THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

CHARACTERIZING NEURAL RETICULON 1 AND DETERMINING THE MECHANISM OF MAINTAINING A DIFFUSION BARRIER IN THE AXON INITIAL SEGMENT IN DROSOPHILA MELANOGASTER NEURONS

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Abstract

Drosophila melanogaster neurons provide an excellent model to study proteins associated with neurodegeneration and regeneration. Among the families of proteins associated with axon regeneration is the general endoplasmic reticulum (ER) protein family, reticulon (rtnl). This family is conserved from Drosophila and humans. It has been implicated in limiting the regrowth of axons in the CNS after injury, but their true function, as of yet, has been undiscovered. The mammalian genome contains 4 rtnl genes, while the Drosophila genome only contains 2. Because there appears to be less redundancy in the Drosophila genome, it has been suggested that it may be easier to study rtnl proteins in Drosophila (Wolfe, 2003). In the first part of this study, I hypothesized that rtnl 1 is an ER structural protein. This is because the decrease of NogoA, an isoform of rtnl 4 in mammals, appears to allow axons to regrow past the site of injury while maintaining its shape, which is dependent upon the cytoskeleton (Schwab, 2004). I found that rtnl 1-GFP colocalizes with microtubules, and is less mobile compared to another ER protein, KDEL-GFP, and a general membrane protein, mcd8-GFP in the cell body by utilizing fluorescence recovery after photobleaching (FRAP). I also found that rtnl 1-GFP had approximately the same mobility in the axon initial segment (AIS) as it did in the cell body. Surprisingly, mcd8-GFP exhibited less mobility in the plasma membrane of the AIS compared to itself in the cell body.

The second part of the study is dedicated to elucidating the existence of a barrier to diffusion in the plasma membrane of the AIS and its mechanism. A diffusion barrier has been found in the AIS of mammalian neurons, and it has also been found that it is dependent upon the structural protein ankyrin G (ankG) (Nakada et al, 2003). A diffusion barrier has been found in the distal portion of the axon in the plasma membrane in immature Drosophila neurons, but no

barrier has been characterized in the AIS similar to that found in mammals (Katsuki et al., 2009). In this study, I found that there is a diffusion barrier in the AIS that does not appear to exist in other parts of the neuron and that the diffusion barrier is dependent on ank2, a structural protein similar to ankG.

These studies help characterize rtnl 1 in the Drosophila neural system via confocal microscopy. Moreover, a diffusion barrier is identified in the AIS and its mechanism is partially elucidated, revealing its similarity to the barrier in mammals. This shows that Drosophila neurons are more similar to mammalian neurons than previously believed, and Drosophila can be used as a better model for neural studies.

Table of Contents

Introduction

Reticulon

Reticulon (rtnl) proteins are membrane proteins that localize to the endoplasmic reticulum (ER) of the cell. They are conserved in all eukaryotes from yeast to mammals and all contain a common C-terminal domain with two long transmembrane domains and a loop between the transmembrane domains (Oertle et al., 2003). They are ubiquitous throughout the organism but are highly expressed in neurons (Huber et al., 2002).

Mammals contain four rtnl genes and there is no known molecular function for the majority of genes and isoforms. The most widely studied is an isoform of rtnl 4, NogoA. It has been observed that the introduction of antibodies to NogoA can cause regrowth of axons past the site of injury and reduce the inhibition of axon growth by myelin in vitro (Schwab, 2004). This evidence seems to point towards NogoA as a possible therapeutic target for axon injury in the central nervous system. Studies have shown that rtnl proteins may function in membrane trafficking and maintaining ER shape in non-neuronal cells, but their exact function in neurons remains unknown (Geng et al., 2005; Voeltz et al., 2006).

It has been proposed that the difficulty in studying the rtnl proteins in the mammalian nervous systems is the redundancy with other rtnl proteins (Woolf, 2003). Drosophila contain two rtnl genes, rtnl 1 and rtnl 2. Like NogoA, rtnl 1 is highly expressed in the nervous system (Wakefield and Tear, 2006). Rtnl 2 is uncharacterized. Because Drosophila contain fewer rtnl genes and can be manipulated with such a wide array of genetic tools, it may be easier to study rtnl proteins in a Drosophila model system.

The Diffusion Barrier in the Axon Initial Segment

Neurons are highly polarized cells in both mammals and invertebrates. These cells are divided into three major subcellular compartments or two active domains. Dendrites either receive signals from other neurons or the organism's environment. The cell body is where cellular processes take place, such as transcription and translation, and the axon relays the signal to the appropriate location (Figure 1). The proper function of each of these compartments in the neuron is dependent on the specific proteins present.

There are several ways that neurons can isolate proteins to these domains that help maintain the neurons proper function. Cells can keep proteins and phospholipids in a certain area by the use of cytoskeletal tethering, such as the postsynaptic protein AMPA being anchored to actin in the post-synapse in rat neurons (Allison et al., 1998). There could be transcytosis that moves proteins and lipids into or out of areas of the cell. An example of this is the insertion of the membrane protein NgCAM in the somatodendritic membrane, which then accumulates in the axon plasma membrane by transcytosis (Wisco et al., 2003). Lastly, a diffusion barrier could exist that prevents diffusion across a specific point in the cell, similar to that of a tight junction in epithelial cells (Winckler et al., 1999).

Diffusion barriers have been implicated in restricting proteins from entering the axon from the cell body and vice versa in mammalian neurons (Winckler et al., 1999, Nakada et al., 2003). It has also been shown that rat hippocampal neurons form a diffusion barrier in the cytoplasm that prevents large macromolecules from entering the axon (Song et al., 2009). In vertebrate axons, the AIS is characterized by high concentrations of voltage-gated Na+ and K+ channels in the most proximal portion of the axon (Kole et al., 2008; Kole et al., 2007; Goldberg et al., 2006). The high concentration of voltage gated channels in the initial portion of the axon

has been found to be typically 35-45 μm in length (Hu et al., 2009; Yang et al., 2007). Neurons intrinsically assemble an AIS without external cues from glial cells (Ogawa et al., 2007). The major organizing protein of the AIS diffusion barrier is ankyrin G (ank G) (Kordeli et al., 1995).

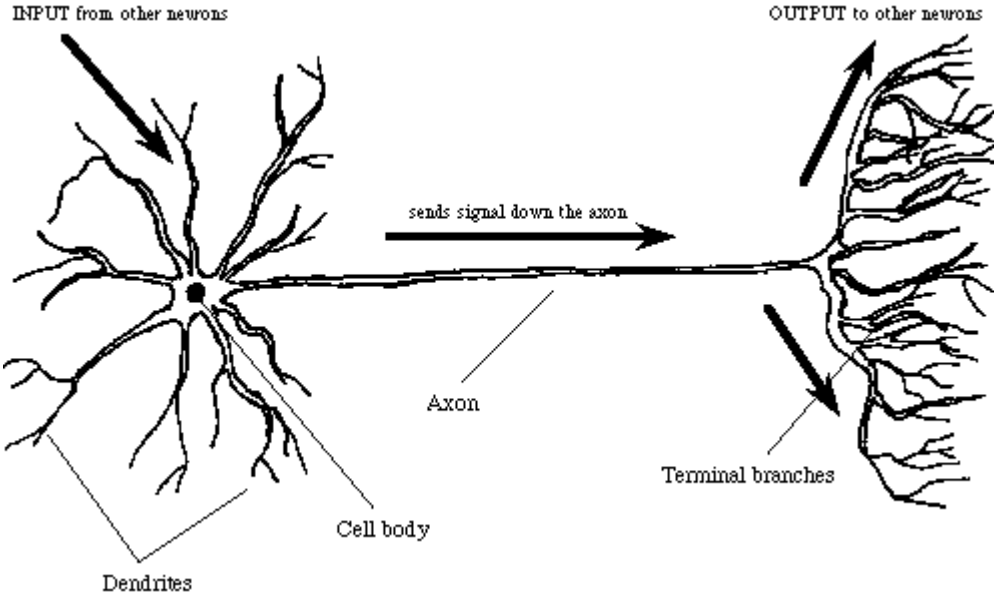
Ank is vital to the proper function of the cytoskeleton and helps to anchor proteins to the cytoskeleton via intermediary proteins, such as spectrin (Figure 2). Vertebrates contain 2 genes that encode ank B and ank G. It has been shown that ank G is localized in the AIS and the accumulation of voltage-dependent channels as well as the initiation of the action potential is dependent on the presence of ank G (Zhou et al., 1998). It also has been proposed that membrane proteins anchored to ank G create a fence in the AIS which prevents the diffusion of proteins across the AIS (Nakada et al., 2003).

Drosophila have two ank genes as well, ank 1 and ank 2. Ank 1 is ubiquitously expressed, whereas ank 2 is neuron specific. It has been shown that in Drosophila the decreased expression of ank 2 affects axon morphology as well as the stability of presynaptic microtubules (Yamamoto et al., 2006; Pielage et al., 2008). Recently it was shown that developing Drosophila neurons contain an intrinsic barrier in the axon which isolates axon guidance receptors to different domains within the axon, but as of yet it has not been shown whether Drosophila contain a barrier in the proximal axon similar to that in vertebrates (Katsuki et al., 2009).

Drosophila melanogaster as an Experimental Model

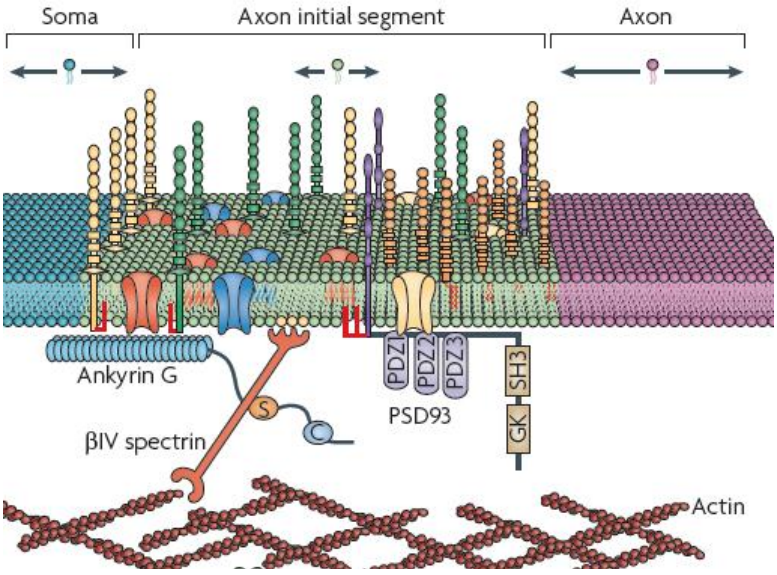
Drosophila melanogaster is used for research because it has a short life cycle, is maintained easily, and can be manipulated with many powerful genetic tools. More specifically, it is good for neural research because the neurons are easily accessed due to the larvae being transparent. *In vivo* research can also be done easily in Drosophila, which allows for greater versatility in studies.

Figure 1. **Illustration of neuron structure**. Sensory input to the neuron enters through the dendrites and passes through the cell body. The signal is then sent down the axon into the terminal branches to the other neurons or other target cells.



(Source: http://biomed.brown.edu/Courses/BI108/BI108_2001_Groups/Nerve_Regeneration/)

Figure 2. **Schematic of a vertebrate axon initial segment**. Ank G binds to βIV spectrin which then binds to the actin cytoskeleton. Ank G causes a high density of specific membrane proteins to accumulate in the AIS, which then reduces other proteins' mobility within the AIS.



(Source: The axon initial segment and the maintenance of neuronal polarity)

Moreover, many Drosophila proteins and features of neuron structure are conserved between flies and humans. This allows for results found in Drosophila to be extrapolated to vertebrates and humans. Among the features shared by Drosophila and mammals is the microtubule orientation within axons. It has been found that both mammals and Drosophila compartmentalize the neuron in a similar fashion by having uniformly oriented microtubules that are plus end out, toward the distal end of the axon (Rolls et al., 2007). Also as it has already been stated, rtnl and ank proteins contain shared regions in the amino acid sequence, which suggests similar functions. Thus, the easy manipulation of Drosophila and the conservation of both neural structure and proteins make it an ideal candidate to do neural research.

A specific type of neuron that is easily studied is the dorsal dendritic arborization (dda) neuron. These neurons are sensory multipolar neurons found on the dorsal side of larvae just beneath the cuticle. Their superficial position allows them to be easily imaged live using confocal microscopy (Anderson et al., 2005). These neurons are classified by the extent of dendritic branching, with class I being the least branched and class IV being the most branched (Grueber et al., 2002). Because these neurons are easily found and classified, they are optimal to study neural structure.

Drosophila have been invaluable in the discovery of therapeutic targets to help research into neurodegenerative diseases. One of these diseases is Parkinson's disease (PD). It was found that mutations in DJ-1α, DJ-1β, among other proteins that are homologous to proteins associated with PD in humans produced PD associated phenotypes (Meulener et al., 2005). This study among others elucidated possible therapeutic targets and illustrates Drosophila's potential in neural research.

Overview of Study

Based on the previous evidence that mouse axons regrow after injury when NogoA is inhibited, it has been hypothesized that rtnl proteins are structural proteins. It is also believed that this function is conserved from Drosophila to humans because of the many conserved regions in the rtnl structure and amino acid sequence. To gather evidence for this, rtnl 1's location within neurons was examined to see if it was possible for rtnl to function as a structural protein. In this experiment, microtubules were marked with cherry-zeus, which is a red fluorescent protein (RFP) tagged to a microtubule associated protein (MAP), and rtnl 1 was tagged to a green fluorescent protein (GFP). Rtnl 1-GFP and cherry-zeus were coexpressed in Drosophila neurons *in vivo* to see if they colocalized. Proteins involved in maintaining structure usually follow along microtubules and if rtnl 1 has a structural function it should also localize to the same areas as the microtubules.

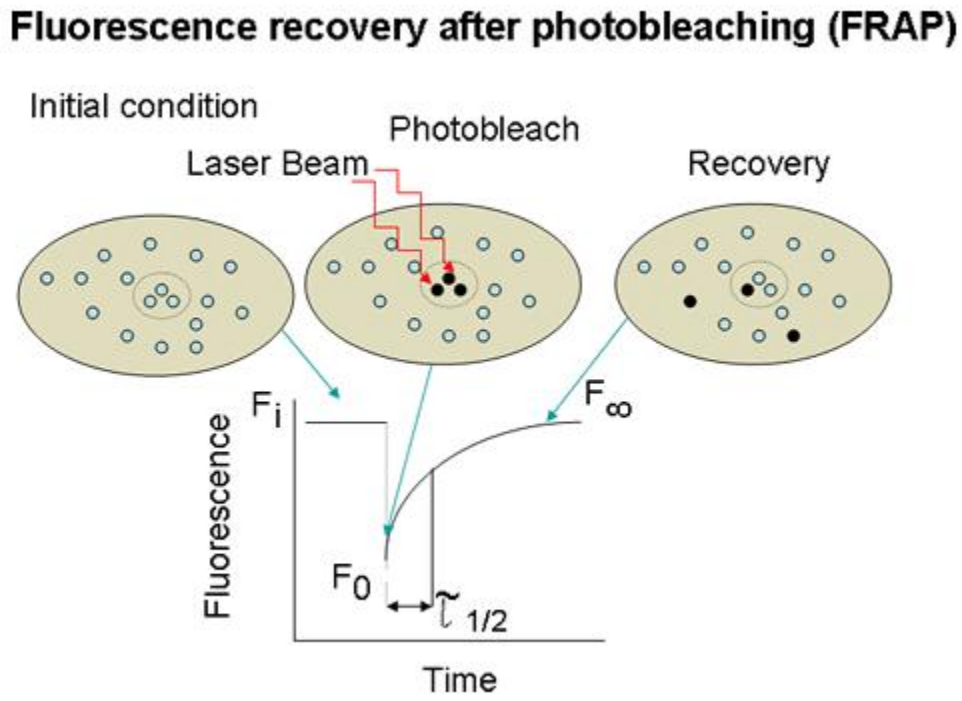
Moreover, structural proteins are usually less mobile within membranes because of their association with microtubules. Showing this would also provide evidence that rtnl 1 is a structural protein. To examine this, the mobility of rtnl 1 was measured in both the cell body and the axon *in vivo* in Drosophila larvae neurons. This was then compared to the mobility of another ER protein, KDEL, and a plasma membrane protein, mcd8, in the same system. Each of these proteins was tagged with a GFP to see their location and measure their diffusion via fluorescence recovery after photobleaching. If rtnl 1 is a structural protein, then it should be less mobile compared to the other proteins because of its association with the cytoskeleton. Thus, I hypothesize that reticulon will be less mobile in both the cell body and axon than the control protein.

The AIS has been greatly studied in vertebrate neurons, but little research has focused on invertebrate neurons. Currently it is believed that in invertebrates, such as D. melanogaster, there is a barrier in a distal portion of developing axons and is an evolutionary stepping stone towards a diffusion barrier in the AIS. However, there is no known diffusion barrier in the AIS of mature invertebrate neurons (Reviewed in Rasband, 2010). I hypothesize that a diffusion barrier in the AIS does exist in mature Drosophila neurons and the mechanism that maintains it is similar to that found in mammals. To determine if a diffusion barrier is present, the mobility of a transmembrane protein was analyzed in different parts of the neuron, also via florescence recovery after photobleaching. The mechanism used to maintain the barrier was tested by performing the same analysis while lowering levels of a candidate protein via the introduction of RNA hairpins.

Fluorescence Recovery After Photobleaching

Throughout this study, the mobility of proteins will be determined and compared. The mobility of proteins within the membranes will be measured using the method of Fluorescence recovery after photobleaching (FRAP). The process of FRAP consists of three parts. First, the intensity of the GFP signal in the neuron is measured to get a base line reference. Then a high intensity laser is focused on a specified area within the neuron. This bleaches out the specified area and turns it black without affecting the surrounding area, which remains green. Finally, the intensity of the area is recorded over time. Because the signal is destroyed in the specified area, any recovery of the signal is due to the movement of unbleached proteins into the area (Figure 3).

Figure 3. **FRAP Overview**. (Top) An illustration of the FRAP process. Molecules tagged with GFP are represented as light colored filled in circles and bleached GFP bound molecules are represented as black circles. After the area is bleached, unbleached molecules eventually diffuse into the area. (Bottom) Graphical representation of FRAP. The initial reading corresponds to the Fi and the initial bleached measurement corresponds to F0. The fluorescence recovers to point F∞ over the course of the measured time period.



(Source: http://www.cellmigration.org/resource/imaging/imaging_approaches_photomanipulation.shtml)

Materials and Methods

Fly Stocks and Crosses

Drosophila lines were maintained at 25^{o}C in standard Drosophila media. In order to visualize rtnl 1 location and mobility, the trap line rtnl1-GFP on 2; elav-Gal4 on 3 was used. The line used to visualize microtubules was W^{-}; pUAST-cherry-zeus. The diffusion of rtnl 1-GFP was then compared to that of the elav-Gal4; mcd8-GFP and the progeny of the cross W*; pUAS-GFP:KDEL}15.2/TM6, tb (KDEL-GFP) and elav-Gal4 on 3 via FRAP analysis. The fly lines used to study and structure of the diffusion barrier in the AIS were the progeny of the cross of Dicer2; 221 Gal4 mcd8 GFP/ TM6 and either ank 2 RNAi on 2 (VDRC #107369 kk), or rtnl2 RNAi on 2 (33320). To prepare for crosses, virgin female Dicer2; 221 Gal4 mcd8 GFP/ TM6 flies were collected for the diffusion barrier experiment. These virgins were collected after allowing the line to incubate at room temperature for 6 hours or at 18^{o}C for 12 hours. The virgin females were then crossed to males of one of the RNAi lines at a time. Once every 24 hours the larvae were collected on a food cap and allowed to age for 3 days at 25^{o}C. Once the larvae were aged, they were imaged by confocal microscopy. The same cross procedure was used to prepare the progeny of the elav-Gal4 on 3 and KDEL-GFP, and elav-Gal4 virgin flies were collected.

Confocal Microscopy

To view Drosophila larvae, whole third instar larvae were placed in Schneider's media and then on a dried agarose pad on a glass slide. The larvae were positioned dorsal side up, and a cover slip was placed on top of the larvae, which was secured to the slide via scotch tape. The larvae were viewed immediately after mounting on a Zeiss LSM 510 microscope. Videos were taken using a 63x oil objective lens.

FRAP Analysis

FRAP was performed using the Zeiss LSM 510 microscope under the 63x oil objective. A bleach scan with the laser at full power with a time frame of 2 was used for all experiments where the mobility of either mcd8-GFP line was used. To study the mobility of rtnl 1-GFP, a time frame of 5 seconds was used. A time frame of 0.2 milliseconds was used when measuring the mobility of KDEL-GFP. Quantification of all videos was done using the Image J program. The intensity of a 1 µm x1 µm area of the bleached region was measured for all time frames using the Time Series Analyzer plugin for Image J. This method was used to compare the mobility of rtnl 1-GFP, mcd8-GFP, and KDEL-GFP in the cell body and rtnl 1-GFP and mcd8-GFP in the AIS. To compare the recovery rates of mcd8-GFP with the RNAi lines, the intensities were normalized using the following method. After the area in the bleached region was measured, an area of the same size was also measured for all time frames in the unbleached distal axon. The intensity measurement of the bleached region when it was initially bleached was then subtracted from the values of the bleach and unbleached intensity measurements for all time frames. The ratio of the resulting values of the bleached to unbleached regions was then determined for each time frame. This value was then divided by the prebleach ratio. The ratios that corresponded to the axon being in focus were graphed over time. This method was used to correct for the bleaching effect of the laser and compare the extent to which the mcd8-GFP recovered in each neuron.

Results

Rtnl 1-GFP colocalizes with microtubules in the axon and dendrite branches

Many structural proteins are located along microtubules inside the cell because their function is to help maintain the microtubules. If rtnl 1 was a structural protein, it may also be located along microtubules. To examine this, microtubules were visualized by using a red fluorescently tagged MAP (zeus-RFP) and rtnl 1 was visualized by utilizing rtnl 1-GFP. These proteins were then expressed together in neurons.

When this was done, it was found that rtnl-1 GFP and the zeus-RFP colocalize in both the axons and dendrite branches. Images were analyzed qualitatively by visually checking if rtnl 1-GFP was located in the same regions of the cells as zeus-RFP. All axon bundles that were imaged showed that rtnl 1-GFP followed along the microtubules (n=5) (Figure 4). Also it was shown that in all dendrite branches that were imaged, rtnl 1-GFP was located along microtubules (n=5). This provides evidence that rtnl 1 is a structural protein because it colocalizes with microtubules in both axons and dendrite branches, which are regions where microtubules converge and split, respectively.

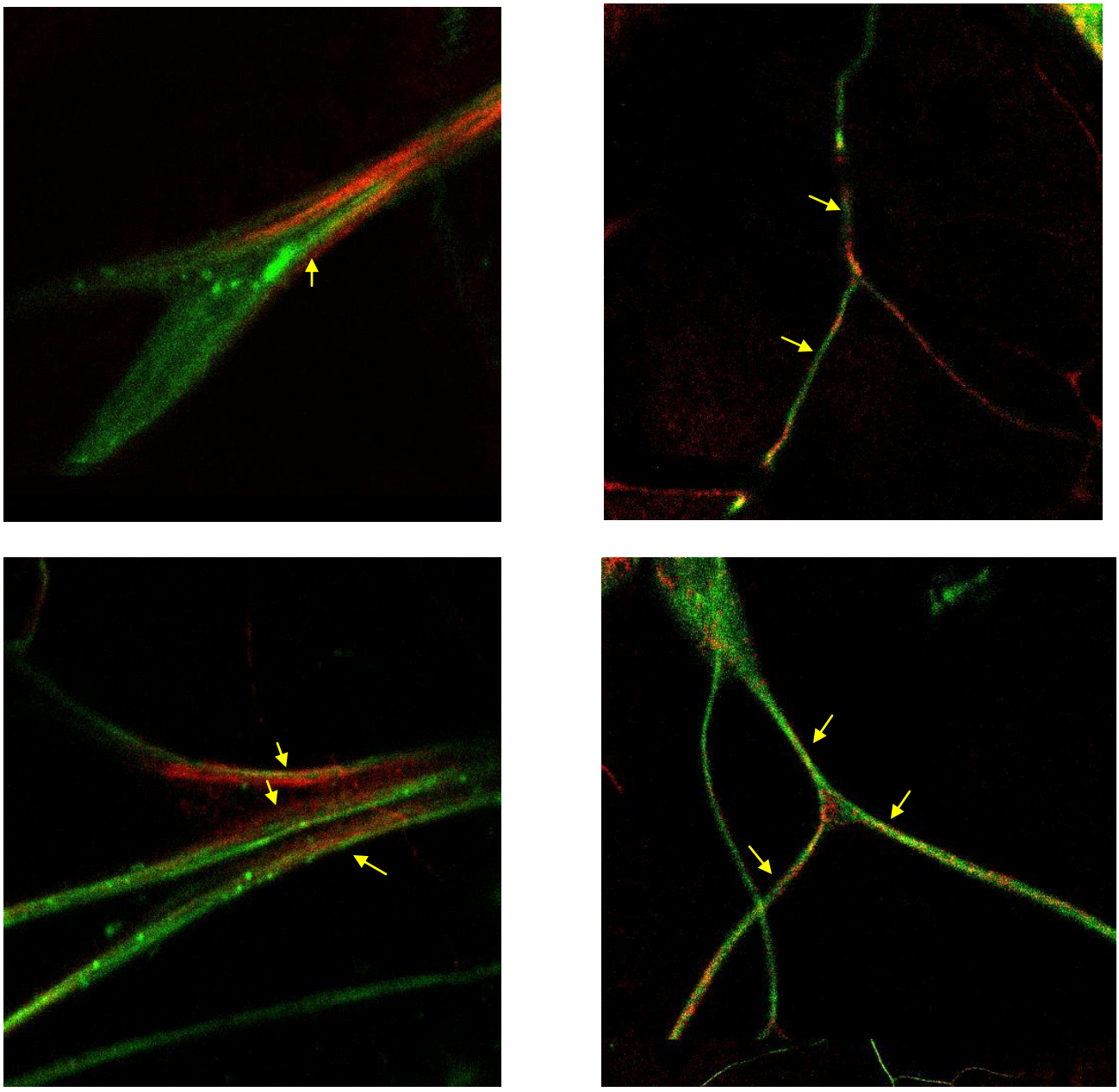
Rtnl 1-GFP is less mobile in the cell body ER compared to another ER protein and a general membrane protein

To further show that rtnl 1 may be a structural protein, its rate of diffusion was compared to the diffusion of other membrane proteins and hypothesized to be slower. Since structural proteins are associated with microtubules, they tend to be more stable and less mobile than other membrane proteins (Dr. Rolls, Personal communication). This was examined by using FRAP to show the rate of diffusion of rtnl 1-GFP, KDEL-GFP, and mcd8-GFP. KDEL-GFP is an ER protein involved in ER/Golgi trafficking that was tagged with GFP and was predicted to

Figure 4. **Colocalization of rtnl 1-GFP and zeus-RFP**. Rtnl 1 is shown in green and zeus, a MAP, is shown in red. Rtnl 1-GFP and zeus-RFP exist in the same regions of the neuron. Arrows point to where rtnl 1-GFP and zeus-RFP show the greatest colocalization.

Axon Bundles

Dendrite Branches



Rtnl 1-GFP

Zeus-RFP

diffuse the fastest of the 3 proteins. Mcd8-GFP is a general membrane protein from mammals, which was inserted into the Drosophila genome to tag all membranes and believed to diffuse the second fastest.

After FRAP was conducted in the cell body and the intensities graphed without being normalized, it was observed that rtnl 1-GFP did in fact diffuse at a slower rate compared to KDEL-GFP and mcd8-GFP (Figure 5). Rtnl 1-GFP recovered to its maximal recovery value in an average of 50 seconds while mcd8-GFP recovered in about 30 seconds on average and KDEL-GFP recovered in approximately 1 second on average (Table 1). This shows that rtnl 1-GFP was the least mobile of the membrane proteins tested. This also suggests that rtnl 1 could be a structural protein because its location is the most stable of the proteins tested.

Table 1. Recovery times of rtnl 1-GFP, mcd8-GFP, and KDEL-GFP

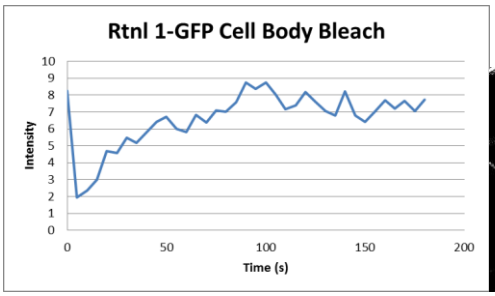
Fluorescent Protein	**n**	**Range of recovery times (s)**	**Average recovery time (s)**
Rtnl 1-GFP	5	35-75	50
Mcd8-GFP	5	18-40	30
KDEL-GFP	5	1-3	2

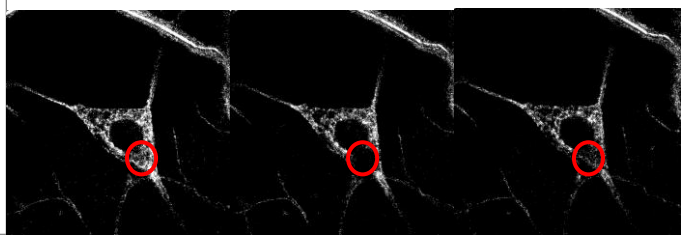
Rtnl 1-GFP exhibits the same diffusion rate in the AIS compared to the cell body, but mcd8-GFP diffuses to a lesser extent in the AIS

Previous research has shown that a diffusion barrier exists in the plasma membrane in the AIS of mammalian neurons. It has also been proved that this diffusion barrier is dependent on structural proteins. Furthermore, our unpublished results show that KDEL-GFP, an ER protein like rtnl 1-GFP, is prevented from entering the axon by some unknown mechanism. Based on this information, I hypothesized that rtnl 1-GFP would be less mobile in the AIS than it was in the cell body and mcd8-GFP would be unaffected. I hypothesized this because if rtnl 1-GFP was

Figure 5. **FRAP analysis of rtnl 1-GFP, mcd8-GFP, and KDEL-GFP**. A corresponds to the FRAP experiment of rtnl 1-GFP in the cell body. B corresponds to the FRAP experiment of mcd8-GFP in the cell body, and C corresponds to the FRAP experiment of KDEL-GFP in the cell body. Red Circles indicate the bleach area.

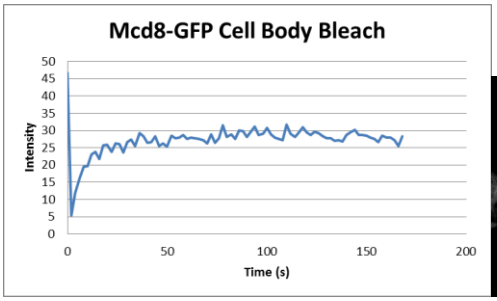
A.

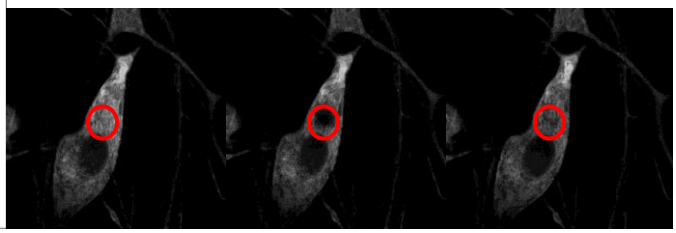




Pre-Bleach Post-Bleach 50 Sec Post-Bleach

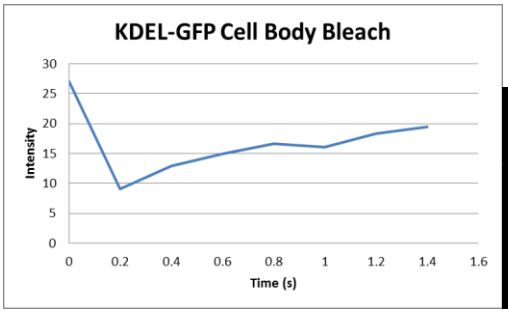
B.

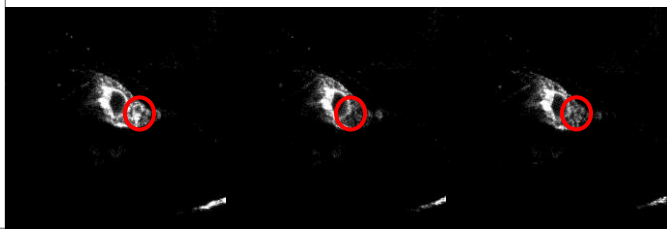




Pre-Bleach Post-Bleach 30 Sec Post Bleach

C.





Pre-Bleach Post-Bleach 1 Sec Post Bleach

a structural protein and present in the ER, it could be what is causing a barrier to KDEL-GFP from entering the axon where rtnl 1-GFP is able to enter the axon.

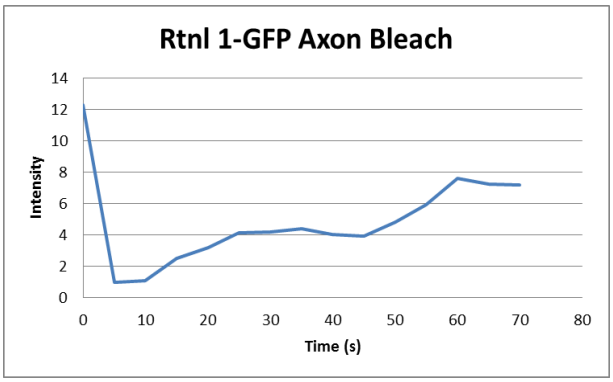
When FRAP was done in the AIS, rtnl1-GFP recovered at approximately the same rate as it did in the cell body. Rtnl1-GFP recovered to it maximum level in approximately 60 seconds (n=5) (Figure 6A). When mcd8-GFP was tested, it was found that 2 of the 5 neurons imaged showed a decreased diffusion maximum compared to the other neurons imaged (Figure 6B). The mcd8-GFP did not recover to anywhere near the same level in those 2 neurons as the mcd8-GFP measured in the cell body in the same time span in the other neurons. This suggests that rtnl 1-GFP is not involved in an ER diffusion barrier because there was no evidence of decreased mobility in the AIS. Surprisingly, the data does suggest that there may be a diffusion barrier in the plasma membrane of the AIS. Because mcd8-GFP displays decreased diffusion in the AIS compared to the cell body, there may be some mechanism that is inhibiting the diffusion of mcd8-GFP in that area.

Mcd8-GFP recovers almost completely in all regions of the neuron except the AIS in ddaE neurons

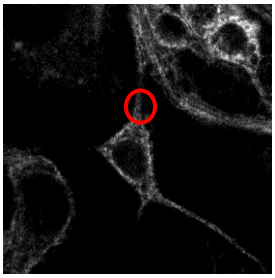
Mcd8-GFP is located in both the plasma membrane and the ER in the cell body but is only located in the plasma membrane in the axon and dendrites (our unpublished results). The previous experiment suggested that mcd8-GFP is less mobile in the plasma membrane of the AIS compared to its mobility in the ER and plasma membrane in the cell body. To determine if mcd8-GFP is in fact subject to a diffusion barrier in the AIS and this is not some inherent property of the plasma membrane in neurons, FRAP was conducted on mcd8-GFP and normalized for the bleaching effect of the laser. This was done so that the relative recovery of mcd8-GFP could be determined over a given amount of time, which shows how mobile mcd8-

Figure 6. **FRAP analysis of rtnl 1-GFP and mcd8-GFP in the AIS**. A corresponds to the FRAP of rtnl 1-GFP in the AIS, and B corresponds to FRAP of mcd8-GFP in the AIS. Red circles indicate the bleach area.

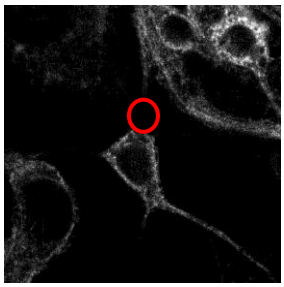
A.



