Journal of Trace Elements in Medicine and Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.com/locate/jtemb

Nutrition

Localization of the zinc binding tubulin polymerization promoting protein in the mice and human eye

Robert G. Tripon[a,b], Judit Oláh[c], Tajwar Nasir[a], Lajos Csincsik[a,d], Chee Lok Li[a], Sándor Szunyogh[c], Haiyan Gong[e], Jane M. Flinn[f], Judit Ovádi[c], Imre Lengyel[a,d,*]

[a] UCL Institute of Ophthalmology, University College London, London, EC1Y 8TB, UK
[b] Department of Histology, University of Medicine and Pharmacy, Tîrgu Mureş, Romania
[c] Institute of Enzymology, Research Centre for Natural Sciences, Hungarian Academy of Sciences, Budapest, 1117, Hungary
[d] Center of Experimental Medicine, The Queen's University Belfast, BT9 7BL, UK
[e] Department of Ophthalmology, Boston University School of Medicine, MA, USA
[f] Department of Psychology, George Mason University Fairfax, VA, USA

ARTICLE INFO

Keywords:
Tubulin polymerization promoting protein
Retina
Inner plexiform layer
Amacrine cell
Zinc
Myelin sheath

ABSTRACT

Tubulin Polymerization Promoting Protein (TPPP/p25) modulates the dynamics and stability of the microtubule network by its bundling and acetylation enhancing activities that can be modulated by the binding of zinc to TPPP/p25. Its expression is essential for the differentiation of oligodendrocytes, the major constituents of the myelin sheath, and has been associated with neuronal inclusions. In this paper, evidence is provided for the expression and localization of TPPP/p25 in the zinc-rich retina and in the oligodendrocytes in the optic nerve. Localization of TPPP/p25 was established by confocal microscopy using calbindin and synaptophysin as markers of specific striations in the inner plexiform layer (IPL) and presynaptic terminals, respectively. Postsynaptic nerve terminals in striations S1, S3 and S5 in the IPL and a subset of amacrine cells show immunopositivity against TPPP/p25 both in mice and human eyes. The co-localization of TPPP/p25 with acetylated tubulin was detected in amacrine cells, oligodendrocyte cell bodies and in synapses in the IPL. Quantitative Western blot revealed that the TPPP/p25 level in the retina was 0.05–0.13 ng/μg protein, comparable to that in the brain. There was a central (from optic nerve head) to peripheral retinal gradient in TPPP/p25 protein levels. Our *in vivo* studies revealed that the oral zinc supplementation of mice significantly increased TPPP/p25 as well as acetylated tubulin levels in the IPL. These results suggest that TPPP/p25, a microtubule stabilizer can play a role in the organization and reorganization of synaptic connections and visual integration in the eye.

1. Introduction

Tubulin Polymerization Promoting Protein (TPPP/p25) is a 25 kDa disordered microtubule-associated protein that was first identified in the brain [1] where it plays a crucial role in the differentiation of oligodendrocytes [2] and is involved in the development, maintenance and stability of oligodendrocyte projections due to the modulation of the dynamics and stability of microtubule network [3–5]. Oligodendrocytes are the major constituents of the myelin sheath. TPPP/p25 directly interacts with histone deacetylase 6, inhibits its activity, and therefore regulates acetylation of tubulin [5] and influence differentiation, cellular polarization and migration [6–9].

While the normal function of TPPP/p25 is to modulate and stabilize the microtubule network [3–5] by it bundling and acetylation enhancing activity due to its interaction with tubulin deacetylases (histone deacetylase 6, sirtuin 2), it can also directly interact with α-synuclein [10], hallmark protein of synucleinopathies, and play a role in its pathological oligomerisation/aggregation. TPPP/p25 is implicated in the development of synucleinopathies such as Parkinson's disease and Multiple System Atrophy, when α-synuclein and TPPP/p25 are co-localized and co-enriched in the inclusions [11–14].

TPPP/p25 has a zinc binding motif [15,16], the specific binding of

Abbreviations: BSA, bovine serum albumin; GCL, ganglion cell layer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; INL, inner nuclear layer; IPL, inner plexiform layer; ON, optic nerve; OPL, outer plexiform layer; PBS, phosphate buffered saline; RNFL, retinal nerve fibre layer; TPPP/p25, Tubulin Polymerization Promoting Protein

* Corresponding author at: Room 01.057, The Wellcome-Wolfson Building Centre for Experimental Medicine, School of Medicine, Dentistry and Biomedical Science, Queen's University Belfast, 97 Lisburn Road, Belfast BT9 7BL, UK.

E-mail addresses: robert.tripon@umftgm.ro (R.G. Tripon), olah.judit@ttk.mta.hu (J. Oláh), tajwar.nasir.12@ucl.ac.uk (T. Nasir), Lcsincsik01@qub.ac.uk (L. Csincsik), chee.li.13@alumni.ucl.ac.uk (C.L. Li), szunyogh.sandor@ttk.mta.hu (S. Szunyogh), hgong@bu.edu (H. Gong), jflinn@gmu.edu (J.M. Flinn), ovadi.judit@ttk.mta.hu (J. Ovádi), i.lengyel@qub.ac.uk (I. Lengyel).

https://doi.org/10.1016/j.jtemb.2017.12.009
Received 6 November 2017; Received in revised form 15 December 2017; Accepted 21 December 2017

the bivalent zinc cation to TPPP/p25 induces structural changes/dimerization coupled with stabilization that counteracts its proteolytic degradation [17]. Thus, zinc may control the intracellular level of TPPP/p25, playing role in its physiological as well as its pathological functions [17]. Zinc levels can be altered in different diseases of the eye [18] and a recent study showed that optic nerve (ON) injuries can elevate zinc levels not only in the ON but also in the retina [19]. In this paper, we set out to investigate whether the intracellular TPPP/p25 level could be affected by the zinc level.

2. Materials and methods

2.1. Antibodies

The list of antibodies used for the experiments can be found in Supplementary Table 1.

2.2. Human eyes

Five whole human eyes with no known pathological conditions were obtained from the Boston University and the UCL Institute of Ophthalmology Eye Depositories within 24 h of death. In addition, paraffin sections from six eyes from the UCL Institute of Ophthalmology pathological archive were also used. Full Local Research Ethics Committee approval and appropriate consent was obtained in each case. The protocol of the study adhered to the tenets of the Declaration of Helsinki regarding research involving human tissue.

2.3. Mouse eyes

Eyes from 6 months old female mice were used in this study from a previous experiment in which zinc supplementation had a significant behavioural effect [20]. The eyes were dissected from double transgenic mice overexpressing human APP and ApoE4 as well as wild type litter mates [20]. Four wild type and 4 transgenic animals were given drinking water without zinc supplementation to establish baseline. In addition, 4 transgenic mice were given drinking water supplemented with 10 ppm (0.153 mM) zinc carbonate from the age of 2 months The zinc-enhanced water was prepared by dissolving 10,000 mg/L zinc in 5% HNO_3 and adding Na_2CO_3 to buffer the solution to pH 7.0 [20].

2.4. Preparation of human retina extract

Cell extracts were prepared from three unfixed human eyes that were flat mounted before peripheral and central retina tissues were excised. After excision, the tissues were homogenized in buffer A (10 mM tris(hydroxymethyl)aminomethane, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid pH 7.4 containing 1 μg/ml leupeptin, 1 μg/ml pepstatin, 10 μM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1 mM benzamidine, 1% Triton X-100) at a 1:4 ratio of tissue and buffer by a Potter-homogenizer. The homogenates were centrifuged at 17000*g* for 10 min at 4 °C, and the supernatants were used for further experiments. Bovine brain extract was prepared similarly. The protein concentration of the retina/brain extract was determined by the Bradford method [21] using the Bio-Rad protein assay kit.

2.5. Purification of human wild type TPPP/p25 and bovine tubulin

Human recombinant full length TPPP/p25 with a His-tag was expressed in E. coli BL21 (DE3) cells and isolated on HIS-Select™ Cartridge (Sigma-Aldrich) as described previously [11,22]. Tubulin was prepared from bovine brain according to the method of Na and Timasheff [23]. TPPP/p25 concentration was determined from the absorbance at 280 nm using an extinction coefficient of 10095 M^{-1} *cm^{-1}.

2.6. Western blot

Retina samples were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis, and were electrotransferred onto Immobilon-PSQ transfer membranes and labelled with antibodies: TPPP/p25 in a dilution of 1:5000 [11] or with tubulin (1:5000; Sigma T9026), acetylated tubulin (1:5000; Sigma T6793) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in mouse (1:25000 Calbiochem CB1001) (Supplementary Table 1). Antibody binding was revealed by using anti-rat or anti-mouse IgG coupled with peroxidase, ECL® (enhanced chemiluminescence) Western Blotting Detection reagents (Amersham Biosciences) and Chemidoc Image system or 3-amino-9-ethylcarbazole as substrate. Images were analysed by Image-J using mean pixel intensity value tool kit.

2.7. Immunocytochemistry

Eyes from human as well as mice were embedded in paraffin and 10 μm sections were collected on glass slides. Immunohistochemistry was performed using monoclonal and polyclonal antibodies against several relevant proteins (see list in Supplementary Table 1). Primary antibodies were applied after antigen retrieval in citrate buffer (heating for 2 × 3 min for mice and 3 × 3 min for human samples in microwave at 750 W) followed by blocking for 1 h in goat serum (1:20, Sigma G9023). Primary and secondary antibodies were applied for 1 h at room temperature, washed 3 × 5 min with phosphate buffer saline (PBS). TPPP/p25 was visualized using Alexa-Fluor 488 Goat anti-rat secondary antibody (1:1000; Invitrogen A11006). Acetylated tubulin, calbindin and synaptophysin labelling were visualized using Alexa-Fluor 568 Goat anti-mouse secondary antibodies (1:1000; Sigma T7782). Nuclear staining was performed using Hoechst (0.001 g/ml, Life Technologies H3570). Samples were imaged using a Zeiss LSM700 confocal microscope through 40 x or 63x/1.2 NA Zeiss Neofluar objectives and images generated using the ZEN software package (Zeiss). Central and peripheral retinal images were taken from each section. Sections from lab water or zinc fed animals were processed in parallel and microscope setting was kept standard between samples for the comparison of immunofluorescence intensities. Images were imported into Image-J and fluorescence intensities were analysed using mean pixel intensity value tool kit.

2.8. Immunogold staining

Retina from an unfixed human eye was flat mounted and fixed in 3% formaldehyde and 0.2% glutaraldehyde overnight at 4 °C, then washed in PBS. The macular region was dissected and embedded in 3.6% agarose solution and vibratome sections of 50 μm thickness were cut and collected in a 24 well plate in PBS. To visualize ultrastructural localization of TPPP/p25 in the retina, sections were processed for pre-embedding immunogold labelling using methods modified from [24,25]. The sections were washed in 50 mM glycine solution for 2 h, then incubated in 10 mM sodium citrate buffer (pH 6.0) for 15 mins using a variable wattage microwave (power at 200 W, sample temperature at 45 °C; Pelco BioWave, Ted Pella) for antigen retrieval. After rinsing (3 × 10 mins in PBS), sections were incubated in blocking solution (10% bovine serum albumin (BSA), 5% normal goat serum (NGS), 0.025% Triton-X in PBS) for 1 h at room temperature. Sections were incubated with the rabbit polyclonal primary antibody against TPPP/p25 (1:100, Novusbio NBP1-80962), first in the microwave (5 mins at 150W, 35 °C) and then at 4 °C overnight. Sections were rinsed (3 × 10 mins in 10 mM phosphate buffer with 0.2% Aurion BSA-c) and then incubated in a 6 nm gold-conjugated goat anti-rabbit secondary FAB2 IgG (1:50, Aurion) in the microwave (5 mins at 150W, 35 °C), followed by incubation overnight at 4 °C. The antibody was diluted in PBS, 0.2% acetylated BSA (BSA-c; Aurion) and 0.025% Triton-X. After antibody incubation steps, sections were rinsed in PBS with 0.2% BSA-