2016

Cardiac Consequences of Selective Adrenergic Cell Ablation in Mice

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CARDIAC CONSEQUENCES OF SELECTIVE ADRENERGIC CELL ABLATION IN MICE

by

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A thesis submitted in partial fulfillment of the requirements
for the Honors in the Major Program in Molecular and Microbiology
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

ABSTRACT

Phenylethanolamine-N-methyltransferase (Pnmt), is the enzyme that catalyzes the conversion of noradrenaline to adrenaline. It has been found in the embryonic heart and in certain adult heart cells, including intrinsic cardiac adrenergic cells, intracardiac neurons, and cardiomyocytes, but their physiological role in the heart is not well understood. To determine the function of Pnmt-expressing cells in the developing heart, a novel genetically-targeted mouse model that causes selective cellular suicide of Pnmt-expressing cells was created by mating *Pnmt-Cre Recombinase* knock-in mice ($Pnmt^{Cre/Cre}$) with *ROSA26-eGFP-DTA* ($R26R^{+/DTA}$). The "cellular suicide" allele is the *Diptheria Toxin A* (DTA) gene fragment. Activation of the *DTA* suicide allele is dependent upon *Cre* expression, which is under the control of the endogenous Pnmt gene locus (i.e., expression is restricted to adrenaline-producing "adrenergic" cells). Ongoing studies in Dr. Ebert's laboratory have shown that *Pnmt-Cre/DTA* mice have a loss of adrenergic cells in the adrenal gland and begin developing serious cardiac and neurological deficits within one month after birth. The purpose of my project is to examine the potential cardiac consequences of selective adrenergic cell ablation in this model. Aim 1 of this study is to analyze echocardiography data from mice with genetic ablation of adrenergic cells compared to age-matched (littermate) controls over the first 6-months after birth. Preliminary evidence indicates that there is substantial loss of function that progressively worsens with age in the ablation group compared to controls. Aim 2 of this study seeks to uncover evidence of adrenergic cell ablation in the heart using histological and immunofluorescence staining techniques. We predict that these experiments will provide physiological and anatomical evidence showing that Pnmt-expressing cells in the heart make significant contributions to

cardiac development and function. This knowledge is expected to increase our basic understanding about the specific roles adrenergic cells play during heart, and could lead to the development of novel treatment strategies for certain types of cardiac defects in the future.

ACKNOWLEDGMENTS

This thesis would not be possible without the encouragement and support of my principal investigator Dr. Steven Ebert who believed in me and encouraged me to pursue my passions. I will forever be thankful to have such a wonderful mentor who started my research interests by giving me the opportunity to work in his lab. I would like to thank my committee members, Dr. Dinendar Singla and Dr. Ulas Bagci, for taking time from their busy schedules to help me with this thesis. I would like to thank all of the lab members who have worked with me these past two years for teaching me and providing me with all I need to grow in the lab. Lastly, I want to thank my family for encouraging me to reach higher and higher each day. I am truly blessed to have worked on this project with all of these amazing individuals.

DEDICATIONS

To every teacher in my life.
To my first teachers, my parents.
To my lab members.
Thank you for instilling within me the knowledge, passion, and determination I need each and every day, to get me one step closer to my dreams.

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CHAPTER 1: INTRODUCTION

Cardiovascular disease is the number one cause of death globally (World Health Organization, CVDs). One in four deaths in the United States are due to cardiovascular problems (World Health Organization, CVDs). Cardiac pathologies can be caused by a large number of factors including muscle contraction and conduction problems. Heart muscle contains various receptors that function in combination with hormones to result in contraction. One such enzyme involved in the biosynthesis of epinephrine is phenylethanolamine-N-methyltransferase (Pnmt). Epinephrine plays both conductive and contractive roles in the myocardium.

Overview of the Nervous System

The nervous system can be divided into several different subgroups. The somatic nervous system, which contains the central and peripheral nervous systems, involves actions that are voluntary (Bear, 2007). The central nervous system is composed of the brain and spinal cord (Bear, 2007). The peripheral nervous system is composed of the neurons outside of the brain and spinal cord (Bear, 2007). Another division of the nervous system is the autonomic nervous system which, contains the sympathetic and parasympathetic systems, controlling involuntary functions (Bear, 2007). The parasympathetic and sympathetic nervous systems oppose each other in action. While one elicits an excitatory effect the other serves to calm (Bear, 2007).

