NOVEL ESTROGEN RECEPTOR BETA LIGAND THERAPEUTICS

By

Devang Deshpande

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University of California, Riverside

Approved

Dr. Seema Tiwari-Woodruff

School of Medicine: Biomedical Sciences

Dr. Begoña Echeverria, Howard H Hays, Jr. Chair

University Honors

ABSTRACT

Multiple sclerosis (MS) is an autoimmune, neurodegenerative disease of the central nervous system in which the blood brain barrier is compromised and autoimmune inflammatory response damages myelin, a protective cover that encases axons leading to abnormal information processing and neuronal damage. Without myelin protecting the axons, signals that are sent between neurons are significantly weakened, and in some cases fully inhibited. Due to research over the years, there are now various therapies in the market that are used to treat MS. However, these drugs decrease the inflammatory response but do not initiate functional recovery or neuroprotection. At most, these drugs ease the symptoms of MS and decrease the progression of the disease. The Tiwari-Woodruff lab and others have shown the superior efficacy of a class of estrogen receptor beta ligands that help add new myelin in demyelinating axons in therapeutically treated mouse models of MS. This project will test the efficacy of novel estrogen receptor β (ERβ) ligands to induce functional remyelination using a demyelinating mouse model. In this study, we fed mice a toxic cuprizone diet to induce chronic demyelination in parts of the CNS for 12 weeks. Afterwards, the mice were returned to normal diet and treated with our novel ERβ ligands for 3 weeks, allowing us to evaluate the degree of remyelination, and thus, the efficacy of the drug over a period of time. To analyze the degree of remyelination, we performed immunohistochemistry (IHC) on brain sections that contain the largest white matter area in the brain called the corpus callosum. The corpus callosum is a bundle of nerve fibers that connect the left and right brain hemispheres and is demyelinated with cuprizone diet and remyelinated after switching to normal diet. The results showed an overall increased amount of myelination of callosal axons in the ERβ ligand treatment groups accompanied by a decrease in inflammation

as compared to the vehicle treated group. Overall, the treatments seem to be promising, suggesting future applications as potential therapeutic agents.

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I would like to extend my gratitude to my mentors: Dr. Seema Tiwari-Woodruff, Moyinoluwa Ajayi, Micah Mae Feri, and Alyssa Anderson, for guiding and supporting me throughout my time in the STW lab. Being a part of this lab gave me my first insight into what the field of research is like. From technical skills like laboratory techniques and designing experiments, to academic skills like engaging with research papers and creating research posters, this lab gave me the chance to explore and learn all of these facets. These are skills I am grateful to have learned, and are skills that I will carry forward throughout my academic career.

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INTRODUCTION

Multiple sclerosis (MS) is an autoimmune, neurodegenerative disease of the central nervous system. It is responsible for affecting approximately 2.8 million people worldwide and is a leading cause of non-traumatic disabling diseases in young adults (Dobson & Giovannoni 2013). In MS, the cells in the body that are responsible for immunity to outside pathogens attack myelin, a protective cover that encases the axons on a neuron. Axons are responsible for the transmission of nerve signals from one neuron to another. When myelin is damaged, signal transduction can occur at a lower rate or can be blocked completely, leading to a number of potential symptoms such as motor and cognitive impairment (Dendrou et al. 2015). Due to the research that has evolved over the years, current therapeutics are used to mitigate symptoms such as Betaferon and Glatiramer acetate. Betaferon reduces relapse rates by blocking gamma interferon signaling, therefore reducing inflammation and disease (Filippi et al. 2018). Glatiramer acetate is a compound that mimics components of myelin and modulates the immune response by shifting T cell populations from pro-inflammatory helper T cells (Th1) to anti-inflammatory helper T cells (Th2). This shift reduces inflammation in the central nervous system and helps prevent further tissue damage (Tselis et al. 2007). This aids in mitigating inflammation, and similar to interferon beta medications, slows down the progression of the disease. While these therapies are effective at managing symptoms and reducing inflammation, they do not prevent the underlying neurodegeneration or actively promote remyelination and repair of damaged tissue.

