2011

Cross-talk of retinoic acid and adrenergic hormone signaling may influence development of cardiac conduction and rhythmicity in utero

Sabikha Alam
University of Central Florida

CROSS-TALK OF RETINOIC ACID AND ADRENERGIC HORMONE SIGNALING MAY INFLUENCE DEVELOPMENT OF CARDIAC CONDUCTION AND RHYTHMICITY IN UTERO

by

SABIKHA ALAM

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 2011

Thesis Chair: Dr. Steven Ebert

ABSTRACT

Stress hormones, adrenaline and noradrenaline, have been shown to be critical for heart development. Mice lacking dopamine β-hydroxylase (Dbh), an enzyme responsible for synthesis of these adrenergic hormones, die during mid-gestation due to cardiac failure. Prior research showed that adrenergic cells are found within the electrical conduction system of the heart, and adrenergic deficiency leads to slowed cardiac conduction during embryogenesis. Microarray analysis of wild-type ($Dbh^{+/+}$) and knockout ($Dbh^{-/-}$) mouse hearts revealed significant differences in expression of retinoic acid (RA) signaling genes. RA signaling has also been shown to be critical for heart development. These data suggest that heart failure due to adrenergic deficiency may be dependent upon RA signaling. This led to the hypothesis that adrenergic hormones promote the development of the electrical conduction system through modulation of RA signaling. To test this, embryonic mouse hearts were cultured with LE 135, a RA receptor blocker. Heart rate, arrhythmic index (AI) and conduction time were measured. Under these conditions there was a marked increase in arrhythmias. Hearts treated with LE 135 showed a mean AI of 0.232±0.057 after 24 hours of treatment while when untreated had an AI of 0.083±0.028 ($p<0.05;n=15$). In contrast, there was no significant change in heart rate or conduction speed after 24 hours with or without the retinoic acid receptor blocker. To determine if adrenergic stimulus influences retinoic acid response, an established RA-sensitive reporter cell line was employed. These F9-RARE-LacZ cells were treated with forskolin (cAMP regulator) and isoproterenol (β-agonist) to measure changes in RA signaling. Evaluation of RA signaling showed an increase in retinoic acid responsiveness when treated with an adrenergic signaling agonist. These results suggest that proper retinoic acid signaling is essential for maintaining

cardiac rhythmicity during embryonic development and adrenergic stimulation can influence this response.

DEDICATIONS

For my parents who have made me who I am today.

ACKNOWLEDGMENTS

I would sincerely like to thank my thesis committee chair, Dr. Steven Ebert for believing in me and allowing me to undertake this project. I feel honored to have been given the opportunity to work in his lab and thankful to have such a wonderful mentor. I would also like to thank my committee members, Dr. Kiminobu Sugaya and Dr. Laurence von Kalm. I am also grateful for all of the lab members who became great friends and teachers throughout my journey. Additionally, I could not have taken on this project without the support of my family, who has pushed me through every obstacle in life. I am truly appreciative to have worked on this project through the guidance of all these individuals.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADH – alcohol dehydrogenase

AI – arrhythmic index

ALDH – aldehyde dehydrogenase

BCMO – beta-carotene 15,15'-monooxygenase

cAMP – cyclic adenosine monophosphate

DMEM – Dulbecco's modified Eagle's media

DMSO – dimethyl sulfoxide

E – embryonic

EPI – epinephrine

FK – forskolin

ISO – isoproterenol

MEA – microelectrode array

NE – norepinephrine

PNMT – phenylethanolamine n-methyltransferase

RA – retinoic acid

RAR – retinoic acid receptor

RARE – retinoic acid response element

RXR – retinoic X receptor

SA – sinoatrial

CHAPTER ONE: INTRODUCTION

According to the Center for Disease and Control, heart disease is the leading cause of death in the United States and every minute an individual dies from this terrible disease ("CDC Features – February is American Heart Month," 2011). Heart disease can have several causes from congenital heart defects to obesity. Key cardiac disorders that can cause a heart attack and ultimately lead to heart failure are conduction disruptions and arrhythmias. The cardiac conduction system is responsible for synchronized, rhythmic contractions. The heart has a natural pacemaker called the sinoatrial (SA) node which is located in the right atrium. It is responsible for the electrical stimulus which causes the heart to beat. There can be disturbances from the SA node which lead to abnormal conduction ("Heart block," 2011). Problems with conduction can lead to increases in arrhythmias. Arrhythmias are characterized by a disorder that causes irregular rhythmic beating of the heart ("Arrhythmias," 2009). These arrhythmias can indicate serious problems with the electrical conduction system of the heart. Understanding the molecular mechanisms that cause problems in the electrical conduction system of the developing heart can give a better understanding of heart development and ultimately aid in combating heart disease and failure. An important potential mechanism is the role of adrenergic hormones during cardiac development and their possible connection to retinoic acid signaling to maintain proper cardiac conduction and rhythmicity during embryogenesis.

Overview of Retinoic Acid

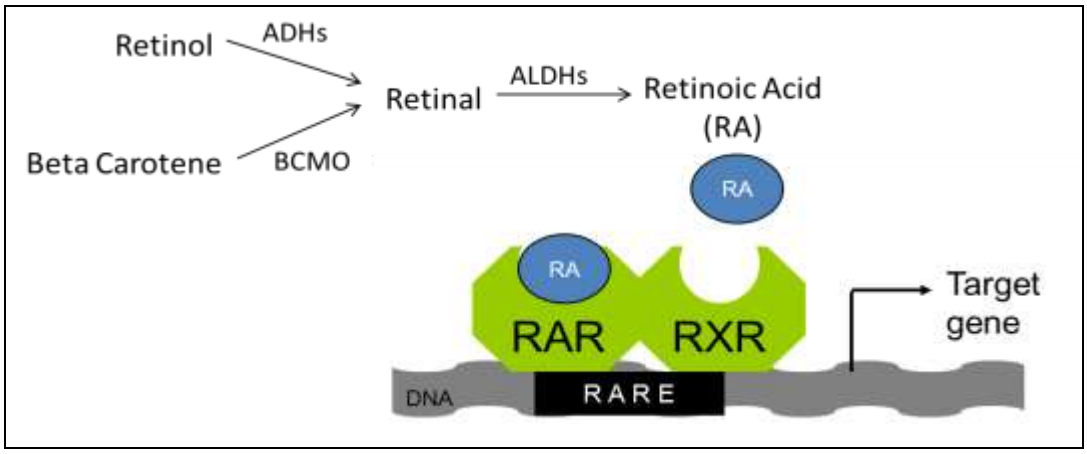

Figure 1: Overview of retinoic acid synthesis and signaling

Retinoic acid is a derivative of vitamin A (retinol). The function of vitamin A in tissues is usually carried out by its acidic form, retinoic acid, and at the cellular level it functions as a hormone. The synthesis of retinoic acid involves two main enzymatic steps. First, a member from the alcohol dehydrogenase (ADH) family oxidizes retinol into retinaldehyde (retinal). Also, through a different pathway β-carotene 15,15'-monooxygenase (BCMO) can convert beta carotene into retinal (Boulanger et al., 2003). Once retinal is produced, class I aldehyde dehydrogenase (ALDH) family members convert retinaldehyde into retinoic acid (Niederreither et al., 2001). Retinoic acid is then utilized as a signaling molecule, acting through nuclear receptors. Retinoic acid receptor (RAR) and the retinoid X receptor (RXR) function as heterodimers, binding to retinoic acid response elements (RARE) on the target DNA (Figure 1). Three genetic isotypes are found of each receptor, in the form of either RARα, β, or γ and RXRα, β, or γ. Each receptor gives a unique pattern of expression during embryogenesis. Once retinoic

acid binds to each of these receptors, transcription of target genes can be initiated (Gruber et al., 1996).

Retinoic acid plays important roles in the body and has been shown to be essential for vision, organ, and immune function throughout the life of an adult. It plays its main function in areas where vitamin A is known to have a significant role such as the kidney, liver, and plasma. Retinoic acid is also found in smaller amounts in areas of the body like the eye and epithelial tissues (McLaren & Frigg, 2001). Retinoic acid is also essential as an anti-inflammatory response and can have a therapeutic effect on autoimmune diseases (Mucida et al., 2007). Although not much is known as to the exact role and amount of retinoic acid in the adult organism, it is essential in areas where vitamin A is necessary.

