Journal of Neurochemistry JNC

Tau regulates the localization and function of End binding proteins 1 and 3 (EB1/3) in developing neuronal cells

Journal:	*Journal of Neurochemistry*
Manuscript ID:	JNC-2014-0815.R2
Manuscript Type:	Original Article
Date Submitted by the Author:	03-Mar-2015
Complete List of Authors:	Sayas, Carmen; Centre for Biomedical Research of the Canary Islands, Institute of Biomedical Technologies, University of La Laguna, Anatomy, Pathological Anatomy, Histology and Physiology Tortosa, Elena; Centro de Biología Molecular "Severo Ochoa", CSIC-UAM; Utrecht University, Faculty of Sciences, Div. of Cell Biology Bollati, Flavia; Grenoble Institut des Neurosciences, Centre Inserm U836; Universidad Nacional de Córdoba, Facultad de Ciencias, Dep. de Farmacología Ramírez-Ríos, Sacnicte; Grenoble Institut des Neurosciences, Centre Inserm U836 Arnal, Isabelle; Grenoble Institut des Neurosciences, Centre Inserm U836 Avila, Jesús; Centro de Biología Molecular "Severo Ochoa", CSIC-UAM
Area/Section:	Brain Development & Cell Differentiation
Keywords:	neuronal development, tau, EBs, microtubule dynamics, MAPs, +TIPs

Tau regulates the localization and function of End binding proteins 1 and 3 (EB1/3) in developing neuronal cells

Carmen L. Sayas[a,b,c], Elena Tortosa[a,c,*], Flavia Bollati[d,¥], Sacnicte Ramírez-Ríos[d], Isabelle Arnal[d] and Jesús Avila[a,c]

[a]Centro de Biología Molecular "Severo Ochoa" (CSIC-UAM), Madrid, Spain; [b]Centre for Biomedical Research of the Canary Islands (CIBICAN), Institute for Biomedical Technologies (ITB), University of La Laguna (ULL), Tenerife, Spain; [c]Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain; and [d]INSERM U836, Grenoble Institut des Neurosciences, Grenoble, France

Present address: *Division of Cell Biology, Faculty of Sciences, Utrecht University, Utrecht, The Netherlands; ¥IFEC-CONICET, Departamento de Farmacología, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina.

To whom correspondence should be addressed: Jesús Ávila: Tel: (34) 91196 4564; Fax: (34) 911964715 ; E-mail: jesus.avila@csic.es, and Carmen L. Sayas: Tel: (34) 922316502; ext. 6459; Fax: (34) 922660253; E-mail: laura.sayas@ull.es

Running title: Tau is a regulator of EBs in neurons

Keywords: tau, EBs, microtubule dynamics, neuronal development, MAPs, +TIPs

Abbreviations:

Microtubules: MTs; microtubule-associated proteins: MAPs; microtubule plus-end tracking proteins: +TIPs; End binding proteins: EBs

ABSTRACT

The axonal microtubule-associated protein (MAP) tau, is a well-known regulator of microtubule stability in neurons. However, the putative interplay between tau and End binding proteins 1 and 3 (EB1/3), the core microtubule plus-end tracking proteins (+TIPs), has not been elucidated yet. Here, we show that a crosstalk between tau and EB1/3 exists in developing neuronal cells. Tau and EBs partially colocalize at extending neurites of N1E-115 neuroblastoma cells and axons of primary hippocampal neurons, as shown by confocal immunofluorescence analyses. Tau downregulation leads to a reduction of EB1/3 comet length, as observed in shRNA-stably depleted neuroblastoma cells and *TAU*-/- neurons. EB1/3 localization depends on the expression levels and localization of tau protein. Overexpression of tau at high levels induces EBs relocalization to microtubule bundles at extending neurites of N1E-115 cells. In differentiating primary neurons, tau is required for the proper accumulation of EBs at stretches of microtubule bundles at the medial and distal regions of the axon. Tau interacts with EB proteins, as shown by immunoprecipitation in different non-neuronal and neuronal cells and in whole brain lysates. A tau/EB1 direct interaction was corroborated by *in vitro* pull-down assays. FRAP assays performed in neuroblastoma cells confirmed that tau modulates EB3 cellular mobility. In summary, we provide evidence of a new function of tau as a direct regulator of EB proteins in developing neuronal cells. This crosstalk between a classical MAP and a core +TIP may contribute to the fine-tuned regulation of microtubule dynamics and stability during neuronal differentiation.

INTRODUCTION

Regulation of microtubule (MT) dynamics and stability is crucial for the proper development of neurons. Classical MT-associated proteins (MAPs), which bind along MTs and stabilize them, and MT plus-end tracking proteins (+TIPs), which interact specifically with MT-growing ends, participate in the regulation of the dynamic state of MTs during neuronal differentiation (Conde and Caceres 2009; Lowery and Van Vactor 2009).

Tau is a classical neuronal MAP that binds directly to and stabilizes MTs, promotes MT assembly and regulates MT dynamics (Brandt and Lee 1993; Panda et al. 1995; Paglini et al. 2000; Feinstein and Wilson 2005). During development, an increase in both the expression levels and molecular complexity of tau takes place, reaching a peak of expression upon neuronal maturation (Mareck et al. 1980; Francon et al. 1982; Knops et al. 1991; Schoenfeld and Obar 1994; Kanai and Hirokawa 1995; Vanier et al. 1998; Avila et al. 2004). In developing neurons, tau is prominently located in growing axons and is crucial for

establishing neuronal polarity and neurite and axon outgrowth (Drubin and Kirschner 1986; Caceres and Kosik 1990). Hippocampal neurons from tau deficient mice present a delay in polarization and maturation (Dawson et al. 2001). However, tau knockout mice are viable and show normal brain development and only modest morphological and behavioral deficits (Harada et al. 1994; Ikegami et al. 2000; Dawson et al. 2001; Fujio et al. 2007; Lei et al. 2012). Mild phenotypes observed in tau knockout mice may be explained by compensatory mechanisms of MT stabilization and/or dynamics of other microtubular proteins, either classical MAPs, such as axonal MAP1B (Harada et al. 1994; Takei et al. 2000) or other types such as +TIPs.

End-binding proteins (EBs), with three family members, EB1-3, are considered as the 'core' +TIPs, (reviewed in (Galjart 2010)). EB1/3 accumulate at the plus-ends of polymerizing MTs (Lansbergen and Akhmanova 2006; Bieling et al. 2007; Bieling et al. 2008; Dixit et al. 2009; Komarova et al. 2009). Every identified +TIP forms a complex with EB1/3 at MT growing-ends (reviewed in (Galjart 2010)). Both EB proteins regulate MT dynamics locally, during neurite and axon extension. EB1 knockdown in neuroblastoma cells reduces neurite length (Stepanova et al. 2010). While EB1 is ubiquitous, EB3 is enriched in brain, mostly in neurons (Nakagawa et al. 2000). EB3, which is highly expressed in growth cones, plays a role in the interaction between MTs and filopodial F-actin during neuritogenesis (Geraldo et al. 2008).

Not much is known about the interplay between classical MAPs and EBs during neuronal differentiation. In this regard, we recently reported that MAP1B, another axonal MAP, interacts directly with EB1/3 and sequesters them in the cytosol of developing neuronal cells, keeping MTs highly dynamic during neuronal development (Tortosa et al. 2013). In this work, we show that tau also controls EB1/3 localization and function in differentiating neuronal cells. Reduction of tau levels by shRNA knockdown or gene knockout decreases EBs accumulation at MT plus-ends. Conversely, tau overexpression at high levels leads to the recruitment of EB1 and EB3 (and consequently of other +TIPs) to MT bundles in neuroblastoma cells. Moreover, tau is necessary for the local accumulation of EB proteins at stretches of MT bundles in the medium-distal axon shaft in developing primary neurons. *In vitro* pull-down assays reveal that tau and EB1 interact directly. Coimmunoprecipitation of tau and EBs in neuroblastoma cells, cortical neurons, and brain, points to a physiological role for this interaction. Moreover, EB3 cellular mobility is regulated by tau. Overall, from our data tau protein emerges as a direct regulator of EB1/3 during neuronal development, Contrary to MAP1B, tau regulates the effective concentration of EB1 and EB3 by recruiting them to MT bundles. This may contribute to the tight control of MT dynamics/stability in developing neurons.

MATERIALS AND METHODS

Primary Antibodies

Primary antibodies (Abs) used in this study were: mouse anti-α-Tyrosinated-tubulin, mouse-anti-α-Acetylated-tubulin, rabbit anti-GST and mouse anti-β-actin (Sigma); mouse anti-GFP and anti-tau (goat-N17) (Santa Cruz Biotechnology); mouse anti-EB1 (BD Transduction), rat anti-EB3 (Abcam); mouse anti-His tag and mouse anti-tau (Tau5) (Millipore). Rabbit anti-EB1 antibody was a gift of Dr. Moutin and EB3 polyclonal antiserum was generated in Dr. N. Galjart´s lab, We also used the previously described Abs.**:** anti-CLIP-170 (# 2360, (Coquelle et al. 2002)) and anti-CLASP2 (clone 12H2, gift of Dr. N. Galjart).

Cell culture

N1E-115 mouse neuroblastoma cells and fibroblast-like monkey COS-7 cells (ATCC) were routinely grown in DMEM (Dulbecco's Modified Eagle's medium), containing 10% fetal bovine serum (FBS), 2 mM of L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 37ºC in a 5% CO_2 atmosphere. In some experiments, cells were differentiated by overnight serum starvation, or by treatment with 1.25 % DMSO in the presence of 2% FBS. Cultures of hippocampal pyramidal neurons obtained from embryonic brain tissue of E18 wild-type (wt) and Tau knockout mice (*TAU-/-*) (Dawson et al. 2001) were prepared as described (Banker and Cowan 1977). Papain and DNase (Worthington Biochemical Corporation) were used to treat dissected hippocampi after dissection. Specific culturing protocols used have been previously described (Tortosa et al, 2013). Media and supplements were acquired from Gibco Invitrogen Corporation. Neurons were maintained in culture for either 1 or 3 DIV (days in vitro) in a humidified 37ºC incubator with 5% CO_2. Cultures of mouse cortical neurons were obtained from fetal mice at 15 days of gestation as described previously (Leveille et al. 2008). Cerebral cortices were dissected, dissociated, and resuspended in DMEM supplemented with 5% FBS, 5% horse serum and 2 mM glutamine and plated on 24-well plates, previously coated with poly-d-lysine and laminin. Cultures were kept at 37°C in a humidified atmosphere containing 5% CO_2.

Plasmids and transfection

EB constructs used (human EB3-mCherry, EB1-GFP and EB3-GFP) have been previously described (Komarova et al. 2002; Stepanova et al. 2010). pLV-GFP-tau (human tau-4R2N) was a gift of Dr. I Santamaría. GFP-DCL was a gift of Dr. E. Vreugdenhil. COS-7 and NIE-115 cells were transfected with

LipofectamineTM 2000 (Invitrogen), following the manufacturer's protocol. Cells were harvested, fixed, or recorded, 36 hours post-transfection, depending on the experiment to be performed.

shRNA plasmids and lentiviral transduction

The effect of different tau shRNA lentiviral vectors (Mission, Sigma-Aldrich) was checked in transfected N1E-115 cells. The two most effective ones in downregulating tau were selected and used as a pool. A plasmid bearing a scrambled shRNA sequence was used as a control. Recombinant lentiviral particles were obtained by cotransfection of subconfluent 293T cells with Lipofectamine 2000 (Invitrogen), with each of the selected shRNA plasmids and both pCMVdR8.74 (Addgene) and pMD2G (Addgene) plasmids. Lentiviruses were collected 48 hours post-transfection. N1E-115 cells were infected with either the scramble shRNA virus (control) or a pool of tau-shRNA viruses for 24 hours. Cells were subjected to puromycin selection (Sigma-Aldrich) for 2-3 weeks to generate stable lines.

Cell/brain extracts, immunoprecipitation, and Western blotting

COS-7 or N1E-115 were harvested in cold lysis buffer and centrifuged at 4ºC at 13.000 rpm for 15 minutes. Lysis buffer contained 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1mM orthovanadate, 1mm okadaic acid, 0.5 % Triton X-100, supplemented with 1× CompleteTM protease inhibitor cocktail (Roche). All subsequent procedures were performed at 4°C. A total amount of 500 µg of protein per cell extract was used in each immunoprecipitation assay, with the ImmunoCruz™ IP/WB Optima System (Santa Cruz Biotechnology). Mouse brain extracts were prepared from 2 days old–mice (P2). Brain tissues were homogenized in 50 mM Tris at pH 8.0, 150 mM NaCl, 0.05% deoxycholic acid, 1% Triton X-100, 10% glycerol and protease inhibitors (Complete Mini EDTA-free, Roche), followed by centrifugation at 20000 g at 4°C for 10 min. For immunoprecipitation, brain lysates were incubated 4h at 4°C with antibodies and pre-incubated overnight with protein G- or protein A-Sepharose respectively. The immune complexes were recovered by centrifugation at 13000 g for 3 min and washed three times with 50 mM Tris at pH 7.4, 150 mM NaCl, 0.05% Triton X-100 and protease inhibitors. Finally, the complexes were resuspended in Laemmli SDS-PAGE loading buffer containing 5 mM DTT and boiled for 5 minutes before SDS-PAGE and Western blotting analysis. Cortical neurons were harvested after 14DIV and lysed in 50 mM Tris at pH 7.0, 150 mM NaCl, 1% Triton X-100, 10% glycerol and protease inhibitors, followed by centrifugation at 20000 g at 4°C for 10 min. Immunoprecipitations were performed as described above for mouse brain lysates in the presence of each antibody and pre-incubated overnight with protein A-

Sepharose. Immunocomplexes were analyzed by SDS-PAGE and Western blotting, as described previously (Tortosa et al. 2013).

Expression constructs and *in vitro* pull-down assays

His-tagged human tau (1N4R tau-412 isoform) and GST-tagged human EB1 plasmids were kindly provided by Drs. N. Sergeant (Lille, France) and I. Hayashi (Yokohama, Japan) (Hayashi et al. 2005) respectively. Proteins were purified either on Glutathione Sepharose (Thermo scientific) and/or Talon metal affinity resin (Clontech), according to the manufacturer´s instructions. Proteins were further processed by a size exclusion chromatography in BRB80 buffer (80 mM Pipes, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.8) supplemented with 50 mM NaCl, concentrated and ultracentrifuged at 230000 g for 10 min. Proteins were frozen in liquid nitrogen and stored at -80°C. Protein concentrations were determined by a Bradford assay (Sigma) using BSA as a standard. For the pull-down experiments, His-tagged tau and GST-tagged EB1 (or GST as a control), were incubated in BRB80 buffer supplemented with 50 mM NaCl and 0.05 % Triton, in the presence of Dynabeads His-Tag (Life technologies). After 4 hours of incubation at 4°C on a rotating wheel, beads were washed three times in the same buffer by repeated pelleting using a magnetic particle concentrator (Invitrogen). The proteins bound to the beads were analyzed by SDS-PAGE and Western blotting.

Immunofluorescence, confocal microscopy and image processing

Cells were either fixed with cold methanol (10 min. at -20ºC), or with cold methanol and then with 4% paraformaldehyde in PBS at room temperature (15 min.). Cells were permeabilized/blocked at room temperature for 1 hour with a solution of PBS containing 3% (w/v) bovine serum albumin (BSA) and 0.1% Triton X-100. Then, cells were incubated overnight at 4ºC with specific primary antibodies. Cells were then washed three times with PBS and incubated with secondary antibodies (Invitrogen), for one hour at room temperature. Immunostained cells were analyzed with a Leica TCS-SP8 confocal microscope or with a Zeiss LSM 510 Meta or Inverted confocal microscopes mounted on an Axiovert 200M or Axioskop2 plus, equipped with a CCD camera. EB comet number and length were quantified using ImageJ free software. EB1/3 comets were counted in areas of 100µm2, defined in different cells. The length of EB comets was defined as the distance from the peak fluorescence intensity at the tips of MTs to the baseline lattice intensity. Blind quantification of a number of EB comets, defined in the figure legends, was performed in several cells per condition (~5) in each experiment.

FRAP experiments

FRAP assays were performed on serum-starved N1E-115 cells, coexpressing EB3-mCherry and either GFP (control) or GFP-tau. Recordings of fluorescence recovery were performed using a LSM510 Multiphoton Laser Scanning and AxioImager M1 (Zeiss) confocal microscope with a 63X objective and a 1.6X zoom. Specific procedures used have been previously reported (Tortosa et al. 2013).

Statistical analyses

Experiments were repeated three to five times in each case. GraphPad Prism5 and SPSS17 software were used to perform statistical tests and graphs. Shapiro-Wilk test was used to check normality of the data set (significance level of $p \leq 0.05$). Depending on normality, parametric (Student t) or non-parametric (Mann-Whitney) tests were used. * in bar graphs indicate statistically significant *P*-values. ($P< 0.05$). *= $p < 0,05$; **= $p < 0,005$ and, ***=$p<0,0005$, as stated in bar graphs. Error bars represent the standard error of the mean in every case (SEM).

Institutional approval:

All the institutions involved in this work have approved the methods used in this study.

RESULTS

Localization of tau and EB1/3 proteins partially overlaps at extending neurites

We first examined the localization patterns of endogenous tau and EB1/3 in differentiating murine neuroblastoma N1E-115 cells. Overnight serum deprivation leads to cell flattening and neurite extension, resembling the first stages of neuronal polarization and differentiation observed in primary hippocampal neurons. In flat cells, tau was mostly present along the MT lattice (Figure 1B-D), whereas both EB1 and EB3 accumulated at comet-like structures at MT growing-ends (Figure 1C and D, for EB1 and Figure 1B and D, for EB3). Higher levels of tau were detected at MT bundles in elongating neurites, in correlation with an increase in both dynamic (tyrosinated) and stable (acetylated) MTs and MT-bundle formation (Figure 1A). Both EB1 and EB3 were also enriched along extending neurites, where they accumulated at stretches that partially colocalized with tau-positive MT bundles (Figures 1D and E). These results indicate that in flat cells, tau and EB1/3 localization differs: EB1/3 bind to the plus-ends of tau-decorated MTs. However, tau and EBs are enriched and their localization partially overlap along MT-bundles in neurites. These data suggest that tau and EBs might crosstalk in the regulation of MT dynamics and stability during differentiation in these neuronal cells.

Tau downregulation reduces the binding of EBs to MT plus-ends

To address whether tau and EB1/3 interplay in differentiating neuronal cells, we generated N1E-115 cell lines in which tau was stably depleted either by one or by a pool of two specific shRNAs (Figure 2A, B, C and (Tortosa et al. 2013)). Differentiated N1E-115 cells (3 days), express low and high molecular weight tau (see two bands in Figure 2A). Both tau isoforms were downregulated by the specific shRNAs (Figure 2A). We started by analyzing the localization of EB1 and EB3 in serum-starved tau-depleted cells (tau-shRNA pool) and in control (scrambled shRNA) cells (Figure 2C). Although EB1/3 comet pattern did not substantially differ between control and tau-depleted cells (Figure 2C), tau stable-knockdown induced a significant reduction in the amount of EB comets (insets in Figure 2C; graph in Figure 2D), indicating that the number of growing MTs is decreased. This correlated with a decrease in MT density in tau-deficient N1E-115 cells that we previously showed (Tortosa et al. 2013). However, EB1/3 comets were shorter upon tau downregulation (Figure 2E). Overall, EB1/3 comet number and length were reduced in tau-depleted cells. The found reduction in the amount of EB comets was most likely due to the decrease in MT number (Tortosa et al. 2013). These data indicate that tau modulates the localization of EBs at MT plus-ends in differentiating neuronal cells.

Tau recruits EBs to MT bundles

To confirm whether tau influences EBs interaction with MTs, we examined EBs localization upon overexpression of GFP-tagged tau in N1E-115 cells. In transfected cells expressing low-to-medium levels of the protein, the MT network was decorated by GFP-tau, while EB1/3 showed a normal **comet** localization at plus-ends (Figure 3A, B, C and F), as we previously described (Tortosa et al. 2013). Another population of transfected cells presented high GFP-tau expression and the subsequent formation of MT bundles (Figure 3D-F) and, which in some cases correlated with neurite extension (Figure 3D). Of note, in these cells, a prominent change in EB1/3 localization was found, switching from MT plus-end comets to a fibrillary MAP-like pattern along MT bundles, in which endogenous EB1/3 colocalized with GFP-tau (Figure 3D-F). The effect of tau on EB1/3 localization was quantified (Figure 3G), showing that the distribution pattern of both EB1 and EB3 directly correlated with tau expression levels and localization in cells: while low-to-medium levels of tau (present at the MT lattice) did not affect the localization of EB1/3 at MT plus-ends, higher tau levels localized at MT bundles, led to the recruitment of both EBs to these MT bundles. When extremely high levels of tau were expressed in cells, EB1/3 were mostly diffuse in the cytosol (Figure 3G).

