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A preliminary study of nerve growth factor as a
regenerative factor for degenerating neurons

Adam Hinthorne

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Introduction

Alzheimer's Disease (AD) is a neurodegenerative disease that is characterized by the degeneration of neurons in the central nervous system that results in memory loss and loss of cognitive function (Kandel et al, 2013). Currently, treatments are focused on managing symptoms and maximizing cognitive function for patients (Mayo Clinic, 2017). Research for potential treatments is focused on determining what causes the disease and how changes in the neurons can be controlled. Researchers have found that in neurons degenerating due to AD, the protein Tau is considered tangled preventing transport of necessary molecules to and from the cell body via the axon (Gong and Iqbal, 2008). Levels of Brain Derived Neurotrophic Factor (BDNF) also appear to be connected to the commencement of the disease. Laske et al (2006) found that BDNF levels spike in early stages of the disease and slowly decrease as the disease progresses.

Nerve Growth Factor (NGF) is a compound commonly used in neuronal cell cultures to aid in axon growth. NGF has shown promise in the potential to be used as a treatment of AD. It has also been found that NGF has the ability to regenerate and restore function to degenerating neurons (Rafii et al, 2014). In a study conducted by Kromer (1987), NGF was used to treat a model of a central nervous system injury. NGF, when continuously administered exogenously, can reduce retrograde cell death of neurons. Kromer suggests that NGF has the potential to be used as a treatment for central nervous system injuries because of its ability to promote neuronal survival (Kromer, 1987). NGF has also been reported to cause the sprouting of axons and stimulate cell communication (Tuszynski et al, 2015). AAV2-NGF in clinical trials to treat patients because of the potential regenerative ability (Rafii et al, 2014). AAV2-NGF is a viral vector that delivers the NGF coding gene through the blood brain barrier because the NGF molecule itself is too large to pass through the blood brain barrier (Bishop et al, 2008 & Tuszynski et al, 2015).

Alzheimer's Disease research has found a relationship between the protein Tau and the folding in of itself, which results in what is referred to as tau tangles. Acetylcholine (ACh) acts as a neurotransmitter in neurons and associated with learning and memory pathways (Oddo and LaFerla, 2006). If cells are exposed to excess levels of ACh, it can result in the hyperphosphorylation of Tau, which in turn can result in the Tau disassociating from the microtubules (Gong and Iqbal, 2008). This study will use ACh to try to cause the aggregation of Tau in neuronal cell culture. If the Tau dissociates from the microtubules, the transport of necessary molecules down the axon to the growth cones is reduced and the axons will be stunted.

The hypothesis of this study is if NGF has a regenerative property, than it may regenerate degenerating neurons affected by the presence of excess ACh.

Materials and Methods

Dissection and Coverslip Preparation

Dissections of day 10 chicken embryos and the treatment of coverslips were conducted according to the procedure provided by R.L. Morris of Wheaton College based on protocol from Peter J. Hollenbeck of Purdue University (Morris, 2017). The purpose of the dissections was to isolate the sympathetic nerve chain and the dorsal root ganglia of the chick embryos. Neurons were then plated into sparse, dense, mixed, and ganglia dishes. For this experiment, only sparse dishes were used. Two dishes acted as a control and six dishes were treated at ACh at varying levels with three of the six dishes being treated with NGF.

Stock and Working Solutions

The neurons were treated with varying levels of ACh at either 5 μ M, 10 μ M, or 20 μ M. To create the different dosage levels, a stock solution was made up 363 mg of Acetylcholine Chloride in 1 ml of H₂O. These three doses were selected by my collaborator to see how the neurons react to varying levels of excess ACh. The solution equaled 363 mg/ml and had a molar concentration of 2000 μ M. Each dosage was diluted into growth medium to reach the desired concentration. To make each dosage, 10 μ l of the stock was added to 1 ml of growth medium to create the 20 μ M dose, 5 μ l to 1 ml of growth medium to create the 10 μ M, and 2.5 μ l to 1 ml of growth medium to create the 5 μ M. This study was done in collaboration with a study on the effects of ACh on neurons. In this study as well, ACh was added to the cultures to hopefully result in the hyperphosphorylation of Tau instead of adding exogenous Tau directly.

The NGF solution was determined by the suggested levels of Tuszynski (2007), who showed primates treated with the AAV2-NGF viral vector are positively affected by 5 fold increase of NGF concentration. This was used to determine how much supplemental NGF should be added to the cells. 100 μ g of NGF added to 1 ml of H₂O was mixed to create the stock solution. In 1 ml of growth medium, 2.5 μ l of the stock solution was added to get a concentration of 250 ng/ml, 5 fold greater than the 50 ng/ml concentrated solution used in original growth medium.

Experimental Procedure

Dissections of neurons were performed in accordance to protocol provided by R.L. Morris and neurons were allowed to grow in growth medium for one day. The growth medium used was a combination of Leibowitz-15, fetal bovine serum, NGF and Glutamine. After one day, the first doses of ACh were administered to cells and placed into incubator to sit for 20 hours. The doses were administered by adding the ACh dose to growth medium and conducting a full exchange of growth medium. After the 20 hours, a second dose of ACh was administered to the cells in another full exchange and placed back into the incubator. Chip chambers were made by using one culture exposed to each level of concentration of ACh and one control culture. In total 4 chip chambers were made, one with 5 μ M, one with 10 μ M, and one with 20 μ M, as well as a control. Protocol for making chip chambers was provided by R.L. Morris (2015b). The remaining three experimental cultures were fully exchanged with growth medium that included supplemental NGF and placed into the incubator to sit for 24 hours before imaging. The remaining control culture was fully exchanged with new growth medium to allow cells to be exposed to similar conditions to the experimental except for ACh and supplemental NGF. After twenty four hours

in the incubator, chip chambers were made with the cultures treated with NGF and the remaining control condition to be imaged.

Imaging and Quantification

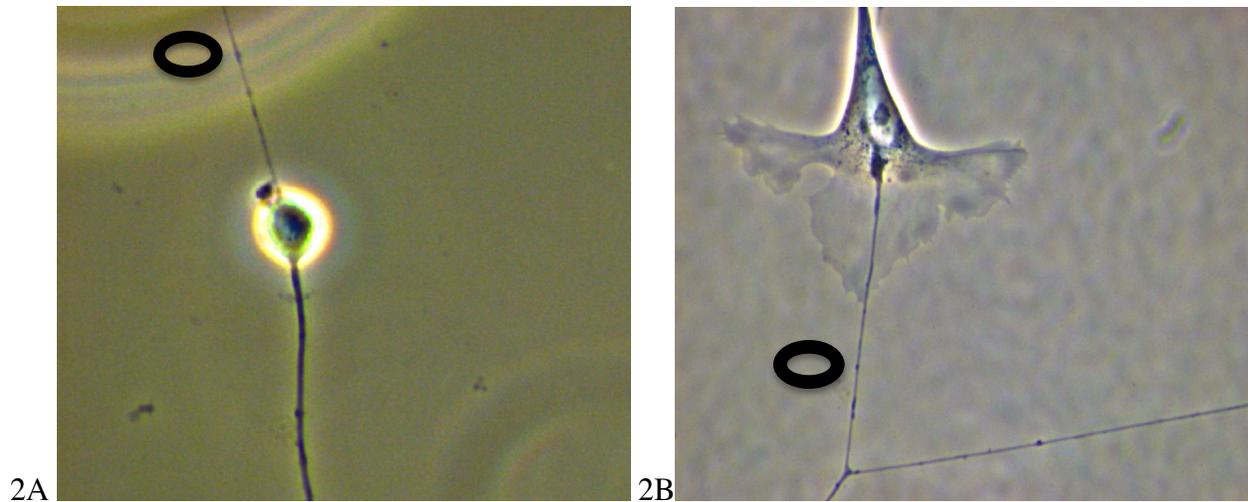
Images were taken in the ICUC at Wheaton College with a Sony DFW-X700 Color Digital Camera with 1x magnification on a Nikon Eclipse E200 microscope using 40x magnification on phase 2 on a Macintosh computer- "Sagitarius." Images were quantified using the imaging software Fiji version 1.0 using the Line tool. The images were converted into a .jpeg file and the width was measured, to determine uniform caliber of axons, and the length of the axons were measured using the Line tool. The number of varicosities was counted on each measured axon. A varicosity in this study is defined as a swelled region on the measured axon or a large dark tapered section on the axon and is distinctly one vesicle. Exclusions may occur if it is unable to be determined the number of varicosities or if the varicosity is not clearly defined in the image, i.e. the contrast of the varicosity against the axon is low and it is not possible to be able to define the boundaries of the varicosity. An axon is defined as a single axon of uniform caliber. To determine uniform caliber in this study measurements were made of the width of the axon to check tapering. Some tapering of axon width, that did not fit the definition of a varicosity, was accepted but all accepted values stayed in the range around .8 μm and 1.2 μm .

Results

Cells exposed to ACh only as well as excess NGF were made into chip chambers and brought into the ICUC for imaging. The control image, Figure 1, shows axon development and varicosities on the axons. Compared to the experimental data taken from the images of both with or without NGF shows more varicosities on average than the control. Between the different concentration groups with or without NGF there is not much variation in the data (Figures 4A, 4B, 5A, and 5B). According to figures 4B and 5B the average axon length for 5 μM is greater in cells treated with NGF after ACh than in cells just treated with ACh. The cells that were not treated with NGF look to have more varicosities on the axons, but the way varicosities is defined in this study resulted in the exclusion of many of them. Looking at Figure 3, the axon of the ACh treated only cell seems to have more cellular aggregation around the cell body than when treated with NGF. A one-way ANOVA was used to analyze the data of the single trial and the results only showed statistical significance between concentration groups with a P value of 0.033. However, this study tested the hypothesis that dose of NGF to which a neuron is exposed will affect the number of varicosities present in its axons. Because of the results of the ANOVA and the interests of the study, there is no statistical significance. This means that NGF may not have a substantial affect on degenerated neurons.

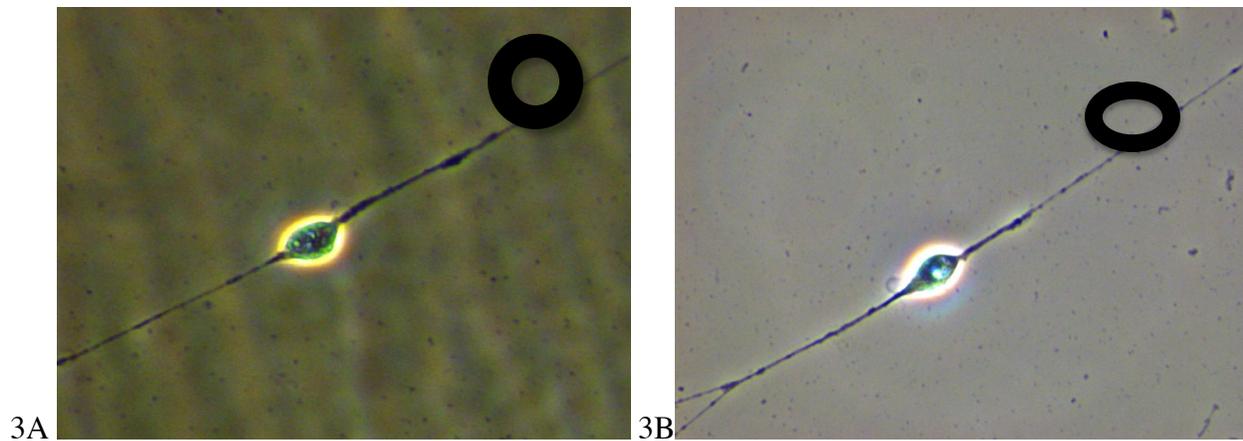


Figure 1.
Image of control culture. The control was not treated with ACh or NGF but was incubated for the same length of time as the NGF cells. In the image above examples of what were considered a single axon in this study are marked with black arrow and a glial cell in the top left corner of the image.



Figures 2A and 2B

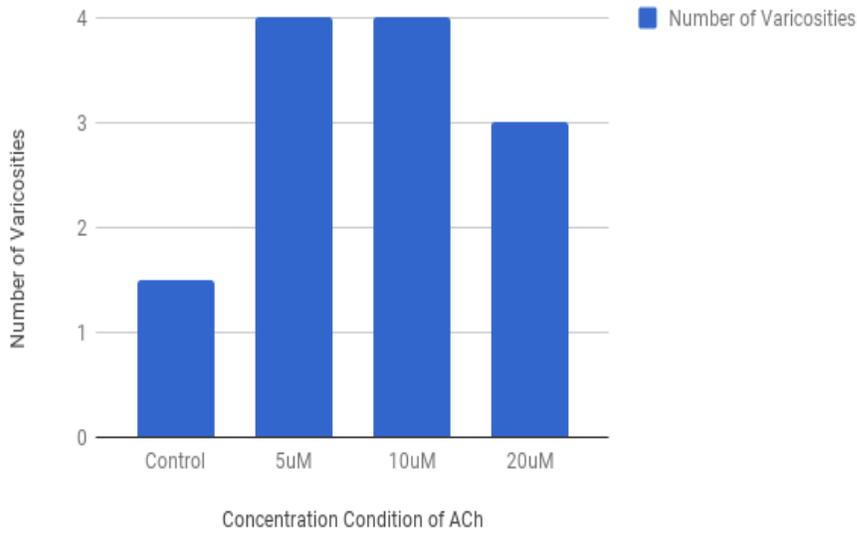
Images taken of 5 μ M concentration conditions. Figure 2A on the left is a neuron in the 5 μ M solution of ACh taken by Hannah Southard. Figure 2B on the right shows axons and a glial cell that was treated in 5 μ M of ACh then treated with NGF. An example of a varicosity in each image is indicated with a black circle.



Figures 3A and 3B

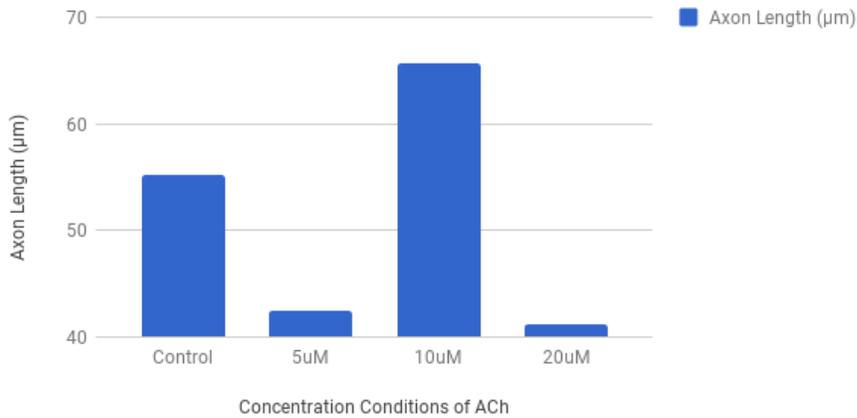
Images taken from 10 μ M concentration conditions. Figure 3A on the left shows a neuron in the 10 μ M ACh solution taken by Hannah Southard. Figure 3B on the right shows a neuron that was treated with 10 μ M of ACh, then treated with NGF. Examples of varicosities are indicated with a black line.

Average Number of Varicosities in Cells Treated Only with ACh



4A

Average Axon Length in μ meters in Cells Treated Only with ACh

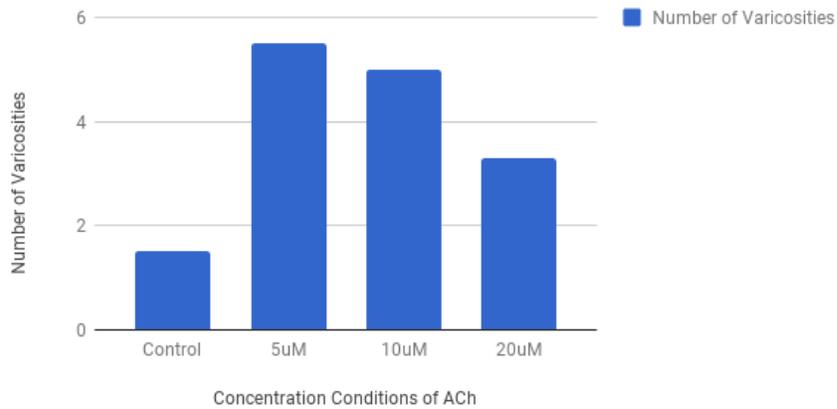


4B

Figures 4A and 4B.

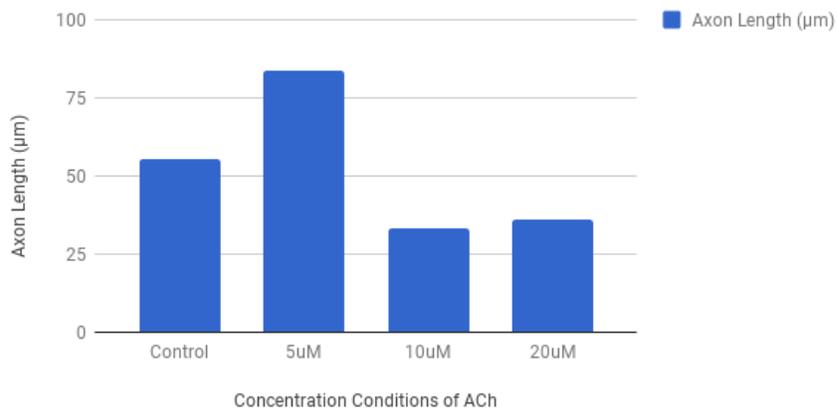
Average number of varicosities (4A) and average axon length (4B) are shown above for the cells treated only with ACh. The control was not treated with ACh. Average axon length is measured in μ m.

Average Number of Varicosities In Cells Treated With Excess NGF



5A

Average Axon Length In μ meters In Cells Treated With Excess NGF



5B

Figures 5A and 5B

Average number of varicosities (5A) and average axon length (5B) are shown above for the cells treated with ACh and NGF. The control was not treated with NGF or ACh. Average axon length is measured in μ m.

Discussion

This study shows the potential benefits to using NGF as a treatment for AD. While the data are not statistically significant, observations of the images show a trend in support of the hypothesis as seen in Figures 2 and 3. From observations of the images alone, there is a visual trend that the cells seem to be morphologically different between treatment with NGF or not. Cells treated with ACh only in Figures 2A and 3A appear to have more varicosities around the cell bodies than the cells in Figures 2B and 3B. However, many of these varicosities could not be counted due to the working definition of varicosity in this study.

This study is still a preliminary study and because of this has a low sample size. While statistical significance would provide evidence that NGF can help regenerate neurons, being a preliminary study, more investigation would be required. Data collection was also different between collaborators for the cells exposed to NGF and ACh and the cells exposed to just ACh. Both researchers took images differently. Originally timeslapse photos were taken for this study whereas single images of neurons were taken for the collaborative study. As a result, I was not able to compare timeslapse images as I had originally intended. I instead was limited to the images from this study that could be used. I was not able to compare timeslapse series images to singular still images and thus looked at varicosities on axons instead. It is also possible that the neurons in the acidic environment were shocked and the presence of NGF did not make a difference as to whether or not normal cell function was restored to the cell. This would require further research to be able to determine what levels of ACh would be too much and result in killing the cells.

Further research is required to be able to determine the full effect of NGF on neurons in cell culture. For future studies, it is advised to add a pH buffer to the ACh solution to prevent the solution from becoming too acidic. More trials are recommended to determine if the results found in this study are consistent but also to be able to better determine statistical significance. As mentioned above, more trials to better determine what levels of ACh would be too high. Another recommendation for future studies is to wash the cells with growth medium when exchanging the ACh growth medium with growth medium plus extra NGF. This would better ensure that ACh will no longer be present in the cell culture when the cells are exposed to NGF.

While many treatments exist to treat symptoms of the disease, nothing exists to treat the disease itself. The idea that NGF has a regenerative property makes it a good candidate for research into its full treatment potential (Rafii et al, 2014). The study conducted here can help create a model for treating AD at the cellular level. There is a focus at treating AD at the organismal level because as people, there is more significance given to making another person suffer less. Because of this nature, treatments tend to treat symptoms and not the disease. By focusing on treating the cells, there can be more focus on what a singular cell needs in order to be cured before attempting to cure the patient. This study, and studies like this in the future can open the door for researchers to better understand what NGF's full capabilities are and how treatments act on a cellular level.

Acknowledgements

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Collaborators: Hannah Southard