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On the contribution of MMP-2 and MMP-9 to the postnatal cerebellar corticogenesis

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On the Contribution of MMP-2 and MMP-9 to the Postnatal Cerebellar Corticogenesis

Albert E. Ayoub

**Dissertation submitted to the
School of Medicine
at West Virginia University
in partial fulfillment of the requirements
for the degree of**

Doctor of Philosophy

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2003**

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Abstract

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Albert E. Ayoub

The development of the cerebellar cortex is dependent on orchestrated cell-cell, cell-matrix interactions, as well as intrinsic genetic programs. Matrix metalloproteinases (MMPs) are proteolytic enzymes that degrade ECM molecules and are involved in neuronal migration, process extension, and synaptic plasticity. The current study investigated (1) the developmental expression and activity of MMP-2 and MMP-9, and (2) the effect of blocking the activity of MMP-2 and MMP-9 by a specific inhibitor on the histogenesis of the cerebellar cortex. Cerebella were collected from rat pups every three days starting at postnatal day 3 (P3) until P25 – a period equivalent to the third gestational trimester in humans. Temporal analysis of MMP activity by gelatinase assay showed that both the active and latent forms were present in the developing cerebellum. The mRNA analysis by real-time quantitative PCR indicated that the expression pattern of MMP-2 and MMP-9 mRNA agreed with their gelatinase activity. *In situ* zymography was localized in the EGL, the PCL, and IGL. The EGL, Bergmann glial fibers, and the PCL showed immunoreactivity for MMP-2, whereas MMP-9 immunoreactivity was detected only in PC and the EGL. Exposure of organotypic slices to a specific MMP-2/MMP-9 inhibitor (1.6, 3.2 or 6.4 μ M) altered the activity of MMP-2 and 9 and caused a significant increase in the thickness of the EGL, and a decrease in migrating granule neurons in the molecular layer. Our study suggests that MMP-2 and MMP-9 play a role in the postnatal cerebellar histogenesis.

Dedication

To my family in Lebanon, to my wife

and

to the loving memory of my father

Elias I. Ayoub

who passed away December 25, 2001.

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List of Abbreviations

BC	Basket cells
BG	Bergmann glia
BGF	Bergmann glial fiber
BL	Basal lamina
Cbl	Cerebellum
CGN	Cerebellar granule neuron
Ctx	Cortex
CF	Climbing fibers
dEGL	Deep external granule layer
DIV	Days <i>in vitro</i>
ECM	Extracellular matrix
EGL	External granule layer
EtOH	Ethanol
GC	Golgi type II cells
HSPG	Heparan sulfate proteoglycans
Hip	Hippocampus
IHC	Immunohistochemistry
IGL	Internal granule layer
ISZ	<i>In situ</i> zymography
ML	Molecular layer
MMP	Matrix Metalloproteinase
O.C.T.	Optimum cutting temperature compound
OSC	Organotypic slice culture
P	Postnatal day
PBS	Phosphate buffer saline
PC	Purkinje cells
PD	Purkinje cell dendrite
PCL	Purkinje cell layer
PF	Parallel fibers
sEGL	Superficial external granule layer
TX	Triton X-100
WM	White matter

Chapter One: Introduction

The development of the cerebellar cortex involves the timely generation of different types of cells with a precise connection pattern that produces highly conserved tissue architecture. The cerebellum develops from the rhombic lip on the roof of the fourth ventricle. Cells in the cerebellum are generated from two germinal zones – the ventricular germinal zone and the external germinal layer (Figure 1.1). Precursor cells in the ventricular germinal zone generate cerebellar neurons and glial cells starting with Purkinje cells around embryonic day 11 in rodents and embryonic day 55 in humans (Gilthorpe et al., 2002; Yachnis and Rorke, 1999). Progenitors of granule neurons migrate from the rhombic lip and form a thin external germinal layer that generate granule neurons during the first two postnatal weeks in rodents and the last trimester of human fetal development. The organization of the cerebellar cortex and its development are dependent on a myriad of intrinsic and extrinsic factors. The orchestration of the activity of genes, gene products, interactions among cells, and between cells and the extracellular matrix is crucial for neurogenesis, migration, and differentiation. Each of these developmental processes is vulnerable to disruption. Numerous gene mutations and neurotoxic agents are known to cause developmental abnormalities in the cerebellum by interfering with these processes (Ferguson, 1996; Millen et al., 1999). Analysis of mutations, like *reeler* and *weaver*, and toxin-induced abnormalities, like exposure to lead and methylmercury (Howard and Mottet, 1986; Lorton and Anderson, 1986), helped uncover an array of molecular and genetic mechanisms that drive the development of the cerebellum.

The histogenesis of the internal granule layer has been extensively studied and is an excellent model to study neurogenesis, migration, process extension, and

differentiation. Progenitor cells in the external granule layer proliferate rapidly then migrate radially towards the ventricular zone and form the internal granule layer. As they descend, they extend two apical processes forming the parallel fibers that synaptically connect to the dendritic spines of Purkinje neurons. Granule neurons migrate through an already formed molecular layer, past the Purkinje cell layer and stop before reaching the white matter. Bergmann glial processes help guide the migration of granule neurons through the molecular layer. Extensive development of Purkinje cell dendritic trees and maturation of Bergmann glial processes occur during the same period. The molecules involved in remodeling the extracellular matrix for migrating granule neurons and extending processes are not well understood.

Extracellular matrix-degrading enzymes, matrix metalloproteinases (MMP), have been studied in other systems and been shown to be involved in cell proliferation, process extension, migration and cancer metastasis. These enzymes are normally tightly regulated due to their potent proteolytic activity. Specific tissue inhibitors of matrix metalloproteinases (TIMPS) are expressed concomitantly with MMPs and regulate the activation of matrix metalloproteinases. Emerging evidence connects MMPs, especially MMP-2 and MMP-9, to events like normal brain development, tissue remodeling and various diseases and injuries affecting the central nervous system (Yong et al., 2001). For example, Vaillant et al (1999) showed that MMP-2 and MMP-9 are expressed in the cerebellum at postnatal days 10 and 15. However, the functional contribution and developmental expression and temporal activity of MMP-2 and MMP-9 in the developing cerebellar cortex are not known.

Figure 1.1 Formation of the cerebellar primordium. Progenitors of granule neurons migrate from the rhombic lip on the surface of the cerebellar primordium (curved arrows) and form the external granule layer (EGL) of Obersteiner.

The central hypothesis of this investigation is that MMP-2 and MMP-9 contribute to the development of the rat cerebellar cortex, with the implication that the disruption of MMP-2 and MMP-9's function would cause developmental abnormalities. The study tested the hypothesis by pursuing the following objectives: 1) localize and determine the temporal pattern of expression of MMP-2 and MMP-9 and their spatiotemporal activity in the developing cerebellum, 2) to determine whether blocking MMP-2 and MMP-9's activation by a specific inhibitor hinders the proliferation and migration of granule cells. In order to establish a developmental pattern for the expression of MMP-2 and MMP-9,

we performed an immunohistochemical study, real-time quantitative RT-PCR, and zymographic analysis on tissue collected from neonatal rats every 3 days from postnatal day 3 until postnatal day 25. Finally, we investigated the effect of inhibition on MMP-2 and MMP-9 in an organotypic slice culture system. We measured the activity of these enzymes, the thickness of the external granule layer and the number of migrating cells in the molecular layer in thinly sectioned slices after exposure to a specific MMP-2/MMP-9 inhibitor.

Chapter Two: Literature Review

A review of the structure and function of the cerebellum

The cerebellum contributes significantly to planning and coordinating movement, maintaining muscle tone and equilibrium, and motor learning and plasticity. Among cerebellar dysfunctions, motor disorders like dysmetria, dysdiadochokinesia and hypotonia are most prevalent. Several developmental and genetic disorders riddle the cerebellum such as Joubert's syndrome (Yachnis and Rorke, 1999), Walker's lissencephaly and neu-laxova syndrome (Lyon et al., 1993). Ethyl alcohol consumption and exposure is also a major cause of damage to the cerebellum (Bendszus et al., 2001; Brodie, 2002; Tagliaferro et al., 2002; White et al., 2000a), especially during development (Bonthius et al., 1988; Bonthius and West, 1988; Cragg and Phillips, 1985; Olney et al., 2002; Pierce et al., 1999).

The cerebellum is located in the posterior cranial fossa below the dural tentorium cerebelli. The cerebellum constitutes part of the roof of the fourth ventricle. It lies behind the isthmus, the boundary between the midbrain and the hindbrain, and dorsal to the pons and medulla oblongata. The cerebellum is divided into a narrow medial portion called the vermis and two lateral hemispheres. Numerous transverse sulci create thin folia (leaflets) that extend over the entire external surface of the cerebellum (Conradi, 1985). Deeper fissures subdivide the surface of the cerebellum into lobules, which are grouped into three principal lobes -- anterior, posterolateral and flocculonodular. The flocculonodular lobe is the phylogenetically oldest part of the cerebellum and is called the archicerebellum. This division is primarily involved in maintaining equilibrium and is connected with the

vestibular system. The second oldest division of the cerebellum is called the paleocerebellum and contains the anterior lobe. This part maintains muscle tone and coordinates limb movements. The posterolateral lobe is considered the neocerebellum and is responsible for coordinating fine movements (Altman and Bayer, 1997; Whiting and Barton, 2003; Dillon and Atkins, 1970; Parma, 1969; Nieuwenhuys, 1967).

In the cerebellum, white matter lies below the cortical mantle and encapsulates four pairs of deep cerebellar nuclei. From medial to lateral, they are called the fastigial, the globose, the emboliform, and the dentate (Korneliussen, 1968). These nuclei receive output from the cerebellar cortex and collaterals from climbing and mossy fibers. Cortical projections to the deep nuclei are all inhibitory, GABAergic neurons, which originate in Purkinje cells. The dentate, emboliform, and globose send projections via the superior cerebellar peduncle to the red nucleus, ventral lateral and ventral posterolateral nuclei of the thalamus, other thalamic nuclei, and the contralateral inferior olivary nucleus. Efferent fibers from the fastigial nucleus target contralateral brain stem nuclei, various nuclei in the reticular formation, motor neurons in the upper cervical spinal cord, bilateral vestibular nuclei, superior colliculus, and ventral lateral and ventral posterolateral nuclei of the thalamus (Figure 2.1) [for more details refer to (Kandel et al., 2000; Altman and Bayer, 1997)].

Three major pairs of fiber bundles connect the cerebellum to the rest of the nervous system. The superior cerebellar peduncle has cerebellar efferents to the thalamus, the red nucleus, vestibular nuclei, the reticular formation, the oculomotor nucleus, and contains a few afferent fibers from the spinal cord. The middle cerebellar peduncle contains efferents from contralateral pontine nuclei, while the inferior cerebellar peduncle

links the cerebellum with the inferior olive, brain stem nuclei, vestibular nuclei and the spinal cord (Carpenter, 1991; Altman and Bayer, 1997; Hayle, 1973; Jansen, 1972; Ito, 1968; Eccles, 1967).

Figure 2.1 An illustration of the major cerebellar pathways. Refer to the text for description. (Ctx: cortex; D: dentate nucleus; DSCT: dorsal spinocerebellar tract; E: emboliform; F: fastigial; G: globose; ICP: inferior cerebellar peduncle; IO: inferior olive; MCP: middle cerebellar peduncle; SCP: superior cerebellar peduncle; VSCT: ventral spinocerebellar tract; WM: white matter) (adapted from Carpenter, 1991).

The organization of the cerebellar cortex is noticeably conserved among vertebrates, especially with regard to the connection pattern among cortical neurons (Lange, 1971; Lange, 1972; Nieuwenhuys, 1967). However, the ratio of granule neurons to Purkinje neurons increases with evolutionary advancement (Han and Bell, 2002; Glickstein and Voogd, 1995; Hayle, 1973; Schleifenbaum, 1973; Kanan, 1973; Hackethal, 1971; Shafranov, 1969). The mature cerebellar cortex contains an outermost

molecular layer, a Purkinje cell layer, and an internal granule layer (Figure 2.2 A and B) (Obersteiner H, 1883; Addison, 1911; Cajal, 1909; Palay and Chan-Palay, 1974; Rakic and Sidman, 1970; Jansen, 1972; Hackethal, 1971).

Figure 2.2 Histology of the cerebellum. An illustration of a cerebellar folium. Granule neurons extend parallel fibers along the long axis of the folium. They synapse with the dendrites of Purkinje cells. Also shown are BC: Basket cell; CF: Climbing fibers; GC: Golgi cell; gr: granule neuron; Gran: Internal granule layer; MF: Mossy Fibers; Mol: Molecular layer; Pur: Purkinje cell; PF: Parallel fiber; SC: Stellate Cell. [from:(Jacobson, 1991)].

The molecular layer in the adult animal contains stellate cells, basket cells, the unmyelinated parallel fibers of granule cells, a small population of astrocytes, Bergmann glia and the immense dendritic trees of Purkinje neurons (Cajal, 1909; Rakic, 1971;

Goldowitz and Hamre, 1998). Several extracellular matrix molecules have been found in the granule and molecular layer, including chondroitin sulfate, laminin, versicans, cytostatin, tenascin-R, hyaluronic acid and hyaluronic acid-binding proteins, and thrombospondin (Bandtlow and Zimmermann, 2000; Bignami et al., 1993a; Bignami et al., 1993b; Grumet et al., 1985; Llano et al., 2000; Loeb and Fischbach, 1995; O'Shea et al., 1990). Mounting evidence suggest that these molecules play an important role in the formation of the cerebellum. For example, abnormal positioning of neurons and glia in the cerebellum and cerebral cortex is observed in animals suffering from a mutation to the extracellular protein reelin (Bignami and Dahl, 1974; Yuasa et al., 1993).

Purkinje neurons are the first neurons to reach in the cerebellar cortex right below the thin external germinal layer of Obersteiner, where they aggregate in a three to four-cell layer (Athias, 1897; Yuasa et al., 1991). After birth in rodents, Purkinje cells progressively begin to align in a one-cell layer and their dendrites start to grow from small bifurcated stumps into branched dendritic trees (Addison, 1911; Altman and Bayer, 1985). As the dendritic trees begin to receive parallel fibers from migrating granule cells, they grow and begin to flatten in a plane perpendicular to the long axis of the folium. In the adult, the Purkinje cell layer has a single row of Purkinje cell somata. The dendrites of Purkinje cells receive climbing fibers arising in the contralateral inferior olive and axo-dendritic excitatory connections from parallel fibers. Purkinje cells synthesize γ -aminobutyric acid as a neurotransmitter and their axons constitute the only output of the cerebellar cortex. Purkinje cells express a variety of molecules including the calcium-binding protein calbindin D28k, the NgCAM-binding protein FAR-2, and the cell

adhesion molecule Tenascin-R (Amenta et al., 1994; Plagge et al., 2001; Woodworth et al., 2002).

Bergmann glia, also called Golgi epithelial cells, have their cell bodies surrounding the Purkinje cell somata (Shiga et al., 1983; Reichenbach et al., 1995). Bergmann glial cells extend processes through the molecular layer to reach the pial surface. These processes are believed to help guide the migration of granule neurons during development. Bergmann glial fibers persist into adulthood and their membranes become closely apposed to Purkinje cell dendritic spines (Figure 2.3) (Castejon, 1990; Spacek, 1985). Several groups studied the relationship between Bergmann glia and Purkinje cells, which led to the proposition that Bergmann glia help with the metabolic demands of Purkinje cells. For example, the enzyme 3PGDH, which is required for the biosynthesis of several membrane proteins, nucleotides, and the neuroactive peptide glycine is expressed in Bergmann glia, but not in Purkinje cells (Yamasaki et al., 2001). Furthermore, Bergmann glia express the Ca^{2+} -permeable AMPA-type glutamate receptor (Iino et al., 2001). This receptor is indispensable for the removal of glutamate released at synaptic clefts between climbing fibers and Purkinje cells. Mounting evidence suggests that Bergmann glia in the adult cerebellum fulfill a role similar to that of astrocytes (Yamasaki et al., 2001; Iino et al., 2001; Seil, 2001; Yuasa et al., 1996; Hatten, 1984; Castejon, 1990).

Figure 2.3 Relationship between Bergmann glia and Purkinje neurons. A Drawing depicting the relationship of Bergmann glia to Purkinje cells in the rat cerebellar cortex. In the second and third postnatal weeks, the external granule layer diminishes in thickness due to cell migration and cell death. As Purkinje cells mature and their dendritic trees grow, Bergmann glial processes increase their coverage of Purkinje dendrites and begin to fulfill a role similar to that of astrocytes. PC: Purkinje cell; BG: Bergmann Glia; BGF: Bergmann Glial fibers; EGL: external granule layer.

Granule neurons are tightly packed in the internal granule layer. These neurons form the parallel fibers that comprise most of the molecular layer in the adult animal. Parallel fibers run in a parallel direction to the long axis of the folium. In this configuration they run in a perpendicular path to the dendritic trees of Purkinje cells (O'Leary et al., 1968). In humans, more than 400,000 parallel fibers are thought to intersect and synapse with each Purkinje cell dendritic tree (Jansen, 1972). Parallel fibers also make excitatory synapses with the dendrites of stellate cells, basket cells, and Golgi type II neurons (see below). Lugaro cells are located just below the Purkinje cell layer and send their process upward into the molecular layer, where they connect with basket cells and stellate cells (Melik-Musyan and Fanardzhyan, 1998; Laine and Axelrad, 1998).

Another type of interneurons in the internal granule layer is the unipolar Brush cells. These receive excitatory collaterals from mossy fibers and synapse with granule neurons in the internal granule layer (Dino et al., 2000a; Dino et al., 2000b). Golgi cells are dispersed among the granule neurons in the internal granule layer and in much smaller numbers. Their axons meet with the claw-like axonal endings of granule neurons and the terminal endings of mossy fibers. The intersection of the three axonal endings is ensheathed by a glial capsule to form a glomerulus (O'Leary et al., 1968; Mugnaini and Forstronen, 1967; Eccles et al., 1967). Several hypothesis [reviewed in (Kandel et al., 2000)] have been proposed to explain the countless computations performed by the cerebellar circuitry, which permit the coordination of muscle movement and the maintenance of balance. Most importantly, the development of these circuits is essential to proper motor movements, which if disturbed can significantly affect the functioning of the organism as a whole.