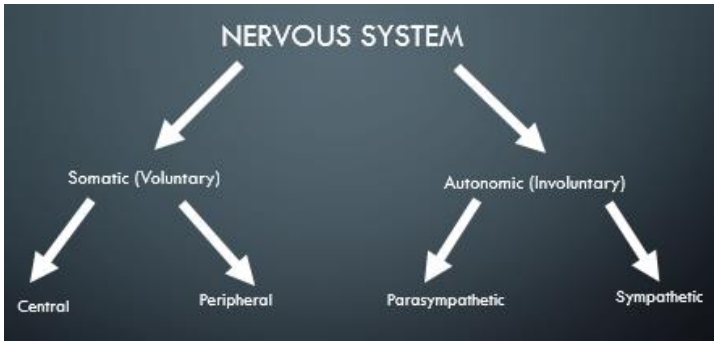

Figure 1: Breakdown of the Nervous System

The sympathetic nervous system is most know for the flight or fight response (Bear, 2007). When an individual feels threatened the adrenal glands are stimulated to release epinephrine which, then acts on the receptors of the heart to increase heart rate and dilate blood vessels for increased blood circulation to distal tissues (Bear, 2007). Cardiac contraction rate is influenced by neurons and hormones acting in conjunction with the heart. Cardiac smooth muscle contains receptors that bind ligands. When a ligand binds to a receptor this elicits an electrical impulse to travel down the neuron resulting in contraction (Costanzo, 2014). In the heart the ligands that influence contraction are epinephrine and norepinephrine (Costanzo, 2014). Epinephrine is secreted from the adrenal medulla and binds to β_2 receptors (Costanzo, 2014). Norepinephrine is secreted from the adrenal medulla and sympathetic nerve endings (Costanzo, 2014). It stimulates the α_1 receptors. α_1 receptors are located in the smooth muscles of blood vessels and aid in blood vessel contraction (Costanzo, 2014). β_1 receptors are located in cardiac muscle and stimulate contractions of heart walls (Costanzo, 2014). β_2 receptors are located in the smooth muscle of blood vessels and stimulation of these receptors results in blood vessel dilation (Costanzo, 2014).

Overview of Cardiac Conduction

Cardiac tissue has a separate nervous system that is only influenced by external nervous stimulation when heart rate needs to be adjusted. The pace maker of the heart is the sinoatrial node (SAN) (Costanzo, 2014). The SAN is located in the external wall of the right atrium (Costanzo, 2014). The atrioventricular node (AVN) causes a significant delay in electrical conduction, which allows sufficient time for the atria to release blood into the ventricles prior to ventricular contraction (Costanzo, 2014). Apart for the AVN delay, the AVN works as a secondary pacemaker in cases where the SAN is damaged (Costanzo, 2014). From the AVN, electrical conduction flows further through the interventricular septum (Costanzo, 2014). The bundle of fibers in this region is referred to as the Bundle of His (Costanzo, 2014). This bundle splits at the base of the interventricular septum into the left and right bundle branches the end in the walls of the left and right ventricles, respectively (Costanzo, 2014). Through SAN stimulation, electrical impulses travel down the Purkinje fibers thereby stimulating coordinated contractions of heart chambers (Costanzo, 2014).

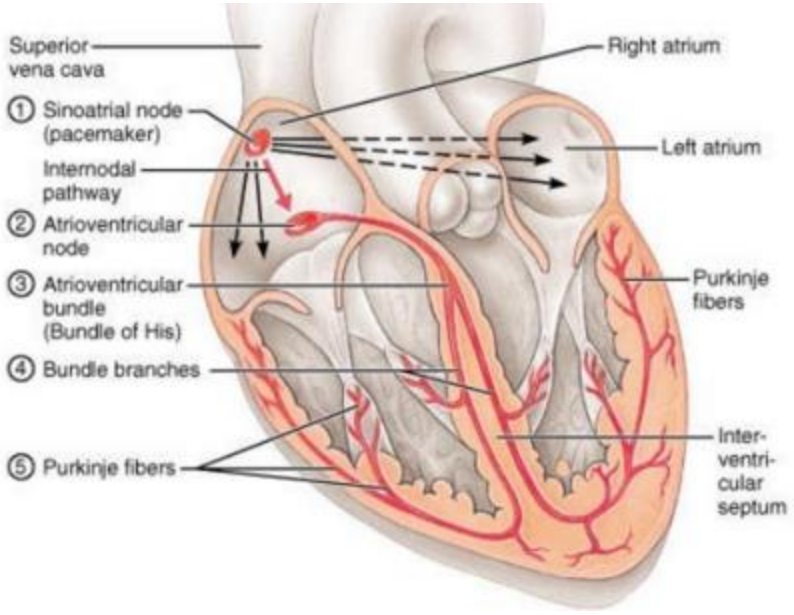

Figure 2: Cardiac Conduction System

Overview of Ablation Model

Phenylethanolamine-N-methyltransferase (Pnmt) is an enzyme that catalyzes the conversion of norepinephrine (NE) into epinephrine (EPI). Thus, cells that express Pnmt typically produce EPI (also known as adrenaline) and are, therefore, referred to as adrenergic cells. Pnmt is known to be found in the adrenal medulla (Kvetnansky et al., 2012). The adrenal medulla is the center portion of the adrenal glands, where adrenal hormones are stored. The Ebert lab has previously shown that Pnmt-producing cells are not solely in the adrenal glands, but can also be seen in high concentration in the left ventricle of the heart as shown in Fig. 1 (Ebert et al., 2004) (Ebert et al., 2008). Previous studies have shown that epinephrine deficienes in mice effects stress response (Bao, 2007).