In the past decade, there has been promising research on the effect of estrogen on the onset of MS disease (Charabati et al. 2023). Studies over the years have shown that estrogen has a positive impact on the development and progression of the disease. Most specifically, estrogen

receptor beta (ERβ) ligand mechanism has been studied and has shown much promise. Chloroindizone, also referred to as indazole chloride, is a selective ERβ ligand that has been well investigated by our lab. This specific ligand has been shown to promote remyelination and reduce the inflammatory response in the EAE mouse model of MS (Karim et al. 2018). Experimental autoimmune encephalomyelitis (EAE), is a clinically relevant model used to examine demyelination induced by an autoimmune response. In the EAE model, mice are injected with myelin protein alongside an adjuvant that contains killed bacteria. This combination causes the immune system to mistakenly recognize myelin as a foreign invader, triggering a strong inflammatory response. As a result, the immune system targets and attacks myelin, leading to demyelination and inflammation in the central nervous system.

As it pertains to this project, we will be looking at chloroidazole analogues and their effect on remyelination and inflammation in the context of multiple sclerosis. Chloroindazole analogues differ from chloroindazole with regards to R groups added onto the base of chloroindazole. These added R groups are the key to enhancing the efficiency of the drugs and aiding in the recovery of the disease. Along with the EAE mouse model to study multiple sclerosis, our lab additionally utilizes a second model called the cuprizone model to study the effects of the disease. In the cuprizone model, mice are fed a diet containing cuprizone, a copper-chelating agent that selectively targets and kills oligodendrocytes which results in chronic demyelination. The cuprizone model is ideal to measure demyelination because it doesn't result in a primary inflammatory response. This results in less confounding variables being present, allowing us to isolate demyelination activity. We will be focusing on a 12 week cuprizone diet, as compared to a six or nine week diet, in order to examine the maximum amount of demyelination taking place in the brain tissue. The focus of our study will be the corpus callosum

region of the brain, composed of the most white matter tracts containing highly concentrated myelinated axons. We will be using our therapeutics of chloroindole analogues to then measure the differences in the tissue when switched to a normal diet. This will allow us to measure the efficacy of the therapeutics in question.

MATERIALS AND METHODS

In this study, female C57BL/6 mice (8–10 weeks old) were fed a 0.2% cuprizone diet for 12 weeks to induce chronic demyelination. Cuprizone is a copper chelating agent that kills oligodendrocytes and is an effective model to look at demyelination. Following the 12-week exposure, mice were returned to a normal diet and treated with two estrogen receptor beta (ERβ) ligand analogues, K102 and K110, for three weeks to assess their potential in promoting remyelination and recovery.

A total of five groups were used in the study, with 4–5 mice per group. The control group (Normals) received neither the cuprizone diet nor treatment and represented healthy, non-diseased brain tissue. The demyelination group (12wk+DM) was fed cuprizone for 12 weeks without any subsequent treatment, serving as the disease model to measure the extent of demyelination. The vehicle group (3wkRM+V) also underwent 12 weeks of cuprizone exposure and was then given the treatment vehicle for three weeks instead of the active compounds. This group allowed us to confirm that the vehicle itself had no therapeutic effect. Finally, the two treatment groups (3wkRM+K102 and 3wkRM+K110) received the same cuprizone diet for 12 weeks and were then treated with K102 or K110, respectively, for three weeks. These groups allowed us to evaluate the therapeutic potential of each ERβ ligand analogue in reversing demyelination and promoting neural repair.

After this time period, the mice were sacrificed and perfused. The brains were collected, and were embedded in gelatin. After embedding the brains, the brains were frozen in a -20°C freezer. Freezing the brains allowed us to make the tissue firm enough to be able to cut through it with a cryostat. The process of sectioning the brains using a cryostat involved mounting the frozen brains on the chuck of the cryostat. After mounting onto the chuck, the brains were cut into thin coronal sections of 40µm. The sections were collected in 24-well plates, and upon collection, each well was filled with a solution of PBS and azide. PBS stands for phosphate buffered saline, and is used to hydrate tissue, while sodium azide prevents bacterial growth, allowing us to store the tissue for long periods of time. After collection, the plates were put in a 4°C fridge for storage. Multiple plates were made with each row containing our five groups. These plates were used to then perform immunohistochemistry.

Immunohistochemistry is a technique that allows us to visualise specific proteins in tissue through fluorescent markers. In regards to the scope of this study, markers via immunohistochemistry were used in order to measure and account for the extent of demyelination, remyelination, and inflammation that took place in the brain tissues. After cutting the brains using the cryostat and sectioning them into 24-well plates, the brain sections were washed multiple times using PBS (phosphate buffered saline) in order to remove debris and any additional extraneous particles present. The brain sections were incubated with a permeabilization buffer containing NGS (normal goat serum) and triton-X, and phosphate buffered saline in order to make the cell membrane more porous for binding to antigens. After the permeabilization buffer, the sections were incubated with blocking buffer, containing normal goat serum and phosphate buffered saline in order to minimize non-specific binding of antigens. Next, the tissues were washed and incubated with primary antibodies, following which they were

then incubated with secondary antibodies with washes in between. After the staining process, the sections were mounted on slides, dried overnight, and then coverslipped with fluoromount. These slides were used to then put under a fluorescent microscope and image the region of interest.

