K+ Induced Exocytosis at Localized Regions of En Passant Synapses in Chick Embryonic Ganglia Cells

Lauren M. Suarez '04, April 2004.

Abstract

The presence of luminescent plasma membrane suggests the presence of exocytosis and endocytosis at the en passant regions imaged. Embryonic chick dorsal root ganglia and sympathetic neurons were stained with FM 1-43 dye to label the membrane, and induced with a 50mM concentration of K+ to cause depolarization. The cells were imaged before and after the K+ induction at regions of en passant and the luminosities were quantified using digital imaging systems. Quantification was measured at both en passant regions and regions lacking synapses and branching to compare differences. Results showed that at regions of en passant, the umination of the FM 1-43 was higher after the K+ elevation, and in regions of non-synapse the membrane was more luminescent after the K+ elevation. These results suggest that no endocytosis or exocytosis occured at the en passant regions and that the K+ may have had a negative effect on the synaptic regions. The results also suggest that the K+ concentration was not high enough and did not cause the cell to depolarize.

Introduction

Regions of the axons in nerve cells are thought to be responsible for many intercellular activities needed in the communication of different cells. The communication between cells is not completely understood, however studies show that synapses are likely to be the primary link between the cells and the region of communication. During cell growth axons from different nerve cells can grow in the same region and overlap causing what is called an en passant region. These regions of en passant regions have been shown to possess synaptic activity in addition to the nerve terminals (Maravall, Koh, Lindquist, & Svoboda 2004).

Communication between neurons at synapses involves a series of vesicle transfer between presynaptic and postsynaptic cells. Neurotransmitter release at the region of synapse is necessary for neurons to communicate. Neurotransmitters are synthesized in the cytoplasm and packaged in vesicles (Harris, Schuske, & Jorgensen 2001). Exocytosis, the process of neurotransmitter release, begins with the arrival of an action potential, which causes an influx of calcium ions allowing the vesicle to dock at the active zone. Once the vesicle has bound to the synaptic protein, it is then free to move toward active zones (Williams College Neuroscience 1998). The synaptic vesicle protein is recruited to a specified region of the plasma membrane, which is marked as a site of endocytosis. Certain proteins known as NSF and SNAPs are responsible for membrane targeting. The binding of the NSF and the SNAPs to receptors (SNAREs) on the plasma membrane form he joining between the vesicle, known also as an endosome, and the cell membrane (Revest & Longstaff 1998). Once the endosome containing neurotransmitters has docked with the plasma membrane of the cell, the membranes fuse forming a small opening, which grows larger and eventually joins the plasma membrane of the cell and exocytosis occurs (Williams College Neuroscience 1998). A temporary ion channel is formed when a second influx of calcium at the active zones causing the membranes to fuse. Once this initial pore is formed dilation occurs and neurotransmitters are released into the synaptic cleft during exocytosis (Williams College Neuroscience 1998). After neurotransmitter release through exocytosis, the vesicular membrane moves away from the cellular membrane and is pinched off into the cytoplasm of the cell where it is recycled and exocytosis begins again (Williams College Neuroscience 1998).

The styryl dye FM1-43 when applied to a cell will permeate the exterior of the lipid bilayer (Avery, 2003). The dye is commonly used in identifying firing neurons and vesicle cycling. The dye is inserted into the outer leaflet of the plasma membrane where it becomes fluorescent. In neurons that are actively releasing neurotransmitters, the dye becomes internalized with the recycled synaptic vesicles from endocytosis and the area of synaptic activity becomes brightly stained (Molecular Probes 2004).

If there is a presence of exocytosis in the en passant region of the axon, then by staining the neuron with FM1-43 fluorescent dye would become internalized inside the vesicles during endocytosis. In order to achieve an action potential in the cell, the cell must reach threshold at the trigger zone. A K+ induction of ions through the extracellular fluid would cause depolarization in the cell and further produce an action potential at the trigger zone sending it down the axon to the region of synaptic activity. When excess K+ is induced into the cell, the intracellular K+ level raises causing a depolarization in the membrane potential of the cell. In theory, if a nerve cell is induced by K+ at the appropriate concentration, a depolarization will occur within the cell causing an action potential to fire along the axon to the area of synaptic activity. Once the action potential is received at the synapse, an influx of Ca++ occurs initiating the beginning stages of exocytosis. By labeling the plasma membrane with FM1-43 fluorescent dye, any endocytic activity within the synapse would become illuminated using a fluorescent microscope and imaging system. In addition to imaging the level of brightness at the en passant regions to determine the presence of endocytosis and exocytosis, imaging regions of the axon that possess no synapses or branching will give evidence as to whether the or not any activity in the synapse is different from activity in the remainder of the axon, which would suggest or reject the presence of endocytosis and exocytosis.

