The Presence of Acetylated Alpha-Tubulin in Differentiating Neurons

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Introduction:

Microtubules are macromolecules that are found in all cells and are composed of a combination of two proteins: α-tubulin and β-tubulin (Weisenberg, 1972). In neurons, α-tubulin serves an important role during differentiation as a site for acetylation. In 1987, a study by MM Black and P Keyser found neurons from sympathetic nerve chains to undergo acetylation as a posttranslational modification to α-tubulin, and found this modified protein present ubiquitously throughout the neuron (Black & Keyser, 1987). A study of cortical projection neurons found the acetylation of α-tubulin to have an effect on the ability of those neurons to mature (Creppe et al., 2009). Another study linked acetylated α-tubulin to Huntington's disease, finding less acetylated tubulin in those who were affected by the disorder (Dompierre, 2007). These studies were just a few of many that suggest acetylation of α-tubulin holds significant importance in cells.

The great presence of acetylated α-tubulin in all neurons suggests that it may be a good target to be considered in studying neurobiology. Acetylated α-tubulin, specifically, has been found to exist in stable microtubules (Janke & Kneussel, 2010), which are a component of almost all living cells. The true function of acetylation in microtubules is still unknown and under investigation. Acetylated α-tubulin was found not to be the cause of microtubule stability (Janke & Kneussel, 2010), nor the driving factor of Kinesin-1 translocation in axons (Hammond et al., 2010). What has not been specifically and deeply addressed in research is the presence and temporal behavior of acetylated α-tubulin in growing neurons and glial cells. The findings of Creppe et al. suggested that acetylation was important for neuronal development, as it was found to be involved in extension and arborization of dendrites. Creppe et al. also suggested that acetylated α-tubulin could play a part in neuronal migration and synaptic targeting, however, these postulations were not further studied. The presence of acetylated α-tubulin in differentiating neurons (and/or glia) could indicate a role earlier on in development and help give insight to the explanation of the purpose of acetylation.

The present study investigates the location of acetylated α-tubulin during the differential growth stage of both neurons and glia. In fully grown neurons, acetylated α-tubulin was found to be concentrated in distinct areas (those poorly populated by tyrosinated-tubulin) (Brown et al., 1993). Considering solely the α-tubulin, the current study aimed to investigate whether a similar compartmentalized pattern would be seen in differentiating cells based on the perceived age of different parts of the neuron. Cells in this study were fixed at a young stage of growth, differing from the subject cells in other studies. For the purpose of the study, it was assumed that neurons and glia located further away from the initial cell tissue were "younger" than neurons and glia closer to the initial cell tissue. Based off previous literature, we hypothesized that locations closer to the original ganglion tissue were "older" and thus would show more acetylated α-tubulin than locations further away, demonstrating a negative correlation between distance from the initial cell tissue and brightness.

This study uses primary cultures of neurons from domestic chick embryos, *Gallus gallus*, as they are a convenient, accessible, and relevant model that can be applied to the human population. The abundance of chickens in today's society makes chicken eggs easy to obtain and chickens' genomes overlap greatly with humans', meaning research can be more meaningfully cross-applied to human biology (JoVE Science, 2014). The presence of acetylated α-tubulin was tested using immunofluorescence imaging after staining with an anti-acetylated-alpha-tubulin antibody. Greater brightness was assumed to indicate a higher level of immunofluorescence, which is considered to mean that there is a greater presence of acetylated α-tubulin. Two concentrations of primary antibody were used, with an expectation that the more concentrated solution would stain more tubulin, and thus yield brighter results.

Materials and Methods:

Materials: The sympathetic nerve chain from one *Gallus gallus* embryo were obtained and dissected using a dissecting microscope, forceps (2 sizes), nerve growth factor (NGF), Dulbecco's Modified Eagle's Medium (DMEM), Hank's Balanced

Salt Solution (HBSS), poly-lysine, and laminin. Rehydrated sea urchin embryos, MeOH EGTA fixative, and formaldehyde/glutaraldehyde buffer were also used to set up samples for fixation. Samples were fixed using block buffer, phosphate-buffered saline (PBS), phosphate-buffered saline + Triton X-100 (PBS-T), Hoechst stain, F-2168-0.2ml anti-alpha-tubulin stain (DM1A) from Sigma, T-6793-0.2ml anti-acetylated-tubulin stain from Sigma, and 2762 Tetramethyl Rhodamine-conjugated goat anti-mouse IgG from Molec Probes. A -80C freezer, 4C refrigerator, incubator, sterile hood, Nikon Eclipse 200 microscope, InSight FireWire camera, SPOT, ImageJ, and the Gemini Mac computer were also employed during the experiment.

Methods: The experiment was conducted over the course of ~4 weeks for one trial of seven different samples. First, primary cultures of *Gallus gallus* embryos were obtained and mounted on poly-K and laminin-treated coverslips following the procedure outlined in "Primary Culture Of Chick Embryonic Peripheral Neurons 1: DISSECTION" (Morris, 2014a) by Dr. Robert L. Morris, creating five coverslips of samples. Methanol fixation was performed one day later on three of the five coverslips of samples and Formaldehyde/Glutaraldehyde fixation was performed on the other two using procedures outlined in "Immunofluorescence of Chick Neuronal Primary Cultures" (Morris, 2014d). On that same day, two more coverslips were fixed with sea urchin embryos (serving as a positive control for immunofluorescence staining) using the procedure outlined in "Immunofluor staining of SU embryos - MeOH fixation" (Morris, 2014c).

One week later, antibody solutions were created by mixing 1000µl of block buffer to 1µl of each antibody stock. An additional stock composed of 500µl block buffer and 1µl anti-acetylated-tubulin ("high concentration") and a 1:1000 dilution of the antibody ("low concentration") were also created. Humidity chambers were then created by placing a sheet of parafilm on the bottom of each of two 150mm petri dishes, lining the edges with rolled up kimwipes (saturated with water), placing a water-saturated kimwipe folded in half in the cover of each dish, and cutting off the tops of eppendorf tubes and pushing them evenly spaced into the parafilm (no more than four caps per dish). All fixed coverslips were placed on epitube caps in humidity chambers, noting which were fixed with MeOH and which were fixed with formaldehyde/glutaraldehyde. A p200 pipetman was used to add 200µl of low concentration anti-acetylated-tubulin antibody to two of the MeOH-fixed coverslips and one of the formaldehyde/glutaraldehyde-fixed coverslips that contained neurons from *Gallus gallus* embryos. On the coverslips containing the sea urchin embryos, 200µl of DM1A antibody was added. The remaining two coverslips were treated with 200µl of block buffer. Humidity chambers were then stored overnight in 4 degree (Celsius) refrigerator. One day later, all coverslips were removed and washed three times by adding PBS-T to coverslips in separate, labeled wells of a six-well plate. All coverslips were placed back on epitube caps in humidity chambers second and a second round of antibodies was added, where 200µl of DM1A was added to all experimental coverslips, 200µl of low concentration anti-acetylated-tubulin antibody was added to one positive control, 200µl of high concentration anti-acetylated-tubulin antibody was added to the other positive control, and 200µl of block buffer was added to negative controls. All coverslips were again stored in humidity chambers in the refrigerator overnight. The next day the same washing/staining procedure was followed again, using 200µl Tetramethyl Rhodamine-conjugated goat anti-mouse IgG on all seven coverslips.

Four days after the last stain, coverslips were mounted on slides first adding coverslip chips (to one positive control only), adding a drop of block buffer, lifting out each coverslip and wiping off the back, placing the coverslip cell side down on the drop of block, wicking away all excess buffer, and sealing each coverslip to its respectively labeled slide with nail polish (2 coverslips per slide). To image cells, a Mac computer (Gemini), Nikon Eclipse E200 microscope, and Insight Firewire camera were used. Images of an area with new growth of neurons and glia were photographed on each slide in a dark room using settings for immunofluorescence of red, green, and blue wavelengths. These images were then analyzed using ImageJ software, measuring two pixels on axons and two pixels on glial cells (one ~100µm from the initial cell tissue and one at ~900µm) for each picture. The brightness values at each point were then taken using a selection tool in the software. For each value measured an additional value was measured of the immediate background to the cell, therefore allowing for a ratio to be created and account for background noise. The brightness ratios were then compared among samples in terms of brightness compared to distance from the initial cell tissue.

Results:

Of the seven conditions fixed, only five showed any growth by the time of imaging (the Positive Control stained with low concentration and Experimental stained with high concentration showed no new cellular growth). Those coverslips with growth showed differential levels of brightness as a result of immunofluorescence imaging. Brightness was considered as a ratio between the value at the point of interest on the axon or glial cell and the value of the immediate background. The neuronal axons and glial cells analyzed on the formaldehyde/glutaraldehyde-fixed coverslip showed a positive correlation between distance from the original cell tissue and brightness level of Tetramethyl Rhodamine-conjugated goat anti-mouse IgG immunofluorescence (Figure 1); the neuronal axons and glial cells on the coverslip fixed with MeOH showed overall lower immunofluorescence of Tetramethyl Rhodamine-conjugated goat anti-mouse IgG, with the axons showing a slight positive correlation and the glial cells showing a negative correlation when comparing distance from the initial cell tissue with

brightness levels of immunofluorescence (Figure 2A). When observing immunofluorescence of the anti-α-tubulin stain, the neuronal axons and glial cells imaged on the formaldehyde/glutaraldehyde-fixed coverslip showed lower overall brightness levels compared to the axons and glia imaged on the MeOH-fixed coverslip, with the glial cells showing a slight positive correlation and the axons a slight negative correlation between distance from original cell tissue and brightness of immunofluorescence; the axons and glia on the MeOH-fixed coverslip both showed a positive correlation between distance from the initial cell tissue and brightness with overall higher levels of immunofluorescence (Figure 2B). The Positive Control stained with the high concentration of anti-acetylated-tubulin antibody and both Negative Controls all showed some degree of fluorescence either from antibody labeling or background noise. In the Negative Controls, prolonged exposure during imaging may have accounted for the high level of brightness.

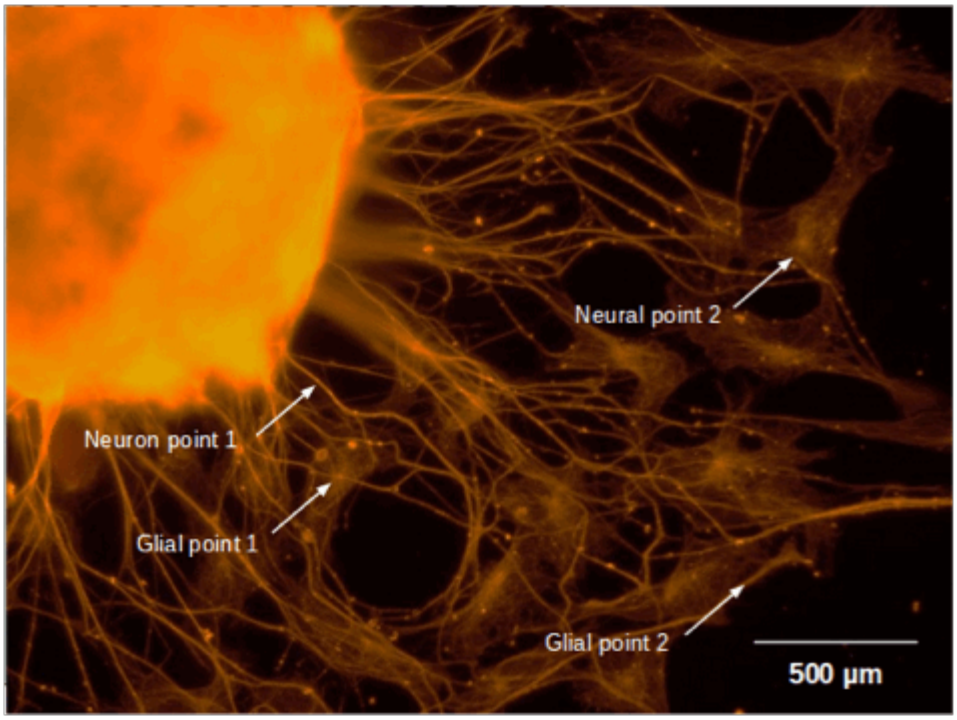
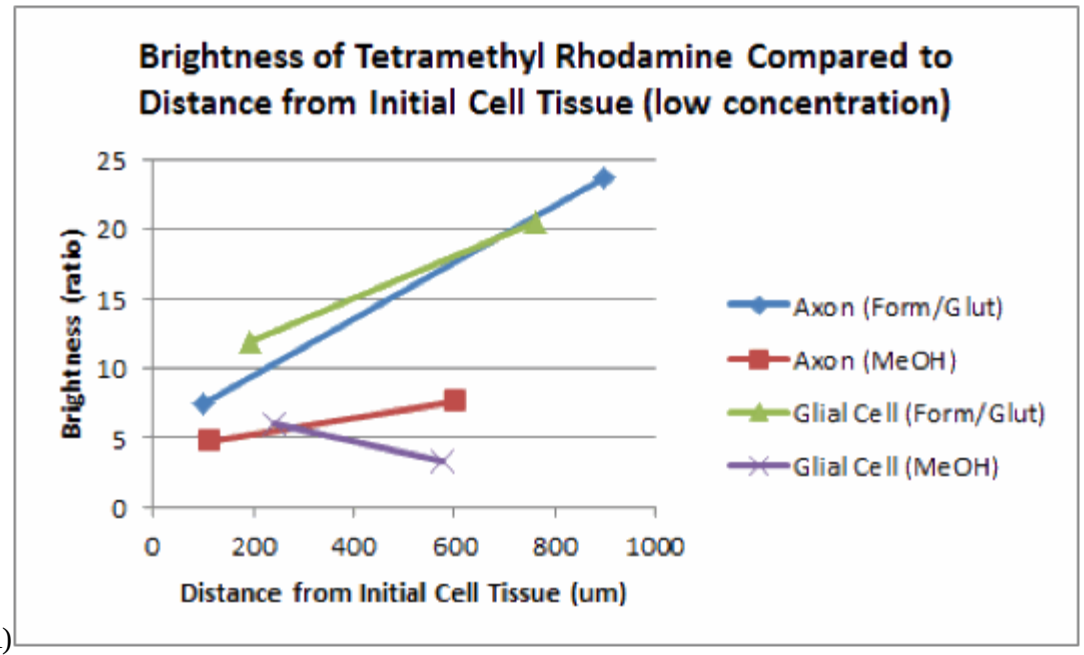
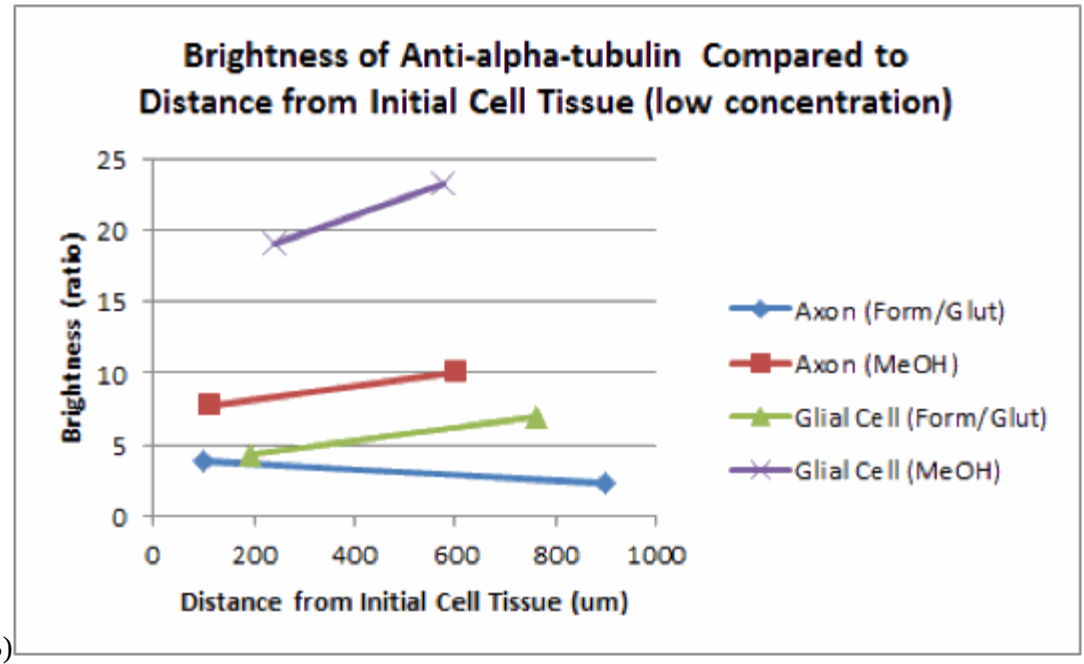


Figure 1. Immunofluorescence of Tetramethyl Rhodamine-conjugated goat anti-mouse IgG on formaldehyde/glutaraldehyde-fixed neuronal tissue. Brightness values of pixels indicated were measured in tandem with a pixel from the immediate surrounding background to create a brightness ratio for each region.

A)





B)

Figure 2. Immunofluorescence by distance from initial cell tissue. Brightness levels of immunofluorescence of A) Tetramethyl Rhodamine-conjugated goat anti-mouse IgG and B) anti-alpha-tubulin antibody stain in both glia and neuronal axons of experimental samples treated with the low concentration of anti-acetylated-tubulin. Experimental sample treated with 1:500 dilution of anti-acetylated-tubulin was not included in analysis because no cells were found on the coverslip during imaging.

Discussion and Conclusions:

The comparison of distance from original cell tissue and brightness of immunofluorescence in neurons and glia was found in most cases to have a positive correlation, contradicting the negative correlation hypothesized. This could be interpreted to mean that either the younger regions of the cells have more acetylated α-tubulin than older regions or that our parameters for which cells can be considered “old” and “young” were incorrect. It is also difficult to make conclusive statements about the relationships seen in the analysis of images since there was a failure of reliable positive controls. A positive control treated with low concentration anti-acetylated-tubulin antibody would be necessary for comparison to the experimental samples analyzed, considering that the experimental sample treated with the high concentration did not show any growth. The positive correlation was also seen in all experimental cells analyzed except for the glial cell grown on the MeOH-fixed coverslip, which could have been a result of the fact that it was treated with slightly less DM1A antibody than the other samples due to a shortage of antibody solution. Although normally this would not affect Tetramethyl Rhodamine-conjugated goat anti-mouse IgG labeling directly, because the DM1A antibody was added after the anti-acetylated-tubulin primary there could have been interference with the binding of DM1A. It was also interesting to note that within the cells on each coverslip there was a trend of increased immunofluorescence of acetylated α-tubulin and decreased regular α-tubulin with distance from initial cell tissue (Figure 2), which would make sense considering some of the regular α-tubulin would have to undergo acetylation to produce the acetylated α-tubulin present. A number of repeat trials would likely show the same trends and result in a similar conclusion in terms of presence of acetylated α-tubulin considering distance from the initial cell tissue.

The true function of acetylated α-tubulin remains unclear. The higher presence of acetylated α-tubulin in more distal locations relative to the initial cell tissue suggests that acetylated α-tubulin could be present in a greater abundance during the primary stages of differentiation. This means that neurons in fact do use acetylated α-tubulin as a component specific to their growth and development, although it is still retained in a lesser amount post-maturation. The experimental images are consistent with the previous literature in this way, finding acetylated α-tubulin to play a role in both growing and mature neuronal cells, seen in different amounts in the regions of newer/older growth. Creppe et al. had also suggested that acetylated α-tubulin could play a part in neuronal migration and synaptic targeting, which is a postulation supported by the current data as there was assumed to be a greater concentration of acetylated α-tubulin at the regions nearest the growth cones of the axons.

There were many sources of error in the experiment, leaving much room for improvement. First of all, the coverslip with the sea urchin embryo treated with low concentration of anti-acetylated-tubulin antibody and coverslip with *Gallus gallus* cells treated with the high concentration of anti-acetylated-tubulin antibody were dropped during the experiment, therefore the side on which the cells were adhered was unclear. Also, after the third antibody staining coverslips were left for four days in the 41 degree refrigerator, during which time some of the antibody solution dried on the coverslips. During imaging, careful consideration was also not taken in making sure that the objective lens and phase of the condenser were matched, which could have affected the brightness readings of certain images and caused a lack of consistency across images. A repetition of this

experiment would have to involve more careful following of all procedural steps. More samples for each condition would also be ideal to improve the data pool of results and DM1A would be added as the first primary to avoid any interference between antibodies. Future experiments could involve monitoring the growth of specific regions of neurons and glia as they differentiate to more definitively determine which areas are “youngest”.

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