We used multiple stains in order to evaluate the extent of factors such as demyelination, remyelination, axonal integrity, and neuroinflammation. We used myelin basic protein (MBP) as a marker for measuring the amount of myelin present in healthy tissue compared to demyelinated tissue and treated tissue. Decreased MBP staining signifies demyelination, which is expected to be seen in the diseased animals in response to the cuprizone diet, while high MBP staining indicates the presence of myelin, which should be seen in healthy brain sections. CC1, an antibody responsible for recognizing adenomatous polyposis coli (APC) protein, was used to mark mature oligodendrocytes, which are glial cells responsible for myelinating axons. Increased CC1 staining signifies oligodendrocyte survival and differentiation of precursor oligodendrocytes into mature oligodendrocytes, whereas decreased staining signifies oligodendrocyte loss. Additionally, we used NFM (neurofilament marker) to measure axonal integrity. Increased staining of NFM shows preserved axonal structure, while decreased staining suggests axonal degeneration. To assess the level of neuroinflammation, we used GFAP (glial fibrillary acidic protein) to mark reactive astrocytes. Increased levels of GFAP signify astrocyte activation as a response to inflammation, whereas decreased levels of GFAP signify non-reactive astrocytes. Lastly, we used CD45 (cluster of differentiation 45) as a pan-leukocyte marker to determine immune cell infiltration. High staining would indicate an increased immune response with peripheral immune cells infiltrating into the brain parenchyma, while low staining would indicate a decreased immune response.

Imaging was done using the software slidebook 6 with 10x, 20x, and 40x objectives. Images were taken in Z-stacks, a method in which brain section images are captured at various different focal points in respect to the thickness of the tissue. After taking images at a range of various focal points, a composite image is constructed that overlays all the images at various points together. Images were captured using two fluorescence channels, cy3 (emission range: 550 nm – 575 nm) and cy5 (650nm-670nm), each corresponding to the different emission spectra of the secondary antibodies used in the immunohistochemistry staining process.

After imaging, analysis was done using the software ImageJ. Two different methods for quantification were used: density analysis and cell count. Density analysis involved selecting a region of interest (ROI) in the corpus callosum, and afterwards measuring the intensity of fluorescence for that region of interest. This method was particularly useful for comparing relative expressions of MBP, measuring myelin; NFM, measuring neurofilament; and GFAP, measuring reactive astrocytes. The second method of quantification used was cell count, in which individual cells were manually counted and compared across the five treatment groups. This method was particularly useful for markers CC1, measuring mature oligodendrocytes; and CD45, measuring immune cells. Using a combination of density analysis and cell counts as distinct methods for quantification, we were able to obtain a more comprehensive view of changes occurring in brain tissue across our various groups.

RESULTS

ERβ ligand treatment increases myelination in the CPZ model: Density analysis revealed significant differences in MBP staining across the five experimental groups. The control group, which did not receive cuprizone or any treatment, showed the highest level of MBP expression, with an average percent area of 52.0%. This is evidence of the abundant presence of myelin as marked by healthy brain tissue. In contrast, the demyelination group (12wk DM), which was a result of the 12 week cuprizone diet, showed significantly lower MBP staining, with the average percent area of the stain being 25.5%, nearly half the percent staining when compared to the normals. The vehicle-treated group (3wk RM+V), which was put on the same 12 week cuprizone diet to induce chronic demyelination but instead of an active compound received a vehicle treatment, showed very similar MBP percent area (25.1%) to the demyelinated group. This suggests that the vehicle solution alone had no significant impact on promoting myelin repair or preventing further myelin degradation, validating its use as a neutral control. Moving on to the treatment groups, the first treatment group, (3wk RM+K102), was shown to have a significant increase in MBP staining, with a percent area of 39.5%, showing evidence of myelin repair. Similarly, the second treatment group, (3wk RM+K110), showed an MBP area of 34.9%, also higher than the disease and vehicle groups. Both treatment groups were shown to have a considerable increase in the myelin content when compared to the disease groups. This increase in MBP expression indicates that both ERβ analogues had a significant effect on remyelination.