Not only is retinoic acid important in maintenance of an adult individual, it has been shown to have a significant impact on embryonic development. In general, retinoic acid during embryogenesis is essential for epithelial differentiation, organ maturity, embryonic circulation, central nervous system development, lymphoid organ functions, as well as immune and vision development (Ross, McCaffery, Drager, & De Luca, 2000). During embryogenesis, both an over-abundance and lack of retinoic acid can have deleterious effects on development. When there is an excess of retinoic acid, studies have shown that the fetus becomes malformed which can change epithelial characteristics that can eventually lead to death. When there are insufficient amounts of retinoic acid during embryogenesis, growth of the embryo begins to fail and vascularization begins to decline which too can lead to death of the embryo (Ross et al., 2000). Thus, maintenance of retinoic acid homeostasis is a vital component during fetal development.

Changes in retinoic acid can have deleterious effects on embryogenesis, specifically with regards to cardiac development. Most notably, retinoic acid has been shown to be essential in

the development of cardiac structures, but too much retinoic acid can lead to serious cardiac problems as well (Dyson et al., 1995). When embryos were treated with excess retinoic acid, malformations of cardiac structures such as aortic arch abnormalities and ventricular septal defects were noted. The contraction rates and α-actinin expression decreased significantly. This could indicate that retinoic acid effects myofibril formation as well as the overall cardiac structure formation of the developing heart (Dickman & Smith, 1996).

Many studies have been conducted that block retinoic acid signaling and have also shown to have negative consequences on the embryonic heart. In one study, mice that were RXRα -/- and thus unable to propagate retinoic acid signaling, developed cardiac septal defects, ventricular chamber hypoplasia, and eventually died in utero during midgestation (Dyson et al., 1995). Serious defects of the conduction system of the developing heart were noted in RXRα -/- mice. Compared to wild-type mice, the RXRα -/- mice developed atrioventricular block, a slower heart rate, and the ventricular contraction rate decreased (Dyson et al., 1995). Also, when retinoic acid signaling decreased by blocking the same previous receptor, aortic sac abnormities, ventricular muscle, and atrioventricular cushion defects also resulted (Gruber et al., 1996). Thus, lack of retinoic acid leads to critical cardiac defects.

Adrenergic Hormones Essential for Cardiac Development

The stress hormones, epinephrine (adrenaline) and norepinephrine (noradrenaline) are mainly produced by the sympathetic nervous system and the adrenal medulla. These catecholamines have been shown to have an essential role in the body. They are often referred to as “flight or fight” hormones because they lead to the activation of the sympathetic nervous system and have their various effects on several organ systems such as the lungs, liver, and

skeletal muscle through different subsets of adrenergic receptors. Metabolic effects include glucose release, glycogenolysis, and lipolysis. Adrenergic hormones act through adrenergic receptors. There are two types: alpha (α) and beta (β) with specific subtypes (α_1, α_2, β_1, β_2, β_3) depending on the effector organ. For example, β_1 adrenergic receptors primarily increase cardiac pumping, heart rate, and contractility, while other adrenergic receptors exist for other functions (Fox, 2006).

Adrenergic hormones are produced in the adrenal medulla or through the nervous system stimulation acting as a neurotransmitter (Fox, 2006). The hormones bind to their G protein coupled receptor which triggers a signaling cascade involving cyclic adenosine monophosphate (cAMP) which prompts the activation of a protein kinase. This has the effect of activating or inactivating enzymes and opening ions channels, having the fundamental effect of regulating physiological responses (Rochais et al., 2006).

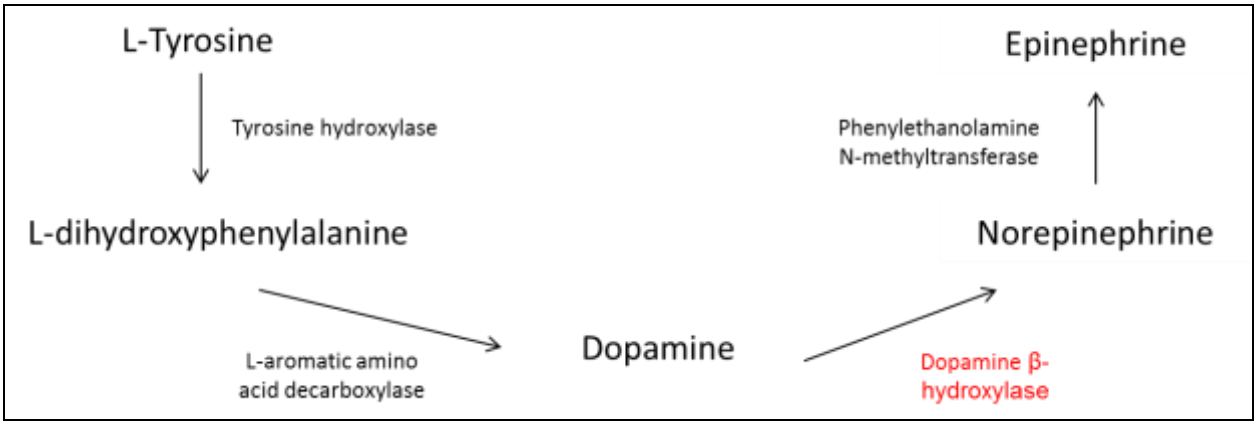

Figure 2: Pathway of catecholamine biosynthesis. Key enzyme dopamine β-hydroxylase (red) essential for norepinephrine synthesis

The enzyme dopamine β-hydroxylase (Dbh) is essential for the production of adrenergic hormones. It has the ability to convert dopamine into norepinepherine (NE), a key step in

catecholamine synthesis (Figure 2). Norepinephrine can further be converted to epinephrine (EPI) using the enzyme phenylethanolamine n-methyltransferase (Pnmt). Mice lacking the *Pnmt* gene are unable to produce epinephrine; however, they are viable and appear to have no development defects. Once the *Dbh* gene is knocked out, mice can no longer synthesize norepinephrine and most of those embryos die before the sympathetic nervous system even develops. Most of the mice lacking Dbh died after embryonic day 10.5 (Thomas, Matsumoto, & Palmiter, 1995) Two key points can be noted, norepinephrine is essential in embryonic survival and there is also a critical window in which adrenergic hormones are needed for cardiac development (Ebert & Taylor, 2006). Consequently, catecholamines are a fundamental component of cardiac embryogenesis.

Previous research has shown that intrinsic cardiac adrenergic (ICA) cells capable of producing adrenergic hormones are found in and around the electrical conduction system of the heart. It has been observed that catecholamines are present during early embryonic development and may be needed for proper rhythmic contractions since the cells are shown to be localized in areas associated with cardiac pacemaking (Ebert & Thompson, 2001). Ongoing research from the laboratory of Dr. Steven Ebert showed that mice which were adrenergic deficient ($Dbh^{-/-}$) had slower atrioventricular conduction compared to wild-type ($Dbh^{+/+}$) embryonic mouse hearts. Also, the adrenergic deficient hearts have increased atrioventricular blockage around E10.5 (Dr. David Taylor, Dr. Steven Ebert, unpublished).

Preliminary microarray data from the laboratory of Dr. Steven Ebert lab was performed to analyze gene expression changes of embryonic mice which were lacking dopamine β-hydroxylase ($Dbh^{-/-}$). This method looked at all the genes of the $Dbh^{-/-}$ mouse genome and were compared to $Dbh^{+/+}$ mice. When comparing the knockout mouse with the wild-type, 23 genes

showed significant changes in expression that was 2-fold or greater. Further analysis also showed that several of the genes that had altered gene expression were directly related to retinoic acid synthesis such as beta-carotene 15,15'-monooxygenase (BCMO), a key enzyme involved in one of the first steps of retinoic acid biosynthesis. Other genes related to retinoic acid also showed changes in the microarray study such as *CRABP-1*, *CRABP*-2, *RALDH1* and *RBP-1* (Kingsley Osuala, Dr. Steven Ebert, unpublished). These findings imply a possible correlation between mice that are unable to produce norepinephrine and retinoic acid regulation, and suggest that the lethality of *Dbh* $^{-/-}$ mice might be a result of alteration of retinoic acid synthesis.

A potential mechanistic connection between retinoic acid signaling and adrenergic hormones may have to do with cyclic adenosine monophosphate (cAMP). As previously mentioned, cAMP is essential in the signaling cascade of adrenergic stimulation. The cAMP response element binding protein can regulate the gene expression of *BCMO1*. The human *BCMO1* promoter has been found to include a cAMP response element. cAMP is able to active the Cre binding protein which can help drive transcription of the *BCMO1* gene, promoting retinoic acid production (Lietz, Lange, & Rimbach, 2010). Given that both adrenergic and retinoic acid signaling involve cAMP, regulation of cAMP could be a possible link between their pathways, although other viable connections may still exist.

