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TITLE: A Novel Stress-Activated Inhibitor of Myelination

PRINCIPAL INVESTIGATOR: Maria Laura Feltri

CONTRACTING ORGANIZATION: Research Foundation for the State University, Amherst, NY

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14. ABSTRACT

In Multiple Sclerosis (MS) myelin and axons are damaged. At the beginning of the disease, the brain can repair itself by 'remyelination' of damaged axons. With time, however, the repair ability of the brain deteriorates, and remyelination is no longer efficient or sometimes even possible. We discovered a novel inhibitor or remyelination that is present in MS plaques and we will test if it can be targeted to promote remyelination and protect axons.
So far, we have discovered that this novel inhibitor delays the ability of oligodendrocyte progenitors (the precursors of the cells that will make myelin) to proliferate, migrate and differentiate. We are currently studying the molecular mechanisms by which the inhibitors exert these effects, because this may reveal novel ways to promote myelination. In addition, we have discovered that this inhibitor accelerate also remyelination after injury, using a toxin that deplete myelin-forming cells in an *in-vivo* model. These latest findings confirm that our novel inhibitors could be relevant to promote remyelination after a demyelination attack in Multiple Sclerosis patients.

15. SUBJECT TERMS
None listed.

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1. INTRODUCTION:

In Multiple Sclerosis (MS) myelin and axons are damaged. At the beginning of the disease, the brain can repair itself by 'remyelination' of damaged axons. With time, however, the repair ability of the brain deteriorates, and remyelination is no longer efficient or sometimes even possible. We discovered a novel inhibitor or remyelination that is present in MS plaques and we will test if it can be targeted to promote remyelination and protect axons.

2. KEYWORDS:

MAPKP38gamma, myelin, axons, remyelination, cuprizone, mice.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Abbreviations used: OPC= Oligodendrocyte Progenitor Cells, CPZ= Cuprizone, BrdU= BromodeoxyUridine, ISH= In situ Hybridization; PDGFrα= Platelet Derived Growth Factor Receptor alpha, GFAP= Glial Fibrillary Astrocytic Protein.

Specific Aim 1: Study the cellular and molecular mechanism by which p38 gamma accelerate myelination.

Major Task 1: <u>Study the cellular and molecular mechanism by which p38γ accelerate myelination</u>

Subtask 1: Delete p38gamma in OPCs using SOX10CreER^{T2} mice in a TdTomato (Td) background and analyze myelination by optic, electron microscopy and immunofluorescence. (months 3-9) 100% completed
Subtask 2: Delete p38gamma in OPCs using SOX10CreER^{T2} mice in a TdTomato (Td) background. Study OPC proliferation *in vivo* by BrdU incorporation and apoptosis by TUNEL. (months 3-15) 100% completed
Subtask 3: Delete p38gamma in OPCs using SOX10CreER^{T2} mice in a TdTomato (Td) background and study OPC migration by imaging acute brain slices *ex-vivo.* (months 12-24) 100% completed

Major Task 2: <u>Study the cellular and molecular mechanism by which p38γ accelerate myelination</u>

Subtask 1: Measure p38alpha expression in p38 gamma null OPCs and downstream effectors of both isoforms to study if the lack of p38γ signalling enhances its p38α counterpart. (months 24-36) 0% completed

Subtask 2: Overexpress a KETXL truncated isoform of p38gamma in p38gamma null OPCs and study the role of the unique KETXL binding domain on OPC differentiation and the network of factors regulated by p38gamma. (months 24-36) 30% completed

Subtask 3: Delete DICER in p38gamma null OPCs and study the control of p38gamma on microRNA production (months 24-36) 0% completed

Specific Aim 2: Study if p38 gamma influences remyelination in the cuprizone (CPZ) model of acute or chronic demyelination.

Major Task 1: <u>Study if p38 gamma influences remyelination in the cuprizone (CPZ) model of acute or chronic demyelination</u>

Subtask 1. Subject Sox10CreER^{T2}//p38 gamma$^{f/f}$//Td mice to the acute CPZ model and study the susceptibility to demyelination (after 6-week intoxication) and efficiency of remyelination (after 2-week recovery). (months 3-15) 100% completed

Subtask 2. Subject Sox10CreER^{T2}//p38 gamma$^{f/f}$//Td mice to the chronic CPZ model (12 weeks intoxication plus 4-week recovery) and ask if if p38 gamma deficiency can overcome the resulting myelination impairment. (months 12-24) 10% completed

Specific Aim 3: Study of the expression of p38MAPKγ in white matter lesions of post mortem multiple sclerosis brain necropsies

Major Task 1: <u>Study the expression of p38MAPKγ in leukocortical MS lesions</u>

Subtask 1: To explore the presence or absence of p38γ in white matter MS lesions when compared to healthy white matter. (months 9-12).. 100% completed

Subtask 2: we will identify the cells that express p38γ by performing ISH for p38γ followed by immunohistochemistry for PDGFrα (Oligodendrocyte precursors - OPCs), major histocompatibility complex II and Iba1 (microglia and antigen presenting cells), GFAP for astrocytes, CD4/CD8 (T cells) and CD19/CD30 (B cells). (months 12-15) 100% completed.

What was accomplished under these goals

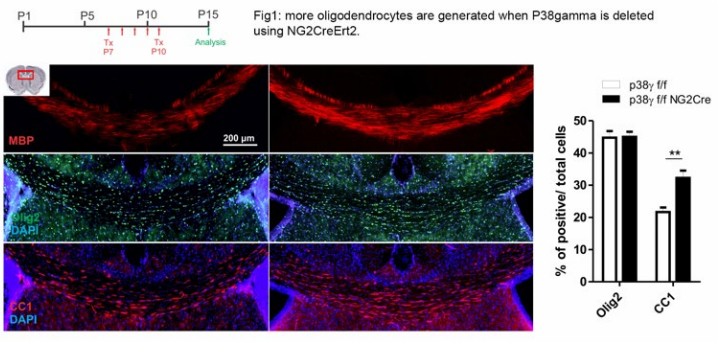


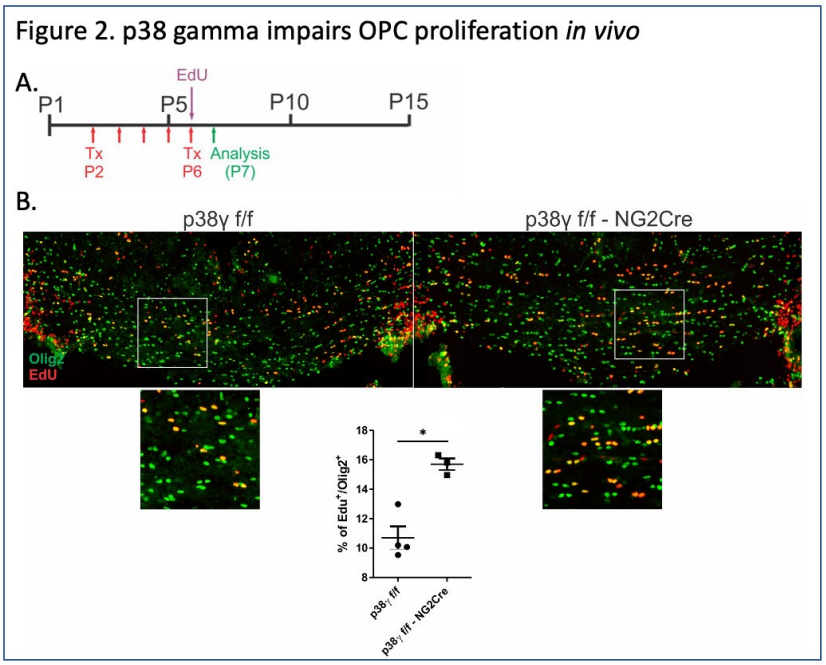
Fig1: more oligodendrocytes are generated when P38gamma is deleted using NG2CreErt2.

Sp. Aim 1: **Study the cellular and molecular mechanism by which p38γ accelerate myelination**

Major Task 1: Study the cellular and molecular mechanism by which p38γ accelerate myelination

Subtask 1: Delete p38gamma in OPCs using SOX10CreER^{T2} mice in a TdTomato (Td) background and analyze myelination by optic, electron microscopy and immunofluorescence. (months 3-9) 100% completed

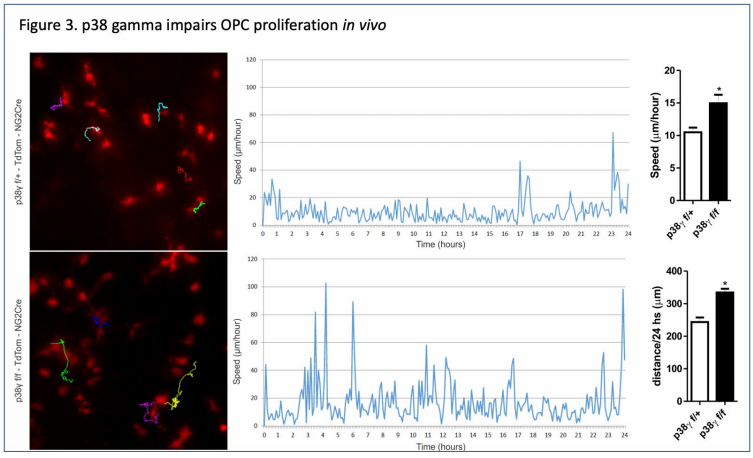
We injected TdTomato – NG2CreER^{T2} mice with tamoxifen from P7 to P11, sampled brains at P15 and performed immunohistochemical analysis on coronal brain sections to investigate the presence of CC1/TdTomato double positive cells. Our findings showed that most CC1^{+} cells in the corpus callosum of P15 animals were TdTomato^{+} (not shown). Next, we used p38γ flox/flox - NG2CreER^{T2} mice, injected tamoxifen from P7 to P11 and sampled brains at P15. We performed immunohistochemical analysis on coronal brain sections and found increased amount of MBP, similar number of Olig2^{+} total OLs and increased number of CC1^{+} mature OLs in p38γ cKO mice at P15 (**Figure 1**). The number of myelin sheaths was increased by EM at P15 (not shown).



Subtask 2: Delete p38gamma in OPCs using SOX10CreER^{T2} mice in a TdTomato (Td) background. Study OPC proliferation *in vivo* by BrdU incorporation and apoptosis by TUNEL. (months 3-15) 100% completed

First, we explored OPC apoptosis by TUNEL staining and found almost no positive cells at P5, P10 and P15 in both wt and cKO mice, indicating that apoptosis is not a significant cellular mechanism of p38gamma actions (not shown)..

To study the effect of p38γ ablation in OPC proliferation *in vivo*, we use the timidine analog 5-Ethynyl-2′-deoxyuridine (EdU). First, we tested the dosis and time on injection that labels the higher number of Olig2^{+} OPCs, and found that 50 mg/kg at P6 was the optimal. Next, we injected wt and cKO mice with tamoxifen in between P2 and P6, and 50 mg/kg of EdU at P6 (**Figure 2A**). Next, we performed Olig2 staining followed by EdU development and found that cKO mice have increased number of Edu/Olig2 double positive cells (**Figure 2B**). Thus, we conclude that p38g inhibits both the number of OPC and their differentiation to OL.



Subtask 3: Delete p38gamma in OPCs using SOX10CreER^{T2} mice in a TdTomato (Td) background and study OPC migration by imaging acute brain slices *ex-vivo*. (months 12-24) 100% completed

To study the effect of p38γ ablation in OPC migration, we performed acute brain slice culture of P3 p38γ flox/flox or p38γflox/+ - TdTom – NG2CreER^{T2} mice. We obtained coronal sections of P3 brains and culture them for two days in the presence of 1 µM 4-hidroxy-tamoxifen to induce p38γ ablation and TdTomato expression. Next, we added PDGF-AA (10 ng/ml) and bFGF (10 ng/ml), placed the slices in a stage motorized spinning disk microscope and tracked OPC migration during 24 hs. After the time-lapse acquisitions, we fixed and sectioned the slices and stained with Olig2 and PDGFrα antibodies. All the TdTomato^{+} cells were Olig2 and PDGFrα positive (not shown). The analysis of OPC speed and distance of migration showed that p38γ cKO OPCs migrate faster and longer distances than controls in *ex vivo* explants **(Figure 3).**

Major Task 2: Study the cellular and molecular mechanism by which p38γ accelerate myelination

Subtask 1: Measure p38alpha expression in p38 gamma null OPCs and downstream effectors of both isoforms to study if the lack of p38γ signalling enhances its p38α counterpart. (months 24-36) 0% completed

Subtask 2: Overexpress a KETXL truncated isoform of p38gamma in p38gamma null OPCs and study the role of the unique KETXL binding domain on OPC differentiation and the network of factors regulated by p38gamma. (months 24-36) 30% completed

This set of experiments are aimed to unravel the network of factors regulated by p38 gamma and the steps include the overexpression of wt p38 gamma and p38 gamma mutants lacking kinase activity and KETAL binding domain (**Figure 4A**). In regards to these experiments, we have successfully performed mutagenesis to obtain 3 different kinase deficient candidates (D171A, K56A and D153A) and KETAL truncated constructs. Next, we validated our mutants by evaluating the effect of each construct on PDS-95 phosphorylation (a known target of p38γ that requires physical interaction through the KETAL domain). To do this, we transduced NSC34 cells with lentiviral particles expressing p38γ mutants and wt isoform. Next, we performed western blot analysis of PDS-95 phosphorylation and, as expected, all the mutants reduced the phosphorylation of PDS-95. Among the kinase deficient candidates, D171A showed the most robust decrease of PDS-95 phosphorylation and thus, was selected for future experiments (not shown).

To explore the effect of the different mutants on OPC differentiation, we isolated OPCs from wild type (WT) P7 mice cortices, transduced them with lentiviruses expressing the mutants or WT construct and allowed the OPCs to differentiate for 3 days *in vitro* (**Figure 4B**). The transduction efficiency was evaluated by eGFP expression relative to DAPI and was over 90% for all the constructs (**Figure 4D**). Next, western blot analysis showed robust p38γ overexpression of all of the constructs (**Figure 4C**). Finally, we performed immunocytochemical analysis with MBP and Olig2 antibodies to evaluate OPC differentiation. Our results showed that FL-p38γ reduced by half the number of MBP+/Olig2+ cells when compared to the control. In regards to Kd and ΔC- constructs, Kd-p38γ showed similar number of MBP+/Olig2+ cells as the controls and ΔC-p38γ showed more MBP+/Olig2+ cells than FL- p38γ but less than the control (**Figure 4D and E**)). Altogether, this data indicates that p38γ overexpression reduces OPC differentiation and this effect is partially dependent on targets that require the specific binding domain of p38γ.

To start elucidating the network of events triggered by p38γ, we explored the activation of relevant OPC differentiation pathways such us ERK1/2, JNK, PI3/AKT and mTOR *in vitro* (**Figure 5 and 6**). To do this, we isolated OPCs from P7 p38γf/f and p38γf/f-NG2Cre mice cortices and induced p38γ ablation by adding 4 hydroxy tamoxifen (4OH-Tx) for 24 hs. Next, we removed 4OH-Tx, allowed the OPCs to proliferate for another 24 hs (DIV3 - proliferation) and triggered differentiation for 24 hs (DIV4 – differentiation) (**Figure 5A and Figure 6A**). Next, we obtained cell lysates during proliferation (DIV3) or after 1 day of differentiation *in vitro* (DIV4) and performed western blot analysis.

First, we evaluated the phosphorylation state of ERK1/2, JNK, canonical AKT (Ser407). We found no differences during proliferation (DIV3) or differentiation (DIV4) in the phosphorylation of JNK and AKT (Ser407) indicating lack of interaction of these pathways and p38γ (**Figure 5B and C**). In regards to ERK1/2 phosphorylation, we found that p38γ cKO OPCs show increased levels of phosphorylated ERK1/2 during proliferation (DIV3) but no during differentiation (DIV4) (**Figure 5B and C**) suggesting a role of p38γ during OPC differentiation but also during proliferation.

Next, we explored the interaction of p38γ with mTOR pathway. To do this we used cell lysates from OPCs during proliferation (DIV3) or differentiation (DIV4) (**Figure 6A**) and perform western blot analysis for 4E-BP1, S6 and AKT Thr308 phosphorylation. Our findings showed that p38γ ablation has no effect on mTOR pathway during proliferation of differentiation (**Figure 6B and C**).

Next, we explored the interaction between p38γ and p38α/β. Previous work done on p38α/β showed that the α isoform promotes OPC differentiation and chemical inhibition of this isoform limits OPC differentiation and myelination. Thus, we aimed to test if p38α/β inhibition limits the differentiation of p38γ deficient OPCs and thus, focus on designing chemical inhibitors that inhibit p38γ but do not affect p38α. To do this, we isolated OPCs from P7 p38γf/f and p38γf/f-NG2Cre mice cortices. Next, we induced p38γ ablation by adding 4OH-Tx for 24 hs. After removing 4OH-Tx, we allowed the OPCs to proliferate for another 24 hs and next, triggered OPC differentiation and added the p38α/β inhibitor SB203580 or DMSO (vehicle) (**Figure 7A**). OPCs were allowed to differentiate for 2 days and to evaluate the effect of SB203580 of OPC differentiation, we performed MBP/Olig2 stainigs. Our results showed that, as previously shown, SB203580 reduced the number of $MBP^{+}/Olig2^{+}$ cells of control cells and had the same effect on p38γ OPCs. Altogether these data highlight the importance of find specific inhibitors that target p38γ without affecting p38α/β.

Subtask 3: Delete DICER in p38gamma null OPCs and study the control of p38gamma on microRNA production (months 24-36) 0% completed. Not started yet

Specific Aim 2. Study if p38 gamma influences remyelination in the cuprizone (CPZ) model of acute or chronic demyelination.
Major Task 1: Study if p38 gamma influences remyelination in the cuprizone (CPZ) model of acute or chronic demyelination

Subtask 1. Subject Sox10CreER^{T2}//p38 gamma$^{f/f}$//Td mice to the acute CPZ model and study the susceptibility to demyelination (after 6-week intoxication) and efficiency of remyelination (after 2-week recovery). (months 3-15) 100% completed

We subjected Sox10CreER^{T2}//p38 γ $^{f/f}$//Td mice (cKO mice) to the acute CPZ model and studied the susceptibility to demyelination (after 2-week intoxication) and efficiency of remyelination (after 2-week recovery). After 7 weeks of CPZ feeding (demyelination), wt and cKO mice showed no differences in the content of MBP both in the corpus callosum and cortex (**Figure 8. CPZ 7**). Next, we evaluated the effect of p38 gamma on remyelination and found that p38 γ KO mice remyelinate faster as shown by increased content of MBP in the corpus callosum and cortex (**Figure 8. CPZ 7 +2).**

Next, we use electron microscopy (EM) to study myelin ultrastructure in the corpus callosum of wt and cKO mice. Our findings confirmed a similar and robust extent of demyelination achieved by wt and cKO mice as evidenced by the similar number of myelinated axons in the corpus callosum after 7 weeks of CPZ feeding and a drastic reduction when compared to NO CPZ (**Figure 9A and B**. CPZ 7 and NO CPZ). After remyelination, cKO mice showed an increased number of myelinated axons (**Figure 9A and B**,

CPZ 7 + 2) and thus, confirms our previous findings indicating improved remyelination in cKO mice. Finally, we studied myelin thickness by measuring g-ratio (relation of axon diameter/myelin diameter) which oscilates in between 0 and 1, with 0 indicating thicker myelin and 1 thiner myelin. As expected, CPZ feeding induced a robust reduction of myelin thicknes and increased g-ratio (**Figure 9A, C and D**, NO CPZ vs CPZ7) with no differences in between wt and cKO mice (**Figure 9A, C and D**, CPZ 7). Finally, we found that after remyelination, cKO mice have thicker myelin and reduced g-ratio (**Figure 9A, C and D**, CPZ 7 + 2). Altogether, our findings show that p38gamma is an OPC specific inhibitor of remyelination.

Subtask 2. Subject Sox10CreER^{T2}//p38 gamma$^{f/f}$//Td mice to the chronic CPZ model (12 weeks intoxication plus 4-week recovery) and ask if p38 gamma deficiency can overcome the resulting myelination impairment. (months 12-24) 10% completed. we are breeding the animals necessary for the experiment and we will perform the CPZ intoxication and analysis.

Specific Aim 3: Study of the expression of p38MAPKγ in white matter lesions of post mortem multiple sclerosis brain necropsies

Major Task 1: Study the expression of p38MAPKγ in leukocortical MS lesions

Subtask 1: To explore the presence or absence of p38γ in white matter MS lesions when compared to healthy white matter. (months 9-12)..100% completed. We found that P38 γ is indeed upregulated in some MS lesions (**Figure 10A**), and that P38 γ expression correlates inversely with remyelination.

Subtask 2: we will identify the cells that express p38γ by performing ISH for p38γ followed by immunohistochemistry for PDGFrα (Oligodendrocyte precursors - OPCs), major histocompatibility complex II and Iba1 (microglia and antigen presenting cells), GFAP for astrocytes, CD4/CD8 (T cells) and CD19/CD30 (B cells). (months 12-15) 100% completed. By performing ISH with specific cell markers, we determined that p38γ is expressed by OPC and microglia in MS lesions (**Figure 10B**) .

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

We submitted a presentation for the ASN 51st Annual Meeting that was going to be held from April 18 to 22, 2020, St. Louis/St. Charles, Missouri. Unfortunately, due to the current pandemic, the conference was cancelled.
PS01-08. P38MAPKγ INHIBITS OPC DIFFERENTIATION DURING DEVELOPMENT AND REMYELINATION AND IS PRESENT IN MS LESIONS. Leandro Marziali1, Marilena Palmisano1, Yoonchan Hwang1, Ana Cuenda2, Rajan Dutta3, Bruce Trapp3, Lawrence Wrabetz1, M. Laura Feltri1

What do you plan to do during the next reporting period to accomplish the goals?

In the next year, in no-cost extension, we will perform proteomic analysis tagged p38gamma after immunoprecipitation. We will also delete DICER in p38gamma null OPCs and study the control of p38gamma on microRNA production

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Our project on P38gamma was selected by the Empire Discovery Institute (EDI)
https://www.buffalo.edu/innovate/all-resources1.host.html/content/shared/www/innovate/resources/business-and-entrepreneur-partnerships/empire-discovery-institute.detail.htmlas
as one of four program for accelerated translation. The EDI will provide funds and expertise to develop an inhibitor of P38gamma to promote remyelination in Multiple Sclerosis.

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

The laboratory has been working at reduced capacity at times during the Covi-19 pandemic. For this reason we asked for a no cost extension of one year.

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Nothing to report

Books or other non-periodical, one-time publications.

Other publications, conference papers and presentations.

Nothing to report

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

no change:

Laura Feltri (PI)
Edward Hurley (technician). Ed Hurley Is performing electron Microscopy, Funding support DOD and other funds
Leandro Marziali (post-doc). Dr. Marziali is performing all the work. His funding is provided from other that this award.
Pablo Paez 2% co-I Dr. Paez is helping with the in vivo imaging. His remaining funding is provided from other that this award.

Fraser Sim 2% co-I. Dr. Sim is helping with the demyelination paradigm. His remaining funding is provided from other that this award.

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Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

M. Laura Feltri: new grant received:

Charcot-Marie-Tooth Association
Mechanisms of axonal degeneration in late onset CMT1B neuropathies: molecular pathways and therapeutic approaches The goal is to understand the mechanism by which CMT1b mutations cause axonal demise.
Overlap: There is no overlap.
Role: Co-Investigator

Charcot-Marie-Tooth Association 5/1/2021 – 4/30/2023
"Improving proteasome function to treat Charcot Marie Tooth 1A and 1B
The goal is to try an experimental therapy for Charcot Marie Tooth 1b in mice
Overlap: there is no overlap
Role: PI

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: