MORPHOLOGY OF PURKINJE CELLS IN CEREBELLA OF EPILEPTIC MICE

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Morphology of Purkinje Cells in Cerebella of Epileptic Mice. (May 2013)

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Calcium ion (Ca^{2+}) homeostasis is a critical component normal neuronal development. Abnormal Ca^{2+} concentrations can disrupt neuron development and nervous system function and lead to various neurological disorders, such as epilepsy. Leaner and tottering mice, which are spontaneous animal models of human absence epilepsy, exhibit reduced Ca^{2+} current density in neurons known as Purkinje cells within their cerebellum. This is a result of unique mutations that each type of mutant mouse carry in the P/Q-type calcium channel α_{1A} subunit gene, and highly expressed in Purkinje cells. Consequently, both mutant mice exhibit severe juvenile onset of ataxia, paroxysmal dyskinesia, and absence seizures. Previous studies have revealed significant loss of Purkinje cells in leaner mice. However, the specific structural effects of these mutations on existing cerebellar neurons remain unclear. We examined the neuronal morphology of Purkinje cells in adult (6-8 months) male and female mice of both mutant genotypes. We found significant reduction in dendritic growth and arborization in Purkinje cells of leaner mice and a trend towards reduction in dendritic growth and arborization in Purkinje cells of tottering mice when compared to wild type mice. No difference in somatic area was observed between any two of the genotypes. Hence, we conclude that there are significant morphological deficits present in Purkinje cells of leaner mice compared to wild type mice that could, in part, explain the symptoms seen in the mice, but the lack of structural differences in Purkinje cells of tottering

mice indicate that there are likely to be underlying functional impairments present that require study beyond the structural level.

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CHAPTER I

INTRODUCTION

Apoptosis, or programmed cell death, occurs in many vertebrate body systems, including the central nervous system (CNS), often to eliminate unwanted or excess neurons. While failure of proper neuronal cell death during development can result in CNS disorders, excessive amounts of apoptosis have been found to take place in a wide range of disorders such as Alzheimer's disease and epilepsy (Lau *et al.*, 2004). One underlying pathogenic process commonly observed among these disorders is disruption of calcium ion (Ca^{2+}) homeostasis (Kim *et al.*, 2007).

In the nervous system, Ca^{2+} influx via voltage-gated Ca^{2+} channels regulates many different and important Ca^{2+}-dependent processes including neurotransmitter release, neuronal excitability and plasticity, excitation-transcription coupling, synaptogenesis, and dendritic growth (Moosmang *et al.*, 2007). The voltage-gated Ca^{2+} channel is typically composed of four major subunits, the largest of which is the α_{1A} subunit that acts as the pore-forming subunit for the channel (Catterall & Few, 2008) (Figure 1A). The other subunits act to modulate overall activity of the channels. P/Q-type calcium channels are a specific classification of voltage-gated Ca^{2+} channels that are activated by strong depolarizing currents. Among the different voltage-gated Ca^{2+} channels, P/Q-type channels play an important role in the influx of Ca^{2+} at the presynaptic terminal of central nervous system neurons, which is essential for release of neurotransmitters from the presynaptic neuron (Catterall & Few, 2008) (Figure 1B). Mutations in these channels have been observed to lead to marked decrease in calcium ion currents through these channels and altered neuronal calcium ion homeostasis (Dove *et al.*, 1998; Lorenzon *et al.*, 1998).

Studies in human patients with ataxia have often revealed loss of cerebellar neurons. Some of these studies have pointed to gene mutations in the α_{1A} subunit of human calcium ion channels as one possible cause for these symptoms. More recently, there also have been reports of early-onset absence epilepsy also being manifested as a symptom of this cerebellar mutation (Lau *et al.*, 2004). Studies of cerebellar mutant mice as genetic animal models may provide significant insight into the various possible mechanisms and characteristics that may result in ataxic and epileptic phenotypes (Heckroth & Abbott, 1994).

