The Role of Alpha-7 Nicotine Acetylcholine Receptors in Angiogenesis After In Vivo Nicotine Administration

Monica Rodriguez
Abstract

Key Words: (Angiogenesis, nicotine, zebra finch, heat shock protein 70, alpha-7 nicotinic acetylcholine receptor)

Coronary Heart Disease (CHD) is one of the deadliest heart diseases in the United States. CHD is described as the narrowing of capillaries. Risk factors that contribute to this disease are smoking and stress. In the Cappendijk Lab, the effects of nicotine are studied in zebra finches, an established model to study cognitive processes. Nicotine acts on nicotinic acetylcholine receptors (nAChRs) to induce effects. Of interest are alpha-7 nAChRs, which are involved in cognitive processes and found on endothelial heart cells (ECs). The number of ECs is positively linked with the process of angiogenesis. Angiogenesis is a physiological process for cell survival and development. There are indications that alpha-7 nAChRs on ECs are a target for revascularization in therapeutic angiogenesis of heart disease. During angiogenesis an increased expression of heat shock protein (HSP) 70, is observed. The aim of the project was to identify the role of alpha-7 nAChRs and HSP70 in angiogenesis in the adult male zebra finch heart following single and repetitive administrations of nicotine. We found that alpha-7 nAChR expression and wall thickness increased in nicotine-exposed animals immediately after nicotine (single and repetitive) treatment. Wall thickness increased only in mid-sized vessels 3 months (long-term) following the cessation of nicotine, but not after 24hr withdrawal from nicotine. The expression of HSP70 was dependent on vessel size (diameter) and decreased after repetitive exposures to nicotine. These results suggest that nicotine could induce a long-term effect on heart tissue and the process of angiogenesis in the adult male zebra finch.
THE ROLE OF ALPHA-7 NICOTINIC ACETYLCHELINE RECEPTORS IN ANGIOGENESIS AFTER IN VIVO NICOTINE ADMINISTRATION

By

MONICA RODRIGUEZ

A Thesis submitted to the Department of Chemical-Biomedical Engineering in partial fulfillment of the requirements for graduation with Honors in the Major

Degree Awarded:
Spring Semester, 2012
The members of the Defense Committee approve the thesis of Monica Rodriguez defended on April 6, 2012.

______________________________
Dr. Susanne Cappendijk
Outside Thesis Director

______________________________
Dr. Rufina Alamo
Committee Member

______________________________
Dr. Jingjiao Guan
Committee Member
Acknowledgments

I would like to thank many people who made the completion of my honors thesis possible. First and foremost Dr. Susanne Cappendijk, whom after four and half years has given me the skills necessary to manage a laboratory facility and the ability to think like a researcher. Outside of the laboratory she has been an amazing mentor who has always guided me through the toughest decisions. Dr. Susan Blessing and the Women in Math, Science and Engineering program at FSU has also played a big role in my research endeavors, without the program I would have not had the opportunity to perform undergraduate research with Dr. Cappendijk.

Also, I would like to thank Jenny Bartels who helped cut the experimental tissue needed to complete this project. Kirsten Brown, Jordan Burdick, and David Alarcon who helped count the immunocytochemistry (ICC) protein expression needed for quantitative analysis of the tissue. Lastly, my fellow lab members through the past years who have encouraged and supported me in and outside the lab: Will Perry, Chris Jones, Jessica Andrews, Jessica Peoples, Melanie Rucci Sheldon Brown, Daniel Pirvan, Priscilla Givens, David Page, Heather Drew, Patrick Yount and Geoffrey Miller.
# Table of Contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Background of the Study</td>
<td>6</td>
</tr>
<tr>
<td>A. Heart Disease in the United States</td>
<td>6</td>
</tr>
<tr>
<td>B. Angiogenesis and alpha-7 nicotinic acetylcholine receptors (nAChRs)</td>
<td>7</td>
</tr>
<tr>
<td>C. Angiogenesis and Heat Shock Protein 70 (HSP70)</td>
<td>8</td>
</tr>
<tr>
<td>D. Nicotine and the Zebra Finch Model</td>
<td>8</td>
</tr>
<tr>
<td>II. Hypothesis of the Study</td>
<td>9</td>
</tr>
<tr>
<td>III. Aim of the Study</td>
<td>10</td>
</tr>
<tr>
<td>IV. Materials and Methods</td>
<td>10</td>
</tr>
<tr>
<td>A. Animals</td>
<td>10</td>
</tr>
<tr>
<td>B. Recorded Parameters and Housing Conditions during the Experiments</td>
<td>10</td>
</tr>
<tr>
<td>C. Tissue Preparation</td>
<td>12</td>
</tr>
<tr>
<td>D. Immunocytochemistry (ICC) procedure</td>
<td>13</td>
</tr>
<tr>
<td>E. Biochemical mechanisms behind an ICC</td>
<td>14</td>
</tr>
<tr>
<td>I. Basics of histological staining</td>
<td>15</td>
</tr>
<tr>
<td>II. Mechanisms of the Masson Trichrome Staining (MTS)</td>
<td>15</td>
</tr>
<tr>
<td>F. Tissue Analysis</td>
<td>16</td>
</tr>
<tr>
<td>V. Results</td>
<td>18</td>
</tr>
<tr>
<td>VI. Discussion and Summary</td>
<td>29</td>
</tr>
<tr>
<td>VII. References</td>
<td>33</td>
</tr>
<tr>
<td>VIII. Appendices</td>
<td>34</td>
</tr>
</tbody>
</table>
I. Background of the Study

