Mitochondrial Density in Regions of Axonal Overlap Compared to Axonal Regions Distal to Overlap

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Introduction

Background

When regions of axon from growing neurons overlap there are a few possibilities that may or may not be occurring due to their interaction. The first possibility may be that there is no interaction and the activities of one axon are completely independent of axons that appear to the viewer, from the perspective of a transmitted light microscope, as crossing the path of another axon from a different neuron. If indeed the neurons are interacting with each other, via interaction of crossing axons, then perhaps a gap junction as been formed and has created an electrical synapse, in which an electrical current can be passed from one cell to another. Yet another contingency that may be occurring is that an *en passant* synapse has been created, and chemical signals, via neurotransmitters, are being transported from a presynaptic neuron to a postsynaptic neuron. The later possibility would be the most interesting to cell biologists and neurobiologists as there would be many different activities and cell structures that could be monitored if the interaction was that of *en passant*. It is such monitoring that will assist researchers to discover what type of interaction, if any, is happening in these areas of axonal overlap.

One cell structure that could play a role in identifying the possibility of an *en passant* chemical synapse is that of the mitochondria. Mitochondria exist in all neuronal compartments, including the axon, where they are selectively distributed (Kandel et al, 2004). Cells have evolved complex and strictly controlled means to transport and position different organelles, like mitochondria, to particular regions of the cytoplasm. Mitochondria use molecular oxygen to generate ATP, the chief, molecule by which cellular energy is transferred or spent (Ho et al., 1989; Hollenbeck and Swanson, 1990; Lee et al., 1989; Corthesy-Theulaz et al., 1992; Kandel et al., 2004). Studying the localization of mitochondria in axons will help solve the ambiguity to the interaction of overlapping axons of growing neurons of sympathetic nerve chains of chick embryos growing in culture.

Hypothesis

If indeed the areas of axonal overlap are *en passant* synapses than I think the region of overlap will display a higher density of mitochondria than the region 10um distal from the region of overlap. In the same fashion, I believe that the region 10um distal from the adjacent region will contain an even lesser density of mitochondria, and so on and so on. Because chemical synaptic transmission depends on the release of neurotransmitter from the presynaptic neuron (Kandel et al, 2004), these areas of axonal overlap, should they be examples of *en passant*, will contain exocytic vesicles because *en passant* synaptic transmission depends on the release of neurotransmitter from the presynaptic neuron (Kandel et al, 2004). (See collaboration with Lauren Suarez and Whitney Sirois). Exocytosis in an energy dependent process involving many ATP-dependent activities such as the formation of vesicle membranes, the RABS responsible for recycling vesicles, the motor proteins responsible for pinching off the membrane of an exocytic vesicle from the plasma membrane of the neuron, etc. Because the distribution of mitochondria is thought to be controlled by local energy and metabolic demand, a task that becomes exceedingly difficult in large polarized cells such as neurons, one can image that if these areas of axonal overlap are indeed *en passant*, then mitochondria will be localized in increasing fashion in approaching regions of the axon toward the area of axonal overlap.

Methods

Materials

- high density growing neurons on Laminin and poly-K-lysine treated coverslips in petri dishes with ~3.5 ml growth medium
- 2 plastic pipettes and waste beaker
- 2 ul of 1mg/ml Rhodamine 123 in DMSO stock
- ~15 ml Hanks
- 37 degrees Celsius incubator
- 4 ml growth medium
- glass slide and crushed coverslips for chip chamber
- sharp forceps
- Kim-Wipes
- Heated VaLap and paintbrush
- 2 ml distilled water
- fluorescence microscope with Spot Insight Camera, Model # 4.2, Serial # 221237
- Mac G4 computer
- Spot Advanded Software
- Image J Analysis Software
- Photoshop 7 Softwware

Procedure

The first step in the experiment for both the control and experimental conditions was to obtain growing neurons plated on a laminin and poly-K-lysine treated coverslips stored in petri dishes with growth medium. The dishes were kept in 37 C incubators. The neurons were taken from sympathetic nerve chains and dorsal root ganglion of 8-15 day chick embryos. The neurons were plated in high density and were allowed to growth from 12-24 hours.

Experimental

From the plated neuron petri dish of 4x density, the growth medium was removed with a plastic pipette and place in a waste container. Approximately 2 ml of 1µl/1mL Rhodamine-123 dissolved / DMSO stock in growth medium was added to petri dish and incubated at 37 C for 10 minutes. Next the coverslips were washed 3 times with Hanks at time intervals of 1 min/wash. Following the final Hanks wash, growth medium was added to the coverslip for 1 minute. One drop of this growth medium was placed onto a chip chamber slide. The remaining growth medium was removed from coverslip and coverslip was placed, cell-side down, on to chip chamber slide. The coverslip was VaLaped and rinsed with distilled water.

