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5HT and the 5HT Receptors- Relevance to Cardiac Valve Disease

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By

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Abstract

Recent studies have revealed that elevated levels of serotonin or 5HT lead to valve dysfunction. A mouse model was successfully established to investigate the effects on the aortic valve in subjects with elevated 5HT levels along with cardiovascular hypertension. For each two-week trial, eight C57BL/6J mice were divided into the following expermental groups: control, 5HT, angiotensin II, and 5HT+angiotensin II. The drug was delivered to the mice via osmotic pumps (Ang II: 0.4ng/g/min, 5HT:0.0025ng/g/min) or direct injections(Ang II: 0.1 mg, 5HT: 2.12 mg, every other day) and the blood pressure of the mice was continually monitored via the CODA tail cuff system. The direct injection model was determined to be a more effective model as the drug delivery was more controllable and there were far less adverse events with the mice. At the end of two weeks, the mice were sacrificed and one heart from each group was used for western blotting and the other for histology. Western blotting for the $5HT_{2A/2B}$ receptors, along with FGF-1, FGF-2, FGF-R1 revealed some trends, but more trials are needed to confirm the significance of these trends. Future studies using this model could successfully reveal the mechanism by which elevated 5HT levels can lead to a diseased aortic valve when under high mechanical stress.

1 Introduction

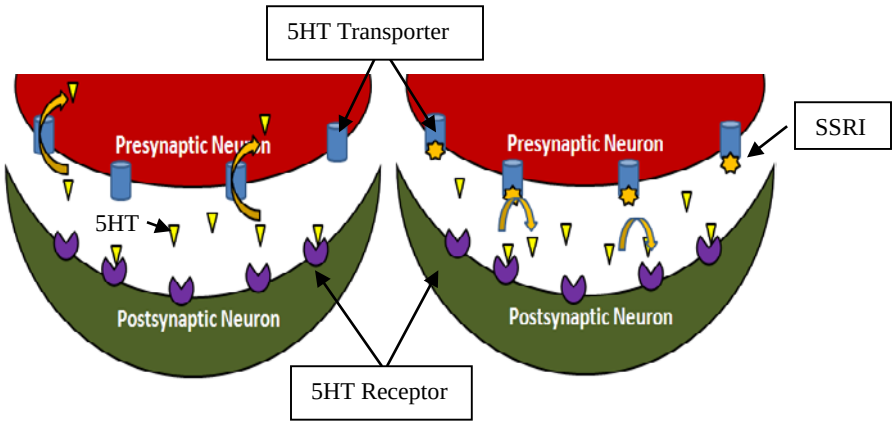
1.1) Serotonin

Serotonin or 5-hydroxytryptamine (5HT) is a neurotransmitter synthesized in the central nervous system.[3,6] The synthesis pathway is shown below in figure (1c).There is always serotonin present in the human body as it is a neurotransmitter that is heavily involved in the regulation of the nervous and gastrointestinal systems. However, when serotonin levels drop too low, a person goes into clinical depression. Of particular interest to our study is the fact that serotonin has multiple effects on the cardiovascular system due to the numerous receptor subtypes present. There are 5HT receptors that control vasoconstriction and vasodilation, and others that are variably expressed in the valve tissues. Significantly, the aortic valve is innervated as shown by staining for acetylcholine in figure (1a). The presence of nerves also implies the presence of the neurotransmitter serotonin. Furthermore, the valve itself has 5HT receptors which certainly means that 5HT is present in the aortic valve. Recent studies have pointed towards 5HT as causing valve dysfunction if present at elevated levels.[4,6,-10,12] This dysfunction results in pathologic conditions within the valve that can often require a valve replacement to repair.

Another relevant fact about serotonin is that when its bodily levels drop below the normal value, the functions of the nervous system are negatively affected. In fact, low serotonin levels are associated with depression. It is not yet known whether these low serotonin levels cause depression or if low serotonin is a byproduct of depression. Still, doctors often prescribe antidepressants (Zoloft, Prozac, Lexapro, Celexa, etc.) that are selective serotonin reuptake inhibitors (SSRIs). These SSRIs prevent the reuptake of serotonin back into the presynaptic neuron, thus increasing the levels of circulating serotonin in the body. This process is depicted in

figure (1b). These drugs that result in an increased level of serotonin are believed to be a viable treatment for depression.

a)



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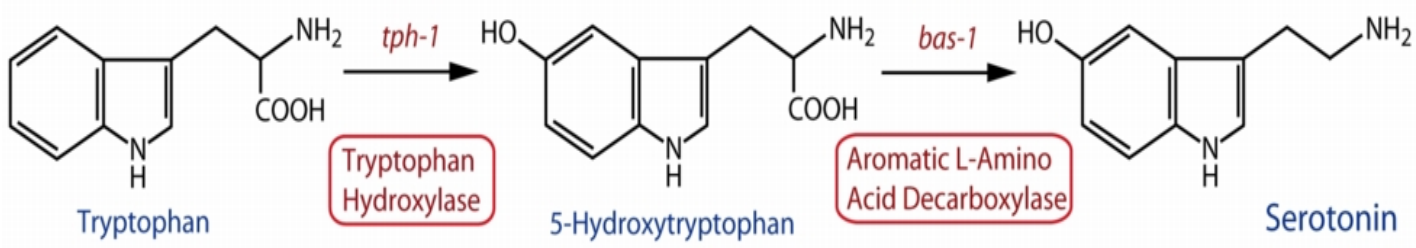


c)

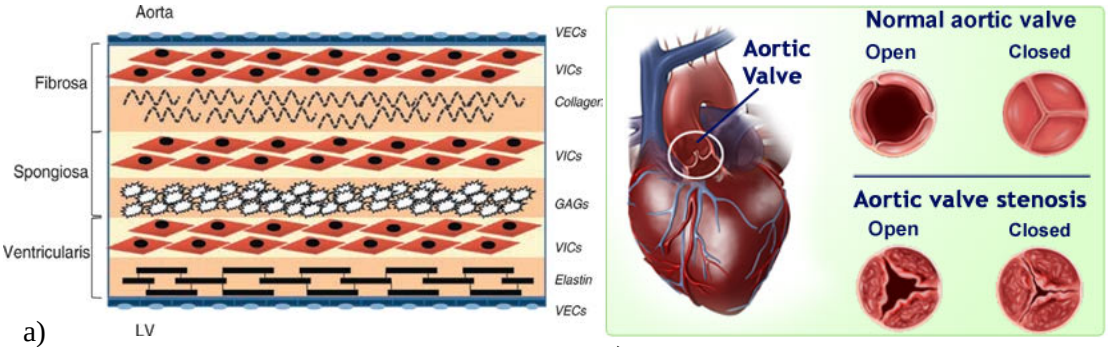
Figure 1: (a) The image in shows an aortic valve that was stained for acetylcholine, a common neurotransmitter. The black lines represent the acetylcholine and thus outline the nerves.[16]

(b) The image on the right shows the mechanism of SSRIs on 5HT pathway in the synapses between nerves.[15]

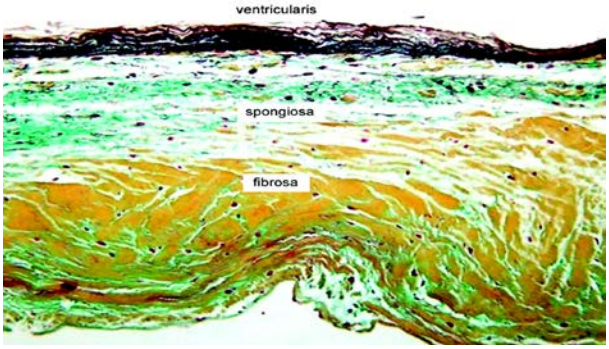
(c) Serotonin synthesis pathway. This occurs naturally in the human body.[17]

1.2) Aortic Valves

The aortic valve is found between the right ventricle and the aorta, the largest artery in the body. This valve is open in systole, allowing the flow of oxygenated blood through the aorta and to the rest of the body. During diastole, the valve remains closed to prevent backflow of blood from the aorta back into the ventricle. This is pictured below in figure (2c), which also illustrates that the aortic valve has three leaflets. Each leaflet or cusp of the aortic valve has three easily identifiable and distinguishable layers.[13] These layers are the lamina fibrosa, the lamina spongiosa, and the lamina radialis. These layers are pictured in figure 2a and 2b. The lamina fibrosa is the widest layer and resides on the arterial side of the valve. This layer mostly contains densely packed collagen. The lamina radialis or ventricularis is the thinnest layer and borders the ventricular side of the valve. This layer contains the majority of the valve's elastin as well as a significant amount of densely packed collagen fibers. Finally, the lamina spongiosa lies in between the fibrosa and the ventricularis and has a few loosely arranged collagen fibers along with most of the aortic valve's glycosaminoglycans (GAGs).[13] Additionally, a thin layer of valvular endothelial cells (VECs) covers the entire leaflet. Finally, within the extracellular matrix of each of the layers lie myofibroblasts and valvular interstitial cells (VICs) that are similar to smooth muscle cells.



a) c)



b)

Figure 2: Panels (a)[18] and (b)[19] show the layered structure of an aortic valve leaflet. Figure (b) is histological sample showing the layers. Panel (c)[20] shows the placement of the aortic valve in the heart (left), along with the normal and stenotic aortic valve (right).

In the bottom right panel of figure 2c, a stenotic aortic valve is shown. As defined by the Mayo Clinic, aortic valve stenosis occurs when the aortic valve narrows, preventing the valve from opening or closing fully. This reduced functionality of the valve, if untreated, can lead to serious heart problems or even death. In general, the only viable treatment option for severe aortic valve stenosis is valve replacement. Naturally, the ideal situation for a patient would be to intervene in the process that leads to stenosis such that a valve replacement never even needs to be considered.

Significant to our study, as figure (1) shows, the aortic valve is innervated. This is important, because as discussed above, serotonin is a neurotransmitter. The conclusion can directly be drawn that serotonin is present in the valve. Additionally, it is known that there are two families of 5HT receptors that are abundantly present and active in the valves of the heart. The question

of interest to this study is what is the effect of elevated serotonin levels on the heart valve? These receptors are the $5HT1B_{/IC}$ and $5HT_{2A/2B}$ receptors.[5,11] Previous work has demonstrated that 5HT signaling in the aortic valve is mediated primarily by the $5HT_{2A}$ and $5HT_{2B}$ receptor subtypes.[2] Recent data also shows that under elevated cyclic mechanical stretch gene expression for both receptor types increases.[1,2] These same studies show that further elevation of mechanical stretch prompted signaling via the 5HT2A but not the 5HT2B receptor subtypes, suggesting that the 5HT receptors have sensitivity that is based upon stress magnitude. This increase in stretch magnitude on a valve is a direct result of cardiovascular hypertension.

1.3) Motivation for this Study

As discussed, the aortic valve can become dysfunctional as a result of elevated serotonin. Additionally, recent studies have pointed out that the serotonin receptors on the aortic valve become more sensitive under increased mechanical stress or hypertension. This directly implies that hypertension in combination with elevated 5HT levels put a person at high risk for aortic valve stenosis. If the mechanism of action for this aortic valve pathology becomes well understood, prevention, rather than reaction would become a plausible treatment. Specifically, if we understood how receptor activity changed with varying cyclic stretch magnitudes, it would point to possibilities for targeting particular 5HT receptors for treatment depending on the hemodynamic stress state of the patient. This is the direct motivation for the study.

A primary example of this in the real world was the FenPhen trial. This drug combination was an anti-obesity treatment that utilized two anorectics. Fenfluramine acts a serotonin releasing agent and phentermine acts as a norepinephrine, dopamine, and serotonin releasing agent. The problem

with this drug trial was that individuals treated with this combination developed aortic insufficiency and valve fibrosis. Significantly, these patients also demonstrate symptoms of pulmonary hypertension. This example simply asserts the importance of studying the interaction of 5HT and heart valves due to the possible severe consequences.

1.4) Objectives and Hypothesis

There are two overall objectives for this research project. First, we want to determine the synergy between cyclic stretch and 5HT signaling that may potentially result in aortic valve disease and dysfunction. The second goal is to understand the effects of this receptor signaling on valve cell mechanics and functions.

Questions to answer:

1) Doe 5HT signaling depend on the mechanical environment of the valve?
2) What are the immediate functional consequences of 5HT signaling in the valve?

The hypothesis is that the aortic valve responds to elevated levels of 5HT via mechanosensitive 5HT synthesis, signaling, and reuptake. It is additionally hypothesized that the $5HT_{2A}$ receptor subtype increases in responsiveness and expression with increased mechanical stretch.

2: Methods

2.1) General

One of the main objectives for my personal project was to set up an animal model that would allow for testing of the hypotheses in a biological system. Female C57BL/6J mice were chosen as the animal for our study and ordered from the Jackson Laboratory. These animals were chosen because hypertension models were available and knockouts were available for purchase if necessary. Additionally, low cost and a mouse facility that was near the lab made mice a good choice. The mice were housed and maintained under pathogen-free conditions in microisolator cages. The Institutional Animal Care and Use Committee at the University of Arkansas approved all experimental procedures.

In order to generate an animal model that generated heart valves exposed to high serotonin levels at high degrees of mechanical stretch, several drugs for treatment of the mice were necessary. First, to increase the serotonin levels, serotonin was administered directly to the mice. This is the easiest and most efficient way to expose the mice to elevated serotonin levels in the body. To achieve high mechanical stress on the valve, a hypertensive environment needed to be established. Angiotensin II was our drug of choice for creating the high blood pressure. Angiotensin II is a hormone that plays a central role in cardiovascular homeostasis.[14] It is a vasoconstrictor and has been shown to play important roles in mediating hypertension, heart failure, and cardiac remodeling.

Upon arrival, the powder form of these drugs was dissolved in the appropriate amount of filter-sterilized saline solution to create a stock solution. These stock solutions were generally at 0.1M for the serotonin and 0.01M for the angiotensin II. These stock solutions were divided into 1mL

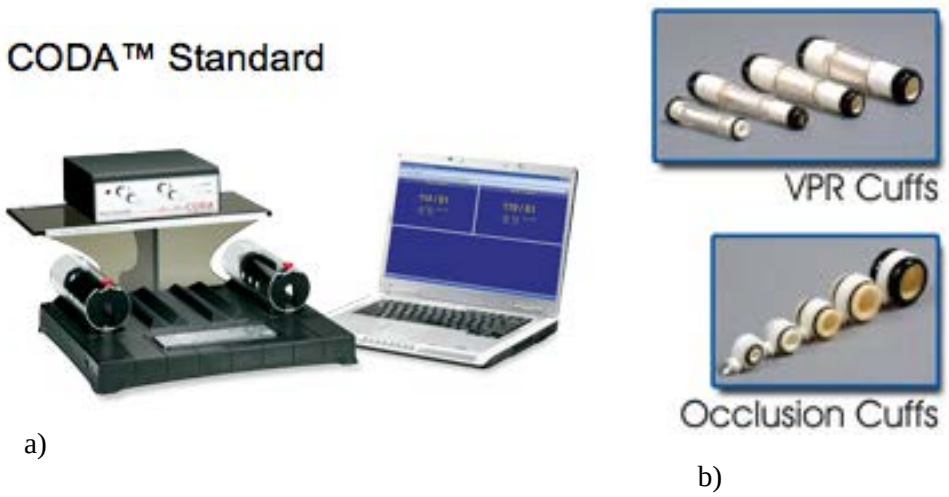
aliquots which were frozen in the -20°C freezer. When the drugs were needed for treatment of the mice, the frozen drug was diluted to the appropriate concentration.

2.2) Blood Pressure Analysis

For the animal model, it was essential to monitor the blood pressures of the animals in order to ensure that the aortic valves had been subjected to the high mechanical stress that is associated with hypertension. Through literature research, it was determined that the best way to monitor the blood pressure of the mice in short term experiments was using a volume pressure recording tail cuff method. This method is a clinically validated technique used to measure the blood pressure in mice and rats according to the Kent Scientific Website. The principle behind the tail cuff method of blood pressure measurement is very similar to the arm cuffs that are commonly used on humans. As seen in figure (3b) the tail cuff system is comprised of two separate cuffs that are placed on the tail. The O-cuff is the smaller, circular cuff that is pushed up right next to the body of the mouse, while the VPR cuff is the longer cuff that goes on the tail of the mouse distal to the O-cuff. At the beginning of each cycle, the VPR cuff pushes the blood out of the tail and then the O-cuff inflates to prevent the flow of blood back into the tail. Similar to the human arm cuff, the O-cuff slowly deflates as the measurement is taken. When the O-cuff deflates to a pressure equivalent to the systolic blood pressure, blood begins to flow back into the tail, thus increasing the tail volume. This first increase in tail volume is recorded as the systolic blood pressure as the mouse. As the O-cuff continues to deflate, the blood flow into and out of the tail will eventually equalize. The pressure at which this occurs is recorded as the diastolic blood pressure. Research suggests that the systolic blood pressure of the VPR tail cuff method is

generally more accurate since the initial increase in volume is more easily detected than the point at which the volume equalizes.

The CODA Standard Non-Invasive Blood Pressure System produced by Kent Scientific was purchased for use in the mouse experiments. This system consisted of tail cuffs, a warming platform, a CODA controller, and two rodent holders as pictured in figure (3). We additionally ordered two extra rodent holders to make the process more efficient and accurate.



a) b)

Figure 3: (a) Components of the CODA Standard blood pressure analysis system.[21]
(b) VPR and O Cuffs[21]

The blood pressure of each animal was routinely measured throughout each of the animal trials. The procedure involved placing the mice in the animal holders, placing the tail cuff device on the tail of the device, and utilizing the CODA software to take the blood pressure measurement. In our particular study, we used 5 acclimation cycles and 10 regular cycles for each animal in order to gather sufficient data to ensure accurate pressure readings.

After the data had been collected from the tail cuff system, it was exported to excel in for processing. The tail cuff system determined if each cycle demonstrated an “accepted” or “rejected” reading. The rejected readings and acclimation cycle pressures were removed from the data set. Next the average of the remaining systolic and diastolic pressures were calculated and plotted in a graph. Additionally, the standard deviation of the blood pressure readings was calculated and added to the graph.

The complete and detailed protocol for usage of the CODA tail cuff system is in appendix A.

2.3) Osmotic Pumps

One method that was used to administer the drugs to the mice was osmotic pump implantation. Alzet osmotic pumps (model 1004, 2004) were used for this method of drug administration. The osmotic pumps simply utilize principles of diffusion to deliver the drug solution that they are filled with. The rate at which the pump delivers the solution within the pump is constant and depends on the model of pump that is purchased. For this reason, the only way to control the time release rate of the drug is to vary the concentration of the drug solution that is injected into the micro osmotic pumps prior to implantation into the animal.

Gathering knowledge from previous published studies, particularly from our collaborator Dr. Kilic of UAMS, we decided to administer angiotensin II and serotonin at 0.4ng/g/min, 5HT:0.0025ng/g/min respectively. Knowing the rate of diffusion from the pump, the reservoir volume of the pump, and the desired duration of the trial, we were able to fill the pumps with the correct concentration of each drug to administer it at the rate that we desired.

After calculating the necessary drug concentration, solutions containing these concentrations of drug were created and used to fill the pumps with the solutions. Following the filling of the

pumps, the pumps were placed in saline solution and incubated at 37 degrees celsius in order to prime the pump. This was simply done to ensure that the pump was already actively pumping the correct amount of drug upon implantion into the mice.

Next was the actual implantation of the pumps into the mice. First, the mice had to be anesthetized with 100uL of a 1:1:4.6 ketamine/xylene/ddH_2O solution. This amount could vary slightly depending on the reactions of the mice to the drug, which depended on the weight and size of the mouse. The mice were shaved at the site of implation and an incision was made that was just large enough to insert the pump. This incision was always made just above and a bit posterior to one of the front shoulders of the mouse. A subcutaneous pocket was then gently opened up with small surgical scissors. The pump was next slid into the prepared pocket in the subcutaneous layer and the incision was closed with stainless steel surgical clips from a the Reflex skin closure system 9. After this, the mice were left on a heating pad until they began to regain consciousness. The surgical procedure was very short and efficient, only taking around 5-10 minutes for each mouse.

2.4) Injection Trials

Besides osmotic pumps, the method of direct injections was explored as an alternative drug delivery method for the mouse model. The purpose exploring this drug delivery method was to reduce the complexity of the model and to ensure that the desired amount of drug was delivered to the mouse each day. Literature was consulted to determine the ideal amounts of 5HT and angiotensin II to include in each injection. It was decided that 2.12 mg of 5HT would be injected every other day, but an ideal amount of angiotensin II for each injection was not found.

However, knowing the suggested amount of angiotensin II used for the osmotic pumps allowed for calculation of an approximate daily value of delivered angiotensin II.

The injection trial to determine the optimal amount of aniotensin II delivered in the injections consisted of two experimental groups of three mice each. One group was injected every other day with 10ug of aniotensin II and the other group was injected every other day with 100 ug of angiotensin II. Each of these injections were a 100uL mixture of angiotensin II and filter sterilized saline solution.The blood pressures of these mice were monitored using the CODA tail cuff system for 9 days. After analysis of the collected blood pressure data, it was decided that 100ug of angiotensin II would be used for the mouse trials utilizing direct injections of the drug.

