Reviewer #2

Comments

The manuscript, although clearly written and quite relevant to the field, is not adequately presented and needs further experimental confirmation. The work was mainly conducted in vitro and using only the technique of real time PCR to assess the expression of receptors and certain intracellular targets activated following stimulation of these with agonists and antagonists.

Major Revisions

1) I believe that talking about expression of certain receptors using only the real time PCR technique is not completely adequate as post-translational modifications could affect receptor expression. Therefore, assays such as Western blot and immunofluorescence analysis to verify the actual expression and membrane localisation of receptors are necessary for the message of the work. Since specific antibodies for the different receptors are commercially available, I ask that this will be verified, at least for SP1 and SP3 receptors.

Thanks to the reviewer for the comment. It was already demonstrated that rat as well as human Schwann cells express S1P1 protein *in vitro* and *in vivo* (Köhne *et al.,* 2012). Nevertheless, we did another qRT-PCR analysis to verify that Schwann cells which were prepared and cultured in different manner also expressed S1P1 (and other S1P receptors). This verification of receptor expression in our system was a prerequisite for follow up experiments. On protein level, it is known that S1P1 is internalized after binding to Fingolimod, hence protein turnover and localization could be highly dynamic depending on extrinsic signals. Therefore, future experiments have to address the temporal and spatial S1P1 localization as well as posttranscriptional/-translational regulation in cultured Schwann cells as well as *in vivo*. In addition, S1P2-S1P5 should be investigated in more detail on protein level, especially S1P3, which is one of the main receptors regulating the Schwann cell repair phenotype. However, detailed analysis of receptor protein localization and turnover was out of scope of the study. From our point of view, for the present study, verification of S1P1 receptor expression was sufficient for subsequent experiments.

We amended the discussion mentioning the limitations of our receptor analysis (see page 11 and 12 of the revised manuscript).

2) In Figure 1, only the SP3 receptor is shown to be statistically significant. SP2 is not significant? In general, in every graph where the statistic is not present is it because the data is not significant? This should be added and specified in the figure legends.

S1P2 is indeed not significantly higher expressed compared to S1P1 which basically depends on the variance of the relative gene expression between each experiment (fold change from 53.78 to 308.38). However, S1P2 could have a *biological relevance* despite S1P2 upregulation compared to S1P1 is not *statistically significant*. Since S1P2 is out of scope of the manuscript, its role and relevance in Schwann cells will be addressed in future studies.

We agree with the reviewers' comment and added a more detailed explanation in the section *statistical analysis* (see page 16 of the revised manuscript).

3) In figures 2G and 4G housekeeping (such as GAPDH or actin) control is missing for quantification of cJun and PTEN. Even if authors in MM say they loaded the same amount of protein in my opinion a housekeeping protein is necessary.

Thanks to the reviewer for the comment. Perhaps we were not clear enough in describing the method we used to analyse relative protein amounts in Western Blot experiments. The authors have many years of experience in the analysis of proteins (see, e.g., PMID 30592632, Schira *et al.* 2019, *Secretome analysis of nerve repair mediating Schwann cells reveals Smad-dependent trophism*; PMID 26183719, Schira *et al.* 2015, *Characterization of Regenerative Phenotype of Unrestricted Somatic Stem Cells (USSC) from Human Umbilical Cord Blood (hUCB) by Functional Secretome Analysis*; PMID Heinen *et al.*, 2015; *Fingolimod induces the transition to a nerve regeneration promoting Schwann cell phenotype*; PMID: 31202002, Schira-Heinen *et al.*, *Pitfalls and opportunities in the characterization of unconventionally secreted proteins by secretome analysis*; PMID: 32094412, Schira-Heinen *et al.*, 2020, *Functional omics analyses reveal only minor effects of microRNAs on human somatic stem cell differentiation*), thus they know that for reproducible and reliable Western Blot results normalization is a critical step. The most frequently used method for normalization is based on using a housekeeping protein as internal loading control. However, reliability of this normalization method depends on the range where there is a linear relationship between the amount of protein on the membrane and the signal intensity. Since most of the housekeeping proteins, such as GAPDH or actin, are highly abundant, detection signals are mostly out of the linear range which makes a reliable quantification impossible (for normalization saturated bands cannot be used because the strength of detected signal is not proportional to the amount of protein in the corresponding band). To prevent this technical issue we here used the *total protein normalization*, which is based on normalization of the abundance of target proteins to the total amount of protein in each lane. This method is independent of a single loading control and is recognized as the gold standard for quantitative Western Blot analysis (Diller *et al.*, 2021, J Biotechnol. 2021, PMID 33373626; see also PMID: 33950481, PMID: 25059473).

