Reduced Reflectance of Retinal Nerve Fiber Layer as a Marker of Microtubule Axonal Damage in Experimental Autoimmune Encephalomyelitis

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Introduction

Microtubules (MT) are the major component of cytoskeleton and is crucial for axonal transportation in neurons [1]. Axonal transport deficits have been shown to precede substantial neurodegeneration in multiple sclerosis (MS) and its model experimental autoimmune encephalomyelitis (EAE) [2]. Reflectance under optic coherence tomography (OCT), a measure of signal intensity reflected from retinal tissue, is proportional to its ultrastructural directional homogeneity, which in axons relies upon the integrity of MT network [3]. Reflectance of retinal nerve fiber layer (RNFL) has been suggested as a marker of axonal MT integrity [4]. Here we induced EAE in dark Agouti rats with myelin oligodendrocyte glycoprotein (MOG) and performed OCT, visual-evoked potential (VEP), and histology of retinas and optic nerves at 14(N=7 rats), 21(N=17), 35(N=13) and 47 (N=10) days post immunization (dpi), aimed at validating the relationship between RNFL reflectance and MT in EAE.

Methods and Results

Forty seven rats were used as the EAE group and 12 kept as healthy controls in the experiment (Figure 1). Since disease-related changes of reflectance may cause segmentation error of RNFL in OCT (Figure 2), we segmented the neuronal ganglion cell complex (NGCC) instead, which contains the dendrites, cell bodies, and axons of retinal ganglion cells (RGCs). RNFL reflectance index (RRI), thickness of NGCC (tNGCC), and VEP latency were quantified for further analysis (Figure 3). The eyes were separated into non On eyes (without delay) and ON eyes (with delayed VEP latency) based on the cutoff calculated from the healthy eyes. Already at 21 dpi, RRI was reduced in both non ON (RRI= 0.95±0.22,p=0.01) and ON (RRI =0.90±0.16, p=0.001) eyes compared with healthy (RRI=1.10±0.12). Compared with healthy, ON eyes showed lower RGC counts (ON:146±61, healthy:325±19, p<0.001), less percentage of neurofilaments (ON:21.84±10.67%, healthy:40.53±12.46%, p=0.005), and β-tubulin (ON:33.24±14.15%, healthy:48.08±5.66%, p=0.004) within RNFL, and more severe demyelination (ON:45.52±42.79%, non ON: 6.45±7.14%, healthy: 0.00±0.00%, p<0.001), microglia infiltration (ON:505.45±408.69, non ON: 92.75±59.54 healthy:76.40.±2.41, p<0.001), and axon loss in optic nerves (ON:30.12±30.56%, non ON: 11.91±8.65%, healthy:0.00±0.00%, p=0.01). In ON eyes, RRI correlated with RGC counts(r=0.621, p=0.002), percentage of β-tubulin(r=0.931, p<0.001), but not with neurofilament (p=0.067). A correlation between β-tubulin and neurofilament was found (r = 0.538, p = 0.032). No correlation was found between RRI and tNGCC.

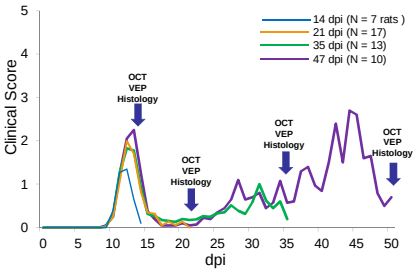

Figure 1 - *The clinical scores of the EAE rats*. The EAE onset at 10 days post injection.

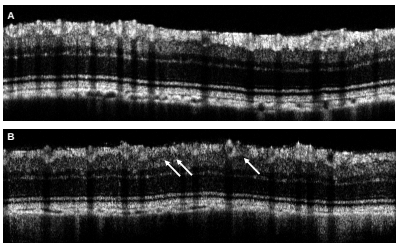

Figure 2 - *Examples of focal reduction of RNFL reflectance.* A: RNFL of a healthy rat. B: RNFL of an EAE rat at 14 dpi. Arrows: areas with focal reduction of RNFL reflectance.

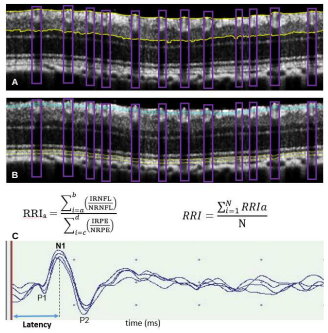

Figure 3 – *Quantification of OCT and VEP parameters.* A: Segmentation results of the NGCC (between yellow lines). The thickness of NGCC was averaged and areas with vessels (purple boxes) were removed from the analysis. B: RNFL reflectance index was sample from the center of RNFL (between blue lines), then normalized with the signal from retinal pigment epithelium (RPE, between yellow lines).Vessel areas (purple box) were not included in the analysis. RRIa: RRI of each a scan; N: number of a scans; a: begging of the measure site; b end of the measure site; I: signal intensity. C: VEP latency were calculated from the P1-N1-P2 complex.

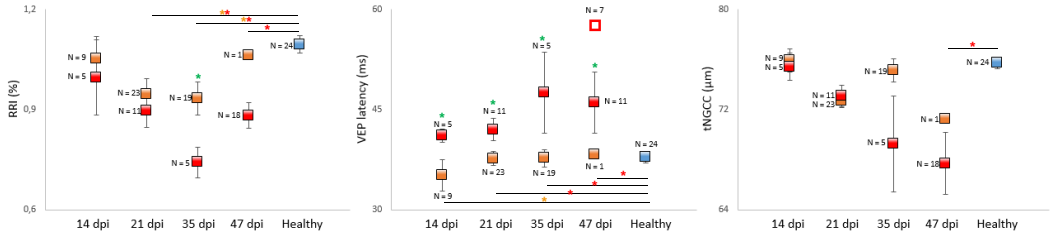

Figure 4 – Results of 2-way ANOVA (group by time). The EAE eyes were separated into non ON (without delay) and ON (delayed VEP latency) eyes. Main effects of group and time were found in all three parameters, no interaction was found. Post-hoc analyses were employed with the α level set with 0,01. * : significant difference in post-hoc between healthy and non ON eyes.
* :significant difference between healthy and ON eyes. *: significant difference in post-hoc between ON and non ON eyes. Error bar: standard deviation.