A. Heart Disease in the United States

In 2008, the Centers for Disease Control and Prevention (CDC) received death reports for over 616,000 people and it was reported that 1 out of 4 people (25%) died of heart disease. Heart disease is a general term used for many different disorders with the heart. Coronary Heart Disease (CHD) is the deadliest of all and it caused death to about 405,309 people in 2008. CHD is described as the narrowing of small blood vessels that supply blood and oxygen to the heart. This narrowing could be caused by a plaque build-up that blocks the blood flow to the heart. The plaque is formed from cholesterol and fatty deposits. These deposits could be caused by a fatty diet. The true etiology of CHD is not clearly understood, however some risk factors for the disease are smoking, hypertension, and an inability to manage stress. In 2010, CHD was projected to cost the United States about $108.9 billion, which includes all health care service costs, medications, and loss of productivity. Plaque is usually removed surgically to restore the flow in the blood vessels. However, new non-invasive techniques are being investigated to restore blood vessel function. Medications are available to minimize the risk of CHD and other heart diseases by cholesterol reducing agents, however, medications that restore the function of clogged blood vessels are not currently available.

Angiogenesis is a biological phenomenon where new blood vessels grow from small blood vessels, known as capillaries. In order to help restore proper blood flow to the heart, researchers are investigating ways to target and gain control over the process of angiogenesis in a non-invasive manner. If researchers can direct the process of angiogenesis then perhaps it will allow patients who suffer from CHD a new treatment option. In this project we focus on two key
factors that take part in the process of angiogenesis, alpha-7 nicotinic acetylcholine receptors (nAChRs) and a specific class of heat shock protein called HSP70.

**B. Angiogenesis and alpha-7 nicotinic acetylcholine receptors (nAChRs)**

In the central and peripheral nervous systems, the alpha-7 nicotinic acetylcholine receptor (nAChR) is widely expressed. In addition, this receptor is found in several non-neuronal tissues, such as the endothelial heart cells (ECs, Figure 1). The number of ECs is positively correlated with the process of angiogenesis. Angiogenesis is a critical physiological process for cell survival and development. Non-selective cholinergic agonists such as nicotine have been shown to induce angiogenesis (Heeschen et al., 2002). There are indications that the alpha-7 nAChRs located on ECs may be a new endothelium target for revascularization in therapeutic angiogenesis of coronary and ischemic heart disease (Arias et al., 2009; Pillai and Chellapan, 2012). However, the underlying intracellular mechanism by which alpha-7 nAChR activation mediates this part of the vital process of angiogenesis is currently unknown.
C. Angiogenesis and Heat Shock Protein 70 (HSP70)

Coinciding with the increased activity of the ECs, an increased expression of heat shock protein 70 (HSP70) has been reported (Zhan et al., 2009). HSP70 is referred to as a powerful antiapoptotic protein and is secreted to help protect a cell from death. Cell death can occur when cells are under stress, experience cancer toxicity or experience high temperature fluctuations (Schmitt et al., 2007). Mechanisms of HSP70 secretion are complex and partially understood; so far studies have shown that HSP70 is secreted by a classical protein secretory route along with other non-classical routes such as, lysosomal and exosomal pathways (Schmitt et al., 2007, Zhan et al., 2009).

D. Nicotine and the Zebra Finch Model

Adult male zebra finches are unique in that they are able to vocalize. The vocalization pattern develops when the animals are born and at the age of approximately 120 days their sole song is crystallized and remains unchanged throughout their life. The process in which these songbirds develop their song pattern is similar to how a human develops the ability to speak. The process of speech is a mental attribute associated with cognition. Nicotine is said to affect cognitive behavior by mechanisms involving the alpha-7 nAChRs.

