Retinal glymphatic system: an explanation for transient retinal layer volume changes?
Axel Petzold

The most extensive exchange of letters in the history of *Brain* (Gelfand et al. 2012). was about a minuscule region of the eye, the retinal inner nuclear layer (INL). With the advent of optical coherence tomography (OCT), a forgotten autopsy observation was restored to the top of the current research agenda in multiple sclerosis (MS) (Gelfand et al. 2012). Severe atrophy of the retinal nerve fibre layer (RNFL) and ganglion cell layer (GCL) was associated with subtle changes in the INL whilst the outer retinal layers were completely preserved (Müller 1857). Whilst very cautious about potential INL changes[1], Heinrich Müller was howevermore convinced about the existence of secondary (retrograde) axonal degeneration. Histological analysis of the macaque monkey eye following lesions to the chiasm and occipital cortex provided experimental evidence for retrograde trans-synaptic degeneration reaching the GCL with associated changes in the INL (van Buren 1963). Lesions of the optic chiasm led to thinning of the RNFL and GCL with the formation of cystic spaces in the INL which "were irregularly rounded and the larger contained fine strands of tissue and debris". Occipital cortex lesions, by contrast, did not affect the INL or any of the outer retinal layers. These INL changes are the likely histological equivalent of what has been called "microcystic macular oedema" (MMO) (Gelfand et al. 2012). I will later return to the debate surrounding MMO in an attempt to explain the next relevant observation, the increase in INL thickness in the absence of obvious MMO. Increased thickening of the pooled outer plexiform and inner nuclear layers was found to be associated with signs of inflammation in MS (Saidha et al. 2012). Together these studies raised the possibility that MMO and INL thickening might be comparable to an MRI "T2 lesion" and could be useful as a surrogate outcome for treatment trials in MS (Petzold 2012). In this issue of *Brain*, Knier *et al.* report that disease-modifying treatment in patients with MS efficiently controlled inflammatory disease activity and was associated with INL volume reduction (Knier et al., 2016).

Recruitment for the present study started right at the time of publication of the two landmark INL papers by Gelfand and Saidha (Saidha et al. 2012). Three years later 121 patients suffering from relapsing-remitting MS and 40 healthy control subjects had been enrolled. Patients were grouped into those who had received no disease-modifying therapy (no DMT, n=36), a first line drug (1st DMT, n=47) or a second line drug (2nd DMT, n=25). Not surprisingly the latter subgroup consisted of patients who had significantly more disease activity based on clinical and radiological criteria.

A frequent criticism of OCT studies is the potential bias caused by inclusion of MSON.About 80%eople of patients with MS will at some time experience an episode of MS-associated optic

1 "Die sogenannten Körner erschienen etwas körnig, doch war mir sehr zweifelhaft, ob dies als pathologische Veränderung anzusprechen sei, da man Aehnliches auch sonst zu Gesicht bekommt." This case was discussed on pages 92-98 and the citation taken from page 93 (Müller 1857). For the contemporary translation it is important to refer back to the terminology of the time. There were three "Körnerschichten" which correspond to the present day "Ganglion cell layer", "Inner nuclear layer" and "Outer nuclear layer". Müller was not able to see the Ganglion cell layer and his comment is on the next nuclear layer, the inner nuclear layer: "The so called nuclei seemed more grainy, but I am rather doubtful if this should be interpreted as a (specific) pathological finding as one can see similar features in other situations."

neuritis (MSON). These patients have more severe damage to the inner retinal layers and more frequently MMO. A frequent criticism of OCT studies is the potential bias caused by inclusion of MSON. In fact, in the present study MMO was only observed in patients with MSON. In total MMO was present in 3/121185 eyespatients (1.6%), which is comparable to previously reported values of 1% (Balk et al. 2012), 4.7% (Gelfand et al. 2012) and 6% (Saidha et al. 2012). Knier *et al*. are therefore to be commended for having excluded patients with MSON prior to enrolment or during the study period before moving on to the exciting statistical analysis of INL volume changes.

Consistent with the literature, there was more severe atrophy in the inner retinal layer in patients with MS compared to controls (see Table 1 in Knier *et al*). In patients, a larger INL volume was predictive of a greater number of new contrast-enhancing and T2 MRI lesions (see Figure 1 in Knier *et al*). Whilst both observations must come as a reassurance, the truly novel finding is shown in the next figure. Even those unfamiliar with OCT will immediately spot that the standardised effect size for one retinal layer stands out, the INL (Figure 2A in Knier *et al.*). Volume reduction of the INL was related to clinical and radiological evidence of reduced inflammatory disease activity. A potential bias owing to the presence of new optic tract T2 lesions was carefully excluded (Figure 2B in Knier *et al*). Even a critical examination of the corresponding tables reveals odds ratios with narrow confidence intervals and highly significant findings for the INL.

Having prepared the ground, the authors present the effect of treatment on individual retinal layers. Particularly striking is the trend shown by the relative changes in three-monthly longitudinal OCT data (Figure 3D in Knier *et al.*). In patients in whom inflammatory disease activity completely ceases, one observes a reduction of the INL volume within 6 months. This trend continues for the entire observation period up to 18 months. In contrast, no changes of the INL volume were observed in those patients in whom inflammatory disease activity could not be controlled. So is INL volume reduction a response marker for successful treatment of inflammation in MS?

