Archives Italiennes de Biologie, 148: 137-146, 2010.

The prospective white matter: an atypical neurogenic niche in the developing cerebellum

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ABSTRACT

Cerebellar GABAergic interneurons comprise heterogeneous phenotypes located at strategic levels of the local networks. Recent findings indicate that they all derive from a common population of multipotent progenitors whose fate choices are determined by instructive information provided by the PWM environment. Here we review about the atypical neurogenic strategy operated within such postnatal niche and we discuss possible instructive mechanisms governing interneuron specification and differentiation.

Key words

Cerebellum • GABAergic interneuron • Cell specification • Neural development • Postnatal niche

Introduction

Neurogenic processes during cerebellar development are characterized by a peculiar evolution of the germinal territories. Although all types of cerebellar neurons derive from neuroepithelia located at the met-mesencephalic junction, the progressive delamination of dividing progenitors leads to the formation of secondary germinal sites, where neurogenesis continues during late ontogenetic stages. The primary neuroepithelia appear at embryonic day 9 (E9), just above the opening of the fourth ventricle. They comprise two major subdivisions: the rhombic lip (RL), located close to the roof plate, is the origin of all glutamatergic types (projection neurons of deep cerebellar nuclei (DCN), unipolar brush cells and granule cells); the ventricular zone (VZ), placed at the inner germinal layer, generates GABAergic neurons (Purkinje cells, nucleo-olivary projection neurons and inhibitory interneurons of the cortex and DCN) (Hoshino et al., 2005; Hoshino, 2006; Machold and Fishell, 2005; Wang et al., 2005; Englund et al., 2006; Fink et al., 2006).

The primary neuroepithelia generate projection neurons (i.e DCN neurons and Purkinje cells) during embryonic life. By contrast, both glutamatergic and GABAergic interneurons derive from progenitor cells that emigrate either from the VZ into the cerebellar prospective white matter (PWM), or from the RL over the cortical surface to form the external granular layer (EGL).The classical view of cerebellar development considered the EGL as the source of granule cells and molecular layer interneurons (Ramón y Cajal, 1911; Altman, 1972). More recently, however, it was unequivocally demonstrated that EGL cells exclusively generate granule neurons (Hallonet et al., 1990; Hallonet and LeDouarin, 1993; Gao and Hatten, 1994). Furthermore, retroviral injection experiments (Zhang and Goldman 1996a,b) together with clonal analyses (Mathis et al., 1997; Milosevic and Goldman, 2002; Mathis and Nicolas, 2003; Milosevic and Goldman, 2004) have shown that inhibitory interneurons originate from VZ progenitors that proliferate in the PWM.

Projection neurons are thus generated at the outset of cerebellar neurogenesis by progenitors that pro-

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liferate and become specified within the primary neuroepithelia. In contrast, the different classes of inhibitory and excitatory interneurons are produced at later ontogenetic stages, by precursors that proliferate in secondary germinal niches (Carletti and Rossi, 2008). However, while EGL cells are fate-restricted to the granule neuron phenotype (Gao and Hatten, 1994; Alder et al., 1996), the PWM is the origin of the variety of GABAergic interneurons, characterized by precise numbers, morphology and position in the cerebellar cortex and DCN (Schilling, 2000; Schilling et al., 2008).

Because of these features, the study of cerebellar germinal niches, and namely the secondary ones that are active during postnatal life, is suitable to address some crucial issues relating to the generation of phenotypic diversity in the CNS. In particular:

– Are progenitors within the niche already committed toward precise phenotypes and positions or are they multipotent and naïve with respect to their final fates?
– Do progenitor cells undergo a progressive restriction of their fate potential?
– Is the last mitosis critical for phenotype specification?
– Which are the intrinsic and extrinsic influences that determine final fate choices?

In other CNS regions, GABAergic interneurons derive from progenitors residing in precise germinal sites. Therefore, comparison with the cerebellum can highlight both different and common traits. The next section briefly outlines the main features of GABAergic interneuron genesis in different CNS regions. Thereafter, neurogenic processes in the cerebellar PWM will be discussed.