Tottering (*tg/tg*) and leaner (tg^{la}/tg^{la}) mutant mice each carry a different homozygous, autosomal recessive mutation, but both are located in the gene encoding the α_{1A} subunit of P/Q-type voltage-gated Ca^{2+} channels (Lau *et al.*, 2004). Both mice manifest varying degrees of a triad of neurological disorders that are initiated during early postnatal development: (1) ataxia; (2) paroxysmal dyskinesia; and (3) absence seizures, with leaner mice being more severely affected (Cicale *et al.*, 2002; Lau, *et al.*, 2004). It is know that the mutation in tottering mice is a point mutation located in an extracellular region of the α_{1A} gene, resulting in a proline-to-leucine amino acid substitution. Leaner mice, on the other hand, exhibit an out-of-frame splice site mutation in the carboxy terminus of the α_{1A} protein, occurring as a result of a splice donor consensus sequence of the α_{1A} subunit (Rhyu *et al*., 1999; Fletcher *et al.*, 1996). These mutations are located in the same subunit as seen in humans with a specific form of cerebellar ataxia and thus validate this model. Furthermore, leaner and tottering mice also are considered to be spontaneously occurring animal models of human absence epilepsy. The absence seizures of

these mutant mice closely resemble *petit mal* epilepsy seen in humans, showing similar symptoms and electroencephalographic recordings (Cicale *et al.*, 2002)

Based on work by Dove *et al.* (1998, 2000), acutely dissociated Purkinje cells from the leaner mouse exhibit a 60% reduction in calcium ion current into the cell. Tottering mice have been reported to exhibit a reduction in calcium ion current of up to 40% in dissociated cerebellar Purkinje cells (Wakamori *et al.*, 1998). Calcium ions are important for cell proliferation, axon growth and dendritic sprouting (Schmitz *et al.*, 2009; Lohman, 2009). Therefore, reduced Ca^{2+} current density entering into neurons can potentially affect the extent of neurotransmitter release from presynaptic neurons and alter other Ca^{2+}-mediated cellular functions (Tsien & Tsien, 1990; Moosmang *et al.*, 2007). It also has been known for a long time that changes in neuronal function are often correlated with changes in neuronal morphology.

This study delves into the morphological complexity of Purkinje cells of the cerebellum of tottering and leaner mice and provides a structural analysis of cerebella from tottering and leaner mice that may, in part, explain the differing degrees of cerebellar atrophy seen in these voltage-gated calcium ion channel mutant mice (Frank *et al.*, 2003). We entered this investigation with the hypothesis that Purkinje cells of tottering and leaner mice will both show significantly reduced overall morphological growth and complexity when compared to wild type mice, with the leaner showing a more severe reduction.

CHAPTER II

METHODS

Golgi-Cox Solution

The Golgi-Cox solution was prepared as follows (Rhyu et al., 1999). Solution A (5% potassium dichromate), solution B (5% mercuric chloride) and solution C (5% potassium chromate) were all made using distilled water. Solution C was freshly prepared on the day the Golgi-Cox solution was prepared for use. Five parts of solution A and five parts of solution B were mixed. In a separate flask, four parts of solution C were diluted with 10 parts of distilled water. Then the mixed solution of A and B was mixed with the diluted solution C, while it was being stirred continuously. Then the combined solution was filtered using Whatman #42 filter paper, and the solution was stored at room temperature in aluminum foil wrapped flask.

Animal Procedure and Tissue Sectioning

Wild type, tottering, and leaner male and female mice were anesthetized with isoflurane and decapitated. The whole brain was rapidly removed and placed in a glass jar containing Golgi-Cox solution and a piece of gauze in the bottom of the jar. The volume of Golgi-Cox solution used was 50X the volume of the whole brain. The brains were stored in the Golgi-Cox solution at room temperature away from light exposure for approximately four to six months for neuronal fixation. After complete saturation of the brain was achieved, the brain was immersion fixed for 5 days at 4°C in 4% phosphate-buffered paraformaldehyde (pH 7.4) before embedding in paraffin. The paraffin-embedded brains were coronally sectioned at 60 µm, with the help of tissue softener solution (Molliflex™), and placed on plus-coated slides. The sections were

processed with 5% ammonium hydroxide to form a black precipitate in the stained neurons and then the sections were dehydrated through a graded series of ethanol, several changes of xylene and then mounted on glass microscope slides and cover-slipped with Permount.

Data Analysis

Three male and three female mice of each genotype were analyzed separately comparing males to females. Neurons with complete cellular and dendritic staining from Purkinje cells of the cerebellar cortex were selected for analysis. These neurons have a prominent tree-like dendritic branching outward from the body of the cell (Figure 2A). Approximately 10 neurons were analyzed for each mouse. Each neuron was traced using Image J (1.46r) NIH software (Abramoff *et al.*, 2004) before being analyzed (Figure 2B). The morphological parameters measured for each neuron were: (i) total dendritic length, (ii) branch order/complexity, and (iii) total area occupied by the soma or cell body. The complexity of dendritic arborization was obtained from Sholl analysis (Sholl 1953) by calculating the number of dendritic branches that intersect defined concentric circles spaced 25 μm apart starting from the center of cell body as a function of distance from soma. Sholl analysis was carried out using a Sholl Analysis "plugin" from the Ghosh Lab Website at UCSD: http://wwwbiology.ucsd.edu/labs/ghosh/software/ShollAnalysis_.class. Traced neurons were converted into 8 bit images using Image J (1.46r) NIH software, where the automated Sholl analysis software was added as plugin. The mean of the number of intersections at each circle was generated for further statistical analysis.

CHAPTER III

RESULTS

We averaged the measurements for all three mice for each sex and genotype. Through statistical analysis, we determined that there were no significant differences in values between male and female mice within each genotype for every measurement. Thus, we averaged the values for all 6 mice (3 male and 3 female) for each genotype to derive a single average value per genotype for every measurement.

Purkinje cells of leaner, but not tottering, mice exhibited significantly reduced average total dendritic lengths

Through Image J software measurement of dendritic length, we observed a trend towards reduced average total dendritic length for Purkinje cells of tottering mice and a significant, more severely reduced average total dendritic length for Purkinje cells of leaner mice when compared to Purkinje cells of wild type mice (Figure 3A). We were able to calculate that the difference between values for tottering and wild type mice was statistically insignificant, whereas the difference in values between leaner and wild type mice was statistically significant ($p < 0.05$). Additionally, the difference between values for leaner and tottering mice also was statiscally significant.

Purkinje cells of neither leaner nor tottering mice displayed significantly different average somatic areas from wild type

Using the Image J software measurement of somatic area (area of the cell body or soma), we saw a slight decrease in the average somatic area of Purkinje cells in tottering mice and a slight increase in the average somatic area of Purkinje cells in leaner mice when compared to Purkinje cells of wild type mice (Figure 3B). Although there were slight differences in values between genotypes, we determined that they were statistically insignificant.

Purkinje cells of leaner, but not tottering, mice showed significantly reduced complexity of dendritic arborization

In the Sholl analysis, we observed a trend towards a smaller average number of intersections between dendrites and concentric circles for Purkinje cells of tottering mice and a signficantly and obviously reduced average number of interesections in Purkinje cells of leaner mice when compared to those of wild type mice in the range of circles between 65µm and 165µm radii (Figure 3C). We determined the difference between values of leaner and wild type mice to be statistically significant ($p < 0.05$) within this range of concentric cirlces. This analysis essentially indicates significantly reduced complexity of dendritic arborization in Purkinje cells of leaner mice compared to wild type mice.

CHAPTER IV

CONCLUSIONS

In this study, we compared the morphological characteristics of Purkinje cells between three experimental groups of mice: wild type (+/+), tottering (*tg/tg*), and leaner (tg^{la}/tg^{la}). Interestingly, the results of this study reveal, for the first time, significant fundamental morphological differences between Purkinje cells in the cerebella of leaner mice and those in the cerebella of wild type mice. Specifically, overall dendritic complexity and development in leaner mice were reduced when compared to wild type mice. Similar, but not statistcally significant, changes in structural characteristics were also observed in Purkinje cells of tottering mice compared to wild type mice. Furthermore, the unexpected lack of significant difference in somatic size in Purkinje cells between all genotypes points more definitively to dendritic morphology as the fundamental difference.

The observations revealed in this study yield interesting insights concerning the role of Ca^{2+} in dendritic growth and development. Our findings correlate well with studies that have revealed a 40% reduction and a 60% reduction of Ca^{2+} current in tottering mice and leaner mice, respectively (Dove *et al.*, 1998, 2000; Wakamori *et al.*, 1998). Along with these previously published studies, our results showing a trend towards decreased dendritic length and arborization in tottering mice and a significant decrease in leaner mice with respect to dendritic length and arborization of Purkinje cells support the notion that Ca^{2+} influx into neurons is critical for neuronal development. In addition, our results also suggest that the previously

reported mutations in the P/Q-type voltage-gated Ca^{2+} channels (Lau *et al.*, 2004) are the most likely underlying cause of the morphological deficits observed in this study.

Since changes in neuronal function can often be correlated with changes in neuronal morphology, it is possible to conclude that the structural deficits seen in Purkinje cells of leaner mice may, in part, explain the symptoms (i.e.: absence seizures, cerebellar ataxia) exhibited by leaner mice. However, the fact that tottering mice also display the same symptoms as leaner mice, albeit with reduced severity, yet did not show significant signs of structural impairment of Purkinje cells insinuates that there may be a degree of neuronal functional loss present that is not reflected by obvious changes in neuronal structure. Hence, the significant deficits in morphology seen in leaner mice compared to wild type mice can better be attributed to the increased severity of the symptoms in leaner mice rather than the symptoms themselves.

In summary, our results indicate significant morphological impairment in Purkinje cells of leaner mice that cannot be ignored and that may possibly play a role in the phenotypic expression of the voltage-gated Ca^{2+} channel mutation. In other words, the reduced growth and arborization of dendrites seen in leaner mice could have possibly weakened the communication and function of the Purkinje cells. This finding in itself provides a path for further investigation regarding the biological cause of the observed structural deficits. Moreover, the failure of this study to reveal similar conclusive structural deficits in Purkinje cells of tottering mice should at least promote future studies with focus on a neuronal functional characteristics that could underlie any structural differences observed.

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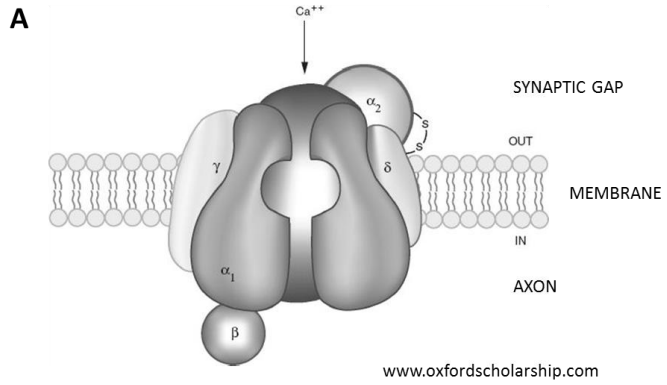
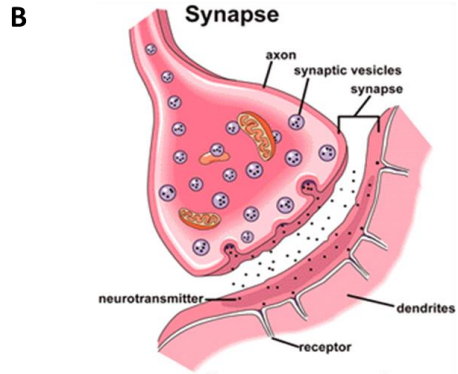
Figures:

Figure 1. Voltage-Gated Calcium Ion Channel and Synapse





http://www.tokresource.org/tok_classes/biobiobio/biomenu/nerves_hormones_homeostasis/index.htm

Figure 1: A) Anatomy of a voltage-gated Ca^{2+} channel. The biggest subunit, α_{1A}, is the pore-forming subunit and is the location of the mutations of focus in this study. **B)** Typical anatomy of a synapse. Voltage-gated Ca^{2+} channels (not depicted) would be embedded along the membrane of the axon terminal.

Figure 2. Purkinje cell

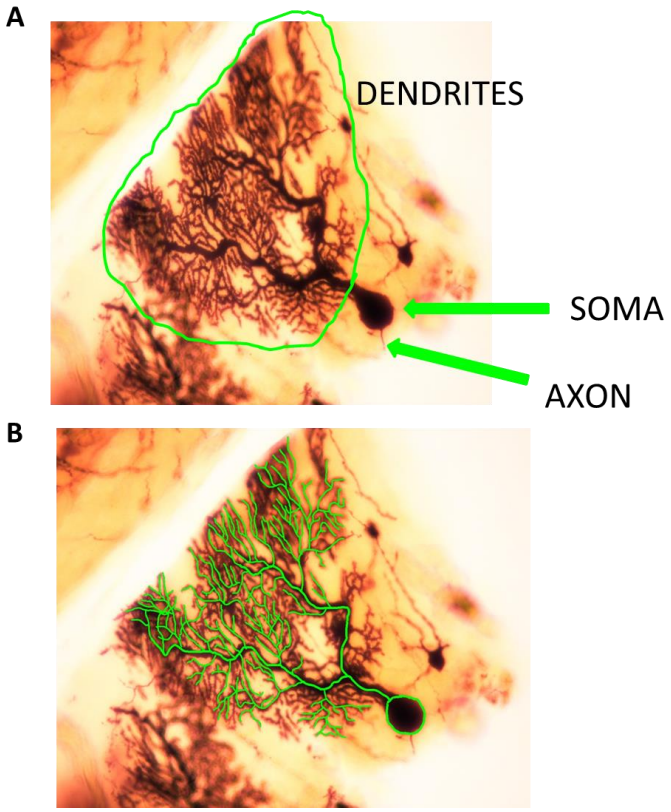


Figure 2: A) A Purkinje cell (stained black) as seen under the microscope. The soma and prominent tree-like branching of dendrites can be seen. Only a small part of the axon can be clearly seen in this image. **B)** Purkinje cell traced using Image J software. The tracing was used for all measurements and Sholl analysis.

Figure 3. Values for Dendritic and Somatic Measurements

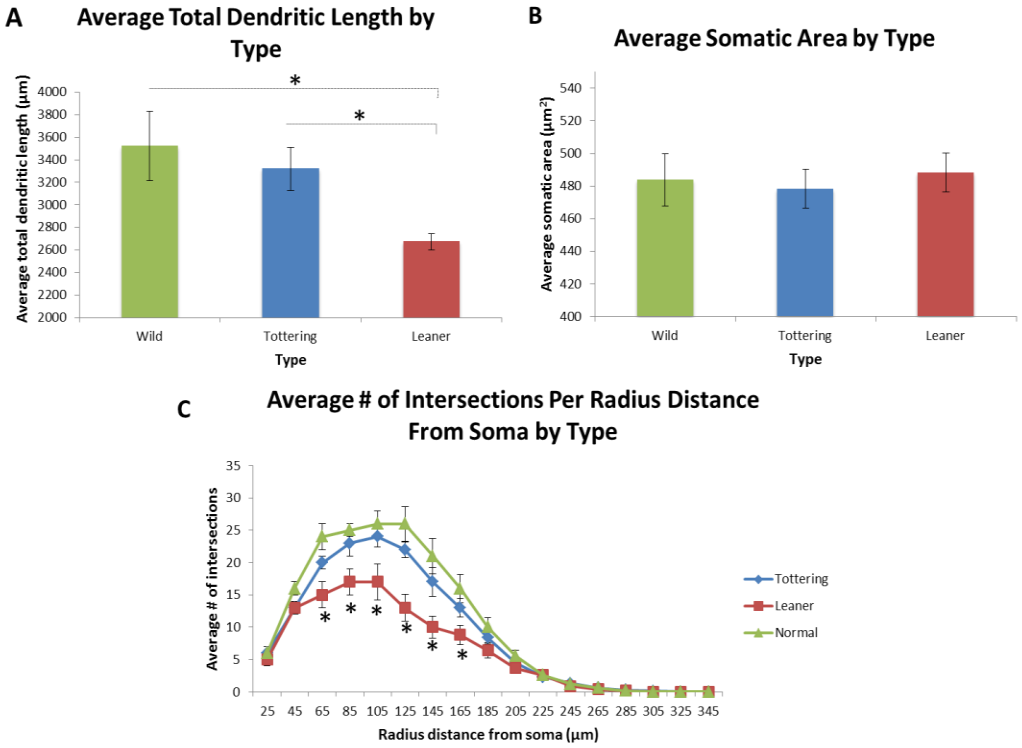


Figure 3: A) Average total dendritic length for the three mice types. Asterisks indicate statistically significant difference between values. **B)** Average somatic area for the three mice types. **C)** Average number of intersections of dendritic branches with defined concentric circles at different distances from the soma (Sholl analysis). Asterisks indicate values significantly different from wild type. One way ANOVA and Tukey post-hoc were used for all comparisons (*, $p \leq .05$).