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The extracellular matrix provides directional cues for neuronal migration during cerebellar development

M.A. Porcionatto

Departamento de Bioquímica, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brasil

Abstract

Normal central nervous system development relies on accurate intrinsic cellular programs as well as on extrinsic informative cues provided by extracellular molecules. Migration of neuronal progenitors from defined proliferative zones to their final location is a key event during embryonic and postnatal development. Extracellular matrix components play important roles in these processes, and interactions between neurons and extracellular matrix are fundamental for the normal development of the central nervous system. Guidance cues are provided by extracellular factors that orient neuronal migration. During cerebellar development, the extracellular matrix molecules laminin and fibronectin give support to neuronal precursor migration, while other molecules such as reelin, tenascin, and netrin orient their migration. Reelin and tenascin are extracellular matrix components that attract or repel neuronal precursors and axons during development through interaction with membrane receptors, and netrin associates with laminin and heparan sulfate proteoglycans, and binds to the extracellular matrix receptor integrins present on the neuronal surface. Altogether, the dynamic changes in the composition and distribution of extracellular matrix components provide external cues that direct neurons leaving their birthplaces to reach their correct final location. Understanding the molecular mechanisms that orient neurons to reach precisely their final location during development is fundamental to understand how neuronal misplacement leads to neurological diseases and eventually to find ways to treat them.

Correspondence
M.A. Porcionatto
Departamento de Bioquímica
UNIFESP/EPM
Rua Três de Maio, 100, 5º andar
04044-020 São Paulo, SP
Brasil
Fax: +55-11-5573-6407
E-mail: marimelia.bioq@epm.br

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Introduction

The name cerebellum comes from "little brain" (Latin). The cerebellum is the smallest part of the central nervous system, comprising only 10% of the total volume of the brain but has more than half of its total number of neurons. The functions of the cerebellum are related to the motor system and include motor coordination and the control of eye movement and balance. It is a function of this part of the brain to evaluate disparities between intention and action during movement, and to adjust the operation of motor centers in the cortex and brain stem while a movement is in progress as well as during repetitions of the same movement. For more than a century, all cerebellar cell types and their connections have been known due to the beautiful studies of Rámon y Cajal

(1889) using Golgi staining. Further studies contributed to the understanding of the genetic and molecular mechanisms involved in several events that occur during cerebellar development, including proliferation and migration of neuronal precursors, and synapse formation (reviewed in Ref. 1).

During embryonic development, starting at embryonic days 13-14 (E13-14) in mice, neuronal progenitors migrate dorsally from the rhombic lip, generated at the junction between the neural tube and the expanded roofplate of the fourth ventricle, to form the cerebellar anlage (2). Cell-fate maps demonstrate that cells that will form the cerebellar anlage derive from both mesencephalic and metencephalic vesicles. On the other hand, cells that will give rise to Purkinje cells, interneurons of the molecular layer, and glial cells of the cerebellar cortex arise via radial migration from the neuroepithelium (3-5). Progenitors of the cerebellar granule cells migrate away from the roofplate via tangential movements to form the external germinal layer (5,6).

The final development of the cerebellum occurs after birth. In rodents, postnatal cerebellar development occurs during the first 2 weeks of age, and in humans during approximately the first 18-24 months after birth. At this time precursors of the most abundant type of cerebellar neurons, the granule cells, proliferate in a secondary germinal zone, the external granule cell layer, and migrate inwards to form the internal granule cell layer giving rise to the multilayered structure of the cerebellum. Cells from the external granule cell layer located in the premigratory zone, the inner portion of the layer, first elongate their neurites bidirectionally parallel to the cerebellar surface, forming the parallel fibers, before migrating inwards into the internal granule cell layer. To migrate out of the external granule cell layer, granule cell precursors use Bergmann fibers as tracks, passing the Purkinje layer, to form the mature internal granule cell layer (7).

Extracellular matrix molecules provide support and orientation for neuronal migration

Two types of migration occur during cerebellar development, tangential migration and radial migration. Tangential migration refers to a migration path parallel to the surface and occurs in early cerebellar development, when progenitors of the granule cells migrate away from the rhombic lip due to the combined action of chemorepellent and chemoattractant cues. The radial migration is observed during postnatal cerebellar development, when granule cell precursors migrate out of the external granule cell layer, perpendicular to the surface, using Bergmann glial fibers as support.

Several extracellular cues are necessary to coordinate and orient neuronal migration during brain development, and some of these molecules also play important roles in axonal guidance. Chemoattractants and chemorepellents act on guiding neurons using basically the same molecular mechanisms used in axonal guidance. Some of these cues are soluble factors such as the neurotrophin brain-derived neurotrophic factor, which is a chemoattractant factor for cerebellar granule cell precursors during postnatal migration away from the external granule cell layer (8). Other soluble factors and adhesion molecules act coordinately to orient, stimulate or inhibit migration during cerebellar development (reviewed in Ref. 9).

Molecular guidance cues such as those given by netrin, tenascin and reelin provide neurons with active attraction or repulsion signals, while other molecules such as extracellular matrix chondroitin sulfate proteoglycans apparently give “where not to go” signals. In this way, when the extracellular matrix is concerned, neuronal migration is governed by two distinct, cooperative mechanisms: inhibitory molecules establish boundaries between permissive and non-permissive areas, while active attraction and

repulsion signals orient neuronal migration.

Molecules that give support for migration and form boundaries between permissive and non-permissive areas: laminin, fibronectin, vitronectin, and proteoglycans

Laminins consist of a very important family of extracellular matrix glycoproteins that have binding sites for integrins, and for other extracellular matrix components such as collagen and proteoglycans. A typical laminin molecule consists of three polypeptide chains, A, B1, and B2, linked via disulfide bonds to form the typical laminin asymmetric cross-structure (10). Cerebellar granule cells require laminin to migrate *in vitro* (11), and *in vivo* (12). Laminin is detected in punctate deposits along the radial Bergmann glial fibers in the premigratory phase (E18-P0), in a higher concentration when compared to the postnatal cerebellum, suggesting that deposits of laminin could be used by granule cells to initiate migration (13,14). *In vitro* experiments have shown that granule cell precursors migrate on fibronectin- but not on collagen-coated surfaces (15).

Cells located in the inner part of the external granule cell layer, the premigratory zone, and in the upper molecular layer of the neonatal cerebellum elongate their neurites bidirectionally before and during migration into the internal granule cell layer. The integrin αvß5, a receptor for vitronectin, and vitronectin itself, are expressed in parallel fibers in the external granule cell layer and molecular layer at postnatal days 3-20, but not in migrating granule cells or mature parallel fibers. *In vitro* experiments show that granule cell precursors elongate parallel fibers in response to vitronectin, but do not migrate on this extracellular matrix component (16).

Proteoglycans interact with several extracellular matrix components stimulating or inhibiting their biological effects. Exogenous chondroitin 4-sulfate, chondroitin 6-sulfate, and keratan sulfate inhibit neurite outgrowth of cerebellar neurons on laminin-coated surfaces, whereas heparan sulfate has no effect on neurite outgrowth (17). The inhibitory effect of chondroitin sulfate proteoglycans on axonal outgrowth during developmental processes and injury has been well documented in the literature. Chondroitin sulfate proteoglycans are among the inhibitory molecules synthesized by reactive glia during glial scar formation at an injury site in the central nervous system (reviewed in Ref. 18).

Although changes in extracellular matrix composition, spatially and with time, are not yet completely understood, the notion that dynamic changes occur and that those modifications are responsible in part for the correct positioning of neurons is well accepted. Not only the expression and location of extracellular matrix molecules are modified during development, but the expression of extracellular proteases, such as MMP-2 and MMP-9, which are implicated in matrix remodeling, have been shown to be developmentally regulated in the cerebellum (19).

Active signaling molecules: reelin, tenascin and netrin

As discussed above, some extracellular matrix components support and stimulate neuronal migration, but do not provide direction for these cells. On the other hand, there are several extracellular molecules that orient neuronal migration. Some of these molecules are soluble factors, such as the neurotrophins that diffuse from a source to form a gradient stimulating chemotaxis while others are components of the matrix, such as reelin, netrin and tenascin, which attract or repel neurons by molecular mechanisms not fully understood.

Reelin is an extracellular glycoprotein responsible for the phenotype of a spontane-

ous mutant mouse named *reeler*, that presents severe motor defects, including ataxia, tremors, and balance and locomotion problems, and was first described in 1951 (20). Analysis of the development of the central nervous system of *reeler* mice showed that these animals have impaired migration of cortical neurons, leading to an inversion of the cerebral cortex organization (inverted cortex) (reviewed in Refs. 21 and 22). Defects in the *reeler* cerebellum include lack of foliation, lack of Purkinje cells, and decreased size (23,24).

Identification of the gene that causes the *reeler* phenotype led to the identification of the extracellular protein reelin (25). Analysis of the reelin gene and protein structure shows that this glycoprotein has several features of extracellular proteins, such as a cleavable signal peptide and several putative glycosylation sites for N-glycosylation and, to a lesser extent, O-glycosylation (26,27).

During early central nervous system development, reelin mRNA is expressed by the Cajal-Retzius cells of the cerebral cortex (E10-12), by the Cajal-Retzius-like cells in the marginal zone of the developing hippocampus (E13-14), and by rhombic lip cells, external neuroepithelium, differentiating Purkinje cells, and deep nuclear neurons as well as elements of the cerebellar peduncles (E13-14). During postnatal cerebellar development, reelin mRNA is expressed by the internal granule cell layer cells and by cells present in the inner layer of the external granule cell layer (26). Purkinje cells and Bergmann glia do not express reelin, but because Purkinje cells accumulate reelin bound to the membrane, it has been suggested that these cells would express a still unknown receptor (28).

Reelin binds to a few transmembrane receptors such as cadherin-related neuronal receptors (29), α3ß1 integrin (30), and two members of the lipoprotein receptor family, apolipoprotein E receptor 2 and very-low density lipoprotein receptor (31,32). In very-low density lipoprotein receptor-knockout mouse cerebella, Purkinje cells are found ectopically, probably due to their inability to respond to reelin synthesized by granule cells (31).

Clustering of lipoprotein receptors seems to be important for signaling by reelin, and the participation of cadherin-related neuronal receptors and integrins as co-receptors is not excluded (33). Downstream signaling by the lipoprotein receptors upon binding of reelin leads to tyrosine phosphorylation of disabled-1, a cytosolic adapter protein, resulting in the activation of signaling pathways through tyrosine kinases of the Src family (34,35). In addition to activation of Src family members, disabled-1 phosphorylation also leads to activation of Akt (36) and redistribution of Nckß from the cell body to distal sites of neuronal processes (37). Although the molecular mechanisms involved in the signaling of reelin are partially known, the role of these events in the correct positioning of neurons during migration is still poorly understood.

Other molecules actively involved in signaling to orient neurons to reach their correct positions are the members of the tenascin family. These are large extracellular matrix glycoproteins consisting of an amino-terminal cysteine-rich region involved in oligomerization, followed by linear EGF-like segments, fibronectin-type III repeats and a fibrinogen-like region at the carboxy-terminal (38). In humans there are 4 members of the family, known as tenascins-C, -Y, -W, and -R, a facultative chondroitin sulfate proteoglycan. Tenascins participate in different cellular processes, including cell adhesion and migration, and interestingly, can either stimulate or inhibit cell migration depending on the cell type (39-41).

During cerebellar development, Purkinje cells migrate radially from the neuroepithelium of the fourth ventricle towards the cortical surface between E13 and E17. At E14, astroglia extend fibers from the ventricle to

the pial surface, and their cell bodies migrate after E15 towards the cortex, shortening the radial processes whose end-feet are attached to the pia mater. During migration of the Purkinje cells, radial glial fibers express tenascin oriented according to the migratory direction of these cells. These findings suggest that the arrangement of radial glia and the expression of tenascin, among other adhesion molecules, are involved in the control and guidance of Purkinje cell migration during early cerebellar development (42).

The cellular receptor for tenascins appears to be the facultative chondroitin sulfate proteoglycan receptor-type protein tyrosine phosphatase ζ/ß (RPTPζ/ß). Two other chondroitin sulfate proteoglycans can also bind tenascin, neurocan and the soluble form of RPTPζ/ß, phosphacan (43). In early postnatal and adult cerebellum, neurocan and phosphacan show immunoreactivities that follow different developmental time courses. Neurocan is seen in the prospective white matter and in granule cells, Purkinje cells, and molecular layer, whereas phosphacan is associated with Bergmann glial fibers in the molecular layer and their cell bodies below the Purkinje cells (44). Besides the chondroitin sulfate proteoglycans, tenascins also bind heparin through their fifth fibronectin type III domain, although it is not clear if this *in vitro* binding capacity is also valid for binding to heparan sulfates that would be found *in vivo*, and if this is somehow related to neuronal migration (45).

Another extracellular molecule that plays a role in cerebellar neuronal migration is netrin. Netrins are laminin-related molecules that exert their biological functions by interacting with the transmembrane receptors deleted in colorectal carcinoma (DCC) (46), and UNC5 (47). Signaling by netrin binding to DCC results in attraction whereas intracellular signaling by UNC5 after netrin binding leads to repulsion. Growth cone attraction to netrin can be converted to repulsion by UNC5, but this process requires the presence of DCC (48). The cytoplasmic domain of UNC5 is sufficient to induce this conversion, and repulsion can be triggered by activation of either receptor. Apparently, netrin-1 can induce the formation of a DCC/UNC5 receptor complex, converting DCC-mediated attraction to DCC/UNC5-mediated repulsion.

The DCC intracellular signaling pathway involves the activation of the mitogen-activated protein kinase family members ERK-1 and -2, which are recruited to the DCC receptor complex (49). Second messengers such as Ca^{2+}, cAMP and cGMP are known to regulate attractive and repulsive axonal guidance by ligands like the netrins. The cAMP:cGMP ratio seems to be responsible for the polarity of axonal guidance by netrin-1; a high cAMP:cGMP ratio favors attraction, whereas a low ratio favors repulsion (50).

Besides DCC and UNC5, netrins also bind to integrins (51), and knockout mice for α3 and α6 integrin subunits exhibit defects in neuronal adhesion and migration (52,53). Thus, integrins and DCC/UNC5 could have complementary functions in the response of neurons to netrin migration signaling. Further evidence for the cooperative roles of integrins and DCC/UNC5 is that laminin is able to convert netrin-mediated attraction to repulsion in neurons from *Xenopus*, and a soluble peptide fragment of laminin (YIGSR) is sufficient to exert the same effect (54). Netrin-1 and its receptor DCC also interact with heparin/heparan sulfate, although the functions of these interactions in the migration of neurons remain unknown (55,56).

The dual effect of netrin-1, i.e., attraction of some precursors and repulsion of others, seems to be specific for the regions where the neurons are born. Floor plate netrin-1-expressing cells have been shown to attract commissural axons coming from dorsal parts of the neural tube (57,58). In a similar way, cells from the lower rhombic lip are also attracted by netrin-1 and migrate away to

form the precerebellar nuclei, while cells derived from the upper rhombic lip, among which are the granule cell progenitors, do not respond to netrin-1. In contrast, netrin-1 repels migrating granule cells exiting explants taken from the external granular layer from the postnatal cerebellum (59).

Mutations in the *Unc5h3* gene give rise to defects in cerebellar development, resulting in reduction of the cerebellar size, abnormal foliation and ectopic Purkinje and granule cells. Migration of granule cell precursors is affected in *Unc5h3* mutant mice, whereas initial migration of Purkinje cell progenitors is not affected. At E13.5, a subpopulation of granule cell and Purkinje cell precursors appears to be abnormally located in the rostral areas of the mutant brain stem, where the granule cell precursors proliferate until the postnatal period. These data suggest that the establishment of the rostral cerebellar boundary is coordinated by chemorepulsive signaling by netrin-1 and requires UNC5 receptors expressed by cerebellar neurons (60).

Normal central nervous system development relies on accurate intrinsic cellular programs as well as on extrinsic informative cues provided by extracellular molecules. Migration of neuronal progenitors to their correct final locations after they proliferate in defined proliferative zones is a key event during embryonic and postnatal development. Guidance cues provided by soluble factors and by extracellular matrix components play a fundamental role in the oriented migration of neuronal progenitors. Extracellular guidance molecules and their respective receptors must follow a well-organized pattern of expression, restricted in time and space, in order to fulfill their role in the coordination of the correct positioning of mature neurons. Based on what is known, the extracellular matrix of the developing cerebellum is composed of molecules that will support neuronal migration (laminin, fibronectin, vitronectin), molecules that will function as barriers for migration (chondroitin sulfate proteoglycans), and molecules that will orient the migrating neurons to reach their correct locations (reelin, netrins and tenascins).

However, we do not have a clear picture of how the extracellular matrix changes temporally and spatially during cerebellar development and much work still needs to be done to further understand how neurons reach their correct positioning in the mature brain.

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