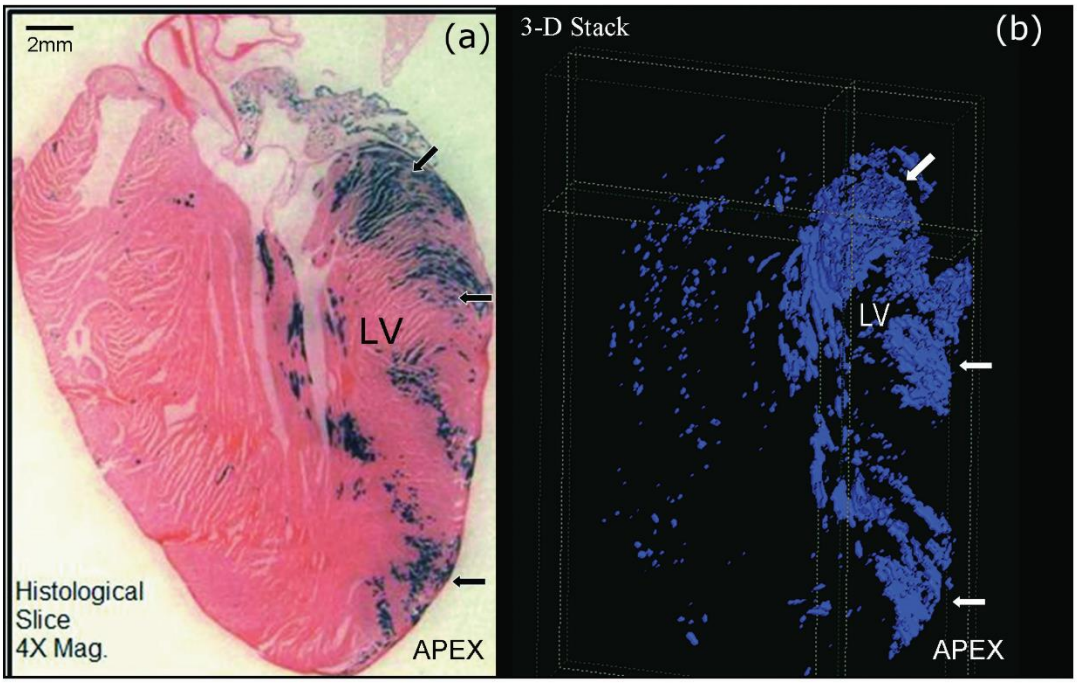

Figure 3: Left ventricle concentration of Pnmt expressing cells. Three dimensional (3D) reconstruction of the XGAL+ staining in the Pnmt+/Cre, ROSA26+/βgal heart

Image: Osuala, K., K. Telusma, et al. PLoS One 6(7)

The ablation of pnmt cells is achieved through activation of DTA expression triggered by Pnmt-dependent Cre expression. DTA is an AB toxin where the A subunit induces apoptosis of the cell (Collier, 2001). In this model the A subunit is expressed directly within cells when Cre-recombinase is activated, selectively ablating pnmt cells (Owji et al., 2015).

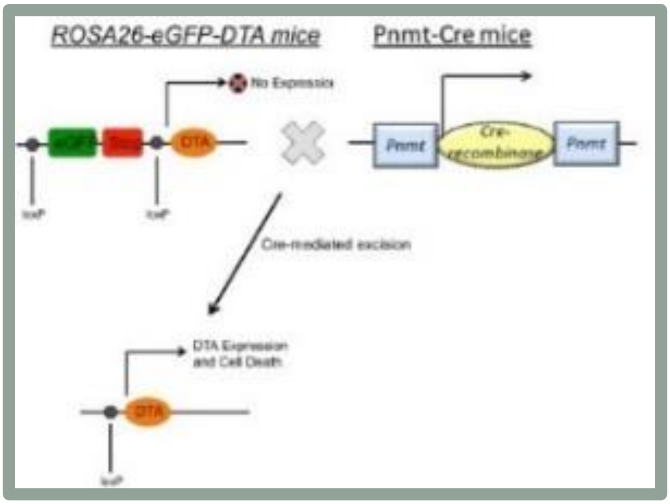

Figure 4: Generating of *Pnmt-Cre/DTA* mice from *ROSA26-eGFP-DTA* and Pnmt-Cre. Pnmt cell ablation caused by DTA expression in *Pnmt-Cre/DTA* mice.

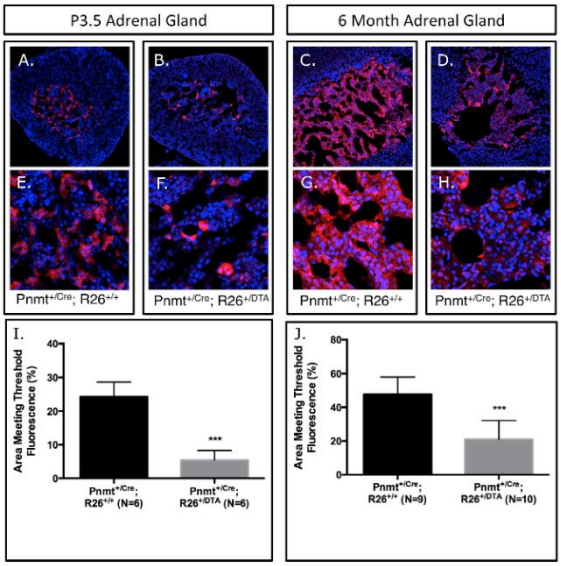

Figure 5: Genetic ablation of adrenergic cells leads to significant losses of Pnmt+ cells mRNA content in mouse adrenal glands. Pnmt protein levels significantly down in *Pnmt-Cre/DTA* mice at postnatal day 3.5 and 6 months.