The Cappendijk lab investigates the effects of nicotine on cognitive functions by observing the finch’s song pattern during and after nicotine administration. Since a side effect of nicotine is hypertension, we need to investigate if the zebra finches are experiencing any type of cardiac disease when we administer nicotine as this could be fatal to the birds and ultimately the study.
II. Hypothesis of the Study

In 2008, the American Heart Association (AHA) found that “nicotine causes a short-term increase in blood pressure, heart rate and the flow of blood from the heart. It also causes the arteries to narrow.” AHA observed these side effects by examining the health of patients who smoke.

The Cappendijk lab examines the cognitive and biochemical effects of nicotine on zebra finches and I hypothesize that hypertension and vessel narrowing will occur in nicotine-exposed finches.

I base my hypothesis on the following: Nicotine-induced cell stress induces vasodilation in the blood vessels, which increases the blood flow and heart rate. This can lead to high blood pressure (hypertension), caused by the thickening of the left ventricular wall. The extra work needed to pump the blood elevates the temperature of the body. This then leads to the activation of HSP70. Therefore, I hypothesize that this protein as well as alpha-7 nAChRs should be expressed dose-dependently after the exposure to nicotine.

One of my previous research projects in the Cappendijk lab showed that the in vivo administration of nicotine dose-dependently affected the morphology of the collagen tissue in the zebra finch heart (Rodriguez and Cappendijk, 2009). This finding pointed toward an increased activity of the ECs, which results in a remodeling of the collagen matrix. From this finding, I formed a second hypothesis, which states that the collagen around the blood vessel structure will thicken due to the elevated activity of ECs brought on by repetitive exposures to nicotine.

Based on these two preliminary hypotheses, I formed a new hypothesis for this honors thesis project: Single and repetitive exposures to nicotine not only induce the expression of alpha-7 nAChRs and HSP70 in the adult male zebra finch heart in a synergistic manner but also...
increase the activity of ECs leading to collagen thickening around blood vessels.

III. Aim of the study

The objective of this project is to identify the role of the alpha-7 nAChR and HSP70 in the process of angiogenesis in the adult male zebra finch heart tissue after single and repetitive exposures of nicotine.

IV. Materials and Methods

A. Animals

Adult male zebra finches (*Taeniopygia guttata*, Figure 2) with body weights between 12 and 15 grams were purchased from a breeder (Acadiana Aviaries, LA). Animals are housed in groups of 3 to 4 for two weeks in a temperature controlled room set at 26 ± 2 °C, under 14L/10D (lights on at 8:00am) conditions. Food and water are available *ad libitum* (Cappendijk et al., 2010).

B. Recorded Parameters and Housing Conditions during the Experiments

After the acclimation period, the zebra finches are transferred to an experimental room. The zebra finches are singly housed in custom-built recording cages (Figure 3). These cages are equipped with a microphone to record both the quality and quantity of individual song patterns during the experiment. Sensors on the perches, food and water...
trays were installed to record the number of times and the length of each event, the finch approached each tray and moved from perch to perch. Body weight and amount of food consumed are also measured during experimentation. This data allows the Cappendijk lab to quantitively and qualitively monitor and analyze the behavior of the zebra finches.

A second acclimation period of one week in the recording cages is taken into account. During this acclimation period, lab members will come in and perform the daily duties of replenishing food and water. After acclimation, subcutaneous injections (s.c) of saline commence for 5 days, twice a day at 7am and 7pm. This is done to familiarize the finches with handling and injections. This process is where the zebra finch undergoes the most intense stress. The stress is generated by their reluctance to handling and injection. After a period of 5 days of saline administration, nicotine is given. Nicotine injections are also administered at 7am and 7pm, for a total of 7 days. During the whole length of the experiment, food consumption, weight and song data are recorded daily. Song expression is recorded for a period of 6 hours per day, starting at 8am when the lights turn on, until 2pm. Husbandry takes place at 2pm, daily for the length of the whole experiment.

**Short-term Nicotine Experiment**

After the saline acclimation period, adult male zebra finches (n=16) were divided at random into 4 equal groups and either treated with saline, (0.03 ml saline/10 gram of body weight), with a single injection of nicotine, or with repetitive injections of nicotine (7 day exposure). A fourth group of animals was treated with a single nicotine dose and sacrificed 24 hours later, this group is referred to as a nicotine withdrawal group. All animals were recorded until the last drug treatment with an exception to the nicotine withdrawal group which was recorded for an
additional day. Based upon our previous results, we used a dose of 0.18 mg nicotine/kg s.c 2x/day, which corresponds to a nicotine intake of 8-10 cigarettes per day (Cappendijk et al., 2010).