Generation of GABAergic interneurons in different CNS sites

In the spinal cord, interneurons are generated according to two main neurogenic waves from eight genetically distinct progenitor pools distributed over the dorsal domain (Caspary and Anderson, 2003; Helms and Johnson, 2003). As in the cerebellum, GABAergic specification requires Ptf1-a expression to prevent glutamatergic differentiation (Cheng et al., 2004; Glasgow et al., 2005; Hori et al., 2008). Dorsal inhibitory interneurons comprise two early born populations – dI4, dI6 – and the late born dL1^{A}. All these classes derive from Lbx1-positive cells (Gross et al., 2002; Müller et al., 2002; Cheng et al., 2004), further characterized by the expression of the transcription factors Pax-2, Lhx1 and Lhx5 (Gross et al., 2002; Müller et al., 2002). It has been shown that the non overlapping expression domains of the proneural genes Math-1, Ascl-1, Ngn1 and Ngn2 can be considered as fate determinants of dorsal progenitors already at E10 (Helms and Johnson, 1998; Bermingham et al., 2001; Gross et al., 2002; Müller et al., 2002). Thus, the initial position of progenitor cells in the dorsal neural tube and their time of birth precisely regulate interneuron identity (Caspary and Anderson, 2003). However, there is evidence that fate choices can be reversed also in postmitotic cells, thanks to specific transcription factor activation. For instance, in absence of Lbx1, normally expressed by postmitotic dI4-dI6 and dIL$^{A/B}$ neurons, cells are mis-specified and acquire typical traits of dI2 and dI3 interneurons (Gross et al., 2002; Müller et al., 2002).

In the cerebral cortex, different interneuron classes derive from distinct domains of the subpallial neuroepithelium. Their developmental potentialities depend on their position (Flames et al., 2007; Fogarty et al., 2007; Wonders et al., 2008) and time of generation (Miyoshi et al., 2007; Rymar and Sadikot, 2007). In particular, the subpallial location is the major determinant of the mature interneuron class, while the time of birth dictates subtype-specific traits (Butt et al., 2005; Miyoshi et al., 2007). The birthdate of cortical interneurons is also predictive of their laminar fates: early-born cells principally populate lower cortical layers, whereas late-born ones adopt progressively more superficial locations (Valcanis and Tan, 2003). Heterochronic transplantation experiments revealed that interneuron progenitors of the medial ganglionic eminence maintain broad developmental potentialities. Cells isolated from donors at early or late developmental stages are able to acquire laminar positions congruent with the age of the host, provided that they are transplanted before their last mitosis (Valcanis and Tan, 2003).

In the olfactory bulb (OB), different interneuron phenotypes derive from a mosaic of spatially-restricted germinal domains distinguished by specific transcription factor profiles (Merkle et al., 2007; Kelsch et al., 2007; Young et al., 2007). The time of birth also defines interneuron identity, as distinct subtypes are

preferentially produced at different ages (De Marchis et al., 2007; Batista-Brito et al., 2008). Genetic fate mapping of OB interneuron progenitors highlighted the prevalence of cell autonomous mechanisms in the specification of different interneuron subpopulations (Batista-Brito et al., 2008). In addition, transplantation experiments have shown that donor cells differentiate independently of the host age, indicating that progenitors at different ontogenetic stages are intrinsically committed toward specific lineages (De Marchis et al., 2007). On the whole, a common feature of GABAergic interneuron genesis in multiple CNS regions is that different neuron categories derive from progenitors that reside in discrete germinal domains, where they become specified at the time of their last mitosis (Helms and Johnson, 2003; Bovetti et al., 2007; Batista-Brito and Fishell, 2009). We will now ask whether the same principles apply for the cerebellum.