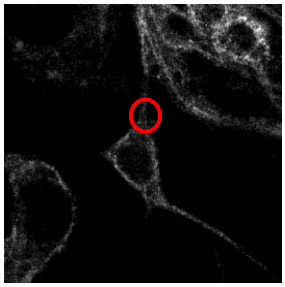
Pre-Bleach



Post-Bleach



65 Seconds Post-Bleach



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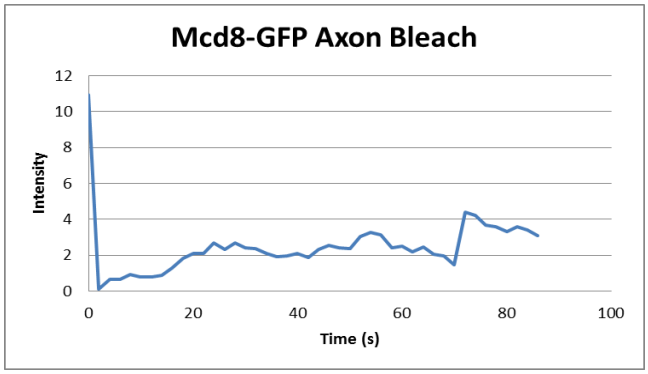
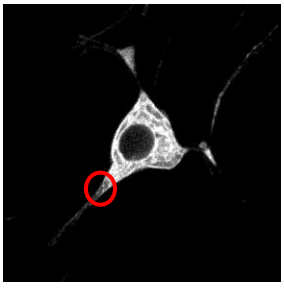
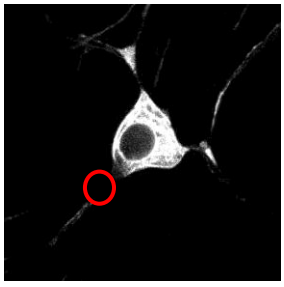


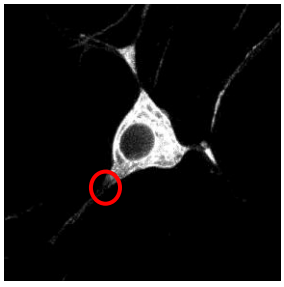
Pre-Bleach



Post-Bleach



30 Seconds Post-Bleach



GFP is in that region of the cell. Moreover, this was done specifically in ddaE neurons to control for any natural differences between neurons by using the 221-Gal4 driver. The areas measured were the cell body, dendrite branch, and initial dendrite trunk. These areas were chosen to reveal the extent mcd8-GFP recovers in different areas of the neuron.

My results show that mcd8-GFP recovers to 100% in one minute in the cell body and in the dendrite branches in 100% of the images (Figure 7A, 7C). There was a population size of 8 for cell body bleaches and 4 for dendrite branch bleaches. In the initial portion of the dendrite trunk, mcd8-GFP recovered to above 80% in 85% of the dendrites imaged (Figure 7B) (n=9). Of the one that did not recover to 80%, it recovered to 60%. These values show that mcd8-GFP is able to recover almost completely throughout the cell, which was not observed in the AIS.

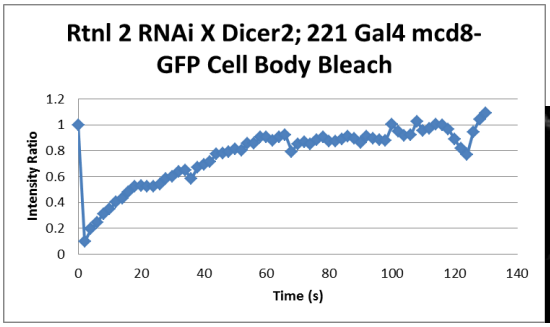
This data suggests that the plasma membrane in the AIS has a property that is inherent to it and not to any other part of the plasma membrane. Mcd8-GFP is not able to diffuse as rapidly in the plasma membrane in the AIS compared to the other regions of the cell. There appears to be some barrier in the AIS plasma membrane preventing mcd8-GFP from diffusing as easily as it is able to in the rest of the plasma membrane.

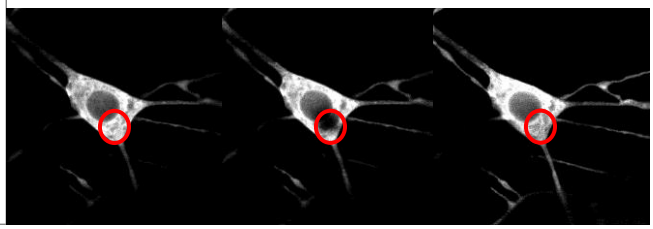
The diffusion barrier in the AIS of ddaE neurons is ank2 dependent

Research in mammalian neurons has shown that a diffusion barrier exists in the AIS and that the barrier is dependent on ankG to maintain it (Nakada et al., 2003). The previous experiment in this study has shown that mcd8-GFP is subject to a barrier to diffusion in the same region, but the mechanism behind this barrier is unknown. Since the barrier observed in the diffusion of mcd8-GFP is also in the AIS and Drosophila contain ank2, a protein with similar properties to that of ankG, I hypothesize that the barrier found in Drosophila will be ank2 dependent.

Figure 7. **Normalized FRAP analysis of mcd8-GFP in the cell body, initial dendrite trunk, and dendrite branch**. A corresponds to the normalized FRAP of mcd8-GFP in the cell body. B corresponds to the normalized dendrite bleach, and C corresponds to the normalized dendrite branch bleach. Red circles indicate the bleach areas.

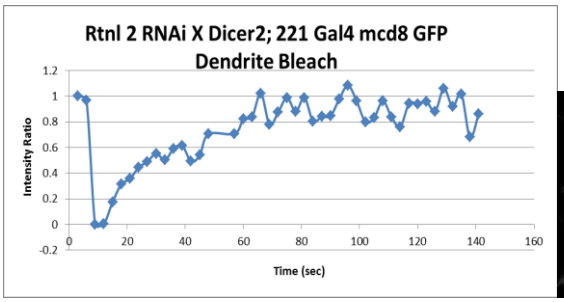
A.

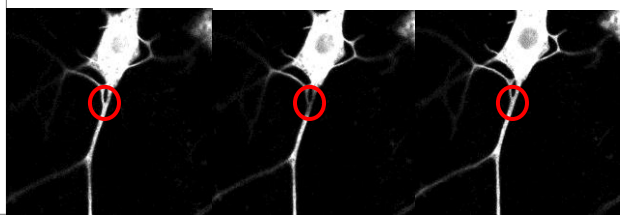




Pre-Bleach Post-Bleach 50 Sec Post-Bleach

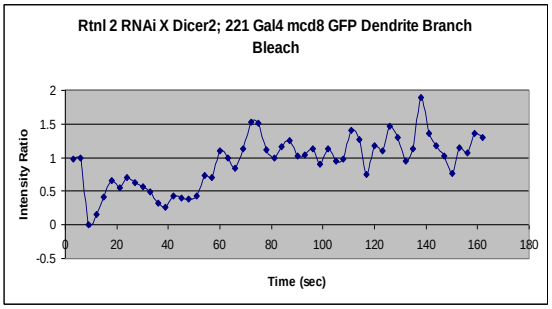
B.

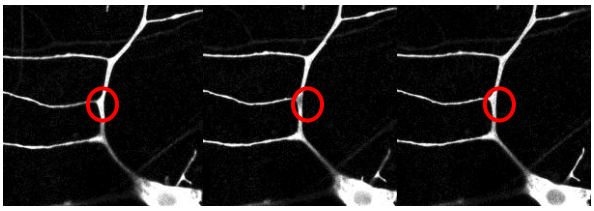




Pre-Bleach Post-Bleach 50 Sec Post-Bleach

C.





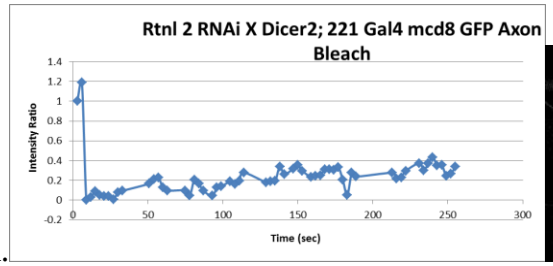
Pre-Bleach Post-Bleach 50 Sec Post-Bleach

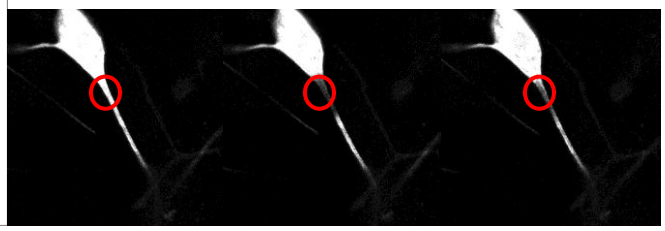
FRAP was conducted in the AIS of ddaE neurons of Drosophila larvae expressing mcd8-GFP and either rtnl2 RNAi or ank2 RNAi. Rtnl2 RNAi was used as a negative control to ensure the RNAi did not affect the diffusion barrier. These intensities were normalized and graphed to show the relative recovery rates and to see if the ank2 RNAi did decrease the effect of the diffusion barrier.

Larvae expressing the control rtnl2 RNAi showed recovery of only 20-30% at one minute in 81% of those imaged (Figure 8A) (n=11). The larvae expressing ank2 RNAi showed 30% recovery in 37% of the larvae imaged (Figure 8B) (n=11). This is significant at a level of $p<.05$ based on a one-tail t test. This data suggests that there is a diffusion barrier in the AIS and it is ank2. This shows that the depletion of ank2 caused a significant increase in mcd8-GFP's mobility in the plasma membrane of the AIS. It is also interesting to note that of the 11 larvae imaged 2 had extensions from the axon, which was never found in the control rtnl 2 RNAi, and the AIS of these neurons recovered past 30% (Figure 8C).

Figure 8. **Normalized FRAP analysis of mcd8-GFP in the AIS expressing rtnl2 RNAi or Ank2 RNAi.** A corresponds the normalized bleach of mcd8-GFP with rtnl 2 RNAi in the AIS. B corresponds to the normalized bleach of mcd8-GFP with ank2 RNAi in the AIS, and C corresponds to normalized bleach of mcd8-GFP with ank2 RNAi with an extension from the axon. Red circles indicate the bleach area.

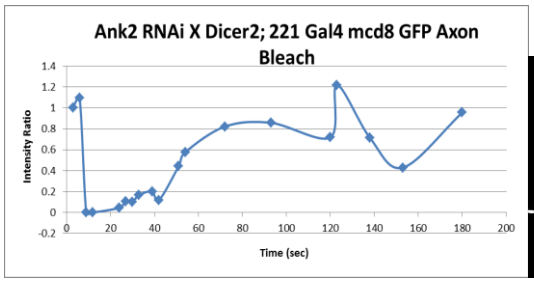
A.

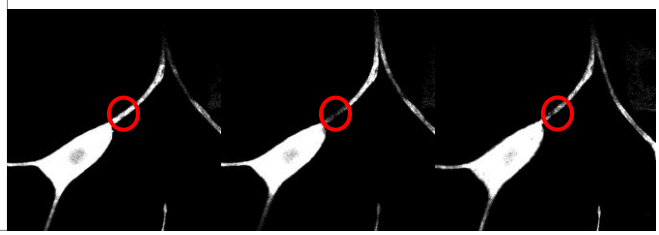




Pre-Bleach Post-Bleach 50 Sec Post-Bleach

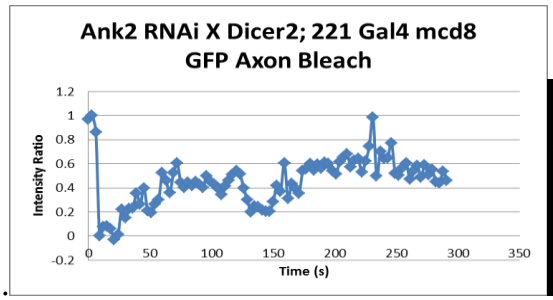
B.

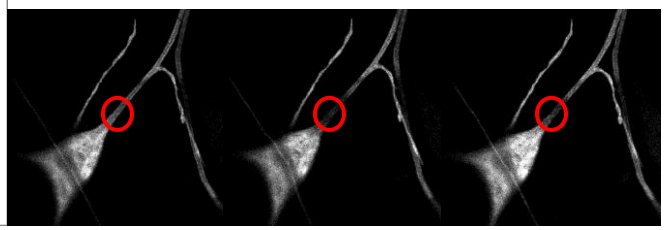




Pre-Bleach Post-Bleach 50 Sec Post-Bleach

C.





Pre-Bleach Post-Bleach 60 Sec Post-Bleach

Discussion

In this study, I first sought to determine if rtnl 1 could be a structural protein in Drosophila neurons, and then I sought to find the mechanism by which a diffusion barrier was maintained in the plasma membrane of the AIS. Rtnl proteins have been implicated in inhibiting axon regrowth after injury, but otherwise many of the isoforms of rtnl in mammals and Drosophila remain relatively uncharacterized (Schwab, 2004). Rtnl 1-GFP was characterized by colocalization and FRAP in this study. In the process of attempting to characterize rtnl 1-GFP, I found that the plasma membrane protein that was used as a comparison to rtnl 1-GFP in mobility was actually less mobile in the AIS compared to the cell body. This prompted me to examine this further by the use of FRAP, which had to be normalized to account for the bleaching out effect the laser has on the fluorescent protein over time.

Rtnl 1 has no known function in Drosophila neurons. Because rtnl proteins are conserved by evolution from Drosophila to humans, it stands to say that rtnl proteins should serve a function, and consequently rtnl 1 should serve a function. I attempted to find this function in Drosophila because the Drosophila genome only contains 2 genes of rtnl proteins while the mammalian genome contains 4 genes (Wolfe, 2003). This makes Drosophila an ideal candidate to study the function of rtnl, which we believe to be structural due to its effect on axon regrowth after injury.

The first part of this study was dedicated to characterizing rtnl 1-GFP in neural cells to gather data suggesting whether rtnl 1-GFP could function as a structural protein. This was done via confocal microscopy and FRAP analysis. In this portion of the study, I assumed that the addition of the GFP did not affect the functionality of rtnl 1. This of course was not known because the true function of rtnl 1 is unknown, and thus this could have affected the results

obtained in the experiments involving rtnl 1-GFP. When looking through various types of neurons in Drosophila larvae, I found that rtnl 1-GFP did appear to track along microtubules in the dendrite branches and the axons. These areas were specifically looked at because they are areas were the microtubules diverge and converge. Microtubules also diverge and converge in the cell body, but zeus-RFP does not allow for the resolution necessary to observe this in the cell body. Moreover, it was found that rtnl 1-GFP was the least mobile of the membrane proteins tested in the cell body. This data appears to suggest that rtnl 1 could function as a structural protein in neural cells because rtnl 1-GFP appears to follow microtubules and has a more stable position than other membrane proteins.

In the process of measuring the mobility of KDEL-GFP, I realized that KDEL is not present in the dendrites or the axon of the neurons. A barrier has not been found in the ER, but there is evidence of a diffusion barrier in the plasma membrane of the AIS in mammalian neurons (Nakada et al., 2003, Winckler et al., 1999). If rtnl 1-GFP was a structural protein, it could be what was preventing KDEL-GFP from entering the ER in the axon just as ankG prevents proteins from diffusing across the AIS in mammalian neurons. The mobility of mcd8-GFP was also measured in the AIS as a negative control to ensure that there was no difference in the membrane that may affect diffusion. Contrary to my hypothesis, I found that 2 out of the 5 neurons imaged showed decreased diffusion in the AIS where rtnl 1-GFP showed consistent diffusion. This data suggests that there is a difference in the plasma membrane in the AIS.

Because mcd8-GFP is located in just the plasma membrane in the axons and dendrites and in both the plasma membrane and ER in the cell body, I needed to check if this diffusion restriction was not due to just mcd8-GFP's presence in the ER in the cell body. Mcd8-GFP diffusion was measured via normalized FRAP in the dendrite, dendrite branch, and cell body in a

single cell type. DdaE neurons were chosen because they are easy to locate and had been previously characterized (Grueber et al., 2002). I found that mcd8-GFP recovered to either 100% or close to 100% in each of these areas. This data shows that mcd8-GFP is capable of near full recovery throughout the neuron, which was not observed in the AIS. This experiment was done with rtnl 2 RNAi. As mentioned before, rtnl 2 is uncharacterized. Because rtnl 2 is uncharacterized, I assumed that the depletion of rtnl 2 should not have an effect on the cells.

Finally, I attempted to characterize the diffusion barrier by determining its mechanism. This was done by finding what percentage of neurons expressing mcd8-GFP and rtnl 2 RNAi exhibit the diffusion barrier in the AIS plasma membrane. I found that 81% of the cells show a diffusion barrier in the AIS because mcd8-GFP was never able to recover past 30%. This was also done solely in ddaE neurons, and it is unknown why there were cells that did not exhibit the diffusion barrier. One of the neurons that did not exhibit the diffusion barrier was morphologically different with a large bulge in the distal portion of the axon, but the other neuron did not show any morphological abnormalities. There was some diffusion present in the AIS even after normalization but it was much less then that seen in the other parts of the neuron. This shows that some kind of mechanism is inhibiting the mcd8-GFP from diffusing as freely as it does in the plasma membrane in the rest of the cell. When ank2 was depleted via RNAi, I saw a similar diffusion barrier in only 37% of the neurons surveyed, and this difference was found to be statistically significant. This suggests that the diffusion barrier is dependent on ank2. It was interesting to find that 2 of the neurons that expressed ank2 RNAi and exhibited greater diffusion in the AIS also had extensions from the axon. This was not observed in any of the control axons. This appears to suggest that a decrease in the effectiveness of the diffusion barrier gives axons greater dendritic character by promoting the growth of extensions from the axon. It is important

to note that the RNAi may not have been completely effective because ank2 is a necessary structural protein. Structural proteins tend to be very stable in the cell because of their necessity to the cells survival. This property may have inhibited the RNAi's ability to deplete the cell of ank2.

In this study, I show that a diffusion barrier exists in the plasma membrane of the AIS of mature Drosophila neurons. This shows that the diffusion barrier is not only present in mammals, as previously believed. The existence of a diffusion barrier reveals that Drosophila neurons are more similar to mammalian neurons then would have been admitted before. Drosophila neurons can now be used as a better model for mammalian neurons.

This line of research remains unfinished. I hope to continue this by searching for more proteins that this diffusion barrier could be dependent upon. An example is the protein neuroglia (nrg). Nrg is a protein that interacts with ank2, and I hypothesize that if nrg was depleted via RNAi then ank2 would be destabilized and there would be more diffusion in the AIS. Moreover, we could examine how the diffusion barrier is affected during axon injury. Currently it is known that when axons are injured in the peripheral nervous system, a dendrite may be converted to an axon and grow towards the site of injury (Stone et al, 2010). It may be that a diffusion barrier is created in this region as part of this differentiation process. Moreover, an unanswered question from this line of research was how far down the axon does the diffusion barrier exist. It has been difficult to study this because the axons of ddaE neurons only remain on the same plane for a short time and then dive into the larvae (Dr. Rolls, personal communication). It may be easier to study this in ddaE neurons where the axon does not dive down, such as the neurons further towards the head. Thus, I hope to repeat this study in those neurons with shorter axons that do not dive into the larvae to show how far down the axon the diffusion barrier exists.

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Education

Norwin High School — Graduated, May 2007
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B.S. in Biochemistry and Molecular Biology — Anticipated Graduation, May 2011
Option in Molecular Biology — Minors in History and Psychology
Honors in Biochemistry and Molecular Biology
Schreyer Honors College
The Pennsylvania State University, University Park, PA

Research Experience

Undergraduate Honors Thesis Research — Spring 2008- Present
McNair Summer Research Program — Summer 2009
Research Advisor: Dr. Melissa Rolls, Department of Biochemistry and Molecular Biology
Pennsylvania State University

- Maintained fly lines and lab equipment
- Presented research each semester and a journal article each summer to lab group
- Studied the function of reticulon proteins in Drosophila neuronal endoplasmic reticulum
  - Utilized genetic crosses and RNAi to decrease the function of particular genes
  - Used immunofluorescence on dissected Drosophila brains
  - Performed fluorescence recovery after photobleaching (FRAP) on Drosophila larvae peripheral neurons
  - Analyzed data using the Image J program
- Determined the presence of a diffusion barrier in Drosophila neurons and its mechanism
  - Employed fluorescent confocal microscopy and RNAi to perform FRAP on sensory neurons in Drosophila larvae
  - Utilized the Image J program to graph amount of fluorescent recovery
  - Developed graphing method to accurately show recovery
- Generating a Green Fluorescent Protein (GFP) and Red Fluorescent Protein (RFP) of the Rdl protein
  - Isolated mRNA and converted to cDNA to produce template for cloning
  - Applied molecular cloning techniques to clone construct into vectors

Teaching Experience

Teaching Assistant for Introductory Biochemistry Lab Fall 2010 - Present

- Lecture on topics covered in experiment that day
- Write and grade exams as well as grade lab reports

Oversee students as they go through the experiment

GRE Math Review Instructor Summer 2010

- Prepared lecture for class
- Provided private tutoring and supplementary problems for outside of class practice

Awards and Scholarships

Discovery Summer Grant
Lighthouse Trustee Scholarship
Thompson Science Scholarship
Collins Trustee Scholarship
Foster Scholarship
Bunton Waller Scholarship
Phi Beta Kappa Honor Society
Phi Kappa Phi Honor Society
Golden Key Honour Society
National Residence Hall Honorary
Dean's List, all semesters (Fall 2007 - Spring 2010)

Oral Presentations

"Diffusion Barriers in Drosophila Neurons" Presentation offered at Penn State McNair Research Conference, July 17, 2010

"Diffusion Barriers in Drosophila Neurons" Presentation offered at The University of Wisconsin McNair Research Conference, November 7, 2009

"Diffusion Barriers in Drosophila Neurons" Presentation offered at Penn State McNair Research Conference, July 18, 2009

Leadership and Service

Resident Assistant Fall 2009 - Present

- Plan floor events to build community on the floor
- Deal with any problems, emotional or legal, that occurred with the residents on the floor or in the building
- Make residents aware of various opportunities occurring around campus

McNair Post-Baccalaureate Achievement Program Spring 2008 - Present

- Participate in weekly professional development workshops and seminars to help in graduate school preparation
- Fulfill program requirements, including maintaining a minimum 3.0 GPA, completing a research methodology course, and participating in a nine-week summer research internship

AXΣ: Chemistry Fraternity, Professional Chair Spring 2009 - Fall 2009

- Organized events between members of the fraternity and those who use science in their careers, such as professors or those in industry
- Attended local American Chemical Society meetings

South Halls Residence Association, Vice President Spring 2008 - Spring 2009

- Chose committee chairs for each committee from all those that applied
- Supervised Events and Diversity Committees to ensure all paper work was filled out properly and events were planned properly

South Halls Residence Association, Events Chair Fall 2007 - Spring 2008

- Planned and put on events for the South Halls Residence Area
- Helped other members with their events

Skills and Certifications

Chemical Storage and Waste Management Training
Proficient in Spanish