Image: Owji, A., et al. AHA

Rational

One of the most well-known methods for understanding the role of a particular enzyme in a biochemical pathway is by removing the enzyme and characterizing the defects seen in the ablation model. When an initial ablation model is created the phenotypic defects are characterized through various techniques. In this study defects in cardiac function were studied through echography, threshold analysis, and visualizing stained heart sections.

Aims

Aim 1 of this study is to analyze echocardiography data from mice with genetic ablation of adrenergic cells compared to age-matched (littermate) controls over the first 6-months after birth. Preliminary evidence indicates that there is substantial loss of function that progressively worsens with age in the ablation group compared to controls.

Aim 2 of this study seeks to uncover evidence of adrenergic cell ablation in the heart using histological and immunofluorescence staining techniques. We predict that these experiments will provide physiological and anatomical evidence showing that Pnmt-expressing cells in the heart make significant contributions to cardiac development and function. This knowledge is expected to increase our basic understanding about the specific roles adrenergic cells play during heart, and could lead to the development of novel treatment strategies for certain types of cardiac defects in the future.

CHAPTER TWO: MATERIALS AND METHODS

Animals

All procedures and handling of mice were conducted in accordance with the University of Central Florida Institutional Animal Care and Use Committees. Mice were handled by PI and Graduate Student. The collected samples will be used for data analysis, histology, and Immunofluorescence studies. The strains of mice are: *Pnmt* $^{+/Cre}$, *Pnmt* $^{+/+}$, *Pnmt* $^{+/Cre}$, and *Pnmt*-$^{Cre/Cre}$.

Echocardiography

Echocardiography will be performed on the mice to visualize left ventricular contractions. This data will then be analyzed using VisualSonics software, in the Vevo2100 equipment, where two individuals blind to the genotypes traced the left ventricle. The software generates data on various cardiac functions based on the left ventricular tracing. The various cardiac functions will be measured blind and then separated by genotype. The average data for each genotype will be used to calculate averages on various cardiac functions that could then be used to better understand the phenotypic defects seen in cardiac contraction across different genotypes.

Histology

The heart tissues were sectioned and embedded in paraffin. A hematoxylin and eosin stain will be performed to visualize the various structures of the heart sections. The following protocol will be used to rehydrate the sectioned tissue. First they are incubated at sixty degrees for one hour. After this the tissues go through a rehydration protocol where they are dipped in an

ethanol gradient ending in xylene. Once the tissues on rehydrated the hematoxylin and eosin stain will be performed. The rehydrated tissues are placed in hematoxylin for 3 minutes followed by tap water, acid alcohol, tap water, ammonium hydroxide, 90% ethanol, Eosin Y for 20 seconds dipping, and finally an ethanol gradient prior to ending in xylene. The hematoxylin stains the nuclei of cells while, the eosin stains the cytoplasm. This stain will be used to visualize structures of the heart and SAN regions to determine any phenotypic defects present.

Immunofluorescence

Adrenal and heart sections were embedded in optimal cutting temperature compound (OCT) prior to staining. Immunofluorescence staining will be performed. Initially, the OCT is removed and the tissue is rehydrated in PBS. After this primary rabbit anti-pnmt antibody with blocking solution is added. The tissue is then incubated for one hour at room temperature and at four degrees overnight. Next the tissue sections are washed in PBS three times for ten minutes each. Lastly, the secondary antibody is added and the tissues are incubated for two hours. DAPI, which stains the nuclei of cells, is included in the adrenal gland stains. Both the nuclei and hCN4, which are ion channels found in pace maker cells, will be stained along with Pnmt in the heart sections. The tissue is then washed three times with Phosphate Buffered Saline (PBS) for 10 minutes each. These sections will then be visualized with confocal microscopy. The adrenal images will be used to generate threshold data to determine the amount of pnmt expressed in each genotype. The heart sections will be used to analyze the SA nodal regions for pnmt cells. This was done through counting the various cells that light up as pnmt positive in the SA nodal regions. First a 500x500 box will be generated and the cells within that area that are positive for HCN4 (green) counted. Then the pnmt positive (red) cells are counted to determine if there is

any difference in the number of pnmt cells in the SA nodal area that could account for the conduction problems seen in the ablation mouse model. DAPI nonspecifically stains nuclei and was used to visualize individual cells.

Statistical Analyses

Statistical analysis of echocardiography data will be determined through t-test analysis to compare cardiac functional markers of *Pnmt* $^{+/Cre}$, *Pnmt* $^{+/+}$, *Pnmt* $^{+/Cre}$, and *Pnmt*-$^{Cre/Cre}$. Immunofluorescence images will be analyzed through counting of the number of Pnmt positive pacemaker cells within the Sinoatrial Node. Comparative analyses between the genotypes will be used to determine any differences seen between the volume of pacemaker cells present in each genotype. P-values were set at less than 0.05.

Expected Results

We expect to that these experiments will provide physiological and anatomical evidence showing that Pnmt-expressing cells in the heart make significant contributions to cardiac development and function.

Alternative Strategies

If the analysis shows that there is no significant difference between the genotypes we will begin exploring alternative mechanisms that could be compensating for the Pnmt ablation.

