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ORIGINAL ARTICLE

Upregulated Retinal Neurofilament Expression in Experimental Optic Neuritis

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ABSTRACT

In optic neuritis (ON), transient thickening of the macular retinal nerve fibre layer (RNFL) can be observed. This optical coherence tomography-based observation is not understood. The axonal diameter correlates with the neurofilament (Nf) protein content, but there are no data on the retinal tissue concentration of Nfs. The myelin-oligodendrocyte-glycoprotein (MOG) induced experimental autoimmune encephalomyelitis (EAE) model was used to investigate the retinas of Brown Norway rats with (i) visual evoked potentials (VEP) confirmed ON, (ii) VEP confirmed absence of ON and (iii) control animals. Twenty retinas were collected from MOG-EAE and control rats 27 days after immunisation. Retinal tissue Nf concentrations per total protein (μg/mg) were significantly higher in MOG-EAE rats with ON (median 4.29, interquartile range [IQR] 3.41–5.97) compared with MOG-EAE rats without ON (1.14, IQR 1.10–1.67) or control rats (0.93, IQR 0.45–4.00). The data suggest that up-regulation of Nf expression in the retinal ganglion cells precedes development of RNFL atrophy and plausibly explains the transient increase of axonal diameter and RNFL thickening.

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Introduction

Acute optic neuritis (ON) causes axonal degeneration, which can be quantified from the blood by neurofilament protein (Nf) levels.[1,2] Within about 3 months atrophy of the retinal nerve fibre layer (RNFL) follows.[1,3] It remains challenging to explain why there is also transient thickening of the macular RNFL in acute ON, not related to optic disc swelling, in some patients in the studies reviewed.[3,4] Transient RNFL swelling has also been reported in Leber's hereditary optic neuropathy (LHON),[5] macular hole surgery[6] and retinal photocoagulation.[7]

We hypothesised that because of the association of axonal diameter with Nf concentration,[2] this transient phenomenon could be related to a change of retinal tissue Nf levels. The concentration of retinal tissue Nf levels following ON is not known. Therefore, we chose an experimental model to investigate this further. The known advantage of the myelin-oligodendrocyte-glycoprotein (MOG) induced experimental autoimmune encephalomyelitis (EAE) rat model is that there are spontaneous relapses, but not all of them will result in ON.[8] This choice permitted study of retinal tissue in controls, but also in diseased rats with and without ON.

Methods

This study was approved by the ethical committee of the participating centres. The animal experiments were conducted in Tübingen, Germany (RW, TH) and the protein quantification in London, United Kingdom (AP). We followed the ARRIVE 2.0 guidelines (https://arriveguidelines.org/sites/arrive/files/documents/ARRIVE%20guidelines%202.0%20-%20English.pdf).

Animals and experimental procedures

Female Brown Norway (BN) rats were chosen for MOG EAE.[9] The rats were 6–8 weeks of age at arrival. The rats were weighed and scored for clinical signs of EAE every second day from day 1 after immunisation. A disease severity score was recorded where: grade 1 indicates tail weakness or tail paralysis; 2 indicates hind-leg para-paresis or

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hemiparesis; 3 indicates hind-leg paralysis or hemiparalysis; and 4 indicates complete paralysis, moribund or death.[9] Because not all rats develop MOG-induced ON, we used visual evoked potentials (VEP) to confirm development of MOG-induced ON.[10] For this purpose we implanted three cortical screws and performed serial VEP examinations until there was evidence of ON. Control rats also had three cortical screws implanted. All rats were sacrificed by inhalation of carbon dioxide. Both eyes were immaculately removed, the retinas dissected and snap frozen in liquid nitrogen within 5 minutes.

Retinal Nf analysis

In total, 20 rat retinas were collected (seven EAE, four control). Two EAE retinas were damaged during surgical removal and were not processed further. The remaining samples were stored at −80°C until analysis. On receipt in London the retinas were dry-weighed then suspended 1:60 water:weight in the enzyme-linked immunosorbent assay sample buffer containing a protease inhibitor cocktail. Next, retinas were thawed and homogenised on ice using a Sonipre 150 (power 14 for 1 minute). The homogenate was spun down (4°C, 150,000 rpm for 10 minutes) and the supernatant used for analysis as described before.[11]

All 20 retinas were batch analysed in one microtitre plate, which was coated overnight with 50 μL of the SMI35 capture antibody, diluted 1/5000 in 0.05 M carbonate buffer, pH 9.5. The plate was then washed with barbitone buffer containing 0.1% bovine serum albumin (BSA) and 0.05% Tween 20 (pH 8.6). The plate was blocked with 150 μL of barbitone buffer containing 1% of BSA. After washing, 25 μL of barbitone buffer, 6 mM EDTA, 0.1% BSA were added as sample diluent to each well. Twenty-five μL of standard or retina homogenate were then added in duplicate to the plate. The plate was incubated at room temperature (RT) for 1 hour. After washing, 50 μL of the second antibody diluted 1/1000 in barbitone buffer were added to each well and the plate was incubated for 1 hour at RT. The microtitre plate was washed and horseradish peroxidase-labelled swine anti-rabbit antibody, diluted 1/1000, was added and incubated for 1 hour at RT. After a final wash, 50 μL of 3,3′, 5,5”-tetramethylbenzidine substrate were added. The plate was incubated for 20 minutes at RT in the dark, then the reaction was stopped by adding 25 μL 1 M hydrochloric acid and the absorbance was read at 450 nm with 750 nm as the reference wavelength on a Wallac Victor2 ELISA plate reader. Adhering to a previously proposed nomenclature[12] we indicated the capture antibody used for NfH quantification (SMI35) in the superscript NfH^{SMI35}.

Statistical analysis

For Gaussian data, we show the mean and standard deviation (SD); for non-Gaussian data, the median and interquartile range (IQR). Two variables were compared by the Kruskal-Wallis tests and for then two variables by general linear models (SAS v9.4m7).

Results

The description of the rats used in the experiment are summarised in Table 1. Of the MOG-immunised rats, 70% developed ON, which was mostly bilateral. The retinas where grouped into: (i) controls who did not have MOG immunisation; (ii) MOG-EAE which did not develop ON as confirmed by VEP and (iii) MOG-EAE which developed ON as confirmed by VEP. Table 2 summarises the severity score for each group and the biomarker

Table 1. Description of animals and number or retinas used for NfH^{SMI35} studies. The mean ± standard deviation and numbers (percentage) are shown.

	Control rats	MOG-EAE rats
Number (animals)	4	7
Gender (F:M)	4:0	7:0
Days from immunisation	n/a	27 ± 1
Severity score	0	1 ± 0.96
ON in OD	0	1
ON in OS	0	0
ON in ODS	0	10
ON (total)	0/6 (0%)	7/10 (70%)
Retinas OD	3 (50%)	8 (57%)
Retinas OS	3 (50%)	6 (43%)
Retina weight (mg)	8.0 ± 2.60	8.6 ± 2.53

F = female.
M = male.
MOG-EAE = myelin-oligodendrocyte-glycoprotein induced experimental autoimmune encephalomyelitis.
OD = right eye.
ODS = both eyes.
ON = optic neuritis.
OS = left eye.

Table 2. Retinal NfH^{SMI35} concentration per µg total protein. The median (interquartile range) are shown.

	Control	MOG-EAE without ON	MOG-EAE with ON
Number	6	3	11
Score	0 (0–0)	0 (0–0)	2 (0–2) *
Total protein [g/L]	2.05 (1.09–2.38)	1.17 (1.13–1.84)	1.76 (1.37–1.93) **
NfH^{SMI35}[mg/L]	2.07 (0.75–9.31)	**1.95 (1.25–2.09)**	**6.78 (5.40–11.54)** ***
NfH^{SMI35} µg/mg per total protein	**0.93 (0.45–4.00)**	1.14 (1.10–1.67)	**4.29 (3.41–5.97)** ****

MOG-EAE = myelin-oligodendrocyte-glycoprotein induced experimental autoimmune encephalomyelitis.
ON = optic neuritis.
* Control vs no-ON p > .05; Control vs ON p > .0021; no-ON vs ON p = .0119.
** Control vs no-ON p > .05; Control vs ON p > .05; no-ON vs ON p > .05.
*** Control vs no-ON p > .05; Control vs ON p > .05; **no-ON vs ON p = .0231.**
**** Control vs no-ON p > .05; **Control vs ON p = .0496**; no-ON vs ON p = .208.

measurements. Total protein was comparable between groups and Nf was elevated in the ON group. Adjusting for the total protein concentration there was a significant, about four-fold increase of NfH^{SMI35} total protein in the MOG-EAE with ON retinas (4.29 µg/mg) compared with the other two groups (0.93 µg/mg in controls and 1.14 µg/mg in MOG-EAE without ON). One MOG-EAE rat developed unilateral ON. In this rat the concentration of NfH^{SMI35} total protein in the eye without ON (left eye) was 1.13 µg/mg (comparable with controls) and in the eye affected by ON (right eye) 5.04 µg/mg.

Discussion

This study demonstrated that there is an increase in Nf proteins in the retina of eyes with VEP proven MOG-EAE-associated ON. Importantly, this increase can be observed not only compared with control rats, but also within the same rat that developed only unilateral ON. The observation of increased retinal Nf tissue levels within an average of 27 days from induction of MOG-EAE associated ON is consistent for inter-group and intra-animal retina comparisons.

