Role of Adrenergic Neurons in Motor Control: Examination of Cerebellar Purkinje Neurons in Mice Following Selective Adrenergic Cell Ablation in Vivo

2016

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ROLE OF ADRENERGIC NEURONS IN MOTOR CONTROL: EXAMINATION OF CEREBELLAR PURKINJE NEURONS IN MICE FOLLOWING SELECTIVE ADRENERGIC CELL ABLATION IN VIVO

by

MONICA MANSOUR

A thesis submitted in partial fulfillment of the requirements for the Honors in the Major Program in Biomedical Sciences in the College of Medicine and in the Burnett Honors College at the University of Central Florida Orlando, Florida

Spring Term, 2016

Thesis Chair: Dr. Steven Ebert, PhD
ABSTRACT

Phenylethanolamine-N-methyltransferase (Pnmt) is the enzyme that catalyzes the conversion of noradrenaline to adrenaline. These catecholamines are synthesized in the medulla of the adrenal gland and by some neurons of the central nervous system. The precise location of Pnmt action in the brain and its physiological significance are unknown. Prior studies led by Aaron Owji, a graduate student in Dr. Ebert’s laboratory, showed that mice with selectively ablated Pnmt cells show signs of neurological defects such as abnormal gait, weakened grip strength, lack of balance, reduced movement, and defective reflexes during tail suspension tests.

The cerebellum is a small section of the brain that is responsible for fine-tuning motor commands. Since the Purkinje cells of the cerebellum act as the sole source of output from the cerebellar cortex, impairment of these cells could possibly account for the motor deficits seen in the mice models. The purpose of this project is to determine if there is indeed a change in Purkinje cells between wild type mice and Pnmt-ablated mice. The first aim is to identify quantitative differences in cell count between both genotypes. The second aim is to determine any morphological changes in the Purkinje cells. The main technique used in this project is immunohistochemistry in which cerebellum tissue from mice models are stained with Calbindin (a cellular marker for Purkinje neurons) and imaged with a confocal microscope. Results showed a slight reduction in the Purkinje cells of the ablated mice compared to the control genotype, accompanied with observable differences in cell structure. Understanding catecholamine pathway mechanisms in the nervous system is imperative for elucidating and targeting key players in neurodegenerative disorders.
DEDICATION

For the family members who have continuously encouraged me and supported my education.
   For the mentors who have inspired me and ignited my desire to pursue knowledge.
   For the people who have challenged me to exceed my capabilities.
   Thank you.
ACKNOWLEDGMENTS

This work would have not been possible without the help of Dr. Steven Ebert. My sincerest and deepest gratitude for all of his endless support, advice, and dedication. Thank you for allowing me to make a contribution.

I would also like to thank Dr. Kiminobu Sugaya and Dr. Stephen Berman for serving on my committee and directing my thesis.

Special thanks to my parents, Maged Mansour and Gigi Habib, and to my sister, Marina Mansour. Your support and confidence in me is truly appreciated.
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CHAPTER ONE: INTRODUCTION

Role of Adrenergic Hormones in the Nervous System

The catecholamines shown in Figure 1 below can all be derived from the amino acid tyrosine. Stress hormones norepinephrine and epinephrine (also referred to as noradrenaline and adrenaline, respectively) are commonly known due to their integral role in the nervous system.

Figure 1: Pathway of catecholamine biosynthesis. Key enzymes that are important for each step are shown in blue.

The nervous system is composed of the central nervous system (CNS) and the peripheral nervous system (PNS). The central nervous system is made up of the brain and spinal cord, with the brainstem serving as a connection between both structures. The spinal cord is a collection of nerve fibers that communicate commands from the brain to the rest of the body. Motor and
sensory nerves from the cerebral cortex must pass through the brainstem in order to reach the peripheral nervous system. The PNS is made up of the nerves that connect the CNS to the rest of the organs in the human body.

The autonomic nervous system, a division of the peripheral nervous system, can be further divided to the sympathetic nervous system and the parasympathetic nervous system. These systems are antagonistic to one another. Adrenergic hormones noradrenaline and adrenaline are key players in the “fight or flight” response by the sympathetic nervous system. In times of stress, adrenaline is released by the adrenal gland into the blood stream where it acts on other cells. This catecholamine is capable of increasing heart rate, dilating pupils, and altering metabolism among other modifications. The parasympathetic nervous system is responsible for restoring the body’s original homeostasis following the stressful event. Hence, it is referred to as the “rest and digest” system.

Adrenaline stimulates the sympathetic nervous system’s fight or flight response by binding to G-protein coupled receptors (adrenergic receptors) on cells. There are two main types of adrenergic receptors: alpha (α) and beta (β). Each receptor has specific subtypes (α₁, α₂, β₁, β₂, β₃) that are responsible for targeting certain pathways and organs to elicit a particular response when incited by adrenaline hormones (Fox, 2006). For example, Figure 2 shows that adrenaline binding to the α₁ receptor causes smooth muscle contraction. Whereas, when adrenaline binds to a β receptor, the opposite is observed and smooth muscle relaxes (Berki et al., 2011).
As depicted in Figure 1, Phenylethanolamine N-methyltransferase (Pnmt) is the main enzyme responsible for converting noradrenaline to adrenaline in the biosynthesis pathway. Although Pnmt and adrenaline are mainly produced in the adrenal medulla, Pnmt is also found in other sites in the body, such as the heart and brain (Ziegler et al., 2002). In addition to the medulla oblongata, Pnmt was also found in the pons and medulla of the brainstem (Goodchild et al., 2000). To determine the exact location of synthesis for these neurotransmitters, immunohistochemistry experiments are conducted using Calbindin, which is a widely-distributed protein.
protein that binds to calcium. The immunoreactivity of Pnmt in these locations is clear, but the physiological significance is still unresolved.

Description of Original Experiment

This project is a deviation from an original experiment led by Aaron Owji in Dr. Ebert’s lab. Aaron’s project centered around the observation that Pnmt cells can be found in the embryonic heart before the adrenal glands have even developed. Using this observation, Aaron developed two aims: (1) to create a genotype in mice that ablates Pnmt cells and (2) to determine if Pnmt enzymes are important for cardiovascular functioning.

Aaron was successfully able to develop a genetic model that ablates Pnmt cells in vivo. In fact, Pnmt mRNA levels in adrenal glands of mice with the Pnmt-ablated genotype were 97.6% lower than those of the wild-type control mice (Owji, 2015). Although Aaron also determined that there was cardiovascular dysfunction in mice with Pnmt-ablated cells (specifically, diminished left ventricular function), other unexpected results manifested during his experiment. By 5-6 months of age, the mice with Pnmt-ablated cells exhibited phenotypes that suggested a development of neuromuscular dysfunction. Mice were examined for motor dysfunction via indicators such as altered gait seen in their walking and kyphosis, which is a curvature of the spine creating a hunched back (Guyenet et al., 2010).

A tail-suspension reflex test was conducted. This test is performed by picking up the mouse via the tail and observing the reaction. A normal response would be for the mouse to
spread apart his legs away from the abdomen (Figure 3A). However, mice with Pnmt-ablated cells reacted by tightly clasping their legs together as seen in Figure 3B (Owji, 2015).

Upon using a BIO-GS3 grip strength testing device, it was determined that mice with Pnmt-ablated cells had significantly weaker grip strength measurements than their control counterparts (Figure 4) (Owji, 2015).
Overall, Aaron’s results initiated a further investigation on the role of Pnmt enzymes, specifically in the brain. While his results clearly demonstrate that ablation of Pnmt cells lead to cardiovascular and neurological dysfunction, the precise location and mechanism of Pnmt action in the brain is still unknown. Literature regarding this topic is sparse and limited. It was previously shown that Pnmt inhibitors lowered spontaneous motor activity (Katz et al., 1978). This project hypothesizes that Pnmt action affects the Purkinje cells in the cerebellum. Following this line of reasoning, if Pnmt cells are ablated then Purkinje cells should be negatively impacted (reduced or damaged), as well.
Overview of the Cerebellum

Latin for “little brain,” the cerebellum sits underneath the occipital lobe and the temporal lobes at the back of the brain (Figure 5). Although the cerebellum only makes up about 10% of the brain, more than half of the neurons that are found in the brain are localized in the cerebellum (Knierim, 1997). The cerebellum does not generate motor commands, but is responsible for fine tuning those commands in order to make movements fluid and accurate. Damage to the cerebellum thus leads to motor deficits. Common disorders linked to damage or inflammation of the cerebellum include Parkinson’s Disease, Cerebral Ataxia, and Dandy-Walker Syndrome.

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*Figure 5: Parts of the Human Brain. Image taken from Haig, H (2016).*
Parkinson’s Disease (PD) is a widely known chronic neurodegenerative disease characterized by tremors, abnormal gait and unstable posture. PD is listed as the 14th leading cause of death in the U.S. (Murphy, Xu, and Kochnanek, 2012). It occurs when brain cells don’t produce enough dopamine. Referring back to Figure 1, dopamine is responsible for synthesizing noradrenaline and adrenaline. It is due to this relationship that some researchers are linking PD to cerebellar damage and to declining Pnmt activity (Wu and Hallett, 2013) (Gearhart, Neafsey, and Collins, 2002).

Cerebral Ataxia is characterized by poor coordination, stumbling when walking, loss of balance, and difficulty with fine motor tasks. It is a result of damage or loss of brain cells in the cerebellum (Mayo Clinic Staff, 2016). Dandy-Walker Syndrome occurs when the cerebellum does not develop properly. The cerebellar vermis located in the center of the cerebellum is either missing or partially formed. There is a build up of fluid between the cerebellum and brainstem that causes an enlargement that is palpable in the back of the head. Other symptoms include slow motor development and lack of muscle coordination (National Institute of Neurological Disorders and Stroke, 2016). These are just a few maladies that are commonly linked to the pathology of the cerebellum. Understanding key components of the cerebellum will aid in treating these pathologies.

The cerebellar cortex has 3 distinct layers: the granule layer, the Purkinje cell layer, and the molecular layer (Figure 6). Purkinje cells from the Purkinje cell layer possess dendrites that extend to the molecular layer. There, the dendrites are positioned at right angles and receive input from parallel fibers. Purkinje cells are crucial components of a functioning cerebellum because they are the only output source from the cerebellar cortex (Purves et. al., 2001). Since
the Purkinje cells are such important structures in the cerebellum, it is highly probable that the neuro-motor dysfunction seen in mice with Pnmt-ablated cells can be attributed to damage or reduction of Purkinje cells.

Figure 6: Layers of the Cerebellar Cortex. Image taken from Atlas Catalog (2016).

Rational

Given that Purkinje cells are the sole source of output for the cerebellar cortex, it is possible to conclude that they play a significant role in motor control and coordination. If Pnmt is localized in these neurons, then it is also probable that they mediate these motor activities, as
well. This study thus hypothesizes that the Pnmt enzyme is expressed in the Purkinje cells that line the cerebellum. Multiple histological experiments will be performed to test whether or not such a connection exists. Results of this study will provide novel insight regarding the role of Pnmt in neurological regulation and motor control, and could thus have a long-range impact for patients that suffer from neuro-motor disorders such as Parkinson’s Disease, Cerebellar Ataxia, and others.

**Aims**

The first aim of this project was to determine if the Purkinje cells in the cerebellum of Pnmt-ablated mice models are depleted in comparison to those of the wild-type mice models. This will be done via manual counting of Purkinje cells following immunohistochemistry staining of both types of mice. If they are indeed depleted, then this would suggest that the Pnmt enzyme is localized in the Purkinje cells. This would further suggest that the neurological defects seen in the Pnmt-ablated mice models can be attributed to a lack of functioning Purkinje cells.

The second aim of this project was to determine if there are any morphological changes in the Purkinje cells of the Pnmt-ablated mice models. A morphological change would indicate that the Pnmt enzyme possesses an integral role in the growth and development of Purkinje cells. Thus, inhibiting Pnmt will damage Purkinje cells and result in the neuromuscular dysfunction observed in the mice with Pnmt-ablated cells. These aims give researchers the opportunity of understanding the significance of Pnmt activity and its mechanism in the brain. Only by elucidating such knowledge can researchers begin targeting key steps in the pathway in order to
design drug-intervention therapies capable of compensating for these motor deficits. This will impact the lives of many and improve the quality of life of people suffering from neuromuscular dysfunction.
CHAPTER TWO: MATERIALS AND METHODS

Overview of Methodology

The procedure of this experiment relied on histological processes and analysis to obtain data. The first part was to cryosection the brain tissues from mice with different genotypes. The sectioned slides then underwent a series of immunofluorescence experiments before being imaged by a confocal microscope. A maximum number of 30 slides per brain were cryosectioned and stained. Each slide contained 3 brain sections. The analysis consisted of examining every 3rd slide of the sectioned brains, resulting in a total of 10 slides per mouse genotype. The experimenter reduced possibility of bias by being blind to the genotypes of each mouse brain.

Successful analysis of data relied on consistent procedures and measurements. Each 3rd slide that was analyzed was imaged at 10X magnification. A region of interest consisted of a 500 μm by 500 μm rectangular box that was placed over the piece of tissue on a well-cut position. Once the data was obtained, the total number of Purkinje cells in the cerebellum was examined using statistical analysis to determine if there was a change in the amount of cells between different genotypes.

Animals

All procedures that required handling and physical contact with the mice were conducted by trained and authorized personnel in accordance with NIH guidelines and the University of
Central Florida Institutional Animal Care and Use Committees. Experienced personnel were also in charge of supervising the cryosectioning of the tissues and programming of the confocal microscope. Using primers, PCR, and isolated mice tail snips, the genotypes of the mice were identified. The mice used had the same genotypes as the ones used by Aaron Owji (2015). The genotype of the wild-type mice in the control group was $Pnmt^{+/Cre}; R26^{+/+}$. The genotype of the experimental mice group which caused an ablation of Pnmt cells was $Pnmt^{+/Cre}; R26^{+/DTA}$.

**Histology**

The procedures of handling mice tissue and sectioning equipment occurred under the direct supervision of trained personnel. The tissue was fixed in 4% paraformaldehyde and kept overnight at 4 degrees Celsius. The tissue was then washed 3 times with PBS. It was stored for at least 2 days in 30% sucrose in PBS at 4 degrees Celsius. To section using a cryosect, the tissue was placed in a mold and covered with Optimal Cutting Temperature (OCT) until frozen. Cryosectioning conditions were constant, cutting the tissue at 12 micrometers at a temperature of -19 degrees Celsius. To conserve slides, 3 sections were placed on each slide. The slides were stored in a slide box at -20 degrees Celsius until staining.
**Immunofluorescence**

Immunofluorescence is a process in which a primary antibody attaches to a protein on the surface of the cell. The primary antibody will only attach given that the it is the complement to the protein. For example, in order for the primary antibody to bind to the protein Calbindin, the antibody must be anti-Calbindin. The secondary antibody then binds to the primary antibody. Upon being hit with a specific wavelength of light, the secondary antibody releases a colored fluorophore response that can be visualized using a confocal microscope (Figure 7).

![Immunofluorescence Schematic](image)

*Figure 7: Immunofluorescence Schematic.*

A basic 2-day protocol for immunostaining was employed (summarized in Table 1). The first day consisted of air drying the slides for an hour at room temperature before removing excess OCT with forceps. Next, a Pap pen was used to encircle the tissues. This created a
hydrophobic barrier to prevent the tissues from drying up and preserve reagents. The tissues were then rehydrated with Phosphate-buffered saline (PBS) for 10 minutes and blocked for 2 hours at room temperature. The blocking solution consisted of 5% w/v dry nonfat milk, 0.3% v/v Triton X-100, 0.02% sodium azide, and 0.02% donkey serum in PBS. Primary anti-Calbindin antibody, which was mixed with the blocking solution at a 1:100 dilution, was added to the tissue and left at room temperature for 1 hour before being incubated at 4 degrees Celsius overnight. To ensure that the tissues did not dry up overnight and cause a lot of background fluorescence, the slides were left in a humidified chamber.

The following day, sections were washed 3 times for 10 minutes each with PBS to remove the primary antibody. The primary was saved and reused as needed. Secondary antibody was diluted with the blocking buffer at 1:750 and left for 2 hours at room temperature. DAPI, which stains the nucleus of cells, was also included with the mixture of the secondary antibody as a positive control. The slides were kept in a dark and humidified chamber during this time. Finally, the secondary was washed off with PBS 3 times for 10 minutes each and mounted with FluoroGel. When the slides hardened, they were imaged using the Zeiss 710 Confocal Microscope. Specific settings were saved on the microscope’s computer to ensure that data remained consistent. As a negative control, some sections were not given a primary. These sections followed the exact same procedure as previously outlined except that they remained in the blocking solution overnight, rather than in the primary antibody solution.
Table 1: Summary of Immunohistochemistry Protocol

<table>
<thead>
<tr>
<th>DAY 1</th>
<th>DAY 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Air dry (1 hr)/ Remove OCT</td>
<td>1. Wash sections with PBS (3 times, 10 min each)</td>
</tr>
<tr>
<td>2. Pap-Pen/ Humidified Chamber</td>
<td>2. Secondary Antibody with DAPI (2 hrs, dark conditions)</td>
</tr>
<tr>
<td>3. Rehydrate with PBS (10 min)</td>
<td>3. Wash sections with PBS (3 times, 10 min each)</td>
</tr>
<tr>
<td>4. Blocking (2 hrs)</td>
<td>4. Mount with FluoroGel</td>
</tr>
<tr>
<td>5. Primary anti-Calbindin (1 hr at RT, O/N at 4 degrees C)</td>
<td>5. Store for imaging with confocal microscope</td>
</tr>
</tbody>
</table>

Data Analysis

Once data was obtained as described above, specific criteria were used to analyze the results. To quantitatively measure if there is a difference in the amount of Purkinje cells between genotypes, a specific region of interest (500 µm X 500 µm) was established over properly imaged and sectioned tissues. This means that the tissue of the region was not folded over itself and was a true representation of the entire section. The number of Purkinje cells in that region were then counted. The average number of cells per micrometer (X) was calculated for each slide. The mean of these averages was then taken for each mouse brain. This resulted in an average number of Purkinje cells per brain and is equivalent to n =1. The experimenter was blind to the genotypes of the brains until data was analyzed in order to reduce bias.

For an example of how data was quantified, see the example in Figure 8 below. The yellow box surrounding the tissue is 500 µm X 500 µm. The Purkinje cells lining the cerebellum are counted. The image of the stained tissue is Cer 33 Slide 16, image a. In that image, the
experimenter counted 38 cells. After counting the number of cells for each image, the average of the slide was calculated. Taking the mean of every slide’s average number of cells resulted in the generation of an overall genotype average for each mouse.

![Figure 8: Example of Data Analysis Protocol.](image)

To determine if there is a morphological change in the Purkinje cells of different genotypes, a qualitative analysis is used. Properties such as cell size, shape, and location will be taken into account.
Statistical Analysis

To determine if quantitative results were statistically significant, the experimenter imported the values to Graphpad Prism. This program generated the bar graph seen in the results section (Figure 9). It also established the p-value that determines the significance of the data. A p-value less than 0.05 is needed in order to ensure that correlation is significant and not just due to chance or coincidence. If the p-value is greater than 0.05, then the experimenter must reject the hypothesis and accept the null hypothesis, meaning that the data is not statistically significant.
CHAPTER THREE: RESULTS

Results of Aim 1

The goal of Aim 1 was to use a manual cell counting technique to determine if there was a quantitative change in Purkinje cells between wild-type mice and mice with ablated Pnmt cells. The hypothesis of this aim was that the amount of Purkinje cells would be higher for control mice rather than their experimental counterparts.

A total of 8 mice were examined. Four of these mice were wild-type while the other four were DTA mice with ablated Pnmt cells. While the hypothesis was correct in that the mice with P-nmt ablated cells had fewer Purkinje cells than control mice, the null hypothesis must be accepted. The average number of Purkinje cells for the wild-type mice was 34.796 cells while the average number of the Purkinje cells for DTA mice was 32.772 cells (Table 2). After determining the calculations via the Graphpad Prism program, the p-value was 0.4868. Since this value is greater than 0.05, the data was not statistically significant (Figure 9).
Table 2: Average Number of Purkinje Cell in Mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Average Number of Purkinje Cell</th>
<th>Total Average Number of Purkinje Cells Per Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cer 17: DTA</td>
<td>39.39048</td>
<td></td>
</tr>
<tr>
<td>Cer 28: DTA</td>
<td>29.97470</td>
<td></td>
</tr>
<tr>
<td>Cer 31: DTA</td>
<td>30.73333</td>
<td></td>
</tr>
<tr>
<td>Cer 32: DTA</td>
<td>30.98750</td>
<td>32.771503</td>
</tr>
<tr>
<td>Cer 34: CTRL</td>
<td>39.00000</td>
<td></td>
</tr>
<tr>
<td>Cer 25: CTRL</td>
<td>32.00000</td>
<td>34.795833</td>
</tr>
<tr>
<td>Cer 33: CTRL</td>
<td>35.53333</td>
<td></td>
</tr>
<tr>
<td>Cer 37: CTRL</td>
<td>32.65000</td>
<td></td>
</tr>
</tbody>
</table>

Figure 9: Average Number of Purkinje Cells per Genotype.
Results of Aim 2

The purpose of Aim 2 was to qualitatively analyze the cells in order to determine if there were any morphological changes seen in the Purkinje cells between wild-type mice and mice with ablated Pnmt cells. It was predicted that the DTA mice would have altered or damaged Purkinje cell structure that would disturb the normal functioning of the cells. This would attribute to the neuromuscular function dysfunction observed in mice with Pnmt-ablated cells (Owji, 2015).

Purkinje cells have established dendrites that allow the cells to act as the sole output source of the cerebral cortex. These dendrites extend to the molecular layer of the cerebral cortex in order to carry out this function (Figure 6). Purkinje cells were first discovered by Jan Evangelista Purkyně in 1837. Figure 10 below depicts a drawing by Santiago Ramón y Cajal of healthy Purkinje cells in the cerebellum. As you can see, the Purkinje cells possess a large and established network of dendrites. These branched structures are responsible for generating action potentials in the cerebellum. The axons are aligned next to each other, forming a single layer. Due to their important function in the cerebellum, the architecture of these cells is relatively maintained through evolution.
Qualitative analysis of stained Purkinje cells gave the experimenter an opportunity to
determine if morphological changes in the architecture of the cell were observed when Pnmt
cells were ablated. The experimenter remained blind to the true genotypes of each image until
the comprehensive analysis was complete.

One key qualitative observation when comparing both genotypes was the diminishing of
dendritic branches in the DTA models. As previously described, these branches should be largely
defined and extend into the molecular layer. Figure 11 below shows the lack of defined dendrites seen in DTA mice (Figure 11A-11B) compared to the clear network of branches observed in control mice (Figure 12A-11B). As you can see in Figure 11A-11B, there are gaps present in the molecular layer where the Purkinje cell dendrites should be (emphasized by the yellow circle).

Figure 11: Stained Image of DTA Mouse Cerebellum. (A) Cer 31, Slide 10, Image C. (B) Cer 31, Slide 9, Image F.

Contrary to the DTA mice, control mice with intact Pnmt cells also portray intact Purkinje cell dendrites as seen in Figure 12A-12B (emphasized by the yellow circle).
Another observation that was brought to the attention of the experimenter were the clustered cells depicted in DTA mice. As previously mentioned, Purkinje cell axons are aligned next to each other in a single file to form one layer. DTA mice showed axons being tightly packed together, sometimes appearing in multiple layers (Figure 13) compared to control mice (Figure 14).
Figure 13: Stained Image of DTA Mouse. Cer 31, Slide 19, Image E. Purkinje neurons are closely grouped together, almost creating multiple layers.

Figure 14: Stained Image of Control Mouse. Cer 25, Slide 14, Image C. Purkinje neurons are evenly aligned in a single layer.
CHAPTER FOUR: DISCUSSION

This study examined the effects of ablating Pnmt cells on Purkinje neurons lining the cerebellum. These effects were examined quantitatively via manual cell counting and qualitatively via comparison of immunofluorescence images. It was predicted that mice with ablated Pnmt cells would have a reduced amount of Purkinje cells compared to that of control mice with intact Pnmt cells. They were also expected to have damaged or deteriorated Purkinje cells.

It was determined that control mice did indeed have a greater amount of Purkinje cells. Although, this data had a p-value greater than 0.05, making the connection statistically insignificant. When viewing morphological distinctions in Purkinje cells, the experimenter determined that DTA mice had underdeveloped Purkinje dendrites. The cells were also tightly packed together in clusters, instead of being aligned in a single file around the cerebellum.

Future Considerations

The results generated in this study were only preliminary and barely scratched the surface of the potential it can have. Although data was statistically insignificant, qualitative examination suggests that Pnmt does have a role in the function or development of Purkinje cells. To expand on this project, more mice should be examined to ensure data is significant and not spontaneous. The experimenter only used a total of 8 mice (4 control mice and 4 experimental mice). To truly test the significance of ablating the Pnmt on cerebellar Purkinje cells, more samples are required.
Another future direction that this project can take is optimizing immunostaining protocol for different cellular markers. Rather than staining for Purkinje cells by using Calbindin, a more preferred method would be to stain for Pnmt cells directly. Currently in the lab, a protocol is developed and optimized for successfully staining Pnmt in the adrenal glands and in the heart. However, when the same protocol is applied to the brain of the mice, results are inconclusive.

Researchers should also attempt to examine effects of Pnmt ablation on other parts of the brain. Specifically, investigating the hypothalamus might yield significant results regarding the role of Pnmt action in the brain. The hypothalamus links the nervous system to the endocrine system via the pituitary gland. Since hormones are released by the endocrine system, this structure of the brain warrants further investigation. An analysis of the brain stem can also be significant since it links motor commands from the CNS to the nerves of the PNS. Understanding the mechanistic pathway of key enzymes such as Pnmt in the brain can help biochemists develop intervention therapies that combat the neuro-motor disorders affecting many people today. Similarly, examining the spinal cord can provide further insight regarding the hindlimb paralysis that mice with ablated Pnmt cells portray.
REFERENCES