Rational

Considering previous literature and the microarray data, a correlation between retinoic acid signaling and cardiac conduction is formulated. Embryonic mice lacking the enzyme Dbh began dying of heart failure around embryonic day 10.5 and additionally, preliminary data shows that *Dbh* $^{-/-}$ have conduction defects. Microarray data has already shown that *Dbh* $^{-/-}$ have

abnormal retinoic acid signaling. Therefore, it may be inferred that the cardiac conduction problems found in adrenergic deficient mice may be the result of altered retinoic acid signaling. Consequently, the goal of this study was to determine if changes in retinoic acid signaling effect cardiac electric conduction properties by measuring heart rate, conduction and rhythmicity during embryogenesis, and to test whether there is a mechanistic connection between adrenergic hormones and retinoic acid signaling.

Aims

The first aim was to determine if blockage of retinoic acid signaling would lead to changes in cardiac rate, rhythm, and electrical conduction properties in *Dbh* $^{+/+}$ wild-type mice. If blockage of retinoic acid signaling caused arrhythmias and conduction defects in the *Dbh* $^{+/+}$ mice this would suggest retinoic acid is essential for maintaining proper cardiac conduction and rhythmicity during embryonic development and may also suggest that the lethality seen in *Dbh* $^{-/-}$ could be from alteration in retinoic acid signaling.

The second aim was to determine if there is a connection between adrenergic hormone production and retinoic acid signaling. This was done using the F9-RARE-*LacZ* cell line which has been constructed with the *LacZ* reporter system. Cells were cultured in different concentrations of retinoic acid, forskolin (cAMP regulator), and isoproterenol (β-agonist), and changes in retinoic acid response was measured. If changes occur with the addition of the adrenergic mimics it will illustrate a mechanistic connection between retinoic acid signaling and adrenergic hormones.

CHAPTER TWO: MATERIALS AND METHODS

Overview of Methodology

The procedure was divided into two main parts. The first part of the experiment used the *Dbh* $^{+/+}$ mice and the retinoic acid receptor antagonist. The embryonic hearts were obtained at embryonic (E) day 10.5. The specific antagonist that was used in the experiment was LE 135, which competitively binds to retinoic acid receptors and will be used to mimic blockage of retinoic acid signaling (Li, Hashimoto, Agadir, Kagechika, & Zhang, 1999). A maximum number of hearts were isolated and the embryonic hearts were then allowed to culture for 24 hours in drug treatment. Before any drug was placed on the hearts, a video was taken for at least 90 seconds. After the hearts have cultured for 24 hours, another video was taken for at least 90 seconds.

Due to experimental timing and scheduling, data was analyzed using two methods: photodiode technology and microelectrode array (MEA). First, the photodiode system was used which utilizes diodes measuring the light changes on the video screen; a data read out with heart rate and rhythmicity measurements was then obtained using this system. In the next set of experiments, hearts were transported on MEA's to the NanoScience Technology Center at the University of Central Florida and a computer software was used to read the electrical impulses produced. Once the data was obtained, heart rate, conduction measurements and overall rhythmicity of the developing heart was analyzed using statistical analysis.

F9-RARE-*LacZ* cells were then employed for the second part of experimentations. These cells were cultured with different drug concentrations and a fluorescent substrate was used to measure changes in fluorescence in photons/mg/mL.

Animals

The strain of mice used were wild-type mice which were able to produce Dopamine β-hydroxylase, *Dbh* $^{+/+}$. The animal surgeries were all performed by trained individuals and all procedures were in accordance with NIH guidelines and approved by the University of Central Florida Animal Care and Use Committee.

The vaginal plug dates of the mice were checked and noted. Noon of the first day plug day was assigned E0.5. 10 days later the mice were ready for surgery. E10.5 was used because it corresponded to the date when the most of the Dbh $^{-/-}$ would die because of the lack of adrenergic hormones (Thomas et al., 1995). As surgeries were performed and the embryos isolated, size of the embryo was also noted. The specific embryos isolated were the same size, corresponding to the 10.5 embryonic day.

Heart Cultures (Ex-Vivo)

Once the embryos were obtained they were isolated under aseptic conditions. They were cultured in cardiac differentiation media which consisted of Dulbecco's Modified Eagle's Media (DMEM) containing v/v of 15% charcoal stripped fetal bovine serum, 1 % PenStrep, 0.1% 2-mercaptoethanol, 0.01 % non-essential amino acids, and 0.01 % glutamine (All products from Invitrogen Inc., Carlsbad, CA).

Drug Treatment

For the first component, embryonic hearts were isolated. Once obtained they were placed in the heart culture on petri dishes with MEAs, corresponding drug treatment also needed to be placed in the culture. 10 μL of 100 nM of LE 135 (Tocris biosciences, Ellisville, MO) was used

for the retinoic acid receptor antagonist treated hearts. The control hearts were given the same volume but treated with 10 μL of dimethyl sulfoxide (DMSO, Sigma Aldrich, St. Louis, MO).

Video Recording

Once the culture was ready and the corresponding drugs were placed in each heart culture, the plates were put into a custom glass chamber which was temperature regulated to about 37 °C. The plate was placed on an Olympus CK2 inverted microscope which was connected with a camera. This recording would play on a nearby computer screen. All hearts were recorded for approximately 90 seconds on 10x magnification. They were recorded at 0 hours and after 24 hours of LE 135 or DMSO treatment. Any of the embryonic hearts which were not beating at this point were discarded for no further use. After all the hearts were recorded, they were placed in a 37 °C incubator for 24 hours supplied with 95% air and 5 % CO_2.

Photodiode Technology

From the computer screen, a photodiode system was connected. This consisted of two diodes which were connected to an oscilloscope that reported heart rate and arrhythmia measurements. One diode was placed on a portion of the screen that had no light change, this would serve as the control. The other diode was placed on the screen where the embryonic heart was located. The diodes could pick up on the changes in light on the screen as the heart would beat. As it recorded, a readout of the heart rate and cycle length was obtained through software acquisition (Ratcell Recorder, LabVIEW v4.0.1, National Instruments, Austin, TX). After all of the hearts were recorded, a final readout with the calculated heart rate in beats per minute and arrhythmia measurements could be obtained for analysis.

Microelectrode Array

For conduction measurements, MEA plates with the embryonic hearts were carefully transported to the NanoScience Technology Center of the University of Central Florida. There a similar readout was obtained but with conduction time measurements. At the center of the 8x8 MEA (#200/10iR-Ti, Multichannel Systems, Reutlingen, Germany) was a gelatin coating. The hearts were placed at the center of the MEA, making sure that they touched the electrodes. The Clampfit v10.0 software (Molecular Devices, Sunnyvale, CA) and MC_DataTool v2.6.0 (Multichannel Systems, Reutlingen, Germany) was used to analyze the electrode array data. Atrioventricular conduction was measured as the time for impulse to travel from the SA node to the apex of the heart (Figure 3). The cycle length was measured from one ventricular beat to the other.

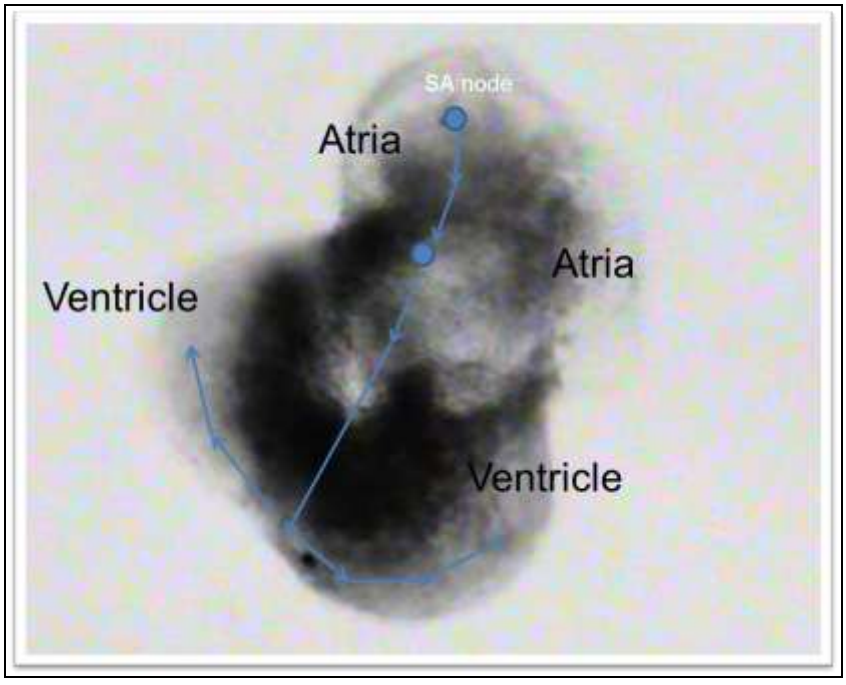

Figure 3: Isolated embryonic mouse hearts extracted at E10.5. Normal conduction flows from SA node toward the apex of the heart

Obtaining Data and Analysis

Once the data was obtained from both the photodiode system and the MEA plates, it had to be analyzed. Hearts rates were already obtained from recording before and after drug treatment. The heart rate was obtained in beats per second and then converted to beats per minute. Conduction velocity was obtained using the MEA analysis which takes into account depolarization from one cycle event to the next. The major indication of rhythmicity was the arrhythmic index (AI). This was calculated by obtaining the cycle length from all of the recordings and putting the data into the program Prism (Graphpad Software, La Jolla, CA). Once in the program, statistics were obtained, specifically, the median and standard deviation. The AI could be calculated by dividing the median by the standard deviation (Fink et al., 2009). A greater AI was indicative of a more arrhythmic heart.

