8-1-2017

The vascular aging of the cerebellum

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The Vascular Aging of the Cerebellum

Chris McElroy, B.S.

THESIS

Presented to the Graduate Council of the
Graduate School of Biomedical Sciences
University of North Texas
Health Science Center at Fort Worth
in Partial Fulfillment of the Requirements

For the Degree of
MASTER OF SCIENCE

By

Christopher L. McElroy, B.S.
Fort Worth, TX
July 2017
ACKNOWLEDGMENTS

I would like to thank Dr. Kunlin Jin for graciously allowing me into his lab. He has been a very patient mentor and allowed me a great deal of autonomy. He has also guided me through many difficulties and roadblocks. His advice has allowed me to become a better critical thinker, problem solver, and overall scientist. The help he has provided is greatly appreciated, and undoubtedly, will be of great benefit in the future.

My committee members, Dr. Meharvin Singh, Dr. Tom Cunningham, Shaohau Yang, and Andras Lacko, have provided immense support. They have taken the time to sit with me and answer my many questions that has allowed me to overcome several issues. They have given suggestions and encouragement beyond what would be considered normal. Without their help I would be lost.

I couldn’t have done this without my lab mates Changhong Ren, Brian Wang, Johnny Hu, Lefu Chen, Hongxia Zhang, Chenqi Yang, and Jixian Wang also. They have helped give me advice and have helped me to develop and learn new skills.

Finally, I would like to thank my mother, C.J. McElroy, and brother, Michael McElroy, for always being there for me. I would not have been able to get to this point without their support. They have provided wonderful support, have listened to my many frustrations, have encouraged me always, and shown great patience and understanding.
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SPECIFIC AIMS

LONG TERM GOALS:

To identify structural and functional changes in the vasculature of the cerebellum caused by aging which may contribute to its physical and functional deficits and to eventually discover why these changes take place and whether not some or all of these changes could be attenuated.

OBJECTIVE:

Determine if aging causes any significant changes to the structure and functional of the vasculature in the cerebellum of mice.

HYPOTHESIS:

The vasculature in young mice will have a greater surface area, volume, length, diameter, amount of branching, and blood flow compared to aged mice.
AIM #1: Identify vascular structural changes in the mouse cerebellum during the aging process through traditional 2D immunofluorescence and 3D methods using CLARITY technology in the cerebellum and the hippocampus.

AIM #2: Find whether or not there is a functional difference of the vasculature in the cerebellum caused by aging using Laser Doppler Flowmetry.

LIST OF ABBREVIATIONS

AD- Alzheimer’s disease
BOLD MRI- blood oxygen level dependent magnetic resonance imaging
CCT- cuneocerebellar tract
CSF- cerebrospinal fluid
D1- dopamine 1
D3- dopamine 3
DCST- dorsospinocerebellar tract
diSia- 8-linked diSialic acid
DTI- diffusion tensor imaging
FEF- frontal eye field
fMRI- functional magnetic resonance imaging
HSF1- heat shock factor 1
M1- motor cortex
MMLD- mean median luminar diameter
PCT- pontocerebellar tract
PD- Parkinson’s disease
PET- positron emission tomography
PICA- posterior inferior cerebellar artery
PSEA- Population Specific Expression Analysis
SCA- superior cerebellar artery
ST8siaIII- 8- sialtransferase III
Topo IIβ- Topo IIβ
VCT- vestibulocerebellar tract
VOR- vestibule-ocular reflex
WML- white matter lesions
CHAPTER I
INTRODUCTION AND BACKGROUND

Recent Progress in the Study of the Aging Cerebellum

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1.2 Anatomical Structure of the Cerebellum and its Cellular Makeup
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Abstract

The cerebellum plays an important role in the aging process, as it is well known that with the aging of the cerebellum that there is a decline in balance and motor function, particularly fine motor skills. In recent years, there have been numerous discoveries on the role of the cerebellum in general. It has several roles beyond balance and fine motor skills, such as cognitive function, memory, and it plays a role in many neurodegenerative diseases.

The cerebellum ages in a more accelerated fashion compared to other brain regions such as the hippocampus. It is important to understand why and how that is. In this review, we will summarize the cerebellum and its function, and will also look in depth in how the cerebellum ages on a cellular, molecular, and functional level. Also, it will look at a possible vascular mechanism as well as exploring the role of the cerebellum in recent research on diseases such as Alzheimer’s, Parkinson’s, Multiple Sclerosis, and the effects of alcoholism on the aging cerebellum.

Key Words

Cerebellum, Aging, Alzheimer’s, Parkinson’s, Multiple Sclerosis, Alcoholism
Introduction

History

Around 335-280 BC Herophilus recognized that the cerebellum is a distinct part of the brain (Holmes, 1939). Galen around (131-200 A.D) later described the vermis as a wormlike outgrowth. The first detailed description of the cerebellum however was by Malacarne in 1780. He created several of the terms to describe the cerebellum we still use today such as pyramid, tonsil, lingual, and uvula (Holmes, 1939, Glickstein et al., 1987). Determining the function of the cerebellum is on-going but was firstly described through lesion studies. Rolando in 1809 removed the cerebellum in several species of animals and recorded how this affected voluntary movements; he noted that it does not affect sensation (Holmes, 1939; Ito, 2002). Flourens in 1822 saw that animals moved spontaneously but not smoothly and lacked coordination. In 1844, he further reported stiff legged locomotion and, in birds, there was retraction of the head when the cerebellum was destroyed (Holmes, 1939; Ito, 2002). In 1902 Babinsky described dysmetria as being a particular pathology of the cerebellum. Dysmetria can be tested by trying placing one's finger on their nose with eyes closed. Individuals that have cerebellar damage cannot do this very accurately (Ito, 2002). After World War I, Holmes observed slower voluntary arm movement on the side where the cerebellum was damaged by a gunshot wound (Ito, 2002). He also described four main functions of the cerebellum as shown through lesions and resulting dysfunction:

“1) Postural hypotonia and impairment of certain reactions of the toneless muscles; 2) mild degree of asthenia and fatigability of the muscles; 3) abnormality of rate, regularity and force of voluntary movements, and 4) failure of certain associated movements (Holmes, 1939).”
**Cerebellar structure and Function**

**Gross Structure of the Cerebellum**

The cerebellum can be divided into three different major sections depending on the input. The largest section is the cereberocerebellum and makes up most of the lateral cerebellar hemisphere. It receives its input from several parts of the cerebral cortex. This part of the cerebellum has to do with complex movements. It includes the planning of intricate spatial and temporal sequences which includes speech (Purves et al., 2001; Ackerman et al., 2007). The vestibulocerebellum is phylogenetically the oldest part of the cerebellum and is composed of the caudal lobes that include the flocculus and nodulus. It receives input from the vestibular nuclei and mainly regulates posture. The spinocerebellum is the subdivision that directly obtains input from the spinal cord. The lateral portion of the spinocerebellum is involved with distal motor movements, and the middle, the vermis, is involved with the eye movements and other close muscle groups (Purves et al., 2001). This is only a very basic breakdown of the cerebellum, but it is a starting place how it has been understood for a long time (Fig 1).

Recently there are studies linking the subsections of the cerebellum to other non-motor related functions. It is hypothesized that the anterior lobe and lobule VIII represents the sensorimotor part of the cerebellum. Also, lobules VI and VII of the posterior lobe makes up the part of the cerebellum dealing with cognition, and the posterior vermis has involvement with the limbic system (Stoodly and Schmahmann, 2010). The review of Stoodly and Schmahmann (2010) explains this in exhaustive detail.

**Anatomical structure of the Cerebellum and its Cellular Makeup**
The cerebellum is a complex machine. One way to have a better understanding of how it works is going through it by each layer, starting with the inferior olive which, of course, is not part of the cerebellum but inherently involved. The inferior olive is well known to provide the cerebellum with climbing fibers and can act as a timing mechanism for movement (Duo et al., 2006; De Zeeuw et al., 1998). These fibers go through the inferior peduncle to the cerebellum. The inferior peduncle also consists of the dorsospinocerebellar tract (DSCT), the cuneocerebellar tract (CCT), and the vestibulocerebellar tract (VCT). The middle cerebellar peduncle contains the pontocerebellar tract (PCT), and lastly the efferents of the cerebellum in the superior peduncle come from the cerebellar nuclei eventually going to the thalamus (Harting, 1997). The inferior olive is the sole source of climbing fibers that are excitatory synapses on Purkinje cells; it also synapses to the deep cerebellar nuclei. The axons of the DSCT, CCT, VCT, and PCT are mossy fibers that pass through the cerebellar white matter and are excitatory synapses onto the granule cells in the cerebellar cortex. In turn the granule cells have parallel fibers that run laterally in the cerebellar cortex and have weak excitatory synapses onto the dendrites of the Purkinje cells which, with enough synapses, will cause an action potential and a simple spike, rather than the climbing fibers which causes a complex spike. There are also inhibitory cells in the cortex of the cerebellum; Golgi cells, and basket cells, are both excited by parallel fibers. The Golgi cell inhibits the granule cell dendrites which is called feedback inhibition. The basket cells axons terminate and inhibit the soma of the Purkinje cells directly which is called feedforward inhibition (Harting, 1997). Also there are stellate cells, aspiny stellate cells are inhibitory, and spiny stellate cells are excitatory onto the dendrites of the Purkinje cells (Stellate Cells-Neuronbank, 2009) (Fig 2). Finally, the Purkinje cells project to the deep cerebellar nuclei, different nuclei for different zones. The lateral zone Purkinje neurons project to the dentate. The
interpositus gets its’ projections from the intermediate zone. The fastigial nucleus gets its projections from the medial zone. Lastly, the floculonodular zone projects to the “surrogate” deep cerebellar nucleus called the vestibular nuclei (Harting, 1997). This review will discuss mainly about how each of these cellular components (Purkinje cells, stellate cells, Golgi cells, and basket cells) are affected by the aging process.