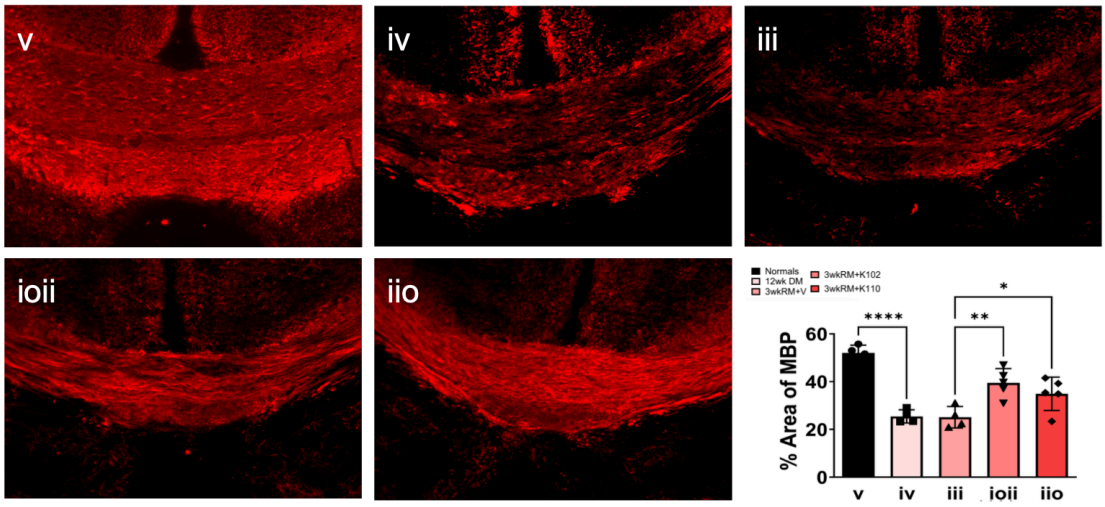

Figure 1: MBP staining and quantification

ERβ ligand treatment promotes oligodendrocyte survival in the CPZ model: Cell counts revealed significant differences in CC1-positive cells across the five experimental groups. The control group, which did not receive cuprizone or any treatment, showed the highest level of CC1 expression, with average cell counts of 205.3 cells. This is evidence of a healthy oligodendrocyte population as marked by healthy brain tissue and standard physiological conditions. In contrast, the demyelination group (12wk DM), which was a result of the 12 week cuprizone diet, showed significantly lower CC1 staining, with the average cell counts being 8.8 cells. This marked a severe loss of oligodendrocyte cells. The vehicle-treated group (3wk RM+V) that underwent the 12 week cuprizone diet to induce demyelination followed by administration of vehicle treatment showed a similar loss of oligodendrocyte cells, with the average cell count being 11.7 cells. Again, this suggests that the vehicle solution alone had no significant impact on protection of oligodendrocyte cells or promoting precursors to mature into mature oligodendrocytes, once again validating its use as a neutral control. Moving on to the treatment groups, the first treatment group, (3wk RM+K102), was shown to have a significant

increase in CC1-positive cell counts, with average cell counts extending to 183 cells, showing evidence of restoration of oligodendrocyte cells. Similarly, the second treatment group, (3wk RM+K110), also showed a significant increase in cell counts, extending to average cell counts of 196.5 cells. Given these results, we can conclude that both treatment groups were shown to restore oligodendrocyte populations when compared to the disease groups by means of offering protection of existing oligodendrocyte cells and promoting the maturation of precursor cells. This increase in CC1-positive cell expression indicates that both ERβ analogues are viable treatments for oligodendrocyte restoration.