**Long-term Nicotine Experiment**

After the saline acclimation period, adult male zebra finches (n=16) were treated with saline (0.03 ml saline/10 gram body weight n=8) or nicotine (0.18 mg nicotine/kg n=8) for 7 days, twice a day. Animals were recorded for 90 days following the last drug treatment.

**C. Tissue Preparation**

After the completion of each experiment finches were sacrificed by an overdose of Equithesin (0.08 ml/10 gram body weight). The heart tissue was extracted and preserved in 4% paraformaldehyde until further analysis. I used a paraffinization protocol developed by XiXi Jia, Dept. of Psychology Florida State University, and modified this protocol in previous semesters (Appendix A). Once the protocol was established, the tissues from both experiments were paraffinized to prepare them for microtome sectioning (Figure 4). Paraffinization of the tissue is required in order to ensure that the tissue does not lose its morphological structure and to prevent tissue degradation.

*Figure 4: Leica 2255 Microtome used to cut paraffinized zebra finch heart tissue.*
The tissue was removed from the paraformaldehyde, dehydrated by ethanol washes, and fixed with paraffin washes. After the tissue was fixed internally, it was molded into a cassette with paraffin. After approximately 2 days, the mold is dry and it is ready for cutting. The heart tissue was cut at a thickness of 5 micrometers on the microtome. Five micrometers is the thickness chosen because previous research in our laboratory showed that at 20 micrometers thickness cut on a vibratome it is not possible to detect any morphological characteristics such as blood vessels and individual cell structures (Figure 5). The 20 micron section was cut on a vibratome where fresh tissue was used, while the 5 micron section was cut on a microtome where paraffinized tissue was used.

Tissue ribbons formed from the microtome were placed on gelatin potassium chromate subbed slides (protocol, Appendix B). The slides were prepared by a protocol according to Phillips (1998) and left to dry overnight. The gelatin potassium chromate slides provide an adhesive film to ensure that the paraffin ribbons remain on the glass slides during the staining procedure.

**D. Immunocytochemistry (ICC) procedure**

One of the ICCs was performed with the alpha-7 nAChR antibody (anti-alpha-7 nAChR mAb 318, kindly provided by Dr. Jon Lindstrom from the University of Pennsylvania, Dept. of
Neuroscience). The other was performed with a HSP70 antibody (anti-HSP70 Rabbit pAb Calibiochem® Cat# 386035). These antibodies were used to detect the expression of alpha-7 nAChR and HSP70 in heart sections cut on a microtome. Since there is not a specific ICC procedure for zebra finch heart tissue, I adapted procedures from Vector Labs® and other procedures used for animal and human tissues to compile one specifically for zebra finch heart tissue (Kosel et al., 2001; Heeschen et al., 2002; Appendix C). The ICC was performed using a VECTASTAIN® ABC Elite IgG Goat kit and DAB enzyme substrate kit (Vector Labs®).

**E. Biochemical Mechanisms behind an ICC**

As primary antibodies, which will allow the binding to the protein site, anti-alpha-7 nAChR or anti-HSP70 are used. A biotinylated secondary antibody reacts with the primary antibody. The biotinylation allows for a more precise binding to that specific antibody. A third layer is added of Avidin/Biotinylated Enzyme Complex (ABC), which binds to the secondary antibody. A brown tinted enzyme substrate is then added to bind to the ABC, which allows the visualization of the binding site.

**I. Basics of histological staining**

Dye structure is an important component when it comes to staining tissue. The dye must be in a specific chemical form before it is able to ionize and form a chemical bond with the area of interest. There are two main structural components needed in a dye, a chromophore and an auxochrome. Figure 6 shows the mechanism how Picric acid, a component in the Bouin’s solution used in the Masson Trichrome Stain (MTS), is formed. Organic compound benzene absorbs UV light strongly, however, it appears white to the human eye. Benzene must be altered
chemically in order to become a visible dye. In order to achieve this, a chemical group that can color organic compounds is needed, known as a chromophore. If a nitro group is added to the benzene, the compound becomes a light yellow. With every addition of a nitro group the color of the compound becomes more intense. However, the colored compound is not yet considered a dye because it will not bind to tissues. In order to turn a colored compound into a dye the addition of an ionizable group is needed. This ionizing group is called an auxochrome. In the case of benzene, if three nitro groups and a hydroxide group are added, the compound becomes trinitrophenol, also called picric acid. The OH auxochrome’s ionization will allow the dye to successfully bind to tissue by forming an ionic chemical bond (Cook 2006).

II. Mechanisms of the Masson Trichrome Staining (MTS)

The Masson Trichrome stain that the Cappendijk lab uses is based off a multi-step process, in which each of the sequential process allows for the staining to be optimized at each step (Appendix D). This particular trichrome stain uses acid solvents i.e., dyes. The acidity of the dyes allows for the maximum amount of dye to attach to the tissues’ amino groups, this controls how the ionized acid dyes react with ionized basic tissues.

The staining procedure starts with a staining preparation, which involves cleaning. The cleaning is performed with xylene washes that remove oils and paraffin from the tissue. The xylene washes are followed by a dehydration and rehydration process. The dehydration process
starts by 3 serial washes of declining ethanol concentration (100-80%). The gradual dehydration allows for many of the tissue’s cellular membrane to dissolve and allows for the molecular components in the cell to be vulnerable for staining. The first step in the staining procedure starts with the addition of the Bouin’s solution, which is a fixative that allows the erythrocytes (red blood cells) to lyse or burst. This permits for higher quality staining because most of the blood in the tissue will no longer be present. The remaining blood cells will be stained a yellow color, an ideal contrast for the upcoming red smooth muscle dye.

Once the tissue is fixed overnight in the Bouin’s solution, it is put into a Working Weigerts solution, which specifically stains the nuclei. The Working Weigerts is washed off with distilled water. Then the Scarlet Fuschin acid is added, which stains smooth muscle, cytoplasm, and collagen. The red blood cells are resistant to this stain because they have been soaked with Bouin’s solution. When the tissue is completely stained, it is put into a Phosphomolybidic and Phosphotungstic acid solution. The large molecules present in this solution allow for the removal of the Scarlet Fuschin acid stain from the collagen fibers. Now that the collagen fibers are available for staining, the Aniline Blue stain is added. This large molecule is used to stain the unstained collagen tissue (Cook 2006).

F. Tissue Analysis

During each ICC, experimental tissue was divided into two slides with at least 5 tissue sections per slide. One slide was treated with a control and the other with an antibody solution. When the slides were dry, the tissue was analyzed on a Leica DM5500 B microscope using various magnifications.

The general morphology was analyzed first to determine changes in the gross appearance of the
tissue. This includes morphological changes such as the space between muscle striations and the condition of the striation. The striations in muscle tissue allow the heart to extend and contract in order to pump blood. Worn out or torn striations indicate that the heart could have not been pumping well.

After the gross morphological analysis was performed, a quantitative analysis was done to determine the amount of alpha-7 nAChR and HSP70 protein expression. As stated earlier, nAChRs are found around the endothelial layer of a blood vessel. Three unbiased undergraduate students located 5 vessels with different thicknesses in one heart section. Then they located the same blood vessels in three other sections on the same slide. They used a microscopic ruler to measure vessel thickness and inside/outside diameter on the Leica microscope at 400X magnification, shown in Figure 7. Then they counted the ICC labeled proteins around the endothelial layer in images at 400X magnification. In order to determine the protein expression at different blood vessel sizes, all counted sections were grouped by the diameter of the vessels. Once they were grouped, average thicknesses and protein expression for each vessel were taken to determine the changes in vessel wall thickness between experimental conditions.
V. Results

ICC Alpha-7 nAChRs expression: Short-term

The aim of this experiment was to examine the short-term effects of nicotine on the zebra finch heart by observing the general morphology and Alpha-7 nAChR protein expression.

![Figure 8: Results of the alpha-7 ICC. Top row shows control groups for each type of short-term experiment. The bottom row shows the tissue treated with the alpha-7 antibody. These pictures were taken at 400X magnification except for the control saline group which was taken at 100X magnification. The black scale bar represents 50 µm for all except for the saline control which represents 100 µm.](image)

As shown in Figure 8, the general morphology was examined between saline, nicotine 1x exposure, and 24 hour withdrawal animals. The striations in the nicotine 1x looked similar to that of the saline, very long and lean. However, the 24hr withdrawal showed a more broken striation pattern around the blood vessel. Also, the space between the striations appeared narrow.
Figure 9: This figure shows that a single administration of nicotine generally increases vessel wall thickness. The five vessels were ranked from smallest (1) to largest (5), based on the inner diameter (34 µm, 60 µm, 88 µm, 109 µm, and 158 µm respectively). The increase in vessel thickness is not due to the administration of antibody as the control groups also show a general increase in vessel thickness.