High amounts of exocytosis, as judged by increased endocytosis recovering the membrane, in localized small regions is expected. An action potential triggered by K+ elevation should cause the cell to depolarize and the indication of endocytosis following the depolarization, by the use of fluorochromes would suggest the presence of exocytosis at the synaptic terminal of the axonal growth cones.

Materials and Methods

Embryonic Chick DRG or sympathetic chain neurons in vitro
35mm petrie dish
Valap and brush
2 Pasteur Pipettes
37º C incubator
22 cm cover slips
75 x 25 glass slide
Forceps
Nikon Optiphot-2 microscope
SPOT 2E digital camera, SPOT 2E digital imaging program
Adobe Photoshop7 software program
Small area heater
50mM K+ (Savage, Biffin, & Martin)
10 mL FM1-43 in 2 ml solution (Stevens & Williams)
Hanks Balance Salt Solution
Distilled Water
Power PC G4 Macintosh
4mL F+ medium

Cell Preparation

Ten-day-old eggs were dissected and DRG's and sympathetic chains were plated out on cover slips. Five cover slips were plated with 1/5 sympathetic chain, 5 cover slips with 1/3 DRG per plate, 5 plates 2 DRG's, and 5 plates with 4 DRGÕs to achieve different densities. The cover slips were treated with 1mg/mL Poly K treatment in 35mm petrie dishes for 60 minutes, then rinsed and dried. Four-year-old Laminin was added to HBSS solution in a concentration of 1 mg/ml. Nine mls of HBSS modified (Ca++/Mg++ free) was added to 1 ml Trypsin EDTA solution to make 10mls 1x Trypsin in modified HBSS. HBSS was aspirated off the cells in 35 mm petrie dishes and approximately 1 ml trypsin was added. The cells were incubated at 37º C for 20 minutes. Trypsin solution was drained off and 1 ml HBSS

solution and 2.5 ml F+ medium was added to dishes.

Staining

2mM FM1-43 was diluted 1:200 to create a 10 mL solution in 2 ml. The F+ medium was drained and the 10mL FM1-43 was added to the dish and let sit for 10 minutes. The FM1-43 was drained and the cover slip was plated onto a flow chamber.

Imaging

Cell regions were chosen based on their classification as en passant. En passant synapses were defined by no potential distance between the two axons using phase optics in a light microscope at 40x magnification. Cells were imaged prior to FM1-43 exposure, post-FM1-43 exposure (pre-KCl), and post-KCl exposure. All images were taken using the Nikon Optiphot-2 microscope and SPOT 2E digital camera and software. Images were taken within approximately 2 minutes or less of the initial KCl+ induction at an en passant synapse. One image was taken at each region, and 3 regions were imaged. Images taken were in both phase and fluorescent contrast (blue excitation, yellow emission) at 40x magnification.

K+ Induction

In order to maintain osmolarity and cause cell depolarization, 37.3mg KCl was added to 5.2 ml and 4.8 ml dH20 to create a 10ml solution. 500 ml of KCl solution was flowed through the flow chamber at approximately a 45^{o} angle over the duration 1.5 minutes. The cells were imaged at en passant junctions.

Quantification

The en passant region defined was freehand outlined box on the fluorescent image using the Adobe Photoshop 7 software on a PowerPC G4 Macintosh computer. The regions drawn were chosen and classified by their interactions of axonal overlap, defined as a region of uniform caliber through the region of interest. The angle of crossing was chosen by the cells possession of a 70 - 110 ^{o} angle of incidence from adjacent axons. An additional freehand outline was defined on the background of the fluorescent image to compare the brightness. The background region was taken from a nearby area that possessed no cells. A histogram was computer generated for both the synaptic region and the background. The histogram provided the count of pixels in the given region, and the mean luminosity. The mean luminosity of the background was then subtracted from the mean luminosity of the synaptic region and the background subtracted mean brightness was recorded. This method of quantification was used for all regions of en passant defined in the images. The same procedures were repeated on regions of the axon that contained no synapses or branching. These regions were classified by a single axon in the direct cntact of no other axons or cells, and no branching of additional axons.

Results

Materials:

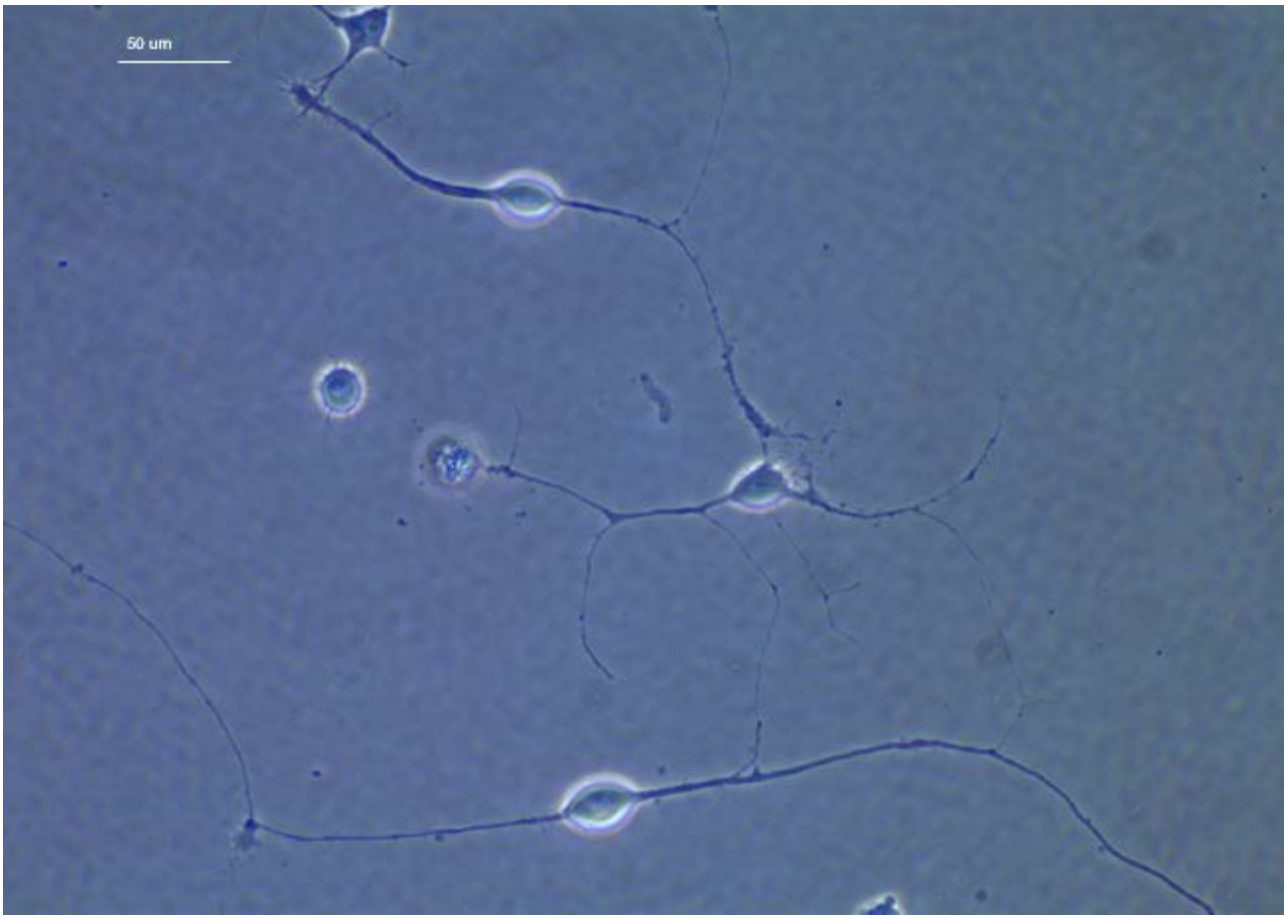
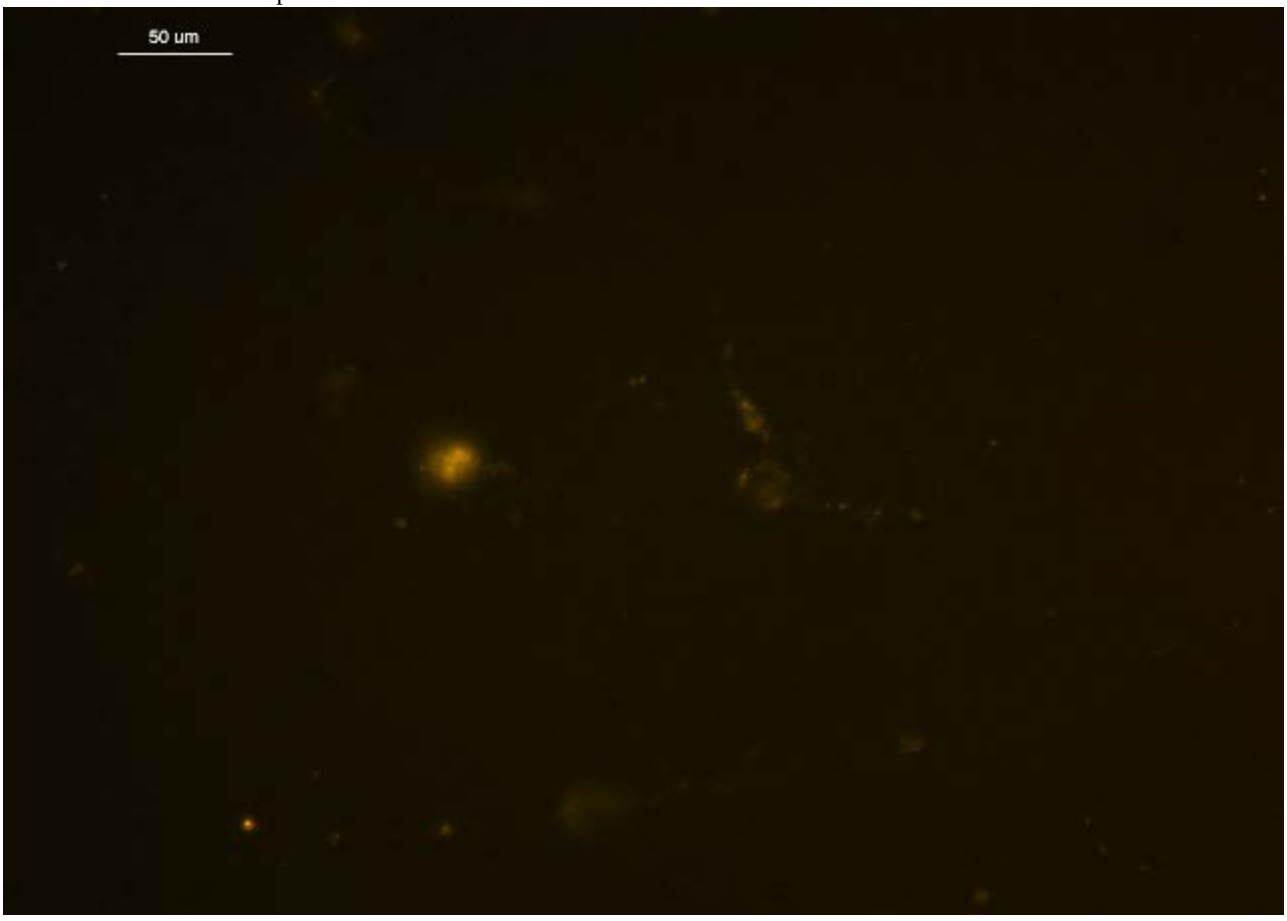


Figure 1
DRG cells taken in phase optic dimaging at 40x exposure directly following a 10 minute exposure to FM1-43. The image was taken before K+ induction and serves as a phase control.



Materials:

Figure 2
Fluorescent image of the DRG cells in Figure 1, after a 10 minute FM1-43 buffer application. Image was taken at 40x blue excitation yellow emission imaging. The image was taken before the K+ induction and serves as a fluorescent control.

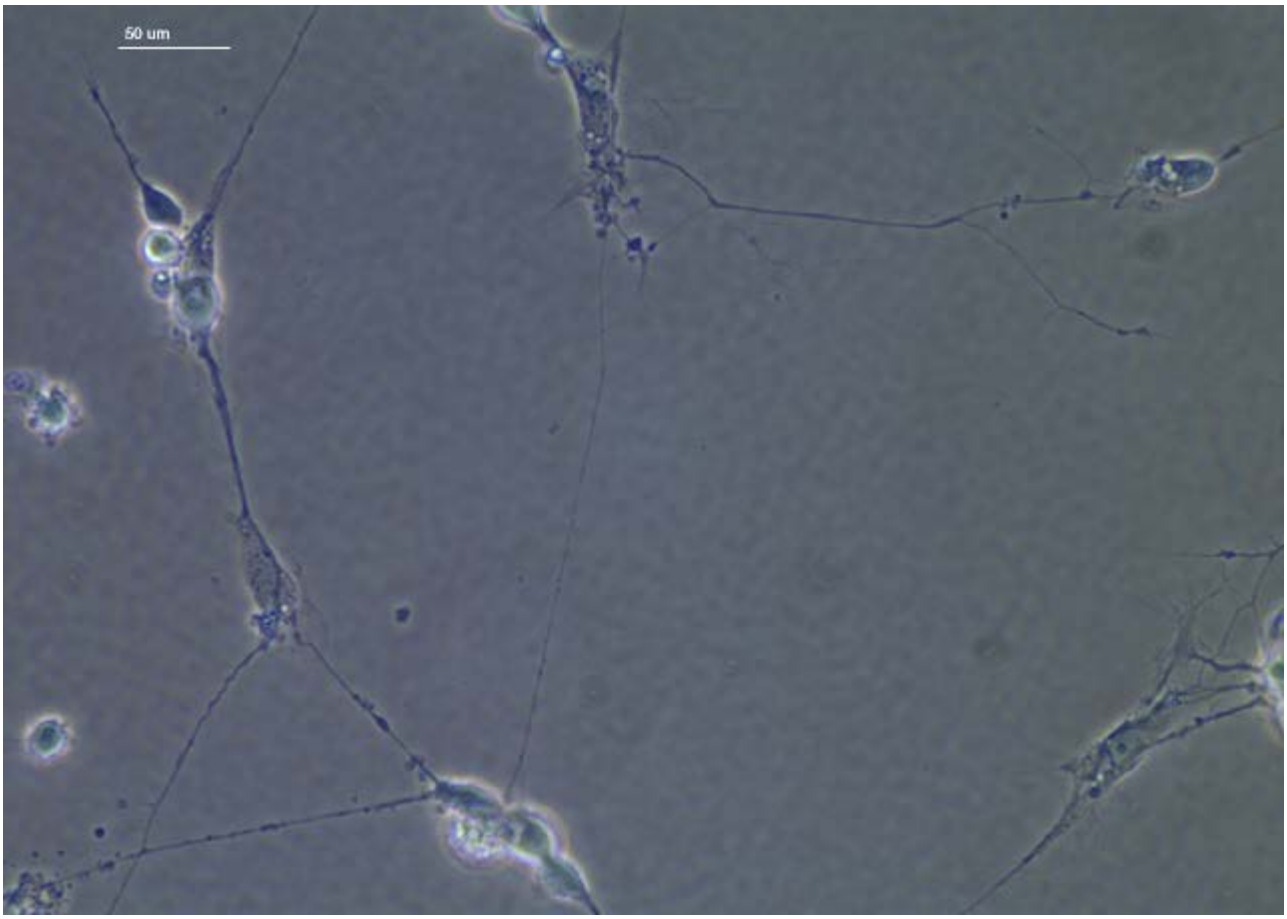


Figure 3
DRG cells taken in phase optics at 40x exposure after a 1.5 minute K+ induction. The image serves as the experimental phase image.

Materials:

