Diffusion Barriers in Drosophila Neurons

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Abstract

Neurons are highly specialized cells that require the specific localization of proteins within the cell to function. If the correct localization is compromised it can lead to neurological diseases. One of the proteins implicated in keeping proteins in their proper location is Ankyrin. Here we use RNAi to decrease the expression of Ankyrin 1 and 2 in Drosophila to see how it affects the axonal diffusion barrier in ddaE neurons. We are also creating a GFP and RFP of Rdl, a protein isolated to the dendrites, to view protein restriction in dendrites.

Introduction

Neurons are highly polarized cells in both mammals and invertebrates. These cells are divided into three major subcellular compartments. Dendrites receive signals from either 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. The proper function of these compartments in the neuron is dependent on the specific proteins present.

There are several ways that neurons can isolate proteins in specific compartments of the cell that help maintain the neurons proper function. Cells can keep proteins and phospholipids in a certain area by the use of cytoskeletal tethering. Also, there could be endocytosis that moves proteins and lipids into or out of areas the cell. Lastly, a diffusion barrier could exist that prevents diffusion across a specific point in the cell, similar to that of a tight junction (Winckler et al., 1999). It has 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). This same phenomenon has been seen in the plasma membrane of the axon initial segment (AIS) (Nakada et al., 2003; Winckler et al., 1999). Nakada was able to show that not only was there a decreased diffusion rate in the AIS but that the cytoskeletal protein ankyrin played a role in maintaining this barrier.

Ankyrin is vital to the proper function of the cytoskeleton and helps to anchor proteins to the cytoskeleton via a spectrin adaptor protein. Vertebrates contain 2 forms of

ankyrin, ankyrinB and ankyrin G. It has been shown that ankyrin G is expressed 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 ankyrin G (Zhou et al., 1998). It also has been proposed that anchored protein pickets in the AIS prevent the diffusion and that ankyrin G is associated with the anchored protein picket (Nakada et al., 2003).

Drosophila have two forms of ankyrin, ankyrin 1 and ankyrin2. Ankyrin-1 is ubiquitous, where as ankyrin-2 is neuron specific. It has been shown that in Drosophila, the decreased expression of ankyrin-2 affected dendrite and axon morphology as well as the stability of presynaptic microtubules (Yamamoto et al., 2006; Pielage et al., 2008). There is no evidence that action potentials are initiated or that there exists an anchored protein picket in the AIS of Drosophila.

We hypothesize that ankyrin plays a role in maintaining a diffusion barrier in the plasma membrane of the AIS in Drosophila neurons. To test this hypothesis we used fluorescence recovery after photobleaching (FRAP) to show that mcd8-GFP freely diffuses in the cell body and does not recover in the axon of neurons of 3rd instar larvae. We go on to use ank1 RNAi and ank2 RNAi to see if ankyrin is one of the anchored membrane proteins responsible for the diffusion barrier in the plasma membrane of the AIS.

Also we are trying to create a fluorescent construct of a protein that is isolated to the dendrites. This is to try and look at a diffusion barrier in the dendrites in future studies. We are generating a green fluorescent (GFP) and red fluorescent protein (RFP) of the dendritic marker Rdl.

Methods and Materials

Fly Stocks and Crosses

The fly lines used in this study were Dicer2; 221 Gal4 mcd8 GFP/ TM6, ank1 RNAi on 2, ank 2 RNAi on 2, and rtnl2 RNAi on 2. All RNAi lines were purchased from VDRC. Males from each RNAi line were crossed to virgin female Dicer2; 221 Gal4 mcd8 GFP/ TM6, and third instar larvae from these crosses were collected on food caps after aging for three days and imaged by confocal microscopy.

Confocal Microscopy

To view Drosophila larvae, whole third instar larvae were placed in a drop of Schneider's media on a dried agarose pad on a glass slide. The larvae were then positioned dorsal side up, and a cover slip was placed on top of the larvae. The cover slip was then taped down to the glass slide. Whole larvae were viewed immediately after mounting on a Zeiss 510 LSM microscope. Images were taken using a 63x oil objective lens and analyzed using Image J.

