Development of cerebellar GABAergic interneurons: origin and shaping of the “minibrain” local connections

Cerebellum (2008) 7:523–529
DOI 10.1007/s12311-008-0079-z

Development of Cerebellar GABAergic Interneurons: Origin and Shaping of the "Minibrain" Local Connections

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Published online: 12 November 2008

Abstract The cerebellar circuits comprise a limited number of neuronal phenotypes embedded in a defined cytoarchitecture and generated according to specific spatio-temporal patterns. The local GABAergic network is composed of several interneuron phenotypes that play essential roles in information processing by modulating the activity of cerebellar cortical inputs and outputs. A major issue in the study of cerebellar development is to understand the mechanisms that underlie the generation of different interneuron classes and regulate their placement in the cerebellar architecture and integration in the cortico-nuclear network. Recent findings indicate that the variety of cerebellar interneurons derives from a single population of multipotent progenitors whose fate choices are determined by instructive environmental information. Such a strategy, which is unique for the cerebellum along the neuraxis, allows great flexibility in the control of the quality and quantity of GABAergic interneurons that are produced, thus facilitating the adaptive shaping of the cerebellar network to specific functional demands.

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Keywords Inhibitory interneurons · Neurogenesis · Neuronal specification · Differentiation

Introduction

All cerebellar neurons derive from two germinative neuroepithelia with distinct developmental potentialities. Glutamatergic lineages—projection neurons of the deep nuclei (DCN), unipolar brush cells, and granule cells—derive from Math-1-expressing progenitors that emigrate from the rostral rhombic lip (RL) and undergo subsequent waves of differentiation [1–6]. GABAergic phenotypes—Purkinje cells (PCs), nucleo-olivary neurons, and the different classes of inhibitory interneurons—are produced by pancreas transcription factor 1-a (Ptf1-a) positive precursors of the ventricular zone (VZ; [7, 8]). Starting from such spatially segregated germinal layers, the different cerebellar phenotypes are generated according to precise temporal schedules. Projection neurons (DCN neurons and PCs) are born at the onset of cerebellar neurogenesis, while all excitatory and inhibitory interneurons are sequentially produced during late embryonic and early postnatal life [9–12]. An impressive increase of the total cell number occurs during this period [13], so that the adult cerebellum takes up about 10% of the cerebral volume but holds more than 50% of the neurons.

GABAergic interneurons comprise about half of the cerebellar neuronal phenotypes. Each class of inhibitory interneurons is characterized by distinctive morphological and neurochemical features (Table 1), a precise position in the cerebellar architecture, and highly specific connections [14–19]. Given such a phenotypic complexity and diversification, a major open question concerns the mechanisms

Table 1 Distribution of markers for cerebellar GABAergic interneurons in adult rodents

	Calretinin	Neurogranin	MGluR2	Pax-2	Parvalbumin	Rorα	Neu-N	References
DCN	− + +	− −	− −	− −	− −	− −	+ + + +	[17–19]
Golgi	+ + +	+ + +	+ + + +	+ + + +	− −	− −	− −	[17, 19, 33, 34, 77–81]
Lugaro	+ + +	− −	− + +	− −	− −	− −	− −	[17, 77, 80–82]
Basket	− −	− −	− −	− −	+ + + +	+ + /	− −	[16, 17, 83]
Stellate	− −	− −	− −	− −	+ + + +	+ + /	− −	[16, 17, 83]

Bold mouse, *regular* rat, + + frequent, + less frequent, − absent, / unknown

underlying the acquisition of interneuron identities and, particularly, the relative contribution of cell-intrinsic properties and environmental cues.

Where Are Cerebellar Interneurons Coming From?

The origin of cerebellar inhibitory interneurons has been controversial for a long time. Until a few years ago, it was accepted that deep nuclei and granular layer (GL) interneurons derived from the VZ, whereas molecular layer (ML) interneurons were supposed to come from the external granular layer (EGL), the only germinal layer that is active during postnatal development [14, 20]. Quail-chick chimeras and transplantation experiments [21–23], together with developmental studies on mutant mice [24], unequivocally demonstrated that EGL cells exclusively give rise to granule cells and suggested that at least a fraction of the ML interneurons actually derive from the VZ [21, 25].