Since all known +TIPs interact with EB1/3 proteins at MT plus-ends (reviewed in Galjart, 2010), relocalization of EBs to tau-induced MT bundles might alter the localization pattern of other +TIPs. Indeed, the +TIP CLASP2, also relocalized to MT bundles in cells expressing high levels of tau protein (Figure 3H).

To confirm that the effect of tau-bundles on EBs was specific, we overexpressed Doublecortin-like protein (DCL), another MAP that induces MT bundle formation in N1E-115 cells (Fitzsimons et al. 2008) and analyzed EB1 localization. Interestingly, EB1 was not recruited to MT bundles induced by high expression of GFP-DCL, but, on the contrary, it was displaced from MT plus-ends and relocated to the cytosol (Figure 3**I**). This result suggests that the relocalization of EB proteins to tau-induced MT bundles is a specific effect of tau, not of bundles themselves.

Taken together, these results suggest that the localization of EB1/3 in neuronal cells varies depending on the localization and expression levels of tau protein. Thus, EBs localization patterns can be summarized as follows: a) EB1/3 present a normal comet localization in control (non-transfected cells) or when low-to-medium levels of tau interact along the MT lattice; b) EB1/3 are recruited to MT bundles (e.g., in neurites), and colocalize with tau**,** when high levels of tau crossbridge MTs and induce MT bundle formation, and c) EB1/3 are present in the cytosol when saturating levels of tau are reached and a high amount of tau protein is present in the cytosol.

Tau interacts with EB1 and EB3

Our results indicate that the subcellular distribution of EBs is regulated by tau. We therefore hypothesized that the action of tau on EBs may be mediated by protein-protein interaction. We started addressing this issue by performing co-immunoprecipitation (co-IP) assays in COS-7 cells -which do not express endogenous tau-, cotransfected with tau and GFP-tagged EBs (Figure 4A, B). Co-IPs were performed with either anti-GFP or anti-tau antibodies. Ectopically expressed tau interacted with EB1-GFP and EB3-GFP, but not with GFP, as indicated by reciprocal IPs (Figure 4A and B). We found that endogenous tau (both low and high molecular tau isoforms) coimmunoprecipitated with GFP-tagged EBs in transfected N1E-115 cells (Figure 4C). Moreover, endogenous tau and EB1 were present in protein complexes in 14DIV mouse primary cortical neurons (Figure 4D) and in postnatal (P2) murine brain (Figure 4E).

Since both tau and EBs bind MTs, the interaction between tau and EBs might be mediated by MTs. To test this possibility, we performed *in vitro* pull-down assays. Notably, our data showed that EB1-GST, but not

GST, interacted with His-tagged tau, indicating that the interaction between tau and EB1 was direct (Figure 4F).

Taken together, these results indicate that: 1) a complex between tau and EBs is found in differentiating neuroblastoma cells, developing primary neurons, and brain and 2) tau and EB1 interact directly. Hence, tau emerges as a new partner of EB proteins in neurons. This supports a potential physiological role for the tau/EBs complex during neuronal development.

Tau modulates cellular mobility of EB3 in living cells

To get further insight into the functionality of the direct interaction between tau and EBs in differentiating neuronal cells, we analyzed whether EBs cellular mobility/dynamics were influenced by tau. For that purpose, we performed fluorescence recovery after photobleaching (FRAP) assays. N1E-115 cells were cotransfected with EB3-mCherry along with either GFP (control) or GFP-tau. Since EB3 is generally used as a marker of MT growth in living cells, we focused on EB3 in these assays. As both EB1 and EB3 interact with tau, it is most likely that results obtained with EB3 apply also to EB1. Cells extending neurites and expressing moderate levels of both GFP-tau and EB3-mCherry were chosen (with GFP-tau present along MTs and EB3-mCherry at MT plus-ends) (Figure 5). Bleaching of EB3-mCherry was performed in extending neurites. Provided that binding reactions with other proteins influence the slow phase of the fluorescence recovery curve, we focused on this region in the FRAP analysis. We found that fluorescence recovery of EB3-mCherry was significantly delayed in GFP-tau expressing cells as compared to control cells (Figure 5A, B), as confirmed by the k and the $t_{1/2}$ parameters ($k_{control}$= 0,6291 ± 0,1168 sec^{-1}; $k_{GFP\text{-}Tau}$=0,3233 ± 0,0067 sec^{-1} and $t_{½\text{-}\ control}$=1,80 ± 0,3641 sec.; $t_{½\text{-}\ GFP\text{-}Tau}$= 2,656 ± 0,3521 sec., where $t_{1/2}$= ln(2)/K) (Figure 5C, D).

These data show that tau regulates EB3 cellular mobility in extending neurites. This adds further evidence for a functional interaction and interplay between tau and EB proteins during neuronal differentiation.

Tau regulates EBs binding to MTs during neuronal development

To confirm the existence of a tau/EBs crosstalk in developing primary neurons, the localization of tau and EB1 was analyzed in wt and *TAU-/-* neurons (1DIV) (Figure 6). We focused on EB1 because its levels are high in immature neurons while EB3 levels are low (Leterrier et al., 2011). In wt neurons, EB1 was mostly present at comet-like structures in cell body, neurites, parts of the axon (Figure 6A, left panels) and growth cones (Figure 6A, left panels, and Figure 6B), not showing colocalization with tau (Figure 6B). However, in some regions at the medium-distal axon shaft, EB1 was accumulated at longer fibrillar

stretches, where it colocalized with tau (Figure 6A, left panels). In tau-deficient cells, EB1 comets were present throughout the neuron, indicating that MT polymerization still occurred in the absence of tau (Figure 6A, right panels). However, as found in tau-depleted N1E-115 cells, EB1 comets were overall shorter in tau knockout neurons (Figure 6C-D). Reduction in EB1 comet length was significant in every cell compartment analyzed (axon, cell body and (axonal) growth cones (Figure 6D). Remarkably, in neurons lacking tau expression, most EB1 localized at comets and only scarce EB stretches were present along the axon shaft (Figure 6A, right panels, and insets). Partial colocalization of tau and EB1 at MTs along the medium-distal axon shaft was confirmed in further differentiated wt neurons (3DIV) (Figure 6E). In addition, impaired distribution of EB1 along the axon shaft was also observed in 3DIV tau-deficient neurons (Figure 6E). These data indicate that: a) tau and EB1 partially colocalize in axon segments at different stages during neuronal differentiation, and b) tau modulates the localization of EBs at MT plus-ends and along MT-bundles in the medium-distal axon shaft of developing neurons. These data confirm that tau/EBs interaction occurs during axon outgrowth in primary neurons and suggest an important physiological role for this interplay.

DISCUSSION

In this work, we describe a novel function of tau as a regulator of EB proteins, the core +TIPs. We show that tau interacts directly with EB1 and controls EBs localization and mobility in neuronal cells. In primary developing neurons, tau is necessary for EB1/3 accumulation at tau-positive stretches (probably MT bundles), along the axon shaft. Our data provide evidence for an interplay between tau and EBs in differentiating neuroblastoma cells and primary neurons.

Tau and EB3 are mostly neuronal proteins (Goedert et al. 1989; Nakagawa et al. 2000) whereas EB1 is ubiquitous, but has some specific functions in neurons (Jimenez-Mateos et al. 2005; Gu et al. 2006; Rosenberg et al. 2008; Mattie et al. 2010; Stepanova et al. 2010; Leterrier et al. 2011; Alves-Silva et al. 2012; Moughamian et al. 2013). While EB1 levels remain constant, expression levels of tau and EB3 increase as neurons become mature (Mareck et al. 1980; Schoenfeld and Obar 1994; Jaworski et al. 2009). Tau and EBs are key regulators of MT dynamics in developing neuronal cells (Brandt and Lee 1993; Panda et al. 1995; Feinstein and Wilson 2005; Stepanova et al. 2010). We describe here that in flat neuroblastoma cells with moderate tau levels, no colocalization between tau and EBs is found. However, as neuroblastoma cells differentiate further, tau and EBs become enriched in extending neurites and partially colocalize. Localization of tau/EBs partly overlaps at stretches in axons of primary neurons (1DIV and 3DIV), mostly at medial and distal axonal regions. Since tau depletion impairs EB1

accumulations at MT-bundles at the medial-distal axon shaft, tau seems necessary for the proper localization of EBs in axons. This may be due to the fact that tau exhibits a proximal-to-distal gradient, with the highest concentration at the distal axon (Kempf et al. 1996). Other factors, such as the axonal MAP MAP1B (see below), are involved in the regulation of EBs localization in axons (Tortosa et al. 2013). Moreover, enrichment of EB1/3 at the MT lattice has been reported in the Axon Initial Segment (AIS) in mature neurons through their interaction with ankyrin G (Leterrier et al. 2011). EBs localization at different axon regions seems to be tightly controlled by different proteins in neurons.

We further show that modifications in expression levels and localization of tau leads to altered localization of EB1/3. Thus, a reduction in tau levels by either stable knockdown (neuroblastoma cells) or knockout (primary hippocampal neurons), decreases concomitantly the accumulation of EBs at MT plus-ends. Conversely, an increase of tau levels upon ectopic expression (neuroblastoma cells), with the subsequent formation of MT bundles, induces extensive recruitment of EBs to these bundles. In fact, in axons tau has a crucial role in the organization of MT bundles, which participate in several neuronal functions, including promoting axon outgrowth, maintaining mechanical integrity and shape of the axon, and facilitating cargo transport (Conde and Caceres 2009)**.** Thus, MT bundles induced by tau ectopic expression in our neuroblastoma cells resemble the physiological situation of bundle formation during neurite/axon extension in primary neurons. Hence, tau-mediated localization of EB1/3 to MT bundles may play a role in MT stabilization during axon outgrowth. In this regard, a delay in axon formation has been reported in tau-deficient neurons (Dawson et al, 2001; Gomez de Barreda et al. 2010). Nevertheless, since tau knockout mice are viable and present no obvious defects in brain structure (Harada et al. 1994; Dawson et al. 2001), the role of tau in neurite/axon growth during neuronal development remains a matter of debate. Since both EB1 and EB3 have been involved in neurite extension (Stepanova et al., 2010; Geraldo et al., 2008) it is possible that mislocalization of EBs in the absence of tau may contribute to the delay in axon formation observed in tau-knockout mice.

The effects of tau on EBs seem to be specific, given that EB1 does not relocalize to MT bundles promoted by ectopic (high) expression of DCL (another MAP), but it is displaced from MT plus-ends and localized to the cytosol (neuroblastoma cells). Furthermore, we recently reported that MAP1B, another axonal MAP, also interacts with EB1/3 and modulates their localization in neuronal cells (Tortosa et al. 2013). However, while MAP1B sequesters EB1/3 in the cytosol, tau recruits EB1/3 to MT bundles, in developing neuronal cells. Since expression profiles of MAP1B and tau are not overlapping but sequential during neuronal morphogenesis (Tucker 1990), it is tempting to speculate that MAP1B and tau may act on EBs at different stages of neuronal development to control differentially the levels of available EBs, free to exchange at MT plus-ends. Further studies are required to address these issues.

Tau can form oligomers along MTs, through intermolecular interactions (Makrides et al. 2003; Duan and Goodson 2012). At very high concentrations, tau molecules can also aggregate on top of each other on the MT surface (Ackmann et al. 2000). We hypothesize that this could be happening in our cell system when tau is expressed at high levels that induce MT bundling and at MT bundles in the axon shaft of primary neurons. In this situation, these tau oligomers might recruit EB1/3 proteins at MT bundles. It has been shown that a tau molecule is able to interact with more than a tubulin dimer (Kar et al. 2003). It is, therefore, possible that tau could interact with itself (oligomers), with MTs and with EBs at the same time. On the other hand, when tau is expressed at extremely high levels and MTs get totally covered by tau, a big pool of tau remains in the cytosol. Notably, in this case, a high amount of EBs is found diffusely localized in the cytosol of these cells. It seems likely that the crosstalk between tau and EB proteins depends on tau expression levels and localization and, probably, on the binding of other MAPs to the MT.

What is the physiological significance of tau-induced relocalization of EBs during neuronal development? Remarkably, MAP2, a dendritic MAP that belongs to tau family, has been shown to recruit EB3 to MT bundles as well (Kapitein et al. 2011), pointing to a shared mechanism of EBs recruitment by tau/MAP2 proteins. One possibility is that, as suggested for MAP2 in dendrites (Kapitein et al. 2011), tau may function in axons as a trapping factor for EBs. In this way, tau would partially deplete the soluble pool of EB1/3 available to exchange at MT plus-ends and promote growth of dynamic MTs. This would provide an extra mechanism of MT stabilization by tau, which may contribute further to the consolidation of the newly formed axon.

We provide evidence for a direct interaction between tau and EB1, supporting that EBs recruitment by tau to MT bundles is mediated by direct binding of the two proteins and is neither mediated by MTs nor an indirect effect of tau actions on MT structure. Although most EB partners can track MT plus-ends, tau does not show plus-end tracking behavior. This is most probably due to the fact that tau is tightly bound to MTs, this hampering its localisation at MT growing ends. As a consequence, tau is the one that actively recruits EBs to stable MTs, not the other way around. We furthermore find that the interaction between tau and EBs occurs not only in developing neuronal cells but also in primary neurons and postnatal mouse brain, indicating that the tau/EBs interaction is functional *in vivo*. In addition, the mobility of EB proteins in living neuroblastoma cells is modulated by tau. Thus, it is likely that the formation of the tau/EBs protein complex is physiologically relevant for neuronal development (e.g. MT stabilization required for axon consolidation). Provided that tau (both overexpressed and endogenous) and EBs colocalize along MT bundles, the interaction between tau and EB1/3 seems to occur preferentially at MTs. However, we cannot rule out the possibility that in certain conditions in which tau is present in the cytosol, tau could be sequestering EBs there.

MT plus-end tracking of most known +TIPs relies on their interaction with EB proteins (reviewed in (Galjart 2010)). Our data indicate that in neuroblastoma cells, the +TIP CLASP2 is also recruited to tau-induced MT bundles, most probably via its direct interaction with EBs. In neurons, CLASP2 is present in axons and play important functions, such as regulation of MT dynamics during neuronal polarization or axon extension (Hur et al. 2011). In this way, it is possible that, as in the case of EBs, the function of CLASP2 would switch to a purely stabilizing action, similar to that of some classical MAPs, as a result of tau/EBs-recruitment. Other +TIPs may also be recruited by EBs to tau-positive bundles, enhancing MT stability. This may happen in situations or locations of the axon in which extra rigidity is needed during development.

Tau is the substrate of numerous kinases and phosphatases (reviewed in (Hashiguchi and Hashiguchi 2013).The association of EBs with other classical MAPs (MAP1B and MAP2), is regulated by phosphorylation (Kapitein et al. 2011; Tortosa et al. 2013). Hence, the formation of the complex tau/EBs might also be modulated by phosphorylation events which occur in different signaling pathways that take place during neuronal development. Since tau and EB1/3 levels remain high in adult neurons, the tau/EBs interplay may also occur in mature neurons, in which it could also be regulated by similar phosphorylation processes. Abnormally hyperphosphorylated tau is the main component of aggregated/oligomeric tau and of neurofibrillary tangles and a hallmark of Alzheimer´s disease (AD) (reviewed in (Iqbal et al. 2010). Hyperphosphorylated tau is also present in other tauopathies (reviewed in (Iqbal et al. 2010). It would be of interest to investigate whether this abnormal hyperphosphorylation induces a dysregulation of the localization and function of EBs and of other EB-interacting +TIPs in AD and other tauopathies.

In conclusion, from our data, tau emerges as a direct regulator of EBs in differentiating neuronal cells. This further suggests that a coordinated interplay between axonal MAPs and EBs is crucial for the proper regulation of microtubule dynamics/stability during neurite/axon extension

ACKNOWLEDGEMENTS

We are grateful to Drs. Galjart; Santamaría, Sergeant, Hayashi and Moutin for providing reagents. This work was supported by grants from the Spanish Research Council (CSIC), Plan Nacional, CIBERNED and Comunidad de Madrid (Spain) and by the Institut National pour la Santé et la Recherche Médicale (INSERM) and the Centre National de la Recherche Scientifique (CNRS) joined ATIP-Avenir program, and the Agence Nationale de la Recherche (ANR MALAAD, program MALZ 2011) (France). C.L. Sayas and E.T. were supported by CSIC and CIBERNED. C.L. Sayas is currently supported and funded by the IMBRAIN project (FP7-REGPOT-2012-CT2012-31637-IMBRAIN, Framework Programme 7 (Capacities)), awarded to the Institute of Biomedical Technologies and the Centre for Biomedical Research of the Canary Islands at ULL. Some images were acquired with Leica TCS-SP8 microscope,

partially supported by IMBRAIN grant. F.B. was supported by a post-doctoral fellowship from the INSERM and S.R.R. was supported by a post-doctoral fellowship from the ANR. The authors declare to have no conflict of interest.

REFERENCES

Ackmann M., Wiech H. and Mandelkow E. (2000) Nonsaturable binding indicates clustering of tau on the microtubule surface in a paired helical filament-like conformation. *The Journal of biological chemistry* **275,** 30335-30343.

Alves-Silva J., Sanchez-Soriano N., Beaven R., Klein M., Parkin J., Millard T. H., Bellen H. J., Venken K. J., Ballestrem C., Kammerer R. A. and Prokop A. (2012) Spectraplakins promote microtubule-mediated axonal growth by functioning as structural microtubule-associated proteins and EB1-dependent +TIPs (tip interacting proteins). *J Neurosci* **32,** 9143-9158.

Avila J., Lucas J. J., Perez M. and Hernandez F. (2004) Role of tau protein in both physiological and pathological conditions. *Physiol Rev* **84,** 361-384.

Banker G. A. and Cowan W. M. (1977) Rat hippocampal neurons in dispersed cell culture. *Brain research* **126,** 397-342.

Bieling P., Kandels-Lewis S., Telley I. A., van Dijk J., Janke C. and Surrey T. (2008) CLIP-170 tracks growing microtubule ends by dynamically recognizing composite EB1/tubulin-binding sites. *The Journal of cell biology* **183,** 1223-1233.

Bieling P., Laan L., Schek H., Munteanu E. L., Sandblad L., Dogterom M., Brunner D. and Surrey T. (2007) Reconstitution of a microtubule plus-end tracking system in vitro. *Nature* **450,** 1100-1105.

Brandt R. and Lee G. (1993) Functional organization of microtubule-associated protein tau. Identification of regions which affect microtubule growth, nucleation, and bundle formation in vitro. *The Journal of biological chemistry* **268,** 3414-3419.

Caceres A. and Kosik K. S. (1990) Inhibition of neurite polarity by tau antisense oligonucleotides in primary cerebellar neurons. *Nature* **343,** 461-463.

Conde C. and Caceres A. (2009) Microtubule assembly, organization and dynamics in axons and dendrites. *Nat Rev Neurosci* **10,** 319-332.

Coquelle F. M., Caspi M., Cordelieres F. P., Dompierre J. P., Dujardin D. L., Koifman C., Martin P., Hoogenraad C. C., Akhmanova A., Galjart N., De Mey J. R. and Reiner O. (2002) LIS1, CLIP-170's key to the dynein/dynactin pathway. *Molecular and cellular biology* **22,** 3089-3102.

Dawson H. N., Ferreira A., Eyster M. V., Ghoshal N., Binder L. I. and Vitek M. P. (2001) Inhibition of neuronal maturation in primary hippocampal neurons from tau deficient mice. *Journal of cell science* **114,** 1179-1187.

Dixit R., Barnett B., Lazarus J. E., Tokito M., Goldman Y. E. and Holzbaur E. L. (2009) Microtubule plus-end tracking by CLIP-170 requires EB1. *Proceedings of the National Academy of Sciences of the United States of America* **106,** 492-497.

Drubin D. G. and Kirschner M. W. (1986) Tau protein function in living cells. *The Journal of cell biology* **103,** 2739-2746.

Duan A. R. and Goodson H. V. (2012) Taxol-stabilized microtubules promote the formation of filaments from unmodified full-length Tau in vitro. *Molecular biology of the cell* **23,** 4796-4806.

Feinstein S. C. and Wilson L. (2005) Inability of tau to properly regulate neuronal microtubule dynamics: a loss-of-function mechanism by which tau might mediate neuronal cell death. *Biochim Biophys Acta* **1739,** 268-279.

Fitzsimons C. P., Ahmed S., Wittevrongel C. F., Schouten T. G., Dijkmans T. F., Scheenen W. J., Schaaf M. J., de Kloet E. R. and Vreugdenhil E. (2008) The microtubule-associated protein doublecortin-like regulates the transport of the glucocorticoid receptor in neuronal progenitor cells. *Molecular endocrinology* **22,** 248-262.

Francon J., Lennon A. M., Fellous A., Mareck A., Pierre M. and Nunez J. (1982) Heterogeneity of microtubule-associated proteins and brain development. *Eur J Biochem* **129,** 465-471.

Fujio J., Hosono H., Ishiguro K., Ikegami S. and Fujita S. C. (2007) Tau phosphorylation in the mouse brain during aversive conditioning. *Neurochem Int* **51,** 200-208.

Galjart N. (2010) Plus-end-tracking proteins and their interactions at microtubule ends. *Curr Biol* **20,** R528-537.

Geraldo S., Khanzada U. K., Parsons M., Chilton J. K. and Gordon-Weeks P. R. (2008) Targeting of the F-actin-binding protein drebrin by the microtubule plus-tip protein EB3 is required for neuritogenesis. *Nature cell biology* **10,** 1181-1189.

Goedert M., Spillantini M. G., Jakes R., Rutherford D. and Crowther R. A. (1989) Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. *Neuron* **3,** 519-526.

Gomez de Barreda E., Perez M., Gomez Ramos P., de Cristobal J., Martin-Maestro P., Moran A., Dawson H. N., Vitek M. P., Lucas J. J., Hernandez F. and Avila J. (2010) Tau-knockout mice show reduced GSK3-induced hippocampal degeneration and learning deficits. *Neurobiology of disease* **37,** 622-629.

Gu C., Zhou W., Puthenveedu M. A., Xu M., Jan Y. N. and Jan L. Y. (2006) The microtubule plus-end tracking protein EB1 is required for Kv1 voltage-gated K+ channel axonal targeting. *Neuron* **52,** 803-816.

Harada A., Oguchi K., Okabe S., Kuno J., Terada S., Ohshima T., Sato-Yoshitake R., Takei Y., Noda T. and Hirokawa N. (1994) Altered microtubule organization in small-calibre axons of mice lacking tau protein. *Nature* **369,** 488-491.

Hashiguchi M. and Hashiguchi T. (2013) Kinase-kinase interaction and modulation of tau phosphorylation. *International review of cell and molecular biology* **300,** 121-160.

Hayashi I., Wilde A., Mal T. K. and Ikura M. (2005) Structural basis for the activation of microtubule assembly by the EB1 and p150Glued complex. *Mol Cell* **19,** 449-460.

Hur E. M., Saijilafu, Lee B. D., Kim S. J., Xu W. L. and Zhou F. Q. (2011) GSK3 controls axon growth via CLASP-mediated regulation of growth cone microtubules. *Genes Dev* **25,** 1968-1981.

Ikegami S., Harada A. and Hirokawa N. (2000) Muscle weakness, hyperactivity, and impairment in fear conditioning in tau-deficient mice. *Neurosci Lett* **279,** 129-132.

Iqbal K., Liu F., Gong C. X. and Grundke-Iqbal I. (2010) Tau in Alzheimer disease and related tauopathies. *Current Alzheimer research* **7,** 656-664.

Jaworski J., Kapitein L. C., Gouveia S. M., Dortland B. R., Wulf P. S., Grigoriev I., Camera P., Spangler S. A., Di Stefano P., Demmers J., Krugers H., Defilippi P., Akhmanova A. and Hoogenraad C. C. (2009) Dynamic microtubules regulate dendritic spine morphology and synaptic plasticity. *Neuron* **61,** 85-100.

Jimenez-Mateos E. M., Paglini G., Gonzalez-Billault C., Caceres A. and Avila J. (2005) End binding protein-1 (EB1) complements microtubule-associated protein-1B during axonogenesis. *J Neurosci Res* **80,** 350-359.

Kanai Y. and Hirokawa N. (1995) Sorting mechanisms of tau and MAP2 in neurons: suppressed axonal transit of MAP2 and locally regulated microtubule binding. *Neuron* **14,** 421-432.

Kapitein L. C., Yau K. W., Gouveia S. M., van der Zwan W. A., Wulf P. S., Keijzer N., Demmers J., Jaworski J., Akhmanova A. and Hoogenraad C. C. (2011) NMDA receptor activation suppresses microtubule growth and spine entry. *J Neurosci* **31,** 8194-8209.

Kar S., Fan J., Smith M. J., Goedert M. and Amos L. A. (2003) Repeat motifs of tau bind to the insides of microtubules in the absence of taxol. *The EMBO journal* **22,** 70-77.

Kempf M., Clement A., Faissner A., Lee G. and Brandt R. (1996) Tau binds to the distal axon early in development of polarity in a microtubule- and microfilament-dependent manner. *J Neurosci* **16,** 5583-5592.

Knops J., Kosik K. S., Lee G., Pardee J. D., Cohen-Gould L. and McConlogue L. (1991) Overexpression of tau in a nonneuronal cell induces long cellular processes. *The Journal of cell biology* **114,** 725-733.

Komarova Y., De Groot C. O., Grigoriev I., Gouveia S. M., Munteanu E. L., Schober J. M., Honnappa S., Buey R. M., Hoogenraad C. C., Dogterom M., Borisy G. G., Steinmetz M. O. and Akhmanova A. (2009) Mammalian end binding proteins control persistent microtubule growth. *The Journal of cell biology* **184,** 691-706.

Komarova Y. A., Akhmanova A. S., Kojima S., Galjart N. and Borisy G. G. (2002) Cytoplasmic linker proteins promote microtubule rescue in vivo. *The Journal of cell biology* **159,** 589-599.

Lansbergen G. and Akhmanova A. (2006) Microtubule plus end: a hub of cellular activities. *Traffic (Copenhagen, Denmark)* **7,** 499-507.

Lei P., Ayton S., Finkelstein D. I., Spoerri L., Ciccotosto G. D., Wright D. K., Wong B. X., Adlard P. A., Cherny R. A., Lam L. Q., Roberts B. R., Volitakis I., Egan G. F., McLean C. A., Cappai R., Duce J. A. and Bush A. I. (2012) Tau deficiency induces parkinsonism with dementia by impairing APP-mediated iron export. *Nat Med* **18,** 291-295.

Leterrier C., Vacher H., Fache M. P., d'Ortoli S. A., Castets F., Autillo-Touati A. and Dargent B. (2011) End-binding proteins EB3 and EB1 link microtubules to ankyrin G in the axon initial segment. *Proceedings of the National Academy of Sciences of the United States of America* **108,** 8826-8831.

Leveille F., El Gaamouch F., Gouix E., Lecocq M., Lobner D., Nicole O. and Buisson A. (2008) Neuronal viability is controlled by a functional relation between synaptic and extrasynaptic NMDA receptors. *FASEB J* **22,** 4258-4271.

Lowery L. A. and Van Vactor D. (2009) The trip of the tip: understanding the growth cone machinery. *Nat Rev Mol Cell Biol* **10,** 332-343.

Makrides V., Shen T. E., Bhatia R., Smith B. L., Thimm J., Lal R. and Feinstein S. C. (2003) Microtubule-dependent oligomerization of tau. Implications for physiological tau function and tauopathies. *The Journal of biological chemistry* **278,** 33298-33304.

Mareck A., Fellous A., Francon J. and Nunez J. (1980) Changes in composition and activity of microtubule-associated proteins during brain development. *Nature* **284,** 353-355.

Mattie F. J., Stackpole M. M., Stone M. C., Clippard J. R., Rudnick D. A., Qiu Y., Tao J., Allender D. L., Parmar M. and Rolls M. M. (2010) Directed microtubule growth, +TIPs, and kinesin-2 are required for uniform microtubule polarity in dendrites. *Curr Biol* **20,** 2169-2177.

Moughamian A. J., Osborn G. E., Lazarus J. E., Maday S. and Holzbaur E. L. (2013) Ordered Recruitment of Dynactin to the Microtubule Plus-End is Required for Efficient Initiation of Retrograde Axonal Transport. *J Neurosci* **33,** 13190-13203.

Nakagawa H., Koyama K., Murata Y., Morito M., Akiyama T. and Nakamura Y. (2000) EB3, a novel member of the EB1 family preferentially expressed in the central nervous system, binds to a CNS-specific APC homologue. *Oncogene* **19,** 210-216.

Paglini G., Peris L., Mascotti F., Quiroga S. and Caceres A. (2000) Tau protein function in axonal formation. *Neurochem Res* **25,** 37-42.

Panda D., Goode B. L., Feinstein S. C. and Wilson L. (1995) Kinetic stabilization of microtubule dynamics at steady state by tau and microtubule-binding domains of tau. *Biochemistry* **34,** 11117-11127.

Rosenberg M. M., Yang F., Giovanni M., Mohn J. L., Temburni M. K. and Jacob M. H. (2008) Adenomatous polyposis coli plays a key role, in vivo, in coordinating assembly of the neuronal nicotinic postsynaptic complex. *Mol Cell Neurosci* **38,** 138-152.

Schoenfeld T. A. and Obar R. A. (1994) Diverse distribution and function of fibrous microtubule-associated proteins in the nervous system. *Int Rev Cytol* **151,** 67-137.

Stepanova T., Smal I., van Haren J., Akinci U., Liu Z., Miedema M., Limpens R., van Ham M., van der Reijden M., Poot R., Grosveld F., Mommaas M., Meijering E. and Galjart N. (2010) History-dependent catastrophes regulate axonal microtubule behavior. *Curr Biol* **20,** 1023-1028.

Takei Y., Teng J., Harada A. and Hirokawa N. (2000) Defects in axonal elongation and neuronal migration in mice with disrupted tau and map1b genes. *The Journal of cell biology* **150,** 989-1000.

Tortosa E., Galjart N., Avila J. and Sayas C. L. (2013) MAP1B regulates microtubule dynamics by sequestering EB1/3 in the cytosol of developing neuronal cells. *The EMBO journal* **32,** 1293-1306.

Tucker R. P. (1990) The roles of microtubule-associated proteins in brain morphogenesis: a review. *Brain Res Brain Res Rev* **15,** 101-120.

Vanier M. T., Neuville P., Michalik L. and Launay J. F. (1998) Expression of specific tau exons in normal and tumoral pancreatic acinar cells. *Journal of cell science* **111 (Pt 10),** 1419-1432.

FIGURE LEGENDS

Figure 1. Localization of tau and EB1/3 partially overlaps at extending neurites in differentiating N1E-115 neuroblastoma cells. Confocal images of serum-starved N1E-115 cells, either flat or bearing neurites. **A.** Cells were stained with antibodies against tau (red), α-Tyrosinated-tubulin (dynamic MTs, green) and α-Acetylated-tubulin (stable MTs, blue). Tau was enriched at growing neurites, along MTs. In flat cells, tau decorated the MT lattice (anti-tau, red; anti-α-Tyrosinated-tubulin, blue) whereas EB3 (**B**) or EB1 **(C)** accumulated at MT plus-ends and showed a comet-like pattern (anti-EB1, green; anti-EB1, green). **D.** Confocal images showing EB1/3 comets (anti-EB3, green; anti-EB1, red) at plus-ends of dynamic MTs (anti-α-Tyrosinated-tubulin, magenta) decorated with tau (anti-tau: blue). Arrowheads point to EB1/3 comets at MT plus-ends **(B, C, and D). E.** EB1 and EB3 are enriched at tau-positive MT stretches at elongating neurites (anti-EB3, green; anti-EB1, red; anti-tau, blue; anti-α-Tyrosinated-tubulin, magenta). Arrows point to neurite segments highlighted by EB3, EB1, and tau. Scale bars= 10 μM.

Figure 2. Tau stable knockdown leads to a reduction in EB1/3 binding to MTs. Tau expression was stably downregulated in N1E-115 cells by lentiviral transduction either using a single tau shRNA construct or a pool of two different tau shRNAs. The pool of shRNAs was used in panels **B-E**. In **A,** cells were differentiated for three days. Differentiation induced an increase in both low and high molecular

weight tau (around 115kDa). Lentiviruses with a scrambled shRNA were used to generate a control cell line Reduction of tau levels was confirmed both by Western blot (the expression of both tau isoforms was reduced) (**A**) and immunofluorescence (**B**), using an antibody anti-tau. **C**. Confocal images of control and tau-depleted cells, stained with anti-EB1 (red), anti-EB3 (green) and anti-α-Tyrosinated-tubulin (blue). Tau-knocked-down cells showed fewer and shorter EB1/3 comets. Scale bar=10μm. Details are presented in insets. **D**. Quantification of the average number of EB1-comets/100μm2 (± SEM) in control (n= 418 comets in 19 cells) and tau-knocked-down cells (n= 269 comets in 16 cells).Comet number is significantly reduced upon tau downregulation. **E.** Average comet length (± SEM) of EB1 or EB3-positive MT-distal ends in control (n= 296 EB1-comets in 5 cells; n= 293 EB3-comets in 6 cells) and tau-deficient cells (n=252 EB1-comets in 5 cells; n= 114 EB3-comets in 3 cells). EB1 and EB3 comet length was significantly reduced upon tau depletion.

Figure 3. Tau overexpression at high levels recruits EBs to MT-bundles. Confocal pictures of N1E-115 cells transfected with GFP-tau (green) and stained with antibodies against EB1 (**A**, **D**) or EB3 (**B**, **E**), or both **(F).** In cells expressing low-to-medium levels of GFP-tau, EB1 (**A, F**) and EB3 (**B, F**) present the usual comet-like pattern. Insets showing details are presented in **A, B, D**, and **E. C**. Average length of EB3-comets in non-transfected cells (control; n=162 comets in 6 cells) and in cells expressing low-to-medium levels of GFP-tau (n= 256 comets in 4 cells). Error bars are SEM. Comet length is not altered upon ectopic expression of tau at moderate levels. Tau overexpression at high levels promotes neurite extension (**D**), MT-bundle formation (**D**, **E** and **F**) and a concomitant recruitment of EB1 (**D, F**) and EB3 (**E, F**) to MT bundles. Both EB1 and EB3 relocalize similarly to tau-positive MT bundles (**F**). Details are shown in insets in each case. Scale bars= 10μM. **G**. Quantification of the percentage of cells that present different localization patterns of EB1 and EB3, depending on the levels and localization of ectopically expressed GFP-tau. High tau levels lead to MT bundle formation and the subsequent relocalization of both EB1 and EB3 to these bundles. A total of 227 cells transfected with GFP-tau were counted: 88 cells with low-to-medium tau expression levels (38.8%); 132 cells with high tau levels (58.1%), and 7 cells with extremely high tau expression (3.1%). **H**. CLASP2, another +TIP, is relocalized to MT bundles induced upon tau overexpression. **I**. GFP-DCL overexpression leads to MT bundle formation and EB1 displacement from MT plus-ends but no EB1 relocalization to MT bundles.

Figure 4. Tau interacts directly with EB1. A and B. COS-7 cells were co-transfected with tau (non-tagged) and GFP (control) or GFP-tagged EB1 or EB3 (at their N-termini). Co-IP assays were performed with an antibody against either tau (**A**) or GFP (**B**). Expression of each construct was confirmed by

Western blot using anti-GFP or anti-tau antibodies (Inputs). Tau was coimmunoprecipitated with both EB1-GFP and EB3-GFP, but not with GFP, as shown in reciprocal IPs (**A** and **B**). **C**. Co-IP of GFP-tagged EB1 and EB3 with endogenous tau in N1E-115 cells. Assays were performed in lysates of cells transfected with EB1 or EB3-GFP and differentiated for 3 days (with DMSO). **D.** Co-IP of endogenous tau and EB1 proteins in 14 DIV mouse cortical neurons. **E** Endogenous tau and EB1 were present in a complex in P2 mouse brain, as shown by reciprocal coIP assays. SN= Supernatant. **F**. *In vitro* pull-down assay of His-tau with either GST (control) or GST-EB1. GST-EB1 but not GST was pulled-down by tau, this showing a direct interaction between tau and EB1.

Figure 5. Tau modulates EB1/3 localization and mobility in living cells. N1E-115 cells were cotransfected with EB3-mCherry and either GFP (control) or GFP-tau and serum-starved overnight. **A-D.** Analyzes of slow FRAP of EB3-mCherry in control (GFP) and GFP-tau expressing cells. Bleaching was performed in proximal regions of the neurites. **A.** Representative example of time-lapse pictures of FRAP of EB3-mCherry in proximal neurites of control (GFP) and GFP-tau transfected cells. Cells expressing moderate levels of GFP-tau and EB3-mCherry, bearing neurites, were chosen for the studies. **B**. Curves of actual examples of FRAP data in control (GFP) and GFP-tau transfected cells. .Parameters corresponding to the slow phase of EB3-mCherry fluorescence recovery obtained from control (GFP) and GFP-tau transfected cells. k (s^{-1}) is shown in **C** and $t_{1/2}$ (s) in **D**. Data were collected from 3 different sets of experiments (~ 8-15 cells/experiment) and normalized and fitted with a two-phase association equation.

Figure 6. Tau regulates the interaction of EBs to MTs in developing neurons. Embryonic wild-type (wt) and *TAU* -/- primary hippocampal neurons were cultured for 1 day (**A-D**) or 3 days (**E**), fixed and stained with different antibodies. **A**. Confocal images of wt and *TAU*-/- neurons stained with anti-EB1 (red) and anti-tau (green) antibodies. Tau and EB1 partially colocalize at the medial-distal axon shaft. Few EB1 accumulations at stretches are found in *TAU*-/- neurons. **B**. Confocal pictures of a wt distal axon tipped by a growth cone showing double-staining of EB1 (red) and tau (green). Tau and EB1 colocalize at the distal axon but not at the growth cone. **C.** Average length of EB1-comets in wt (n= 1002 comets in 16 neurons) versus *TAU*-/- neurons (n= 1261 comets in 22 neurons). **D**. Quantification was performed in different cell compartments (axon, cell body and axonal growth cones (GC)). Comets counted per cell compartment: wt: n= 464 in axons, n=457 in cell body and 81 in growth cone (16 neurons); *TAU*-/-: n=377 in axons; n= 791 in cell body, and n= 93 in growth cones (22 neurons). EB1 comets were shorter in tau-deficient neurons in all neuronal compartments analyzed. **E.** Confocal images show that 3DIV tau-

knockout neurons present an impaired localization of EB1 along MT-bundles in axons, mostly at medial-distal regions (anti-EB1: red; anti-tau; green; anti-α-Tyrosinated-tubulin: blue). Scale bars= 10μm

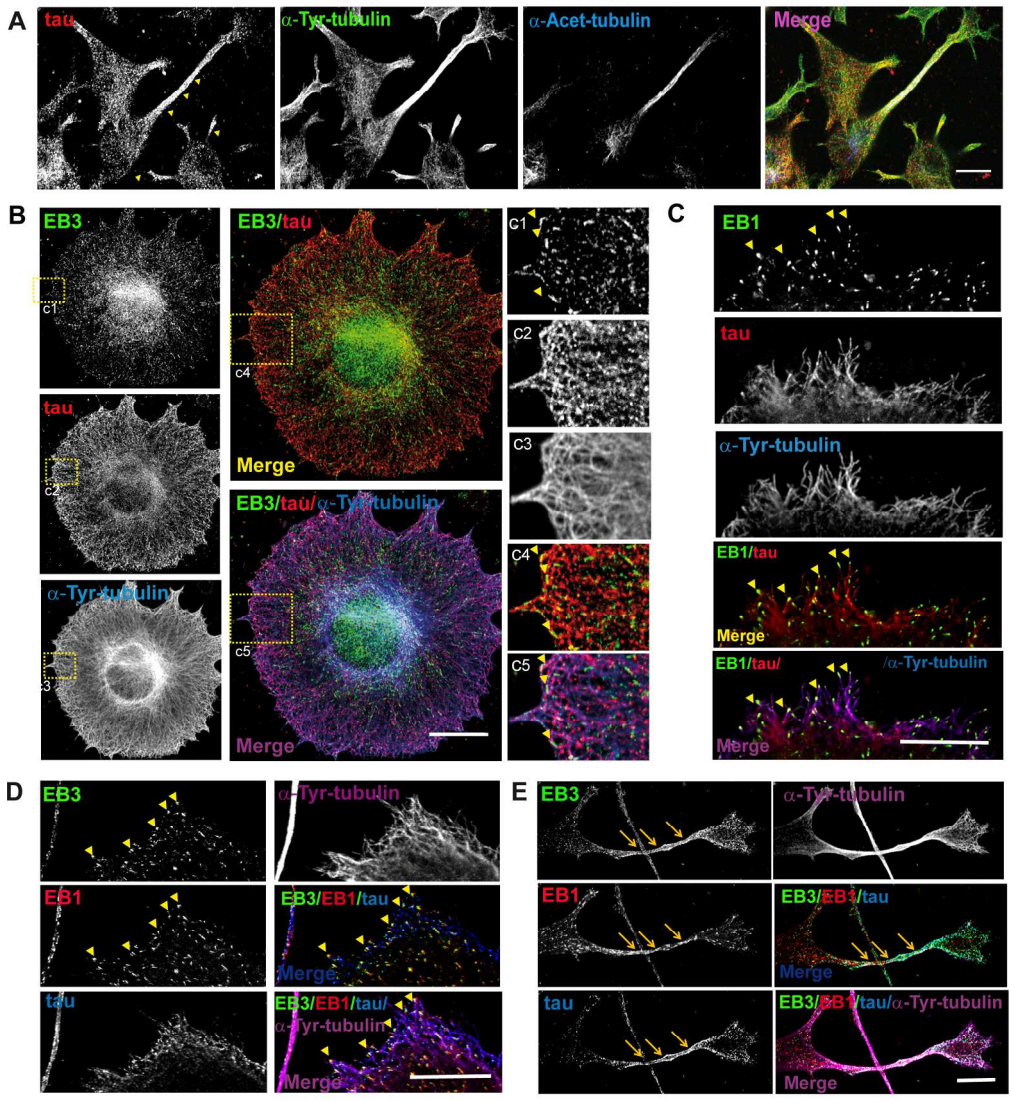


Figure 1

194x224mm (300 x 300 DPI)

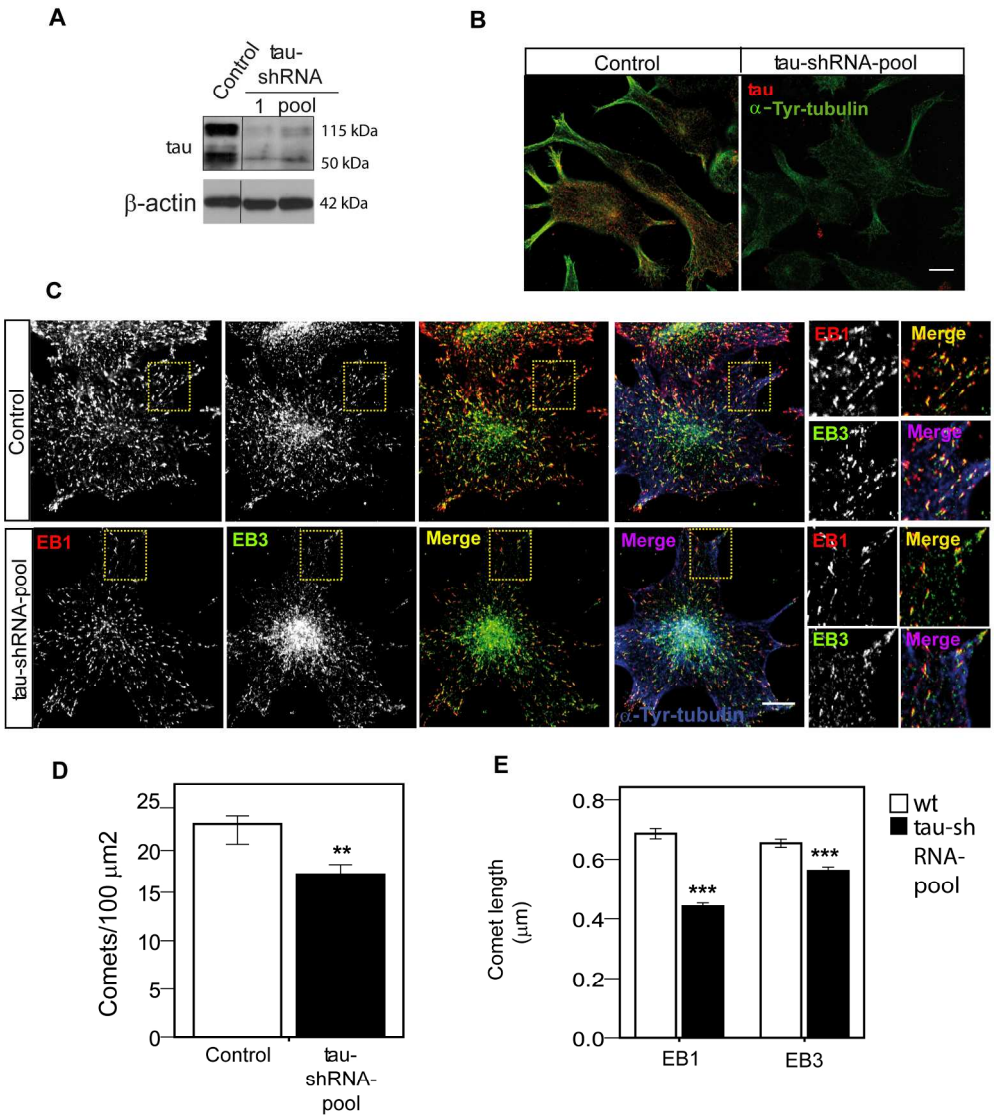


Figure 2

207x245mm (300 x 300 DPI)

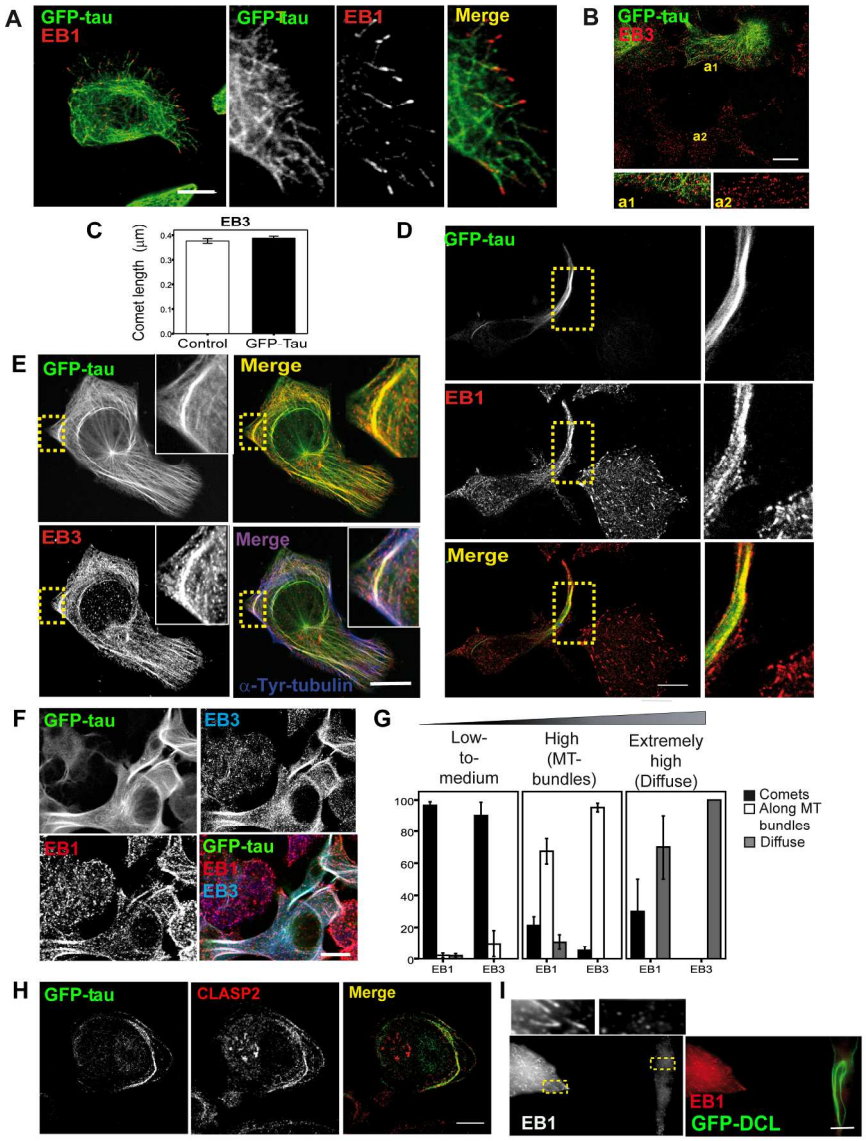


Figure 3

182x252mm (300 x 300 DPI)

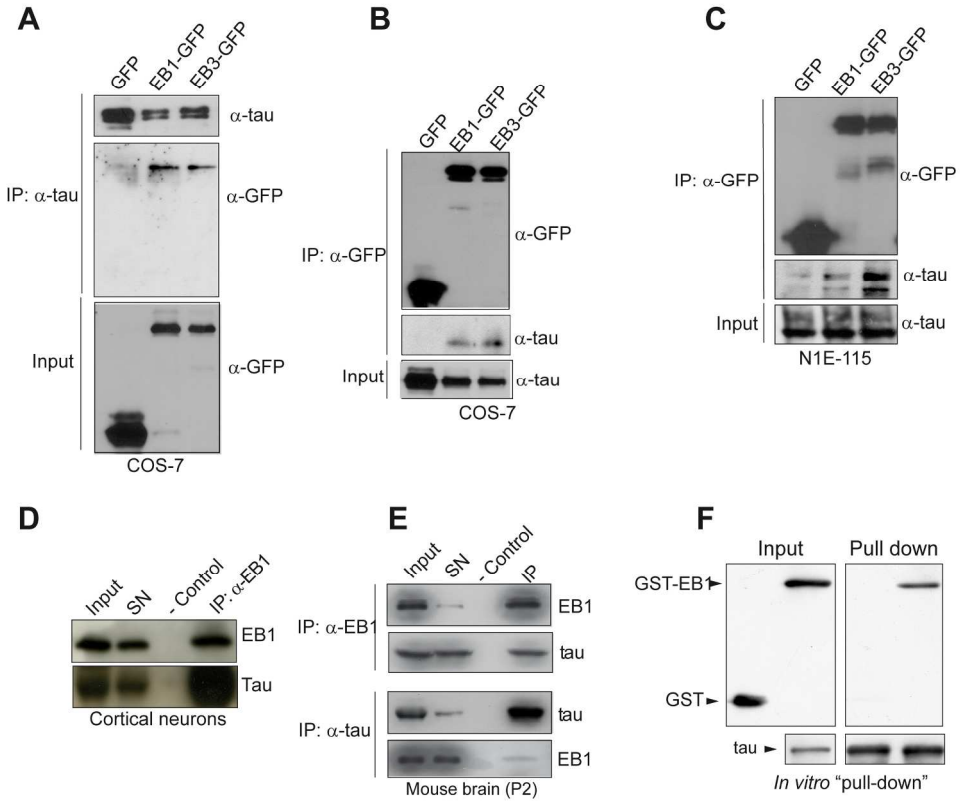


Figure 4

206x181mm (300 x 300 DPI)

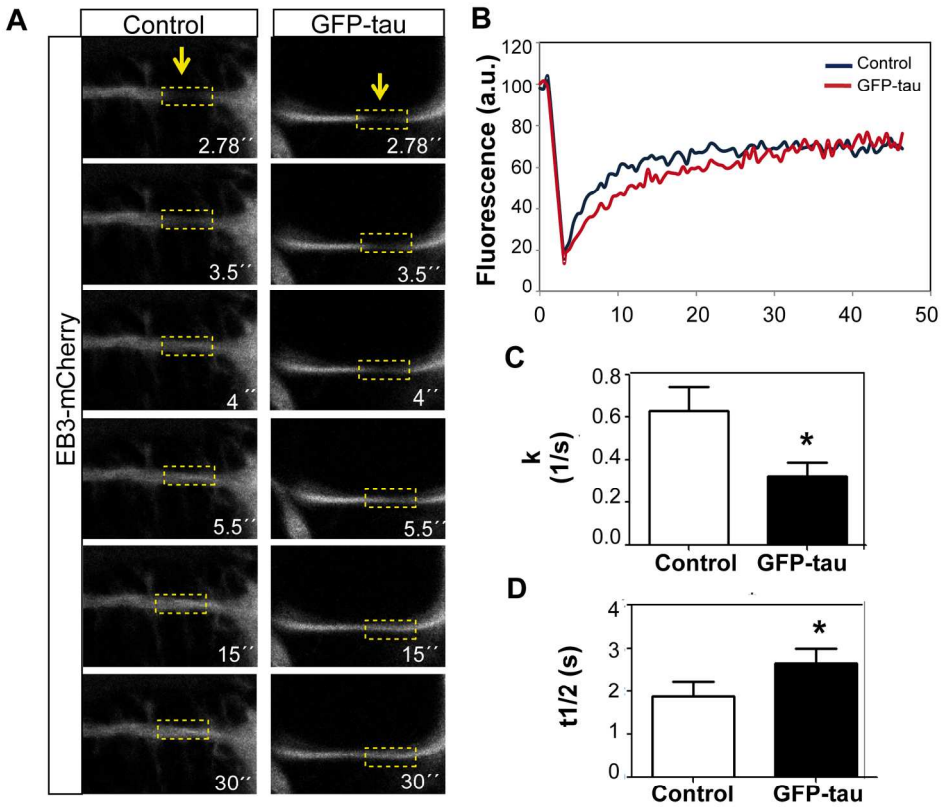


Figure 5

166x148mm (300 x 300 DPI)

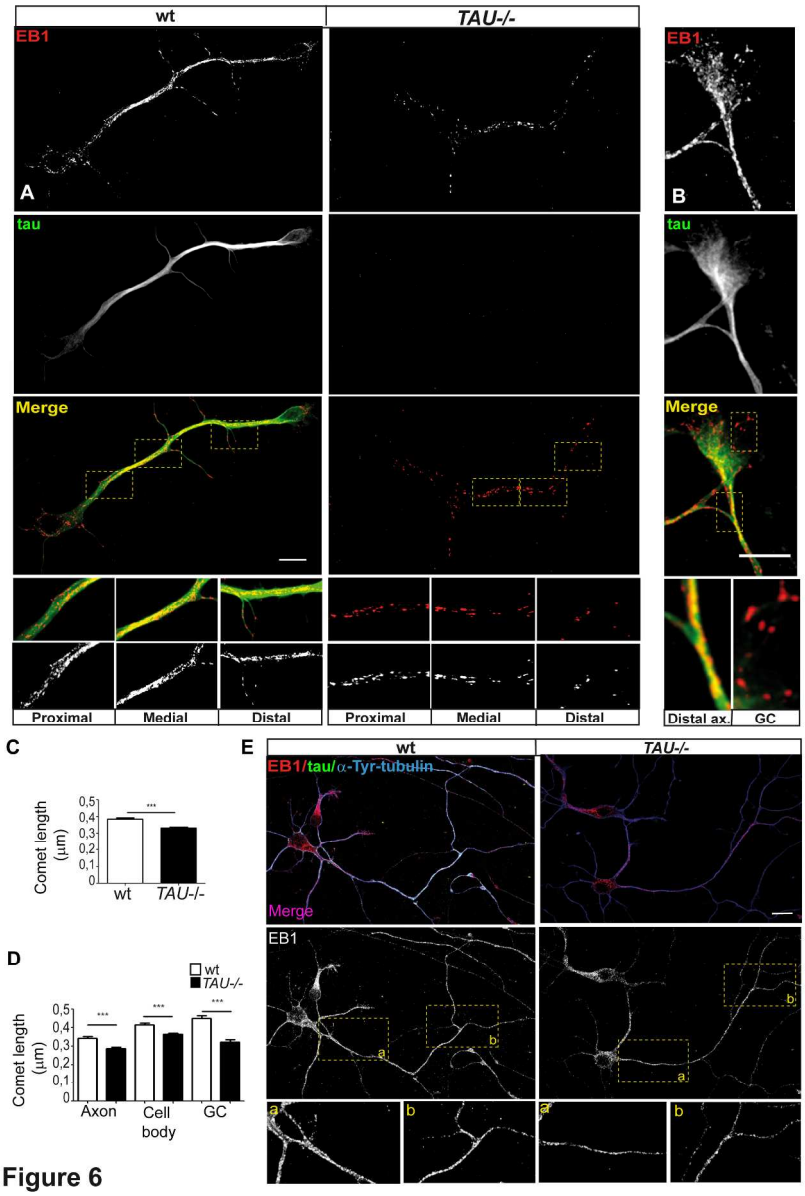


Figure 6

202x294mm (300 x 300 DPI)

MS number	Full Paper	Date received
JNC-2014-0815.R2	Full paper	28/11/14

Special issue / comments

All authors
Avila, Jesús (contact); Sayas, Carmen; Tortosa, Elena; Bollati, Flavia; Ramírez-Ríos, Sacnicte; Arnal, Isabelle

Title
Tau regulates the localization and function of End binding proteins 1 and 3 (EB1/3) in neuronal cells

Corresponding author
Dr. Jesús Avila

Address

Tel

FAX

E-mail
jesus.avila@csic.es

Date of first decision	Date received 1st revision	Date received 2nd and further revisions
22/12/14	23/02/15	03/03/15

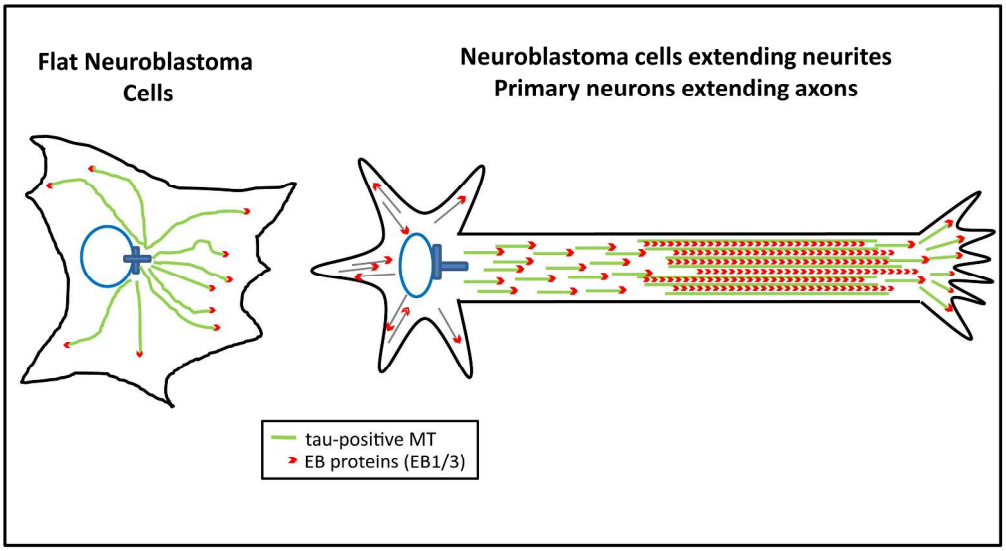
Final Decision	Days to 1st decisio	Days submission to final decisio
Accepted on 05/03/15	24	27

Copyright Form	Disk Enclosed	Colour Figures
No	N/A	Yes

ISN member - Confirmation	Category
No	Brain Development & Cell Differentiation

Tau regulates the localization and function of End binding proteins 1 and 3 (EB1/3) in neuronal cells

We describe here a novel function for tau as a direct regulator of End binding (EB) proteins in differentiating neuronal cells. EB1/3 cellular mobility and localization in extending neurites and axons is modulated by tau levels and localization. We provide new evidence of the interplay between classical microtubule-associated proteins (MAPs) and "core" microtubule plus-end tracking proteins (+TIPs) during neuronal development.



241x131mm (300 x 300 DPI)