Axonal Outgrowth of a Neuron with an Encroaching Neuron

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Abstract:

Cytoskeletal structures, including microtubules, neurofilaments, and microfilaments along with other molecules all contribute to the outward growth of the axon and growth cone. Filopodia have the greatest influence on where the axon and growth cone move. When filopodia bind calcium ligands, the calcium channels open and there is an influx of calcium. This influx changes the structure of the cytoskeleton aiding in the growth cone advancement. Actin polymerization also creates an actin poor space in which the microtubules push forward, advancing the growth cone and axon. In this experiment, the effects of a proximal neuron on axonal outgrowth of another neuron were tested. Plated sympathetic nerve chain cells and dorsal root ganglion cells at 1x concentration were used to observe growing neurons. A neuron with a proximal neuron was time lapsed using a Nikon Eclipse E200 microscope and a Sony DFW Y700 camera. It was hypothesized that the proximal growth of another neuron would inhibit the axonal outgrowth rate of the encroached neuron. It was found that there was an inhibitory effect on the neuron due to the proximity of the encroaching neuron, and the eventual contact of its growth cone with the axon of the other neuron. Therefore, our hypothesis was supported. The average rate of growth for the uncontacted neuron was 1.53 micrometers per minute, and the average rate of growth for the neuron after it was contacted was 1.36 micrometers per minute.

Introduction:

There are many molecules and structures that compose an axon and growth cone of a growing neuron. Each of these structures and/or molecules plays an important role in the axonal outgrowth of the neuron.

There are three main cytoskeletal structures that compose the axon and the growth cone of a growing neuron. One such structure is the microtubule. These microtubules run the entire length of the neuron. Microtubules are made of protofilaments and each of the protofilaments is made up of alpha and beta tubulin subunits. These subunits arrange themselves to create a polar structure. Microtubule associated proteins known as MAPs assist in the polymerization of the microtubules as well as their arrangement (Kandel et.al. 2000). Microtubules are arranged in a parallel fashion with the plus end of the microtubule facing the cell body. The orientation of the microtubule allows organelles to move down its track in only one direction.

Another prominent structure in the neuron, especially in the axon, is the neurofilaments. The neurofilaments are the most fibrous part of the axon. These structures are much more stable than microtubules and are completely polymerized in the cell, unlike the microtubules (Kandel et. al. 2000).

A third structure present in neurons that composes the cytoskeleton of the neuron is known as the microfilament. Between these three structures, the microfilament is the thinnest. The microfilament is a polymer of actin monomers. Unlike the neurofilament, which is concentrated in the axon of the neuron, the microfilaments, or actin, are concentrated at the periphery of the cell in the cortical cytoplasm, mainly in the filopodia that extend from the growth cone. The microfilaments form a dense network under the plasma membrane of the cell along with actin binding proteins. This dense network plays a key role in the motility of the growth cone (Kandel et.al. 2000)

The form in which actin is present within the cell depends upon which binding proteins are also present. The proteins present help to assemble and block polymer growth. They do this by, either capping the end of the polymer or cutting it off completely. This unique polymerization method allows the neuron to extend new processes while at the same time retracting the old ones (Kandel et. al. 2000). There are actin motors known as myosins. The myosins assist in the cell motility, especially in the extension of cell processes: mainly the extension of filopodia from the growth cone (Kandel et.al. 2000).

The filopodia are actin rich and are depended upon greatly for the growth cones sensing capabilities. They are extremely motile structures that have receptors for themolecules that act as directional cues for the growth of the axon. These receptors act as mediators to adhesion cues, as well as signal transducing receptors that are connected directly with second messanger molecules. Calcium is an important second messanger molecule. The motility of the growth cone is prime at the set point, which is a specific calcium concentration. When a receptors on the filopodia are activated, the calcium concentration shifts. This flux of calcium causes the cytoskeleton to alter its confirmation and thus assist in growth cone motility (Kandel et. al. 2000)

There are three movements that the axon can perform if one of the filopodia comes in contact with a signal: advance, retract, or turn. The filopodia contact an adhesive cue causing a retraction. This retraction moves the growth cone forward. The polymerization of actin in the filopodia creates a force enough to push the filopodia forward. The actin polymerization creates an actin poor space in which the microtubules then advance in to. The microtubules create a tight bundle which causes a collapse of the cytoplasm. This event creates a new length of the axon (Kandel et.al. 2000).

Axons terminate at a structure known as the growth cone. The growth cone is a sensory motor structure that is thought to be responsible for axonal pathfinding. It is known as a hand-like structure that has abilities to detect

directional cues. The interpretation of the guidance cues that growth cones do are extremely important in the wiring of the peripheral and central nervous system. Guidance cues are known as attractive or repulsory, but scientists are not sure whether or not a growth cone can detect whether a guidance cue is repulsive or attractive (Mueller 1999). Some scientists have done experiments proving that extracellular matrix molecules promote axon outgrowth as well as guide the behavior of growth cones in the presence of diffusible guidance cues. Hopker et. al have shown, using retinal neurons, that growth cones display behaviors that would suggest that they know what chemoattractive and chemorepulsive signals are (Hopker et.al. 1999). The growth cones exhibit a chemoattractive turn towards netrin-1. This attractive cue leads the growth cones into the optic nerve head. In Xenopus, laminin-1 converts the attraction of the netrin-1 into repulsion. Low levels of cyclic AMP in the growth cone lead to this conversion from attractive to repulsive (Hopker et.al. 1999). It was proven by Ramon Y Cajol that growth cones do receive directional cues from the environment as well as a motor structure that leads to axonal outgrowth. Growth cones have three regions: The central core, the filopodial extensions, and the lamellipodia. The central core contains microtubules, mitochondria, and other various organelles. The filopodia are long extensions from the growth cone, and the lamellipodia are located between the filopodia and have a ruffled appearance (Kandel et.al. 2000).

Actin, myosin, and other membrane molecules contribute to the advancement of the growth cone, however, it is the pushing of the microtubules into the new extension that moves the growth cone forward. The lamellipodia then fill in the new growth cone and from there the formation of processes regenerates (Kandel et.al. 2000). Cyclic AMP also promotes axonal outgrowth through the activation of protein kinase A. The axonal outgrowth promotion by cyclic AMP and protein kinase A is mediated by the phosphorylation of synapsins (Kao et.al 2002).

In this study, we sought to address how the rate of growth cone advancement/axonal outgrowth was affected in the presence of a proximal neuron. We specifically watched the overall growth of the axon and growth cone using a time lapse movie on a light microscope. No studies were found to indicate that proximal neuron advancement inhibited axonal outgrowth, but we believe that proximal neuron growth does inhibit axonal outgrowth. It was reported that axonal outgrowth is inhibited by proximal neuron growth after contact between the growth cone and axon of the other neuron are made.

Hypothesis:

It was hypothesized that the rate of axonal outgrowth of a neuron would be inhibited by an encroaching growth cone of

another neuron as well as the interaction of a growth cone with the axon of another neuron.

Methods and Materials:

Materials

The materials for this experiment were very simple. A Nikon Eclipse E200 microscope with a Sony DFW-Y700 camera was needed for imaging the neurons during their growth. The microscope and the camera were connected to a Macintosh computer that contained the programs BTV Pro, Adobe Photoshop, and Image J. All of these programs were used during the course of the experiment to image the neurons as well as for analysis after. A 1x concentration of cells in F-medium, on a coverslip, were used for imaging. In order to keep the slide warm, an air curtain was used in which a space heater was connected to a cut Styrofoam box in order to create a warm air current across the microscope stage. A ring stand was used along with a ring clamp to attach a mercury thermometer which was then placed just hovering above the microscope stage.

Methods

Laboratory Work

Cells were first plated out using 10 day old chick embryos. A mix of dorsal root ganglion and sympathetic nerve chains were plated out and made into varying concentrations as per Robert L. Morris 3/1/2004 p.1 class notebook. After being plated out, the cells remained in 37 degree Celsius incubation for 20-24 hours. This allowed for growth to occur. For our purposes, a 1x concentration of cells was used to increase the likelihood of us finding an isolated neuron. After 20-24 hours of incubation, the cells are ready to be viewed. The microscope apparatus was set up. The air curtain was turned on low heat, and placed in front of the cut Styrofoam box to create a warm air current over the microscope stage. The thermometer was then attached to the ring stand via a ring clamp and placed just hovering over the microscope stage. The stage was allowed to heat to 37 degrees Celsius, and then maintained at that temperature. When the temperature on the stage was at a constant 37 degrees Celsius, a chip chamber was made as per Jessica Allegra 2/18/2004 p.19 for the slide. The slide was taken out of the F-medium via tweezers, and the bottom was wiped dry. It was then flipped over onto the coverslip within the chip chamber. It was then sealed by placing warm wax on the edges between the coverslip and the slide. The coverslip is then coated with a little distilled water and then wiped clean to ensure a clean slide. The slide was then ready to be viewed.

Viewing and Measuring Axonal Outgrowth

The slide used in this experiment was one of a neurons growth cone encroaching on the axon of another neuron perpendicularly. A Nikon Eclipse E200 microscope on phase optics mode with a Sony DFW Y700 camera was used for imaging. The control and the experimental conditions were both in this slide. The control condition was the isolated neuron, and the experimental condition was the encroaching neuron and eventual attachment of the neuron to the other one. Using BTV Pro and Adobe Photoshop, half hour to hour time lapse movies were taken as well as fixed images of the neuronal growth. These movies and photos were then used later for axonal outgrowth analysis. The movies were taken at one frame per ten seconds. There were three hundred and ninety frames taken over this sixty-five minute movie, so in order to get even intervals to measure change in length over the duration of the movie, the three hundred and ninety frames were divided by five. This determined at what frames to take measurements. It turned out that three hundred and ninety divided by five was every seventy-eight frames. The movies were opened up in BTV Pro. File was then clicked on, and frame export was chosen from this drop down menu. The image was saved as a jpeg image on the desktop. Frame 1, frame 78, frame 156, frame 234, frame 312, and frame 390 were saved on the desktop in the folder labeled jallegra. Refer to figures three, four, five, six, seven, and eight for neuron images.

The desktop image was then opened in Image J. In this program, the straight line selection was chosen and the image was then traced with a line. Analyze was chosen from the drop down menu and measure was clicked. In the box that appeared, the length of the axon and growth cone appeared in pixels. That number was then taken and converted to micrometers using a conversion factor found using Adobe Photoshop. Refer to Jessica Allegra 4/13/2004 p59-61. From here the slope, or the rate of growth, was determined between each of the frames using the slope equation: the change in Y/ change in X. The change in Y is the micrometer change between each of the frames, and the X is the change in time between frames. After, the average rate of growth between the frames, where there was no contact between the neurons was taken, as well as the average rate of growth between the frames where there was proximal growth as well as contact between the neurons was taken.

The image was dragged into Adobe Photoshop, and a box in the lower right corner was outlined. Apple C and then Apple N was clicked. This brought up a box that showed how many pixels the box was. By using a conversion factor of 3.3 pixels per micrometer, found previously using a ruler on a slide at 40x. magnification, it was found that there are 165 pixels in 50 micrometers. Refer to Jessica Allegra 4/13/2004 p59-62. The number of pixels in the box was then changed to 165 pixels wide by 10 pixels high. After hitting enter, another box with a box 165 pixels by 10 pixels appeared. This box was copied and pasted onto the image. Then, Image, on the drop down menu was chosen and flatten image was clicked. From here, the image, with the scale bar on it was saved on the desktop under file jallegra. See

figure one for graph showing axonal growth.

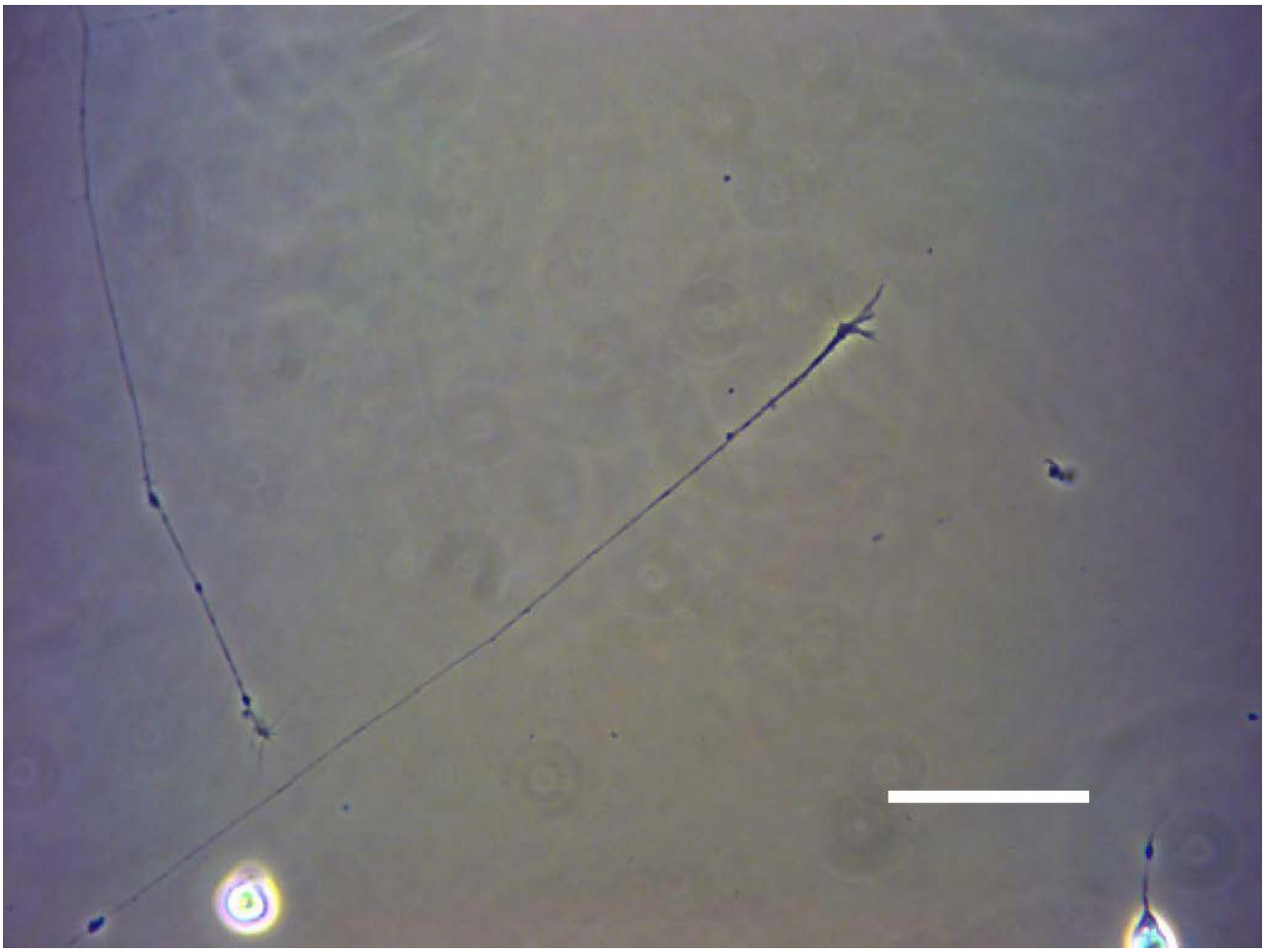
Results*:*

When first analyzing the images using the number of pixels to determine the length of the axon and whether or not it grew, it was found that as the number of frames went up, the axon and growth cone length did increase in the number of pixels contained in the outlined image. Frame one contained 862 pixels, frame seventy-eight contained 928 pixels, frame one hundred and fifty-six contained 976 pixels, frame two hundred and thirty-four contained 1047 pixels, frame three hundred and twelve contained 1091 pixels, and the last frame, frame three hundred and ninety contained 1164 pixels. These numbers indicate that there was axonal outgrowth present in this neuron with another neuron encroaching on it. Using these measurements and ones determined as per Jessica Allegra 4/13/2004 p.61-62, the pixel lengths were then able to be converted to micrometers. Frame one was 261 micrometers, frame two was 281 micrometers, frame three was 295 micrometers, frame four was 317 micrometers, frame five was 330 micrometers, and frame six was 352 micrometers. The time between each of the six frames was determined as per Jessica Allegra 4/6/2004 p.43. It was found to be thirteen minutes between each of the frames. Refer to figure one for graph of the length of each image in micrometers in relation to the time in minutes.

The slope was also determined for the interval between each frame. The determination of the slope gives the rate of axonal outgrowth between each of the six frames. The slope, or rate of outgrowth between frame one and frame seventy-eight was found to be 1.53 micrometers per minute; the slope between frame seventy-eight and frame one hundred fifty-six was found to be 1.07 micrometers per minute; the slope between frame one hundred fifty-six and frame two hundred thirty-four was found to be 1.69 micrometers per minute; the slope between frame two hundred thirty-four and frame three hundred twelve was 1.00 micrometers per minute; and the slope between frame three hundred twelve and frame three hundred ninety was 1.69 micrometers per minute.

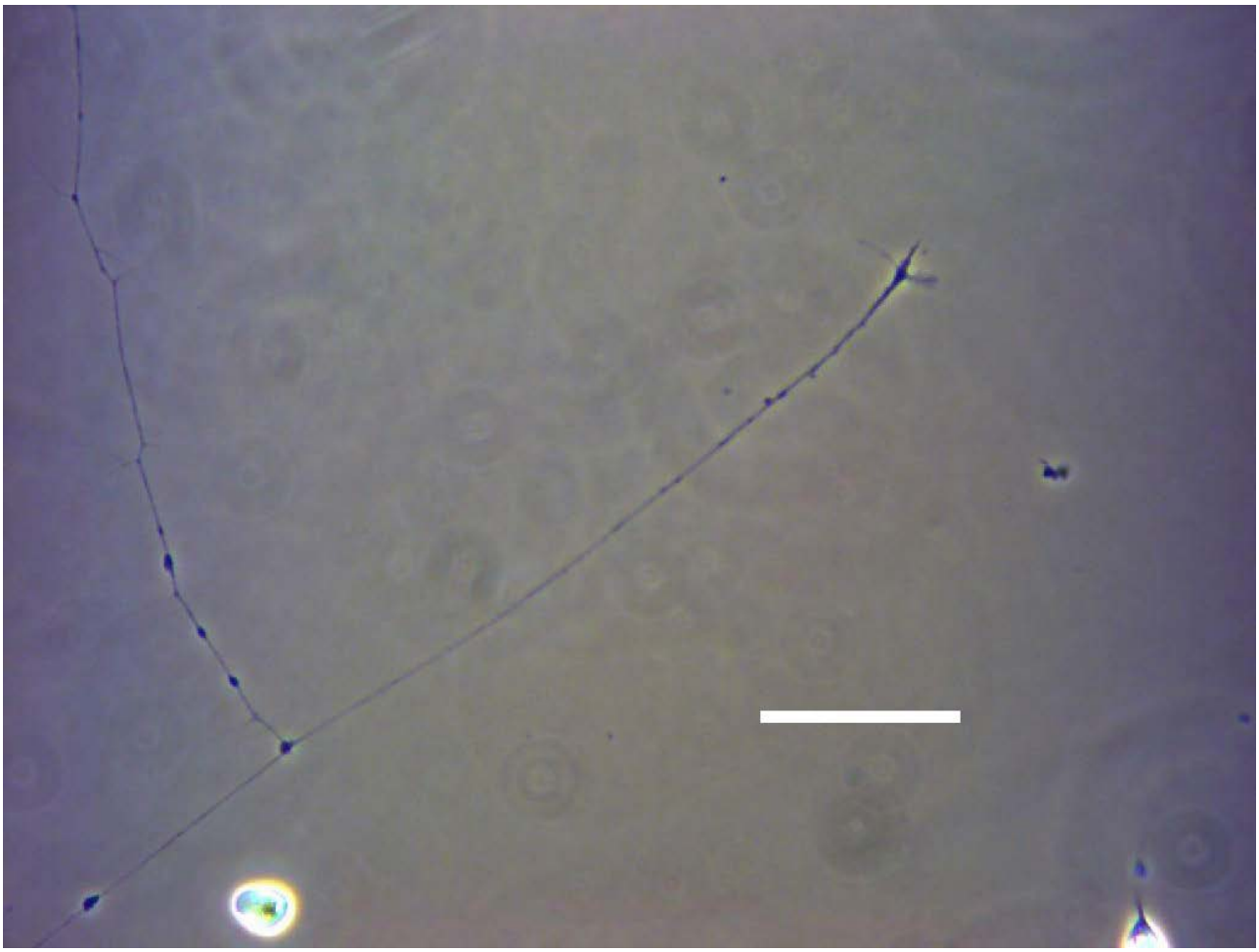
The average rate of growth between the frames, where there was no growth cone to axon contact, and the average rate of growth between the frames where there was proximal growth as well as growth cone to axon contact was taken. It was found that only the first frame had no interaction between the two neurons. The average for this was 1.53 micrometers per minute. The average of frame seventy-eight through frame three hundred ninety was 1.36 micrometers per minute.

Figure 3:



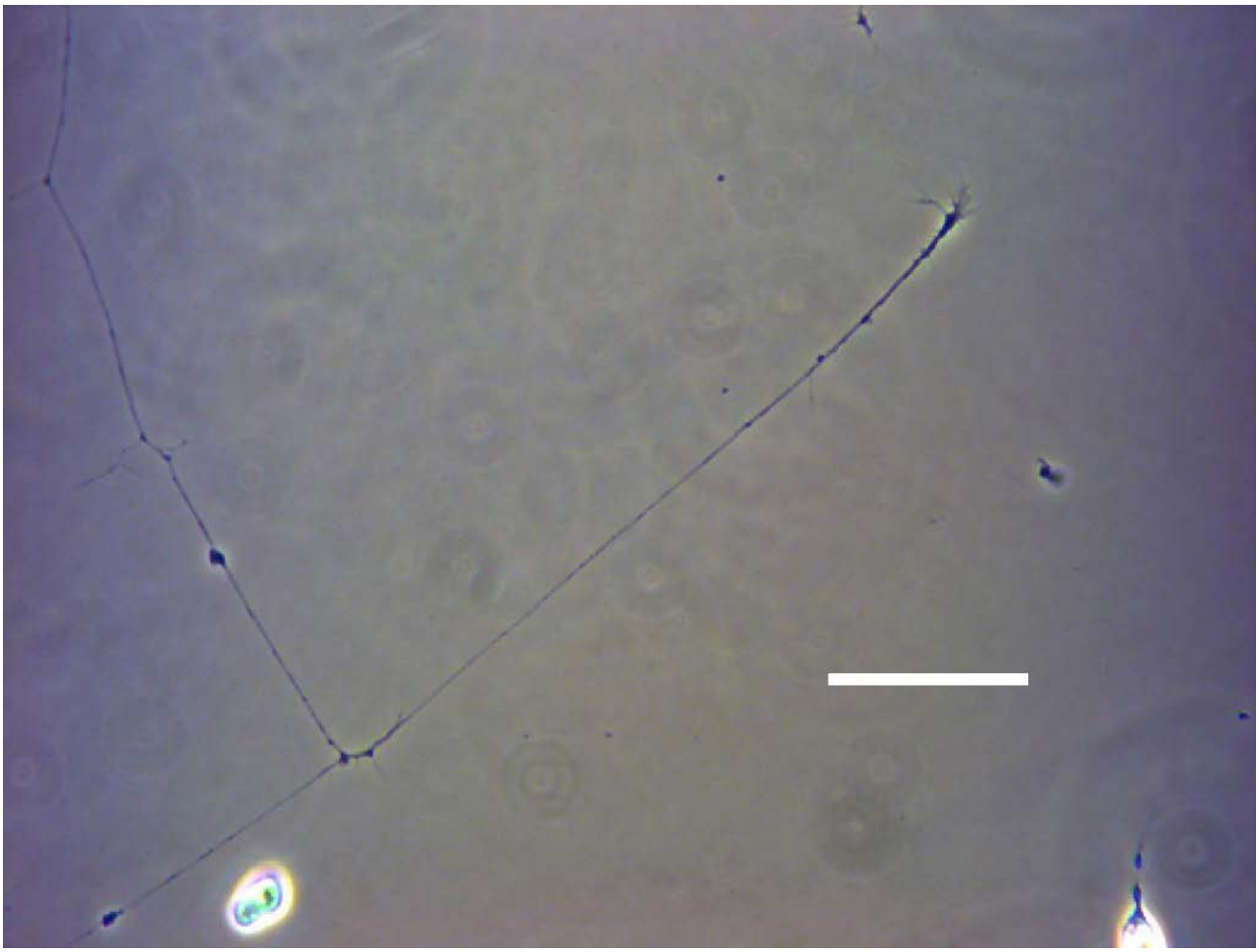
This is an image of an encroaching neuron on another neuron. This image was taken at 40x magnification on a Nikon Eclipse E200 microscope with a Sony DFW Y700 camera. A frame was taken every ten seconds for sixty-five minutes. This is frame one taken at 0 minutes. The length of this axon, at the start of the movie, is 261 micrometers, and the scale bar on this image is 50 micrometers.

Figure 4:



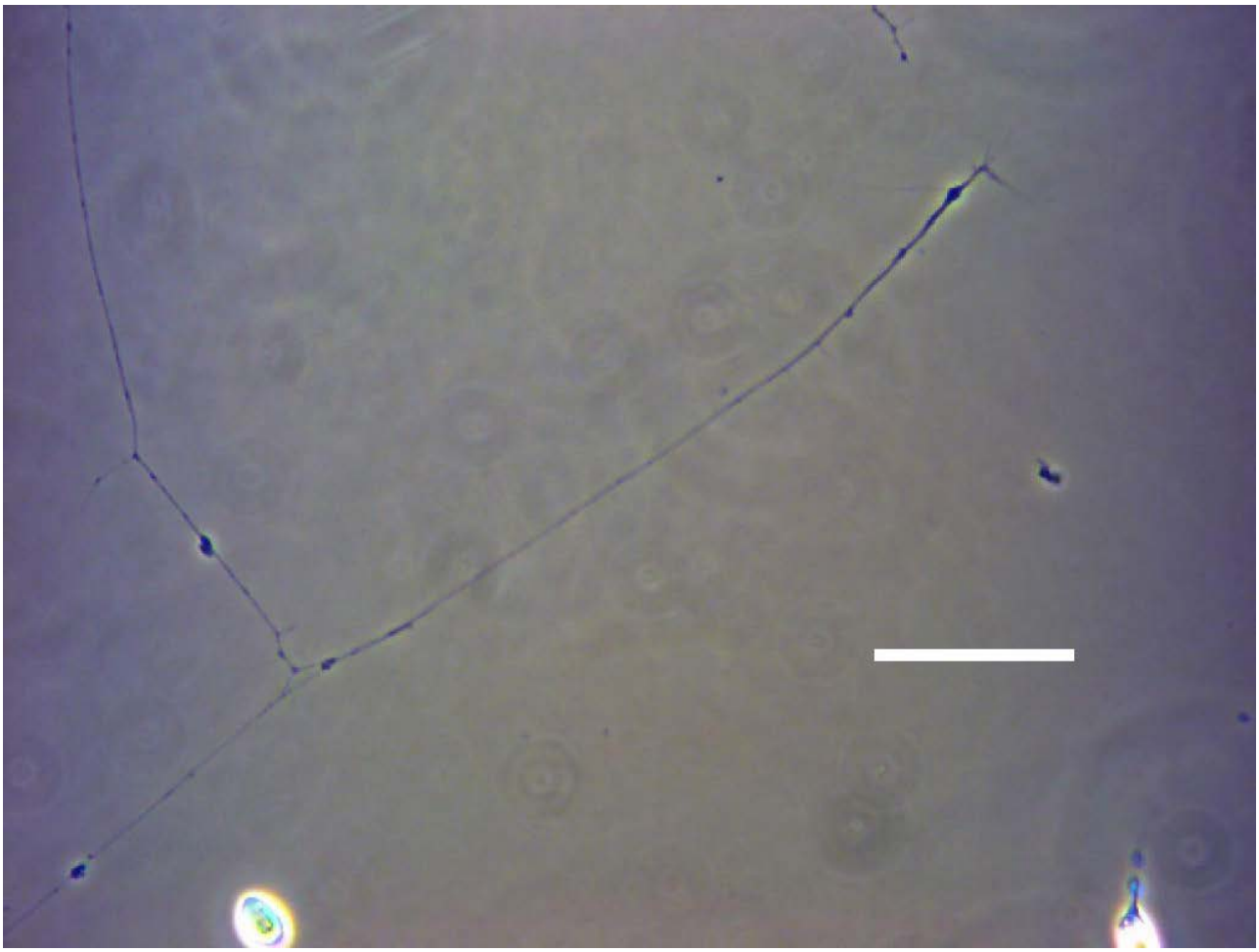
This image as well was taken at 40x magnification with a Nikon Eclipse E200 microscope and a Sony DFW Y700 camera. This is frame seventy-eight in the series at 13 minutes. This frame shows the encroaching neuron just after it has made growth cone to axonal contact. The length of this axon and growth cone is 281 micrometers, and the scale bar on this figure is 50 micrometers. From figure 3, there is a rate of growth of 1.53 micrometers per minute.

Figure 5:



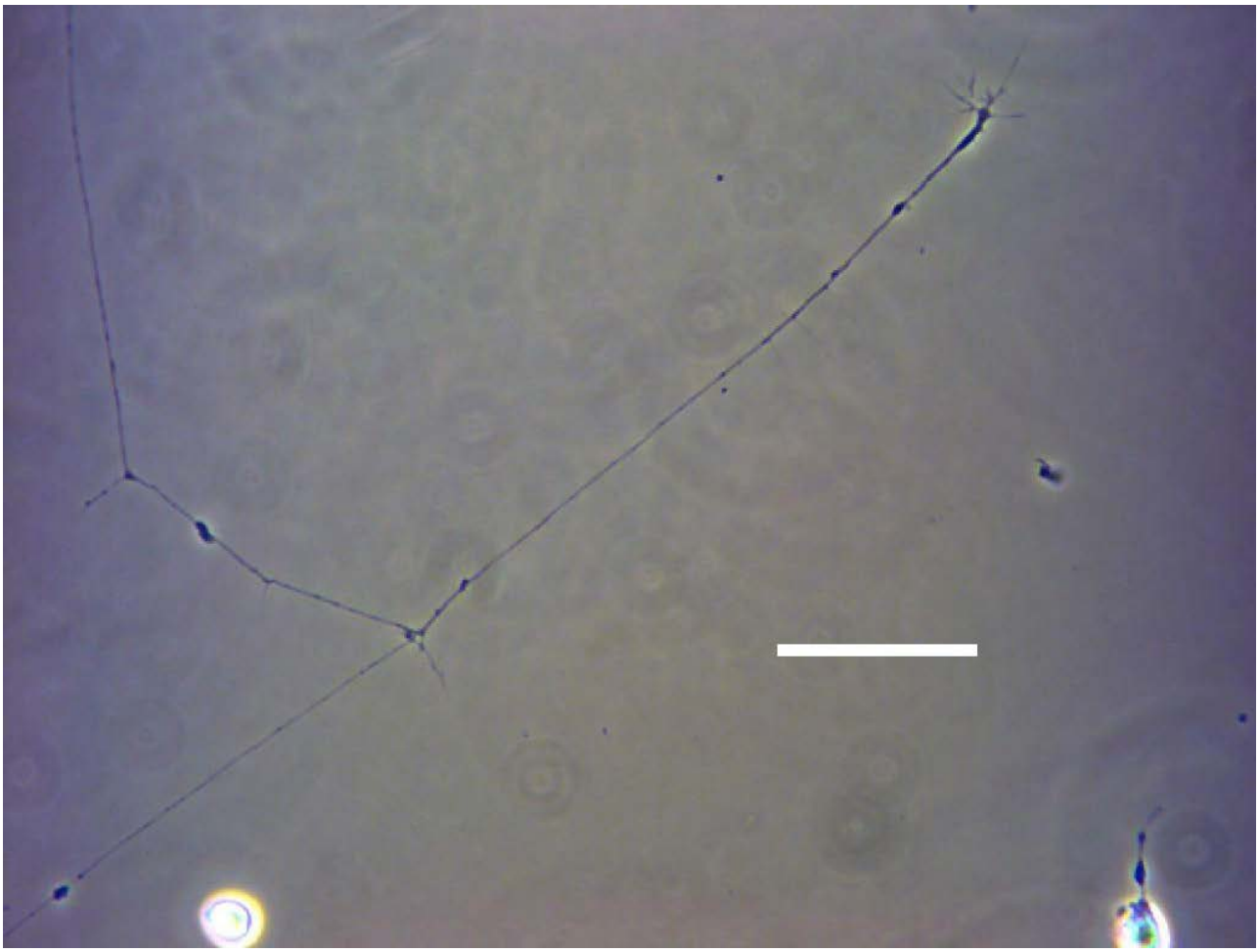
This image was also taken at 40x magnification with a Nikon Eclipse E200 microscope and a Sony DFW Y700 camera. This image is on frame one hundred and fifty-six of the series at 26 minutes. If one looks closely, one can see the growth cone of the encroaching neuron (already contacted now) beginning to grow laterally along the axon of the other neuron. The length of this axon and growth cone is 295 micrometers, and the scale bar on this image is 50 micrometers. From figure 4, there is a rate of growth of 1.07 micrometers per minute.

Figure 6:



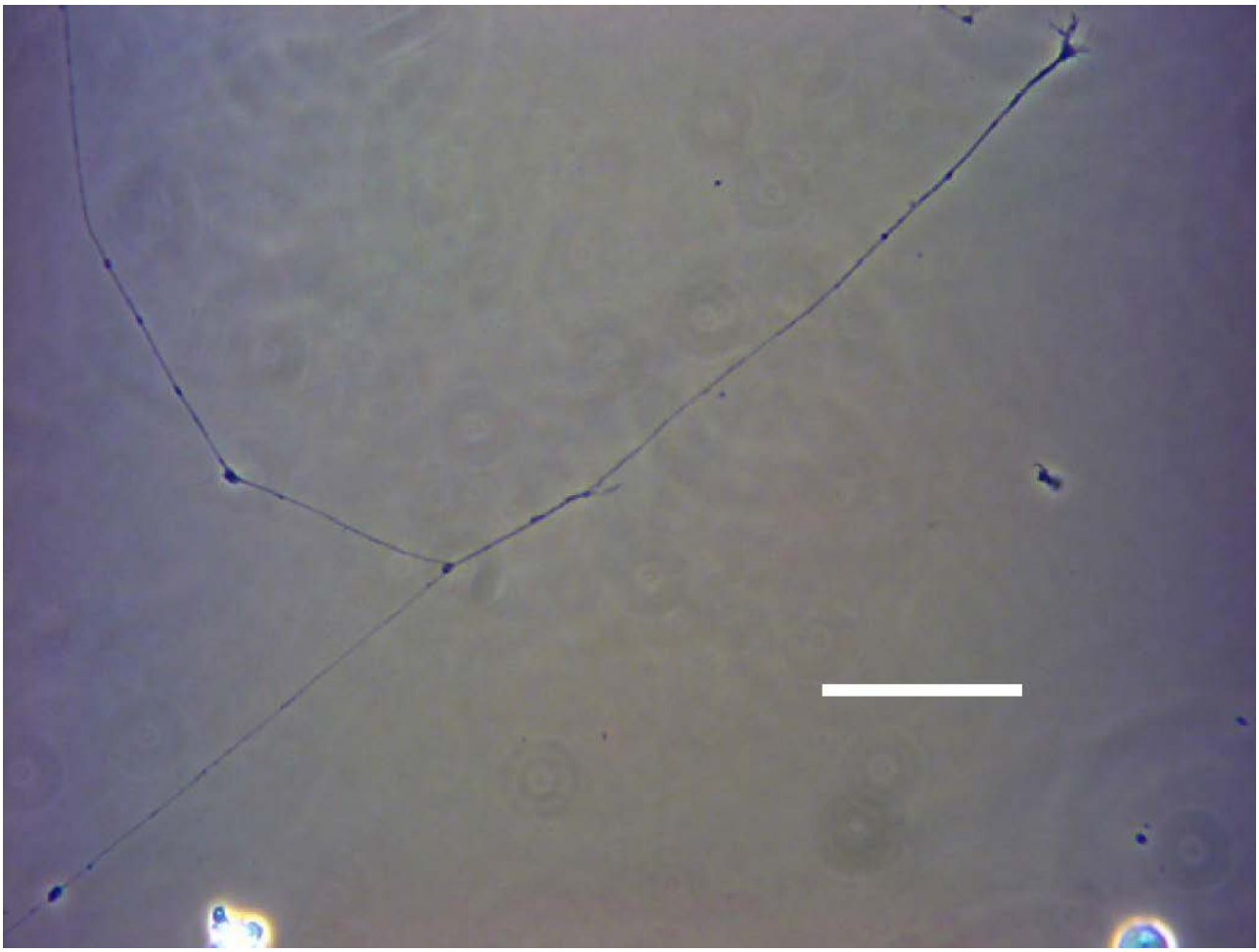
This image was taken at 40x magnification as well with a Nikon Eclipse E200 microscope and a Sony DFW Y700 camera. This is frame two hundred and thirty-four of the series at 39 minutes. This image is the same image as figure 5, except, the growth cone of the encroaching neuron has grown further laterally along the axon of the other neuron. In this figure, the encroaching neuron is pulling slightly up on the axon of the other neuron creating a slight angle. The length of this axon and growth cone is 317 micrometers, and the scale bar on this image is 50 micrometers. From figure 5, there is a rate of growth of 1.69 micrometers per minute.

Figure 7:



This image was also taken at 40x magnification with a Nikon Eclipse E200 microscope and a Sony DFW Y700 camera. This is frame three hundred and twelve of the series at 52 minutes. This image again shows the growth cone of the encroaching neuron growing laterally along the other neuron. The length of this axon and growth cone is 330 micrometers. The scale bar on this image is 50 micrometers. From figure 6, there is a rate of growth of 1.00 micrometers per minute.

Figure 8 :

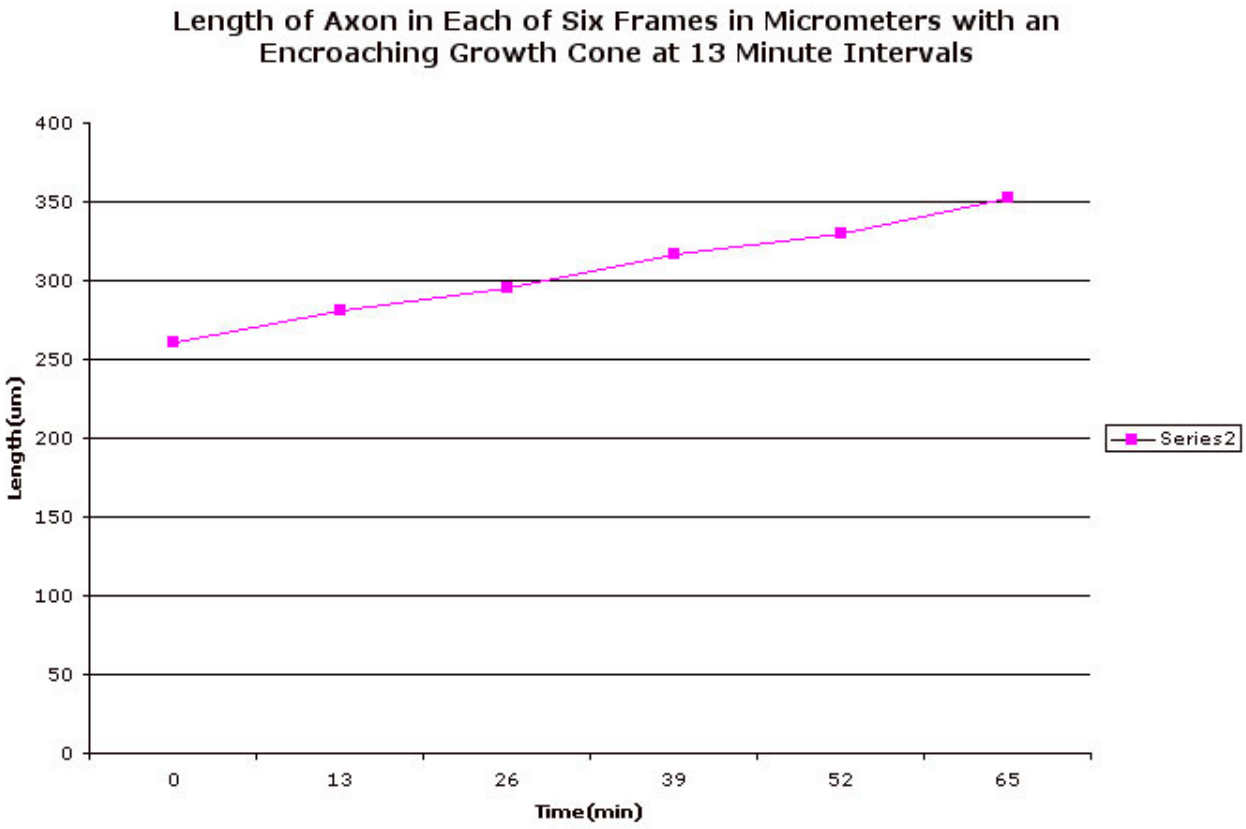


This image was taken at a 40x magnification with a Nikon Eclipse E200 microscope and a Sony DFW Y700 camera. This is frame three hundred and ninety (the last frame) in the series at 65 minutes. This image, like the previous four show the encroaching growth cone growing laterally along the axon of the other neuron. The length of the axon and growth cone is 352 micrometers long. This image has a 50 micrometer scale bar. From figure 8, there is a rate of growth of 1.69 micrometers per minute.

Discussion:

A growth cone has been defined in this experiment as the terminal end of an axon where there is a strong appearance of filopodia and lamellapodia. A lateral interaction has been defined when there is no perceptible distance between the axons growing along side of each other using phase optics on a light microscope at 40x power. A proximal neuron has been defined as a neuron that is encroaching another neuron, as in figure one, as well as a neuron that is interacting with the other neuron, as in figure two. Looking at the original calculations of the length of each image in each frame, one can say that our hypothesis was not supported. If one refers to figure one, the rate of axonal outgrowth is directly proportional to the time. As the time increases, the axonal outgrowth continues to increase proportionately even though there was proximal neuron growth as well as growth cone to axon contact.

Figure 1 :



This figure shows the length of the axon in each of the six frames taken. There is thirteen minutes between each of the six frames. Each axon and growth cone was measured using the straight line selection in the IMAGE J program. Then the outlined axon and growth cone were analyzed and then number of pixels outlined was converted to micrometers. This graph indicates that between each frame the axon and growth cone were longer than in the previous frame. When the slope between each frame was taken, and then the average of the slopes of the frames with no contact versus the average of the slopes of the frames with growth cone to axon contact, it was found that the rate of growth was inhibited. The average rate of growth for the neuron with no contact was 1.53 micrometers per minute, and the average rate of growth for the neuron with contact was 1.36 micrometers per minute.

Although our hypothesis was not supported by the length calculations in figure one, when the slope between each point was calculated our hypothesis was supported. The slope, or rate of growth calculated for the interval between frame one and frame seventy-eight was 1.53 micrometers per minute, and the slope calculated for the interval between frame seventy-eight and frame one hundred and fifty-six was 1.07 micrometers per minute. This calculation was done at the point where the encroaching growth cone contacted the axon of the other neuron. Because there is a decrease in the rate at this point, it is thought that the rate of axonal outgrowth is inhibited by proximal neuron growth. Between the next two frames there is an increase in the growth rate: 1.69 micrometers per minute, but between the following two frames, there is another decrease in growth rate: 1.00 micrometers per minute. It was thought that the lateral growth of the

growth cone along the axon would be inhibiting the growth of the neuron, but the rate of growth between the last two frames increases again to 1.69 micrometers per minute. If one takes an average of the rate of growth of the frames where there is no growth cone to axon contact, the rate of growth is found to be 1.53 micrometers per minute. If one takes the average of the rate of growth of the frames where there is growth cone to axon contact, frames seventy-eight to three hundred ninety, the rate of growth is found to be two tenths lower than that of non-contacting neurons: 1.36 micrometers per minute. This finding supports our hypothesis that axonal outgrowth is inhibited by proximal neurons.

Although it can not be seen with phase optics on a light microscope at 40x, synapses may be forming where the two axons are interacting as well as where the growth cone interacts with the axon. These jumps in the rate of axonal outgrowth could be due to the formation of synapses enpassant. If synapses are forming enpassant, there is a chance that these synapses are extracting energy normally saved for the axonal outgrowth, but is instead being used in the synapse formation.

There was not much time between the time that the axon was isolated to the time that it was interacting laterally with another axon. The little time that there was an isolated neuron, there was only enough information to have one calculation. There were many calculations for the laterally interacting axons. This is one thing that could have been done differently. Next time that this experiment is done, an isolated neuron should be observed and analyzed as well as a neuron with a proximal growth cone or a neuron with a lateral interaction. That way, the control and the experimental conditions are not on the same slide, and they are able to be analyzed separately.

Looking back on this experiment, there are some methods that could be changed. If this experiment was performed again, some things to be changed include: the number of frames taken for quantification. There should be more frames taken to obtain more data. The more data taken, the more accurate the graphs and calculations. A further experiment could be to test the rate of axonal outgrowth of a neuron with a proximal neuron, but also to test the rate of axonal outgrowth of the encroaching neuron and to test whether or not the growth of the encroaching neuron is faster while approaching the neuron, just when it contacts the other neuron, or when it is growing laterally along the axon of the other neuron.

It should be noted that these measurements may not be entirely accurate because none of the axons were measured from the cell body. When they were put up on the screen, the entire axon from the cell body to the growth cone was too much to fit on the screen, so the half of the axon with the growth cone present on it was put halfway into the screen, and growth was observed that way. Measurements were taken from the exact place where the axon entered the corner of the screen to the end point of the growth cone. If this experiment is done again, a suggestion would be to pick a neuron that has a small axonal outgrowth: one that has a visible cell body and an axon with a growth cone on the

screen. Then one would be able to measure from the cell body to the end of the growth cone, which would in turn give more accurate axonal outgrowth measurements.

Overall, our hypothesis was supported when looking at the data referrring to the rate of growth. This data set is a clear indicator that proximal neurons have an inhibitory effect on growing neurons and the rate of their axonal outgrowth.

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