A single administration of nicotine increased the vessel wall thickness in comparison to the saline treated animals in all vessels except for 5. The largest vessel (5) did not have a vessel around 158 µm in the nicotine 1x control slide, therefore, it is not shown in the figure.

Figure 10: This figure shows alpha-7 nAChR expression in the saline and the single administration of nicotine group. The five vessels were ranked from smallest (1) to largest (5), based on the inner diameter. Approximately the vessel sizes for 1, 2, 3, 4, and 5 were 34 µm, 60 µm, 88 µm, 109 µm, and 158 µm respectively. Control and alpha-7 antibody treated slides were given for comparison.

A single administration of nicotine increased the alpha-7 nAChR expression in the vessels 2-4, but did not affect the expression in the smallest and the largest vessel. However, as there is some unspecific binding registered for alpha-7, the effect is mainly observed in vessel 2.
Nicotine withdrawal seems to affect the wall thickness in the vessels 1-3, however when compared to the tissue that was treated with alpha-7 antibody this effect seems to be reversed.

Alpha-7 nAChRs expression examined in all blood vessels did not change when comparing the saline treated group with the nicotine 24 hr withdrawal group. There is a high background expression of nAChRs expression in vessel 4, which could be due to unspecific binding.
ICC Alpha-7 nAChRs: Long-term

The aim of this experiment was to examine the long-term effects of nicotine on the zebra finch heart by observing the general morphology of the heart tissue.

As shown in figure 13, the striations in one of the nicotine-control treated tissue seemed long and lean without much striation breakage, however in the tissue of the same animal treated with the antibody for alpha-7 nAChR, broken and spread out striations were observed around the blood vessel. In all saline treated tissue similar broken and spread out striations around the blood vessel were observed. We conclude that there is a possibility that nicotine could serve as a neuroprotector and is able to conserve the general morphology, as measured by striations, of the heart tissue.
In the long-term study, the wall thickness in nicotine treated animals seem to decrease in smaller vessels (2), while an increase was observed in the largest vessel (5) as compared to saline treated animals.

A long-term exposure of nicotine did not affect the expression of alpha-7 in any of the vessels as compared with the saline treatment.
**ICC HSP70 expression Short-term**

The aim of this experiment was to examine the short-term effects of nicotine on the zebra finch heart by observing the general morphology and HSP70 protein expression.

![Image showing control and HSP70 treated groups for nicotine 1x, nicotine 7x, nicotine 24hr withdrawal, and saline.](image)

Figure 16: Results of the HSP70 ICC. Top row shows control groups for each type of short-term experiment. The bottom row shows the tissue treated with the HSP70 antibody. These pictures were taken at 400X magnification. The scale bars represent 50 µm.

As seen in Figure 16, the general morphology was examined between saline, nicotine 1x and 7x exposure, and 24 hour withdrawal animals in the short-term experiments. It was observed that the striations around the blood vessels were smooth and lean for the saline and nicotine 1x treated animals. In the nicotine 24hr withdrawal and nicotine 7x, broken striations were observed around the blood vessels.
Figure 17: This figure compares how vessel wall thickness changes with saline and a single administration of nicotine. The five vessels were ranked from smallest (1) to largest (5), based on the inner diameter. Approximately the vessel sizes for 1, 2, 3, 4, and 5 were 30 µm, 60 µm, 75 µm, 120 µm, and 160 µm respectively. Control and HSP70 antibody treated slides were given for comparison.

A single administration of nicotine induced an increase in the vessel wall thickness of vessels 2-5 as compared to the saline treated group.

Figure 18: This figure compares how HSP70 expression changes with saline and a single administration of nicotine. The five vessels were ranked from smallest (1) to largest (5), based on the inner diameter. Approximately the vessel sizes for 1, 2, 3, 4, and 5 were 30 µm, 60 µm, 75 µm, 120 µm, and 160 µm respectively. Control and HSP70 antibody treated slides were given for comparison.

The HSP70 protein expression increased in the larger vessels (4-5) after nicotine administration, while a decreased expression was observed in the smaller vessels.
Figure 19: This figure compares how vessel wall thickness changes with saline and a 7 day administration of nicotine. The five vessels were ranked from smallest (1) to largest (5), based on the inner diameter. Approximately the vessel sizes for 1, 2, 3, 4, and 5 were 35 µm, 50 µm, 70 µm, 100 µm, and 150 µm respectively. Control and HSP70 antibody treated slides were given for comparison.

A 7-day exposure of nicotine showed an overall increase in vessel wall thickness in all vessels.

Figure 20: This figure compares how HSP70 expression changes with saline and a 7 day administration of nicotine. The five vessels were ranked from smallest (1) to largest (5), based on the inner diameter. Approximately the vessel sizes for 1, 2, 3, 4, and 5 were 35 µm, 50 µm, 70 µm, 100 µm, and 150 µm respectively. Control and HSP70 antibody treated slides were given for comparison.

A 7-day exposure of nicotine decreased the expression of HSP70 in the smaller vessels, while in the larger vessel (5) an increased expression of HSP70 was observed as compared to the saline treated group.
Figure 21: This figure compares how vessel wall thickness changes with saline and a 24 hr withdrawal of nicotine. The five vessels were ranked from smallest (1) to largest (5), based on the inner diameter. The vessel sizes for 1, 2, 3, 4, and 5 were approximately 30 µm, 40 µm, 50 µm, 56µm, and 100 µm respectively. Control and HSP70 antibody treated slides were given for comparison.

The wall thickness of animals 24 hr after withdrawal of nicotine and saline did not show any differences.

Figure 22: This figure compares how HSP70 expression changes with saline and a 24 hr withdrawal of nicotine. The five vessels were ranked from smallest (1) to largest (5), based on the inner diameter. The vessel sizes for 1, 2, 3, 4, and 5 were approximately 30 µm, 40 µm, 50 µm, 56 µm, and 100 µm respectively. Control and HSP70 antibody treated slides were given for comparison.

The expression of HSP70 was decreased in the nicotine 24 hr withdrawal group in the vessels 2-4 as compared to the saline group.
ICC HSP70 expression: Long-term

The aim of this experiment was to examine the long-term effects of nicotine on the zebra finch heart by observing the general morphology of heart tissue and HSP70 protein expression.

![Figure 23: Results of the HSP70 ICC. Top row shows control groups for the long-term experiment. The bottom row shows the tissue treated with the alpha-7 antibody. These pictures were taken at 400X magnification. The scale bar represents 50 µm.](image)

As seen in Figure 23, the general morphology was examined between saline and nicotine treated animals in the long-term experiment. The striations in the nicotine treated animal seemed to have larger gaps between striations than that of the saline treated group.
Figure 24: This figure compares how vessel wall thickness changes with a long-term saline and nicotine exposure. The five vessels were ranked from smallest (1) to largest (5), based on the inner diameter. The vessel sizes for 1, 2, 3, 4, and 5 were approximately 30 µm, 45 µm, 60 µm, 70 µm, and 90 µm respectively. Control and HSP70 antibody treated slides were given for comparison.

The wall thickness of the vessels 4 and 5 seems to thicken following the exposure to nicotine as compared to the saline exposed animals.

Figure 25: This figure compares how HSP70 expression changes with saline and a 24 hr withdrawal of nicotine. The five vessels were ranked from smallest (1) to largest (5), based on the inner diameter. The vessel sizes for 1, 2, 3, 4, and 5 were approximately 30 µm, 45 µm, 60 µm, 70 µm, and 90 µm respectively. Control and HSP70 antibody treated slides were given for comparison.

The HSP70 expression was affected mostly in the smallest and in the largest vessels, with a decreased and an increased expression in nicotine exposed animals, respectively.
VI. Discussion and Summary

The objective of this study was to observe the effects on vessel wall thickness and alpha-7 nAChR and HSP70 expression after short- and long-term nicotine administration of nicotine. The objective was completed by observing the general morphology, vessel wall thickness and protein expression in ICCs performed with alpha-7 nAChR and HSP70 antibodies.

Discussion

Short-term: A single administration of nicotine increased the alpha-7 nAChR expression in the vessels 2-4, but did not affect the expression in the smallest and the largest vessel. The HSP70 protein expression increased in the larger vessels (4-5) after nicotine administration, while it decreased the wall thickness in the smallest vessel as compared to the saline-treated group. The striations in the nicotine 1x looked similar to that of the saline, very long and lean.

In the nicotine 24hr withdrawal and nicotine 7x, broken striations were observed around the blood vessels. Nicotine withdrawal seems to affect the wall thickness in the vessels 1-3, however when compared to the tissue that was treated with alpha-7 antibody this effect seems to be reversed. Alpha-7 nAChRs expression examined in all blood vessels did not change when comparing the saline treated group with the nicotine 24 hr withdrawal group. There is a high background expression of nAChRs expression in vessel 4, which could be due to unspecific binding. In a personal communication with a research group at the University of Alabama, Dr. Cappendijk discussed this non-specific binding and this group confirmed that they had similar problems with the specificity of this alpha-7 antibody when it was used in their mouse studies.

