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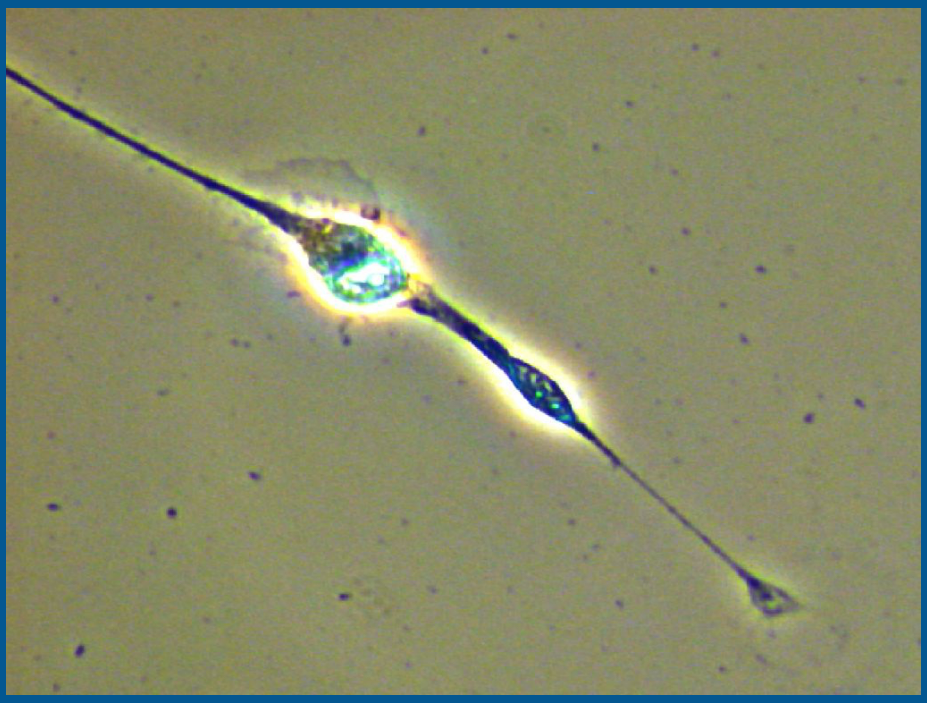

A preliminary study of the negative effect of acetylcholine on axon morphology in *Gallus gallus* embryonic sympathetic neurons as a possible indicator of tau aggregation

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Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease of our time, affecting more than 5 million individuals in the United States alone in 2017 (Alzheimer's Association, 2017a). Neurodegenerative diseases such as AD are characterized by their effect on the neurons in the brain leading to dementia, and are presently considered incurable (JPND Research, 2017). However, recent studies of AD and its pathology have led to the development of treatments and therapies that could slow the rate of disease progression, and potentially lead to prevention (Alzheimer's Association, 2017b). This possibility has pushed studies of the disease to the forefront of research, leading to several important discoveries within the past few years to try and understand the mechanisms of Alzheimer's development. From these studies, the Tau protein has emerged as a major player in the development of AD in the brain (Lippens et al., 2011). Tau is a microtubule-associated protein (MAP) that stabilizes microtubules within neurons allowing for axons to extend during cell growth while still maintaining their uniform caliber and structural integrity (Buée, Bussiérem Buée-Scherrer, Delacourte & Hof, 2000).

In AD pathology, the Tau protein aggregates to form "Tau tangles" a type of neurofibrillary tangle (NFT) (Shahani & Brandt, 2002). These structures weaken microtubules and can interfere with neuron signaling leading to learning and memory issues, hallmarks of AD (National Institute on Aging, 2017). What causes the Tau protein to form these tangles is less well known, and is the main subject of this study. We treated neurons, taken from the dorsal root ganglia of *Gallus gallus* embryos at seven days, with Acetylcholine (Ach) to attempt to trigger the aggregation of Tau. If this aggregation were to occur, it could potentially be observed through morphological changes in the axons of the neurons.

Since Ach naturally occurs within the brain as a neurotransmitter that is associated with learning and memory, it has the potential to be one of the factors associated with AD pathology (Francis, 2005). Previous studies have shown that Ach levels in the brain may affect the phosphorylation of Tau, leading to microtubule instability, but the actual morphological changes seen in the structure of the axon are less well studied (Rubio, Ávila, & Pérez, 2006). Other studies have shown that these morphological changes may be most visible close to the cell body of the axon, since during immunofluorescence imaging, the Tau protein aggregations were

particularly abundant in this area (de Calignon et al., 2017). Also, since axon growth stems initially from the centrosome in the cell body, the effects of microtubule instability should be more clearly visible closer to their origin (Joshi & Baas, 1993).

In the current study, we tested the hypothesis that the addition of Ach would negatively affect axon morphology in otherwise healthy neurons, as seen through changes in axon taper near the cell body. This change in taper may perhaps be due to the aggregation of the Tau protein, but this level of analysis is beyond the scope of the current study. While Ach is not the only player in AD pathology, and is less well researched than other factors such as β-amyloid, its potential involvement in the hyperphosphorylation of the Tau protein cannot be overlooked. If it were to be shown that Ach does play a role in Tau aggregation leading to the development of AD, then it could be potential starting point for research into therapies and treatments for those suffering from the disease. The ability to simulate the effects of AD in healthy neurons through the addition of compounds naturally found in the brain such as Ach would open new frontiers in the fight against AD as a platform for testing new therapies outside of human trials.

Materials and Methods

Materials

For this study, four poly-lysine and laminin treated coverslips with sparse neurons from seven-day *Gallus gallus* embryos were used. The coverslips were grown in four separate 35mm petri dishes in F+ growth medium. Acetylcholine chloride ordered from Sigma Aldrich as a powder in a 25g bottle.

Dissection of Neurons

Neurons were dissected from seven day *Gallus gallus* embryo dorsal root ganglia as described by Morris (2015a). Neurons were plated sparsely with minimal glia on coverslips treated with poly-lysine and laminin and placed in 35mm petri dishes containing F+ growth medium. Four coverslips were created for this experiment for each of the experimental conditions: control, 5μM, 10μM, and 20μM. These concentrations are based off the concentrations used in the 2006 study by Rubio, Álvia, and Pérez. This study used a 10μM concentration as the base of their work, which showed a change in axon behavior (Rubio, Álvia, & Pérez, 2006). The other two concentrations were decided upon through combined work with collaboration partner Adam Hinthorne as suitable experimental conditions based on the 10μM concentration.

Concentrations

To begin creating the three separate concentrations, a stock was created by mixing 363mg of the Ach powder with 1ml of sterile water in an Eppendorf tube. This created a 2000μM stock concentration. To create the 20μM concentration, 10uL of this stock was added to 1ml of F+ growth medium in an Eppendorf tube. For the 10μM concentration, 5uL of stock was added to 1ml of F+ growth medium, and the 5μM was created by adding 2.5mL of stock to 1mL of F+, in the same way. Two 1mL Eppendorf tubes were made at each concentration and stored in the refrigerator until needed.

Treatment

Neurons were dissected and left to grow for twenty-four hours in 37°C incubator. After this incubation, a full exchange was completed using sterile pipettes, replacing the F+ in the

three experimental dishes with 1ml of a modified F+ containing increasing concentrations of acetylcholine as described above. Dishes were returned to the incubator for twenty hours at 37°C, until the next morning. It was then replaced with fresh 1ml doses of the same concentrations of the acetylcholine to counteract any depletion of the compound that may have occurred overnight. At this point dishes were quickly examined using a light microscope to ensure that cells were alive and axons were showing signs of growth. Dishes were then returned to the incubator for four additional hours. The control dish was left in the original growth medium for the duration of these treatments.

After this time, dishes were removed from the incubator. Coverslips with neurons were removed from the growth medium and mounted on microscope slides as chip chambers using pieces of broken coverslips separate the coverslip from the slide to avoid crushing cells. A few drops of growth medium were used to mount the slips and create an environment that the neurons could live in. Coverslips were sealed to the slides using melted VALAP (a mixture of equal parts Vaseline, lanolin, and paraffin) and a small paintbrush. Slides were rinsed with sterile water to remove any remaining salts from the growth medium and dried before being taken to the imaging center. Slides which were not being immediately imaged were kept in the incubator at 37°C.

Imaging

Cells were imaged using a Nikon Eclipse E200 light microscope and a Sony DFW-X-700 (Digital Interface) camera with a 1.0x magnification camera mount. A small heater and a temperature monitor were used to keep the microscope stage at 37°C while the cells were being imaged. Photos were taken at 40x magnification using BTV, software version 6.0b1 on the iMac labeled Sagittarius running OS X Yosemite version 10.10.5 in the Imaging Center for Undergraduate Collaboration (ICUC) at Wheaton College in Norton, MA. These images were focused near the cell body capturing as much of the axon as possible to observe any morphological changes between the conditions. Photos were saved in separate folders on the computer, to keep experimental conditions separate from each other. Photos were then uploaded to Google Drive in similar folders to be accessed on a personal computer.

Data Analysis

Once pictures had been taken of the cells in all conditions, they were analyzed using ImageJ software downloaded to a PC from http://imagej.net. To determine if there were any changes to the axon morphology, the region of axon closest to the cell body was analyzed. Axons were defined as the projection from the cell body which laid straight, without branching, and had a thicker base attachment from the cell body as compared to any other projections. Using the program, a line was drawn measuring from the inside edge of the phase ring around the cell body, out to 400 pixels along the length of the axon. A mark was made at the beginning and end of this line to show where to measure the area. An example of this is shown in Figure 1. Then using the polygon tool of the same program, the outside edge of the axon was traced from the one end of the line to the other, containing the whole area of the axon in this area. The area of this polygon was measured using the "measure" function of the imaging software. The areas of each measured axon were recorded in a separate document so that they could be accessed outside of the ImageJ program. This was done for every viable cell body and axon photograph that was taken at each concentration including the control.

The 400-pixel length was converted into microns using a stage micrometer, which was imaged at 40x on the same computer with the same microscope and camera setup that was used to image the cells themselves. Using ImageJ, the distance from the left-side edge of one mark on the micrometer to the left-side edge of the line 120 microns to the right. The length of this line was measured using the "measure" function to determine the number of pixels this distance was equal to, in this case this was 782 pixels. The number of microns to pixel was calculated by dividing 120 microns by 782 pixels, for this study the calculated value was 0.1533 microns/pixel. This number was then multiplied by 400pixels to determine the distance measured in microns rather than pixels. When the length in pixels was converted to microns it was shown that 400 pixels at 40x in ImageJ is equivalent to 61.32 microns. This information could be used in future studies that do not use the same image analysis software

Minitab Express 18 software was used to run a one-way ANOVA test on the data to conclude whether the changes in axon taper and area were large enough to be considered significant in this experiment. The same program was used to generate figures representing the results of this test and visual represent the results quantitatively.

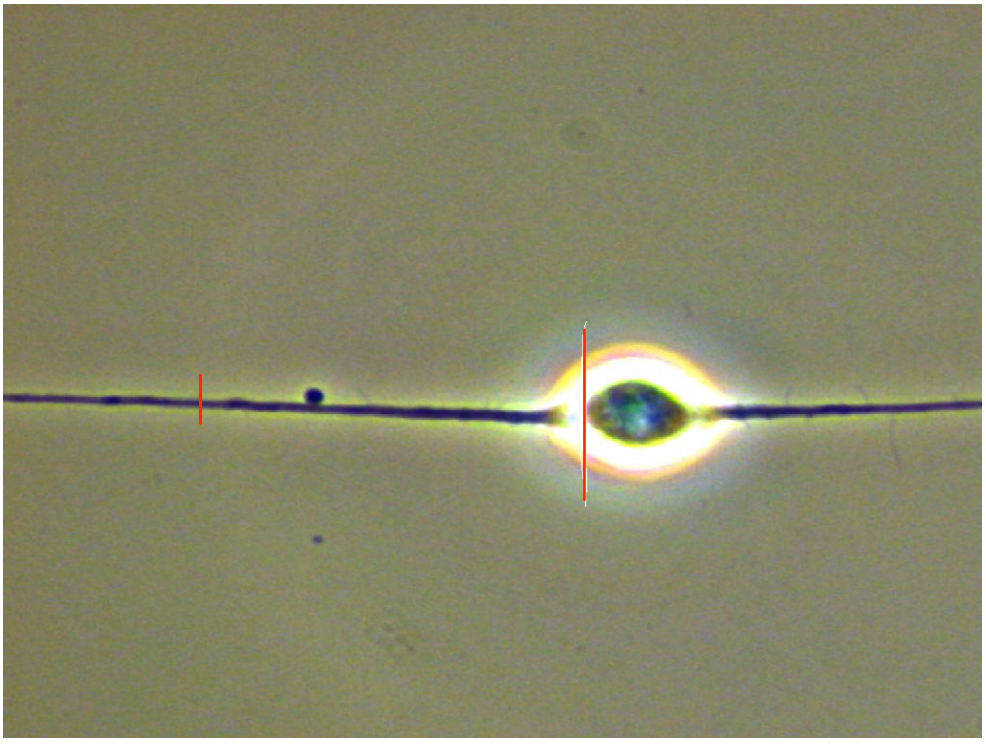

Figure 1 Example of measurement from inner edge of phase halo out to 400 pixels (61.32 microns). The white lines represent where the polygon containing the area of the axon would begin and end.

Results

Figures 2 and 3 represent an average light microscopy image collected from a control slide and a slide treated with a 10µM Ach solution respectively. They show what the average image of the axon morphology looked like at these conditions for this experiment. Note how in Figure 3 there are visible differences to the morphology of the axon as compared to the uniform caliber seen in Figure 2. Figures 4 and 5 show results of statistical tests run using Minitab Express software. Figure 4 shows a boxplot representing the differences in the average axon area at 400 pixels, as well as the minimum and maximum area found at each of those conditions. Figure 5 shows the results of a Tukey test, looking at statistical significance in the differences of area between all experimental conditions.

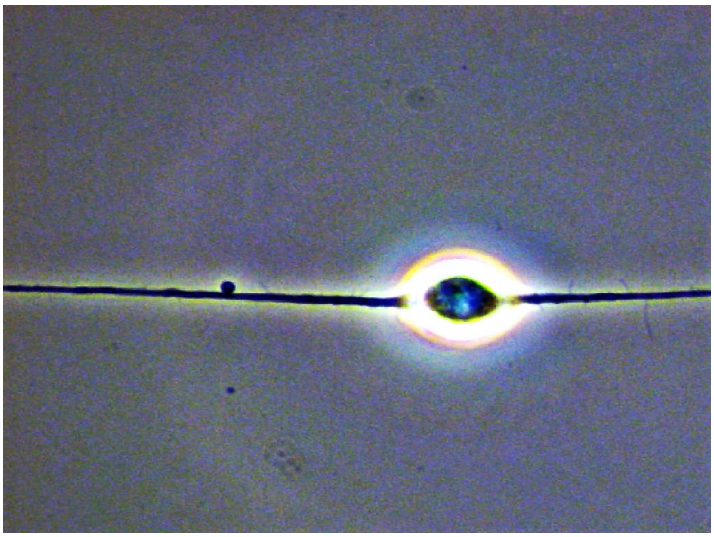

Figure 2 Healthy neuron from control dish, grown in F+ medium for 48 hours at 37°C. Notice the even caliber of the axon extending towards the left-hand side of the frame

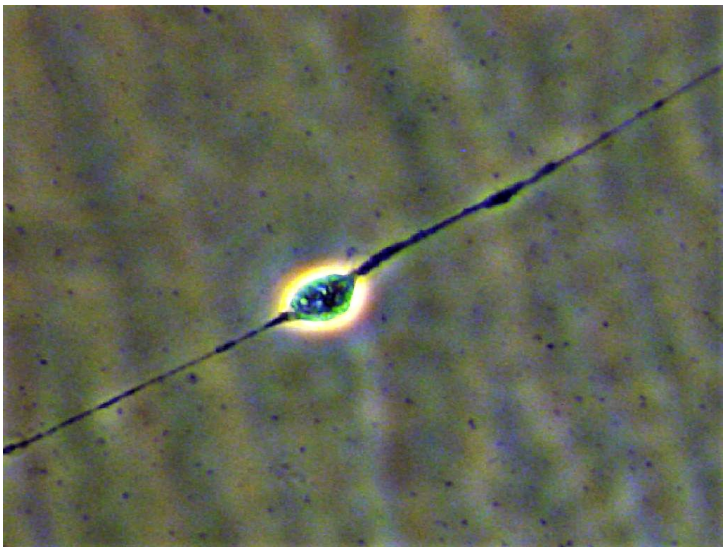

Figure 3. Neuron grown in 10µM Ach solution for 24 hours after an initial 24 hours of growth in F+ growth medium. Notice the uneven appearance of the axon extending towards the right-hand side of the frame. Background noise caused by VALAP on coverslip.

The axons which were treated with the Ach solution showed more severe changes in morphology at 10µM and 20µM concentrations. This was reflected in the decreased area of axon taper at a set distance for these concentrations as compared to the same measurements at 5µM and the control. The uneven appearance seen in this figure can be compared with Figure 1 in the 2014 Lim, Haque, D. Kim, D. J. Kim, & Y. K. Kim study which shows the possible effect of microtubule stability on axon structure. This same study credits this microtubule instability to the presence of Tau tangles (Lim, Haque, D. Kim, D. J. Kim & Y. K. Kim, 2014)

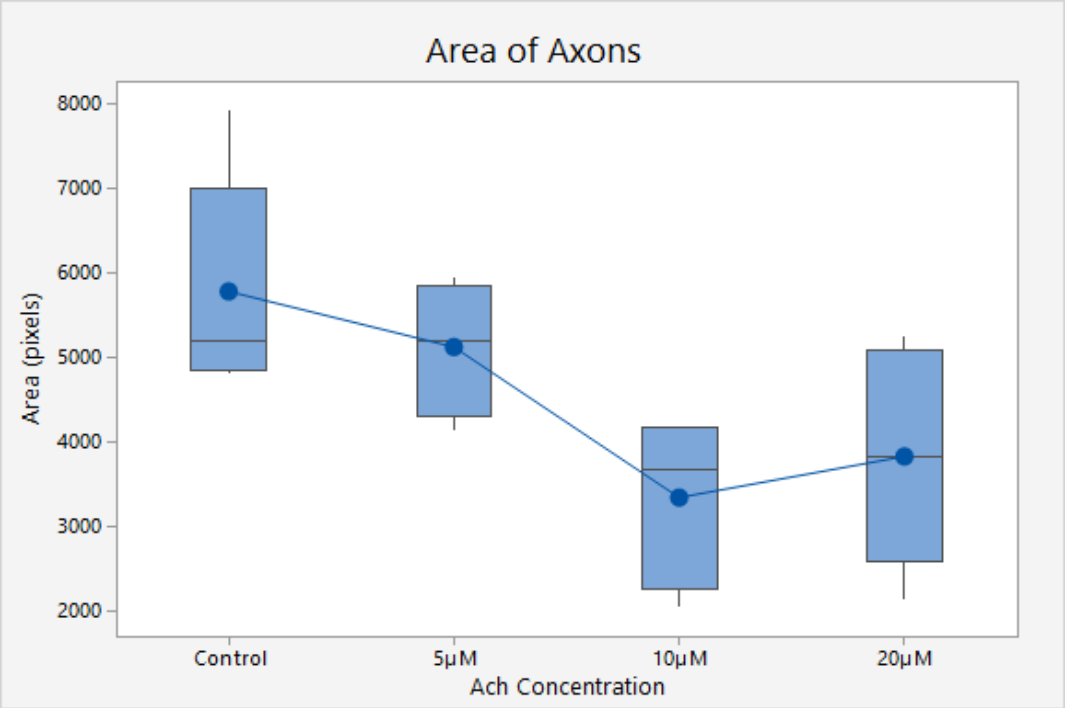

Figure 4. Boxplot of area of axon to 400 pixels (61.32 microns) from cell body. Whiskers indicate the minimum and maximum areas seen at each concentration. Dots represent the average area of the axon from the inside edge of the phase halo to 400 pixels (61.32 microns) as shown in Figure 1 at each concentration. Standard deviation for the control, 5µM, 10µM, and 20µM concentrations were 1253.4, 804.4, 931.2, and 1226.4 respectively. N-values for the control, 5µM, 10µM, and 20µM concentrations were 6, 4, 8, and 6 respectively. This n-value refers to the number of axons measured at each concentration each from a different neuron. Notice the decrease in axon area after the 5µM. Also notice the minimal variance in the three experimental conditions as compared to the more pronounced variance in the control condition as indicated by the error bars.

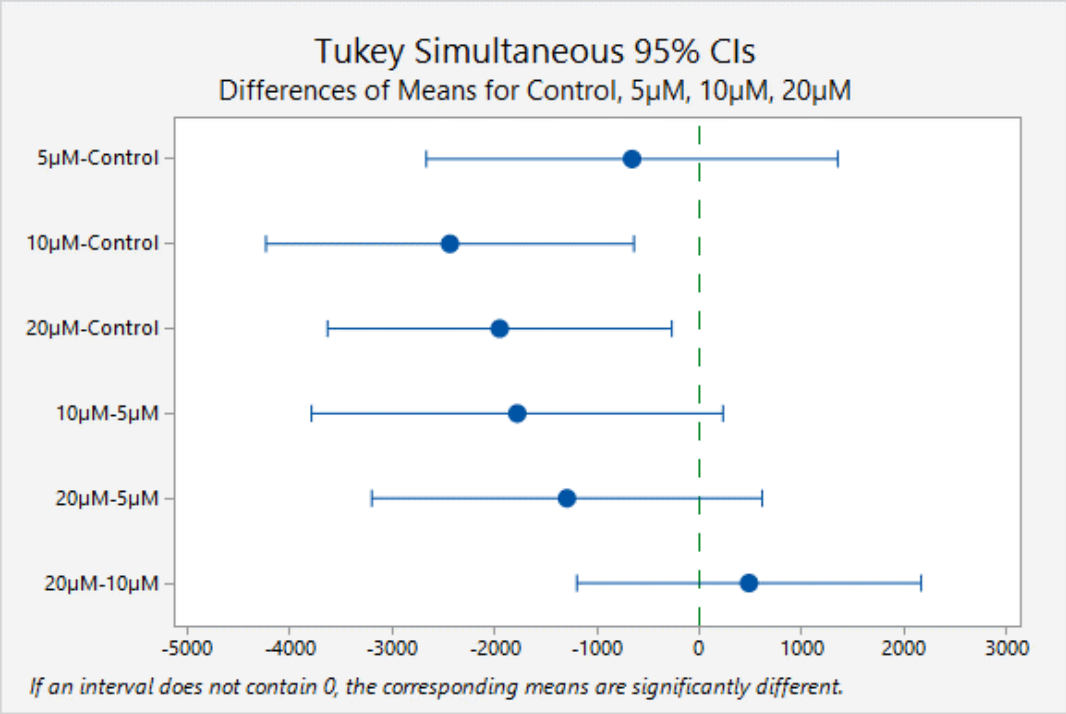

Figure 5. Results of Tukey test at 95% confidence interval. Note that a statistically significant difference is demonstrated in the difference between the control and 10µM comparison and the control and 20µM comparison.

Results of the Tukey test show that there was a significant difference between the control and the 10µM slide, as well as the control and 20µM slide. However, the difference in axon area between the control and the 5µM slide was not shown to be significant.

Discussion

Based on the results of this study it is suggested that Acetylcholine, when added to healthy neurons in culture, may perhaps affect axon growth. This potential effect is seen in the differences of axon taper close to the cell body at differing concentrations. Based on the results of this study this hypothesis is supported if the concentration of Ach is at least 10µM. This could vary if experimental conditions were varied (such as temperature, length of incubation, etc.), but in the context of this study, no significant difference was seen between the control and the 5µM slide, whereas it was for the other two experimental conditions. From the data, it may be suggested that Ach does perhaps play a role in changing cell morphology.

The data pool that was collected for this experiment was relatively small, and so the effect of human variability in measurement is higher than it would be with a larger data set. To counteract this variability, all data was averaged within each experimental condition. If this experiment was done with a larger data set, and all the trends remained the same, then it could be said that Ach may in fact be a player in the development of neurodegenerative diseases. From a cellular standpoint, the results suggest that proper Tau function may be necessary for the growth of healthy, stable axons. Ach can cause hyperphosphorylation of the Tau protein, which may lead to aggregations of the protein, a hallmark of Alzheimer's disease (Gong & Iqbal, 2008). The

results of this study suggest that hyperphosphorylation and aggregation of the Tau protein due to the presence of Ach may lead to microtubule instability while the axon is growing in culture. If the morphological changes observed in this study were to signify microtubule instability, the axons in the dishes with higher concentrations of Ach may not have grown in the same manner as the axons in the control dish. This could be one reason for the observed decrease in axon size, and visible morphological differences. While not tested in this experiment, the function of these axons (including transport) may be impacted by the possible decrease in microtubule stability as well.

One possible extraneous factor in this study was failure to consider the acidity of the Ach when adding it to the F+ growth medium. F+ contains a pH buffer, which gives it its pink color, but when Ach was added, the growth medium turned yellow in color indicating a lower pH. While the cells were still able to grow in this environment, any results gathered must be understood with the acidity of the growth medium considered. Since it has been previously shown that a pH in the range of 7.38 to 7.87 is optimal for cell growth, and that a higher acidity can retard growth, this effect may have been felt in the pretense of this study (C. Mackenzie, J. Mackenzie, & Beck, 1961). Therefore, changes to axon growth cannot be attributed only to the presences of Ach in the growth medium. Should this experiment be reproduced, an additional pH buffer should be added to the F+ to have all the cells growing in the same environmental pH conditions as the control group.

Based on the results of this study, future research could be done looking at a wider variety of concentrations of Ach to determine the exact minimum concentration necessary for Tau to aggregate in the neurons. The Tau protein itself could be stained using immunofluorescence to determine where in the neuron the Tau is aggregating, and how big these tangles are. This would allow for targeted treatments directly to areas where Tau was aggregating. Research could also be done using affected neurons to test new drugs to reverse the effects of the Ach and regenerate the neurons to a healthy state. Similar experiments could also be produced with compounds instead of (or in conjunction with) Ach, to determine other key elements of AD pathology. This could include compounds not naturally occurring in the brain but rather ones that we are subject to over the course of our lifetime such as pesticides, artificial sugars (like aspartame), and certain heavy metals including aluminum and fluoride (Hardick, 2016). By pursuing any one of these directions we could provide more evidence that could lead to larger human trials in the study of AD. Considering the prevalence of AD in today's world, the impact of a new treatment, or even a cure, would be enormous in the lives of millions of individuals and their families.

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Methods were created and data shared with collaboration partner Adam Hinthorne.