Cell proliferation in the VZ ceases at birth, but dividing progenitors, initially identified as glial precursors [9, 26], persist in the prospective white matter (WM) during postnatal development. Zhang and Goldman [27] labeled such cells by retroviral injections and showed that they also generate GABAergic interneurons. Clonal analyses suggested that neuronal, astrocytic, and oligodendrocytic lineages are largely separated during postnatal development [28, 29], while cortical interneurons share lineage relationships with other VZ-derived neurons, such as PCs and DCN neurons [30, 31].

A conclusive contribution to this point was provided by Hoshino and colleagues [7], who used genetic fate mapping analysis to reveal that all cerebellar GABAergic phenotypes derive from VZ progenitors expressing Ptf1-a. Interestingly, recent observations suggest that this transcription factor is required to specify GABAergic identities but also to suppress the glutamatergic differentiation program [32].

Dividing progenitors for GABAergic interneurons in the cerebellar parenchyma can be identified by the expression of Pax-2 [33, 34]. A discrete cluster of Pax-2-positive cells can be detected in the medial aspect of the E12 mouse cerebellar anlage. Thereafter, these cells spread throughout the prospective WM and eventually settle in the deep nuclei and cortical layers. After P16, when the genesis of interneurons ceases, Pax-2 transcripts are only expressed in Golgi neurons [33]. Therefore, the current view is that inhibitory interneurons originate from Ptf1-a-expressing VZ progenitors, which are the common ancestors for all cerebellar GABAergic types. Interneuron progenitors delaminate from the germinal layer during early embryonic development and continue to divide into the overlying cerebellar tissue. Thus, similar to granule cell precursors which originate from the RL and form a secondary neuroepithelium (the EGL), interneuron precursors stem from the VZ but continue their developmental process in the prospective WM.

Temporal Axis of Interneuron Neurogenesis: An Inside–Out Model

Classical studies based on 3H-tymidine labeling demonstrated that cortical interneurons are produced according to a precise progression, first in the GL and then in the ML, basket cells preceding stellate cells [9, 10]. Neurogenesis in the DCN was originally thought to be completed between E13 and E15 in the rat [10, 11, 35]. However, recent birthdating analysis showed that, while projection neurons (including both glutamatergic neurons and GABAergic nucleo-olivary neurons) are generated within this period, inhibitory interneurons are produced during a longer time window extended to early postnatal life [19]. On the whole, the different classes of GABAergic interneurons are generated during largely overlapping developmental periods according to an inside–out sequence, starting from the deep nuclei to the granular and molecular layers (Fig. 1).

Taking advantage of transgenic Pax-2-green fluorescent protein (GFP) mice [36], it has been estimated that the number of GABAergic interneuron precursors increases during development to a total of some 905,000±77,000 cells. The peak is around P5, and the production of 75% of all inhibitory interneurons occurs prior to P7 [34]. Between P0 and P3, the numerical increase of interneuron precursors is due to the proliferation of a Pax-2-negative precursor population, whereas beyond this age, the mechanisms regulating the amplification of interneuron progenitors

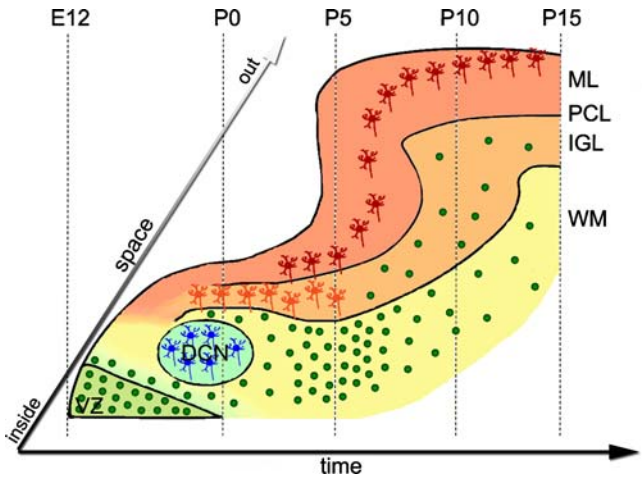

Fig. 1. Inside–out sequence of cerebellar GABAergic interneuron neurogenesis. VZ-derived inhibitory interneuron precursors give rise to DCN interneurons mainly during embryonic life, while during postnatal development, they migrate through the prospective WM, generating cortical interneuron phenotypes (first in the GL, then in the ML). *VZ* ventricular zone, *DCN* deep cerebellar nuclei, *WM* white matter, *IGL* internal granular layer, *PCL* Purkinje cell layer, *ML* molecular layer

remain to be clarified, and it is possible that the number of precursor may be also determined by the proliferation of Pax-2-positive cells [34].

