

Fingolimod does not enhance cerebellar remyelination in the cuprizone model



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ABSTRACT

Fingolimod (FTY720) is approved for treatment of relapsing–remitting multiple sclerosis. In vitro studies have found that fingolimod stimulates remyelination in cerebellar slices, but in vivo animal studies have not detected any positive effect on cerebral remyelination. The discrepant findings could be a result of different mechanisms underlying cerebral and cerebellar remyelination. The cuprizone model for de- and remyelination was used to evaluate whether fingolimod had an impact on cerebellar remyelination in vivo. We found that fingolimod did not have any effect on cerebellar remyelination, number of mature oligodendrocytes, microglia or astrocytes when fed after cuprizone exposure.

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1. Introduction

Fingolimod (FTY720) is a non-selective sphingosine 1-phosphate (S1P) receptor modulator that regulates lymphocyte trafficking and retains lymphocytes within the lymph node. It is widely used for the treatment of relapsing–remitting multiple sclerosis (RRMS). S1P receptors are also expressed on neuroglia and fingolimod could therefore have a role in neuroprotection and remyelination independent on its role on peripheral lymphocytes (Brinkmann et al., 2010; Groves et al., 2013; Sobel et al., 2015). It has been demonstrated that fingolimod enhances oligodendrocyte survival (Miron et al., 2008a,b), as well as remyelination in organotypic cerebellar slices in vitro (Miron et al., 2010). However, fingolimod is not able to promote remyelination in the corpus callosum (Hu et al., 2011; Kim et al., 2011; Slowik et al., 2015) or cerebral cortex (Slowik et al., 2015) of mice after experimentally induced demyelination. Different mechanisms seem to underlay cortical and white matter

remyelination (Gudi et al., 2009), as well as cerebellar remyelination (Skrípuletz et al., 2010), suggesting that the discrepant findings could have resulted from comparisons of different brain areas.

The cuprizone model is a T cell independent experimental model of toxic CNS demyelination. The copper chelator *bis*-cyclohexanone oxaldihydrazone (cuprizone) induces apoptosis of mature oligodendrocytes with subsequent myelin disruption, microglia activation, astrogliosis and infiltration of blood monocytes (macrophages) (Blakemore, 1973a; Torkildsen et al., 2008; Praet et al., 2014). The model demonstrates acute, selective demyelination with subsequent spontaneous remyelination after five weeks of cuprizone exposure (Blakemore, 1973b; Skripuletz et al., 2011; Wergeland et al., 2012). Although most studies using this model have focused on corpus callosum demyelination, demyelination and remyelination in the cerebellum have been well-studied and described (Groebe et al., 2009; Skripuletz et al., 2010), making it an ideal model to study the effects of fingolimod on cerebellar remyelination in vivo.

2. Methods

2.1. Mice

Five-week-old female c57Bl/6 mice (total $n = 32$) were purchased from Tacom, Tornbjerg, Denmark. Mean weight was 18.5 g \pm SD

Abbreviations: S1P, sphingosine 1-phosphate; Wr, weeks of recovery; β -APP, amyloid β precursor protein; IHC, immunohistochemistry; PL, Purkinje layer; GL, granule layer.

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1.1. The mice were acclimatized for 12 days prior to the experiment. They were housed by six together in GreenLine type II cages with open top (Scanbur, Karlslunde, Denmark), in standard laboratory conditions. Cage maintenance was performed once a week and the animals were handled by the same individuals throughout the experimental period. Food and tap water were available ad libitum throughout the acclimatization and experimental period. The experiment was carried out in accordance with the European Laboratory Animal Science Associations recommendations, and the protocol was approved by the Norwegian Animal Research Authority (permit 2013*5682).

2.2. Study design, cuprizone administration and fingolimod treatment

To induce demyelination, all mice were fed with 0.2% cuprizone (Sigma, St. Louis, MO, USA) mixed into ground mouse chow for six weeks. Cuprizone exposure was then discontinued. Control group (no demyelination) got normal mouse chow for the entire period. To study the effect on cerebellar remyelination, fingolimod was reconstituted in distilled water and given orally 1×/d by gavage at 1 mg/kg (Hu et al., 2011; Kim et al., 2011; Deshmukh et al., 2013) body weight from week five. Cuprizone exposure and fingolimod treatment overlapped with one week to make sure that the drug was taken up and phosphorylated to its active compound while cuprizone was still present. For comparison, animals in the cuprizone control group (maximal demyelination) were given the same volume of water (vehicle) by gavage. To study the dynamic effect of fingolimod on remyelination, animals in each group (n = 4) were sacrificed at weeks 5, 6 (1 wr), 7 (2 wr) and 9 (4 wr) as illustrated in Fig. 1. The animals were anesthetized with midazolam (Dormicum; F. Hoffmann-La Roche AG, Basel, Switzerland) in combination with fentanyl/fluanisone (Hypnorm, VetaPharma Ltd., UK) and sacrificed by cardiac puncture. Cerebelli were removed, post-fixed in 4% paraformaldehyde (PFA) and cryo-preserved.

2.3. Immunohistochemistry

Sagittal 8 µm sections were cut on a Leica CM1960 cryostat. Antigen retrieval was performed using the 2100 Retriever and Diva decloaker buffer as described by the manufacturer (Dako, Glostrup, Denmark), unless otherwise specified. Antibodies used: Iba1 (1:1000, Wako chemicals 019-19741), GFAP (1:1000, Sigma G3893), NOGO-A (1:500, Millipore AB5664P), β-APP (1:1000, Abcam ab32136), PLP1 (1:1000, AbD Serotec MCA839G), MBP (1:500, without antigen retrieval, Abcam ab24567), and neurofilament (1:1000, Millipore MAB1615). Secondary antibodies were Alexa Fluor 488 and 594 anti-mouse or anti-rabbit. Pictures were taken with a Nikon TE2000, with a 10× or 40× objective, or a Leica Confocal SP2 with 40× or 63× objective. Myelin was analysed by visual scoring of demyelination on a scale from 0 (no demyelination) to 3 (total demyelination), as previously described (Skripuletz et al., 2010; Wergeland et al., 2011). Results are given as a mean between the score for PLP1 and MBP. For cell number analysis, numbers are given as a mean from 2 pictures within the subcortical

region and 2 pictures from the cerebellar cortex (Fig. 1b). β-APP was measured by counting particles in the range of 10–600 pixels using the FJI software. 2–4 sections were analysed for each animal per antibody. All analyses were done blinded.

2.4. Statistics

One-way analysis of variance (ANOVA) was used to analyse parametric data, followed by Fisher's least significant difference (LSD) for post-hoc analysis when applicable. Kruskal–Wallis H-test was used to analyse non-parametric data. Statistical analyses were done using IBM SPSS statistics 22.

3. Results

3.1. Mice

After five weeks of cuprizone exposure, cuprizone-exposed mice had a mean weight 18.5 g ± 1.1 (SD) compared to 22.1 ± 1.2 (SD) in healthy controls (p < 0.0001). There were no significant differences between mice randomized to fingolimod or vehicle treatment (p = 0.23). After ending cuprizone exposure, no significant weight difference between fingolimod and vehicle treated mice was observed at any time points (data not shown). One mouse died of unknown cause.

3.2. Remyelination

To evaluate cerebellar de- and remyelination, PLP1- and MBP- staining was scored in the subcortex and two areas of rostral parts of cerebellar cortex as shown in Fig. 1b. The myelin scores are provided as the mean of PLP1 and MBP scores. Subcortical demyelination of the cerebellum was robust and significant in animals exposed to cuprizone (p < 0.0005) (Fig. 2). After 2 weeks of recovery, there was a mild and significant subcortical remyelination for both placebo (p < 0.0005) and fingolimod (p = 0.05). After 4 weeks of recovery, remyelination was clearer (p < 0.0005 for both placebo and fingolimod compared to the cuprizone control group), although not complete (significant demyelination for both groups compared to the control group, p < 0.0005). Cuprizone exposure led to mild demyelination of the cerebellar cortex that did not reach significance on group level (p = 0.054). Single comparison between the control group and cuprizone control group showed significant demyelination. Similar single comparison showed significant remyelination after 4 weeks (both placebo and fingolimod) compared to the cuprizone control group. There were no significant effects of fingolimod at any time points, neither in the cerebellar subcortex nor in the cerebellar cortex (Fig. 2).

3.3. Axonal damage

Accumulation of β-APP was measured to study acute axonal damage. Cuprizone exposure led to a significant increase in β-APP positive

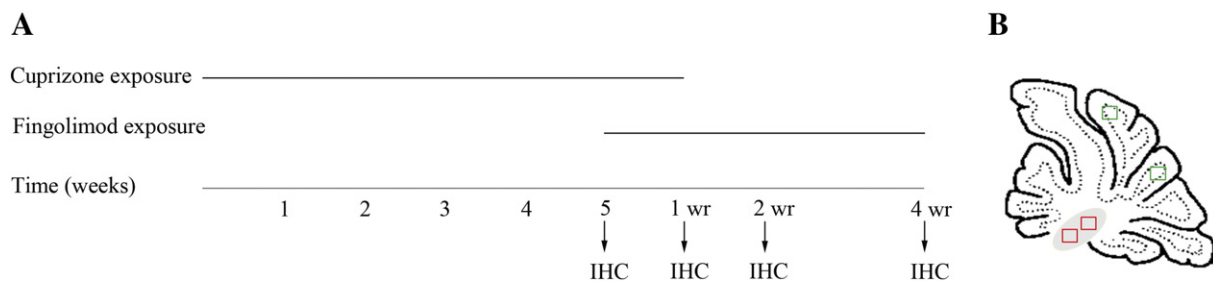


Fig. 1. Study design. Mice were fed with cuprizone for 6 weeks. From week 5, either fingolimod or placebo (vehicle) was given by gavage. Mice were sacrificed and cerebellum taken for IHC at 5 weeks cuprizone exposure (cuprizone control), 1 wr, 2 wr, and 4 wr (A). Mouse cerebellum with rectangles showing the regions examined with IHC; red; subcortical region, green; Purkinje cell and internal granule layer (B). Wr: weeks of recovery. IHC: immunohistochemistry.