The injections for the actual trial using the four experimental groups were performed every other day. Again, the desired amount of drug was dissolved in 100 uL of filter sterilized saline solution. The injections were administered intraperitoneally in the lower left abdominal region of the mice as pictured below in figure(4). The blood pressure of the mice was measured on the alternate days when the mice were not injected.

Figure 4: Intraperitoneal injection technique used on mice.

2.5) Mice Experiments

In order to experimentally analyze the effects of high serotonin levels on an aortic valve that is under high mechanical stress, four experimental groups were needed. Each trial consisted of two mice in each of the four groups for a total of eight mice per trial. The control group received treatment with filter sterilized saline solution.

Each trial consisted of 8 C57BL/6J mice divided into the following four experimental groups: Control (saline), 5HT, Angiotensin II, and 5HT+Angiotensin II. Each of the groups received treatment with the drug corresponding to the name of the group. At the beginning of each trial, the amount of each drug to be administered was decided based on whether the trial utilized Alzet osmotic pumps or every other day injections to deliver the drug. The quantities of drug delivered in each trial are shown in the chart below.

	Control (Saline)	5HT	Angiotensin II	5HT+Angiotensin II
Osmotic Pump Model 2004 Trial 1	1x DPBS @ .25uL/hr	0.0025 ng/g/min	0.04ng/g/min	Ang II: 0.04 ng/g/min 5HT: 0.0025 ng/g/min
Osmotic Pump Model 1004 Trial 2	1x DPBS@ .12 uL/hr	0.0025 ng/g/min	0.04ng/g/min	Ang II: 0.04 ng/g/min 5HT: 0.0025 ng/g/min
Osmotic Pump Model 1004 Trial 3	1x DPBS @ .12uL/hr	0.0025 ng/g/min	0.4ng/g/min	Ang II: 0.4 ng/g/min 5HT: 0.0025 ng/g/min
Injections	100 uL 1X DPBS	2.13 mg/injection in 100uL	100 ug/injection in 100 uL	Ang II: 100ug/injection 5HT: 2.13mg/injection In 100 uL

Table 1: Description of Mouse Trials

Before implantation of the pumps or the beginning of injections, at least one blood pressure measurement was performed to establish a baseline pressure for each mouse. Next, eithe r the pumps were implanted or the injections began. Blood pressure data was recorded for each animal at a minimum of three times per week. For the injection trial, the blood pressure data was always recorded on the days on which the mice were not injected. After two weeks, the pressure data was analyzed to determine if the drugs had taken the desired effect. If so, the trial was ended on day fourteen of treatment. Only trial three was extended to a twenty one day trial.

When it was time to conclude the trial, the mice were sacrificed by cervical dislocation. Immediately, the hearts were dissected out and washed in saline solution. One heart from each experimental group was frozen in Tissue-Tex Opitmal Cutting Temperature (O.C.T.) compound for histology. The second heart from each group was grossly trimmed down to a small section surrounding the aortic valve and immediately placed in a cryovial that was frozen in liquid nitrogen. Some of the ventricle was cut away in order to give better resolution in western blot data. In each case, the hearts were stored in the -80°C freezer.

2.6) Histology

In order to analyze the structural condition of the heart and to measure the concentration of $5HT_{2A/2B}$ receptors, several histology techniques were implemented. Examples include H&E staining, picrosirius red staining, and immunostaining for $5HT_{2A/2B}$ receptors along with FGF-1 and FGF2 receptors. Another undergraduate student that works in Dr. Balachandran's lab, Jessica Morales, was in charge of the histology and the data is part of her research.

2.7)Western Blotting

To test our hypotheses about the response of the aortic valve to the combination of elevated 5HT levels and hypertension, several proteins of interest were analyzed via western blotting. As a major hypothesis was that the $5HT_{2A/2B}$ receptors were mechanosensitive, these two receptors were quantified by western blotting. Addtionally, to examine the structural health of the valve, western blotting for FGF1, FGF2 was completed. B-actin expression was also evaluated via the western blot and then used to normalize the expression of the other proteins of interest.

The western blotting process can be broken down into six distinct sections as follows:

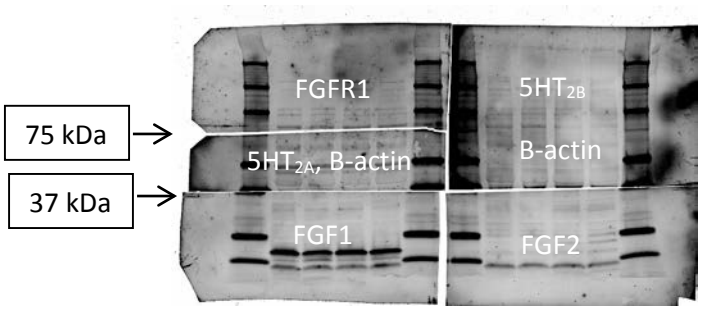
1. Tissue collection, weighing, and grinding into powder
2. BCA Protein Assay (Thermo Scientific)
3. Gel Electrophoresis with 80 ug tissue per lane
4. Western Blotting Transfer Step
5. Staining
6. Imaging

The gel elctrophoresis and western blotting transfer step were completed with a bio-rad western blotting kit. This kit includes the necessary tanks, running buffers, and a PowerPac to use for these steps. The gel used was a Bio-Rad Criterion TGX precast gel with twelve lanes. The organization of samples and controls in the twelve lane gel electrophoresis is shown below. Each sample was run in duplicate to allow for western blotting of multiple proteins from each sample.

1	2	3	4	5	6	7	8	9	10	11	12
C	S1	S2	S3	S4	C	C	S1	S2	S3	S4	C

Table 2: Gel Electrophoresis layout

Next, the western blotting transfer step was completed. . The organization of the proteins of interest on the PVDF membrane are shown below along with the expected moleculat weights of each protein. The lines separating the regions denote where cuts were made to allow for separate staining of the different regions.



Protein	Weight (kDa)
$5HT_{2A}$	53-55
$5HT_{2B}$	50-55
FGF-1	15.5
FGF-2	18/21/24
FGFR-1	92
B-actin	42

Figure 5: Western blot data and expected protein molecular weight

Now that the proteins were on a transfer membrane, they were blocked using Licor blocking buffer and a primary antibody incubation was performed for the proteins of interest. The secondary antibody was then completed using Licor IR dyes that would allow for infrared imaging. The transfer membrane was then imaged using the LICOR Odyssey Infrared Imaging system. This system also included software that allowed for quantification of the bands by integration intensity.

2.8) Statistics and Analysis

2.8.1) Blood Pressure Analysis

. In order to get a good idea of the blood pressure variation over the osmotic pump trials, it was decided to analyze and statistically compare both the systolic and diastolic blood pressures at three time points in the trial. The first time point was in the preliminary readings, where a baseline was established for the blood pressure of each mouse. The second time point was after seven days, which is the very middle of each trial except for trial 3. The third point for analysis was the last blood pressure taken before the sacrifice of the animals. Because trial three was extended to three weeks instead of two, a time point was also included at 21 days for this trial only. In the event that a mouse lost the osmotic pump or died, the pressure data was not analyzed for this particular mouse.

The data was grouped by treatment. For example, every preliminary blood pressure reading from the saline group was put in one, single column. The same was done for the time points that fell at the middle and end of each trial. The mean systolic and diastolic blood pressures were calculated as well as the standard error for each column. The same process was repeated for each treatment.

Additionally, each the data from each time point within each group was statistically compared using a one-way ANOVA statistical test using the SigmaPlot software. Occasionally, a one-way ANOVA by ranks test was used when the data was not suited for the one-way ANOVA. P values are reported in the results section and any P-value less than 0.05 is considered statistically significant.

2.8.2) Western Blot Analysis

Similar to how the blood pressure data was processed, the western blot data was separated by treatment group and analyzed. The data from each western blot was consolidated by treatment group, and the averages and standard errors were calculated. The data from each group was also used to conduct a one-way ANOVA or one-way ANOVA on ranks (if the data was not normally distributed) to compare the protein expression between the different groups. For the one-way ANOVA, the P-values are reported and the Q-values are reported for the one-way ANOVA on ranks.

The injection dosing trial data and the injection trial data was consolidated and analyzed separately, but using the same procedure as for the osmotic pump trials.

3: Results and Discussion

3.1) Trial Notes

Osmotic Pump Trial 1

- Saline group: No complications
- Angiotensin II group: One mouse lost its pump by day 12
- 5HT: One mouse lost its pump by day 12
- Angiotensin II+5HT group: No complications
- The pumps used in this trial (model 2004) seemed too big for our mouse trial. It appeared to stress the mice out, particularly when animal was placed in the animal holder for blood pressure readings.

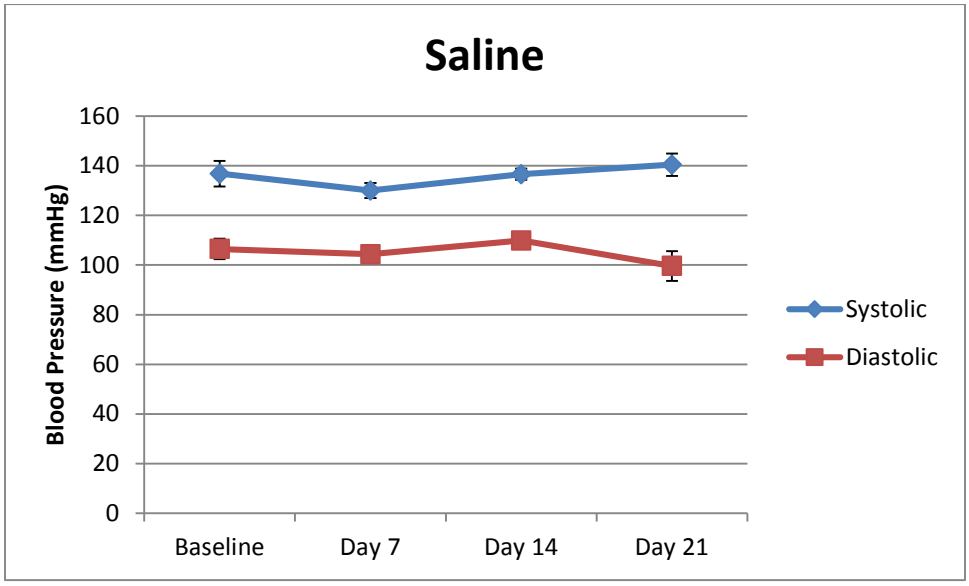
Osmotic Pump Trial 2

- Saline group: One mouse lost its pump by day 6 and died on day 11. The other mouse had lost its pump by day 9.
- Angiotensin II group: no complications
- 5HT Group: One mouse lost its pump by day 9.
- Angiotensin II+5HT group: One mouse lost its pump by day 9.

Osmotic Pump Trial 3

- Angiotensin II+5HT group: One mouse died on day 5 of the trial.
- No other complications

Osmotic Pump Trial Data

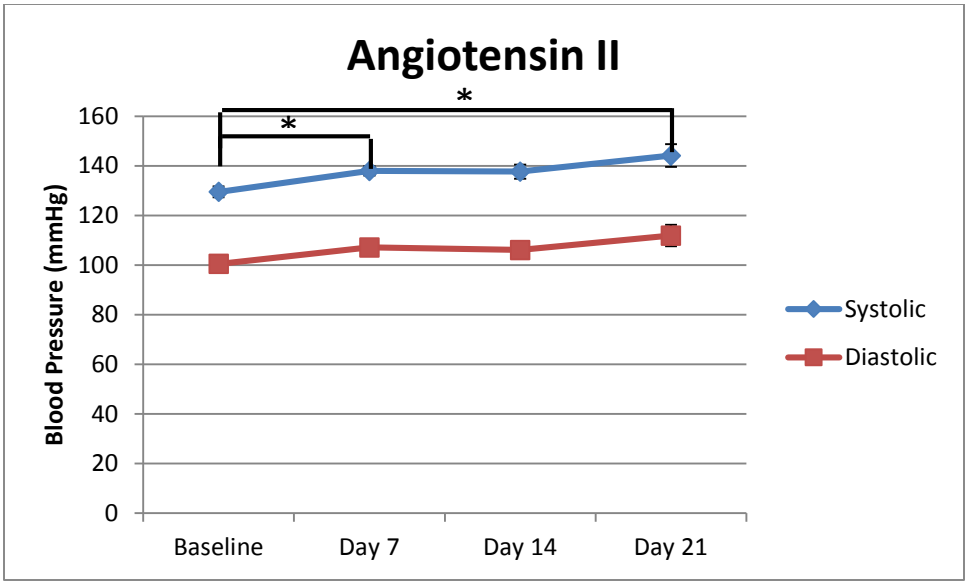


Saline	Systolic % Change	Diastolic % Change
Baseline→Day 7	-5.00	-2.03
Day 7→ Day 14	+5.09	+5.31
Day 14→Day 21	+2.80	-9.34
Total	+2.89	-6.06

Figure 6: Osmotic pump trials, saline group blood pressure data. * Denotes statistical significance. n= 4 mice

There was no statistically significant difference in any of the changes of systolic or diastolic blood pressures between the analyzed time points in the saline group (P=0.266 for systolic, P=0.417 for diastolic).

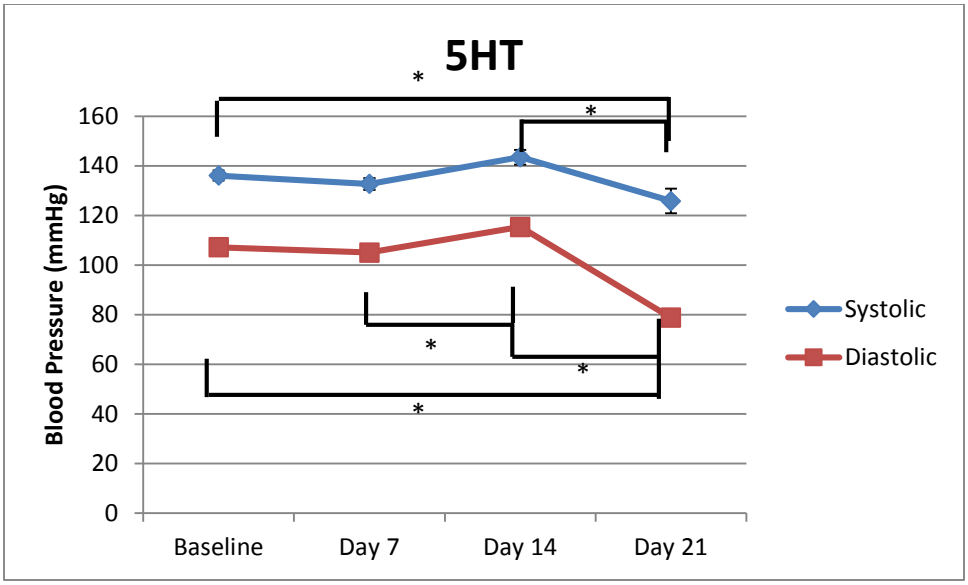
The blood pressure of the saline group behaved as expected overall. As depicted in the graph, both the systolic and diastolic pressures stayed similar throughout the trial. Again, this was expected, since it was the control group that was administered saline solution rather than any drug.



Angiotensin II	Systolic % Change	Systolic Q-Value	Diastolic % Change	Diastolic Q-Value
Baseline→Day 7	+6.53	3.147*	+6.59	2.621
Day 7→ Day 14	-0.18	0.739	-0.91	0.863
Day 14→Day 21	+4.65	1.027	+5.45	0.886
Total	+11.00	2.945*	+11.14	2.284

Figure 7: Osmotic pump trials, angiotensin II group blood pressure data. * Denotes statistical significance. n= 5 mice

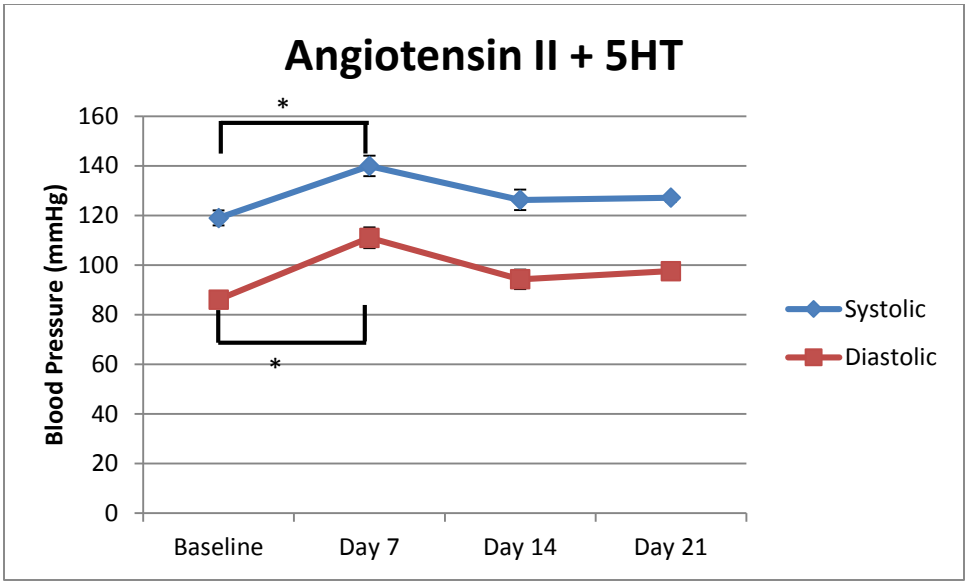
The angiotensin groups did show some significant increase in blood pressure, but not as large of an increase as desired. There are several possible explanations for this behavior of the blood pressures. One major reason is that the angiotensin II dose was simply too low to elicit the desired results. This was certainly the case in the first two osmotic pump trials, as the dose was 0.04 ng/g/min as opposed to the 0.4 ng/g/min that was used in the third osmotic pump trial. Still, the third trial produced results similar to the first two. Another possible explanation would be the fact that the pumps did not always stay in the animal to the end or could have potentially been damaged or not functioned properly. After the pump is implanted, the researcher loses control of the drug delivery process and just has to expect it to work. If it malfunctions, the results could easily change.



5HT	Systolic % Change	Systolic Q-Value	Diastolic % Change	Diastolic P-Value
Baseline→Day 7	-2.50	1.073	-1.97	0.496
Day 7→ Day 14	+8.12	2.499	+9.78	0.004*
Day 14→Day 21	-12.30	4.088*	-31.67	<0.001*
Total	-6.68	2.851*	-23.86	<0.001*

Figure 8: Osmotic pump trials, 5HT group blood pressure data. * Denotes statistical significance. n= 4 mice

The 5HT group showed some up and down variance for the systolic and diastolic blood pressures throughout the trials. While there doesn't seem to be any clear trend, some change in blood pressure can be expected as 5HT is a vasoactive hormone. The data displayed above does not affect the hypothesis or the testing of the hypothesis. It does seem that any effect that 5HT had on the blood pressure was reduced after 14 days.



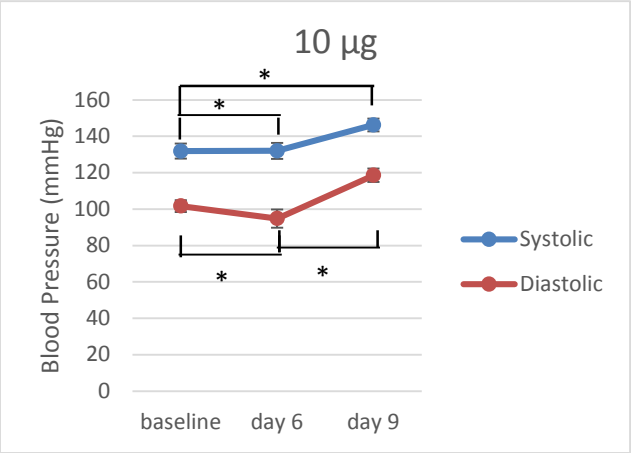
Angiotensin II + 5HT	Systolic % Change	Systolic Q-Value	Diastolic % Change	Diastolic Q-Value
Baseline→Day 7	+17.63	3.529*	+28.90	4.191*
Day 7→ Day 14	-9.79	2.393	-15.09	2.567
Day 14→Day 21	+0.74	0.709	+3.50	1.080
Total	+8.58	1.475	+17.31	2.176

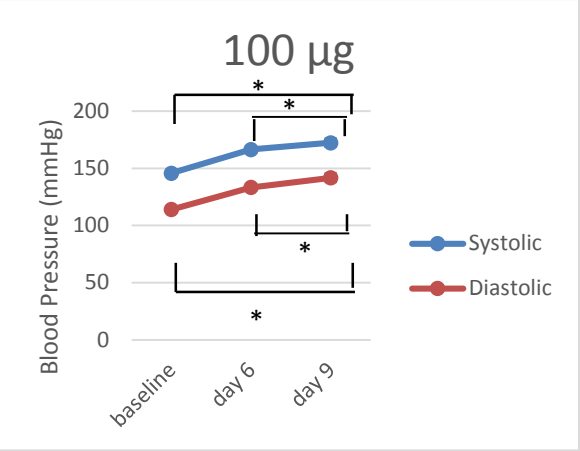
Figure 9: Osmotic pump trials, angiotensin II + 5HT group blood pressure data. * Denotes statistical significance. n=4 mice

This group was the key group to the testing of the hypothesis. It was essential that the mice had hypertension as well as were exposed to elevated levels of serotonin. While the mice were certainly subjected to increased serotonin levels, the blood pressure data does not reveal the marked increase to hypertension that was desired. There are really two plausible explanations for this observation. One, the dose of angiotensin II could be too low as mentioned in the analysis of the angiotensin II treatment group. The second possible explanation is that there is some interplay between angiotensin II and 5HT that prevents the angiotensin II from having its full effect. For this reason, it might be the best option to find another method to increase the blood pressure or purchase genetically hypertensive mice rather than trying to induce hypertension via angiotensin II.

Injection Dosing Trial

- No complications in this trial





10ug	Systolic % Change	Systolic P Value	Diastolic % Change	Diastolic P Value
Baseline→Day 6	+0.09 %	<0.001*	-6.8%	<0.001*
Day 6→ Day 9	+10.8%	.179	+25.1%	0.04*
Total	+10.9%	<0.001*	+18.3%	<0.001*

100ug	Systolic % Change	Systolic P-Value	Diastolic % Change	Diastolic P-Value
Baseline→Day 6	+14.1 %	0.0984	+16.8%	Q
Day 6→ Day 9	+3.6%	0.045*	+6.4%	Q*
Total	+17.7%	0.045*	+23.2%	Q*

Figure 10: Injection dosing trial blood pressure data. * Denotes statistical significance. n= 3 mice for each dose

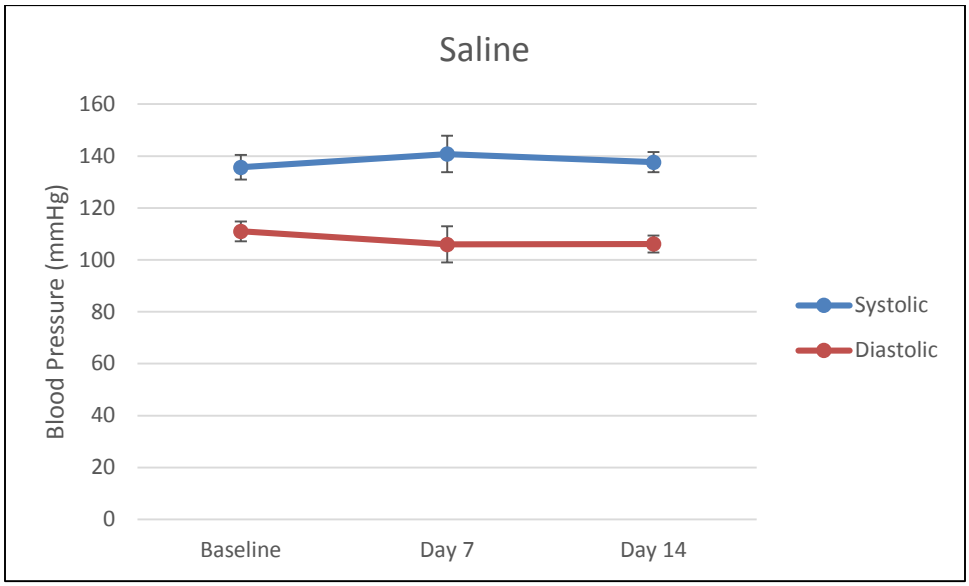
The data from this trial that was used to choose a dose of angiotensin II for the injections shows that both the 10 ug and 100 ug doses of angiotensin II are effective. The total changes in systolic and diastolic pressure for the 10 ug treatment were 10.9% and 18.3% respectively as outlined above in the table. For the 100 ug treatment group the total changes in the systolic and diastolic blood pressures were slightly higher at 17.7% and 23.2% respectively. The major difference in these two treatments reveals itself in the change between the baseline blood pressure and the blood pressure that is measured at day 6. In the 10 ug group, the pressures stayed stable or even

decreased between the baseline pressure and day 6. However, in the 100 ug group, the effect of the angiotensin II seems to be more immediate as the pressure rises noticeably in the first six days of the trial. While this change isn't marked as statistically significant, the P-value is 0.0984, which suggests that more trials would make this a statistically significant jump. This immediate rise of the blood pressure is what is desired in the short, two-week, trials that were used to test the hypothesis. It is necessary for the mice to have hypertension as soon as possible in the two weeks so that the study reflects the combination of elevated 5HT levels and hypertension.

For this reason, it was decided that the future injection trials would utilize a dose of 100 ug of angiotensin II per injection. This trial suggests that the dose should provide the hypertension model in the mice that was desired to test the hypothesis.

Injection Trial

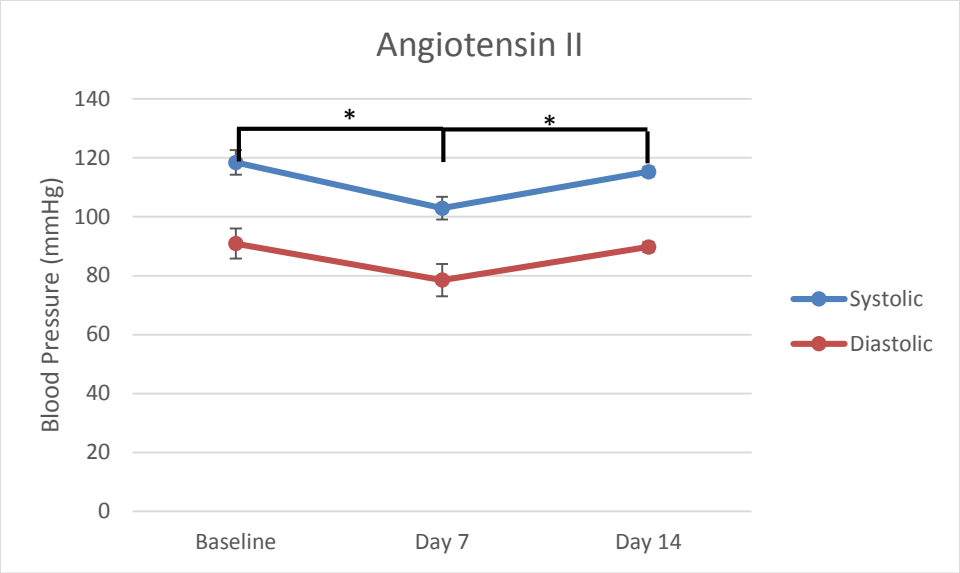
- No complications in this trial



Saline	Systolic % Change	Diastolic % Change
Baseline→Day 7	+3.8%	-4.6%
Day 7→ Day 14	-2.2%	+0.2%
Total	+1.5%	-4.4%

Figure 11: Injection trials, saline group blood pressure data. * Denotes statistical significance. n=2 mice

According to a one-way ANOVA statistical test, the systolic and diastolic value were not significantly different between any of the three time points. For systolic blood pressure the P-value was 0.892 and for the diastolic blood pressure the P-value was 0.616. This is exactly what would be expected from the control group of the experiment. The blood pressure should not increase or decrease significantly over the trial from injecting PBS into the mice.



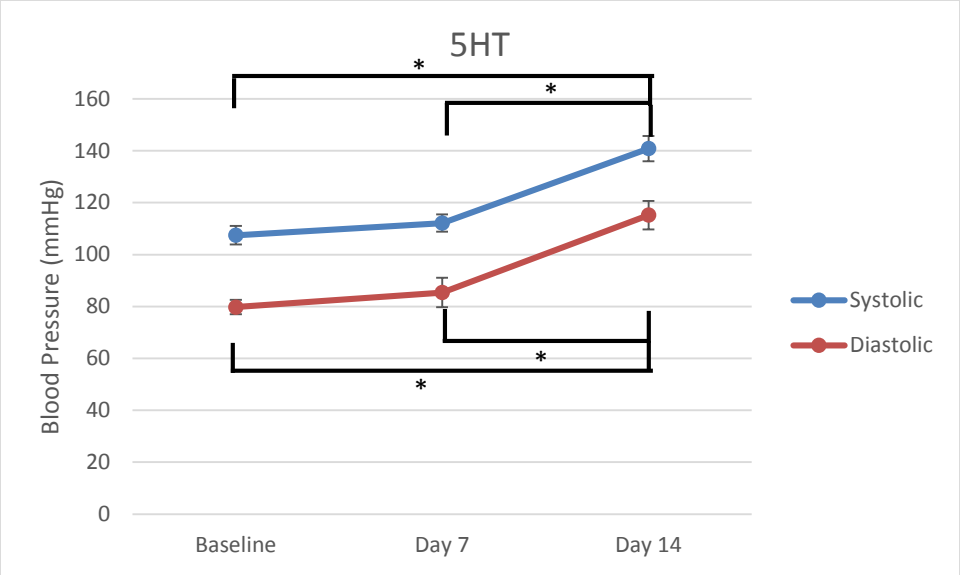
Angiotensin II	Systolic % Change	Systolic P-Value	Diastolic % Change
Baseline→Day 7	-13.1%	0.016*	-13.7
Day 7→ Day 14	+12.0%	0.034*	+14.3
Total	-1.1%	0.743	+0.7

Figure 12: Injection trials, angiotensin II group blood pressure data. n= 2 mice

There was no significant difference in the diastolic blood pressures at the three measured points (P=0.22). Since this was the angiotensin II group, these results do not follow the expected trend. The injection dosing trial revealed an increasing blood pressure from injecting this dose of angiotensin II every other day. However, this data shows a 13.1% drop in systolic blood pressure from the baseline to day 7 and then it rises back up by 12% between day 7 and day 14. The increase in systolic and diastolic blood pressure between day 7 and day 14 is expected, but the drop in blood pressure was not. One possible explanation for this is that the mice initially displayed a higher blood pressure from stress associated with the tail cuff system and having the blood pressure measurements taken. As the animals acclimate to this, a drop in pressure could occur. The second possible reason that the pressure dropped is related to the timing of the injections and blood pressure readings. If each injection only had effect for a short time until the

mice could adjust to the raise in angiotensin II, then measuring blood pressure at different times on the alternate days could really effect the blood pressure data that is collected. It could also potentially explain a decrease in the blood pressure such as the one seen in this data set.

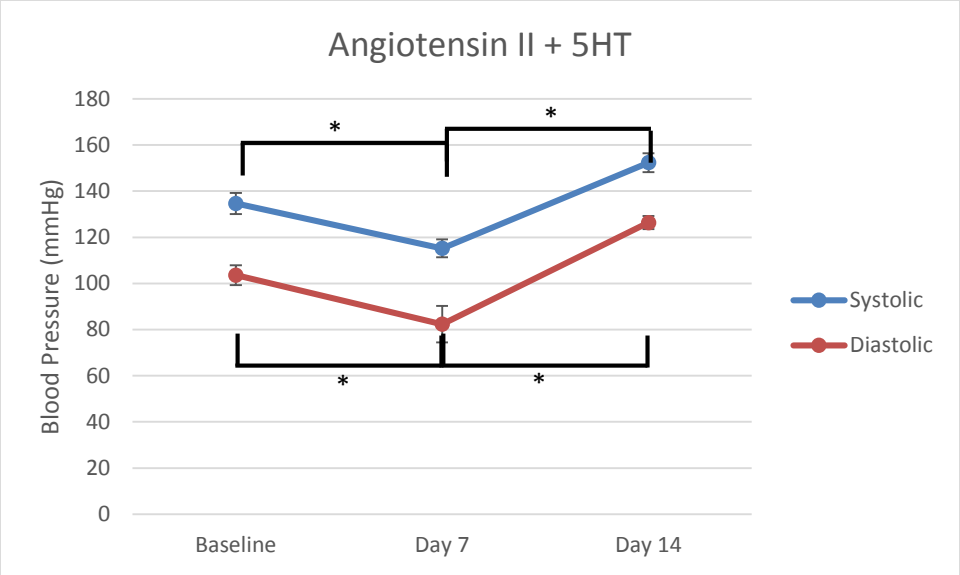
In order to test if the effectiveness of the angiotensin II injections varies over time, I suggest a kinetic study of the angiotensin in the blood of the mouse. If the free level of angiotensin II could be monitored over two days following an injection, the action of the drug could be better understood. In addition to monitoring the free level of angiotensin II in the blood, it would be necessary to monitor the blood pressure every few hours over these two days. If these two studies were completed then there would be a good idea of the kinetics of both the drug and the blood pressure of the mice following each injection. This could lead to important information regarding the timing of blood pressure readings used to collect data for an experimental trial. If the blood pressure does vary over the next day or two, it would become essential to measure the blood pressure of the mice at the same amount of time after an injection in order to gather accurate data.



5HT	Systolic % Change	Systolic Q-Value	Diastolic % Change	Diastolic P-Value
Baseline→Day 7	+4.3%	0.792	+7.0%	0.4
Day 7→ Day 14	+25.6%	3.500*	+34.9%	<0.001*
Total	+29.9%	4.263*	+41.9%	<0.001*

Figure 13: Injection trials, 5HT group blood pressure data. n= 2 mice

For the 5HT injected mice in this trial, another interesting blood pressure trend was observed. The expectation for the 5HT group is to have some variance in blood pressure, but not any huge differences one way or another. Previous studies in other labs have reported both increases and decreases in blood pressure from 5HT administration to animals. Still, increases of 29.9% and 41.9% for the systolic and diastolic blood pressures over two weeks seems fairly large. Again, it would probably help to perform the blood pressure readings at a set time after the injections to ensure consistency. Overall, it would be expected that these results will level out with the completion of more injection trials.



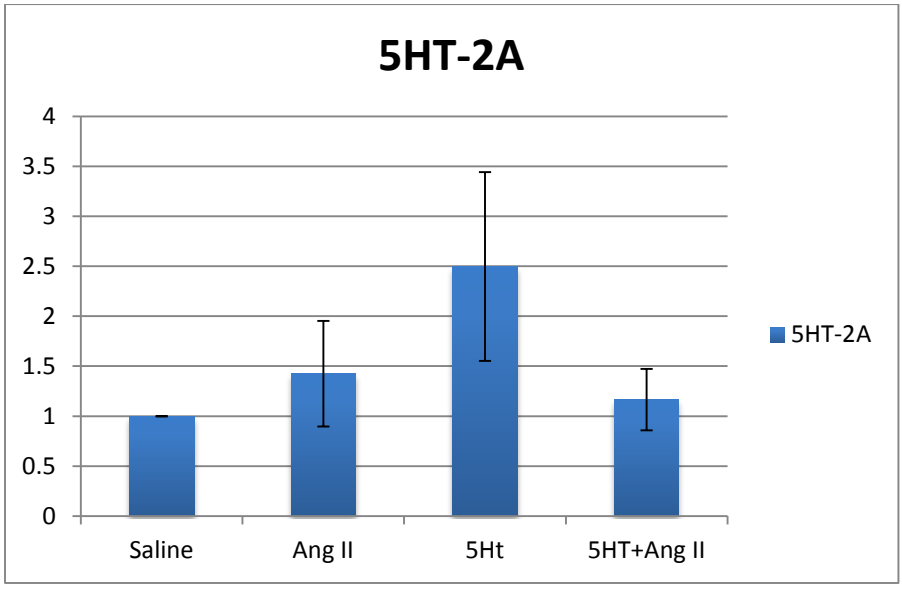
Angiotensin II+ 5HT	Systolic % Change	Systolic P-Value	Diastolic % Change	Diastolic P-Value
Baseline→Day 7	-14.4%	0.007*	-20.5%	<0.001*
Day 7→ Day 14	+32.2%	<0.001*	+53.5%	<0.001*
Total	+17.8%	0.008*	+32.9%	<0.004*

Figure 14: Injection trials, angiotensin II + 5HT group blood pressure data. n= 2 mice

The same trends that were observed in the angiotensin II group are observed in the group of the injection trial. Although the trend is not the expected one, it is good to see that the angiotensin II effect seems to take precedence over the effect of the 5HT in regards to blood pressure. The good news is that this group did exhibit an overall increase in blood pressure that was significant. This data should work well for the testing of the hypothesis. The only desired change is an earlier hypertensive state within the two week trial. For this reason, analysis of the angiotensin group as outlined in the discussion would probably benefit this group as well.

Western Data

1) **$5HT_{2A}$**



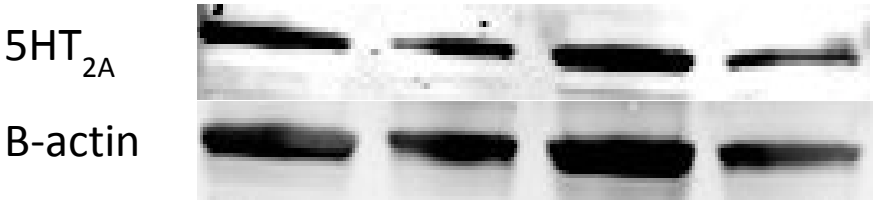


Figure 15: $5HT_{2A}$ western blot data. n= 4 western blots,

There are no statistically significant differences in the $5HT_{2A}$ receptor expression between the four experimental groups (P=0.282). Still, a trend is visible in the data. As expected, the 5HT group exhibits the highest level of $5HT_{2A}$ receptors. It is noteworthy that the angiotensin II + 5HT group does not reveal this same increase in expression of the receptor. This could be related to some interplay between the angiotensin II and 5HT. It would be interesting to run longer trials and see if the angiotensin II + 5HT group didn't reveal higher expression over time.

2) $5HT_{2B}$

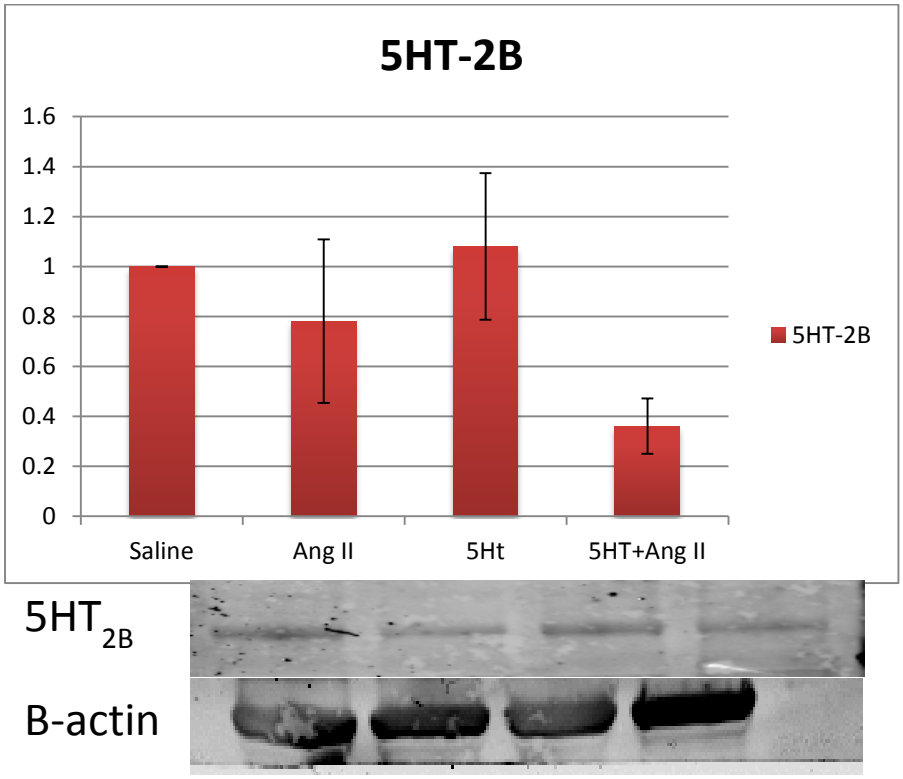


Figure 16: $5HT_{2B}$ western blot data. n= 2 western blots

There are no statistically significant differences in the $5HT_{2B}$ receptor expression between the four experimental groups (P=0.476). Again, as expected, the 5HT treated group shows, on average, the highest expression of the receptor. Additionally, the angiotensin II + 5HT group showed much lower expression of the receptor than the 5HT group similar to the results of the $5HT_{2A}$ receptor. Unfortunately, the $5HT_{2B}$ receptor did not always appear in western blotting. For this reason, more data is certainly needed to outline any trends.

3) FGF-1

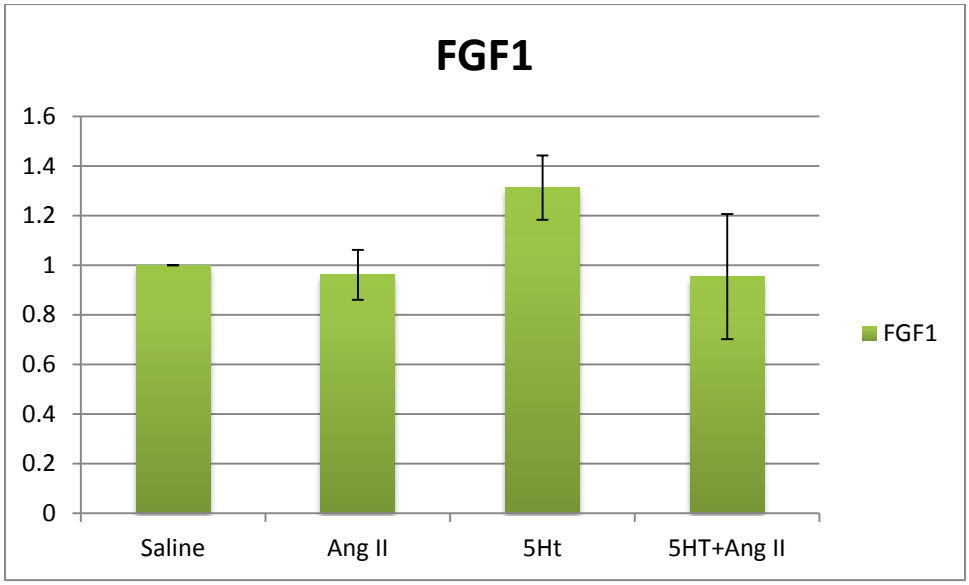


Figure 17: FGF-1 western blot data. n= 4 western blots

There are no statistically significant differences in the FGF-1 expression between the four experimental groups (P=0.318). The saline, angiotensin II and angiotensin II + 5HT groups express roughly the same amount of FGF-1. The 5HT group expressed a higher amount of FGF-1 than the control group by 31%. The initial hypothesis was that, as the angiotensin II + 5HT group began to act more like a diseased valve, then the amount of normal remodeling by FGF-1, and thus the expression of FGF-1 would decrease. Again, this would be interesting to watch over a longer trial, but it is important to be able to catch this in the beginning in order to suggest any treatment. Furthermore, more data needs to be collected to observe any statistically significant trends.

4) FGF-2

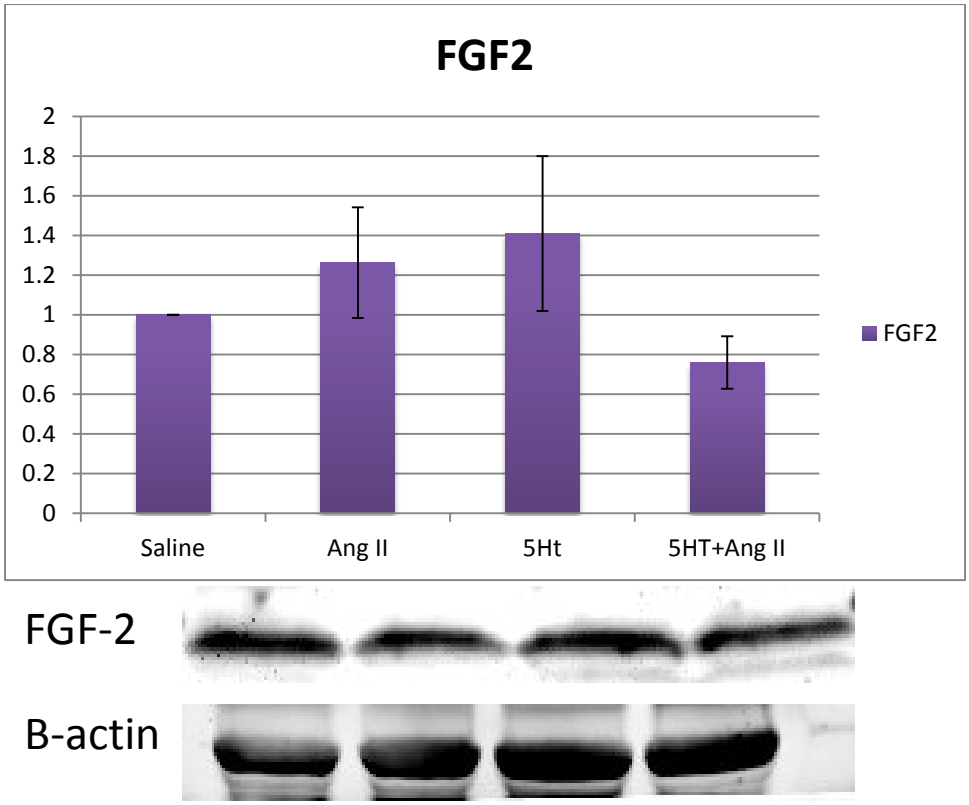


Figure 18: FGF-2 western blot data. n=4 western blots

There are no statistically significant differences in the FGF-2 expression between the four experimental groups (P=0.172). Similar to the other proteins of interest, there needs to be more data collected in order to observe trends or statistically significant data. Much like the expectation for FGF-1 expression, the expression of FGF-2 would be expected to decrease as the valve began to exhibit pathological conditions. The data above does show the lowest amount of FGF-2 expression in the angiotensin II + 5HT group, which does line up with the expected results. Still, more data is needed to ensure that the difference is not from random variation.

5) FGFR1

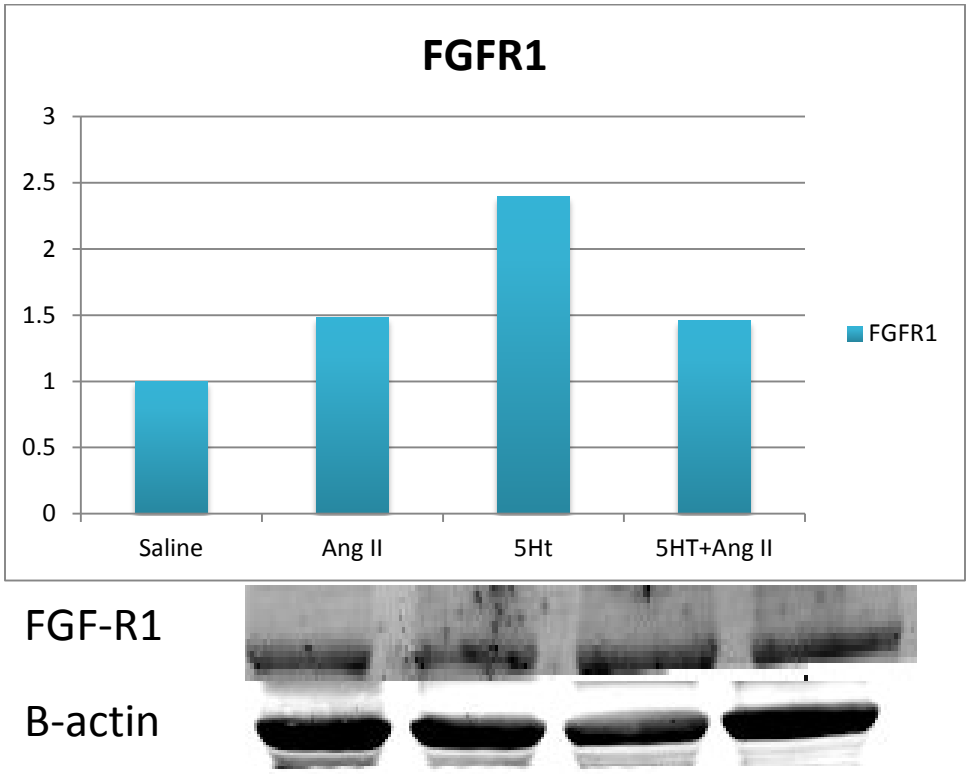


Figure 19: FGF-R1western blot data. n= 1 western blot

There can be no statistical comparison of the FGF-R1 expression as it only showed up in the western blot from one trial. Even when it did show up, it is very dim as can be seen from figure (19) above. Future experimentation with this project will hopefully reveal more data. As the data comes, the same trend is expected as that of FGF-1 and FGF-2.

4: Conclusions

A mouse model was successfully established to investigate the effects on the aortic valve in subjects with elevated 5HT levels along with cardiovascular hypertension. Based on the results of this study, a daily direct injection model is suggested going forward with this project. This method, rather than osmotic pumps, reduces complications and allows for dosage adjustment if needed during a trial. Additionally, the pumps seemed to make the mice uncomfortable when placed in the rodent holders for blood pressure readings. The injection model removes this stress and improves the accuracy of the blood pressure measurements. Drug and blood pressure kinetics should be analyzed to further improve and confirm the viability of this injection model. Long term studies should also be considered to see if the effects become more pronounced over a longer time period.

The western blot data that is gathered from this model shows some trends, but more heart samples will be needed for western blotting to validate these trends are statistically significant. Additionally, the histology analysis of the heart tissues will either further confirm or reject these observed trends.

Overall, the setup of the animal model was a success. Future studies using this model could successfully reveal the mechanism by which elevated 5HT levels can lead to a diseased aortic valve when under high mechanical stress. Knowledge of this mechanism could be directly applied or used to improve in vitro experimentation used to find a treatment for this type of valvular pathology.

Acknowledgements

First, I would like to thank my faculty advisor, Dr. Kartik Balachandran, for giving me the opportunity to participate in undergraduate research in his lab. I appreciate very much all of the time he has used for personal instruction and direction to help me accomplish this research. Beginning to get involved in the lab, near when Dr. Balachandran arrived at the University of Arkansas provided me with a tremendous learning opportunity and a terrific chance to see how research works even from the beginning at a new school. I can certainly say that I have gained a lot of knowledge and many skills that would not have been possible to acquire without the help of Dr. Balachandran. Thank you Dr. Balachandran for all of the help and support you provided throughout the duration of my research.

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Additionally, I would like to thank Dr. David Zaharoff for showing us how to implant the osmotic pumps into the mice and allowing us to use time and space in the lab and room where the mice were kept. I also thank the graduate students in his lab, particularly Sean Smith, who showed me multiple aspects of how to work with the mice and supplied me with mice to practice the dissection technique on. Thank you all for the direction.

Finally, I must thank my family and friends for supporting me throughout my collegiate journey. I could not have experienced the success at college without the continual supply of motivation provided. I truly could not have done this without all of you.

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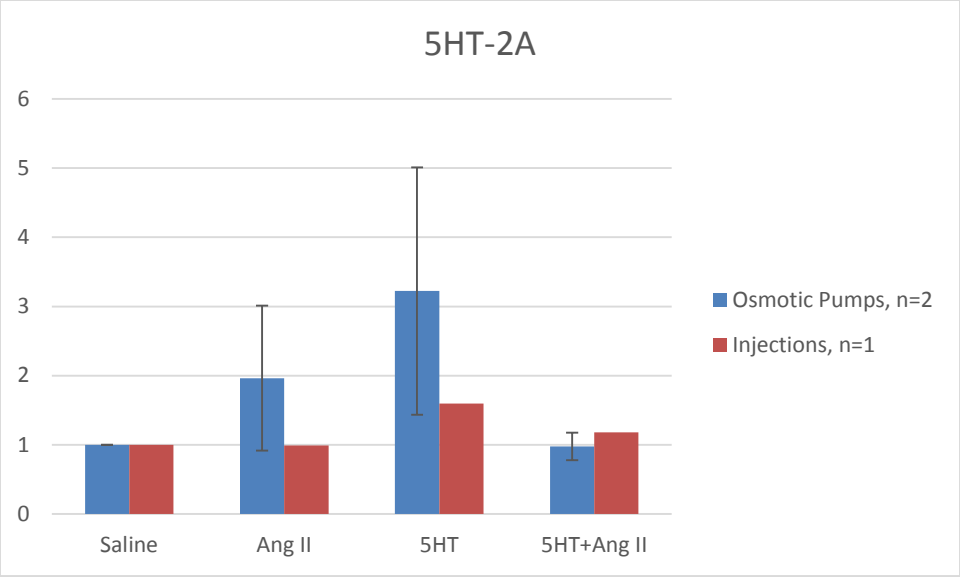
Appendix

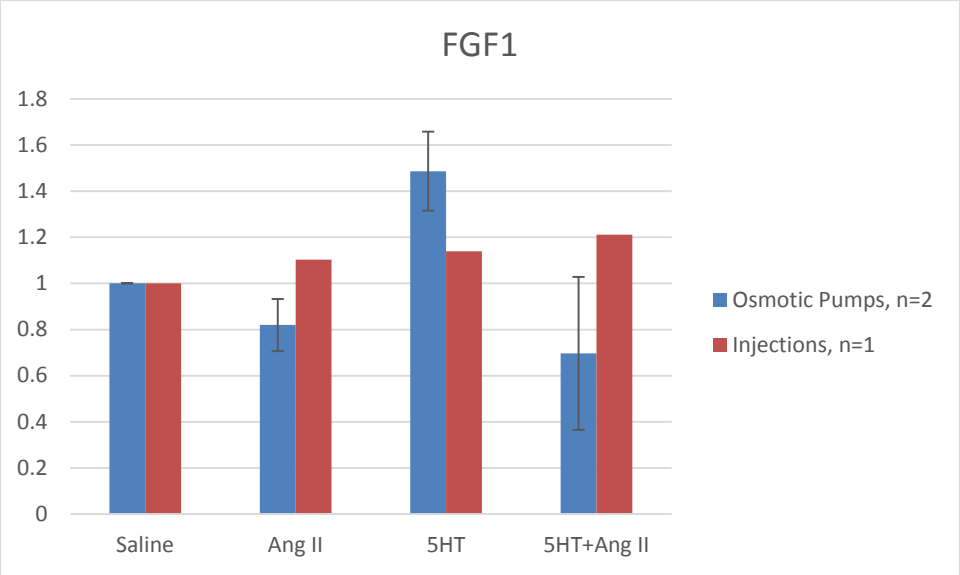
Comparing Western Blot Data Between Pump and Injection Trials

1) $5HT_{2A}$



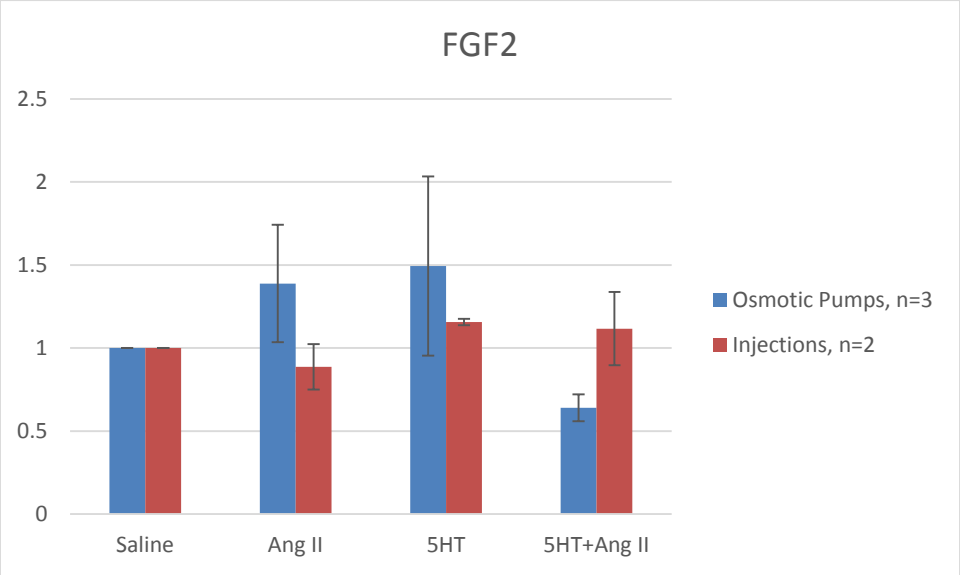
The trends seen in the $5HT_{2A}$ receptor expression are similar between osmotic pumps and injections, but the differences seem to be magnified with the osmotic pump model. Overall, the highest expression of the receptor is seen in the 5HT group. The lowest in the osmotic pump trial is seen in the 5HT + Ang. II group and in the Ang. II group in the injection trial. As mentioned in the western blot analysis before, it is difficult to know why the expression drops so markedly between the 5HT group and the 5HT + Ang. II group. This trend should be confirmed with more data and should be investigated using genetically hypertensive mice. The fact that there is only one western blot using the injection method suggests that more data must be gathered before this comparison is finalized. However, it is suggested that the comparison between the two drug delivery methods be continued as data continues to be produced by the model.

2) FGF-1



The trends in FGF1 expression do not match up between the injection and osmotic pump trials. In the osmotic pump trials, the relative order of expression in the different treatment groups is as follows: 5HT + Ang. II < Ang. II < Saline < 5HT. On the other hand, the relative order in the injection trials is saline < Ang. II < 5HT< 5HT + Ang. II. These relationships really do not line up at all. The expected results would fall more toward the osmotic pump averages as a valve turning toward the diseased state would be hypothesized to express a lower amount of FGF1. In this model, the group that should be changing to a diseased state would be the 5HT + Ang. II group. Certainly, as the sample sizes are only between 1 and 2, more data needs to be acquired before a good comparison can be made between the two drug delivery methods with regard to FGF-1 expression.

3) FGF-2



Similar to the trends seen in FGF-1, the FGF-2 trends do not match up between the osmotic pump trial western blots and the injection trial western blots. The hypothesis would be to see the lowest level of expression in the 5HT + Ang. II group as the valve would likely not be restructuring normally if it were progressing to a diseased state. Still, in a two week trial, it is difficult to determine exactly how and to what degree the FGF-2 expression should change. For this reason, a major suggestion would be to investigate the variance of the FGF-2 expression over a longer time frame. This would ensure that the valve had enough time to begin exhibiting the behavior of a diseased valve. Much like the FGF-1 expression, the osmotic pumps seem to show an expression pattern more similar to the expected pattern than the injection trials. Still, more data is required for a strong comparison to be made between the two different drug delivery models.

*The other proteins analyzed in the western blot data that were not compared here were either not seen in the injection or osmotic pump trials. Therefore, there was no way to compare the two drug delivery methods.

Osmotic Pump Loading Protocol

1. Prepare desired concentrations of different drugs
    a. For 5HT and Angiotensin II, the desired concentrations can be calculated on excel. Access the sheet by: Research Projects→Mouse Studies→Drug Concentration
    b. Input parameters at the top of the sheet and the amount of PBS and drug needed to fill the pump will be calculated.
    c. Make sure to prepare extra solution since some will inevitably be lost.
2. Weigh the empty pump and flow moderator.
3. Using a syringe and the provided filling tube, draw up the desired amount of drug and remove any air bubbles.
4. Holding the pump with the opening facing up, insert the filling tube through the opening at the top of the pump until it can go no further.
5. Slowly empty the syringe, maintaining the upright position of the pump. When the solution appears at the outlet, stop filling and carefully remove the tube.
6. Wipe off the excess solution and insert the flow moderator until the cap is flush with the top of the pump.
7. Weigh the filled pump with the flow moderator in place.
    a. For most solutions, the weight change in milligrams is approximately the same as the volume of solution you have added in microliters.
8. To prime the pumps, place them in saline preferably for 24-48 hours at 37° C. Make sure to label the containers with PBS to ensure that you remember which pump is which.

Blood Pressure Measurement and Injection Protocols

1. Pull out the two rolling carts and place side by side with the coda control box facing the big table.
2. Place the heating platform on the large cart
3. Plug in the power strip and then plug the adapters into the coda control box and the heating platform. Turn the heating platform to level 3 and 1.5 for the time. Cover the heating platform with the warming cover.
4. At this point, I would advise putting the first two mice in the holders and putting them on the heating platform, under the cover to let them warm up.
5. Attach the tail cuffs to the control box. One of the bags containing the cuffs is marked with a "1." This is the cuff that goes with channel one. The other goes with channel two.
6. Slide the tail cuffs onto the mice and make sure that the O-ring is closest to the body of the mouse. Also make sure you note the channel number that you are hooking the mice up to. Generally, I hook the lower number mouse up to channel one, and the higher number to channel two. Again, leave the mice under the cover.
7. Now place the next two subject mice in the holders and place them under the cover so that they can begin warming.
8. Make sure and plug the USB cord into the computer and open the CODA software.
9. Press "new session wizard"(looks like a blank page in upper left corner), pick the appropriate trial(#4 in this case), select the mice you have (guide in red notebook), leave the settings as they are, and then you should see the "begin" option.
10. For the first two mice, I generally allow another 5 minutes for relaxation and warming.
11. Press begin.
12. When the experiment ends, the data will pop up and you should click "save to excel." Save it somewhere that you can find it again.
13. Repeat for all the mice. You shouldn't have to wait on the next groups to warm since they have been on the platform for a while by the time you need them. Still, I generally hook them up to the tail cuff, then take out the previous two mice from the holders and place the next two mice in the holders before beginning the next experiment.
14. After you have measured the pressures of all the mice, clean everything thoroughly with 70% alcohol and paper towels.
15. Disconnect the tail cuffs and place them in the appropriate bags.
16. Unplug everything and put it up on the carts like you found it.

Injections:

We have stock of 10^{-3} M angiotensin and 0.1 M serotonin. We also have saline. For each of these experimental groups you simply inject 100uL to each specimen of the mentioned drugs.

For the mixture, add 10uL of 10^{-2}M angiotensin per 100uL of 0.1M serotonin. This gives the amount of each drug necessary. In general, make more than you need as exactly 220uL will not be enough with the

syringes. (I generally try 250uL of serotonin and 25uL of angiotensin. If you are careful with the injections, this is enough.)