Inhibition of Muscarinic Receptor Subtypes and Effects on Oligodendrocyte Differentiation

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Abstract

Oligodendrocytes are the myelinating cells of the central nervous system, and these cells and their myelin sheaths are the autoimmune target in multiple sclerosis (MS). Increasing the differentiation of OPCs to myelinating OLs is a promising method for treating MS. Recent clinical trials have revealed a positive effect of clemastine, an antihistamine/muscarinic antagonist, in stimulating myelin repair in patients with MS. This study is investigating the combinatorial effects of muscarinic receptor antagonists darifenacin (M1) and pirenzepine (M3) on the rate of oligodendrocyte (OL) maturation. Combinatorial treatment of cultured oligodendrocyte progenitors increased myelin-specific gene expression and increased the percentage of mature OLs in the cultures compared to control. Combination treatments were also studied in the larval zebrafish model. RNA was isolated and analyzed for changes in expression of myelin-specific genes MPZ and MAG. Several dosages of combinatorial treatment and time points were tested, but no significant changes in larval zebrafish gene expression were identified. However, muscarinic agonist, cevimeline, was shown to reduce expression of myelin-specific genes MPZ and MAG, which supports the hypothesis of involvement of the muscarinic pathway in myelination. Effects of muscarinic antagonists were evident in an incomplete maturation model (cultured OLs), but do not appear to enhance the vigorous myelination program of zebrafish larvae.

Introduction

Significance

Multiple Sclerosis (MS) is a currently incurable autoimmune disease that affects over 2.3 million people worldwide[1]. There is no cure for MS, there are only treatments to try to manage the symptoms and prevent more damage from being done. The overall goal of this research is to discover new ways to repair damage done by demyelinating diseases such as MS, as well as new ways to encourage recovery on a cellular level.

Model Organisms

For this research, an *in vivo* and an *in vitro* model were used. The *in vivo* model was larval zebrafish, which have a transparent larval stage that allows for easier visualization of internal structures. An advantage to using zebrafish is their rapid development. Oligodendrocyte progenitor cells (OPCs) begin differentiation at 24 hours past fertilization (h.p.f.), followed by migration at 36 h.p.f. and begin producing myelin at 72 h.p.f.[2] Zebrafish are similar in genetic structure to humans, sharing approximately 70% of genes with them.[3]

Pharmaceutical Information

Currently, research is being done to determine how to promote remyelination. So far, it has been found that using a muscarinic receptor antagonist such as Darifenacin increases the rate at which remyelination occurs in demyelinated cultures.[4] Antagonists to both the M1 receptor and M3 receptor have shown to promote remyelination in Darifenacin and Pirenzepine are both muscarinic antagonists, Darifenacin is an M1R antagonist, whereas Pirenzepine is an M3R antagonist. To determine impact of different receptors, each were tested independently. A combinatorial test was performed as well to determine if there was a significant effect. Studies[5] have shown that agonizing the muscarinic pathway prevents efficient remyelination in culture and mice models, to replicate this result, cevimeline, a muscarinic agonist was tested. Research has also shown that antagonizing histamine pathways can increase the rate of myelination. To replicate this, Clemastine, which is a histamine antagonist, as well as a M1R and M3R muscarinic receptor reverse antagonist, was tested. Clemastine has been seen to increase remyelination in previous studies. [6]

Methods

In vitro model

Mixed Glial Cell Preparation: Cortices were dissected from 1-day postnatal rat pups, and meninges were removed. The tissue was digested and mechanically dissociated to obtain glial cells. Cells were cultured for 10-12 days.

OPC Isolation and Culture: Differential shakes were used isolate OPCs. The OPCs were plated on poly-d-lysine coated plates and flasks. OPC cultures were fed every two days with media containing growth factors PDGF and FGF to inhibit differentiation. After reaching desired confluency, cells were plated on 60 mm dishes and in wells for treatment, then isolated for RNA analysis and immunostaining, respectively.

Treatment of Cells: Cells were treated with M1R and M3R antagonists Darifenacin and Pirenzepine (Dar + Pir), alone and in combination, or DMSO, which served as the control.

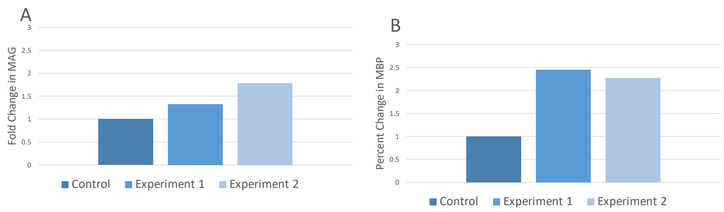
Gene Expression Analysis: RNA was isolated using Trizol then purified using a PureLink RNA Mini kit. RNA was converted to cDNA using a ThermoFisher High-Capacity RNA-to-cDNA kit. Taqman probes MAG and MBP were used to analyze gene expression using a StepOnePlus Real Time PCR system.

Immunostaining: Cells were fixed using paraformaldehyde and then stained using A2B5 or O1 primary antibodies, which were identified with fluorescently-labeled secondary antibodies.

In vivo model

Treatment for zebrafish followed flow chart depicted in Figure 2. Experimental procedures were done by altering the time when treatment began, what treatments were used, the dosages of the treatments, and the duration of the treatment. A summary table is seen in Figure 3 with the what the exact treatment conditions were.

Effect of Muscarinic Antagonists on Maturation of Cultured OPCs



C

Treatment	% O1+ Cells
DMSO	84.6
Dar.	97.8
Pir.	93.4
Dar./Pir.	97.6

Figure 1. Darifenacin (Dar) and Pirenzepine (Pir) combinatorial treatment increases MAG (A) and MBP (B) expression in oligodendrocytes and percentage of O1+ cells (C). OL cultures were treated with Dar and Pir in combination for four days.OPCs treated with Dar or Pir alone had no significant increase in expression of MAG or MBP (data not shown). Gene expression is quantified in terms of RQ value. Percentage of O1+ was quantified using primary and secondary antibody for immunostaining.

A

B

trial number	Tx Start	Tx End	Dar + Pir	Clemastine	Cevimeline	PP2
1	2 dpf	4 dpf	1 uM			
2	2 dpf	4 dpf	1 uM, 10 uM			
3	2 dpf	4 dpf	10 uM			
4	1 dpf	4 dpf	10 uM, 50 uM			
5	4 dpf	8 dpf	10 uM, 50 uM			
6	4 dpf	8 dpf	10 uM, 50 uM			
7	4 dpf	8 dpf	50 uM		200 uM	
8	2 dpf	4 dpf				1 uM, 15 uM
9	4 dpf	8 dpf			200 uM	
10	3 dpf	7 dpf	50 uM	20 uM		

Figure 2. Treatment Paradigm for In Vivo Model Zebrafish. Flowchart (A) was followed for all experiments. Summary table (B) depicts the range of different treatment protocols that were used during this study.

Drug Treatment Effects on Myelin-specific Gene Expression in Larval Zebrafish

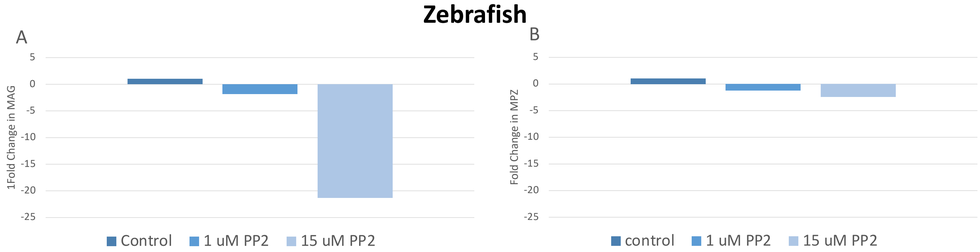


Figure 4. Inhibition of SRC kinase family decreases expression of MAG (A) and MPZ (B) in larval zebrafish using DMSO and PP2 treatment (2 – 4 dpf). Drugs were replaced daily. Gene expression is analyzed with the **ΔΔ**Ct method and displayed in terms of Fold Change (RQ value). There was a (21-fold decrease in MAG, as well as a slight (2-fold) decrease in MBP.

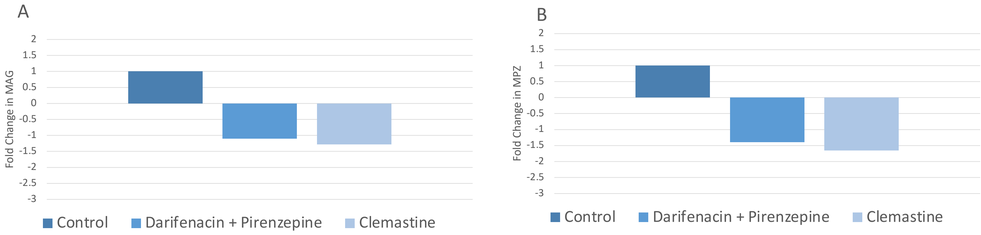


Figure 5. Expression of MAG (A) and MPZ (B) decreases in larval zebrafish following treatment with muscarinic and histamine antagonists (2-4 dpf). Drugs were replaced daily. Gene expression is analyzed with the **ΔΔ**Ct method and displayed in terms of Fold Change (RQ value). Treatment resulted in a slight (<2-fold) decrease in MPZ expression.

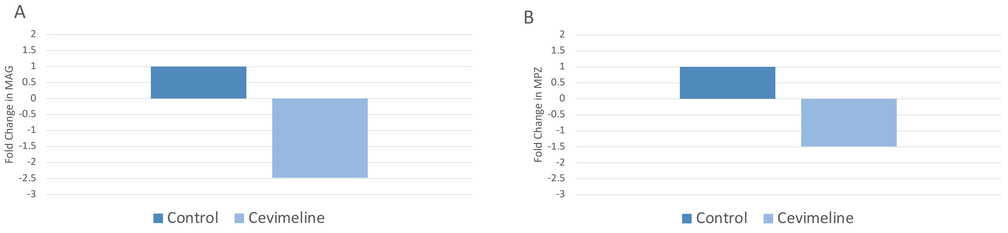


Figure 6. Muscarinic agonist Cevimeline decreases expression of MAG (A) and MPZ (B) in larval zebrafish following treatment (4 – 8 dpf). Drugs were replaced daily. Gene expression is analyzed with the **ΔΔ**Ct method and displayed in terms of Fold Change (RQ value). Cevimeline had a greater effect on MAG expression than MPZ expression.

Conclusions

- The combination of M1 and M3 muscarinic receptor antagonists had the greatest effect on oligodendrocyte maturation in culture.
- This study replicated previous studies demonstrating a negative effect on the myelination program by Src kinase inhibitor PP2 and the muscarinic agonist Cevimeline. Myelin gene expression has inhibited by both compounds in our larval zebrafish model.
- Myelination in the larval zebrafish was not enhanced by muscarinic antagonists during normal development.

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Sources

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