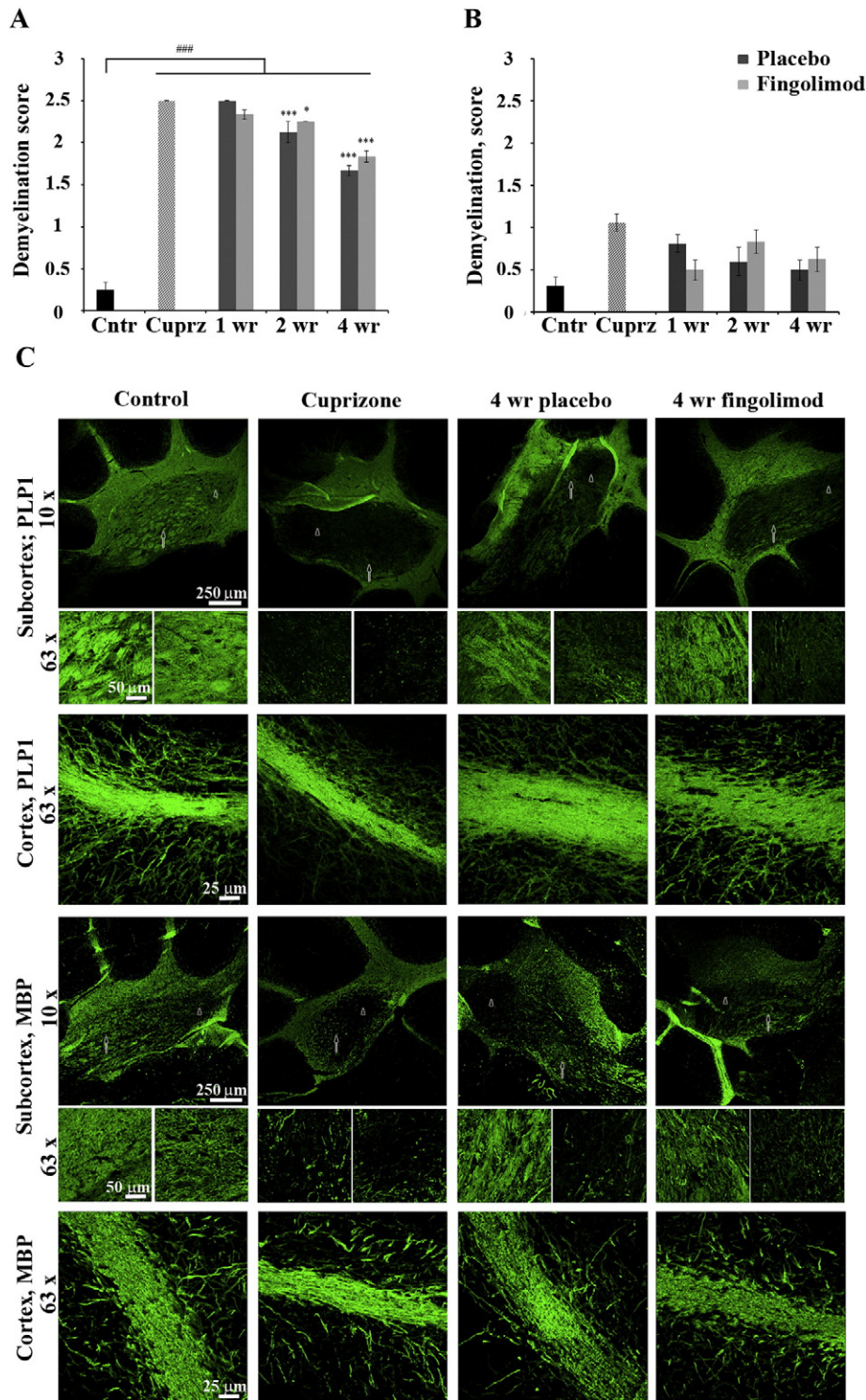


Fig. 2. Effect of fingolimod on remyelination in cerebellum of cuprizone-exposed mice. Robust subcortical demyelination was apparent after 5 weeks of cuprizone exposure compared to untreated controls. After 2 weeks of recovery, both placebo and fingolimod groups were significantly remyelinated and remyelination proceeded throughout the recovery period (A). Cuprizone exposure led to a mild, but not significant, demyelination of the cerebellar cortex (B). Representative pictures show myelination (PLP1 and MBP) in the subcortex and cortex of mouse cerebellum for control, 5 weeks of cuprizone exposure and after 4 weeks of fingolimod/placebo treatment. For subcortex, higher magnification pictures (63 \times) of the region with highest degree of myelination (arrow) and lowest degree of myelination (arrow head) are given (C). 2–4 sections were analysed per animal ($n = 3-4$). ### $p < 0.001$ to control group. *** $p < 0.001$, * $p < 0.05$ to cuprizone group. Scale bars: 250 μm for subcortex (10 \times), 25 μm for subcortex (63 \times) and 50 μm cortex (63 \times).

axons in the subcortical region ($p < 0.0005$) (Fig. 3a). There was no loss of neurofilament positive axons after cuprizone treatment (Fig. 3b). After 1 week of recovery β -APP positive axons were almost not detectable, and the levels remained low and close to baseline in the recovery period

($p < 0.0005$ for all groups compared to 5 weeks of cuprizone exposure). There were no differences in the number of β -APP positive axons between fingolimod- and placebo treated mice. In cerebellar cortical regions, no β -APP positive axons were detected (results not shown).

