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Inflammatory changes in optic nerve after closed-head repeated traumatic brain injury: Preliminary study

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Abstract

Background: Closed-head, repeated, mild traumatic brain injury (r-mTBI) leads to inflammatory and degenerative changes in the optic nerve of young wild type mice. This work has investigated whether similar changes may be present when the same model is applied to htau mice, a transgenic mouse in which the non-mutated human tau gene is expressed on a null murine tau background.

Methods: This study investigated neuropathological changes in the optic nerve in both young (15 weeks) and old (65–70 weeks) htau mice at 24 hours after r-mTBI or anaesthesia only (r-sham). Change in the level of cellularity, myelin content and astroglial reactivity were evaluated in optic nerve samples.

Results: Increased cellularity and areas of demyelination were clearly detectable in the intracranial portion of the optic nerve in both young (10–15 weeks) and old (65–75) htau r-mTBI mice at 24 hours post-injury, in contrast to r-sham. Increased astroglial reactivity was also observed, together with increased tau phosphorylation.

Conclusions: Localized inflammatory and degenerative response of the intracranial part of the optic nerve was detected in htau mice after r-mTBI. Further studies to clarify the cause and consequences of this phenomenon are warranted.

Keywords

Traumatic brain injury, vision, animal models, closed head injury

Introduction

Previous studies in this lab have found that closed-head, repeated mild traumatic brain injury (r-mTBI) leads to specific and localized inflammatory and degenerative changes in the optic nerve of wild type mice at 10–13 weeks post-injury [1]. These optic nerve changes observed in relatively young (10-week old) mice have the hallmarks of ‘cavernous’ optic nerve degeneration [2,3] and appear to be progressive, as were also observed at an ultrastructural level at a later time point post injury—6 months [4]. Furthermore, similar changes were observed, to a lesser extent, at an earlier time point—3 weeks post-injury [5]. In addition to the neurodegenerative and inflammatory signs in the optic nerve characteristic of the cavernous degeneration process, at all time points tested after injury signs of optic nerve demyelination were also present, indicating localized myelin breakdown, most prominent in the area in and around the site of the ‘cavernous’ degeneration. All these changes were observed in wild type mice.

Tau proteins are intracellular proteins expressed in CNS neurons and astrocytes and provide a stabilization and assembly of microtubules. Although they could be detected in dendrites, their primary neuronal localization is in axons and in oloigodendrocytes and it is believed that they have a role in axonal transport [6,7]. Tau is expressed in the human optic nerve and likely plays a role in some eye diseases, like diabetic retinopathy and glaucoma [8], and also in the visual abnormalities caused by Alzheimer’s disease [9]. Various mouse models expressing different forms of the human tau protein have been developed to study the consequences of its over-expression on mouse development and health. In one of these models, where mice express a mutated human tau (P301S mutation) it has been demonstrated that accumulation of phospho-tau was already present in the nerve fibre layer of retinas from 1 month old mice [10] and abnormal axonal enlargements, which accumulated phospho-tau and disordered filaments and degenerating mitochondria and organelles, were found at 5 months of age. In the same mice, retrograde and anterograde transport axonal transport within the optic nerve was reduced at 3 and 5 months of age, when compared to C57BL/6 control mice [11]. However, presently it is unclear whether mutated tau protein plays any role in modifying the outcome from clinical cases of TBI, as genetic studies in this area are lacking [12].

Another important factor that should be considered when developing animal models of injury that best recapitulate the human scenario is to use appropriate mouse models with the same non-mutated human tau isoform background present in equimolar concentration. In humans, the tau protein has six isoforms, generated by alternative splicing and distinguishable by the
exclusion or inclusion of a repeat region of exon 10, which are referred to as 3-repeat (3R) and 4-repeat (4R) tau. In contrast with humans, most adult mice almost exclusively express isoforms with 4R tau [13]. This model used mice that express all six human tau isoforms on a null murine background. These mice (to be referred to as ‘htau mice’) develop progressively insoluble tau paired helical filaments in neurons, decrease in cortical thickness, cortical neuronal loss (beginning at 8, but more pronounced after 14 months of age), decrease in thickness of corpus callosum and accumulation of phosphorylated tau in the hippocampal regions [14–16].

More recently, it was demonstrated that pS422, a marker of advanced tau hyperphosphorylation, was dramatically elevated (10–25-times) in retinal ganglion cells bodies after repeated TBI in both transgenic mice (expressing a P301S mutated human tau) and htau mice [17] at 10 weeks post-injury. That increase was paralleled by a reduction (30–40%) in retinal ganglion cell density, indicating an ongoing degenerative process in the retina.

Thus, there are indications for possible tau involvement in neurodegenerative processes occurring in the optic nerve after traumatic brain injury, especially in older htau mice. This prompted the authors to look into the pattern of inflammatory changes in the optic nerve of htau mice [14], at an acute time point—24 hours post r-mTBI.

Materials and methods

All procedures were carried out according to the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research, the Association for Assessment and Accreditation of Laboratory Animal Care and were approved by the local Institutional Animal Care and Use Committee. Adult C57BL/6 male htau mice aged 10–15 weeks (n = 11), referred in the text further as ‘young’ htau mice or aged 65–75 weeks (n = 11), referred in the text as ‘old’ htau mice were randomly assigned to either r-mTBI or repetitive sham (r-sham; anaesthesia only) groups. Five consecutive hits with an inter-concussion interval of 48 hours were applied, sham mice received anaesthesia of equal duration to the injured group. Adult C57BL/6 male mice aged 10–15 weeks (n = 5) were obtained from Jackson Laboratories (Bar Harbor, ME) and were used as a reference group for histology (determination of optic nerve cellularity).

Tissue preparation was carried out as described [1]. Paraffin blocks were sectioned at a thickness of 3 μm and mounted on positively charged glass slides (Erie Scientific, Portsmouth, NH). Haematoxylin and eosin (H&E) staining was applied to the optic nerves and eye cross-sections. In addition, Luxol fast blue (LFB) staining with cresyl violet (CV) as a counterstain was applied to the optic nerve cross-sections.

Estimating cellularity in the optic nerve

Cellularity in the optic nerve was estimated in the paraffin-embedded, H&E-stained longitudinal sections of nerves in method similar to one described before [1] with slight modifications. Briefly, optic nerve cell nuclei were counted using ImageJ software by converting to gray-scale images, background subtraction, brightness and contrast adjustment and conversion to binary format with a manual optimization of the threshold and watershed separation of nuclei. The cellularity was then estimated in two ways. First, global cellularity was estimated by generating rectangular areas equivalent in size to a ×40 microscopic field along the entire length of the optic nerve such that they did not overlap but incorporated as much surface area of the optic nerve as possible. Second, the length of each optic nerve was divided into boxes of 1000 micrometres length and the cellularity within this box was determined. Cell density (in cells per square millimetre) was calculated for each area based on cell nuclei count and the area of region of interest.

Immunohistochemistry

Optic nerve specimens were obtained, processed, and mounted on slides as described above. Sections were deparaffinized in xylene and rehydrated in a decreasing gradient of ethanols. Sets of adjacent sections were stained for glial fibrillary acidic protein (GFAP, 1:10 000; Dako, Glostrup, Denmark, ZO334), phospho tau antibody CP13 (pSer-202) (1:500; Gift from Peter Davies). As a negative control, one section was incubated with all reagents except the primary antibody. Tissue sections were subjected to antigen in citrate buffer (Dako, S1699) under pressure. Endogenous peroxidase activity was quenched with a 15 minute H2O2 treatment (3% in water). Each section was rinsed and incubated with the appropriate blocking buffer (ABC Elite kit, MOM kit, Vector Laboratories, CA) for 20 minutes, before applying the appropriate primary antibody overnight at 4°C. Then, the diluted biotinylated secondary antibody from the ABC Elite Kit was applied to the optic nerves and eye cross-sections. In addition, luxol fast blue (LFB) staining with cresyl violet (CV) as a counterstain was applied to the optic nerve cross-sections.

One section from each animal in each group was available for immunohistochemistry and LFB staining and quantitation. Quantitation was done using ImageJ software (US National Institutes of Health, Bethesda, MD). Images were separated into individual colour channels (DAB chromatagen) using the colour deconvolution algorithm [22]. The area within the
optic where GFAP or CP13 immunoreactivity was detected, was calculated and expressed as a percentage of the total optic nerve tissue within the microscope field. For LFB quantitation, images were optically segmented and analysed using CellSens morphometric image analysis software (Olympus, Center Valley, PA). A semiautomated RGB histogram-based protocol (specified in the image analysis programme) was used to determine the optimal segmentation (threshold setting) for LFB staining. Positive staining was discriminated in this manner and used to determine the specific LFB positive pixel percentage area (see Ojo et al. [20]).

Results

Haematoxylin and eosin staining of longitudinal sections of optic nerves from htau mice after injury showed a tendency for the presence of a larger number of nuclei compared to optic nerves after anaesthesia for both young and older mice (Figure 1). Systematic counting of the nuclei along the total length of the optic nerve (including all areas shown in Figures 1(a–d)) confirmed this impression and showed a statistically significant difference between r-sham and r-mTBI animals for young (2290 ± 181 vs 2875 ± 266 cells mm⁻², p < 0.01, Mann-Whitney test) or older mice (1726 ± 232 vs 2938 ± 787 cells mm⁻², p < 0.05, Mann-Whitney test) (Figure 1(f)). Of note, there was no statistically significant difference between the cellularity in young r-sham htau mice vs naïve mice (2290 ± 181 vs 2550 ± 469, p > 0.05, Mann-Whitney test). When cellularity was estimated in a more topographically focused manner by estimating it within 1000 micrometre long segments along the length of the nerve, the segments closest to the chiasm (B1) showed a significantly increased cellularity after injury compared to sham controls for both young mice (2117 ± 298 vs 2906 ± 359 cells mm⁻², p < 0.01, Mann-Whitney test) and older htau mice (1533 ± 340 vs 3453 ± 649 cells mm⁻², p < 0.05, Mann-Whitney test, Figure 1(g)). In younger mice, such a difference was observed also for the third segment furthest from the chiasm (B3) (2271 ± 261 vs 2943 ± 141, p < 0.05, Mann-Whitney test).

In general, optic nerves of older r-sham mice demonstrated lower cellularity compared to younger ones overall (p < 0.05) and also for segments B1 and B2 (p < 0.05), whereas there

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**Figure 1.** Longitudinal cross-sections and estimation of cellularity in mouse optic nerves at 24 hours after repeated mTBI or sham (H&E stain). (a, c) Examples of optic nerves from 15-weeks and 70-weeks old r-sham mice, respectively. (b, d) Examples of optic nerves from 15-weeks and 70-weeks old r-mTBI mice, respectively. Scale bar = 50 μm. All areas presented in (a–c) were part of the quantitation analysis shown in (g) and reflected tissue located in the area closest to the chiasm (Box B1 in (e)). (e) Representative example of the appearance of three areas (each of 1000 μm length) for local estimate of cellularity along the length of the optic nerve, the dashed curve line indicates the approximate location and size of the vertical projection of the contact area on the skull during injury in relation to the horizontal plane of optic nerves and chiasm; scale bar = 1000 μm. Blue arrows indicate the approximate centre of the focal lesions in r-mTBI mice. (f) Average values for optic nerve cellularity for the whole length of the nerve for young (15 weeks) and old (70 weeks) htau and naïve (C57BL/6) mice; the dotted line is placed as a visual anchor for the mean of the cellularity in naïve mice in comparison to the rest of the mean values. (g) Average values within the areas represented in B for young (15 weeks) and old (70 weeks) mice; the dotted line is placed as a visual anchor for the mean of the cellularity in the three areas (B1, B2 & B3) in young r-sham mice in comparison to the rest of the mean values.
was no significant difference between the cellularity after injury, although some tendency was present in older mice after r-mTBI for cellularity to decrease with increased distance from the chiasm. Thus, cellularity in older r-mTBI mice in segment B1 was significantly higher compared to segment B3 (3453 ± 649 vs 2126 ± 836 cells mm$^{-2}$, $p < 0.05$, Mann-Whitney test).

When optic nerve tissue was stained for myelin with Luxol Fast Blue, samples from both younger and older mice after r-mTBI revealed clearly areas of reduced uptake of the stain (Figures 2(b) and (d)) compared to sham control mice (r-sham) (Figures 2(a) and (c)), indicating areas of incipient demyelination. Although in most mice the location of the area of demyelination was medial, as illustrated in Figure 2(b), occasionally the location of the area of demyelination was more lateral as shown in Figure 2(d). It should be emphasized, however, that in all cases there was excellent correspondence between the area of demyelination and the areas of increased cellularity assessed by H&E stain.

Optic nerve tissue also demonstrated increased astrocyte reactivity after r-mTBI compared to r-sham for both younger and older htau mice, as evidenced by more intense GFAP labelling, a sign a classic glial reactivity after injury (Figure 3). Of note, GFAP staining was slightly more pronounced in older r-sham mice compared to young ones (Figure 3(a) vs Figure 3(c)).

Staining with antibody against CP13, which detects tau phosphorylation at serine 202 [14] demonstrated increased immunoreactivity for phosphorylated tau in young r-mTBI mice compared to r-sham. In contrast, similar immunoreactivity was observed in optic nerve tissue of older mice with or without r-mTBI (Figure 4). It should be cautioned that the quantification of the immunohistochemical reactivity in the staining for GFAP and CP13 (Figures 3 and 4) and the LFB intensity (Figure 2), although in agreement with the visual impression, is limited to the samples presented in these figures. Larger sample sizes are needed for a more definitive conclusion about the quantitative differences between the groups, which is part of future plans.

**Discussion**

Several studies have shown an increase in both oligomers and phosphorylated tau after different types of TBI. Thus, Hawkins et al. [23] found oligomeric and phosphorylated tau proteins in rat brain at 4 hours, 24 hours and 2 weeks post-controlled cortical impact (CCI) injury. Similarly, Kondo et al. [24] found an increase in phosphorylated tau in the brains of wild type mice undergoing either a closed-head blunt or blast TBI. They noted that a mild injury resulted in an elevation in phosphorylated tau levels in cortical axons at 48 hours post-injury, but a return to baseline at 2 weeks, while severe TBI or repeated TBI resulted in robust and persistent tau induction.

Although the studies mentioned above indicate that the tau protein and its phosphorylated form increase after certain r-mTBIs in brain tissue of wild type rodents, the results have been varied depending on the severity, paradigm and genotype of the mice [20,25]. Thus, studies from this group did not detect any TBI-dependent tau pathology in wild type mice up to 24 months after r-mTBI (B. Mouzon, unpublished data). Generation of TBI-dependent tau pathology which
Figure 3. Astroglial reactivity of the optic nerve after r-mTBI. Glial fibrillary acidic protein (GFAP) immunostain of longitudinal sections of optic nerves at 24 hours after r-sham for young (a) and old (c) r-sham mice and for young (b) and old (d) r-mTBI mice. There is prominent GFAP immunoreactivity indicating astrogliosis in both (b) and (d). The percentage areas showing GFAP-immunoreactivity determined by image analysis were 15.3, 21.1, 18.2, 32.2 in (a)–(d), respectively. All areas presented correspond to tissue located in the area closest to the chiasm (Box B1 in (e)). Scale bar = 20 μm.

Figure 4. Older htau mice show less increase in phosphorylated tau in optic nerve tissue compared to younger mice after repeated mTBI. Representative longitudinal sections of the optic nerve stained with CP13 at 24 hours after r-sham for young (a) and old (c) mice and for young (b) and old (d) r-mTBI mice. The percentage areas showing CP13-immunoreactivity determined by image analysis were 1.4, 3.4, 2.6, 5.7 in (a)–(d), respectively. The insets represent images of representative areas taken at higher magnification. All areas presented correspond to tissue located in the area closest to the chiasm (Box B1 in (e)). Scale bar = 20 μm.
persists after cessation of injury has been difficult to model [25,26]. For example, Ojo et al. [21] found increased phosphorylation for tau at 24 hours in the cortex of 18-month-old htau mice after r-mTBI, but not any persistent TBI-dependent tau pathology. On the other hand, Brody et al. [26] did not detect any effect of mTBI on tau pathology in either htau or P301S transgenic mice. Similarly, Xu et al. [17] did not find increased tau phosphorylation at 10 weeks post-TBI of young (4-months-old) P301S or htau mice in the cortex.

Previous studies indicated that isoflurane anaesthesia alone does not induce re-organization in microtubules of the optic nerve at an ultrastructural level in htau mice, despite the presence of hyperphosphorylated tau [4]. The present study observed increased immunoreactivity of phosphorylated tau 24 hours after r-mTBI in young htau mice and similar levels of immunoreactivity in older mice whether injured or sham. An increase in phosphorylated tau with age and tau pathology located in the neocortex and hippocampus was demonstrated by the team that generated the mice used in the present study [14] and has been demonstrated in a different transgenic mouse model expressing human tau [27]. The findings are consistent with these observations and indicate similarity between age-related and post-traumatic increase in tau phosphorylation. However, it should be noted that, because of the age-related increase in tau protein, any TBI-dependent tau response in the older mice may be masked.

Indirect evidence about the connection between tau accumulation and myelin degradation and breakdown in vivo comes from studies of transgenic mice expressing human tau exclusively in the oligodendrocytes [28]. Axonal transport impairment in the optic nerves of these mice was evident at 6 months of age, preceding the onset of overt axonal degeneration and accumulation of tau aggregates. Of note, although fast axonal transport was suppressed uniformly throughout the length of the optic nerve, slow axonal transport was suppressed only in the most distal part of the nerve closest to (or at) the chiasm, indicating that this may be part of the tissue which is particularly vulnerable to a disruption in axonal transport as a result of tau over-expression. This part was also found to be most susceptible to damage in a model of a controlled elongation in a guinea pig [29]. Similar findings in terms of importance of tau for the process of myelination in the CNS was reported in studies of the myelin-deficient rat, an animal model of demyelination [30].

Over-expression of wild-type murine tau protein by two-fold in a transgenic mouse model resulted in a progressive increase in hyperphosphorylated tau pathology with age accompanied by gliosis in the brain [31], constant with the observation of more widespread GFAP immunoreactivity in older r-sham mice, although in this case this age-related change was subtle, in contrast to the dramatic increase in astroglial proliferation reported in the over-expressing murine model. Of note, a decrease in normal tau concentration and GFAP immunoreactivity in older r-sham mice decreases with age is a novel one and has been demonstrated in a different transgenic mouse model [32], supporting previous findings of increased vitreous levels of tau in glaucoma patients [33]. Similar results were found recently in a tau rat model of glaucoma [34], underscoring a possible common mechanism between the two conditions. This hypothesis is further supported by radiological findings of reduced optic nerve diameter throughout its length and reduced chiasm dimensions in two major forms of glaucoma: primary open angle glaucoma [35] and low-tension glaucoma [36], indicating that the axonal degeneration extends at least to the chiasm and likely beyond that anatomical location.

The increased cellularity observed in optic nerve tissue in htau mice after r-mTBI in the present study is not surprising and is in accordance with previous findings at a later time points in wild type mice [1,5]. The likely mechanism for that is the formation of a focal lesion of the white matter, a process that has been described before as a result of mTBI, with collapse of myelin sheaths, triggered protracted neuroinflammation and stimulated microglial activation [37]. Similar to TBI, the triad of increased cellularity, astrogial proliferation and demyelination was observed also in optic nerves of DBA/2NNia mice, a mouse model of angle-closure glaucoma [38], indicating a possible common pathogenic mechanism between the two conditions.

The observation that cellularity in the optic nerve in r-sham htau mice decreases with age is a novel one and somewhat surprising, given that studies have demonstrated an increase in the number of astrocytes in rat optic nerve [39], increased number of oligodendrocytes and astrocytes in monkey optic nerve [40] and an increased number of astrocytes in human optic nerve [41] with age. Whether or not this age-related change is limited to this particular mouse strain or it applies to other mouse strains is currently unknown and will be the subject of further studies.

Topographical differences in degree of cellularity in the optic nerve were noted in older, but not in younger mice after r-mTBI. Thus, cellularity was much more pronounced in the area containing the location of ‘cavernous’ degeneration, within 1000 micrometres from the chiasm compared to areas further away from the chiasm (Figure 1). The reason for this novel observation, likely due to age-related changes in the optic nerve tissue, is currently unknown. One possible explanation may be the increased phosphorylated tau reactivity in brain tissue, accompanied by strong microglial activation after r-mTBI reported previously in these mice [21]. This activation may be much more pronounced in the brain tissue and circulation, while present to a much lower extent in the peripheral circulation which supplies the intra-orbital parts of the optic nerve further away from the chiasm. It is unclear why the optic nerve degeneration is localized to such a small area, especially when compared with the larger impact area on the skull. This may be related to the geometry of the propagation of the impact shockwaves throughout the brain, or to an anatomical and biochemical vulnerability of this particular part of the optic nerve, but clearly warrants further investigation.

This pilot study warrants further investigation in a larger number of animals; additionally, cellularity was analysed in individual slices (one per animal) and that provides an estimate of the cellular distribution in only one plane (typically—close to a mid-horizontal section in the centre of the optic nerve). A stereological approach combined with a 3D reconstruction would have provided a more detailed and accurate estimate of the changes in the optic nerve, something that should be a subject of further studies. Finally, tau expression
in the optic nerve determined by immunohistochemistry, by other analytical techniques (e.g. Western blot) or a combination of both would help clarify the contribution of the tau protein to the optic nerve pathology observed in this model.

The present study may have direct clinical implications. The pathological involvement of the optic nerve after TBI was recognized for a long time. Crompton [42] published the first large series of pathological analysis of the optic nerves of 84 patients with closed head injuries (likely moderate-to-severe). He found ischaemic necrosis and shearing lesions of the nerve to be the most common finding in the intracranial portion of the nerve and in the chiasm (in 31 cases) and to be quite frequent in the intracanicular portion of the nerve (30 cases), similar to an earlier observation based on a smaller number of cases (43) by Turner [43]. Crompton concluded that ‘These are probably the lesions producing the bulk of the visual deterioration’ (p. 789). This finding and hypothesis have received surprisingly little attention since their communication. Although it is now widely accepted that diffuse axonal damage is a mechanism underlying the pathogenesis of indirect traumatic optic neuropathy [44], there is still a lack of understanding about the details related to the natural course of the disease and treatment options, especially with regard to the effects of multiple injuries. Additionally, the present findings underscore the similarity between the pathological processes in the optic nerve in glaucoma and after r-mTBI and enhance understating of the mechanism underlying both conditions.

In conclusion, this study has demonstrated an age-related change in topographic distribution of inflammatory changes after r-mTBI along the length of the optic nerve in htau mice, with higher inflammatory activity closer to the chiasm in older mice. This was accompanied by a similar pattern of incipient demyelination in the optic nerve and astroglial proliferation. These findings expand upon previous observations in wild type mice and will allow comparison between different animal models of repeated TBI in future studies.

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Declaration of interest
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