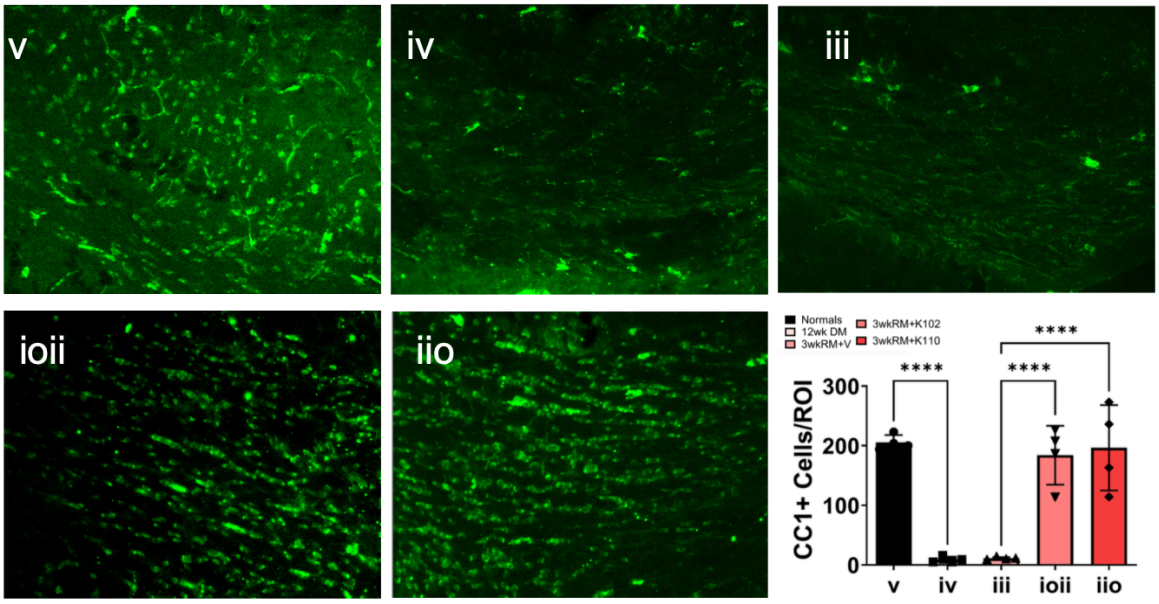

Figure 2: CC1 staining and quantification

ERβ ligand treatment preserves axonal integrity in the cuprizone model: Density analysis of NFM (neurofilament medium chain) staining revealed significant differences in axonal integrity across the five experimental groups. The control group, which did not receive cuprizone or any treatment, showed the highest level of NFM expression, with an average percent area of 55.1 percent. This high level of NFM staining is consistent with intact and

healthy axonal networks, as expected for mice under normal physiological conditions. In contrast, the demyelination group (12wk DM) marked with 12 weeks of demyelination showed a marked reduction in NFM expression with an average percent area of 23.9 percent, indicating substantial axonal disruption. The vehicle-treated group (3wk RM + V), which received vehicle solution following the 12-week cuprizone diet, similarly showed low NFM expression, averaging 15.50 percent area. Treatment with ERβ analogues resulted in a notable increase in NFM expression. The 3wk RM + K102 group showed an average percent area of 36.29 percent, while the 3wk RM + K110 group displayed a 39.02 average percent area, both significantly higher than the disease and vehicle groups. These findings suggest that both treatments provided axonal protection and supported axonal recovery. The increase in NFM expression in both treatment groups indicates that ERβ activation may play a role not only in remyelination but also in preserving and restoring neuronal structural integrity.

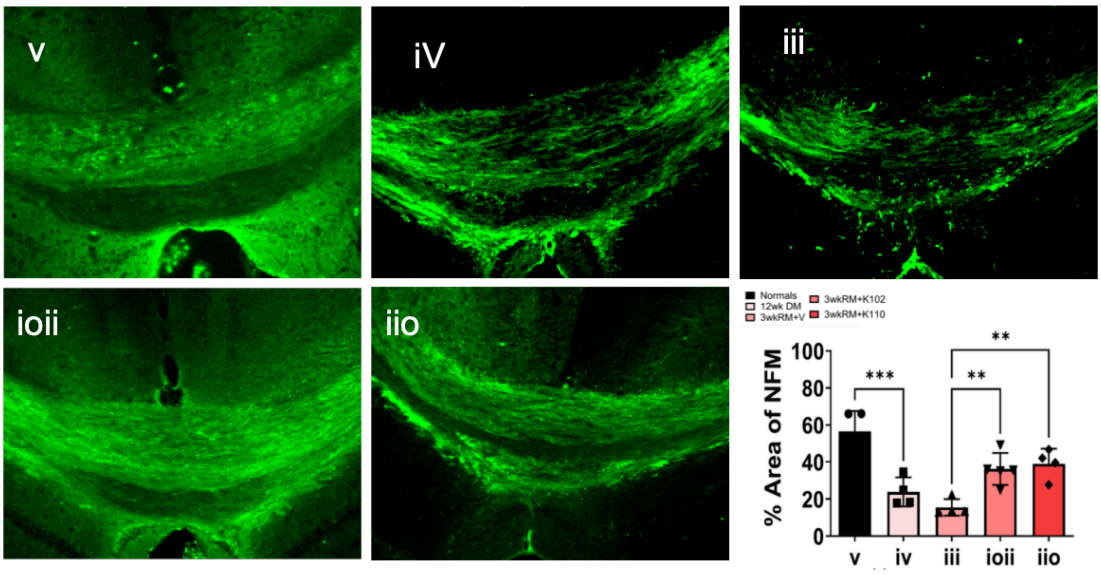

Figure 3: NFM staining and quantification

ERβ ligand treatment does not significantly reduce astrogliosis in the cuprizone model: Quantification of GFAP (glial fibrillary acidic protein) staining via density analysis,