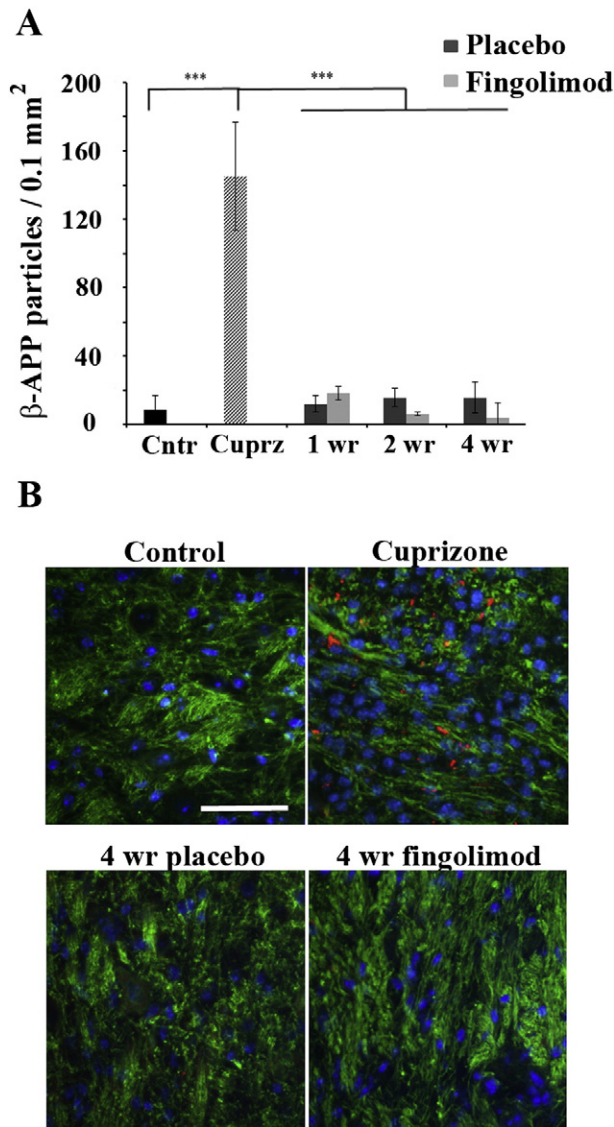


Fig. 3. Effect from fingolimod on accumulation of subcortical β -APP accumulation and neurofilament integrity. Five weeks of cuprizone exposure led to an increased subcortical accumulation of β -APP with no change of neurofilament integrity. After 1 week of recovery β -APP positive axons were hardly detected. Fingolimod did not affect the accumulation of β -APP particles throughout the recovery period (A). Subcortical β -APP (red) and neurofilament (green) staining of control, after 5 weeks of cuprizone exposure and after 4 weeks of recovery (B). 2–4 sections were analysed per animal ($n = 3$ –4). β -APP: amyloid β precursor protein. *** $p < 0.001$. Scale bar 25 μ m.

3.4. Mature oligodendrocytes, astrocytes and microglia

Cuprizone exposure led to a significant subcortical and cortical loss of NOGO-A positive mature oligodendrocytes ($p = 0.003$ and $p = 0.001$). In both regions, the number of NOGO-A positive oligodendrocytes increased during the recovery period, reaching normal levels by 4 weeks of recovery. Fingolimod did not have any significant effect on NOGO-A positive (mature) oligodendrocytes (Fig. 4a and b).

There was a subcortical increase in GFAP-positive astrocytes after 5 weeks of cuprizone treatment (>4 -fold, $p < 0.0005$) (Fig. 4c). During remyelination, the number of GFAP-positive astrocytes remained high in both placebo- and fingolimod-groups, with no significant differences at any time points. In the cerebellar cortex, there were no changes in number of GFAP-immunopositive astrocytes at any time points.

In line with previous studies (Groebe et al., 2009; Ingwersen et al., 2012), 5 weeks of cuprizone exposure led to a robust subcortical increase in Iba1-positive microglia/macrophages (12-fold, $p < 0.0005$) (Fig. 5a). It has previously been found that cuprizone-induced microgliosis is a combination of strong local proliferation of brain resident microglia and infiltration of blood-derived monocytes (macrophages) (Praet et al., 2014). In this study, microglia and infiltrated macrophages were not distinguished and are referred to as microglia. During the recovery phase, the number of microglia steadily decreased, although not to normal levels. In the cerebellar cortex, there was no significant microgliosis (Fig. 5b). Fingolimod did not have any effect on microglia numbers at any time points.

4. Discussion

The aim of this study was to investigate the possible effect of fingolimod on cerebellar remyelination in vivo by using the cuprizone model. We analysed three time points during the recovery phase without detecting any effect of fingolimod on cerebellar remyelination. We did not detect any effect of fingolimod on maturation of oligodendrocytes, microglia numbers or GFAP positive astrocytes in the recovery period after cuprizone induced demyelination.

The cuprizone mouse model is a highly reproducible and well described animal model used to study mechanisms underlying demyelination and remyelination (Torkildsen et al., 2008; Wergeland et al., 2012; Praet et al., 2014). Previous work from our group has shown that a vitamin D rich diet can reduce demyelination (Torkildsen et al., 2009; Wergeland et al., 2011) and promote remyelination (Nystad et al., 2014) in the corpus callosum of cuprizone-exposed mice.

In line with previous studies (Groebe et al., 2009; Skripuletz et al., 2010), cuprizone exposure induced cerebellar demyelination. Subcortical regions were severely demyelinated, while demyelination was minor in the cerebellar cortex after five weeks of cuprizone exposure. We did not detect remyelination after the first week of recovery, suggesting that cerebellar remyelination is delayed compared to the rapid remyelination seen in the corpus callosum (Skripuletz et al., 2008; Wergeland et al., 2012). Remyelination is known to be initiated by proliferation and migration of oligodendrocyte precursor cells towards the lesion site where they mature into myelin forming mature oligodendrocytes in a process that is dependent on a plethora of growth factors and signalling molecules (Praet et al., 2014). S1P is considered a survival factor for mature oligodendrocytes (Jaillard et al., 2005) and when fingolimod is given together with cuprizone, it protects mature oligodendrocytes from apoptosis (Kim et al., 2011). In our study, fingolimod was given after cuprizone-induced demyelination, at a time point where hardly any mature oligodendrocytes were present in the demyelinated area. The number of NOGO-A positive mature oligodendrocytes steadily increased during the recovery phase, corresponding to subcortical remyelination, without any influence from fingolimod.