The expression of HSP70 was decreased in the nicotine 24 hr withdrawal group in the vessels 2-4 as compared to the saline group.
**Long-term:** The striations in one of the nicotine-control treated tissue seemed long and lean without much striation breakage, however in the tissue of the same animal treated with the antibody for alpha-7 nAChR, broken and spread out striations were observed around the blood vessel. In all saline treated tissue similar broken and spread out striations around the blood vessel were observed. Similar observations were observed in the tissue used for the HSP70 analysis. We conclude that there is a possibility that nicotine could serve as a neuroprotector and is able to conserve the general morphology, as measured by striations, of the heart tissue.

A long-term exposure of nicotine did not affect the expression of alpha-7 in any of the vessels as compared with the saline treatment. The HSP70 expression was affected mostly in the smallest and in the largest vessels, with a decreased and an increased expression in nicotine-exposed animals, respectively. The thickness of the vessel wall seemed to increase in the large vessels and decrease in the small vessels.

The first part of my hypothesis stated that both alpha-7 nAChR and HSP70 expression would increase synergistically with the exposure of nicotine. From this data I concluded that alpha-7 nAChR and HSP70 expression seemed to not increase synergistically with exposure to nicotine.

The second part of my hypothesis stated that vessel wall thickness would increase synergistically with the exposure of nicotine. From this data I concluded that the size of the vessel also determined expression level and that the thickness in smaller vessels decreased synergistically with exposure to nicotine.
Summary

The two factors that contribute to the formation of angiogenesis, alpha-7 nAChR activation and vessel wall thickening were present in the adult male zebra finch heart vessels. Alpha-7 nAChR seemed to not increase synergistically with exposure to nicotine. This conclusion was based off the data collected, however it was shown the alpha-7 antibody caused unspecific binding. Therefore, we cannot say for certain that it does not increase synergistically with exposure to nicotine.

The thickness in smaller vessels decreased synergistically with exposure to nicotine, while the larger vessels were prone to thickening in the long-term experiment. The thickening of the blood vessel could be induced by EC migration, therefore, EC migration would decrease in the small vessels and increase in the large vessels with exposure to nicotine. At this time point, we cannot link the intensity of the EC migration with the exposure of nicotine.

Conclusion

EC migration and alpha-7 nAChR activation are necessary to induce the process of angiogenesis. From the observation in this honors thesis project, EC migration was found in the large vessels in the long-term study, however, the expression of alpha-7 nAChR in the long-term experiment could not be linked to nicotine exposure. Therefore, I conclude, that angiogenesis could potentially occur with repetitive long-term exposures to nicotine.

HSP70 expression is dependent on vessel size and after repetitive exposures to nicotine the expression decreases.
Future Studies

- Different antibodies that are labeling alpha-7 nAChR subunits are becoming available, and I suggest that we use some of these newer available antibodies in order to avoid the unspecific binding. This is however, not an easy task, as none of these antibodies are tested in zebra finches so preliminary studies will be required.

- In order to further examine the possibility that nicotine acts as a neuroprotector in zebra finch heart tissue, the morphology of all vessels labeled in all the nicotine exposed animals and all the saline exposed animals need to be examined and linked with the diameter of the vessel it surrounds. This type of data would give us a better indication whether nicotine affects capillaries more pronounced compared to main vessels. This is an important issue as angiogenesis is most commonly found in the capillaries, which can be demonstrated by injecting magnetic dye into the capillaries and using MRI imaging to visualize the in vivo process of angiogenesis. Vast amounts of funding would be needed to perform this study, however, we do consider an option for future investigation.

- The inclusion of other angiogenic agents such as vascular endothelial growth factor protein (VEGF) is recommended for further studies. This protein is released during angiogenesis, therefore it would be a good marker to show the presence of angiogenesis.

- As the whole genome of the zebra finch was recently released, and the Cappendijk lab has designed their own gene arrays, factors playing a role in angiogenesis can be examined and then it can be decided which factors specifically to examine using the ICC method.

- Specific alpha-7 nicotinic agonists and antagonists are available and could be used in a follow up study to examine the role of this specific subunit in the process of angiogenesis in the zebra finch.
VII. References


[10] Pillai S., Chellappan. Alpha7 nicotinic acetylcholine receptor subunit in angiogenesis and epithelial to mesenchymal transition. Curr. Drug Target 2012, only epub available.


VIII. Appendices

Appendix A
Paraffinization Protocol

Appendix B
Subbed Slides

Appendix C
ICC Protocol

Appendix D
Masson Trichrome Stain Procedure

Appendix E
BBB Poster Competition

* Appendices available upon request. Contact Dr. Susanne Cappendijk of FSU COM*