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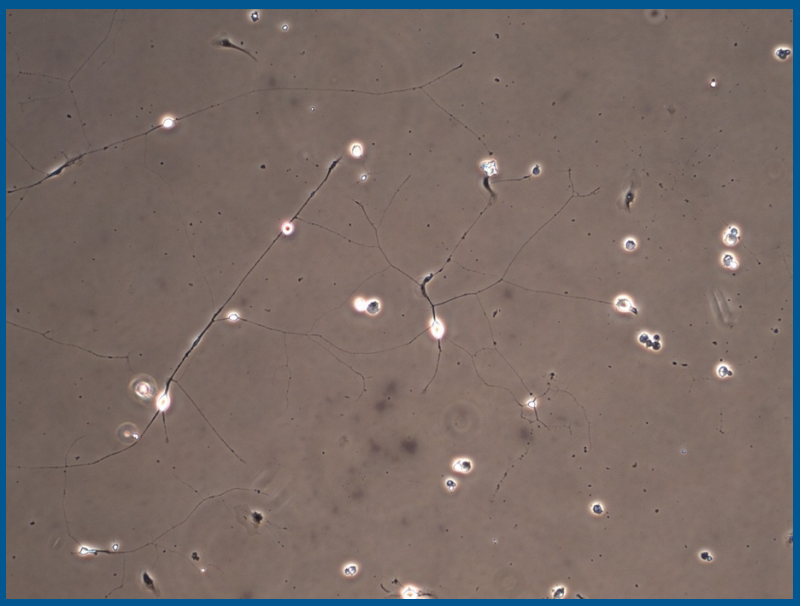

Evidence of the effect of cortisol solution on axonal growth in embryonic *in vitro Gallus gallus* sympathetic neurons

Trevor L. Ragas

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Introduction:

The biological process of stress inhibits several cellular processes in neurons (Kasai & Yamashita, 1988), such as inducing dendritic atrophy in hippocampal CA3 pyramidal neurons (Watanabe, Gould, & Mcewen, 1992). To date, research on chronic or prolonged stress on organisms provides evidence that this form of stress can lead to issues with health and other negative effects (Schulz, Kirschbaum, Prüßner, & Hellhammer, 1998; Hartig, Zhu, King, & Coffman, 2016). The process of stress itself can be a model in *in vitro* experiments by the addition of cortisol into cell cultures, as done in previous research on myelination and the inhibition of microglial cells (Bohn & Friedrich, 1982; Drew & Chavis, 2000). There are different levels of stress and cortisol in organisms however, for example, a newborn human baby on average might contain 1-24 mcg/dL of cortisol while an adult might contain anywhere from 0 to 25 mcg/dL depending upon the time of day (Consult, n.d.). This fluctuation occurs due to a variety of factors such as levels of anxiety, disease, or simply genetics; it's natural for a rise and fall to occur in all organisms. Nonetheless, it is important in this type of research to recognize this and use an appropriate amount of cortisol similar to amounts found in similar organisms for research.

Based on previous research, one quantifiable approach to investigate cortisol as a model for stress is by measuring the potential effects of the reagent on axons and their growth. Axonal growth can be measured by observing branching in neuronal cells (Acebes & Ferrús, 2000). Axon branching provides a neuron with the ability to establish synaptic connections with multiple targets, making the process critical for assembly of highly interconnected networks (Kalil et al., 2000). For this experiment, however, the method of branching was not particularly relevant, only that branching occurred.

The primary objective of this preliminary study was to examine the relationship between cortisol and its effects on neuronal cells. The hypothesis for this proposed preliminary study is that if neurons harvested from a *Gallus gallus* embryo are exposed to cortisol for a prolonged amount of time, then a decrease in overall axon growth will be observed. The main significance of this study is that the molecule cortisol can act as a model for stress in an experiment such as this, which will help determine important aspects about the neuronal cells being grown. For example, conducting this experiment might suggest if the peripheral cells of *G. gallus* contain glucocorticoid receptors. The importance of these receptors has been studied in previous research

which provided evidence that these receptors are directly affected by glucocorticoids, such as cortisol, and negatively affect cells in a variety of ways (Uno et al., 1994; Dai, Buijs, & Swaab, 2004). If this experiment provides preliminary evidence suggesting that chick embryo neuronal cells also contain these receptors, then there is the potential that these cells will correlate with the previous research in which atrophy and decreases in axonal and dendritic growth, as well as a decrease in myelination, were observed. Further investigation into this field could also benefit overall research into the negative effects of stress on developing neurons and eventually potential ways to lessen cortisol's effects on organisms.

Materials and Methods:

Primary Cultures of Chick Sympathetic Neurons

In this study, dorsal root ganglia were harvested from the spinal cords of embryotic *G. gallus* specimens and incubated on coverslips treated with laminin and poly-lysine, following the procedure modified by Dr. Robert L. Morris (Morris, 2019). Cell cultures were plated on 22x22mm coverslips cleaned with 100% ethanol. After plating of sympathetic neurons, the cell cultures were incubated for 24 hours at 37ºC. Control and experimental doses were administered after incubation. Professor Morris provided research teams with new cells on coverslips in petri dishes on 10/16/19, 10/23/19, and 10/30/19. For this experiment, the coverslips in the petri dishes contained plated single cell neurons of high density.

Petri Dish Labeling

For this study, a labeling system was developed in order to more accurately locate the same areas on the coverslips in the petri dishes when imaging with the microscope. Before the application of either cortisol solution (Experimental Condition) or DMSO solution (Control Condition), the caps of the petri dishes were labeled with a cross using a ruler and a sharpie pen, this would split up into four even quadrants which were then labeled as either A, B, C, or D. The sides of the cap and dish were also labeled with quadrant letters and lines connected to the lines on the cap so that when the dishes were under the microscope accurate quadrant identification occurred. The caps and dishes were label with the initials of the collaborators and were labeled depending upon the condition they would undergo (ex. Ex for Experimental or Con for Control), as shown in Figure 1.

Figure 1: Labeling Strategy for Petri Dishes. The figure above is an example of how the petri dishes were labeled in this experiment for a more accurate cell imaging procedure. Notice the straight and dotted lines used to create even quadrants on the cap of the dish and that the edges of the dish were labeled with letters like A, B, C, and D for more effective placement location. Images were generated in collaboration with Jocelyn Mora.

Creating Solutions

Following research conducted on the ratio of the average amount of cortisol levels due to chronic stress in the adult human body of 13nmol/L and research on cortisol treatment in zebrafish (Schulz, Kirschbaum, Prüßner, & Hellhammer, 1998; Hartig, Zhu, King, & Coffman, 2016), it was determined by the research team that the specific cortisol reagent to be used would be Hydrocortisone 21-hemisuccinate sodium salt which came in 100mg bottles (Sigma Aldrich catalog number H4881-100MG). The solvent added to the hydrocortisone was 2 mL of DMSO, stirred, to create a stock solution of 0.103M or 103mM in 50mg/mL. After initial trial runs with the stock to create a working solution, it was determined that a 1:4 dilution needed to occur. Once diluted, the final working solution of the cortisol solution that would be used in experimental treatments was 4uL of cortisol added to 0.5mL growth media solution with a final concentration of 2 uM. The solution created for the control would be 4uL of DMSO added to 0.5mL growth media solution. Subsequent dilutions into the culture medium were made using the culture medium F+ medium. F+ medium was mixed according to the recipe for F+ medium used in Morris (2019). Reagents were shared with collaborators Michael Parrella, Sara McLeman, and Jocelyn Mora

Solution Application and Incubation

Once the solutions were made, Professor Morris added 1.5ml of growth media to petri dishes for research teams. For this study, trial 1 and 2 contained only 2 petri dishes in each trial with trial 1 containing 2 experimental dishes and trial 2 containing 1 experimental dish and 1 control dish. Trial 3 contained 4 dishes in total with 2 dishes as controls and 2 dishes as experimental. After the dishes were received, labeled, and imaged (as described previously and in the following section), a pipette was used to transfer the 0.5mL of growth media containing 4uL of cortisol solution into the experimental dish(es) and another pipette was used to transfer the 0.5mL of growth media containing 4uL of DMSO into the control dish(es). The petri dishes were then transferred into the incubator where they would remain for 48 hours before being imaged again on Day 2 of the trial and again on Day 4 of the trial.

Axon Imaging

Imaging took place before solution application in the cell cultures, then took place every 48 hours after initial treatment for 4 days. Images were collected by taking single still frame images of the neuronal cultures using a single transmitted light phase microscope at the ICUC. The microscope used was the Nikon Eclipse E200 equipped with a SPOT 5.3 imaging software on ICUC "LEO" computer Mac OS 10.13.6, aligned for proper Koehler Illumination, at a 10X magnification and Ph2 Phase setting. This process took place through the Wheaton College Imaging Center for Undergraduate Collaboration (ICUC). Image collection was completed in collaboration with Michael Parrilla, Jocelyn Mora, and Sara McLeman. These microscope and objective lens were chosen because the study was investigating the effects of cortisol solution on overall axon growth for numerous cells, which would be difficult at a higher magnification.

Data Collection and Analysis

After the images were collected, the FIJI program was used to measure axonal growth in the cell cultures. This was completed by using the "Plugins" section to select the "Analyze" subsection and in turn select the "Cell Counter" section. This program allowed for the axonal growth to be measured by counting the number of neurons and branch points in the collected images. In this experiment, "axonal growth" was defined as the number of axonal branch points or locations where axons cross or interact with one another, as this shows growth patterns in the axons. Using the "Cell Counter" program in FIJI the nuclei of the neurons were labeled with a red 1 and the branch points or axonal crossings were labeled with a blue 2 (as shown in Figures 2 and 3). The process for using the "Cell Counter" program in FIJI followed the procedure detailed by Arpan Parichha (Parichha, 2018). For this study, data was collected from three trials over the course of 3 weeks, all of which were pooled together for final data analysis as the same treatment and imaging conditions were used throughout the three trials. Specifically, control data was derived from axonal growth measured in cells exposed to a DMSO solution for 96 hours in three trials and experimental data was derived from axonal growth measured in cells exposed to a cortisol solution for 96 hours in three trials.

Results:

The peripheral neurons, harvested from *Gallus gallus* embryos, were allowed to incubate for 24 hours before the experimentation phase began. Following those initial 24 hours, one cell culture served as the control group and received a DMSO solution treatment (as shown in Figure 2) and the other served as the experimental group which received the cortisol solution treatment (as shown in Figure 3). The cell cultures were then placed back into incubation. Every 48 hours, the cells were observed under a single-phase light microscope and imaged, those images were later analyzed using the FIJI program. The "Cell Counter" application in FIJI was used, as demonstrated in Parichha (2018), to label and count the number of neurons and the number of axonal branching points observed in the image.

To calculate results, the number of axonal branching points was divided by the number of neurons counted in each image in order to generate an overall average of branching points per cell for each image. Figure 2A contained an overall average of 2 axonal branches per neuron and Figure 2B contained an overall average of 4 axonal branches per neuron. Figure 3A contained an overall average of 2.615 axonal branches per neuron and Figure 3B contained an overall average of 2 axonal branches per neuron. These averages are compared to one another over the course of 48 hours and are shown in Figure 4. Overall, the general analysis shows the average number of

axonal branching points as a means to quantify axonal growth in chick neurons over the period of 48 hours and there was a decrease in axonal branching observed in the experimental condition and an increase in axonal branching observed in the control condition.

An interesting observation found in some of the cell cultures imaged was the presence of what appears to be flattened glial cells. As they were only observed in some of the cell images however, they were largely ignored in this study as they did not provide enough evidence to have to do with the overall experimental hypothesis. This study also focused more on the initial 48 hours of neuronal cell exposure to control and experimental conditions as images captured in this time window provided a much better documentation of the effects of the experimental and control conditions (as shown in Figures 2 and 3). Other interesting observations made in this study are that the axons growing in the experimental solution appeared to have more complex axonal shapes (see Figure 3) whereas those cells in the control condition had more simplistic axonal shapes (see Figure 2). It was also observed that the cells in the control condition started off with less branch points that the experimental condition at Day 0, and by Day 2 the control condition cells only gained more branch points while the cells in the experimental condition decreased in branch points, with the control cells having almost double the branch points by Day 2 (see Figure 4).