used to assess astrocyte activation and gliosis, revealed elevated expression across all groups exposed to cuprizone, without significant differences between the disease and treatment groups. The control group, which did not receive cuprizone or treatment, showed the lowest GFAP expression, with an average percent area of 16.6%, consistent with baseline astrocyte activity in healthy, uninjured brain tissue. In contrast, the demyelination group (12wk DM) exhibited a significant increase in GFAP expression, with an average percent area of 37.7 percent, while the vehicle-treated group (3wk RM + V) having an average percent area of 31.1 percent, both indicating a strong presence of reactive astrocytes in response to the demyelinating effects of cuprizone. Both treatment groups, 3wk RM + K102 and 3wk RM + K110, showed GFAP percent areas of 31.0% and 27.8%, respectively. These values were either slightly lower or about the same as compared to the demyelinated groups, and thus not significantly enough to conclude that the treatments had a substantial effect on reducing inflammation and astrocyte reactivity in the region of interest. This could be due to the fact that inflammation could still be present during the recovery phase as astrocytes still remain reactive during repair. Another possibility is that the ERβ ligand treatments have limited effects on astrocytic pathways.

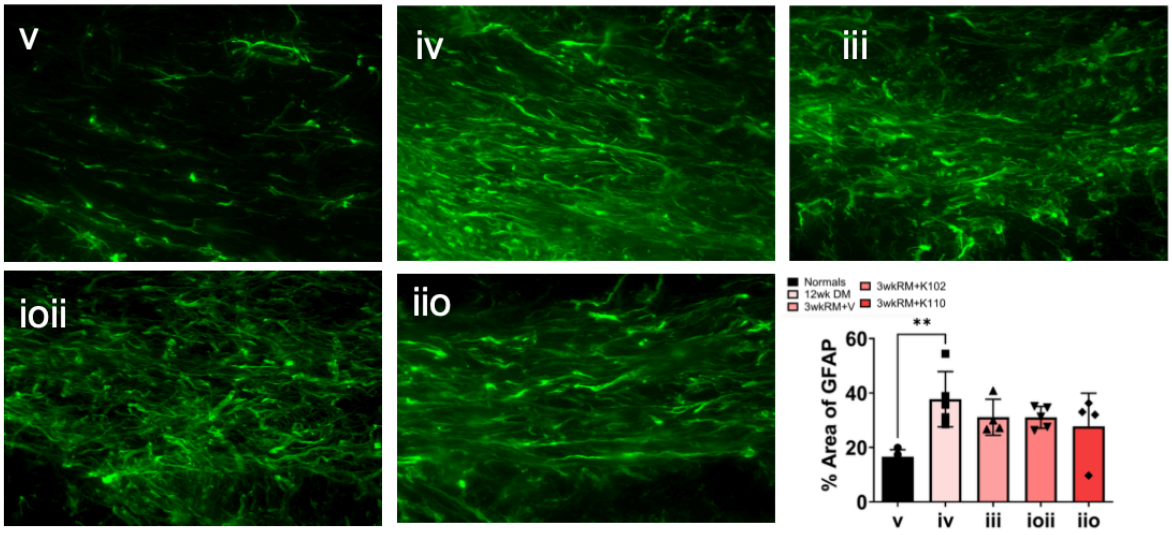

Figure 4: GFAP staining and quantification

ERβ ligand treatment reduces immune cell infiltration in the cuprizone model: To assess immune cell infiltration, we quantified CD45, a pan-leukocyte marker that labels infiltrating peripheral immune cells. The control group exhibited minimal CD45 staining, with an average cell count of 0.25 cells in the region of interest, indicating extremely low baseline presence of immune cells under normal conditions. The demyelination group (12wk DM) showed a significant increase in CD45 expression, with an average cell count of 12.4 cells, confirming that the cuprizone diet not only induces demyelination but also provokes a strong immune response. The vehicle-treated group (3wk RM + V) had a similarly high CD45 cell count, with the average being 12.0 cells, once again exhibiting that the vehicle alone had no impact on reducing immune cell infiltration. Treatment with ERβ analogues resulted in significant reduction in CD45 expression. The 3wk RM + K102 group had an average cell count of 6.5 cells, showing reduction of immune-cell infiltration, while the 3wk RM + K110 group also demonstrated significant decrease, with cell counts averaging 3.2 cells. These findings indicate that both compounds were effective in reducing the immune response, and potentially limiting

the recruitment of immune cells. These results are in strong support of the immunomodulatory potential of ERβ ligands in chronic demyelination models.