The timing of the NfH^{SMI35} increase is consistent with earlier data on retinal Nf expression.[13] After injection of L-[2,3–^{3}H]proline into the vitreous of mice the amino acid was incorporated by the retinal ganglion cell into the Nf isoforms. Retinal Nf levels peaked 9 days later. Over the following 76 days all three Nf isoforms where transported continuously from the retina into the optic nerve. Based on these radio-isotope experiments, we interpret the present data as evidence for intra-retinal up-regulation of Nf after ON. This is then followed by anterograde axonal transport of Nf towards the proximal stump of the degenerating axon. The concept explains the sustained release of Nf into body fluids for about 3 months after a relapse of multiple sclerosis (MS).[2] An accumulation of Nf proteins in the RNFL can also explain the transient thickening of the RNFL observed in some individuals, a finding generally masked by the group-level data of optical coherence tomography (OCT) cohort studies.[3] Axonal transport of Nf proteins towards the optic nerve then explains normalisation of the RNFL over time. In MOG-EAE, 39.1% of lesions affect the optic nerves, tracts and chiasm.[14] It can however take up to 80 days in this model for optic nerve atrophy to develop.[15] This is consistent with the approximately 3 months delay for RNFL atrophy to be reliably quantifiable on OCT.[3]

It is also likely that our finding helps to explain the previously measured transient increase of RNFL thickness in macular hole surgery.[6] Within 1 month after surgery there was a significant increase of the peri-papillary RNFL (pRNFL) from 93.3 µm to 98.7 µm ($p < .05$) before returning to baseline. Similar observations were made following retinal photocoagulation; the average pRNFL increased from 108 µm at baseline to 117.4 µm after 2 month ($p = .006$), to return to near baseline levels 2 months later.[7] Barboni et al. was first to propose that axonal stasis may precede de-compensation of retinal ganglion cells in LHON.[5] Bielschowsky silver impregnation of the retina in MOG-EAE did indeed

show swollen axons with frequent spheroids.[10] This interpretation is consistent with our retinal NfH^{SMI35} data.

A limitation of the present study is that we only quantified one of the three retinal Nf isoforms.[13] Another shortcoming is that we did not investigate the adjacent body fluid and tissue compartments to the retina. Future studies may consider including the vitreous, retina, proximal and distal optic nerve and serial blood samples,[1] ideally, to be combined with retinal OCT[3] with focus on the optic disc where the dynamic development of peripapillary hyperreflective ovoid mass-like structures represent a novel OCT finding, which is of interest for the investigation of axonal stasis.[16] Finally, the presence of conduction block in more severely affected eyes made it impossible to perform correlative analyses between VEP peak latencies or amplitudes and NfH^{SMI35}. Future studies investigating this question will need much larger numbers, and ideally combine biomarkers for axonal degeneration with those for demyelination. Another limitation is that because of the two damaged retinas and the small numbers it was not feasible to statistically correct for inter-eye differences.

The concentration of NfH^{SMI35} total protein in controls (0.93 μg/mg) was marginally lower than what is found in human brain control grey matter (1.15 μg/mg).[17] Likewise, the concentration in the MOG-EAE ON retina of 4.29 NfH^{SMI35} total protein is marginally lower than what is found in the human MS grey matter (5.15 μg/mg; all data are median). This strengthens the argument on similarities between the experimental model and human post-mortem data.[14] Finally, in human MOG antibody disease there were elevated Nf levels in the cerebrospinal fluid and serum of 14 cases with MOG-ON from a deep phenotyped cohort.[18]

In conclusion, we present a biologically plausible concept which helps to integrate structural and biomarker observations in ON.[1–4,18] The proposed sequence of pathology in ON is that, following the inflammatory damage to optic nerve axons, retinal ganglion cells react in a compensatory way, which includes up-regulate expression of Nf proteins. This then leads to a transient increase of the axonal diameter explaining the OCT observation of RNFL thickening. This phase is followed by one of two options: preservation of axonal integrity with normalisation of the RNFL or progression of retrograde axonal degeneration with atrophy of the RNFL. During this latter phase, which lasts for about 3 months, the intra-retinal up-regulation of Nf isoforms combined with anterograde axonal transport sustains the increase of Nf isoforms levels measured in the patients' blood.[1,18]

Author's contributions

RW, TH and AP contributed to the conception and design of the study; RW, TH and AP contributed to the acquisition and analysis of data; AP drafted the text and prepared the Tables. All authors reviewed the manuscript. RW revised the draft and contributed to the final version of the manuscript.

Declaration of interest statement

AP is part of the steering committee of the ANGI and ARI networks which is sponsored by ZEISS, steering committee of the OCTiMS study which is sponsored by Novartis and reports speaker fees from Heidelberg-Engineering. RW and TH have nothing to disclose.

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