It has been shown that fingolimod may have neuroprotective properties, reducing axonal damage in the corpus callosum after acute and chronic cuprizone-induced demyelination (Slowik et al., 2015). However, we did not detect any effect of fingolimod on axonal damage in the cerebellum, as subcortical β -APP positive axons were almost at baseline levels after one week of remyelination and there were no differences in neurofilament levels or number of APP-positive axons between placebo and fingolimod groups throughout the recovery phase.

Cuprizone exposure resulted in sustained subcortical astrogliosis throughout the remyelination period. This is in line with previous studies (Groebe et al., 2009; Hibbits et al., 2012), and has been suggested that astrocytes promote remyelination by supporting oligodendrocyte differentiation and recruitment of microglia/macrophages to lesion sites (Nair et al., 2008; Praet et al., 2014; Tanaka and Yoshida, 2014). Five weeks of cuprizone exposure induced extensive subcortical microgliosis, which declined during the recovery phase independent of fingolimod treatment. This is in line with recent results from the corpus callosum

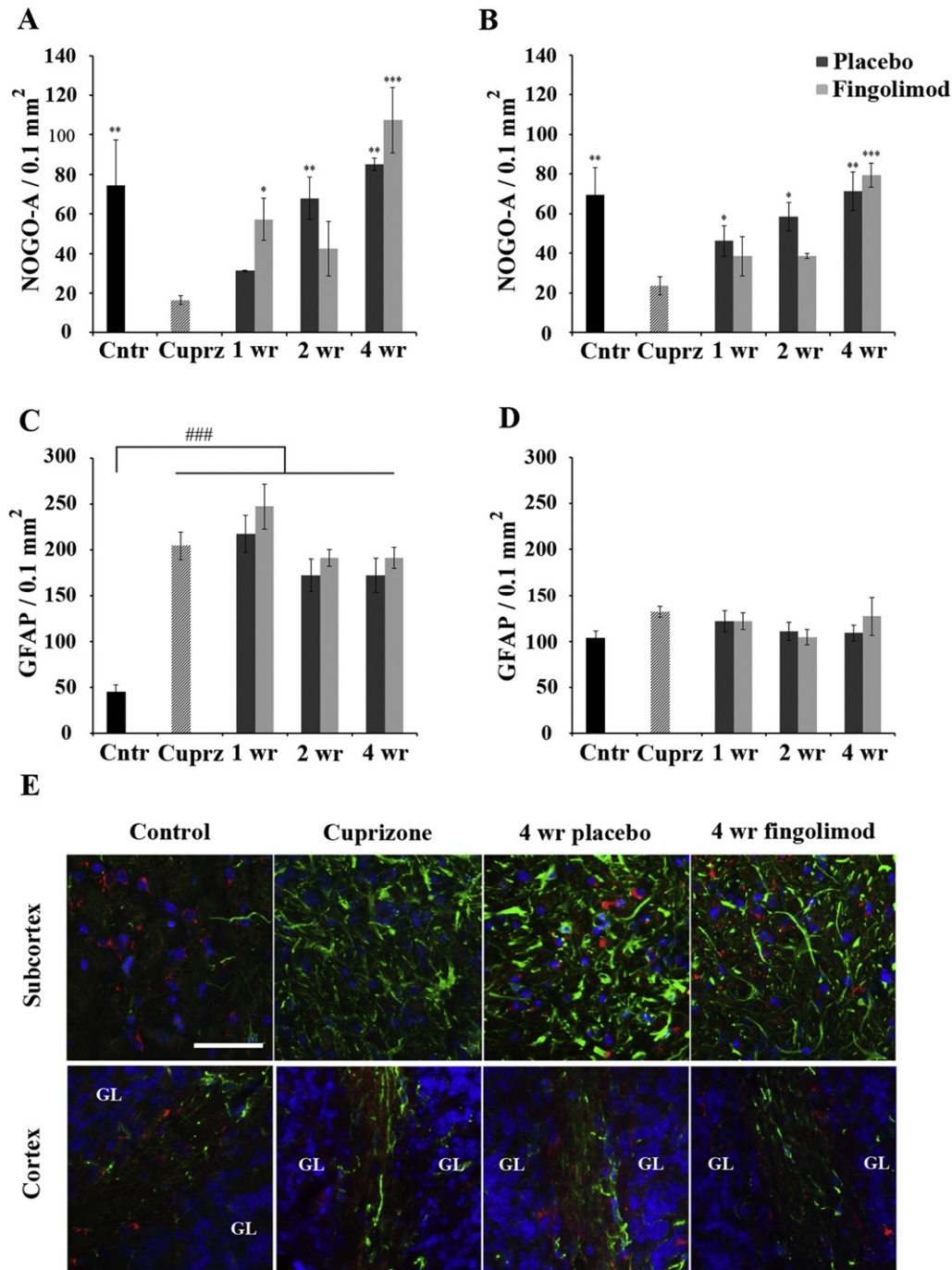


Fig. 4. Effect from fingolimod on mature oligodendrocytes and astrocytes in mouse cerebellum after cuprizone exposure. Numbers of NOGO-A positive oligodendrocytes were significantly reduced in the subcortex (A) and cortex (B) after 5 weeks of cuprizone exposure. During the recovery period, there was a steady increase in NOGO-A positive oligodendrocytes in both regions, reaching normal levels after 4 weeks of recovery (A and B). Cuprizone exposure led to robust subcortical astrogliosis that sustained during the recovery phase (C). No astrogliosis was seen in the cerebellar cortex (D). NOGO-A positive mature oligodendrocytes (red) and GFAP-expressing astrocytes (green) in subcortex and cortex in control, 5 weeks of cuprizone exposure and after 4 weeks of recovery (E). 3–4 sections were analysed per animal ($n = 3-4$). $***p < 0.001$, $**p < 0.01$, $*p < 0.05$ to cuprizone, $###p < 0.001$ to control group, GL: granule layer. Scale bar 25 μm .

