Tubulin post-translational modifications in developing dog primary neurons obtained with methods according to the 3Rs principles

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A B S T R A C T

Microtubules play a crucial role during neuronal morphogenesis regulating many functions. In the study of these phenomena in vitro cellular models have been employed, mainly resorting to housed experimental animals. Among alternative models in neurobiological study, recently dog caught particular attention. In fact, the com- plexity of the canine brain, the lifelong span and the neurodegenerative pathologies render the dog a species closer to humans than rodents. Lately, growing interest in the limitation of the use of experimental animals, has stimulated the search for alternative experimental protocols. Starting from fetal dog brain, obtained by alternative way of sampling, we set neuronal primary cultures. Through immunofluorescence, we examined the presence and the cellular distribution of tubulin post-translational modifications as tyrosinated and acetylated α- tubulin, as markers of dynamic and stable microtubule respectively. In addition, we evaluated the pattern of two associated proteins which may slide on these two tubulin modifications, i.e. CLIP-170 and Kinesin-1. A clear positivity for tyrosinated and acetylated α-tubulin, was found. As far as the motor proteins are concerned, we detected a prevalence of CLIP-170 compared to kinesin-1 with a better overlapping between tyrosinated α- tubulin and CLIP-170. Our findings highlighted some original data about the role of the microtubular network during early phases of canine neuronal morphogenesis. In addition, the experimental protocol underlined the utility of this alternative model that allows to bypass both the scarcity of commercial canine neuronal cell lines and the need to resort to experimental dogs, respecting the 3Rs principles (reduction, refinement, and re- placement).

Keywords:
Tubulin post-translational modifications; Neuronal morphogenesis; Dog Primary neuronal culture; 3Rs principles

1.Introduction

Microtubules (MTs), cellular polymers composed by the assembly of α- and β-tubulin heterodimers, may exhibit a high complexity and crucial importance in neurons which are involved in cargo traffiking, neuronal polarization and morphogenesis during the early phases of neurogenesis (Janke and Kneussel, 2010). These functions are made possible thanks to at least three main MTs patterns: i) the presence of different encoded isoforms; ii) the action of specific motor proteins associated with them (MAPs); iii) several post-translational modifica- tions (PTMs) generating on a microtubule a complexity of signals that known as tubulin code (Janke, 2014; Gadadhar et al., 2017). Tubulin PTMs include acetylation, tyrosination, detyrosination, Delta 2 (Δ2) modification, polyglutamylation, polyglycylation, palmitoylation, and phosphorylation (Song and Brady, 2015; Magiera and Janke, 2014; Westermann and Weber, 2003). Most of PTMs take place in the car- boxy-terminal tubulin tail and seem to affect the α-tubulin (acetylation, tyrosination/detyrosinatione and Δ2 modification), whereas glutamylation glycylation may target both α- and β-tubulin (Li and Yang, 2015). In addition, some PTMs are more present on stable MTs (acetylation, detyrosination and Δ2 modification), whereas others, like tyrosination, feature on more recent and more dynamic MTs (Fukushima et al., 2009). Among other, the role of PTMs seems to be crucial during the neuronal morphogenesis. Indeed, although it was thought that MTs had a secondary role in neurogenesis for several years, it is now established which MTs are essential regulators of neu- ronal morphogenesis phenomena (Poulain and Sobel, 2010; Yu et al., 2001). It seems to be now asserted that axonogenesis and den- dritogenesis, the essential processes by which neurons acquire their typical functional morphology, comes into play with a mix of stable and dynamic MTs (Burnette et al., 2008; Shea, 1999). Moreover, at this stage, the activity of MTs is regulated by a large number of associated proteins (MAPs), some of which may characterize the plus end of the most dynamic microtubules (like CLIP-170) while others such as the motor protein Kinesin-1, prefer to associate with stable microtubules (Galjart, 2010; Reed et al., 2006; Witte et al., 2008; Gomis-Ruth et al., 2008; Hammond et al., 2010; Verhey and Hammond, 2009). Many of the advances in the study of neurogenesis have been made possible by the use of in vitro experimental models, most part of which are based on murine embryo cultures (Dotti et al., 1988; Yu et al., 2001; Dehmelt et al., 2003). Despite the vast use of the murine experimental model, it presents some limitations compared to high mammals (humans in- cluded) such as the inbred nature of certain rodent strains, a short life span and size brain limitations (Lim et al., 2012). In recent years, several experimental models have been suggested in the study of many neurobiological aspects, pointing to other animal species, much closer to the human than the murine model, such as cow, sheep and dog (Cummings et al., 1993; Hashimoto et al., 2000; Duittoz and Hevor, 2001). The use of these alternative models for in vitro study have shown unquestionable advantages. Firstly, it allows some physio- pathological aspects, typical of some species, to be studied directly on the cells obtained from that same species (think for example of neuronal cultures from bovine brains to study biological aspects related to BSE). Secondly, many of these experimental models are set up with materials taken from slaughterhouses or removed during surgery for

other rea- sons (therefore destined for destruction), allowing to set up any kind of experimental protocol, without however, resorting to housed experi- mental animals (Peruffo and Cozzi, 2014; Panin et al., 2015). Thirdly, by doing so it is often possible to obtain cell lines from species and organs, which are not always present even in the most popular com- mercial cell banks. Among other alternative species for experimental models, over the last years dogs have been considered an excellent translational model for human medical research. Indeed, the canine nervous system is much more similar in size to that of humans than to that of rodents and it may develop several neurological diseases overlapping with those observed in the human brain (Milward et al., 1997; Kirk, 2003; Starkey et al., 2005; Thomas et al., 2009). The aim of this work was to assess the suitability of dogs' primary neuronal cells obtained through alternative methods of sampling to study the early phases of canine neuronal morphogenesis. The pattern of tubulin acetylation and tyrosination, as markers of stable and unstable micro- tubules respectively was monitored. In addition, in order to better evaluate the behavior of posttranslational modifications of the tubulin during neuronal morphogenesis, we evaluated the pattern of distributions of some associated proteins that preferentially may slide on dynamic and stable microtubules, like CLIP-170 and Kinesin-1 respectively.

2.Materials and Methods

2.1.Primary Cell Culture

The sampling of the canine fetuses was made thanks to the colla- boration between the Normal Anatomy Section and the Clinical Obstetrics of the Veterinary Teaching Hospital of our department. During routinary ovariohysterectomy procedures, whenever pregnant uteri were found, they would be immediately refrigerated and provided to our laboratory. Isolation of canine primary neuronal cells was per- formed as previously described (Mura et al., 2016). Briefly, cryopre- served brain tissues obtained from 25 gestation days dog fetuses were thawed, grossly minced and treated with Earle's balanced salt solution (EBSS) containing papain and DNase I for 40 min at 37 °C and cen- trifuged. The pelleted cells were resuspended in Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12; Euroclone,) supplemented with penicillin (30 mg/l) with streptomycin (50 mg/l), sodium bi- carbonate (2.4 g/l), insulin (10 µg/ml), transferrin (10 µg/ml), sodium selenite (10-8 M), 10% fetal calf serum. As neuronal growth promoter we added 10 ng/ml GDNF (glial cell line derived neurotrophic factor- Sigma, St.Louis, MO, USA) and 2 ng/ml of BDNF (brain derived neu- rotrophic factor- Sigma, St.Louis, MO, USA) to the medium 100 ng NGF (nerve growth factor- Sigma, St.Louis, MO, USA). Cells were plated into Petri dishes with a cover-glass slide on the bottom, previously coated with polylysine (Sigma, St.Louis, MO, USA) and placed in incubator (37 °C; 5% CO2).

2.2.Immunofluorescence Staining

Single and double indirect immunofluorescence were performed. After 16 days of culture, cells were fixed in cold 100% methanol for 20 min. After washing with PBS, fixed cells were incubated overnight with the following antibodies diluted with PBS containing: 3% bovine serum albumin (BSA) and 0,1% tryton X-100) anti βIII-tubulin (monoclonal, clone DM1A, 1:1000, Merck KGaA, Darmstadt Germany); 2) anti-total α-tubulin (monoclonal, clone DM1A, 1:1000, Merck KGaA, Darmstadt Germany); 3) anti-tyrosinated α-tubulin (monoclonal, clone TUB-1A2, 1:1000, Merck KGaA, Darmstadt Germany); 4) anti-acety- lated α-tubulin (monoclonal, clone 6-11B-1, 1:1000, Merck KGaA, Darmstadt Germany); 5) anti kinesin-1 (polyclonal, Merck KGaA, Darmstadt Germany); 6) anti-Clip 170 (polyclonal, Merck KGaA, Darmstadt Germany). After rinsing with PBS, cells were incubated for 1 h at 37 °C with secondary anti-rabbit and anti-mouse fluorescein/ tetramethylrhodamine isothiocyanate-conjugated antibodies (AlexaFluor 488, AlexaFluor 594, 1:400, Termo Fisher, Waltham, MA, USA). Images were obtained through a confocal laser scanning micro- scope from Leica (TCS SP5 DMI 6000CS, Leica Microsystems GmbH, Wetzlar, Germany) using a 40/60× oil objective. FITC was excited at 488 nm and emission was detected between 510 and 550 nm. Rhodamine was excited at 568 nm and emission was detected between 585 and 640 nm. Nuclear counterstaining was performed using Hoechst blue-33,342 (1:5000, Merck KGaA, Darmstadt Germany). During the acquisition process we hold constant the laser intensity, gain, and offset (Pinhole [airy] 1.00; Zoom 1.0; Digital offset: 0; Power Laser Line UV [405] 21.00%; Power Laser Line Visible [488] 38.00%; Power Laser Line Visible [543] 35.00%).

2.3.Fluorescent Intensity and Colocalization

The quantitative analysis of fluorescence intensity was carried out through the Leica LAS AF Lite image analysis software package (Leica Microsystems GmbH, Wetzlar, Germany). On the images taken with confocal microscope, converted to grayscale and by appropriate mag- nifications we designed a ROI on cell profiles (or on the cell processes or on the cell body) turning off the channel 1 (Hoechst blue) so that the software read the pixel average value on the remaining channel 2 and 3 which were switched on (AlexaFluor 488 or 594) and we recorded the mean values of pixel, to which we subtracted the value of the possible background. The trend of colocalization was performed using the same software package as indicated above. Chosing the picture of double immunofluorescence the software made automatically a scatterplot of the pixel intensity values of the two channels (red and green labeling), calculating the Pearson's correlation coefficient, the percentage of co- localization rate and the Mander's overlap coefficient (M1 and M2). As indicated by Dunn et al. (2011) the more the value of the Pearson's coefficient approaches to 1, the more the two molecules are coloca- lizing.

2.4.Statistical Analysis

Statistical analyses were carried out through the software program Statgraphics (Statpoint Technologies, Warrenton, VA). Data are ex- pressed as a mean ± standard error (SEM) and the inter-group analysis was done by Student's t-test or ANOVA. Statistical significance was accepted when $p < 0.05$.

3.Results

The brain tissue with 25 days of gestation chosen for this study provided a large amount of proliferating neural cells. Indirect im- munofluorescence was performed on fixed cells after 16 days of culture. The anti-β III antibody, a well-known neuronal marker (Dráberová et al., 1998), revealed high immunopositivity, confirming the neuronal character of the majority of the cells obtained in the culture. Also, the total α-tubulin antibody homogeneously marked the neuronal cells with equal distribution in the cell body and cytoplasmic prolongations (Fig. 1). The characterization of tubulin post-translational modifica- tions through single immunofluorescence labeling revealed well de- tectable immunopositivity for both tyrosinated and acetylated α-tu- bulin (Fig. 2). In order to better understand the pattern of cellular distribution of the two PTMs examined, we performed a quantification of the fluorescence intensity on cytoplasmic processes and around the cell body region. We detected slightly higher immunopositivity in cy- toplasmic prolongations than in the cell body for tyrosinated α-tubulin, although this difference did not appear significant (P = .8). On the contrary, the analysis of the cellular distribution of acetylated α-tubulin showed significant fluorescence intensity in cellular prolongations in comparison with that detectable in the cell body region (P = .02; Fig. 3). When we compared tyrosinated and to acetylated distribution, acetylation resulted significantly higher (P = .004) than tyrosination, both close to the cell body and along cytoplasmic processes (Fig. 4). Regarding the proteins associated with microtubules, immuno- fluorescence for CLIP-170 and Kinesin-1 highlighted a significant (P = .002) signal emission for CLIP in comparison with Kinesin immunopositivity (Fig. 5). Colocalization estimation on double im- munostaining with tyrosinated α-tubulin and CLIP-170 antibodies showed intense immunopositivity throughout the cell prolongation with a good degree of overlapping (PCC = 0.760; M1 Red/ Green = 0.993; M2, Green/Red = 0.998; Fig. 6). Diversely, double immunofluorescence for acetylated α-tubulin and Kinesin-1 highlighted a low colocalization trend between the two molecules (PCC = 0,430; M1, Red/Green = 0.445, M2, Green/Red = 0.600) (Fig. 7).

4.Discussion

Primary dissociated neurons can be considered a useful metho- dology to perform several studies on morphology, functions, survival, and toxicology on large number of homogeneous cell (Gibbons and Dragunow, 2010; Schock et al., 2010; Humpel, 2015). As far as the studies on neuronal development and differentiation are concerned, scientific literature is rich in in vitro work conducted in primary culture of various species, e.g. rats, mice, fish (Grondona et al., 2013; Darbinyan et al., 2013). Over these past years, particularly when dealing with studies on the biology of the nervous system, the need to seek an experimental model closer to humans has increased, especially considering some aspects such as brain size and complexity, life span expectancy and presence of pathologies similar to those present in humans (Mak et al., 2014; Hofman, 1985). In this context, the dog has lately started to represent an ideal candidate as a useful experimental model (Studzinski et al., 2005; Cotman and Head, 2008). In the present work we found interesting data both regarding the pattern of the mi- crotubular network and some associated motor protein during dog neuronal morphogenesis, as well as data regarding the utility of an alternative experimental model without the use of housed experimental dogs. In our experiment, we focused our attention on two tubulin PTMs patterns such as tyrosination and acetylation since they are distinctive of dynamic and stable microtubules, respectively, and play a funda- mental role during neuronal morphogenesis (Westermann and Weber, 2003; Poulain and Sobel, 2010). Immunofluorescence revealed a marked immunopositivity for tyrosinated and acetylated α-tubulin. Although it is well known that tyrosinated tubulin is present in most dynamic regions of the neuron (growth cone) and the cellular body (Marcos et al., 2009) while not abundant in cell prolongations, our data showed a good level of fluorescence intensity both towards the cellular body and along the neurites. This is a behavior that we have already observed in other types of neural cells (Gadau, 2015). That pattern of tyrosinated distribution may be in accordance with the fact that tyr- osination is a modification that characterizes the plus end of the most dynamic microtubules, which are not only present in the growth cone (Webster et al., 1987). Dynamic instability characterizes microtubules that tend to evolve rapidly by exploring three-dimensional space in order to allow fast adaptation of the cytoskeleton network (Cai et al.,
2009). Moreover, evidences reported that subset of dynamic micro- tubules are present along the neurites during the early stages of mor- phogenesis before their stabilization (Stiess and Bradke, 2011). To- gether with tyrosination, we wanted to evaluate the presence and distribution pattern of a microtubule-associated motor protein, the CLIP-170. CLIP-170 is part of a family of + TIPs protein accumulating at the end of the growing microtubules (Arnal et al., 2004; Folker et al., 2005). Double fluorescence and quantification showed intense marking for CLIP-170 and a perfect overlay with tyrosinated tubulin. This data is consistent with what has already been shown about CLIP-170. Indeed, that +TIP displays a tendency to bind microtubules that are growing and therefore dynamic, and has a high affinity to the richly tyrosinated

microtubules (Bieling et al., 2008). In regards with the second PTMs evaluated, the acetylated α-tubulin, we found a strong fluorescence intensity along the shaft of neuronal cells in contrast with the lesser intensity close to the cell body. It is well known which acetylated mi- crotubules are more present in neuronal compartment that require more stability like the axons, where long-lived MTs are abundant (Fukushima et al., 2009). The pattern of distribution of acetylated α- tubulin, i.e. the presence of stabilized MTs along the neuronal processes may confirm both an early polarization during neuronal development and a need for the developing neurons to possess stable MTs during morphogenesis (Hammond et al., 2008; Witte et al., 2008; Peris et al., 2009). Still referring to the tubulin acetylation, we also found another interesting result. Indeed, the neurons used in our experiment were grown in culture until the day 16 before being fixed for immuno-fluorescence. So, observing our data on fluorescence intensity, we can
assume which acetylated α-tubulin levels were still high on that day.

This is somewhat contradictory to what reported in Audebert et al. (1994) where in primary neurons from mouse, a decrease of acetylated tubulin after day 12 was found. This data surely deserves further in- sights but may suggest that the developmental pattern of PTMs may be different in different species. Even for acetylated tubulin we wanted to evaluate the behavior of a motor protein that seems to have a high affinity with it, i.e. Kinesin 1. Kinesin-1 is part of the large family of kinesins, motor protein involved in axonal transport (Hirokawa et al., 2009). It is well known which Kinesin-1 preferentially moves on to stable microtubules (Vale, 2003). Moreover, acetylation of tubulin seems to promote the linkage and transport of kinesin 1 (Reed et al., 2006). Our results have confirmed the good distribution of Kinesin-1 but without a high grade of overlay with acetylated tubulin and with a low quantification of fluorescence, especially when compared to CLIP-170 data. It would seem that on the 16th day of culture, compared to the high intensity of acetylated tubulin fluorescence, we have seen a decrease in Kinesin-1 activity. We know from the literature that Ki- nesin-1 is crucial during assogenesis and that its scarcity may be fatal during neurogenesis as it may cause a defects growth in axons (Lu et al., 2013; Winding et al., 2016). In addition, authors have pointed out that a decrease in Kinesin-1 in neurons may occur due to a Tau competition with microtubule protofilament. The more tau binds to the microtubule, the more the kinesin levels may decrease (Dixit et al., 2008; McVicker et al., 2011). Unfortunately, we did not consider these aspects in our study, so we are absolutely aware that the pattern of Kinesin-1 observed deserves further insights.

Our experimental protocol has revealed some aspects of tubulin PTMS and motor proteins such as CLIP-170 and Kinesin-1 during dog neuronal development. Although some data are not entirely original (the presence of tyrosinated and acetylated α-tubulin and motor pro- teins are certainly not a novelty in neural cells), they have highlighted the patterns of distribution and amount of tubulin PTMs and certain associated protein, not previously evaluated in dog neurons. In addi- tion, we have confirmed the utility of our experimental model for several reasons. Indeed, considering that the ideal animal model for studies on human diseases does not

exist (Hartung, 2017; Daneshian et al., 2015; Gregory et al., 2012), it is always advisable to choose an animal model that is closer to the human species so as to avoid failures in translating the results obtained, the dog seems to be a good candidate for this (Patterson, 2000; Pasquali, 2018). Moreover, thanks to cryo-preservation and starting from occasionally sampling, we have been able to store canine fetal brain tissues, which can be easily cultivated even after a long time from storage. This method certainly allows ad- vantages such as not having to resort to the use of housed experimental animals as hard to manage as the dog and to bypass the difficulty of finding commercial neuronal primary/continuous canine cell lines. Our experimental model would also allow the study on neurodegenerative pathologies of the dog, shedding light on cellular molecular aspects, otherwise not evaluable with other types of clinical analysis. Finally, work like the present can highlight the possibility of integration be- tween veterinary practice and basic experimental research, too often considered quite far from each other.

Conflict of interest

The author declares that there are no conflicts of interest.

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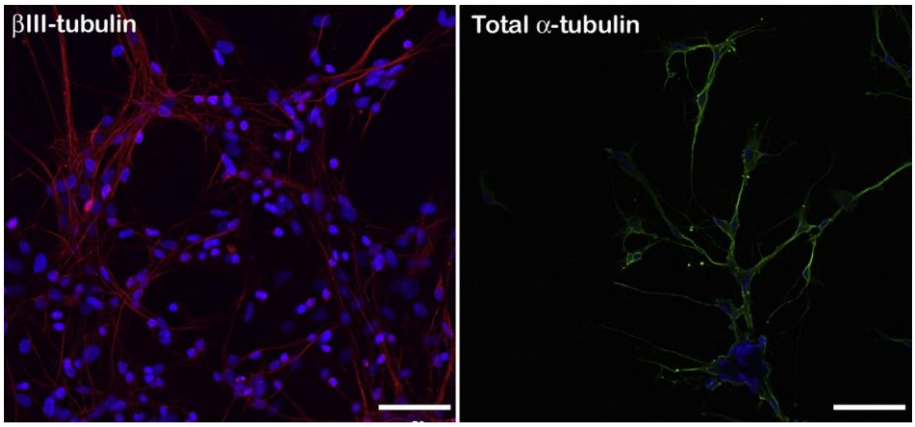

Fig. 1. Immunofluorescence staining to tubulin β-III and total α-tubulin on 16 days dog primary neuronal cultures. Bars = 50 µm.

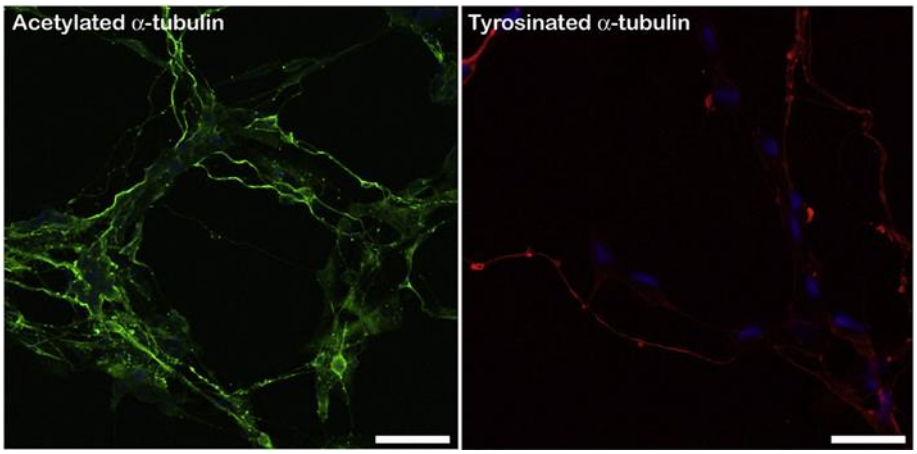

Fig. 2. Immunolabeling with acetylated and tyrosinated α-tubulin antibodies. Bars = 50 µm.

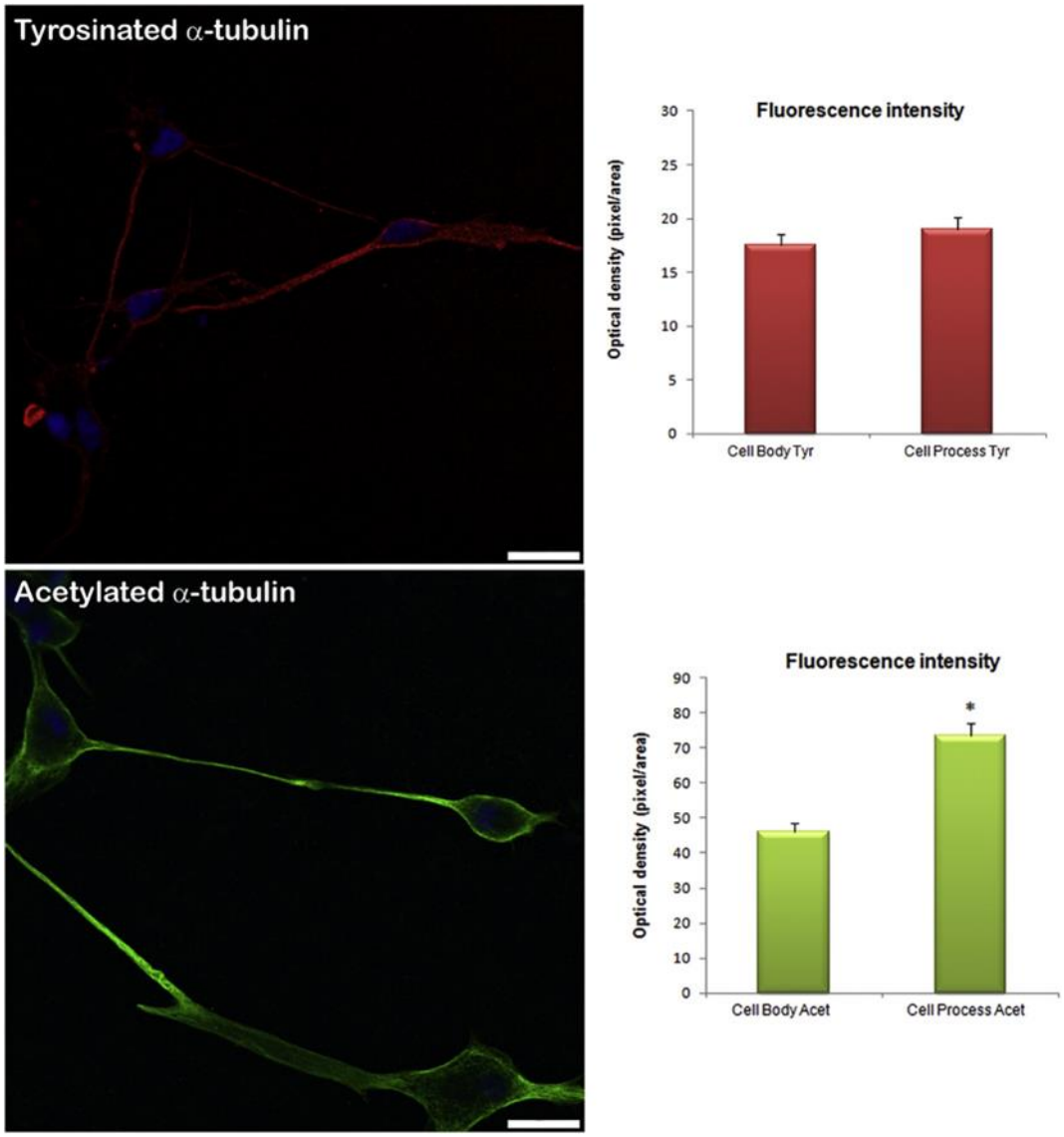

Fig. 3. Confocal images. Immunofluorescence staining revealed different cellular distribution of tubulin PTMs. By fluorescent intensity quantification (diagrams), tyrosinated α-tubulin (white arrows) showed no immunostaining differences in both cellular processes and cell body. On the contrary, acetylated α-tubulin intensity was stronger in cytoplasmic processes than the cell body. Data are expressed as means ± SEM of five experiments. * = $p < 0.0001$. Bars = 25 µm.

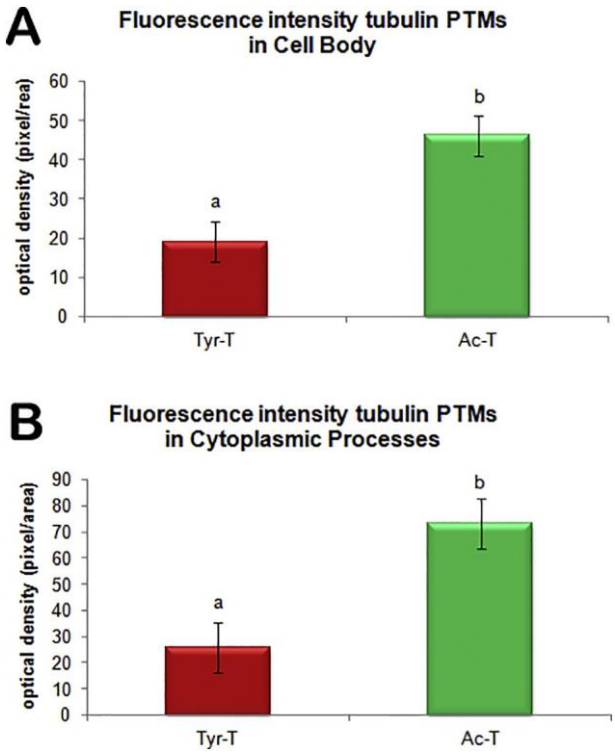

Fig. 4. Anova one way. Diagrams underlined a prevalence of acetylated α-tu- bulin respect to tyrosinated in both cell body (A) and cytoplasmic processes (B). Lower case letters indicate statistical difference among tyrosinated and acetylated α-tubulin (ANOVA $p < 0.01$). Data are expressed as means ± SEM of five experiments. Bars = 25 µm.

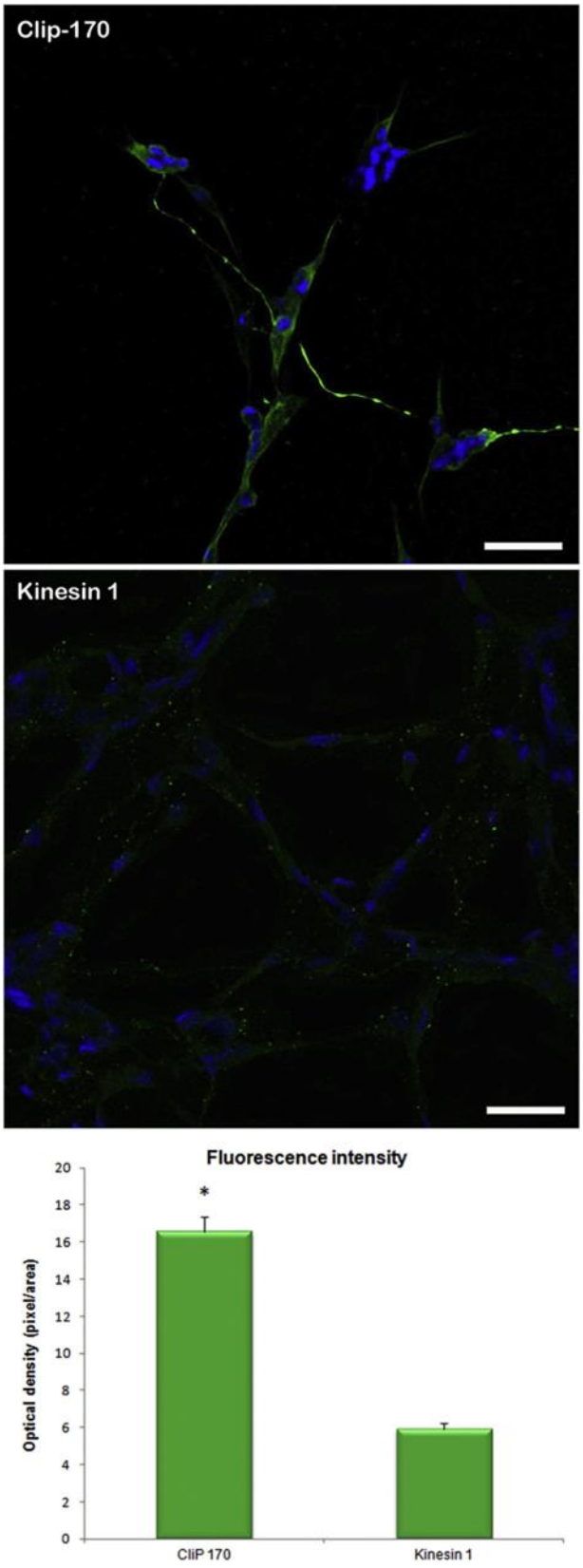

Fig. 5. Confocal images of immunofluorescence staining for CLIP-170 and Kinesin 1 microtubule associated protein. The diagram displayed a significant prevalence of fluorescence intensity for Clip −170 respect to Kinesin 1. Data are expressed as means ± SEM of five experiments. * = $p < 0.002$. Bars = 25 µm.

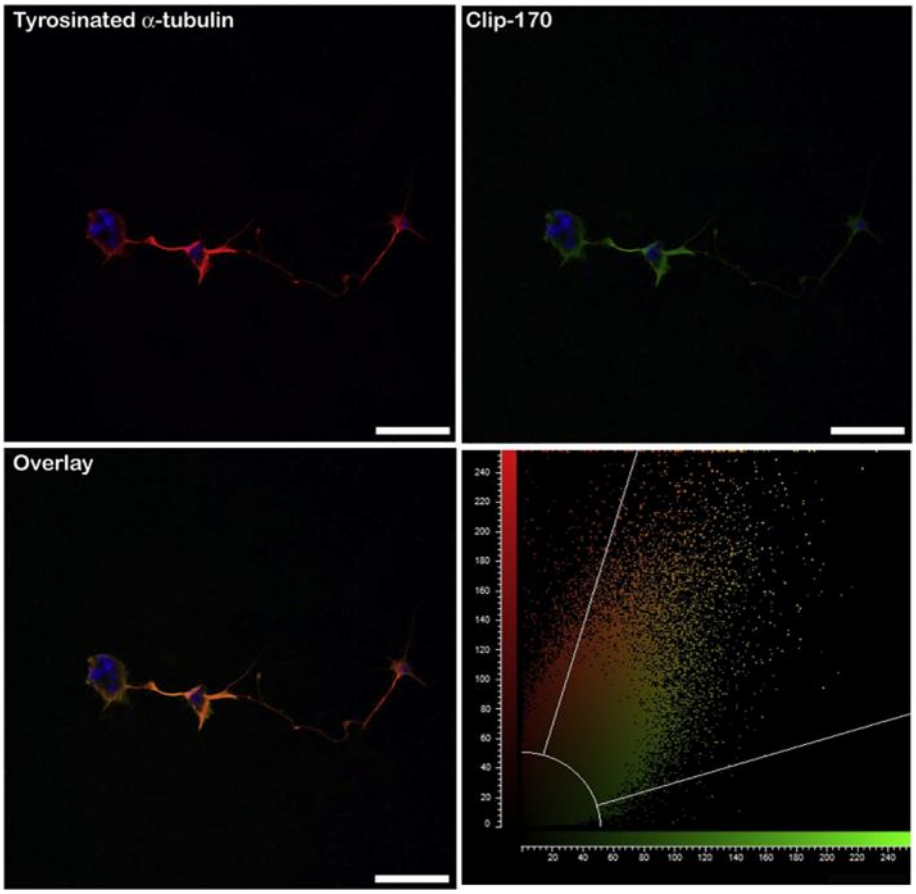

Fig. 6. Immunofluorescence staining to tyrosinated α-tubulin (top, on the left) and Clip-170 (top on the right) and overlay of both (bottom on the left). The diagram represents the degree of colocalization between the tubulin PTMs and the associated protein. Data are expressed as means ± SEM of five experiments. Bars = 25 µm.