CHAPTER THREE: RESULTS

Echocardiography

Echocardiography was used to measure cardiac functional outputs in the left ventricle to see if any significant difference was observed between genotypes. Pnmt-Cre mice here are the control group in comparison to Pnmt-Cre/DTA, adrenergic ablation model. The measurement were made at baseline cardiac parameters. During month one and two little difference is seen in functional output values. Starting at month three significant differences ($p<0.001$) in heart rate, stroke volume, and cardiac output was observed. During month four, five, and six the ablation mice started showing a significant decrease in heart rate, stroke volume, ejection fraction, and cardiac output in comparison to the control mice.

Table 1 Monthly Echocardiography Data

	1 Month			2 Month			3 Month		
	$Pnmt^{+/Cre}; R26^{+/+}$	$Pnmt^{+/Cre}; R26^{+/DTA}$		$Pnmt^{+/Cre}; R26^{+/+}$	$Pnmt^{+/Cre}; R26^{+/DTA}$		$Pnmt^{+/Cre}; R26^{+/+}$	$Pnmt^{+/Cre}; R26^{+/DTA}$	
	N=26	N=24	p-value	N=11	N=7	p-value	N=15	N=10	p-value
Heart Rate (BPM)	428 ± 8	400 ± 8	0.019	440 ± 11	412 ± 7	0.085	442 ± 10	383 ± 7	<0.001
Stroke Volume (µL)	35 ± 1	31 ± 1	0.04	35 ± 2	28 ± 3	0.035	39 ± 2	25 ± 2	<0.001
Ejection Fraction (%)	67 ± 2	57 ± 2	<0.001	64 ± 3	51 ± 5	0.033	60 ± 3	49 ± 3	0.029
Fractional Shortening (%)	37 ± 1	30 ± 1	<0.001	35 ± 2	26 ± 4	0.054	32 ± 2	24 ± 2	0.022
Cardiac Output (mL/min)	15 ± 1	13 ± 1	0.006	16 ± 1	12 ± 1	0.011	17 ± 1	10 ± 1	<0.001
Systolic Diameter (µm)	2.3 ± 0.1	2.6 ± 0.1	0.007	2.4 ± 0.1	2.7 ± 0.2	0.209	2.7 ± 0.1	2.7 ± 0.2	0.967
Diastolic Diameter (µm)	3.6 ± 0.1	3.6 ± 0.1	0.431	3.6 ± 0.1	3.6 ± 0.2	0.985	3.9 ± 0.1	3.5 ± 0.2	0.042
	4 Month			5 Month			6 Month		
	$Pnmt^{+/Cre}; R26^{+/+}$	$Pnmt^{+/Cre}; R26^{+/DTA}$		$Pnmt^{+/Cre}; R26^{+/+}$	$Pnmt^{+/Cre}; R26^{+/DTA}$		$Pnmt^{+/Cre}; R26^{+/+}$	$Pnmt^{+/Cre}; R26^{+/DTA}$	
	N=18	N=14	p-value	N=17	N=12	p-value	N=18	N=12	p-value
Heart Rate (BPM)	447 ± 9	387 ± 9	<0.001	452 ± 8	410 ± 13	0.005	477 ± 11	388 ± 15	<0.001
Stroke Volume (µL)	41 ± 1	27 ± 2	<0.001	43 ± 1	30 ± 1	<0.001	42 ± 2	29 ± 2	<0.001
Ejection Fraction (%)	63 ± 2	51 ± 2	0.001	66 ± 2	52 ± 3	<0.001	66 ± 2	53 ± 5	<0.001
Fractional Shortening (%)	34 ± 2	26 ± 1	0.002	36 ± 2	27 ± 2	<0.001	36 ± 1	28 ± 3	0.013
Cardiac Output (mL/min)	18 ± 1	11 ± 1	<0.001	19 ± 1	12 ± 1	<0.001	20 ± 1	11 ± 1	<0.001
Systolic Diameter (µm)	2.6 ± 0.1	2.6 ± 0.1	0.738	2.5 ± 0.1	2.7 ± 0.1	0.114	2.4 ± 0.1	2.6 ± 0.1	0.278
Diastolic Diameter (µm)	3.9 ± 0.1	3.5 ± 0.1	<0.001	3.9 ± 0.1	3.7 ± 0.1	0.090	3.8 ± 0.1	3.6 ± 0.1	0.125

