapping analysis of Ptf1-a derivatives with Ptf1-a$^{cre/+}$/R26R mice shows that, in addition to GABAergic neurons, the reporter gene is also expressed by some astrocytes, suggesting that the latter lineage may also stem from the Ptf1a-expressing precursors (Hoshino et al., 2005). Similarly, fate mapping of Neurog2 derivatives also includes a small number of astrocytes (Florio et al., 2012). However, the occasional frequency of astroglia observed in these studies indicates that the bulk of cerebellar astrocytes do not derive from these cell populations. On the other hand, glial cells may be rare in the progeny of Neurog2 or Ptf1a-expressing precursors, because these markers are related to neuronal specification of VN progenitors.

Ascl1 is also transiently expressed in the VN between E10.5 and E13.5. Its distribution overlaps that of Ptf1a and of the Neurogenins, but it is mutually exclusive with Math1 (Kim et al, 2008; Zordan et al., 2008). In addition, Ascl1 is expressed during late embryonic and postnatal development in two non-overlapping cell populations of the prospective white matter (PWM), distinguished by the expression of Olig2 or Pax2 (Grimaldi et al., 2009). A recent fate mapping study, using tamoxifen-inducible GFP under control of the Ascl1 promoter, indicates that multiple lineages derive from Ascl1 expressing cells (Sudarov et al.,

2011). Notably, concerning glial types, BG are labelled when tamoxifen is administered at E13.5, whereas at later times (between E14.5-P7) GFP-positive cells include different fractions of parenchymal astrocytes and oligodendrocytes. On the contrary, other studies describing the derivatives of Ascl1-expressing cells reported GABAergic neurons and oligodendrocytes, but not astrocytes (Kim et al., 2008; Grimaldi et al., 2009). Indeed, Ascl1 ablation results in reduced quantities of GABAergic interneurons and oligodendrocytes, whereas astrocytes are increased (Grimaldi et al., 2009; Sudarov et al., 2011). In addition, overexpression of Ascl1 in VN cells, by intraventricular injection and electroporation of an expression vector at E14.5, results in enhanced generation of Pax2-positive interneurons at the expense of GFAP-expressing astrocytes (Grimaldi et al., 2009). Taken together, these results suggest that Ascl1 regulates fate choices in bipotential progenitors of the VN (and plausibly of the PWM, see below section 5.1) inducing their commitment towards the neuronal lineage, while suppressing their astrocytic specification (Grimaldi et al., 2009).

On the whole, the available evidence suggests that cerebellar neurons and astrocytes derive from common progenitors residing in the VN, and possibly also in the RL. However, most of the transcription factors so far identified in these germinal layers are related to neuronal specification and, not surprisingly, they are not suitable to distinguish the gliogenic progenitors. Thus, while the multipotent precursors remain to be identified, it is plausible that proneural genes act by suppressing default gliogenic differentiation programs in these cells (Bertrand et al., 2002).

2.5. Lineage relationships between neurons and astrocytes in the cerebellar prospective white matter

A salient feature of cerebellar development is that dividing progenitors leave the primary neuroepithelia and populate secondary germinal sites: the EGL, formed by RL-derived

precursors, and the PWM, containing progenitors that delaminate from the VN or immigrate from extracerebellar sources (Carletti and Rossi, 2008; Mecklenburg et al., 2011; Hoshino, 2012). While EGL cells appear committed to become granule neurons, the PWM generates multiple phenotypes, including GABAergic interneurons, astrocytes and oligodendrocytes. The cellular composition of the PWM is not homogeneous and includes several subsets of progenitor cells (Milosevic and Goldman, 2002; Leto et al., 2010), whose developmental properties are still scarcely characterized. There is evidence indicating that specification of interneuron subtypes also occurs in the PWM (Leto et al., 2006, 2009). On the other hand, scant information is available about the mechanisms underlying neuronal vs glial specification as well as the generation of the variety of astrocyte phenotypes. In this respect, it is important to establish whether the PWM progenitors are multipotent cells that make their final fate choices *in loco* or they are already fate-restricted at the time when they leave their native neuroepithelium.

A few years ago, two parallel studies reported the isolation of stem cells from the postnatal cerebellar parenchyma (Lee et al., 2005; Klein et al., 2005). In the postnatal PWM, Lee et al. (2005) identified a population of cells, characterized by the expression of prominin/CD133, whose proliferation was stimulated by the basic Fibroblast Growth Factor (bFGF). These cells formed neurospheres and differentiated into neurons, astrocytes and oligodendrocytes both *in vitro* and following transplantation to newborn mice. Klein et al. (2005) isolated neurosphere forming cells from the embryonic (E14.5), perinatal and adult mouse cerebellum. These cells also produced different types of neurons and astrocytes both *in vitro* and following transplantation to P4 cerebella. While these studies suggested that multipotent progenitors reside in the postnatal cerebellar parenchyma, they did not define whether and how these cells contribute to the generation of different mature phenotypes during physiological development.

Maricich and Herrup (1999) reported that Pax2 is expressed in proliferating progenitors of GABAergic interneurons in the PWM. Later, however, it was shown that Pax2 is upregulated at the time of the last mitosis (Weisheit et al., 2006; Leto et al., 2009) and, hence, this marker essentially identifies postmitotic interneurons. Accordingly, Pax2-expressing cells exclusively differentiate into inhibitory interneurons and do not contribute to any other phenotype (Maricich and Herrup, 1999; Leto et al., 2009). Another study suggested that CD44-positive cells that reside in the postnatal PWM are fate-restricted precursors of the astrocyte lineage (Cai et al., 2012). These cells express markers of juvenile astrocytes BLBP, GLAST and vimentin, but not GFAP or S100, which are featured by mature astroglia (but see Silbereis et al., 2009, 2010). *In vitro*, the CD44-positive cells proliferate following stimulation with bFGF, but fail to form neurospheres and exclusively differentiate into astrocytes. Their developmental potential *in vivo* has not been investigated.

These studies indicate that the PWM contains distinct pools of fate-restricted precursors committed towards neuronal or astrocytic phenotypes. Other reports, however, suggest that more strict relationships link the two lineages in this germinal niche. Silbereis et al. (2009) examined GFAP-CRE::ErT mice, in which the expression of the GFP reporter can be selectively induced in GFAP-expressing cells by tamoxifen administration. Two days after reporter induction at P5, GFP highlights a population of proliferating cells expressing astrocyte markers, such as S100, nestin, BLPB, Sox2, plus occasional elements displaying interneuron or oligodendrocyte traits. The frequency of GFP-tagged interneurons, however, progressively increases during the following days and, at longer survival times, GFP-positive cells comprise significant fractions of astrocytes (BG and parenchymal astrocytes) and GABAergic interneurons (basket and stellate cells). The neurogenic activity of GFAP-positive precursors ceases at more advanced ages and induction at P12 exclusively yields astroglia. Taken together, these findings indicate that cerebellar astroglia and GABAergic interneurons

derive from progenitor cells that proliferate in the PWM and are distinguished by the expression of astrocyte lineage markers (GFAP, Sox2, BLBP, nestin). However, it remains to be established whether these cells are true astrocytes or represent a population of proliferating progenitors that express astrocytic traits, as shown for stem cells in several CNS regions (Deutsch, 2003). In addition, it is not clear whether both interneurons and astrocytes derive from a single population of multipotent progenitors or from distinct pools of fate-restricted precursors (Fig. 1). Analysis of Ascl1 expression, ablation and overexpression in developing cerebellum suggests that fate choice between neuronal and astroglial identities occurs in the postnatal cerebellar PWM (Grimaldi et al., 2009). On the other hand, clonal analysis of postnatal cerebellar progenitors *in vitro* yielded a majority of mono-lineage clones and a minority of mixed neuronal-glial clones (Milosevic and Goldman, 2004). It is difficult to relate the latter findings to the situation *in vivo*, where a small subset of multipotent precursors may generate fate-restricted progenies that are amplified to produce the relevant phenotypes in due quantities. Therefore, further (clonal) analysis *in vivo* is definitely required to clarify these issues.

2.6. Generation of the variety of cerebellar astrocyte phenotypes

Birthdating studies consistently indicate that most cerebellar astrocytes are generated during late embryonic and postnatal development (Miale and Sidman, 1961; Altman and Bayer, 1997; Sekerková et al., 2004). Notably, a BrdU pulse to the postnatal cerebellum clearly defines three discrete sites of cell proliferation: the EGL, the PWM and a thin cellular rim at the interface between granular and molecular layers, where juvenile BG divide (Yuasa, 1996). Not considering the possible origin of glial cells from the EGL (Silbereis et al., 2010), both PWM progenitors and BG may be the source of different types of cerebellar astrocytes. The classical view (Ramón y Cajal, 1911; Rakic, 1984; Yuasa, 1996; Altman and Bayer, 1997;

Yamada and Watanabe, 2002) posits that BG derives directly from radial glia, through the retraction of ependymal processes, the progressive displacement of the cell body towards the cortex and the maintenance of basal processes abutting the pial surface (see below). On the other hand, parenchymal astrocytes are thought to be generated by local progenitors that emigrate from the VN into the PWM during embryonic life (Altman and Bayer, 1997; Yamada and Watanabe, 2002).

Although these processes and the underlying mechanisms have not been directly demonstrated, the available evidence suggests that proliferating BG and PWM cells represent different progenitor pools with distinct potentialities and fates. This idea is also supported by the differential cycling rhythms of BG and PWM cells (our unpublished observations), and by the complementary distribution of cyclinD1 and cyclinD2 (Leto et al., 2011). In the embryonic cerebellar primordium, cyclinD1 is expressed by VN cells, whereas cyclinD2 is observed in the overlying parenchyma. After birth, cyclinD1 is expressed in BG, whereas cyclinD2 is present in PWM progenitors. Finally, while bFGF promotes PWM cell proliferation (Lee et al., 2005), very little is known on the regulation of the expansion of BG. It associates with the enlargement of the granule cell pool (Shiga et al., 1983a). However, Sonic hedgehog (Shh), the main morphogen orchestrating granule cell proliferation, does not appear to affect BG mitoses (Dahmane and Ruiz y Atalba, 1999).

In other CNS territories, such as spinal cord and forebrain, positional identities and subtype heterogeneity of astroglial subsets appear determined by different sites of origin in the VN (Tsai et al., 2012). Moreover, there is evidence that the transcriptional code utilised by ventricular progenitors to specify spinal cord neuronal identities is employed later in development to determine astroglial subtypes (Hochstim et al., 2008). Whether the categories of cerebellar astrocytes are also derived from distinct domains of the germinal niches is unknown. Similarly, it remains undefined whether discrete transcriptional combinations in

VN or PWM progenitors produce specific astroglial cerebellar subtypes, and whether so-far-undetermined transcription factors identify multipotent progenitors at the origin of both neuronal and astroglial lienages.

Taken together, these observations indicate that BG and parenchymal astrocytes share common ancestors in the VN, but have distinct natural histories. BG initially derives from the morphological transformation of radial glia and later proliferate to match the concomitant expansion of the cerebellar cortex (Fig. 2). On their side, parenchymal astrocytes derive from the mitosis of radial glia, whose daughter cells emigrate into the PWM, where they continue to divide and eventually acquire mature identities of white matter or cortical astrocytes (Fig. 2). Nevertheless, it cannot be excluded that dividing BG in the postnatal cerebellum also contribute to other astrocyte populations (Miyake et al., 1995; Yuasa, 1996) and, viceversa, that PWM progenitors can also differentiate into BG (Fig. 2). This possibility has still to be tested and, in fact, the actual developmental potential of dividing BG and PWM precursors has to be determined. Further studies are definitely warranted to elucidate these points.

2.7. *Astrocyte differentiation*

In early co-culture studies Mary Hatten (Hatten, 1985) and Melitta Schachner (Nagata et al., 1986) independently showed that the morphology, antigenic profile and proliferation of cerebellar astroglia are strongly dependent on influences exerted by cerebellar neurons. When added to the cultures, cerebellar neurons reduced astroglial proliferation, induced polarised BG-like or multipolar shapes instead of flattened morphologies, and down-regulated specific antigens, revealing that neurons are key regulators of cerebellar astroglial differentiation. Over time, several of the underlying cellular and molecular mechanisms have been elucidated. Yet, the available evidence almost exclusively concerns BG differentiation, while the processes of maturation of parenchymal astroglia remain undetermined.

After withdrawal of their apical process and displacement of the cell bodies toward the cerebellar cortex, radial glial cells undergo morphological changes and proliferate to produce BG. Differently from radial glia that possesses a single basal branch, BG cells exhibit multiple ascending processes (usually three to six per cell) crossing the molecular layer and forming palisades parallel to the long axis of the folium. In the rat, the multiple fibres emerge after birth, increase in number until the end of the first postnatal week and then decrease, in parallel with the expansion and reduction of the EGL (Shiga et al., 1983b), suggesting that granule cells contribute to regulate BG process formation. At early maturation stages BG fibres are rather smooth with beady enlargements and tiny bud-like excrescences that progressively grow to bushy expansions covering most of the radial process (Shiga et al., 1983b) and establishing tight interactions with Purkinje cell synapses (see section 3.6.).

Available evidence shows that crucial factors for the correct occurrence of these morphogenic changes reside in the interaction of juvenile BG with three components of the cerebellar milieu: the subpial basement membrane, Purkinje neurons and granule cells. Essential for BG polarization, process outgrowth and layering is the tight anchorage of BG endfeet to the basement membrane lying on the surface of the cerebellar cortex, which is deposited by meningeal elements during early development (Sievers et al., 1994b), and actively maintained and remodelled by neural cells (Graus-Porta et al., 2001). If endfoot anchorage is defective, as a consequence of abnormal membrane composition or altered functioning of BG, fibre formation and orientation are perturbed and BG cell bodies translocate to the molecular layer, thereby severely disrupting cerebellar foliation and layering (see section 3.2.). Mechanistically, several receptors and intracellular transduction pathways expressed by BG are implicated in the correct adhesion to the basement membrane (Fig. 3A,C). They include β1-integrins (Graus-Porta et al., 2001, Frick et al., 2012), the integrin-linked kinase ILK (Belvindrah et al., 2006), the Abl family of tyrosine kinases (Abl and Arg; Qiu

et al., 2010), as well as ric-8a (a guanine nucleotide exchange factor in the G-coupled receptor pathway; Ma et al., 2012). These molecules are associated with the intracellular domain of β1-integrin and provide link with rho-type GTPases, such as CDC42 (Belivindrah et al., 2006), to regulate F-actin assembly at the tips of outgrowing BG processes.

A generalized disruption of BG endfeet anchoring as well as disorganised and truncated BG fibres have been reported also following ablation of α-dystroglycan (Moore et al., 2002; Satz et al., 2008) or dystroglycan-glycosiltransferase (Qu and Smith 2005), both expressed by BG, indicating that the dystrophin-dystroglycan complex is another player in the regulation of BG maturation through the interaction with the subpial basement membrane. The analogous phenotypes induced following genetic ablation of components of either $\beta 1$ integrin o α-dystroglycan signalling cascades imply that both pathways are necessary for the accomplishment of a critical ontogenetic step. These receptors, expressed on the BG membrane, mediate a bidirectional interaction with components of the extracellular matrix (most likely laminin; Fig. 3A,C), which is required both to stabilize the subpial basement membrane and to regulate the growth of Bergmann fibres, the formation of the endfeet and their anchoring to the basement membrane.

In agreement with a general mechanism where neuron-derived signals contribute to astrocyte differentiation, Notch in BG (Stump et al., 2002) controls the formation of radial fibers and BG layering by interacting with the ligand Delta/Notch-like EGF-related receptor DNER present in Purkinje cells (Eiraku et al., 2005). Experimental DNER deletion or γ-secretase and Deltex-dependent activation of Notch signalling indicate that DNER promotes the formation of multiple processes and contributes to proper positioning of BG cell bodies (Eiraku et al., 2005). Yet, the aberrant phenotype induced by experimental DNER deletion appears compensated after the third week of life, suggesting that DNER action is transient, and that additional Notch ligands present in the BG surroundings vicariate DNER functions.

Notably, the canonical ligand Jagged1 is broadly expressed in the postnatal cerebellum by both granule cells and BG itself (Stump et al., 2002). Jagged1 deletion from neuroepithelial progenitors at the midbrain-hindbrain boundary results in loss of BG contact with the pial surface, truncated fibers and reduction in BG number with no effect on monolayer formation (Weller at al., 2006). Clear defect in BG monolayer formation accompanied by impaired differentiation of BG fibres, especially evident after P14, have been also observed upon conditional deletion of Notch1, 2 or the downstream target RBPJ in precursor cells with active human (h)GFAP promoter (Komine et al., 2007). Interestingly, similar phenotypes occur after ablation of the endoribonuclease Dicer1, which also interferes with Notch1 signalling (Kuang et al., 2012). Taken together, these findings show that multiple and partially redundant signals issued by neighbouring neurons (Purkinje and granule cells), and possibly by BG itself, activate the Notch pathway in BG to promote various cell maturation processes, such as fibre formation and BG layering.

Purkinje cells also promote BG differentiation by releasing Sonic hedgehog (Shh; Dahmane and Ruiz y Atalba, 1999). However, despite its strong proliferative effects on granule cell precursors, Shh does not appear to influence the expansion of BG. Further differentiation-promoting signals are issued by granule cell precursors via the neuregulin1 (NRG) pathway and secretion of FGF9. *In vitro* observations show NRG interacts with the tyrosine kinase receptor erbB exposed by BG to induce the acquisition of polarised morphologies in cerebellar astrocytes (Rio et al., 1997). Notably, Notch has been proposed to act synergistically with NRG by upregulating the expression of ErbB and thus enhancing the response to NRG signals (Schmid et al., 2003; see section 3.5 for the role of NRG signalling in granule cell migration along BG).

Genetic manipulations and *in vitro* approaches showed that FGF9 is secreted by granule neurons and acts on the FGFR1 and FGFR2 in BG to control fibre formation and

marker expression (Lin et al., 2009). In addition to morphological abnormalities, reduction in BG number has been reported upon double FGFR1 and 2 deletion in hGFAP positive cells, likely due to a decreased number of embryonic progenitors (Müller Smith et al., 2012). It has been proposed that the Notch signalling may interact with the FGF pathway (Yoon et al., 2004) to promote the differentiation of radial glia from neuroepithelial cells and that it may act upstream of FGF9 in BG (Lin et al., 2009). Interestingly, both *in vivo* and *in vitro* evidence indicates that ablation of FGF9 signalling results in star-shaped multipolar astroglia (Lin et al., 2009), suggesting that this factor participates in the fate choice between BG (or radial glia) and multipolar stellate astrocytes.

Few cell-intrinsic determinants are known that take part in cerebellar astroglial differentiation. The SRY-box (Sox) transcription factors C family member including Sox4 is transiently expressed in immature neuronal and glial neural precursors of the CNS subventricular zones prior to terminal differentiation (Hoser et al., 2007). When Sox4 protein was overexpressed under the activity of the hGFAP promoter, radial glia appeared normal but BG maturation was defective and was accompanied by architectural defects and ataxia. Conversely, multipolar astrocyte morphologies did not appear to be affected. Thus, the correct maturation of the BG monolayer requires Sox4 down-regulation, suggesting that this transcription factor interferes with the molecular cascades implicated in BG differentiation.

An opposite function has been unveiled for the phosphatase and tensin homolog PTEN and the ubiquitin ligase Huwe1. PTEN deletion in hGFAP-positive cells induces the disappearance of glia with the typical BG morphology, revealing that active PTEN signalling is intrinsically required for correct BG differentiation and maintenance of a polarized phenotype (Yue et al., 2005). In its absence, only astrocytes with a star-shaped multipolar morphology are present. It is thus plausible that the PI3K/AKT pathways, normally antagonised by PTEN, actively promote the acquisition of multipolar phenotypes in astroglial precursors at the

expenses of the polarised morphology of BG. Ablation of the ubiquitin ligase Huwe1, that acts on oncoproteins such as N-Myc, leads to misaligned BG and abortive formation of radial fibres that often lack contact with the pial surface (D'Arca et al., 2010). However, no clear transition to a multipolar stellate morphology occurs as for PTEN deletion. Further, HUWE ablation also reduces the global number of GFAP expressing astrocytes in the white matter, indicating that this pathway may have a general positive function in astroglial differentiation, in line with general prodifferentiative properties of this tumor suppressor. Another tumor suppressor gene, the adenomatous polyposis coli (APC), appears instead implicated in the active maintenance of BG morphology. After APC deletion in GFAP expressing cells, BG differentiation proceeds normally during the first postnatal week, but at later stages BG cell bodies translocate to the molecular layer, then lose contacts to the pial surface and concominantly acquire a stellate morphologies (Wang et al., 2011). In line with loss of inhibition of the Wnt pathway, these changes are accompanied by progressive beta-catenin accumulation specifically in BG. Accordingly, specific activation of the Wnt pathway in glial cells in postnatal cerebellar explants resulted in defects of glial fiber outgrowth and favoured reactive phenotypes (Pöschl et al., 2013). Thus, the BG phenotype not only has to be actively promoted after birth, but also actively maintained, at least at juvenile ages. Importantly, the effects of APC deletion exhibit both cell type and regional specificity among GFAP-positive astroglia, highlighting astroglia heterogeneity.

On the whole, available data indicate that BG morphogenesis requires tight and timely–regulated interactions with the surrounding cerebellar microenvironment. Impairment of these regulatory mechanisms results in the acquisition a stellate morphology, which may thus represent a default differentiation pathway for cerebellar astroglial precursors. Yet, it is likely that the fine refinement of the variety of stellate morphologies is tuned by the interplay with local cues.

3. Function of Bergmann glia during cerebellar corticogenesis

During CNS development, radial glia exerts a dual function as a progenitor for neurons and glia and as a structural scaffold to guide migration and homing of postmitotic cells. Radial glia of the cerebellar primordium makes no exception to this rule. In addition, however, in the context of cerebellar corticogenesis radial glia (and its derivative BG) acquires another fundamental role, essential for the correct patterning of cortical foliation and layering.

Radial glia follows a peculiar evolution during cerebellar development. The original radial glia cells, which span the entire width of the embryonic cerebellar anlage, undergoes a progressive morphological transformation into BG (Ramón y Cajal, 1911; see above sections 2.6, 2.7). This evolution of radial glia reflects the two fundamental steps of cerebellar corticogenesis: the formation of the Purkinje cell plate and the ensuing expansion of granular and molecular layers, due to migration and maturation of granule cells and inhibitory interneurons. Throughout these developmental phases, radial glia and BG provide trails to guide the migration of different cell types, but also regulate the directional elongation of axons and dendrites.

3.1. Radial glia and the formation of the Purkinje cell plate

The main classes of cerebellar projection neurons, Purkinje cells and deep nuclear neurons, are generated at the outset of cerebellar neurogenesis (Miale and Sidman, 1961; Altman and Bayer, 1997; Calretti and Rossi, 2008). Nuclear neurons are born first in the RL and follow a tangentially-oriented subpial migratory route to aggregate at the nuclear transitory zone, before attaining their final position (Altman and Bayer, 1997; Sotelo, 2004). Purkinje cells, which are born slightly later, initiate cortical morphogenesis by moving radially from the VN

towards the pial surface, where they gather into a several cell-thick layer, the Purkinje cell plate (Fig. 4A,B; Altman and Bayer, 1997; Sotelo and Rossi, 2012). The classical view posits that Purkinje cell migration is regulated by attractive cues issued by overlying structures combined with physical trailing provided by the radial glia scaffold (Sotelo and Rossi, 2012). Purkinje cells navigation towards the nascent cortex and the subsequent arrangement into a monolayer are regulated by reelin, released first by nuclear neurons in the nuclear transitory zone, and then by granule cell progenitors in the EGL (Miyata et al., 1996; Schiffmann et al., 1997). Migrating Purkinje cells are physically apposed to radial glia processes (Rakic and Sidman, 1970; Yuasa et al., 1991, 1996), and a number of adhesion molecules have been suggested to mediate their guidance and targeting to specific cortical domains (Yuasa et al., 1991; Hatten, 1999; Redies et al., 2011). However, the molecular mechanisms underlying these processes still remain largely unexplored. In addition, a recent study showed that Purkinje cells originating from the posterior regions of the cerebellar anlage initially follow a tangential migratory route and appear to interact with BG only during the last phases of their homing in the cortex (Miyata et al., 2010). It is not clear whether all Purkinje cells may share this behaviour. If so, however, the essential role of the glial scaffold might be restricted to the formation of the Purkinje cell monolayer.

All the phases of cerebellar corticogenesis are characterized by complex interactions between Purkinje cells and BG that reciprocally orchestrate their harmonic maturation (e.g. Dahmane and Ruiz i Altaba, 1999; Eiraku et al., 2005). In this context, the transformation of radial glia into BG lags Purkinje cells migration (Fig. 4B,C). Notably, migrating glial cell bodies never exceed the front of migrating Purkinje cells (Yamada and Watanabe, 2002), suggesting that the two processes are interdependent and that young Purkinje cells may regulate the morphological transition of the radial glial scaffold. This point, however, remains to be investigated.

3.2. Role of BG in foliation and layering of the cerebellar cortex

After the formation of the Purkinje cell plate and the appearance of BG, cerebellar corticogenesis progresses through two parallel processes, foliation and layering, both of which require guidance provided by the glial scaffold (Fig. 4C,D). The most comprehensive model of cerebellar foliation posits that localized changes in the behaviour of granule cell progenitors, at precise spots along the external granular layer, induce the anchoring of nearby Purkinje cells to the underlying white matter, thus defining the base of the fissures (Sudarov and Joyner, 2007). From such "anchoring centres", the expansion of folia progresses through the coordinated action of maturing granule cells and BG. At anchoring centres Bergmann fibres converge towards a single point assuming a fan-like arrangement. This orientation of the glial scaffold, in turn, directs the distribution of migrating granule cells all around the base of the fissure, thus determining the initial folding of the cortex. The outgrowth of the folium apex then proceeds in a self-sustained manner, also including addition of newly-born BG, required to match the tangential expansion of the cortical mass.

BG is necessary to initiate the foliation process, which is abortive if the radial glia to BG transition is prevented by interfering with the execution of the cell-intrinsic ontogenetic program (Hoser et al., 2007). On the other hand, foliation occurs if astrocytes are selectively ablated just after birth (P1, Delaney et al., 1996). In this condition, however, cortical layering is severely disrupted, with significant loss and ectopia of granule cells. This phenotype is progressively less pronounced if astrocytes are killed at more advanced ages during the first postnatal week (P3 and P5). Together, these observations indicate that BG is required to initiate the process of foliation, whereas later it is essential to develop the normal layering.

The role played by BG in cerebellar corticogenesis is more complex than just guiding directional migration of granule cells. A critical step is the development of glial endfeet and

their anchorage to the basal membrane at the pial surface, to form the glia limitans. This process appears to be mediated by reciprocal interactions with components of the extracellular matrix and of the basement membrane (see section 2.7). Disruption of this interplay leads to a characteristic phenotype (Fig. 3B,D), in which: i) the folia are formed but fuse to each other along the cortical surface; ii) granule cells are decreased in number and remain ectopically located along the fused folia and in the molecular layer; iii) Purkinje cells are preserved, but the final monolayer arrangement is not achieved. These features are a consequence of defective anchoring of BG endfeet to the subpial basement membrane. In this condition, the extracellular matrix is disorganized and the glial scaffold is not properly assembled. As a consequence, adjacent folia fuse along the pial surface, while young granule cells fail to migrate radially through the cortex and eventually die or remain ectopically positioned. The final outcome is the disrupted cortical layering and the misalignment of Purkinje cells (Fig. 3B,D). In most reports, neuronal abnormalities appear consistently secondary to glial defects (see section 2.7.), although there is evidence that some of the involved factors may also play a direct role in neuronal development (e.g. ILK, Mills et al., 2006).

3.3. BG and the migration of granule cells

The function of BG as scaffold for granule cell migration is probably the first example of glial-guided neuronal migration (Rakic, 1971), which has been extensively studied over the last decades. A comprehensive review of the literature on the mechanisms underlying the radial migration of granule cells is beyond the scopes of this article and can be found elsewhere (Hatten 1999, 2002; Chédotal, 2010; Solecki, 2012). Here, we will briefly discuss the reciprocal crosstalk between granule neurons and Bergmann fibres during the migratory process.

The interaction of migrating granule cells with BG consists of two distinct phases: granule cells first induce guidance properties in BG (Hatten, 1985; Rio et al., 1997) and, then, specific recognition and adhesion mechanisms regulate cell navigation along glial axes (Hatten, 1999; Solecki, 2012). The regulation of scaffolding properties of BG will be treated in section 3.5, whereas here we will focus on cell migration. A number of molecules have been identified that mediate this process, including astrotactin1 and 2 (Edmondson et al., 1988; Fishell and Hatten 1991; Wilson et al., 2010), NRG (Rio et al., 1997), and profilin1 (Kullmann et al., 2011; Rust et al., 2012). On the contrary, other cell-adhesion molecules, such as β1-integrin, L1, N-CAM or TAG-1 are not directly involved (Fishell and Hatten, 1991; Belvindrah et al., 2007; Frick et al., 2012).

Astrotactin1 was discovered by examination of cerebellar microcultures, where it was shown that, following application of neutralizing antibodies against this protein, granule cells were unable to take contact with BG and to stimulate the elongation of glial processes (Edmondson et al., 1988). Further *in vitro* analysis led to identify the astrotactin1 gene and demonstrated its prominent role, among other adhesion molecules, in glial-guided navigation of granule cells (Fishell and Hatten, 1991; Zheng et al., 1996). The generation of astrotactin1 null mice revealed reduced adhesion of granule cells to astroglia, leading to significant delay of granule cell migration, associated with moderate alterations of cerebellar cortical cytoarchitectonics (Adams et al., 2002). More recently, a new member of the astrotactin family, astrotactin2, has been identified (Wilson et al., 2010). Astrotactin2 is also expressed in granule cells and forms complexes with astrotactin1, to regulate the placement of the latter protein on the neuronal membrane. Live imaging experiments show that astrotactin1 undergoes a cyclic process of internalization and exposure on the cell surface, concomitant with the forward advancement of the cell body and the formation of new contacts along the glial axis. These findings reveal a mechanisms underlying directional navigation of the

migrating neuron, in which receptor trafficking and recycling continuously generate new adhesions at the front of the advancing cell (Wilson et al., 2010).

Selective ablation of profilin1 in the CNS results in disrupted cerebellar cortical layering, due to severe impairment of radial migration of granule cells (Kullmann et al., 2011). Profilin1 is expressed by both granule cells and BG and mediates homophilic adhesion between the two cells, which is needed for proper formation and functioning of specialized junctions (Ziegler et al., 2006). *In vitro* assays show that profilin1 expression is necessary on both neuronal and glial partners. Disruption of this interaction reduces granule cell adhesion to glial processes. As a consequence, migration speed is reduced, granule neurons fail to complete their way to the internal granular layer, and remain ectopically positioned in the molecular layer (Kullmann, 2011).

On the whole, these findings indicate that the role of BG in granule cell migration is to provide an adhesive substrate and a conducive scaffold. Notably, specific recognition and contact with a defined set of adhesive molecules appears to be crucial to guide the migrating cell, but also to guarantee the specificity of the migratory route and the direction of navigation. In addition, the speed of migratory movements, which is related to the efficiency of adhesion mechanisms (e.g. Adams et al., 2002; Kullmann et al., 2011), is another crucial parameter. Granule cell migration occurs within a defined ontogenetic time-window, regulated by an intrinsic clock (Yacubova and Komuro, 2002; Komuro et a., 2012). Reduced migratory speed determines the premature arrest of migration, leading to granule cell ectopia in the molecular layer.

3.4. Bergmann glia and the migration and homing of molecular layer interneurons

Following the discovery that molecular layer interneurons do not originate from the external granular layer, but derive from VN progenitors that continue to divide in the PWM (Hallonet

and Le Douarin, 1993, Zhang and Goldman 1996a; Maricich and Herrup, 1999), it has been proposed that young basket and stellate cells migrate tangentially up to the edge of the EGL and become progressively integrated in the expanding molecular layer (e.g. Yamanaka et al., 2004). While a purely radial trajectory may be consistent with BG-guided navigation, time-lapse video microscopy has revealed that the migration of molecular layer interneurons is actually more complex and involves multiple radial and tangential phases (Cameron et al., 2009). Accordingly, it has been proposed that migrating interneurons actually follow bundles of ascending granule cell axons and parallel fibres (Cameron et al., 2009). Direct apposition of migrating interneurons to BG has been observed both *in vivo* (Guijarro et al., 2007; Simat et al., 2007) and in cerebellar explants *in vitro* (Guijarro et al., 2007). Hence, it is likely that young interneurons follow Bergmann fibres along their way from the white matter towards the EGL. However, it is still uncertain as to whether this is an exclusive route and if the different phases of interneuron migration are regulated by interactions with different cell types in the molecular layer.

BG glia also exerts a prominent role in the differential guidance and targeting of basket and stellate axons. Basket axons grow along Purkinje cell dendrites and somata attracted by a gradient of neurofascin 186, a member of the L1 family of cell adhesion molecules, distributed along the soma and the initial segment of the Purkinje axon (Ango et al., 2004). On their hand, stellate axons and dendrites elongate following the BG scaffold and preferentially form synapses at intersections between Bergmann fibres and Purkinje dendrites (Ango et al., 2008). This guidance is mediated by another member of the L1 family, the Close Homologue of L1 (CHL1), which is expressed in BG and stellate cells during postnatal cerebellar development. Analysis of CHL1-null mice reveals aberrant growth of stellate axons and reduced numbers of GABAergic synapses on Purkinje cell dendrites with signs of synaptic degeneration (Ango et al., 2008). This phenotype is essentially reproduced following selective

ablation of CHL1 in BG. Therefore, BG provides a physical scaffold, which also bears type-specific recognition cues to direct the targeting of stellate axons towards defined domains of the Purkinje cell dendritic tree.

3.5. Regulation of the scaffolding properties of Bergmann glia

The ability of radial glia for sustaining neuronal migration is transient and, at the end of migratory processes, radial glia turns into mature astrocytes. The scaffolding phenotype is actually induced, or re-induced, and maintained by migrating neurons themselves, who regulate the expression of a specific set of molecules, needed to establish contact and to direct navigation (Hunter and Hatten, 1995; Hatten, 1999; Sotelo, 2004). One crucial aspect of this process concerns specificity. In order to reach their appropriate destinations, different types of neurons should be able to recognize and selectively interact with specific subsets of radial glia. Nevertheless, *in vitro* assays for gliophilic migration indicate that neurons from a certain CNS region can migrate along glial axes from another site, leading to the concept that radial glia may function as a "passive generic guidance" for different neuronal populations (Hatten, 1990).

BG exerts a transient scaffolding function during cerebellar development (Fig. 4) and, eventually, achieve the functional properties of astrocytes, although they retain the radial morphology. This process is dependent on the execution of an intrinsic ontogenetic program, whose time-course is determined by interactions with other cell types. Accordingly, genetic manipulations that induce the conversion of BG into astrocytes severely impair granule cell migration (Yue et al., 2005).

It is now well established that the expression of scaffolding properties in BG is regulated by granule cells. In co-cultures of postnatal cerebellar cells, granule neurons induce the acquisition of polarised morphologies by astrocytes (Hatten, 1985). This process is

mediated by multiple molecular mechanisms (see section 2.7.), also including the interaction between NRG ad its receptor Erbb4, which are expressed by granule cells and BG respectively, during the period of granule cell migration (Rio et al., 1997). Application of a soluble form of NRG induces the radial glia phenotype in astrocytes *in vitro*, and this effect is blocked by the overexpression of a dominant negative form of Erbb4 (DN-Erbb4) in the glial cells. Furthermore, DN-Erbb4 bearing astrocytes are unable to support granule cell migration. These results indicate that NRG/Erbb4 interaction is critical first to induce the radial glia phenotype in BG and, then, to sustain granule cell navigation along the glial axis. However, while the former effect appears to be directly induced by NRG, the latter could be actually mediated by other molecules (e.g. astrotactin1), whose expression is regulated by NRG signalling. A similar mechanism has been described in the cerebral cortex, where neuronal NRG interacts with the Erbb2 receptor to induce migration conducive properties in radial glia (Anton et al., 1997: Schmid et al., 2003). These observations point to NRG-Erbb interaction as a general mechanism, by which migrating neurons regulate the guidance properties of the glial scaffolds. Nevertheless, selective deletion of Erbb2 and Erbb4 in the CNS does not result in impaired layering of the cerebral and cerebellar cortices (Barros et al., 2009), indicating that other mechanisms may compensate for the loss of NRG-Erbb signalling.

The scaffolding function of BG can be re-induced in the adult, when Bergmann fibres can support the migration of transplanted neurons. Notably, during their radial migration through the molecular layer, grafted embryonic Purkinje cells transiently induce the re-expression of developmentally regulated markers (e.g. nestin), associated with the scaffolding function of BG (Sotelo et al., 1994). A similar effect, accompanied by a conspicuous outgrowth of Bergmann processes, is observed when Cajal-Retzius cells are placed on the cerebellar surface (Soriano et al., 1997). Interestingly, this effect is not mediated by reelin and cannot be obtained with other types of neocortical cells. These observations indicate that defined cell

types, from different CNS sites, are capable of reverting adult BG to the radial glia phenotype expressed during development. This might suggest that mature BG may actually function as a generic passive guidance for different categories of neurons. However, comparison of the migratory behaviour of donor cells isolated from different CNS sites and transplanted to the cerebellum reveals the highly specific character of this neuron-glia interaction.

Embryonic Purkinje cells grafted to adult cerebella exploit Bergmann fibres to penetrate into the host molecular layer, moving along the opposite direction than that followed during development (Sotelo and Alvarado-Mallart, 1987, 1991; Carletti et al., 2008). Donor granule cells also use the same route to become integrated in the recipient granular layer, both in immature and adult hosts (Williams et al., 2008). On the contrary, extracerebellar neurons appear to be unable to navigate along Bergmann fibres. Embryonic neocortical neurons migrate along the surface of the adult cerebellum, but fail to undertake the radial route across the host molecular layer and remain clustered just beneath the pial surface (Borsello et al., 1994). This outcome could be explained by the inability of embryonic neocortical neurons to induce scaffolding properties in adult BG (Soriano et al., 1997). However, a complete failure to migrate along BG or settle in the molecular layer also invariably occurs when donor cells from different extracerebellar sources, from the telencephalon to the spinal cord, are transplanted to embryonic or postnatal cerebella (Carletti et al., 2004; Rolando et al., 2010). At these developmental stages BG glia should be supportive for migrating cells, independently from the influence of the grafted cells. Therefore, although BG may sustain migration throughout the entire lifespan, conducive properties appear to be strictly selective for cerebellar neurons. This suggests that the expression of characteristic features of radial glia, which can be also induced by extracerebellar neurons, such as Cajal-Retzius cells (Soriano et al., 1997), is not sufficient to allow neuronal migration. Rather, region- or cell type-specific recognition mechanisms

restrict the use of glial axes to defined neuron categories, thus determining the specificity of gliophilic migratory pathways in different CNS subdivisions.

3.6. Role of BG in the development of Purkinje cell dendrites and synaptic investment

The last phases of BG differentiation, with acquisition of mature traits and properties, typically follow a proximo-distal direction, concomitant with the outgrowth of Purkinje cell dendrites and their synaptic investment (Rakic, 1971; Altman, 1975; Das, 1976; Shiga et al., 1983b; Hanke and Reichenbach, 1987; Yamada et al., 2000). Starting from the deepest regions of the molecular layer, Bergmann fibres emit numerous processes and develop a fine reticular meshwork that gradually enwraps Purkinje cell dendrites and their synapses. Although it is well established that major instructive stimuli for Purkinje cell dendritic growth and orientation come from granule cells (Altman and Bayer, 1997; Sotelo, 2004; Sotelo and Rossi, 2012), the intimate relationship between BG and Purkinje dendrites suggests that BG may also contribute to shape the Purkinje cell dendritic tree. Indeed, both histological examination of fixed material (Yamada et al., 2000) and time-lapse video microscopy in organotypic slices (Lordkipanidze and Dunaevsky, 2005) have shown that outgrowing Purkinje dendrites are tightly apposed to the radially oriented Bergmann fibres. This suggests that the glial processes provide physical and trophic support to the elongating processes, and facilitate their contact with incoming inputs (Ango et al., 2008).

At the molecular level, this interaction could be mediated by phosphacan/6B4 proteoglycan, a chondroitinsulphate proteoglycan expressed on the surface of Purkinje cells (Maeda et al., 1992). Phosphacan is a component of the protein tyrosine phosphatase zeta (PTP ζ) receptor, that mediates Pleiotropin (PTN) and Midkine (MN) signalling (Maeda et al., 1996, 1999). These molecules are both expressed and released by BG (Matsumoto et al., 1994; Wewetzer et al., 1995), and perturbation of PTN/MN signalling by application of anti-PTP ζ

neutralizing antibodies in organotypic cerebellar cultures induces significant abnormalities of Purkinje cell dendritogenesis, with disrupted polarity (i.e. increased numbers of PCs with multiple primary dendrites) and abnormal orientation (Tanaka et al., 2003). Antibody treatment does not affect granule cell development, whereas it reduces GLAST immunoreactivity on Bergmann fibres. On the whole, these observations indicate that BG contributes to regulate Purkinje dendrite morphogenesis through PTP ζ -PTN/MN signalling. This effect could be mediated both by direct activation of PTP ζ receptors exposed on the neuronal membrane and by activation on the same receptors on BG. The latter mechanism would modulate dendritic growth by modifying GLAST expression and the rate of glutamate clearance from extracellular spaces (Tanaka et a., 2003).

The final maturation of glial sheaths surrounding synaptic contacts on Purkinje dendrites also regulates the formation and function of Purkinje cell synapses. Indeed, knockout mice for major astrocytic molecules, such as GLAST (Watase et al., 1998) or GFAP (Shibuki et al., 1996), show no overt anatomical abnormalities, but display defects in synaptic signalling and plasticity, accompanied by behavioural or motor dysfunction. Two-photon analysis of the reciprocal dynamics of glial ensheathing and synapse formation on Purkinje cell dendritic spines indicates that the maturation of glial coverage leads to reduced motility of both glial processes and dendritic spines, suggesting that glial maturation may contribute to stabilize synaptic contacts (Lippman et al., 2008). However, impairment of the ensheathing process by misexpression of MGluR2 (Iino et al., 2001), does not alter spine motility, but increases the number of parallel fibre synapses, suggesting that glial differentiation is more important to control synapse numbers than spine motility (Lippmann Bell et al., 2010). Nevertheless, other studies using MGluR2 misexpression in maturing BG reported significant defects in the synaptic development of Purkinje cells, including maintenance of multiple climbing fibre innervation (Iino et al., 2001), and delayed maturation of parallel fibre

synapses (Saab et al., 2012). Therefore, although the precise mechanisms are still uncertain, it is clear that the completion of BG enwrapping of neuronal processes in the molecular layer is required for the correct development and adaptive function of synaptic contacts impinging upon Purkinje cell dendrites.

3.7. The cooperative function of Purkinje cells and BG in cerebellar corticogenesis

The findings reported in the previous sections highlight a prominent role played by BG in virtually all phases of cerebellar corticogenesis, including foliation, assembly of the physical framework required to develop cortical layering, guidance and support for migrating neurons and outgrowing neurites, and regulation of synaptogenesis. In all these processes BG appear to act both by constituting a physical scaffold and by providing trophic support and regulatory cues to modulate different ontogenetic processes. On the whole, these observations indicate that BG acts as primary organizers of cerebellar corticogenesis, together with Purkinje cells. Purkinje cells orchestrate cortical development by regulating proliferation, survival and maturation of other cell types, including BG itself, and by directing the ingrowth and topographic distribution of afferent inputs (see Sotelo and Rossi, 2012). On its side, BG contributes to this process by constituting the physical framework upon which the cerebellar cortex is constructed and by issuing instructive signalling to direct the correct placement of the different cortical neurons and the proper assembly of the cortical circuitry.

4. Cerebellar-specific functions of Bergmann Glia and parenchymal astrocytes

4.1. Structure of BG and their relationship with the neuronal elements in the molecular layer

In the adult cerebellar cortex, each individual BG cell bears several Bergmann fibres (Ramón y Cajal, 1911; Palay and Chan-Palay, 1974), which are parasagittally oriented, along the

direction of Purkinje cell dendritic trees. On the other hand, the fibres from different cells form longitudinally oriented palisades that partition bundles of parallel fibres in the molecular layer (Palay and Chan-Palay, 1974). Each Purkinje cell is surrounded by eight BG cells on the average (Reichenbach et al., 1995), that together envelop the entire non-synaptic surface of the neuronal perikaryon and dendritic tree (Palay and Chan-Palay, 1974). Each glial cell takes contact with several thousand synapses (Reichenbach, 1995). However, the dendritic territories covered by different BG cells are extensively interdigitated (Livet et al., 2007; Ango et al., 2008), so that discrete, cell-specific domains cannot be readily disclosed.

Each Bergmann fibre bears a huge number of processes, which intertwine with the elements of the surrounding neuropile. Such processes cover the surface of Purkinje cells and inhibitory interneurons, and take contact with blood vessels of the molecular layer. Most importantly, astrocytic processes almost completely enwrap parallel fibre-Purkinje cell synapses as well as climbing fibre-Purkinje cell synapses (Palay and Chan-Palay, 1974; Spacek 1985; Xu-Friedmann et al., 2001; Yamada and Watanabe, 2002). These perisynaptic structures, named "microdomains" (Grosche et al., 1999), are made of fine stalks that stem from Bergmann fibres and terminate with cabbage-like arrangements of fine leaflets enwrapping one or a few synapses (see detailed descriptions and modelling in Grosche et al., 1999, 2002). On the whole, this anatomical arrangement of Bergmann glia is suggestive of a strategically positioned device, able to sense synaptic activity and, at the same time, to provide functional connection with local circulation.

4.2. BG-mediated regulation of signalling at Purkinje cell synapses

Activation of BG can be elicited by stimulation of both parallel (Clark and Barbour, 1997) and climbing fibres (Bergles et al., 1997). Brief pulses of parallel fibre stimulation in acute cerebellar slices induce discrete calcium transients, confined to individual microdomains

(Groshe et al., 1999). The effect is abolished by application of tetrodotoxin or inactivation of voltage-gated calcium channels, indicating that neuronal activity and synaptic transmission are required. The close apposition of glial processes to synapses of the molecular layer, suggests that the microdomains contribute to regulate synaptic signalling. BG shows high content of glutamate/aspartate transporter (GLAST; Rothstein et al., 1994), which is particularly enriched at perisynaptic membranes (Chaudhry et al., 1995). Electrical stimulation of climbing fibres induces rapid-onset glutamate-transporter currents in BG (Bergles et al., 1997) and pharmacological block of the glutamate transporter prolongs the duration of EPSCs in Purkinje cells (Barbour et al., 1994). Together, these features indicate that the activation of microdomains is related to removal of glutamate from extracellular space to prevent spillover of the neurotransmitter at Purkinje cell synapses.

In addition to GLAST, BG also expresses a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) type glutamate receptors. Notably, AMPA receptors of BG comprise subunits GluR1 and GluR4, but lack the GluR2 subunit, which confer inward rectification and high Calcium permeability (Müller et al., 1992; Burnashev et al., 1992). These glutamate receptor properties turn out to be crucial for the function of BG. Calcium permeability has been abolished in AMPA receptors of BG by introducing the GluR2 gene, via adeno-viral mediated transfer (Iino et al., 2001). In this condition, the glial processes retracted from their perisynaptic position, while the nearby boutons swelled (Fig. 5). Most importantly, electrophysiological recordings from *in vitro* slices revealed that the decay of EPSCs at parallel fibre-Purkinje cell synapses was significantly prolonged, consistent with a reduced efficacy of glutamate removal from the synaptic cleft. In addition, multiple climbing fibre innervation of Purkinje cells was maintained in the treated cerebella, suggesting a role for BG in the regulation of synaptogenesis (see also Lipmann et al., 2008).

These findings have been corroborated by subsequent observations on cultured BG (Ishiuchi et al., 2001), showing that abolishing Calcium permeability by introduction of the GluR2 gene in AMPA receptors induces the retraction of glial processes. Conversely, overexpression of Calcium-permeable AMPA receptors triggered process elongation that could be counteracted by pharmacological blockade of the same receptors (Fig. 5). Together, these findings indicate that glutamate released by parallel fibre or climbing fibre terminals acts through calcium-permeable AMPA receptors to regulate the degree of perisynaptic envelopment by BG (Fig. 5). This, in turn, modulates the rate of glutamate removal from the synaptic cleft and ultimately affects the dynamics of synaptic signalling. The observation of $GABA_A$ receptors on the BG membranes adjacent to contacts made by molecular layer interneurons with Purkinje cells, suggests that a similar mechanisms may also operate at inhibitory synapses (Riquelme et al., 2002).

It was initially assumed that AMPA receptors on BG were activated by diffusion of glutamate released at nearby climbing fibre or parallel fibre synapses. However, Matsui and Jahr (2003) provided an elegant demonstration that climbing fibre stimulation induces quantal current transients in BG, characterised by fast kinetics that are not consistent with receptor activation by low concentrations of neurotrasmitter diffusing from the synaptic cleft. Most importantly, by simultaneous recordings from pairs of Purkinje cells and BG, following desynchronization of vesicular release, it was shown that the events evoked by climbing fibre stimulation in the glial cell were not coincident to Purkinje cell responses, suggesting that AMPA receptors on BG membranes are activated by dedicated extrasynaptic release sites, rather than spillover of neurotransmitter from the synaptic cleft.

Later it was demonstrated that ectopic release differs from synaptic release for molecular mechanisms and dynamics (Matsui and Jahr, 2004). Moreover, it was shown that AMPA receptors are enriched in perisynaptic locations and presumptive neuron-glia junctions

were identified, characterized by close apposition between the facing membranes and some synaptic vesicles clustered near to the inner face of the neuronal membrane (Matsui et al., 2005). It has been proposed that extrasynaptic release of glutamate signals to the BG the position of the synapse, so that the astrocyte can effectively enwrap the junction and efficiently remove glutamate from the synaptic cleft.

These findings have been mostly obtained by *in vitro* preparations. More recently, however, novel technological approaches allowed the analysis of the same phenomena *in vivo*. Inducible knockout mice were generated to selectively ablate GluR1 and GluR4 subunits in astrocytes at defined ages (Saab et al., 2012). Loss of AMPA receptors in juvenile animals (3 weeks of age) induced retraction of perisynaptic processes and changes in duration and decay of parallel fibre-evoked Purkinje cell EPSCs, consistent with disrupted clearance of glutamate from the synaptic cleft. These modifications were accompanied by a delay in the maturation of parallel fibre synapses.

Retraction of glial processes was also observed when AMPA receptors were inactivated in adult mice, although in this case the phenotype was only detectable after 3 months. Strikingly, both immature and mature animals displayed some moderate, but significant, defects in motor function and learning. These observations underscore the role of BG in cerebellar synaptogenesis, but also highlight a crucial role for AMPA receptors in the fine tuning of synaptic signaling and Purkinje cell firing, required to ensure correct output from the adult cerebellar cortical network.

4.3. Activation of BG through purinergic signaling

In addition to activation of AMPA receptors, calcium signaling in BG can be also mediated by release from intracellular stores, regulated by purinergic signaling acting through the P2Y metabotropic purine receptor (Kirischuk et al., 1995, Beierlein and Regher, 2006; Piet and

Jahr, 2007). More recently, however, it has been shown that BG also expresses functional ionotropic receptors of the $P2X_7$ subtype (Habbas et al., 2011). Upon stimulation, these receptors form pores permeable to small molecules, suggesting that they might be involved in the release of neuroactive gliotransmitters (reviewed in Tozaki-Saitoh et al., 2011).

Purine-elicited events, which are usually slower and larger in amplitude than those triggered by activation of Calcium-permeable AMPA receptors, can be evoked by brief bursts of parallel fibre stimulation and require activation of metabotropic glutamate and purinergic receptors (Beierlein and Regehr, 2006). Parallel fibres activate metabotropic glutamate receptors on molecular layer interneurons and induce release of ATP. This, in turn, acts on purinergic receptors located on BG and triggers the release of calcium from intracellular stores (Piet and Jahr, 2007, reviewed in Hoogland et al., 2007). In this condition molecular layer interneurons appear to be responsible to relaying parallel fibre activity to BG, although astrocytic release of ATP, as shown in other systems (Guthrie et al., 1999), cannot be completely ruled out. The functional significance of this phenomenon is not fully clear. In addition, since spontaneous activation of parallel fibre beams *in vivo* is a rare event, the physiological pattern of activity that triggers this mechanism is still uncertain (Hoogland et al., 2007).

4.4. In vivo *analysis of Bergmann glia activation*

The introduction of two-photon confocal microscopy combined with calcium indicators recently allowed the analysis of calcium dynamics in BG *in vivo* (Hoogland and Kuhn, 2010). Spontaneous calcium waves in BG have been first recorded in adult anaesthetized rats (Hoogland et al., 2009). Each wave encompasses several tens of Bergmann fibres from different cells and display characteristic ellipsoidal shapes and diffusion dynamics. The events can be induced by local injection of ATP and are abolished by application of purinergic

antagonists, thus highlighting the role of purinergic signalling in the initiation and propagation of the transglial waves.

Recent recordings in awake, behaving animals showed that BG networks can be activated by motor behaviour (Nimmerjahn et al., 2009). In these conditions, three distinct types of calcium transients were distinguished and named sparkles, flares and bursts (Fig. 6). Sparkles and flares are affected by anaesthesia and they are related to synaptic transmission, being sensitive to application of tetrodotoxin or block of glutamate receptors, but not to application of purinergic antagonists. Sparkles are highly localized events that involve single Bergmann fibres and occur spontaneously in awake animals. They might be related to the activation of microdomains (Grosche et al., 1999), although the current resolution of *in vivo* imaging techniques does not allow exact matching between these events and the cellular compartments involved.

Flares are induced by motor behaviour and involve large networks of glial cells (hundreds of Bergmann fibres over a range of several hundred microns). Although they appear to be related to flares, they cannot be reduced to the mere sum of multiple smaller events. Most importantly, flare dynamics matches activity-related changes of blood flow in the molecular layer, suggesting a role in the regulation of blood supply during motor function (Fig. 6).

Finally, bursts are spontaneous events that are not influenced by tetrodotoxin or glutamate antagonists, but are abolished following block of purinergic receptors. They expand radially to involve up to several tens of Bergmann fibres (Fig. 6). Because of their pharmacological features and spreading dynamics, bursts likely correspond to the spontaneous waves recorded in anaesthetised animals (Hoogland et al., 2009), and to the purine-mediated calcium transients elicited by parallel fibre stimulation *in vitro* (Beierlein and Regehr, 2006; Piet and Jahr, 2007).

4.5. Physiological significance of Bergmann glia regulation of signalling at synapses in the molecular layer

Although many issues remain to be clarified, the picture that emerges from these reports indicates that in addition to the typical tasks of gray matter protoplasmic astrocytes, BG has acquired a number of highly peculiar functional roles, specifically designed to support information processing in the molecular layer. Indeed, the available knowledge highlights the role of BG in K^+ buffering, clearance of neurotransmitters that diffuse from synaptic clefts and even coupling of neuronal activity with energy supply and blood flow. These functions, and particularly removal of glutamate, may be relevant for physiological operation, but also for protection against excitotoxic insults (Rothstein et al., 1996; Watase et al., 1998).

In addition to these "classical" astrocytic tasks, however, the highly specific structural arrangement and functional features of the microdomains suggest a very peculiar function in the modulation of signalling at parallel fibre and climbing fibre synapses (see also Reichenbach et al., 2010). This view is further corroborated by the observation that following parallel or climbing fibre stimulation BG responses can undergo both short-term (Matsui and Jahr, 2004; Bellamy and Ogden, 2005; reviewed in Bellamy 2006) and long-term plasticity (Bellamy and Ogden, 2006; Balakrishnan and Bellamy, 2009). In particular, long-term depression of BG responses to parallel fibre stimulation appears to be due to the lack of fast recycling mechanisms at the ectopic release sites that would become easily exhausted (Balakrishnan et al., 2011). Together, these findings indicate that BG microdomains are able to dynamically sense patterns of neural activity and modulate the effectiveness of the glutamate clearance mechanisms, thus influencing synaptic signalling. Such a fine-tuning in the operation of the cortical network has clear implications for the proper execution of motor behaviour (Saab et al., 2012).

4.6. Function of velate protoplasmic astrocytes

Compared to BG, very little is known about the function of velate protoplasmic astrocytes of the granular layer. It is conceivable that, in all cortical layers, the different types of astrocytes carry out typical "astrocytic" tasks in the regulation of tissue homeostasis (Kimelberg, 2010). On the other hand, the anatomical relationship with synaptic contacts is strongly different. Contrary to the strict ensheathment of BG on molecular layer synapses, astrocyte processes in the granular layer border small groups of granule cells and completely enwrap the glomeruli, the synaptic structures comprising mossy fibre rosettes, Golgi neuron boutons and granule cell dendrites, but fail to penetrate within these junctional complexes (Chan-Palay and Palay, 1972; Palay and Chan-Palay, 1974). It has been proposed that this arrangement may serve to isolate synaptic complexes and partition subsets of mossy fibres conveying different types of information (Chan-Palay and Palay, 1972; Palay and Chan-Palay, 1974; Hoogland and Kuhn, 2010). While direct evidence for this function is still missing, it is clear that the influence of velate protoplasmic astrocytes on synaptic signalling in the granular layer is very different from the fine regulation exerted by BG in the molecular layer. Nonetheless, this also awaits elucidation and, to date, very little is known about the physiology of parenchymal astrocytes.

Spreading calcium waves have been recorded from velate protoplasmic astrocytes. These events encompassed spontaneous subcellular transient that could diffuse to involve nearby elements and were influenced by purinergic signalling (Hoogland et al., 2009; Hoogland and Kuhn, 2010). While these features are reminiscent of similar phenomena occurring in BG, their significance is unclear. In addition, considering the important dynamic differences shown by "radial" or "star-shaped" astrocytes *in vitro* (Fiacco and McCarthy, 2006), it is most likely that distinctive features will become evident as soon as our understanding of velate astrocytes will be extended.

5. Origin and differentiation of cerebellar oligodendrocytes

5.1. Origin of cerebellar oligodendrocytes

Specification and genesis of oligodendrocytes occurs at multiple locations along the neuraxis (Rowitch, 2004), including both ventrally and dorsally located germinal sites (Vallsted et al., 2005). Quite surprisingly, however, the origin of cerebellar oligodendroglia remained uncertain for a long time. One of the first reports devoted to this issue suggested that oligodendrocytes derive from subependymal layers of the fourth ventricle and migrate into the cerebellum through the superior medullary velum and the cerebellar peduncles (Reynolds and Wilkin, 1988). Later, Ono et al. (1997) described a ventro-dorsal dispersion of O4/O1-positive oligodendrocyte precursors in the chick embryo, suggesting that these cells originate in the medial pons and move to the lateral and dorsal metencephalon.

Recent fate mapping analyses showed that the cerebellar progeny derived from precursors expressing the proneural gene Ascl1 includes GABAergic neurons (Purkinje cells, deep nuclei neurons and interneurons) and oligodendrocytes, but not glutamatergic neurons and astrocytes (Kim et al., 2008; but see Sudarov et al., 2011). Ascl1 is involved in the specification of oligodendroglia at many CNS sites (Parras et al., 2004, 2007), where it may regulate the choice of bipotent progenitors between neuronal and oligodendroglial fates (Petrinyak et al., 2007; Jessberger et al., 2008). Given the exclusive origin of cerebellar neurons from intracerebellar germinal neuroepithelia (Carletti and Rossi, 2008; Hoshino, 2012), these findings suggest that cerebellar oligodendrocytes may also derive from an endogenous germinal site and share common origins with other cerebellar phenotypes. Notably, during cerebellar embryogenesis, Ascl1 is first expressed in the VN, whereas later (around E15 in the mouse) Ascl1 expressing cells appear scattered through the PWM (Kim et

al., 2008; Zordan et al., 2008; Grimaldi et al., 2009). A subset of these cells in the PWM also expresses Olig2, thus witnessing their oligodendroglial identity (Kim et al., 2008).

Analysis of late embryonic and postnatal cerebella of Ascl1-GFP mice reveals that green fluorescent cells in the PWM comprise two non-overlapping populations, expressing either Olig2 or Pax2, a specific marker for cerebellar GABAergic interneurons (Grimaldi et al., 2009). In Ascl1-null mice both oligodendrocytes and interneurons are dramatically reduced, whereas astrocytes are increased (Grimaldi et al., 2009; Sudarov et al., 2011). A similar phenotype is present in mice knockout for Bmi1 (van der Lugt et al., 1994; Jacobs et al., 1999; Zhang et al., 2011), a component of the Polycomb repressor complex that modulates glial specification (Zhang et al., 2011). Together, these findings suggest that the generation of different cerebellar lineages is strongly interdependent, so that altered regulatory mechanisms lead to increased numbers of one type (e.g. astrocytes) at the expense of the other ones (oligodendrocytes and interneurons).

To ask whether interneurons, astrocytes and oligodendrocytes originate from common ancestors, a GFP-expression vector was electroporated in the fourth ventricle of E14 mouse embryos, to selectively label progenitors residing in cerebellar germinal neuroepithelia (Fig. 7A). Surprisingly, many Pax2-positive interneurons and GFAP-positive astrocytes were labelled, while olig2-positive oligodendrocytes were extremely rare. The latter cells, by contrast, were frequently visualized following intraparenchymal application of the same expression vector. Furthermore, overexpression of Ascl1 in the ventricular zone produced a marked increase of Pax2-positive cells accompanied by a dramatic reduction of astrocytes, with no effect on oligodendrocytes (Grimaldi et al., 2009).

Together, these experiments indicate that, although oligodendrocyte precursors are present in the cerebellar parenchyma at E14 in the mouse, this type of glia does not derive from local germinal sites and has no common origin with interneurons or astrocytes. Transplantation of

solid cerebellar grafts, that develop fully differentiated minicerebellar structures (Sotelo and Alvarado-Mallart, 1991; Rossi et al., 1992), confirms the exogenous derivation of oligodendrocytes (Grimaldi et al., 2009). In addition, recent fate mapping analyses of GFAP::CreER^{T2} mice (Silbereis et al., 2009) and GLAST::CreER^{T2} (our unpublished observations) corroborate the conclusion that the majority of cerebellar oligodendrocytes has no lineage relationship with cerebellar neurons and astrocytes.

An extracerebellar source of cerebellar oligodendrocytes has been recently identified by examining chick-quail chimeras and *in ovo* transplants in the chick brain (Fig. 7B, Mecklenburg et al., 2011). By this approach, it was shown that most cerebellar oligodendrocytes, plus a small contingent of astrocytes, are generated in the parabasal bands of the mesencephalic neuroepithelium. This region is characterized by high expression of Nkx2.2, a transcription factor necessary for the specification of oligodendroglia in the spinal cord (Fu et al., 2002). From this germinal site, oligodendrocyte precursors migrate tangentially towards the isthmus, enter the cerebellum through the velum medullare and eventually disperse throughout the PWM. An additional source of cerebellar oligodendrocytes from germinal sites in the dorsal hindbrain cannot be ruled out (Fu et al., 2003; Vallsted et al., 2005), but no direct evidence for this possibility is currently available. In the chick brain, oligodendrocytes are generated around E5, whereas invasion of the cerebellar territory occurs between E9 and E12 (Mecklenburg et al., 2011). A homologous extracerebellar source of oligodendroglia in the mammalian brain has still to be identified.

Although these findings point to an exogenous origin for most cerebellar oligodendrocytes, a minor but consistent fraction of these cells descends from local progenitors (Grimaldi et al., 2009; Sudarov et al., 2011; Zhang et al., 2011). The latter cells are likely generated by multipotent precursors residing in the prospective cerebellar white matter, that might be able to differentiate along the three main neural lineages (Klein et al.,

2005; Lee et al, 2005). However, the precise origins of these oligodendrocytes as well as the relative ontogenetic significance of these alternative sources are unknown.

5.2. Maturation of cerebellar oligodendrocytes

Oligodendrocyte precursors enter the cerebellum through the velum medullare and disperse throughout the cerebellar parenchyma (Reynolds and Wilkin, 1988; Meklenburg et al., 2011). They first occupy the central mass, surrounding the deep cerebellar nuclei, and progressively invade the nascent cortical lobuli, where they settle both in the axial white matter and in the cortical layers. Following this *migratory wave*, oligodendrocyte differentiation follows a typical *maturation wave* that parallels the spatio-temporal pattern of colonization of the cerebellum (Reynolds and Wilkin, 1988). Starting from the central mass and progressing towards the lobule tips, maturing oligodendrocytes pass through a typical sequence of marker expression (the ganglioside GD3, GalC, CNP, NogoA and eventually MBP), which reflects their progressive acquisition of mature traits. Accordingly, myelin formation follows a similar centrifugal course (Reynolds and Wilkin, 1988; Kapfhammer and Schwab, 1994; Gianola et al., 2003; Rossi et al., 2006), to be completed at about the end of the second postnatal week in rodents (Bouslama-Oueghlani et al. 2003; Gianola et al., 2003). Notably, however, because of this peculiar maturation pattern, corticofugal Purkinje axons are myelinated in a retrograde manner (from their terminal field to the cell body), whereas cortical afferents (olivocerebellar axons and mossy fibres) are ensheathed in the opposite direction.

The maturation of oligodendrocytes is regulated by a complex interplay between cell-autonomous processes and interactions with other elements in the surrounding microenvironment, involving both cell-to-cell contact and diffusible factors (see Li et al., 2009; Emery, 2010a, 2010b; Chatonet et al., 2011 for reviews). While most of these mechanisms appear to be common to different CNS sites, a particular role for the maturation of cerebellar

oligodendrocytes has been attributed to thyroid hormones. It is well known that hypothyroidism is associated with severe impairment of cerebellar development and significant delay in the maturation of cerebellar oligodendroglia and myelin (Balász et al., 1971; Barradas et al., 2001; see Koibuchi and Ikeda, 2012; Chatonet et al., 2011, for reviews). More recently, however, following the analysis of knockout mice for specific isoforms of the thyroid hormone receptors, a dual action has been proposed for these hormones on cerebellar oligodendrocytes. At early developmental stages thyroid hormones would indirectly influence the proliferation and maturation of oligodendroglial precursors by inducing the release of trophic substances from Purkinje cells and astrocytes. In adult life, the same hormones would act directly on the remaining precursors, to regulate their ability to divide or acquire mature traits (Fauquier et al., 2011; Picou et al., 2012).

Further insights on the cellular/molecular interactions underlying oligodendroglial development in the cerebellum come from a recent study showing that Purkinje cells regulate different phases of oligodendrocyte maturation by releasing diffusible factors (Bouslama-Oueghlani et al., 2012). During early postnatal development Shh stimulates the proliferation of Olig2-positive oligodendrocyte progenitors. By the end of the first postnatal week, Purkinje cells down-regulate Shh and produce vitronectin, which induces oligodendrocyte maturation and myelin formation. While these observations further highlight the role of Purkinje cells as the organizers of the entire cerebellar morphogenesis (Sotelo and Rossi, 2012), the mechanisms that orchestrate the centrifugal progression pattern of oligodendrocyte development have not been addressed directly. However, it is likely that this precise spatio-temporal schedule results from the proximo-distal gradient of Purkinje cell maturation along the outgrowing cortical lobules (Inouye and Murakami, 1980; Altman and Bayer, 1985, 1997; Gianola et al., 2003).

Another relevant feature refers to the specific myelination of defined axon subsets: while oligodendrocytes are present in the white matter and in all cortical layers, myelin formation is restricted to white matter and granular layer (Palay and Chan-Palay, 1974; Reynolds and Wilkin, 1988). Moreover, the only myelinated processes present in the molecular layer are branches of the recurrent supraganglionic plexus of Purkinje axons (Palay and Chan-Palay, 1974; Rossi et al., 2006). This peculiar pattern reflects some type-specific interactions between oligodendrocytes and different categories of cerebellar cortical neurons that have still to be elucidated.

5.3. Developmental potential of cerebellar oligodendrocyte progenitors

Oligodendrocyte progenitors (OPCs), characterised by the expression of the chondroitin sulphate proteoglycan NG2, are dispersed also at adult stages throughout the cerebellar white matter and granular and molecular layers. These cells derive from OPCs generated at developmental stages and maintain proliferative activity in the adult brain. They are capable of differentiating into oligodendrocytes if myelin is damaged, and likely sustain a certain degree of myelin remodelling throughout life (Nishyiama et al., 2009; Young et al., 2013).

Genetic fate mapping studies tracking NG2 expressing cells in the forebrain have shown that they differentiate into myelinating oligodendrocytes and to protoplasmic astrocytes in the ventral gray matter (Zhu et al., 2008a). Similar studies in the cerebellum revealed that in this area NG2-expressing progenitors produce exclusively oligodendroglial cells (Zhu et al., 2008b). Some capability of NG2 expressing cells for astrogliogenesis has been reported in *ex vivo* studies, indicating that the capability to produce astrocytes could be activated also in cerebellar NG2 positive glia in defined conditions (Leoni et al., 2009). Another fate-mapping study based on perinatal tracking of cells with active Plp or Olig2 promoters was interpreted as proving that NG2 expressing cells generate astroglia in addition

to oligodendrocytes (Chung et al., 2013). However, the precise phenotype and real NG2 positivity of precursor cells tracked in this study has not been established, thereby weakening its conclusions. Yet, bipotent gliogenic precursors capable to produce both astroglia and oligodendroglia have been reported also in former *in vitro* clonal analyses (Milosevic and Goldman, 2004).

5.4. Oligodendrocyte contribution to cerebellar development

There is evidence that oligodendrocytes also contribute to regulate the correct maturation of the surrounding cerebellar tissue. Selective ablation of this cell type during the first weeks of postnatal life produces severe disruption of the cortical cytoarchitecture and neuronal network (Mathis et al., 2003; Collin et al., 2007; Doretto et al., 2011). Although the mechanisms underlying such a severe effect have not been elucidated, these findings suggest that oligodendroglia may be required for correct neuronal development. In line with this view, application of neutralizing antibodies against the myelin-associated growth inhibitory protein Nogo-A during the second postnatal week impairs the physiological pruning of supernumerary intracortical collaterals of Purkinje axons, thus disrupting the normal compartmentation of these neurites within defined cortical laminae (Gianola et al., 2003). The same effect can be also induced in adult animals (Buffo et al., 2000), where it is associated with upregulation of axotomy-related genes in intact Purkinje cells (Zagrebelsky et al., 1998). Taken together these studies suggest that oligodendrocytes mediate a lifelong regulation of neuritic plasticity, needed to maintain specific laminar distributions and connection patterns of cerebellar axons (Rossi et al., 2006, 2007).

5.5. Interactions between oligodendrocyte progenitors and neurons

Recent research showed that OPCs possess receptors for both glutamate and GABA (Mangin and Gallo, 2011; Vélez-Fort et al., 2012) and respond to the gliotransmitter ATP, which is the functional substrate of neuron-astroglial units and promotes their differentiation (Wigley et al., 2007). Receptor activation evokes depolarising currents, whose functional relevance in shaping the activity of neuronal circuits remains so far undefined. Notably, while glial cells are able to detect transmitter molecules that have accumulated in the extracellular space, OPCs discern single quanta of neurotransmitter released from individual neurons. Neurotransmitter-based interactions with neurons have been proposed to regulate the rate of oligodendrocyte progenitors proliferation, maturation and migration in physiology and pathology (Gallo et al., 2008; Mangin and Gallo, 2011; Vélez-Fort et al., 2012). Further, glutamate and ATP-mediated changes modulate the surface expression and conductance of AMPA receptors (Zonouzi et al., 2011). However, the function(s) of these interactions remain elusive.

Several features of the integration of NG2 cells in cerebellar neuronal circuits are known. Release of glutamate from climbing fibres produces robust activation of calcium-permeable AMPA receptors with rapid kinetics in NG2 cells of the molecular layer (Lin et al., 2004). NG2 expressing cells may receive up to 70 discrete inputs from one climbing fibre and, unlike mature Purkinje cells, are often innervated by multiple olivary axons. Paired Purkinje cell-NG2 cell recordings showed that one climbing fibre can innervate both cell types. These electrophysiological studies were confirmed by ultrastructural analyses that revealed direct synaptic contacts of climbing fibres with NG2 cell processes. Inputs from parallel fibres were also observed, but much weaker compared to those of climbing fibres (Lin et al., 2004). Synapses on OPC processes are not enwrapped by BG lamellae. NG2+ cells were also in direct apposition with boutons that made no synaptic junctions, as well as with PF axons, Bergmann glial processes, and interneuron dendrites (Lin et al., 2004). While the existence of GABAergic

inputs on cerebellar OPCs has not been determined so far, acitivation of AMPA receptors and blockade voltage-gated potassium channels influence the maturation of OPCs in organotypic cerebellar slices (Yuan et al., 1998).

Recently, a study on rat cerebellar white matter uncovered the existence of a subpopulation of NG2 cells firing action potentials (Karadottir et al., 2008), suggesting that these cells could be more similar to neurons than previously though, and that spikes could derive from synaptic inputs on these cells. These findings, however, remain uncertain, as they were not confirmed in mouse studies (Clarke et al., 2010) and in other reports (De Biase et al., 2010). Cerebellar OPCs also contact nodes of Ranvier in the anterior medullary velum (Butt et al., 1999), suggesting a direct communication with axons, as further indicated by axon-OPC synaptic structures as well as responses evoked by vesicular release of glutamate from unmyelinated axons in other white matter tracts (Ziskin et al., 2007). Yet, whether OPCs do more than passively responding to neuronal activity is so far unknown.

Overall, evidence of a physiological response of OPCs to neuronal network activity supports the view that in the adult brain these cells are not progenitors only. However, both the nature and the implications for neuronal functioning of their crosstalk with neurons have still to be elucidated.

6. Conclusion

The findings discussed in this essay witness the increasing interest of the scientific community for issues related to the ontogenesis and functional role of cerebellar glia. Over the last few years we have learned that cerebellar astrocytes and oligodendrocytes are endowed with some very peculiar features, which are beyond the usual attributes of glial cells of the CNS. In part, this is because the particular organization and functioning of the immature and adult cerebellum has made it possible to carry out targeted experiments and

manipulations that were not feasible in other sites. Many questions remain unanswered. However, the currently available knowledge shows that glial cells provide some essential contributions to cerebellar development and function. As a consequence, a full understanding of these processes requires an integrated knowledge of the ontogenetic and working properties of both neurons and glia.

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Figure legends

Fig. 1. Possible lineage relationship of cell types derived from progenitors of the cerebellar prospective white matter (PWM). Progenitors that proliferate in the PWM and generate cerebellar astrocytes (blue), interneurons (green) and part of the oligodendrocytes (red) originate from radial glia of the ventricular neuroepithelium (VN, gray) and delaminate in the PWM during late embryonic development. However, it is still unclear whether PWM progenitors represent a single population of multipotent cells able to generate all the different phenotypes (in gray, on the right side of the figure), or if they comprise different subsets of fate-restricted precursors (in red, green and blue, on the left side of the figure). The dashed red arrow indicates that part of the oligodendrocytes derive from extracerebellar source.

Fig. 2. Possible origins of intraparenchymal astrocytes and Bergmann Glia (BG). All types of cerebellar astrocytes derive form radial glia of the VN. Intraparenchymal astrocytes (i.e. protoplasmic and fibrous astrocytes) are generated by progenitors of the prospective white matter (PWM, green arrows on the left side of the picture). It is unclear whether these progenitors also contribute to produce BG (dashed green arrow on the left side of the figure). The latter phenotype is though to originate from direct transformation of radial glia of the ventricular neuroepithelium (VN). BG continue to proliferate during late embryonic and postnatal development and generate more cells of the same type to match the concomitant expansion of the cerebellar cortex (red arrows on the right side of the figure). It is unknown whether proliferating BG may also contribute to the genesis of intraparenchymal astrocytes (dashed red arrows on the right side of the figure).

Fig. 3 A-D. Anchorage of Bergmann glia (BG) endfoot to the basement membrane (BM) of the pial surface and its role in cerebellar corticogenesis. A shows the molecular cascade linking the BG endfoot and BM at the pial surface of the cerebellar cortex. This signalling mechanism is thought to be mediated by BG expressed integrin or dystroglycans (α-DG, βDG) interacting with laminin of the extracellular matrix. Known components of this molecular cascade also comprise: integrin-linked kinase (ILK), focal adhesion kinase (FAK), Abl family non-receptor tyrosine kinases (Abl/Ab1), the adaptor protein Crk (Crk). This BG-BM reciprocal interaction is required to stabilise the basal membrane, regulate the growth of BG processes and establish the scaffolding framework needed to achieve the normal layering of the cerebellar cortex (depicted in B). If this mechanism is disrupted (indicated by gray shading in C), the endfeet fail to anchor to the BM, which becomes disorganized. This results in multiple defects of cortical morphogenesis (depicted in D), including fusion of the adjacent lobules, loss of granule cells and granule cell persistence in ectopic positions, impaired formation of the Purkinje cell monolayer. A, is modified from Qiu et al., 2010.

Fig. 4 A-D. The scaffolding role of radial glia/BG during cerebellar corticogenesis. During embryonic development (A), Purkinje cells (blue) are generated by proliferating radial glia (red) in the ventricular neuroepithelium (VN). Newly born Purkinje cells migrate along radial glial processes towards the prospective cerebellar cortex, following attractive cues released by overlying structures such as the granule cell precursors (green) in the nascent external granular layer (EGL). At the end of embryonic life (B), the VN vanishes and radial glia turns into BG, by losing apical processes and translocating cell bodies towards the cortex. Migration of radial glia cell bodies strictly lags that of Purkinje cells. At the end of this phase (C), Purkinje cells are clustered in the Purkinje cell plate (PCP), just beneath the EGL, whereas BG send multiple radial processes up to the pial surface. During postnatal development (D), the array of BG fibres is used by newborn granule cells as a guide to migrate beyond the position

of Purkinje cells, which progressively acquire the final monolayer arrangement. Granule cell migration and maturation, directed by the BG scaffold, leads to the expansion of the molecular layer (ML) and internal granular layer (IGL) of the mature cerebellar cortex.

Fig. 5. The role of Calcium permeable AMPA receptors in regulating the interaction between BG and parallel (PF)-Purkinje cell (PC) synapses. BG processes enwrap synaptic contacts in the molecular layer. BG processes bear Calcium permeable AMPA receptors that sense glutamate release from the adjacent synapse and also express glutamate/aspartate transporters (GLAST) that actively remove glutamate from the synaptic cleft. Overexpression of Calcium permeable AMPA receptors induces an enlargement of the BG processes, whereas their substitution with a Calcium impermeable isoform induces retraction of the same processes. Such changes of the synaptic coverage by the astrocytic process modulate the ability for glutamate removal. In this way, BG microdomains sense glutamate release and regulate signalling at PF-PC synapses.

Fig. 6. Different types of Calcium transients that can be recorded from BG in awake, behaving mice, as described by Nimmerjahn et al., 2009. Sparkles are highly localized events (highlighted by red shading of the BG process), which are likely related to the activation of individual BG microdomains, impinging upon single parallel fibre (PF)-Purkinje cell (PC) synapses, that are active (indicated by orange shading). Flares are associated with motor behaviour and involve the activation of numerous astrocytic processes and synaptic contacts. Their dynamics matches activity-related changes in blood flow (BV, blood vessel), suggesting that these events may be related to the regulation of energy supply during neuronal activation. Bursts are not related to the activation of PF-PC synapses, but are likely induced by Purinergic signalling, also involving molecular layer interneurons, metabotropic glutamate receptors expressed on BG and Calcium release from intracellular stores.

Fig. 7 A-B. Extracerebellar origin of cerebellar oligodendrocytes. A illustrates the results of overexpression of Ascl1 in the cerebellar primordia of E14 mice. Electroporation of a control expression vector (GFP) injected in the ventricle labels both interneurons and astrocytes, whereas green fluorescent oligodendrocytes are extremely rare. Overexpression of Ascl1 (GFP-Ascl1) in the same conditions labels numerous Pax2-positive interneurons, but no astrocytes. Injection of the expression vector directly into the PWM results in labelling of oligodendrocytes, indicating that most of the latter cells do not originate from the VN. B depicts the results of chick-quail chimera experiments showing that cerebellar oligodendrocytes derive from the parabasal bands (pb, highlighted by shading) of the mesencephalic neuroepithelium. Dashed arrows indicate the migratory route followed by the oligodendrocytes from the ventral mesencephalon to the cerebellum of the E12 chick embryo. B is modified from Fig. 7 of Mecklemburg et al., 2011.

Figure 1

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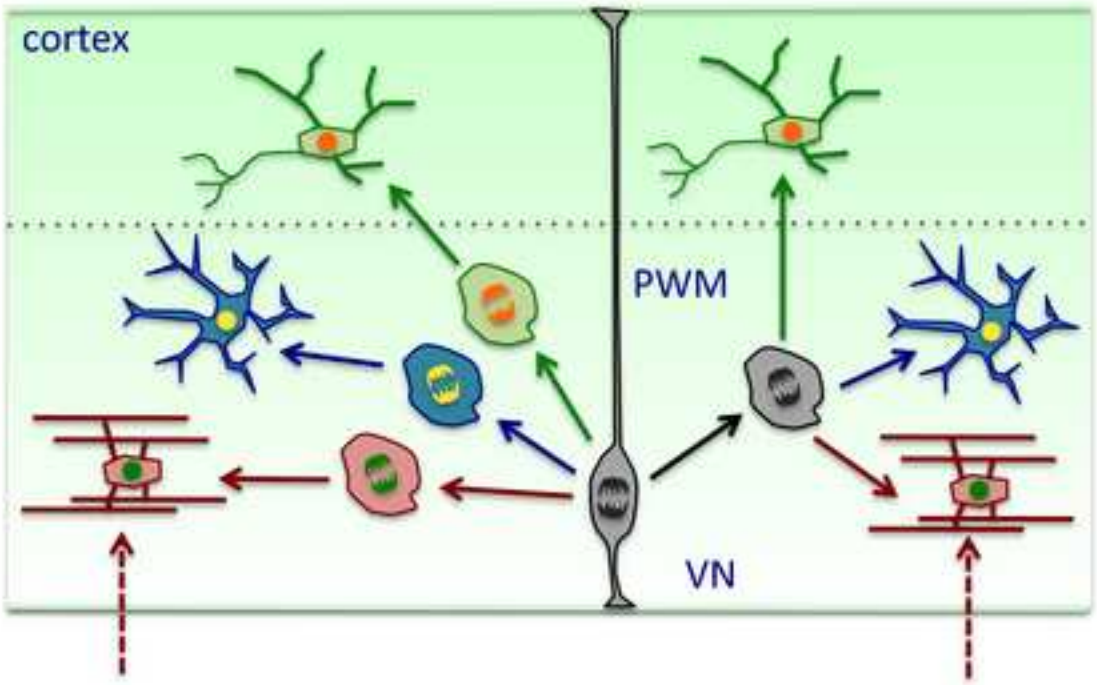

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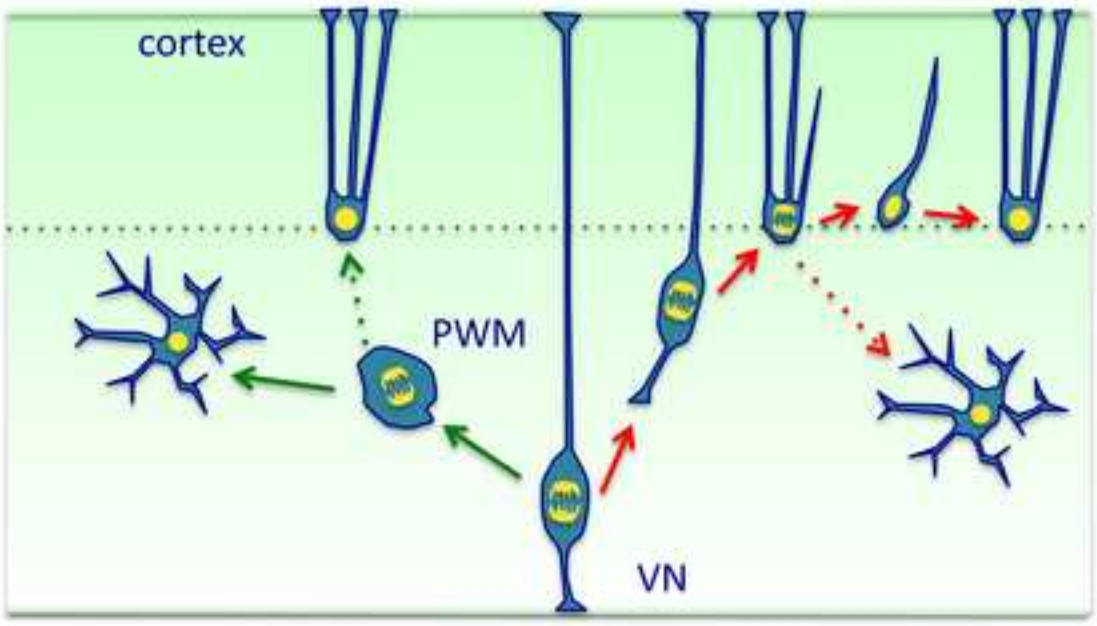

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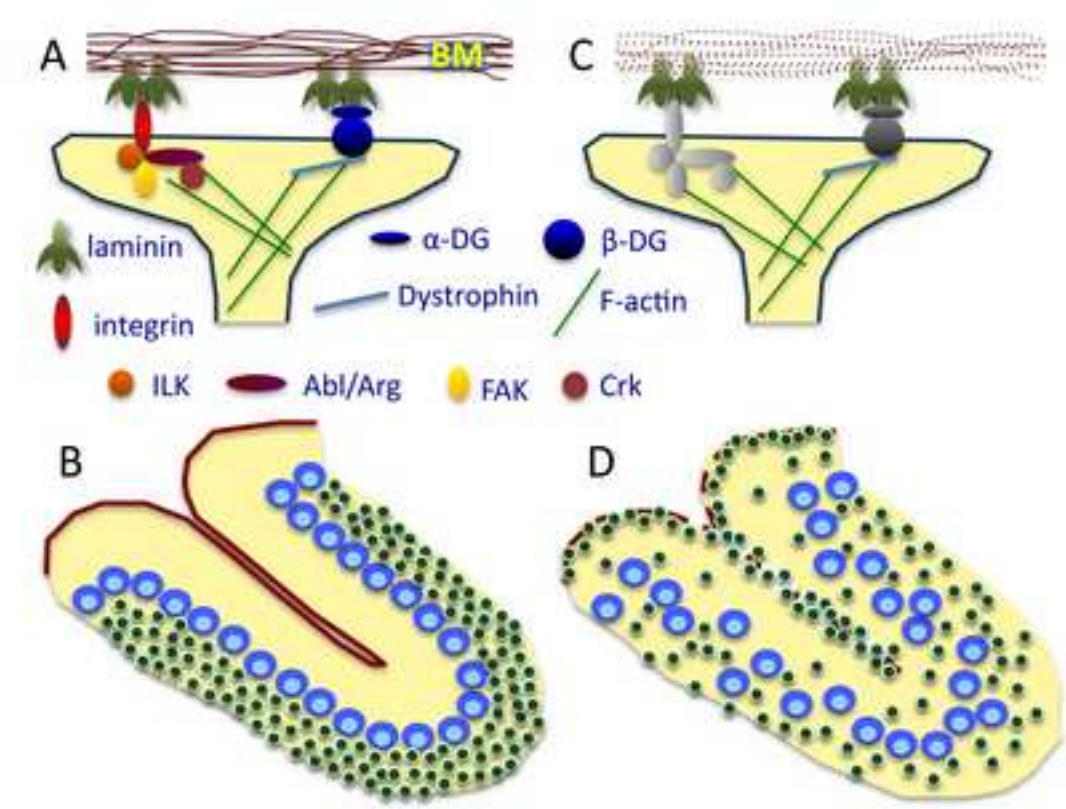

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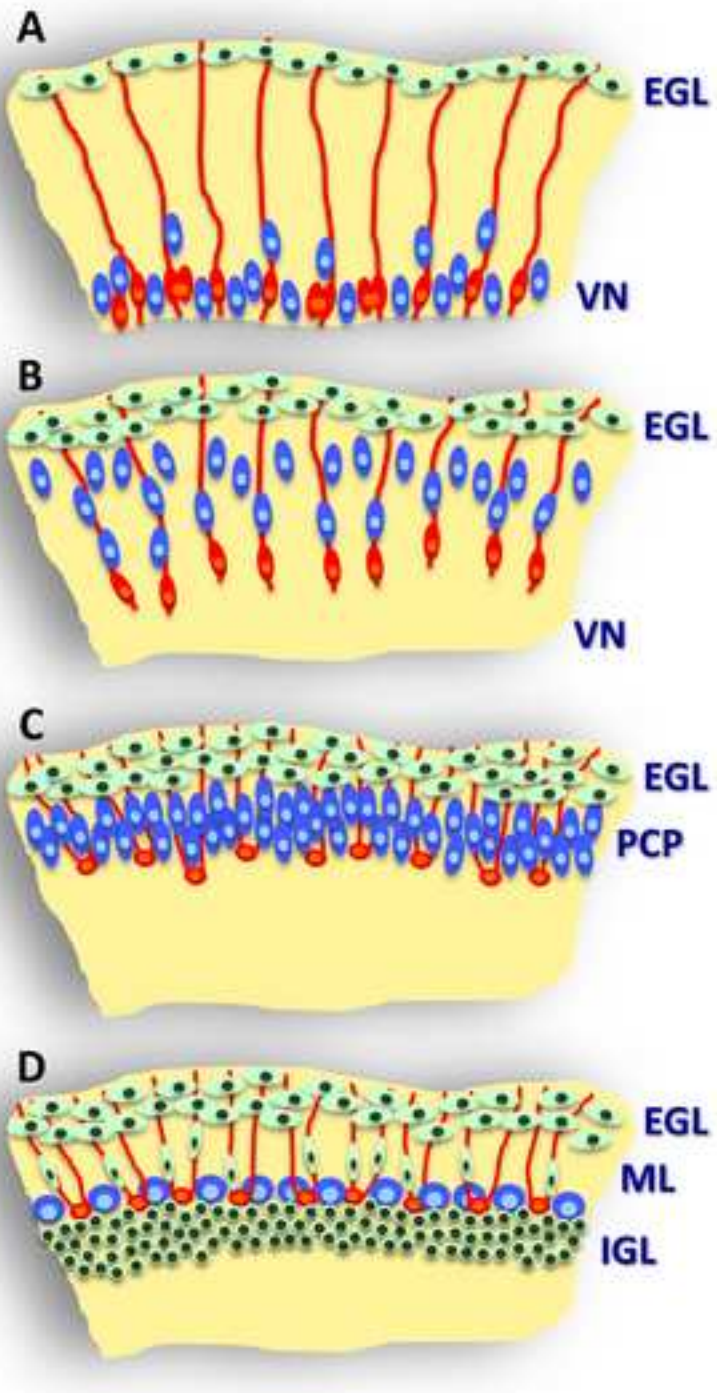

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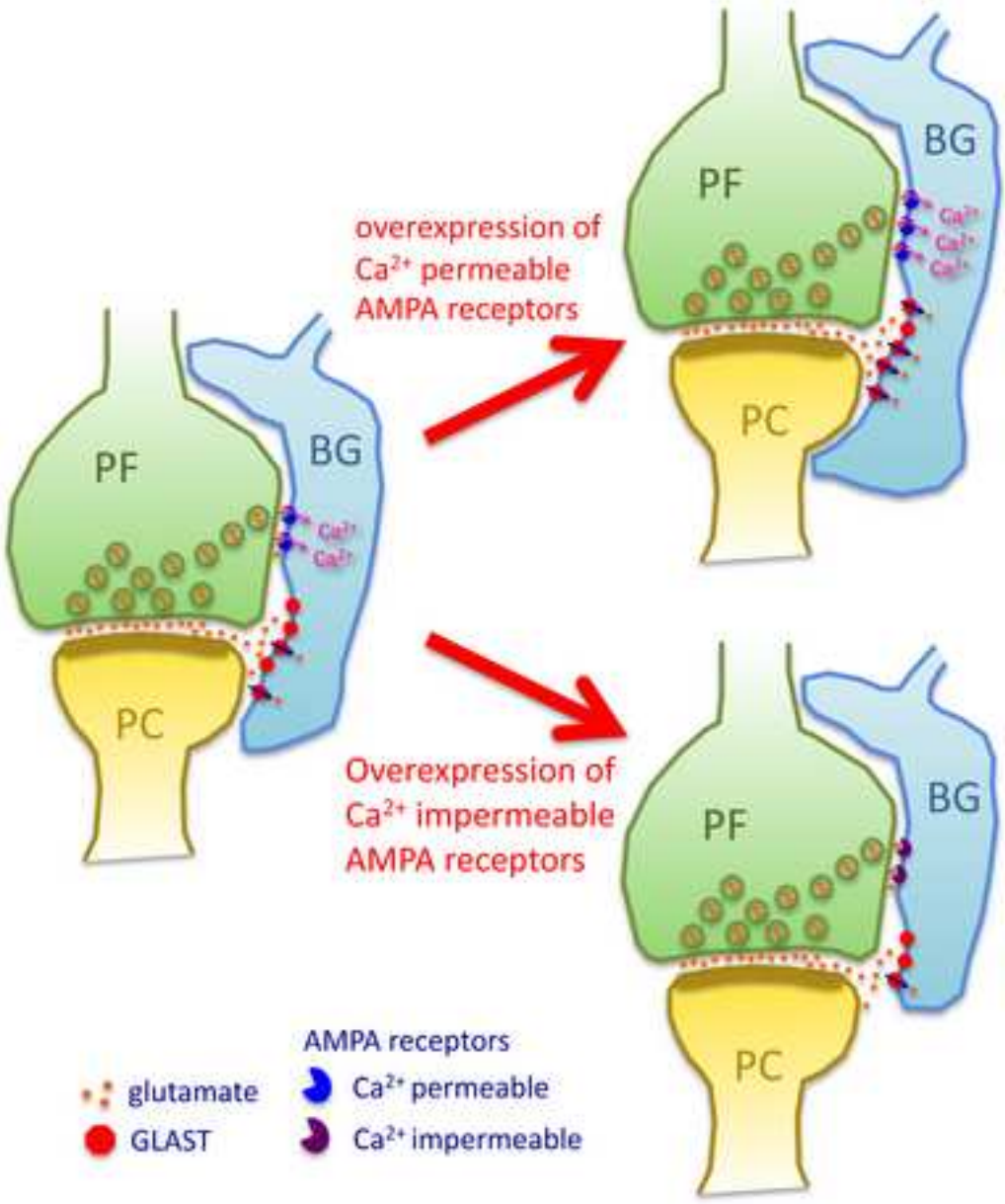

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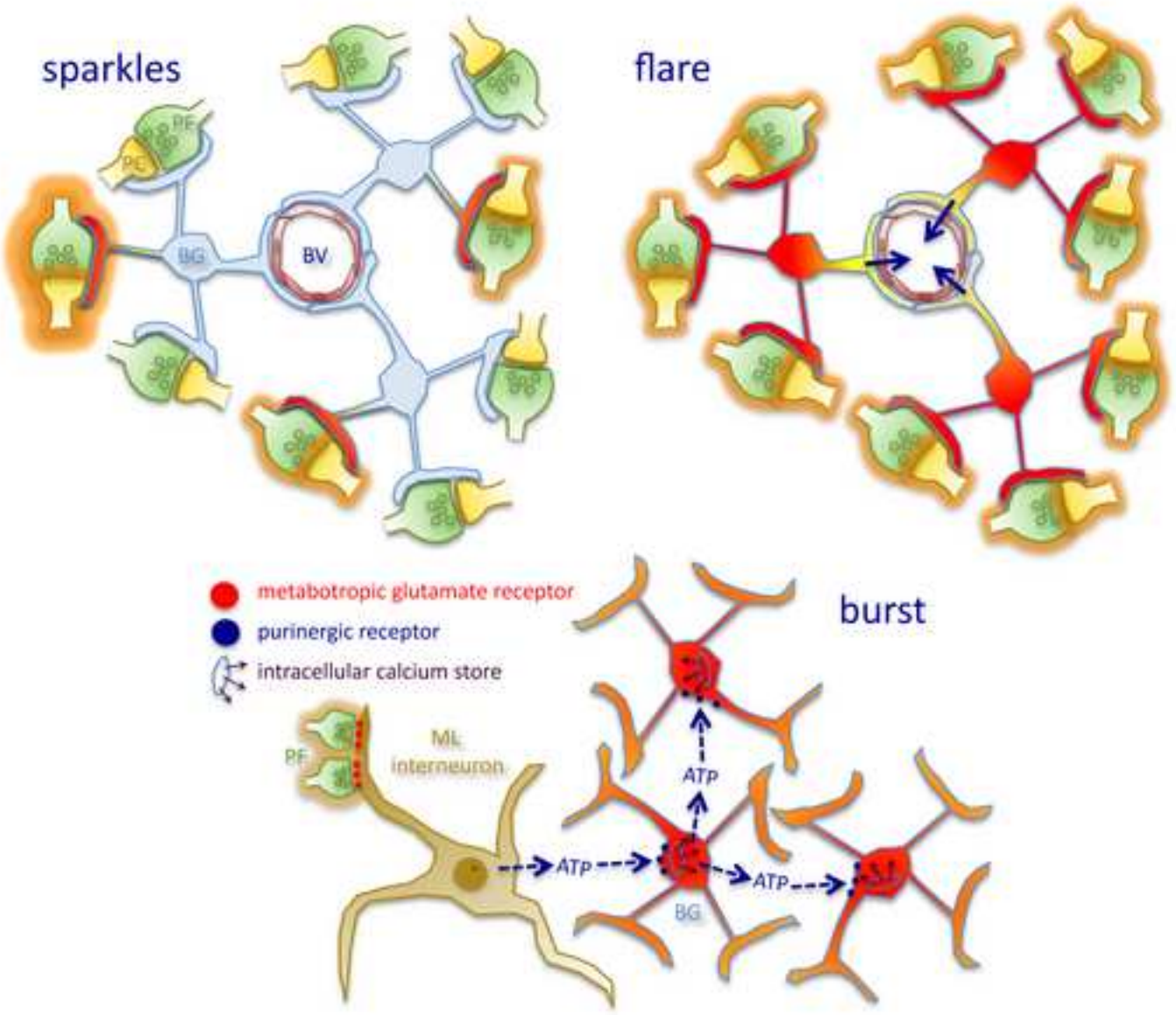

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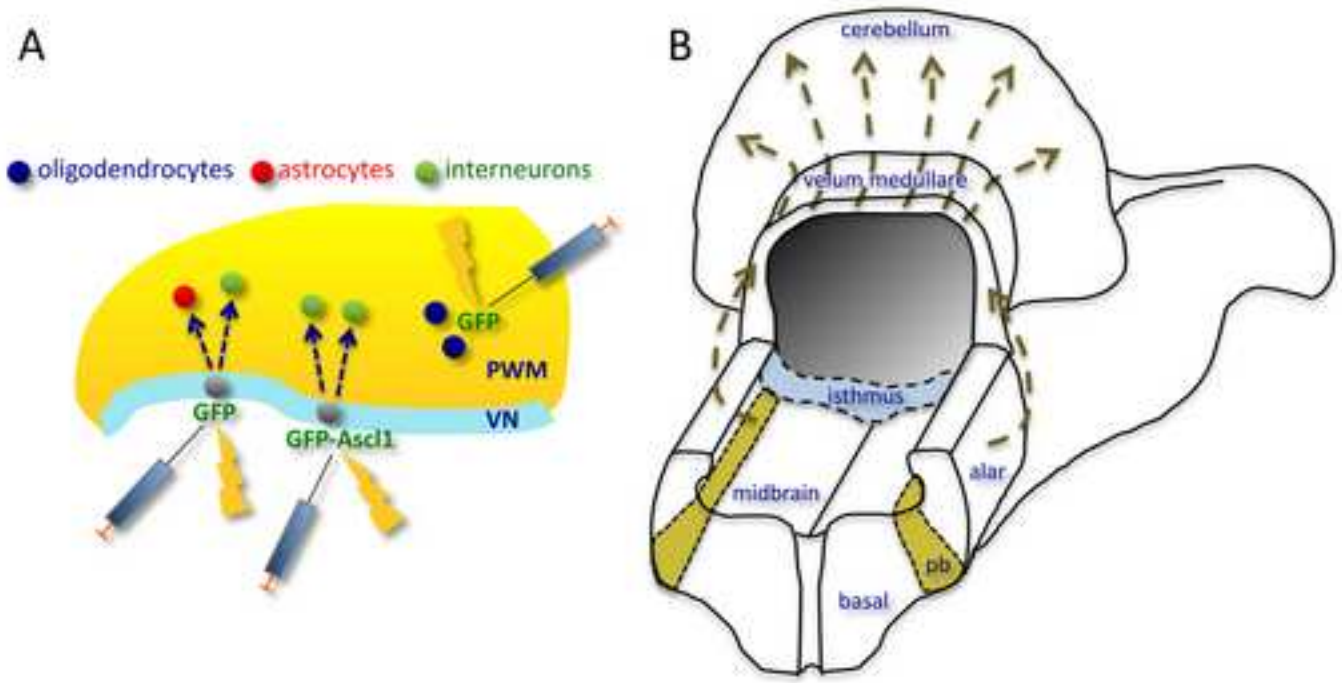

Figure 7

***Abbreviation List**

AMPA	a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BG	Bergmann glia
EGL	external granular layer
FGF	Fibroblast Growth Factor
GLAST	glutamate/aspartate transporter
MN	Midkine
NRG	Neuregulin
PTN	Pleiotropin
PTP ζ	protein tyrosine phosphatase zeta
OPC	oligodendrocyte precursor cell
PWM	prospective white matter
RL	rhombic lip
VN	ventricular neuroetiphelium