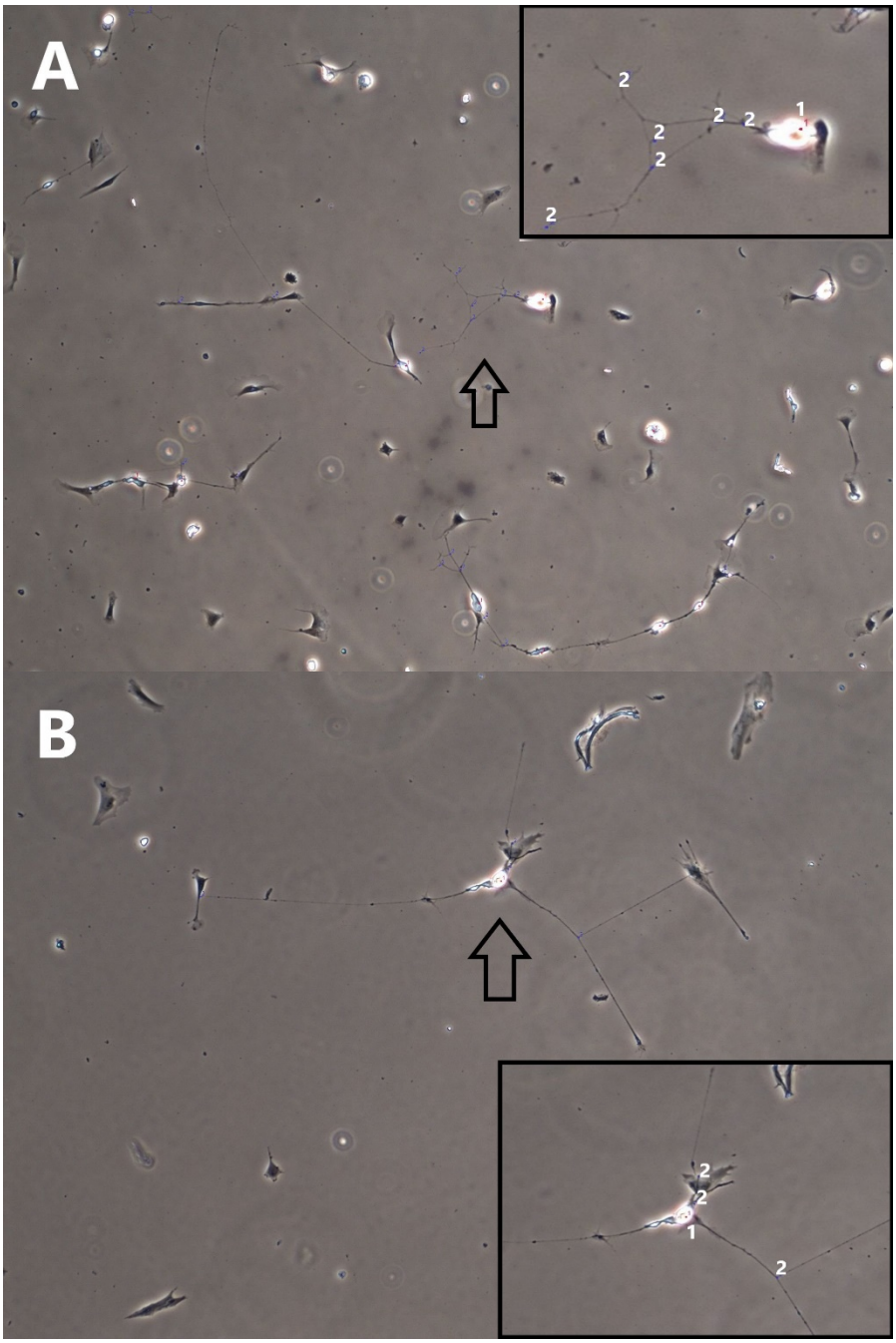

Figure 2: Axons and Neurons Representative of Control Condition. The transmitted light image in Panel A (all images in this figure were captured with an objective 10X lens), depicts a neuronal cell culture at Day 0, 24 hours after being harvested and plated on a coverslip, and before being exposed to DMSO solution. Panel B depicts the neuronal cell culture at Day 2, 48 hours after being exposed to DMSO solution. The arrows in these images serve as a reference point for the enlarged images in the corners of the original panels for greater clarity. Notice the bright and stretched physiologies of the locations marked as "1" as the nucleus of neuronal cells are often stretched due to outgrowing axons.

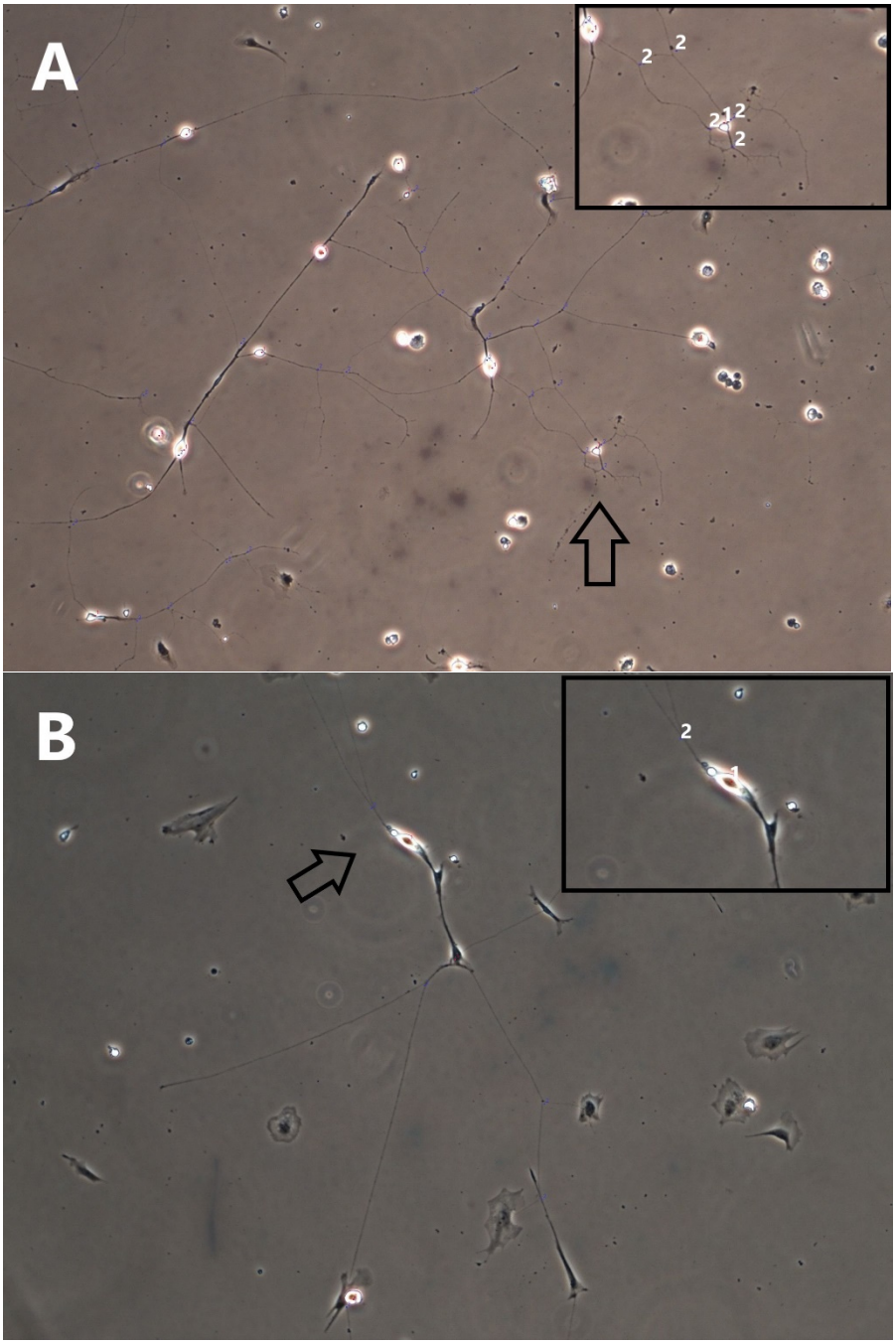

Figure 3: Axons and Neurons Representative of Experimental Condition. The transmitted light image in Panel A (all images in this figure were captured with an objective 10X lens), depicts a neuronal cell culture at Day 0, 24 hours after being harvested and plated on a coverslip, and before being exposed to cortisol solution. Panel B depicts the neuronal cell culture at Day 2, 48 hours after being exposed to cortisol solution. The arrows in these images serve as a reference point for the enlarged images in the corners of the original panels for greater clarity. Notice the more complex axonal shapes created by the cells in Figure 3 as compared to the ones in Figure 2, as well as the number of branch points in comparison to the number of neuronal nuclei.

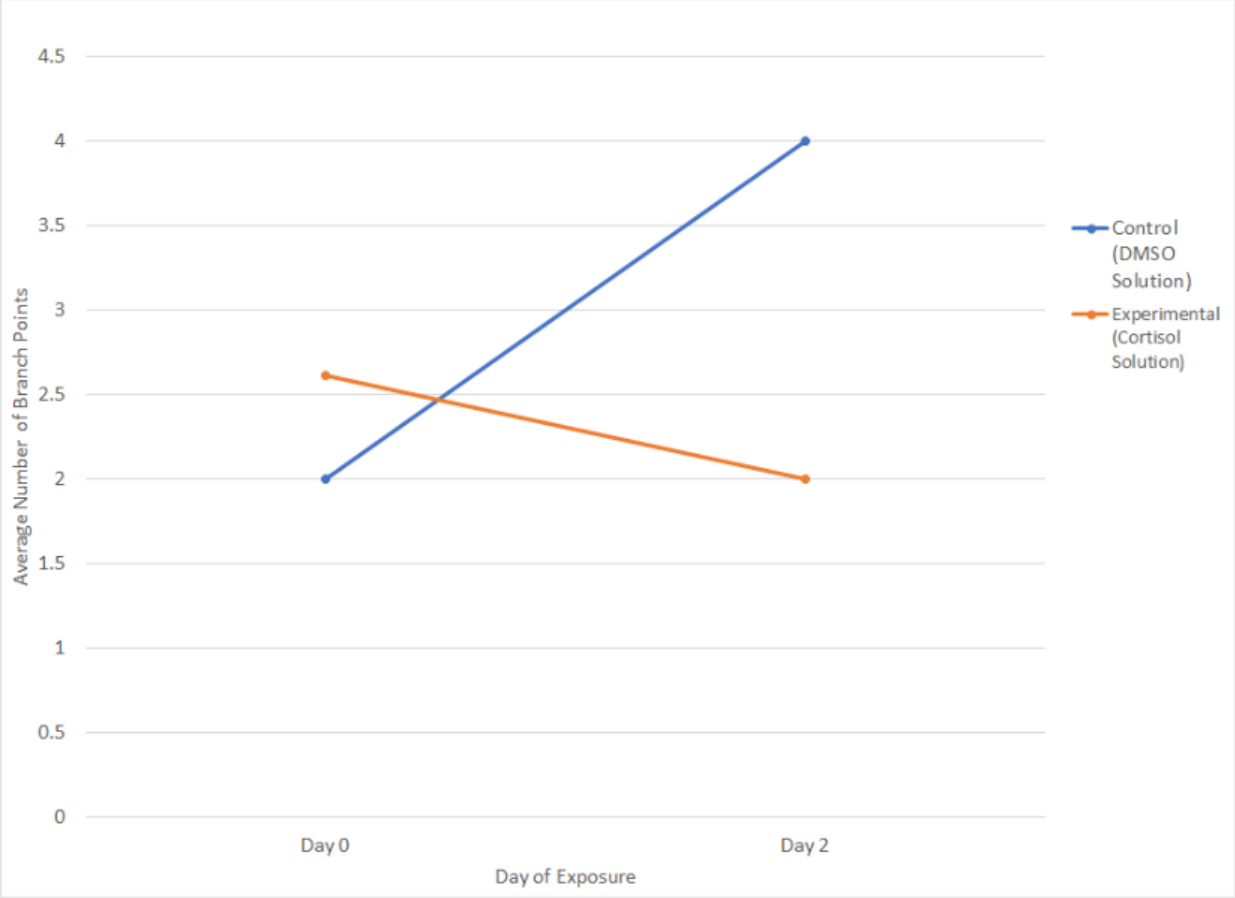

Figure 4: Average Number of Axon Branch Points in Both Control and Experimental Conditions. The total number of neurons and axon branch points were counted, then averaged in order to calculate a more uniform number of branch points by the number of neurons in each image captured above. Notice the different number of branch points at both the beginning and the end for both the experimental and control conditions. Also notice that in the control condition, the average number of branch points increased by Day 2, yet in the experimental condition the average number of branch points decreased by Day 2.

Discussion:

Based on previous research, it was hypothesized that neurons exposed to a solution of cortisol will undergo a decrease in axonal growth. The data collected and analyzed from this preliminary study supports that hypothesis. From the data collected in this preliminary trial, it can be concluded that there is evidence that neuronal cells exposed to cortisol solution experience a decrease in axonal growth, measured by number of axonal branch points per number of neurons counted. It can also be concluded that neuronal cells exposed to a control solution containing DMSO experience an increase in axonal growth, measured by number of axonal branch points per number of neurons counted. This conclusion however is based solely on this research however as there is no prior research to support or refute this finding.

If this experiment was repeated and if more research was completed, and the same results were observed, it could be concluded that peripheral neurons, in a manner similar to neurons collected from the central nervous system (CNS), specifically hippocampal CA3 pyramidal neurons, undergo dendritic and axonal atrophy and decreases in axonal growth when treated with a cortisol solution that simulates a practical model for stress (Watanabe, Gould, & Mcewen, 1992; Šťastný, Pokorný, Lisý, & Krechler, 2009). If this were true, this would also support previous research about the toxicity of glucocorticoids like hydrocortisone and the detrimental effects it can have on neuronal cells such as decreases in myelination and axonal atrophy (Bohn & Friedrich,1982; Dai, Buijs, & Swaab, 2004).

As this was a preliminary study however, it should be stated that there are a few reasons as to why these results should be considered with hesitation and further experimentation. Mainly that, for each image only one location on the coverslip was captured and analyzed for each cell culture, this means that only a small sample of the whole cell culture was analyzed and may not be a significant representation of all the neuronal cells in the culture. This potentially means that perhaps only this one area imaged experienced such increases or decreases in axonal growth.

To refine this experiment if it were to be repeated and improved, a more precise method of locating and imaging the same neuronal cells in the cultures should be developed so that the data collected can be more accurate. Specifically, that future studies should continue to pool data, but also image multiple sections in order to get a wider sampling of data, and potentially increase the concentrations for experimental and control reagents and incubation periods. As axonal growth can be defined multiple ways, one potential strategy proposed by Professor Morris would be to observe and count the number of flattened glial cells in captured images. Future studies should endeavor to observe these flattened glial cells and find evidence to support that they can be used as a measure to indicate axonal growth, and if so, potentially use this strategy to measure cortisol's effect on these glial cells and axonal growth in neurons. Regarding the current experiment however, further research into the effects of cortisol exposure on developing neuronal cells may lead to the advancement of more knowledge about the dangerous effects of stress in organisms, and how it can potentially be diminished.

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I have abided by the Wheaton College Honor Code in this work.
Trevor Ragas 12/4/19