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Localized filopodial activity as evidence of differential regions of endocytic activity

Abstract

Filopodial activity is essential to axonal outgrowth and neuronal pathfinding as they act both as a mechanism of advancement as well as a receptor for cues for directing growth. A high amount of endocytosis is associated with the creation, maintenance and extension of filopodia which in our experiment ranged from as few as 2 to as many as 12 within our region of interest. There was no evidence of the formation of *en passant* synapses but there was evidence that the number of axonal filopodia and the number of growth cone filopodia are roughly inversely proportional to each other, implying that increased endocytic activity in one area cause a decrease in endocytic activity in another area. While it does not show the existence of *en passant* synapses it does provide evidence that their formation could be manifested as a decease in filopodial activity.

Introduction

Growth cones are essential structures for neuronal pathfinding and the creation of neuronal networks. In order of a two neurons to communicate a signal they need to have some area of axonal proximity that allows for the creation of a chemical or electrical synapse across which an signal can be sent. Subsequently, cells may have to search for a target cell to innervate. This is done primarily through axon growth which is guided by the growth cone. The growth cone is the terminus of a growing axon and is comprised of three primary areas: filopodia, lamellipodia, and the central core (Bridgman and Dailey 1989). The central core is an area dense in microtubules which structure the axon (Letourneau and Ressler 1984).

While axonal growth is caused by microtubule advancement, before these filaments can move forward it is necessary for the filopodia in the growth cone to advance, effectively pulling the axon along and creating room for further axonal growth (Kandel 2000). The advancement of the growth cone occurs in three primary steps. First integrin proteins on the surface of the filopodia adhere to the surface of adhesion which in our case is a coverslip treated with poly-K and laminin (Kandel 2000). These transmembrane proteins are linked inside the cell by actin filaments that extend throughout the filopodia and give them their structure (Williamson et. al. 1995). Parts of the filopodial membrane proximal to the dense core are endocytosed and transported to the tip of the filopodia where they are integrated with the distal membrane, creating a small area of advancement which is still unsupported by actin filaments (Kandel 2000). Actin molecules are then polymerized at the leading edge of the actin filament, extending it and pushing the filopodia forward (Gordon-Weeks 1991). Microtubules then bundle and the cytoplasm collapses around them, forming a new length of axon (Gallo and Letourneau 2004).

These filopodia are extremely important for pathfinding not only because of the crucial role they play in terms of advancing the axon, but they are also sensitive to chemotropic signals as well as cell adhesion glycoproteins and other extrinsic guidance cues (Hynes and Lander 1992). These may be signals from other neuronal cells or from specific target cell types such as muscle cells, optic cortex cells, or other specialized cell types (Smith 1988).

The role of filopodia visible along the existing axon has not been heavily explored in published research, but although they are apparently not involved in axon growth or growth cone advancement, such an expenditure of energy should have some justification. It is likely that axonal filopodia not involved in active outgrowth still perform pathfinding functions, but in a more passive manner than the aggressively seeking growth cone. These filopodia may act to signal other growth cones or serve as a kind of net, hoping to increase the chances of associating with another growing axon and forming a synapse.

In this paper I hope to explore the possibility of an abnormal class of synapse called an *en passant* synapse which is

formed by regions of axonal membrane proximal to each other without any termini or butons present. l The morphological picture of an *en passant* synapse would have the neuronal orientation of an electrical synapse with the interaction seen in chemical synapses. The formation of these synapses has not yet been well documented and while my experiment may not precisely visualize such interactions, it may provide evidence of their existence. This kind of neuronal synapse would be most prevalent in neural nets where several axons and somas cross each other, creating multiple possible sites for such associations, but such probability does not rule out the possibility of their inception along two proximal axons with still advancing growth cones. In comparison an isolated neuron, the development of an *en passant* synapse should produce a marked decrease in the number of filopodia created over time in neurons observed both before and after proximal association with one another.