To clarify which quantification method was used description of the method was revised (see materials and methods section, page 16 of the revised manuscript).

4) In line 328-329 the authors argue that Fingolimod mediated induction of p-cJUN is likely to be S1P3. This aspect is not completely clear to me. First, as mentioned above, western blot analyses need to be normalised with housekeeping proteins. In addition, Fingolimod appears to promote both SP1R- and SP3R-dependent cJUN synthesis (Fig 2H and 4H). Furthermore, p-cJUN appears to be dependent on both receptors as the use of specific antagonists causes reduced expression of p-cJUN after treatment with Fingolimod (Fig2K and Fig4K) even if statistical analysis is missing (or is not significant). Therefore, these results need a better explanation.

As already described above, total protein normalization was the method of choice for reliable Western Blot data. Statistical analysis was performed, however, differences were not significant.

We agree with the reviewers comment that c-Jun phosphorylation depends on both S1P1 and S1P3. However, it is known that S1P3 acts via Gαi/0, Gαq and G12/13, the latter of which is known to regulate Jun kinase (JNK) which in turn phosphorylates c-Jun. Therefore, we suggested that c-Jun phosphorylation is directly dependent on S1P3 whereas S1P1 indirectly leads to c-Jun phosphorylation. Since we can only speculate that this is the case, we revised our statement and refer to future studies which should include a detailed analysis of involved pathways.

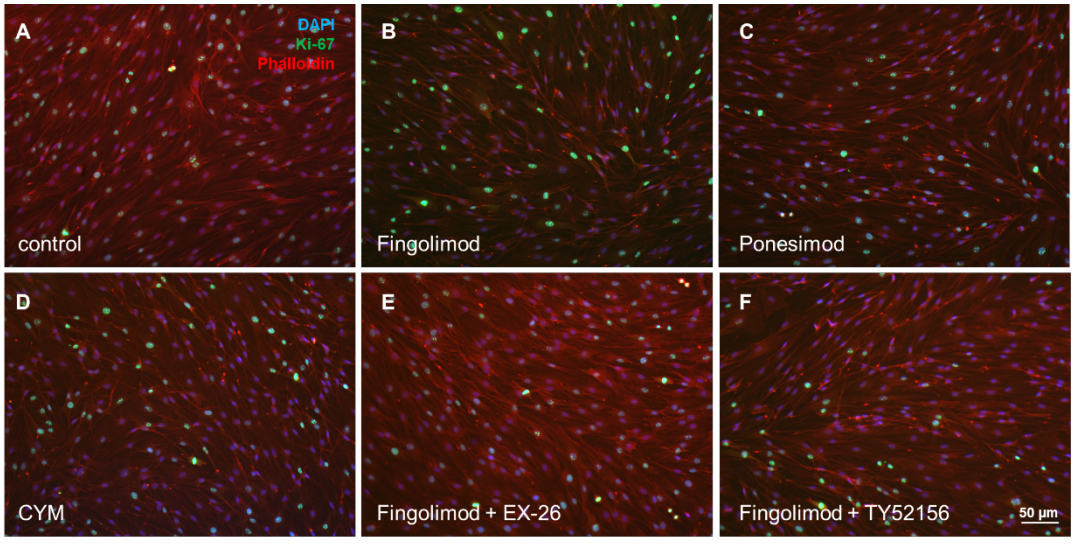
We revised the discussion referring to future experiments investigating c-Jun pathway in more detail (see page 13 of the revised manuscript).

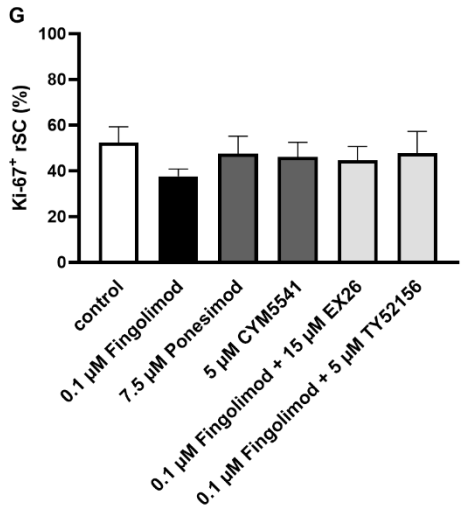
5) Previous results have revealed that S1P induces remodelling of the actin cytoskeleton in Schwann cells, promoting cell migration. These are essential processes for Repairs Schwann cells. It would be interesting to test whether Fingolimod induces a similar morphological change (with IF analysis).

