

Activation of nuclear receptors RAR, RXR and LXR does not reduce cuprizone-induced demyelination in mice

Davina **Kruczek**¹, Tim **Clarner**², Cordian **Beyer**², Markus **Kipp**^{2,3*} and Jörg **Mey**^{1,4,5*}

1 Institut für Biologie II, RWTH Aachen, Germany

2 Institut für Neuroanatomie, Universitätsklinikum Aachen, Germany

3 Lehrstuhl II – Neuroanatomie, Ludwig-Maximilians-Universität München, Germany

4 Laboratorio Regeneración Nerviosa, Hospital Nacional de Paraplégicos, Toledo, Spain

5 EURON Graduate School of Neuroscience, Maastricht University, Netherlands

* joint supervision and corresponding authors

Prof. Dr. Jörg Mey

Laboratorio Regeneración Nerviosa, SESCOAM, Hospital Nacional de Paraplégicos

Finca La Peraleda s/n, E-45071 Toledo, Spain

Tel.: +34-925-247754

e-mail: jmey@sescam.jccm.es

Prof. Dr. Dr. Markus Kipp

Department of Anatomy II - Neuroanatomie, Ludwig-Maximilians-Universität

Pettenkoferstr. 11, 80336 München, Germany

Tel.: +49-89-218072637

e-mail: markus.kipp@med.uni-muenchen.de

Abstract

Experiments with animal models of multiple sclerosis have shown that the expression of retinoid X receptors (RXR) increases during demyelination and that RXR α is involved in the regulation of remyelination. After ligand binding RXR form heterodimeric transcription factors with other nuclear receptor (NR) families including the retinoic acid receptors (RAR) and liver X receptors (LXR). We tested whether activation of these nuclear receptor complexes reduces pathological demyelination using the cuprizone mouse model. Cuprizone, which causes oligodendrocyte degeneration, was given for three weeks as a food additive. For the activation of nuclear receptors mice were treated with daily i.p. injections of agonists for RXR (9-*cis* RA), RAR (all-*trans* RA) and LXR (T0901317). Myelin status, oligodendrocyte survival, astrogliosis, microglial activation and axon density were monitored with immunohistochemistry and evaluated quantitatively. Three weeks of cuprizone feeding caused severe demyelination and significantly raised the number of Iba1 immunoreactive microglia cells in the caudal corpus callosum. This increase of microglia activity was reduced with 9-*cis* RA treatment but enhanced with all-*trans* RA and was not affected by T0901317. Nuclear receptor activation did not influence the degree of demyelination, oligodendrocyte survival, astrogliosis or axonal preservation. We conclude that RXR activation, although affecting Iba1-positive microglia, does not protect oligodendrocytes from cuprizone toxicity and does not induce compensatory mechanisms in the initial phase of demyelination.

Key words cuprizone; retinoic acid; nuclear receptor; astrocyte; myelin; microglia

Introduction

Recently, ligand activated transcription factors of the retinoid X receptor family (RXR) have been shown to be involved in the endogenous responses to toxin induced demyelination [1, 2]. The RXRs form heterodimers with other nuclear receptor (NR) families, including retinoic acid receptors (RAR) and liver X receptors (LXR). Experimental administration of agonists of these receptors was suggested as a potential treatment option for Alzheimer's disease, multiple sclerosis, ischemia and spinal cord injury [3]. Anti-inflammatory functions of retinoic acid (RA), the natural ligand of RAR/RXR, have been described for astrocytes [4-6] and microglia [7, 8]. In three animal models of multiple sclerosis, i.e. experimental allergic encephalomyelitis (EAE), systemic treatment with cuprizone, and focal application of lysolecithin, the oral administration of ligands of RAR [9-11], PPAR [12, 13] or n-3 polyunsaturated fatty acids (which activate RXR) [14] resulted in therapeutic benefits. The studies evaluated T-cell function, pro- and anti-inflammatory cytokines, histological signs of demyelination and clinical scores of disease progression. Since **LXR/RXR signaling regulates cholesterol homeostasis in oligodendrocytes [15] and modulates the activity of macrophages [16] and T-cells [17], their ligands are likely to be involved in the physiological processes during remyelination as well.**

Using acute demyelination combined with laser capture microdissection the groups of Franklin, French-Constant and Chambon demonstrated that RXR α controls the differentiation of oligodendrocyte precursor cells and promotes remyelination *in vivo* [1]. The involvement of RXR α in the process of demyelination was demonstrated by us using the copper chelator cuprizone [2]. In this model 0.2% cuprizone is fed to young male mice. The treatment causes apoptosis of mature oligodendrocytes, which is closely followed by microglia and astrocyte activation. The blood-brain barrier remains intact and a hematogenic immune response with cerebral T-cell infiltration is not elicited [18]. In the present work we investigated whether activation with ligands of RAR (all-*trans* RA), RXR (9-*cis* RA) and LXR (T0901317) affected the process of demyelination in this animal model. Since we observed that three weeks of cuprizone treatment led to a strong degree of demyelination of the *Corpus callosum* (Cc) this treatment period and fiber tract were selected in the present study.