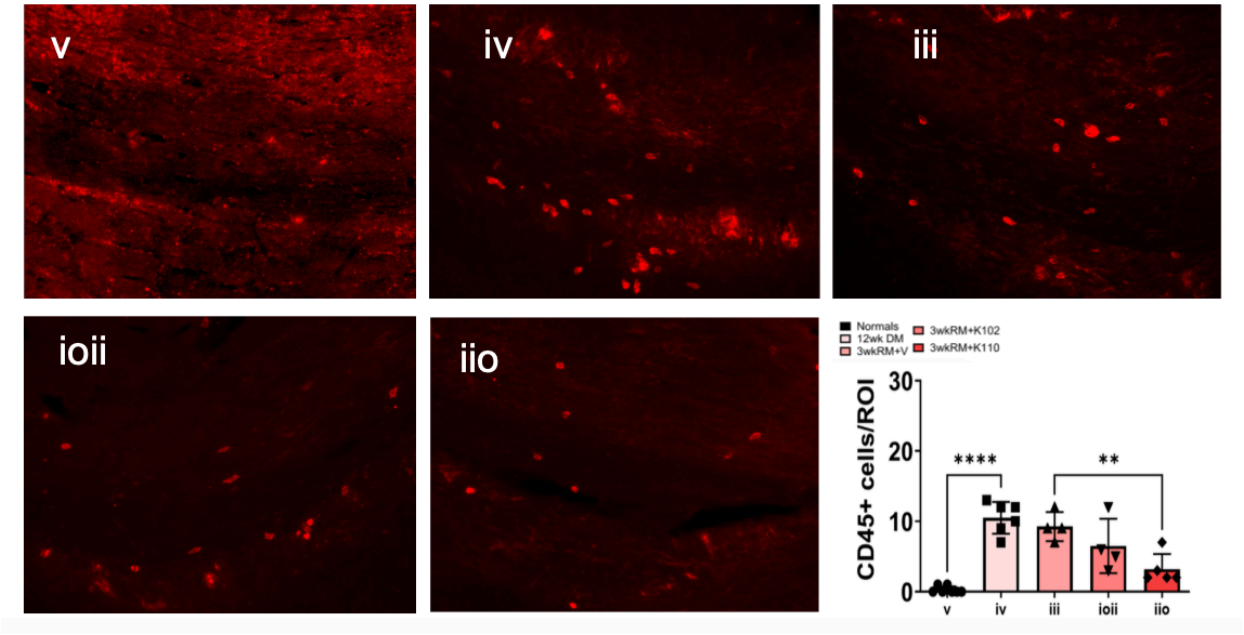

Figure 6: CC1 staining and quantification

DISCUSSION

In this project, we set out to examine whether two ERβ ligand analogues, K102 and K110, could help in reducing damage done by multiple sclerosis in the central nervous system by replicating disease conditions using the cuprizone model. By looking at various markers of remyelination, inflammation, and those that represent general health of brain cells, we were able to better distinguish how well these compounds work in supporting recovery after demyelination, as done so by the cuprizone diet. Multiple sclerosis is characterized by the loss of myelin, which is the insulating layer that surrounds axons and is involved in transmitting effective neuronal signals. This demyelination is largely due to damage to oligodendrocytes, cells responsible for myelin production. Oligodendrocytes go through various stages of development, starting as proliferating oligodendrocyte precursor cells (OPCs) to then differentiating into immature

oligodendrocytes and then eventually into fully myelinated cells. When oligodendrocytes are damaged or degraded, as seen in MS and in the cuprizone model we used in this study, neuronal signals are severely affected, often leading to motor and cognitive defects. Myelin degradation also has effects on axonal health, making neurons more susceptible to degeneration. In response, glial cells such as astrocytes become reactive and contribute to inflammation. Though inflammation generally helps in tissue repair, uncontrolled and chronic inflammation often inhibits this process. Furthermore, immune cells such as microglia and leukocytes infiltrate the brain and contribute to further damage by releasing pro-inflammatory signals. Our study aims to look at the effects of the proposed therapeutics, K102 and K110 to determine whether our ERβ analogues could promote remyelination, support oligodendrocyte survival, preserve axonal integrity, and reduce neuroinflammation.