(Slowik et al., 2015). Whether fingolimod has any clinical significant effect on microglia is debated (Groves et al., 2013). The use of different experimental model systems, inducing CNS damage with different aetiologies, could be a plausible explanation for the discrepant findings. Regenerative functions of microglia depend on their phenotype. Microglia in a pro-inflammatory state are considered inhibitory to remyelination while microglia in an anti-inflammatory state could promote remyelination by phagocytosis of myelin debris and secretion of cytokines and growth factors (Miron and Franklin, 2014). In this study we

analysed the total number of microglia without distinguishing between different functional phenotypes and it is therefore possible that fingolimod has functional effects on microglia that we did not detect. However, regardless of any effects on microglia functions, we did not find that fingolimod promotes remyelination in the cuprizone model.

Fingolimod is effective in preventing acute attacks in RRMS by internalising T cells in the lymph nodes (Brinkmann et al., 2010; Ingwersen et al., 2012). From murine models it has been found that fingolimod is distributed to and phosphorylated to its active form

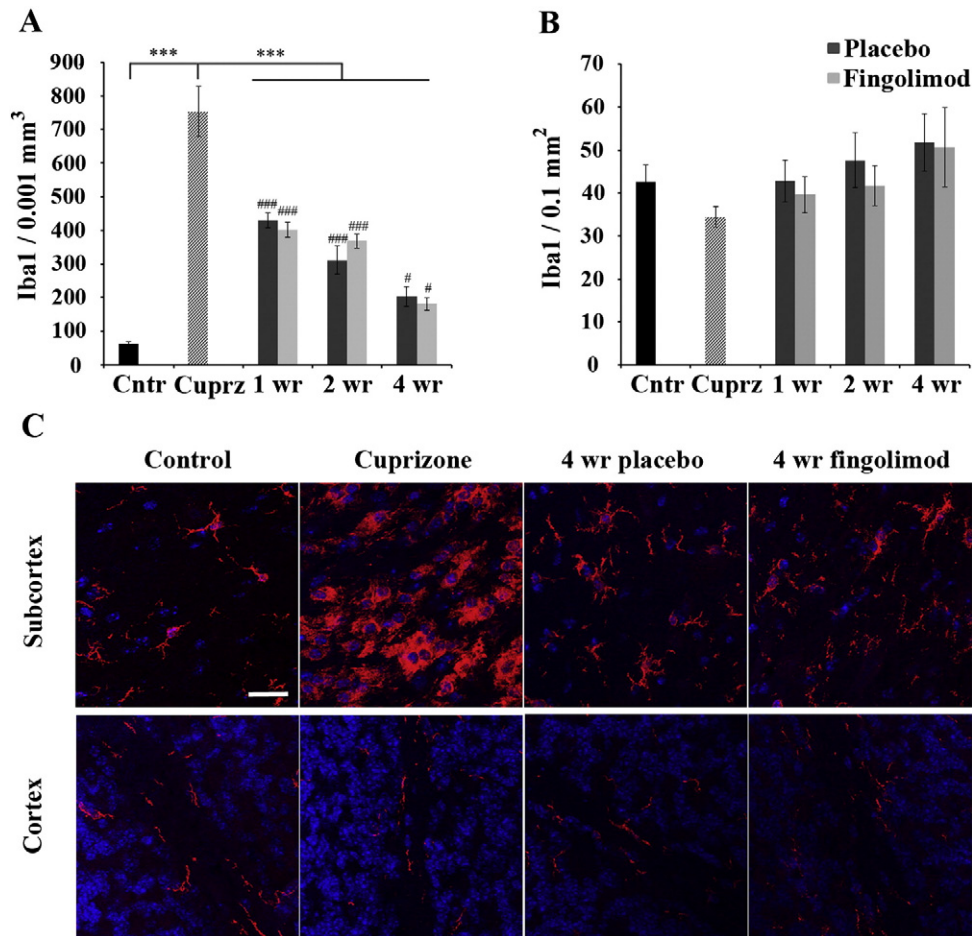


Fig. 5. Effect from fingolimod on microglia in mouse cerebellum after cuprizone exposure. There was extensive subcortical microgliosis (Iba1) after 5 weeks of cuprizone exposure which steadily decreased during the recovery period. There were no differences between fingolimod and placebo groups (A). No microgliosis was detected in cerebellar cortex after 5 weeks of cuprizone exposure (B). Representative pictures show microglia in the subcortex and cortex in control, 5 weeks of cuprizone exposure and after 4 weeks of recovery (C). 3–4 sections were analysed per animal ($n = 3-4$). *** $p < 0.001$ to cuprizone. # $p < 0.05$, ### $p < 0.001$ to control group. PL: Purkinje layer. GL: granule layer. Scale bar 25 μm .

within the brain (Meno-Tetang et al., 2006). It has therefore been suggested that fingolimod could modulate immune responses and promote CNS regeneration by targeting neurons and neuroglia (Miron et al., 2008; Groves et al., 2013). As S1P is involved in immune cell trafficking, vascular homeostasis and cell communication in the CNS, it has been suggested that S1P can mediate activation and proliferation of neuroglia during inflammatory responses (Brinkmann, 2007). Results from the present study show that there is no effect of fingolimod on maturation of oligodendrocytes, astrogliosis or microgliosis in the cerebellum after cuprizone exposure. This indicates that modulating neuroglial S1P receptors by fingolimod does not have any clear regenerative effects although it has been found to have some neuroprotective effects during cuprizone induced CNS damage (Kim et al., 2011).

5. Conclusions

We show that fingolimod does not affect cerebellar remyelination, number of mature oligodendrocytes, microglia or astrocytes in the recovery phase after cuprizone-induced demyelination. This suggests that fingolimod does not have any effect on cerebellar remyelination in vivo. Our conclusions are in line with the previous reported in vivo studies on the corpus callosum (Hu et al., 2011; Kim et al., 2011; Slowik et al., 2015), but differ from the in vitro study using organotypic cerebellar slices (Miron et al., 2010). We suggest that the discrepant results are caused by the use of different experimental models and not by different effects from fingolimod on cerebral and cerebellar remyelination.

Conflict of interest

MNA and AEN have received unrestricted grants from Novartis. SW has received unrestricted grants and honoraria as a speaker from Alexion Pharmaceuticals and Novartis.

LB has participated on scientific advisory boards for Novartis Norway; received funding for travel from Sanofi-Aventis, Novartis, Merck-Serono and Biogen Idec; received speaker honoraria from Bayer, Genzyme, Sanofi-Aventis, Novartis, Merck-Serono and Biogen Idec; and received unrestricted research support from Bayer, Sanofi-Aventis, Novartis, Merck-Serono and Biogen Idec.

KMM has participated on scientific advisory boards for Novartis Norway, Biogen Idec, and Genzyme; received funding for travel from Bayer, Novartis, Merck-Serono and Biogen Idec; received speaker honoraria from Bayer, Genzyme, Sanofi-Aventis, Novartis, Merck-Serono and Biogen Idec; and received unrestricted research support from Bayer, Sanofi-Aventis, Novartis, Merck-Serono, Biogen Idec, Pronova Biocare and Norwegian MS Society.

ØT has participated on a scientific advisory board for Biogen Idec and received speaker honoraria and travel grants from Genzyme, Merck-Serono, Novartis and Biogen-Idec.

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References

- Blakemore, W.F., 1973a. Demyelination of the superior cerebellar peduncle in the mouse induced by cuprizone. *J. Neurol. Sci.* 20, 63–72.
- Blakemore, W.F., 1973b. Remyelination of the superior cerebellar peduncle in the mouse following demyelination induced by feeding cuprizone. *J. Neurol. Sci.* 20, 73–83.
- Brinkmann, V., 2007. Sphingosine 1-phosphate receptors in health and disease: mechanistic insights from gene deletion studies and reverse pharmacology. *Pharmacol. Ther.* 115, 84–105.
- Brinkmann, V., Billich, A., Baumruker, T., Heining, P., Schmouder, R., Francis, G., Aradhye, S., Burtin, P., 2010. Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. *Nat. Rev. Drug Discov.* 9, 883–897.
- Deshmukh, V.A., Tardif, V., Lyssiotis, C.A., Green, C.C., Kerman, B., Kim, H.J., Padmanabhan, K., Swoboda, J.G., Ahmad, I., Kondo, T., Gage, F.H., Theofilopoulos, A.N., Lawson, B.R., Schultz, P.G., Lairson, L.L., 2013. A regenerative approach to the treatment of multiple sclerosis. *Nature* 502, 327–332.
- Groebe, A., Clarner, T., Baumgartner, W., Dang, J., Beyer, C., Kipp, M., 2009. Cuprizone treatment induces distinct demyelination, astrocytosis, and microglia cell invasion or proliferation in the mouse cerebellum. *Cerebellum* 8, 163–174.
- Groves, A., Kihara, Y., Chun, J., 2013. Fingolimod: direct CNS effects of sphingosine 1-phosphate (S1P) receptor modulation and implications in multiple sclerosis therapy. *J. Neurol. Sci.* 328, 9–18.
- Gudi, V., Moharreggh-Khiabani, D., Skripuletz, T., Koutsoudaki, P.N., Kotsiari, A., Skuljec, J., Trebst, C., Stangel, M., 2009. Regional differences between grey and white matter in cuprizone induced demyelination. *Brain Res.* 1283, 127–138.
- Hibbits, N., Yoshino, J., Le, T.Q., Armstrong, R.C., 2012. Astroglialosis during acute and chronic cuprizone demyelination and implications for remyelination. *ASN Neuro* 4, 393–408.
- Hu, Y., Lee, X., Ji, B., Guckian, K., Apicco, D., Pepinsky, R.B., Miller, R.H., Mi, S., 2011. Sphingosine 1-phosphate receptor modulator fingolimod (FTY720) does not promote remyelination in vivo. *Mol. Cell. Neurosci.* 48, 72–81.
- Ingwersen, J., Aktas, O., Kuery, P., Kieseier, B., Boyko, A., Hartung, H.P., 2012. Fingolimod in multiple sclerosis: mechanisms of action and clinical efficacy. *Clin. Immunol.* 142, 15–24.
- Jaillard, C., Harrison, S., Stankoff, B., Aigrot, M.S., Calver, A.R., Duddy, G., Walsh, F.S., Pangalos, M.N., Arimura, N., Kaibuchi, K., Zalc, B., Lubetzki, C., 2005. Edg8/S1P5: an oligodendroglial receptor with dual function on process retraction and cell survival. *J. Neurosci.* 25, 1459–1469.
- Kim, H.J., Miron, V.E., Dukala, D., Proia, R.L., Ludwin, S.K., Traka, M., Antel, J.P., Soliven, B., 2011. Neurobiological effects of sphingosine 1-phosphate receptor modulation in the cuprizone model. *FASEB J.* 25, 1509–1518.
- Meno-Tetang, G.M., Li, H., Mis, S., Pyszczyński, N., Heining, P., Lowe, P., Jusko, W.J., 2006. Physiologically based pharmacokinetic modeling of FTY720 (2-amino-2[-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride) in rats after oral and intravenous doses. *Drug Metab. Dispos.* 34, 1480–1487.
- Miron, V.E., Franklin, R.J., 2014. Macrophages and CNS remyelination. *J. Neurochem.* 130, 165–171.
- Miron, V.E., Hall, J.A., Kennedy, T.E., Soliven, B., Antel, J.P., 2008a. Cyclical and dose-dependent responses of adult human mature oligodendrocytes to fingolimod. *Am. J. Pathol.* 173, 1143–1152.
- Miron, V.E., Jung, C.G., Kim, H.J., Kennedy, T.E., Soliven, B., Antel, J.P., 2008b. FTY720 modulates human oligodendrocyte progenitor process extension and survival. *Ann. Neurol.* 63, 61–71.
- Miron, V.E., Schubart, A., Antel, J.P., 2008c. Central nervous system-directed effects of FTY720 (fingolimod). *J. Neurol. Sci.* 274, 13–17.
- Miron, V.E., Ludwin, S.K., Darlington, P.J., Jarjour, A.A., Soliven, B., Kennedy, T.E., Antel, J.P., 2010. Fingolimod (FTY720) enhances remyelination following demyelination of organotypic cerebellar slices. *Am. J. Pathol.* 176, 2682–2694.
- Nair, A., Frederick, T.J., Miller, S.D., 2008. Astrocytes in multiple sclerosis: a product of their environment. *Cell. Mol. Life Sci.* 65, 2702–2720.
- Nystad, A.E., Wergeland, S., Aksnes, L., Myhr, K.M., Bo, L., Torkildsen, O., 2014. Effect of high-dose 1.25 dihydroxyvitamin D3 on remyelination in the cuprizone model. *APMIS* 122, 1178–1186.
- Praet, J., Guglielmetti, C., Berneman, Z., Van Der Linden, A., Ponsaerts, P., 2014. Cellular and molecular neuropathology of the cuprizone mouse model: clinical relevance for multiple sclerosis. *Neurosci. Biobehav. Rev.* 47, 485–505.
- Skripuletz, T., Lindner, M., Kotsiari, A., Garde, N., Fokuhl, J., Linsmeier, F., Trebst, C., Stangel, M., 2008. Cortical demyelination is prominent in the murine cuprizone model and is strain-dependent. *Am. J. Pathol.* 172, 1053–1061.
- Skripuletz, T., Bussmann, J.H., Gudi, V., Koutsoudaki, P.N., Pul, R., Moharreggh-Khiabani, D., Lindner, M., Stangel, M., 2010. Cerebellar cortical demyelination in the murine cuprizone model. *Brain Pathol.* 20, 301–312.
- Skripuletz, T., Gudi, V., Hackstette, D., Stangel, M., 2011. De- and remyelination in the CNS white and grey matter induced by cuprizone: the old, the new, and the unexpected. *Histol. Histopathol.* 26, 1585–1597.
- Slowik, A., Schmidt, T., Beyer, C., Amor, S., Clarner, T., Kipp, M., 2015. The sphingosine 1-phosphate receptor agonist FTY720 is neuroprotective after cuprizone-induced CNS demyelination. *Br. J. Pharmacol.* 172, 80–92.
- Sobel, K., Monnier, L., Menyhart, K., Bolinger, M., Studer, R., Nayler, O., Gatfield, J., 2015. FTY720-P activates sphingosine-1-phosphate receptor 2 and selectively couples to G α 12/13/Rho/ROCK to induce myofibroblast contraction. *Mol. Pharmacol.* 87 (6), 916–927.
- Tanaka, T., Yoshida, S., 2014. Mechanisms of remyelination: recent insight from experimental models. *Biomol. Concepts* 5, 289–298.
- Torkildsen, O., Brunborg, L.A., Myhr, K.M., Bo, L., 2008. The cuprizone model for demyelination. *Acta Neurol. Scand. Suppl.* 188, 72–76.
- Torkildsen, O., Brunborg, L.A., Milde, A.M., Mork, S.J., Myhr, K.M., Bo, L., 2009. A salmon based diet protects mice from behavioural changes in the cuprizone model for demyelination. *Clin. Nutr.* 28, 83–87.
- Wergeland, S., Torkildsen, O., Myhr, K.M., Aksnes, L., Mork, S.J., Bo, L., 2011. Dietary vitamin D3 supplements reduce demyelination in the cuprizone model. *PLoS One* 6, e26262.
- Wergeland, S., Torkildsen, O., Myhr, K.M., Mork, S.J., Bo, L., 2012. The cuprizone model: regional heterogeneity of pathology. *APMIS* 120, 648–657.