Given the function of endocytosis both in chemical synapses as well as filopodial growth, I hypothesize that the formation of *en passant* synapses in proximal growing neurons may be manifested as a decrease in filopodial activity due to a change in the localization of endocytic activity. To observe this interaction we defined our region of interest as a visual field which captured the growth of terminal growth cone while still allowing it room to extend and at the same time trying to maximize the amount of axon visible.

Methods and Materials

Preparation of cells and coverslips

In order prepare neuronal cells, 10 day old chicks were dissected for their dorsal root ganglia (DRG). These ganglia were then trypsinized and put on coverslips treated with poly-K and laminin in 1 inch Petri dishes filled with F+ medium and Hanks Balanced Salt Solution (HBSS). The cells were put onto the coverslips in relative concentrations of 1x, 2x and 4xDifferent concentrations of these cells were then placed on cover slips treated with poly K to aid in cell adhesion. The F+ medium was made up of Leibovitz L-15 medium, 2mM glutamine, 0.6% glucose, penicillin and streptomycin, 10% fetal bovine syrum and 20ng/ml Neuronal Growth Factor (NGF).

Preparing chip chambers for cells

To examine the affect of interaction on growth cone and filopodial activity the cells were put into chip chambers. This was done by crushing a glass coverslip into several pieces and arranging 4-8 shards on a slide in such a way as to support another coverslip. The F+ medium was then pipetted off of the cultured coverslip and the bottom of the slip was wiped dry with a chem wipe. Then 2 drops of the F+ medium were pipetted onto the slide and the treated coverslip was lowered gently face down onto the slide, being careful to keep the broken shards underneath. The edges of the coverslip were then brushed over with valap wax and the surface was cleaned with de-ionized water by placing a chem wipe on the slide so as to cover 3 of the 4 inside edges of the coverslip. The chem wipe was then slowly drawn away, careful not to streak any wax over the coverslip.

Imaging axonal growth

The slides were then observed under a Nikon Eclipse E200 microscope which used a Sony DFW-X700 Digital Interface Camera which connected with a G4 Macintosh computer running OSX. BTv Pro was used to display the video input and record time lapse photography or single still shots. Adobe Photoshop was used for size analysis. All our observations were made at 40X objective magnification under a phase II condenser which produced a much shaper image than simple light microscopy. Two time lapse movies were made: One recorded an neuron lacking any proximal cell-cell contact while the other recorded an encroaching growth cone associating and interacting with a target axon. The isolated neuron movie was taken for 30mins with 15sec lapsing between each frame. The movie showing axonal association was taken for 90mins with 10sec passing between each frame. The only reason for the difference in frame rate between the two movies is that I forgot to change the settings for the second movie.

Analyzing the data

Once the videos were taken I watched them frame by frame to record the number of visible filopodia extending from the growth cone as µover time to look for trends**.**

Results

There are no error bars because only one measurement was performed.

Figure 1

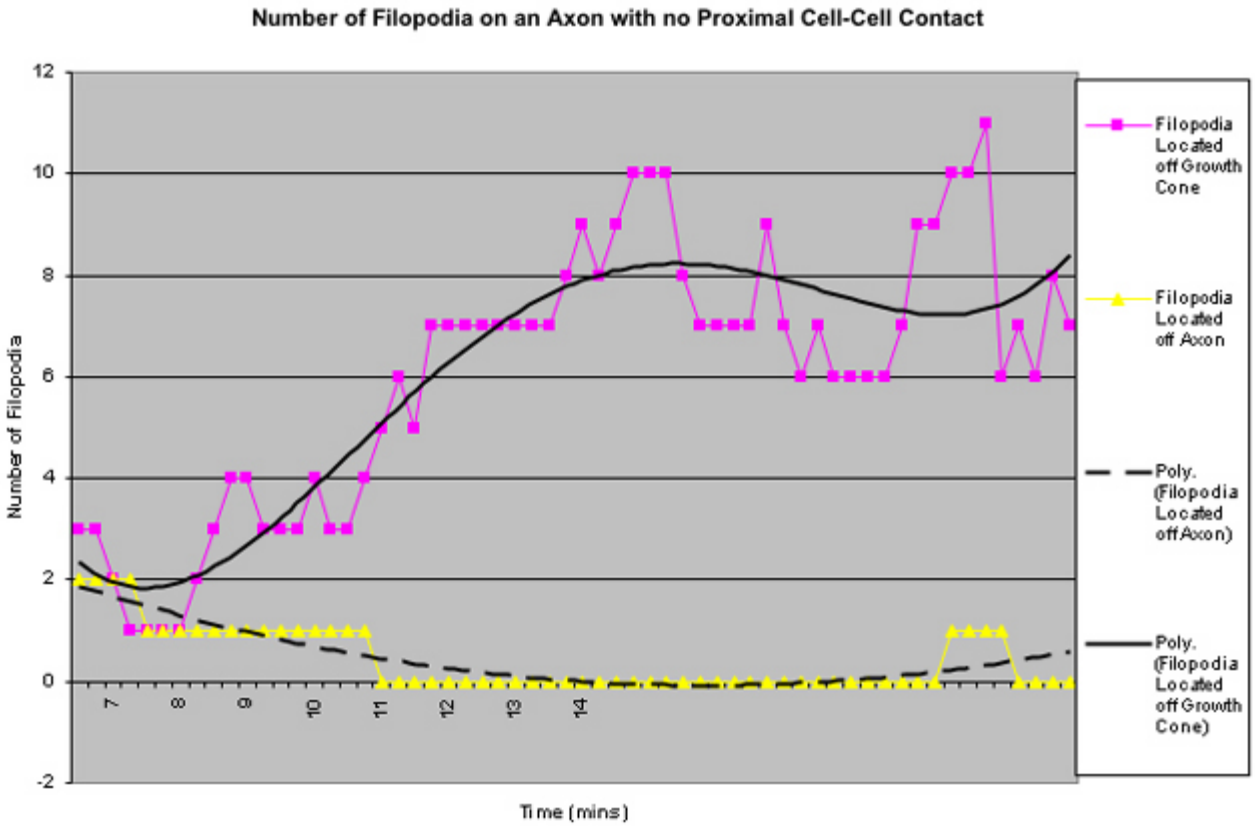

Figure 1 shows a graph of the data collected for the isolated neuron. The first 15 minutes of the video was analyzed frame by frame to count the number of filopodia apparent at the growth cone and the number appearing along the axis. The graph of the data is shown below in Figure 1. While the number of growth cone filopodia (GCF) vacillates greatly, it averages 6.5. The number of axonal filopodia (AF) remains consistently low, averaging 0.5.

Figure 2

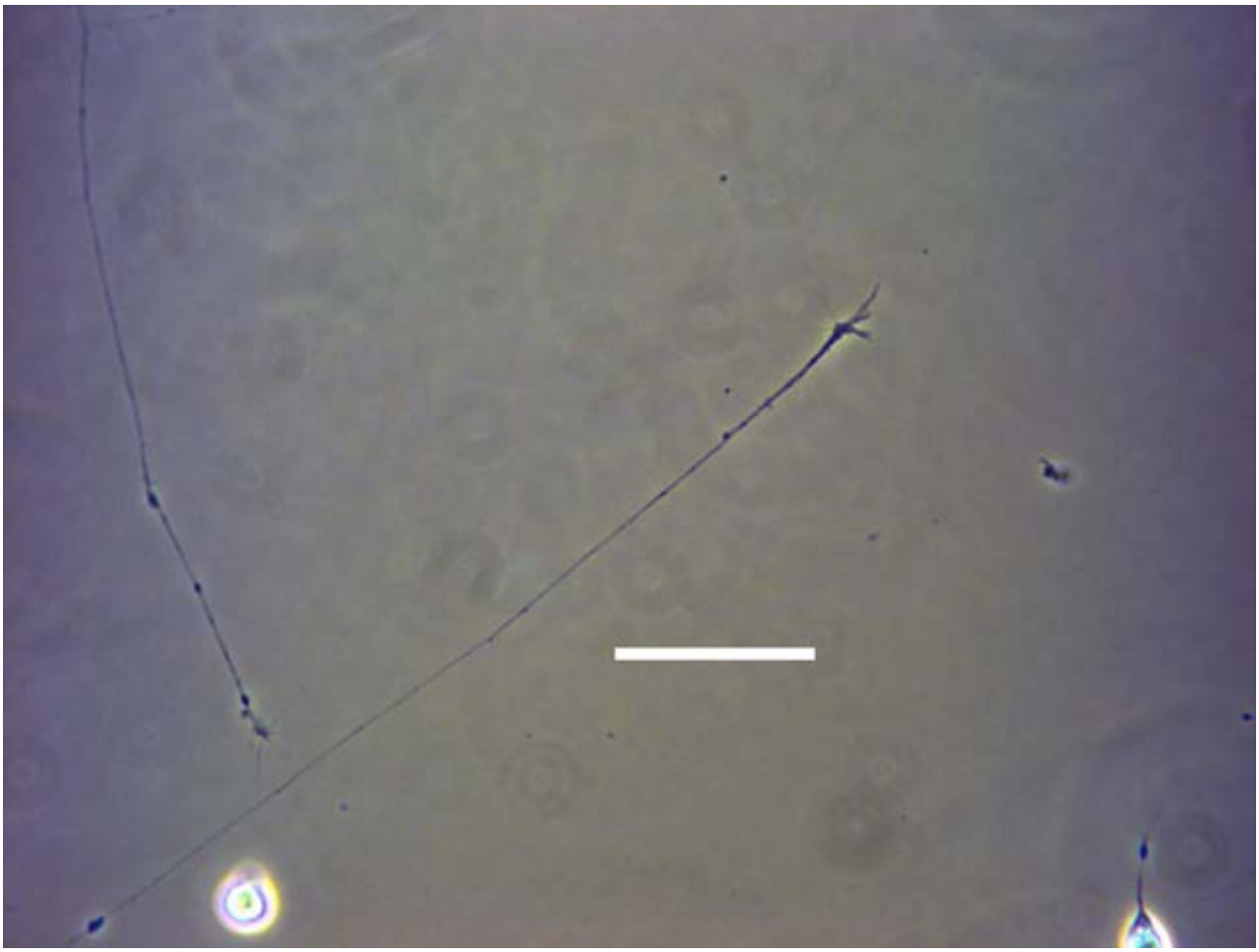

Figure 2 shows the first frame of the video of axonal association. The encroaching axon runs vertically along the left side of the shot, obviously on a path to intersect with the target axon which extends from the bottom left hand corner of the screen towards the upper right. The video for the isolated neuron would resemble figure 2 if only one of the neurons were visible. Because the length of each axon on the screen was roughly equal to the other, I was not concerned that I was seeing more of one axon and, thus, fewer filopodia. The scale bar is 50**μm.**

Figure 3

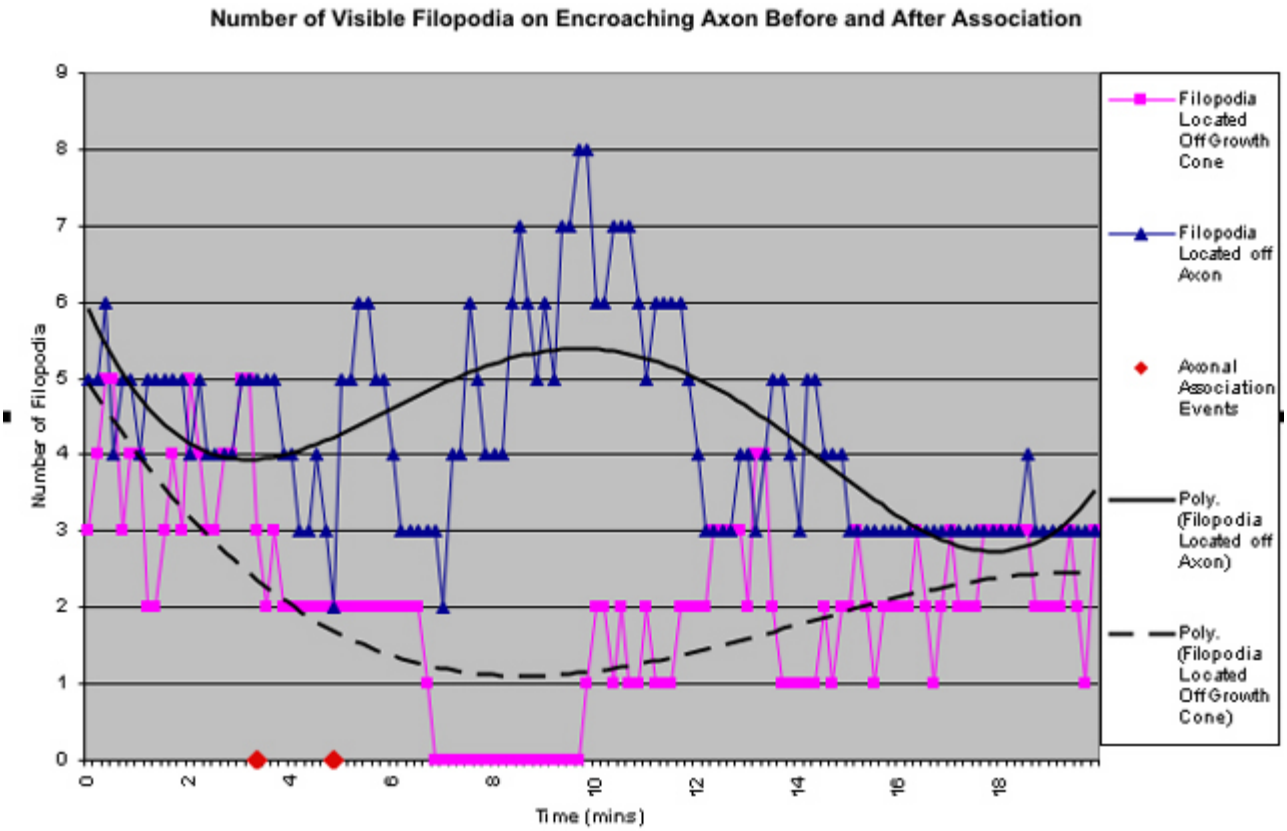

Figure 3 shows the results for the data taken from the encroaching neuron in the video which recorded axonal interaction. The average number of GCF was only 2.0 while the AF averaged 4.3. Contact between the encroaching neuron and the target neuron was made by individual filopodia at 21 frames and at 26 frames, but were still distinctly separated until 30 frames at which point separation was no longer visible.

Figure 4

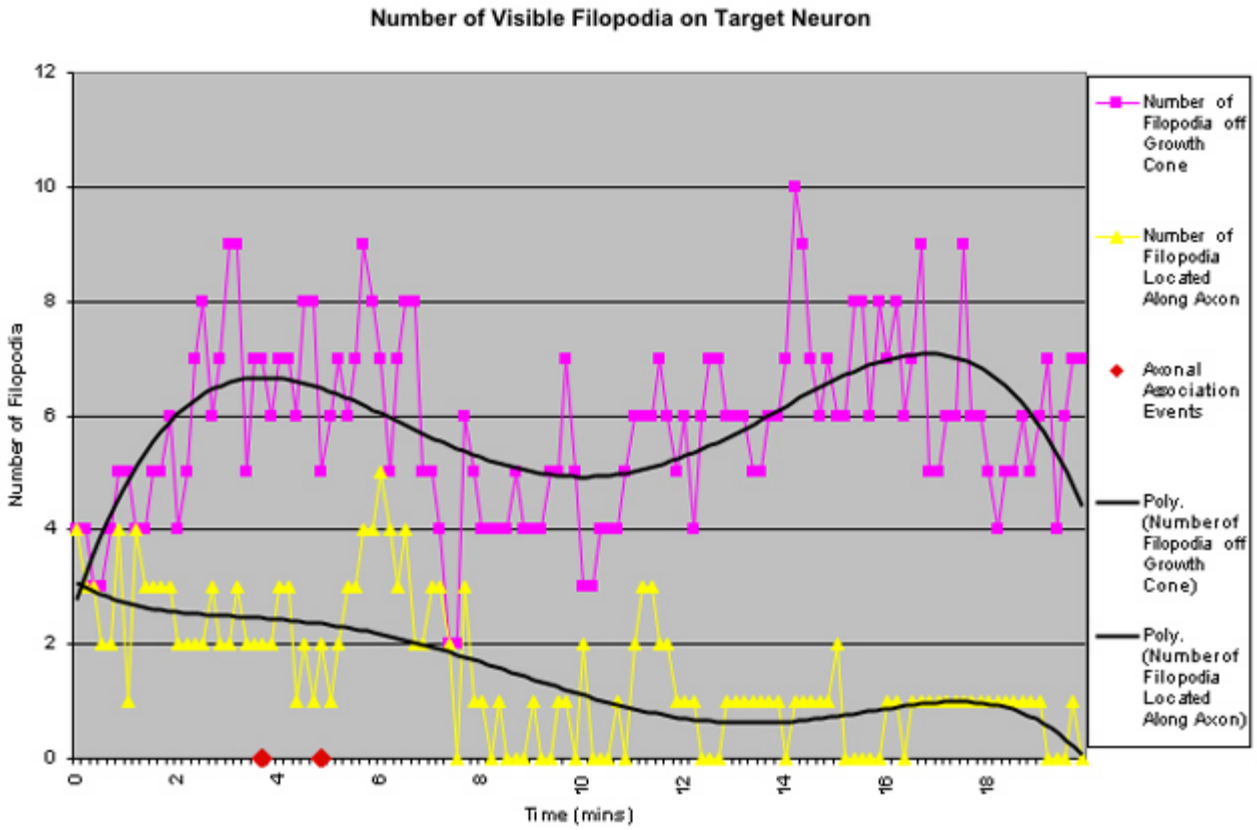

Figure 4 shows the number of AF and GCF counted on the target neuron both before and after the time of association. The average number of GCF remained 5.8 while the average number of AS was 1.5. Initial contact occurred at 21 and 26 frames, and by 30 frames the two axons were indistinguishable.

Discussion

Filopodial activity was used in this experiment as a measure of neuronal growth. While a growth cone may be relatively still, it may have very active filopodia which suggests that it is still engaged in pathfinding because the cell is still devoting energy to the restructuring of the cytoskeletal actin filaments at the terminus of the axon. If this is the case, then it is also reasonable to look at filopodial activity along the axon as that may also be a sign of active network building behavior. If axons form synapses *en passant*, then the localized endocytosis associated with synaptic activity may manifest itself as a decrease in the amount of filopodial activity in the growth cone and along the axon because of the endocytic activity associated with these pathfinding behaviors.

The isolated neuron served as our control against which we could measure the impact of neuronal interaction on filopodial activity. Over the course of 15 minutes, the growth cone of the isolated neuron generally increased in the number of filopodia present while the number of filopodia on the axon stayed consistently low. For a period of approximately 7 minutes the number of axonal filopodia drops to 0. It is unclear whether this represents a significant reallocation of energy, or whether it was simply part of the cycle of the cell. It may have even been a return to normalcy, since we do not know how long the two filopodia observed in the first frame were actually in existence. The filopodia on the axon at the beginning of the film were present until they were reabsorbed into the axon at 4.5min. When contrasted to the GCF which were frequently changing in size and arrangement by combining with one another, splitting, being broken down and built up, it shows that even though AF were present, they were not very active. In terms of the size and thickness of the filopodia, those at the growth cone were generally both longer and more sizable than those observed at the axon. All of this evidence suggests that the primary area of activity for pathfinding in a chick DRG cell is the growth cone. Yet the appearance of an AF at 28mins also suggests that there is at least some energy invested in axonal pathfinding. Because I wanted to allow the maximum room for growth cone outgrowth, the field of view was decided upon with the growth cone in the upper right hand corner of the screen growing towards the center. This limited the amount of axon visible in relation to the length observed in the video depicting interaction, and so I may have counted fewer filopodia on the isolated axon than on the associated axons.

In the video depicting neuronal interaction, the number of GCF and AF seem inversely proportional to each other. On the encroaching neuron, after association and fusion occur between 3.5mins and 4.5mins the number of AF drastically increases from an average of 4.5 to a peak of 8. The growth cone was the region of greater interest as that was the area of association. After a single GCF associated with the target axon, the number of GCF remained low, and the activity of GCF became involved only in associating and fusing the growth cone and the target axon. This suggests that once contact was made, the primary function of the GC was to solidify the connection between the two axons. GCF activity increased again as the growth cone of the encroaching axon began to move along the target axon towards the growth cone of the target neuron.

The times of the greatest AF activity occurred at times when GCF activity was least visible. Even though AF were more numerous than GCF in this case, they were still less pronounced, shorter and seemingly less dense than those at the growth cone. The AF were also less mobile than GCF. While the encroaching growth cone was fusing with the target axon, the number of AF on the encroaching axon was at its peak. This suggests that the relationship between GCF and AF activity in associated cells is similar to the inversely proportional relationship observed in the isolated neuron.

In the target neuron, GCF activity dropped by as much as 50% and by an average of about 2.5 filopodia/10sec. Yet there are similar degrees of change found in the isolated neuron so that does not signify any impact caused by neuronal association. Similarly, in regards to the AF, even though they are rare or not-visible from about 8mins on, there was a long time in which the isolated neuron did not show any AF activity. So there does not appear to be any evidence to impeded filopodial growth as a direct result of interaction with another neuron.

While these results do not explicitly support our hypothesis, neither do they negate it. We were testing to see if endocytic activity at *en passant* synapses could be recorded as a measure of decreased endocytic activity in other highly active regions of the cytoskeleton, namely growth cones and extending axons. Our results show no evidence of the formation of *en passant* synapses or any synapses at all, and so it follows that because the situation was never observed, then its significance in regards to other areas of the cell could not be measured.

We did show that endocytic activity in one region of the cell may come at the expense of such activity occurring in other regions. In our case this was manifested by GCF and AF activity being inversely proportional to one another. If this is indeed true, then it is reasonable to hypothesize that endocytic activity in the case of *en passant* synapses would impact the endocytic activity of the rest of the cell. It is unclear how much such a synapse would affect GCF or AF, and whether it would even be measurable by time lapse photography.

We may have problematized our results by choosing a red-herring situation. We were looking for synapsed neurons that were still engaged in pathfinding. It may be that the activity of a radically changing axon needed for growth cone outgrowth would directly inhibit the formation of synapses. Further research should be done to determine whether synapse formation and pathfinding can occur simultaneously.

Our results were also impacted by the physical limitations of the relationship between microscope magnification, resolution, and field of view. Because our field of view was small at a high magnification it was difficult to monitor both the soma and the axon simultaneously, so other neurons may have been interacting with either of the conditions we observed, impacting our results without our knowledge. Yet if we had used a weaker magnification the resolution would have been less clear and it would have been nearly impossible to see smaller filopodia. Before recording the isolated neuron we looked around its soma to ensure that there were no other proximal cells interacting with it, but there was no way to tell if other axons grew onto the soma or a part of the axon not in the field of view during observation.

While our hypothesis was not supported, our results still provided useful evidence for the relationship between different regions of endocytosis.

Works Cited

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