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**TITLE: Promoting Recovery by Inhibiting PDE10A-Mediated Inflammation**

**PRINCIPAL INVESTIGATOR: Bradford C. Berk**

**CONTRACTING ORGANIZATION: University of Rochester, Rochester, NY**

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## 14. ABSTRACT

The first goal was to determine the effects of TP-10 treatment in nerve and muscle recovery after sciatic nerve injury. Using sciatic nerve crush injury, we obtained reproducible damage as measured by muscle force and CatWalk gait analyses. To find the optimal dose of PDE10A inhibitor to improve these parameters, we used two different PDE10A specific inhibitors, MP-10 and TP-10. We optimized the dose using an in vitro macrophage model. Specifically, we stimulated inflammasome activation and pyroptosis to show the importance of PDE10A in macrophage mediated inflammation, inflammasome activation and pyroptosis (a specific type of programmed cell death that releases high amounts of inflammatory cytokines such as IL-b and IL-18. To stimulate pyroptosis, we incubated macrophages with lipopolysaccharide (LPS) and nigericin. Optimal protection was a 50% reduction in cell death with 3 μM MP-10 which correlated with a 60% reduction in IL-Ib release. We studied the process of pyroptosis that requires the clustering an adaptor protein called ASC in freshly isolated peritoneal macrophages. TP-10 at 3 μM inhibited ASC clustering and pyroptosis by 66%. The second goal was to define the effect of PDE10A inhibition on cytokine expression and after nerve injury. We performed immunohistochemistry on sham and crush injury sciatic nerve sections. There was a 5-fold increase in PDE10A after crush injury. Next, we performed a cytokine array on injured nerve and found significantly increased IL-1b, IL-18, and myeloperoxidase. We confirmed increases in these cytokines by performing an immunoblot. In summary, we showed that pyroptosis and "cytokine storm" were present after sciatic crush injury. TP-10 or MP-10 reduced inflammasome formation and activation.

**15. SUBJECT TERMS**
PDE10A and its inhibitors, inflammation, Macrophage, Sciatic Nerve Crush Injury, Gait Analysis, Pyroptosis

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## 1. INTRODUCTION:

The first goal was to determine the effects of TP-10 treatment in nerve and muscle recovery after sciatic nerve injury. Using sciatic nerve crush injury, we obtained reproducible damage as measured by muscle force and CatWalk gait analyses. To find the optimal dose of PDE10A inhibitor to improve these parameters, we used two different PDE10A specific inhibitors, MP-10 and TP-10. We optimized the dose using an in vitro macrophage model. Specifically, we stimulated inflammasome activation and pyroptosis to show the importance of PDE10A in macrophage mediated inflammation, inflammasome activation and pyroptosis (a specific type of programmed cell death that releases high amounts of inflammatory cytokines such as IL-b and IL-18. To stimulate pyroptosis, we incubated macrophages with lipopolysaccharide (LPS) and nigericin. Optimal protection was a 50% reduction in cell death with 3 μM MP-10 which correlated with a 60% reduction in IL-Ib release. We studied the process of pyroptosis that requires the clustering an adaptor protein called ASC in freshly isolated peritoneal macrophages. TP-10 at 3 μM inhibited ASC clustering and pyroptosis by 66%. The second goal was to define the effect of PDE10A inhibition on cytokine expression and after nerve injury. We performed immunohistochemistry on sham and crush injury sciatic nerve sections. There was a 5-fold increase in PDE10A after crush injury. Next, we performed a cytokine array on injured nerve and found significantly increased IL-1b, IL-18, and myeloperoxidase. We confirmed increases in these cytokines by performing an immunoblot. In summary, we showed that pyroptosis and "cytokine storm" were present after sciatic crush injury. TP-10 or MP-10 reduced inflammasome formation and activation.

## 2. KEYWORDS:

PDE10A, Inflammation, macrophage, sciatic nerve crush injury, gait analysis, pyrotosis, TP-10, MP-10

## 3. ACCOMPLISHMENTS:

### What were the major goals of the project?

Aim 1: To determine the effects of TP-10 treatment in nerve and muscle functional recovery after sciatic nerve injury.