Cerebellar corticogenesis: with emphasis on the ontogenesis of granule neurons and the contribution of the ECM

The development of the cerebellar cortex has been extensively described in several species by Obersteiner (1883), Cajal (1911), Addison (1911), and Miale and Sidman (1961) to name but a few. The formation of the cerebellar primordium starts around embryonic day 55 in human fetuses and embryonic day 11 in rodents (Sidman and Rakic, 1973; Altman and Bayer, 1985) and continues until the postnatal period. The ventricular germinal zone at the edges of the rhombic lip, between rhombomere 1 and 2, generates the precursors of deep cerebellar neurons as a first wave, followed by the generation of the precursors of Purkinje cells and of Bergmann glia (Figure 2.4). The latter two types populate the mantle layer. Golgi type II neurons, basket cells, and stellate cells are generated afterwards. Golgi type II neurons settle in the internal granule layer, whereas the basket cells and stellate cells settle in the molecular layer. Astrocytes and oligodendrocytes are generated later during the development.

A restricted region in the ventricular zone of the rhombic lip generates precursors that form the external granule layer of Obersteiner. Neuroblasts migrate dorsomedially on the surface of the cerebellar anlage and form the one-layer-thick external germinal zone (Gilthorpe et al., 2002). This layer generates the mature granule neurons that populate the internal granule layer. Purkinje neurons are generated long before granule neurons. Purkinje cells first aggregate to form a thick layer below the external germinal zone. By the time granule neurons start to migrate, Purkinje cells have already aligned themselves

in a single-cell layer. In newborn rats, the dendritic tree of Purkinje cells is limited to a few branches.

The differentiation and growth spurt of the Purkinje dendritic trees occur during the period of migration of granule neurons (Addison, 1911; Altman, 1972a; Zecevic and Rakic, 1976), which takes place between postnatal day 4 (P4) and P20 in the rat, and between the 4th fetal month and the end of the 1st postnatal year in humans. The relationship between the growth of the Purkinje dendritic tree, and the migration of granule neurons and the formation of parallel fibers was noted by Cajal (Cajal, 1909), who proposed an interdependence between these cells. Indeed, destruction of the external granule layer, as in x-irradiated neonatal rats, results in underdeveloped Purkinje dendrites (Altman et al., 1969b; Altman et al., 1969a); whereas the destruction of Purkinje neurons causes a comparable loss of granule neurons. Purkinje neuron somata receive synapses from climbing fibers from the contralateral inferior olive. In human fetuses, these fibers initially aggregate in a cell-free layer called the lamina dissecans before contacting Purkinje neurons. As the Purkinje dendritic trees grow and branch, climbing fibers start to synapse with the apical dendrites (Rakic and Sidman, 1970). Later during development, basket cells extend processes and surround Purkinje somata with an array of fibers that resemble baskets.

The lamina dissecans is a thin acellular layer composed of cytoplasmic outgrowths from immature Purkinje cells and granule neurons. Axonal endings of mossy fibers and climbing fibers also contribute to this layer. This layer appears in the

Figure 2.4 Cerebellar histogenesis. Starting at embryonic day 9 (E9), the cerebellar primordium is composed of a marginal layer (a) and a mantle layer (b). By E13, precursor cells in the internal germinal zone (d) start generating cerebellar cells, and cells from the edge of the rhombic lip migrate to the surface of the cerebellar anlage and form the external germinal layer of Obersteiner (c). Between E14 and P3, immature neurons migrate to form deep cerebellar nuclei (e) followed by Purkinje neurons (f) and Bergmann glia (g). Around the time of birth, interneurons (h) -- Golgi type II, stellate, and basket cells -- settle in the cerebellar cortex. By P3, the EGL increases in thickness due to proliferating progenitor cells (i). During the same period, Purkinje neurons line up and form a one-layer thick Purkinje cell layer. Postmitotic granule neurons start to migrate by P6 (j, k) and descend through the molecular layer and settle in the IGL. Granule neurons extend parallel fibers (k) as their nuclei translocate centripetally. They maintain close association with Bergmann glial fibers. Purkinje cell dendrites mature (l) as they receive synaptic contacts from granule neurons. By postnatal day 20, the EGL disappears and granule neurons form the internal granule layer. The development of the cerebellar cortex is near completion as myelination begins (n).

developing human fetus by week 21 and disappears by week 30 (Rakic and Sidman, 1970). This layer has been demonstrated in cetaceans (blue whale) (Korneliussen, 1967) and is thought to exist in primates, but not in other vertebrates. Rakic and Sidman (1970) proposed that afferent fibers occupy and form this layer during their wait to receive their targets. It is possible that afferent fibers are supplied with trophic support that prevents their degeneration for several weeks, as well as a “stop” signal that halts their progress before reaching the Purkinje cell layer. The mechanisms and signals that might contribute to the formation of this layer are unknown.

Bergmann glia, also called Golgi epithelial cells, were described by Bergmann (1857), Golgi (1885), Athias (1897), Cajal (1911) and later by Miale and Sidman (1961), Gobel (1967), Mugnaini (1967), Rakic (1971), Palay and Chan-Palay (1974) and others. These cells are generated early during development in the ventricular zone and take on a radial orientation. A small number of these cells move to the mantle layer by embryonic day 14 in the mouse, as described from *in situ* hybridization, and immunohistochemistry for tenascin and the glutamate transporter GLAST (Bartsch et al., 1992; Yuasa, 1996; Yamada et al., 2000; Yamada and Watanabe, 2002). Once in the mantle layer, they extend long fibers that terminate as conical end-feet that make contact with the pial surface. These cells form a dense layer below the Purkinje cell layer, and after birth move to position themselves among the Purkinje cells. During the first two postnatal weeks in the rat, Bergmann glial cells appear to continue their proliferation (Das, 1976). Furthermore, they continue to form a scaffold through their processes that facilitate the migration of granule neurons (Das et al., 1974; Das, 1976). Immature granule neurons start to differentiate and migrate centripetally in a close association with the Bergmann

glial fibers (Rakic, 1971). In electron microscopy, the membranes of glial fibers appear tightly attached to the spindle-shaped migrating neurons. Evidence presented from *in vitro* experiments and time-lapse video microscopy suggests that a mechanism more complex than just sliding is in effect in these migratory granule neurons (Edmondson and Hatten, 1987; Hatten M, 1990). Intracellular cytoskeletal organization is evident by the formation of a shell of microtubules that extends to the tip of the leading process and allows for vesicle trafficking. Furthermore, cortical actin wraps the nucleus and extends into the filopodia and the leading process. Migrating neurons form temporary junctions, called “interstitial densities”, with the glial fiber (Gregory et al., 1988). The connecting densities associate closely with the microtubular system and seem to differ from desmosomes seen in stationary neurons. During the first twenty postnatal days, Bergmann glia increase their membrane appositions with developing Purkinje dendrites. The surface in contact with Purkinje dendrites and budding spines appears more complex and dense than the surface in contact with migrating granule neurons. Bergmann fibers extend GLAST-positive digitations that progressively cover Purkinje cell dendrites (Yamada et al., 2000). Following the cessation of granule neuron migration, Bergmann glia gradually assume a role similar to that of protoplasmic astrocytes (Spacek, 1985).

The formation of the internal granule layer from the external germinal zone was initially described using the Golgi impregnation method (Obersteiner H, 1883; Addison, 1911; Cajal, 1909). Further support emerged in the mid 1960s with the use of autoradiography (Miale I and Sidman R, 1961; Hanaway J, 1967; Altman, 1972a). Even though some evidence suggests that Golgi cells and basket cells, as well as granule neurons, are generated from the external granule layer (Hausmann et al., 1985), recent

evidence suggests otherwise. Clonal analysis using retroviral probes in chicks and rats allowed the determination of detailed lineage maps and provided additional support for the hypothesis that only granule neurons originate in the EGL (Ryder and Cepko, 1994; Zhang and Goldman, 1996b; Zhang and Goldman, 1996a). Ryder and Cepko (1994), followed later by Zhang and Goldman (1996), injected retroviruses carrying the β -Galactosidase gene in the developing cerebellum at various ages and provided direct evidence for the origin of granule neurons. Injections in the EGL labeled progenitor cells that matured to become granule neurons only, whereas injections in the white matter labeled glial cells, basket, stellate and Golgi neurons. It is now accepted that neurons in the cerebellar cortex are generated from two germinal zones via opposing modes of migration. An outward migration from the ventricular zone generates all the neurons and glia of the cerebellar cortex, except the granule neurons. The later neurons are generated in the external germinal layer and migrate inwardly to populate the internal granule layer.

Ramon y Cajal (1911) treated the developmental stages of granule cells in great length and tallied the knowledge learned from his contemporaries. According to Cajal, the maturation of granule neurons encompasses four developmental phases (Figure 2.5). The outermost zone in the external granule layer (of Obersteiner) contains proliferating undifferentiated progenitor cells. The deeper zone of the external granule layer contains fusiform bipolar cells that extend tangential parallel processes. These cells are postmitotic and pre-migratory. Progressively these cells descend into the plexiform or molecular layer. The third phase, the *Radial Bipolar Stage*, contains cells with fully extended processes. A dendrite protrudes from the cell body and extends centripetally towards the Purkinje cell layer. The nucleus translocates downward following the leading tip of the

dendrite until it reaches the internal granule layer. Thus, the parallel fibers or axons of granule cells are fully developed by the time the cells reach their destination. When granule cells pass the Purkinje cell layer, their leading dendrite is resorbed and replaced with several thin dendrites that emanate from their perikarya in all directions. Only three or four of these dendrites develop varicosities as they start receiving mossy fiber rosettes. These dendritic varicosities eventually develop into claw-like digitations.

Figure 2.5 A schematic representation of the developmental stages of granule neurons by Ramon y Cajal (1911). Progenitor cells proliferate rapidly in the EGs. Daughter cells assume a pre-migratory phenotype (c) in the EGd and start growing parallel processes. As granule neurons move through the molecular layer (d), they extend their parallel process and make synaptic contacts with Purkinje cell dendrites. A leading process extends from the forward tip of the granule cell (e, f) and crawls towards the internal granule layer. The nucleus translocates down (g, h), past the Purkinje cell layer and settles in the internal granule layer. The cell differentiates further and grows claw-like dendrites that receive mossy fibers (i, j). Abbreviation: EGs, superficial external granule layer; EGd, deep external granule layer; Mol, molecular layer; Pur, Purkinje cell layer; IG, internal granule layer.

The advent of molecular biology has helped shed light on the molecular machinery that drives this process. An internal genetic clock has been proposed to dictate the migration of granule neurons (Trenkner et al., 1984; Zagon et al., 1985; Komuro and Rakic, 1995). Indeed, there is growing evidence in support for an intrinsic genetic program with the potential to maneuver the migrating granule neurons in a time and age dependent manner (Komuro and Rakic, 1995; Komuro and Rakic, 1998; Komuro et al., 2001). Recently, Yacubova and Komuro (2002) showed in an elegantly designed set of experiments that cultured granule neurons exhibit sequential alterations in morphology and migratory behavior in the absence of ECM signaling (Yacubova and Komuro, 2002). The observed movements, however, did not perfectly mimic those normally observed *in vivo*, which suggests a role for extracellular signals and cell-cell interactions. Hausmann and Sievers (1985) showed that granule neurons *in vivo* maintain contact with the basal lamina during their proliferative phase. They observed that proliferating cells in the superficial EGL make contact with the basal lamina through their soma or with short radial processes from embryonic day 16 until postnatal day 17 (Hausmann and Sievers, 1985). Growing evidence supports a role for the extracellular matrix and extracellular signaling in addition to the innate genetic program. For example, Hager et al (1995) reported that granule neurons in organotypic cerebellar slices can migrate centripetally with or without contact with glial elements (Hager et al., 1995). A follow-up study by the same group showed the involvement of the extracellular molecule laminin in the migration process. Blocking the activity of laminin with a specific antibody against the B2 chain of laminin in living cerebellar slices inhibited neuronal migration (Liesi et al.,

1995). Additionally, laminin interacts and enhances the mitogenic effect of Sonic hedgehog (Shh) signaling on proliferating granule neurons *in vitro* (Pons et al., 2001).

The proliferation of granule progenitors in the sEGL has been shown to be induced by Purkinje cells through Shh signaling (Wallace, 1999). Secreted Shh causes the progenitor cells in the sEGL to upregulate the transcription factors Gli1 and Gli2 (Dahmane and Altaba, 1999), a mechanism that promotes proliferation and inhibits differentiation (Rowitch et al., 1999). ECM molecules like HSPG, laminin and vitronectin, regulate the response of granule progenitors to Shh. HSPG and laminin enhance Shh-induced proliferation of granule progenitors (Pons et al., 2001; Rubin et al., 2002), whereas vitronectin induces cell-cycle exit and differentiation (Wechsler-Reya, 2001). Vitronectin stimulates differentiation through the c-AMP/CREB signaling cascade (Pons et al., 2001; Wechsler-Reya, 2001).

We are just beginning to understand the role that interactions with the extracellular environment play in the shaping of the nervous system. The extracellular matrix is a product of its inhabitants and seems to affect their behavior dramatically. Several extracellular molecules emerged as modulators of cell behavior like laminin, proteoglycans, integrins, reelin, tenascin, cytotactin, hyaluronic acid and matrix degrading enzymes (Grumet et al., 1985; Werz and Schachner, 1988; Bignami et al., 1993a; Pires Neto et al., 1999; Bandtlow and Zimmermann, 2000; Pesheva and Probstmeier, 2000; Schwartz, 2001; Del Bigio et al., 1999). Proteoglycans are large glycoproteins that form a main component of the extracellular matrix in all tissues including the brain. Some proteoglycans, like neurocan, versican v2, brevican and neuroglycan, are secreted exclusively in the CNS by neurons or glial cells. Others are

available in other tissues like syndecans, NG2, agrins and testicans. These molecules bind to a plethora of molecules including growth factors, cell surface molecules, adhesive and repellent molecules, and other constituents of the ECM such as laminin, tenascin, fibronectin and thrombospondin (Bandtlow and Zimmermann, 2000; Bruckner and Grosche, 2001; Ohtsuka et al., 2000; Oohira, 2001; Sobel, 2001). The distribution pattern of proteoglycans (e.g. glypican 1 and NG2) during development points to their direct involvement in neurogenesis and gliogenesis, axonal pathfinding and differentiation.

Some ECM molecules, like hyaluronic acid, went unnoticed for a long time because they readily dissolve during tissue preparation. Hyaluronic acid exerts its influence partly through hyaluronic-acid-binding proteins (HA-BP) like versicans, aggrecans, and the glial HA-BP (Bignami et al., 1993a; Bignami et al., 1993b). ECM molecules bind adhesive and repellent molecules and modulate their function. For example, Ng-CAM and cytactin are expressed in migrating granule cells and glial cells respectively (Chuong et al., 1987). Anti-Ng-CAM antibodies arrest migratory granule neurons in the external granule layer, whereas anti-cytactin antibodies arrest granule neurons in the molecular layer. The adhesive function of Ng-CAM and cytactin is promoted by chondroitin sulfate proteoglycans, but inhibited by heparin and heparan sulfate (Milev et al., 1995; Werz and Schachner, 1988; Hoffman et al., 1988). Conversely, in peripheral nerves and DRG neurons, chondroitin sulfate proteoglycans inhibit laminin-stimulated neurite growth (Zuo et al., 1998). This effect can be reversed *in vitro* by the addition of recombinant MMP-2, a matrix-degrading enzyme also thought to be expressed in the cerebellar cortex. Another example is netrin-1 which acts as an

attractant to growth cones and changes to repulsion when it binds laminin-1 (Hopker et al., 1999). Another adhesion molecule, astrotactin 1, is expressed in migrating neurons in the cerebellum. This molecule is involved in the formation of temporary junctions between the leading process of migrating neurons and glial cells (Adams et al., 2002; Zheng et al., 1996). *In vitro* assays show that blocking astrotactin with specific antibodies caused a significant 60% decrease in the migration rate of granule neurons (Fishell and Hatten, 1991).

Thrombospondin (TSP), is a trimeric glycoprotein that is expressed at the neuromuscular junction and around synapses in the retina and the adult cerebellum (Arber and Caroni, 1995). Thrombospondin is first apparent in the rhombic lip and contributes to the formation of the external germinal zone (O'Shea et al., 1990). TSP appear to be involved in the initial interactions between leading axons of granule cells and Bergmann glial cells, to promote neurite outgrowth and help mediate the migration of granule cells across the molecular layer in explants of neonatal cerebellum. Activation of cellular events by TSP-1 is thought to involve the extracellular receptor Integrin alpha 3 beta 1 (DeFreitas et al., 1995). Studies on the function of thrombospondins in neovascularization and tumor metastasis show that TSP-1 upregulates the matrix degrading enzyme MMP-9 (Albo et al., 2002; Qian et al., 1997). The intermediate steps may include signaling via cell surface receptors like integrins but are not clear at the present time.

The effect of several soluble and insoluble ECM molecules is exerted through the integrin family of cell surface receptors. Integrins have extracellular domains that bind extracellular molecules, transmembrane domains, and an intracellular domain involved in

cell signaling (Damsky and Werb, 1992; Schwartz, 2001). The binding of integrins to the ECM is relatively weak and allows for rapid formation and dissociation of contacts, which are necessary processes in cell migration. Integrins associate with growth factor receptors and are thought to modulate and potentiate their downstream signaling (Eliceiri, 2001; Martin et al., 2002). Binding of integrins to ECM molecules, like fibronectin and laminin, results in the rapid phosphorylation of FAK (focal adhesion kinase) and Src proteins (nonreceptor protein-tyrosin kinases). Phosphorylation of the SH2 domain of FAK induces the PI-3K signaling pathway and recruits the Grb2-Sos complex, which activates the Ras-ERK-MAP kinase signaling cascade. More than 20 proteins, including vinculin and myosin II, are thought to associate with the cytoplasmic domain of integrins. These molecules help trigger the reorganization of the actin cytoskeleton and the formation of stress fibers in motile cells. Motility requires the dissociation of connections between integrins and their ECM ligands. This process is facilitated by de-adhesive molecules like disintegrins, ADAMs (a disintegrin and metalloproteinase), and ADAMTSs (ADAM with thrombospondin repeats). Disintegrins have binding motifs that block the interactions with ECM molecules and can sequester matrix metalloproteinases that degrade the ECM (Shrimali and Reddy, 2000; Wu et al., 2003). ADAMs are membrane-anchored disintegrins that catalyze the breakdown of ECM and membrane molecules in the vicinity of the cell surface (Huang et al., 2003; Fourie et al., 2003). ADAMTS have a structure similar to ADAMs except they have thrombospondin-1 like repeats (Tang, 2001). Proteolytic cleavage of extracellular domains allows the cell to control the activity of membrane receptors and release factors. For example, the ADAM

member TACE (TNF- α converting enzyme) can release the inflammatory cytokine TNF- α from the cell surface (Moss and Lambert, 2002).

Autocrine and paracrine diffusible factors like neurotrophins and cytokines have been shown to be involved in various aspects of CNS development. In the cerebellar cortex, neurotrophins promote cell differentiation, proliferation, and inhibit cell death (Vaudry et al., 2003). IGF-1 (insulin-like growth factor 1) is secreted by Purkinje cells and exerts such a trophic effect on granule cells. IGF-1 triggers the phosphoinositide 3-kinase (PI-3k), which phosphorylates Akt and upregulates survival signaling (D'Mello et al., 1993; Bonthius et al., 2003). Glutamate released at synapses also activates the PI-3k/Akt pathway and enhances the survival of differentiating cells. On the other hand, glutamate has an opposite effect on mature granule neurons; glutamate can activate the p38-Rb, which in turn activates caspase-3 death signaling (Felici et al., 1989). Some cytokines like TGF- β and TNF- α act as death signals by activating the JNK-p53 pathway, which results in the activation of caspase-3 and the initiation of cell death. Bone morphogenic proteins -- BMP-4, 6 and 7 -- are also members of the TGF- β family, are expressed in the postnatal cerebellum and are thought to promote differentiation (Ming et al., 2002; Yabe et al., 2002; Alder et al., 1999). BMP binding to cell surface receptors induces the phosphorylation of smad1 and its association to smad-4. The smad1/sm4 complex translocates to the nucleus activating genes involved in cell differentiation (Angley et al., 2003).

The growth factor Neuregulin, secreted by neurons in the cerebellum (Chen et al., 1994), affects their migration, and induces the maturation of glial radial process (Ozaki et al., 1998; Rio et al., 1997) and the formation of synapses between mossy fibers and

granule neurons (Ozaki et al., 2000). Blocking the interaction of neuregulin with its receptor erbB4 *in vitro* causes impairment in the development of glial radial fibers and the migration of granule neurons. Furthermore, differential expression of the neuregulin receptors -- erbB-1, -2, -3, -4 and NRG beta-1-- by granule neurons and other cells in the cerebellum may promote proliferation or differentiation. The receptors erbB-2 and -1 are not normally expressed in the EGL. The erbB-3 receptor is observed in postmitotic cells, whereas erbB-4 is thought to inhibit differentiation (Pinkas-Kramarski et al., 1997). In medulloblastoma, a tumor of the EGL, a deregulated expression of the erbB-2 receptor, which is normally absent in the EGL, and the erbB4/NRG beta-1 is thought to promote tumorigenesis and metastasis (Gilbertson et al., 1998).

Neurotrophins have varying effects on granule neurons in the cerebellum depending on their developmental stage. BDNF, NT-4 and NT-5 promote the survival of immature granule progenitors (Yacubova and Komuro, 2003; Dieni and Rees, 2002; Borghesani et al., 2002; Carter et al., 2002). The PI-3k pathway, via the PLC- γ and the SHC-Ras-Raf pathway, mediates the trophic effect of BDNF, NT-4, and NT-5. The latter two pathways activate the MAPK cascade, which results in the phosphorylation of transcription factors in the nucleus and the initiation of gene translation. On the other hand, NT-3 seems to promote cell cycle exit and the differentiation of granule cells (Seabold et al., 1998; Condorelli et al., 1998; Maisonpierre et al., 1990). In various cases, it is becoming apparent that neurotrophin signaling *in vivo* is enhanced by integrin signaling, thus affecting the organization of the cytoskeleton, cell motility, cell cycle regulation, as well as survival and differentiation (Mills et al., 2003; Martin et al., 2002) .

A considerable understanding of the morphogenesis of the cerebellar cortex and the interdependence of cells has been learned from studies of mutant mice. To date, about fifty mutations have been identified that result in aberrant development and a spectrum of clinical deficits. A comprehensive review of mutations affecting the cerebellum is beyond the scope of this discussion [reviewed in (Sotelo, 1990; Goffinet, 1990; Katsuhiko et al., 1984; Sidman, 1983; Caviness, Jr. and Rakic, 1978; Landis and Landis, 1978)]. Of particular interest is the mutation affecting the extracellular protein reelin in *reeler* mice (D'Arcangelo et al., 1997). Reeler mutants have abnormal patterning and neuronal positioning in laminated structures of the CNS, including the cerebral cortex, hippocampus, the dorsal cochlear nucleus, the inferior olivary complex, and the cerebellar cortex. Purkinje neurons in the cerebellum of reeler mice are ectopically located and reduced in number (Yuasa et al., 1993; Heckroth et al., 1989), and so are granule neurons and Bergmann glia (Terashima et al., 1985; Bignami and Dahl, 1974). Despite the disorderly arrangement of cells in the cerebellar cortex, the synaptic connections appear normal between synaptic partners.

Reelin is expressed in pioneering neurons and forms an integral part of the cortical matrix (cortical plate) (Tissir et al., 2002). Reelin binds the membrane receptors VLDLR (very-low-density lipoprotein) and ApoER2 (apolipoprotein E receptor type 2), which phosphorylate the cytoplasmic protein Dab1 (disabled-1) and trigger presently-unknown signaling cascades (Rice et al., 1998; Howell et al., 1997). Interestingly, mutations affecting the genes of both receptors VLDLR and ApoER2, as well as the cytoplasmic protein Dab1, cause abnormalities similar to those observed in reelin mutant mice (Trommsdorff et al., 1999; Ohshima et al., 2001). The sequencing of genes and

identification of gene products of the *reeler* and other cerebellar mutants, like the *weaver* mutant, will provide additional insight about the mechanisms contributing to the histogenesis of the cerebellar cortex.

The cerebellar cortex is a marvelous processing powerhouse that easily outpaces any available manmade superprocessor. The microstructure of the cerebellum has been known since the beginning of the twentieth century, but the molecular machinery that pieces it together is largely unknown. Evidence is accumulating in support for both an intrinsic genetic machinery, as well as intercellular and intracellular signaling molecules. New work is unraveling the role of extracellular-matrix molecules and the mechanisms cells use to degrade and reconstruct the extracellular matrix. For a developing cerebellar cortex, remodeling the extracellular matrix is a balance between proteolysis and synthesis that facilitates cell migration and process extension, yet holds all cellular elements together. Close proximity allows for intercellular communication and triggers more downstream events, events that culminate in the formation of the highly specialized circuits of the cerebellar cortex.

Matrix metalloproteinases and the extracellular matrix in the CNS

The extracellular matrix contributes to the integrity of the CNS, although not as extensively as other tissues like bone and cartilage. The extracellular matrix contains invaluable structural and signaling molecules needed for proliferation and differentiation, but too rigid and invariable a matrix would hinder developmental and homeostatic mechanisms like cell migration, neurite extension, and synaptic plasticity. Fortunately, the same cells that secrete extracellular matrix proteins secrete proteolytic enzymes that degrade them. This allows for tissue remodeling around extending neurites and filopodia, permitting the establishment of new connections as well as cell motility. As one can envision, the proteolytic activity of matrix-degrading enzymes has to be precisely controlled such that the enzyme is active only in front of growth cones, for example, and not at sites of attachment with the basal lamina. Matrix metalloproteinases are zinc-dependent endopeptidases that degrade all extracellular molecules. Their activity is regulated at various levels by activators and inhibitors. Over 24 MMPs have been classified to date and seven have been found to be expressed in the nervous system. The expression of MMP-1 (Agapova et al., 2001; Schutz et al., 2002), MMP-2 (Costa et al., 2002; Rosenberg et al., 2001), MMP-3 (Vaillant et al., 1999; Oh et al., 1999), MMP-7 (Rosenberg et al., 2001; Conant et al., 1999), MMP-9 (Conant et al., 1999; Tanney et al., 1998), MT1-MMP (Tanney et al., 1998; Agapova et al., 2001), MT5-MMP (Sekine-Aizawa et al., 2001; Llano et al., 1999) and their inhibitors, TIMP-1 through 4, have been observed in normal CNS development and in pathological conditions. The expression of MMPs in the CNS does not appear to be cell-specific but function-specific. For example,

glial cells as well as neurons in various regions of the CNS express MMP-2 and MMP-9 during process or neurite outgrowth (Costa et al., 2002; Ferguson and Muir, 2000; Oh et al., 1999; Uhm et al., 1998).

Numerous studies found that abnormal expression (over- or under-expression) and uncontrolled activity of MMPs accompanies various CNS pathologies like Alzheimer's disease, aneurisms, ischemia/reperfusion, multiple sclerosis, meningioencephalitis, disruption of the blood-brain barrier, and Wallerian degeneration (Khuth et al., 2001; Vecil et al., 2000; Yoshiyama et al., 2000; Leib et al., 2000; Azeh et al., 1998; Planas et al., 2000; Clark et al., 1997; Gokaslan et al., 1998; Liuzzi et al., 2000; Fujimura et al., 1999; Rosenberg et al., 1996; Matsuura et al., 2000; Rosenberg et al., 1998; Lim et al., 1997; Hughes et al., 2002; Maquoi et al., 2002). Outside the nervous system, MMPs have been studied extensively in bone remodeling, nephrogenesis, implantation and human fetal development, lung tissue and lung cancer, spermatogenesis, tumor invasion and viral infections like HIV-1 (Huppertz et al., 1998; Tanney et al., 1998; Bord et al., 1999; Kanwar et al., 1999; Furuya et al., 2000; Vliagoftis et al., 2000; Ghorpade et al., 2001; Marchenko et al., 2001; Bischof et al., 2002; Schutz et al., 2002; Hunter and Spector, 2003; Baumgart et al., 2002; Buchman-Shaked et al., 2002).

With few exceptions, all MMPs described to date share conserved structural domains, including an N-terminal signal peptide, a propeptide region, a catalytic domain that contains the Zn^{2+} binding site, fibronectin repeats, a short hinge region, and C-terminal hemopexin-like repeats (Becker et al., 1995; Bode et al., 1993; Hayashita-Kinoh et al., 2001; Sekine-Aizawa et al., 2001). MMPs differ mostly in their content of hemopexin-like and fibronectin repeats, with MMP-7 lacking all repeats and MMP-9

having the most (Figure 2.6) (Dhanaraj et al., 1996; Van Wart and Birkedal-Hansen, 1990).

The signal peptide is a hydrophobic region with about 25 residues. This sequence targets the enzyme for secretion and is cleaved before activation. Between the signal peptide and the catalytic domain is a propeptide region. The propeptide forms 3 α -helices that contain about 80 residues and a highly conserved “Cystein switch” sequence PRCGVDP. Removal of the cystein residue activates the enzymes (Springman et al., 1990; Vallee and Auld, 1990). The attachment of the propeptide region is essential in keeping the enzyme in an inactive state (Fisher et al., 2002). The importance of the propeptide region to maintaining latency was determined by mutational studies for several MMPs (Murphy et al., 1999). The presence of 20 residues at the C- and N-termini proved crucial to the function of the propeptide; enzymatic or mutational removal of these sequences cause autoactivation of the enzyme (Freimark et al., 1994). The integrity of the propeptide region is also essential in binding tissue inhibitors of MMPs (TIMP) and the subsequent activation of MMPs (Pavlaki et al., 2002; Cao et al., 1998).

The catalytic domain is highly conserved among MMPs and contains the zinc-binding sequence HEXGHXXGXXHS (Bode et al., 1994; Willenbrock et al., 1995). Of special importance are the histidines in this sequence because their disturbance by point mutations inactivates the catalytic domain (Windsor et al., 1994). A second Zn^{2+} is found in some MMPs but its importance is uncertain at this point. Downstream from the catalytic Zn^{2+} is a Ca^{2+} molecule that binds flanking His and Asp and is thought to help stabilize the structure (Bode et al., 1994; Willenbrock et al., 1995). The catalytic domain of gelatinases (MMP-2 and MMP-9) contains 3 tandem fibronectin inserts. These repeats

fold into double stranded antiparallel β -sheets (Clark and Cawston, 1989; Lowry et al., 1992; Overall, 2002) and are thought to allow cooperative binding to substrates (Banyai et al., 1994).

Figure 2.6 Structural domains of gelatinases. MMP-2 and MMP-9 are among the largest MMPs. They contain a signal peptide (A), a pro-domain (B), a catalytic domain (C), Fibronectin-repeats (D), a linker domain (E), and hemopexin-repeats (F). Other MMPs may lack the fibronectin-repeats and the hemopexin-repeats, while membrane-tethered MMPs have an additional hydrophobic domain (G).

Following the catalytic domain is a hinge region of variable length that connects to the C-terminal hemopexin repeats. The structure of the hemopexin domain was resolved by X-ray crystallography and is arranged like a four-blade propeller structure with 4 β -sheets (Li et al., 1995; Libson et al., 1995). This domain allows the binding to substrates like collagen I and II, TIMPs, and heparin (Allan et al., 1991; Crabbe et al., 1993). An additional highly hydrophobic N-terminal region is found in membrane

tethered MMPs, e.g. MT1-MMP, and allows the enzyme to attach to the plasma membrane (Okumura et al., 1997; Sato et al., 1996).

From expression to activation, MMPs are tightly regulated to prevent deleterious effects. Several signal transduction cascades have been implicated in activating MMP gene expression including the ERK-MAP kinase, PI-3 kinase, PKC, and p38 pathways (Wang et al., 2002; Hussain et al., 2002; White et al., 2000b). Mounting evidence suggests that these pathways induce homo- and heterodimerization of c-fos and c-jun, members of the proto-oncogene family, which form the intranuclear transcription activator protein AP1 (Illman et al., 2001; Sato et al., 2002). Binding sites for AP1 are found in the promoter sequence of most MMPs. Moreover, most MMPs have binding sites for other nuclear factors like NF κ B, Smad, and Ets (Hall et al., 2003; Eberhardt et al., 2002). In addition to MMPs, tissue inhibitors of MMPs (TIMPs) seem to be regulated by the same factors. For example, inhibition of AP1 binding by specific inhibitors suppresses the expression of MMP-1 and MMP-9 and increases the expression of TIMP-1 (Sato et al., 2002). The gene expression of TIMP-1 is also induced by TGF- β 1 by binding AP1, while concomittent interaction of Smads with the AP1 binding site on the MMP-1 promotor region represses the later's gene expression (Hall et al., 2003). In a mouse model for hydrocephalus resulting from the overexpression of TGF β 1, a recent study found abnormally high expression of TIMP-1 and a simultaneous decrease in the expression of MMP-9 (Zechel et al., 2002). Conversely, multiple cytokines could interact and increase the expression and activation of MMP-9. In human skin keratinocytes, for example, TGF β 1 and TNF α bind to TGF β -RE (TGF β 1 response element) and NF κ B binding site and induce increased MMP-9 expression (Han et al., 2001). Similarly, in malignant

gliomas and transformed keratinocytes, TGF- β is thought to induce the expression of MMP-9 (Santibanez et al., 2002; Platten et al., 2001). In human breast cancer cells, heuregulin- β 1, a growth factor that promotes the survival of breast cancer cells, induces the expression of MMP-9 through the ERK-MAPK, PKC, and p38 signal transduction pathways. However, the downstream signaling molecules and transcription factors leading to the activation of MMP-9 gene expression are yet to be identified. Furthermore, our knowledge of the regulation of gene expression of MMPs and their inhibitors is still in its early stages.

Figure 2.7 Activation of MMP-2 and MMP-9's gene expression. Several signal transduction pathways modulate the expression of MMP-2 and MMP-9, and the simultaneous release of TIMP-1 and TIMP-2.

Once secreted, MMPs are activated by a variety of compounds including organomercurial, phorbol esters, SDS, reactive oxygen species like HOCl $^{\cdot}$, H $_2$ O $_2$, ONOO $^{\cdot}$,

acidic pH, and other proteinases (Birkedal-Hansen and Taylor, 1982; Okada et al., 1992; Owens et al., 1997; Saari et al., 1990; Sopata and Wize, 1979). The activation step invariably involves the cleavage of the Zn^{2+} --Cys bond, which makes the catalytic domain free to interact with substrates (Springman et al., 1990). MMPs are activated on the cell surface by membrane tethered MMPs, like MT1-MMP, by furin and plasmin, and by stepwise activation of other MMPs (Fillmore et al., 2001; Gveric et al., 2001; Makowski and Ramsby, 1998; Murphy et al., 1999; Nagase, 1997). For example, pro-MMP-2 is activated on the cell surface by MMP-1, plasmin, and MT1-MMP. The tissue inhibitor TIMP-2 binds pro-MMP-2 and presents it to MT1-MMP (Maquoi et al., 2000). This reaction is dependent on stoichiometric concentrations of TIMP-2. Furin and plasmin also activate MT1-MMP (Monea et al., 2002; Sato et al., 1996), which cleaves the prodomain of pro-MMP-2. A similar process for the activation of pro-MMP-2 is mediated by integrin $\alpha(v)\beta(3)$, which binds pro-MMP-2 and presents it to TIMP-2 and MT1-MMP (Hofmann et al., 2000a; Hofmann et al., 2000b). After the activation of MMP-2, the MT1-MMP/TIMP-2 complex is quickly internalized and degraded inside lysosomes and endosomes (Jiang et al., 2001).

Pro-MMP-9 is activated by trypsin, plasmin, cathepsin G, and chymotrypsin (Morodomi et al., 1992). Other MMPs, like MMP-1 (Sang et al., 1995), MMP-2 (Fridman et al., 1995), MMP-3 (Goldberg et al., 1992), MMP-7 (Imai et al., 1995), MMP-10 (Nakamura et al., 1998), and MMP-13 (Knauper et al., 1997) cleave the prodomain of MMP-9 and generate an ~82 kDa active zymogen. MMP-9 autolysis can also occur *in vivo* and results in smaller 80 and 67 kDa active zymogens (Fillmore et al., 2001; Fridman et al., 1995; Makowski and Ramsby, 1998; Murphy et al., 1999; Nagase,

1997). The generation of smaller fragments eventually deactivates the enzyme. Activation of pro-MMP-9 proceeds in a stepwise fashion through other MMPs (Ogata et al., 1992) and requires the dissociation of TIMP-1 – a necessary step for activation (Itoh and Nagase, 1995). The stepwise mode of activation allows the formation of active intermediates and a greater control over the degradation of ECM molecules (Suzuki et al., 1995).

Abnormal activation or secretion of MMPs is invariably associated with CNS pathologies. The functions of MMP-9, MMP-1, and MMP-3 have been shown to be disturbed in human brains affected with Alzheimer's disease (AD). A study on postmortem tissue from Alzheimer's patients, found MMP-9 in pyramidal neurons in the hippocampus, but not in granule neurons and glia (Backstrom et al., 1996). Active MMP-9 can degrade β -amyloid proteins *in vitro*, but accumulates in a latent form around β -amyloid plaques in tissue collected from Alzheimer's patients. A similar observation was made in canine brains with β -amyloid plaques (Lim et al., 1997), which supports the proposition that accumulation of β -amyloid proteins is partly due to insufficient activation of MMP-9. A recent study investigating the distribution of MMP-9 and uPA (urokinase plasminogen activator), an activator of pro-MMP-9, in postmortem tissue collected from Alzheimer's and vascular dementia patients revealed a segregation in the expression pattern (Asahina et al., 2001). In Alzheimer's tissue, MMP-9 colocalised in neurons, senile plaques and the vascular wall whereas uPA was found only in the vascular wall. On the other hand, MMP-9 and uPA were colocalized to the vascular wall in vascular dementia tissue. MMP-3 also has reduced expression around senile plaques (Yoshiyama et al., 2000). Whether MMP-3 contributes to the pathology of AD, or not, is

contingent on MMP-3's ability to degrade β -amyloid proteins, which is not known at the present time. In contrast to MMP-9, MMP-1 expression in AD tissue is elevated compared to normal tissue especially around capillaries (Leake et al., 2000), which suggests a role in damaging the blood-brain barrier (BBB).

Elevated levels of MMP-9 have been reported in studies of transient focal ischemia (Clark et al., 1997; Fujimura et al., 1999; Planas et al., 2000). Increased expression of MMP-9 is first seen after 4 hrs of the ischemic insult, and is sustained for almost 4 days. MMP-2 expression showed a delayed response in that it increases after 4 days of the ischemic injury (Heo et al., 1999; Planas et al., 2001). The rise in the expression of the active MMP-9 is believed to contribute to the damage caused to the blood-brain barrier and myelin membranes. Several components of the BBB, like laminin, and a major component of the myelin membrane, myelin basic protein, are degraded by MMP-9. Interestingly, MMP-9 knock-out mice show reduced damage after ischemic and traumatic brain injuries compared to wild type mice (Wang et al., 2000; Asahi et al., 2001). Administration of the broad spectrum MMP inhibitor batimastat (BB-94) significantly decreased the damage caused by ischemic injury in wild type mice, but not in MMP-9 knockouts (Asahi et al., 2000). The use of BB-94 also reduces hemorrhagic volumes, a known complication with the administration of tPA in ischemic and embolic injuries (Lapchak et al., 2000; Sumii and Lo, 2002). Moreover, in clinical studies of stroke patients, MMP-9 plasma levels proved to be a reliable predictor of hemorrhagic infarcts and parenchymal hematomas (Montaner et al., 2001; Castellanos et al., 2003).

Increased MMPs', especially MMP-9, activity and expression are also seen in inflammatory demyelination and malignant gliomas (Rosenberg et al., 1996; VanMeter et al., 2001; Yong et al., 2001). Secretion of matrix degrading enzymes by invading immune cells is thought to exacerbate the damage to the ECM and myelin in MS (Sobel, 2001; Sobel and Ahmed, 2001). Malignant and metastatic brain tumors invariably show increased expression of MMPs, which are believed to facilitate tumorigenic invasion and subsequent vascularization (Fillmore et al., 2001; Sameshima et al., 2000; Friedberg et al., 1998). Therapies based on MMP inhibition are under development but have to overcome the problems that plagued early therapies for stroke, arthritis, MS and cancer (Marder and Greenwald, 2003; Scappaticci, 2003; Fingleton, 2003; Zheng et al., 2003). One of the hurdles is the redundancy of MMPs and their wide spectrum of substrates and functions. A successful therapeutic approach minimizes damage caused by overexpression of certain MMPs, like gelatinases, without interfering with the homeostatic function of other MMPs. Significant advances have been made in designing specific inhibitors or "suicide substrates" that exhibit high affinity and specificity (Brown et al., 2000; Kleinfeld et al., 2001). A better understanding of the mechanism of action of MMPs and their distribution is of utmost importance in order to design therapies that are more effective.

Chapter Three: Expression of MMP-2 and MMP-9 and their role in the histogenesis of the postnatal cerebellar cortex

Introduction

Matrix metalloproteinases 2 and 9 belong to the metalloendopeptidase family and are known to catalyze the degradation of ECM proteins including collagens, proteoglycans, fibronectin, and elastin (Chandler et al., 1997; Huppertz et al., 1998). Excess MMP production and activation is thought to be a key feature of several CNS pathologies. For example, remodeling of dendritic trees in the hippocampus is accompanied by an increased expression of gelatinases in a model of temporal lobe epilepsy (Szklarczyk et al., 2002; Zhang et al., 1998). MMP-9 has also been shown to be involved in the pathogenesis of Alzheimer's disease (Asahina et al., 2001; Backstrom et al., 1996; Lim et al., 1997), ischemic injuries (Fujimura et al., 1999; Planas et al., 2001) and Multiple Sclerosis (Lee et al., 1999; Yushchenko et al., 2000). Accumulating evidence suggests that MMP-2 and MMP-9 are involved in normal developmental processes as the differentiation of neurons and glia (Agapova et al., 2001; Frolichsthal-Schoeller et al., 1999), migration, and process extension by oligodendrocytes during development (Oh et al., 1999; Uhm et al., 1998; Vu and Werb, 2000). In the peripheral nervous system, neurite extension over Schwann cell basal lamina is thought to be facilitated by MMP-2 and MMP-9 (Ferguson and Muir, 2000). MMP-2 and MMP-9 have been shown to be expressed in the developing cerebellum (Vaillant et al., 1999). The expression of MMP-2 and MMP-9 coincided with the formation of the internal granule layer and the maturation of neurons and glia but the nature of their contribution is not understood.

During the first three postnatal weeks, the cerebellar cortex of the rat is the stage for major developmental changes. The postnatal histogenesis of the cerebellar cortex has been described by Obersteiner (1883), Cajal (1911), Addison (1911), Mugnaini and Forstronen (1967), and several others. Progenitors of granule cells proliferate rapidly in the EGL between P3 and P9 and start their migration towards the internal granule layer by P6. Proliferating granule neurons maintain their contact with the basal lamina (Hausmann and Sievers, 1985). Migrating neurons extend parallel fibers that make synaptic contact with Purkinje dendrites. Granule cells have to pass through an already developed molecular layer, past the Purkinje cell layer, to reach their final destination in the IGL. This tortuous journey is facilitated by the interactions between granule cells and Bergmann glial fibers (Rakic, 1971). During the same period the dendritic trees of Purkinje neurons grow extensively as they synapse with parallel fibers (Addison, 1911; Zecevic and Rakic, 1976). Purkinje cells also interact with neighboring Bergmann glial fibers (Riquelme et al., 2002; Seil, 2001). Between P7 and P21 in rats, Purkinje dendrites become increasingly ensheathed by Bergmann glial processes and membranous digitations (Seil, 2001; Yamada and Watanabe, 2002). These events are known to involve various ECM molecules including hyaluronic acid, versicans, tenascin-R, laminin, proteoglycans and others (Aspberg et al., 1995; Bignami et al., 1993a; Mendis et al., 1994; Thelen et al., 2002; Tucker et al., 1994). These molecules have adhesive or repulsive properties that permit migration, process extension, and plasticity of neuronal and glial elements. Moreover, many of these extracellular proteins are substrates for MMP-2 and MMP-9 (Chandler et al., 1997; Schutz et al., 2002).

The important role of MMPs in the regulation of interaction between cells and the ECM enticed us to test the hypothesis that MMP-2 and MMP-9 contribute to postnatal cerebellar histogenesis. To better understand the developmental pattern of expression and the distribution of gelatinases, we performed realtime quantitative RT-PCR, *in situ* zymographic analysis, and immunohistochemistry on cerebellar tissue from pups at the postnatal age of 3 until P25. We investigated the contribution of these gelatinases to the developmental events that shape the cerebellar cortex by determining the effect of a specific gelatinase inhibitor on MMP-2 and MMP-9's activity in cerebellar organotypic slice culture.

Materials and Methods

Animal surgeries. We used four Sprague Dawley rat pups from the postnatal age of 3 (P3), P6, P9, P12, P15, P18, P21 and P25 for each experiment. Animals were housed and bred in an ACUC-accredited animal facility at West Virginia University Health Sciences Center. All animal protocols complied with the Society for Neuroscience guidelines for animal care and use. For the immunohistochemistry experiments, animals were anesthetized with sodium pentobarbitol (150 mg/kg body weight) then perfused intracardially with 4% paraformaldehyde. Brains were dissected and post-fixed in the same solution for 12 hrs at 4°C, cryprotected in 30% sucrose solution for 3 additional days, then embedded in O.C.T. compound (Optimum Cutting Temperature; Cat.# 25608-930, VWR, Westchester, PA) and frozen on powdered dry ice. For the remaining procedures, brains were dissected and immediately frozen in liquid nitrogen. Tissue collected was stored at -80°C until further processing.

Real-time Quantitative PCR. The cerebellum was dissected under a dissecting scope and immediately frozen in liquid nitrogen. For comparison purposes, cortical tissue from the cerebrum was dissected from the same animals by making a shallow tangential cut through the sagittal plane. Total RNA was extracted using TRIZOL reagent according to the protocol provided by the manufacturer (Life Technologies, Grand Island, NY). The solution was then treated with DNase I (Ambion Inc., Austin, TX) for 30 minutes at 37°C. DNase I was inactivated and removed from the reaction with the addition of DNase inactivation reagent (Ambion). RNA concentration was determined by spectrophotometric analysis at A_{260} and A_{280} using a DU 640 Spectrophotometer

(Beckman Coulter Inc., Fullerton, CA). Oligonucleotide primers and Taqman probes for rat MMPs were designed using Primer Express Software (Perkin-Elmer, Boston, MA) and were synthesized by Perkin-Elmer. Primer and probe sequences (5'-3') were as follows: MMP-2 forward primer, CCCATGAAGCCTTGTTTACCAT; MMP-2 reverse primer, CTGGAAGCGGAACGGAAA; MMP-2 probe, 6FAM-CAATGCTGATGGACAGCCCTGCA-TAMRA. MMP-9 forward primer, CGTGGCCTACGTGACCTATGA; MMP-9 reverse primer, GCACCGCTGAAGCAAAAGA; MMP-9 probe, 6FAM-CTCCTGCAGTGCCCTTGAAGCT-TAMRA; Primers and probes for rat 18S were purchased from Perkin-Elmer. Real-Time quantitative TaqMan PCR analysis was used to determine the relative levels of MMP-2 and MMP-9 mRNA. RT-PCR and TaqMan PCR were performed according to the manufacturer's instructions (PE Biosystems, TaqMan Gold RT-PCR protocol and TaqMan Universal PCR Master Mix). Sequence-specific amplification was detected with an increase fluorescence signal of the reporter dye FAM during the amplification cycle. Amplification of the gene for rat 18S was performed in the same reaction on all samples tested as an internal control for variations in RNA amounts. Levels of the different mRNAs were subsequently normalized to 18S mRNA levels, and were represented as fold difference of various postnatal ages against age of day 3.

Culture of cerebellar granule neurons. Dissociated cerebellar cell culture were used to ascertain the presence of and determine the probable source of gelatinases in the cerebellum. Cerebellar granule neurons and glial cells were isolated from P4 rat cerebella, cultured, and then assayed for gelatinase activity. Primary cultures of CGNs and glial cells were generated as previously described (Luo et al., 2001; Li et al., 2001).

Briefly, the cerebellum was dissected out and quickly placed in a 2 ml conical tube filled with 1H buffer (50 ml EBSS, 0.125 g Glucose, 0.15 g BSA, 0.5 ml of 3.82% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$). All tissue was finely chopped with a razor blade on a glass slide and moved to a 50 ml conical tube full of solution A (0.1 ml of 25 mg/ml trypsin, 9.9 ml 1H buffer). After vigorous shaking, the tube was placed in the incubator/shaker at 37°C for 15 min to allow ample time for trypsinization. Next, solution C was added (9.78 ml 1H, 160 μl of 4 mg/ml trypsin inhibitor, 30 μl of 200 U/ml DNase and 30 μl of 3.82% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) and centrifuged the mixture at 1000 rpm for 5 minutes. After removing the supernatant, trypsin inhibitors (buffer B: 8.6 ml 1H, 1 ml of 4 mg/ml trypsin inhibitor, 0.2 ml of 200 U/ml DNase, 0.2 ml of 3.82% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) was added to the tube, swirled the mixture thoroughly and left to settle for 5 min. The supernatant was transferred to a 15 ml tube, combined gently with solution D (9.8 ml 1 H, 400 mg BSA, and 0.2 ml 3.82% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), and the mixture was spun down for 5 min at 2000 rpm. The solution was discarded again and the pellet, which contained CGNs, was suspended in culture media and plated over PDL-coated culture dishes. For glial culture, the pellet was transferred to a flat culture bottle with 10 ml DMEM and incubated for the same period. After 48 hrs, cultured Cells and their conditioned media were collected in separate tubes and processed for gelatinase assay.

Organotypic slice culture. Organotypic slice cultures were prepared from postnatal day 5 cerebella. The animals were numbed on ice for several minutes, and then the cerebellum was rapidly extracted and immersed in oxygenated-ACSF (artificial cerebrospinal fluid). Cerebella were sectioned at 500 μm thickness in the parasagittal plane with a Vibraslicer (WPI, Sarasota, FL). Slices were cultured on collagen-coated

Nucleopore membrane (Millipore, Bedford, MA) at the interface between air and culture medium according to the method of Kunimoto and Suzuki (1997) and Tanaka et al. (1994), with minor modifications (Jin et al., 2001). Two slices were placed on every membrane, and four membranes in each sterile six-well culture plate, each over 1 ml of culture media. Culture media was changed every two days and 24 hrs before each harvest. The slices were incubated at 37°C and 5% CO₂ atmosphere.

Gelatinase Assay. Zymographic analysis was performed on proteins extracted from freshly frozen tissue, cerebellar granule neuronal cultures, mixed glial cultures, and conditioned media from cerebellar organotypic slice cultures. Cerebella were dissected out from P3 to P25 rat pups and quickly frozen in liquid nitrogen. Proteins were extracted as described earlier (Li et al., 2001). Samples were collected from three animals at each time point and three cell culture replications, and gelatinase assays were repeated 5 times for each sample. In the case of *in vitro* preparations, culture media containing secreted proteins were collected in addition to the cell lysates. The concentration of proteins in extracts was determined by a Lowry assay then equal amounts of protein were loaded in the electrophoretic gel. The gel was prepared with substrate (1 mg/ml gelatin) copolymerized with 10% SDS-polyacrylamide. Equal volumes (25 µl) of the proteins were prepared with RIPA buffer and 4x non-reducing sample buffer. All samples were kept on ice during the whole procedure until loaded in the gel to minimize denaturation of proteins and enzymes. After 3 hrs of electrophoresis, gels were washed with 2.5% Triton X-100 for 30 min at room temperature, then with a rinse buffer (50mM Tris-HCL pH7.4, 0.1M NaCl, 2.5% Triton-100) for an additional 60 min, followed by an overnight incubation at 37°C in reaction buffer (50mM Tris-HCL pH7.4, 10mM CaCl₂). Gels were

then stained with a solution of Coomassie blue (0.5% Coomassie blue R in 45% MeOH, 10% acetic acid) for 60 min, then de-stained in a solution of 10% MeOH and 10% acetic acid until the digested bands are clear. To further ascertain equal loading in gels, we cut the top 2 cm from each gel and stained it with Coomassie blue for 5 min. Gels were photographed on a trans-illuminator using a Sony CCD Camera and digitally captured and processed using the Eagle Sight® Software (Stratagene). Densitometric quantification of the digested bands was performed on FluorChem SA (Alpha Innotech Corp., San Leandro, CA) and graphed using MS Excel (Microsoft Corp., Seattle, WA). Densitometry measurements were collected after calibration for background intensity and loading controls, and then data were analyzed using SigmaSTAT statistical software (SPSS Inc., Chicago, IL; see statistical analysis for more details).

Reverse Zymography. The levels of gelatinase inhibitors in tissue affect the availability and effectiveness of MMP-2 and MMP-9 in digesting ECM molecules. Reverse zymographic analysis was performed on protein extracts to monitor for the presence of gelatinase inhibitors. Briefly, the experiment proceeds in a similar fashion to the gelatinase assay, except that a 12% SDS-polyacrylamide gel (containing 1 mg/ml Gelatin) was prepared with a mixture of pro-MMP-2 and pro-MMP-9, which were activated with APMA (*p*-Aminophenylmercuric Acetate) following manufacturer instructions (Cat.# 444213, 444231, 164610; Calbiochem, San Diego, CA). This procedure uncovered bands of gelatinase inhibitors, TIMP-1 and TIMP-2, where the added enzyme did not digest the gelatin. Loading controls and densitometry were performed as described for the gelatinase assay.

***In situ* zymography.** Brains were embedded and frozen in O.C.T. compound directly after the dissection and without fixation. Specimens were sectioned using a cryostat at 20 μ m thickness, then stored at -20°C until stained. All slides were incubated in a solution containing 0.05 M Tris-HCl, 0.15 M NaCl, 5 mM CaCl₂, 0.2 mM NaN₃ (pH 7.6) and 40 μ g/ml of FITC-labeled gelatin (Molecular Probes, Eugene, OR), on a shaker at 37°C for 24 hrs. Slides were coverslipped with Vectashield mounting media after blotting excess fluids, and without any rinses. This technique allowed endogenous gelatinases to digest the gelatin producing a fluorescent reaction product, which was detected using conventional fluorescent microscopy as in Oh et al (1999). We pre-incubated control sections in 20 mM EDTA for 1hr, or 4% formaldehyde for 10 min, to determine the specificity of the enzymatic activity (Mook et al., 2003). We detected the fluorescence with excitation at 460-500 nm and emission at 512-542. Slides were imaged on an Olympus AX70 microscope (Olympus America Inc., Melville, NY) equipped with a Spot 2e digital camera (Diagnostic Inst. Inc., Sterling Heights, MI). Camera settings, including optical gain, exposure, and brightness control were the same for captured images. Pictures were imported directly into Adobe Photoshop 5.5 (Adobe Systems Inc., San Jose, CA) for cropping and labeling.

Immunohistochemistry. Brains were sectioned parasagittally on a LEICA cryostat at 12 μ m final thickness and collected on superfrost plus slides (Fisher); they were then stored at -20°C until processing. Sections representing all experimental ages and controls were stained at the same time to minimize variability. Slides were rinsed and cleared in 25% ethanol solution for 5 min to remove excess tissue freezing compound. This step was followed by 5 min rinse in ddH₂O and 10 min in PBS containing 0.1%

Triton-X 100. Slides were incubated in primary antibodies against MMP-2, MMP-9 (all goat-anti-rat; sc-6838, sc-6840; Santa Cruz Biotech. Inc., Santa Cruz, CA) diluted 1:1000 in PBS with 1% normal horse serum (Wagner et al., 2003). Primary antibodies against GFAP and Calbindin (mouse anti-rat; Sigma G3893 and C9848) were prepared at a final dilution of 1:1000 and 1:3000 respectively. After 24 hours at 4°C, slides were placed at room temperature for 15 min to equilibrate, and rinsed 3 times in PBS for a total of 30 min. Slides were then incubated in FITC-labeled anti goat secondary antibody or FITC-labeled anti-mouse (1:200 in PBS; Zymed Laboratories Inc, South San Francisco, CA) for 2 hrs at room temperature. The nucleic acid stain TOTO-3 iodide (T-3604, Molecular Probes, Eugene, OR) was diluted with the secondary antibody at a final concentration of 1 µg/ml. After three rinses in PBS, slides were covered with Vectashield (Vector labs) solution and coverslipped. We processed negative control sections, by omitting the primary antibody, with every batch of stain. Furthermore, to test the specificity of the primary antibodies, we incubated sections with MMP-2 antibody preabsorbed with the specific blocking peptide (sc-6838p, Santa Cruz Biotech), and with MMP-9 antibody preabsorbed with MMP-9 blocking peptide (sc-6840p). All immunohistochemically prepared slides were imaged using a Zeiss LSM 510 confocal microscope (Carl Zeiss Imaging Inc., Thornwood, NY). The same settings for filters, pinhole size, stack size and resolution, were used for all captured images. All images were cropped and labeled using Adobe Photoshop 5.5.

Treatment with gelatinase inhibitor. Organotypic slices were prepared from P5 pups, as described earlier, and treated with a selective MMP-2/MMP-9 inhibitor (1.6 µM, 3.2 µM and 6.4 µM; SB-3CT; 80512-564; VWR) or without inhibitor (Controls) diluted

in culture media for 24hrs, 4 days *in vitro* (DIV), 7DIV, and 10DIV. This gelatinase inhibitor functions as a substrate analogue, and binds with specificity and high affinity to the enzyme active site ($K_i = 13.9$ nM for MMP-2; $K_i = 600$ nM for MMP-9) (Brown et al., 2000; Kleifeld et al., 2001; Rosenblum et al., 2003). We collected slices after 1DIV, 4DIV, 7DIV, and 10DIV (table 3.1).

	Treatment			
	1DIV-C	1DIV-1.6 μ M	1DIV-3.2 μ M	1DIV-6.4 μ M
	4DIV-C	4DIV-1.6 μ M	4DIV-3.2 μ M	4DIV-6.4 μ M
	7DIV-C	7DIV-1.6 μ M	7DIV-3.2 μ M	7DIV-6.4 μ M
	10DIV-C	10DIV-1.6 μ M	10DIV-3.2 μ M	10DIV-6.4 μ M

Table 3.1 Slice culture treatment groups. Serial slices were distributed in four treatment groups and incubated for 1, 4, 7 and 10 days *in vitro*. Slices were treated with SB-3CT at increasing concentrations for the entire culture period. We collected the culture media for gelatin zymography and the slices were sectioned for immunohistochemistry and *in situ* zymography.

For each treatment group and time point, we collected the culture media, since it contains secreted proteins, and stored it at -80°C until further processing with gelatin zymography. The slices were embedded in O.C.T. compound and frozen at -20°C between 2 glass slides on a flat metal block (Haydar et al., 1999). This procedure helped us maintain the slices flat and allowed us to section the slices thinner for immunohistochemistry and *in situ* zymography.

Morphometric analysis. several parameters were monitored to assess the effect of MMP inhibitor on the histogenesis of the cerebellar cortex in organotypic slices (n = 6 per treatment group). The thickness of the EGL was measured and the number of granule

cells in the molecular layer was counted. Measurements were taken from three lobes in every section. Lobes were assigned by systematic random sampling. Sampling areas ($325.7\text{ }\mu\text{m} \times 325.7\text{ }\mu\text{m}$) were predefined and equivalent in all treatment groups to minimize variability. Three lines were drawn to define the EGL and the ML. We measured the thickness of the EGL as the minimum distance between tangents to the lines defining the EGL and counted the number of cells with migrating morphology (fusiform somata) in the ML (see Appendix A10 for an example).

Statistical analysis. Parametric data were analyzed data by an ANOVA followed by post-hoc Tukey's t-test comparisons between groups when appropriate, using SigmaSTAT statistical software (SPSS Inc.). Bartlett's statistic was used to identify nonparametric data, which were analyzed with a Kruskal-Wallis test followed by Dunn's multiple comparisons.

Results

Developmental mRNA expression of MMP-2 and MMP-9

The gene expression of MMP-2 and MMP-9 is modulated by a variety of factors expressed during development including growth factors, cytokines, and ECM molecules (Vu and Werb, 2000). We measured the relative mRNA expression of MMP-2 and MMP-9 in cerebella and tissue from cerebral cortices of rat pups from P3 to P25. As measured by real-time quantitative PCR MMP-2 mRNA decreased by 40% between P3 and P6, and by an additional 20 % between P6 and P25 (Figure 3.1A). In comparison, MMP-2 expression in the cerebral cortex gradually decreased from P3 to P25 (Figure 3.1B). The expression of MMP-9 in the cerebellar cortex decreased by 3-fold from P3 to P6 and remained constant thereafter (Figure 3.1A). In comparison, the mRNA expression of MMP-9 in cerebral tissue increased dramatically after postnatal day 12 and continued to rise until postnatal day 25 (Figure 3.1B). Since tissue were dissected from the same animals, prepared, and analysed at the same time to reduce variability, then the observed changes are specific to each of these cortical areas.

Developmental changes in MMP-2 and MMP-9 activity

Matrix metalloproteinases are secreted as inactive enzymes into the extracellular matrix (Corcoran et al., 1995). Their activation is the last step in a highly controlled process and is needed to start their proteolytic activity (Murphy et al., 1999; Nagase,

1997). It is thus important to distinguish between the levels of pro-enzyme and the proteolytically active enzyme. To determine the spatiotemporal levels of activation of MMP-2 and MMP-9 in the cerebellar cortex between P3 and P25, we performed a zymographic assay on protein extracts from freshly frozen tissue, on cultured cerebellar granule neurons (CGN) and on glial cell cultures prepared from same-age cerebella. This was followed by an *in situ* zymographic assay on freshly frozen cryostat sections. We were able to detect bands of pro-MMP-2 at 72 kDa and weaker bands of active MMP-2 at 68 kDa. The gelatinase activity showed a progressive decrease in the concentration of pro-MMP-2 from P3 until P15 (Figure 3.2A, G). Since zymographic assays are sensitive enough to detect picogram amounts of gelatinases (Troeberg and Nagase, 2003), then the weak MMP-2 activity suggests a limited tissue-spread and a focused role for MMP-2. As for MMP-9's expression, the predominant form before P12 is the active form (Figure 3.2B, H). After P12, the latent pro-MMP-9 increased in proportion while the overall expression profile of MMP-9 (active and latent) started to decrease. We also measured the activity of the tissue inhibitors of MMPs, TIMP-1 and TIMP-2, by reverse zymography. We detected TIMP-2 (21 kDa), but not TIMP-1 (28 kDa), in the reverse zymographic assay (Figure 3.2 C, I). TIMP-2 binds to and controls the activity of MMP-2. The activity of TIMP-2 increased between P3 and P9 and leveled afterwards.

To determine the source of the gelatinolytic activity in the cerebellum, we performed a gelatinase assay on conditioned media and cell lysate from cultured cerebellar granule neurons (CGN) and glial cells prepared from P4 cerebella. In culture, CGN showed high activity for pro-MMP-2 (72kDa), two active fragments of MMP-2 at 68kDa and 65 kDa, as well as pro-MMP-9 (92kDa) and the active MMP-9 at 80kDa

(Figure 3.2E). In glial culture, we detected pro-MMP-2 and MMP-2 (68 and 65 kDa), and a relatively weaker band of pro-MMP-9 (Figure 3.2F).

We performed an *in situ* zymography assay on frozen cerebellar slices from P3 to P25, which allowed us to visualize the distribution of active forms of the gelatinases. Even though this method is sensitive to minute amounts of the gelatinases, it does not offer the high resolution achieved with immunohistochemical methods. In order to detect developmental changes, we used the same optical settings to capture all images. Briefly, we took 20 μ m cryostat sections from fresh-frozen brains from P3 to P25. After 24 hr incubation with FITC-quenched gelatin, we detected fluorescence in areas of the sections where active MMPs cleaved gelatin. In the cerebellum, the fluorescence was mostly present in the EGL, the Purkinje cell layer and the IGL (Figure 3.3). The fluorescence in the pial basal lamina and the EGL was more intense than in the ML and the white matter. From P3 to P15, the distribution of gelatinase activity changed to include more of the internal granule layer and not just the EGL and PCL. During the same period, we observed a progressive decrease in proteolytic activity limited to the cerebellar cortex. Gelatinase activity was still detectable in the same sections in the hippocampal formation at P15 (Figure 3.3A) and persisted beyond P21 (not shown). We processed control sections as described by Mook et al. (2003) to ascertain the specificity of the fluorescent signal. Briefly, we incubated sections with gelatin-DQ® (Molecular Probes) as a substrate after blocking the gelatinase activity with 20mM EDTA or 4% formaldehyde. Sections treated with EDTA and formaldehyde showed greatly reduced fluorescence (Figure 3.3B).

Developmental changes in MMP-2 and MMP-9 immunoreactivity

To determine the distribution of MMP-2 and MMP-9, we performed immunohistochemical staining on serial sections from the cerebellum. Briefly, for this experiment, fixed tissue was sectioned in the sagittal plane at 12 μm thickness and probed for MMP-2 and MMP-9 with specific antibodies, followed by FITC-tagged secondary and counterstained with TOTO-3 iodide, a fluorescent nucleic-acid binding dye. Sections were imaged using a LSM510 confocal microscope under the same optical settings (see methods) and optical stacks were later collapsed into one image. In cerebellar sections, staining with TOTO-3 was noticeably more intense in the cytoplasm of Purkinje cells than in their nuclei (Figure 3.4). TOTO-3 iodide binds with specificity to nucleic acids, nuclear DNA and cytoplasmic RNA, which may have contributed to the cytoplasmic staining in Purkinje neurons. This diffuse staining seems more noticeable in cells with higher cytoplasmic RNA content and bigger somata (Boutonnat et al., 2000; Suzuki et al., 1997). Sections of similar age groups were also stained for Calbindin D28k, which stains Purkinje neurons in the cerebellum. We detected calbindin immunoreactivity in Purkinje cell bodies, dendritic trees, and axons (Figure 3.4). We also stained sections with anti-GFAP antibody, which is expressed in Bergmann glia in the cerebellar cortex and astrocytes in the white matter (Levitt and Rakic, 1980; Dahl and Bignami, 1973). The immunoreactivity of GFAP increased in the first postnatal week and was stable afterwards. Bergmann glial endfeet stained intensely with GFAP, whereas Bergmann fibers stained more intensely as they approached the EGL. Immunostaining was present in perikarya (although less numerous in our sections) and absent in short bushy lamellate expansions seen in silver impregnation preparations; a comparable pattern was reported

by other authors (for details see Levitt and Rakic, 1980). Artifacts and weaker stain were noticeable in some specimens and might be due to the age of animals, the condition of the tissue, and other conditions. However, the use of GFAP and calbindin allowed us to compare the cellular sources of MMP-2 and MMP-9, and tentatively identify cellular elements. Double or triple stain for GFAP, calbindin, and MMP (-2 or -9), would have presented better cellular identification; however, we were unable to preserve the integrity of the tissue and get consistent results using this approach.

We detected MMP-2 immunoreactivity in Purkinje somata and initial dendritic trunks, in radial fibers resembling Bergmann glial fibers and in the EGL (Figure 3.4 - 5). MMP-2 immunoreactivity was strongly reduced in Bergmann glial fibers after P12 and in Purkinje cells after P15. Immunoreactivity of MMP-9 staining coincided with Purkinje neurons and the EGL (Figure 3.4 - 6). Diffuse staining was also noticeable around the Purkinje dendrites, which indicates the detection of MMP-9 in the extracellular space. MMP-9 staining in Purkinje cells was below detection after P18. Figures 3.5 and 3.6 show representative fields from sections treated with MMP-2 and MMP-9 antibodies at higher magnification. We processed negative control sections, where we omitted the primary antibody, with every batch of stain. To further ascertain the specificity of the antibodies, we incubated sections with the primary antibody pre-absorbed with the corresponding specific blocking peptide. Figure 3.7 shows representative control sections from P15 stained with MMP-9 antibody pre-absorbed with increasing dilution of MMP-9 blocking peptide (Santa Cruz Biotech). As can be noticed, the immunoreactivity increased as the concentration of the blocking peptide was reduced in relation to the antibody from 1:10, 1:20, 1:50, and was comparable to full antibody staining when we

omitted the blocking peptide (0:1). Similar results were observed with MMP-2 preabsorbed with the MMP-2 blocking peptide (not shown).

Effect of specific MMP-2 and MMP-9 inhibitor on cerebellar morphology

We used organotypic slice cultures from cerebella of P5 rat pups. This technique has proved very useful in studying the developmental mechanisms in cerebellar cortex without sacrificing the role of the ECM and intercellular communication (Haydar et al., 1999). We cultured cerebellar slices for 1 day (1DIV = 1 day *in vitro*), 4DIV, 7DIV, and 10 DIV as described earlier (Jin et al., 2001; Tanaka et al., 1994). At each time point, serial slices were divided in four groups, three of which received treatment with a selective MMP-2/MMP-9 inhibitor (SB-3CT) at increasing concentrations (1.6, 3.2 and 6.4 μ M) and a control group. This inhibitor was shown to bind specifically to MMP-2 and MMP-9 in a manner similar to TIMP-1 and TIMP-2 (Kleifeld et al., 2001). At the end of the treatment period (24hrs, 4 days, 7 days, and 10 days) we collected and stored the culture media, which contains secreted enzymes, and the slices at -80°C. We performed a zymographic assay on the culture media and *in situ* zymography on sectioned slices. We also sectioned and stained cerebellar slices to quantify the effect of inhibitor treatment on the histogenesis of the cerebellar cortex.

We processed alternate serial sections with immunohistochemistry for MMP-2, MMP-9, Calbindin D28K, and GFAP, and with *in situ* zymography from treatment groups and controls. The immunoreactivity of MMP-2 and MMP-9 followed an expression pattern similar to that observed *in vivo*. Figure 3.8 (A, B, C, and D) shows sample sections from control slices at 7DIV immunostained for MMP-2, MMP-9,

Calbindin D28k and GFAP. Figure 3.8 (E and F) shows a sample from sections processed for *in situ* zymography taken from adjacent slices at 7 days *in vitro*. The distribution of the gelatinase activity in the section in (F) treated with 3.4 μ M SB-3CT was reduced and less uniform compared to the section in (E), which was taken from the adjacent control slice and imaged under the same optical settings. We observed a similar pattern of reduced and irregular activity in other slices exposed to SB-3CT at other concentrations.

We performed a zymographic assay on the culture media collected from inhibitor treated slices and controls (Figure 3.9A-F). The concentration of proteins in conditioned media was determined by a Lowry assay and the same amount of proteins was loaded in all wells (see methods). A progressive decrease in gelatinase activity, similar to the expression pattern detected *in vivo*, was measured for both MMP-2 and MMP-9 in control slices at all time points (Figure 3.9 A and F). Treatment with the specific inhibitor SB-3CT was accompanied by a progressive decrease in gelatinase activity. At 7 DIV and 10DIV (Figure 3.9 B, E, F), the activity of MMP-2 was still detectable, whereas the activity of MMP-9 was drastically reduced. To ascertain equal loading in the gels, we cut and stained the top 2 cm of each gel, after electrophoresis and without incubation, with coomassie blue for 5 min.

To assess the effect of MMP-2/MMP9 inhibitor treatment on the development of the cerebellum, we measured the thickness of the EGL before and after inhibitor treatment (Table 3.2; Figure 3.10 A). The EGL is composed of a dense population of cells between the pial basal lamina and the molecular layer, which provides a convenient method to delineate its outer and inner boundaries. This method has been successfully used to assess the effect of exogenous compounds on the proliferative activity of

progenitors and migratory activity of immature postmitotic neurons (Ohmori et al., 1999; Doughty et al., 1998). To minimize error and bias, we chose the counting fields by systematic random sampling. All fields were equivalent in dimension (325.7 μm x 325.7 μm) at 20x magnification regardless of age and treatment conditions. Treatment with inhibitor caused a significant dose-dependent increase in the thickness of the EGL compared to controls at 1DIV 1.6 μM , 3.2 μM , 6.4 μM ($F_{(3,53)} = 38.42$; all $p < 0.001$), and 4DIV 3.2 μM ($F_{(3, 53)} = 11.84$; $p < 0.01$) and 6.4 μM ($p < 0.001$). At 7DIV, the thickness of EGL was significantly thicker in 7DIV 1.6 μM , 7DIV 3.2 μM , and 7DIV 6.4 μM ($F_{(3, 53)} = 112.5$; all $p < 0.001$). At 10 DIV, slices treated with 1.6 μM and 3.2 μM inhibitor had significantly thicker EGL ($F_{(3,53)} = 368.0$; $p < 0.001$); while slices treated with 6.4 μM inhibitor had a significantly thinner EGL ($p < 0.001$).

Percent difference compared to control slices				
<i>Days in vitro</i>		1.6 μM Inhibitor ($\pm\text{SEM}$)	3.2 μM Inhibitor ($\pm\text{SEM}$)	6.4 μM Inhibitor ($\pm\text{SEM}$)
	1DIV	+14.1% (± 1.66)	+26.2% (± 0.98)	+30.4% (± 0.67)
	4DIV	+8.2% (± 2.37)	+17.1% (± 1.58)	+24.2% (± 1.61)
	7DIV	+58.6% (± 0.73)	+42.3% (± 1.31)	+22.8% (± 0.61)
	10DIV	+17.4% (± 0.36)	+11% (± 0.32)	-30.9% (± 0.35)

Table 3.2 Percent change in the thickness of EGL after treatment with SB-3CT. Comparison of the average percent change in the thickness of the EGL in treated slices compared to in-group controls.

We also counted the number of fusiform-shaped cells, presumed granule cells, in the molecular layer (Table 3.3; Figure 3.10 B). We limited our counting to cells with round or fusiform nuclei smaller than 7.5 μm in diameter to exclude the bigger nuclei of other interneurons. The number of fusiform-shaped granule cells in the molecular layer was also affected by the inhibitor treatment. There was a dose-dependent decrease in the number of migrating cells in all treatment groups.

Percent difference compared to control slices				
<i>Days in vitro</i>		1.6 μM Inhibitor ($\pm\text{SEM}$)	3.2 μM Inhibitor ($\pm\text{SEM}$)	6.4 μM Inhibitor ($\pm\text{SEM}$)
	1DIV	-2.5% (± 1.99)	-16.4% (± 1.92)	-22.3% (± 1.71)
	4DIV	-17.4% (± 2.56)	-27.3% (± 1.52)	-46.6% (± 1.08)
	7DIV	-19.9% (± 1.68)	-28.5% (± 1.47)	-62.1% (± 0.96)
	10DIV	-28.8% (± 1.06)	-48.7% (± 1.14)	-72.1% (± 0.83)

Table 3.3 Percent change in the number of migrating cells in the ML after treatment with SB-3CT. Comparison of the average percent change in the number of migrating granule neurons in the ML in treated slices compared to in-group controls.

The effect was significant in 1DIV 3.2 μM ($F_{(3,53)} = 7.348$; $p < 0.05$), 4DIV 1.6 μM ($F_{(3, 53)} = 38.31$; $p < 0.01$), and highly significant ($p < 0.001$) in 1DIV 6.4 μM , 4DIV 3.2 μM , 4DIV 6.4 μM , 7DIV 1.6 μM ($F_{(3, 53)} = 65.82$), 7DIV 3.2 μM , 7DIV 6.4 μM , 10 DIV 1.6 μM ($F_{(3, 53)} = 129.8$), 10 DIV 3.2 μM , and 10DIV 6.4 μM respectively. Blocking the activity of gelatinases caused an increase in the thickness of the EGL and a decrease in the number of granule neurons in the ML. These results are indicative of either altered

proliferation of progenitor cells in the EGL, altered initiation of migration of immature CGNs from the EGL, or disrupted migration through the ML.

Discussion

We examined the expression, activity, and contribution of the matrix metalloproteinases 2 and 9 in the postnatal rat cerebellar cortex using a variety of experimental approaches. Temporal patterns of expression and activity pointed to the involvement of MMP-2 and MMP-9 in the cellular events that shape the cerebellar cortex. During the first two postnatal weeks, granule neurons complete their migration from the external granule layer to the internal granule layer. Before the end of the second postnatal week, the rate of granule cell migration significantly decreases while other cerebellar interneurons, stellate, Golgi, and basket cells complete their maturation (Altman, 1972a; Altman, 1972b). This period is also important for the maturation of Bergmann glial fibers and the growth of Purkinje cell dendritic trees (Seil, 2001; Zecevic and Rakic, 1976).

Developmental mRNA expression of MMP-2 and MMP-9

We determined the level of mRNA expression in the cerebellar cortex by realtime quantitative RT-PCR (Figure 3.1). The level of MMP-2 mRNA expression in the cerebellar cortex decreased sharply between P3 and P6 and more gradually between P6 and P25. In the cerebral cortex during the same period, MMP-2 mRNA progressively declined from P3 until P25. The transcript levels of MMP-9 remained unchanged between P6 and P25 after an initial decrease from P3 to P6. In comparison, the level of

MMP-9 mRNA expression increased significantly in cerebral tissue from P6 to P25. During this period in the rat neocortex, dendritic trees of cortical neurons are expanding and axons are beginning to acquire a myelinating sheath. Both, dendritic remodeling and process extension of oligodendrocytes, are known to involve gelatinases (Oh et al., 1999; Szklarczyk et al., 2002; Uhm et al., 1998). The mRNA expression for MMP-9 was previously studied by Vaillant et al. (1999). They observed MMP-9 mRNA in the cerebellum at P10 and P15 by *in situ* hybridization; however, they did not detect MMP-2's mRNA. Their observations in the cerebellum at P10 and P15 are in accordance with our data, except that we detected a preceding decrease between P3 and P6. The decline in MMP-9's mRNA may have an impact on the level of pro-MMP-9 and, subsequently, the active MMP-9 available for the degradation of ECM proteins. Other regulatory mechanisms may be at play as well, especially the possibility that MMP-9 is controlled post-transcriptionally or post-translationally. Modulation and expression of the transcript of gelatinases, MMP-2 and MMP-9, can occur on a localized level, within a cell type or cell layer, and not at the organ level. Studies on gliomas, astrocytomas, and other metastatic brain tumors give ample support for this proposition (Raithatha et al., 2000; Vince et al., 1999; Lampert et al., 1998). In these studies, gelatinase transcripts were detectable only within cancerous tissue and not in the surrounding normal tissue. Evidence from these studies and other work on lung, liver, skin, and ovarian cancers links this expression to migration, invasiveness, and to neovascularization (Gokaslan et al., 1998; Platten et al., 2001; Furuya et al., 2000; Hah and Lee, 2003; Rooprai and McCormick, 1997).

Developmental changes in MMP-2 and MMP-9 activity

The physiological roles of MMP-2 and MMP-9 are dependent on the availability of the latent enzymes and their subsequent activation (Vu and Werb, 2000). We examined the gelatinase activity of MMP-2 and MMP-9 in the cerebellar cortex from rat pups and found that the activity gradually decreases in the first 25 postnatal days (Figure 3.2 A-D, G-I). The gelatinase activity of MMP-2 decreased progressively from P3 until P18 and was constant afterwards. Vaillant et al. (1999) reported gelatinase assays for only three time points (P10, P15 and adult) with somewhat different results. Even though Vaillant and colleagues reported a constant level of MMP-2 throughout development, close inspection of their results suggest a modest decrease between P10 and P15; however, no quantitative data was reported to clarify this discrepancy. In the current study, we studied eight time points covering the period from birth to P25. Furthermore, to address differences with published results (Vaillant et al. 1999), we performed densitometric analysis on zymographic gels (n=5 per time point), which supported our observations (Figure 3.2 A, G). MMP-2 activity gradually decreased between P3 and P18 and stabilized afterwards.

During the same period, reverse zymography assays showed constitutive activity for TIMP-2 (21 kDa) between P9 and P25, after an initial slow increase between P3 and P9 ($p<0.05$; Figure 3.2 C, I). This suggests that the activity of MMP-2 is being controlled at yet another level. In fact, TIMP-2 binds and inhibits MMP-2 (Nagase, 1997), and functions as an adaptor protein between pro-MMP-2 and its activators, MT-MMPs. High levels of TIMP-2 saturate all the binding sites on MT-MMPs (membrane tethered MMPs) and block the activation of MMP-2, whereas low levels favor binding and activation

(Murphy et al., 1999). The availability of TIMP-2, which may limit the activation of pro-MMP-2, combined with a progressive decrease in MMP-2's gelatinase activity suggests a more significant developmental role for MMP-2 in the first postnatal week than in the second and third postnatal weeks.

As for MMP-9 (Figure 3.2 B, H), the active form was predominant between P3 and P12. The proportion of the latent form increased on and after P12, however, the total MMP-9 activity (latent and active) gradually decreased after P18. These data suggest a switch in the available form of MMP-9; the active enzyme was predominant until P9, while the latent form increased afterwards. Vaillant et al. (1999) reported a parallel pattern, with MMP-9's activity strong at P10 but hardly noticeable at P15 and in adults. Quantitative analysis of our gels by densitometry supports our qualitative observations ($p < 0.05$; Figure 3.2 H). In reverse zymographic assays, we did not detect TIMP-1 (28 kDa), which normally binds to and inhibits MMP-9. The absence of the inhibitor TIMP-1 and the availability of the catalytically active form of MMP-9 suggest the involvement of MMP-9 in the biochemical and cellular events between P3 and P15, and a declining role afterwards. The activity of these enzymes persists longer than their RNA message, which supports the idea of translational, post-translational or post-transcriptional regulation of the activity and expression of MMP-2 and MMP-9.

To determine the potential source of MMP-2 and MMP-9 activity in the developing cerebellum, we isolated and cultured CGNs from P4 cerebella. After 48 hrs in culture, we performed a zymographic analysis on the conditioned media, which contained secreted proteins (Figure 3.2 E). We also performed a zymographic analysis on conditioned media from glial cultures isolated and cultured from same age cerebella.

Both CGNs and glial cells *in vitro* expressed pro-MMP-2 (72 kDa) and MMP-2 (68 kDa and 65 kDa). On the other hand, pro-MMP-9 and MMP-9 expression was more prominent in CGNs than glial cells. This suggests that granule neurons and glial cells are a potential source of gelatinases in the developing cerebellar cortex, with CGNs expressing both MMP-2 and MMP-9.

We used an *in situ* zymography assay to determine the spatial distribution of active gelatinases (Figure 3.3). This technique has been successfully used to study the distribution pattern and changes of gelatinases in the developing and adult nervous system (Oh et al., 1999; Szklarczyk et al., 2002). In cerebellar sections, the distribution of gelatinase activity in the cerebellar cortex gradually increased between P3 and P12. Initially the activity was present in the EGL and basal lamina. Between P9 and P12, the distribution of gelatinase activity progressively increased to include the Purkinje cell layer and the IGL. By P15, however, the gelatinase activity decreased to a minimal level in all layers of the cerebellar cortex. In contrast, gelatinase activity in the hippocampal formation was still detectable at P15 in the same section (Figure 3.3), demonstrating that the decrease in activity was localized to the cerebellum. This decrease in activity from P12 to P15, as detected with *in situ* zymography, mirrors the pattern observed in gelatinase assays (previous section). Several studies have documented the contribution of gelatinase activity to retinal development, but none have examined the cerebellum. In the retina, MMP-2 and MMP-9 activity was observed in Müller cells, which form a “scaffold” structure like Bergmann glia, in growing neurites and growth cones, in the retinal pigment epithelium and the ECM surrounding photoreceptors (Limb et al., 2002; Padgett et al., 1997; Sheffield et al., 1994). There is strong evidence for the participation

of gelatinases in synapse formation (Taishi et al., 2001), differentiation (Chambaut-Guerin et al., 2000), migration through glial scars (Muir et al., 2002) and tumor metastasis (Rooprai and McCormick, 1997). In the cerebellum, during the first three postnatal weeks, three of these processes (synapse formation, differentiation, and migration) are underway. Granule neurons are migrating from the EGL to the IGL, they are forming connections with Golgi cells and mossy fibers, and PCs, interneurons and glia are still differentiating (Altman, 1972a; Sotelo, 1990). *In situ* gelatinase activity is enhanced within the EGL, PCL, and the IGL, which suggests a role for MMP-2 and MMP-9 in some of these critical cellular events.

Developmental changes in MMP-2 and MMP-9 immunoreactivity

Inspection of the expression of MMP-2 and MMP-9, over time, by immunohistochemistry helped us understand the distribution of the enzymes relative to cells and cell layers (Figure 3.4-6). Staining with a polyclonal antibody against MMP-2 coincided with the Purkinje cell layer, especially the soma and proximal dendritic trunks of PCs. This pattern was different from the immunoreactivity detected by calbindin D28k, which as expected labeled entire PCs including their dendrites. MMP-2 immunoreactivity was also associated with radial processes that strongly resemble Bergmann glial fibers in size, location, and arrangement. Moreover, staining for MMP-2 was present in the EGL. Staining for MMP-2 decreased below detection level at P15. Vaillant et al. (1999) observed MMP-2 immunoreactivity in the PCL, but not in the EGL or ML. This difference is probably due to several factors including the specificity of the

antibody we used (Santa Cruz), which recognizes the C-terminal domain common to both pro-MMP-2 and MMP-2. We performed several controls to ascertain the specificity of the antibody including negative controls, pre-absorption with a specific MMP-2 blocking peptide (data not shown), and by western blot analysis (Dr. Luo, unpublished data). The specificity of this antibody has been independently confirmed and successfully used by other authors (Wagner et al., 2003). Furthermore, CGN and glial cultures (Figure 3.2 E, F) showed strong gelatinase activity for MMP-2, which further supports our observations. Several developmental events occur during this period, including the migration of granule neurons and the extension of parallel fibers, the maturation of Bergmann glial fibers, and the ensheathment of Purkinje cell bodies by glial processes (Yamada and Watanabe, 2002; Altman, 1972a; Altman, 1972b). The expression of MMP-2 in the EGL, Bergmann glial fibers, and the Purkinje cell layer suggests the involvement of MMP-2 in these developmental events.

MMP-9 immunoreactivity was most intense in the EGL and in the Purkinje cells. Side by side comparison of MMP-9's immunoreactivity to calbindin immunostaining, revealed a difference in the distribution. Whereas calbindin stained the entire Purkinje cell, MMP-9 was distributed in somata and dendrites but did not extend to smaller dendrites and spines. Furthermore, punctate staining for MMP-9 could be observed in the immediate pericellular space surrounding PCs. Similar punctate immunoreactivity for MMP-9 and other MMPs have been reported in trophoblasts of the human placenta and in malignant gliomas (Huppertz et al., 1998; Rooprai et al., 1998). MMP-9 has been shown to contribute to process extension of oligodendrocytes, to neurite extension, and dendritic remodeling in the hippocampus (Oh et al., 1999; Pittman and Williams, 1989; Uhm et al.,

1998; Zhang et al., 1998). Diffuse and punctate staining around the dendrites (Figure 3.6) suggests a role in the degradation of ECM around extending neurites or synapse formation. The pattern of expression of MMP-9 also may suggest a role in the maturation of Purkinje cells and the branching and expansion of their dendritic trees.

The effect of inhibiting gelatinases on cerebellar development in organotypic slice culture

We next tried to demonstrate possible effects of diminishing MMP-2 and MMP-9 on the histogenesis of the cerebellar cortex by exposing cerebellar slices to a selective MMP-2/MMP-9 inhibitor (Figure 3.8-10). Organotypic slice culture has been successfully used to study the development of the cerebellar cortex (Hager et al., 1995; Kunitomo and Suzuki, 1997). The cytoarchitecture of the cerebellar cortex is maintained (Tanaka et al., 1994; Bruckner and Grosche, 2001), even in organotypic slices prepared from P8 pups and cultured for 11 days *in vitro* (Takacs and Metzger, 2002). In our experiments, sections from cerebellar slices showed a developmental pattern that is comparable to what we observed *in vivo* (Figure 8A-C). Several experimental modifications seemed to improve the integrity of cultured slices like replacing the culture media every 48 hrs instead of 36 hrs, adding 10 μ l of culture media on top of the slice, and placing slices side by side on the filter insert. The inhibitor we used (SB-3CT, VWR) has been shown to mimic the binding of TIMP-1 and TIMP-2 (Kleinfeld et al., 2001). *In situ* zymography in sectioned cerebellar slices showed aberrant activity in slices treated with the gelatinase inhibitor compared to controls (Figure 7E and F). This altered

gelatinase activity was most noticeable in the EGL and the IGL. We used two parameters to quantify the effects of MMP-2 and MMP-9 inhibitor on cerebellar cortical histogenesis in organotypic (Figure 3.10A and B). First, we measured the thickness of the EGL, which is an indirect indicator of the density of granule cells in the EGL. Secondly, we counted the number of small fusiform cells, presumed to be migrating granule neurons, in the ML. The first parameter pertains to the proliferative activity of progenitor cells and the rate of CGN exodus from the EGL. Combining this information with the number of migratory profiles allows us to assess the effect of inhibiting the function of gelatinases. A decrease in the thickness of the EGL could be due to lengthening of the cell cycle or an increase in the rate of migration. The first condition leads to a decrease in the number of migrating profiles, while the second leads to a transient increase followed by a decrease due to reduction of the premigratory population in the deep EGL. An increase in the thickness of the EGL would indicate shortening of the cell cycle or a decrease in the rate of migration. Shortening the cell cycle will increase the packing density of cells and thickness of the EGL without affecting the number of migratory profiles in the ML, while a decrease in the number of migrating cells in the ML would be due to a decrease in the rate of migration.

Inhibitor treatment resulted in a significant dose-dependent increase in the thickness of the EGL in slices cultured for 1 day (1DIV) and 10DIV compared to untreated controls. The number of fusiform cells in the ML, presumed to be migrating, decreased significantly in a dose dependent fashion in all treatment groups compared to controls. We speculate that the increase in the thickness of the EGL can be explained by an increase in the number of cells unable to start migration, whereas the decrease in

thickness at 10 DIV may be due to an increase in cell death, particularly in the postmitotic-premigratory population. A similar decrease in the thickness of the EGL was observed in cerebellar slices treated with methylmercury, which inhibited neuronal migration and caused an increase in pyknotic nuclei in the deep EGL as identified by BrdU pulse labeling and TUNEL (Kunimoto and Suzuki, 1997). Kunimoto and colleagues proposed that the retardation of migration may cause an increase in naturally occurring cell death that is thought to reduce the number of granule neurons (Wood et al., 1993).

The reduction in the number of migrating cells in the ML and the increase in thickness of the EGL was accompanied by a time- and dose-dependent decrease in gelatinase activity detected in cerebellar slices (Figure 9A-F). These data support the proposition that MMP-2 and MMP-9 may contribute to the migration of CGNs. The concentration of inhibitor we used (1.6 μ M - 6.4 μ M), has been shown to have minimal effect on other MMPs (Brown et al., 2000) and on the viability of cells *in vitro* (Fingleton, 2003; Rosenblum et al., 2003). Other issues remain to be clarified about the effect of inhibitor treatment. It is not clear whether the decrease in migratory cell number is due to the decrease in the rate of migration, or is secondary to a decrease in the ability of postmitotic neurons to initiate migration. Gelatinases have been shown in other systems to promote cell motility and tumor metastasis (Gokaslan et al., 1998; Tokuraku et al., 1995). Gelatinase inhibitors has been shown to reduce the migration of Langerhans cells, skin dendritic cells (Ratzinger et al., 2002), and the migration of vascular smooth muscle cells (Pross et al., 2002). Although inhibitor treatment did not completely stop the migration of granule neurons, it decreased it significantly. Since the development of the

cerebellar cortex is dependent on intact interactions and normal development of its cellular constituents, then a role for gelatinases is strongly supported.

Disturbing the development of granule neurons, by mutations or toxic compounds, is known to cause stunting of Purkinje dendrites and damage to Bergmann glia; disrupting the development of Purkinje neurons can have even gravier consequences (Ferguson, 1996; Bignami and Dahl, 1974; Adams et al., 2002; Altman and Anderson, 1972; Llinas et al., 1973). The pattern of immunoreactivity of MMP-2 localizes around fusiform-shaped cells in the ML, presumably migrating granule neurons, and around granule neurons in the deep EGL, which supports a role in migration. MMP-2 immunoreactivity was also detected in Bergmann glial fibers. Additionally, purified CGNs and glial cells from P4 cerebella had strong MMP-2 expression. Temporary “interstitial densities” have been shown to attach migrating granule neurons to BG fibers (Gregory et al., 1988). We speculate that Bergmann glia and/or granule neurons secrete MMP-2 to dissolve these densities and allow for further advancement of cells or processes. Gelatinases have been shown to facilitate the migration of gliomas (Uhm et al., 1996), and of oligodendrocyte progenitors *in vitro* on an astrocytic ECM by degrading temporary cell attachments (Uhm et al., 1998; Oh and Yong, 1996).

We detected MMP-9 activity in purified CGNs, and immunoreactivity in the EGL and in Purkinje cell dendrites. This expression pattern indicates a role for MMP-9 in the migration of CGNs and in the growth of Purkinje cell dendrites. We speculate that the effect on migrating cells may be secondary to impeding the growth of Purkinje dendritic trees. This could be resolved by measuring the growth of Purkinje dendritic trees in treated slices compared to control slices. Damage to Purkinje cells by X-irradiation or

mutations is known to cause reduction in granule neuron numbers (Altman et al., 1969b; Bradley and Berry, 1978). Another possibility is that the inhibition of gelatinases reduced the ability of cells to degrade the ECM, which might have resulted in an increase in the packing density of cells in the EGL. Furthermore, the expression pattern in the EGL suggest a role for MMP-2 and MMP-9 in neurogenesis; a function suggested for MMP-9 (Canete et al., 1995) but not for MMP-2. These possibilities, however, will require experimental evaluation.

Concluding remarks and future directions

Several studies reported the absence of MMP-2 transcript in normal adult tissue and elevated mRNA levels only in malignant tissue (Raithatha et al., 2000; Vince et al., 1999; Lampert et al., 1998). The decrease in MMP-2 mRNA from developmental levels to adult levels has been reported to occur before birth (Casasco et al., 1995). Our data suggest that this decrease occurs postnatally in rats (Figure 3.1). Transcript levels of MMP-2 in the cerebellar and cerebral cortex declined progressively between P3 and P25. The possibility remains that MMP-2's transcripts are expressed locally in certain cells, which could be verified by *in situ* hybridization. Furthermore, new technologies like laser capture microdissection (LCM, Acturus) or laser microdissection and pressure catapulting (LMPC, Carl Zeiss Inc.) allow the dissection of cells or small regions of tissue for RNA or DNA analysis. These techniques would allow the study of the expression of MMP-2 and MMP-9 by RT-PCR in cortical tissue, cortical layers, and in specific cell populations.

MMP-9 transcript showed an increase during the first two postnatal weeks in cerebral tissue, which consistently contained parts of the corpus callosum. The contribution of MMP-9 to process extension of oligodendrocytes and to myelination, have been previously proposed (Oh et al., 1999; Uhm et al., 1998). Myelination in the corpus callosum increases during the second postnatal week, but is delayed until after postnatal day 25 in the cerebellum (Wiggins, 1986; Raval-Fernandes and Rome, 1998; Hamano et al., 1998). Therefore, the increase in MMP-9 mRNA in cortical tissue may be partly due to the increase in myelination. If this speculation were true, we would expect to observe an increase in MMP-9 mRNA in the cerebellum after P25. A progressive decrease to adult MMP-9 mRNA levels would be expected as myelination subsides. Another possibility is that MMP-9 contributes to dendritic growth and plasticity. Evidence presented by Szklarczyk and colleagues (2002) suggests a role for MMP-9 in these functions in the adult nervous system but whether MMP-9 fulfills the same function during development remains to be explored.

Genetic manipulations of the expression of MMPs provided ample knowledge about the mechanisms of activation and the consequences of abnormal expression. Knock-out studies of individual MMPs show no lethal mutants, which was attributed to the redundancy of MMPs and their broad spectrum of substrates (Shapiro, 1998). Overexpression of MMPs, such as MMP-2 and MMP-9, has been shown to contribute to the degradation of myelin and the BBB in MS, cancer metastasis, and other diseases (Beliveau et al., 1999; Wang et al., 2000; Yong et al., 1998). On the other hand, reduced expression and activation of MMPs has been linked with several brain pathologies especially Alzheimer's disease (Asahina et al., 2001; Yong et al., 2001). Evidence from a

variety of experimental approaches suggests that pathologies arise from the disruption of more than one MMP, particularly MMPs like MMP-2 and MMP-9 that degrade common substrates. Inhibition of gelatinases has been shown to limit the extension of retinal growth cones by stopping the formation of filopodial processes (Sheffield et al., 1994). Outside the nervous system, the inhibition of gelatinases has been shown to reduce the migration of Langerhans cells, skin dendritic cells, and vascular smooth muscle cells (Pross et al., 2002). Furthermore, synthetic inhibitors of gelatinases have been successfully used to limit the metastasis of tumors in animal models, and are potential candidates for treating invasive tumors in humans (Brown et al., 2000; Marder and Greenwald, 2003; Rooprai and McCormick, 1997).

The defects we observed in cerebellar slices after inhibitor exposure are probably due to the combined inhibition of MMP-2 and MMP-9. MMP-9 knock-out mice do not exhibit significant abnormalities in the development of the cerebellum (Shapiro, 1997). Gelatinases are similar to other MMPs in that they are zinc-dependent enzymes. Abnormalities in the development of the cerebellar cortex have been described in zinc-deficient mice (Dvergsten et al., 1983). The damage to the cerebellar cortex, especially in the number of granule neurons and the thickness of EGL, was more severe in zinc-deficient pups than in undernourished pair-fed pups. Toxic compounds are also known to damage the cerebellum during development (Antonio et al., 2002; Nielsen et al., 2003; Stoltenburg-Diding et al., 1990). In rodents, *in vitro* and *in vivo* exposure of the cerebellum to methylmercury, an animal model for Minamata disease, causes a severe decrease in the number of migrating granule neurons as seen in our study (Howard and Mottet, 1986; Kunitomo and Suzuki, 1997; Sager et al., 1982). Mercuric compounds

modulate the activity of MMPs, especially MMP-2 and MMP-9. Some organic mercurial compounds are known to induce gelatinase activity, whereas mercuric salts suppress the activity of gelatinases (de Souza et al., 2000; Sopata and Wize, 1979). The damage caused by a zinc-deficiency and exposure to methylmercury involves a myriad of molecules, most of which are unknown. The damage, nevertheless, could possibly be due, at least in part, to disruption of the activity of MMPs.

Several additional aspects of these results need to be addressed, especially the role of gelatinases in the extension of parallel fibers. MMP-9 contributes to process extension of oligodendrocytes and to neurite outgrowth in the retina (Letourneau et al., 1992; Uhm et al., 1998; Ferguson and Muir, 2000; Buttery and French-Constant, 2001). We did not observe immunoreactivity for MMP-9 in association with parallel fibers. This might be due to our use of the parasagittal plane in all sections. As a result, our slices were all perpendicular to the direction of parallel fibers. The proposition that gelatinases contribute to the extension of parallel fibers might be addressed experimentally by performing immunohistochemistry for gelatinases on frontal sections. Furthermore, depositing small particles of DiI on the surface of the EGL, followed by immunohistochemistry for gelatinases in the frontal plane, would permit the validation of this proposition. We also observed MMP-2 and MMP-9 expression in the EGL and treatment with the MMP-2/MMP-9 inhibitor caused an increase in the thickness of the EGL. The increase in thickness could be due to an increase in proliferation, a decrease in migration, a decrease in cell death, or a combined effect. These possibilities are addressable by a variety of available techniques including the determination of the growth fraction (population of actively cycling cells) by BrdU labeling of proliferating

cells and determining the percentage of newborn BrdU-positive neurons that migrate after treatment with the gelatinase inhibitor. Furthermore, immunostaining of migrating granule neurons with doublecortin will reduce the errors associated with the morphometric analysis. Determining the amount of cell death by TUNEL staining will help us assess the possible neurotoxic effect of blocking gelatinase activity on the cerebellar cortex. Furthermore, MMP-2 activity and expression coincided with EGL and fusiform-shaped cells, resembling migrating granule neurons, in the ML (Figure 3.5). Whether MMP-2 contributes to the migration of progenitor neurons, between E14 and E17, from the rhombic lip to the EGL is yet to be determined.

Matrix metalloproteinases, especially MMP-2 and MMP-9, have emerged as key contributors to several homeostatic mechanisms, including process extension and cell migration. These processes are essential to the formation of the cerebellar cortex and the normal development of the nervous system. More research is needed to clarify how these matrix-degrading enzymes are controlled and coordinated to serve such complex situations. The investigation of the contribution of gelatinases to earlier events in cerebellar corticogenesis are also important, especially the migration of progenitor cells from the rhombic lip to populate the EGL, and the migration of immature Purkinje neurons and deep cerebellar neurons from the ventricular zone.

Figures

A

B

Figure 3.1 Real-time quantitative RT-PCR. A) The relative level of MMP-2 and MMP-9 mRNA in the cerebellar cortex (top graph) gradually decreased from P3 to P25. B) The expression pattern was different for MMP-9 but not for MMP-2 in the cerebral cortex (see text for details; Bars: $\pm$ SD).

Figure 3.2 Gelatinase activity in the cerebellar cortex between P3 and P25. The cerebellum was dissected from rat pups at P3 until P25 and proteins were extracted from the tissue. Gelatin zymography showed digested bands of (A) pro-MMP-2 (72kDa) and MMP-2 (68 kDa); (B) pro-MMP-9 (92 kDa) and MMP-9 (82 kDa). We also performed a reverse zymographic assay to determine the presence of the gelatinase inhibitors TIMP-1 (28kDa) and TIMP-2 (21kDa) in tissue extracts. Only TIMP-2 activity was present between P3 and P25 (C). As a loading control, we cut the top 2 cm of every gel following the electrophoresis and stain it with Coomassie blue stain for 5 min, which revealed bands of loaded proteins (D). We also performed gelatinase assay on cerebellar granule neurons (E) and mixed glial cells cultured from P4 cerebella (F). Gels were quantified with a densitometric analysis. (G) Shows densitometry for MMP-2, (H) shows densitometry for MMP-9, and (I) shows densitometry for TIMP-2. Experiments were repeated 3 times and gels were repeated 5 times (densitometry in arbitrary units; bars: $\pm$ SEM).

G

H

I

Figure 3.3 *In situ* Zymography in the developing cerebellum. Frozen tissue sections were incubated with Gelatin-FITC as a substrate (see methods). The digested gelatin fluoresces on tissue sections. (A) Gelatinase activity was present in the EGL and the Purkinje cell layer from P3 to P12. The activity increased in the IGL between P6 and P12. By P15 the activity decreased significantly in the cerebellum but was still detectable in the hippocampal formation in the same sections (scale: 800 μ m). Inset shows gelatinase activity in the EGL, the Purkinje cell layer, and the IGL. Cells scattered in the WM also showed gelatinase activity, but the identity of these cells is not known (scale: 250 μ m). Panel (B) shows serial sections treated with 20 mM EDTA and 4% formaldehyde, which abolish gelatinase activity, and an adjacent untreated section processed for *in situ* zymography. Reaction product was detectable only in the untreated section. Pictures were taken with the same optical settings.

P6 (FITC-Gelatin ®) **P6 (20mM EDTA / FITC-Gelatin ®)** **P6 (4% Formaldehyde/ FITC-Gelatin ®)**

Figure 3.4 Immunohistochemical expression of MMP-2 and MMP-9. Panels showing representative sections from P6, P9, P12, P15, and P21 stained using anti-MMP-2, anti-MMP-9, GFAP, and Calbindin D28k. All antibodies were detected with FITC-tagged secondary antibody (in green). In red is the nucleic-acid counterstain TOTO-3. (see results section for details). (EGL: external granule layer; ML: molecular layer; IGL: internal granule layer; PCL Purkinje cell layer; and WM: white matter; scale: 50 μ m).

Figure 3.5 Detailed view of MMP-2 IHC. Sample images from P6 and P9 stained for MMP-2 (scale: 50 μm). Insets show from P6 and P9-MMP-2 at higher magnification in combined 2 channel images (FITC-TOTO3), and single channel images (Green = FITC). White arrows point to Bergmann glial fibers (see results section for details; scale: 20 μm ; EGL: external granule layer, PC: Purkinje neuron).

Figure 3.6 Detailed view of MMP-9 IHC. Sample images from P9 and P12 stained for MMP-9; scale: 50 μm). Insets show from P9 and P12-MMP-9 at higher magnification in combined 2 channel images (FITC-TOTO3), and single channel images (Green = FITC). White arrows point to Purkinje dendrites (see results section for details; scale: 20 μm ; EGL: external granule layer, PC: Purkinje neuron).

Figure 3.7 Control sections for MMP-9 IHC from P15 rat cerebellum. To determine the specificity of the antibodies used, serial sections were stained with the primary antibody pre-absorbed with an antibody-specific blocking peptide (see methods). Sample sections were incubated with the anti-MMP-9 alone (0:1) or with increasing dilution of the anti-MMP-9 blocking peptide (1:10, 1:20, and 1:50). As the concentration of the blocking peptide was reduced, the immunoreactivity of MMP-9 increased. Similar controls were done for MMP-2 (not shown; Mag bar: 100 μ m).

Figure 3.8 Expression and activity of MMP-2 and MMP-9 in organotypic slice cultures. Sample sections from a control slice at 7 DIV stained with anti-MMP-9 (A), anti-MMP-2 (B), anti-Calbindin D28k (C), and anti-GFAP (D) then counterstained with the TOTO-3 (red; scale: 50 μ m). MMP-2 and MMP-9 immunoreactivity in organotypic slices was similar to that observed *in vivo*. We performed *in situ* zymography on organotypic slices treated with a selective MMP2/MMP9 inhibitor. (E) Control section cultured for 7DIV without inhibitors. (F) Sample section from an adjacent slice to (E) and treated with 3.2 μ M inhibitor for 7DIV shows reduced and aberrant gelatinase activity (scale: 1 mm).

Figure 3.9 The effect of inhibitor treatment on the gelatinase activity of MMP-2 and MMP9 *in vitro*. Slices in culture were exposed to SB-3CT for 24hrs, and the conditioned media was collected and assayed for MMP activity. (A) Shows the gelatinase activity in control slices. (B) Shows the gelatinase activity in treated slices from 1DIV, 4DIV, 7DIV and 10DIV. (C-G) show densitometric analysis for zymographic gels for 1DIV, 4DIV, 7DIV, and 10DIV (densitometry in arbitrary units; bars: $\pm$ SEM)

A

B

C

D

E

F

G

Figure 3.10 The effect of treatment with MMP-2/MMP-9 inhibitor on cerebellar corticogenesis. (A) Treatment with inhibitor caused an initial increase (1DIV and 4DIV), followed by a reduction in the thickness of the EGL. (B) The inhibitor reduced the number of migrating cells in the molecular layer in a dose-dependent manner. (n=3; p values denote statistical difference from control samples).

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Appendix 1: Detailed experimental protocols

A1. Animal surgeries

1. Prepare, clean and sterilize surgery tools.
2. Sterilize work area with 5% Chlorox, then with 75% EtOH
3. Anesthetize pups on ice (P3 and P6) or with 120 mg/kg injection of pentobarbital (P9 and older)
4. Decapitate the pup and gently make a midline incision and deflect the scalp. Do not cut too deep, this will damage underlying neural tissue. Also do not apply pressure because it will cause the soft brain tissue to ooze out of the foramen magnum.
5. Gently score the cranium with a scalpel and remove it with a pair of small forceps.
6. Do the following step over a dish full of cold PBS. Turn the head upside down and sever the cranial nerves. The brain will drop into the PBS.
- N.B: For biochemical processing experiments, use ddH₂O instead of PBS. Separate cerebellum from cerebrum and freeze tissue liquid nitrogen. Label tube (Cbl = cerebellum; Ctx = cerebral cortex; plus age) and store at -80°C.
7. Very carefully remove remaining meninges and place the brain in fixative.
- N.B: For *in situ* zymography experiments, **do not use fixative**.
8. Cut a small cork and place it in a small embedding cube. Label the cube (IHC Px = immunohistochemistry + postnatal day; ISZ Px: *in situ* zymography + postnatal day). Cover the cork with tissue freezing compound

(O.C.T.), place brain on its side and cover entirely with O.C.T. Place the cube in powdered dry ice and cover with a thin layer of dry ice.

9. Store cubes in -80°C freezer until sectioning.
10. Important: use superfrost plus (Fisher 12-550-15) glass slides or coated (2%saline or gelatin) slides. Tissue will detach easily from regular slides.

A2. Immunohistochemistry

1. Store cryostat sections, 12 µm thick, in plastic slide boxes wrapped with plastic wrap at -20°C.
2. Slides are inserted in plastic slide container (4 each; leave one slot empty for negative control) and placed on shaker for all subsequent steps. Need to prepare 12 ml of solutions to submerge the sections. Never let slides dry out between steps.
3. Rinse slides in 25% ethanol in ddH₂O for 15 min to remove all O.C.T. compound residues.
4. Rinse slides in PBS-TX (0.1M PBS + 0.1% Triton-X 100) for 30 min.
5. Incubate slides in primary Ab solution for 24 hrs at 4°C on shaker. Dissolve goat-anti-MMP-9 and goat-anti-MMP-2 in PBS (1:1000). Dissolve mouse-anti-Calbindin D28k (1:3000) and mouse-anti-GFAP (1:1000) in PBS/ PBS-TX (1:1).
6. Transfer antibody solutions individually into 50 ml conical tubes, label the tubes, and store them at 4°C. Antibody solutions are good for a maximum of

1 month and can be reused 2 to 3 times.

7. Rinse slides 3 times in PBS for a total of 30 min. During this time prepare secondary antibodies. For MMP-2 and MMP-9, dissolve anti-goat-FITC (1:300) and TOTO-3 (1:500) in PBS. For Calbindin D28k and GFAP, dissolve anti-mouse-FITC (1:300) and TOTO-3 (1:500) in PBS. Protect working solutions of secondary antibody from light by wrapping the tube with aluminum foil. Working solutions of secondary antibody can be reused 2-3 times if stored at 4°C. Remember to rinse the Control section in 25% ethanol for 15 min, followed by 30 min in PBS. Add control section to slide container and incubate all slides with secondary antibody
8. Incubate slides in secondary antibody solution for 2 hrs at room temperature. Wrap the slide container with aluminum foil to prevent bleaching of secondary antibodies.
9. Rinse slides 3 times in PBS for a total of 30 minutes.
10. Gently blot excess fluid from around the sections (do not let them dry), add 2-3 drops of mounting media (PBS-Glycerol 1:1 or Vectashield) and coverslip. Blot excess fluid from around the coverslips and seal the edges with a coat of clear and fast-drying nail polish.
11. Store slides in a slide box at 4°C.

A3. In situ zymography

1. Brains are dissected, embedded in O.C.T. compound and frozen without

fixation

2. Section brains and store sections in slide box at -20°C
3. Prepare reaction buffer: 0.05 M Tris-HCl, 0.15 M NaCl, 5 mM CaCl₂, 0.2 mM NaN₃ (pH 7.6). Dissolve DQ™ gelatin-FITC (40 µg/ml; D-12054; Molecular Probes Inc., Eugene, OR) in reaction buffer in 50 ml conical tube. Place tube in warm water bath and shake for 30 sec or until the gelatin dissolves completely.
4. Add staining solution to slide container and wrap it with aluminum foil. Place slide container on a shaker at 37°C for 24 hours.
5. Retrieve slides and blot excess fluid from around the sections. Don't let the slides dry out. Add few drops of wet mounting media over the sections and coverslip the slides. Blot excess fluid from around the coverslips and seal the edges with fast-drying nail polish.
6. Store slides at 4°C.

A4. Cerebellar cell culture

Cerebellar granule neuron cell culture protocol:

1. Dissect the cerebellum out and put the fresh tissue in a small tube of 1H solution
2. When finished, finely chop all tissue with a razor blade on a glass slide
3. Move chopped tissue to a 50ml conical tube and add solution A. lock tube well, shake several times, then incubate/shake at 37°C for 15 min.

4. Then, add solution C directly to the tube, shake and centrifuge for 15 sec at 500 rpm
5. Remove supernatant, add 5 ml solution B, swirl and mix well and let sit for 5 min.
6. Transfer supernatant to a 15ml tube; gently add solution D to about 12 ml
7. Then spin down for 3 min at 2000 rpm
8. Remove supernatant, keep pellet and add 5 ml of culture media and divide over dishes
9. For glial culture, transfer supernatant to a flat culture bottle and add 10 ml DMEM and incubate. Change media every 3 days

Stock solutions:

1. trypsin: 25 mg/ml (T8003, sigma)
2. trypsin inhibitor: 4 mg/ml soybean trypsin inhibitor (T9003, sigma)
3. DNase: 200 U/ml DNase I (D-4527, sigma)
4. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 3.82% (w/v)

N.B: prepare all solutions in ddH₂O

1H buffer (50 ml): 50ml EBSS + 0.125 g Glucose + 0.15 g BSA + 0.5 ml MgSO_4 stock

A buffer (10 ml): 0.1 ml trypsin stock + 9.9 ml 1H buffer

B buffer (10 ml): 8.6 ml 1H + 1 ml trypsin inhibitor Stock + 0.2 ml DNase stock + 0.2 ml MgSO_4 stock

C buffer (10 ml): 8.4 ml 1H + 1.6 ml B buffer

D buffer (10 ml): 9.8 ml 1H + 400 mg BSA + 0.2 ml MgSO_4 stock

Culture media stock solutions:

1. KCl 2M: 7.2g + 50 ml ddH₂O
2. Glutamine 0.2M: 2.92 g + 100 ml ddH₂O
3. Glucose 1.33M (24%): 12 g + 50 ml
4. Poly-D-Lysine 50 µg/ml in ddH₂O (for coating slides and cover slips)

For 100 ml medium: 1ml KCl + 1ml Glutamine stock + 2.5ml Glucose stock + 10ml FBS
+ 1ml Pen/Strep (0.12ml gentamicin) + 85ml EMEM

A5. Protein extraction

(Monolayer cell culture)

1. Remove culture medium with care. (Some cells easily detach from plate)
2. Add 0.5 ml (the amount of buffer added may be increased if the cell culture is too dense) of RIPA buffer with freshly added inhibitors to cell culture plate.
3. Scrap with a cell scraper and 1 ml pipetter up and down several times to shear membranes. Incubate on ice for 10 min.
4. Spin down at 10,000g for 10 min at 4°C.
5. Transfer the supernatant fluid, which is the total cell lysate, to clean eppendorf tubes and freeze at -20°C or run lowly assay to check protein concentration.

RIPA buffer:

1 x PBS

1% P-40

0.5% sodium deoxycholate

0.1% SDS

RIPA buffer with inhibitors (make fresh):

10 mg/ml PMSF at 10ml/ml RIPA

Aprotinin (Sigma #A6279 as liquid) at 30 µl/ml RIPA

100mM sodium orthovanadate at 10ml/ml RIPA

Lowly assay (for microplate assay) 40:

1. Make A': Add 20 ml of A to 1 ml of S.
2. Prepare 5 dilution of protein standard (BSA) from 0.0875, 0.175, 0.35, 0.7, 1.4 mg/ml. Standard curve should be prepared each time the assay is performed.

N.B: prepare all samples and standards in triplicates

3. Add 5 µl of standard and samples into a clean microtiter plate.
4. Add 25 µl of A' into each well.
5. Add 200 µl of B into each well; gently agitate the plate to mix the reagents.
(Avoid air bubbles)
6. Incubate at room temperature for 15 min and read absorbance at 750nm
(program 33)
7. Calculate average protein concentration in µg/µl.
8. Maximum volume to load in a gel with 0.75 mm wells is 25 µl and 60 µl in a gel with 1.5 mm wells.

9. Prepare aliquots with 16 μ l (protein + RIPA buffer) + 5 μ l sample buffer minutes before loading in the gel

Tissue	Age	Ave. Conc. (μ g/ μ l)	Protein (μ g)	Protein (μ l)	RIPA buffer (μ l)
Cerebellar Cortex	P3	34.13	853.25	5.9	10.1
	P6	30.82	770.5	6.5	9.5
	P9	45.57	1139.25	4.4	11.6
	P12	49.46	1236.5	4	12
	P15	36.46	911.5	5.5	10.5
	P18	40.37	1009.25	4.9	11.1
	P21	44.6	1115	4.5	11.5
	P25	34.13	853.25	5.9	10.1
	P30	35.62	915.5	5.5	10.5
Cerebral Cortex	P3	25.89	647.25	7.7	8.3
	P6	29.38	734.5	6.8	9.2
	P9	29.27	731.75	6.8	9.2
	P12	33.64	841	6	10
	P15	37.47	936.75	5.3	10.7
	P18	36.74	918.5	5.4	10.6
	P21	21.7	542.5	9.2	6.8
	P25	19.38	484.5	10.3	5.7
	P30	37.78	944.5	5.3	10.7

Table 1. Protein concentration for gelatinase assay. Example from Tuesday, Nov. 27 2001

A6. Gelatinase assay

Note: Zymographic analysis = Gelatinase assay

Gelatinolytic activity = Gelatin degrading activity

10% Separating Gel: 15ml

Dissolve 15mg gelatin in 7.5ml ddH₂O by heating in stir plate.

Add 3.75 ml 4X separating buffer after cooling to RT

Add 3.75 ml 40% 37.5:1 acrylamine/Bisacrylamine

Add 100 µl 10% APS, mix well

Add 20 µl TEMED

Stacking Gel:

37.5:1 Acrylamine/Bisacrylamine	1 ml
4X stacking buffer	2.5 ml
ddH ₂ O	6.5 ml
10% APS	50 µl
TEMED	10 µl

Samples preparation and electrophoresis:

1. Prepare samples in 4X sample buffer (non-reducing, 40mM Tris-HCL pH 8, 80% Glycerin, 8% SDS, 0.4% Bromophenol blue). Do not heat samples before loading.
2. Fill the container with Protein Running Buffer. Load samples in the electrophoretic gel. Max load: 25 µl in 0.75 mm plate.
3. Attach the gel container to the power supply and adjust the current intensity to 0.32 mA. Let the gel run for 2 to 3 hours (until the blue dye starts to run down).
4. Wash gel in 2.5% Triton-100 for 30 min
5. Switch to 50mM Tris-HCL pH 7.4, 0.1M NaCl, 2.5% Triton-100 for additional 60 min
6. Incubate in reaction buffer (50mM Tris-HCL pH 7.4, 10mM CaCl₂) overnight at 37°C.
7. Stain the gel with 0.5% coomassie blueR in 45%MeOH, 10% acetic acid for

30 min at room temperature on shaker.

8. De-stain the gel in destaining solution (10% MeOH, 10% acetic acid)
9. Take picture of gel on the Eaglesight® workstation.

A7. Reverse zymography assay

Gel preparation:

Separating Gel (12%):

1. Dissolve 15 mg gelatin in 6.75 ml ddH₂O. Put tube in hot water bath until gelatin dissolves.
2. 4.5 ml of 40% 37.5:1 Acrylamine/Bisacrylamine
3. 3.75 ml 4X Separating Buffer
4. 15 µl of 0.2 µg/ml pro-MMP2 (Calbiochem 444213)
5. 75 µl 10% APS
6. 15 µl TEMED

Overlay gel with ddH₂O

Stacking Gel:

1. 1 ml of 40% 37.5:1 Acrylamine/Bisacrylamine
2. 2.5 ml Stacking Buffer
3. 6.5 ml ddH₂O
4. 50 µl 10% APS
5. 10 µl TEMED

Let the gel run for 2 hours

N.B: sample preparation, incubation time and staining are the same as in Gelatinase Assay

A8. Organotypic slice culture

Solutions:

1. Culture media: 46% Eagle's basal medium + 25% Earl's balanced salt solution + 25% horse serum + 1% penicillin-streptomycin-glutamine (penicillin 10^5 units/ml; streptomycin 10 mg/ml; 29.2 mg.ml⁻¹) + 6.5 mg/ml glucose. Adjust pH to 7.2. Store culture media in an autoclaved bottle at 4°C until use.
2. ACSF: 126.0 mM NaCl + 3.0 mM KCl + 1.3 mM MgSO₄ + 2.5 mM CaCl₂ + 1.2 mM NaH₂PO₄ + 26 mM NaHCO₃ + 20 mM dextrose. Keep ACSF at 4°C. Bubble ACSF in O₂:CO₂ gas mixture (95%:5%) for 15 min before use.
3. Autoclave all paper towels, surgery tools and glassware
4. Perform all steps under sterile conditions in a laminar flow hood. Clean space with a mixture of 25% ethanol and 2% chlorox before use.

Procedure:

5. Prepare six plates. Put 1 ml of culture medium in the well. Place Millicell membrane-inserts (Cat# PICMORG50, Millipore, Bedford, MA) over the culture media and try to avoid air bubbles.
6. Anesthetize rat pups (P5) by cooling on ice. Rub head with 75% ethanol.
7. Decapitate pups and quickly dissect the hind brain including, the cerebellum, out.

7. Place the cerebellum in a Petri dish filled with ice cold ACSF.
8. Under magnifying glass, make a cut behind the inferior colliculus and gently separate the cerebellum from the pons. Make a sagittal cut 2 mm lateral to the vermis.
9. Glue flat surface down on the stage of vibraslicer (WPI, Sarasota, FL), and immerse in ACSF.
10. Section the cerebellum at 500 μm thickness. Collect slices from the vermal region and few millimeters around it.
11. Gently transfer slices to the millicel membrane. Arrange only 2 slices over every membrane and place 20 μl of culture media on top of the slices. Use only 4 wells from the six-well culture plate. One well is for control slices, while the other three are for treatment groups.
12. Incubate culture plates at 35°C with 5% CO_2 atmosphere.
13. Replace culture media every 2 days. Warm the culture media to 35°C before adding to the culture plate.

A9. MMP inhibitor treatment

1. Dilute the gelatinase inhibitor (SB-3CT) in culture media to a final concentration of 1.6 μM , 3.2 μM , and 6.4 μM . Prepare inhibitor solution from aliquots 30 min before treatment and allow the solution to warm up to 37°C.
2. Start treat slices with the inhibitor 6 hrs after the beginning of the slice culture.

3. For 1DIV, harvest the slices 24 hrs after the initial treatment. Place metal block in -80°C freezer the night before. Place glass slides on the metal block and place few drops of O.C.T. compound on them. Sandwich the O.C.T. compound between two cold glass slides and let it freeze (Figure 1, a-c). The O.C.T. compound should freeze flat like a coin. Detach the glass slides from the frozen O.C.T. coin by rubbing the surface lightly with your finger. Place the frozen O.C.T. coin flat on the metal block. Cover a tissue slice with O.C.T. compound and gently place it on the frozen O.C.T. coin using a paint brush (Figure 1d). Cover the slice with powdered dry ice and let it freeze (Figure 1e).

Figure 1. Freezing slices for IHC and ISZ. Refer to text for details.

4. Pipette out the culture media from the culture plate and place in pre-labeled eppendorf tubes. Store tubes at -20°C.

5. For the remaining treatment groups, replace the culture media with the inhibitor every two days or 24 hrs before collecting the slices, and repeat the procedure in (3).

A10. Morphometric analysis

1. Sampling frames were chosen by systematic random sampling. All frames are of equal size using a 20x objective. Fame size= 325.7 x 325.7
2. Using the LSM510 software, choose “Overlay” from the menue and pick the following: line thickness, color (white), ruler, and curved line.

Figure 2. Morphometric analysis in cerebellar sections. Sample frame from 4DIV 1.6 μ M section. We measured the thickness of the EGL and the number of migrating cells in the ML. (*: migrating cells)

3. Draw 3 lines: line 1 borders the EGL-pia, line 2 borders the EGL-ML, and line 3 through the nuclei of Purkinje cell (Figure 2).
4. Draw 2 circles between line 1 and line 2. The circles have to touch the lines at 2 points, one point on each line. Record the area (A) of each circle
5. To determine the distance (D) between the lines, apply the following formula in Excel: $D = 2 * \text{SQRT}(A / \text{Pi} ())$
6. Count the cells between line 2 and 3. Choose only clearly visible cells with ovoid nuclei, not exceeding 8 μm in diameter

Curriculum vitae

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Present Position and Address

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Biography

Date of Birth	May 7, 1973
Citizenship	Lebanon

Education

2003	Ph.D., Neurobiology and Anatomy Department of Neurobiology and Anatomy WVU School of Medicine, Morgantown WV 26506
1998 (Dec.)	B.A. Biology (minor in Physics). <i>Summa cum laude</i> Department of Biology West Virginia University, Morgantown WV 26506

Honors and Awards

2002	Doctoral student travel fellowship (award: \$ 1,400)
2000	Doctoral student travel fellowship (award: \$ 1,200)
1999-2000	Swiger doctoral student fellowship (award: \$ 1,000/yr)
1998	Summer research fellowship (award: \$ 2,200)
1998	Graduated with High Honors, WVU Eberly College of Arts and Science
1998	Dean's Award for Scholarly Achievements, Eberly College of Arts and Sciences

Professional Membership

1999-	American Association for the Advancement of Science (AAAS)
2000-	Society for Neuroscience
2000-	American Association of Anatomists
2000-	FASEB
2001-	Bioinformatics.Org

Academic and University Committees

2003	Admissions committee for the Biomedical Science Graduate Training Program
2001- 2003	Graduate student representative, Department of Neurobiology and Anatomy
2001-	Student Leadership Committee, WVU School of Medicine

Teaching Experience

Spring 2003	Medical Neurobiology (CCMD 775)
Spring 2002	Medical Neurobiology (CCMD 775)
Spring 2002	Continuing Education for Psychiatry Residents at Chestnut Ridge Hospital (Internal structures of the CNS)
Spring 2001	Medical Neurobiology (CCMD 775)
Spring 2000	Medical Microanatomy (ANAT 723)

Seminar Presentations

2003	The matrix metalloproteinases 2 and 9 are expressed in the developing cerebellar cortex and are disrupted by ethanol exposure, Van Liere Research Symposium.
2003	Contribution of MMP-2 and MMP-9 to the postnatal development of the rat cerebellar cortex, Dept. of Neurobiology at Yale University School of Medicine.
2002	Contribution of MMP-2 and MMP-9 to the development of the cerebellar cortex and the effect of ethanol exposure, Dept. of Neurobiology and Anatomy.
2001	Structural Plasticity of Microglia in the hypothalamic SON, Dept. of Neurobiology and Anatomy.
2000	Microglial plasticity in the Supraoptic Nucleus, Van Liere Research Symposium.
1999	Neurobiology of Neurotrophins (A Review), Dept. of Neurobiology and Anatomy.

Research Activities

2001-2003	Contribution of MMPs to the development of the cerebellar granule neurons and the effect of ethanol exposure. Advisor: J. Luo, Ph.D.
2001	Developmental neurobiology of oligodendrocyte progenitors. Research Rotation; Laboratory of R. C. Wiggins, Ph.D.
2000	Plasticity of Microglia in the hypothalamic SON Research Rotation; Laboratory of A. K. Salm, Ph.D.
1999	Molecular aspects of ROS-induced oligodendrocyte injury. Research Rotation; Laboratory of G. W. Konat, Ph.D./L.M.
1999	Structural organization of auditory brain stem (SOC) circuitry. Research Rotation; Laboratory of A. S. Berrebi, Ph.D.
1998	Glial plasticity in the hypothalamic SON Summer Research Fellowship; Laboratory of A. K. Salm, Ph.D.
1998	Feeding competition and dominance in the shrimp <i>Palaemonetes vulagris</i> . Co-investigator (33 % effort). Undergraduate Research Honors Project; Department of Biology.

Publications

Ayoub A. E. and Luo J. (2003) On the Contribution of MMP-2 and MMP-9 to the Postnatal Cerebellar Corticogenesis (in preparation)

Ayoub A. E. and Salm A. K. (2003) Increased morphological diversity of microglia in the activated hypothalamic supraoptic nucleus. *J Neurosci* 23(21):7759-66

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