We began by looking at the effect of our treatments on myelination using the stains MBP and CC1 to measure how the treatments affected myelin content and myelin producing cells–oligodendrocytes. We noticed significantly increased levels of MBP and CC1 than that of the disease or vehicle groups. We saw an increase of MBP staining by nearly 30-40% from the disease groups to the treatment groups, demonstrating significant reversal of demyelination and promotion of myelin restoration. A similar trend was seen with the CC1 stain, with significantly higher CC1-positive cells in the treatments compared to the disease groups, exhibiting oligodendrocyte survival and differentiation of precursor cells into mature oligodendrocytes. Together, the combined increase of MBP and CC1 staining supports that ERβ ligand treatment enhances both the rebuilding of myelin, as well as supporting underlying machinery–oligodendrocytes–responsible for myelination. These findings are crucial as current therapeutics on the market do not offer remyelinating properties, and as a result patients with

multiple sclerosis are unable to experience a reversal in the progression of their disease. With the therapeutics in this study showing promising results of remyelination, they may address the unmet need for directly promoting myelin repair in the central nervous system in the scope of multiple sclerosis treatments.

We then looked at axonal health using NFM staining. The control mice had strong NFM signals, indicative of uninjured and healthy mice, but those signals were significantly weaker in the disease groups. This suggests that axons were affected as a secondary result of demyelination. As demyelination takes place, axon degeneration is prevalent as the protective cover that encases axons gets stripped off of these structures, and this is what we saw in our study as well. However, in the treatment groups, we saw much better NFM expression, indicating that the treatment compounds may have helped keep axons intact and supported their recovery. This was further evidence that our ERβ therapeutics were effective in not just remyelination but also in protecting and strengthening underlying structures as a result of it.

One of the areas where we didn't see as big a difference was in GFAP expression, which we used as a marker for reactive astrocytes. GFAP was high in all the cuprizone-exposed groups, including the treatment groups. And while there was a slight decrease in the K110 treatment group, it was not of much significant value. This may be due to the fact that astrocytes remain active even during the recovery phase, and as a result GFAP expression may not decrease in this phase due to their sustained reactivity. It could also signify that these particular treatments do not strongly affect astrocytic pathways. It is also possible that three weeks of treatment wasn’t long enough to see a decrease in gliosis, and a longer recovery window might be needed.

On the other hand, CD45 staining—a marker for immune cell response—showed significant results. There was a large increase in CD45-positive cells in the demyelinated groups,

which was expected of diseased mice as myelin loss creates a pro-inflammatory environment, causing peripheral immune cell activation and infiltration into the brain parenchyma. However, both the K102 and K110 treatment groups showed a noticeable drop in immune cell infiltration, pointing to the fact that these compounds have strong anti-inflammatory effects, either by way of reducing the inflammatory response or limiting immune cell infiltration.

Overall, these findings strongly suggest that ERβ ligand analogues had positive effects with respect to several metrics we set out to observe: it helped boost remyelination, restore mature oligodendrocyte numbers, reduce axonal damage and strengthen axons, and limit immune cell infiltration. In regards to not seeing any significant difference in GFAP expression and astrocyte reactivity, it would be interesting to see how that changes if we raise the treatment period from three weeks to something like five or six weeks. Perhaps we may observe decreased inflammation and astrocyte reactivity further along into the recovery phase. Regardless however, this study proved to be successful in the majority of the metrics we set out to observe, and the therapeutics as a result prove to be strong candidates for further testing and clinical trials.

CONCLUSION

In conclusion, this study showed that the ERβ ligand analogue treatments, K102 and K110 had strong potential in helping with recovery and reversing effects of multiple sclerosis after chronic demyelination caused by a cuprizone diet. While current therapeutics on the market primarily focus on suppressing the immune response and slowing disease progression, they do not offer many options of repairing damage and restoring lost function. As a result, patients are left with long term motor and neurological deficits that seldom return to baseline functional levels. With the results of our study, we were able to conclude that the ERβ ligand therapeutics showed potential not just in decreasing the rate of disease progression as many therapeutics on

the market today do, but also contributed to recovery in the central nervous system and reversal of many of the effects of multiple sclerosis by promoting remyelination and offering neuroprotection, which are lacking in current treatment options. Given the positive effects of the therapeutics we tested in this study, we are optimistic and enthusiastic to see how they span further in research and clinical application, and hopefully one day in the near future can improve the quality of life of many patients experiencing multiple sclerosis across the world.

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