BrdU incorporation experiments show that dividing Pax-2 positive cells are exclusively localized in the prospective WM [19, 33]. However, after pulse administration of this nucleotide analogue in GAD67-GFP transgenic mice, in which all GABAergic neurons are fluorescent, the reporter gene is detectable in WM cells only 2 days after the last mitosis [37]. Together, these observations indicate that newly born interneurons remain in the prospective WM for a certain time after their terminal division. These postmitotic cells also show protracted expression of the cell cycle marker Ki-67 that persists during the ensuing migratory phases [34]. It has been proposed that this peculiar behavior reflects a quiescent phase of inhibitory interneuron development before the final acquisition of the mature phenotype [34].

Beyond Space and Time: Derivation of Interneurons from a Single Pool of Multipotent Progenitors

Different strategies can be envisaged to regulate the generation of the variety of cerebellar inhibitory interneurons. It may be possible that Pax-2-positive interneuron progenitors actually comprise distinct subsets of fate-restricted precursors, each destined to a specific identity. Nevertheless, the clonal relationship linking Golgi, basket, and stellate cells [30, 31] suggests that all inhibitory interneurons share a common ancestor. Then, it is possible that the sequence of interneuron generation is obtained by progressively restricting in space and time the developmental potentiality of initially multipotent progenitors. Such a mechanism is functioning during cerebellar neurogenesis, since postnatal precursors are unable to adopt projection neuron identities, even when exposed to the embryonic environment [38–40]. Alternatively, however, interneuron progenitors may retain full potentialities throughout development and make their phenotypic choices in response to extrinsic cues.

Heterotopic/heterochronic transplantation of interneuron progenitors is the most suitable approach to distinguish between these possibilities. At birth, when all interneuron categories are being generated, proliferating progenitors are located both in the periventricular region and in the subcortical WM. To ask whether these represent spatially segregated pools of fate-restricted precursors, periventricular or cortical cells, isolated from β-actin GFP transgenic animals, were transplanted to cerebella of different ages, from embryonic life to adulthood [19]. The result of this experiment shows that both donor populations share the same developmental potentialities: regardless of their origin, they yield the same interneuron repertoires, which are strictly dependent on the host age and engraftment site.

To assess whether interneuron progenitors show lineage restriction in time, embryonic or postnatal progenitors were grafted to different-aged hosts [19]. The results are again clear-cut. Both embryonic and postnatal progenitors maintain full multipotency and generate interneuron types appropriate for the developmental stage of the recipient. Even P7 cortical progenitors, which are normally fated solely to the stellate cell phenotype [10], generate the complete repertoire of nuclear and cortical interneurons when exposed to embryonic environment but only adopt ML phenotypes in isochronic recipients [19]. Therefore, all GABAergic interneuron categories are generated by a common pool of multipotent precursors. These cells maintain their full competence up to late developmental stages and develop specific phenotypic traits in response to environmental instructive signals.

Intrinsic Versus Extrinsic Regulation of Interneuron Identities

To achieve full understanding of the processes underlying the generation of the variety of cerebellar interneurons, it is necessary to identify the nature and the mechanisms of action of the instructive cues that influence the fate of multipotent precursors. Clonal analysis of progenitor lineages may provide useful information on the time and place where the naive cell makes its final choice [41]. In addition, it is important to precisely define when the specification machinery is active in the precursors, as their sensitivity towards external signals may vary during the cell

cycle or according to the developmental stage [42–45]. In vitro essays demonstrated that the acquisition of appropriate identities by cerebellar interneuron progenitors does not depend on the precise timing of their last mitosis and on the completion of a predetermined number of divisions [46]. However, the responsiveness of these precursors to extrinsic signals changes during the cell cycle, as cells that are in the S phase at the moment of cultivation poorly survive or fail to differentiate.

In spite of these considerations, the identity of the signals that determine interneuron differentiation remains obscure. Considering the available information, two points appear particularly relevant: (1) interneuron progenitors exclusively proliferate in the prospective WM; and (2) the Pax-2-positive cells remain in the WM for a rather long time after their terminal division. These observations suggest that the specification of multipotent progenitors occurs in the prospective WM, where the newly generated interneuron also undergoes a process of maturation before moving towards its final destination. This scenario implies that the fate choices of interneuron progenitors are imposed by spatio-temporally patterned signals that act on the WM cells. Such signals might come either from the WM milieu itself or from neighboring structures.

The cell composition and microenvironment of the prospective cerebellar WM are scarcely known. In addition to afferent and efferent cortical axons, it contains newly born neurons and glia, the relative progenitors, and likely, some stem cells with broader potentialities [47, 48]. The relationships and interactions between local WM elements may be important to regulate the balance between proliferation and differentiation, as demonstrated for the EGL [49]. Indeed, dissociation and plating induce almost immediate differentiation of interneuron progenitors [46], suggesting that local cell–cell interactions regulate their cycling properties. In addition, expression analysis in the postnatal cerebellum indicates that patterned expression of hyaluronan [50] or of different types of cadherins [51] regulate the sorting and migration of GABAergic interneurons.

Although local variations and/or temporal evolution of the cellular/molecular composition of the prospective WM could be sufficient to regulate the production of interneurons at precise times and places, it is conceivable that signals issued from neighboring cell populations contribute to this process (Fig. 2). According to this view, during late embryonic development, DCN neurons may induce progenitors in the periventricular zone to acquire the phenotype of nuclear interneurons. At the same time, precursors in the subcortical WM differentiate into GL interneurons under the influence of the neighboring Purkinje cell plate. After birth, granule cells that progressively populate the nascent internal granular layer are in a good position for switching the fate of WM progenitors to ML interneuron phenotypes. Indeed, following heterotopic transplantation of granule cells in the deep parenchyma of postnatal cerebella, host interneurons that remain intermingled with donor cells acquire the phenotype of basket/stellate cells, suggesting that grafted granule cells can dictate the fate choices of nearby host progenitors [19]. In addition, there is evidence that granule cells influence the survival of ML interneurons [5, 52] and regulate their migration and cortical placement through Netrin1 signaling [53]. Although these hypotheses still wait for sound experimental evidence, it is likely that specification of interneurons is determined by the interplay between local interactions in the WM environment and spatio-temporally patterned signals coming from nearby developing structures.

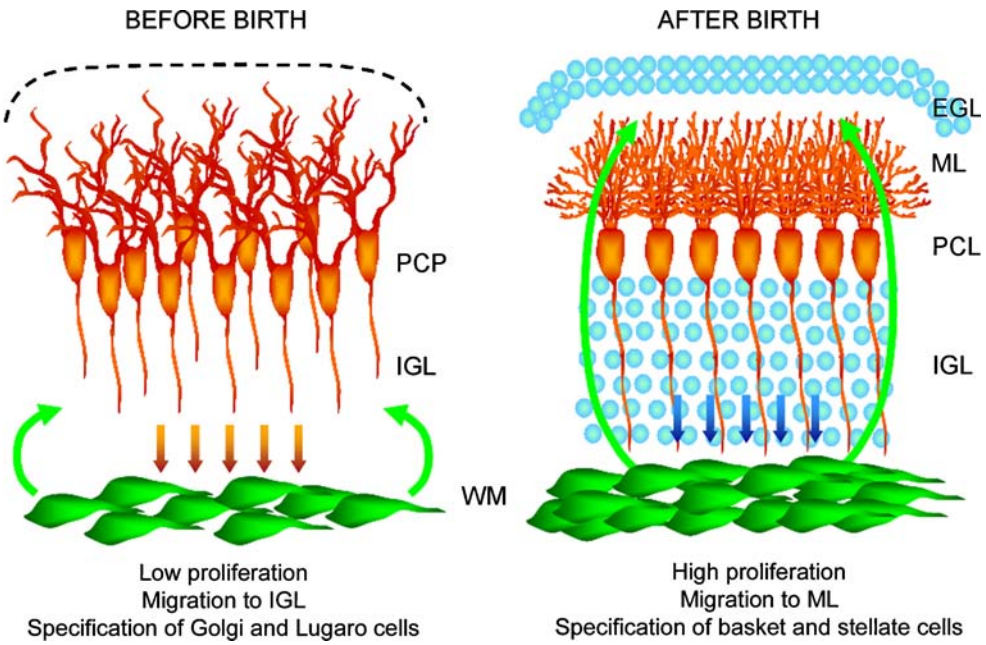

Fig. 2. Extrinsic influences from neighboring cells on the specification of GABAergic interneuron precursors. The cartoon proposes the hypothesis that neuron populations that are adjacent to the prospective white matter influence the fate choices of interneuron precursors. During late embryonic development, Purkinje cells in the Purkinje cell plate would induce the differentiation of granular layer interneurons. After birth, maturing granule cells would boost the proliferation of WM precursors, elicit their migration to the molecular layer, and induce their differentiation into basket and stellate cells. *PCP* Purkinje cell plate, *WM* white matter, *IGL*, internal granular layer, *PCL* Purkinje cell layer, *ML* molecular layer, *EGL* external granular layer

In addition, the contribution of more general regulatory cues, such as hormones [54] or activity-dependent mechanisms [55], should not be disregarded.

Concluding Remarks: A Peculiar Neurogenic Strategy

Cerebellar GABAergic interneurons are generated through a highly peculiar strategy, different from the one applied for other cerebellar types or for inhibitory interneurons in other regions of the neuraxis. RL-derived glutamatergic phenotypes originate from discrete pools of fate-restricted progenitors [1, 4–6, 56]. On the other hand, the genesis of GABAergic neurons in the VZ progresses in two steps [56]. Projection neurons are generated locally at the onset of neurogenesis. In addition, dividing progenitors delaminate into the overlying parenchyma, where they will become inhibitory interneurons [27, 33, 34]. Most importantly, the latter precursors maintain the ability for generating the full spectrum of interneuron phenotypes up to the latest developmental stages, and their fate choices are entirely dependent on environmental instructive information [19].

This mechanism is unique also when the origin of GABAergic interneurons at different central nervous system (CNS) levels is considered. Similar to other sites, in the cerebellum, projection neurons are generated first, and local interneurons follow. In addition, transcription factors relevant for the specification of cerebellar GABAergic types, such as Ptf1-a or Pax-2, also participate to the same processes in other CNS regions [7, 8, 32, 57–60]. Nevertheless, a common theme in the genesis of interneurons along the neuraxis is that different phenotypes are generated from distinct subsets of fate-committed progenitors. For instance, interneurons of the spinal cord derive from eight genetically distinct progenitor pools distributed in the dorsal domain [61]. In the cerebral cortex, different interneuron categories originate from defined subsets of subpallial progenitors, whose developmental potentialities depend on their location [62–64] and on the time of their generation [45, 65, 66]. Finally, the production of olfactory bulb interneurons is sustained by separate progenitor pools, restricted in space [67–70] and time [71–73]. Thus, while the most common strategy to generate the variety of CNS interneurons relies on dedicated precursors that differentiate according to cell-intrinsic programs, those of the cerebellum are produced by naive progenitors under the influence of extrinsic instructive cues.

The significance for this unique feature of cerebellar neurogenesis remains unclear. It can be speculated that its long duration is not compatible with a neurogenic mechanism in which types and numbers of interneurons are largely predetermined by the developmental potentialities of fate-restricted precursor pools. Rather, the dramatic increase of neuronal number that occurs postnatally requires a more flexible mechanism, able to dynamically regulate the production of inhibitory elements to be inserted in the maturing circuitries. This could be obtained by modulating the proliferation rate and fate choices of a single population of multipotent progenitors. Considering the relevance of inhibition for shaping neural networks and preventing a range of developmental disorders [74–76], such a strategy may offer great advantages to adjust the properties of cerebellar circuits to specific developmental constraints or environmental demands.

Acknowledgements This work was supported by grants from Ministero dell'Università e della Ricerca Scientifica e Tecnologica (PRIN, nr 2005055095); Ministero della Salute (Nuove terapie cellulari per le malattie neurodegenerative, nr 533F/G1 to FR & LM), European Community (nr 512039); Compagnia di San Paolo (Neurotransplant Project, 2004.2019–2007.0660), Istituto Superiore di Sanità (Convenzione 530/F-A5 to FR and LM); Regione Piemonte (Proj. A14/05 and 865/2006); University of Turin; Ketty Leto is supported by a "Lagrange Project" PhD fellowship from the Cassa di Risparmio di Torino.

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