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Title: "Preventing oxidative stress-induced changes in mitochondrial dynamics - Investigation of neuroprotective effects of S1P receptor modulators in a semi-automated live imaging trial *ex vivo"*

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ABSTRACT

In multiple sclerosis (MS), immune cells and activated microglia produce reactive oxygen species. These may affect the mitochondrial function in neurons and contribute to neuroaxonal damage and disease progression. Fingolimod and siponimod are sphingosine-1-phosphate (S1P) receptor modulators that regulate inflammatory responses and myelination. Fingolimod was reported to prevent oxidative stress-induced alterations in mitochondrial morphology. Siponimod is approved for the treatment of secondary progressive MS because of its anti-inflammatory and less understood neuroprotective effects.

This study aimed to investigate the potential neuroprotective effects of siponimod, compared to fingolimod, on neuronal mitochondrial dynamics in oxidatively-stressed living brain tissue in an *ex vivo* model of chronic hippocampal slice cultures using a semi-automated in-house developed image analysis.

We have established a protocol for chronic hippocampal slice cultures and optimized it for live imaging, enabling semi-automated monitoring of neuroaxonal mitochondrial alterations. The assessment of mitochondria was conducted on brain tissue of the mouse strain B6.Cg-Tg(Thy1-CFP/COX8A)S2Lich/J, allowing for the specific detection of neuronal mitochondria using confocal fluorescence microscopy. Oxidative stress was induced with hydrogen peroxide for a period of 24h. Simultaneously, 1 nM fingolimod or siponimod was applied to the tissue culture to assess the effects of S1P-receptor modulators. After 24h of treatment, the mitochondrial morphology and motility were assessed in 2min time lapses.

Under oxidative stress, mitochondria were shorter and smaller compared to negative controls. They covered slightly smaller distances and the fraction of motile mitochondria decreased. Siponimod was able to prevent oxidatively-induced alterations in mitochondrial morphology, while for fingolimod a similar trend was observed. Siponimod partly prevented the decrease in mitochondrial displacement, while fingolimod did not. In addition, both compounds led to an increase in track speed. In slices treated with siponimod or fingolimod, the fractions of motile mitochondria approached that of control slices.

Thus, both siponimod and fingolimod at 1 nM partially prevented oxidative stress-induced mitochondrial alterations in living brain tissue *ex vivo.*