At the beginning of this commentary, I wrote that the discovery of MMO and volume changes of the INL triggered possibly the most extensive exchange of letters on a single subject in the history of *Brain (full list of references available from author*). It seems about time to review the key points covered in this heated debate. Credit is due to Matthias Abegg for having pointed out that MMO may be caused by retrograde axonal degeneration as a result of any type of lesion in the anterior optic pathways (Abegg et al 2012). He continued to pursue this subject, proposing the term “retrograde maculopathy”. At the same time my own group showed that MMO was not specific to MS, and suggested that an impaired capacity of Müller cells to maintain retinal fluid homoeostasis might be relevant (Balk et al. 2012). The Müller cell hypothesis enabled Abegg to show that INL volume can be reduced by treatment of MMO with acetazolamide, which improves the water-pumping function of Müller cells (Abegg 2016). But it has also been suggested that MMO results from neither inflammatory nor trans-synaptic degeneration (Barboni et al 2013). Might traction at the vitreoretinal interface also be involved (Petzold 2012, Lujan and Horton 2013)? And why was MMO transient in 84% of cases (Burggraaff et al. 2014)?

In summary, the Knier *et al*. study takes the discovery of MMO and INL volume changes (Gelfand et al. 2012, Saidha et al. 2012) to the next level. But because it is essentially correlative, it further emphasises the need for a comprehensive understanding of the underlying mechanisms in the retina. Taken together the observations imply that dynamic fluid shifts, likely related to inflammation, vascular permeability and Müller cell function need to be considered, as well as non-inflammation related changes such as traction and a retrograde maculopathy. The location of

MMO suggests a close relationship to the retinal vasculature (Balk et al. 2012). Müller cell Müller cellprocesses encompass the capillary plexus of the retina (Figure). Above the INL lies the superficial vascular plexus; below the INL lies the deep capillary plexus. Both are readily visualised by OCT angiography and are connected vertically by small vessels. Understanding fluid trafficking through the retina will now be important to better explain volume changes of the INL: the Figure accompanying this commentary summarises the potential mechanisms involved.

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To conclude, patient numbers in the exciting longitudinal data presented by Knier *et al.* (Figure 3D) were small, but this throws down the gauntlet to everyone who can get their hands on existing MS OCT trial data. Can reduction of INL volume be validated as a biomarker for sustained control of autoimmune central nervous system inflammation by therapeutic interventions? Publication of both positive and negative data will be highly informative for answering this question.

Glossary

Optical Coherence Tomography (OCT): A non-invasive, very fast method which permits construction of an image based on the amount of light reflected (back scattered) from tissue. The axial resolution of current spectral domain OCT devices is 3–7 µm. For comparison the thickness of a retinal ganglion cell is 5–20 µm. OCT therefore permits *in vivo* research on a cellular level.

Trans-synaptic axonal degeneration: A key concept underlying OCT research in MS. Trans-synaptic axonal degeneration explains why it is possible to measure atrophy of inner retinal layers with OCT in patients with MS, with or without MSON. Any lesion in a hard wired pathway, such as the projection from the retinal ganglion cell to the primary visual cortex will result in axonal degeneration. The direction of axonal degeneration is described as anterograde (or Wallerian) if it moves away from its neuron and retrograde if it moves towards its neuron. A key question which remains to be answered is why degeneration moves on to the next, supposedly "healthy" neuron. In a "domino effect" all neurons in the hard wired visual pathway eventually succumb to neurodegeneration, including 1st order neurons in the retinal ganglion cell layer 2nd order neurons in the dorsal lateral geniculate nucleus and 3rd order neurons in the primary visual cortex. In the strict definition of this single pathway model, absent excitatory input from presynaptic cells might be an attractive hypothesis. Alternatively one might think of "nexopathies" and transport of "toxic"/damaged proteins or aggregates. From a therapeutic point of view targeting trans-synaptic axonal degeneration will likely be more promising then trying to cure axonotmesis.

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Figure. Schematic illustration of a retinal glymphatic system which can account for the OCT observations made on MMO and INL volume changes.

(A) The Müller cells are shown in green. Water and potassium channels (black dotted lines) are expressed where the foot plates which form the inner limiting membrane (top horizontal black line) are in contact with the retinal blood vessels. The bipolar cells of the INL (grey box) are shown in purple, cones in red and rods in pink. Horizontal (dark blue) and amacrine (light blue) cells contribute to the neuronal network of the INL. The retinal ganglion cell (yellow) extends its axon into what can be seen as the retinal nerve fibre layer (RNFL) by optical coherence tomography (OCT). Channels for potassium (Kir4.1 and Kir2.1) and water (aquaporin 4) are also scattered, albeit at a much lower density, over the entire Müller cell membrane. The superficial vascular and deep capillary plexus (red circles) localise to the upper and lower borders of the INL. Fluid reaches the retina through the internal limiting membrane and the plexus (blue double arrows). Under normal condition both plexus and Müller cells can absorb the interstitial fluid (black double arrows). **(B)** With inflammation, diffusion of fluid from the retinal blood vessels increases. Possibly disruption of the blood-retina barrier (BRB) would permit additional extravasation of osmotic agents, which would increase the oncotic pressure of fluids in the extracellular space. Activation of resting microglia and B-cell migration may contribute further. Together this would make it more difficult for Müller cells to maintain retinal fluid homoeostasis. A new equilibrium would result with potentially transient tissue expansion. This would be detected by OCT as an increase of INL volume (larger grey shared area). **(C)** The possibility of pathology of Müller cells or the retinal vasculature needs to be considered, too. This could impair the absorption of interstitial fluid and buffering of osmotic agents. **(D)** Mechanisms unrelated to a retinal glymphatic system may also be involved. **(E)** The study by Knier *et al.* placed in context. At baseline patients with MS have evidence of inflammatory activity. The retinal glymphatic system is impaired and consequently the INL volume is increased. Treatment with fingolimod, a strong modulator or Sphingosine-1-phosphate (S1P) receptors, and the multilevel anti-inflammatory effect of interferons reduce the systemic inflammatory response, including in the retina. Reduction of the inflammatory response enables the retinal glymphatic system to recover its function, and consequently the INL volume is reduced.

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