Aim 2: To determine the combined effects of TP-10 treatment and exercise training in neuropathic pain after nerve injury.

| **Specific Aim 1**<br>**To determine the effects of TP-10 treatment on nerve and muscle functional recovery after sciatic nerve injury** | **Timeline** (Months) | *percentage of completion* | *Actual/ expected completion dates* |
|---|---|---|---|
| **Major Task 1**<br>**Optimize the dose of TP-10 on nerve and muscle functional recovery after nerve injury.** | | | |
| Subtask 1 Perform sciatic nerve crush injury | 1-2 | 100% | July 1, 2021 |

| | | | |
|---|---|---|---|
| Subtask 2 Perform CatWalk gait analysis | 3-4 | 100% | Aug 1, 2021 |
| Subtask 3 Perform muscle force analysis | 5-6 | 30% | Dec 1, 2021 |
| Milestone Achieved: Identify TP-10 dose that maximally improves functional recovery | 5-6 | 90% | Dec 1, 2021 |
| **Major Task 2**<br>**Define the effect of PDE10A inhibition on cytokine expression and NLRP3 activation after nerve injury** | | | |
| Subtask 1 Perform immunohistochemistry for PDE10A on nerve | 7-8 | 100% | Jan 31, 2022 |
| Subtask 2 Perform cytokine array on nerve | 9-10 | 80% | |
| Subtask 3 Perform protein assay for inflammasome components on nerve | 11-12 | 100% | |
| Milestone(s) Achieved: TP-10 treatment reduces NLRP3 formation and activation | 10-12 | 100% | |

## What was accomplished under these goals?

Traumatic nerve injury induces an inflammatory response followed by axon degeneration and demyelination. To understand the role of PDE10A in traumatic nerve injury, we used two different mouse models; a sciatic nerve crush injury and a spinal cord T9 contusion injury. To analyze PDE10A expression in injured sciatic nerve, we performed immunohistochemistry (IHC) of the sciatic nerve on day 3 after crush injury. PDE10A was highly induced by peripheral nerve injury (PNI), (Fig. 1A). Inflammasomes play an important role in neuroinflammation and are critical in processing IL-1β and IL-18. To determine the effect of nerve injury on inflammasome transcription, we harvested sciatic nerve 3 days after injury and measured inflammasome-related mRNA expression. Expression of NLRP3 and ASC (an inflammasome adapter protein) was significantly increased in injured sciatic nerve compared to uninjured (Fig. 1B). In the studies below, we used two PDE10A inhibitors. TP-10, has been administered systemically in people with schizophrenia, as well as in mouse models of heart failure. MP-10 has been used in several human clinical trials to treat Huntington's disease. Recently, we showed MP-10 treatment decreased wire injury-induced intima formation in femoral arteries. To examine the effects of PDE10A inhibition on motor function following nerve injury, we injected MP-10 (10 mg/kg) or vehicle as a control, subcutaneously 3 hr after injury, and then once daily for seven days. We measured motor recovery after nerve injury using CatWalk gait analysis to determine sciatic functional index (SFI). The SFI was calculated by walking foot print morphology on day 7 after injury (normal SFI range: 0-10, Impaired range: 100-120. We found that the vehicle group exhibited severe impairment with a SFI of 98 while the MP-10 treated group had a significantly improved SFI at 78 (Fig. 1C). To examine the effects of PDE10A inhibition on NLRP3 inflammasome activation, we measured cleaved IL-1β in the sciatic nerve by IHC 3 days after crush injury. Nerve injury dramatically increased IL-1β cleavage in sciatic nerve (Fig. 1D). Importantly, MP-10 treatment significantly reduced cleavage compared to vehicle (Fig. 1D) suggesting that PDE10A inhibition reduced inflammasome activation. Muscle atrophy is associated with increased myogenin and Gadd45a gene expression. Daily MP-10 treatment significantly decreased their expression in gastrocnemius muscle on day 3 after crush injury compared to vehicle (Fig. 1E and 1F). To determine the role of PDE10A in SCI, we performed T9 contusion injury in C57BL/6N mice. Similar to sciatic nerve injury, PDE10A expression in spinal cord was significantly increased after SCI at both acute and late time points (Fig. 1G, 5.5 +0.7 fold). Mac2 IHC demonstrated a large increase in macrophages at 3 days (10 + 2.1 fold) and 42 days (7 + 1.1 fold) after SCI (Fig. 1G). There was not complete co-localization of PDE10A and membrane-associated Mac2 since PDE10A can localize both with membranes and cytoplasm (Fig. 1G). Our finding that PDE10A levels remain high even at 6 weeks post SCI opens

the possibility that PDE10A may contribute to chronic inflammation and thus may be a druggable target even for chronic neuropathic pain.

To examine the effects of PDE10A inhibition on inflammation and muscle atrophy after SCI, T9 SCI and Control mice were treated with vehicle or TP-10 (s.c. injection, 6mg/kg) 3 hr after SCI, and then once daily for 7 days. Spinal cords and gastrocnemius muscles were harvested on day 7 after injury. NLRP3 gene expression in spinal cord was significantly increased in the SCI group (Fig. 1H). TP-10 treatment significantly reduced NLRP3 mRNA expression compared to vehicle treatment (Fig. 1H, 1.5 vs 2.5 fold increase). Gastrocnemius weight at day 7 was reduced from baseline after SCI in the vehicle treated group. However, it was significantly greater in the TP-10 group (Fig. 1I). In summary, PDE10A inhibition with either TP-10 or MP-10 inhibited expression of NLRP3, decreased muscle atrophy, and improved motor function. These results support the concept that SCI-induced PDE10A activation drives inflammasome formation and inflammation leading to motor dysfunction and muscle atrophy.

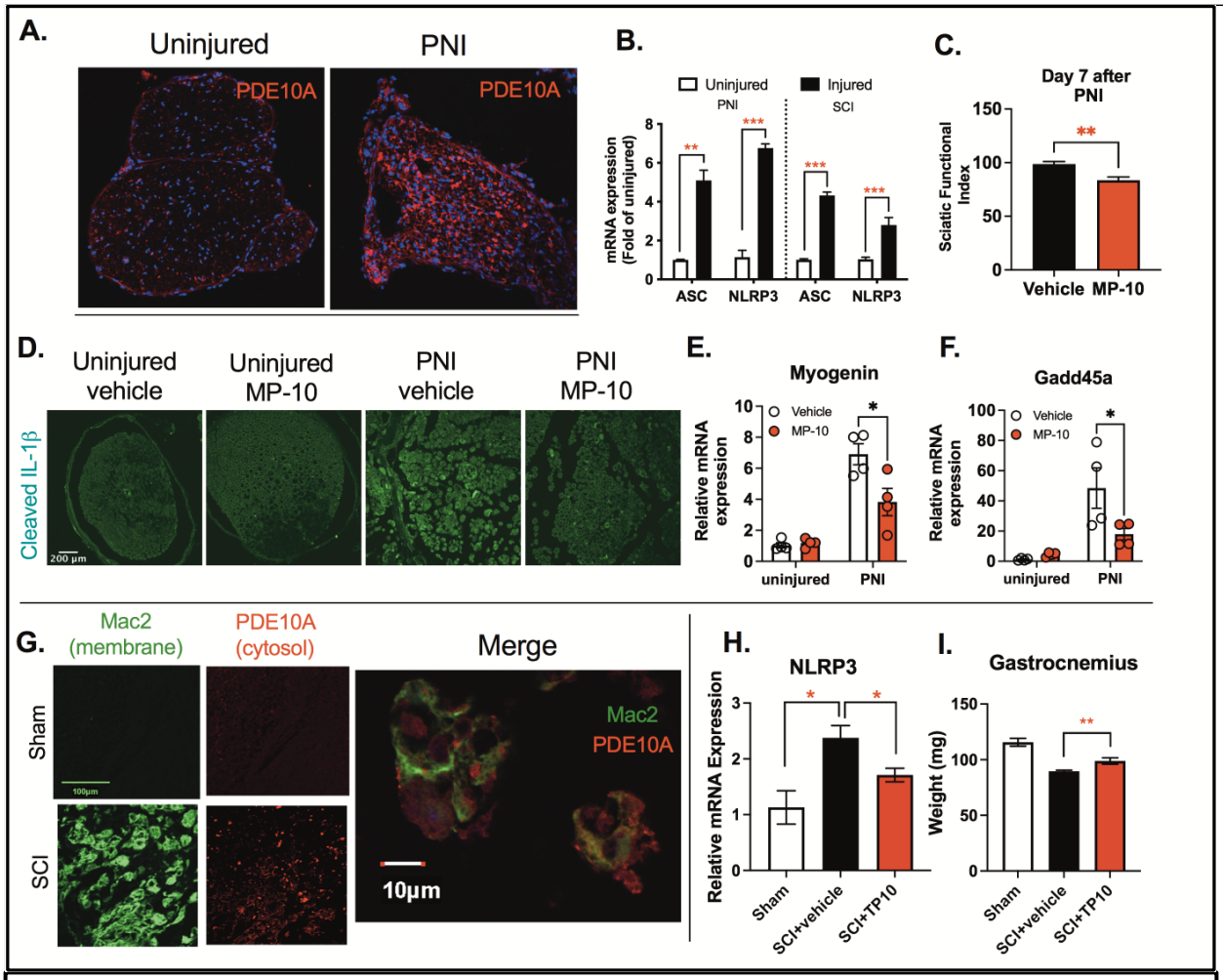


**Fig. 1. PDE10A activity controls traumatic injury-induced inflammasome gene expression and muscle atrophy.** (A) C57BL/6N mice underwent sham surgery and peripheral nerve injury (PNI). Sciatic nerves were harvested on day 3 for (A) PDE10A staining (PDE10A, Red; DAPI, Blue), representative of immunofluorescence images of 3 animals from each group. (B) Gene expression of inflammasome components NLRP3 and ASC after peripheral nerve injury (PNI) and spinal cord injury (SCI). (n=4 from each group) (C) In a separate experiment, C57BL/6N mice underwent peripheral nerve crush injury and treated with vehicle or MP-10 once daily (s.c. injection, 10mg/kg). Sciatic functional index was measured by gait analysis on day 7 after injury. (n=8 for each group). (D) Cleaved IL-1β staining (Green) in sciatic nerve, representative of immunofluorescence images of 4 animals from each group. (E) Myogenin mRNA expression (F) Gadd45a mRNA expression in gastrocnemius muscle (n=4 for each group). (G) C57BL/6N mice underwent T9 contusion injury (SCI).Spinal cord tissues were harvested on day 3 and 42after SCI or sham mice for Mac2 (macrophage marker, green) and PDE10A (Red, white arrows) staining. In a separate experiment (H-I), C57/BJ mice underwent SCI and treated with vehicle or TP-10