2. Material and methods

The cuprizone animal model

To induce demyelination of central fiber tracts, 8-week-old male C57BL/6 mice, 20-25 g body weight (Charles River, Germany), were treated with the copper chelator cuprizone (Sigma-Aldrich, Germany). Demyelination was induced by feeding a diet containing 0.2% cuprizone mixed into a ground of standard rodent chow for three weeks. Animals were maintained in a pathogen-free environment, underwent routine cage maintenance once a week and microbiological monitoring. Food and water were available *ad libitum*. Research and animal care procedures were in accordance with national regulations and guidelines for the care and use of laboratory animals and were approved by the Review Board for the Care of Animal Subjects of the district government in Nordrhein-Westfalen, Germany.

Nuclear receptor activation *in vivo*

To activate RAR, RXR or LXR transcription factors, mice received daily intraperitoneal (i.p.) injections of either 20 µg all-*trans* RA (Sigma-Aldrich), 20 µg 9-*cis* RA (Sigma-Aldrich) diluted in 100 µl of 50/50 DMSO/PBS, or 0.5 mg T0901317 (Cayman Chemicals) dissolved in 100 µl of 10/90 DMSO/PBS. Animals in the cuprizone-treated control group received daily i.p. injections of 100 µl 50/50 DMSO/PBS. The efficacy of NR activation with i.p. application of these listed agonists had been established in previous studies [19-21]. Following an accommodation period and the three weeks of experimental drug application, all animals were sacrificed and further processed.

Tissue preparation and demyelination scoring

For the histological evaluation, mice were transcardially perfused with 4% paraformaldehyde containing saturated picric acid. After decapitation and post fixation over night, brains were dissected and embedded in paraffin. Best staining results were achieved with dehydration for seven days in 70% ethanol followed by 96%, 100% ethanol, acetone and paraffin embedding. Parasagittal sections of 5 µm covered the lateral 0.48 mm – 0.72 mm area according to the stereotaxic atlas [22]. The degree of demyelination was evaluated immunohistochemically and by staining with Luxol fast blue/cresyl violet following the protocol previously described [18]. The myelin status was staged independently by three blinded investigators using a scale between 0 and 100, where a score of 100 was given to the myelin status of reference mice, not treated with cuprizone, whereas zero corresponded to a totally demyelinated corpus callosum.

Analysis of nuclear receptor expression

The expression of nuclear receptors under conditions of cuprizone induced demyelination was assessed with the GeneChip Mouse Gene 1.0 ST Array (Affymetrix, USA) using the Agilent 2100 Bioanalyzer (Agilent, USA). The detailed procedure and treatment regimen have been published before [18]. Effects on nuclear receptors that are likely to mediate RA signaling were confirmed with quantitative RT-PCR using iQ5 real-time PCR detection (BioRad) using the same primers as listed previously [2].

Immunohistochemistry

Paraffin-embedded sections on silan-coated slides were rehydrated, heat-unmasked in citric acid buffer (pH 6.0; 20 min, microwave 180 W) and then processed using the Vectastain Elite ABC-Kit (avidin-biotinylated enzyme complex, Vector Laboratories, PK4010). Sections were blocked with 2% normal serum and incubated over night with mouse monoclonal antibodies against myelin proteolipid protein (PLP; 1/500; Abd Serotec, Germany, catalogue number: MCA839G) adenomatous polyposis coli (APC; 1/250; Calbiochem, Germany, OP80) and pan-axonal neurofilament (smi312, 1/1000; Covance, UK, SMI312-R), with rabbit polyclonal sera against glial fibrillary acidic protein (GFAP; 1/1000; EnCor, USA, RPCA-GFAP) and against ionized calcium binding adaptor molecule-1 (Iba1; 1/200; Wako, Germany, 019-19741). In Iba1 labeling experiments, counterstaining with hematoxylin/eosin was used. Biotinylated secondary antibodies, included in the Vectastain kit, were goat anti rabbit IgG and horse anti mouse IgG (both from Vector Laboratories, BA-1000, BA-2000). For visualization of peroxidase labeling a substrate kit with aminoethyl carbazole and a chromophor was used (Zymed, AEC Red Substrate Kit, 00-2007; Invitrogen). Sections were viewed and documented using a Nikon Eclipse 80i microscope equipped with a Nikon Y-TV55 camera.

Quantification and statistical analysis

Microphotographs were captured and evaluated with Nikon NIS Element D program and NIH *ImageJ*. Immunoreactive cells were manually selected and counted automatically. Three regions of the Corpus callosum (Cc), caudal (splenium), central (adjacent to fornix) and rostral Cc, were assessed independently. Cell densities were normalized to 0.1 mm² tissue area. Using a 40x objective (Iba1) or 20x objective (GFAP, APC) the numbers of positive cells were counted by two blinded observers in four animals per condition except for the group treated with cuprizone only, which contained eight animals. Two sections per mouse were averaged.

Non-parametric data of myelin staging were evaluated with the H-Test after Kruskal and Wallis followed by Wilcoxon/Mann/Whitney post hoc U-test with correction for ties. Cell density data were evaluated with ANOVA and post hoc Dunnett's test. Statistical tests were done using GraphPad Prism 5.01, the U-test manually with Excel. In all graphs means +/- standard error of the mean are shown.

Results

Cuprizone-induced demyelination is not affected by retinoic acid or T0901317

Three weeks of oral cuprizone treatment caused severe demyelination of the Cc, as shown with histological staining (Luxol fast blue) and PLP immunohistochemistry (Fig. 1a, b). In addition to the reduced staining, dark granular myelin debris was visible, as illustrated in the Cc and adjacent cingulum. In contrast to these fiber tracts, myelination of the fornix was not affected. To test whether pharmacological activation of NR influences demyelination, the cuprizone treatment was combined with daily i.p. injections of ligands for RARs (*all-trans* RA), RXRs (*9-cis* RA) and LXRs (T0901317; Fig. 1c-e). Previous studies have established that this treatment alters gene expression in the CNS [19-21]. [Immunoreactivity of RAR \$\alpha\$ and RXR \$\alpha\$ is present in the corpus callosum \[2\]](#). The cuprizone-induced demyelination was not affected by any of the NR agonists ([LFB staining](#)). Quantification of PLP immunoreactivity corroborated the signal reduction due to cuprizone administration (Fig. 1a, b). [Although myelin degradation appeared to be somewhat attenuated after treatment with all-trans RA \(Fig. 1c\) and T0901317 \(Fig. 1e\), the quantification](#) showed no significant effect of any of the NR ligands (Fig. 1f).

Mature oligodendrocyte cell bodies were marked with an antibody against APC (Fig. 2). Numerous oligodendrocytes were homogeneously distributed in the Cc of control mice, and no difference was apparent between caudal, middle and rostral parts of the Cc. In cuprizone-treated animals the numbers were strongly reduced. After three weeks we found the decline to be most pronounced in the caudal area, where the APC positive cell population was reduced to 24% (Fig. 2a, b). The administration of *all-trans* RA, *9-cis* RA or T0901317 did not affect oligodendrocytes (Fig. 2b-e), as [demonstrated](#) by the quantification of cell numbers (Fig. 3a, [no statistical differences between cuprizone-treated animals and the groups that received additional injections of NR ligands](#)). Across the rostro-caudal extent of the Cc and treatment conditions the decline in oligodendrocytes paralleled demyelination.

At three weeks of continued cuprizone treatment the density of nerve fibers, quantified with smi312 immunohistochemistry, was not yet affected. Compared to the neurofilament signal

intensity in the Cc of control animals (normalized mean 100 +/- SEM 1.7), cuprizone treatment caused no change (100 +/- 2.6), and none of the NR agonists affected fiber density either (cuprizone with all-*trans* RA: 99 +/- 1.5; cuprizone with 9-*cis* RA: 99 +/- 3.6; cuprizone with T0901317: 96 +/- 1.0). These are data from the rostral Cc, and similar results were obtained from the central and caudal areas of the fiber tract.

Effect of NR agonists on microglial activation and astrogliosis

Although the cuprizone-induced demyelination differs from the EAE model by not activating the immune system systemically, the tissue damage caused a local inflammatory reaction. In cuprizone treated mice we noted the appearance of a large population of Iba1 expressing microglia (Fig. 4), which was particularly strong in the caudal Cc. The quantification is shown in Fig. 3b. Additional treatment with 9-*cis* RA reduced this increase (Fig. 4g, h), such that the difference with respect to the control condition without cuprizone was no longer significant, however the comparison between 9-*cis* RA treated and non-treated animals under conditions of demyelination fell short of the required level of significance ($0.05 < p < 0.1$). On the other hand, injections of all-*trans*-RA caused a significantly stronger Iba1 immunoreactivity than cuprizone feeding alone ($p < 0.05$). The LXR agonist T0901317 did not modify the response (Figs. 3b, 4c-j).

Cuprizone-induced damage was accompanied with mild signs of local astrogliosis. Immunohistochemical staining against GFAP revealed a moderate increase in the size and staining intensity of astrocytes in the Cc (Fig. 5a, b). The strongest signal was apparent in the caudal area, although it reached significance only in the rostral Cc (Fig. 3c). The quantitative evaluation revealed no influence of all-*trans* RA, 9-*cis* RA and T0901317 under conditions of demyelination ($p > 0.05$ in all cases; Figs. 3c, 5b-e).

Nuclear receptors of the RAR, RXR and LXR families are expressed in the murine corpus callosum under conditions of demyelination

The expression of nuclear receptors that form functional dimers with RXR and would provide the basis for physiological effects of the investigated ligands was detected in the Cc of untreated and cuprizone-fed mice. An affimetrix GeneChip screen indicated high levels of expression of RAR α , RXR α , ROR α , LXR α and PPAR α and a cuprizone-induced increase of the transcripts for RXR α and ROR α (Tab. 1). The observed effects of RA on microglia are likely to be mediated by RAR/RXR heterodimers.

Their expression was confirmed with RT-PCR (Fig. 6). During demyelination we measured a significant increase of RXR α (t-test, $p < 0.05$) and a tendency to increase for RXR β ($p = 0.059$).

Discussion

After three weeks of cuprizone feeding we found that activation of RAR, RXR or LXR had no effect on the degree of demyelination, oligodendrocyte survival or astrogliosis. Cuprizone feeding raised the number of activated microglia cells in the Cc, as shown with Iba1 IR. This increase was reduced with 9-*cis* RA treatment. On the other hand, after exposure to all-*trans* RA microglia activity was enhanced, whereas T0901317 had no effect above the toxin-induced changes.

Previous experiments suggested that tissue damage in the nervous system causes local production of all-trans RA, and we hypothesized that this constitutes a neuroprotective feedback via activation of RAR/RXR [23]. Recent experiments based on RXR α knockdown and the application of RXR α agonists provide convincing data that this receptor is an endogenous regulator of remyelination [1]. In another organ system, cholesterol homeostasis was disrupted in Sertoli cells of RXR α deficient mice [24]. Therefore, we speculated that the regulation of lipid turnover is one of the functions of this receptor in the brain, at least under pathological circumstances with an effect on myelin [2]. In light of the results with RXR α the absence of a treatment effect in the present experiments is surprising. Different experimental models were employed in the two approaches with RA treatment, focal demyelination with lysolecithin in one study [1] versus long-term systemic exposure to cuprizone in the other. While this divergence may explain some difference in the outcome, the more likely explanation is that RXR α activation controls differentiation of oligodendrocytes but does not affect primary demyelination. Using the time period of three weeks we were able to disentangle demyelination from the compensatory processes regulated by RXR α .

A second interesting observation is the opposite effect of 9-*cis* RA and all-*trans* RA on macrophage activation. So far, mainly anti-inflammatory activity of both isomers has been reported [4, 5, 8, 23, 25]. In the EAE mouse model, LXR activation reduced pathological symptoms via a reduction of Th17 mediated autoimmunity [17]. Thus, the lower number of Iba1 positive cells following injections with 9-*cis* RA was to be expected. However, Iba1 immunoreactivity was elevated in the caudal Cc when we combined cuprizone treatment with all-*trans* RA. In the brain, Iba1 is a specific marker of microglia which upregulate that gene during inflammation and when they assume a phagocytotic phenotype [26]. Although most

studies focus on the destructive aspect of excessive microglia activation, it is crucial to bear in mind that different physiological phenotypes of microglia are not adequately described with a linear scale from resting state to inflammation [27]. In experimental models of demyelination, the role of macrophages is controversial [28]. Many studies suggest that the proinflammatory cytokines released by these cells are detrimental. Suppression of macrophages prevented the development of EAE. On the other hand, experiments with monocyte depletion indicate that microglia/macrophages have the potential to clear myelin debris, promote the recruitment of OPC and release neurotrophic factors. A detailed discussion of these aspects is provided in a recent review [29]. The differential regulation of Iba1 described here after prolonged exposure to all-*trans* and 9-*cis* RA indicates that RAR and RXR modulate the activity of microglia cells. Stimulation by all-*trans* RA might foster phagocytosis and clearance of myelin debris and by this mechanism facilitate remyelination. It will be important in the future to study the differential effects of RXR-binding NR families with respect to the multiple physiological functions of microglia cells.

Conclusion: Experimental activation of the transcription factors RAR, RXR and LXR does not directly affect toxin-induced demyelination. [Ligands of these three NR families](#) have differential effects on microglia [activation](#).

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Fig. 1. Myelination of central fiber tracts shown with immunohistochemistry of PLP. Parasagittal sections of the Cc area are shown three weeks after cuprizone treatment. a: Control section of a mouse fed normal chow; b: effect of cuprizone feeding; c: of cuprizone and daily intraperitoneal injections of all-*trans* RA; d: of cuprizone and 9-*cis* RA; e: of cuprizone and T0901317. Reduced myelin staining in all cuprizone treated animals is visible as is dark granular myelin debris. The caudal end of the Cc is in the upper right corner of the photographs; co: cerebral cortex, cc: corpus callosum; spl: splenium; hc: hippocampus; fo: fornix. f: Quantitative evaluation of myelin status in the Cc, based on PLP immunohistochemistry; myelin scale converted to % of control. Bars indicate means and standard error of the mean (SEM). Non-parametric H-test of treatment effect was significant and post hoc U-tests demonstrated a significant effect of cuprizone feeding compared to control (* $p < 0.05$, ** $p < 0.01$) and no influence of the NR agonists.

Fig. 2. Examples of immunohistochemical staining illustrate the effect of cuprizone and NR agonists on oligodendrocytes. Shown are sections of the caudal Cc following treatments a: control condition, normal chow; b: after three weeks of cuprizone feeding; c: combined cuprizone feeding with injection of all-*trans* RA; d: combined treatment of cuprizone with 9-*cis* RA; and e: combined treatment of cuprizone with T0901317. Oligodendrocytes were marked with an antibody against adenomatous polyposis coli (APC). Scale bar 100 μm in panel e is valid for all photographs.

Fig. 3. Quantification of the effect of cuprizone and NR agonists on cell numbers of oligodendrocytes, microglia and astrocytes. a: After three weeks of cuprizone feeding APC positive cell counts were significantly reduced in all regions of the Cc while treatment with the NR agonists had no mitigating or aggravating effect. b: Quantification of microglia activation and c: of GFAP positive cell numbers. Bars indicate means \pm SEM. Following ANOVA, Dunnet's test comparisons vs. control condition are marked with asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$), comparison vs. cuprizone condition with the pound symbol (# $p < 0.05$). Regions of the Cc are abbreviated r: rostral, f: central near fornix, c: caudal.

Fig. 4. Examples of immunohistochemical staining illustrate the effect of cuprizone and NR agonists on microglia cells. Shown are sections of Cc following treatments a, b: control condition, normal chow; c, d: after three weeks of cuprizone feeding; e, f: combined cuprizone feeding with injection of all-*trans* RA; g, h: combined treatment of cuprizone with 9-*cis* RA; and i, j: combined treatment of cuprizone with T0901317. Panels a, c, e, g, i represent areas in the caudal, panels b, d, f, h and j in the rostral Cc. Microglia cells were marked with an antiserum against ionized calcium binding adapter molecule-1 (Iba1). Scale bar 50 μm in panel j is valid for all photographs.

Fig. 5. Examples of immunohistochemical staining illustrate the effect of cuprizone and NR agonists on astrocytes. Shown are sections of caudal Cc following treatments a: control condition, normal chow; b: after three weeks of cuprizone feeding; c: combined cuprizone feeding with injection of all-*trans* RA; d: combined treatment of cuprizone with 9-*cis* RA; and e: combined treatment of cuprizone with T0901317. Astrocytes were marked with an antiserum against glial fibrillary acidic protein (GFAP). Scale bar 100 μm in panel e is valid for all photographs.

Fig. 6. Gene expression analysis RARs and RXRs in the murine corpus callosum. a Agarose gels with RT-PCR products under control conditions, and after 2 and 5 weeks cuprizone feeding. b Quantification with qRT-PCR was normalized to the expression of hypoxanthine phosphoribosyltransferase (HPRT). Error bars indicate SEM, controls n = 4-8 animals/group; t-test: * p < 0.05.