UMKC Health Sciences Student Research Summit

Intracellular Calcium Channel Expression in Autoimmune Encephalomyelitis

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INTRODUCTION

- Multiple sclerosis (MS) involves progressive auto-immune mediated demyelination of the central nervous system.[1]
- MS was modeled in rats through induction of experimental autoimmune encephalomyelitis (EAE) by generating an autoimmune response against myelin sheath.[2,3]
- Alterations to intracellular calcium channels called inositol 1,4,5-trisphosphate receptors (IP_3Rs) may contribute to the pathogenesis of EAE and MS.[4]

We hypothesize that IP_3R expression and function are altered during EAE, optic neuritis and MS development resulting in calcium signaling dysfunction and potential damage to neuronal axons, representing potential novel targets for pharmaceutical treatment of MS.

METHODS

EAE Induction: Female Dark Agouti rats were injected with Freund's complete adjuvant ± recombinant myelin oligodendrocyte glycoprotein (MOG; amino acids 1-125) protein, on experimental day (ED) 0. Tissue was harvested on ED16. EAE was scored on ED 0-16 (See Figure 1; CLM and HMJ).

Immunoblotting: Spinal cords segments C1 to L1 were homogenized in 4% paraformaldehyde/phosphate buffered saline (PBS) containing protease/phosphatase inhibitors, and solubilized in RIPA buffer (HMJ). Proteins were separated on a 10% acrylamide gel, transferred to nitrocellulose membrane, incubated with primary antibodies (see Table 1) and subsequently secondary antibodies. Proteins were visualized using the SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA, USA) and imaged on a G:BOX imaging system (SYNGENE, Frederick, MD, USA).

Immunohistochemistry: Spinal cords segments L1 and S2 were fixed in PBS and cryo-protected in sucrose/PBS before snap-freezing in isopentane/liquid nitrogen for cryo-sectioning on to gelatin-coated slides (HMJ). Sections were incubated with primary antibodies (see Table 1) and subsequently secondary antibodies. Tissue sections were imaged on a Nikon C2+ (Nikon Instruments Inc., Melville, NY, USA) confocal imaging system.

Statistical analysis: Fiji image analysis software[5] was used to quantify protein levels from immunoblot images. Confocal laser scanning microscopy, fluorimetric analysis of immunoreactivity and cell counts were used for statistical analyses (Student's t-test; average of 10 regions of interest with a 62,500µm² area each). Data were plotted as mean values ± standard error of the mean and statistically analyzed using Student's t-test, unless otherwise stated.

Epitopes of primary antibodies	Marker	Company	Catalogue #	Lot #	IHC dilution	IB Dilution
Myelin Basic Protein (MBP)	Myelin/oligodendrocytes	Covance	SMI-94R	E10172EF	1:150	-
Neurofilament (NF-L)	Axon marker	Chemicon	AB1983	24110225	1:500	-
Glial Fibrillary Acidic Protein (GFAP)	Astrocytes	Abcam	ab4674	GR267558-1	1:1000	-
Neuronal Nuclei (NeuN)	Neuronal bodies	Millipore	MAB377	LV1519148	1:500	-
Glyceraldehyde 3-phosphate dehydrogenase (GADH)	Loading control	Santa Cruz	sc-25778	D1014	-	1:5000
Inositol triphosphate receptor 1 (IP3R1)	IP3R1	Pierce	PA3-901	OH189867	1:200	1:500
Inositol triphosphate receptor (IP3R2)	IP3R2	Novus	NB100-2466	P1	-	1:250
Inositol triphosphate receptor 3 (IP3R3)	IP3R3	BD	610312	67600	-	1:1000

Table 1: Antibodies used in immunoblotting and immunohistochemistry

RESULTS

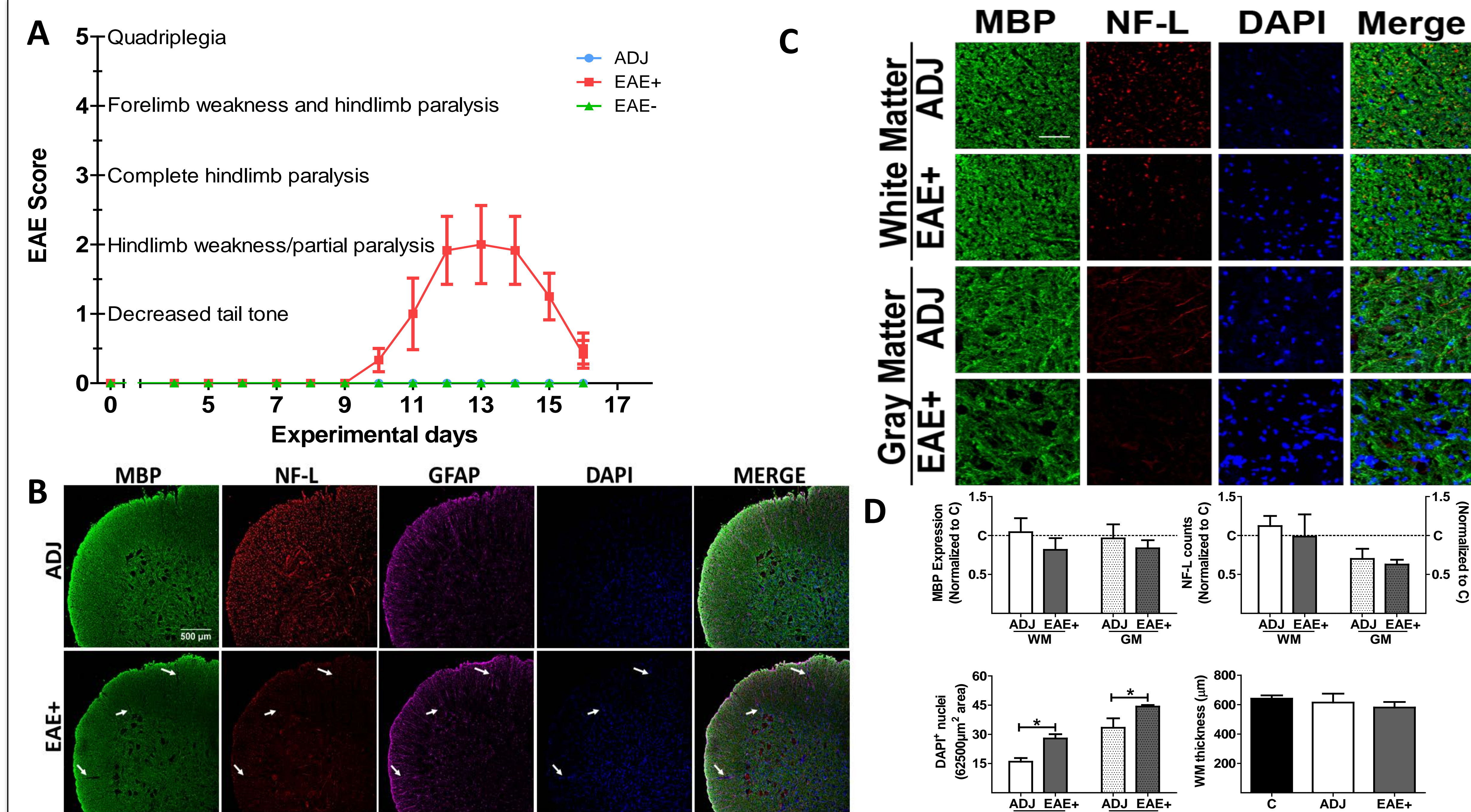

Figure 1. EAE was induced in Female Dark Agouti rats. A) Rats were scored daily for clinical signs of EAE induction. Groups were adjuvant-treated controls (ADJ), six rats developed EAE (EAE+), while the remaining two animals showed no deficits over the experimental observation period indicative of a lack of disease induction (EAE-). B) Oligodendrocytes and myelination (MBP immunoreactivity; green), neuronal cell bodies and axons (NF-L immunoreactivity; red), astrocytes (GFAP immunoreactivity; pink) nuclei (DAPI histochemical stain; blue) were labelled in the lumbar region of the spinal cord of ADJ and EAE+ animals. DAPI labelling shows increased nuclei infiltration throughout the spinal cord, and in particularly high concentrations at small demyelinated lesions subsequent to EAE-induction (white arrows). C) and D) A trend toward decreased levels of MBP immunoreactivity and significant nuclei infiltration was measured in EAE+ compared to ADJ animals. D) WM thickness, axons of spinal cord neurons (counts of NF-L-positive profiles, left Y-axis in the top right diagram) and their somata (NF-L immunoreactivity levels, right Y-axis) were not significantly reduced in EAE+ rats compared to ADJ. Data were normalized to immunoreactivity levels of untreated controls (C). (*, p<0.05, n=3-4 rats).

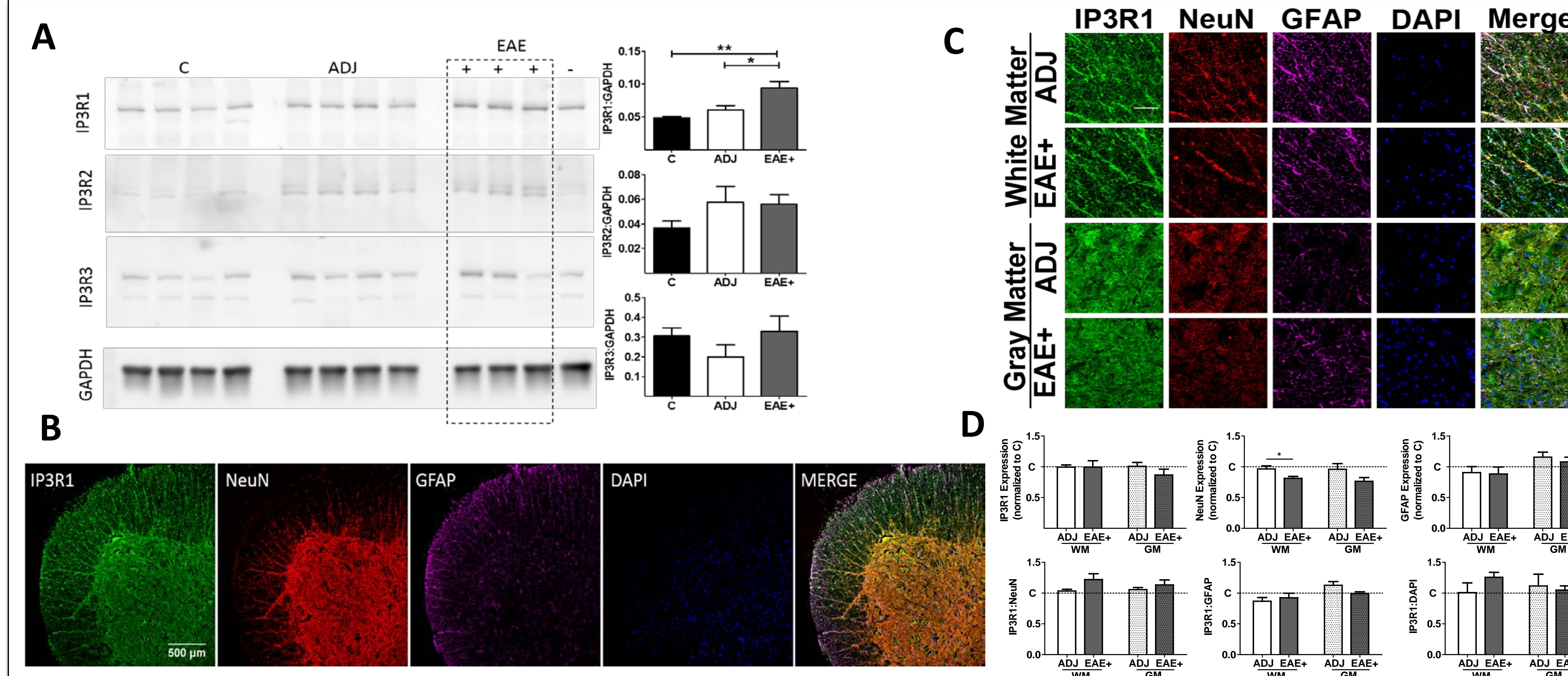

Figure 2. IP_3R1 immunoreactivity levels are increased in spinal cord tissue after EAE induction. A) Immunoblot showing IP_3R (types 1-3) immunoreactivity levels in the spinal cord of untreated (C) adjuvant-treated (ADJ), six rats developed EAE (EAE+), while the remaining two animals did not (EAE-). Expression levels were normalized to GAPDH immunoreactivity levels as a loading control. Each lane represented an individual animal. Data were statistically analyzed using one-way ANOVA with Bonferroni correction for multiple comparisons (*, p<0.05, **, p<0.01). B) IP3R type 1 immunoreactivity was found in neurons (NeuN-immunoreactive cells) and astrocytes (GFAP-immunoreactive cells) in the lumbar region of the spinal cord. C) and D) A trend towards increased IP_3R type 1 immunoreactivity was determined in spinal cord neurons of EAE+ rats when compared to ADJ controls. Data were normalized to immunoreactivity levels of untreated controls. (*, p<0.05, n=3-4 rats).

SUMMARY

- EAE was successfully induced in 75% of rats injected with adjuvant and recombinant MOG_{1-125} protein with an average clinical EAE score of 2, a significant immune cell infiltration in the white and gray matter of the lumbar spinal cord and small unmyelinated lesions containing high numbers of infiltrating immune cells (Figures 1).
- NeuN labelling was significantly decreased in the white and gray matter of the lumbar spinal cord of EAE-induced animals indicating neuronal degeneration and potentially nerve cell loss (Figure 2).
- Concomitant with neuronal degeneration and potentially nerve cell loss, IP_3R type 1 immunoreactivity was increased in somata located in the GM of the spinal cord (Figures 2).

CONCLUSION

An increase in intracellular calcium channel density could contribute to apoptosis of spinal cord neurons and axon damage during disease development due to the resulting dysregulation of calcium signaling and calcium toxicity.

FUTURE DIRECTIONS

As calcium dysregulation appears to play a role in the disease development and progression of multiple sclerosis, the study of intracellular calcium channels and their associated signaling proteins in addition to the ones reported here, appears indicated as a means to determine novel targets for drug development in this field and in optic neuritis.

REFERENCES

[1]Lassmann, H. Mechanisms of white matter damage in multiple sclerosis. Glia 62, 1816-1830 (2014).

[2]Rao, P. & Segal, B.M. Experimental Autoimmune Encephalomyelitis, in Autoimmunity: Methods and Protocols. (ed. A. Perl) 363-375 (Humana Press, Totowa, NJ; 2004).

[3]Kanellopoulos, J. Experimental autoimmune encephalomyelitis. Biomed J 38, 181-182 (2015).

[4]Toldi, G. et al. Lymphocyte calcium influx kinetics in multiple sclerosis treated without or with interferon beta. Journal of neuroimmunology 237, 80-86 (2011).

[5]Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9, 676-682 (2012).