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Post-translational Tubulin Modifications in Human Astrocyte Cultures

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Abstract

The cytoskeletal protein tubulin plays an integral role in the functional specialization of many cell types. In the central nervous system, post-translational modifications and the expression of specific tubulin isoforms in neurons have been analyzed in greater detail than in their astrocytic counterparts. In this study, we characterized post-translational specifications of tubulin in human astrocytes using the Normal Human Astrocyte (NHA; Lonza) commercial cell line of fetal origin. Immunocytochemical techniques were implemented in conjunction with confocal microscopy to image class III β -tubulin (β III-tubulin), acetylated tubulin, and polyglutamylated tubulin using fluorescent antibody probes. Fluorescent probe intensity differences and colocalization were quantitatively assessed with the 'EBImage' package for the statistical programming language R. Colocalization analysis revealed that, although both acetylated tubulin and polyglutamylated tubulin showed a high degree of correlation with β III-tubulin, the correlation with acetylated tubulin was stronger. Quantification and statistical analysis of fluorescence intensity demonstrated that the fluorescence probe intensity ratio for acetylated tubulin/ β III-tubulin was greater than the ratio for polyglutamylated tubulin/ β III-tubulin. The open source GEODATA set GSE819950, comprising RNA sequencing data for the NHA cell line, was mined for the expression of enzymes responsible for tubulin modifications. Our analysis uncovered greater expression at the mRNA level for enzymes reported to function in acetylation and deacetylation as compared to enzymes implicated in glutamylation and deglutamylation. Taken together, the results represent a step toward unraveling the tubulin isotypic expression profile and post-translational modification patterns in astrocytes during human brain development.

Keywords

tubulin; acetylation; polyglutamylation; astrocyte; immunocytochemistry; TUBB3; colocalization; human fetal; post-translational modifications; RNA seq

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Electronic supplementary material

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1. INTRODUCTION

Tubulin is a ubiquitous cytoskeletal component tasked with supporting a complex array of cellular functions such as chromosomal segregation, intracellular transport, and structural mechanics [1, 2]. The diversity of microtubule (MT) functions are attributed, at least in part, to variants of the tubulin alpha and beta isotypes that are expressed preferentially in differentiated tissues or during particular developmental periods [3, 4]. Additional MT specializations arise from post-translational modifications, commonly including tyrosination, acetylation, glutamylation, and glycation [5]. The heterogeneity of tubulin function conferred by differential isotypic expression patterns and post-translational modifications has been referred to as the ‘tubulin code’ or ‘MT code’ [6, 7]. The translation of this code is in its infancy, and the analysis of isotypes and modifications throughout the developmental specification of the organ systems is a subject of elevated interest. In the central nervous system, neuronal tubulin isotypes and post-translational modifications of MTs have received a considerable amount of attention [4, 8–13]. In particular, there is a growing awareness of the relationship between the tubulin code and neurodegenerative disorders: post-translational modifications affect binding of the Alzheimer’s-implicated protein tau, and levels of post-translational tubulin modifications are altered in individuals afflicted with Alzheimer’s [11, 13].

In contrast, tubulin specializations are relatively understudied in astrocytes, the most abundant type of glial cell in the brain. For instance, although the class III β -tubulin isotype (β III-tubulin) encoded by the TUBB3 gene has long been considered a hallmark of neuronal differentiation, it is only recently that TUBB3 expression has been experimentally confirmed in developing human astrocytes [14, 15]. In mature astrocytes, studies have shown that TUBB3 expression is elicited after injury [16, 17]. β III-tubulin is expressed after neoplastic transformation of mature glial cells, such as in astrocytoma/glioblastoma, and in oligodendromal tumors where it is expressed in tumor phenotypes that resemble migrating oligodendrocyte progenitor cells [18, 19]. These findings imply that β III-tubulin may signify plasticity in non-neuronal cell types.

Although a few research teams have evaluated tubulin acetylation in astrocytes, the absence of a thorough characterization of post-translational modifications in astrocytic tubulin is a significant obstacle to deciphering the MT code in the central nervous system [20, 21]. In order to contribute to the translation of the tubulin code in glial cells, we evaluated two tubulin posttranslational modifications, acetylation and glutamylation, in the commercially available Normal Human Astrocyte (NHA) primary cell line of embryonic (fetal) origin. Our experimental design relied on immunocytochemical detection with antibodies in the Research Resource Identifiers (RRID) registry and implemented methods to enhance reproducibility [22]. The expression of enzymes associated with acetylation and glutamylation at the mRNA level was confirmed by mining our open-source RNA sequencing data for NHA (GSE81995). Taken together our findings provide insight into the expression of tubulin isotypes and modifications at mid-gestation in human brain development.

2. METHODS

2.1 Cell Culture

The Normal Human Astrocyte (NHA; Lonza, CC-2565) cell line was chosen for this study because it is a viable *in vitro* system for studying the post-translational modifications of tubulin in human neural cells. Moreover, NHA cells are commercially available and can be implemented by any research group for validation and follow-up experiments. Two lots of NHA (#0000412568, #0000514417) were cultured according to methods previously published in an open source journal [15]. All manufacturer specifications were followed except for the omission of gentamicin, because aseptic technique enabled culture of NHA in an antibiotic-free environment. Three experiments were undertaken with two different vendor lots (#0000514417, passage 1, passage 2; lot #0000412568, passage 1). Cells were plated in BD Falcon 4-well chambered slides and cultured for five days prior to fixation.

2.2 Antibody Selection

β III-tubulin was selected as the base target for our comparison of acetylation and polyglutamylation in NHA because studies reported in the primary literature provide evidence that β III-tubulin is ubiquitously expressed in 100% of human fetal astrocytes [14]. β III-tubulin was detected with rabbit anti- β III-tubulin antibody (Abcam; catalog # ab202519, RRID: AB_2631274). Glutamylation was assessed with the mouse anti-polyglutamylated tubulin antibody (Abcam; catalog # ab11324, RRID:AB_297929) which is reported to detect polyglutamylation of both the α - and β -tubulin isoforms. Acetylation was evaluated with the mouse anti-acetylated tubulin antibody (Sigma-Aldrich; catalog # T7451, RRID:AB_609894) that has been reported to detect the α -isoform.

2.3 Antibody Validation

Western blot analyses were used to validate the antibodies with protein isolated from both NHA lots. Cells cultured in T-25 flasks for 5 days were lysed in radioimmunoassay precipitation buffer (50 mM Tris-HCl pH = 8.0, 150 mM sodium chloride, 0.1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate) with supplemented protease inhibitor cocktail diluted at 1:10 (Sigma-Aldrich; catalog # P8340). Protein concentration was determined with the *RCDC*TM Protein Assay (BioRad; catalog # 5000121). Protein samples (40 μ g) were mixed with Laemmli buffer and heated at 70°C for 10 minutes. Precision Plus ProteinTM WesternCTM Standards (5 μ L) were heated at 37°C for 5 minutes. Samples and molecular weight markers were analyzed with SDS-PAGE (200 V, 40 minutes) with 10 % Mini-PROTEAN® TGX Stain-Free Protein Gels (BioRad; catalog # 4568034S) and pre-chilled (4°C) electrophoresis buffer (25 mM Tris pH = 8.3, 192 mM glycine, 0.1 % sodium dodecyl sulfate) using a HoeferTM MiniVE vertical electrophoresis unit. The same unit was used to electrotransfer protein to nitrocellulose with preheated (70°C) Towbin buffer without methanol (25 mM Tris pH = 8.3, 192 mM glycine), using a modified version (20 V, 25 minutes) of the protocol as described in [23]. Membranes were probed using the Pierce Fast Western Blot Kit (ThermoFisher Scientific; catalog # 35055) according to the manufacturer's specifications, with overnight primary antibody incubation periods at 4°C. Data from at least two separate blots were collected for each antibody. The unconjugated version of the rabbit anti- β III-tubulin antibody (Abcam; catalog # ab52623,

RRID:AB_869991) and the mouse anti-acetylated tubulin antibody were used at a 1:1000 dilution, whereas the concentration of mouse anti-polyglutamylated tubulin antibody required a higher concentration (1:250) to detect a signal. Chemiluminescent signals were detected with a Chemidoc XRS (BioRad).

2.4 Immunocytochemistry

NHA were labelled in accordance with previously published methods with the following modifications for antibody type and dilutions [15]. PBST containing 1% Bovine Serum Albumin was used to dilute primary antibodies to the following final concentrations: rabbit anti- β III-tubulin antibody, 1:300; mouse anti-polyglutamylated tubulin, 1:200; mouse anti-acetylated tubulin, 1:200. The primary antibody against β III-tubulin was incubated simultaneously with either mouse anti-acetylated tubulin or mouse anti-polyglutamylated tubulin.

To determine specificity of the β III-tubulin antibody, 1 μ g/ ml of the β III-tubulin synthetic immunizing peptide was incubated with the diluted β III-tubulin antibody for one hour at ambient temperature (~26 °C) before incubating overnight in chambered slides. Slides were incubated at 4°C overnight in a sealed, darkened, humidified chamber with 250 μ l of antibody solution in each well. Nonspecific binding of the secondary antibody was assessed by omitting the primary antibody from negative controls. Alexa Fluor® 594-conjugated goat anti-mouse IgG (Abcam; catalog # ab150120, RRID: AB_2631447) was diluted at 1:500 in PBS with 1% BSA. Hoescht 33342 (0.1 μ g/ ml) was used to counterstain cell nuclei before mounting slides as described previously [15].

2.5 Confocal Imaging

Confocal images were acquired with an EC Plan-Neofluar 10X/0.3 objective mounted on an inverted LSM 700 microscope (Zeiss). Hoescht 33342 was excited with laser excitation at λ = 405. Alexa Fluor® 555 and Alexa Fluor® 594 were excited with laser excitation at λ = 555. The main beam splitter was MBS 405/488/555/639, and the SP 555 filter used to collect emission from all fluorophores. To separate the emission from Alexa Fluor® 555 and Alexa Fluor® 594 fluorophores, dichroic beam splitters were used at 559 nm and 628 nm in the respective channels. All confocal images were captured with identical settings (Table 1).

2.6 Fluorescent intensity analysis

The ZenLightEdition software package (Zeiss, 2009) was used to create maximum intensity projections and export files with a .tif extension. The image processing package 'EbImage' for the R statistical programming language was used to import .tif files and translate images into a 2 dimensional array of pixel intensity values between 0 (black) and 1 (white) using a 16-bit range (2^{16} values) [24].

The degree of post-translational modification of tubulin (acetylation or polyglutamylation) was estimated as a ratio of the sum of fluorescent signal from acetylated or polyglutamylated tubulin to the sum of fluorescent signal from β III-tubulin with intensity signals corrected as described below. The ratio of modified tubulin/ β III-tubulin fluorescent signal was calculated for each image (n = 10 acetylated; n = 10 polyglutamylated). Potential

differences in mean values of fluorescence intensity ratios for acetylated and polyglutamylated tubulin to β III-tubulin were calculated using the student's t-test with a p-value significance threshold of 0.01.

In order to omit 98% of fluorescent signal that resulted from nonspecific antibody binding, the 98th percentiles of pixel intensities from control images were calculated individually for each image, and averaged separately for both negative control groups (primary antibody against β III-tubulin blocked with immunizing peptide, secondary antibody with primary omitted). Omission of 98% of the background signal was accomplished by subtracting the aforementioned average value for negative control images from the pixel intensity of the corresponding frames of experimental images (value of controls blocked with immunizing peptide was subtracted from pixel intensity values β III-tubulin images; value of secondary antibody with primary omitted was subtracted from pixel intensity values for acetylated tubulin or polyglutamylated tubulin). Pixel intensity values that fell outside the established range from black (0) to white (1) after background subtraction (i.e. below zero) were set at zero prior to calculating the sum of intensity values.

2.7 Colocalization

The statistical programming language R was used to estimate colocalization for all pixels in both modified tubulin (1C3, 2C3) and β III-tubulin (1B3, 2B3) channels corresponding to a single optical section from each condition. Colocalization of signals from post-translationally modified tubulin and β III-tubulin was evaluated using the Pearson's correlation coefficient, Mander's overlap coefficients, and the Costes randomization coefficient (Reviewed in [25]).

A second colocalization analysis was undertaken with the values of the extracellular areas of the image omitted. An extracellular section of approximately 200×200 pixels was cropped from each image. The mean extracellular image pixel values were estimated and used as a threshold for normalizing the corresponding images. This analysis was intended to validate the first colocalization analysis by ensuring that the Pearson's correlation coefficient was not inflated due to correlated extracellular regions. Moreover, because both the Mander's and the Costes' approach are sensitive to background signal, omitting the extracellular pixel values strengthens the validity of these analyses. The second colocalization was estimated for the normalized images as described above.

2.8 Figure Preparation

Figures were prepared using the statistical programming language R, in conjunction with the 'ggplot2' and 'ggthemes' data visualization packages [26, 27]. Alignment, labelling, and assembly of images were completed with Photoshop (Adobe, CS6). The ZenLightEdition software package (Zeiss, 2009) was used to manipulate the contribution of the fluorescence signal from each channel in order to visualize all signals in the merged confocal images, and to export confocal images as TIFF files. Digital images of western blots were converted to TIFF files and digitally enhanced for contrast using the Auto Scale function in Image Lab software (version 6.0; BioRad).

2.9 Compliance with Ethical Standards and Reproducibility Methods

Guidelines for preclinical research set forth by the NIH were used as a model for our experimental procedures [28]. The vendor, Lonza, produced NHA according to national ethics standards, de-identified the cell line, and retains the signed record of informed consent from human donors. The NMSU Institutional Biosafety Committee approved the use of commercially available human cell lines (approval # 1401SE2F0103). De-identified, commercially available human cell lines developed before 2015 are exempt from review by the NMSU Institutional Review Board. The NIH retains a signed copy of the Extramural Institutional Certification form for human cell lines created before January 25, 2015 with the GEO data files previously submitted by our research team (GSE819950).

NHA were used within 3 passages (10 population doublings) according to the guaranteed vendor specifications. The human origin of the cell line was verified by the vendor, and confirmed in previous RNA sequencing experiments by our laboratory (GSE81995) [15].

Alexa Fluor® 555-conjugated rabbit anti-TUBB3 monoclonal antibody (Abcam; catalog # ab202519, RRID: AB_2631274) was validated as described above, by blocking with the immunizing peptide. The unconjugated version of this antibody (ab52623) from the identical clone [EP1569Y] has been validated by the vendor, and previously used to label the NHA cell line [15]. Both the mouse anti-polyglutamylated tubulin antibody (Abcam Cat# ab11324, RRID:AB_297929) and the mouse anti-acetylated tubulin antibodies (Sigma-Aldrich Cat# T7451, RRID:AB_609894) have been validated in previous experiments with murine neural cells [29, 30]. The Alexa Fluor® 594-conjugated goat anti-mouse IgG (Abcam; catalog # ab150120, RRID:AB_2631447) was validated by assessment of fluorescence when the primary antibodies were omitted.

3. RESULTS

3.1 Immunocytochemical detection

The characterization of tubulin post-translational modifications in NHA was undertaken using immunocytochemical methods. A positive label for β III-tubulin was observed throughout optical sections of the cell cultures (Fig. 1, 2). This result is congruent with previous findings that report expression of β III-tubulin in 100% of cultured human fetal astrocytes at the midgestational period [14]. The labelling pattern of acetylated tubulin resembled the labelling pattern of β III-tubulin (Fig. 1). In contrast, the labelling pattern for polyglutamylated tubulin represented a smaller portion of the cultured cells than both acetylated tubulin and β III-tubulin (Fig. 2). Western blot experiments were consistent with these findings; higher concentrations of antibody were required to detect polyglutamylation in the blots. (Online Resource 3).

3.2 Evaluation of fluorescence intensity

The 'Ebimage' package for the statistical programming language R was used to separate the channels from confocal images and assign each pixel an intensity value ranging from 0 (black) to 1 (white). The ratio of intensity values for post-translationally modified (acetylated or polyglutamylated) tubulin label to β III-tubulin label was calculated for each

image. The mean value of the acetylated tubulin/ β III-tubulin ratio (0.42) was almost twice as much as the mean value of polyglutamylated tubulin/ β III-tubulin ratio (0.24; Table 2). The p value from Student's t test comparing the ratio of acetylated tubulin to β III-tubulin to the ratio of polyglutamylated tubulin to β III-tubulin (Table 2) was less than the threshold p value of 0.01 (Table 2), conferring significance to the findings.

3.3 Colocalization analyses

The degree of colocalization between post-translationally modified tubulin and β III-tubulin was calculated for the 3rd optical section from Fig. 1 and 2. Scatterplots of the pixel intensity values from the red (acetylated or polyglutamylated tubulin; x axis) and green (β III-tubulin; y axis) channels were plotted against one another (Fig. 3). The pixel intensity values assigned by 'Ebimage' were used to calculate the Pearson correlation coefficient, the Costes randomization coefficient, and the Mander's overlap coefficient with the statistical programming language R.

Colocalization analysis uncovered a high degree of correlation between acetylated tubulin and β III-tubulin with all three methods (Table 3). Similarly, a strong correlation was indicated for polyglutamylated tubulin and β III-tubulin; however, there is an even greater degree of correlation for acetylated tubulin and β III-tubulin (Table 3). Thresholding to exclude all pixel values below the mean intensity value from a 200×200 extracellular region increased the degree of correlation with the Costes and Mander's techniques, but did not alter the Pearson correlation coefficient. This finding is consistent with the sensitivity to background signal previously reported for these methods [25].

3.4 mRNA levels of enzymes with a putative role in acetylation or glutamylation

RNA sequencing data from NHA (GSE81995) were used to evaluate expression of enzymes that are reported to function as tubulin post-translational modifiers [15]. mRNA expression levels, normalized as Fragments per Kilobase of exon per Million reads mapped (FPKM), were determined for 22 enzymes that are implicated in acetylation/ deacetylation and glutamylation/ deglutamylation of tubulins (Table 4; reviewed in [2, 31, 32]). Enzymes with very low FPKM levels (below 1) are omitted from Table 4 (tubulin glutamylases, TTLL 2,6,9, 13; tubulin deglutamylases, AGBL 1–4). The average FPKM values for enzymes involved in acetylation (~16) and deacetylation (~16) were greater than the average FPKM values for enzymes involved in glutamylation (~2) and deglutamylation (~2).

4. DISCUSSION

Astrocytes are highly variable in both structure and function, particularly in humans [33–36]. However, the role of specified and modified tubulins in imparting this diversity is not well documented. This research was an effort to advance the current understanding of tubulin specifications in human fetal astrocyte cultures. Immunocytochemistry and western blot methods were used to label β III-tubulin, acetylated tubulin, and polyglutamylated tubulin in NHA.

In accordance with previous reports of β III-tubulin expression in human fetal astrocytes, we observed a prevalent label for β III-tubulin in Normal Human Astrocyte cultures [14, 15].

Although it is possible that β III-tubulin expression in cultured NHA may represent a cytoskeletal adaptation of microtubules to *in vitro* culture conditions, our findings are consistent with the *in situ* demonstration of β III-tubulin co-localization with GFAP⁺ and nestin⁺ immature glial cells in formaldehyde-fixed, paraffin embedded histologic sections of human fetal brain [14]. While β III-tubulin has had a long-standing role as a neuronal marker, recently it has been recognized as indicative of more plastic cell types, such as immature astrocytes and different types of cancer [14, 18, 19, 37]. Moreover, upregulation of β III-tubulin has been observed in astrocytes following injury, alluding to a role in neuroglial regeneration [16, 17]. These findings suggest a link between the expression of β III-tubulin and plasticity, a phenotype that has been previously established in NHA [38].

Confocal images also demonstrated a positive label for acetylated tubulin and polyglutamylated tubulin in NHA cultures (Fig. 1, 2). In neurons, acetylation and polyglutamylation have been associated with growth [39, 40] and regeneration [41], respectively. In glial cells, post-translational modifications of tubulin have been implicated in functions that include modulating astrocytic Na⁺ K⁺ ATPase activity [20], and dysfunctions related to the tubulin-associated protein tau [21, 42]. Colocalization analyses revealed a strong correlation between the fluorescence signals from β III-tubulin and acetylated tubulin, as well as between β III-tubulin and polyglutamylated tubulin (Table 3, Fig. 3). Quantification of fluorescence intensity values from confocal images revealed statistically significant differences between fluorescent intensity ratios of acetylated tubulin / β III-tubulin and polyglutamylated tubulin / β III-tubulin (Table 2). The larger ratio of acetylated tubulin / β III-tubulin provides quantitative support of our qualitative visual observations. A similar pattern can be observed in our western blot analyses of acetylation and polyglutamylation (Figs. S1B, S1C).

Recent interest in post-translational modifications of glial cells has prompted investigation of this process in Schwann cells. Gadau (2010) characterized relative amounts of several modified tubulins and found that polyglutamylation occurs at a significantly higher level than acetylation in an immortalized rat Schwann cell line. [43]. In contrast, we found that levels of polyglutamylation are significantly lower than acetylation in NHA. Gadau speculated that the increase in polyglutamylation that they observed could be due to the antibody binding to both the α - and β -tubulin isoforms. Although we used the same antibody in our studies, we observed a decreased intensity of signal for polyglutamylation as compared to acetylation in NHA. The marked difference in relative abundance of acetylation and polyglutamylation in cultured, primary human astrocytes as compared to immortalized rat Schwann cells warrants downstream investigation of the function of acetylation and polyglutamylation in glial cells and the potential role of the tubulin code in the specification of glial cell fate.

We reasoned that detection of post-translational tubulin modifications at the protein level implied the presence of specific modifying enzymes in NHA. We predicted that by mining RNA sequencing data for NHA (GSE819950), we should be able to identify transcripts for enzymes involved in the addition and removal of acetyl and glutamyl side chains. As expected, mRNA expression values were greater than 1 FPKM for 14 of 22 enzymes implicated in acetylation/ deacetylation and glutamylation/ deglutamylation of tubulins

(Table 4; reviewed in [31, 32]). The greater FPKM expression values for enzymes involved in acetylation (~16) and deacetylation (~16) as compared to enzymes involved in glutamylation (~2) and deglutamylation (~2) support the premise that acetylation is more prevalent than polyglutamylation in NHA. However, the amount of post-translational modification also depends on enzymatic activity. Therefore, additional studies are needed to link the amount of post-translational modification of tubulin with the expression levels of the enzymes conferring the modifications.

In mature murine brain tissue, the deacetylating enzymes HDAC6 and SIRT2 are expressed in Purkinje cells and oligodendrocytes, respectively, but not astrocytes [44]. In contrast, RNA-seq data from cultured NHA show that SIRT2 and HDAC6 are expressed in the mRNA of human fetal astrocytes, and our detection of the acetylated tubulin protein implies the presence of acetylating and deacetylating enzymes in NHA. The observation that SIRT2 and HDAC6 are expressed in human fetal astrocyte mRNA, but not mature mouse astrocytes, suggests that the expression of these enzymes in astrocytes may be restricted to an immature developmental time point, or may be species-specific.

In summary, these experiments profiled isotypic expression and post-translational modification patterns of tubulin in human fetal astrocytes. Quantification of fluorescence intensity values from immunocytochemical probes revealed a greater proportion of acetylated tubulin than polyglutamylated tubulin, though both modifications were detectable at the protein level. This trend was supported by data from our western blot experiments and our analysis of RNA-seq experiments with cultured NHA (GSE81995). Results from these experiments illuminate the complexity of expression patterns and modifications of tubulin in human fetal astrocytes, and accentuate the need to explore how the tubulin code contributes to the determination of glial identity in the developing nervous system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

β	beta
BSA	bovine serum albumin
FPKM	fragments per kilobase of exon per million reads mapped
MT	microtubule

NHA	Normal Human Astrocyte
PBS	phosphate buffered saline
PBST	phosphate buffered saline with 0.1% TWEEN® 20
RNA	ribonucleic acid
RNA-seq	RNA sequencing
RRID	Research Resource Identifiers

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Fig. 1. β III-tubulin and Acetylated Tubulin

Confocal microscopy was used to obtain the fluorescence signal from optical sections (1 – 4). The fluorescent nucleic acid probe Hoescht 33342 (a1–a4) was used in conjunction with immunocytochemical methods that labelled β III-tubulin (b1–b4) and acetylated tubulin (c1–c4) in normal human astrocytes cultured for 5 days. Scale bar = 200 μ m. Settings for image capture are described in Table 1. See Online Resource 1 for color representation of the merged images.

Fig. 2. β III-tubulin and Polyglutamylated Tubulin

Confocal microscopy was used to obtain the fluorescence signal from optical sections (1 – 4). The fluorescent nucleic acid probe Hoescht 33342 (a1–a4) was used in conjunction with immunocytochemical methods that labelled β III-tubulin (b1–b4) and polyglutamylated tubulin (c1–c4) in normal human astrocytes cultured for 5 days. Scale bar = 200 μ m. Settings for image capture are described in Table 1. See Online Resource 2 for color representation of the merged images.

Fig. 3. Scatterplots of pixel intensity values

The fluorescent intensity values for β III-tubulin (Y axis) were plotted against values for acetylated tubulin (a series, X axis) and for polyglutamylated tubulin (b series, X axis). Scatterplots (a1, b1) comprise pixel values with 98% of the nonspecific background pixel intensity values subtracted. Scatterplots (a2, b 2) comprise pixel values thresholded to exclude the mean value of an extracellular region ($\sim 200 \times 200$ pixels). β III-tubulin intensity values from Fig. 1b3, 2b3; acetylated tubulin intensity values from Fig. 1c3; polyglutamylated tubulin intensity values from Fig. 2c3

Table 1
Settings for Confocal Image Acquisition

Image capture settings for all channels were kept consistent for images used in this analysis.

	Channel 1 Hoescht 33342	Channel 2 Alexa Fluor® 555	Channel 3 Alexa Fluor® 594
Laser Power	8%	5%	5%
Master Gain	1024	755	864
Digital Gain	1	1	1
Digital Offset	0	0	0
Pinhole	1 Airy Unit	1 Airy Unit	1 Airy Unit

Statistical comparison of acetylated tubulin/ β III-tubulin and polyglutamylated tubulin/ β III-tubulin pixel intensity values
 Students t-test revealed significant differences between the two posttranslational tubulin modifications (acetylation and polyglutamylation; *p < 0.01)

Table 2

	Mean (n = 10)	Standard Deviation	T statistic	D.F.	p Value
Acetylated Tubulin/ β III-tubulin	0.42	0.09	5.25	18	$*2.7 \times 10^{-5}$
Polyglutamylated Tubulin/ β III-tubulin	0.24	0.05			

Table 3**Colocalization analysis**

Pearson correlation coefficients, Costes randomization coefficients, and Mander's overlap coefficients were calculated for acetylated tubulin with β III-tubulin (from Fig. 1B3, 1C3), and polyglutamylated tubulin with β III-tubulin (from Fig. 2B3, 2C3)

	Pearson Correlation Coefficient	Costes Method	Mander's Overlap Coefficient (Red Channel)	Mander's Overlap Coefficient (Green Channel)
Acetylated Tubulin & β III-tubulin (all pixels, nonspecific background subtracted)	0.91	1	0.97	0.9
Acetylated Tubulin & β III-tubulin (extracellular pixel values excluded)	0.91	1	1	1
Polyglutamylated Tubulin & β III-tubulin (all pixels, nonspecific background subtracted)	0.8	1	0.91	0.84
Polyglutamylated Tubulin & β III-tubulin (extracellular pixel values excluded)	0.8	1	1	1

Table 4
Expression levels of putative enzymes that confer post-translational modifications of tubulin

RNA sequencing data from NHA (GSE81995) was mined to determine expression levels (FPKM; mean $\pm$ S.E.) of enzymes postulated to acetylate, glutamylate, deacetylate, and deglutamylate tubulin

ACETYLTATION		GLUTAMYLTATION	
Gene	FPKM	GENE	FPKM
NAA10	52.40 $\pm$ 3.57	TTLL1	5.35 $\pm$ 0.25
NAA50	16.72 $\pm$ 1.59	TTLL5	4.84 $\pm$ 0.41
KAT2A	11.55 $\pm$ 0.49	TTLL7	2.20 $\pm$ 0.07
ATAT1	8.57 $\pm$ 0.67	TTLL4	2.12 $\pm$ 0.10
ELP3	5.98 $\pm$ 0.13	TTLL11	1.30 $\pm$ 0.08
NAT1	2.92 $\pm$ 0.31		
DEACETYLTATION		DEGLUTAMYLTATION	
HDAC6	8.24 $\pm$ 0.61	AGBL5	8.64 $\pm$ 0.16
SIRT2	24.02 $\pm$ 1.48		