The PWM: an atypical neurogenic niche in the postnatal cerebellum

All GABAergic interneurons derive from Pax-2-positive cells

Generation of GABAergic phenotypes in the VZ requires the expression of the pancreatic transcription factor Ptf1-a (Hoshino et al., 2005; Hoshino, 2006). In the absence of this factor, VZ progenitors aberrantly migrate toward the EGL and activate a default program of granule cell specification (Pascual et al., 2007). A host of recent studies, aimed at unraveling the genetic codes responsible for the generation of the different GABAergic types, have shown that the VZ is made of a mosaic of segregated microdomains, defined by specific patterns of gene expression (Chizhikov et al., 2006; Sillitoe and Joyner, 2007; Salsano et al., 2007; Zordan et al., 2008; Mizuhara et al., 2009; Lundell et al., 2009). For instance, two regions within the progenitor domain expressing Neph3, a downstream target of Ptf1-a, have been demarcated on the basis of high or low E-cadherin expression levels and identified as the origin of Purkinje cells and inhibitory interneurons, respectively (Mizuhara et al., 2009). Interneuron progenitors are characterized by the expression of Pax-2: they appear at E12.5 in the medial portion of the VZ and progressively delaminate to the PWM (Zhang and Goldman, 1996a,b; Maricich and Herrup, 1999).

Pax-2 is the first known specific and selective marker for the entire lineage of cerebellar GABAergic interneurons. Nevertheless, recent observations have shown that Pax-2 expression initiates in interneuron precursors close to their last cell division (Weisheit et al., 2006; Leto et al., 2009). As a consequence, the generation of appropriate numbers of interneurons must be sustained by the proliferation of Pax-2-negative cells, and Pax-2 should be perceived as an early marker of commitment towards the interneuron lineage (Weisheit et al., 2006; Schilling et al., 2008). The identity of the Pax-2-negative progenitor remains elusive. The PWM contains different populations of dividing cells, including progenitors for glial types (Grimaldi et al., 2009; Silbereis et al., 2009). However, clonal analyses suggest that the different lineages are already separated during postnatal development (Mathis et al., 1997; Milosevic and Goldman, 2002; Mathis and Nicolas, 2003; Milosevic and Goldman, 2004), and transplantation experiments indicate that oligodendrocytes derive from an extra-cerebellar source (Grimaldi et al., 2009). On the other hand, overexpression of the transcription factor Ascl-1 in the embryonic VZ directs progenitor cells towards the interneuron lineage, while suppressing astrocytic differentiation (Grimaldi et al., 2009). This suggests that interneurons and astrocytes share a common progenitor, whose choice towards either lineage is determined by the expression of specific regulatory genes. Genetic fate mapping analysis of GFAP::CreER^{T2} mice might be also consistent with this conclusion (Silbereis et al., 2009), but sound demonstration that these phenotypes stem from common progenitors is still lacking.

Generation of inhibitory interneurons in the PWM

In the mouse Pax-2-positive cells are continuously generated between E12.5 and P15, with an increase around P5, leading to produce virtually all GABAergic interneurons before P7 (Weisheit et al., 2006; Leto et al., 2006, 2008). In other species (e.g. rabbit), however, the genesis of interneurons may persist for longer times, up to adulthood (Ponti et al., 2008). The different phenotypes are generated during the whole period, with a specific neurogenic

window for each interneuron class. Inhibitory interneurons of the DCN and GL are mainly produced during late embryonic/early postnatal development, whereas ML phenotypes are primarily generated during postnatal life. The ontogenetic sequence follows a similar inside-out positional gradient of lamination, in which the first generated cells are fated to the deepest positions of the cerebellar cortex and the latest ones are directed toward the most superficial levels (Leto et al., 2009).

At all ages young postmitotic interneurons sojourn in the PWM for a variable period, lasting one to several days, which is necessary to develop further GABAergic traits, such as Gad67 expression (Simat et al., 2007; Leto et al., 2009). This scenario discloses a number of alternative strategies suitable to produce defined quantities of different interneuron types in due times. The Pax-2-positive cells may represent a single population of multipotent progenitors, whose fate choices are induced by instructive environmental cues. Such cells may either remain multipotent up to the end of development or become progressively restricted towards late-generated phenotypes. Alternatively, the PWM may comprise distinct subsets of fate-restricted precursors already committed to different identities and laminar positions.

These alternative possibilities have been investigated by heterotopic/heterochronic grafts of GABAergic interneuron progenitors. At birth, dividing interneuron progenitors are present both in the periventricular region and in the subcortical white matter. Following transplantation, however, cells isolated from either site generate the same phenotypic repertoire, thus showing that all interneuron progenitors have the same potentiality regardless of their location in the PWM (Leto et al., 2006). Concerning stage-dependent properties, progenitors isolated at different embryonic or postnatal ages and exposed to a heterochronic environment invariably produce phenotypic repertoires appropriate for the host age. For instance, P7 cortical cells, normally fated to the stellate phenotype (Altman and Bayer, 1997), retain full developmental potentialities and are able to become Golgi cells or DCN interneurons when placed in younger cerebella (Leto et al., 2006, 2009). Furthermore, donor-derived interneurons always acquire laminar locations that match the positions of their endogenous counterparts generated at the time of transplantation (Leto et al., 2009). Therefore, transplanted progenitors fully entrain in the recipient neurogenic mechanism and consistently adopt host-specific identities and positions, showing that interneuron progenitors retain a full developmental potentiality up to the latest developmental stages. Together, these observations indicate that all the categories of cerebellar GABAergic interneurons derive from a single population of multipotent cells whose fate choice is dictate by extrinsic signals.

To ask whether interneuron specification occurs at the time of the last mitosis, dividing progenitors or early-postmitotic interneurons isolated from P7 cerebella have been heterochronically transplanted into E15 or P1 recipients. Both types of donor cells consistently yielded the same phenotypic repertoire typical of the recipient age (Leto et al., 2009). Thus, although the mature identity and position are temporally related to the interneuron birthdate, postmitotic interneurons are still able to switch their fate.

This result, together with the observation that both endogenous and grafted postmitotic interneurons sojourn in the PWM suggests that phenotype specification occurs in this site. This idea is in line with the observation that the different categories of interneurons are present even in mutant cerebella, such as the *reeler*, in which the cytoarchitecture is strongly altered and most of the cells end in ectopic positions (Takayama, 1994). Further support about this conclusion comes from transplantation of solid PWM grafts to the non-neurogenic environment of the adult cerebellum (Grimaldi and Rossi, 2006). In this condition, transplanted interneurons adopt identities typical of the donor age, disclosing the endogenous neurogenic capabilities of the PWM (Leto et al., 2009).

On the whole, the genesis of GABAergic interneurons in the embryonic/postnatal PWM niche is characterized by a number of peculiar features:

– interneuron progenitors constitute a single population, which remains multipotent up to late postnatal development;
– specification occurs in postmitotic cells that transiently sojourn in the PWM before their ultimate translocation;
– age-dependent cues in the PWM environment influence the phenotypic specification of the interneurons.

Given the instructive activity exerted by the PWM on transplanted cerebellar cells, this environment

may be also able to influence the differentiation of extracerebellar cells. Indeed, progenitors from the telencephalic subventricular zone grafted to the postnatal PWM acquire morphological and neurochemical features reminiscent of cerebellar interneurons (Milosevic et al., 2008). Nevertheless, neocortical cells grafted in utero to the cerebellar primordium fail to develop clear cerebellar phenotypes (Carletti et al., 2004). In addition, cells isolated from different brain regions (lateral ganglionic eminence, telencephalic subventricular zone, ventral mesencephalon, dorsal spinal cord) and transplanted to the postnatal PWM acquire morphological features, neurochemical profiles and laminar placements that do not match those of endogenous interneurons (Rolando et al., 2010). Interestingly, the extracerebellar donors fail to upregulate cerebellar-specific regulatory genes such as Pax-2 (Milosevic et al., 2008; Rolando et al., 2010), indicating that they actually differentiate by unfolding cell-autonomous programs typical of their native sites. Therefore, the sensitivity to neurogenic cues provided by the cerebellar PWM appears to be restricted to local elements.

Neurogenic signals in the PWM

The signals that specify interneuron phenotypes may derive from different sources, including cell-cell interactions, molecules anchored to the extracellular matrix or released by local elements, signals issued by nearby cell populations. Most likely, multiple interactions among all these components shape the function of the PWM as a neurogenic site. Even though little is known about the nature of these processes and the underlying mechanisms, we would like to propose a model that may be suitable to design targeted mechanistic experiments (Fig. 1). The cellular composition of the PWM is rather complex. In addition to afferent and efferent cortical axons, it contains progenitors for neurons and glia with their newly-born descendants (Grimaldi et al., 2009; Silbereis et al., 2009), plus a minor fraction of stem/progenitor cells with broader developmental potentialities (Klein et al., 2005; Lee et al., 2005). In spite of this cellular variety, the population of Pax-2 positive cells appears very homogeneous, and the analyses carried out to date failed to identify clear

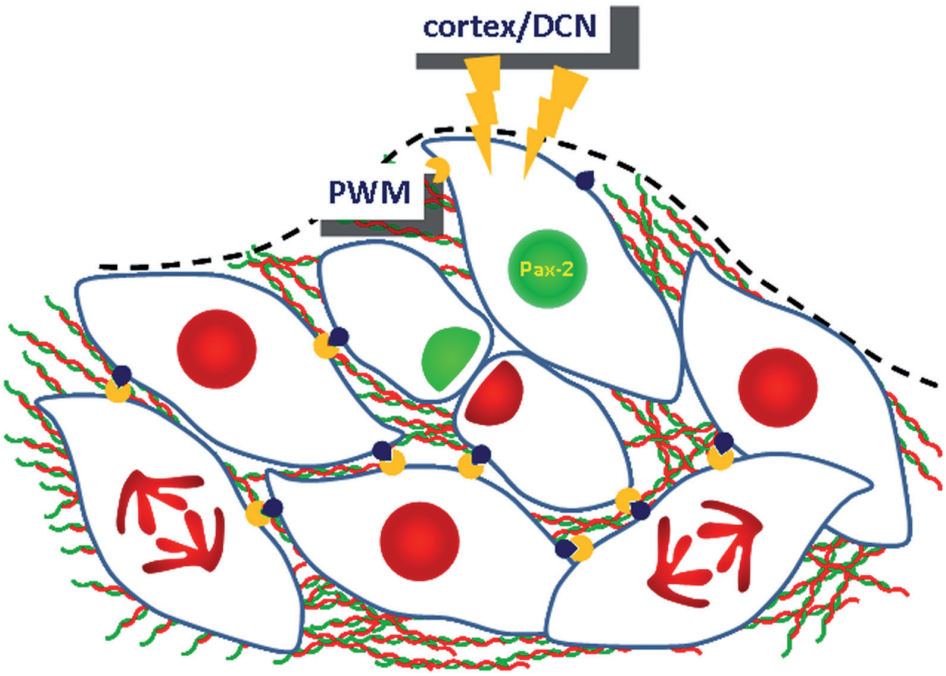

Fig. 1. -Neurogenic signals in the PWM.
According to the model, the generation of GABAergic interneurons is determined by cell-environment interactions within the PWM and regulated by spatio-temporally patterned influences from surrounding cerebellar structures. Signals for proliferation and cell cycle exit of interneuron progenitors (cells with red nucleus) could derive from cell-cell interactions and extracellular matrix components (red and green lines), whereas Pax-2-positive postmitotic interneurons (cell with green nucleus) cold be instructed toward mature phenotypes by signals issued by nearby cell populations of the cerebellar cortex and DCN.

subtypes that could be related to distinct classes of interneurons (Maricich and Herrup, 1999; Weisheit et al., 2006; Glassmann et al., 2009). Now, if the Pax-2-positive cells are all equally sensitive to environmental signals, they might be all specified simultaneously, adopt the same phenotype and, hence, rapidly deplete the reservoir of interneuron progenitors. This paradoxical situation would be avoided if the cells become sensitive to instructive signals only at an advanced stage of maturation, which is consistent with the observation that interneuron fate choice may occur after the last mitosis (Leto et al., 2009). In other words, the timely generation of appropriate numbers of interneurons belonging to different classes could be obtained by producing a sequence of cell cohorts that acquire different mature identities under the influence of spatio-temporally patterned environmental signals. Such a mechanism implies a fine control on the proliferation rates of dividing progenitors as well as on their ability to leave the cell cycle and become sensitive to extrinsic cues. The latter act on the responsive cells to induce their specification.

