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Treatment with relaxin reduces disease symptoms and enhances neuroprotection and remyelination in murine experimental autoimmune encephalomyelitis

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Abstract

The use of glucocorticoid agonists in treating acute attacks of multiple sclerosis is well established. Relaxin, a member of the insulin super family is a pleiotropic hormone capable of influencing multiple pathways which include the glucocorticoid receptor and relaxin family peptide receptors 1 and 2. In addition to the action of relaxin on the glucocorticoid receptor, activation of the relaxin receptors have additional anti-inflammatory and immuno-modulating effects. In the present study we investigated the effectiveness of relaxin in treating a murine model of MS, experimental allergic encephalomyelitis. Disease was induced and the mice were scored daily for clinical signs of disease (0=normal, 3=hind limb paralysis, 5=found dead). When a clinical score of 3 or higher was reached, relaxin was continuously infused for 8 days. Plasma for RT-PCR and spinal cords for histology were collected. The levels of CCR2, CCR5, CCR7, interleukins-6 and 17 were analyzed using quantitect primers and SYBR green based RT-PCR kits. Spinal cords were formalin fixed, paraffin embedded, sectioned and scored for myelin content, macrophage infiltration, neurofilaments, gliosis and markers of remyelination. The results of the study show that continuous infusion of relaxin significantly reduced the clinical signs of disease, decreased mRNA expression of pro-inflammatory cytokines and chemokine receptors. Histological staining and immune-histochemistry of the spinal cords showed that relaxin treatment lead to a decrease in lesion load and size and macrophage infiltration, preserved myelin and neurofilaments, reduced gliosis and promoted remyelination.

Key words

Multiple sclerosis, EAE, remyelination, neuroprotection, relaxin, gliosis.

Abbreviations:

CCR	chemokine receptor
CD68	anti-cluster of differentiation 68
CNS	central nervous system
EAE	experimental allergic encyphalomyelitis
ER	estrogen receptors
ETOH	ethanol
FFPE	formalin fixed and paraffin embed

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GCR	gluccocorticoid receptor
GFAP	glial fibrillary acidic protein
IL	interleukin
MS	multiple Sclerosis
PPARγ	peroxisome proliferator-activated receptor gamma
PTX	pertussis toxin
RT	Room temperature
rRLN	relaxin
RXFP	relaxin family receptor
TBST	Tris buffered saline with Tween 20

Introduction

Multiple Sclerosis (MS) is an inflammatory, auto-immune, neurodegenerative disease of the central nervous system (CNS). It appears to be largely driven by inflammation (Frischer et al, 2009) and auto-reactive T- (Fletcher et al, 2010) and B-lymphocytes (Li et al, 2015). Acute relapses continue to be a major factor in disease progression and patient disability. Current standard of care is to treat relapses with intravenous or oral steroids. Although effective, these treatments produce significant side effects that reduce compliance by up to 31% (Nickerson et al, 2015). Underlying the need for effective and well tolerated therapeutics to increase compliance in treating relapses. This has prompted our investigation of the anti-inflammatory immune-modifying pregnancy hormone relaxin (RLN) as a novel pathway in the treatment of relapses.

A spontaneous remission of symptoms in the later stages of pregnancy has been well characterized in subjects with MS, (Lorenzi and Ford, 2002). These remissions are thought to be due to the production of pregnancy hormones, in particular estrogen (Zhu et al, 2007). In other studies, estrogen has shown positive results when given after onset of experimental allergic encyphalomyelitis (EAE, Jansson et al, 1994) and in MS (Gold and Voskuhl, 2009). While these results support a role for estrogen in disease remission during pregnancy in MS, it does not preclude a role for other pregnancy factors in the spontaneous remission of disease symptoms.

One such pregnancy factor is RLN, a member of the relaxin peptide superfamily (Bathgate et al, 2013) which is also up regulated in the later stages of pregnancy (Goldsmith and Voskuhl, 2009). RLN is pleiotropic in nature, acting as an agonist for the glucocorticoid receptor (GCR, Dschietzig et al, 2004) and the relaxin family peptide receptors 1 and 2 (RXFP1 and RXFP2, Bathgate et al, 2013). Through these receptors RLN can modulate the immune system by suppressing cell adhesion molecules (Bani et al, 2003), increasing cyclic adenosine monophosphate (Nguyen and Dessauer, 2005), regulating differential expression of matrix metalloproteinases (Mu et al, 2010), regulating cytokine and chemokine receptor (CCR) expression (Bathgate 2013) and inhibit cell-mediated pro-inflammatory activity by stimulation of the peroxisome proliferator-activated receptor gamma (PPARγ; Singh and Bennet, 2010) which has neuroprotective effects (Gray et al, 2012). Through these diverse actions, RLN could have a potential to modulate the clinical symptoms of MS, be neuroprotective and promote remyelination.

To test this hypothesis, treatment with a recombinant form of porcine relaxin (rRLN, SKY BioHealth, Inc., Eden Prairie, MN) was investigated in a murine model of MS. Specifically, the C57BL/6 (H-2b) murine model immunized with $MOG_{35–55}$ to develop EAE. This model presents as an immune mediated chronic progressive and demyelinating form of the disease (Robinson et al, 2014). This model allows to study the response to treatment of the inflammatory spinal cord response and assess markers of neuro-protection and remyelination.

Materials and methods

Animals and treatment

Active inflammatory/demyelinating EAE was induced in Female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) with a peptide fragment of myelin oligodendrocyte glycoprotein ($MOG_{35–55}$) as previously described (Lyons et al, 1999; Miller et al, 2007; McCarthy et al, 2012). Lyophilized $MOG_{35–55}$ was dissolved in phosphate buffered saline (PBS) pH 7.0 at a concentration of 4 mg/ml. One milliliter of $MOG_{35–55}$/PBS was added to 1 ml of complete Freund's adjuvant containing 4 mg/ml heat killed desiccated *M. tuberculosis*, H37 RA. This was then mixed to form a thick emulsion, loaded into a 1 ml syringe with a 27 gauge needle.

The mice were immunized with 100 μl of emulsion injected subcutaneously in 3 sites on the back. To induce EAE in the C57BL/6 strain with $MOG_{35–55}$, the animals were primed with pertussis toxin (PTX) in PBS. This was administered intraperitoneally at the time of immunization, on day 0 (75 ng) and day 2 (200 ng). Clinical EAE scoring was done according to the recommendations of the institutional animal care and use committee. Scoring was done on a scale of 0-5 as described in Table 1 (0 = normal, 1 = tail paralysis, 2 = tail paralysis and strong hind limb weakness, 3 = complete hind limb paralysis, 4 = hind limb and forelimb paralysis, 5 = found dead). Treatment was commenced when the animals reached a clinical score of 2. Mean onset of clinical score 2 was 14 days post-immunization.

Subcutaneous osmotic pumps (Alzet, Cupertino, CA) were inserted and the animals were randomly assigned to receive either recombinant porcine relaxin (n=8) or placebo (PBS, n=8) at a rate of 0.11 μl/hr. The rRLN was brought to a concentration of 0.25 mg/ml and administered at a standard dose of 0.66 μg/day, equivalent to the previously described effective dose of 30 μg/kg body weight/day (Hasse et al, 2014). Mice were daily subjected to clinical scoring by an observer blinded to the treatments. Statistical analysis was done using the Student's t-test and P values < 0.05 were considered to be statistically significant.

Euthanasia

Euthanasia was carried out by carbon dioxide asphyxiation, to effect, in conjunction with exsanguination (terminal blood collection by cardiac puncture), followed by thoracotomy.

Table 1. EAE Clinical Scoring Chart.

Score	Clinical Observations
0	No obvious changes in motor function compared to non-immunized mice. When picked up by base of tail, the tail has tension and is erect. Hind legs are usually spread apart. When the mouse is walking, there is no gait or head tilting.
1	Limp tail. When picked up by base of tail, instead of being erect, the whole tail drapes over finger. Hind legs are usually spread apart. No signs of tail movement are observed.
2	Limp tail and weakness of hind legs. When picked up by base of tail, the legs are not spread apart, but held closer together. When the mouse is observed walking, it has a clearly apparent wobbly walk. One foot may have toes dragging, but the other leg has no apparent inhibitions of movement. - OR - Mouse appears to be at score 0.0, but there are obvious signs of head tilting when the walk is observed. The balance is poor.
3	Limp tail and complete paralysis of hind legs (most common). - OR - Limp tail and almost complete paralysis of hind legs. One or both hind legs are able to paddle, but neither hind leg is able to move forward of the hind hip. - OR - Limp tail with paralysis of one front and one hind leg. - OR - ALL of: · Walking only along the edges of the cage, · Pushing against the cage wall, · Spinning when picked up by base of tail.
4	Limp tail, complete hind leg and partial front leg paralysis. Mouse is minimally moving around the cage but appears alert and feeding.
5	Found Dead

Tissue collection and analysis

Plasma

Whole blood (maximal obtainable volume) was collected into EDTA plasma separator tubes, centrifuged at 200 x g at 2-8^{0}C and the plasma recovered. Plasma samples were stored at -80^{0}C until processed.

Spinal cords

The spinal cords were collected from animals post-euthanization in moribund condition at scheduled necropsy. Tissues were examined in situ and dissected free. The lumbar portion of the spinal cord was snap frozen in liquid nitrogen. The thoracic portion of the spinal cord was fixed in 10% neutral buffered formalin for 48

hours. The formalin fixed samples were then transferred to 70% histology grade alcohol prior to analysis.

All animal work was carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals.

Spinal cord analysis

Spinal cords were collected for real time PCR (RT-PCR) and histopathology. Total RNA was isolated from spinal cord tissue using the RNeasy mini-kit protocol (Qiagen, Valencia, CA) and converted to cDNA using oligo (dT), random hexamers, and Superscript RT II, as described by the manufacturer (Invitrogen; Grand Island, NY). RT-PCR was used to determine the levels of CCR2, CCR5 and CCR7, which are elevated in CNS and spinal cord lesions in EAE (Du, 2012), and for the interleukins (IL) IL -6 and IL-17, which are key pro-inflammatory markers in the MS and EAE (Erta, 2008; Gold, 2008). Analysis was done using quantitect primers and SYBR green based qRT-PCR kits, according to the manufacturers instructions (Qiagen, Valencia, CA).

For histology, spinal cords were formalin-fixed and paraffin-embedded (FFPE) as previously described (Canene-Adams, 2013). Briefly, spinal cords were rinsed in PBS to remove blood and fixed in 10% formalin for 48 hours at room temperature. Tissues were then transferred to 70% ethanol for 1 hour, 95% ethanol/5% methanol for 1 hour, then incubated in four absolute ethanol baths (1x1 hour, 2x1½ hours, 1x2 hours. Tissues were then cleared with 2 xylene baths, 1 hour each, and then incubated in 2 paraffin baths at 58°C, 1 hour each, and embedded in cassettes. The FFPE tissues were sectioned into 5 μm slices on a microtome, transferred to glass slides and warmed to 65°C for 20 minutes to bond the tissue to the glass.

Upon deparaffinization in xylene and rehydration in graded ethanol, some spinal cord sections were stained with Kiernan's eriochrome cyanin for myelin content (Kiernan. J.A., 1999). Briefly, the sections were placed in a solution containing 1 g of eriochrome cyanine R, 20 ml of 5.6% ferric chloride solution and 2.5 ml concentrated sulphuric acid brought up to 500 ml with distilled water for 20 minutes. The sections were then rinsed under tap water and de-stained in 5.6% ferric chloride solution until only the myelin remained blue. The sections were then dehydrated with ethanol, cleared in xylene rinse and mounted.

Single label immunohistochemistry was performed using commercially available antibodies (Antibodies Online, Atlanta, GA) following the protocols described by Ramos-vara and Miller (Ramos-Vara JA and Miller MA, 2014). The antibodies used were; anti-CD68, expressed on macrophages (Holness and Simmons, 1993), anti-neurofilaments (Alberts, B. 2002), anti-glial fibrillary acidic protein (GFAP) a marker for glial cells (Jacque CM, et al, 1978). Briefly, tissue sections were dewaxed by incubation in a 60^0C oven for 20 minutes. Following incubagtion the slides were placed in Xylene for 2 minutes with 4 changes of solution, 100% ethanol for 2 minutes with 3 changes of solution and then 95% ethanol for 2 minutes, followed by a 5 minute rinse in deionized waster (dH_2O).

Slides were then placed in a sealed staining container with the antigen unmasking solution (Tris based, Vector Laboratories, Burlingame, Calif.) and put into a pressure vessel and incubated at 15 PSI for 20 minutes. Slides were removed from the unmasking solution and rinsed in dH_2O for 5 minutes, immersed in 3% hydrogen perox-

ide (H_2O_2) for 15 minutes then a 5 minute rinse in dH_2O. Slides were then moved to staining flats and rinsed with 1X Tris buffered saline with tween 20 (TBST) and blocked with 2.5% horse serum in TBST for 20 minutes.

Single labelling with primary antibodies was done by incubating slides overnight, then rinsed in blocking buffer. Detection antibody was added and incubated for 3 hours at room temperature (RT). Tissues were washed in TBST and the conjugated dye developed for visualization.

Double staining for Olig-1 and Olig-2 was done to determine if oligodendrocyte present in the spinal cord were previously existing mature oligodendrocytes or newly recruited. Olig1 is expressed by both mature oligodendrocytes and differentiating oligodendrocyte precursor cells, whereas Olig2 is only expressed on differentiating oligodendrocyte precursor cells (Miron V, et al, 2013).

Briefly, slides were blocked as above and the primary antibody (goat anti-Olig-1, Abcam, Cambridge, Mass.) was added to the slides and incubated for 60 minutes at RT. The slides were rinsed thoroughly in TBST and the detection antibody (ImmPRESS HRP anti-goat IgG (peroxidase), Vector Laboratories, Burlingame, Calif.) was applied to the sections and incubated for 45 minutes at RT. The slides were rinsed in TBST and developed using the DAB peroxidase (HRP) substrate kit (Vector Laboratories, Burlingame, Calif.) according to the manufacturers directions. Briefly, each slide was placed in 2.5 ml of dH_2O with 1 drop of buffer pH7.5, 2 drops DAB substrate and 1 drop H_2O_2 and incubated for 3 minutes, followed by 4 rinses in TBST.

Other slides were blocked as above and the primary antibody (Rabbit anti-Olig-2, Millipore, Billerica, Mass.) was added to each slide and incubated for 60 minutes at RT. The slides were rinsed in TBST and the detection antibody (ImmPRESS AP anti-rabbit IgG (alkaline phosphatase, Vector Laboratories, Burlingame, Calif.) was added to each section and incubated for 45 minutes at RT. The slides were rinsed in TBST and developed with the VECTOR blue (Alkaline Phosphatase) substrate kit (Vector Laboratories, Burlingame, Calif.). according to the manufacturers directions. Briefly, each slide was placed in 2.5 ml of dH_2O with one drop each of reagents 1, 2 & 3 from the kit. The slides were incubated for 10 minutes at RT then rinsed twice in dH_2O and allowed to dry.

Once dry, coverslip were carefully placed over the tissue sections using TBS Shur-Mount toluene-based mounting media (VWR, Radnor, PA). This media was selected to best preserve AP staining while providing clarity of sections.

Results

Disease severity

At the end of treatment, the group receiving rRLN had clinical scores of <0.5. The placebo group had scores of >1.5. The difference in scores between the rRLN and placebo treated group were significant on days 19, 20, & 21 ($p=<0.05$), see Figure 1.

Real-time PCR

In comparison to the placebo group (n=8), the spinal cords of the rRLN-treated group (n=8) showed a significant decrease in the mRNAs for chemokine receptors

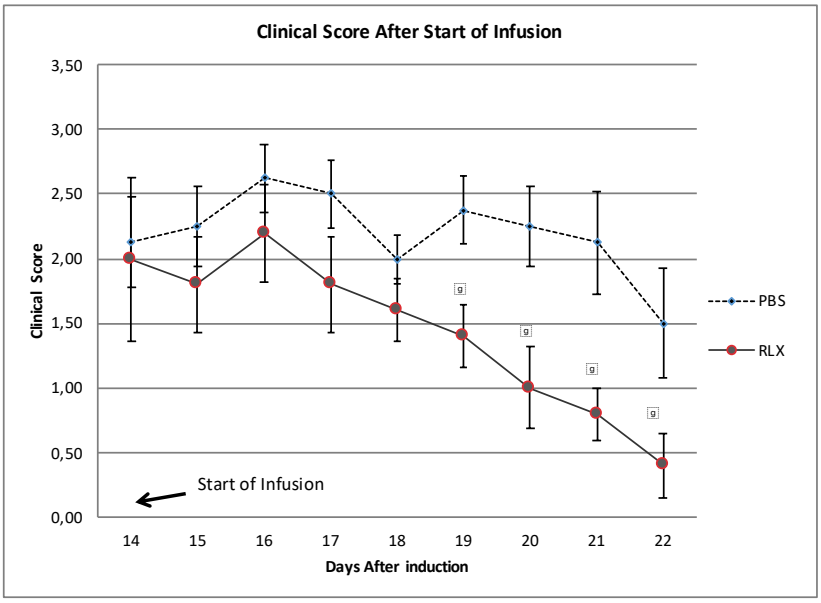

Figure 1. Animals receiving RLN treatment (n=8) showed greater improvement in clinical scores (2.0->0.5) compared to the placebo treated group (n=8, 2.0-1.5). Using a scale of 0-5 (0 = normal, 1 = tail paralysis, 2 = tail paralysis and strong hind limb weakness, 3 = complete hind limb paralysis, 4 = hind limb and forelimb paralysis, 5 = found dead). (*= p<0.05).

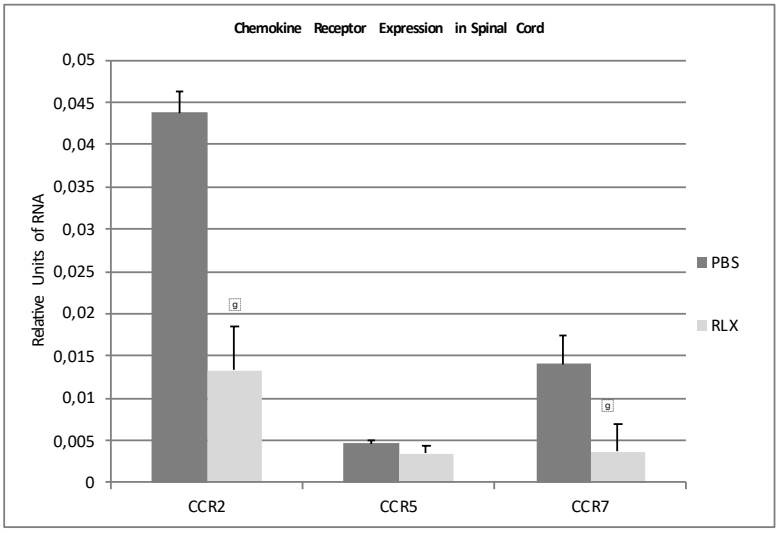

Figure 2. Expression of chemokine receptors in relative units normalized to β-actin. The pro-inflammatory chemokine receptors were decreased in treated mice (n=8) when compared to the placebo group (n=8). CCR2 (0.013 vs 0.043, *= p<0.05), CCR5 (0.0035 vs 0.0046, *= p<0.05) and CCR7 (0.0037 vs 0.014, *= p<0.05).

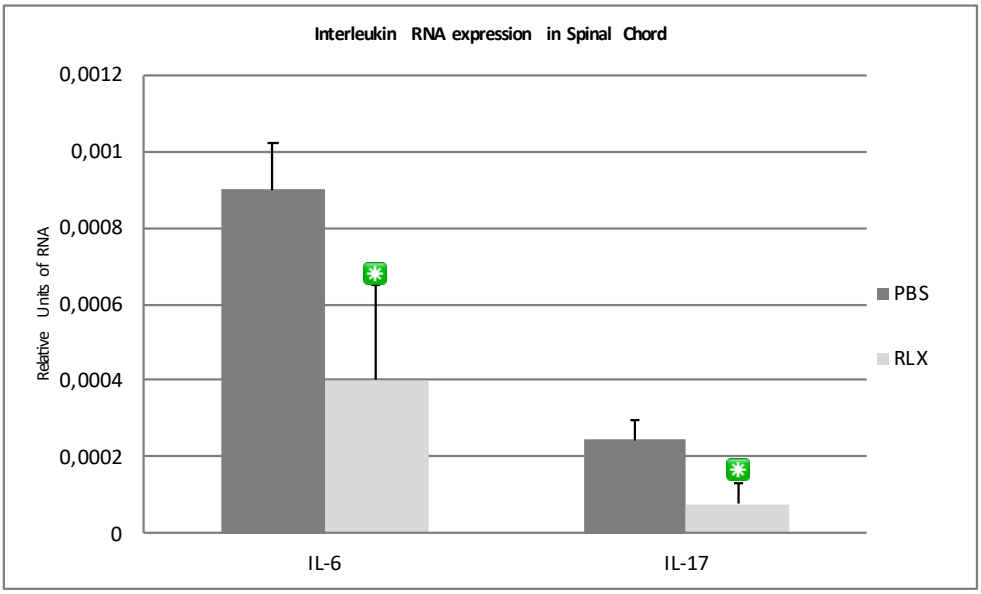

Figure 3. Expression of cytokines in relative units normalized to β-actin. The proinflammatory cytokines were decreased in the treated group (n=8) relative to the placebo (n=8). IL-6 (0.0004 vs 0.0009, *= $p<0.05$) and IL-17 (0.00024 vs 0.00007, *= $p<0.05$).

CCR2 (0.13 vs 0.044, $p<0.05$) and CCR7 (0.003 vs 0.014, $p<0.05$) and a slighter, not-significant decrease in CCR5 (0.0035 vs 0.0046) (see Figure 2). There was also a significant reduction of IL-6 (0.0004 vs 0.0009, $p<0.05$) and IL-17 (0.000078 vs 0.00026, $p<0.05$, see Figure 3).

Histology

Kiernan's eriochrome cyanin for myelin

The spinal cords of untreated mice with EAE showed inflammation, Wallerian degeneration and loss of myelin. Mice treated with rRLN showed more extended and intense myelin staining (see Figure 4).

CD68 immunostaining

Tissue samples immunostained for CD68, a marker for macrophage infiltration, showed a more intense labeling in the sections from untreated mice than in those from the rRLN-treated ones (see Figure 5).

Neurofilament immunostaining

The spinal cords from the untreated mice showed a relatively greater loss of neurofilaments compared to mice receiving rRLN therapy, suggesting the occurrence of neuronal demise (see Figure 6).

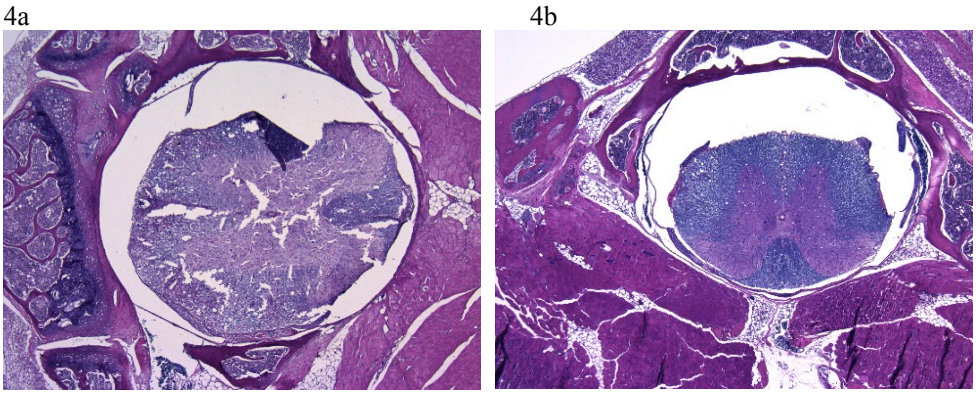

Figure 4. Myelin content in spinal cord cross sections; myelin is stained blue. The decrease in blue color indicates demyelination in untreated mice (1a). Whereas, mice treated with RLN (2b) have normal appearing myelin which stains an intense blue. Images are enlarged 40X.

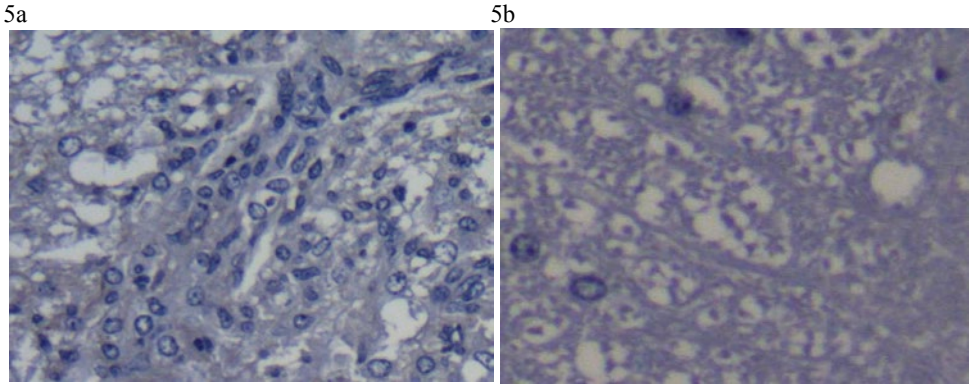

Figure 5. Decreased macrophage infiltration of the spinal cord of RLN treated mice (5b) when compared to untreated mice (5a). Tissue samples were embedded in paraffin and stained for CD68. Macrophages stain blue.

GFAP immunostaining

The spinal cords for rRLN treated mice were negative for GFAP while the untreated mice stained positive for GFAP, suggesting the occurrence of gliosis (see Figure 7).

Olig-1/Olig-2 immunostaining

The Olig-1+ and Olig-2+ oligodendrocytes were determined in sections of spinal cords by placing the stained sections under a grid and manually counting the positive cells for each marker in seven ramdomly selected microscopical fields of the grid. Mice treated with relaxin had an average of 58.92±12.1 (SD) of which 71% co-localised with Olig-1+ cells. Sham treated mice had an average of 38.90 (STD= 6.05) of which 7% co-localised with Olig-1+ cells (see Figures 8 & 9).

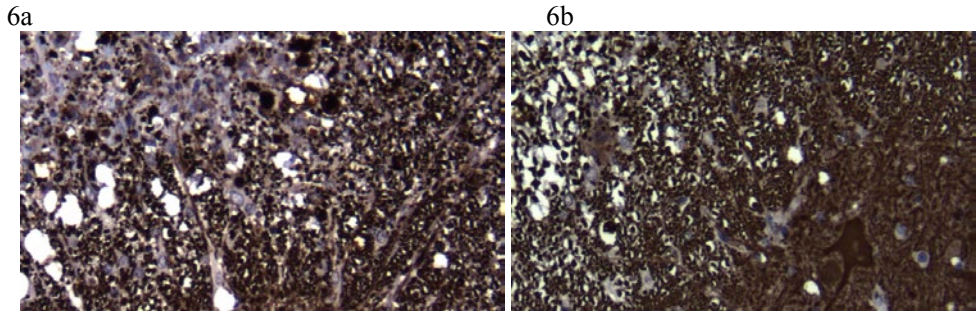

Figure 6. Greater loss of neurofilament in the spinal cords of untreated mice (6a) compared to mice treated with RLN (6b). Loss of the structural neurofilaments indicates a loss of neurons in the untreated mice as compared to those treated with RLN.

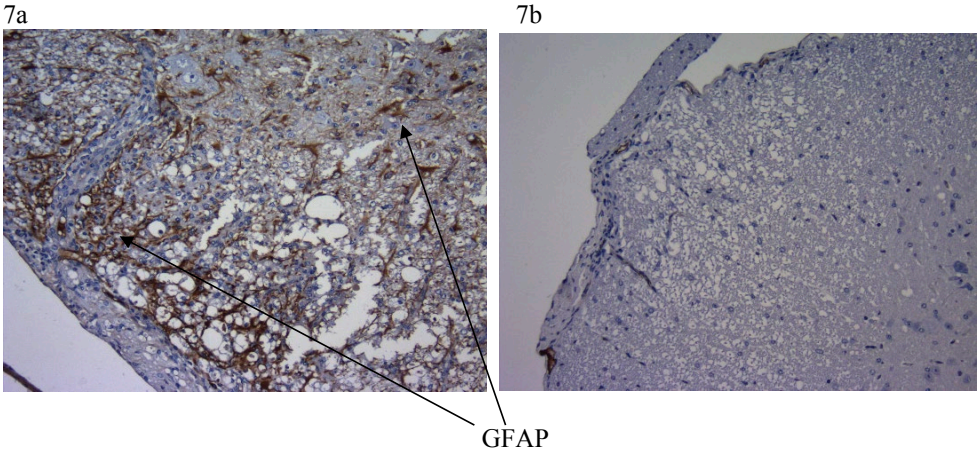

Figure 7. Gliosis in spinal cord slices stained for the presence of GFAP. The untreated sample (7a) has large amounts of GFAP present indicating extensive gliosis. In contrast, the RLN treated samples did not stain positively for GFAP. Magnification 200X.

Discussion

Inflammatory autoimmune diseases of the CNS which include MS and EAE may differ in onset and etiology, but the goals of treatment are the same; control inflammation, regulate autoimmunity and enhance neural protection. Despite the development of agents capable of modulating inflammation and autoimmunity, treatment remains problematic. Low efficacy and severe side effects being the main limiting factors in the use of these agents (Costello et al, 2008).

The discovery that estrogens could reduce the relapse rate and symptoms in MS (Sicotte et al, 2002) provided a novel approach to treatment based on naturally

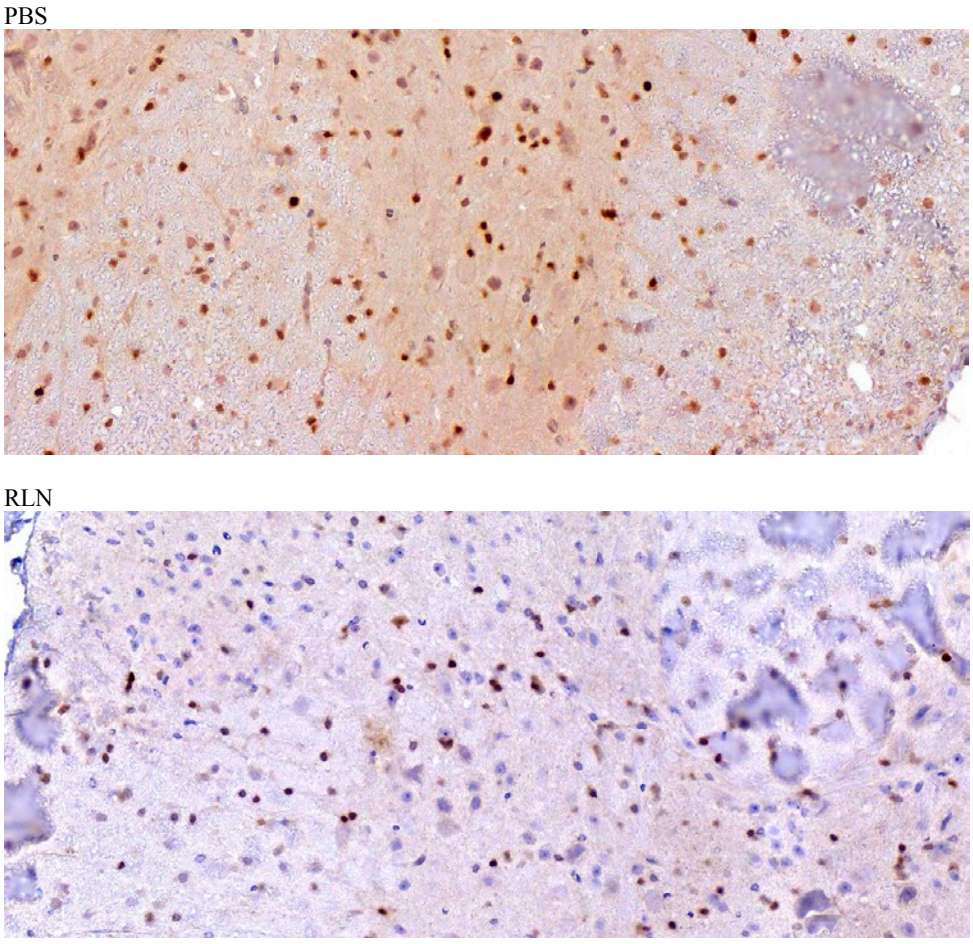

Figure 8. Olig1+/Olig-2+ cell expression. Representative grid of spinal cord slices stained for Olig1 (brown) and Olig2 (blue) used in counting Olig expressing cells. Spinal cords from untreated mice expressed mainly Olig1+ cells. Spinal cords from RLN treated mice expressed both Olig1+ and Olig2+ cells which highly co-localised. Indicating recruitment, differentiation and maturation of myelin producing oligodendrocytes.

occurring sex hormones. However, estrogen administration is also accompanied by unwanted side effects, especially upon prolonged use (Barrett-Connor and Stuenkel, 2001). The challenge of using sex hormone-based therapy for these diseases is to maximize the effects of this treatment on the disease and minimize the effects on the reproductive system. Studies of other molecules capable of modulating GCR and PPARγ may identify potential modulators of inflammation with minimum or no effects on the reproductive system. In this study we investigated the ability of one such hormones, rRLN, in the treatment of experimental EAE in mice *in vivo*. The results of this study show rRLN can decrease the severity of symptoms, modulate inflammation, promote neuro-protection and enhance remyelination. Treatment with

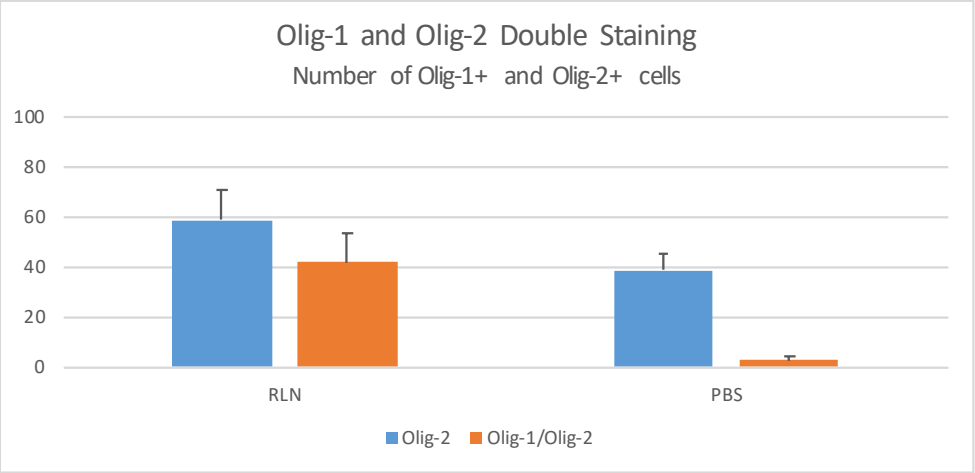

Figure 9. Percent co-localiztion of Olig1+ and Olig2+ cells in spinal cord sections. Animals receiving RLN had a 35% increase in Olig-2+ cells compared to controls. In RLN treated mice over 71% of Olig2+ cells co-localized with Olig1+ cells. In contrast only 7% co-localized in the controls. Indicating that RLN treatment promotes differentiation and maturation of OPC's into mature myelin producing oligodendrocytes and promotes remyelination.

rRLN down regulated the secretion of pro-inflammatory chemokine receptors CCR2 and CCR5. CCR2 has been identified as a key driver of encephalitogenic Th17 cell recruitment into the CNS in MS and EAE. Molecules capable of modulating CCR2 have been identified as possible novel therapies in MS and EAE (Kara et al, 2015). Similarly, CCR5+ Th-1 cells are increased in the cerebral spinal fluid and brain lesions of active demyelinating MS patients. Glatiramer acetate which is widely used for treatment of MS, acts by decreasing the expression of CCR5 and related Th-1 cells (Cheng and Chen, 2014). Th-1 and Th-17 cells are also modulated by CCR7, the presence of which is required for the development of EAE (Kuwabara, 2009).

Other regulators of Th-17 are the cytokines IL-6 and IL-17. Decreased expression of IL-6 and IL-17 by rRLN suggests a possible role for rRLN as a novel therapeutic in MS. Drugs reducing IL-6 levels can also reduce symptoms and disease progression in EAE (Erta, 2016), while therapies designed to block or reduce expression of IL-17 have shown some efficacy in treating EAE (Hofstetter et al, 2005) and MS (Havrdová E, et al, 2016).

The results of histology and immunohistochemistry showed a reduction of infiltrating macrophages, reduced demyelination, preserved neurofilaments and reduced gliosis in the rRLN-treated group, suggesting a neuroprotective effect of rRLN. Immunohistological staining for Olig-1 and Olig-2, specific markers of differentiating and maturating oligodendrocytes, showed a greater co-localisation in rRLN treated mice. This finding suggests oligodendrocyte recruitment, differentiation and maturation possibly related to enhanced remyelination. Conversely, the low level of Olig-1/Olig-2 co-localisation in the untreated mice indicates the presence of many mature oligodendrocytes, which are not committed to remyelination.

Of note, agonist of GCR and PPARγ have shown efficacy in treating EAE and MS. The ability of rRLN to modulate GCR and PPARγ may provide the background to its ability to ameliorate the symptoms of EAE, promote neuro-protection and enhance remyelination in the present experimental mouse model. These notions are of particular interest in the perspective of new therapeutic approaches to MS. Agonists of GCR have long been used in the treatment of MS symptoms. Agonist of PPARγ have previously been shown to promote differentiation and maturation of OPC's in cell culture (De Nuccio, et al. 2011) and rat OPC's (Bernardo et al., 2009). Clinically, a patient with secondary progressive MS was treated with a PPARg agonist for 3 years, which induced apparent clinical improvement. It also stopped any further demyelination based on sequential MRI's (Pershadsingh HA, et al. 2004). Another study of 50 MS patients treated with a PPARg agonist showed a significant decrease in the number of new or enlarging T2 lesions as well as of gadolinium-enhancing lesions in the treated group as compared with the control groups (Negrotto L, et al. 2016).

The present data and the excellent safety profile of rRLN (Smith et al, 2006) support further studies to address the potential efficacy of rRLN in treating inflammatory demyelinating diseases of the CNS.

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Conflict of interest

The author is employed by BVBiomed Ltd.

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