Photobleaching

Fluorescence recovery after photobleaching (FRAP) was performed also using the Zeiss 510 LSM microscope and under the 63x oil objective used in confocal microscopy. A bleach scan with the laser at full power with a time frame of two or three seconds was used for all experiments. Quantification of all movies was done using the Image J program. The intensity of a small area of the bleached region was taken as well as the intensity of a small area of the unbleached region. Background intensity was then subtracted from the values of the bleach and unbleached areas. The ratio of the resulting values of the bleached to unbleached region was then taken. This value was then divided by the initial prebleach ratio to correct for the bleaching affect of the laser.

Generation of Rdl-GFP and RFP Constructs

Rdl-GFP and Rdl-RFP constructs were generated by amplifying an Rdl EST (DGRC) by PCR with Bgl II and Not I primers. The Rdl DNA was cloned into pUAS-TDC, the RFP construct, and pUAS-EMC, the GFP construct, vectors that were digested with Bgl II and Not I restriction enzymes. The resulting plasmid will be inserted into the Drosophila genome via P-element insertion.

Results

A Diffusion Barrier Exists in the Proximal Portion of the Axon and not in the Cell Body or the Dendrites

It has been shown that a diffusion barrier exists in the AIS of mammalian neurons, but not invertebrates (Nakada et al., 2003; Song et al., 2009; Winckler et al., 1999). Thus, we looked into if there is a diffusion barrier in the AIS of the Drosophila motor neurons by using FRAP on larvae expressing a green fluorescent protein (GFP) tagged to the protein mcd8. Mcd8 is a protein that exists in both the plasma membrane and the endoplasmic reticulum (ER) in the cell body but only the plasma membrane in the axon and dendrites (Fig. 1B). Rtnl2 RNA interference (RNAi) was used because the RNAi would inhibit the production of rtnl2, which has no known function. This would allow the diffusion of mcd8-GFP to be accurately measured because the loss of rtnl2 would not have an affect on the mobility of mcd8-GFP in the plasma membrane. The 221 Gal4 driver was used to express the mcd8-GFP in dendritic arborization neurons. Dicer2 is a transgene that was also used to promote the RNAi expression in the larvae.

To get a baseline for the diffusion of mcd8 in neurons, FRAP was done on the cell body of class 1 dorsal dendritic arborization neurons, ddaE, in 3rd instar larvae. A small area of the cell body was bleached and the recovery was measured. A full recovery of the area was observed, after normalizing for the bleaching affect of the laser (Fig. 1A-D). There is an almost 100% recovery after approximately 50 seconds after bleaching. This shows that mcd8-GFP diffuses in the cell body of ddaE neurons at a constant rate and is not inhibited from entering an area of the cell body.

The same experiment was done on the proximal part of the axon to see if it would recover as well. When this was done there was recovery of the axon of about 20% (Fig. 1E-H). This shows that mcd8 does not diffuse as freely into the proximal part of the axon from either the cell body or the distal portion of the axon and that there is a diffusion barrier in AIS of ddaE neurons. This provides evidence that there is a diffusion barrier similar to that of mammals present in 3rd instar larvae ddaE neurons.

Next FRAP was done on the dendrites of ddaE neurons to see if the same diffusion barrier that was seen in the proximal axon exists in the proximal dendrite. After bleaching the proximal dendrite, there was an almost full recovery of the mcd8-GFP (Fig 1I-L). Thus there is a diffusion barrier in the AIS of Drosophila but not in the dendrites. It is interesting to note that there was a slower diffusion of mcd8-GFP in the dendrite than in the cell body. This could be because mcd8-GFP diffuses slower in the plasma membrane than in the ER, which is where mcd8-GFP is present in the cell body.

Ankyrin Plays a Role in Maintaining the Diffusion Barrier in the Axon Initial Segment in ddaE Neurons

The next step was to determine how this diffusion barrier is maintained. It has been previously shown that Ankyrin is a protein associated with the diffusion barrier in the axon initial segment of rat hippocampal neurons, which makes it a logical choice to see if it also has an affect on axon initial segment in Drosophila (Nakada et al., 2003). To test if ankyrin is associated with the diffusion barrier in the AIS of Drosophila, an ankyrin loss of function experiment was done. The AIS of ank1 RNAi and ank2 RNAi third instar larvae ddaE neurons were bleached in the proximal portion of the axon. These RNAi lines were also crossed to the Dicer2; 221 Gal4 mcd8 GFP/ TM6 as in the previous experiment.

When the same axon bleach experiment done in the rtnl2 RNAi is done in larvae with ank1 RNAi, only 1 of 3 images show a possible affect of the RNAi. The graph shows a steady increase and a recovery of approximately 50% (Fig. 2A) This is difficult to see in the images because of the imaging conditions were not ideal because the axon was to dim and bleached out to quickly during imaging, but this image provided evidence that ank1 plays a role in maintaining a diffusion barrier in the AIS.

Photobleaching the AIS in larvae with the ank2 RNAi also showed some recovery, similar to that in larvae with Ank1 RNAi. 4 of 9 larvae show a recovery of 40% to 50% after photobleaching of the AIS (Fig. 2B). This also provides evidence that ankyrin maintains the diffusion barrier in the AIS of Drosophila.

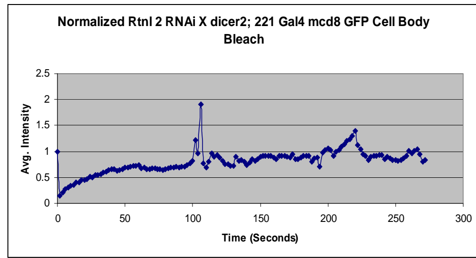
Generating a Rdl-GFP and Rdl-RFP Construct

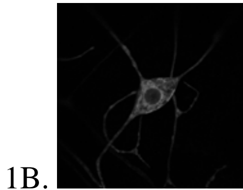
Rdl, stands for resistant to dieldrin, was first identified as an insecticide resistant neurotransmitter receptor (ffrench-Constant et al., 1993). It is isolated to the dendrites of motor neurons, and the purpose of this project is to make a transgenic fly line via p-element insertion that expresses either a green fluorescent protein (GFP) or red fluorescent protein (RFP) of the rdl (Sanchez-Soriano et al., 2005). The rdl gene is

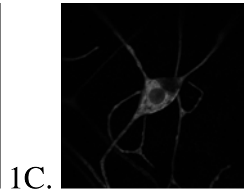
placed in between two p-element ends in a plasmid and injected into an embryo in the presence of another plasmid with a transposase gene. The rdl gene will then be randomly transposed into the Drosophila genome by the transposase and expressed under the 221 Gal4 driver for use in future experiments.

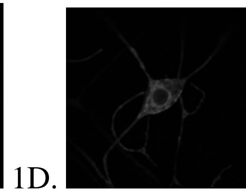
Figure 1.

1A.

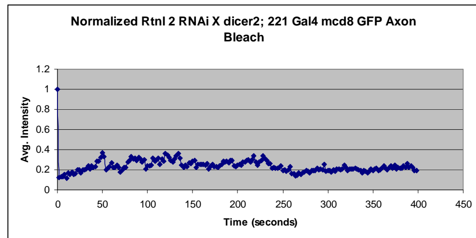


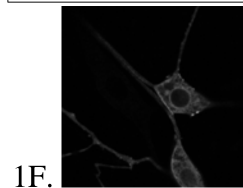
1B. 
Pre-Bleach

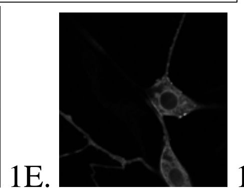
1C. 
Post-Bleach

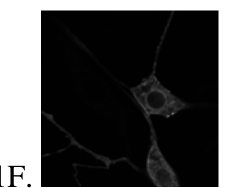
1D. 
50 seconds

1E.

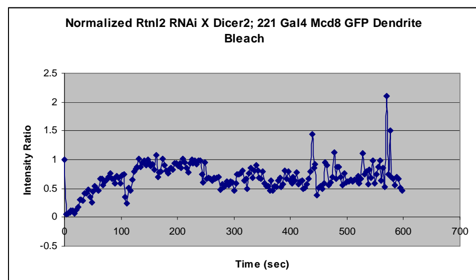


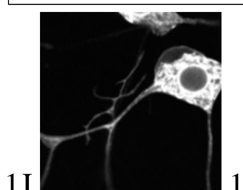
1F. 
Pre-Bleach

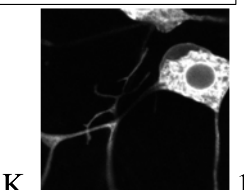
1E. 
Post-Bleach

1F. 
50 seconds

1I.



1J. 
Pre-Bleach

1K. 
Post-Bleach

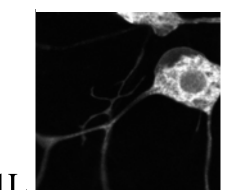
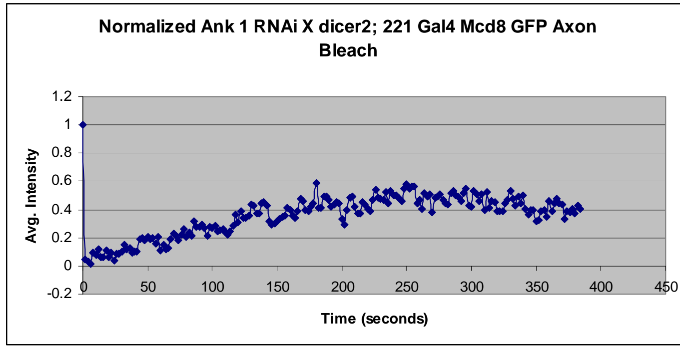
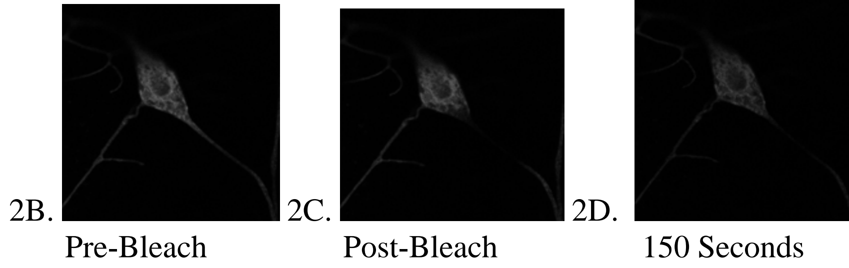
1L. 
50 seconds

Figure 2.

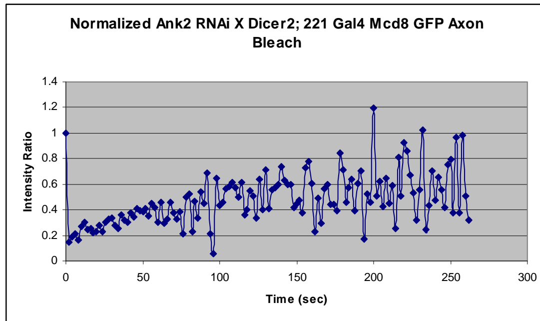
2A.

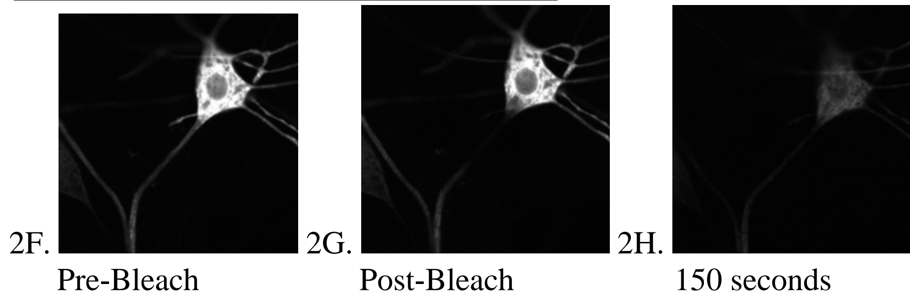




2B. Pre-Bleach 2C. Post-Bleach 2D. 150 Seconds

2E.





2F. Pre-Bleach 2G. Post-Bleach 2H. 150 seconds

Discussion

By using FRAP experiments on the axon, cell body, and the dendrites, we have shown that a diffusion barrier exists in the plasma membrane of axons in Drosophila ddaE neurons and not in the cell body. It was observed that diffusion was slower in the dendrites then in than in the cell body. This could be from the fact that a longer region of the dendrite was bleached compared to the area bleached in the cell body. The longer diffusion rate could also be because that diffusion is slower in the plasma membrane than in the ER. Mcd8-GFP is present in the ER and plasma membrane in the cell body but only present in the plasma membrane in the dendrites. If mcd8-GFP naturally diffuses faster in the ER than in the plasma membrane, it could explain this difference.

Our experiments with the ankyrin RNAi as of yet are not conclusive because it is difficult to see in the videos whether it is membrane diffusion that is being measured or vesicular trafficking. The imaging conditions need to optimized to better show the axon and minimize bleaching over time span of the imaging. There is evidence to show that ank1 and ank2 may play a role in the diffusion barrier in the AIS, but more research needs to be done. Another RNAi line that needs to be looked at is ank1 RNAi with the ank2 RNAi to view its affect on the diffusion barrier in the AIS. It is hypothesized that the loss-of-function of ank1 RNAi and ank2 RNAi will further decrease the diffusion barrier in the AIS and the diffusion of mcd8-GFP will begin to look more like that of dendrites. This is the current hypothesis because both forms of ankyrin seem to have similar effects when one is depleted from the larvae and when both are depleted from the larvae the affects will be more pronounced.

Currently the Rdl-GFP and Rdl-RFP are not completed. The rdl DNA that has been inserted into the RFP construct plasmid, pUAS-TDC, and is being sequenced to ensure that no mutations have occurred over the process. We are in the process of isolated the GFP construct plasmid, pUAS-EMC, so that the rdl DNA can be inserted into it. Once one of the clones has been sequenced and shows no mutations, the rdl DNA that has been inserted into a p-element vector will be injected into embryos with a plasmid containing transposase. This rdl gene with the GFP or RFP tagged on the C-terminus, to not interfere with the function of rdl, will be inserted into the Drosophila genome. This will cause Drosophila larvae to express either rdl-GFP or rdl-RFP once combined with the Gal4 driver.

This data shows that Drosophila do contain a diffusion barrier in the AIS of ddaE neurons. This is relevant because it shows that the Drosophila nervous system is more similar to mammalian nervous system then previously thought. We can use Drosophila to understand how ankyrin loss could affect the nervous system of the organism. It was previously mentioned that loss of AnkG in mammals could cause neurodegenerative diseases because it causes the axon to exhibit dendritic properties (Hedstrom et al., 2008). This evidence provides a jumping point for further research into this topic in Drosophila.

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References

Ffrench-Constant RH. (1993). Cloning of the *Drosophila* cyclodiene insecticide resistance gene: a novel GABAA receptor subtype? Comp Biochem Physiol C. 104, 9-12.

Hedstrom, K. L., Ogawa, Y., and Rasband, M. N. (2008). AnkryinG is required for maintenance of the axon initial segment and neuronal polarity. J. Cell Biol. 183, 635-640

Nakada, C., Ritchie, K., Oba, Y., Nakamura, M., Hotta, Y., Iino, R., Kasai, R. S., Yamaguchi, K., Fujiwara, T., and Kusumi, A. (2003). Accumulation of anchored proteins forms membrane diffusion barriers during neuronal polarization. Nat. Cell Biol. 5, 626-632

Pielage, J., Cheng, L., Fetter, R. D., Carlton, P. M., Sedat, J. W., and Davis, G. W. (2008). A presynaptic giant ankyrin stabilizes the NMJ through regulation of presynaptic microtubules and transsynaptic cell adhesion. Neuron. 58, 195-209

Sanchez-Soriano N, Bottenberg, W., Fiala, A., Haessler, U., Kerassoviti, A., Knust, E., Löhr, R., and Prokop, A. (2005). Are dendrites in Drosophila homologous to vertebrate dendrites? Dev. Biol. 288, 126–138.

Song. A., Wang, D., Chen, G., Li, Y., Luo, J., Duan, S., and Poo, M. (2009). A selective filter for cytoplasmic transport at the axon initial segment. Cell. 136, 1148-1160

Winckler, B., Forscher, P., and Mellman, I. (1999). A diffusion barrier maintains distribution of membrane of proteins in polarized neurons. Nature. 397, 698-701

Yamamoto, M., Ueda, R., Takahashi, K., Saigo, K., and Uemura, T. (2006). Control of axonal sprouting dendrite branching by the nrg-ank complex and the neuron-glia interface. Curr. Biol. 16, 1678-1683

Zhou, D., Lambert, S., Malen, P. L., Carpenter, S., Boland, L. M., and Bennett, V. (1998). Ankyrin$_G$ is required for clustering of voltage-gated Na Channels at axon initial segments and for normal action potential firing. J. Cell Biol. 143, 1295-1304