The slide was imaged on a Nikon Eclipse E400 microscope at 400x-600x magnification with transmitted light to insure neurons were present. See Figure 1. The slide was then imaged with fluorescence at an exposure of 5000 msec and a Gain of 8 at 400x magnification, and 2000 msec with a Gain of 1 at 600x magnification. See Figure 1.

Control

From the plated neuron petri dish of 4x density, the growth medium was removed with a plastic pipette and place in a waste container. Fresh growth medium was added and the cells were incubated at 32 C for 10 minutes. Next the coverslips were washed 3 times with Hanks at time intervals of 1 min/wash. Following the final Hanks wash, growth medium was added to the coverslip for 1 minute. One drop of this growth medium was placed onto a chip chamber slide. The remaining growth medium was removed from coverslip and coverslip was placed, cell-side down, on to chip chamber slide. The coverslip was VaLaped and rinsed with distilled water.

The slide was imaged on a Nikon Eclipse E400 microscope at 400x magnification with transmitted light to insure neurons were present. The slide was then imaged with fluorescence at an exposure of 5200 msec and a Gain of 8.

Analysis

Areas of axonal overlap were defined as regions of axon that appeared to be overlapping from the view of a 400-600x magnified image taken with a transmitted light microscope, see Figure 2. As not to confuse distinct axons from separate cell bodies with axonal branching from a single cell body, each cell body had to be established for each axon imaged.

Transmitted light images from the Nikon E400 were analyzed for regions of distinguishable axon overlap. Once established, transmitted light images were closely compared to their corresponding fluorescent images to insure that the regions to be measured included the entire region of axon. This was done by overlaying the corresponding images with Photoshop 7. Using the scale bar that was stamped into each image with Spot Advanced software, ImageJ was able to establish that 10 microns was equal to 60 pixels with its Measurement tool. Knowing this, ImageJ was employed to measure the luminosity of each axon in regions of 60 pixels, or 10 microns. This gradient of the axon measured the region of axonal overlap, the region 10 um distal to the overlap, 20 um distal to the overlap, and 30 um distal to the overlap. Because ImageJ gives the x- and y- coordinates for where the cursor is place, I was able to avoid accidentally overlapping regions along the axon. Using the Histogram tool, lists of levels of brightness and the number of pixels found for each level of brightness were collected. A background histogram was also collected to serve as a control. The brightness values for the background were subtracted from each region of interest. Once the background was subtracted, the average luminosity for each region was derived. These values of the average luminosity are used to compare to one another of the same axon to come up with a ratio of brightness along the axons.

Because each image may have experienced varied levels of photo-bleaching due to the lack of stability of Rhodamine when exposed to light, the luminosities of each image were not compared to each other with their average brightness values, but instead their ratios of brightness along the length of the axon.

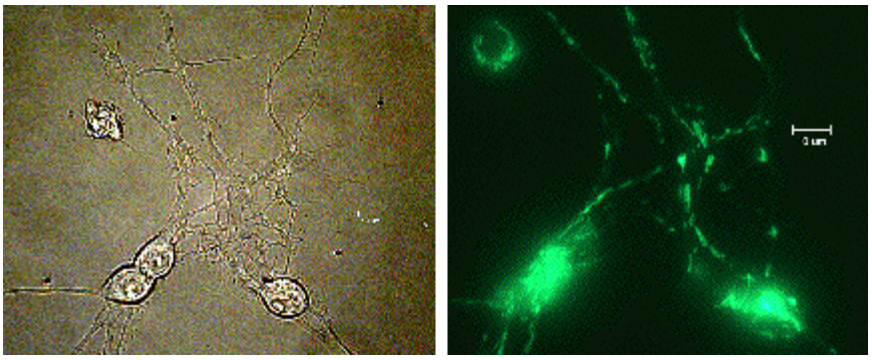

Figure 1. These images are of a transmitted light image taken with Spot Camera on a Nikon E400 microscope and its corresponding fluorescent image taken also taken with Spot Camera on a Nikon E400. Both images are magnified 600 times.

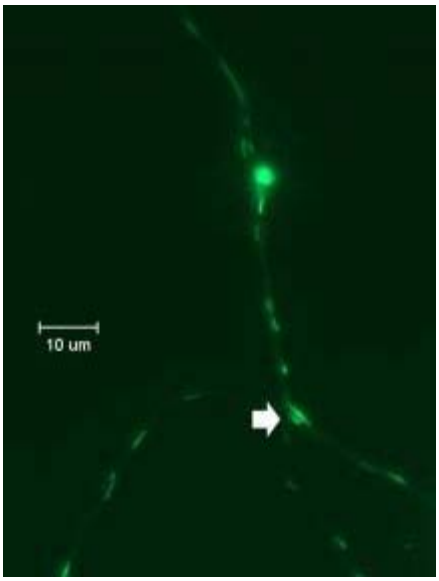

Figure 2. This is a fluorescent image taken with Spot Camera on a Nikon E400 microscope. The regions of green fluorescence are Rhodamine stained mitochondria. The white arrow points to a region of axonal overlap. This image has been magnified 600 times.

Results

The following figures compare the ratio of average luminosity of a gradient of regions along a single axon. Three axons were analyzed. The region of overlap is always displayed first in the graph. The region 10um from the overlap is referred to as region 1, the region 20um from the overlap is referred to as region 2, and the region 30um from the overlap is referred to as region 3.

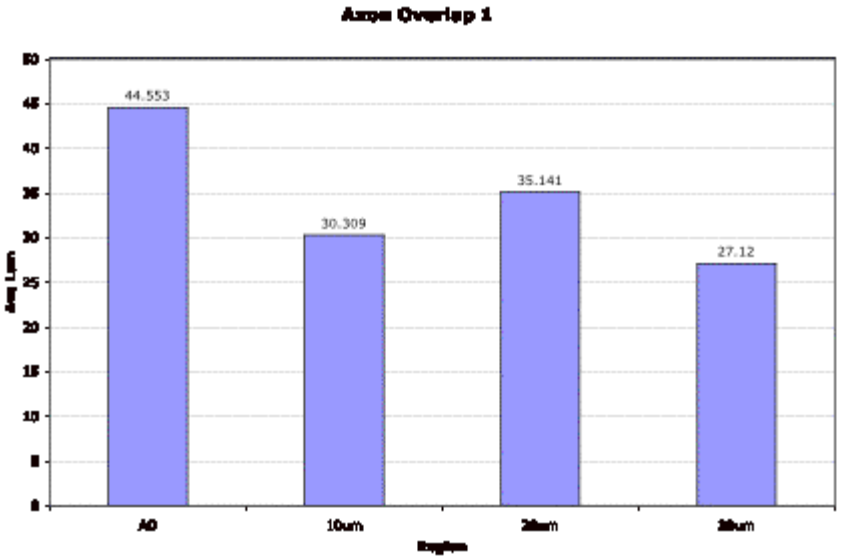

Figure 3. This graph provides a representation of the average luminosity of pixels found at the region of axonal overlap and regions of increasing distal distance from the region of overlap.

Axon 1 shows a high density of mitochondria in the region of axonal overlap as it has an average luminosity value of 44.553. This brightness value is at least 9.412 higher than that of the other regions. The region 30 um distal to the overlap, region 3, shows the lowest average luminosity. The region 10 um distal to the overlap, region 1, has the next lowest value of average luminosity and the region 20 um distal, region 2, has an average luminosity higher than that of region 1 or 3, but is still lower than the region of overlap.

The ratio of brightness from the region of overlap to that of regions 1, 2, and 3 is 1.6:1.1:1.3:1, respectively.

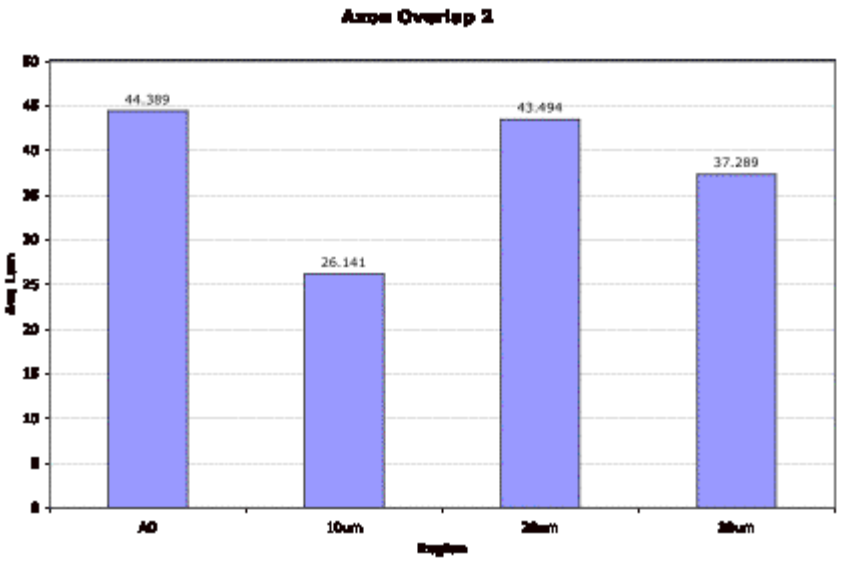

Figure 4. This graph provides a representation of the average luminosity of pixels found at the region of axonal overlap and regions of increasing distal distance from the region of overlap.

Axon 2 shows a high density of mitochondria in the region of axonal overlap as it has an average luminosity value of 44.389. This brightness value is only 0.895 higher than that of region 2. Region 1 shows a large decrease in brightness

from that of the region of overlap, and region 3 has an average luminosity between regions 1 and 2.

The ratio of brightness from the region of overlap to that of regions 1, 2, and 3 is 1.7:1:1.6:1.4, respectively.

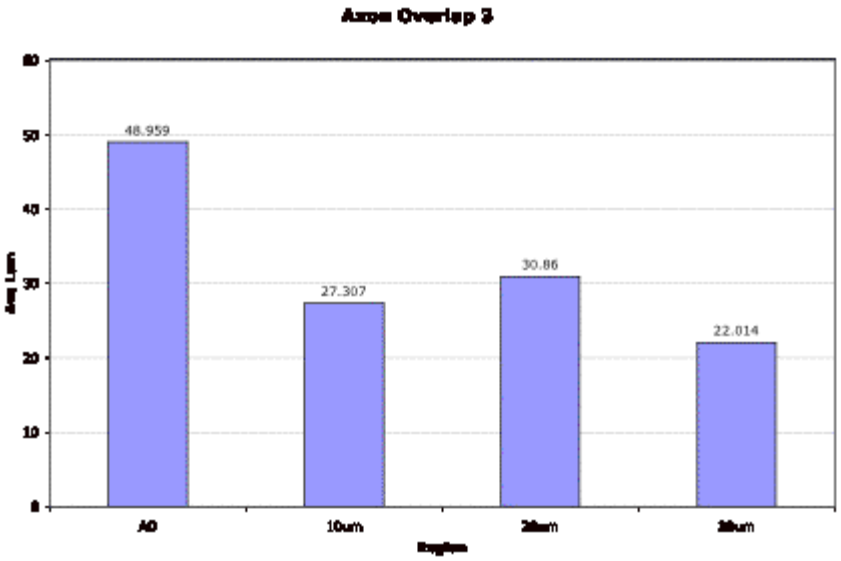

Figure 5. This graph provides a representation of the average luminosity of pixels found at the region of axonal overlap and regions of increasing distal distance from the region of overlap.

Axon 3 shows a high density of mitochondria in the region of axonal overlap as it has an average luminosity value of 48.959. This brightness value is at least 18.099 higher than that of the other regions. Region 3 shows the lowest average luminosity of the axon.

The ratio of brightness from the region of overlap to that of regions 1, 2, and 3 is 1.8:1.2:1.4:1, respectively.

Conclusion/Discussion

Previous to the experimentation documented above, it was thought that the average luminosity axonal regions would decline as the distance away from the region of axonal overlap increased. If the results of the experimentation and analysis agreed with this hypothesis, this would have meant that mitochondrial density decreased as distance away from the overlap increased. The results show that while regions of axonal overlap did have a higher average luminosity than that of other axonal regions, thus having a higher concentration of mitochondria in the overlap, a constant trend of decreasing density of mitochondria with increasing distance did not occur in any of the three axons analyzed.

In each of the three axons, the average luminosity of the axonal overlap stayed consistent, with an average luminosity of 45.967 and a standard deviation of 2.422. This region was the only region that showed consistency in values of luminosity.

Interestingly, region 2, the region 20um from the overlap, was always higher than both regions 1 and 3, but never higher than the overlap. This trend starts a pattern of high density, low density, high density, low density, but would need much more data to make an assumption that this pattern is commonly found in the growing axons of sympathetic nerve chains of chick embryos.

While the data collected does not show a gradient of decreasing density with an increase of distal distance, they fact that regions of axonal overlap were always shown to have a higher value of luminosity, and thus a higher concentration of mitochondria, does allow for speculation of the localization of the ATP generating organelle. Because mitochondria are consistently found to have a higher concentration in regions of overlap with other axons, this supports the hypothesis that this region requires more energy than other regions of axon. This may mean that there are indeed *en passant* synapses in these regions of overlap, but much more research is needed to conclude this hypothesis. More evidence besides that this region is an area of intense ATP consumption is essential. Other such evidence, such as the

presence of exocytic vesicles would support the *en passant* hypothesis (See collaboration with Lauren Suarez and Whitney Sirois).

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