F9-RARE-*LacZ* Reporter Cell Line

F9-RARE-*LacZ* reporter cell line was employed. The cells were donated by Dr. Michael Wagner of Downstate Medical Center. The cells are transfected with the *LacZ* gene downstream from the retinoic acid response element. Therefore, when retinoic acid signaling is stimulated, *LacZ* is transcribed producing the protein product β-galactosidase (Wagner, Han, & Jessell, 1992). Fluorescein di-β-D-galactopyranoside (FDG, Sigma Aldrich, St. Louis, MO) is the substrate whose fluorescence can be measured (Figure 4).

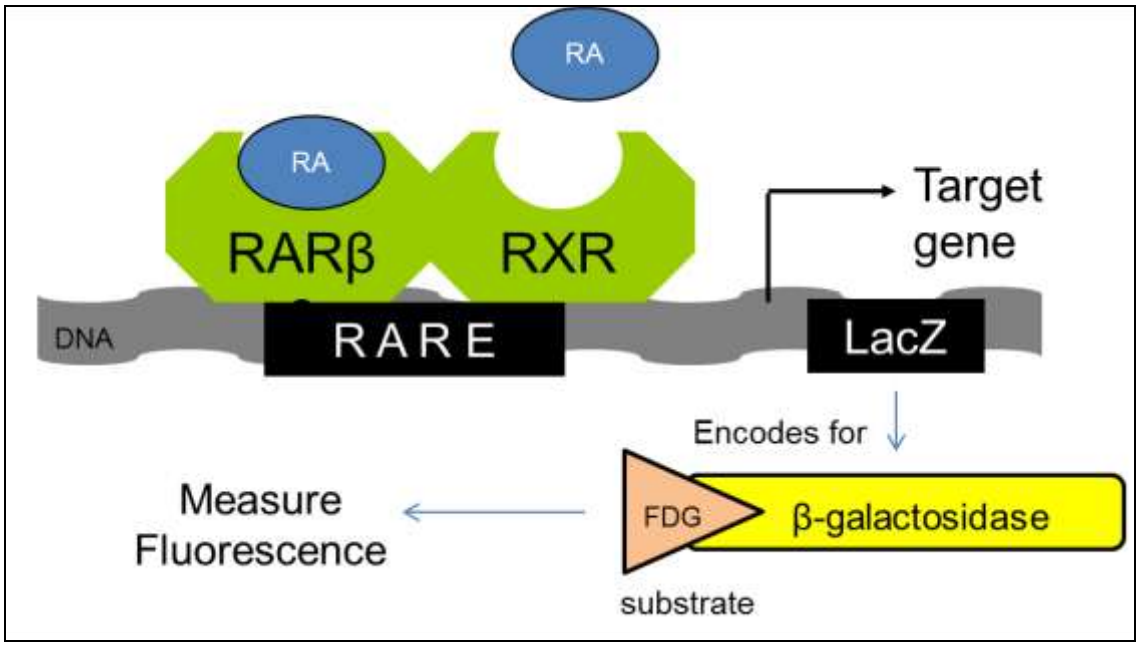

Figure 4: Basic mechanism of F9-RARE-*LacZ* reporter assay

Cells were maintained in media containing 50% L-15 Leiboitz media (Sigma Aldrich, St. Louis, MO) 50% DMEM, and 5% FBS v/v. The cells were grown to confluency in two 60 mm cell culture dishes. They were trypsinized with 0.5 mL of 0.25% trypsin (Invitrogen Inc., Carlsbad, CA) for about five minutes to break up cells. Then, 4.5 mL of warm passage media was added to the dish. The two 60 mm cell culture dishes were combined into one conical tube, making sure all cells where mixed through the tube. They were then added to media to make the desired volume needed for experimentation.

The cells were allowed to grow overnight to confluency on the 24 well plates. They were then treated with varying concentration of drugs, (purchased from Sigma Aldrich, St. Louis, MO) retinoic acid (all-trans retinoic acid, diluted in DMSO), forskolin (diluted in DMSO), or isoproterenol ((−)-isoproterenol hydrochloride, diluted in 0.1 N HCl). The drugs were diluted in

separate media to appropriate concentrations. Old media was removed from plates and media with drugs was then placed into wells with cells.

The cells were allowed to culture in drug treated media for 24 hours. The media was removed and the cells were lysed using 100 µL Glo Lysis Buffer (Promega Corporation, Madison ,WI). A 96 well plate was needed to run assay. 70 µL of PBS was pipetted into each well with 20 µL of supernatant of the lysed cells. Florescence was measured using Wallac Envision 2104 Multilabel Reader (Perkin Elmer, Waltham, MA) which was programmed to read fluorescein activity. Then 2 µL of 1 mM FDG was added and readings were taken every five minutes for sixty minutes. Data was extrapolated from the change in FDG concentration based on the formed linear curve. Data was normalized to include protein concentrations in mg/mL. Fold change could then be calculated by dividing the average from the wells which had no drug treatment.

CHAPTER THREE: RESULTS

Heart Rate

To determine if the retinoic acid receptor antagonist LE 135 effects embryonic heart rate, hearts were isolated into two treatment groups. 18 hearts served as controls, treated with 10 µL of DMSO. The initial heart rate of the DMSO heart at 0 hours was 145.4 BPM with a standard error of the mean (SEM) of 10.1 (145.4±10.10 BPM). After 24 hours of treatment the average heart rate of the DMSO treated heart was 144.8±11.58 BPM. 15 hearts were treated with the retinoic acid receptor blocker, LE 135. The average initial heart rate of the LE 135 treated hearts was 138.8±10.65 BPM and after the 24 hours of treatment the heart rate was 131.9±62.52 BPM. There was no significant change in heart rate of either group between their initial reading and 24 hours after drug treatment (Figure 5).

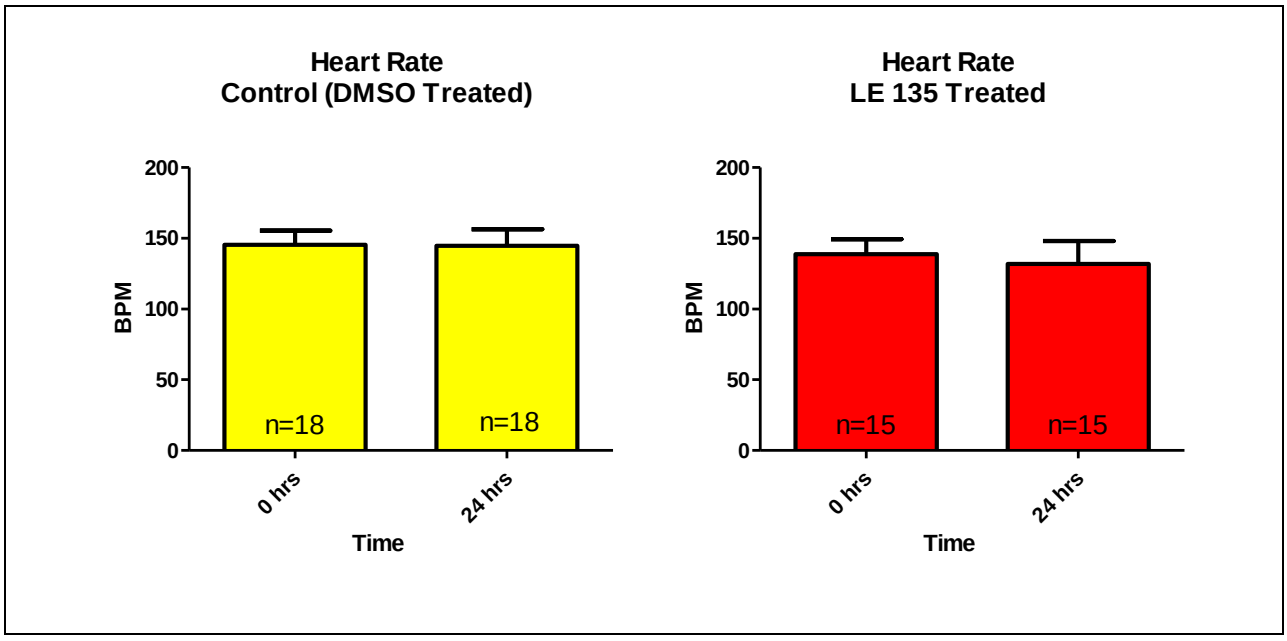

Figure 5: Comparison of heart rates at 0 hours of treatment versus after 24 hours with or without retinoic acid receptor antagonist (100 nM LE 135)

Conduction

Conduction time and cycle length were measured to see the effects of the retinoic acid receptor antagonist on heart conduction. A sample size of 4 hearts was available to obtain conduction time and cycle length measurements. Atrioventricular conduction time and cycle length was measured after 24 hours of treatment (Figure 6). The conduction time after 24 hours of the DMSO (control) hearts was 65.82±10.510 ms while the LE 135 treated hearts had an average conduction time of 78.74±9.359 ms. The cycle length of the DMSO treated heart after 24 hours of treatment was 0.6468±0.155 s while LE 135 treated heart had an average cycle length of 0.7928±0.210 s. While both the conduction time and cycle length increased compared to the control, DMSO treated hearts, the increase was not statically significant.

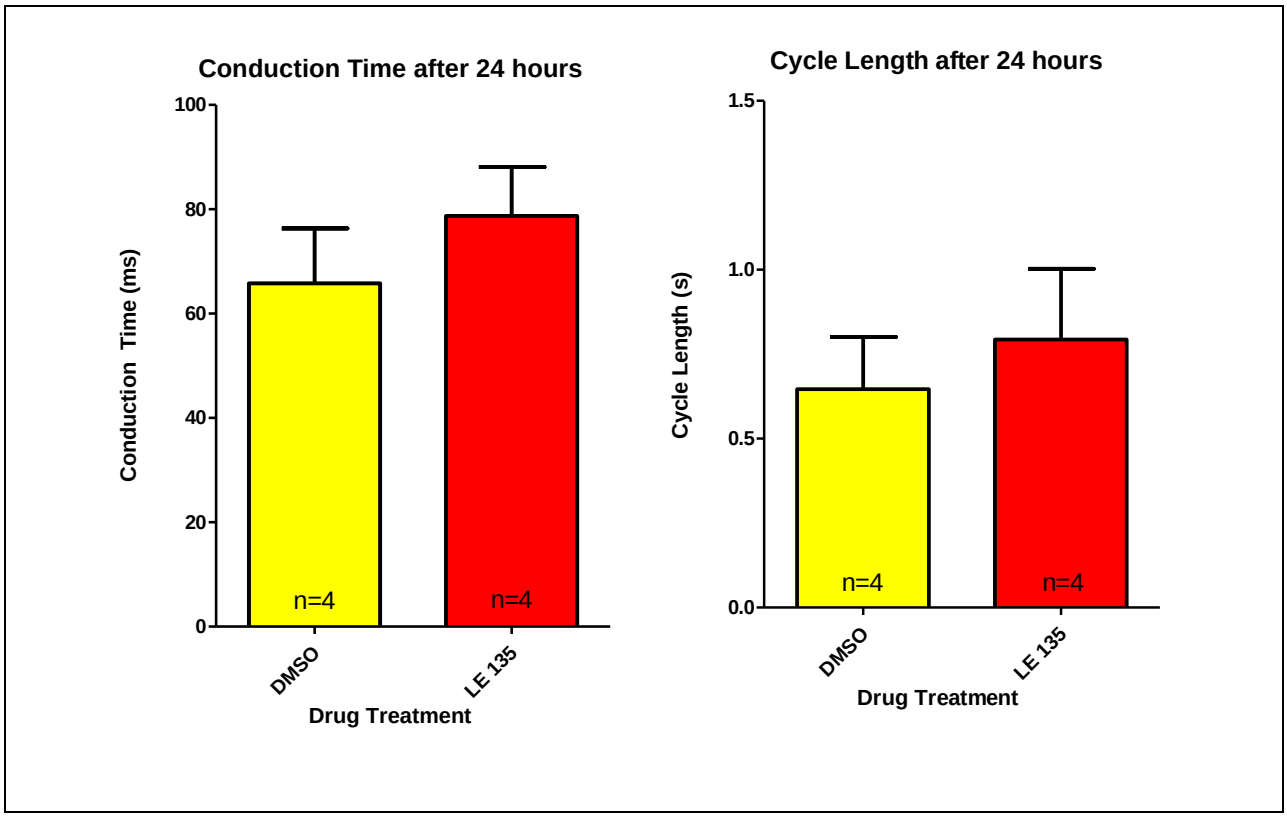

Figure 6: Conduction time and cycle length measurements after 24 hours of treatment with or without retinoic acid receptor antagonist (100 nM LE 135)

Arrhythmias

To assess arrhythmic occurrences, arrhythmias were measured as a function of arrhythmic index (AI) as previously mentioned in the methods sections and graphed comparing initial and 24 hour readings (Figure 7). The higher the AI, the more significant the arrhythmias associated with the heart. 18 hearts were used as control hearts treated with DMSO. The average initial AI of the DMSO treated heart was 0.1224±0.252 and after 24 hours of treatment the AI was 0.1195±0.025. 15 hearts were used to measure AI of the LE 135 treated. The initial AI of the LE 135 treated hearts was 0.08317±0.028 and after 24 hours of treatment the AI increased to 0.2329±0.057. A ttest comparing the difference in AI of the LE 135 gave a p value of 0.0268, indicating a statically significant increase after the addition of LE 135. When fold change was examined, the DMSO treated hearts did not have a significant fold change while the LE 135 treated hearts have almost a 3 fold increase after 24 hours of drug treatment.

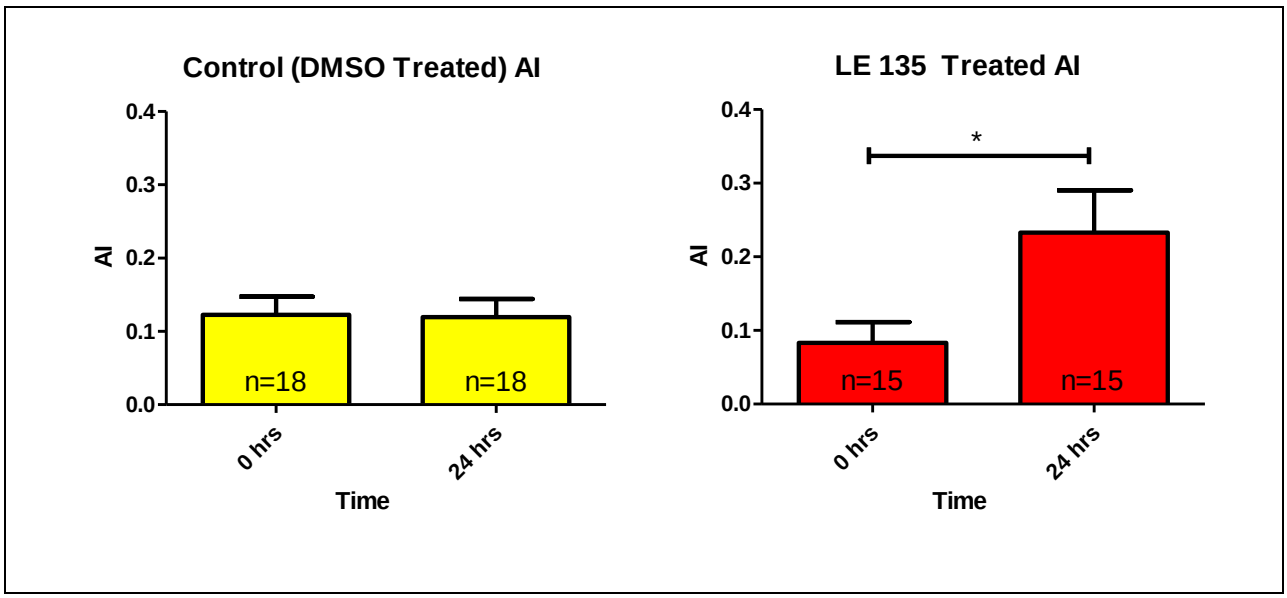

Figure 7: Difference in AI at 0 hours of treatment versus 24 hours with or without retinoic acid receptor antagonist (100 nM LE 135)

Retinoic Acid Dose Response

To evaluate a mechanistic connection between retinoic acid and adrenergic hormones, a reporter cell line was used. Retinoic acid dose response was measured using the F9-RARE-*LacZ* cells. The cells were treated with varying concentrations of retinoic acid as previously described in the methods sections. Different experiments were normalized using fold change compared to cells treated with no drug (Figure 8). 14 wells with the F9-RARE-*LacZ* cells were treated with 1 nM RA and the average fold change was 2.187±0.160. 14 wells were treated with 10 nM RA and the average fold change was 3.255±0.105. 15 wells were treated with 100 nM RA and the average fold change was 4.404±0.467. This data was plotted and a linear line was obtained when the 10 fold increments of RA were plotted against fold change with a R^2 value of 0.9996.

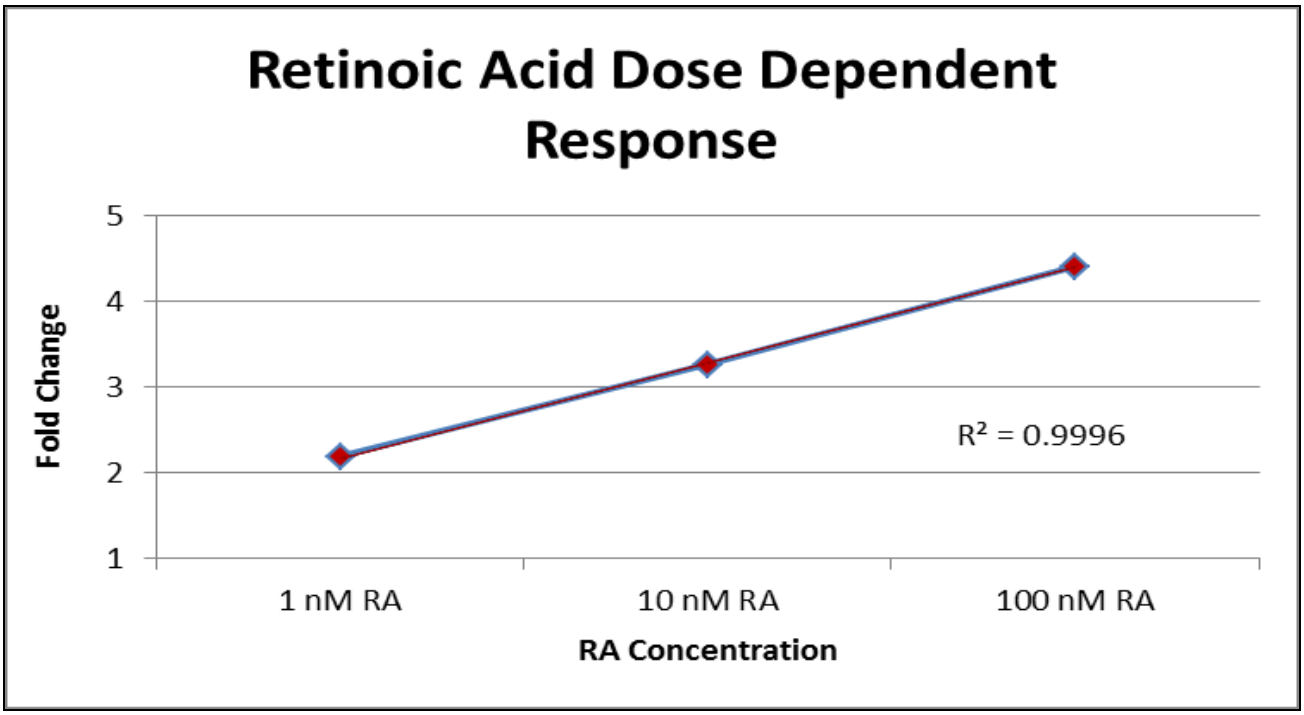

Figure 8: Dose dependent relationship in F9-RARE-*LacZ* cells after treatment of retinoic acid

Different Concentrations of Forskolin and Isoproterenol

In a small initial experiment varying concentrations of forskolin (FK) and isoproterenol (ISO) were used to see if any changes would occur in retinoic acid response using the F9-RARE-*LacZ* cells compared to the same cells with no drug (Figure 9). 0.1 μM FK had a mean fold change of -0.7243±0.656 (n=4), 1 μM FK had a mean fold change of -0.5722±0.620 (n=9), and 10 μM FK had a fold change of 0.1674±0.841 (n=4). 10 nM ISO had a mean fold change of 0.01164±0.669 (n=4), 100 nM ISO had a mean fold change of 0.2111±0.996 (n=9), and 1 μM ISO had an average fold change of -0.7175± 0.584 (n=4). Fold change in the range of -1 to +1 is indicative of no fold and since all values were in this range, it was inferred that varying concentrations of FK or ISO had no effect on retinoic acid concentration alone. Therefore, the middle concentrations of each drug, 1μM FK and 100 nM ISO were used for further experimentation.

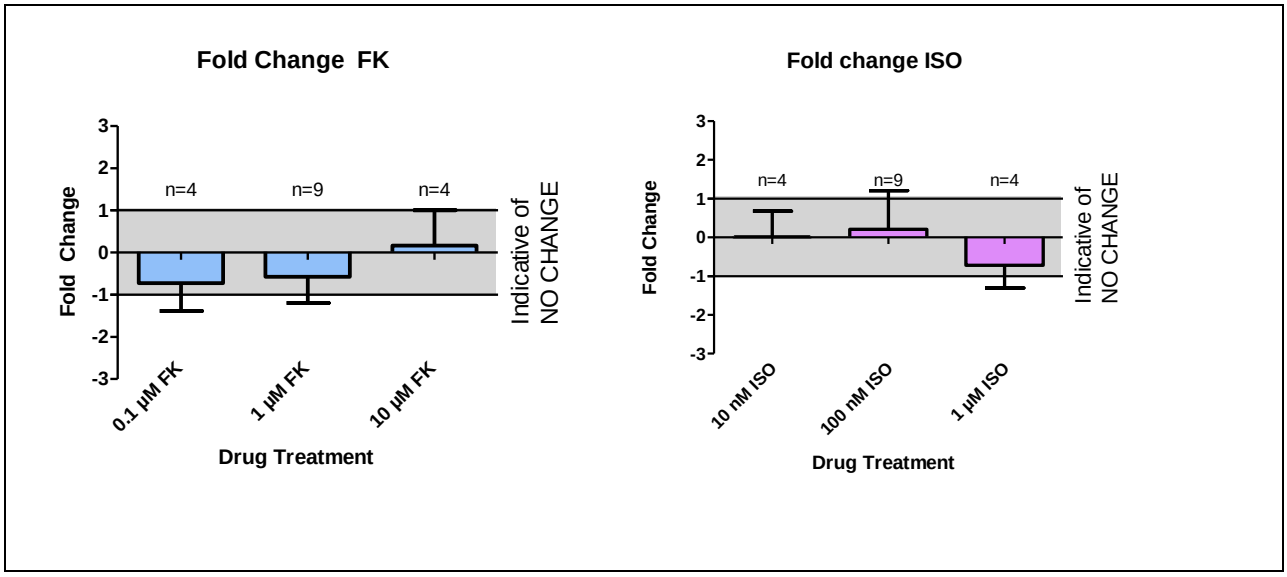

Figure 9: RA response with different concentrations of forskolin and isoproterenol alone on F9-RARE-*LacZ* cells

F9-RARE-*LacZ* Cells Treated with Forskolin

To determine the effects on RA response after the addition of a β-agonist, cells were cultured with the cAMP regulator, forskolin in combination with retinoic to compare changes in retinoic acid response to just retinoic acid alone. Fold change was then calculated and compared to graphs with just retinoic acid (Figure 10). 1 nM RA in combination with 1 μM FK had an average fold change of 2.911±0.313 (n=11). 10 nM RA in combination with 1 μM FK had an average fold change of 3.755±0.270 (n=11). 100 nM RA in combination with 1 μM FK had an average fold change of 5.812±0.336 (n=11). One way ANOVA shows overall means to be statically significant (P<0.0001).

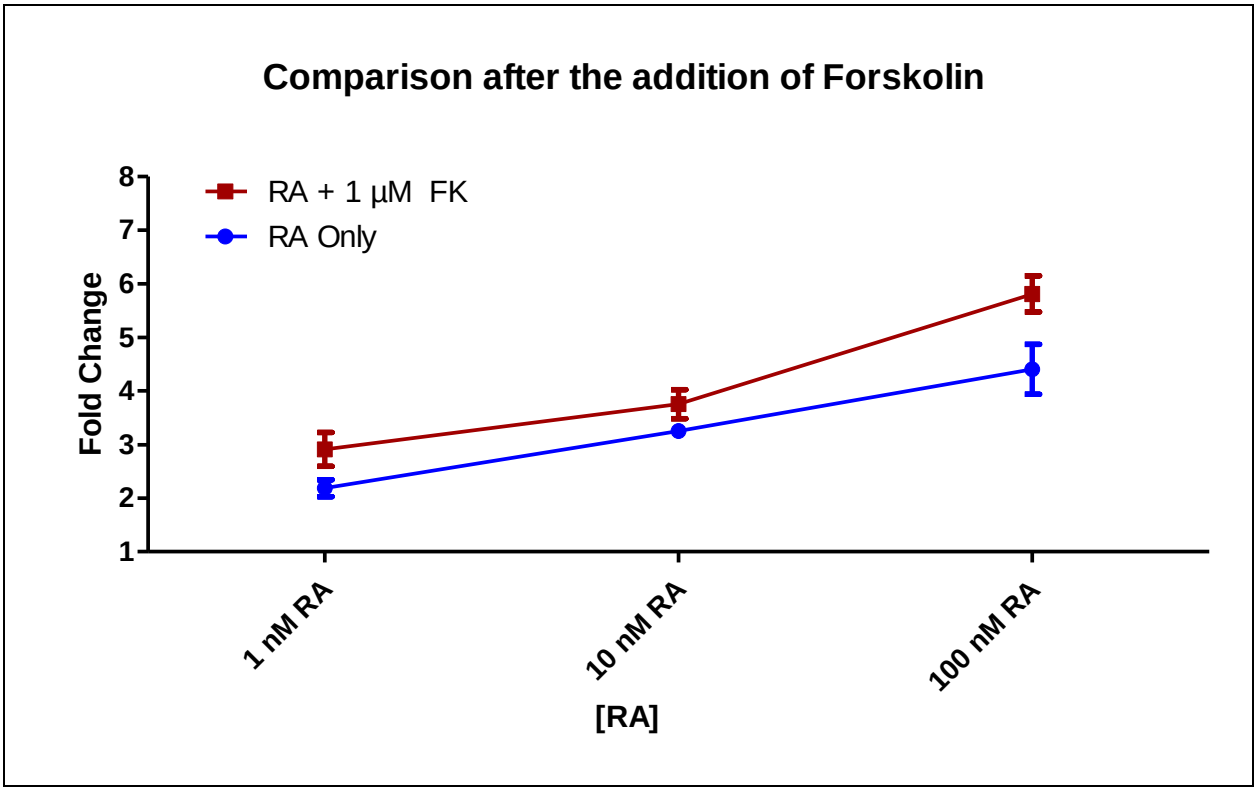

Figure 10: Comparison of fold change in RA response after the addition of forskolin in combination with retinoic acid

F9-RARE-*LacZ* cells Treated with Isoproterenol

Cells were cultured with another β-agonist, isoproterenol in combination with retinoic acid to compare changes in retinoic acid response to just retinoic acid alone. Fold change was then calculated and compared to graphs with cells treated with just retinoic acid (Figure 11). 1 nM RA in combination with 100 nM ISO had an average fold change of 3.168±0.431 (n=12). 10 nM RA in combination with 100 nM ISO had an average fold change of 3.797±0.306 (n=11). 100 nM RA in combination with 100 nM ISO had an average fold change of 5.496±0.539 (n=11). One way ANOVA shows overall means to be statically significant (P<0.0001).

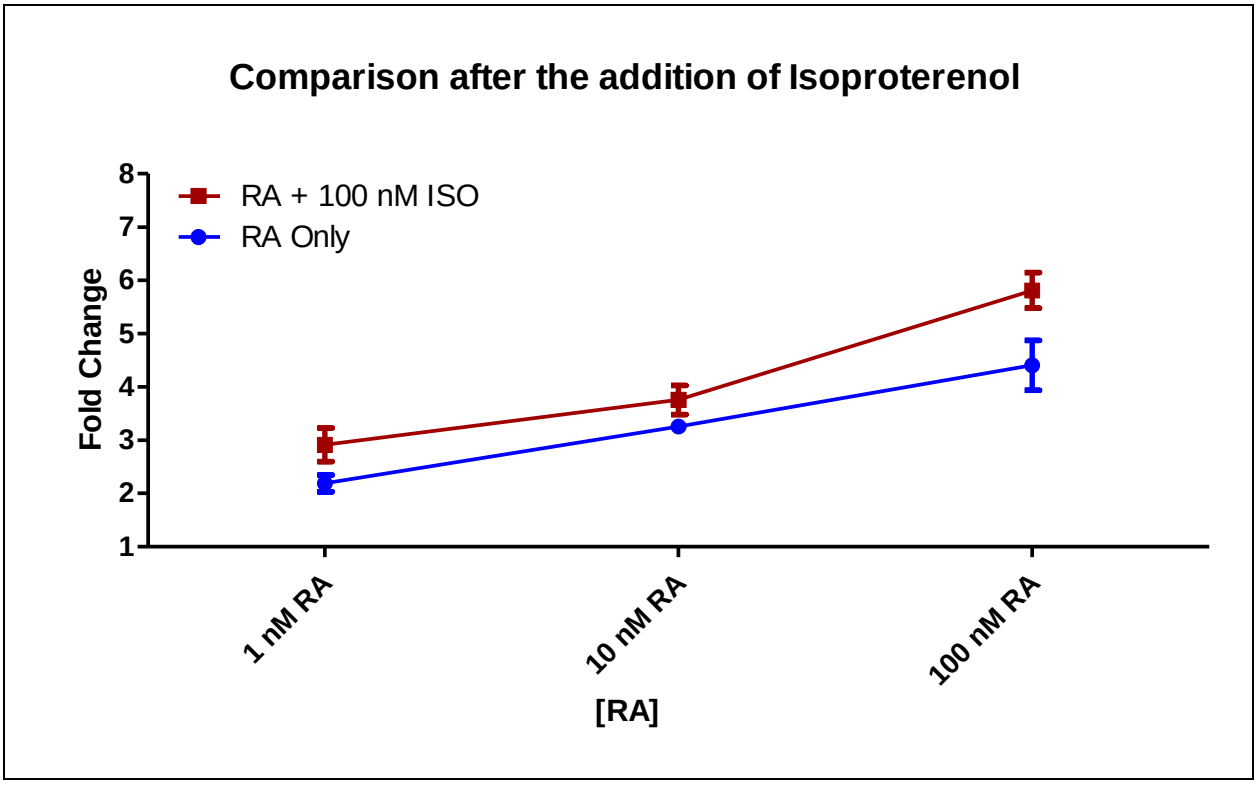

Figure 11: Comparison of fold change in RA response after the addition of isoproterenol in combination with retinoic acid

CHAPTER FOUR: DISCUSSION

This study examined the effects of blockade of retinoic acid signaling on embryonic mouse hearts at day 10.5 to see if similar occurrences in electrical conduction were noted as seen with *Dbh* $^{-/-}$mouse hearts around the same period of time. The second part of the study examined if there was an intrinsic relationship between retinoic acid signaling and adrenergic hormone action.

First, electrical conduction properties were measured in hearts cultured for 24 hours with the retinoic acid receptor blocker, LE 135. There was no significant change in heart rate between the LE 135 treated hearts and the control group, treated with DMSO. Which means on average the number of beats per minute produced by the LE 135 treated hearts and the DMSO treated did not change.

Conduction time and cycle length were calculated using microelectrode arrays. After 24 hours of drug treatment the conduction time and cycle length were obtained. Conduction time was measured from atrial depolarization to next spike intensity, the ventricular depolarization. The cycle length was the time it took to go from one ventricular peak to the next. The results show that there was an increased trend in conduction time and cycle length, but this trend was not statically significant. There was a small sample size used for this portion of the experiment but a larger sample size would be helpful to come to a more accurate conclusion.

Arrhythmia data was obtained as a function of arrhythmic index (AI). To make sure there were no errors between the recordings and data, the videography was checked visually to see if hearts were arrhythmic. When hearts were treated with DMSO for 24 hours there was no significant change in AI. However, the hearts which were treated with the retinoic acid receptor

blocker LE 135 had an almost three fold increase in AI. The results indicate that when retinoic acid signaling is blocked there is an increase in arrhythmias in normal wild-type embryonic mouse hearts. These results suggest that retinoic acid is essential in maintaining cardiac rhythmicity of the developing heart.

To further evaluate if there is any mechanistic connection between retinoic acid signaling and adrenergic hormone action, F9-RARE-*LacZ* cells were employed. When cells were treated with increasing concentrations of RA in 10 fold increments, 1 nM RA, 10 nM RA, 100 nM RA a linear line was obtained from the fold change averages with 10 fold increases in RA concentration. These results indicate proper experimentation and a dose dependent relationship because if there is a higher concentration of retinoic acid applied to the cells there is more retinoic acid available to bind to its receptor. Therefore, more transcription of the *LacZ* gene takes place which will encode for more β-galactosidase, giving a higher fluorescent reading after the addition of the substrate FDG.

In the next experiment, it was shown that different concentrations of forskolin and isoproterenol had no overall effect on transcription of *LacZ* on the F9-RARE-*LacZ* cells compared to cells which had no drug treatment. This indicates that when cells are treated alone with an adrenergic stimulus there was no additional stimulation of the *LacZ* gene, meaning it had no effect on the amount of RA binding to the receptor on the cells.

However, additional experiments were done to measure the effects of beta adrenergic signaling in combination with retinoic acid. The same concentrations of retinoic acid were used (1, 10, 100 nM RA), but a constant concentration of forskolin (1 μM) and isoproterenol (100 nM) was used. These results indicate an increase in retinoic acid response after the addition of one these two adrenergic agonist. It seems that forskolin and isoproterenol are sensitizing RA to

bind to its receptor, increasing RA response because the curve too has a similar correlation but with an increased fold change. Isoproterenol is mimicking adrenergic action which NE and EPI would exhibit (Vaniotis et al., 2011). Forskolin in more downstream of the signaling, meaning it bypasses the point where a direct adrenergic hormone would have to bind to the receptor to initiate a signaling cascade, thus directly regulating cAMP in the process (Barman, Choisy, Hancox, & James, 2011). Since both isoproterenol and forskolin effect adrenergic signaling, it makes sense that their response was similar.

The overall results of the study suggest retinoic acid signaling is essential for proper rhythmicity during embryonic development and may be dependent on adrenergic action. Since *Dbh* $^{-/-}$ mice experienced conduction abnormalities during the same critical time of E10.5, and this study finds that retinoic acid causes a significant increase in arrhythmias. The lethality seen in the *Dbh* $^{-/-}$ could be the direct result of disruption of retinoic acid signaling. While adrenergic dependence is suggested through the F9-RARE-*LacZ* reporter cell line study, further experimentation should be done to fully evaluate this possibility.

For future research, electrical conduction properties of embryonic hearts treated with just adrenergic hormones should be measured, and then after addition of retinoic acid to see if rhythmic changes occur. Also, using the F9-RARE-*LacZ* cell line, further studies should be conducted with different adrenergic stimulus. A constant retinoic acid concentration should also be used with varying concentrations of adrenergic stimulus to see if any changes occur in retinoic acid response. To test whether cAMP is involved in both signaling process, other cAMP regulators like dibutyryl cAMP should be used to treat the cells. Since it is still unclear of the exact connection between retinoic acid signaling and adrenergic hormone action, it is essential to evaluate these and other parameters of research.

REFERENCES

Arrhythmias. (2009). *American Heart* Association. Retrieved December 21, 2009, from http://www.americanheart.org/presenter.jhtml?identifier=10845.

Barman, P., Choisy, S. C., Hancox, J. C., & James, A. F. (2011). beta-Adrenoceptor/PKA-stimulation, Na(+)-Ca(2+) exchange and PKA-activated Cl(-) currents in rabbit cardiomyocytes: A conundrum. *Cell Calcium*.

Boulanger, A., McLemore, P., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Yu, S. S., . . . Redmond, T. M. (2003). Identification of beta-carotene 15, 15'-monooxygenase as a peroxisome proliferator-activated receptor target gene. *FASEB J, 17*(10), 1304-1306.

CDC Features - February is American Heart Month. (2011, January 31). *Centers for Disease Control and Prevention*. Retrieved February 2, 2011, from http://cdc.gov/features/heartmonth.

Dickman, E. D., & Smith, S. M. (1996). Selective regulation of cardiomyocyte gene expression and cardiac morphogenesis by retinoic acid. *Dev Dyn, 206*(1), 39-48.

Dyson, E., Sucov, H. M., Kubalak, S. W., Schmid-Schonbein, G. W., DeLano, F. A., Evans, R. M., . . . Chien, K. R. (1995). Atrial-like phenotype is associated with embryonic ventricular failure in retinoid X receptor alpha -/- mice. *Proc Natl Acad Sci U S A, 92*(16), 7386-7390.

Ebert, S. N., & Taylor, D. G. (2006). Catecholamines and development of cardiac pacemaking: an intrinsically intimate relationship. *Cardiovasc Res, 72*(3), 364-374.

Ebert, S. N., & Thompson, R. P. (2001). Embryonic epinephrine synthesis in the rat heart before innervation: association with pacemaking and conduction tissue development. *Circ Res, 88*(1), 117-124.

Fink, M., Callol-Massot, C., Chu, A., Ruiz-Lozano, P., Izpisua Belmonte, J. C., Giles, W., . . . Ocorr, K. (2009). A new method for detection and quantification of heartbeat parameters in Drosophila, zebrafish, and embryonic mouse hearts. *Biotechniques, 46*(2), 101-113.

Fox, S. I. (2006). *Human Physiology* (9th ed., pp. 238-239). New York: Mc Graw Hill.

Gruber, P. J., Kubalak, S. W., Pexieder, T., Sucov, H. M., Evans, R. M., & Chien, K. R. (1996). RXR alpha deficiency confers genetic susceptibility for aortic sac, conotruncal, atrioventricular cushion, and ventricular muscle defects in mice. *J Clin Invest, 98*(6), 1332-1343.

Heart block. (n.d.). *American Heart Association.* Retrieved March 11, 2011, from http://www.americanheart.org/presenter.jhtml?identifier=4611.

Li, Y., Hashimoto, Y., Agadir, A., Kagechika, H., & Zhang, X. (1999). Identification of a novel class of retinoic acid receptor beta-selective retinoid antagonists and their inhibitory effects on AP-1 activity and retinoic acid-induced apoptosis in human breast cancer cells. *J Biol Chem, 274*(22), 15360-15366.

Lietz, G., Lange, J., & Rimbach, G. (2010). Molecular and dietary regulation of beta,beta-carotene 15,15'-monooxygenase 1 (BCMO1). *Arch Biochem Biophys, 502*(1), 8-16.

McLaren, D., & Frigg, M. (2001). Vitamin A in Nature. *Sight and Life Manual on Vitamin A Deficiency Disorders (VADD)* (pp. 1-8). Basel, Switzerland: Oxford University Press.

Mucida, D., Park, Y., Kim, G., Turovskaya, O., Scott, I., Kronenberg, M., & Cheroutre, H. (2007). Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science, 317*(5835), 256-260.

Niederreither, K., Vermot, J., Messaddeq, N., Schuhbaur, B., Chambon, P., & Dolle, P. (2001). Embryonic retinoic acid synthesis is essential for heart morphogenesis in the mouse. *Development, 128*(7), 1019-1031.

Rochais, F., Abi-Gerges, A., Horner, K., Lefebvre, F., Cooper, D. M., Conti, M., . . . Vandecasteele, G. (2006). A specific pattern of phosphodiesterases controls the cAMP signals generated by different Gs-coupled receptors in adult rat ventricular myocytes. *Circ Res, 98*(8), 1081-1088.

Ross, S. A., McCaffery, P. J., Drager, U. C., & De Luca, L. M. (2000). Retinoids in embryonal development. *Physiol Rev, 80*(3), 1021-1054.

Thomas, S. A., Matsumoto, A. M., & Palmiter, R. D. (1995). Noradrenaline is essential for mouse fetal development. *Nature, 374*(6523), 643-646.

Vaniotis, G., Del Duca, D., Trieu, P., Rohlicek, C. V., Hebert, T. E., & Allen, B. G. (2011). Nuclear beta-adrenergic receptors modulate gene expression in adult rat heart. *Cell Signal, 23*(1), 89-98.

Wagner, M., Han, B., & Jessell, T. M. (1992). Regional differences in retinoid release from embryonic neural tissue detected by an in vitro reporter assay. *Development, 116*(1), 55-66.