Interconnections between the Cerebellum the Cerebrum

Another important aspect of the cerebellum is its interconnections with other parts of the brain. It was thought to that the cerebellum acted as a funnel for cortical areas into the motor system, but the cerebellum has projections to many of the same cortical areas that send projections to it (Middleton and Strick, 2000). Middleton and Strick (2000) injected HSV1 into the arm portion of the motor cortex (M1) in primates; the interpositus and the dentate of the cerebellum were labeled 5 days later. A similar experiment was done to the ventral premotor area and the supplementary area, and they concluded that the cerebellum, particularly the dentate, has projections towards these areas also. Injecting HSV1 into the Frontal Eye Field (FEF), the prefrontal cortex, and the cerebellar thalamocortical pathway which is part of the Walker’s area involved in working memory all showed connections to the dentate of the cerebellum (Middleton and Strick 2000; Dum and Strick, 2003).

Through its interconnections, the cerebellum has also been found to mediate non-motor functions such as working memory and tactile sensation. A functional MRI (fMRI) study revealed that superior cerebellar activation was involved in articulation of speech while inferior cerebellar activation would be for phonological storage. This could bring a feedforward command to the frontal lobe completing a phonological loop (Chen and Desmond, 2005;
Desmond, 1997, Helmuth et al, 1997). Also, the cerebellum has efferent and afferent projections directly to and from the lateral and posterior parts of the hypothalamus. Another non-motor function of the cerebellum has to do with tactile sensation. The cerebellum has several projections to the red nucleus (Flumerfelt et al., 1973; Stanton, 1980). A study found that where there was contralateral coactivation of the right red nucleus with the left dentate nucleus of the cerebellum, and this activation was much greater with tactile discrimination than simply moving a finger (Liu et al., 2000). In lieu of all these connections, probably the most interesting and recently studied interaction and connection of the cerebellum is between it and the prefrontal cortex. This is important because the aging cerebellum could result in deficits in cognition.

**Cognitive Theories of the Cerebellum**

Shmahmann and Pandya (1995) injected tritiated amino acids proline and leucine, which moved in retrograde fashion the lateral medial and ventral parts of the prefrontal cortex. Using an autoradiographic technique, they found that the majority of the pontine efferents were from the dorsolateral and medial prefrontal cortex, with fewer efferents in the ventrolateral portion. This is important, because the feedforward pathway of the cerebrocerebellar system passes through the corticopontine pathway (Shmahmann and Pandaya, 1995). These parts of the prefrontal cortex have implications in higher functioning. The prefrontal cortex is separated into different functional regions. The dorsolateral and medial portions are associated with kinesthetics, motivation, and spatial memory. The inferior prefrontal and orbital areas are responsible for emotional and autonomic response inhibition, stimulus significance, object recognition, and memory (Goldman-Rakic and Friedman, 1991 as quoted in Schmahmann and Pandya, 1995). In 1996 Schmahmann proposed five rules for governing the relationship between the cerebellum and cognitive thought: (1) The incorporation of the limbic and associative regions in the
cerebrocerebellar circuit is part of the cerebellar contribution to cognitive thought and emotions. (2) There is a topographic organization of the cerebellum related to cognition and behavior. (3) Many afferents to the cerebellum from cerebral associative areas helps the cerebellum to regulate supramodal functions. (4) The cerebellum contributes to cognition through modulation. (5) The calculations that the cerebellum does for the sensorimotor movement are the same for the associative and paralimbic functions also (Schmahmann, 1996; Schmahmann and Pandya, 1997). These interconnections with the cerebellum and other portions of the brain, along with the typical motor learning and motor movement aspect, make it an important structure to take note of in the aging process.

**Functions of the Cerebellum**

There are certain important observations that reflect the most basic functions of the cerebellum; these would including posture, smooth voluntary movement, and gait.

Posture is an important aspect that includes muscle tone. Holmes had described through examining individuals with cerebellar lesions as having “postural hypotonia,” or decreased muscle tone (Holmes 1939). There was a particular study that looked at five groups of individuals; each group having a different cerebellar lesion or affliction of some sort. The individuals that had damage to the vermis, the spino-cerebellar afferents, or vestibulo-cerebellum showed that they had a higher sway, improper balance, when asked to stand still with their arms crossed and feet 4cm apart. This was seen when asked to close their eyes rather than have them open except for the vestibulo-cerebellar lesioned individuals which showed that balance was not stabilized by vision as were the others. Also, there was no difference between individuals with
damage to the cerebellar hemispheres and normal individuals as far as balance was concerned (Diener et al. 1983).

Smooth voluntary movement is something that is also attributed to the cerebellum in the way of the cortico-thalamo-cerebellar pathway. There is a lot of speculation about how exactly this pathway controls movements and the involvement of the cerebellum in the pathway. A great review of some of the past theories of how the cerebellum controls different types of motor movement is in a review by Horne and Butler (1995). Another study puts more onus on the cerebral volume having more emphasis on fine motor skills rather than the volume of the cerebellum. The smaller the cerebrum through atrophy caused by aging, the worse the spiral drawing skills were in aged individuals (Hoogendam et al., 2014).

Gait is an important and well known aspect of the cerebellum. It includes our posture and our ability to recognize our limbs in space as well as timing. A very simple study by Stolze et al., (2002) compared healthy individuals with those with moderate to mild cerebellar damage. Two simple tests were conducted, normal locomotion and tandem gait. Both were measured using three dimensional infrared movement analysis system. In the tandem gait experiment individuals were asked to put one foot in front of the other on a red tape line attached to a treadmill. The individuals with cerebellar disease showed a high variability in gait. The intonation was slower for the individuals with cerebellar disease and they had longer contact with the ground. Also, step width was increased in the afflicted patients to try to improve stability.

One of the main functions of the cerebellum found later is motor learning. This is an amazing aspect of the cerebellum considering that our declarative memory can easily be outdone
by most basic computers these days, but the ability to learn and express procedural memories cannot even be matched by the most advanced robots using supercomputers (De Zeeuw and Brinke, 2015). To gain these procedural memories there are two types of coding to processes for motor learning. “Rate coding,” is the main information coding device, while “temporal coding” is spikes in synapses that occur with millisecond accuracy (De Zeeuw and Brinke, 2015; Boyden et al., 2004; Ito et al., 1982; Lisberger, 2009; Walter and Khodakhah, 2009).” Rate coding can be better explained using the Mars- Albus model where the climbing fiber provides the strength regulating signal to the parallel fibers and thus the Purkinje neurons (Boyden et al., 2004; Lisberger, 2009). Albus was actually more accurate in his version of the model, which was proven later, where the climbing fibers actually cause long-term depression in Purkinje cells (Walter and Khodakhah, 2009; Ito et al., 1982).

The model proposed by Albus can be seen in a simple model of the vestibule-ocular reflex (VOR) wherein the eyes moving in the opposite direction of the head, automatically stabilize vision in relative movement of the head in space (Ito, 2002). This is done by the flocculus, which receives primary and secondary signals through mossy fibers and in itself has afferent and efferent signals to and from the flocculus (Ito, 2002). The VOR is important as a means for many studies to test cerebellar function or dysfunction.

There are two major types of motor learning, supervised and reinforced, and these are typically studied individually (Therrien et al., 2011). The field is just beginning to understand how these processes are carried out behaviorally and neurally (Therrien et al., 2011). Error based learning is a type of supervised learning. A good analogy of this would be shooting a basketball at 12 feet instead of 10. One would not be very good at this initially; the motion would be full of errors. Through repetition these errors are corrected and the shot becomes more accurate. In
other words, there are deviations to a predicted movement and these errors are corrected through practice (Therrien et al., 2011; Shadmehr and Mussa-Ivaldi, 1994; Krakauer et al., 2000). Reinforced learning relies on scalar measures that predict success or failure. A lot of real world movements like walking or making a swing go higher do not have errors in each point in time (Therrien et al., 2011). There is an experimental example of this where the hand is covered by a screen and there is a cursor underneath, and when the hand moves straight the cursor moves slightly to the left. There is a target one must hit by adapting to the cursor movements. Now one group was given visual feedback whether or not they hit the target but none on their movement. The other had feedback on their movements as well as whether the target was hit. This allowed for error based learning in the second group. In the rewarded, reinforced learning, group the question posed was whether or not adaptation of motor command would take place without changes in the motor-sensory map (Izawa and Shadmehr, 2011). Reinforced learning is spared in individuals with cerebellar damage, and even in healthy adults the retention rate of learning is better in reinforced learning than in error based learning (Therrien et al., 2015).

Motor learning works by including the contralateral primary motor and premotor cortical areas to the side of movement, and with increased number of neurons involved, along with the ipsilateral cerebellum, can increase “motor acuity.” This is defined as the swap of speed to accuracy in doing a task (Shmuelof, 2014). This increase in neuron recruitment can increase signal to noise ratio and in turn improve feedback corrections. This is a type of motor skill learning not an adaptation. Motor skill learning is the aforementioned swap of speed and accuracy without perturbation (Shmuelof, 2012).

**Cerebellar Aging**
How Aging affects the Gross Structures of the Cerebellum

When the cerebellum ages it starts to shrink in volume as a whole in the mid-fifties of humans, and it shows earlier senescence than the hippocampus (Raz et al., 2005; Woodruff-Pakk et al., 2010; Bernard and Seidler, 2014). The vermis is significantly affected by age along with lobules VI, Crus I, Crus II, and VIIb, and, although it was suspected, it does not significantly affect cognitive decline when controlling for the prefrontal cortex (Abe et al., 2008; Paul et al., 2009; Bernard and Seidler, 2013;). Another study concluded that in men there was a correlation between gray matter of the cerebellum’s vermis and cognition but not with women (Hogan et al., 2010). In addition, a smaller cerebellar volume was seen to correlate with a decline in processing speed, memory, and visual reproduction (MacLullich et al., 2004). Looking particularly at the lobules as a whole, the anterior lobules and Crus I were most significantly affected by aging (Bernard and Seidler, 2013). In one study, the medial hemispheres are not significantly affected by age except for the inferior portion. The lateral portions of the cerebellum were not affected at all (Luft et al., 1999). This is in contrast to the findings of Raz et al. (2003) where the cerebellar hemispheres aged and shrank faster than expected. Also, looking at the white matter using Diffusion Tensor Imaging (DTI), it was suggested that the degraded white matter involving the frontocerebellar and parietocerebellar circuitry can damage the interaction between these brain regions and could relate to a decline in cognitive function (Sullivan and Pfefferbaum 2006).

Procedural learning and non-verbal working memory are associated with reduced volume of the hemispheres of the cerebellum (Raz et al., 2000). It is known that the eye-blink conditioning is also a form of implicit learning that may be attributed to the cerebellum (Bernard and Seidler, 2014). In one study a tone is signaled followed by a puff of air to the eye. After a time there is an anticipatory eye-blink to the tone. There are fewer anticipatory eye-blinks in
older individuals than there are in younger individuals. The older individuals show a correlation between the smaller cerebellar volume and having a weaker link between the tone and the air puff considering anticipatory blinks (Woodruff-pak et al., 2001; Woodruff-pak et al. 1988).

**How Aging affects the Cerebellar Cellular Components**

Aging affects parts of the brain differently. Some regions like the cerebellum have significant neuron loss while other brain regions like the primary motor cortex, primary visual cortex, prefrontal cortex, hippocampus, and entorhinal cortex do not (Peters and Kemper, 2012). Relevant to the cerebellum, the inferior olive does not significantly lose neurons with age (Sjöbeck et al., 1999). There is also no significant age difference in pontine or cerebellar white matter according to a diffusion tensor imaging (DTI) study (Sullivan et al., 2008). More research needs to be done on the synaptic connections of mossy fibers and climbing fibers, but the research thus far suggests that they are not affected nor do they contribute to the aging process. A stereological study reported that the anterior lobe of the cerebellum was the most affected by age with a 40.6% loss of neurons and a 40.9% loss of Purkinje cells in the same area. The overall loss of granule cells in the entire cerebellum was 12.7% and 11.7% for Purkinje cells. Contrary to the DTI study, there was a decrease in white matter (26%) with aging (Anderson et al., 2003). Another stereological study by Tang et al. (1997) showed that there was no significant difference in total white matter volume, but there was a significant difference in total length of the myelinated fibers and the diameter of the myelinated fibers. The diameter increased with age. Using mice up to the age of 31 months a 30% Purkinje cell loss was observed and only the pontocerebellum showed significant loss of granule cells (Sturrock, 1990). Although that is true, the parallel fibers lose up to 79% varicosity in the Fischer 344 rat cerebella (Huang et al., 1999). Interestingly enough there are few studies that address the connections of the climbing fibers and
its role in aging. The Golgi II neurons, at least in an aged mouse model, do not change in number in any part of the cerebellum (Sturrock 1990). There are few studies that looked at the number of basket cells, which tend to decrease with age. There is one study in particular that investigated the changes within the basket cells with age, and it was shown that there was an increase in the volume of Golgi, dense bodies, and ground substance, and a significant decrease of rough endoplasmic reticulum surface area, which suggests a decrease in protein synthesis (Sturrock 1989; Henrique et al., 2001). This study is important, because it points out that there may be differences within other cells apart from the cell number and synapses that could affect function.

Purkinje cells are the most vulnerable cells to aging (Egrova et al., 2016; Symanski et al., 2014; Woodruf-Pak et al., 2010; Anderson et al., 2003). This can be attributed to them being the sole output of the cerebellum (Zhang et al., 2010). Also, it’s because of the importance of Purkinje cells during the aging process on not only motor skills but motor learning (Hilber and Caston, 2001, Mattay et al., 2002) and even intellect (Lee et al., 2005). Older studies studying rats argue that there is not a significant difference in Purkinje cell loss, but more recently as exhibited in Anderson et al. (2003) have shown that there is a substantial loss of Purkinje neurons at least in the anterior lobe (Drüge et al., 1986; Bakalian et al., 1991). There are many circumstances that could cause the age related loss including impaired L-glutamate catabolizing mechanisms in the cerebellar cortex of rats (Felici et al., 1989), loss of neuroglobin (Sun et al., 2005), and even the loss of interaction with parallel fibers (Huang et al., 1999). Not only is the cell number affected by the aging process but also the morphology of the cells themselves.

The soma of Purkinje cells can deteriorate up to 33% in the human cerebellum (Andersen, 2003). There is a restructuring of the dendritic tree of Purkinje neurons also (Quakenbush et al., 1990; Hadj-Sarhaoui et al., 2001; Zhang et al., 2006). This decrease, in
particular the terminal segments of the dendritic tree, reduces the exchange of synaptic
information, which may contribute to the loss of the molecular layer in the aged cerebellum
(Zhang et al., 2006; Zhang et al., 2010). The reason for this is not well understood. It is supposed
that the parallel fibers from the granule cells decrease their inputs on the Purkinje neurons
(Dlugos and Pentney, 1994; Huang et al., 1999, 2006). It has been reported that there are even
organelles that change within the aged Purkinje neuron. One in particular is the mitochondria,
which decrease in number with age and could affect cellular function (Fattoretti et al., 1996).
Succinic dehydrogenase is reduced in aged mitochondria along with the volume and numbers of
mitochondria themselves (Fattoretti et al., 1998). The nucleus becomes pyknotic and the
nucleolus is also reduced, as well as disrupted Cajal bodies which reduce in volume in the aged
cerebellum (Ogata et al., 1984; Baltanas et al., 2011). These deficits in the organelles may
contribute to cellular death and dysfunction. Synaptic plasticity is another factor that could cause
Purkinje cell death. A 33% reduction in Purkinje cell dendritic spines were seen in some older
studies (Glick and Bondareff, 1979; Huang et al., 1999).

**Functional Deficits Attributed to the Aging**

By 2030 1 in 5 U.S residents will be over the age of 65, and those individuals will face a
decline in sensorimotor control (Seidler et al. 2010; U.S. Census data). In aged individuals there
is coordination difficulty (Seidler et al., 2002), loss of fine motor control (Contreras-Vidal et al.,
1998), slowing of choice reaction time (Woodley et al., 2015), and balance and gait issues
(Woollacot and Tang 1997). A simple explanation that there is early cell death in the cerebellum,
particularly in the anterior lobe, is that it disrupts the ability to predict outside and inherent forces
acting on the body (Boisgontier, 2015). In part, the issues with movement in aging is due to the
periphery such as sensory receptors, muscles, nerves, and joints (Seidler et al., 2009). Several
unimanual and bimanual tasks were done to 217 adults to evaluate the relationship between cerebellar morphology, age, and manual motor performance taking into consideration cerebellar white and gray matter volume. In this study, it was observed that there was a correlation of gray matter and white matter volume with dexterity, grip strength, and finger tapping performance declining with smaller white matter and gray matter volumes (Koppelmans et al., 2015).

The cerebellum does not act on its own when performing motor movements. There are two projected motor movement models. The forward model control system imitates the dynamic controlled object (CO) properties in which the error signals are brought about by the output and filtered through the sensitivity system. It then goes to the inferior olive to the forward model of the cerebellum where the signal is modified. There is also the inverse model, which has the feedback of the error signals coming directly from the command signals derived from the motor or prefrontal cortex (Ito, 2008). It is suggested that, in advanced age, forward models would decline particularly, although the inverse model may also be damaged (Bernard and Seideler, 2014).

Possible Underlying Mechanisms Contributing to Cerebellar Aging

Molecular Aging of the Cerebellum

There are many molecular mechanisms that can affect the aging process of the cerebellum, and there are several very interesting recent discoveries. One of these studies refutes the hypothesis that the cerebellum is one part of the brain that deteriorates the quickest (Raz et al., 2005). Instead of the decreased rate of degeneration around 50 years of age, this study looked at individuals above 110 years of age and found that the epigenetic clock, which is an evaluation of DNA methylation through aging, was less in the cerebellum than other brain regions. In other
words, epigenetic age which was also called DNAm has a strong correlation with chronological age in brain tissue, and the cerebellar samples showed less DNAm change compared to non-cerebellar samples. (Horvath et al., 2015).

With aging, the cerebellum has several changes in the metabolic activity and neurotransmitter synthesis with aging. A proteomic screening and immunohistochemistry study on Ercc1 knock-out mice showed that aging affects the expression of many proteins in the cerebellum including proteins involved in neurotransmitter reception (ie. mGluR1, GluRδ2, GABABR1, and GABABR2), signaling molecules (ie. GlurRδ2, Delphilin, and IP3R1), and receptor-signal transduction scaffolds (ie. cGK1, PKCγ, Ahrgef33, RGS8, TN-C, and Ppp1r16b) (de Graff et al., 2013).

DNA repair also suffers in senescence. Topoisomerase IIβ (Topo IIβ), a DNA repair enzyme, is not expressed as highly in aged individuals. On the other hand, several genes are upregulated in senescence that code for inflammatory cytokines and are called senescence-associated secretory phenotype (Davalos et al., 2010). It was reported that genes Slit2 and Npy gradually increase during aging and that the suppression of these two genes increased the viability of in vitro cerebellar granule cells along with the upregulation of Topo IIβ (Gupta et al., 2015).

Further, presenilins are crucial proteins in the aging process. The mutation in presenilins can lead to Alzheimer’s disease, and the differential expression of presenilins in normal aging in the cerebellum contributes in deficits in motor coordination during “healthy” aging (Kaja et al., 2015). This could be a potential therapeutic target. Although, healthy aging involves more than just a couple of proteins being differentially expressed.
An additional study looked at Lipins-1 and -2; they are involved in the synthesis of phosphatidate phosphatase, triglycerides, and phosphatidylethanolamine. Lipin-2 in particular, with concomitant reduction of lipin-1 with age, can cause ataxia in lipin-2 knockout mice (Dwyer et al., 2012). One interesting approach to elucidating the molecular profile of the aging cerebellum is using a method called Population Specific Expression Analysis (PSEA), which is very useful in heterogeneous samples, because it uses genetic profiles to separate cell populations. Using this technique one study found that the astrocyte population showed differential expression of 50 genes in the aging brain (Kuhn et al., 2012). One reason for this differential expression could be the decrease in the endonuclease Dicer which is critical for producing miRNA’s. Ablation of Dicer in Purkinje cells led to the reduction miRNA’s and caused Purkinje cell death and eventual ataxia (Schaefer et al., 2007; Persengiev et al., 2012). These studies demonstrate that genetics are not the only factor affecting the changes caused by aging in the cerebellum.

Sialic acid containing glycoproteins are also affected. In particular, α2, 8-linked disialic acid (diSia), which can attach to glycoproteins such as NCAM or CD166 via α2, 8-sialtransferase III (ST8SiaIII), are involved in cell adhesion and neurite growth. diSia epitopes and ST8SiaIII are both reduced in the aging cerebellum of senile mice (Rinflerch et al., 2012; Sato et al., 2002). This reduction is evident in mice with ataxia and is one consequence of the aging cerebellum. Looking at the cerebrospinal fluid (CSF) of individuals with ataxia, one study revealed that free sialic acid was increased. This could be a possible biomarker for individuals that do not have other obvious issues that could cause ataxia. Unfortunately, sialic acid is only present in the CSF, considering how invasive the procedure is, it is not the best diagnostic option (Mochel et al., 2009).
There several ways to protect oneself from the ravages of the aging process. One of these means of protection could be a calorie restricted diet. This particular study is not necessarily recent, but it shows that we may have some influence on how we age. The mRNA expression for the α2 subunit of the GABA<sub>A</sub> receptor in particular, is significantly decreased in aged rats but not so in calorie restricted aged rats (Mhatre and Tiku, 1998). Another positive is that Mcl-1 is neuroprotective for cerebellar granule cells. However, this protein is phosphorylated and ubiquinated with age. This leaves cells more susceptible to DNA damage and apoptosis. There is a therapeutic means of stopping this degradation by inhibiting JNK. This inhibitor has been shown to be protective against dopaminergic neuron loss and could also probably do the same for cerebellar neurons (Magieri et al., 2013; Ekholm-Reed et al., 2013). Parkin degrades Fbw7β which is a SCF substrate adaptor which can ubiquinate Mcl-1. Increasing parkin in turn increases Mcl-1. This is also a therapeutic target, because parkin can be increased by diaminodiphenyl sulfone which could help increase neuron survivability (Lee et al., 2016; Ekholm-Reed et al., 2013).

**Vascular Aging of the Cerebellum**

The macro- or microcirculation of the brain is susceptible to aging as is the vascular system of the rest of the body. In the cerebrum, there is a failure of several controls such as autoregulation, neurovascular coupling, the BBB leakage, reduced cerebrospinal fluid, and lack of vascular tone that can contribute to neurodegeneration (Kalaria, 2010). Few reports are available with respect to direct effect of aging on cerebellar blood vessels. One study by Akima et al. (1998) compared the vascular aging of the cerebrum compared to the cerebellum. One particular difference that was seen in a few of the advanced aged cerebellum was an intertwining or ropelike appearance of arteries in the cerebellum. This was much more pronounced in the
cerebrum (Akima et al., 1998). As far as function of these blood vessels, the blood flow and oxygen consumption through positron emission tomography (PET) scan was studied on young and aged individuals. It was concluded that there was no significant difference in blood flow or oxygen concentration in the cerebellum between young and old individuals (Marchal et al., 1992). Some older studies have also come to the same conclusion, but no recent work has been done that could dispute their results (Yamaguchi et al., 1986; Leenders et al., 1990). This is why this current study is important. It employs new techniques to reevaluate the question of whether or not there is any vascular differences that could account for some of the physical or functional deficits in the cerebellum.

Cerebellar Compensations in Aging

Another factor to consider with increased age is how differently the brain, including the cerebellum, participates in motor movements. In aged individuals the accuracy of pressing the button in simple button press motor task is not something that declines; it is the reaction time that is affected. The brain actually compensates by adding more cortical and subcortical regions for simple motor tasks (Mattay et al., 2002). In a study by Wu and Hallet, (2004) automaticity was tested between aged and young individuals. In both groups similar areas of the brain were activated when learning the directed finger movements and achieving automaticity, but aged individuals had a higher activity in the bilateral anterior lobe of cerebellum, premotor area, parietal cortex, left prefrontal cortex, anterior cingulate, caudate nucleus and thalamus, and employed more areas, comprising the pre-supplementary motor area and the bilateral posterior lobe of cerebellum (Wu and Hallet, 2004). This means that when one age’s simple tasks aren’t particularly simple anymore. It takes more brain activity to try to perform the same tasks as a young adult and still the results decline. It would be interesting to see if this increased
compensatory activity actually creates more errors in coordination between the cerebellum and other sections of the brain.

**Cerebellar Involvement in Diseases**

**Alzheimer’s Disease**

The cerebellum is unique in how it reacts to different diseases. Fairly recently more attention has been paid to the cerebellum and its involvement in several different diseases such as Alzheimer’s, Parkinson’s, multiple sclerosis, hypertension, and alcoholism.

In Alzheimer’s disease (AD) there are amyloid β plaques in the molecular layer of the cerebellum in Braak stage 3 of Alzheimer’s patients (Serrano-Pozo et al., 2011). For reasons yet unknown the cerebellum is spared from neurofibrillary tangles. The fact that there are plaques in the cerebellum may perhaps just be a symptom of advanced AD, but it is arguable whether there are functional consequences because of this (Larner, 1997). On a cellular level there seems to be a loss of Purkinje cells in the AD brain, and which can be salvaged through the upregulation of heat shock factor 1 (HSF1) which in turn increases the expression of heat shock proteins 60, 70, and 90 (Jiang et al., 2013). Synaptically, Purkinje cells are affected by alterations in their intrinsic excitability, and the release of GABA is lowered in interneurons in the cerebellum (Hoxha et al., 2012). Considering the effects on the Purkinje cells, one could come to the conclusion that AD in the cerebellum accelerates the aging process. Furthermore, it was revealed through MRI that AD and frontal temporal dementia are connected with focal volume loss in the cerebellum. Therefore, although AD’s effect on motor function in the cerebellum may be debatable, it seems to contribute to inevitable dementia related to the disease (Schmahmann, 2016).
Parkinson’s Disease

Parkinson’s disease is ascribed as being a progressive decline in motor skills including gait, posture, rigidity, and resting tremor. Most of the attention has been put on the basal ganglia as far as the pathophysiology and potential treatment for the disease. The reader is directed to Wu and Hallet (2013) for an extensive review. For purposes of this section we will focus on the more recent developments involving the cerebellum.

One of the symptoms of the disease is the change in α-synuclein (Pagonabarraga et al., 2015; Solano et al., 2000). Although α-synuclein is increased in the basal ganglia it is arguably reduced in the cerebellum in Parkinson’s disease patients (Westerlund et al., 2008; Fuchs et al., 2008). More research needs to be done to clarify the role of α-synuclein in the cerebellum.

According to Hurley et al. (2003) there are dopamine 1 (D1) and dopamine 3 (D3) receptors in the cerebellum, as well as the presence of tyrosine hydroxylase, which were found in lobules 9 and 10. In Parkinson’s disease patients the mRNA of D1, D3 receptors, and tyrosine hydroxylase are reduced, suggesting that the cerebellum may have an active role in the motor dysfunction in the disease. There are also morphological changes in the cerebellum due to Parkinson’s disease. Borghammer et al. (2010) found using deformation-based morphometry, which is touted to be more sensitive that voxel based morphometry, that the left cerebellum was smaller than the average of the age-matched controls. The cerebellum also increases in activation in individuals with Parkinson’s based on (blood oxygen level dependent magnetic resonance imaging) BOLD MRI and PET studies when performing different tasks that involve motor learning and motor execution (Wu and Hallet, 2013). The increased activation is also true with individuals with akinesia/rigidity Parkinson’s at rest (Wu et al., 2009). In another experiment rats were subjected to dopaminergic deafferentation via 6-hydroxydopamine lesions and then made to perform
skilled or non-skilled aerobic exercise. The skilled aerobic exercised animals had a greater rCBF in the cerebellum and had greater connectivity between the prelimbic cortex and motor areas. They had changed a functional connection between the midline cerebellum and sensorimotor parts of the brain, particularly the cerebellar-thalamocortical compensatory circuit (Wang et al., 2015). This compensatory reorganization is also seen in individuals that undergo ventral intermediate nucleus thalamotomy (Wen et al., 2016). Interestingly, using levodopa changes this reorganization back to normal (Wu et al., 2009). This begs the question of whether the reorganization of the cerebellum is part of the pathological process of Parkinson’s disease or is merely a compensatory mechanism. To answer this question one would have to suppress the cerebellar activity and observe whether the symptoms become better or worse in a Parkinson’s animal model (Wu and Hallett, 2013).

**Multiple Sclerosis**

Another neurodegenerative disease that begins at a younger age is multiple sclerosis (MS). Although the onset of MS starts earlier in life, the average age of the individual with MS has increased greatly (Sanai et al., 2016). A study out of British Columbia, which has a high rate of MS, reported that the average age increased from 45-49 in the early 90’s to 55-59 years in 2008 (Kingwell et al., 2015). Another study in Genoa, Italy saw 18% of people in their study that had MS over 65 years of age (Solaro et al., 2015). Overall the younger the beginning, the longer the survivability of individuals when switching from exacerbating-remitting inception and the inception of secondary progression, and lower the rate to actually switching to secondary progression MS (Confavreux and Vukusic, 2003; Confavreux and Vukusic, 2006; Sanai et al., 2016). So in essence although MS is not necessarily age dependent like AD or PD, its progression is definitely dependent on age.
Previously the focus has been mainly on the telencephalic brain regions, but the cerebellum received attention only fairly recently (Weier et al., 2015). One study found that there is much cortical demyelination of the cerebellum, especially in individuals with progressive MS. It was shown that there was a moderate loss of Purkinje cells but otherwise a preservation of most of the neurons and their axons, although the axons showed swelling. Purkinje cell loss was only observed in individuals that had leucocortical lesions (Redondo et al., 2014). In MS, demyelination can be quite severe. Normally, demyelination is about 30% to 40% but can go up to around 90%. Even though one would expect to have some type of functional consequence, it is still unclear what the extent of those consequences are. (Kutzelnigg et al., 2007). Individuals that demonstrate signs of cerebellar damage tend to have a quicker and more disabling progression of the disease (Amato and Ponziani, 2000).

One relatively unexplored area of MS affecting the cerebellum is how it is involved in cognitive decline. A study by Valentino et al. (2009) showed that individuals with cerebellar motor deficits also had deficits in attention and verbal fluency compared with individuals with MS that did not show cerebellar dysfunction. Likewise a different study came to a similar conclusion but used an antisaccade task that could assess cognitive function (Kolbe et al., 2013). MS was also found to affect both working memory and processing speed (Weier et al., 2014). One reason for the motor and cognitive deficits is the inflammation associated with MS. CD+, CD8+ T-cells, B cells, macrophages, and plasma cells are involved in this inflammation, and it occurs largely in the meninges and spreads to the subpial cortices (Frischer et al., 2009). Howell et al., (2015) investigated this inflammation in the cerebellum because of its deep folded sulci and found that inflammation in the subarachnoid space contributes to a greater subpial demyelination in the cerebellum. It has also been reported that the lesions in the cortical as well
as the white matter parts of the cerebellum contribute to an increased Kurtzke expanded disability status scale (Favaretto et al., 2016). Not only are the lesions attributed to cerebellar dysfunction but there is also a molecular component. IL-1β is an inflammatory cytokine that is highly expressed in mice models of MS, and it reduces GABA-ergic transmission. One possible direction to look at for treatment is blocking IL-1β via IL-1ra, a receptor antagonist, and in the study this was done, and it alleviated inflammation with the MS mouse model (Mandolesi et al., 2015). An interesting note about MS is that it affects the homogeneity of the left cerebellum in particular like that of Parkinson’s disease. This lack of homogeneity could be due to the reduction of cortico-ponto-cerebellar and spino-cerebellar inputs and could contribute to higher ataxia scores (Dogonowski et al., 2014). There is some hope in some recent research. A study by Shields et al. (2015) found that a blocker for the sodium channel Nav1.8, PF-01247324, is able to be administered per os, which alleviated cerebellar symptoms in an experimental autoimmune encephalomyelitis (EAE) mouse model without noticeable side effects because of its selectivity. Another positive in the research of MS is the finding of certain miRNA biomarkers that can assess the progression, detections, and the remission of the disease and could help to be diagnostic in the future (Kacperska et al., 2015).

**Alcoholism**

Although healthy brain aging is ideal and having a disease such as Parkinson’s disease, AD, or MS cannot necessarily be helped, we do have the choice of reducing the risks of aging by avoiding excessive drinking of alcohol. It is well known that alcoholism can cause ataxia by atrophy of the cerebellum and peripheral neuropathy (Melgaard and Ahlgren, 1986). Complicating alcoholism is a thiamine deficiency that results in ataxia, confusion, and ophthalmoplegia called Wenicke’s encephalopathy. Many also suffer from Korsakoff syndrome
simultaneously which entails agitated delirium and delirium tremens; together it is termed Wernicke's-Korsakoff syndrome (McCormick et al., 2011).

Independent of those complications there are two hypotheses regarding how alcoholism affects how the brain ages. One hypothesis is the “accelerated aging hypothesis.” This model purports that an individual ages cognitively and possibly psychologically before they are supposed to age, which begins early on in adulthood. The second hypothesis is the “age sensitivity hypothesis.” In this model, once an individual starts showing age related cognitive changes around their 40’s, if they abused alcohol they will show greater changes than individuals that did not drink excessively (Ellis and Oscar-Berman, 1989; Harris et al., 1999). In a SPECT study by Harris et al. (1999) cortical/cerebellar blood perfusion was measured under the assumption of the “age sensitivity hypothesis.” The cerebellum was found to be hypoperfused to a larger extent in abstinent alcoholics than their paired controls, and the hypoperfusion was greater in the cerebellum than in the cerebral cortex. This was more pronounced with individuals whose last drink was later in life. Not only is the blood flow impaired in abstinent alcoholics but there is greater shrinkage of the cerebellum vermis in alcoholics (Torvik and Torp, 1986). There is atrophy in the white matter too, which is seemingly disproportionate with the other layers of the cerebellum. This may indicate a particular degeneration of axons of the cerebellum (de la Monte, 1988). The white matter degeneration in the cerebellar hemispheres was evident in individuals with Korsakoff’s syndrome in a study by Sullivan et al. (2000) but not in individuals with uncomplicated alcoholism. In the anterior vermis both gray and white matter were reduced, and the amount of ataxia an individual displayed correlated with the white matter atrophy in this area (Sullivan et al., 2000). GABA_A receptor inhibition has been shown to increase in Purkinje and granule cells with acute exposure to alcohol (Valenzuela and Jotty, 2015). It was seen in
some early studies that the granule cells and interneurons were vulnerable to atrophy from alcoholism, but more recent studies show that there isn’t a significant reduction in number (Tavares and Paula-Barbosa, 1982; Tavares et al., 1987; Tabaa et al., 1999; Pentney et al., 2002). This is only true if the individual does not suffer from Wernicke’s disease, which can induce a 57% loss of Purkinje cells (Baker et al., 1999). Even though there may not be an excessive loss of Purkinje cells in alcoholics without Wernicke’s, there is a change in the dendritic arbor. Pentney (1991) discovered that when twelve month old rats were treated with ethanol for 24 or 48 weeks, there was an elongation of terminal segments of dendrites. A later study concluded that aged ethanol fed rats had a dilation of smooth endoplasmic reticulum in their dendritic arbors (Dlugos, 2006). These could account for the ataxia induced by alcoholism later in life.

Alcoholism is considered to be a disease that affects millions of people. Confronting the problem earlier in life will help reduce the effects of the sensitivity induced by alcoholism and the damage induced by aging considerably.

**Conclusion**

Through the last several decades it has been revealed that the cerebellum is more than just a structure that controls fine motor movements. The cerebellum has recently garnered more attention and for good reason. It has connections to other parts of the brain that are involved in speech, working memory, tactile sensation, and cognition. It also may, depending on the study you look at, be one of the brain structures that age’s the soonest. Given all the implications this may have, this is important to study. It is already established that the cerebellum when it age’s affects balance and how quickly one performs fine motor movements, gait, and posture. What needs to be looked at is how these connections with the other parts of the brain are affected with
aging. Also, the cerebellum plays a role in many neurodegenerative diseases such as AD, Parkinson’s and MS, and it has a considerable reaction to aging combined with alcoholism.

CHAPTER II
MATERIALS AND METHODS

Animals-

All experiments including animals were conducted using the National Institute of Health guidelines. Also, all protocols were approved by the Institutional Care and Use Committee at University of North Texas Health Science Center. Adult male C-57/B6 mice (20-45g body weight, Charles River and NIH) were housed in a temperature controlled environment with the lights on a 12:12 hour cycle. Water and food were given to the animals ad libitum.

CLARITY -

C57-B16 mice ages 3 month (n=3) and 20 month (n=3), which correlate to about 27 and 89 years of age in humans respectively, were anesthetized with isoflurane at 4% (Dutta and Sengupta, 1973). The oxygen and nitrogen percentage was kept at 30% and 70% respectively. Mice were immediately exsanguinated with 60 ml of cold 1X PBS and also perfused with 60 ml of 4% PFA. The cerebellum was collected from each mouse and put in 4% PFA overnight. The next day the samples were washed briefly in 1X PBS. The samples were then individually placed in a conical tube filled with 4% polyacrylamide in 1X PBS and 0.0025 g/ml of VA-044. It is important that no air is in the conical tube as oxygen will impede the polymerization process. It is best to overfill the tubes and put the cap on tightly. Invert the tube see if there is any bubble. A very small one is acceptable. The tubes were then placed in the 4°C refrigerator for 24 hours. The next day samples were set in room temperature for an hour and then placed in a 37°C water-bath for 3
hours. The tubes were then decanted of the polyacrylamide/VA-044 solution as much as possible. The solution should be viscous. The samples were then briefly washed in 10X PBS. Then they should be put in a separate tube with 1X PBS and washed for 5 min. The samples are then placed in a solution of 10ml of 8% SDS and 0.5% 2-mercaptoethanol. Then the samples are put in a 37°C shaker. Do not put shaker at a high because samples become more fragile when they clear. Every 3 days the solution of SDS/2-mercaptoethanol should decanted and refilled. For a mouse cerebellum it takes approximately 5-7 days to completely clear (Fig. 7). The samples are then washed in 1X PBS with 0.1% Tween 20 and 0.1% sodium azide for approximately 24 hours with four changes of the solution. The next day the samples are then placed in a solution of 1X PBS with 0.1% Tween 20, 0.1% sodium azide, 2% donkey serum, and .01 % Triton X (50 ul of 0.3% solution for every 1.5 ml) and lectin conjugated with 488 fluorophore. Wrap tubes in in aluminum foil and place on the shaker at room temperature for 11 days. Samples should then be washed in 1X PBS with 0.1% Tween 20 and 0.1% sodium azide for 3-4 days changing the solution each day. Samples should then be placed overnight in RIMS solution that is made with 40g of histodenz and 30ml of 0.2% PB and 0.1% sodium azide which will have a refractive index of 1.46. The samples will actually become clearer which is normal. Each cerebellum was placed in the cap of a 5ml Falcon round bottom polystyrene tube with fresh RIMS solution overfilling the cap slightly. A round coverslip is placed on the cap and then turned upside down. The glass coverslip should stay on during this process and not slip. A gel type super glue was then used to hold the coverslip onto the cap for approximately 30 or until mostly dry. Then the coverslip is sealed with the cap using epoxy. Wait 5 minutes and turn the cap and coverslip right side up and make sure there are no bubbles or the process must be repeated. Wait approximately 15 minutes for the epoxy to dry mostly. The cap is then placed in a plastic beaker cut to
approximately 3-4cm with a small amount of glue at the bottom. 100ml of a solution of 90% glycerol with 1X PBS should be made for visualization purposes.

Visualization-

Visualization was done on a Fluoview 1200 confocal microscope with a 10X dipping objective for Clarity samples and a regular 10X objective for slides. Clarity samples with the cut plastic beaker were placed under the objective and then filled with the 90% glycerol solution to maintain the refractive index of 1.46 as much as possible. The sample was scanned with 512X512 pixels and at 0.2 s/pixel. The entire cerebellum was scanned at every 5 microns. Slides were also scanned at 512X512 and at 0.2 s/pixel.

Imaris-

Analysis was done for the 2D slices, the 500X500X500 sections, and the entire cerebellum with Imaris 8.1.2 and Imaris 9.0 beta from Bitplane. The ‘Surface’ application was used to gather the volume and surface area of the blood vessels. For each sample smoothing was set at 3 µm and background subtraction was set at 5 µm. The upper and lower threshold was set automatically to avoid any bias. Anything under 27 voxels was considered noise and not taken into consideration in the analysis.

To obtain the length, diameter, and branching of the blood vessels the ‘Filament Tracer’ application was used.

2D Sections-

3 month old mice (n=3) and 20 month old mice (n=3) were anesthetized with 4% isoflurane with 70% nitrogen and 30% oxygen. Mice were then exsanguinated with 60ml of 1X PBS and 60ml
of 4% PFA. The cerebellum was removed from the cerebrum, and then cut in half and placed in
4% PFA overnight. The cerebellum were dehydrated in increasing concentrations of EtOH and
Xylene and then put into paraffin blocks the next day and cut into 5 micron sections sagittally
beginning at the midline. Three sections from each mouse were taken about 10 microns apart,
rehydrated in increasing concentrations of EtOH. Sections were then washed in 1XPBS, blocked
in 10% donkey serum for 1 hour and then stained with Dylight 649 Lycopersicon Esculentum
(Tomato) Lectin at a concentration of 1:500 in blocking buffer (Vector Labs, Burlingame, CA).

3D sections of the vermis and Crus I and Crus II

A 500X500X500 subsection of both the anterior vermis and the Crus I and Crus II of 3
month old mice (n=3) and 20 month old mice (n=4) were calculated. The whole cerebellum was
made transparent via Clarity technique and were processed as stated above. The analysis was
done with Imaris 8.2.1. Automatic thresholding on both upper and lower limits were used to
avoid any type of bias.

3D sections of the hippocampus-

3 month old mice (n=3) and 20 month old mice (n=3) were anesthetized via isoflurane and the
brain was removed and cut into 2mm sections. The anterior part of the hippocampus was
processed using the Clarity technique. Dylight 649 Lycopersicon Esculentum (Tomato) Lectin
(Vector labs, Burmingame, CA) 1:500 was used to stain these smaller sections, and the sections
were only stained for 5 days. The hilus of the hippocampus was scanned and analyzed because it
was easily recognized under the microscope. The hippocampi, like the cerebellum, were also
scanned using the Olympus Fluoview 1200 confocal microscope and evaluated using Imaris 9.0
beta 2.
Laser Doppler Flowmetry

Blood flow was measured using a Perimed 5000 laser Doppler flowmetry machine. 3 month old mice (n=3) and 19 month old mice (n=3) were used for both blood flow and oxygen measurements. Mice were anesthetized with 750 mg/kg of urethane and 50 mg/kg of alpha-chloralose given intraperitoneally. If mice were not anesthetized within 30 minutes another dose was given at 25% of the original dose. Older mice were given 75% of the initial dose young mice were given and then 25% after 30 minutes. The mice were then placed in a stereotaxic device. The laser Doppler probe was placed at 6 mm caudally from Bregma to measure the anterior vermis and at 7mm caudally and 2mm laterally to the right for Crus 1 and Crus2 measurements. Measurements were taken for 5 minutes on both locations on each mouse. Care was taken not to measure on top of any major blood vessels.

Statistical Analysis-

All statistics were done on Prism Graphpad 5. A student’s t test was used to analyze all the results in measuring blood flow, oxygen measurements, and 2D and 3D data from Clarity. Results were considered significant at p=0.05.

CHAPTER III

RESULTS

2D sections-

Sections of 5 microns were visualized using Olympus Fluoview 1200. The sagittal midline of the anterior vermis was scanned, and the region of interest analyzed was between the 3\textsuperscript{rd} and 4\textsuperscript{th} lobe traversing the Preculminate Fissure (Fig 3. A,B). Analysis was done with Imaris
8.2.1 using the ‘Surface’ and ‘Filament’ applications to measure the average and total surface area, volume, count, length, diameter, and branching. The average area of the 3 month old young mice were not significantly larger than the 20 month old mice (t=0.09358, p= 0.3714), and the average volume was not significant either (t=0.9006, p=0.3811) (Fig 3. C, D). Neither the total surface area (t=1.218, p=0.0525) nor the total volume (t=2.094, p=0.2409) was significant, but older tended to be larger than young mice. Interestingly, the older mice had a tendency to have more blood vessels than the younger mice but was not significant (t=1.913, p= 0.0739) (Fig. 4).

Using the ‘Filament’ application of Imaris the other measurements were analyzed. The average length of the blood vessels were almost the same between old and young mice (t=0.6696, p=0.5127) (Fig. 5 A). The average diameter of the young mice though was observed to be slightly larger but not significantly so (t= 1.476, p= 0.1593) (Fig. 5 B). Finally, neither the mean branching points per blood vessel nor the total branching points were significantly different (t=1.144, p=0.2693; t=1.644, p=0.1197) (Fig 6).

3D sections of Vermis and Crus I/Crus II

Clarity was used to obtain 3D samples to evaluate the cerebellum of the 3 month old young mice and 20 month old mice (Fig 7.). A 500X500X500 section of both the anterior vermis and the right Crus I and Crus II was analyzed with Imaris 8.2.1 (Fig 8). Only the average and total surface area, volume, and counts were measured. The average surface area of the vermis was not seen as being significant (t=1.644, p= 0.1612) (Fig. 9A), neither was the average surface area of the Crus I and Crus II (t=1.541, p=0.1840) (Fig. 10 A). Like the 2D sections though, the younger had a tendency to have both a larger total surface area and volume which not significant either for the vermis or Crus I and Crus II (t= 1.466, p=0.2027; t=1.342, p=0.2375) (Fig. 11 B;
Fig 12 B). Also, like the 2D sections there was a tendency for the old mice to have more blood vessels in both the vermis and Crus I and Crus II, but it was not significant either (t=1.975, p=0.1053; t=1.563, p= 0.1789) (Fig. 11). The total area and for the vermis and Crus I and Crus II was only slightly larger for the young mice but again was not significant (t=1.522, p=0.1884; t=2.124, p=0.0871) (Fig. 12 A; Fig 13 A). The same is true for the total volume of the vermis and Crus I and Crus II (t=0.9227, p=0.3985; t=1.361, p=0.2318) (Fig. 12 B; Fig 13B).

3D Sections of Hippocampus-

The blood vessels of the hippocampus were measured to see if the vessels age faster than the cerebellum. Out of the 2mm cut from the young mice (n=3) and old mice (n=3), cleared, stained, and scanned, approximately 1mm of the hilus was evaluated. Like the cerebellum the average surface area and volume were assessed, as well as the total surface area, volume, and counts. Although it was not significant it was unexpected to see that the average surface area and volume was greater in older mice than young mice (t=0.8115, p=0.0795; t=0.7057, p=0.5193) (Fig. 14). The total surface area and volume were not significant either (t=0.1052, p=0.9213; t=0.7840, p=0.4769) (Fig. 15). Like the cerebellum, there tended to be slightly more blood vessels in the old mice than the young (t=0.2335, p=0.8268) (Fig. 16).

Laser Doppler Flowmetry-

Blood flow was measured using Laser Doppler flowmetry. This is not a direct measurement but rather evaluates Brownian motion inside the vessels via a laser that penetrates approximately 2mm below the skull. Although it is difficult to know exactly where major blood vessels lie in the brain, care was taken to avoid them and still measure the blood flow of the anterior vermis and Crus I and Crus II. Evaluation by laser Doppler flowmetry is recorded as
perfusion units. The anterior vermis of the young mice (n=5) and old mice (n=5) was measured 6mm from Bregma. After 5 minutes the mean values were compared (Fig. 17). The young mice did not have a significantly higher mean of perfusion in the vermis compared to old mice (t=0.2748, p=0.7971). A lateral measurement was also taken close to where Crus I and Crus II would be. Although, the older mice presented with a higher mean of perfusion it was not significant (t=1.397, p=0.2052) (Fig. 18).
Figure 1. Diagram of the Cerebellum: The spinocerebellum contains the vermis mainly. The cerebrocerebellum contains the lateral portions of the cerebellum, and the vestibulocerebellum contains the nodulus, ubula, and pyrimidal lobule. Adapted from (http://www.nature.com/nrn/journal/v5/n3/fig_tab/nrn1347_F1.html?foxtrotcallback=true)
Figure 2. Diagram of Cells in the Cerebellum: The inferior olive provides climbing fibres to the Purkinje neurons and can cause a complex spike. The granule cells are provided with synapses from the mossy fibre terminals of the different cerebellar tracts and synapse via the parallel fibre onto Golgi cells stellate cells, Purkinje cells, and basket cells in the molecular layer. Basket cells and aspiny stellate cells are Gaba-ergic and inhibit the Purkinje cells. The Golgi cells are also Gabaergic and are an inhibitory feedback mechanism for the granule cells.
3 month old mouse vermis 2D
Figure 3. 2D Average Surface Area and Volume of the Vermis: DyLight 649 Lycopersicon Esculentum (Tomato) Lectin was used to label 3 month old mice (A) and 20 month old mice Lectin (+) blood vessels (B) in lobules III-V of the anterior vermis. Using the ‘Surface’ application in Imaris the average surface area and volume was determine for 3 month old mice (C) and 20 month old mice (D).
A. Total Area of Lectin (+) Vessels in the Vermis (µm²), 2D

- Young: 200,000 ± 10,000
- Aged: 300,000 ± 15,000

p = 0.2409

B. Total Volume of Lectin (+) Vessels in the Vermis (µm³), 2D

- Young: 200,000 ± 10,000
- Aged: 400,000 ± 20,000

p = 0.0525

C. Count of Lectin (+) Vessels in the Vermis, 2D

- Young: 500 ± 50
- Aged: 600 ± 60

p = 0.0739
Figure 4. 2D Total Surface Area Volume and Count of the Vermis: The ‘Surface’ application was also used to obtain the total surface area, volume, and count of 3 month and 20 month old first (A,B,C).
B.
C.

D.

Figure 5. 2D Average Diameter and Length of the Vermis: DyLight 649 Lycopersicon Esculentum (Tomato) Lectin was also used to detect Lectin (+) blood vessels for 3 month old (A) and 20 month old (B). Using the ‘Filament’ application in Imaris the average diameter (C) and average length (D).
A. Average # of Branching Points of Lectin (+) Vessels in the Vermis, 2D

B. Total # of Branching Points of Lectin (+) Vessels in the Vermis, 2D
Figure 6. 2D Average and Total Branching of the Vermis: The ‘Filament’ application in Imaris was also used to detect the average branching of blood vessels (A) and the total number of branches (B).

Figure 7: CLARITY: Whole cerebellum from C57/B6 mouse embedded using A4PO and VA-044 (B) Same cerebellum cleared using 8% SDS for 5-7 days (A).
A. 3 month old mouse cerebellum
B.
**Figure 8. 3D Lectin (+) Staining of Cerebellum:** 500X500X500 sample of the vermis and Crus I/ Crus II with 3D rendering using Imaris ‘Surface’ application to compare 3 month old (A) and 20 month old mice (B).
Figure 9. 3D Average Surface Area and Volume of the Vermis: DyLight 488 Lycopersicon Esculentum (Tomato) Lectin was used to label 3 month old mice and 20 month old mice blood vessels to evaluate the difference in average surface area (A) and average volume (B) of the vermis.
A. Average Surface Area of Lectin (+) Vessels in the Crus I/Crus II ($\mu m^2$), 3D

B. Average Volume of Lectin (+) Vessels in the Crus I/Crus II ($\mu m^3$), 3D

Figure 10. 3D Average Surface Area and Volume of Crus I/Crus II: DyLight 488 Lycopersicon Esulentum (Tomato) Lectin was used to label 3 month old mice and 20 month old mice blood vessels to evaluate the difference in average surface area (A) and average volume (B) of Crus I/Crus II.
Figure 11. 3D Total Count of the Vermis and Crus I/CrusII: DyLight 488 Lycopersicon Esculentum (Tomato) Lectin was also used to determine the total blood vessel count in both the vermis (A) and Crus I/Crus II.
Figure 12. **Total Surface Area and Volume of the Vermis:** The anterior vermis was labeled with lectin to also obtain the total surface area and volume.
Figure 13. 3D Total Surface Area and Volume of Crus I/Crus II: The Crus I/ Crus II was labeled with lectin to also obtain the total surface area and volume.
A.

B.

**Figure 14. 3D Hippocampus Average Surface Area and Volume:** DyLight 649 Lycopersicon Esculentum (Tomato) Lectin was used to label 3 month old mice and 20 month old mice Lectin (+) blood vessels in the hilus of the hippocampus as well to determine the average surface area and volume determined by the ‘Surface’ application in Imaris.
Figure 15. 3D Hippocampus Total Surface Area and Volume: DyLight 649 Lycopersicon Esculentum (Tomato) Lectin was also used to find the total surface area and volume of the hilus of the hippocampus.
Figure 16. 3D Hippocampus Count: The total number of Lectin (+) blood vessels was evaluated for young and aged mice hippocampus also.
Figure 17. Blood Flow Measurement: The vermis was measured 6mm from Bregma (A) and the average blood flow for 5 minutes was assessed (B).
Figure 18. Laser Doppler Flowmetry: 3 month old C57/B6 with laser Doppler probe 401 set on top of the skull at 6mm posterior to Bregma (A). Also, an example of a 5 min interval of blood flow measured via LDF (B). Using this method it was possible to attain the blood flow via perfusion units of both the vermis (C) and Crus I/Crus II (D).
### Power of Analysis

<table>
<thead>
<tr>
<th>(2D Average) Power of Analysis of the Vermis</th>
<th>Current Sample Size (Young--Aged)</th>
<th>Power Obtained (Young--Aged)</th>
<th>Sample Size Needed for a Power of 80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface Area</td>
<td>9–9</td>
<td>0.162–0.162</td>
<td>68</td>
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<tr>
<td>Volume</td>
<td>9–9</td>
<td>0.136–0.136</td>
<td>88</td>
</tr>
<tr>
<td>Length</td>
<td>9–9</td>
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<td>Diameter</td>
<td>9–9</td>
<td>0.303–0.303</td>
<td>31</td>
</tr>
<tr>
<td>Branching</td>
<td>9–9</td>
<td>0.189–0.189</td>
<td>56</td>
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<table>
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<tr>
<th>(2D Total) Power of Analysis of the Vermis</th>
<th>Current Sample Size (Young--Aged)</th>
<th>Power Obtained (Young--Aged)</th>
<th>Sample Size Needed for a Power of 80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface Area</td>
<td>9–9</td>
<td>0.212–0.212</td>
<td>48</td>
</tr>
<tr>
<td>Volume</td>
<td>9–9</td>
<td>0.542–0.542</td>
<td>16</td>
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<tr>
<td>Branching</td>
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<td>0.382–0.382</td>
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<tr>
<td>Count</td>
<td>9–9</td>
<td>0.502–0.502</td>
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<th>(3D Average) Power of Analysis of the Vermis</th>
<th>Current Sample Size (Young--Aged)</th>
<th>Power Obtained (Young--Aged)</th>
<th>Sample Size Needed for a Power of 80%</th>
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<tr>
<td>Surface Area</td>
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<td>0.137–0.189</td>
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<tr>
<td>Volume</td>
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<th>(3D Total) Power of Analysis of the Vermis</th>
<th>Current Sample Size (Young--Aged)</th>
<th>Power Obtained (Young--Aged)</th>
<th>Sample Size Needed for a Power of 80%</th>
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<tbody>
<tr>
<td>Surface Area</td>
<td>3–4</td>
<td>0.173–0.246</td>
<td>15</td>
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<tr>
<td>Volume</td>
<td>3–4</td>
<td>0.095–0.121</td>
<td>39</td>
</tr>
<tr>
<td>Count</td>
<td>3–4</td>
<td>0.274–0.402</td>
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<tr>
<th>(3D Average) Power of Analysis of the Crus I/II</th>
<th>Current Sample Size (Young--Aged)</th>
<th>Power Obtained (Young--Aged)</th>
<th>Sample Size Needed for a Power of 80%</th>
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<tbody>
<tr>
<td>Surface Area</td>
<td>3–4</td>
<td>0.185–0.264</td>
<td>14</td>
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<tr>
<td>Volume</td>
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<tr>
<th>(3D Total) Power of Analysis of the Crus I/II</th>
<th>Current Sample Size (Young--Aged)</th>
<th>Power Obtained (Young--Aged)</th>
<th>Sample Size Needed for a Power of 80%</th>
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<tr>
<td>Surface Area</td>
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<td>0.222–0.323</td>
<td>11</td>
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<tr>
<td>Volume</td>
<td>3–4</td>
<td>0.151–0.211</td>
<td>18</td>
</tr>
<tr>
<td>Count</td>
<td>3–4</td>
<td>0.147–0.205</td>
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<table>
<thead>
<tr>
<th>Laser Doppler Flowmetry</th>
<th>Current Sample Size (Young--Aged)</th>
<th>Power Obtained (Young--Aged)</th>
<th>Sample Size Needed for a Power of 80%</th>
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<tbody>
<tr>
<td>Blood Flow of the Vermis</td>
<td>5–5</td>
<td>0.059–0.059</td>
<td>393</td>
</tr>
<tr>
<td>Blood Flow of the Crus I/II</td>
<td>3–4</td>
<td>0.12–0.161</td>
<td>26</td>
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</tbody>
</table>

**Table 1: Power of Analysis:** Current power of analysis obtained in this study and the animals needed to obtain at least a power of 80%.
CHAPTER IV

DISCUSSION

According the U.S Center for Disease Control and Prevention one-fourth of individuals over the age of 65 falls each year. Every 11 seconds an older adult is treated in an emergency room for a fall and every 19 minutes an elderly person actually dies from a fall. Falling in the elderly has resulted in over 2.8 million reported injuries treated in emergency rooms each year. In 2013 the cost of these injuries caused by falls grew to $34 billion dollars. This cost is expected to increase to $67.7 billion by 2020 (National Council on Aging; U.S Center for Disease Control and Prevention).

Not only is falling a great issue among the elderly, but fear of falling is also a large factor that inhibits individuals from social interactions and can result in increased falling incidents, and can limit the activity in daily life (Makino et al., 2017; Doi et al., 2012; Zijlistra et al., 2007; Friedman et al., 2002; Cumming et al., 2000). Deficits related to gait in the elderly include slower speed, a smaller stride length, a wider step, a slower rate, and a longer double support time (Hollman, JH., et al. 2011). Moreover, gait characteristics can be differently attributed to gender with aging. A study by Scaglioni-Solano and Aragon-Vargas (2017) presented data that demonstrated that women have a greater vertical and mediolateral pelvis harmonic ratios, as well as better reduction in acceleration, and standing balance. This indicates that men as they age have poorer performance in gait than women and could result in a greater risk in falling. Concomitant with age, other risk factors that increase the chance of falling include those that have difficulty standing from a chair, performing poorly in tandem walk, arthritis, Parkinson’s disease, over three falls the year before, and being Caucasian. There are only weak correlations
for a single fall; these are risk factors for multiple falls (Nevitt et al., 1989). These are important aspects to consider, because, along with the newly discovered cognitive impairments that may be attributed to the aging cerebellum, there is still a great need to understand and hopefully find a way to ameliorate the motor deficits caused by aging.

There are several studies that look at vascular differences in aging and how it affects the ability of individuals maintaining their gait and balance, but they look at the entirety of the brain or several subsections and don’t pay too much attention specifically to the cerebellum. A more recent study by de Laat et al. (2010), showed that white matter lesions (WML) and lacunar infarcts may be responsible for gait deficits and determined that stride length may be a good indicator of small vessel disease related gait impairments. That particular study looked at several brain regions, including the cerebellum and brain stem, which were determined to affect cadence when there was WML and lacunar infarcts. So far there is not a study that looks specifically at the cerebellum and small or large vessel issues that may contribute to functional deficits. The cerebellum is subject to a volume decrease as one ages and white matter hyperintensities may contribute to that decrease in volume (Smith et al., 2015; Raz et al., 2003). So the question becomes how do we prevent or intervene to prevent the deficits caused by aging in the vasculature of the cerebrum and cerebellum. One study looking specifically at the hippocampus and cortex noticed that giving Wistar rats a calcium channel blocker darodipine, starting at old age of 21 months and ending in advanced age of 27 months, increased the capillary length of the Ammon’s horn of the hippocampus and the occipital cortex (Amenta et al., 1995). Insulin like growth factor-1 (IGF-1) is also an important component in the process of cerebrovascular aging. Calorie restriction, which increases the amount of IGF-1, or directly administering IGF-1 can help to repair the damage to the cerebrovasculature and increase microvascular density (Sonntag
et al., 2000). This is something to take note of, because it means there may be a way to prevent and possibly intervene in the process of aging blood vessels in the brain.

The cerebrum and the cerebellum vasculature can also be subject to structural differences such as ropelike braiding of the blood vessels, but older methods of looking at blood flow and oxygen measurements show there isn’t any difference between young and aged individuals (Kalaria et al., 2010; Akima et al., 1998; Marchal 1992; Leenders et al., 19590; Yamaguchi et al., 1986). The current study looked at specific regions of the cerebellum and suggests that there may be some slight differences in the anterior vermis of the cerebellum but not so much in the lateral portions such as the Crus I/Crus II. Possibly an increase in the number of mice would have given a more definite result as far showing any significance.

The current study looked at the very basic structure, and the initial hypothesis was that surface area, volume, length, diameter, count, and branching would all be larger for younger mice. Previous research supports this hypothesis, because vessel dilation is known to be reduced in the microvascular in the skeletal, coronary, and cerebral vascular beds of older individuals (Scioli et al., 2014). There is little to no research done on the counts of blood vessels specifically in the cerebellum. There are several studies that are regionally specific on the amount of vessels in the cerebrum. It’s been observed that mean minimal luminar diameter (MMLD) of capillaries was increased in a strip of cortex of aged Wistrar-Kyoto rats, but the overall number of capillaries did not change. In the same study the number and MMLD of venules were increased in the aged rats, but no change in either number or MMLD of arterioles was observed (Knox and Oliveira, 1980). A study by Bell and Ball (1981) saw that there was an increase in the diameter of both capillaries and arterioles in the hippocampus of aged humans. The study also revealed that the density of capillaries significantly decreases with age, but the density of arterioles
increased. This would indicate a decrease in exchange potential with aging. The average surface area and volume in the hilus of the hippocampus, having a slight increase in the present study, supports that there is possibly an increase in size of both the capillaries and arterioles in the area.

The superior cerebellar artery (SCA) supplies the blood flow to the anterior vermis and the superior part of the cerebellar hemisphere (Stoodley and Schmahmann, 2010; Tatu et al., 1996). The SCA and the posterior inferior cerebellar artery (PICA) do not keep specific lobular boundaries (Stoodley and Schmahmann, 2010). This would also suggest that the branching from these major arteries would be variable. During oxygen measurements (data not shown) when the skull was removed from over the vermis there was variation in the blood vessels visually observed. This may have affected the blood flow measurements making it difficult to parse out an actual difference in blood flow between young and old mice. A study performing a BOLD fMRI on the cerebellum demonstrated that there is an increased BOLD signal in aged individuals performing slower less consistent task performance, and there is a greater variability in aged individuals that could be compensation for lack of network complexity or could be a just a dysfunction (Garrett et al., 2010; Rapkin et al., 2014). In the current study when the mice were anesthetized with isoflurane rather than urethane and alpha-chloralose, there was a far greater increase in blood flow with the aged mice than the young mice in the vermis but not the Crus I/Crus II (data not shown). This may be contributed to the variability in the blood vessels in the cerebellum or the vasodilation caused by the isoflurane (Ida, 1998). With the urethane and alpha-chloralose anesthetic, which is supposed to maintain physiological parameters, showed a slight increase in blood flow of the old Crus I/Crus II but none with the vermis (Hara and Harris, 2002; Tremoleda et al., 2012).
Some of the limitations of the current study was the technology not being available at the time. The 2mm sections of the hippocampus were more successful in the staining than the whole cerebellum. The cerebellum itself was just too large to analyze as a whole. There were instances of the tissue being partly damaged, or there were dim spots where the lectin didn’t quite diffuse properly throughout the tissue. The Imaris software was another limitation. It has vastly improved its rendering capabilities since the start of this project, and in the end, Imaris 9.0 beta was able to analyze larger segments. Still, to analyze the diameter, length, and branching of the larger 3D segments is something that needs to be improved upon. It was just not feasible with the current software. More importantly, the main limitation was that there was not enough animals to get a proper analysis. A power of analysis was done for each parameter after the experiment (Table 1). Many more animals in most cases were needed to conduct the experiment and have at least a power of analysis of 80%.

CHAPTER V

CONCLUSION

The results of this current study supports several of the conclusions of previous studies. Although none of the results showed any significant changes with age, there were certain trends that indicate that there may be some slight changes in the physical structure and functionality of the blood vessels in the cerebellum. There may be underlying changes in the blood vessel ultrastructure or other changes such as WML’s or ropelike braiding of the blood vessels that may contribute to the physical shrinkage of the cerebellum and could contribute to deficits in function.
New methods were used in this study to take another look in an advanced manner of the vasculature of the cerebellum. CLARITY is a new method that allowed a deeper and more detailed look at the blood vessels. This method though has some drawbacks. It takes a considerable amount of time to see the results, and the end product may not be perfect. There were instances of the cerebellum being damaged in the process of clearing or inconsistent staining creating dim areas in several cerebellum. This method, along with using Imaris for analysis, is worth the effort, because it has the ability to replace traditional 2D staining methods that could give inconsistent or skewed results. Each 500X500X500 section that was analyzed was done at 5 microns apart giving one hundred sections to be examined. This would be very difficult to replicate using traditional methods.

Since the start of this project new advances in processing tissue, visualization, and analysis have come to fruition. Some of these advances would include light sheet fluorescent microscopy (LSFM) which uses a plane of light rather than a point as in confocal microscopy to scan samples. This would reduce the time of scanning greatly. Also, the passive clearing method was used to make tissues transparent which takes several days to a week or more depending on the type and size of the tissue. Using electrophoretic tissue clearing such as X-Clarity™ would reduce clearing and labeling and could possibly be more consistent with staining large tissues.

Laser Doppler flowmetry is an established method of measuring blood flow. The results obtained in this study for blood flow are not significant and could be the result of having too small of a sample size to sufficiently compare the old and young mice cerebellum. In the future more advanced techniques, such as BOLD fMRI, should be employed to give a better idea of how blood flow could change in the respect of function between old and young mice. This may better explain the reduction in physical size and function of the cerebellum.
There were several limitations in this study, but overall, it was sufficient to support some of the previous conclusions about the vasculature of the cerebellum. It is important in science to employ new methods to either confirm or reject previous results. Although there were no groundbreaking realizations obtained in this study, it was still necessary to re-evaluate the vasculature of the cerebellum given that previous research has been scant and dated. Hopefully, in the future, the reason why the accelerated aging of the cerebellum happens can be elucidated using more advanced and efficient techniques. These advances will help obtain results that could allow individuals to age in a healthier manner.
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