THE EXPRESSION OF NEURONAL NITRIC OXIDE SYNTHASE DURING POSTNATAL DEVELOPMENT IN THE LEANER AND TOTTERING MOUSE CEREBELLA

A Senior Honors Thesis

by

DANIEL RICHARD ZEVE

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
in partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

April 2003

Group: Life Sciences II

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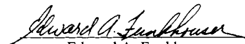
Approved as to style and content by:

Louise C. Abbott
(Fellows Advisor)



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April 2003

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ABSTRACT

The expression of neuronal nitric oxide synthase during

postnatal development in the leaner and tottering

mouse cerebella. (April 2003)

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Tottering and leaner mice carry different mutations in the portion of their genetic information (tottering locus) that encodes for the α_{1A} subunit of P/Q-type voltage-gated calcium ion channels. These channels, located on the cell membrane, allow calcium ions in and out of the cell. The mutations in both mice lead to the production of abnormal calcium channels (mostly in the cerebellum), and these defective channels cause decreased entry of calcium ions into the cell. The tottering mutation is the less severe of the two, with leaner mice exhibiting a more severe cerebellar dysfunction as well as neuronal cell death. In several areas of the central nervous system, neuronal development, synaptogenesis, and neuronal death are thought to be directed in some part by nitric oxide (NO), which is regulated through control of its production by neuronal nitric oxide synthase (neuronal NOS). The expression of neuronal NOS/NO is regulated by calcium ion signaling and it has been shown that abnormal calcium ion flux in adult tottering and leaner mice affects neuronal NOS expression in these mice. We analyzed

the expression of neuronal NOS in wild-type, tottering and leaner mice during postnatal cerebellar development. Using western blotting, NADPH diaphorase histochemistry, an indirect marker for neuronal NOS, we confirmed previous reports of transient expression of neuronal NOS in wild-type cerebellar Purkinje cells at postnatal day (P) 8 and basket cells at P12. We also observed an increase of neuronal NOS expression at P12 in tottering and leaner cerebella compared to the wild-type cerebellum, as well as an increase in neuronal NOS expression at P20 in tottering cerebellum compared to wild-type. This suggests that NO may be involved in early postnatal cerebellar development, and that tottering and leaner postnatal cerebellar development are altered compared to wild type mice. This work was supported in part by CERH NIEHS grant P30-EF09106

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INTRODUCTION

The cerebellum is an integral part of the central nervous system through its control of coordination, balance and fine motor control. Morphologically, the cerebellum is divided into three major parts: left and right hemispheres and the medially-located vermis (Voogd and Glickstein, 1998). The paravermis is a narrow region between the vermis and each cerebellar hemisphere. The cerebellum is connected to the brain stem via left and right peduncles. Peduncles contain efferent and afferent axons to and from the cerebellum (Martin, 1996). The superior cerebellar peduncle carries efferent axons, the middle cerebellar peduncle contains afferent axons, and the inferior cerebellar peduncle contains both types of axons. The vermis does not have peduncles (Martin, 1996).

The cerebellar cortex is divided into three lobes and these lobes are organized into groups known as lobules, which are most easily seen in the vermis (Fig. 1). The rostral lobe of the cerebellum is made up of five lobules: lingual (I & II), central (III) and culmen (IV & V) lobes. The caudal lobe of the cerebellum consists of the declive (VI), follium (VII), tuber (VIII), pyramis (IX) and uvula (X) lobules (Voogd and Glickstein, 1998). The rostral and caudal lobes are both involved in preparation, implementation and control of limb and trunk movement. The third lobe, the flocculonodular lobe, consists of the nodulus and the flocculus, which both play roles in balance and eye movement (Martin, 1996). The deep cerebellar nuclei, which consist

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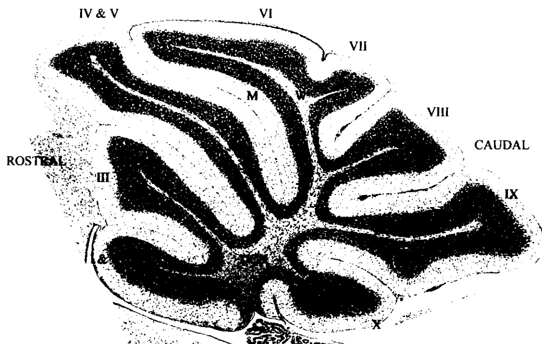

Figure 1. Representative Image of an Adult Mouse Cerebellum
A representative image of a hematoxylin and eosin stained adult cerebellum. M indicates molecular layer, IGC indicates internal granule cell layer, W indicates white matter, and DCN indicates deep cerebellar nuclei. The Purkinje cell layer is located between the molecular layer and the internal granule cell layer.

of fastigial, globose, emboliform and dentate nuclei, are found towards the center of the cerebellum (within the white matter), deep to the cerebellar cortex, and act as cerebellar conduits of information leaving the cerebellum (Martin, 1996).

Functionally, the cerebellum is divided into three regions: the vestibulocerebellum, the spinocerebellum and the cerebrocerebellum (Martin, 1996). The vestibulocerebellum, which corresponds to the flocculonodular lobe of the

cerebellum, receives information from vestibular nuclei and primary vestibular afferents and sends efferent information back to the vestibular nuclei through the inferior cerebellar peduncle (Martin, 1996). The vestibulocerebellum is involved in maintaining balance and controlling eye and head movement (Voogd and Glickstein, 1998). The spinocerebellum, which corresponds to the vermis and the paravermal cerebellar cortex of the cerebellum, receives principal somatic input from the spinal cord. The vermis acts to control axial and trunk muscles, while the paravermal cerebellar cortex controls limb muscles (Martin, 1996). Efferent information from the spinocerebellum travels through two different pathways. The vermis sends information to the fastigial nuclei, which influence motor neurons via synapses with brain stem nuclei and medial descending pathways (reticulospinal and vestibulospinal tracts). The paravermal cerebellar cortex sends efferent information to the interposed nuclei (globose and emboliform nuclei), which synapse with the magnocellular portion of the red nucleus in the midbrain and portions of motor area of the frontal lobe and give rise to the lateral descending pathways, the rubrospinal and lateral corticospinal tracts (Martin, 1996). The cerebrocerebellum is involved in the preparation of movement and corresponds to the lateral hemispheres of the cerebellum (Martin, 1996). The lateral hemispheres receive their afferent information from the cerebral cortex, mainly the contralateral cerebral cortex. Efferent projections from the cerebrocerebellum synapse in the dentate nuclei, which send their axons to the ventral lateral nucleus and to the parvocellular portion of the red nucleus (Martin, 1996).

The histology of mouse cerebellar cortex shows four to five layers of cells within

each lobule, depending on the age of the mouse. Juvenile mice (postnatal day 0 to 14-16) exhibit five layers in cerebellar cortex, in order from superficial to deep: the external granule cell layer, molecular layer, Purkinje cell layer, internal granule cell layer, and white matter (Fig. 1) (Goldowitz and Hamre, 1998). The external granule cell layer is transitory in nature. It is in this layer that cells proliferate and start differentiating, to later reside in the molecular layer (basket and stellate cells) or the internal granule cell layer (granule cells) (Goldowitz and Hamre, 1998). Cells of the external granule cell layer migrate into the cerebellar cortex and as they migrate, they mature to become fully differentiated cells. Once the mouse is approximately postnatal day 20 (P20), the cerebellar cortex cellular layers are fully formed, and thus, the external granule cell layer is no longer present in the normal mouse cerebellum (Goldowitz and Hamre, 1998).

The internal granule cell layer, which is densely packed with granule cells and a small number of Golgi cells, is located between the Purkinje cell layer and the white matter. The dendrites of granule cells synapse with mossy fibers, which are the extracerebellar afferents of the spinal cord, medulla and pons (Altman and Bayer, 1997). Granule cell axons rise vertically past the Purkinje cell body layer into the molecular layer, where they bifurcate into parallel fibers (Voogd and Glickstein, 1998). These fibers form synapses mainly with Purkinje cell dendrites, but also terminate with dendrites of basket, stellate and Golgi cells (Altman and Bayer, 1997). Granule cells are classified as interneurons and, therefore, the excitatory effect of mossy fibers is felt indirectly by the cells that contact granule cell axon terminals (Voogd and Glickstein, 1998). Golgi cells, the other cell type found in the internal granule cell layer, have

dendrites that arborize in the molecular layer and synapse with parallel fibers. Golgi cell axons arborize within the internal granule cell layer and synapse with mossy fibers, providing an inhibitory influence (Voogd and Glickstein, 1998). Furthermore, unipolar brush cells also are present in the internal granule cell layer. These cells have short, stubby, paintbrush-like dendrite and an axon that terminates in areas related to mossy fiber endings (Mugnaini and Floris, 1994).

There are two main types of cells found in the cerebellar molecular layer, basket and stellate cells. Basket cells are located in the lower half of the molecular layer, near Purkinje cell somata. Basket cell dendrites synapse with parallel fibers in the molecular layer, while basket cells axons send out descending branches that wrap around and contact Purkinje cell bodies (Altman and Bayer, 1997). Excited by parallel fibers, basket cells act to inhibit the spontaneous discharge of Purkinje cells (Martin, 1996). Stellate cells are located in the upper half of the molecular layer and, similarly to basket cells, have dendrites that synapse with parallel fibers. Stellate cells also have an inhibitory effect on Purkinje cells, but, unlike basket cells, stellate cell dendrites synapse with Purkinje cell dendrites within the molecular layer (Voogd and Glickstein, 1998).

The Purkinje cell layer is a monolayer of cell bodies, situated between molecular and internal granule cell layers. Purkinje cells characteristically have very large and extensive dendritic trees that form synapses within the molecular layer, while the single axon of each Purkinje cell extends into the white matter and is the major efferent fiber of the cerebellar cortex (Martin, 1996). The Purkinje cell dendrites synapse with many extrinsic presynaptic fibers, including climbing fibers of the contralateral inferior olive

as well as adrenergic fibers of the brainstem (Altman and Bayer, 1997). Climbing fibers have an excitatory effect on Purkinje cells, causing increased firing of action potentials, while adrenergic fibers are believed to have a modulatory affect (Altman and Bayer, 1997). Purkinje cell axons, which are inhibitory, mainly terminate on deep cerebellar nuclei (the major output from the cerebellum to extracerebellar structures). Therefore, excitation of Purkinje cells either directly by climbing fibers or indirectly by mossy fibers produces inhibition of deep cerebellar neuron activity while inhibition of Purkinje cells via Golgi, basket and stellate cells results in increased (excitatory) output from the deep cerebellar neurons (Voogd and Glickstein, 1998). Bergmann glial cells are also present in the Purkinje cell layer. Their processes extend into the molecular layer and are developmentally important in guiding granule cells from their places of origin to their position within the cerebellum (Voogd and Glickstein, 1998).

Neurons are very dependent on the concentration of calcium ions (Ca^{2+}) within the cell, due to calcium's involvement in many physiological functions, including synaptic transmission, cell excitability, neurite growth and plasticity, as well as neuronal survival (Berridge 1998; Clapham 1995; Kano et al., 1992; Konnerth et al., 1992). Calcium ion channels are very important in controlling the influx of Ca^{2+} and therefore are very important in influencing the physiological functions of neurons. These channels are multimeric proteins, involving a pore-forming/voltage-sensing subunit, known as the α_1 subunit, as well as multiple regulatory subunits (α_2, β, γ, and δ). Currently, there are ten different α_1 subunit isoforms known, including S, A, B, C, D, E, F, G, H, and I (Catterall, 2000; Burgess and Noebels, 1999).

Even though there are multiple calcium ion channels (L, N, R, P, Q and T), the α_{1A} subunit has been shown mainly to be characteristic of P and Q type channels (Jun et al., 1999). While P and Q type channels are found in many areas of the brain, they are found in high concentrations within neurons of the cerebellum, with P-type channels making up 90% of cerebellar Purkinje cell calcium channels. Cerebellar granule cells have a calcium ion channel makeup that consists of 11% P-type channels and 35% Q-type channels (Dove et al., 1998; Randall and Tsien, 1995). Mutations in the pore-forming/voltage-sensing α_{1A} subunit of P and Q type channels can, therefore, cause many neurological disorders, especially cerebellar dysfunctions.

In humans, mutations of the α_{1A} calcium channel subunit have been shown to produce many neurological disorders, including familial hemiplegic migraine, episodic ataxia type-2 and autosomal dominant spinocerebellar ataxia (Ophoff et al., 1996; Yue et al., 1997; Zhuchenko et al., 1997; Kraus et al., 2000).

Both tottering (*tg/tg*) and leaner mouse genotypes (tg^{la}/tg^{la}) result from mutations in the tottering (*tg*) locus on chromosome 8 (Fletcher et al., 1996). This locus encodes the α_{1A} subunit of P/Q voltage-gated calcium ion (Ca^{2+}) channels (Fletcher et al., 1996). The tottering mouse mutation results in a non-conservative substitution from proline to leucine on the extracellular region linking the IIS5 and IIS6 regions of the α_{1A} subunit (Fletcher et al., 1996). The leaner mouse mutation results in a single nucleotide substitution at a splice donor region near the 3' end of the α_{1A} subunit (Burgess and Noebels, 1999). This substitution can lead to either a shortened protein due to skipping of the exon/intron or elongation of the protein due to inclusion of the intron into the α_{1A}

mRNA. In either case, the leaner mutation results in expression of an abnormal C-terminus on the α_{1A} subunit (Fletcher et al., 1996).

The mutations present in tottering and leaner mice lead to decreased Ca^{2+} influx through P/Q-type channels. It has been shown that tottering mice have a 40% reduction of calcium current through P/Q-type calcium channels of cerebellar Purkinje cells while leaner mice show a more severe reduction of 60% through P/Q-type calcium channels of the same cells (Dove et al., 1998; Lorenzon et al., 1998; Wakamori et al., 1998). This decrease in current is not due to quantitative changes in mRNA production or protein expression of the mutated α_{1A} subunit, but due to actual abnormalities in the calcium ion channels (Lau et al., 1998). Furthermore, it has been shown that cerebellar Purkinje cells of the leaner mouse have a significant decrease in rapid calcium ion buffering, which functions to immediately reduces the amount of free Ca^{2+} that has entered the cytoplasm (Dove et al., 2000). A reduction in rapid calcium ion buffering, which includes calcium buffering by the endoplasmic reticulum (ER) and calcium binding proteins, is believed to be a compensatory function that results from the already decreased Ca^{2+} influx across the Purkinje cell membrane (Dove et al., 2000; Nahm et al., 2002). The decrease in calcium ion current through P/Q-type channels leads to similar phenotypic traits in both tottering and leaner mice. Approximately 25-28 days after birth, tottering mice begin to display three characteristic neurologic disorders: 1) absence seizure activity similar to *petit mal* epilepsy; 2) ataxia and 3) paroxysmal dyskinesia or intermittent movement disorder (Austin et al., 1992). Leaner mice show similar but more severe symptoms as compared to tottering, with phenotypes appearing

12-13 days after birth (Rhyu et al., 1999). The leaner mutation also has a degenerative effect, with excessive loss of cerebellar granule cells, which peaks at postnatal day 20 (P20), and loss of selective cerebellar Purkinje cells beginning during P25-30 (Herrup and Wilczynski, 1982; Frank et al, 2003). This loss of cerebellar Purkinje cells continues until adult leaner cerebella contain 80% fewer Purkinje cells than wild-type and may, in part, be due to the decreased rapid calcium ion buffering (Dove et al., 2000).

Due to detrimental effects of abnormal calcium ion influx in neurons of the cerebellum, the tottering and leaner mutations also alter development of the cerebellum. In tottering mice the volume of the cerebellar molecular layer is decreased compared to wild-type mice (Isaacs and Abbott, 1994). Furthermore, it has been shown in a previous study that there is altered synapse formation within the cerebellum of adult tottering and leaner mice, which may stem from altered synaptogenesis (Rhyu et al., 1999).

Nitric oxide (NO), a novel neural messenger, interacts with soluble guanylyl cyclase increasing cGMP production and affecting a wide range of cellular functions, including many aspects of nervous system development (Dawson et al., 1998a; Dawson et al., 1998b). The production of NO has been associated with the elongation of axons and dendritic growth and branching as well as synaptogenesis in various parts of the central nervous system (Hölscher, 1997). Specifically in the cerebellum, NO has been shown to affect the rate of migration of granule cells from the external granule cell layer, play a regulatory role in the propagation of granule cells *in vitro*, and have an anti-apoptotic affect on granule cells in culture (Komuro and Rakic, 1998; Lievremont et al., 1999). However, in very high concentrations, NO can be neurotoxic (Samdani et al.,

1997; Hwang et al., 2002).

NO is a novel messenger in that it can affect the function of distant cells due to its diffusibilty (the ability to spread three-dimensionally, regardless of anatomical connectivity). There is no known mechanism for storage and subsequent release of nitric oxide. The concentration of NO is regulated by activation of nitric oxide synthase (NOS) (Brüning, 1993; Contesabile, 2000). NOS is an enzyme that oxidizes arginine forming nitric oxide and cirtrulline (Moncada and Higgs, 1993). Theoretical modeling has predicted that a single point source of NOS creating and emitting NO for just a few seconds can affect cells up to 100μm away, which could potentially influence up to two million synapses (Wood and Garthwaite, 1994). There are three isoforms of NOS that produce NO in the brain. Two of the isoforms, neuronal NOS and endothelial NOS, are both activated by calcium-calmodulin (a calcium-binding protein), and are constitutively expressed, while the third isoform is inducible (inducible NOS), and is not calcium dependent (Huang and Fishman, 1996). Thus, expression of the constitutive isoforms is strictly dependent on and correlated with calcium ion influx as well as intracellular calcium homeostasis (Dawson et al., 1998a). NO function is usually viewed as resulting from stimulation of NMDA receptors, which are ligand-gated ionic channels that have a high conductance for calcium (Dawson et al., 1992). The correlation between voltage-gated ion channels and NO function has yet to be studied.

In the mammalian central nervous system, NOS concentrations are highest in the cerebellum (Förstermann et al., 1990). Neuronal NOS has been shown to have a distinct postnatal developmental expression pattern in the brain (Brüning, 1993; Ohyu and

Takashima, 1998). This includes transient expression in developing cerebellar Purkinje cells in humans and mice (Brüning, 1993; Ohyu and Takashima, 1998). However, it is unclear what role neuronal NOS/NO has in directing cerebellar development. Since neuronal NOS is regulated by intracellular Ca^{2+} homeostasis, we were interested to determine if different severities of P/Q-type calcium ion channelopathies resulted in different levels of neuronal NOS expression during cerebellar development. The two mutant mice used in this experiment, tottering and leaner, exhibit different degrees of cerebellar dysfunction and postnatal cerebellar death. Leaner mice have more severe phenotypic dysfunction compared to tottering mice and display postnatal Purkinje and granule cell death, which is not observed in the tottering mouse cerebellum. It has been previously shown that the expression of neuronal NOS in adult tottering and leaner mice is altered compared to wild-type mice (Rhyu et al., 2003), but expression of neuronal NOS in juvenile mutant mice had not yet been examined. We examined neuronal NOS protein expression indirectly through the use of NADPH-d histochemistry and directly using western blotting. We observed that there are distinct patterns of neuronal NOS expression among the mutant and wild-type mice during postnatal development of the cerebellum.

MATERIALS AND METHODS

Animals

Male and female control mice on the C57BL/6J: +/+ (wild-type) background, and mutant male and female mice, C57BL/6J:*tg/tg* (homozygous tottering) and C57BL/6J: tg^{la}/tg^{la} (homozygous leaner) were housed at the Laboratory Animal Research and Resource facility at Texas A&M University. Heterozygous male and female leaner ($tg^{la}/+$) and tottering (*tg*/+) mice were bred to produce homozygous tg^{la}/tg^{la} and *tg/tg* offspring, respectively. Mice were bred and maintained on a 12-hour light/dark cycle with constant temperature (21-22°C) and allowed free access to food and water. Due to the extreme ataxia, epileptiform seizures and paroxysmal dyskinesia of homozygous leaner pups, many of these young mice die due to a limited ability to move around the cage and find food and water. Accordingly, many of the homozygous leaner (and a few homozygous tottering) pups used in this experiment were fostered to lactating Swiss White Webster female mice and kept alive until the appropriate age. Handling and care of the animals used was in accordance with Texas A&M University and the National Institutes of Health, with a minimum number of animals used for each experiment.

Male and female homozygous +/+, tg^{la}/tg^{la}, and *tg/tg* mice at the ages of P8, P12, and P20 were used in this experiment. Homozygous leaner pups were easily distinguished from heterozygous littermates by the ataxia present starting at P12 and the oligosyndactylism present from birth (Rhyu et al., 1999). Homozygous tottering pups and homozygous leaner pups can be distinguished from heterozygous littermates by oligosyndactylism, or fused digits, present from birth (Green and Sidman, 1962). The

tottering locus on chromosome 8 is closely linked to the *Os* locus, a mutation which produces fused second and third digits, and thus the *Os* mutation was also used as a marker for the presence or absence of the tottering mutation (Green and Sidman, 1962) and leaner mutation. When parents heterozygous for either genotype are mated, either heterozygotes with fused digits are born or homozygous mutant mice with normal digits (unfused) are born. Mice that are homozygous for *Os* die prior to birth (Isaacs and Abbott, 1992).

Tissue collection for NADPH diaphorase histochemistry

Mice were deeply anesthetized with 3.0 mg/kg ketamine plus 0.5 mg/kg xylazine given for every twenty grams body weight intraperitoneally (i.p.), then perfused intracardially with Tyrode's saline (pH 7.2-7.4) followed by 4% phosphate buffered paraformaldehyde (PB-paraformaldehyde; pH 7.4). P8 and P12-aged mice were perfused with 10 mls Tyrode's saline and 20 mls 4% PB-paraformaldehyde. P20 mice were perfused with 50 mls Tyrode's saline and approximately 500 mls 4% PB-paraformaldehyde. After perfusion, the brains were removed and cryoprotected in a 20% sucrose solution and rapidly frozen using powdered dry ice. Using a cryostat, the brains were cut into 25μm sagittal sections and placed on gelatin-coated glass microscope slides and stored at -70°C until used. Serial sections that contained the cerebellar vermis were stained.

NADPH-diaphorase (NADPH-d) histochemistry

It has been previously shown that NADPH diaphorase histochemical staining is co-expressed with neuronal NOS in the mouse central nervous system (Bredt et al., 1991). Sections previously prepared as described above were permeablized in 0.25% Triton-X-100 in 0.1M Tris HCl for 25 minutes and then stained in a solution containing 0.25% Triton-X-100 in 0.1M Tris HCl, 1.0mM β-nicotinamide adenine dinucleotide phosphate (β-NADPH) (Sigma-Aldrich, St. Louis, MO, USA) and 0.5mM nitro blue tetrazolium (NBT. Sigma-Aldrich) for two hours at 37°C. The reaction was terminated using three ten minutes washes of 0.1M Tris HCl (pH 8.0). The sections were then dehydrated through an ascending series of ethyl alcohols and xylenes and coverslipped using Permount mounting media.

1.0% Thionin staining

Thionin (Sigma-Aldrich) stains for chromatin and, thus, for nuclei within cells. Sections created for, but not used in, NADPH-d histochemistry were used for this staining procedure. Six sections per individual were analyzed. The sections were dipped into 1.0% thionin solution, containing thionin, deionized water, 0.2M acetic acid, and 0.2M sodium acetate, for about 7 seconds. Excess stain was removed using brief washes first in 70% ethanol and then deionized water. The sections were then allowed to air dry overnight. The next day the sections were then put through two washes of xylenes and coverslipped using Permount.

Tissue collection for western blotting

Mice were anesthetized with isoflurane, an inhaled anesthetic, and then killed by decapitation. The brains were quickly removed and immediately frozen with powdered dry ice. The frozen brains were stored at -70°C until used. For protein extraction, each brain was thawed and the cerebellum isolated. On a chilled glass plate with a clean razor blade, the cerebellum was minced and added to a 2.0mL microfuge tube containing Pierce mammalian protein extraction reagent with proteinase inhibitor (20,000 μL/g) (Pierce, Rockford, IL, USA) and then mixed. The mixture was then sonicated to disrupt the cell membranes. Extracts were boiled (5 minutes), centrifuged at 12,000g (15 minutes) and the supernatant was collected into a new tube. The protein concentration of each sample was determined by performing a Bradford assay. All extracts were stored at -20°C.

Western blot for neuronal nitric oxide synthase

30μg of each protein extract was mixed with 15μL 2X loading buffer (Sigma-Aldrich), which consists of bromophenol blue, xylene cyanole and sucrose in water, and boiled at 95°C (5 minutes). The samples were then cooled, centrifuged (7500g for 30 seconds) and vortexed, then chilled (5 minutes). 20μL of each sample were then loaded onto a 7% SDS-polyacrylamide gel and separated by electrophoresis. After protein separation, the protein was transferred onto a PVDF membrane using an electrophoretic system. After the transfer, the gel was stained with a 0.025% Coomassie blue stain for 10 minutes to determine the efficiency of protein transfer. The membrane was incubated

in 5% skim milk in Tris-buffered saline plus 0.1% Tween-20 (TBST) for two hours in order to prevent non-specific binding. The membrane was then incubated overnight at 4°C in a solution of 2.5% skim milk in TBST and 0.25μg/ml rabbit neuronal NOS antibody (Transduction Laboratories, Lexington, KY, USA) at a concentration of 1:1,000. The membrane was washed five times in TBST and then placed in a solution of 0.06μg/ml peroxidase-labeled anti-rabbit IgG (Vector, Burlingame, CA, USA) diluted in 2.5% skim milk in TBST to 1:30,000, and incubated at room temperature for two hours. The membrane was again washed in TBST. Neuronal NOS immunopositive bands were visualized using enhanced chemiluminescence substrate (Pierce) and subsequently exposed for 5 minutes and developed using Alpha Innotech equipment.

RESULTS

P8

Wild-type (n=2) mice showed very little overall NADPH-d staining, with most of the reactivity occurring within the Purkinje cell layer (Fig. 2A). The staining of Purkinje cell bodies was noticeable, with some of these Purkinje cells also displaying positive staining in dendrites (Fig. 3A). The external granule cell layer showed some background staining with no cellular staining. The molecular layer also showed very little staining, but there was a tendency of increased staining of portions of the molecular layer adjacent to the Purkinje cell layer in sections of lobules close to the cerebellar deep nuclei (Fig. 4). This staining, which was not located in neuronal somata, may be due to staining of Purkinje cell dendrites or climbing fibers. The internal granule cell layer showed very light, background staining that was consistent throughout the entire cerebellum. The white matter showed no significant staining.

P8 tottering mice (n=2) had a similar staining pattern as wild-type mice, but there was a tendency for increased overall intensity of staining in tottering compared to wild-type mice (Fig. 2C). The most noticeable histochemical staining appeared in the Purkinje cell layer, with both cell bodies and dendrites staining (Fig. 3B). The same staining phenomenon observed in the wild-type cerebellar molecular layer was also present in the tottering mouse cerebellum, with increased background staining occurring in the deep portion of lobules. Also similar to the wild-type cerebellum, the tottering mouse external granule cell layer appeared to have some background staining, with little cellular staining. The internal granule cell layer appeared to have consistent, light

background staining while the white matter also displayed no staining.

P8 leaner mice (n=2) showed the same staining intensity and staining pattern as the wild-type mice (Fig. 2E). Once again, the Purkinje cell layer showed marked staining, with the most intense staining appearing in the Purkinje cell body (Fig. 3C). As with the other two genotypes, the white matter showed no staining, while the molecular and internal granule cell layers also displayed the same types of staining pattern as the other two genotypes (Fig. 4).

Thionin staining revealed that, for all genotypes, the molecular layer contained cell bodies at P8. This means that even though cells are present in the molecular layer at P8, they are not expressing neuronal NOS. For all three genotypes, thionin staining showed that Purkinje cells and cells that occupy the internal and external granule cell layer also were present. Also, when comparing tottering and leaner mice to wild-type mice in both thionin and NADPH-d staining, there appears to be an increased size of the external granule cell layer within the mutant cerebella compared to wild-type mice (Fig. 2B,D,E). All three genotypes showed NADPH-d staining similar to that described by Brüning (1993) and Yan et al. (1993).

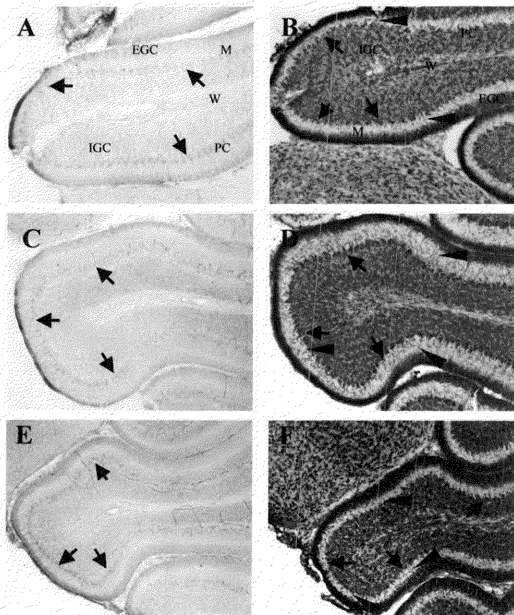

Figure 2. Representative Images of P8 Cerebellar NADPH-d and Thionin Staining
Representative images of NADPH-d histochemistry and thionin staining in P8 wild-type (A,B), tottering (C,D) and leaner (E,F) mice. EGC (external granule cell layer), M (molecular layer), PC (Purkinje cell layer), IGC (internal granule cell layer) and W (white matter). All sections show lobule IV & V (rostral). Note similar thionin staining patterns in all sections (B,D,F), with cells staining within the PC, the EGC and the IGC. In all three sections, cellular thionin staining within the molecular layer is present. The arrows indicate reactive Purkinje cells, while the arrowheads denote reactive cells within the molecular layer. The NADPH-d staining also seemed similar amongst all three sections (A,C,E). Note the highly reactive Purkinje cells in all three genotypes. The molecular layer shows only background staining, even though thionin staining shows that viable cells are present. Magnification of all representative images = 40X.

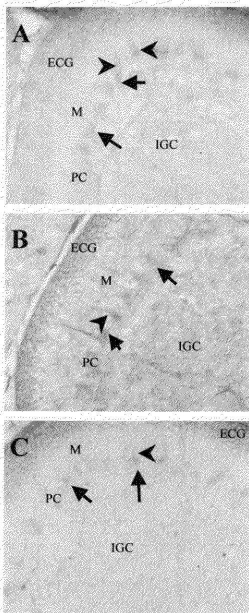

Figure 3. Representative Images of NADPH-d Stained P8 Cerebella – High Magnification
A-C are representative images of P8 cerebella from wild-type, tottering and leaner mice, respectively. EGC (external granule cell layer), M (molecular layer), PC (Purkinje cell layer), and IGC (internal granule cell layer). Purkinje cell staining is the predominant staining at this age. Arrows indicate Purkinje cell somata and arrowheads indicate Purkinje cell dendrites. Magnification for all representative images = 400X.

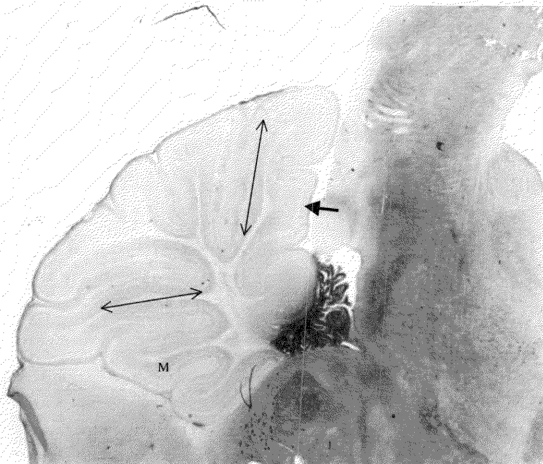

Figure 4. Representative Image of NADPH-d Stained P8 Leaner Cerebellum – Low Magnification
A representative image of NADPH-d histochemical in the P8 leaner mouse. M (molecular layer). Note the intense staining of Purkinje cells (arrows) in the cerebellar lobules as well as the decreased intensity of molecular layer background staining radially (double-headed arrows). The pattern of decreased intensity of molecular layer background staining radially is seen in all three genotypes (wild-type and tottering mice not shown). Magnification of representative image = 20X.

P12

Wild-type mice (n=4) showed NADPH diaphorase staining similar to that described by Brüning (1993) and Yan et al. (1993) (Fig. 5A). The external granule cell layer showed very little staining, while the molecular layer showed marked staining in the basket cell population, which is located adjacent to the Purkinje cell layer. The

upper third of the molecular layer, closest to the external granule cell layer, showed a mild background staining compared to the rest of the molecular layer, with few, if any, basket cells staining in that region (Fig. 6A). The Purkinje cell layer showed a marked decrease in staining compared to the reactivity within the wild-type Purkinje cell layer at P8, with almost all of the Purkinje cells showing no reactivity at all. Staining within the internal granule cell layer was moderate, with some cells within this layer not staining for NADPH-d, and others staining as intensely as basket cells, but overall, the compartmentation described by Hawkes and Turner (1994) of dark and light patches of NADPH-d positive granule cells did not appear to be evident. The white matter displayed no NADPH-d staining.

The tottering (n=3) cerebellum showed a higher overall intensity of staining compared to wild-type mice (Fig. 35C). The external granule cell layer stained similarly to wild-type granule cells and appeared to have very little staining. In the molecular layer, the overall background staining as well as cellular staining of basket and stellate cells appeared more intense compared to the wild-type molecular layer. However, the overall pattern of staining in the tottering molecular layer was similar to that observed in wild-type mice, with the portion of molecular layer adjacent to the external granule cell layer showing less staining compared to the rest of the molecular layer (Fig. 6C). The Purkinje cell layer showed slight staining, which was most likely due to nearby processes from basket cells or parallel and climbing fibers. Staining within the internal granule cell layer was moderate, and appeared to have the same pattern of staining compared to age-matched wild-type mice. The white matter within the tottering

cerebellum, similar to wild-type mice, did not appear to stain for NADPH-d.

Leaner mice (n=3) showed very little staining within the external granule cell layer, but showed a higher concentration of cellular staining in the molecular layer compared to wild-type mice (Fig. 5E). There was a very distinguishable band of intensely stained basket cell bodies located adjacent to the Purkinje cell layer, which was not present in the wild-type mice. Similar to wild-type mice, the upper third of the molecular layer showed very little cellular staining (Fig. 6E). Staining of Purkinje cells was not evident in the P12 leaner cerebellum. In the internal granule cell layer, a staining pattern similar to age matched wild-type mice was evident, and the white matter also appeared to follow the same staining trend as age matched wild-type mice.

In all three genotypes, there was a very noticeable staining pattern throughout the cerebellar lobules. Intense staining was concentrated towards the center of each lobule , with the stain becoming less intense moving radially to the tips of the lobules. Furthermore, the rostral lobules (I-V) seemed, overall, to have a more intense NADPH-d staining when compared to the caudal lobes (VI-X) (Fig. 7A-C). This staining suggested a rostral to caudal pattern of neuronal NOS expression within P12 mouse cerebellum.

Furthermore, thionin staining showed a different staining pattern within the molecular layer when compared to P12 cerebella stained for NADPH-d for all three genotypes. The thionin stained sections showed numerous basket and stellate cells occupying the molecular layer as well as some migrating granule cells, but NADPH-d stained sections from the same individual revealed mostly basket cells staining, with background staining occupying the area where stellate cells reside. For all three

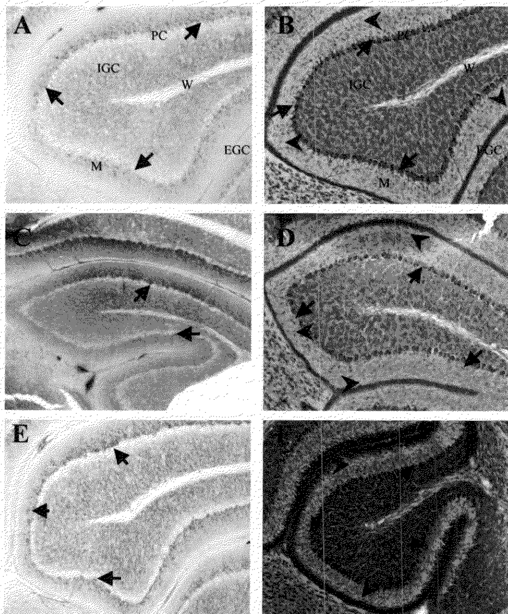

Figure 5. Representative Images of P12 Cerebellar NADPH-d and Thionin Staining
Representative images of NADPH-d histochemistry and thionin staining in P12 wild-type (A,B), tottering (C,D) and leaner (E,F) mice. EGC (external granule cell layer), M (molecular layer), PC (Purkinje cell layer), IGC (internal granule cell layer) and W (white matter). All sections show lobule III (rostral). Note similar thionin staining patterns in all sections (B,D,F), with cells staining within the PC, the EGC and the IGC. The arrows indicate reactive basket cells while the arrowheads indicate reactive stellate cells. Note the lack of Purkinje cell staining in all NADPH-d sections (A,C,E). The IGC as well as the M are both positively stained in all three sections, though the intensities observed appear to differ with genotype. The NADPH-d staining appears more intense in tottering (C) compared to wild-type (A) and leaner (E) while the leaner appears to show a more intense basket cell body staining compared to wild-type. The molecular layer showed a common NADPH-d staining pattern among all three genotypes. Magnification of all representative images = 40X.

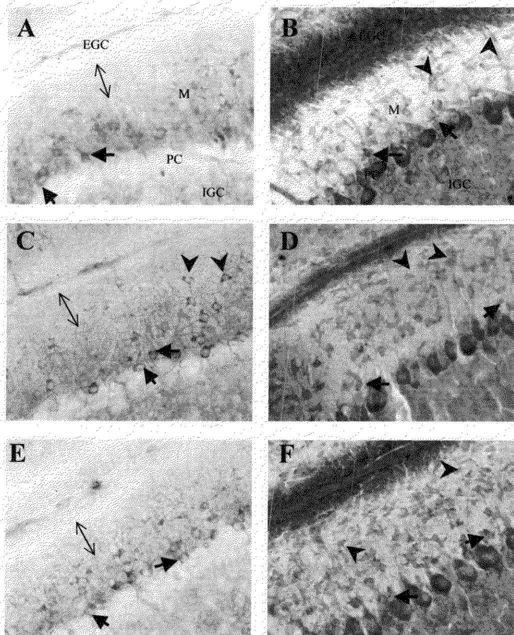

Figure 6. Representative Images of NADPH-d and Thionin Stained P12 Cerebella – High Magnification Representative images of NADPH-d histochemistry and thionin staining in P8 wild-type (A,B), tottering (C,D) and leaner (E,F) mice. EGC (external granule cell layer), M (molecular layer), PC (Purkinje cell layer) and IGC (internal granule cell layer). At this level of magnification, the portion of the molecular layer that contains no NADPH-d cellular staining is very noticeable (double-headed arrow). Baskets cells are indicated by arrows and stellate or migratory cells are indicated by arrowheads. Note the almost total absence of positive stellate cells in the NADPH-d staining of all three genotypes. Magnification for all representative images = 400X

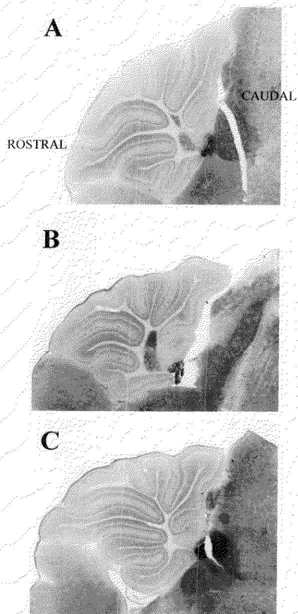

Figure 7. Representative Image of NADPH-d Stained P12 Cerebella – Low Magnification
Representative images of NADPH-d histochemistry in P12 wild-type (A), tottering (B) and leaner (C) mouse cerebella. Notice the overall rostral to caudal NADPH-d staining pattern of all three genotypes, which is most noticeable in the wild-type mouse (A). Also notice the overall increased intensity in the rostral lobules compared to the caudal lobules in all three mouse cerebella. There is also a pattern of decreased NADPH-d staining radially in all three genotypes, which is similar to P8 cerebella NADPH-d expression. Also note the increased intensity of the tottering cerebellum (B) compared to the wild-type cerebellum (A) and the overall increased staining in the leaner cerebellum (C) compared to the wild-type cerebellum (A). Magnification of all representative images = 20X.

genotypes, thionin staining also revealed a typical Purkinje cell population, as well as large population of cells within the internal granule cell layer (Fig. 3B,D,F and Fig. 6B,D,F). Both stains also revealed a tendency for an enlarged external granule cell layer within the mutant mice compared to wild-type mice.

P20

For wild-type mice (n=4), the NADPH diaphorase staining took the pattern described by Brüning (1993) and Yan et al. (1993) (Fig. 8A). Staining in the molecular layer primarily resulted from basket cells bodies and stellate cell bodies. Neuronal processes stained as well, many of which seemed to originate from basket and stellate cells, but could also be parallel and climbing fibers. Furthermore, there was a consistent background staining throughout the molecular layer, which could be reactive Purkinje cell dendrites, climbing and parallel fibers, or stained glial cell fibers (Fig. 9A). The Purkinje cell somata showed no staining. The outline of individual Purkinje cells present was most likely due to structures around the Purkinje cells, which include basket cell dendrites and climbing fibers. The internal granule cell layer showed moderate staining throughout, with some cells within the granule cell layer not staining. This staining pattern of light and dark areas within the internal granule cell layer was consistent with the compartmentation described by Hawkes and Turner (1994). The white matter also showed no staining.

The tottering mouse (n=4) showed a more intense staining pattern compared to wild-type mice, which is in agreement with the findings of Rhyu et al. (2003) (Fig. 8C). In the molecular layer, staining was dominated by basket and stellate somata and

multiple neuronal processes. However, compared to wild-type mice, there was more intense staining of basket cell bodies, stellate cell bodies and processes. The background staining also was more intense compared to the wild-type mouse (Fig. 9B). The Purkinje cell somata lacked staining, which was similar to wild-type Purkinje cell somata. The internal granule cell layer was much more intensely stained in the tottering cerebellum compared to wild-type mice, but the pattern of light and dark staining patches was still evident. The white matter did not display any staining.

The leaner mouse (n=4), overall, did not present any obvious differences in staining compared to the wild-type cerebellum (Fig. 8E). In the molecular layer, staining was due to basket and stellate cell bodies, with staining also occurring from processes originating from basket, stellate, granule, or Purkinje cells, or from climbing fibers. In the leaner mouse molecular layer, there was a tendency towards a higher intensity in the staining of basket and stellate cell bodies compared to the wild-type mouse molecular layer (Fig. 9C). The leaner mouse Purkinje cell layer showed very little staining, appearing similar to the staining of the wild-type Purkinje cell layer, while the staining in the internal granule cell layer also showed a similar pattern and intensity compared to wild-type. Similar to the wild-type cerebellum, the leaner white matter showed no staining.

Thionin staining for P20 cerebella showed that all cells were present, including Purkinje cells, which did not stain for NADPH-d. The other layers, including the white matter, internal granule cell layer and molecular layer displayed the typical cellular make-up of an adult mouse cerebellum (Fig. 8B,D,F). The external granule cell layer

was no longer present in either the NADPH-d or thionin stained slides of all genotypes, which is to be expected in the P20 mouse cerebellum.

We completed two western blots using cerebella from P20 mice, two of each genotype (Fig. 10A-B). The first blot we performed showed signs of protein degradation (Fig. 10A). Multiple bands per individual were observed, which suggested degradation product had formed (neuronal NOS should appear as a single band (110Kda) on western blots). Lack of appropriate chemiluminescence also occurred in several samples, which was likely due to human error during extraction, which may have removed most of the desired protein before the gel was run. The second blot showed that some, if not all, of the errors had been corrected (Fig. 10B). Even though the second band that appeared on the second gel indicated that probably some degradation of neuronal NOS had occurred, the intensity and size of each band were equal to each other, such that a viable comparison of the neuronal NOS bands of the different mice was possible. By comparing samples from two mice of each genotype, we noted some differences between genotypes, but also between individuals within the same genotype as well. This suggests that there is individual variability to the amount of neuronal NOS expression, and to see an overall pattern, more individuals must be examined.

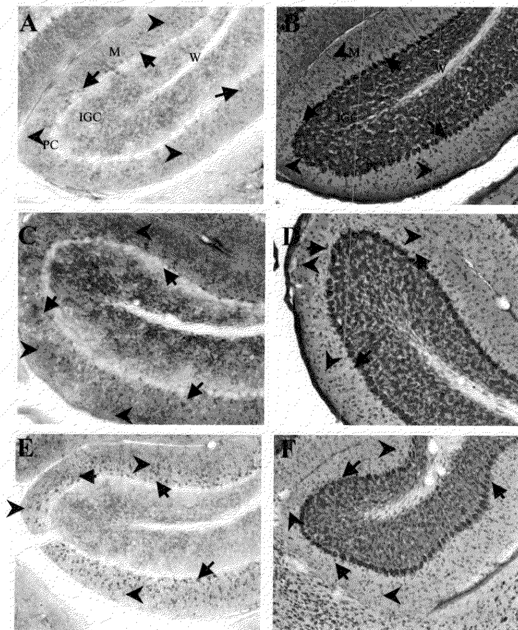

Figure 8. Representative Images of P20 Cerebellar NADPH-d and Thionin Staining
Representative images of NADPH-d histochemistry and thionin staining in P20 wild-type (A,B), tottering (C,D) and leaner (E,F) mice. EGC (external granule cell layer), M (molecular layer), PC (Purkinje cell layer), IGC (internal granule cell layer) and W (white matter). All sections show lobule I & II (rostral). Note similar thionin staining patterns in all sections (B,D,F), with cells staining within the M, PC, and the IGC. Arrows indicate reactive basket cells and the arrowheads indicate reactive stellate cells. The NADPH-d stained sections (A,C,E) all showed positively stained cells within the IGC and the M, and no staining within the PC. The tottering mouse (C) appears to have a more intense NADPH-d staining when compared to the wild-type (A) and leaner (E). The leaner mouse (E) appears to have more intense staining within its basket and stellate cell bodies when compared to wild-type (A). However, this trend was not seen throughout the entire cerebellum. Magnification of all representative images = 40X.

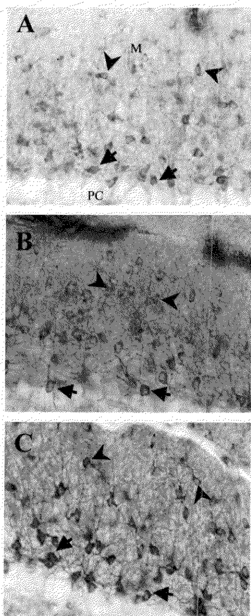

Figure 9. Representative Images of NADPH-d Stained P20 Cerebella – High Magnification
Representative images of NADPH-d histochemistry in P20 wild-type (A), tottering (B) and leaner (C) mice. M (molecular layer), and PC (Purkinje cell layer). The increased intensity of NADPH-d staining in the tottering cerebellum (B) is very noticeable compared to the other genotypes. There also appears to be a tendency for increased cellular staining of the leaner cerebellum (C) compared to wild-type (A). Arrows indicate basket cell somata and arrowheads indicate stellate cell somata. Notice the high amount of processes staining positive for NADPH-d. These processes are either from basket or stellate cells. Also notice the indistinct background staining, which may be Purkinje cell dendrites, climbing fibers or parallel fibers. Magnification of all representative images = 400X.

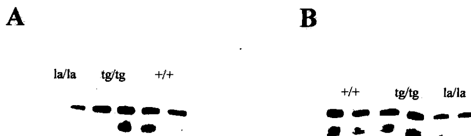

Figure 10. Representative Images of P20 Cerebellar Neuronal NOS Western Blots
Figure A shows the first western blot performed on P20 mouse cerebella for neuronal NOS. +/+ means wild-type, tg/tg means tottering and la/la means leaner. The top band indicates neuronal NOS while the bottom band indicates either non-specific binding of the antibody or degradation product of neuronal NOS. Notice the almost total lack of stained neuronal NOS in the first leaner individual and the presence of the second band in three out of six individuals. Figure B shows the second western blot performed on P20 mouse cerebella for neuronal NOS, using different individuals than the blot in figure A. Note the differences in staining in the neuronal NOS band amongst the wild-type individuals, as well as differences amongst the tottering individuals. Also note the presence of a second band for each individual, meaning either non-specific binding or degradation products are present. Note in figure B that the leaner mouse bands are lighter than the other two genotypes, but more individuals need to be run before any conclusion can be made. Images were adjusted for brightness and contrast using Adobe Photoshop 7.0.

DISCUSSION

Through NADPH-diaphorase histochemistry and western blotting, we observed two significant and altered neuronal NOS expression levels during postnatal cerebellar development of tottering and leaner mice compared to age-matched wild-type mice. In the tottering mouse cerebellum, there was an increased histochemical staining for NADPH-d in the molecular and internal granule cell layer during postnatal days 12 and 20, with a possible increase in staining at P8. In the leaner mouse cerebellum, P12 displayed an increased histochemical staining in both the molecular and internal granule cell layer for NADPH-d compared to control mice, but by P20, the leaner cerebellum appeared to have only a subtle increase in staining in the molecular layer compared to control mice and no difference in staining in the internal granule cell layer (Figs. 2,5,8).

NADPH-d histochemical staining also showed certain cerebellar developmental patterns of neuronal NOS expression that were present in all three genotype. In the P8 cerebellum, there was a tendency for the molecular layer to show increased background staining in portions of lobules located medially in the cerebellum (Fig. 4). Another similar phenomenon occurred in the P12 cerebellum. There was an increased intensity of NADPH-d staining in portions of lobules located near the deep cerebellar nuclei, when compared to more exterior portions of the cerebellum (Fig. 7). This inside-outside pattern may be due to signals being received from the white matter neuronal tracts. Synaptogenesis within the rat molecular layer is said to begin at P8, so perhaps the staining pattern we observed within the molecular layer of P8 and P12 mice is due to signals from the nervous system (via white matter tracts) to begin synaptogenesis (Chen

et al., 2001) in P8 or to increase synapse formation in P12. Since the cerebellum expands outward, the signaling for synaptogenesis would thus start medially and spread laterally. Neurotrimin, a neural cell adhesion molecule, has been shown to have a temporal expression within the white matter of the cerebellum of rats, and was shown to be expressed as a wave, moving from the cerebellar peduncles, to the deep cerebellar nuclei, and finally to the lobules and internal granule cell layer (Chen et al., 2001). Furthermore, the α_{1E} subunit of R-type calcium channels, which has been associated with myelinogenesis, was shown to show a similar wave-like temporal pattern within rat cerebellum, with expression in cerebellar peduncles at P8, lobular white matter by P14 and the internal granule cell layer by P17 (Chen et al., 2000). Thus, perhaps the expression of the α_{1A} subunit of P/Q-type calcium channels and/or calmodulin activation in the developing cerebella of the mice we observed also proceeds in a similar fashion. However, the cerebellar lobules grow in a radial fashion during cerebellar development. (Altman and Bayer, 1997). It is most likely that the NADPH-d staining pattern noticed in the P8 molecular layer is due to the pattern of lobule development and the entry of cells radially as lobules extend outward.

The NADPH-d histochemical staining pattern present in all three P8 mouse cerebella seemed to follow the pattern of staining described by Brüning (1993) and Yan et al. (1993) (Fig. 2). The intense staining present in the Purkinje cell layer may denote that these cells are a main site of neuronal NOS synthesis during synapse development in the mouse cerebellum. The background staining evident in the molecular layer, which may be due to parallel or climbing fibers, or Purkinje cell dendrites, denotes that these

processes may also be important in producing neuronal NOS during development (Fig. 4). The lack of cellular staining within the molecular layer is probably due to the lack of mature basket and stellate cells in the P8 cerebellum. Fully mature stellate cells do not appear in the molecular layer until approximately P12, while fully mature basket cells do not usually appear until around P8 (Altman and Bayer, 1997). Thus, the lack of cellular staining may be due to the lack of mature cells within the molecular layer at P8. Also, the compartmentation in the granule cell layer reported by Hawkes and Turner (1994) was not present at this age. The uniformity present in the internal granule cell layer at P8 may mean that the roles granule cells and parallel fibers are believed to play in synaptic plasticity have not yet begun.

In P12 mice cerebella, a distinct staining pattern was noticeable (Figs. 5,7). The increased staining present towards the center of the cerebellum may be a sign of growth. Nitric oxide has been implicated in many areas of neuronal development and perhaps the increased NADPH-d staining shows an increased activation of neuronal NOS due to a need of nitric oxide as the cerebellum enlarges. There also appears to be a tendency for the P12 cerebella to show a rostral to caudal staining pattern, with decreased staining caudally (Fig. 7). There is a known pattern of development in the cerebellum which extends in a rostral to caudal direction (Altman and Bayer, 1997). This pattern is seen outside of development as well, including Purkinje cell and granule cell death within the adult leaner cerebellum, with more cells dying in the rostral portion of the cerebellum compared to the caudal portion (Frank et al., 2003). Therefore, this rostral-caudal patterning could influence the expression of neuronal NOS/NO in the developing mouse

cerebellum. The distinct staining pattern noticed in the molecular layer may be explained by the pattern of maturation of cells within the molecular layer (Fig. 6). As noted earlier, the molecular layer of all three mice showed a distinct histochemical pattern, whereby a portion of the molecular layer near the Purkinje cell layer displayed NADPH-d cellular staining while the other portion only showed background staining. By P12, basket cells are beginning to develop their axons, and thus should show expression of neuronal NOS due to its role in synaptogenesis and neuron growth (Hölscher, 1997). However, immature stellate cells begin differentiating and migrating around P12 (Altman and Bayer, 1997), and may not need to use NO. Even though NO has been noted to be involved in neuronal development and synaptogenesis, its involvement in neuronal events prior to these activities is questionable, and thus, P12 may just be too early for stellate cells to express neuronal NOS. A second hypothesis for the thionin staining of cells in the upper portion of the molecular layer in P12 mouse cerebella is that these cells could be migrating granule cells as well, which have yet to differentiate. Therefore, maybe it is too early for these migrating granule cells to start expressing neuronal NOS, and thus they do not show up in NADPH-d staining. To see what types of cells are showing the thionin staining, electron microscopy could be done on thionin staining P12 mouse cerebella, and the type of cell could then be determined from structural differences. Since both types of cells are maturing and migrating at P12, it is probably a mixture of both cell types.

In both P8 and P12 cerebella, the tottering and leaner cerebella appeared to have a thicker external granule cell layer when compared to age-matched wild-type cerebella.

This seems to indicate that development of the tottering and leaner cerebella are slower in the mutant mice compared to wild-type mice. A larger external granule cell layer would indicate either that fewer cells have begun their migration and differentiation, or that cells are migrating at a slower rate than wild-type mice. However, this larger external granule cell layer could be an effect of decreased molecular layer size noticed by Isaacs and Abbott (1994). A decrease in the size of the molecular layer could make the external granule cell layer look larger, and thus there may be no actual difference in size between mutants and wild-type.

The background staining of all three genotypes in all three age groups may be produced by several different cellular structures. To determine whether the staining comes from Purkinje cell dendrites, climbing fibers or parallel fibers, NADPH-d staining followed by electron microscopy (EM) could be performed. These processes are structurally different, and thus, the use of EM could help to differentiate which, if any, of these neuronal processes could be producing the background staining noticed in light microscopy.

The western blot of P20 mice shows differences amongst the genotypes, which is similar to results found by Ryhu et al. (2003) (Fig. 10B). However, before we proceed to quantify this data, more individuals need to be tested. The western blots performed thus far show that there are also differences amongst individuals of the same genotype, and thus, an experimental population of two is not enough to reasonably and confidently report quantitative data. Therefore, the use of four individuals per genotype and per age will be used for western blots in future experiments to ensure that there are enough

individuals to account for the variability within a genotype.

NO has been implicated in many different areas of neuronal development, but its definitive role is still not well understood. Nitric oxide has been shown to be involved in increasing the rate of migration of cerebellar granule cells (Tanaka et al., 1994), as well as synaptic plasticity (Hölscher, 1997). It has also been shown that calcium ion channels affect the migration of granule cells from the external to the internal granule cell layer, with a decrease in calcium ion amplitude or frequency yielding decreased speed of granule cell movement (Komuro and Rakic, 1998). There have also been studies showing that elevations in intracellular calcium ion concentration can induce plasticity in Purkinje cells (Kano et al., 1992; Konnerth et al., 1992).

Even though nitric oxide has been shown to affect the migration of cerebellar granule cells, it has also been shown that there are no structural and neurochemical changes that result from *in vivo* inhibition of NOS in neurons during development (Virgili et al., 1999). In the tottering mouse cerebellum, it has been previously shown that there is a 40% reduction in the calcium ion current through P/Q type voltage-gated calcium ion channels of Purkinje cell (Wakamori et al., 1998). 46% of the voltage-gated calcium ion channels of cerebellar granule cells are of the P/Q subtypes (Randall and Tsien, 1995), and thus it should be safe to assume that there is some reduction in the calcium ion current in these cells as well as Purkinje cells. Therefore, the increase in NADPH-d staining intensity, and by inference NOS/NO expression, in the developing cerebella of tottering mice may be, in part, a compensatory action of cells with decreased calcium ion influx to maintain granule cell migration and synaptic plasticity. This would

offer an explanation to the information presented by Virgili et al. (1999) that shows *in vivo* inhibition of NOS has no affect on the brain structure, since calcium ion fluctuations are the main method of granule cell migration, NO being secondary. Furthermore, the increased intensity of NADPH-d histochemistry and thus neuronal NOS expression shown in the adult tottering cerebellum by Rhyu et al. (2003) may be due to the further compensatory effect of nitric oxide for the deficient calcium ion channels present.

The leaner mouse cerebellum, on the other hand, showed an increased intensity of NADPH-d staining at P12 compared to wild-type, but by P20 the histochemical intensity difference was not as pronounced. Rhyu et al. (2003, in press) stained adult (2.5-6 months) leaner cerebella for NADPH-d and NOS expression and noticed a decrease of histochemical staining intensity compared to age-matched controls. Furthermore, the leaner mouse also displays cellular degeneration, with excessive granule cell death starting around P10 and Purkinje cell death starting around P25-P30, while wild-type and tottering mice do not show this rapid decrease in cerebellar cells (Herrup and Wilczynski, 1982; Heckroth and Abbott, 1994; Frank et al., 2003). It is believed that NO may have protective and trophic effects in the cerebellum (Wang et al., 1998). Rhyu et al. (2003, in press), using their data as well as the idea of NO having a trophic role in the cerebellum, hypothesized that leaner mice cannot compensate for their severe P/Q-type channel function via a large increase in neuronal NOS/NO expression, and since there is no increase in NO levels to compensate for the deficient calcium ion channels, cerebellar cells die prematurely.

However, our data shows that at one point in development, there is an increase of neuronal NOS expression at P12 in leaner mice and there is a tendency for increased neuronal NOS expression in molecular layer somata at P20. Following the ideas expressed by Rhyu et al. (2003, in press), it may be possible that the neuronal NOS/NO expression in the leaner cerebellum has reached a maximum by a certain early age that is prior to the age of consistent neuronal NOS production in the wild-type cerebellum. Therefore, over time, the concentration of neuronal NOS and NO may become equal and even decrease when compared to wild-type mice. The production of neuronal NOS may also be reduced due to decreases in calmodulin expression. Calmodulin is a calcium-binding protein that is essential for neuronal and endothelial NOS activation (Huang and Fishman, 1996). It has been shown in a previous study that in leaner Purkinje cells, there is a decrease in the mRNA levels of parvalbumin and calbindin, which are other calcium-binding proteins (Dove et al., 2000). It has also been shown that there is decreased calretinin (another calcium-binding protein) expression in cerebellar granule cells of the leaner mouse (Nahm et al., 2002). Since there is a decrease in expression of these calciuim-binding proteins, it is possible that calmodulin expression could also be decreased. This could lead to a decreased NO expression shown in the leaner compared to tottering at P12 and P20, since activation of neuronal NOS is dependent on calmodulin binding to calcium. Immunohistochemistry and western blots for calmodulin could be done on developing mouse cerebella of all three genotypes to observe if any differences of calmodulin expression exist between mutant and wild-type cerebella.

The compartmentation of NADPH-d staining cerebellar granule cells described

by Hawkes and Turner (1994) is an interesting phenomenon that may be different amongst the three genotypes, especially in the leaner mouse, since it displays cerebellar granule cell death starting around P10. With this granule cell death, it would be interesting to see if the compartmentation of these cells changes to compensate for the loss. Therefore, coronal or frontal sectioning of the cerebellum should be done on developing cerebellum of both mutant (tottering and leaner) and wild-type mice to see how and when the compartmentation of the NADPH-d producing granule cells starts and if the compartmentation is altering the mutant mice. Furthermore, coronal or frontal sectioning should be done on P12 mice to see if the patterns of NADPH-d staining previously noticed are actually present or if they were just artifacts of the sagittal sections and how the tissue was handled.

CONCLUSIONS

The cerebellar development pattern differences observed between tottering, leaner and wild-type mice are one component of a multitude of physiological effects caused by mutations in the tottering locus. Calcium ion concentrations and fluctuations are important in multiple physiological processes within neurons, and thus it is important that the roles of NO in neurons are elucidated. Calcium ions and NO are both very widespread and non-discriminate molecules, and it would be too simplistic to state that the differences in neuronal NOS/NO expression observed in the tottering and leaner mice are due to just the need to direct cerebellar development. Furthermore, calcium ions and NO are not the only molecules involved in cerebellar development. As the role of NO, as well as the roles of other molecules in cerebellar development, becomes more defined, and as the phenotypes of the tottering and leaner mice become better understood, molecularly and cellularly, it will be possible to select targets for pharmacological studies to maintain normal cerebellar development and preserve cerebellar integrity in P/Q-type calcium ion channel deficient individuals.

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