Several studies show that defects of cell cycle regulation have important consequences on the genesis of cerebellar interneurons. For instance, null mice for cyclin D2, which is required for G1/S transition, show reduced amounts of granule neurons and virtually no stellate cells, while other cell types are generally preserved (Huard et al., 1999). A comparable phenotype has been observed after neural-specific inactivation of Xrcc1, a DNA repair protein. The effect has been attributed to a p-53-dependent cell-cycle arrest that occurs when interneuron progenitors start differentiation (Lee et al., 2009). It is still unclear whether these outcomes are due to impaired specification of certain classes of interneurons or to a premature exhaustion of the progenitor pool, which would primarily affect the latest generated molecular layer types. In any instance, these findings indicate that a fine balance of cell proliferation and cycle exit is required to produce the variety of cerebellar interneurons. These processes could be controlled by cell-cell interactions, such as the lateral inhibition mediated by Notch-Delta signaling, where neighboring cells can influence each other abilities to divide or initiate differentiation (Ready et al.,1976; Cagan et al., 1992). As previously described for the EGL (Pons et al., 2001), components of extracellular matrix may also contribute to these mechanisms. Indeed, both the structure of the extracellular matrix (Baier et al., 2007) and the expression of synthetic enzymes for its major components (Ishii and Maeda, 2008) change during cerebellar development, being strictly related to the maturation of inhibitory interneurons. Similar analyses of the expression and function of different types of cadherins in the postnatal cerebellum suggest that these molecules modulate the sorting and migration of interneurons (Gliem et al., 2006).

In addition to signals that regulate the proliferation and cycle exit of GABAergic interneuron progenitors, the sequential generation of the different phenotypes also requires type-specific instructive signals precisely distributed in space and time. Although there is still no direct evidence about the nature and the origin of these signals, they likely derive from the rapidly evolving cerebellar structures that surround the PWM. Namely, while the periventricular PWM is next to the DCN, in the cortical regions it is adjacent to the Purkinje cell plate during embryonic development and to the internal granular layer after birth. These evolving spatial relationships suggest that the generation of different interneuron categories may be directed by signals issued by nearby cell populations. Hence, during embryonic development DCN neurons induce Pax-2-positive progenitors to generate nuclear interneurons (Leto et al., 2006), whereas precursors in the subcortical PWM differentiate into Golgi and Lugaro cells under the influence of the neighboring Purkinje cells. After birth, granule cells that progressively accumulate in the internal granular layer switch the fate choice of PWM cells in favor of molecular layer interneurons. This model implies that, as soon as they become committed to their final phenotype, the young interneurons must activate the molecular machinery needed to reach their final destination. For instance, there is evidence that netrin-1 signaling influences the targeting of interneurons to the molecular layer, and the relevant receptors are differentially expressed in PWM cells (Gujarro et al., 2006). Furthermore, analysis of knockout mice for the ErbB4 receptors suggests a role for the ErbB4/neuregulin pathway in the development of Golgi cells (Tidcombe et al., 2003). In conclusion, although most of the mechanisms and molecular mediators responsible for the generation of the variety of

cerebellar interneurons have still to be elucidated, the available evidence suggests that this process is determined by cell-environment interactions within the PWM itself, which are modulated by additional influences issued by the surrounding structures of the developing cerebellum.

Conclusions: significance and advantages of atypical neurogenic niches

GABAergic interneurons represent critical elements of cerebellar local networks with precise functions in providing direct or indirect modulation on PCs activity. While in most CNS regions interneuron diversity is reached by recruiting precursors from distinct neurogenic territories, in the cerebellum it is achieved by creating diversity from a single source. In this context, the PWM niche provides the substrate for an atypical neurogenic strategy, in which defined numbers of uncommitted cells, produced according to precise time schedules, are instructed by local cues to final phenotypic and layering identities. This mechanism represents a flexible and dynamic way to generate wide interneuron repertoires in appropriate quantities, according to precise spatiotemporal patterns. Furthermore, late specification in the PWM could facilitate the adaptation of the cerebellar networks to specific developmental constraints or evolutionary demands. The fundamental processes acting within the niche remain to be clarified. Our future efforts will be devoted to address these questions.

Acknowledgements

This work was supported by grants from Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MIUR-PRIN 2007 prog. nr. 2007F7AJYJ), Compagnia di San Paolo (Neurotransplant Project 2008; GABAGEN Neuroscience project 2009), Regione Piemonte (Project A14/05; Ricerca Sanitaria Finalizzata, 2008, 2009), Ataxia UK; Fondazione Cavaliere del Lavoro Mario Magnetto of Turin, and the University of Turin. K.L. is supported by fellowships funded by Fondazione per la Ricerca Biomedica Onlus of Turin.

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