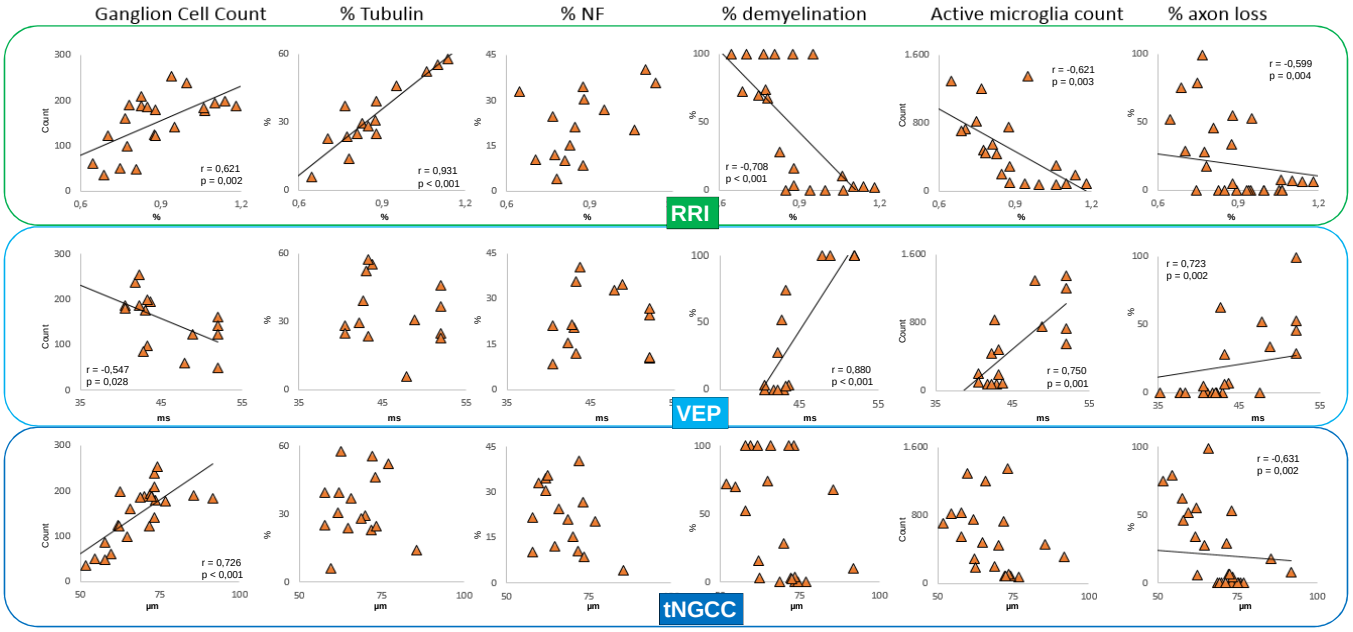

Figure 5. Correlations of OCT, VEP and Histology. The correlations were performed in ON eyes. Retina tissues were stained with BRN3 for ganglion cell, β-tubulin for microtubules, and anti-neurofilament for neurofilament, and further quantified as ganglion cell count, percentage β-tubulin (% Tubulin), and percentage of neurofilament (%NF) within NGCC. Optic nerves were stained with Luxol fast blue for myelin, Iba-1 for microglia, and SMI-312 for axon, then quantified as percentage of demyelination (% demyelination), active microglia count, and percentage of axon loss (% axon loss).

Discussion

Our results consistent with previous *in vitro* studies suggesting that, at OCT wavelength, RNFL reflectance is mainly contributed by microtubules. Lower RRI was found in all the EAE eyes suggesting that cytoskeleton deformation exits even in the eyes without evidence of demyelination from VEP, which consists with literature that pervasive axonal transport deficits in EAE rats [5]. Further, in ON eyes, significant atrophy of tNGCC was found at 47 dpi, meanwhile RRI was higher at 47 dpi compared with 35 dpi. Since no correlation was found between RRI and tNGCC, the result might imply that the MT network reorganization after axon loss. Therefore, RRI and tNGCC should be considered together when monitoring neurodegeneration. RNFL reflectance enables us to monitor the integrity of microtubule *in vivo* and may help testing therapeutic approaches targeting axonal transport in neurodegenerative diseases.

Reference

[1] Millecampus S., Julien J. *Nat Rev Neurosci. 2013 Mar; 14(3): 161-76.* [2] van den Berg, R., Hoogenraad C. C., Hintzen R. Q.. *Acta Neuropathol 2017; 134:1-14.* [3] Huang X., Knighton R. W., Feuer W. J., Qiao J. *Biomedical Optics Express 2015; 7(1): 22-33.* [4] Huang X., Knighton R. W., Cavuoto L. N. *Investigative Ophthalmology & Visual Science 2006; 47(12):5363-67.* [5] Sorbara C. D., Wagner N. E., Ladwig A., Nikic I., Merkler D., Kleele T., Marinkovic P, Naumann R., Godinho L., Bareyre F. M., Bishop D., Misgeld T., Kerschensteiner M. *Neuron 2014; 84: 1183-1190.*