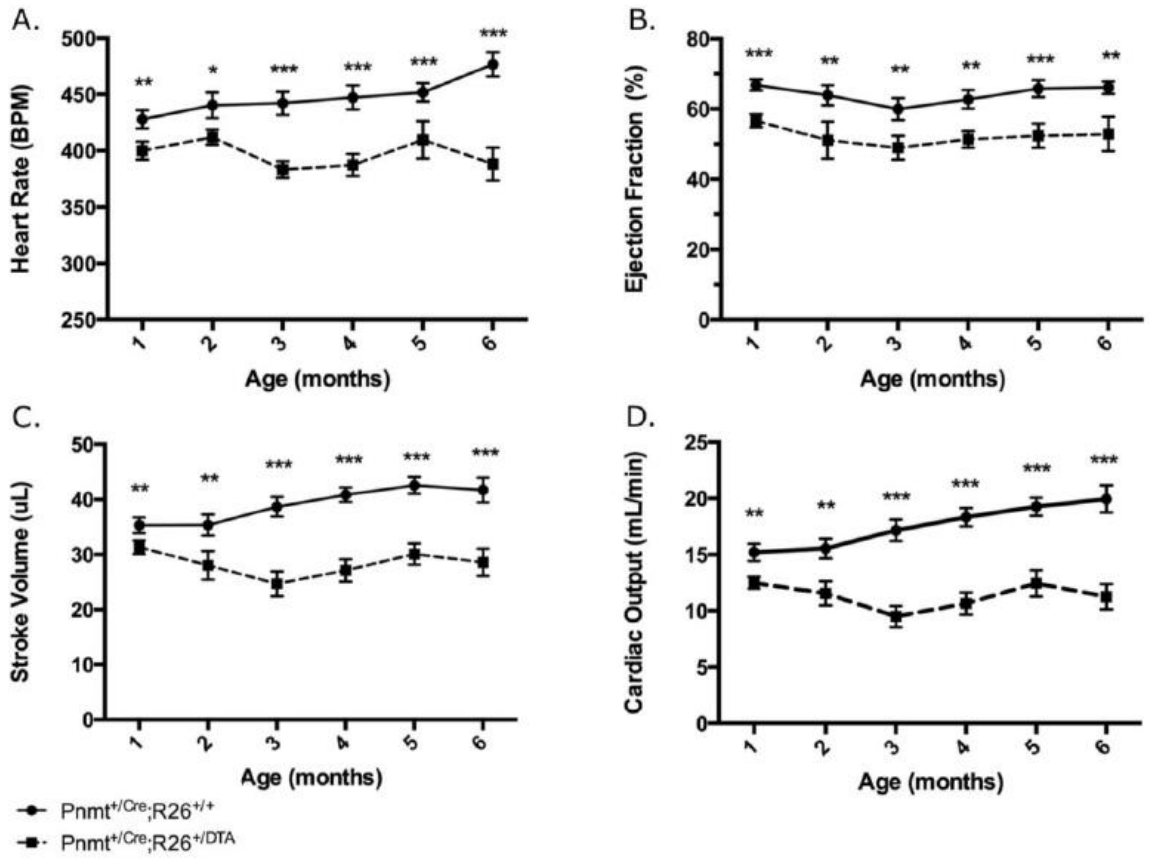

Figure 6: Echocardiography of Left Ventricular Dysfunction in Mice. After month 2 significant differences in heart rate, ejection fraction, stroke volume, and cardiac output can be observed.

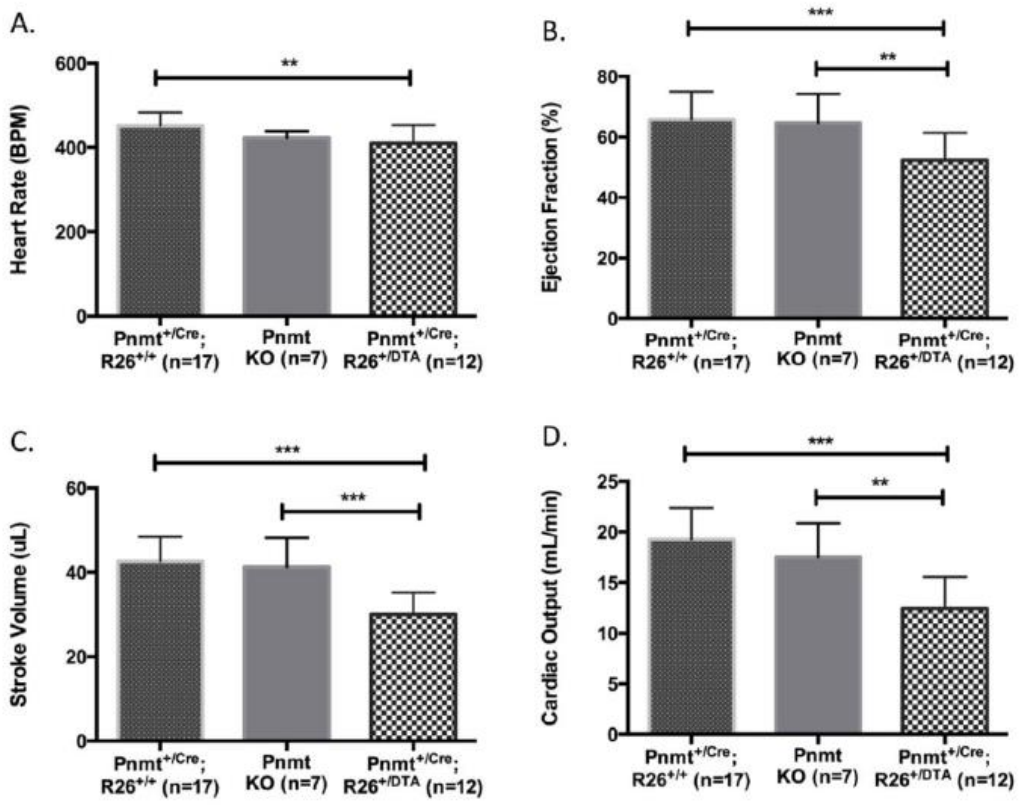

Figure 7: Echocardiography of Left Ventricular Dysfunction in Mice. Significant differences in heart rate, ejection fraction, stroke volume, and cardiac output can be observed in Pnmt KO and Pnmt+Cre/DTA mice in comparison to the controls.

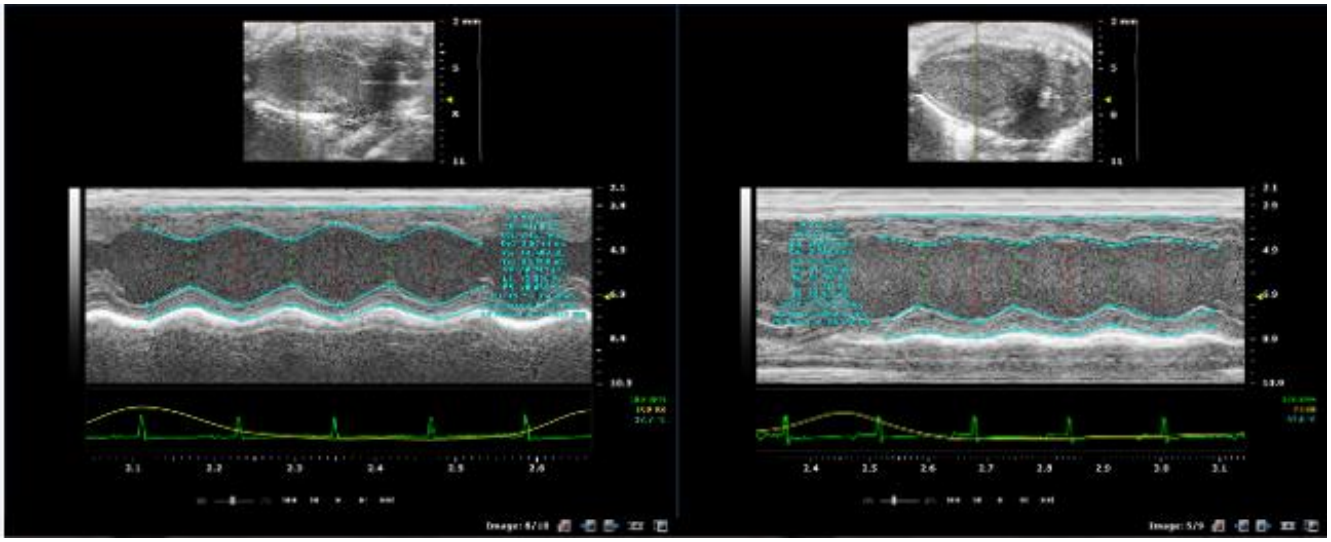

Figure 8: Echocardiography images of left ventricular function of Pnmt-Cre mice (left) and Pnmt-Cre/DTA (right) at baseline. A decrease in contractility of the left ventricle is visible.

Histology

Hematoxylin and Eosin stain was performed on heart tissue sections of both genotypes to identify any morphological defects seen in the tissue of the hearts. Some tissues have a slight thinning look in the posterior left ventricular wall. This could potentially be cardiac muscle hypertrophy and further staining and quantitative analyses must be performed on this data (Papamitsou, 2011).

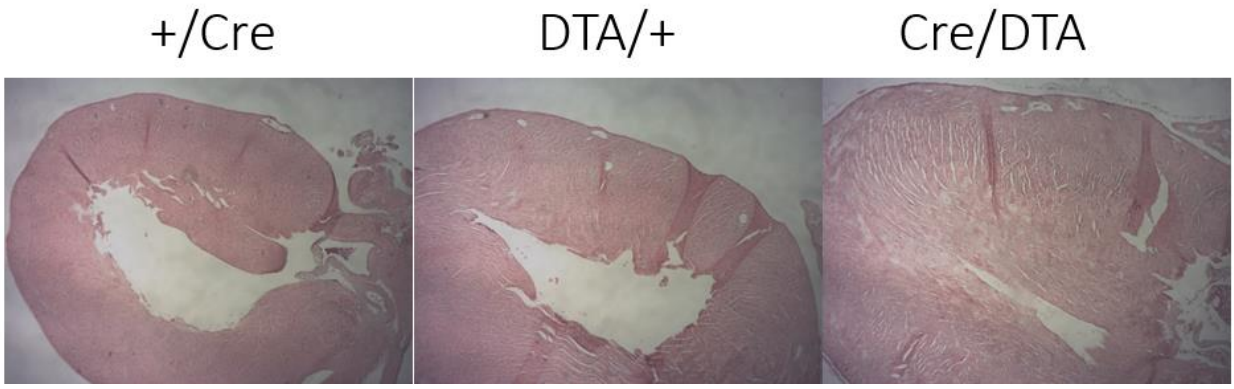

Figure 9: Hemotoxylin and Eosin staining of +/Cre, DTA+ and Cre/DTA mice. Some tissue differences observed. Cre/DTA shows visual thinning of the left ventricle further studies must be performed to see if cell depletion could be a cause.

Immunofluorescence

Immunofluorescence staining was used to count cells in the SA node region of cardiac tissue. No significant difference was seen in the number of overall cells and pnmt cells in the SA nodal region between the Pnmt+/Cre and Pnmt-Cre/DTA mice. The images and numbers need to be reevaluated and confirmed as images seem to contain equal number of HCN4 and Pnmt cells in same regions however, the Velocity Software used for counting purposes is not reflecting this observation. More analysis must be done with comparison to controls.

Table 2: Sinoatrial Node Cell Counts

Pnmt+/Cre	**DAPI (Blue)**	**HCN4 (Green)**	**Pnmt (Red)**
SH 25	485.00	332.00	32.00
SH 27	477.00	264.00	17.00
SH 33	454.33	362.67	3.00
SH 34	488.50	323.00	16.00
SH 38	189.00	183.17	52.00
Average	**418.77**	**292.97**	**24.00**
	-		
Pnmt-Cre/DTA	**DAPI (Blue)**	**HCN4 (Green)**	**Pnmt (Red)**
SH 17	221.00	134.67	26.00
SH 28	274.40	238.80	0.00
SH 32	355.50	356.50	7.50
SH 39	468.00	402.33	1.67
Average	**329.73**	**283.08**	**8.79**
	-		
T-Test	**0.84105548**	**0.840930768**	**0.83635656**

Sinoatrial node cells count of Pnmt positive cells in Pnmt+/Cre and Pnmt-Cre/DTA mice.
$p>0.01$, $N = 9$

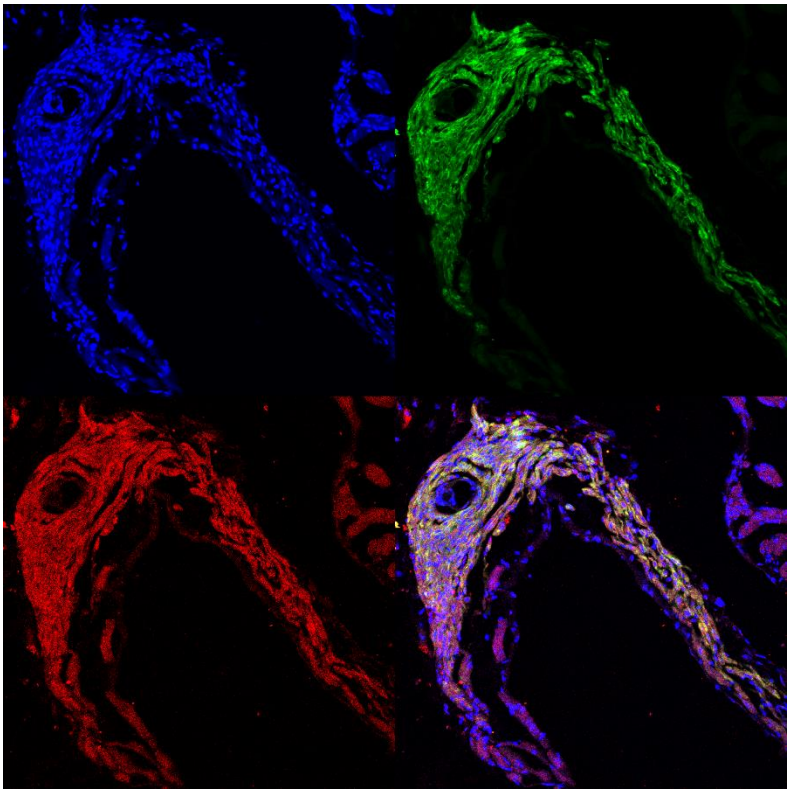

Figure 10: Sinoatrial node immunofluorescence staining. DAPI (top left) indicates nuclei, HCN4 (top right) stains SA nodal region, Pnmt (bottom left) , and composite (bottom right)

CHAPTER FOUR: DISCUSSION

These results suggest that ablation of Pnmt cells shows a significant deficit in cardiac function. The hypothesis was based on the understanding that Pnmt played a vital role in embryonic development. Since the enzyme was thought to be essential it was hypothesized that the lack of Pnmt would cause embryonic lethality. However, the mice, though showing significant deficits especially in terms of stress response, did not die in the fetal stage of development.

This project then began to characterize the role of Pnmt producing cells in the heart. In order to determine this the Pnmt-Cre/DTA mouse was developed. Diphtheria toxin α (DTA) is the α subunit of the diphtheria toxin, which is an AB toxin (Collier, 2001). The α subunit is expressed in the Pnmt-Cre/DTA mice, since the Cre Recombinase causes cleaving at the loxP sites, removing the stop codon and allowing the expression of DTA. DTA is a cytosolic protein that, when expressed, stops all protein synthesis mechanisms in the cell. This cessation induces the cell to go into apoptosis. The final result is a complete ablation of Pnmt producing cells in the Pnmt-Cre/DTA mice. From here, the phenotypic differences were characterized to determine the functional role of Pnmt by understanding the deficits caused by the lack of Pnmt cells, and therefore epinephrine production.

Deficits in heart rate, ejection fraction, stroke volume, and cardiac output are observed starting at 2 months of age. This indicates a contractile deficit in the left ventricle. Further characterization of the left ventricle showed a potential thinning of cells in the posterior left ventricular wall that needs further studies. This could be hypertrophy of the cardiac muscle

(Papamitsou, 2011). Quantitative studies potentially looking to the cell density per area in this region could lead to a better understanding. Sinoatrial analysis for Pnmt cell depletion showed no significant Pnmt cell difference between genotypes in the sinoatrial nodal region. Further studies delving into the brain could be used to further understand the neurological deficits seen in the mice.

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