Thanks to the reviewer for the comment. We performed actin staining using phalloidin to investigate changes in actin cytoskeleton as well as morphological abnormalities. However, no obvious changes were observed, under the here applied culture conditions *(see also answer 6. and figure below).* At this point, we also want to emphasize that the primary scope of this study was not to describe new S1P dependent cellular processes in light of peripheral nerve repair but rather to determine receptors involved in the previously described gene/protein regulations (Heinen et al., 2015; Schira et al., 2019).

6) In Kohne et al., 2012, the authors of Arch Neuro showed that lower concentrations of Fingolimod (10nM and 100nM) showed no effect on cell proliferation or viability, but increased activation of caspase 3 and caspase 7 (two markers of apoptosis). In contrast, 1 µM FTY720P showed a decrease in cell proliferation and viability. The different agonists used in the work were employed at a micromolar concentration. Did authors test whether these concentrations impaired cell viability?

We co-stained actin (using phalloidin) with Ki-67, a marker for cell proliferation. No changes in cell density as well as morphology were observed (see figure below) and Schwann cells were still proliferating. DAPI staining revealed no changes in cell nuclei structures and the number of apoptotic bodies was very low under all culture conditions. In addition, 24h after treatment with S1P receptor modulators no significant difference of the proliferation rate (only Fingolimod tended to lead to decreased proliferation rate) was observed indicating that all treated cultures were viable and proliferative. Summarized, no conspicuous cellular changes were observed after treatment of Schwann cells with S1P receptor modulators.





Morphologies and proliferation rates of S1P receptor modulated Schwann cells. Schwann cells were prepared, cultured and treated with S1P receptor modulators as described in the manuscript. Concentrations were the same as for qRT-PCR and Western Blot analysis. (A-F) Schwann cells were incubated for 24h, fixed and stained using/for phalloidin, Ki-67 and DAPI. Cell cultures showed high densities and no obvious morphological changes were observed. (G) Quantification of Ki-67 positive cells showed a proliferation rate between 37.6% (Fingolimod) - 52.4% (control). For statistical analysis, a one-way ANOVA with Tukey's multiple comparison test was used, n=3 biological replicates.

Minor Revisions

Lines 34-37 need citations. I suggest Jessen KR, Arthur-Farraj P (2019), Glia, 67, 421-437 and Rigoni M, Negro S (2020) Signals Orchestrating Peripheral Nerve Repair. Cells, 9.

We agree and included the suggested citations.

Although the aim of the work is to understand which receptor is responsible for Schwann cell transdifferentiation after Fingolimod treatment, I would discuss, in more detail, (in the discussion section) the role of the natural ligand SP1 in the process. Is SP1 express in vivo after nerve injury? If yes, which cells express it? Is it the axis SP1-SP receptor involved in Peripheral nerve regeneration?

The natural ligand S1P is a product of phosphorylation of sphingosine, which is catalyzed by sphingosine kinase I. Hence, S1P as a bioactive sphingolipid is not "classically expressed". For reliable results, S1P could be analyzed by, e.g., LC-MS/MS based approaches (lipidomics) which is time-consuming, expensive and could only be performed by experts. Today, there is no detailed analysis of S1P in peripheral nerves after injury *in vivo*, which however is necessary to understand the role of S1PR-S1P in peripheral nerves. Interestingly, Meyer Zu Reckendorf et al., (2020, PMID 32358558) performed lipidomics in an *ex vivo* peripheral nerve injury model revealing that S1P is lower abundant in murine Schwann cells after injury, whereas in human Schwann cells the effect on S1P level was not significant.

The role of S1P receptors in peripheral neurons was investigated in greater detail and seem to be involved in neurite retraction and extension and are probably implicated in peripheral nerve regeneration (PMID: 29066950). To our knowledge, the role of S1PRs-S1P axis in Schwann cells after peripheral nerve injury is not investigated yet. In future studies, molecular mechanisms as well as functionality of S1PR in peripheral nervous system regeneration should be addressed.

According to the reviewers' suggestion, we discussed the role of S1P-S1PR axis *in vivo* in more detail (see page 13 of the revised manuscript).

Lines 66-67 the sentence "Additionally, the TGFβ-Smad signaling pathway is activated in response to Fingolimod treatment" needs reference

This has now been amended.

Line 75, sentence "Since Fingolimod targets four out of five S1PRs (S1P1, S1P3-5)" needs references

This has now been amended.