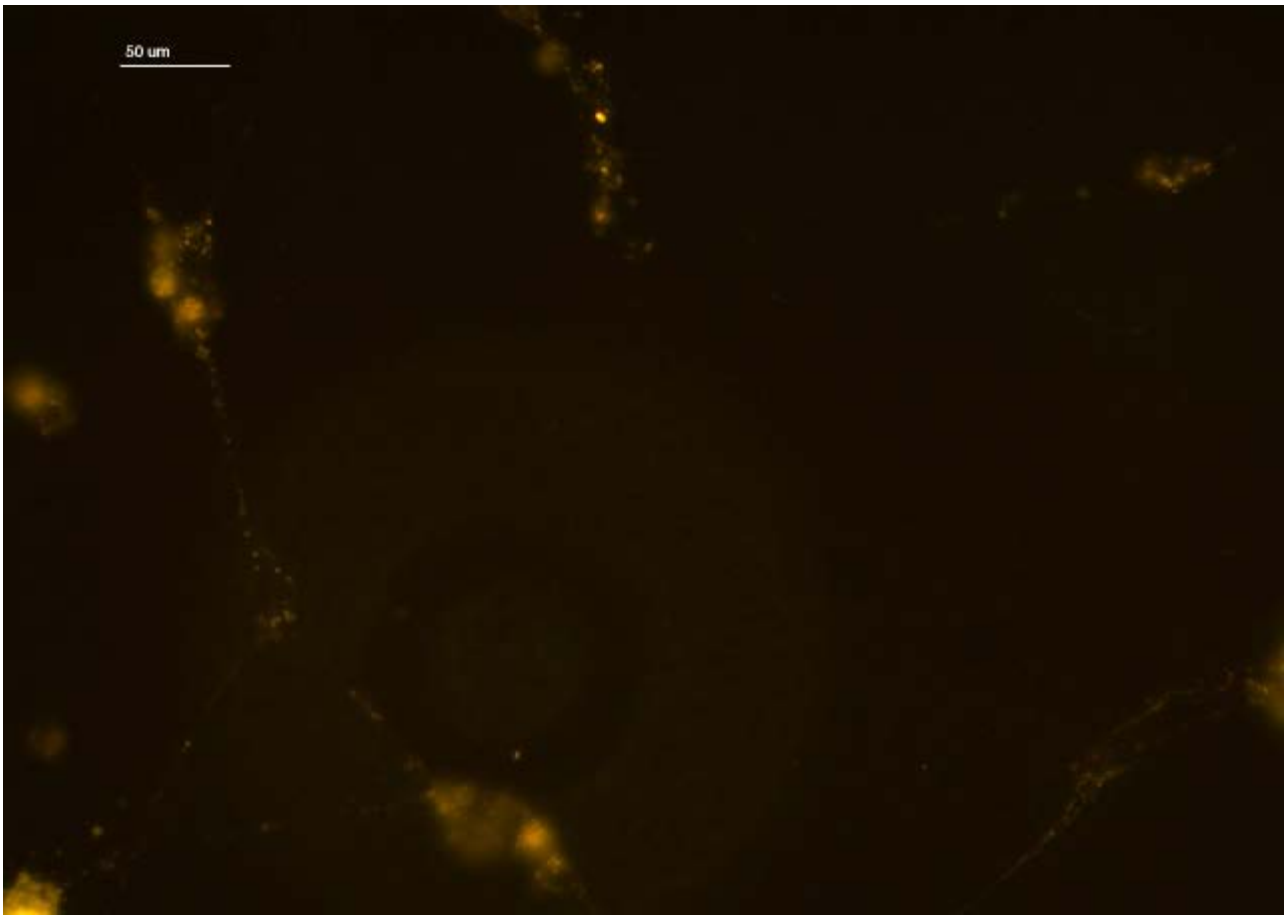


Figure 4
DRG cells imaged after a 1.5 minute K+ induction in fluorescent optics, 40x exposure, blue excitation yellow emission fluorescence.

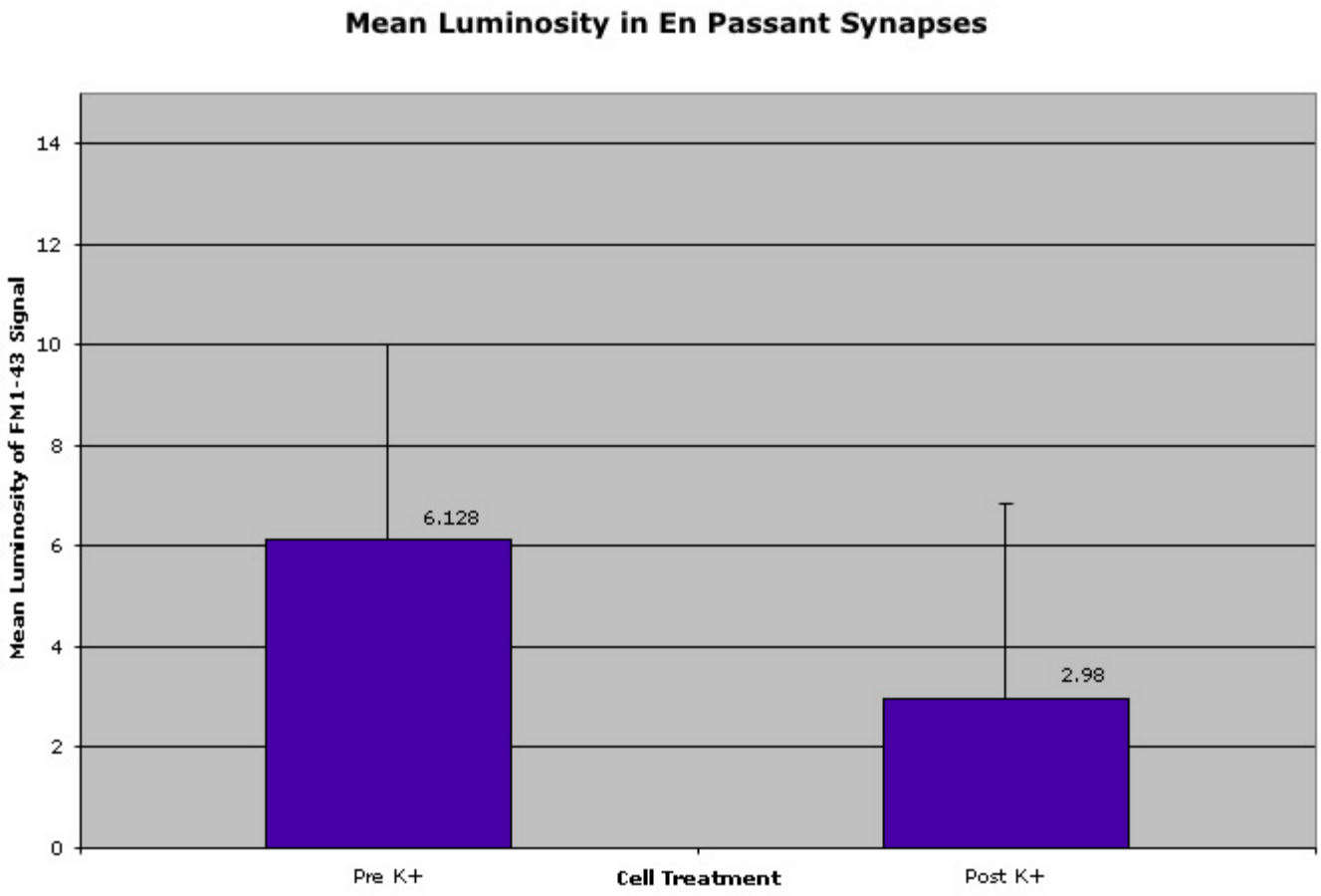


Figure 5
A graph of the average mean luminosity before the application of K+ and after the application of K+ in en passant regions of the axon. The average mean luminosity is measured by the amounts of pixels brighter than the background. Average mean luminosity values are shown above bars.

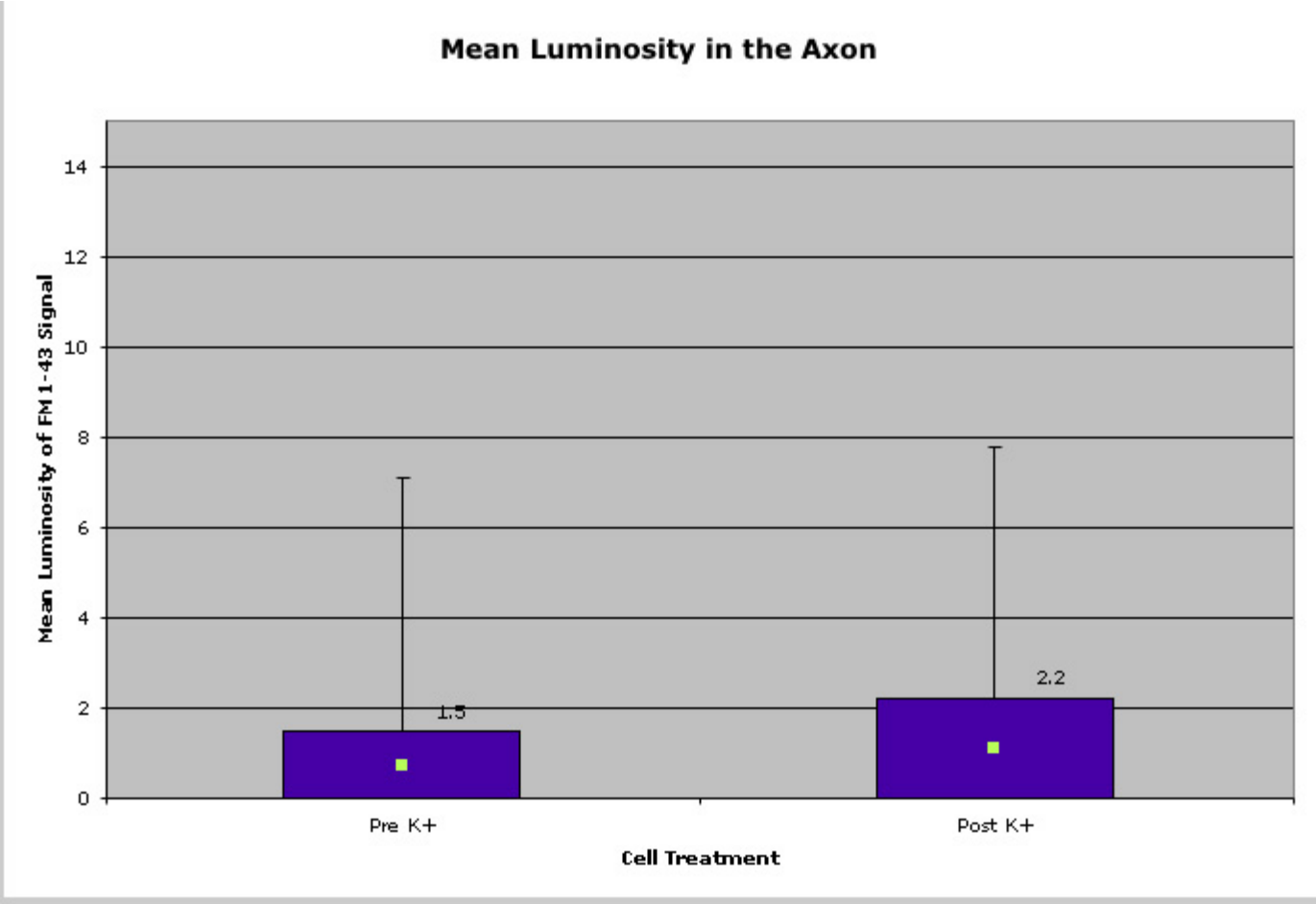


Figure 6
A graph of the average mean luminosity before the application of K+ and after the application of K+ in regions containing no synapse on the axon. The average mean luminosity is measured by the amounts of pixels brighter than the background. Average mean luminosity values are shown above bars.

Discussion and Conclusions

The hypothesis that K+ induction into the cell will cause the cell to depolarize and exocytose was not evident in the results obtained, and the hypothesis was refuted. The luminosity provided by the staining and imaging of FM1-43 was the main determinant in quantifying the data (Figure 1 and Figure 2). A higher luminescent mean after the exposure to K+ would be the indicator that depolarization and exocytosis had occurred and that the FM1-43 was illuminating the plasma membrane of the endosomes taken back up during endocytosis. The average mean luminosity determined before the exposure to K+ was 6.128 pixels, while the mean luminosity after the exposure to K+ was an average of 2.98 pixels (Figure 2). These results are evidence that the luminosity before the K+ exposure was higher than after the K+ exposure. In regions where there was no synapses on the axon, the mean luminosity before exposure to K+ was 1.5 pixels while the mean luminosity after the exposure to K+ was 2.2 pixels. Although there is only a 0.7 pixel difference before and after the exposure to K+, the results show that the luminosity is higher after the K+ exposure. Several conclusions can be made from this data.

It is assumed that since the luminescence was higher before the K+ exposure, that the cell did not depolarize or exocytosis. Depolarization and exocytosis of the cell would have caused changes in the plasma membrane, which would have therefore been reflected in the FM-43 illumination. In comparison to the regions of axon that did not contain synapses, it is clear that the K+ elevation seemed to have a negative effect on the plasma membrane of the en

passant regions. Since the results of the regions of axon without synapses show a higher level of luminosity after the application of the K+elevation (Figure 6), and the regions of en passant synapses show a lower luminosity after the K+ elevation (Figure 5) it can be theorized that the K+ had a negative effect at the area of synapse causing it to be less luminescent. It is likely that since the FM1-43 dye stains the plasma membrane, that the plasma membrane is a primary area in which the K+ had a negaitve effect, possibly causing it to deform.

Considering the cells that were exposed to the K+ solution showed a lower mean luminescence it can be concluded that the K+ solution may have had some form of negative affect on the cells, possibly in the plasma membrane, which was being illuminated. The concentration or time duration of K+ solution could have had a large impact on the outcome of the results. The concentration (50mM) or time duration (1.5 min.) may have been too high therefore damaging the cells, especially the plasma membrane, and maybe even causing cell death. However, there was evidence of luminescence in certain parts of the axons and in the cell bodies, which proves that the cells were likely to have been healthy and that the plasma membrane was intact. Also, there was no evidence of blebs on the cell bodies (Figure 1 a. and c.), which are evident in dead and dying cells therefore proving again, the health of the cells. However, another explanation is that the concentration of the solution (50mM) may have been too low therefore not causing the cell to reach threshold at the trigger zone and not depolarizing or showing evidence of exocytosis. The K+ may have rushed into the cell but the concentration may not have been strong enough to allow the channels to flow through enough K+ to reach threshold. The cells may have depolarized without the presence of an action potential therefore not causing the cell to exocytose, leading to no release or reuptake of endosomes.

The images of the results show that the cell bodies and certain sections of the axons are illuminated (Figure 2 and Figure 4.). This evidence proves that the amount and concentration of FM1-43 and the digital imaging equipment was suitable to the cells.

This experiment suggests further testing of the cells under such conditions, but with altered concentrations and exposure time to the K+ solution. Further testing should include higher and lower concentrations of K+ at different durations of exposure. Since the results show a decrease in luminescense after the K+ it is an appropriate conclusion that the concentration of K+ had a negative effect on the depolarization of the cell. In addition, other possible research could be done by subsitituting the K+ for Ca++ to cause depolarization.

References

Avery, J. (2003). Oh! The Chaos the Chaos! *Bio 219 Student Webpage.* http://icuc.wheatonma.edu:16080/%7Ebio219/javery/

Harris, T.W., Schuske, K., & Jorgensen, E. M., (2001). Studies of Synaptic Vescicle Endocytosis in the Nematode *C. elegans*. *Traffic.* 2: 597-605.

Maravell, M., Koh, I. YY, Lindquist, W.B., Svoboda, K. (2004). Experience-dependent Changes in basal Dendrite Branching of layer 2/3 Pyramidial neurons During a Critical period for Developmental Plasticity in Rat Barrel Cortex". *Cerebral Cortex Advance Access.*

Molecular Probes (2003) "T-3163." www.probes.com/servlets/search (March 3, 2004).

Savage AL., Biffin M., & Martin, BR. (1989). Vasopressin-stimulated Ca2+ influx in rat hepatocytes is inhibited in high-K+ medium. *Biochemical Journal.* 260 (821-827)

Stevens, C., & Williams J.H. (2000). "Kiss and Run" exocytosis at hippocampal synapses. *PNAS.* Vol. 97, no. 23.

Zimmerberg, B. (2000). Multimedia Neuroscience Project. Williams College Neuroscience. <http://www.williams.edu:803/imput/synapse/index.html>

Materials: