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Visualization of inflammation and demyelination in 2D2 transgenic mice with rodent MRI

Jordan C. Bell^a, Qingwei Liu^a, Yan Gan^a, Qiang Liu^{a,b}, Yaou Liu^b, Fu-Dong Shi^{a,b,*}, and Gregory H. Turner^{a,*}

^aDepartment of Neurology and BNI-ASU Center for Preclinical Imaging, Barrow Neurological Institute, St. Joseph's Hospital and Medical Center, Phoenix, AZ 85013, USA

^bDepartment of Neurology and Tianjin Neurological Institute, Tianjin Medical University, General Hospital, Tianjin 300052, China

Abstract

Research tools are urgently needed to elucidate the specificities of NMO and MS due to their clinical similarity at the early stage of the diseases. Herein, using high-field-strength MRI we characterized the optic nerve and spinal cord lesions in 2D2^{tg} mice (MOG 35-55 specific TCR). Specifically, early Blood-Brain Barrier breakdown was observed in 86% of the 2D2^{tg} mice, while the majority of mice showed little to no brain lesions. Further, immunohistology showed inflammatory infiltrates and demyelination in the brain and spinal cord that mirrored sites of MRI lesions, along with a decrease in AQP4 protein at lesion sites. Collectively, 2D2^{tg} mice develop optic and spinal cord lesions that can be visualized by high-field rodent MRI and verified by pathological assessment. The similarity of these lesions with those seen in NMO patients suggests that the 2D2^{tg} mouse might serve as a model for NMO research.

Keywords

Neuromyelitis Optica; multiple sclerosis; TCR transgenic mice; MRI

1. Introduction

Neuromyelitis Optica (NMO) also known as Devic's disease, is the co-occurrence of optic neuritis and myelitis (Cree et al., 2002). The clinical presentation of NMO is very similar to multiple sclerosis (MS), another neuroinflammatory disease (Sinnecker et al., 2012), and due to their similarities, some NMO patients have been misdiagnosed. Recent research however has begun to identify NMO as its own specific disease (Cree et al., 2002; Kinoshita et al., 2009; Pfueller and Paul, 2011; Wingerchuk and Weinshenker, 2003). NMO has been shown to have inflammatory foci that localize specifically to the optic nerve and spinal cord (Cree et al., 2002; Lucchinetti et al., 2002; Wingerchuk et al., 1999; Wingerchuk and

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*Corresponding authors. Department of Neurology and BNI-ASU Center for Preclinical Imaging, Barrow Neurological Institute, St. Joseph's Hospital and Medical Center, 350 W. Thomas Rd, Phoenix, AZ 85013, United States. Tel: + 1 602 406 2649; + 1 602 406 4094; fax: + 1 602 406 8765., Gregory.turner@DignityHealth.org (G. Turner) and fshi@DignityHealth.org (F.-D. Shi).

Disclosures

The authors declare that they have no conflict of interest.

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Weinshenker, 2003). Unlike in MS (Sinnecker et al., 2012), there are very limited lesions in the brain of NMO. An early and correct diagnosis of NMO is imperative due to the poorer prognosis of the disease in comparison to MS. There is also a different treatment regiment for NMO as compared to MS, so early detection and correct treatments are imperative (Mandler et al., 1993; Wingerchuk et al., 2006; Wingerchuk and Weinshenker, 2005). Recently, serum reactivity that targets aquaporin-4 (AQP4) has been used to distinguish NMO from MS patients (Jarius and Wildemann, 2010). This antibody, AQP4-Ab also known as NMO-IgG, is detectable in 60–90% of patients with NMO and has been used as one of the criteria for diagnosing NMO (Lennon et al., 2005; Papadopoulos and Verkman, 2012). However, the pathology of NMO remains largely unknown, and a precise diagnosis is urgently needed.

Animal models that have distinct features that closely resemble that of NMO are important tools to study the disease. Currently, there have been steps taken to develop NMO animal models that utilize the antibody AQP4 as a way to induce NMO. Some of those emerging models include transferring NMO patient's sera to rats to induce NMO and an *ex vivo* spinal cord slice model. However, there are limitations to these models. The use of spinal cord slices provides little information about the systemic effects of the disease. The NMO patient sera model induces experimental autoimmune encephalomyelitis (EAE) first, and then introduces the patient's IgG, thus the effects of sera alone are lost (Kinoshita et al., 2009; Zhang et al., 2011). However, by using AQP4 as the primary antibody for inducing disease, these models can not address the 10–40% of NMO patients that are AQP4 sera negative.

Kuchroo's group developed a TCR (T cell receptor) transgenic mouse (2D2^{tg}), with the majority of CD4 cells recognizing a myelin antigen myelin oligodendrocyte glycoprotein (MOG) derived from an MS patient who showed isolated optic neuritis in the absence of clinical or histological signs of EAE (Bettelli et al., 2003). The 2D2^{tg} animal model shows promise in understanding the 10–40% of NMO patients that present with classic NMO symptoms, but are AQP4 serum-negative. In light of this, myelin antigens such as MOG are suspected as the immune target. Our current study aims to evaluate the potential of the 2D2^{tg} mice as an efficient animal model of NMO, and particularly focuses on examining longitudinal progression of lesions in the optic nerve and spinal cord using high-field MRI in conjunction with histology.

2. Materials and Methods

2.1. Animals

8–10 week old female mice were grouped into 2 groups. Control Group contained wild type (WT) C57BL/6 mice (B6) $n = 10$, and the Experimental Group contained 2D2^{tg} mice on C57BL/6 background $n = 22$. B6 mice were purchased from Taconic (Germantown, NY) and 2D2^{tg} mice were purchased from The Jackson laboratory (Bar Harbor, ME). Clinical assessment of EAE was performed on 2D2^{tg} mice according to the following criteria: 0, no symptoms; 1, flaccid tail; 2, hindlimb weakness or abnormal gait; 3, complete hindlimb paralysis; 4, complete hindlimb paralysis with forelimb weakness or paralysis; 5, moribund or deceased (Miller et al., 2010). 2D2^{tg} mice without any clinical evidence of EAE (Bettelli et al., 2003) were enrolled in this experiment. After the mice received MRI scans, they were sacrificed for histological staining. For the longitudinal study, five 2D2^{tg} female mice and three B6 female mice were scanned every two weeks, starting at four weeks of age until three months of age. All mice were maintained in specific pathogen-free conditions in accordance with the guidelines prescribed by the Institutional Animal Care and Use Committee (IACUC) at the animal facilities of Barrow Neurological Institute, St. Joseph's Hospital and Medical Center. At the end of the three-month period, the mice were sacrificed for histological staining.

2.2. MRI imaging and analysis

All the scans were performed using a 7T small animal, 30-cm horizontal-bore magnet and BioSpec Advance III spectrometer (Bruker, Billerica, MA) with linear transmitter coil and the surface coil was used for mouse brain images. Each animal was induced and maintained under isoflurane anesthesia, 3 % and 1.5 %, respectively, and delivered with 1.5 L/min of 100 % oxygen. Following intravenous (i.v.) administration through the tail vein of contrast agent Gd-DTPA (gadopentetate dimeglumine brand name Magnevist, Schering AG, Berlin, Germany) with dosage 0.2 mmol/kg bodyweight, T1-weighted MRI was performed at 10 minutes or 40 minutes post-Gd injection. During MRI scan, the animal's respiration was continually monitored by a small animal monitoring and gating system (SA Instruments, Stony Brook, NY) via a pillow sensor positioned under the abdomen. Mice were placed on a heated circulating water blanket (Bruker, Billerica, MA) and the normal body temperature (36–37°C) was maintained.

Axial 2D multi-slice T1 weighted images of brain were acquired with TR = 463ms, TE = 10.58ms, 0.5mm slice thickness without slice gap, field of view (FOV) 2.56cm × 2.56cm, matrix 256 × 256, eight averages, 20 slices, total scan time 16 minutes. In addition, sagittal 2D multi-slice T1 weighted images of spine were acquired, with TR = 320ms, TE = 10.58ms, 0.5mm slice thickness without slice gap, FOV 2.56cm × 2.56cm, matrix 256 × 256, eight averages, 8 slices, total scan time 16 minutes. Acquired images were analyzed using ImageJ (NIH, Bethesda, MD).

2.3. Histology

Mice were lethally anaesthetized by isoflurane and perfused intracardially with 50 mL of cold PBS and fixed in 4% paraformaldehyde (PFA). Brain and spinal cord were removed and postfixed in 4% PFA. The tissues were dehydrated and embedded in paraffin. 8 µm serial sections were then prepared. Serial sections were either stained with Hematoxylin and Eosin (H&E) for morphological changes, particularly the infiltration of lymphocytes, or luxol fast blue (LFB) for the detection of demyelination.

2.4. Immunohistochemistry

For further characterization of astrocytes, 8 µm tissue sections were incubated with chicken anti-GFAP antibodies (1:1000) (Neuromics, Edina, MN) and rabbit anti-AQP4 (1:1000) (Proteintech group, Chicago, IL) in PBS overnight at 4°C following preincubation in 10% normal goat serum (Sigma) to block non-specific binding, and then with secondary antibodies (anti-chicken and anti-rabbit, Invitrogen, CA) for 1 h at room temperature. Finally, all slices were counterstained in Hoechst 33258 (Invitrogen, Eugene, OR) for 10 minutes to stain nuclei, then coverslip-sealed with Fluoromount-G fluorescence mounting medium (SouthernBiotech Birmingham, AL). Images were taken using a confocal microscope (LSM 710; Carl Zeiss, Inc.), assembled using Zen software (Carl Zeiss, Inc.), and analyzed using ImageJ (NIH, Bethesda, MD).

2.5. Statistical Analysis

MRI contrast differences were obtained using ImageJ software (NIH, Bethesda, MD). All statistical data was analyzed using Prism software (GraphPad, LaJolla, CA) using the Student's *t*-test for matched pairs or using 2-way ANOVA. *p* < 0.05 was considered as significant.

3. Results

3.1. 2D2^{tg} mice show disseminated contrast-enhancing lesions, specifically in the optic nerve, optic chiasm, optic tract and spinal cord via MRI

Twenty-two 2D2^{tg} mice aged 8–10 weeks were scanned at either 10 or 40 minutes after Gd-DTPA injection to identify any enhancement in the brain and spinal cord. Vessel signal enhancement was found for both time points indicating the continued presence of contrast agent. MR images from these two time points showed that BBB breakdown appeared in 86% ($n = 19$ of 22) of 2D2^{tg} mice (Fig. 1A–C row 2). The optic nerve (Fig. 1A2), chiasm (Fig. 1B2) and tract (Fig. 1C2) were enhanced in 2D2^{tg} mice compared to WT B6 mice (Fig. 1A–C row 1). This enhancement in the area is identified as the BBB breakdown along the optical nerve tract. The difference in the level of enhancement was quantified using region of interest (ROI) analysis in ImageJ and showed significant differences ($p < 0.01$) in optic nerve enhancement in 2D2^{tg} mice compared to WT B6 controls (Fig. 1D). Because of the importance of spinal cord involvement in NMO, MR images of the spinal cord of WT (Fig. 2A, B) and 2D2^{tg} mice (Fig. 2C, D) were acquired. In the 2D2^{tg} mice enhancement of Gd-DTPA in the spinal cord was observed in half the mice (52%, 11 out of 21). The dissemination of enhancement of Gd-DTPA only affected the areas along the optical nerve tract and spinal cord and never produced lesions in the cortex of the brain of the 2D2^{tg} mice. This is similar to what is seen in classic cases of NMO; more posterior parts of the optic nerve are enhanced during an MRI scan in mainly the optic chiasm and optic tract (Khanna et al., 2012).

3.2. 2D2^{tg} mice show progression of lesions starting as early as 6 weeks of age

MRI technology gives us the advantage of tracking progression of disease longitudinally to facilitate the understanding of how the disease advances. To identify lesion development in the brain and spinal cord in the 2D2^{tg} mice, BBB compromise was assessed by the presence of Gd-DTPA post systemic contrast agent application. We observed five 2D2^{tg} mice from four weeks of age up to three months of age and were able to track the initiation and progression of MRI enhancement, which correlates with sites of inflammation and BBB breakdown in the optic nerve and the spinal cord. The spinal cord began to swell in the 2D2^{tg} mice as the mice aged (Fig. 3) along with increase in enhancement around the optic chiasm (Fig. 4).

3.3 2D2^{tg} mice spontaneously acquire inflammatory infiltrates in the optic nerve and spinal cord

To investigate the enhancement seen in the 2D2^{tg} mice MRI scans, we compared tissue sections from WT B6 mice and 2D2^{tg} mice. On histological examination, the presence of cellular infiltrates was seen in the ventricular areas in 86% of 2D2^{tg} mice that showed contrast enhancement in the MRI. The use of H&E staining allowed us to identify more immune cell infiltrates in the 2D2^{tg} mice brain ventricles (Fig. 5B and D) that were not present in the B6 control mice (Fig. 5A and C). The spinal cord of the 2D2^{tg} mice also showed increased presence of inflammatory cell infiltrates (Fig. 5F), while there was little to no inflammation seen in the control spinal cords (Fig. 5E). The increased inflammatory mediators and the enhancement seen after Gd-DTPA on MRI scans prompted us to evaluate the degree of demyelination in the brain and spinal cord. Consistent with the observed pattern of inflammation, the brain (Fig. 5D) and spinal cord (Fig. 5H) from 2D2^{tg} mice had areas of demyelination detected by LFB staining, whereas no demyelination occurred in B6 control mice brain (Fig. 5C) or spinal cord (Fig. 5G). The 2D2^{tg} mice that had lesions showed localized demyelination in the spinal cord that was mainly located in the outer white matter of the ventral spinal cord (spino-cerebellar tracts) and evidenced by a reduced density of myelinated fibers in the animals that had active lesion areas.

We also preformed immunohistochemical staining to analyze the protein expression of AQP4 in the spinal cords of 2D2^{tg} mice in the presence or absence of active lesion sites (Fig. 6). AQP4 expression was dependent upon the level of cellular infiltration at the site of the lesion. We observed a marked increase in protein levels around the edges of the spinal cord where the infiltration was occurring, and the staining with anti-AQP4 antibody was co-localized with anti-GFAP antibody.

3.4. MRI reveals histopathological changes

To determine the sensitivity of the MRI scans we correlated the MR images with histopathological findings (Fig. 7). We found that Gd-DTPA enhanced MRI was able to show areas of compromise. The enhancement seen in the optic nerve and chiasm indicates the compromised optic nerve. We see areas of demyelination in the optic nerve prior to its entrance into the brain cortex. Also, the histology revealed inflammatory infiltrates in the ventricle area that matches the marked enhancement seen in the MRI scans of the 2D2^{tg} mice. However, we did not observe any lesion sites in the cortex of 2D2^{tg} mice with post-Gd T1-weighted imaging.

4. Discussion

We demonstrated the longitudinal development of NMO-like lesions in the 2D2^{tg} mouse model using MRI. We were able to confirm the presence of optic neuritis and spinal cord lesions in these mice as a way to elucidate the 2D2^{tg} mouse potential as a model system for the biological studies of NMO. We were also able to show that histological evidence matches the findings of the MRI. This is the first study to report longitudinal MRI based characterization of 2D2^{tg} mice for their potential use as a model for NMO.

MRI has been demonstrated to be important in diagnosing not only MS, but also NMO. A longitudinal MRI scanning of a NMO patient showed that lesions develop in the optic chiasm first and eventually spread into the spinal cord while showing no signs of brain lesions (Fazekas et al., 1994). This finding was also seen in other reports that followed the original paper (Filippi et al., 1999; Khanna et al., 2012; Pfueller and Paul, 2011). A unique component of NMO diagnosis is the involvement of the spinal cord. It has been reported that the spinal cord when seen in MRI is enlarged in 90% of NMO patients (K yat-Atamer et al., 2012; Pawate and Sriram, 2009; Wingerchuk et al., 2006). A typical MS spinal cord lesion is usually confined to one vertebrae while in NMO the spinal cord is compromised in three or more vertebrae (Cree et al., 2002; Filippi et al., 1999; Pau et al., 2011; Tashiro et al., 1987; Wingerchuk and Weinshenker, 2003; Wingerchuk and Weinshenker, 2005). We have been able to show in our current study the involvement of the optic nerve in disease development as early as six weeks of age. In our longitudinal study we also observed that the spinal cord enlarged over more than one vertebra and the optical nerve was compromised as the 2D2^{tg} mice aged. The MRI scans did not show MS-like brain lesions in 2D2^{tg} mice, usually seen as enhanced areas in post-contrast images of the cortex. We did observe BBB compromise evidenced by the enhancement in the ventricles of the mice. These findings help to further emphasize the relevance of the 2D2^{tg} mouse model as a biological tool and the significance of MRI for future studies of NMO.

In order to further confirm the usefulness of the 2D2^{tg} mouse as a model system for NMO biological studies, histological studies have been used to demonstrate localized inflammatory lesions in these 2D2^{tg} mice. The localization of the inflammatory lesions were found in the spinal cord and optic nerve, which is similar to what was previously reported (Bettelli et al., 2006). We also observed inflammatory infiltrates in the ventricle areas of the 2D2^{tg} mice. The matching between compromised areas in MRI scans with lesions elucidated in histopathological slides shows the sensitivity and power of MRI technology in

neurological disease research in small animals. The infiltration of lymphocytes and monocytes at the sites of lesions in the 2D2^{tg} mice also mimics what has been observed in NMO patients. The optic neuritis and spinal cord lesion and inflammatory infiltration with the lack of clear brain lesions are characteristics found in NMO that help to show the usefulness of this mouse model as a relevant and potentially useful tool for understanding the etiology or mechanisms of NMO disease.

The antibody AQP4-Ab or NMO-IgG was positive in 60–90% of patients diagnosed with NMO (Jarius and Wildemann, 2010; Pfueller and Paul, 2011). The discovery of this antibody in NMO patients has been significant in defining the correct treatment course for those who may have otherwise been diagnosed with MS. In light of this, a few animal models have been created to understand how this antibody plays a role in NMO progression. Zhang et al. have created an *ex vivo* model using spinal cord that is able to identify effector actions under defined conditions and allows for rapid screening of effectors of NMO lesion development (Zhang et al., 2011). The authors note, however, that there are limitations to their model, mainly that an *in vitro* slice model cannot reproduce some of the potential determinants of neuroinflammation. Another animal model being chosen to look more closely at the effects of the AQP4-Ab is the passive transfer model created by Kinoshita et al. (Kinoshita et al., 2009). The mice were initially induced EAE and then subsequently injected with IgG from NMO patients to see if the hallmarks of NMO were present. This model is the first to show *in vivo* the pathogenicity of AQP4-Ab (Kinoshita et al., 2009). Both models use AQP4-Ab as the source of pathogenesis, however, there are 10–40 % of NMO patients that are AQP4-Ab serum negative may have the involvement of a myelin derived antigen as a factor of their disease state. Hence, the 2D2^{tg} mouse model as a research tool shows significant promise for the serum negative population. It has been shown that loss of AQP4 expression is present at the site of lesions in NMO patients (Kinoshita et al., 2009; Misu et al., 2007; Roemer et al., 2007). Our study showed that the AQP4 was increased around the surrounding area of lesions; however, there was decreased expression throughout the sites of lesions in the 2D2^{tg} mouse model. Misu et al. 2009 reported that NMO lesions have a decrease in AQP4 immunoreactivity while MS plaques do not present a loss of reactivity (Misu et al., 2007). We speculate this aspect of the 2D2^{tg} mouse model needs further evaluation, including a serum evaluation for the presence or absence of the AQP4-Ab, or the ability of the activated T-cells in these mice to react to the AQP4.

In conclusion in this study, high-resolution MRI enhanced by Gd-DTPA identified early subclinical disease activity in the 2D2^{tg} mice that was mainly localized to the optical tract and spinal cord which was then confirmed by utilizing histological techniques. Our data further supports the usefulness of Kuchroo's group's 2D2^{tg} mice as a valuable model to determine the factors responsible for the involvement of optic nerve and spinal cord. This report is the first to use MRI to observe the progression of disease longitudinally and to identify sites of lesions in the 2D2^{tg} mice that correlate with what is seen in NMO patients. The ability of the 2D2^{tg} mice to reproduce many of the key pathological features of NMO provides the opportunity to address questions that are specific to NMO that may be reactive due to a myelin based antigen.

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Highlights

- High resolution MRI can be used to identify subclinical neuroinflammatory lesions in 2D2^{tg} mice.
- High resolution MRI can track the progression of disease longitudinally in 2D2^{tg} mice.
- 2D2^{tg} mice produce lesions in optic nerve tract and spinal cord that are similar to NMO.
- 2D2^{tg} mouse is a valuable tool to study NMO disease.

Fig. 1.

Gadopentate dimeglumine (Gd-DTPA) MRI Enhancement of the optic nerve in 2D2^{tg} mice. 8 week old female 2D2^{tg} mice with no clinical signs of EAE were scanned and corresponding coronal T1-weighted images of these mice (A–C row 2) vs. WT B6 mice (A–C row 1) are shown for comparison. Leakage of Gd-DTPA into the cerebrospinal fluid is visible in 2D2^{tg} mice (arrowheads). Bright contrast enhancement (arrows) after Gd-DTPA application in coronal sections shows the lesions in the optic nerve (A2), the optic chiasm (B2) and the optic tract (C2) in the 2D2^{tg} mice. Gd-DTPA contrast observed in the optic nerve was quantified and normalized using region of interest (ROI) in ImageJ software (D). Enhancement was seen in 86% of 2D2^{tg} mice and no brain lesions were seen in these mice we scanned. Line diagram of coronal level of MR images A–C is illustrated (E). 2D2^{tg}, $n = 22$; WT B6, $n = 10$. * $p < 0.01$. Scale bar, 1.0 mm.

Fig. 2. Gd-DTPA MRI Enhancement of spinal cord lesions in 2D2^{tg} mice. Sagittal (**A**, **C**) and axial (**B**, **D**) T1-weighted images are presented post Gd-DTPA application. 2D2^{tg} mice frequently show lesions where Gd-DTPA accumulated along the spinal cord (**C** and **D**, arrows), whereas WT B6 control mice didn't show lesions (**A** and **B**). Gd-enhancement in **B** and **D** was quantified using ROI in ImageJ software and normalized (**E**). Enhancement of the spinal cord was observed in 52% of the 2D2^{tg} mice. 2D2^{tg}, $n = 21$; WT B6, $n = 10$. Scale bar: **A** and **C** 1.0 mm; **B** and **D** 0.2 mm.

Fig. 3. Gd-DTPA MRI Enhancement visualized spinal cord swelling that progressed at 6–12 weeks of age in 2D2^{tg} mice. Time course study of 2D2^{tg} mice revealed spinal cord swelling that increased as the mice aged. Representative images from a 2D2^{tg} mouse with swelling spinal cord are shown (red arrows). Scale bar, 1.0 mm.

Fig. 4.

Gd-DTPA MRI Enhancement visualized optic chiasm enhancement that progressed at 4–14 weeks of age in 2D2^{lg} mice. Time course study of 2D2^{lg} mice revealed optic chiasm enhancement (arrows) that increased as the mice aged. The optic chiasm remained enhanced at 8 weeks of age and older. Representative images from a single 2D2^{lg} mouse are shown (A–F). Region of interest (red circles) calculating enhancement after Gd-DTPA were quantified using ImageJ. The statistic analysis of signal quantification from 2D2^{lg} mice ($n = 5$) is presented (G). Scale bar, 1.0 mm.

Fig. 5.

Inflammatory cell infiltration and demyelination are visible in 2D2^{tg} mice. Serial brain coronal sections and spinal cord axial sections from WT B6 control mice and 2D2^{tg} mice were stained with H&E (**A**, **B**, **E**, **F**) to show inflammation and Luxol Fast Blue (**C**, **D**, **G**, **H**) to show demyelination. Histopathology shows acute inflammatory cell infiltrates in the ventricular space of the 2D2^{tg} mice (**B** and **D**) that is not present in B6 control mice (**A** and **C**). There was also significant demyelination around the ventricular area of the 2D2^{tg} mice (**D**) that was also not seen in the B6 control mice (**C**). The spinal cords of the 2D2^{tg} mice are also inflamed with the presence of infiltrates (**F**) and showed signs of demyelination (**H**), both of which are not seen in control mice (**E** and **G**, respectively). All slides are 8 μ m thick. **A**, **B**, **E**, and **F** are at 4 $\times$ magnification, while **C**, **D**, **G**, and **H** are at 10 $\times$.

Fig. 6.

Aquaporin-4 protein expression in 2D2^{tg} mice is reduced at sites of lesions. The expression of Aquaporin-4 protein was compared between non lesions sites (**A**) and 2D2^{tg} mice with lesions (**B**, **C**). The infiltration of inflammatory cells caused a reduction in AQP4 and GFAP and this reduction was dependent on the level of inflammation seen at the sight of the lesion. Scale bar, 50 μ m. Green: AQP4 positive. Red: GFAP positive astrocytes. Blue: Hoechst 33258 stained cell nuclei.

Fig. 7. Correspondence between MRI and Histological images. Contrast enhancement is shown in the ventricles indicated by small yellow squares in a coronal T1-weighted image (**A**). The same regions at higher magnifications of H&E staining are given (**A**, large yellow squares). The staining revealed sites of inflammation in the area along with blood clot areas. The lesions present in the optical nerve area are shown by H&E (**B**) and Luxol Fast Blue staining (**C**). Inflammatory cell infiltrates in H&E and demyelination areas (yellow outline) along the optic nerve with Luxol Fast Blue were detected at the same localization. Sections, 8 μm . **A** inserts and **B**, **C** are 10 $\times$ magnification.