Inflammatory monocytes and resident tissue macrophages are key mediators of tissue repair, regeneration, and fibrosis during infections and sterile tissue injury. To understand the regulation and function of PDEs in macrophages, we screened for all PDE genes in peritoneal and alveolar macrophages, and found that PDE4B and PDE10A were the two most highly induced by lipopolysaccharide (LPS) (Fig. 2A). PDE4B is a well-known inflammatory drug target, and a number of PDE4 inhibitors have been used clinically to treat inflammatory diseases such as psoriasis and chronic obstructive pulmonary disease (COPD). We chose to study only PDE10A as the magnitude of response was significantly greater for PDE10A than PDE4B, its role in neuroinflammation is less well understood, and it represents a more novel approach. We first investigated the role of PDE10A in macrophage inflammatory responses in vitro. Stimulation of inflammasome-secreted inflammatory molecules requires both priming (e.g., NF-kB mediated expression of NLRP3) and subsequent assembly and activation (e.g., by nigericin or ATP to activate caspases). Inflammasome formation triggers the activation of caspase-1 and processing of IL-1β and IL-18 into their mature forms. GSDMD is a pore-forming protein and the final common effector for the inflammasome downstream of caspase-1, -4, and -11 activation. Active caspases cleave GSDMD to generate an N-terminal cleavage product (GSDMD-NT) that forms transmembrane pores to enable IL-1β release and to drive pyroptosis. To show the importance of PDE10A in macrophage mediated inflammation; specifically, in inflammasome activation and pyroptosis, we treated differentiated human THP-1 macrophages with LPS, with or without MP-10, followed by nigericin. Cell death was measured by real-time nucleic acid staining (SYTOXTM), and secreted IL-1β was quantified by ELISA. MP-10 treatment significantly decreased the magnitude of LPS-nigericin stimulated cell death and IL-1β release in a dose dependent manner (Fig. 2B-C), suggesting that PDE10A inhibition protects macrophages from LPS/nigericin-mediated pyroptosis. Upon NLRP3 inflammasome activation, the adaptor protein ASC is recruited by NLRP3 and forms large multimeric complexes, termed ASC specks. To further characterize the role of PDE10A in inflammasome activation, we overexpressed an ASC-GFP fusion protein in THP-1-differentiated macrophages and stimulated them with LPS followed by nigericin. PDE10A inhibition by TP-10 blocked ASC speck formation, as indicated by reduced ASC speck immunofluorescence (Fig. 2D). Next, we measured the effect of TP-10 on parameters of inflammasome activation in peritoneal macrophages. TP-10 treatment dramatically decreased cleaved caspase-1, cleaved GSDMD, and mature IL-1β (Fig. 2E). Lastly, we harvested spinal cord bone marrow cells and differentiated them into macrophages by incubating in L929 conditioned medium for 7 days. PDE10A inhibition by TP-10 and MP-10 also decreased IL-1β secretion from these macrophages stimulated with ATP (Fig. 2F), further supporting the role of PDE10A in regulating inflammasome activation.

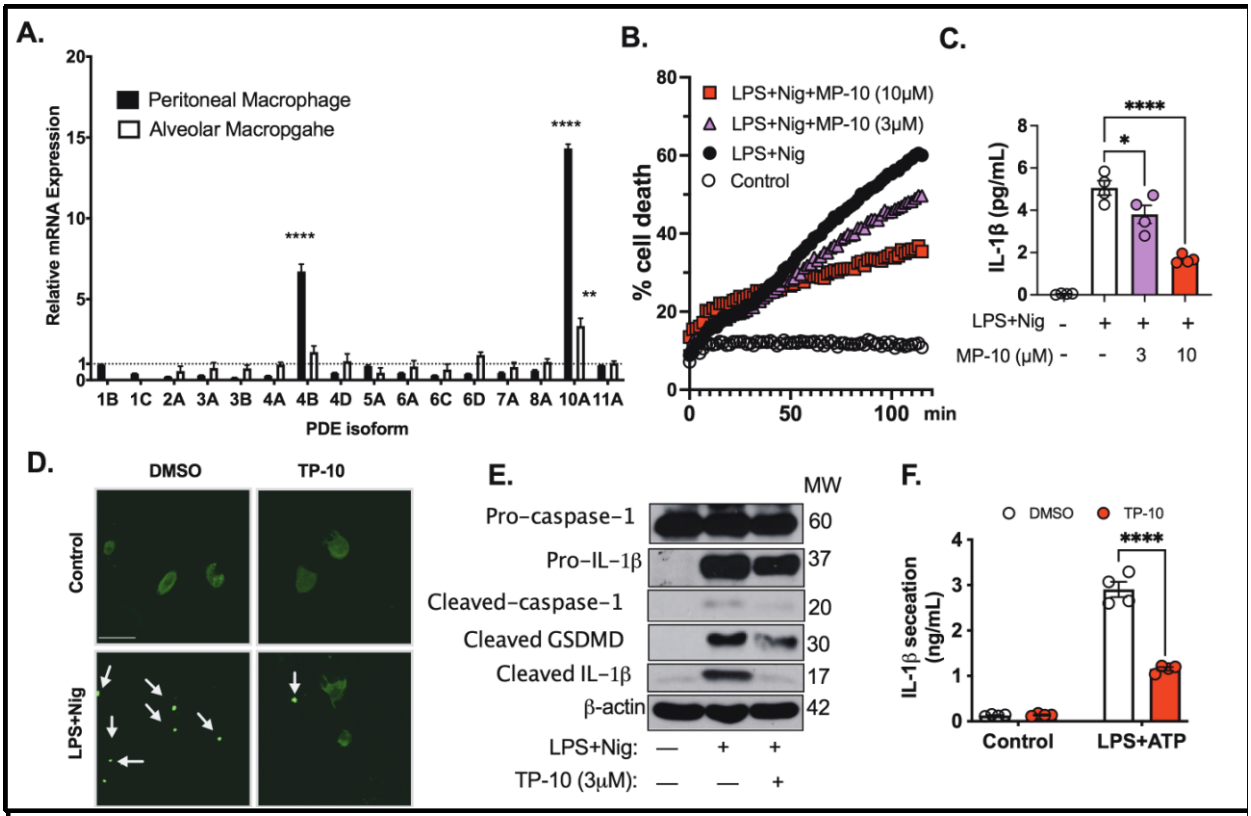


**Fig. 2. PDE10A inhibition reduces inflammasome activation in LPS-stimulated macrophages.** (A) Different PDE expressions after LPS (100ng/mL) stimulation for 6 hours in peritoneal macrophages (Black bar) and alveolar macrophages (White Bar), dashed line: no LPS.(B) THP-1 differentiated macrophages were stimulated with LPS (100 ng/mL) and co-incubated with MP-10 (3 or 10 μM) or vehicle (ethanol) for 3 hr followed by 2 hr nigericin (Nig, 6 μM) treatment. Real-time percentage cell death was determined by normalizing to maximal nuclear acid fluorescent intensity (0.1% Triton-X100 treated cells). (C) Peritoneal macrophages were stimulated with LPS (100 ng/mL) for 3hr followed by nigericin (Nig, 2 μM) stimulation for 1hr. IL-1β in the medium was measured by ELISA. (D) THP-1 ASC-GFP overexpressed macrophages were stimulated by LPS+Nig (nigericin) with or without TP-10. ASC speck formation was measured by confocal microscope. (Arrowheads indicate ASC specks (green)). Scale bar = 50 μm) (E) Peritoneal macrophages were stimulated with LPS for 3 hours followed by nigericin. Pro- and cleaved caspase-1, IL-1β and GSDMD were measured by western blots. (F) Peritoneal macrophages were stimulated with LPS for 3 hours followed by ATP (2mM). IL-1β in the medium was measured by ELISA. Data were expressed as mean ± SEM.

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

We hired a technician, Camila Lage Chavez, to assist with the injury and analysis experiments. She was mentored by the postdoc working full time on this project, Chia Hsu, who taught her numerous techniques. Furthermore she states that the training of the sterile technique and surgery will benefit her as she goes through medical school. Also, her time in the lab made her interest in biomedical research increase significantly. Furthermore, she was able to assist in other projects analyzing and interpreting the data, and then participating in writing the paper. She already has two published papers.

**How were the results disseminated to communities of interest?**
Nothing to Report

**What do you plan to do during the next reporting period to accomplish the goals?**
For specific aim 1, task 1 we need to perform additional catwalk gait analysis, muscle force analysis and treatment of more animals with MP-10. If we have time we will also compare a dose of 3 mg/kg vs 10 mg/kg. We also need to perform additional measurements of SFI, muscle protein atrophy and muscle force analysis.

## *4.* IMPACT:

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

We have shown that crush injury of the peripheral sciatic nerve causes a rapid increase in inflammation mediated by specific cytokines such as IL-1b. We found a critical role for an enzyme called PDE10A that decreases intracellular cyclic AMP. Furthermore, we found that a drug inhibitor of PDE10A called MP-10 was able to inhibit the level of inflammation as measured by expression of IL-1b. We also found that MP-10 improved SFI, a measure of the sciatic nerve function. MP-10 decreased the muscle atrophy of the gastrocnemius showing that PDE10A mediated signal events were important in impaired muscle function.

**What was the impact on other disciplines?**
The data we present will extend to other disciplines in 2 ways. 1) The data show that nerve injury is associated with inflammation which is specifically regulated by the enzyme PDE10A. Because the inflammatory proteins such as IL-1b were inhibited by the specific PDE10A inhibitors, MP-10 and TP-10, they establish a new intracellular mediator for inflammation. 2) The demonstration that components of the NLRP3 inflammasome mediate the production of inflammatory cytokines extends the signals that induce inflammation in the nervous system. These results will influence neuroscience and immunology.

**What was the impact on technology transfer?**
Nothing to report.
The impact on commercial technology will be to develop additional PDE10A inhibitors that are more potent and tissue specific.

**What was the impact on society beyond science and technology?**
Nothing to report.

## 5. CHANGES/PROBLEMS:

We changed TP-10 to MP-10 for in vivo experiments because of the difference in cost, at the time of the grant submission, MP-10 was not readily available. We show that the dose response relationship for inhibiting PDE10A by MP-10 was similar to TP-10. Because they are related to each other we did not consider this a significant change.

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

1) We found more variability in the catwalk gait analysis than expected. We will have to increase the number of animals that we will study to achieve significant results.

**Changes that had a significant impact on expenditures**
The project has remained on budget.

**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**
Nothing to report.

**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.**
  Nothing to report.

**Other publications, conference papers and presentations**.

**Hsu CG**, Chavez CL, Zhang C, Sowden M, Yan C, Berk BC. The lipid peroxidation product 4-hydroxynonenal inhibits NLRP3 inflammasome activation and macrophage pyroptosis. Cell Death Differ. 2022, 29(9), 1790-1803

**Hsu CG**, Li WJ, Chavez CL, Zhang C, Sowden M, Berk BC. Pnpt1 mediates NLRP3 inflammasome activation by MAVS and metabolic reprogramming in macrophages. Cell Mol Immunol. 2023, 20(2):131-142

- **Website(s) or other Internet site(s)**
  Nothing to report.

- **Technologies or techniques**
  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?**

Name: Chia Hsu
Project Role: Postdoc Student
Research Identifier: 0000-0002-1133-4116
Nearest person month worked: 12
Contribution to Project: Dr. Hsu has designed the experiments, performed the surgeries, performed immunoblots, IHC, and catwalk gait analysis. He has trained the technician, Camila Lage Chavez, on the procedures above.
Funding Support:

Name: Camila Lage Chavez
Project Role: Lab Technician
Research Identifier: 0000-0002-5307-1908
Nearest person month worked: 6
Contribution to project: Camila has helped Dr. Hsu with performing the surgeries, as well as immunoblots, IHC and catwalk gait analysis.
Funding support:

Name: Mark Sowden
Project Role:
Research Identifier: 0000-0002-7824-0915

Nearest person month worked: 1
Contribution to project: Dr. Sowden has designed the experiments in collaboration with Dr. Hsu.
Funding support:

Bradford Berk
Project Role: PI
Research Identifier: 0000-0002-2767-4115
Nearest person month worked: 3
Contribution to project: Dr. Berk has designed the experiments, analyzed the data and troubleshooted with Dr. Hsu, Dr. Sowden and Camila.
Funding support: University endowment

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
Nothing to report.

**What other organizations were involved as partners?**
Nothing to report.

## 8. SPECIAL REPORTING REQUIREMENTS

**COLLABORATIVE AWARDS:**

**QUAD CHARTS:**

## 9. APPENDICES: