AWARD NUMBER: W81XWH-17-1-0638

TITLE: Therapeutic Target Identification through analysis of Convergent AD and TBI Pathogenic Mechanisms

PRINCIPAL INVESTIGATOR: Drs. Fiona Crawford/Joseph Ojo

CONTRACTING ORGANIZATION: The Roskamp Institute, Inc.
2040 Whitfield Avenue,
Sarasota, FL
34243-3922

REPORT DATE: September 2021

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE: September 2021	**2. REPORT TYPE: FINAL REPORT**	**3. DATES COVERED: 09/15/2017 – 05/14/2021**

4. TITLE AND SUBTITLE: Novel Therapeutic Target Identification through analysis of Convergent AD and TBI Pathogenic Mechanisms.

5a. CONTRACT NUMBER
W81XWH-17-1-0638

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Fiona Crawford, PhD – Main PI; Joseph Ojo, PhD – Co-PI; Lauren Horne – Grant Coordinator

E-Mail: fcrawford@roskampinstitute.org; jojo@roskampinstitute.org; lhorne@roskampinstitute.org; cgil@roskampinstitute.org.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Roskamp Institute, Inc., 2040 Whitfield Avenue Sarasota, FL 34243-3922

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT: Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES: N/A

14. ABSTRACT

Traumatic Brain Injury (TBI), in particular mild TBI (mTBI) is a major cause of disability in military and in civilian populations, and for many years has been known to be an epigenetic risk factor for Alzheimer's Disease (AD) and other neurodegenerative conditions. However, the precise nature of how TBI leads to or precipitates AD pathogenesis is not understood. To address this problem, we have generated molecular profiles of AD and TBI pathogenesis in mouse models at a range of ages/timepoints post-injury respectively, in order to identify molecules and pathways that are common to both AD and TBI. Extensive datasets have been generated, and our comparison and integration of omic profiles clearly points to overlapping disruption of brain lipids and related protein signaling pathways in AD mice with age and TBI mice with time post injury. These include activation of lipid related leukotriene signaling, deficiencies in the eicosanoid signaling, dysfunctional PI3-kinase/Akt/mTOR/insulin and RXR/PPAR pathway. Lipids, particularly phospholipids are essential components of neuronal and glial cell membranes and axonal myelin, they modulate protein-protein interaction and cell signal transduction. Thus the TBI-induced pathobiology we have observed may precipitate development of AD and lower the threshold for onset of pathogenic mechanisms. We therefore proposed in this study to validate these convergent AD and TBI mechanisms as novel targets to block the negative sequelae of TBI.

15. SUBJECT TERMS: **AD, TBI, Pathology, Animal Models, Novel Therapeutic Targets**

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	54	USAMRMC

TABLE OF CONTENTS

INTRODUCTION: ***Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.***

In this project we plan to identify and provide preliminary validation of novel targets (eicosanoid signaling, dysfunctional PI3-kinase/Akt/mTOR/insulin and RXR/PPAR pathway) for the TBI-AD interrelationship discovered through our molecular level profiling. The goal here is target validation and provision of these therapeutic targets for future studies in drug discovery and preclinical efficacy; i.e. IND enabling studies of new therapeutic approaches to the AD sequelae of TBI.

1. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

AD, TBI, Pathology, Animal Models, Novel Therapeutic Targets

2. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Objective/Hypothesis: Molecular pathways that are triggered in response to both TBI sequelae and AD pathogenesis represent novel molecular targets to mitigate the AD-neurodegenerative sequelae of TBI. From our interrogation of omic datasets of AD pathogenesis and TBI sequelae we have identified coincident lipid disturbances and related pathways which are potential targets for therapeutic intervention. Therefore we propose to first: **Aim 1:** Evaluate target engagement and efficacy in recovering normal molecular profiles in TBI mice using compounds that modulate the TBI and AD coincident molecular changes in a short-term treatment paradigm. **Aim 2:** In vivo validation of therapeutic targets in mice with TBI using chronic intervention with the top two performing compounds for two different targets from Aim 1 to evaluate the long term neurobehavioral, neuropathological and biochemical consequences.

Major Task 1: Validation of potential targets for therapeutic intervention in the pathogenic TBI-AD interrelationship - eicosanoid signaling, PI3-kinase/Akt/PTEN/mTOR/insulin signaling and RXR/PPAR pathway. [100% Complete]

Subtask 1: Obtaining ACURO approval (Roskamp Institute approval for TBI procedures is already in place, and will be amended to include the treatment paradigms). **Subtask 2**: Evaluation of bioactive lipids and downstream signaling in stored TBI and AD mouse model samples to identify targets from Leukotriene and Sphingomyelin signaling. **Subtask 3**: Administration of r-mTBI or r-sham injury to 180-220 male hTau mice (C57BL/6 humanized tau transgenics) at 2-3 months of age. **Subtask 4**: Administration of therapeutic compounds to mice for 2 weeks immediately prior to 3 month timepoint post TBI/sham. **Subtask 5**: Euthanasia of hTau mice at 3 months post-TBI/sham, followed by brain omic and antibody based analyses. **Subtask 6**: Validation of

identified targets in human TBI brains with history of repetitive concussions. **Subtask 7**: Evaluation and selection of therapeutics for each target for use in Task 2.
Task One Deliverable: Acute study validation of 6 coincident TBI and AD molecular targets and verification of up to eleven compounds as engaging those targets *in vivo. In task one, up to 110 hTau mice will receive the r-mTBI procedure, and up to 110 hTau mice will receive the r-sham procedure.*

Major Task 2: Chronic evaluation of the efficacy of two potential therapeutics against the pathogenic TBI-AD interrelationship. [100% Complete]

Milestone Two: Chronic evaluation of the efficacy of three potential therapeutics against the pathogenic TBI-AD interrelationship.
Subtask 1: Administration of r-mTBI or r-sham injury to 180 male mice at 2-3 months of age (60 mice per group). **Subtask 2**: Administration of therapeutic compounds to mice for 6 months beginning at 3 months post TBI/sham. Subtask 2: Administration of therapeutic compounds to hTau mice for 6 months beginning at 3 months post TBI/sham. **Subtask 3**: Neurobehavioral testing of hTau mice in the two weeks prior to 9 months post injury timepoint. **Subtask 4**: Euthanasia of hTau mice at 9 months post-TBI/sham, followed by further evaluation of outcomes. **Subtask 5**: Data analysis and interpretation to identify most potent therapeutic approach.
Task Two Deliverable: Chronic study validation of top two candidate treatments that rescue TBI-dependent sequelae from convergence with AD pathogenic mechanisms. *In task two, 90 mice will receive the r-mTBI procedure, and 90 mice will receive the r-sham procedure.*

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Summary of accomplishments for each Aim.

Aim 1: Validation of 6 potential targets for therapeutic intervention in the pathogenic TBI-AD interrelationship.

We completed target engagement and investigation of the therapeutic efficacy (i.e. ability to ameliorate glial reactivity and white matter changes in our TBI model) of all our potential molecular targets. This study was conducted in a short-term treatment paradigm lasting 14days.

Given the important role of neuronal and glial cells in TBI pathophysiology, we included as part of this Aim an additional in vitro screening platform for our compounds in injured microglia cell lines and primary cultures. We developed a timeline of pathological profiles of inflammatory markers in these cell culture models. We used this platform to screen the efficacy of our top performing

compounds, utilizing this as an additional validation step in the selection of some of our lower tier lists of top performing compounds prior to initiating our long term treatment studies in Aim 2. These studies have all been conducted with our top performing compounds, using microglial cell lines to detail drug induced effects following injury to the cell lines. The top two drug candidates chosen were Pioglitazone and Zileuton.

Aim 2: Chronic evaluation of the efficacy of two top performing therapeutics against the pathogenic TBI-AD interrelationship.

For the second aim of this project, we perform a chronic treatment and post-injury timepoint study in mice using our top two performing compounds, we selected Pioglitazone and Zileuton as the best performing compounds based on our in vivo/in vitro work. We generated behavioral analyses for cognitive function. After euthanasia brain tissue was collected for molecular and pathological studies. Our combined datasets reveal efficacy of Pioglitazone and PPARg as a target for r-mTBI in our model.

SUBTASK DESCRIPTIONS FOR MAJOR TASK 1

Subtask 1: Obtained ACURO approval [Completed]
Subtask 2: Evaluation of bioactive lipids and downstream signaling in stored TBI and AD mouse model samples to identify proposed targets from Leukotriene and Sphingomyelin signaling. [Completed]
Subtask 3: Administration of r-mTBI or r-sham injury to 180-220 male C57BL6/J mice at 2-3 months of age. **[Completed]**
Subtask 4: Administration of therapeutic compounds to C57BL6/J mice for 2 weeks immediately prior to 2 month timepoint post-TBI/sham. **[Completed]**
Subtask 4a-d: Administration of therapeutic compounds to target (eicosanoid signaling, PI3-kinase/Akt/PTEN/mTOR/insulin signaling and RXR/PPAR pathway)
Subtask 4e: Target exploration of downstream eicosanoid pathway and administration of therapeutic compounds to target leukotriene signaling
Subtask 4f: Target exploration of downstream sphingomyelin pathway and administration of therapeutic compounds to target sphingomyelin signaling
Subtask 5: Euthanasia of hTau mice at 3 months post-TBI/sham, followed by brain omic and antibody based analyses. **[Completed]**
Subtask 6: Validation of identified targets in human TBI brains with history of repetitive concussions. **[Completed]**
Subtask 7: Evaluation and selection of therapeutics for each target for use in Task 2. **[Completed]**

MAJOR TASK 1 DELIVERABLE: Acute study validation of coincident TBI and AD molecular targets and verification of 6-8 compounds as engaging those targets *in vivo.*
Selection of Top 2 performing compounds for study in MAJOR TASK 2.

SUBTASK DESCRIPTIONS FOR MAJOR TASK 2

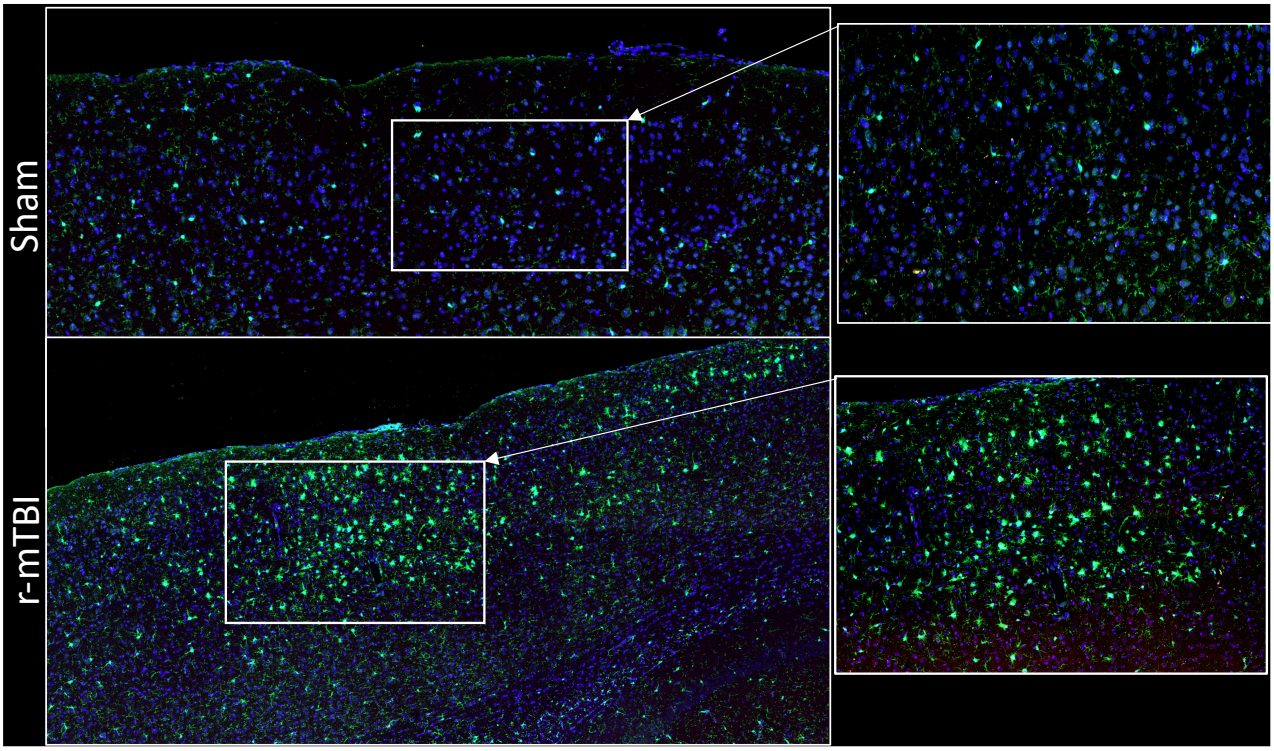
Subtask 1: Administration of r-mTBI or r-sham injury to 120 male mice at 2-3 months of age (60 mice per group) **[Completed]**
Animals will receive closed head injuries over a 1 month period.
For each of 2 potential therapeutics there will be 6 groups– [r-mTBI treated low dose; r-mTBI treated high dose; r-mTBI vehicle; r-sham treated low dose; r-sham treated high dose; r-sham vehicle] each with 12 mice
Subtask 2: Administration of therapeutic compounds to mice for 3 months beginning at 3 months post TBI/sham **[Completed]**
Subtask 2a: Administration of Top performing therapeutic compound I
Subtask 2b: Administration of Top performing therapeutic compound II
Subtask 3: Neurobehavioral testing of mice in the two weeks prior to 6 months post injury timepoint **[Completed]**
At the last two weeks prior to the 6 month post-injury time-point, animals will undergo neurobehavioral testing in the Barnes maze for 7 days, followed by rotarod test , open field and elevated plus maze tests.
Subtask 4: Euthanasia of mice at 6 months post-TBI/sham, followed by further evaluation of outcomes **[Completed]**
Subtask 4a: Brain neuropathological analyses
Subtask 4b: Molecular Omics and antibody based analyses
Subtask 5: Data analysis and interpretation to identify most potent therapeutic approach **[Completed]**

MAJOR TASK 2 DELIVERABLE: Chronic study validation of top three candidate treatments that rescue TBI-dependent sequelae from convergence with AD pathogenic mechanisms.

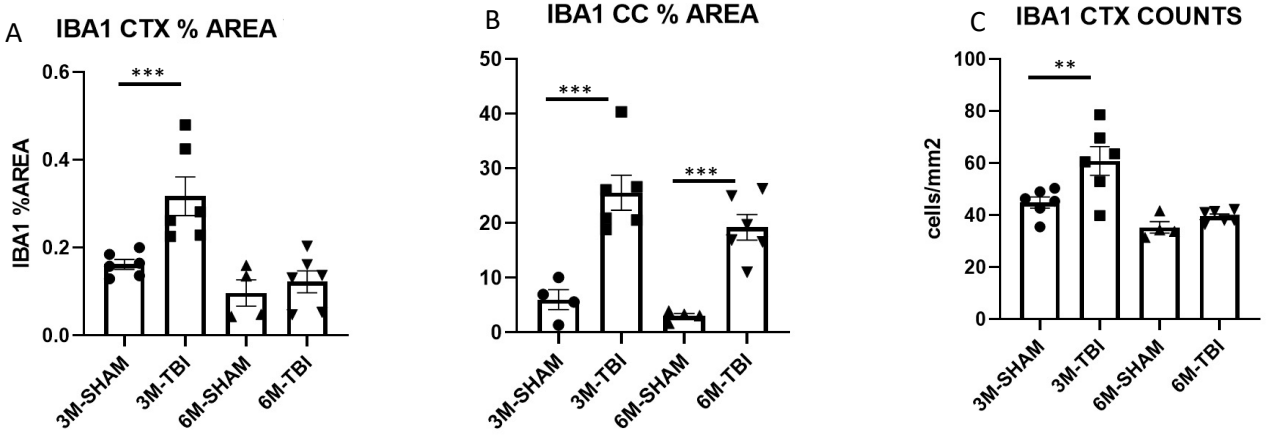
SUMMARY OF THE MAIN FINDINGS FROM STUDIES OVER THE LAST 3YRS/7Mos

1. Microglia reactivity in mice exposed to 20x r-mTBI paradigm (5 injuries/week for 1 months)

1.1. Microglia immunohistochemistry in a mouse model of r-mTBI/r-sham mice

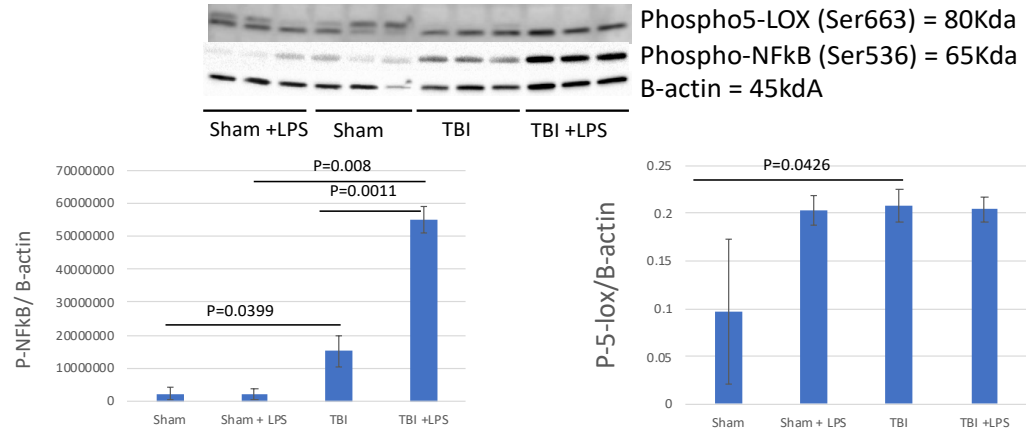


Representative confocal micrograph images showing increased Iba1 (green) expression in the cortex of mice exposed to repetitive mild TBI at 3 months post injury compared to r-sham injury mice

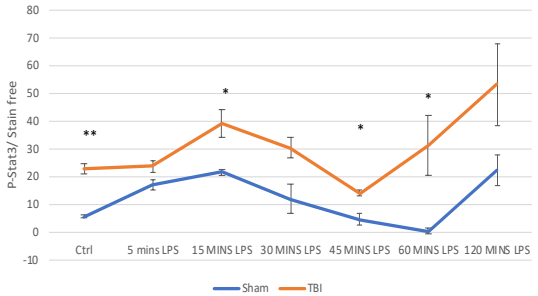


TBI increases Iba1 reactivity in mouse cortex. IHC images of mouse cortex (20x magnification) and corpus callosum (40x magnification) were collected and numbers and % reactive area of Iba1+ve cells were quantified in sham and injured mice at 3- and 6-months post injury or sham procedure. Normal distribution was assessed using Shapiro wilk test and data were assessed statistically using a one-way ANOVA. A. . 3M-Sham Vs 3M-TBI P=0.001. 6M-Sham Vs 6M-TBI P=0.588 B. 3M-Sham Vs 3M-TBI P=<0.001 . 6M-Sham Vs 6M-TBI P=<0.001. C. 3M-Sham Vs 3M-TBI P=0.003. 6M-Sham Vs 6M-TBI P=0.423. Error bars represent SEM.

1.2. Analysis of glial response to LPS (10ng/ml) for 2hrs after isolation from injured or sham mice



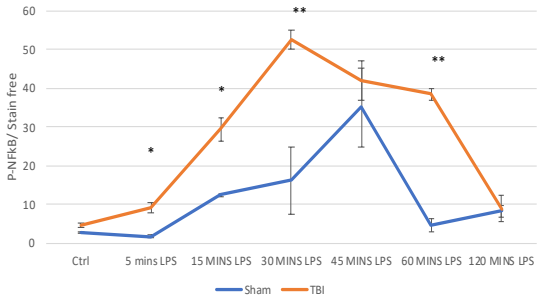
1.3. Inflammatory response profile to the LPS in r-mTBI vs sham primary microglial cells – *Injured microglia show prominent response vs shams – exhibiting primed response to LPS*



A graph showing the protein expression of Phospho- STAT3 (P-STAT3) expression in the cytoplasmic/ nuclear fraction of IMG immortalized microglial cells (N=3/group). Data were normalized to Stain free total protein. Cells were treated with DMEM/F12 media containing LPS and 10ng/ml. Data were assessed for normality using the Shapiro Wilk test and significance was assessed by a students T-test of a treated time point Vs the untreated control.

No LPS control Vs No LPS TBI samples P=0.006 (Indicated by **). 15 Mins LPS control Vs 15 Mins LPS TBI samples P=0.037 (Indicated by *). 45 Mins LPS control Vs 45 Mins LPS TBI samples P=0.027 (Indicated by *). 60 Mins LPS control Vs 60 Mins LPS TBI samples P=0.05 (Indicated by *).

Error bars represent SEM.

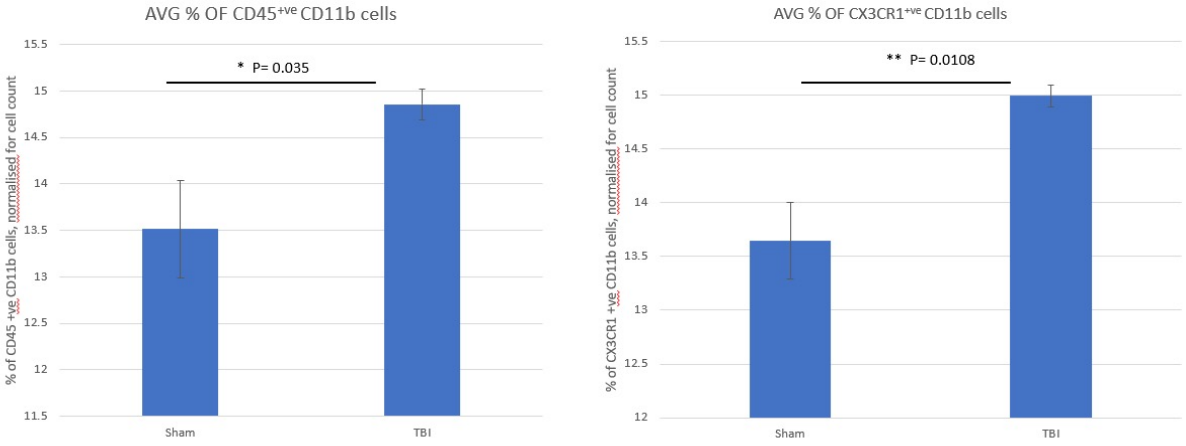


A graph showing the protein expression of Phospho- STAT3 (P-STAT3) expression in the cytoplasmic/ nuclear fraction of IMG immortalized microglial cells (N=3/group). Data were normalized to Stain free total protein. Cells were treated with DMEM/F12 media containing LPS and 10ng/ml. Data were assessed for normality using the Shapiro Wilk test and significance was assessed by a students T-test of a treated time point Vs the untreated control.

No LPS control Vs No LPS TBI samples P=0.0659 . 5 Mins LPS control Vs 5 Mins LPS TBI samples P=0.029 (Indicated by *). 15 Mins LPS control Vs 15 Mins LPS TBI samples P=0.023 (Indicated by *). 30 Mins LPS control Vs 30 Mins LPS TBI samples P=0.0013 (Indicated by **). 60 Mins LPS control Vs 60 Mins LPS TBI samples P=0.004 (Indicated by **).

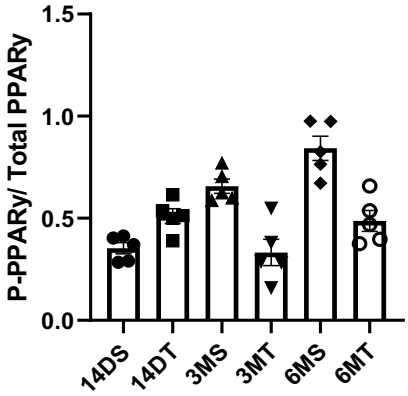
Error bars represent SEM.

1.4. Flow cytometry of CD11b/CD45 and CD11b/CX3CR1 cells at 3 months post-injury

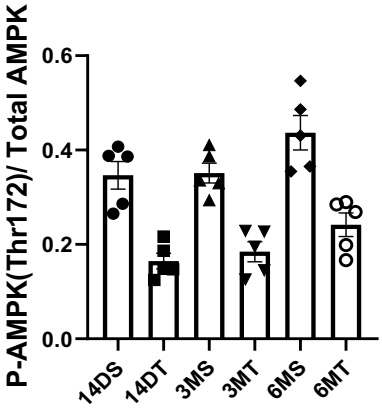


1.5. Validation of Targets (PPARg, PI3K, AKT, PTEN, 5-LOX etc..) in 20hit r-mTBI/r-sham model in the hippocampus

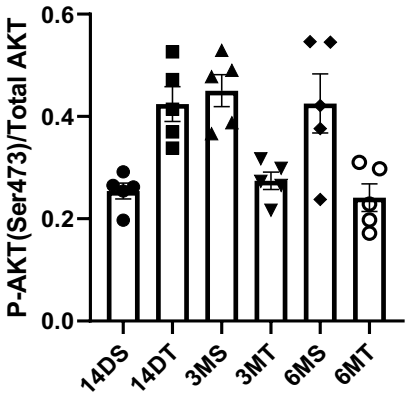
Western blotting data



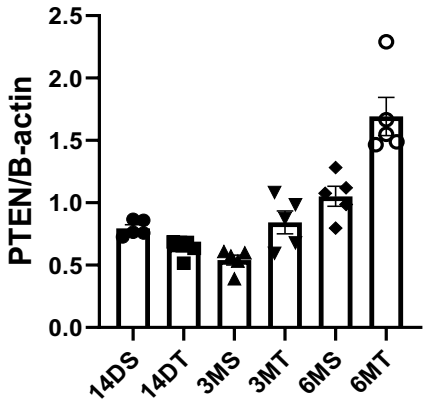
Analysis of hippocampal expression of Phospho-PPAR gamma (Ser112) via immunoblotting. Data were assessed for normal distribution via Shapiro-wilk test and data were assessed statistically using a one-Way ANOVA. 14D sham Vs TBI P=0.218. 3M sham Vs TBI P=<0.001. 6M Sham Vs TBI P=<0.001. N=5/group. Error bars represent SEM



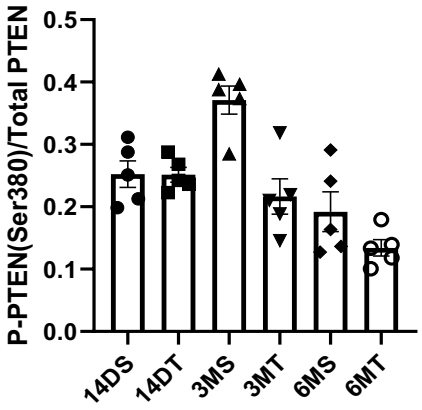
Analysis of hippocampal expression of Phospho-AMPK (Thr172) via immunoblotting. Data were assessed for normal distribution via Shapiro-wilk test and data were assessed statistically using a one-Way ANOVA. 14D sham Vs TBI P=<0.001. 3M sham Vs TBI P=0.001. 6M Sham Vs TBI P=<0.001. N=5/group. Error bars represent SEM



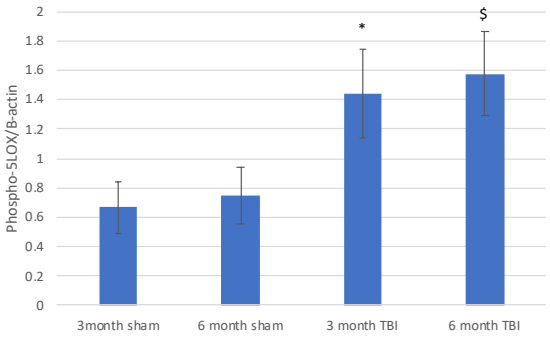
Analysis of hippocampal expression of Phospho-AKT (Ser473) via immunoblotting. Data were assessed for normal distribution via Shapiro-wilk test and data were assessed statistically using a one-Way ANOVA. 14D sham Vs TBI P=0.016. 3M sham Vs TBI P=0.012. 6M Sham Vs TBI P=0.008. N=5/group. Error bars represent SEM



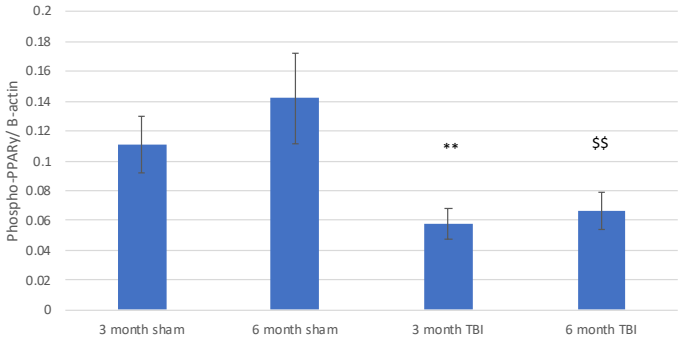
Analysis of hippocampal expression of PTEN via immunoblotting. Data were assessed for normal distribution via Shapiro-wilk test and data were assessed statistically using a one-Way ANOVA. 14D sham Vs TBI P=0.776. 3M sham Vs TBI P=0.147. 6M Sham Vs TBI P=<0.001. N=5/group. Error bars represent SEM



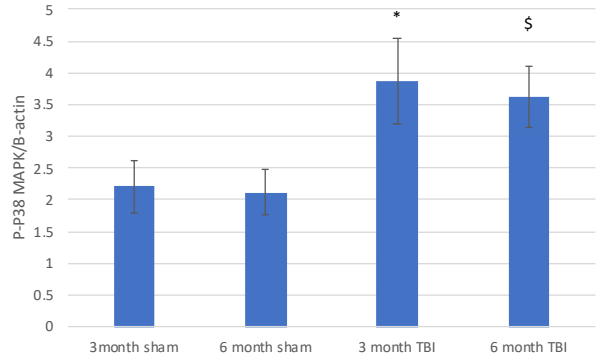
Analysis of hippocampal expression of Phospho-PTEN (Ser380) via immunoblotting. Data were assessed for normal distribution via Shapiro-wilk test and data were assessed statistically using a one-Way ANOVA. 14D sham Vs TBI P=0.999. 3M sham Vs TBI P=<0.001. 6M Sham Vs TBI P=0.486. N=5/group. Error bars represent SEM



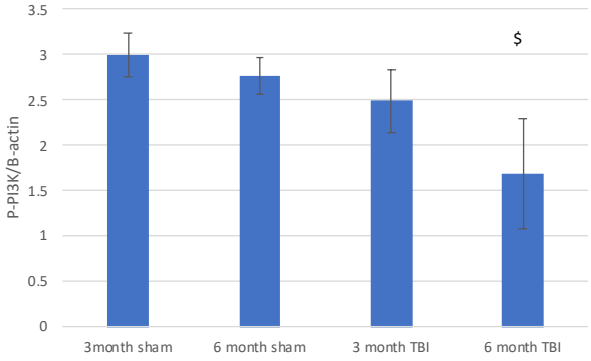
A graph showing the protein expression of Phospho- 5-Lipoxygenase (P-5-LOX) (Serine 663) in the cytoplasmic/ nuclear fraction of cortex homogenate derived from sham and injured wild type mice at 3 and 6 months post injury (N=4 group). Data were normalized to total 5-LOX and to B-actin. Data were assessed for normality using the Shapiro-Wilk test and then analyzed using a one way Anova and then corrected for family wise error rate using the Benjamini Hochberg and Yekutieli correction for false discovery rate. 3 month Sham Vs 3 month TBI P=0.0207 (Indicated by *). 6 month Sham vs 6 month TBI P=0.0114 (indicated by $). Error bars represent SEM.



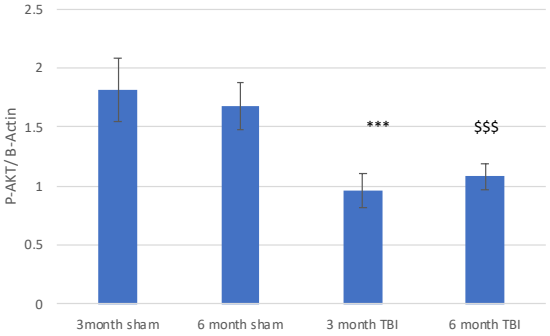
A graph showing the protein expression of Phospho-Peroxisome proliferator activated receptor gamma (PPARγ) (Serine 112) in the cytoplasmic/ nuclear fraction of cortex homogenate derived from sham and injured wild type mice at 3 and 6 months post injury (N=4 group). Data were normalized to B-actin. Data were assessed for normality using the Shapiro-Wilk test and then analyzed using a one way Anova and then corrected for family wise error rate using the Benjamini Hochberg and Yekutieli correction for false discovery rate. 3 month Sham Vs 3 month TBI P=0.00461 (Indicated by **). 6 month Sham vs 6 month TBI P=0.0083 (indicated by $$). Error bars represent SEM.



A graph showing the protein expression of Phospho-P38 Mitogen associated protein kinase (P-P38 MAPK) (Threonine 180/tyrosine 182) in the cytoplasmic/ nuclear fraction of cortex homogenate derived from sham and injured wild type mice at 3 and 6 months post injury (N=4 group). Data were normalized to B-actin. Data were assessed for normality using the Shapiro-Wilk test and then analyzed using a one way Anova and then corrected for family wise error rate using the Benjamini Hochberg and Yekutieli correction for false discovery rate. 3 month Sham Vs 3 month TBI P=0.0194 (Indicated by *). 6 month Sham vs 6 month TBI P=0.0083 (indicated by $$). Error bars represent SEM.

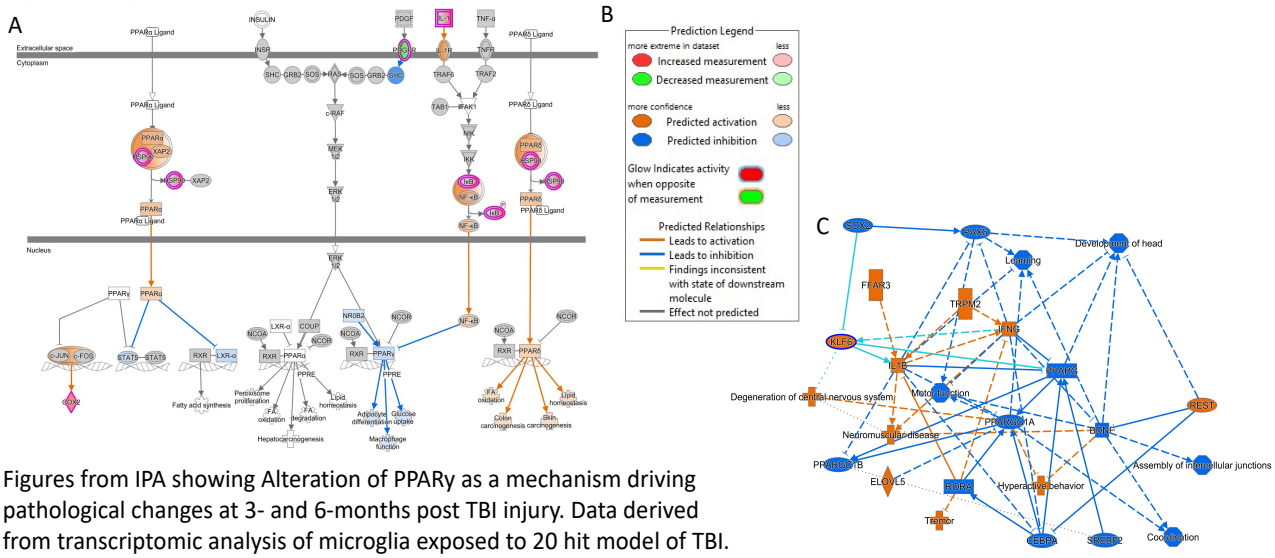


A graph showing the protein expression of Phospho-phosphoinositide 3-kinase (P-PI3K) (Tyrosine458/ Tyrosine199) in the cytoplasmic/ nuclear fraction of cortex homogenate derived from sham and injured wild type mice at 3 and 6 months post injury (N=4 group). Data were normalized to B-actin. Data were assessed for normality using the Shapiro-Wilk test and then analyzed using a one way Anova and then corrected for family wise error rate using the Benjamini Hochberg and Yekutieli correction for false discovery rate. 6 month Sham vs 6 month TBI P=0.0178 (indicated by $). Error bars represent SEM.



A graph showing the protein expression of Phospho-AKT(P-AKT) (Serine 473) in the cytoplasmic/ nuclear fraction of cortex homogenate derived from sham and injured wild type mice at 3 and 6 months post injury (N=4 group). Data were normalized to B-actin. Data were assessed for normality using the Shapiro-Wilk test and then analyzed using a one way Anova and then corrected for family wise error rate using the Benjamini Hochberg and Yekutieli correction for false discovery rate. 3 month Sham Vs 3 month TBI P=<0.001 (Indicated by ***). 6 month Sham vs 6 month TBI P=<0.001 (indicated by $$$). . Error bars represent SEM.

1.6. Transcriptomic analysis of microglia shows support for involvement of chronic PPAR dysregulation as a major contributor to TBI pathology

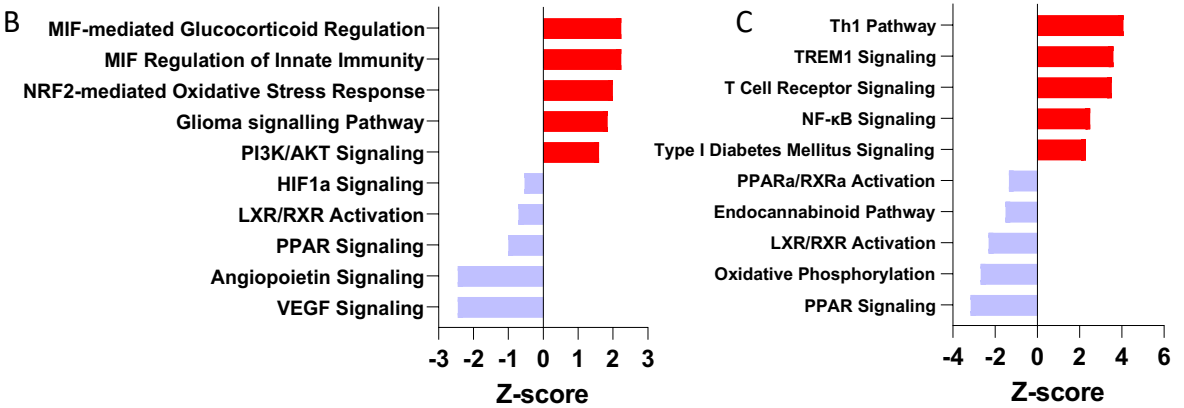


Figures from IPA showing Alteration of PPARγ as a mechanism driving pathological changes at 3- and 6-months post TBI injury. Data derived from transcriptomic analysis of microglia exposed to 20 hit model of TBI.

1.7. Top predicted upstream regulators of microglial phenotype at 3 months post injury

A

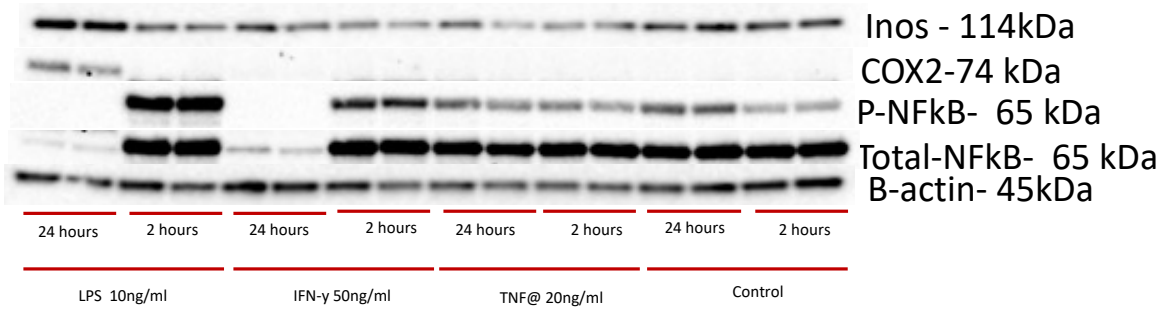
Predicted upstream regulators		
Name	P-Value	Predicted Activation
Lipopolysaccharide (LPS)	1.60x10E-29	Activated
Tumor Necrosis factor alpha (TNFa)	2.28x10E-24	Activated
Interleukin 1-beta (IL1B)	5.11x10E-23	Activated
Interferon gamma (IFNG)	1.25x10E-20	Activated
Amyloid Precursor protein (APP)	7.13x10E-20	Activated

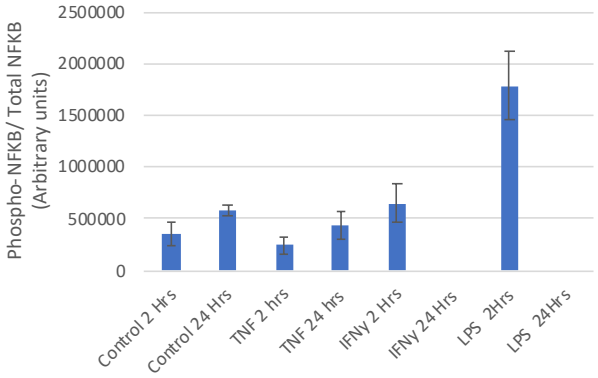


A. Table showing the top predicted upstream regulators of microglial phenotype at 3months post injury. B&C. Analysis of microglial transcriptomic signature at 3(B)- and 6(C)-months post injury showing Top up and down-regulated canonical pathways.

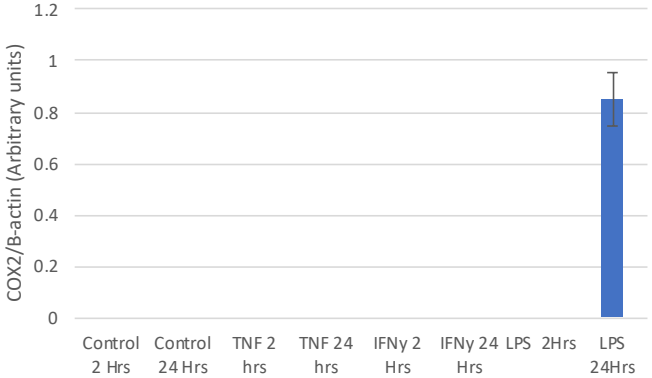
1.8. LPS, IFNg and TNFa were selected as upstream regulators of microglial dysfunction and were exposed to microglial cell culture to generate an in vitro model to screen compounds modulating our targets and mitigating against inflammation.

1.8.1. Immortalized Microglia (IMG) cell culture tissue following stimulation for 2 & 24 hours with LPS (10ng/ml) ,IFN-y (50ng/ml) or TNFa (20ng/ml)

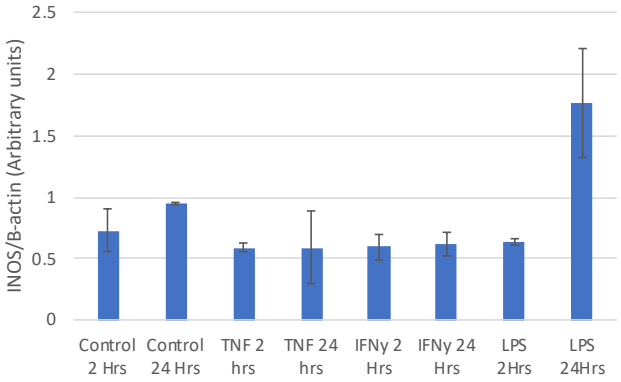




A graph showing the protein expression of Phospho-NFkB (Serine 536) in the cytoplasmic/ nuclear fraction of Immortalised Microglia (IMG) cell culture tissue following stimulation for 2 & 24 hours with LPS (10ng/ml) ,IFN-y (50ng/ml) or TNFa (20ng/ml) (n=2/condition) Error bars represent SD.

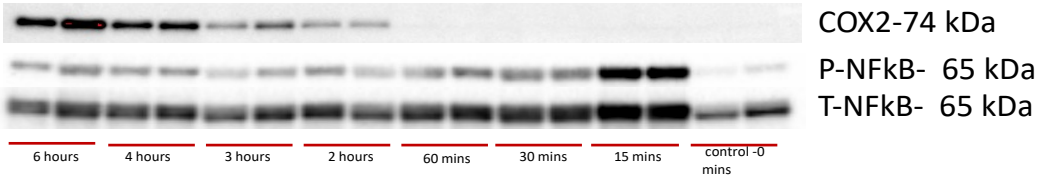


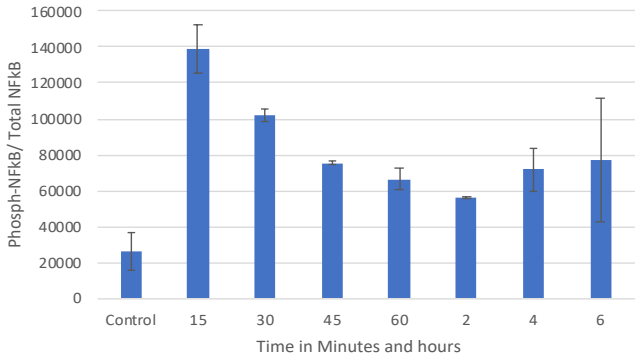
A graph showing the protein expression of cyclo-oxygenase2 (cox2) in the cytoplasmic/ nuclear fraction of Immortalised Microglia (IMG) cell culture tissue following stimulation for 2 & 24 hours hours with LPS (10ng/ml) ,IFN-y (50ng/ml) or TNFa (20ng/ml) (n=2/condition) Error bars represent SD.



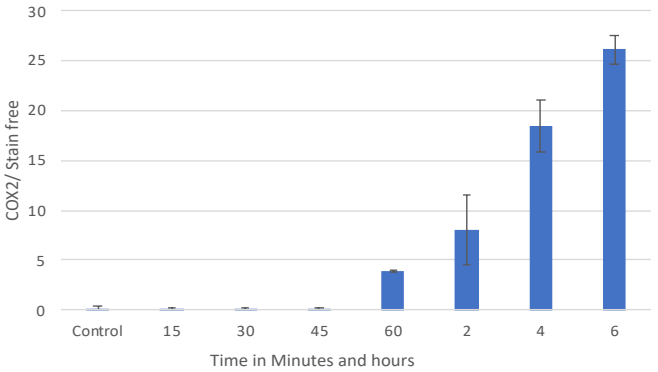
A graph showing the protein expression of Inducible Nitric Oxide synthase (INOS) in the cytoplasmic/nuclear fraction of Immortalised Microglia (IMG) cell culture tissue following stimulation for 2 & 24 hours with hours with LPS (10ng/ml) ,IFN-y (50ng/ml) or TNFa (20ng/ml) (n=2/condition) Error bars represent SD.

1.8.2. IMG immortalized microglial response to LPS (10ng/ml) at 15minutes - 6 hrs of stimulation.



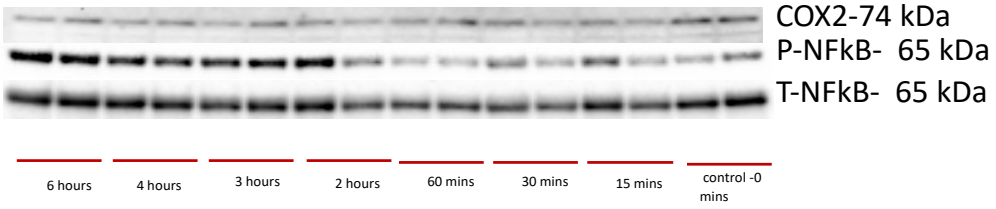


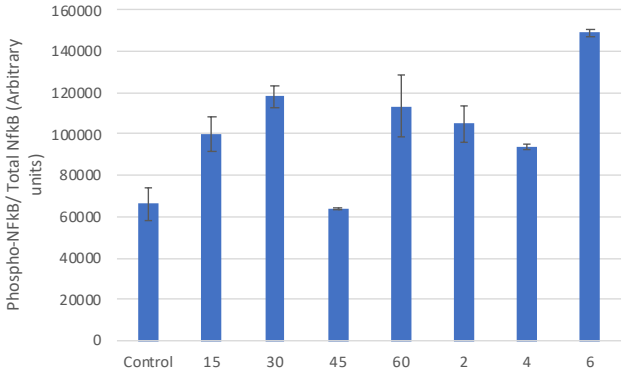
A graph showing the protein expression of Phospho-NFkB (Serine 536) in the cytoplasmic/ nuclear fraction of Immortalised Microglia (IMG) cell culture tissue following stimulation for between 15 minutes and 6 hours with Lipolysaccharide (LPS) (10ng/ml). (n=2/condition) Error bars represent SD. Data are normalised to stain free blot



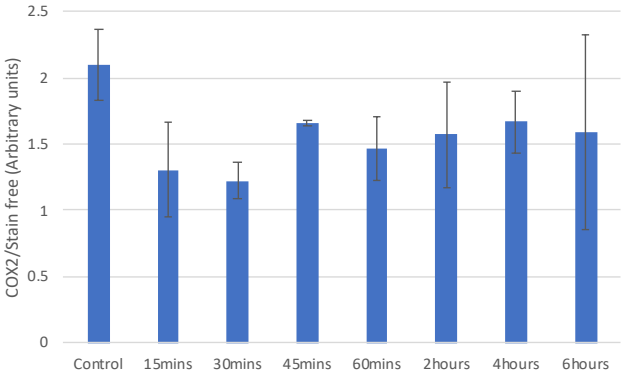
A graph showing the protein expression of cyclo-oxygenase2 (cox2) in the cytoplasmic/ nuclear fraction of Immortalised Microglia (IMG) cell culture tissue following stimulation for between 15 minutes and 6 hours with Lipolysaccharide (LPS) (10ng/ml). (n=2/condition) Error bars represent SD. Data are normalised to stain free blot

1.8.3. IMG immortalized microglial response to IFN-y (50ng/ml) at 15minutes- 6 hrs of stimulation.





A graph showing the protein expression of Phospho-NFkB (Serine 536) in the cytoplasmic/ nuclear fraction of Immortalised Microglia (IMG) cell culture tissue following stimulation for between 15 minutes and 6 hours with IFN-y (50ng/ml) . (n=2/condition) Error bars represent SD. Data are normalised to stain free blot

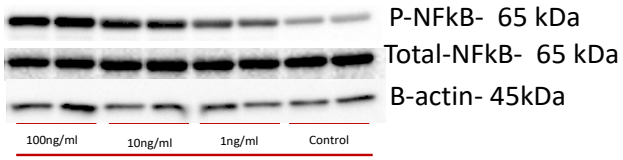


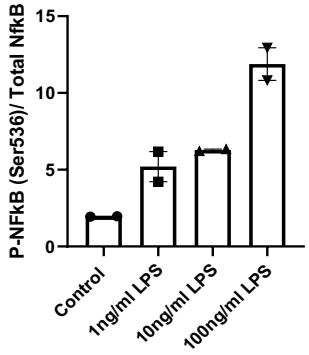
A graph showing the protein expression of cyclo-oxygenase2 (cox2) in the cytoplasmic/ nuclear fraction of Immortalised Microglia (IMG) cell culture tissue following stimulation for between 15 minutes and 6 hours with IFN-y (50ng/ml) (n=2/condition) Error bars represent SD. Data are normalised to stain free blot

We selected LPS due to the performance of targets after exposure.

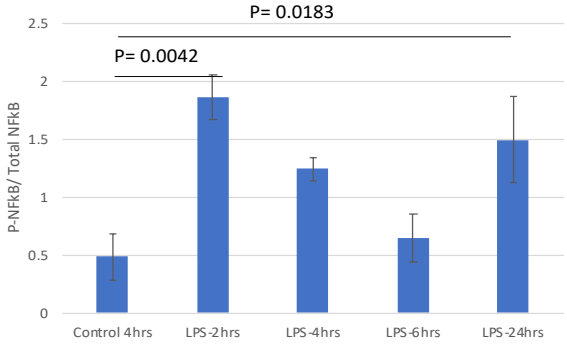
2. In vitro screening of compounds in microglial cell lines exposed to LPS.

2.1. LPS as an inflammatory stimuli to modify targets for in vivo screening in microglia cultures

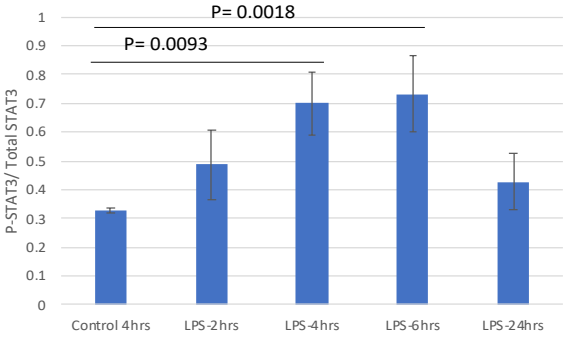




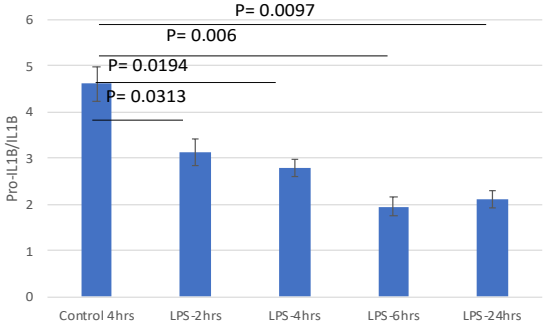
Immunoblot analysis of the effects of exposure to varying concentrations of LPS on the expression of P-NFkB (Ser536) in IMG immortalised microglia. Microglia were treated for 2 hours with either 1,10 or 100ng/ml LPS in cell culture media.



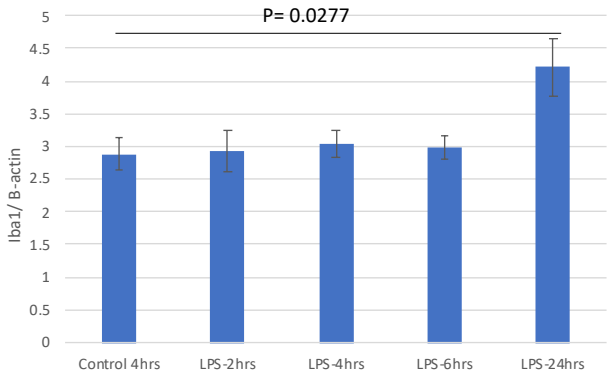
A graph showing the protein expression of Phospho- NFkB (Serine 536) in the cytoplasmic/ nuclear fraction of cell lysate from IMG immortalized microglial cells (n=3/group), data were normalized to Total NFkB and to B-actin. Cells treated with standard media @ 4 hours (n=3) vs Cells treated with LPS @10ng/ml for 2 hours (n=3) P= 0.0042. Cells treated with standard media @ 4 hours (n=3) vs Cells treated with LPS @10ng/ml for 24 hours (n=3) P= 0.0183. Error bars represent SD.



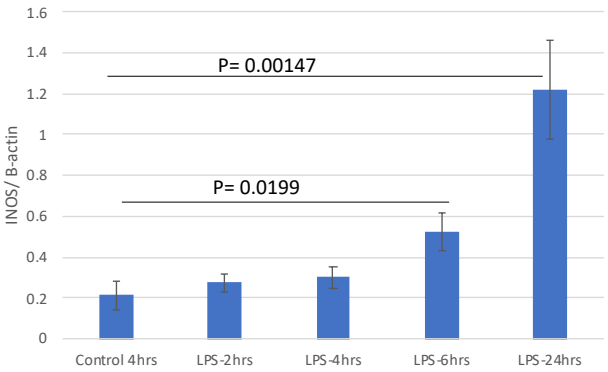
A graph showing the protein expression of Phospho- STAT3 (Tyrosine 705) in the cytoplasmic/ nuclear fraction of cell lysate from IMG immortalized microglial cells (n=3/group), data were normalized to Total NFkB and to B-actin. Cells treated with standard media @ 4 hours (n=3) vs Cells treated with LPS @10ng/ml for 4 hours (n=3) P= 0.00932. Cells treated with standard media @ 4 hours (n=3) vs Cells treated with LPS @10ng/ml for 24 hours (n=3) P= 0.0018. Error bars represent SD.



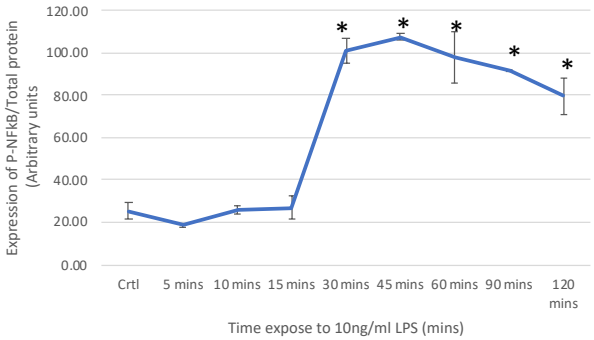
A graph showing the protein expression ratio of Pro-IL1B to mature IL1B in the cytoplasmic/ nuclear fraction of cell lysate from IMG immortalized microglial cells (n=3/group), data were normalized to B-actin. Cells treated with standard media @ 4 hours (n=3) vs Cells treated with LPS @10ng/ml for 2 hours (n=3) P= 0.0313. Cells treated with standard media @ 4 hours (n=3) vs Cells treated with LPS @10ng/ml for 4 hours (n=3) P= 0.0194. Cells treated with standard media @ 4 hours (n=3) vs Cells treated with LPS @10ng/ml for 6 hours (n=3) P= 0.006. Cells treated with standard media @ 4 hours (n=3) vs Cells treated with LPS @10ng/ml for 24 hours (n=3) P= 0.0097. Error bars represent SD.



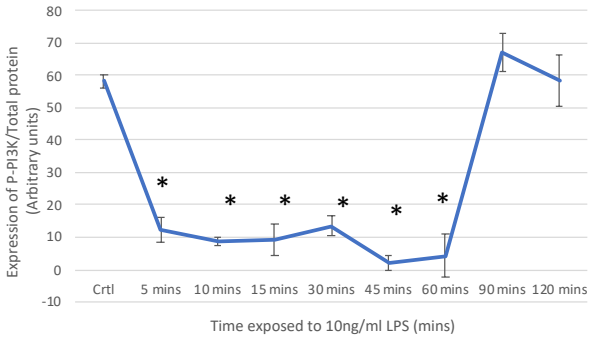
A graph showing the protein expression of Iba1 in the cytoplasmic/ nuclear fraction of cell lysate from IMG immortalized microglial cells (n=3/group), data were normalized to B-actin. Cells treated with standard media @ 4 hours (n=3) vs Cells treated with LPS @10ng/ml for 24 hours (n=3) P= 0.0277. Error bars represent SD.



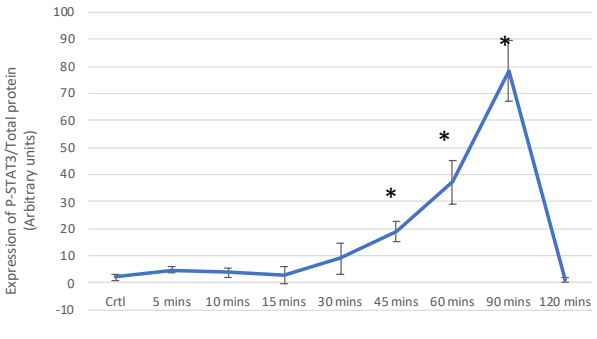
A graph showing the protein expression of Inducible Nitric oxide synthase (INOS) in the cytoplasmic/ nuclear fraction of cell lysate from IMG immortalized microglial cells (n=3/group), data were normalized to B-actin. Cells treated with standard media @ 4 hours (n=3) vs Cells treated with LPS @10ng/ml for 2 hours (n=3) P= 0.0042. Cells treated with standard media @ 4 hours (n=3) vs Cells treated with LPS @10ng/ml for 24 hours (n=3) P= 0.0183. Error bars represent SD.



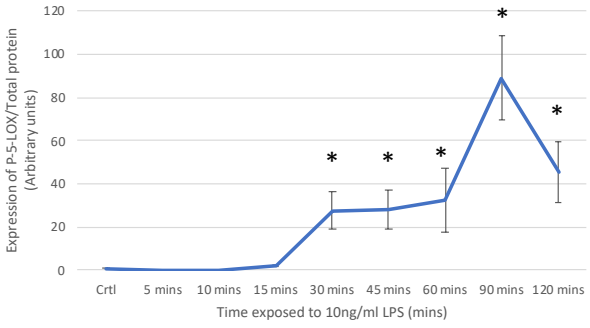
A graph showing the protein expression of Phospho- NFkB (Serine 536) in the cytoplasmic/ nuclear fraction of cell lysate from IMG immortalized microglial cells (n=3/group), data were normalized to total Protein. Cells treated with either control DMEM/F12 media or media containing LPS @10ng/ml for varying lengths of time. Data were analyzed via students T- test significance of treated time point vs control is shown by *. Control vs 30 mins P=<0.001. Control vs 45 mins P=<0.001. Control vs 60 mins P=0.0044. Control vs 90 mins P=0.0051. Control vs 120 mins P=0.0079. Error bars represent SEM.



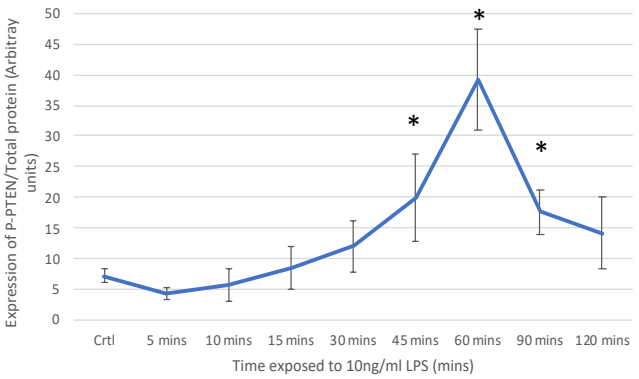
A graph showing the protein expression of Phospho-PI3K (Tyrosine 199) P55 in the cytoplasmic/ nuclear fraction of cell lysate from IMG immortalized microglial cells (n=3/group), data were normalized to total Protein. Cells treated with either control DMEM/F12 media or media containing LPS @10ng/ml for varying lengths of time. Data were analyzed via students T- test significance of treated time point vs control is shown by *. Control vs 5 mins P=0.0067. Control vs 10 mins P=<0.001. Control vs 15 mins P=0.0048. Control vs 30 mins P=0.0053. Control vs 45 mins P=<0.001. Control vs 60 mins P=0.0049. Error bars represent SEM.



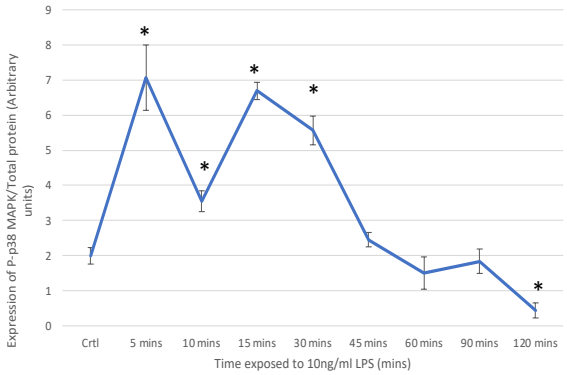
A graph showing the protein expression of Phospho-STAT3 (Tyrosine 705) in the cytoplasmic/ nuclear fraction of cell lysate from IMG immortalized microglial cells (n=3/group), data were normalized to total Protein. Cells treated with either control DMEM/F12 media or media containing LPS @10ng/ml for varying lengths of time. Data were analyzed via two-tailed students T- test significance of treated time point vs control is shown by *. Control vs 45 mins P=0.02114. Control vs 60 mins P=0.0093. Control vs 90 mins P=0.004. Error bars represent SEM.



A graph showing the protein expression of Phospho-5-LOX (Serine 663) in the cytoplasmic/ nuclear fraction of cell lysate from IMG immortalized microglial cells (n=3/group), data were normalized to total Protein. Cells treated with either control DMEM/F12 media or media containing LPS @10ng/ml for varying lengths of time. Data were analyzed via students T-test significance of treated time point vs control is shown by *. Control vs 30 mins P=<0.001. Control vs 45 mins P=<0.001. Control vs 60 mins P=0.017. Control vs 90 mins P=<0.001. Control vs 120 mins P=<0.001. Error bars represent SEM.



A graph showing the protein expression of Phospho-PTEN (Serine 380) in the cytoplasmic/ nuclear fraction of cell lysate from IMG immortalized microglial cells (n=3/group), data were normalized to total Protein. Cells treated with either control DMEM/F12 media or media containing LPS @10ng/ml for varying lengths of time. Data were analyzed via students T-test significance of treated time point vs control is shown by *. Control vs 45 mins P=0.0411. Control vs 60 mins P=0.010. Control vs 90 mins P=0.0239. Error bars represent SEM.



A graph showing the protein expression of Phospho-p38 MAPK (Threonine 180/Tyrosine 182) in the cytoplasmic/ nuclear fraction of cell lysate from IMG immortalized microglial cells (n=3/group), data were normalized to total Protein. Cells treated with either control DMEM/F12 media or media containing LPS @10ng/ml for varying lengths of time. Data were analyzed via students T-test significance of treated time point vs control is shown by *. Control vs 5 mins P=0.0062. Control vs 10 mins P=0.0154. Control vs =15 mins P=<0.001. Control vs 30 mins P=0.0017. Control vs 120 mins P=0.008. Error bars represent SEM.

SCREENING COMPOUNDS IN LPS EXPOSED MICROGLIA CELL CULURES

- IMG - Immortalized microglial cells were grown to 85-90% confluency in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) or DMEM (for astrocytes), with cells cultured at 37C and 5%CO2.
- Cells were treated with **several compounds** with/w.o 10ng/ml LPS exposure which lasted for 15mins to 45hrs
- Cells were Lysed using Mammalian protein extraction reagent (MPER) supplemented with Protease and Phosphatase inhibitors.
- Protein concentration was assessed by (BCA) assay, 15ug of protein was then loaded onto a Bis-tris polyacrylamide gel electrophoresis (PAGE) using a 4-20% gradient gel. Protein was then transferred to a PVDF membrane overnight at 90mA.

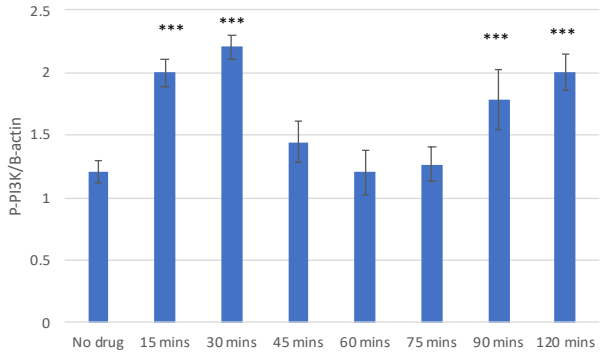
List of compounds and their concentrations screened in our in vitro platform

Drug	Low Dose	High dose
Zileuton	25um	100um
Pioglitazone	2.5um	10um
Montelukast	3um	12um
Fingolimod	50nm	200nm
7'8-DHF	15um	60um
Insulin	50nm	200nm
Bexarotene	50nm	200nm
BisPeroxovanadium (BpV)	50nm	200nm
Rapamycin	100nm	400nm
BpV + Rapamycin	50nm +100nm	200nm +400nm

Below only 4 drugs are presented from this list above.

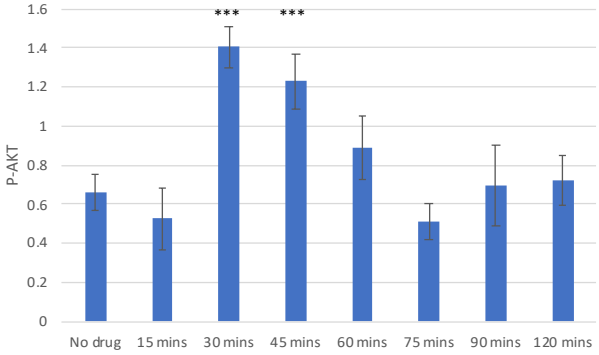
2.2. In vitro screening of Insulin on microglia

2.2.1. Investigating drug timing with response of primary target of Insulin to optimize in vitro drug treatment paradigm



A graph showing the protein expression of Phospho- PI3K (Tyrosine458/ Tyrosine199) expression in the cytoplasmic/ nuclear fraction of IMG immortalized microglial cells (N=3/group). Data were normalized to B-actin. Cells were treated with DMEM/F12 media containing Insulin @25nM for 15- 120 minutes to identify the peak of temporal target engagement. Data were assessed for normality using the Shapiro Wilk test and significance was assessed by a students T-test of a treated time point Vs the untreated control.

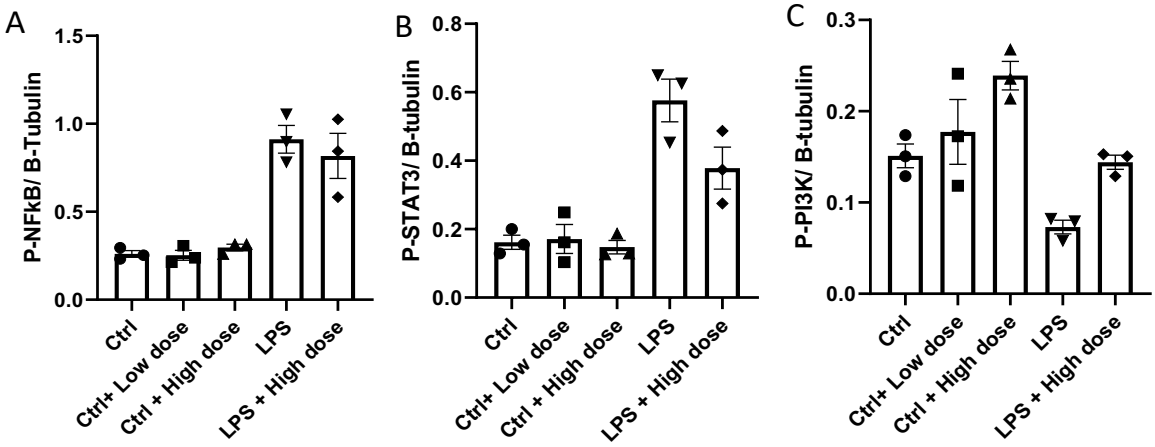
No drug control Vs 15 minutes treated samples P=<0.001 (Indicated by ***).No drug control Vs 30 minutes treated samples P=<0.001 (Indicated by ***). No drug control Vs 90 minutes treated samples P=<0.001 (Indicated by ***). No drug control Vs 120 minutes treated samples P=<0.001 (Indicated by ***). Error bars represent SEM.



A graph showing the protein expression of Phospho-AKT (Serine 473) expression in the cytoplasmic/ nuclear fraction of IMG immortalized microglial cells (N=3/group). Data were normalized to B-actin. Cells were treated with DMEM/F12 media containing Insulin @25nM for 15- 120 minutes to identify the peak of temporal target engagement. Data were assessed for normality using the Shapiro Wilk test and significance was assessed by a students T-test of a treated time point Vs the untreated control.

No drug control Vs 30 minutes treated samples P=<0.001 (Indicated by ***).No drug control Vs 45 minutes treated samples P=<0.001 (Indicated by ***). Error bars represent SEM.

2.2.2. IMG microglia were treated with 10ng/ml LPS and either 50nM (Low dose) or 200nM (High dose) Insulin for 2 hours



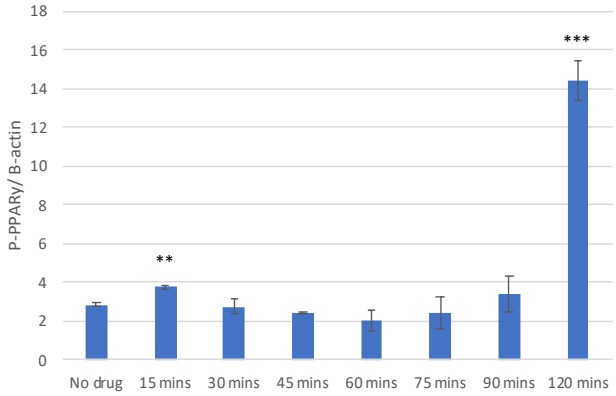
Analysis of expression of Phospho-NFKB (Ser536) via immunoblotting of whole cell lysate. Data were assessed for normal distribution via Shapiro-wilk test and data were assessed statistically using a one-Way ANOVA. Ctrl Vs LPS P=<0.001. LPS Vs LPS + High dose P=0.869. N=3/group. Error bars represent SEM

Analysis of expression of Phospho-STAT3 (Tyr705) via immunoblotting of whole cell lysate. Data were assessed for normal distribution via Shapiro-wilk test and data were assessed statistically using a one-Way ANOVA. Ctrl Vs LPS P=<0.001. LPS Vs LPS + High dose P=0.068. N=3/group. Error bars represent SEM

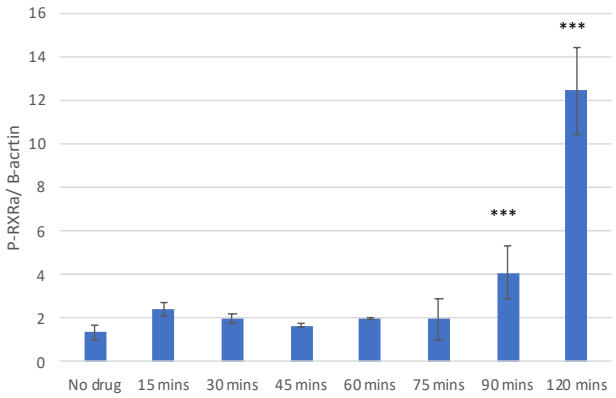
Analysis of expression of Phospho-PI3K (Tyr199) via immunoblotting of whole cell lysate. Data were assessed for normal distribution via Shapiro-wilk test and data were assessed statistically using a one-Way ANOVA. Ctrl Vs LPS P=0.015. LPS Vs LPS + High dose P=0.024. N=3/group. Error bars represent SEM

2.3. In vitro screening of Pioglitazone on microglia

2.3.1. Investigating drug timing with response of primary target of Pioglitazone to optimize in vitro drug treatment paradigm

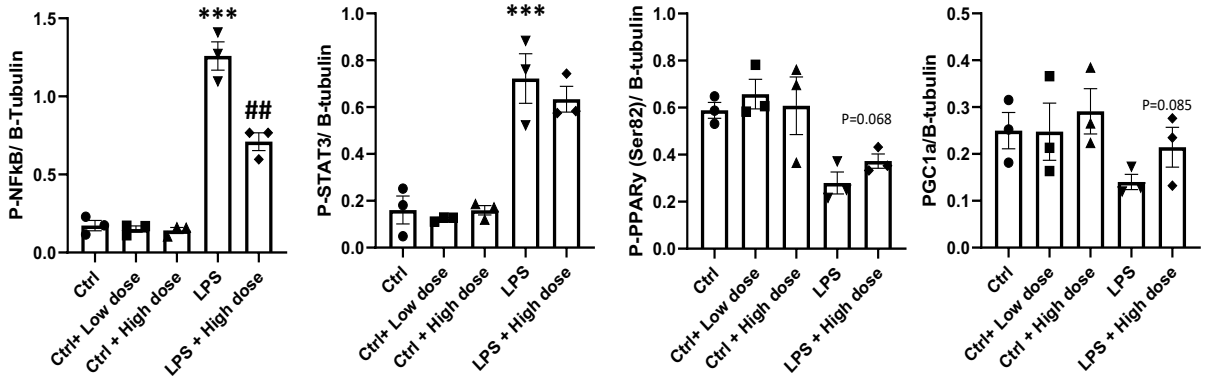


A graph showing the protein expression of Phospho- PPARY (Serine 112) expression in the cytoplasmic/ nuclear fraction of IMG immortalized microglial cells (N=3/group). Data were normalized to B-actin. Cells were treated with DMEM/F12 media containing Pioglitazone @10uM for 15- 120 minutes to identify the peak of temporal target engagement. Data were assessed for normality using the Shapiro Wilk test and significance was assessed by a students T-test of a treated time point Vs the untreated control. No drug control Vs 15 minutes treated samples P=0.001 (Indicated by **) No drug control Vs 120 minutes treated P=<0.001 (Indicated by ***). Error bars represent SEM.



A graph showing the protein expression of Phospho- RXRa (Serine 112) expression in the cytoplasmic/ nuclear fraction of IMG immortalized microglial cells (N=3/group). Data were normalized to B-actin. Cells were treated with DMEM/F12 media containing Pioglitazone @10uM for 15- 120 minutes to identify the peak of temporal target engagement. Data were assessed for normality using the Shapiro Wilk test and significance was assessed by a students T-test of a treated time point Vs the untreated control. No drug control Vs 120 minutes treated P=<0.001 (Indicated by ***)No drug control Vs 90 minutes treated P=<0.001 (Indicated by ***).Error bars represent SEM.

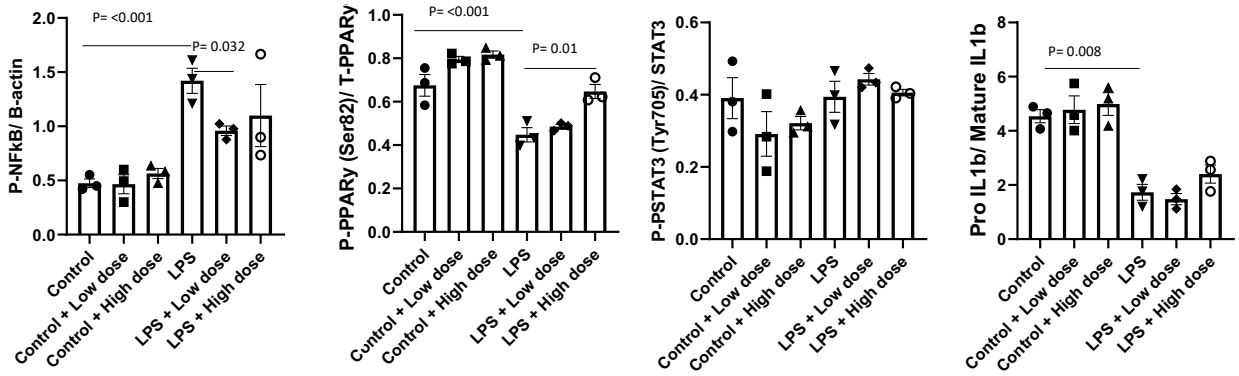
2.3.2. Effect of Pioglitazone on Microglia - 2hrs



A graph showing the protein expression of Phospho-NFkB (Serine536), Phospho-STAT3(Tyrosine705), Phospho-PPARy and PGC1a in the cytoplasmic/ nuclear fraction of cell lysate from IMG immortalized microglia cells treated with Pioglitazone 1uM(Low Dose) or 10uM (High dose) and 10ng/ml Lipopolysaccharide (LPS) for 2 hours(n=3/group), data were normalized to B-tubulin. Data were assessed for normal distribution and significance was assessed using a one-way ANOVA following by post hoc testing with a two-stage linear setup by Benjemini-Hochberg and Yekuteli to control for false discovery rate. Significance vs control is indicated on the graph (*=P<0.05) (**=P<0.01) (***=P<0.001). Significance vs LPS treated is indicated on the graph (#=P<0.05) (##=P<0.01) (###=P<0.001)

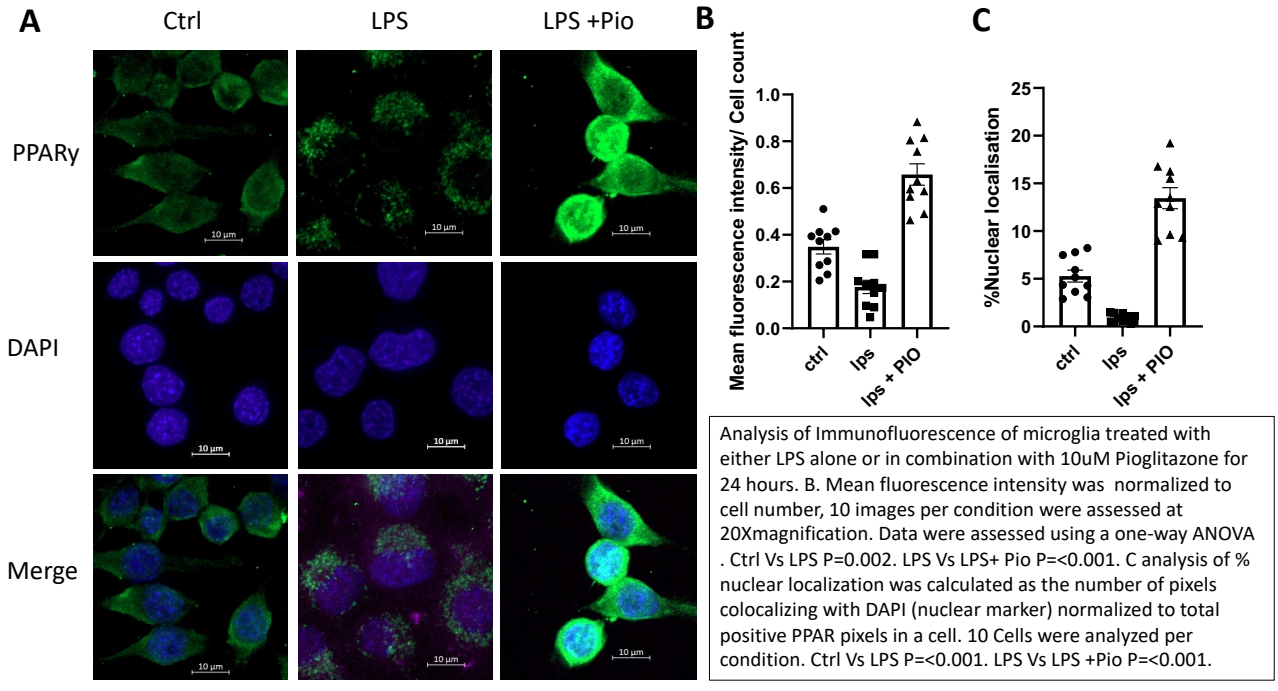
2.3.3. Effect of Pioglitazone on Microglia 24hrs

Biochemistry of cell lysates



A graph showing the protein expression of Phospho-NFkB (Serine536), Phospho-STAT3(Tyrosine705), Phospho-PPARy(Ser112) and PGC1a in the cytoplasmic/ nuclear fraction of cell lysate from IMG immortalized microglia cells treated with Pioglitazone 1uM(Low Dose) or 10uM (High dose) and 10ng/ml Lipopolysaccharide (LPS) for 24 hours(n=3/group), data were normalized to B-tubulin. Data were assessed for normal distribution and significance was assessed using a one-way ANOVA following by post hoc testing with a two-stage linear setup by Benjemini-Hochberg and Yekuteli to control for false discovery rate. Significance vs control is indicated on the graph (*=P<0.05) (**=P<0.01) (***=P<0.001). Significance vs LPS treated is indicated on the graph (#=P<0.05) (##=P<0.01) (###=P<0.001)

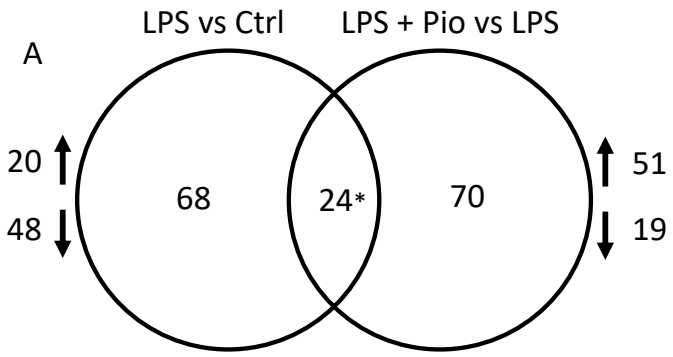
Immunocytochemistry



Analysis of Immunofluorescence of microglia treated with either LPS alone or in combination with 10uM Pioglitazone for 24 hours. B. Mean fluorescence intensity was normalized to cell number, 10 images per condition were assessed at 20Xmagnification. Data were assessed using a one-way ANOVA . Ctrl Vs LPS P=0.002. LPS Vs LPS+ Pio P=<0.001. C analysis of % nuclear localization was calculated as the number of pixels colocalizing with DAPI (nuclear marker) normalized to total positive PPAR pixels in a cell. 10 Cells were analyzed per condition. Ctrl Vs LPS P=<0.001. LPS Vs LPS +Pio P=<0.001.

2.3.4. In vitro – Proteomics – Microglia/LPS w/w.o Pioglitazone

A



* Of 24 Shared genes all were differentially regulated

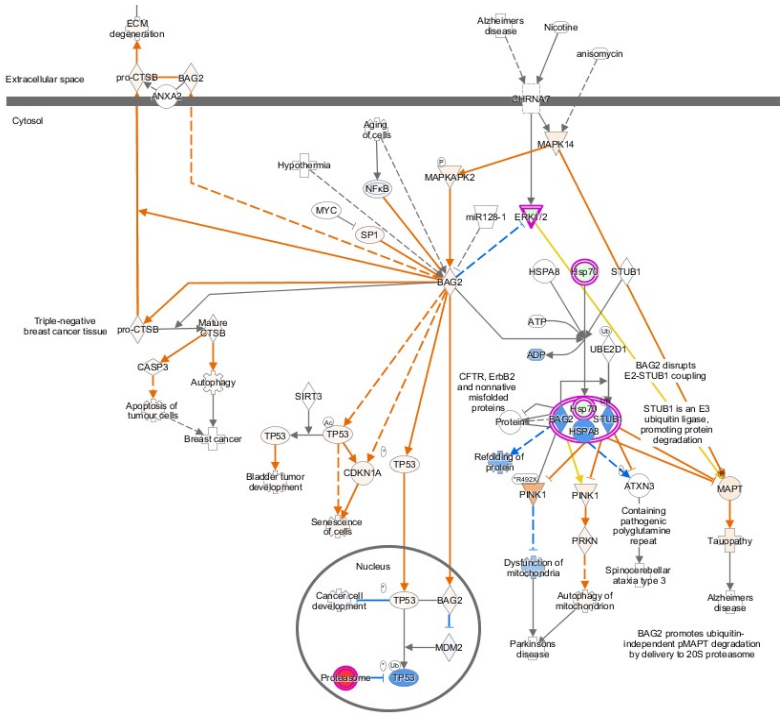
A .Venn diagram of significantly altered proteins (p value =>0.05) from either Control treat IMG microglia (Ctrl), LPS (10ng/ml for 24 hours), or with both LPS (10ng/ml for 24 hours)and Pioglitazone (10uM for 24 hours) (N=3/group). Analysis of LPS Vs Ctrl cells identified 68 significantly altered proteins (20 up and 48 down). Analysis of LPS + Pio Vs LPS identified 70 significantly altered proteins (51 up and 19 down). Analysis between comparison groups identified 24 overlapping altered proteins, all differentially regulated as shown in B.

B

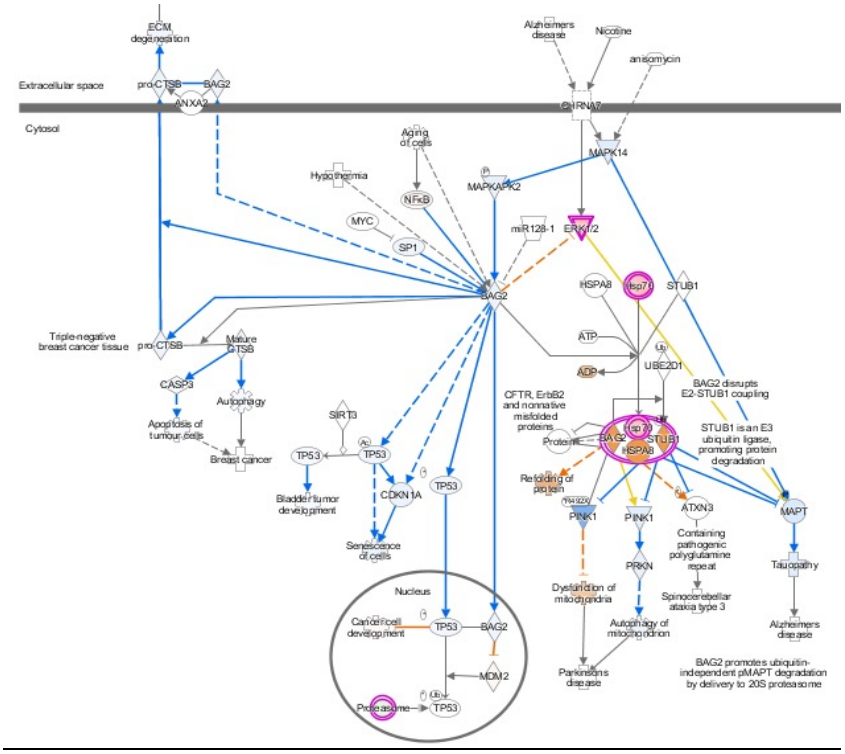
	Direction	
Gene name	LPS Vs Ctrl	LPS + Pio Vs LPS
Tpp2	Up	Down
Farsb	Up	Down
Sgta	Up	Down
Sec31a	Up	Down
env	Up	Down
Esd	Up	Down
Smc1a	Up	Down
Eci1	Up	Down
Ehd1	Up	Down
Septin9	Down	Up
Adss	Down	Up
Idi1	Down	Up
HSPA1A	Down	Up
Mapk3	Down	Up
Anxa3	Down	Up
Raly	Down	Up
Ptma	Down	Up
Myl6	Down	Up
Kctd12	Down	Up
Cab39	Down	Up
Snx6	Down	Up
Nap1l4	Down	Up
Hnrnpdl	Down	Up
Snd1	Down	Up

Identification of significantly altered pathways in microglia treated with LPS or LPS and Pioglitazone

LPS Vs Ctrl

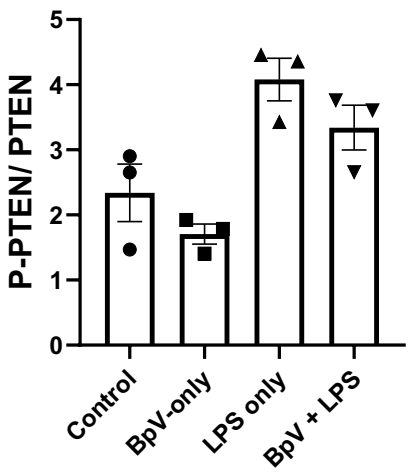


LPS + Pio Vs LPS

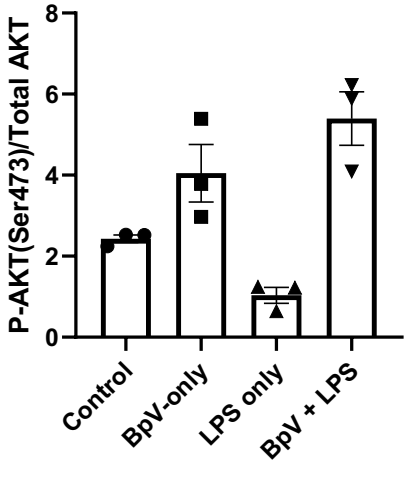


2.4. BvP(ohPic) effect on PTEN inhibition in microglia

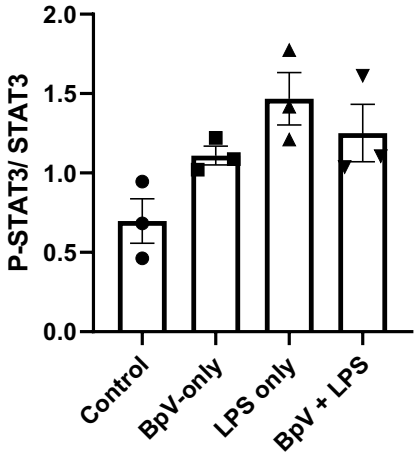
IMG Immortalized microglia were treated with BVP (200nM) and/or LPS 10ng/ml for 2 hours



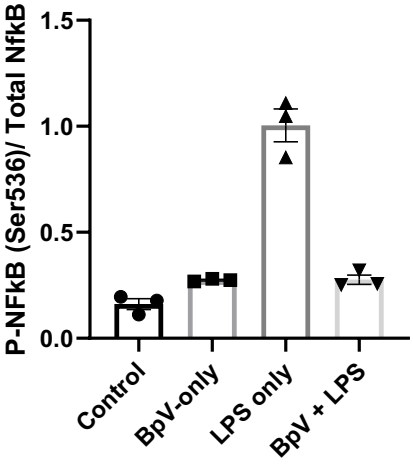
Analysis of expression of Phospho-PTEN (Ser380) via immunoblotting of whole cell lysate. Data were assessed for normal distribution via Shapiro-wilk test and data were assessed statistically using a one-Way ANOVA. Ctrl Vs LPS P=0.026. LPS Vs LPS + Bvp P=0.448. N=3/group. Error bars represent SEM



Analysis of expression of Phospho-AKT (Ser437) via immunoblotting of whole cell lysate. Data were assessed for normal distribution via Shapiro-wilk test and data were assessed statistically using a one-Way ANOVA. Ctrl Vs LPS P=0.269. LPS Vs LPS + Bvp P=0.001. N=3/group. Error bars represent SEM



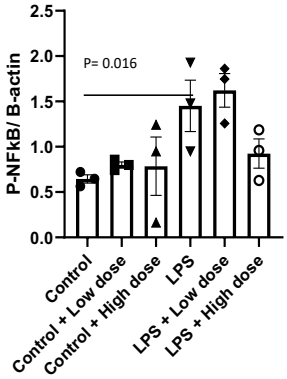
Analysis of expression of Phospho-STAT3 (Tyr705) via immunoblotting of whole cell lysate. Data were assessed for normal distribution via Shapiro-wilk test and data were assessed statistically using a one-Way ANOVA. Ctrl Vs LPS P=0.022. LPS Vs LPS + Bvp P=0.722. N=3/group. Error bars represent SEM



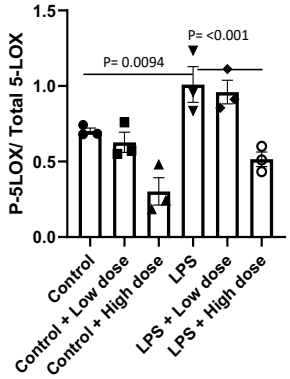
Analysis of expression of Phospho-STAT3 (Tyr705) via immunoblotting of whole cell lysate. Data were assessed for normal distribution via Shapiro-wilk test and data were assessed statistically using a one-Way ANOVA. Ctrl Vs LPS P=<0.001. LPS Vs LPS + Bvp P =<0.001. N=3/group. Error bars represent SEM

2.5. Zileuton effect on 5-LOX inhibition in microglia

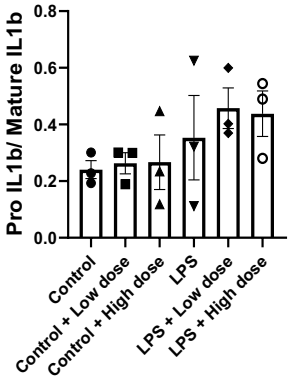
2.5.1. IMG Immortalized microglia were treated with Zileuton (25 or 100uM) and/or LPS 10ng/ml for 24 hours.



A graph showing the protein expression of Phospho- NFkB (Serine 536) in the cytoplasmic/ nuclear fraction of cell lysate from IMG immortalized microglial cells (n=3/group), data were normalized to Total NFkB and to B-actin. Cells treated with either control DMEM/F12 media or media containing LPS @10ng/ml and Zileuton @ 25uM (Low dose) or 100uM (High dose) for 24 hours. Data were analysed via students T-test. Control vs LPS treated P=0.016. Error bars represent SEM.

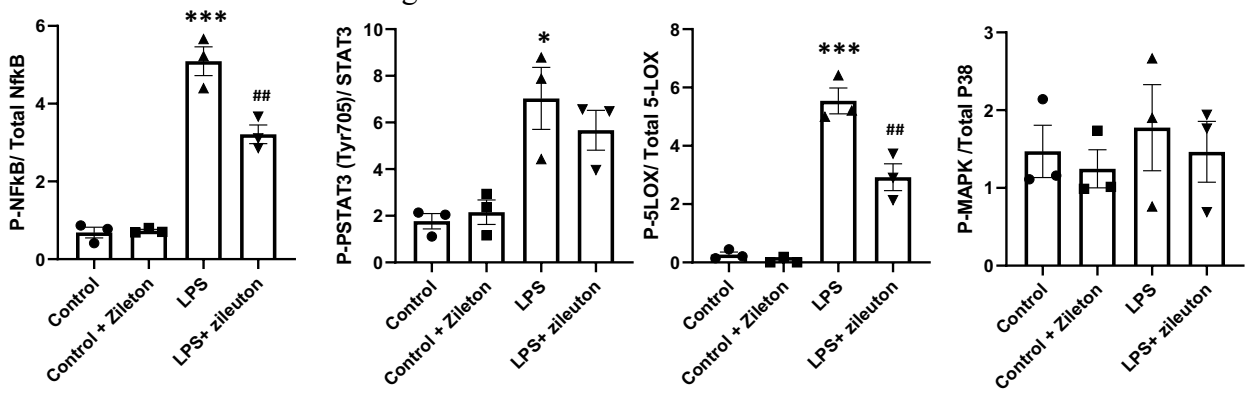


A graph showing the protein expression of Phospho- 5-LOX (Serine 663) in the cytoplasmic/ nuclear fraction of cell lysate from IMG immortalized microglial cells (n=3/group), data were normalized to Total NFkB and to B-actin. Cells treated with either control DMEM/F12 media or media containing LPS @10ng/ml and Zileuton @ 25uM (Low dose) or 100uM (High dose) for 24 hours. Data were analysed via students T-test. LPS vs LPS + 100um Zileuton P=<0.001. Control vs LPS treated P=0.0094 Error bars represent SEM.



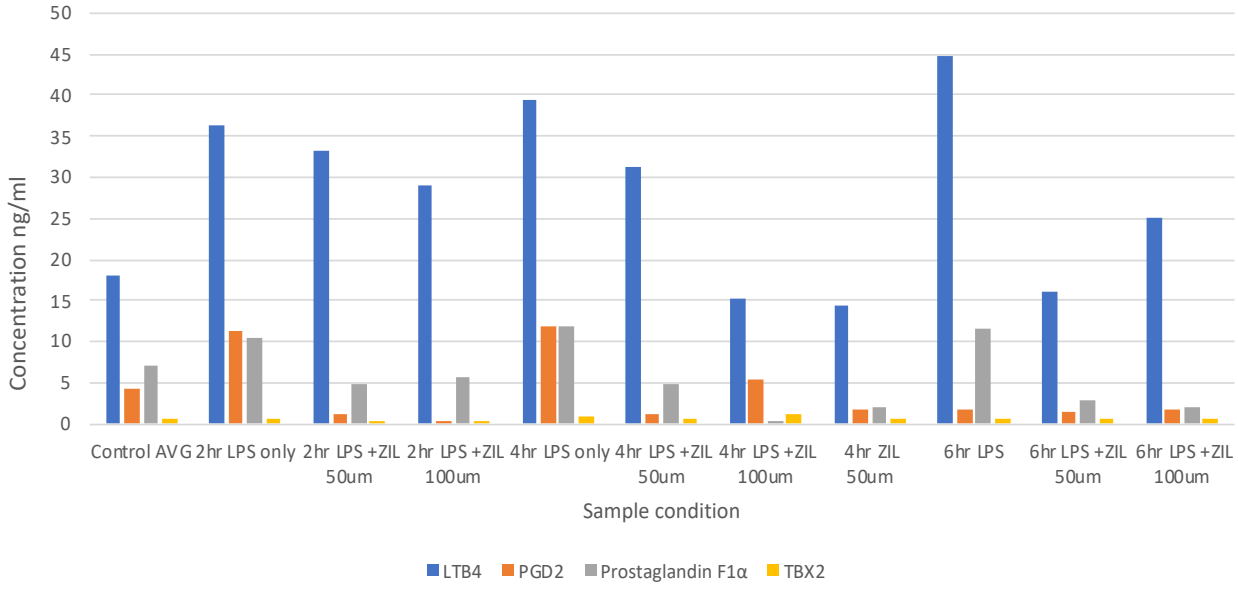
A graph showing the ratio of protein expression of Pro-IL1b against Mature IL1b in the cytoplasmic/ nuclear fraction of cell lysate from IMG immortalized microglial cells (n=3/group), data were normalized to Total NFkB and to B-actin. Cells treated with either control DMEM/F12 media or media containing LPS @10ng/ml and Zileuton @ 25uM (Low dose) or 100uM (High dose) for 24 hours Data were analysed via students T-test. Error bars represent SEM.

2.5.2. IMG Immortalized microglia were treated with Zileuton (100uM) at 90mins after a 30mins pre-treatment with/w.o LPS 10ng/ml



A graph showing the protein expression of Phospho-NFkB (Serine536), Phospho-STAT3(Tyrosine705), Phospho-5-Lipoxygenase (Serine663) (5-LOX) and Phospho-P38 (Thr180/Tyr182) MAPK in the cytoplasmic/ nuclear fraction of cell lysate from IMG immortalized microglia cells following a 30 minute pre-treatment with Insulin at either a low (50nM) or high dose (200nM),the cell culture media was then replaced with fresh media containing 10ng/ml Lipopolysaccharide (LPS) for 45 minutes (n=3/group), data were normalized to B-tubulin. Data were assessed for normal distribution and significance was assessed using a one-way ANOVA following by post hoc testing with a two-stage linear setup by Benjemini-Hochberg and Yekuteli to control for false discovery rate. Significance vs control is indicated on the graph (*=P<0.05) (**=P<0.01) (***=P<0.001). Significance vs LPS treated is indicated on the graph (#=P<0.05) (##=P<0.01) (###=P<0.001)

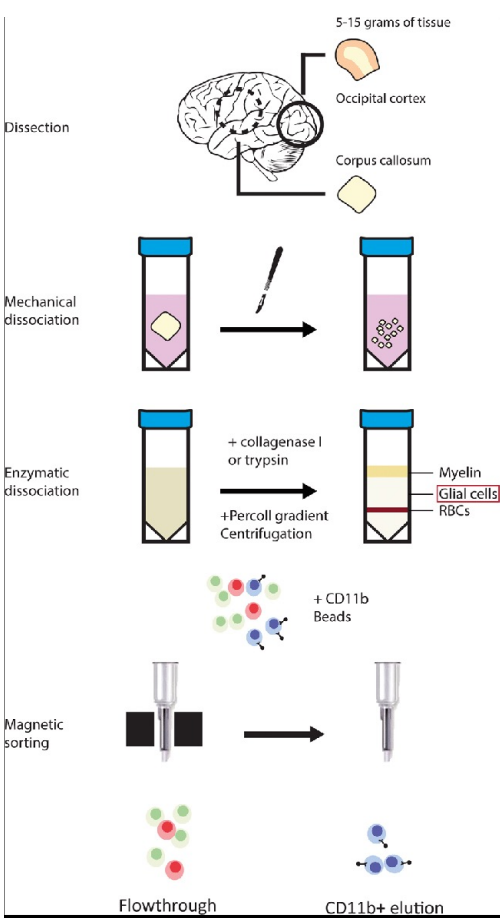
2.5.3. Lipid profiles in microglia cultures exposed to LPS with/w.o Zileuton

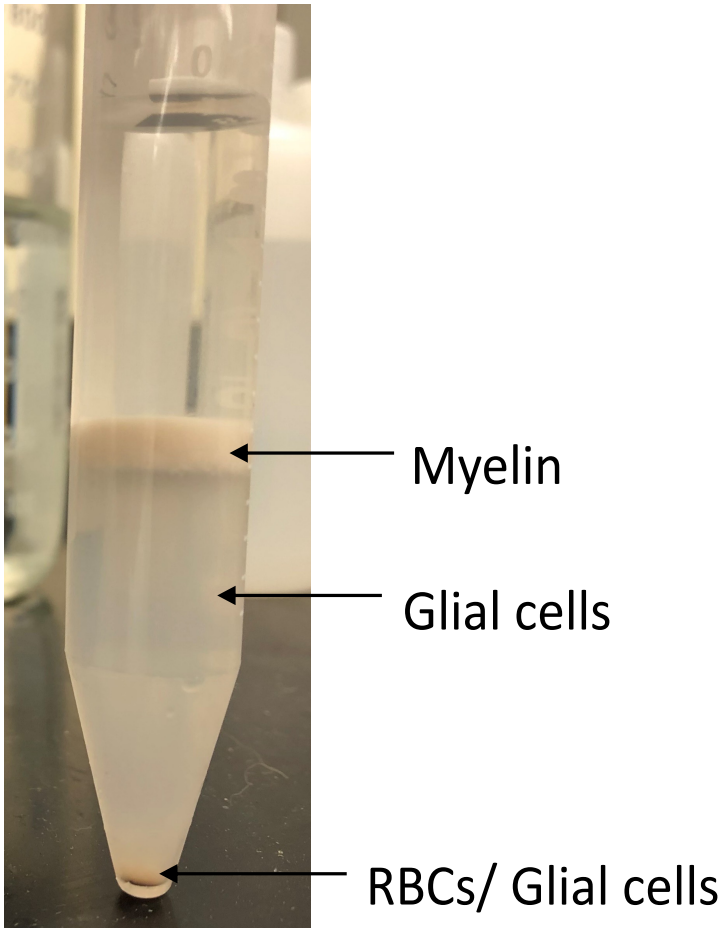


Bioactive lipid profile derived from LCMS/MS data showing the effect of 2,4 and 6 hours of 10ng/ml LPS administration on IMG microglia. Either with or without Zileuton (ZIL) @50 or 100uM LTB4= Leukotriene B4, PDG2= Prostaglandin D2, Prostaglandin F1a and Thromboxane 2. n=2 per group, samples were run in triplicated error bars reported as Standard deviation.

3. Ex vivo study (short-term treatment – 7 days)

3.1. Microglial cell isolation





3.2. Analysis of cells types present in each phase of glial isolation

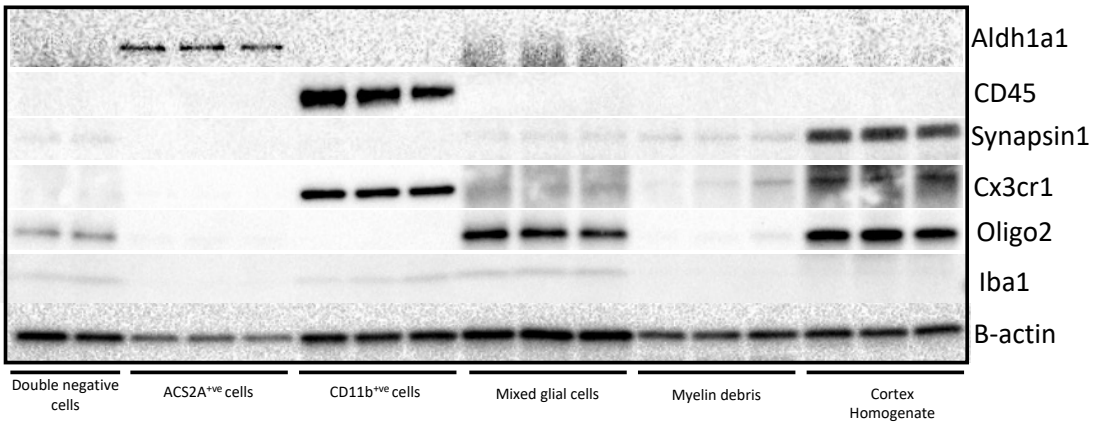
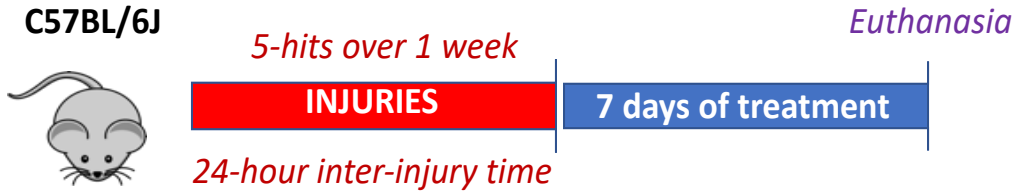
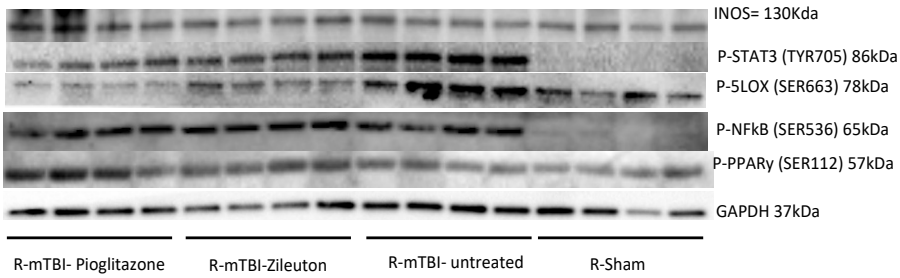


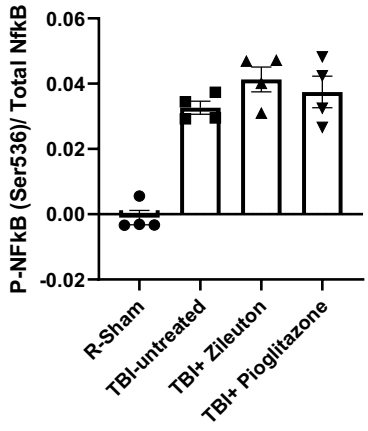
Figure showing confirmation of cell type specific isolation using CD11b and ACSA 2 magnetic labelled antibodies.

3.3. Ex vivo analysis of acutely isolated microglia - Experimental design

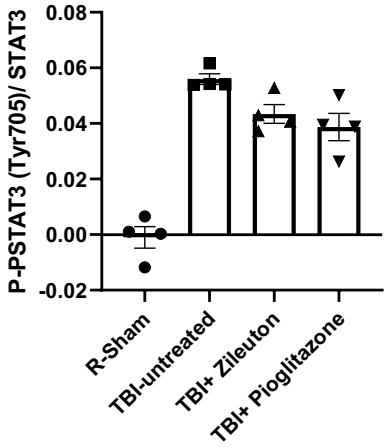


Mice (n=4/group) were exposed to 5 injuries, separated by 24 hours and then treated with pioglitazone (125mg/kg chow) for 7 days to assess target engagement and effect on markers of inflammation in microglia pellets.

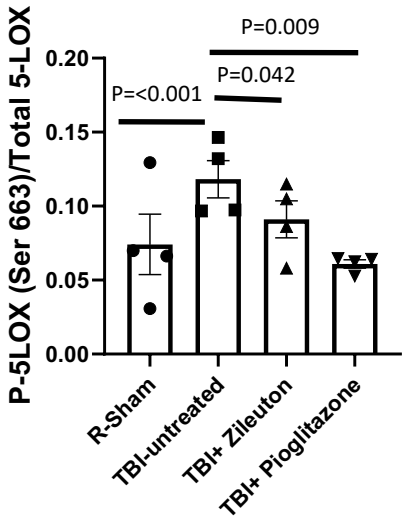




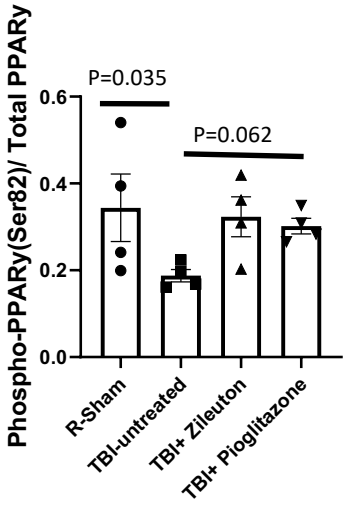
Analysis of expression of Phospho-NFkB (Ser536) via immunoblotting of whole cell lysate. Data were assessed for normal distribution via Shapiro-wilk test and data were assessed statistically using a one-Way ANOVA. R sham Vs R-mTBI P=<0.001. N=4/group. Error bars represent SEM.



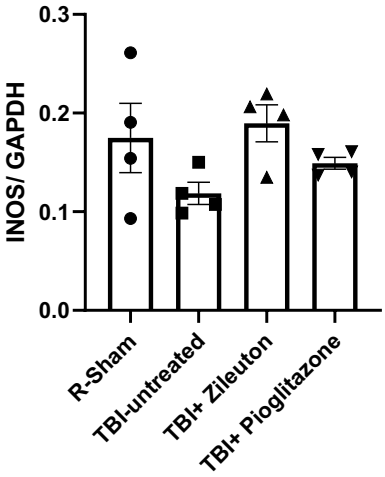
Analysis of expression of Phospho-STAT3 (Tyr705) via immunoblotting of whole cell lysate. Data were assessed for normal distribution via Shapiro-wilk test and data were assessed statistically using a one-Way ANOVA. R sham Vs R-mTBI P=<0.001. N=4/group. Error bars represent SEM.



Analysis of expression of Phospho- 5-LOX (Ser663) via immunoblotting of whole cell lysate. Data were assessed for normal distribution via Shapiro-wilk test and data were assessed statistically using a one-Way ANOVA. R sham Vs R-mTBI P=<0.001.TBI-untreated Vs TBI Zileuton P=0.042. TBI untreated Vs TBI Pioglitazone P= 0.009. N=4/group. Error bars represent SEM.



Analysis of expression of Phospho-PPARy (SER112) via immunoblotting of whole cell lysate. Data were assessed for normal distribution via Shapiro-wilk test and data were assessed statistically using a one-Way ANOVA. R sham Vs R-mTBI P=0.035. TBI untreated Vs TBI Pioglitazone P= 0.062. N=4/group. Error bars represent SEM.



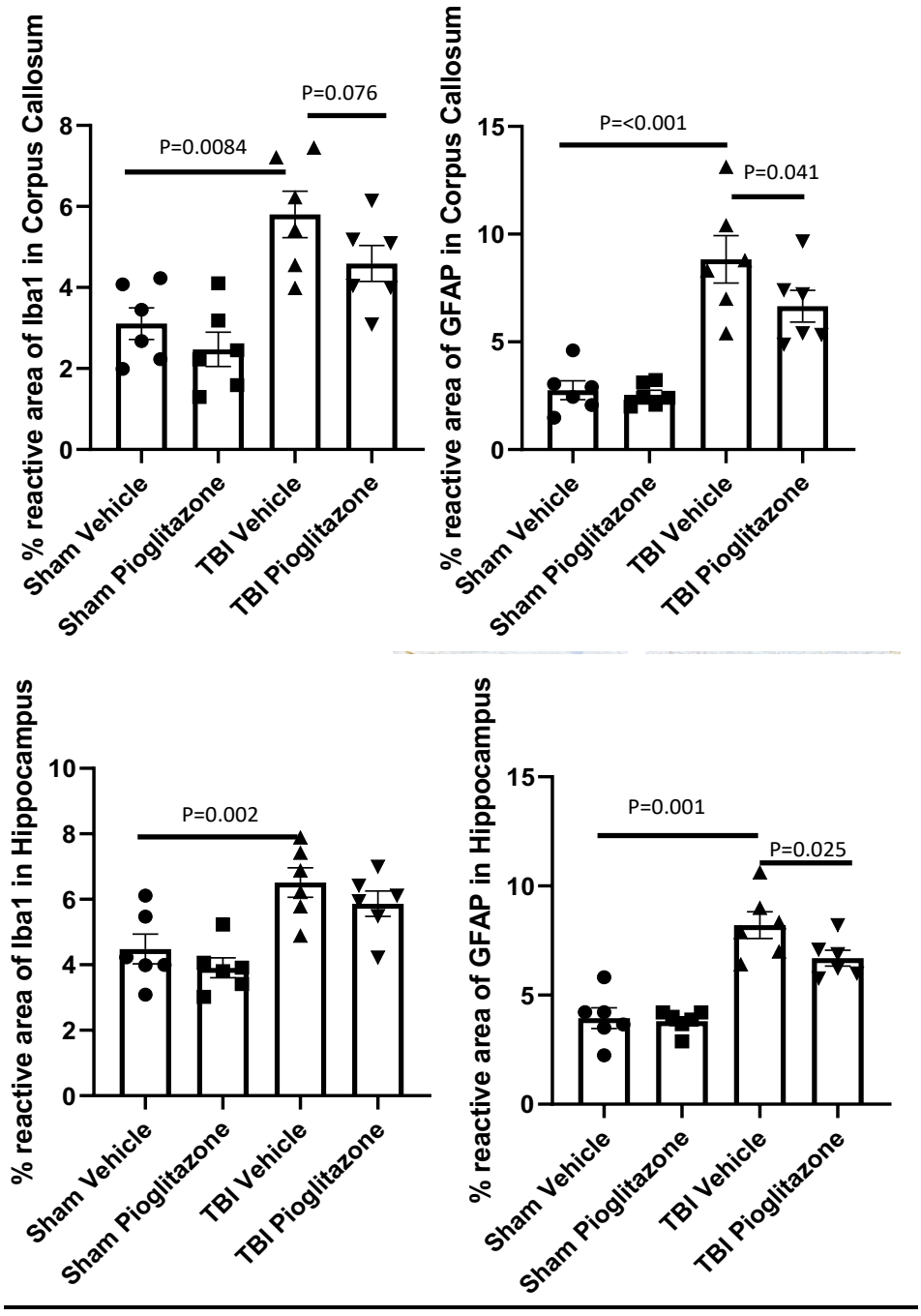
Analysis of expression of Inducible nitric oxide synthase INOS via immunoblotting of whole cell lysate. Data were assessed for normal distribution via Shapiro-wilk test and data were assessed statistically using a one-Way ANOVA. No significant effect was found. N=4/group. Error bars represent SEM.

4. Short term In vivo studies

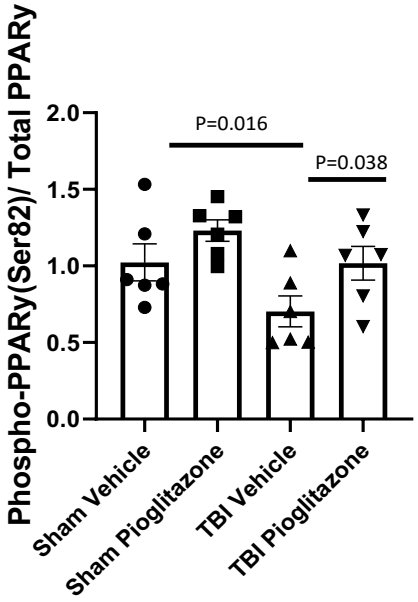
Methodology – Animals were subjected to our injury TBI paradigm, receiving 20 closed head injuries (5 per week over 1 month) with an electromagnetic impounder (see Mouzon et al., 2012; 2014); subsequently allowed to age for 3 months post first-injury, whereby they received treatment with compounds for the last 14 days until euthanasia. Groups per treatment – [r-mTBI treated; r-mTBI vehicle; r-sham treated; r-sham control] each with 6 mice per group.
Western blotting and **immunohistochemistry studies** were conducted using established protocols as previously demonstrated (see Ojo et al., 2016; Mouzon et al., 2018). ELISA/EIA kits followed manufacturers guidelines.

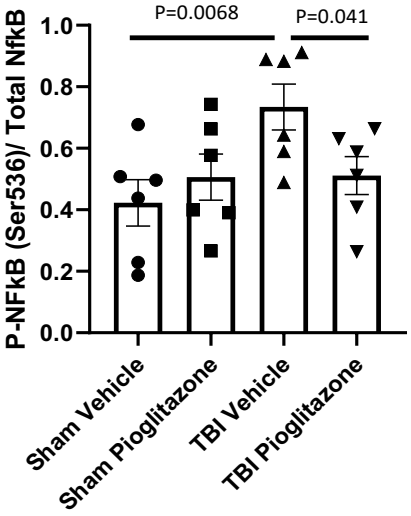
4.1. In vivo Investigation of Pioglitazone on TBI

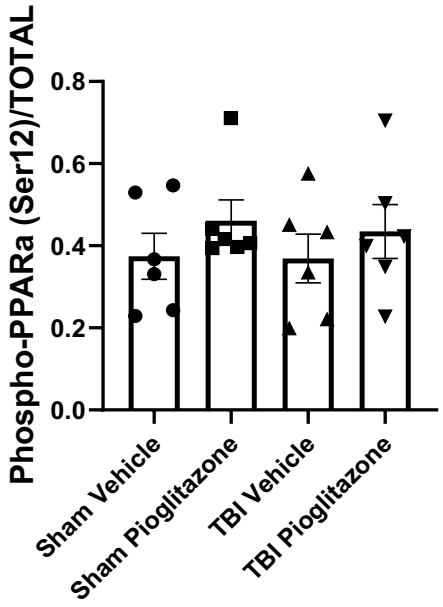
Neuropathology - Immunohistochemistry

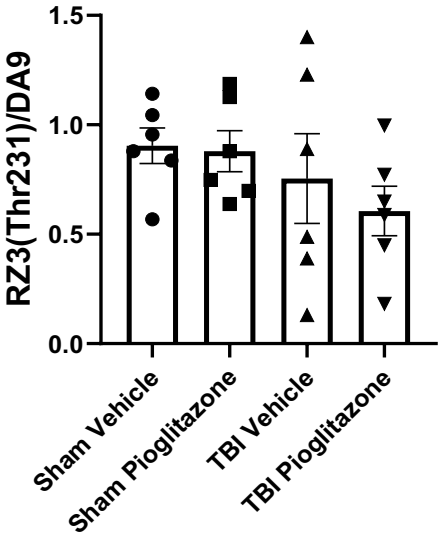


Biochemistry - immunoblotting

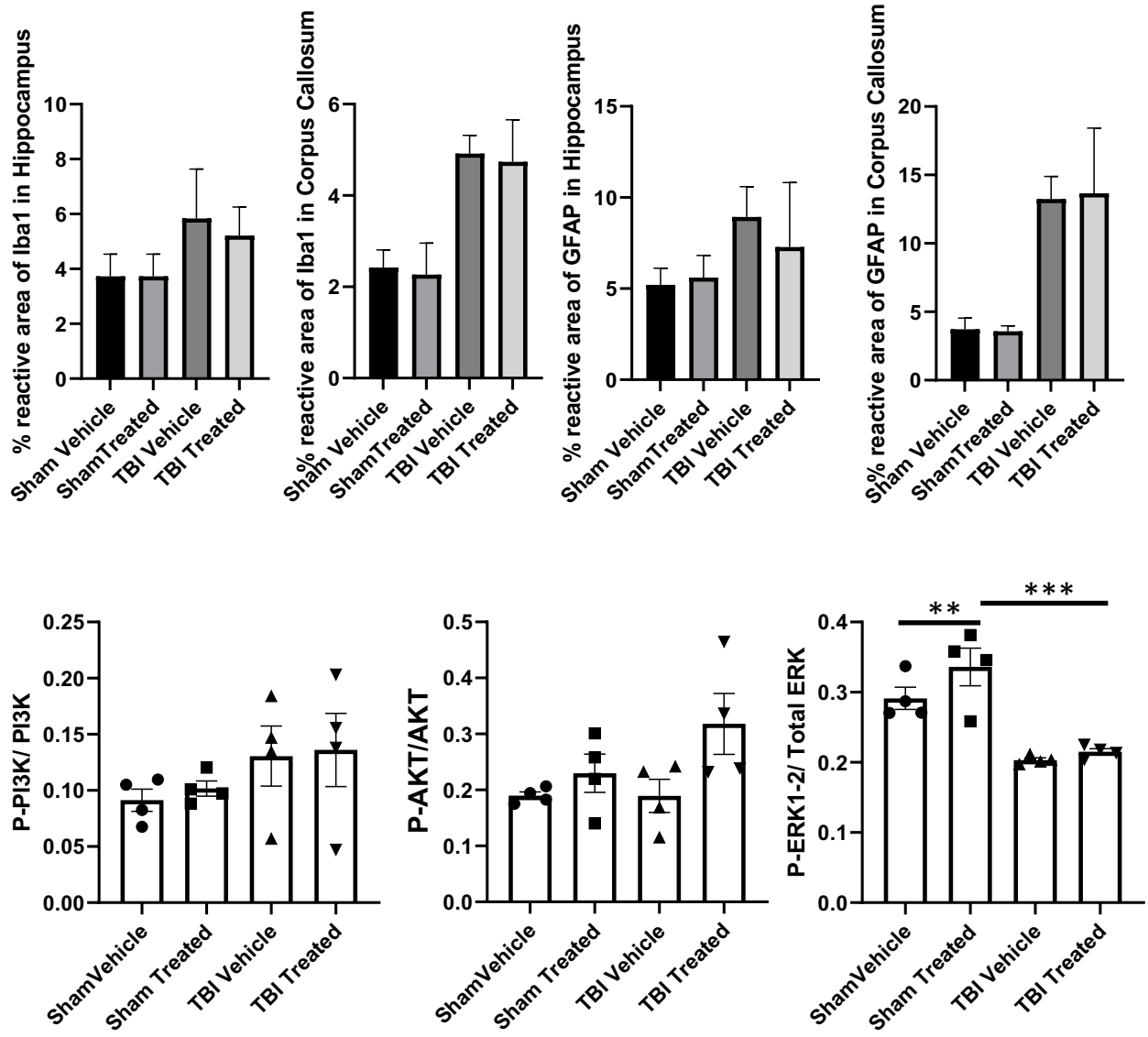








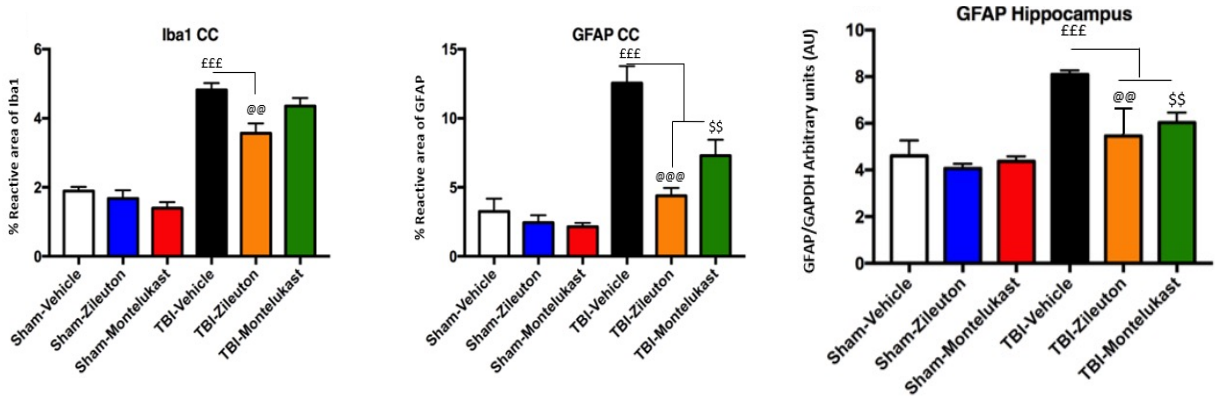
4.2. Analysis of Target engagement following Insulin treatment
Pharmacological blockade of IGF receptor after repetitive mTBI.



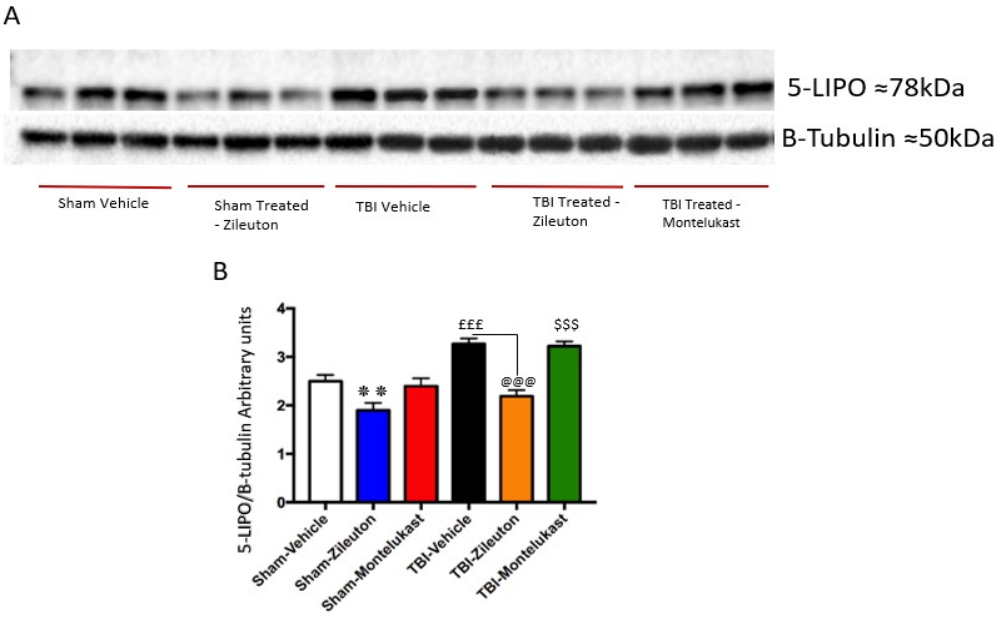
Graph showing Protein expression of PI3K when normalized for GAPDH.
Graph showing protein expression of Phospho-AKT when normalized for GAPDH.
Graph showing protein expression of Phospho-ERK1 when normalized for GAPDH.
n=4 per group, error bars are shown as standard error of the mean (SEM) and data was statistically analyzed using ANOVA.

Lack of significant increase in downstream IGF-signaling by intranasal insulin following injury.
p-AKT ser473 shows a trend towards increase with intranasal insulin following injury

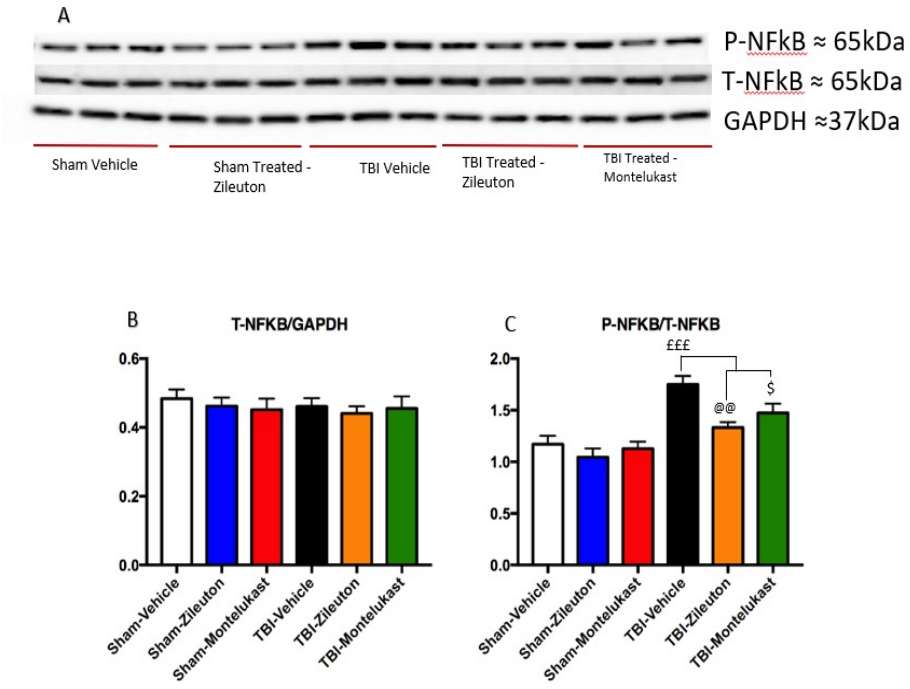
4.3. Analysis of Target engagement following Zileuton treatment
Pharmacological blockade of 5-Lox activity after repetitive mTBI.



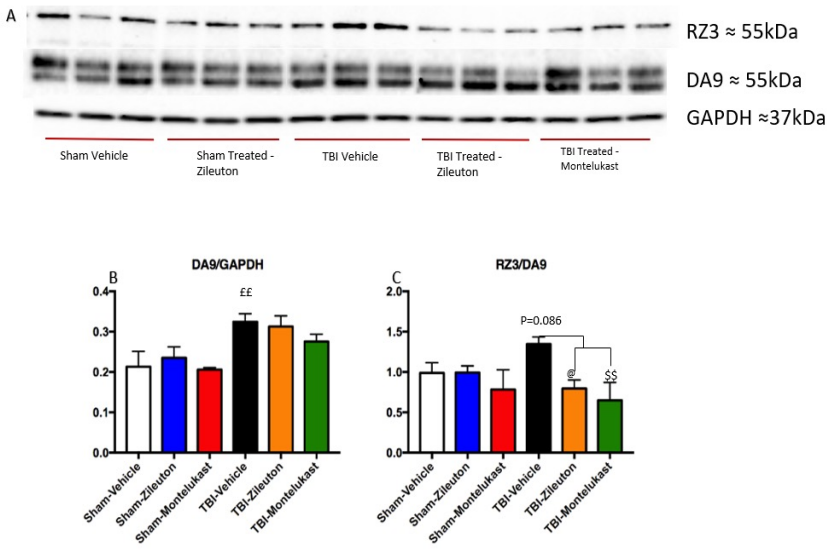
Neuropathological analysis of GFAP and/or IBA1 in the corpus callosum and hippocampus of C57BL/6 (Wild type) mice following 20-hit injury paradigm or sham procedure, and treatment with zileuton(32mg/kg/day) or Montelukast (10mg/kg/day). Quantification of % reactive area of Iba1^{+ve} cells in 6 non-overlapping images taken along the body of the Corpus callosum, data were analysed by One-way ANOVA and p= 0.001. Quantification of % reactive area of GFAP^{+ve} cells in 6 non-overlapping images taken along the body of the Corpus callosum, data were analysed by One-way ANOVA and p =0.0001. Data are expressed as ± standard error of the mean (n=6 per group). Significance between Sham vehicle vs. TBI vehicle group represented by (£), significance assessed to be p=0.001 represented by £££. TBI vehicle vs. TBI treated with Zileuton group is represented by (@), significance assessed to be p=0.001 represented as @@@. TBI vehicle vs. TBI treated with Montelukast group is represented by $, Significance assessed to be p=0.01, represented as $$.



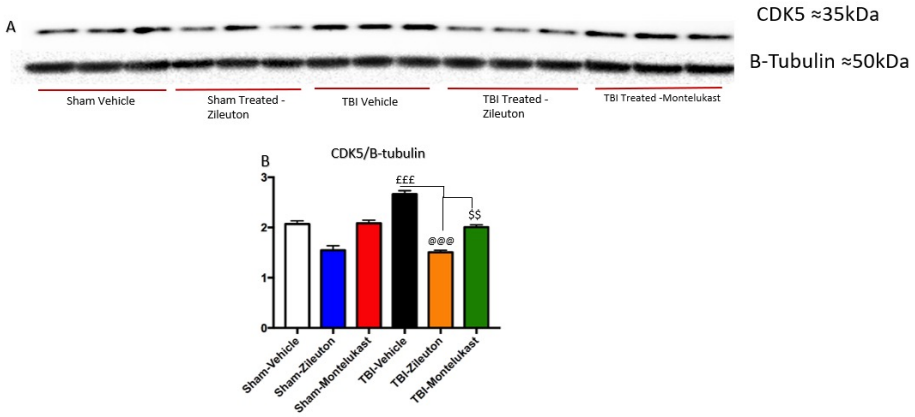
*5-LOX protein expression in C57BL/6 (Wild type) mice following 20-hit injury paradigm or sham procedure, and treatment with zileuton(32mg/kg/day) or Montelukast (10mg/kg/day) analysis of mouse brains following Injury and treatment. (A) Representative western blotting of 20 hit mTBI mice cortex samples. (B) of Figure A, data were analysed by One-way ANOVA and p =0.0001. Data are expressed as ± standard error of the mean (n=6 per group. Significance between Sham vehicle vs. TBI vehicle group represented by (£), significance assessed to be p=0.001 represented by £££. TBI vehicle vs. TBI treated with Zileuton group is represented by (@), significance assessed to be p=0.001 represented as @@@. TBI vehicle vs. TBI treated with Montelukast group is represented by $, Significance assessed to be p=0.001, represented as $$$. Significance between Sham vehicle vs. Sham treated with Zileuton is represented at *, significance assessed to be p=0.01 represented as (**).*



Total NFkB (T-NFkB) and Phospho NFkB (P-NFkB) expression in C57BL/6 (Wild type) mice following 20-hit injury paradigm or sham procedure, and treatment with zileuton(32mg/kg/day) or Montelukast (10mg/kg/day) analysis of mouse brains following Injury and treatment. (A) Representative western blotting of 20 hit mTBI mice cortex samples. (B) of Figure A, data were analysed by One-way ANOVA and p =0.0001. Data are expressed as ± standard error of the mean (n=6 per group). (Figure.C.) Significance between Sham vehicle vs. TBI vehicle group represented by (£), significance assessed to be p=0.001 represented by £££. TBI vehicle vs. TBI treated with Zileuton group is represented by (@), significance assessed to be p=0.007 represented as @@. Significance between Sham vehicle vs. TBI treated with Montelukast is represented by ($) significance assessed to be p=0.0176, represented as ($).



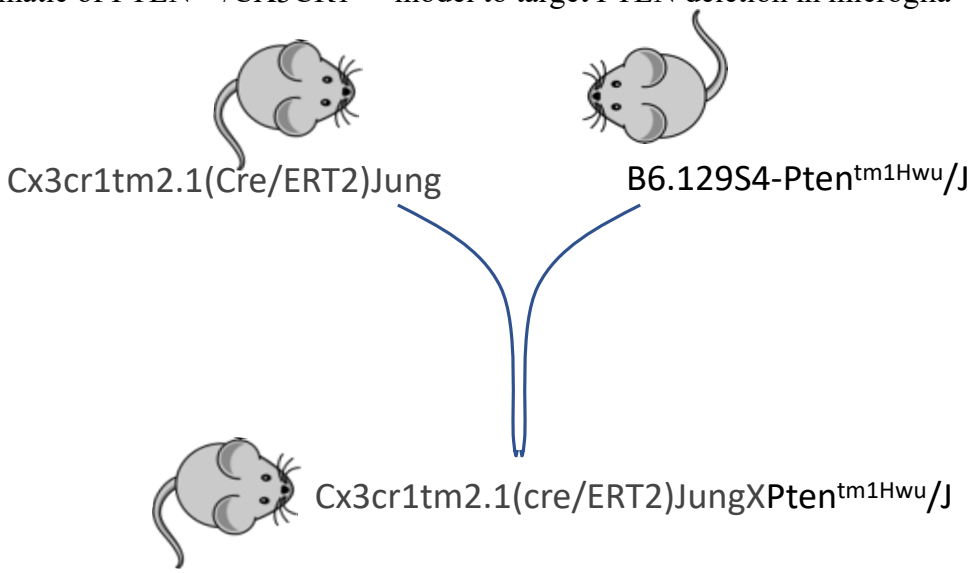
Total TAU (DA9) and Phospho Tau (RZ3) expression in C57BL/6 (Wild type) mice following 20-hit injury paradigm or sham procedure, and treatment with zileuton(32mg/kg/day) or Montelukast (10mg/kg/day) analysis of mouse brains following Injury and treatment. (A) Representative western blotting of 20 hit mTBI mice cortex samples. (B) of Figure A, data were analysed by One-way ANOVA and p =0.0001. Data are expressed as ± standard error of the mean (n=6 per group. Significance between Sham vehicle vs. TBI vehicle group represented by (£), significance assessed to be p=0.0056 represented by ££ (Fig.8.B) and p=0.086 represented (Fig.8.C). TBI vehicle vs. TBI treated with Zileuton group is represented by (@), significance assessed to be p=0.011 represented as @. @@. Significance between Sham vehicle vs. TBI treated with Montelukast is represented by ($) significance assessed to be p=0.002, represented as ($$).



CDK5 expression in C57BL/6 (Wild type) mice following 20-hit injury paradigm or sham procedure, and treatment with zileuton(32mg/kg/day) or Montelukast (10mg/kg/day) analysis of mouse brains following Injury and treatment. (A) Representative western blotting of 20 hit mTBI mice cortex samples. (B) of Figure A, data were analysed by One-way ANOVA and p =0.0001. Data are expressed as ± standard error of the mean (n=6 per group. Significance between Sham vehicle vs. TBI vehicle group represented by (£), significance assessed to be p=0.001 represented by £££ (Fig.9.B) and p=0.086 represented (Fig.8.C). TBI vehicle vs. TBI treated with Zileuton group is represented by (@), significance assessed to be p=0.001 represented as @. @@. Significance between Sham vehicle vs. TBI treated with Montelukast is represented by ($) significance assessed to be p=0.01, represented as ($$).

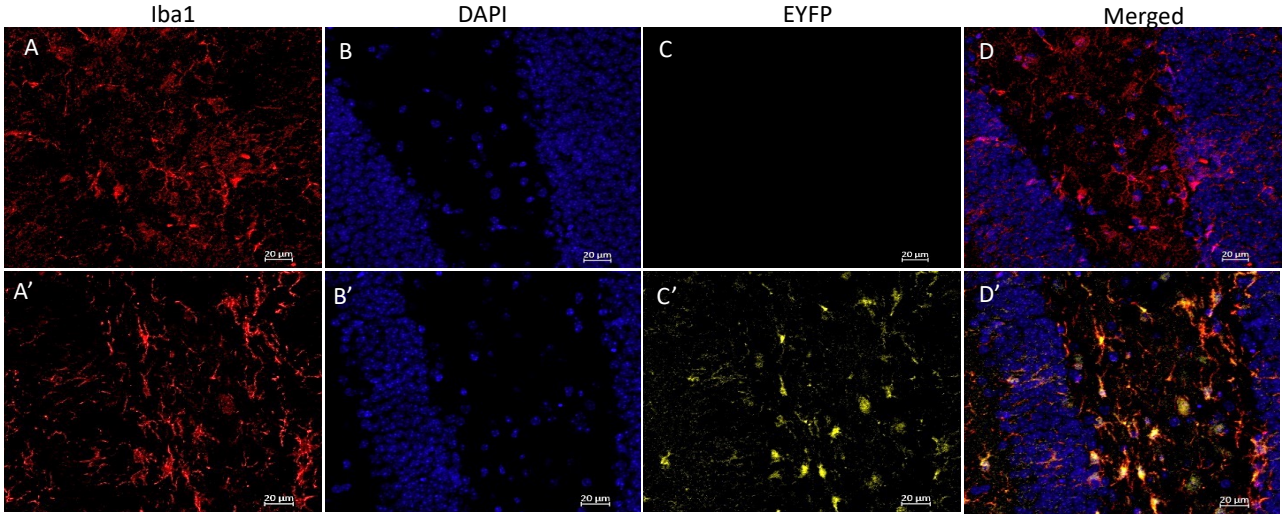
4.4. PTEN inhibition in microglia after r-mTBI (Cre-LoxP model).

4.4.1. Schematic of PTEN$^{fl/fl}$/CX3CR1^{cre+} model to target PTEN deletion in microglia



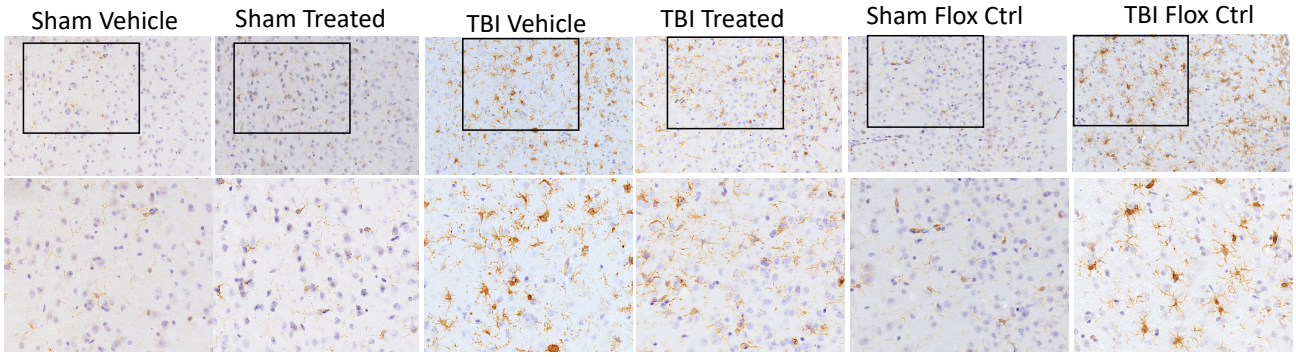
Graphical summary showing the breeding strategy used to generate the PTEN microglial-specific conditional knockout mice. Briefly, we first crossed PTEN $^{fl/fl}$ mice (Jax #006440) with homozygous CX3CR1^{CreERT2} EYFP reporter mice (Jax #021160) to generate hemizygous CX3CR1 $^{+/CreERT2}$PTEN$^{+/fl}$ mice. Hemizygous carriers were then mated to PTEN $^{fl/fl}$ mice to generate CX3CR1 $^{+/CreERT2}$PTEN$^{fl/fl}$ mice (KO, knockout). To generate our experimental cohorts, we mated the KO mice back the PTEN $^{fl/fl}$ mouse line to generate an approximately 50:50 distribution of Cre^{+}PTEN $^{fl/fl}$ (KO) and Cre^{neg}PTEN $^{fl/fl}$ (WT, wild type) littermates. All mice were genotyped by Transnetyx. For all experiments, both male and female mice were used in a 50:50 ratio.

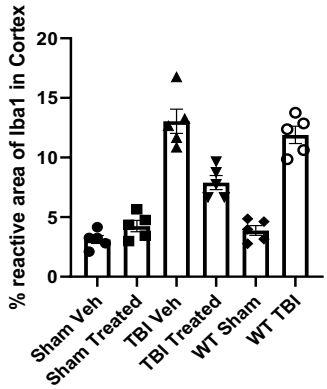
4.4.2. Demonstration of cre-recombination in PTEN$^{fl/fl}$/CX3CR1^{cre+} mice.



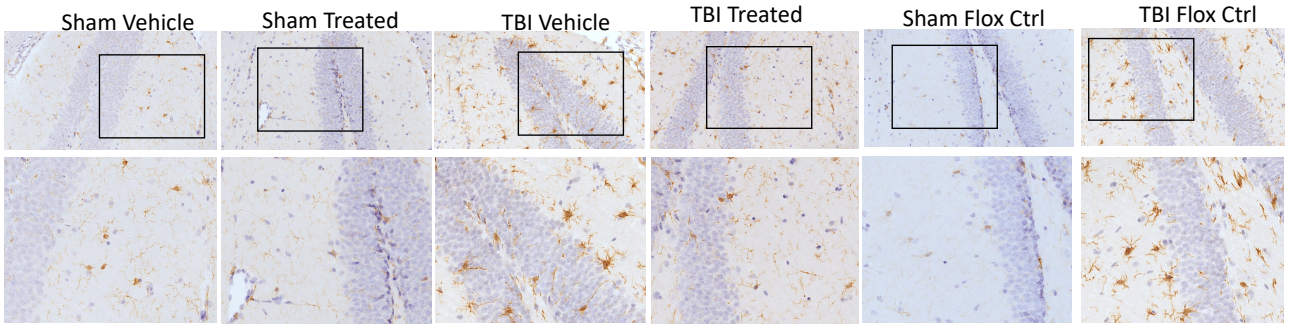
CX3CR1^{CreERT2} EYFP reporter mice were crossed with PTEN $^{fl/fl}$ mice, at 2.5 months post injury or sham treatment mice were fed tamoxifen in chow (Harlan, #TD.130860, 400 ppm) for 14 days to induce eYFP expression. Representative confocal micrographs showing eYFP expression in Iba1-positive microglia upon tamoxifen administration A'-D') or vehicle treatment (A-D).

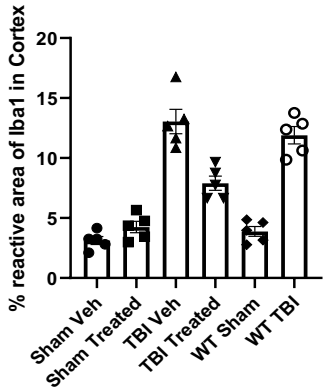
4.4.3. IBA1 – Microglial activation (IHC) in PTEN$^{fl/fl}$/CX3CR1^{cre+} mice after r-mTBI/r-sham injury Data from mice fed on tamoxifen for 14days, at 2.5months post-injury timepoint.



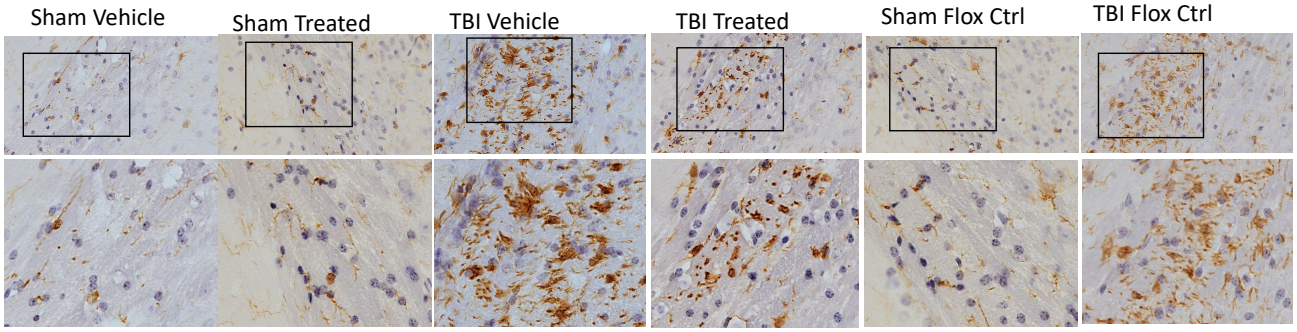


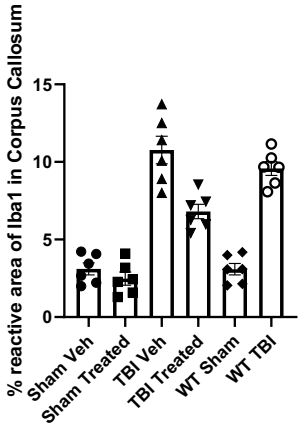
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	
Sham Veh vs. Sham Treated	-1.126	-3.892 to 1.640	No	ns	.803	A-B
Sham Veh vs. TBI Veh	-9.920	-12.69 to -7.154	Yes	***	<.001	A-C
Sham Veh vs. TBI Treated	-4.776	-7.541 to -2.010	Yes	***	<.001	A-D
Sham Veh vs. WT Sham	-0.7597	-3.526 to 2.006	No	ns	.955	A-E
Sham Veh vs. WT TBI	-8.778	-11.54 to -6.012	Yes	***	<.001	A-F
Sham Treated vs. TBI Veh	-8.794	-11.56 to -6.028	Yes	***	<.001	B-C
Sham Treated vs. TBI Treated	-3.649	-6.415 to -0.8836	Yes	**	.005	B-D
Sham Treated vs. WT Sham	0.3665	-2.399 to 3.132	No	ns	.998	B-E
Sham Treated vs. WT TBI	-7.651	-10.42 to -4.885	Yes	***	<.001	B-F
TBI Veh vs. TBI Treated	5.144	2.379 to 7.910	Yes	***	<.001	C-D
TBI Veh vs. WT Sham	9.160	6.395 to 11.93	Yes	***	<.001	C-E
TBI Veh vs. WT TBI	1.143	-1.623 to 3.908	No	ns	.794	C-F
TBI Treated vs. WT Sham	4.016	1.250 to 6.782	Yes	**	.002	D-E
TBI Treated vs. WT TBI	-4.002	-6.768 to -1.236	Yes	**	.002	D-F
WT Sham vs. WT TBI	-8.018	-10.78 to -5.252	Yes	***	<.001	E-F





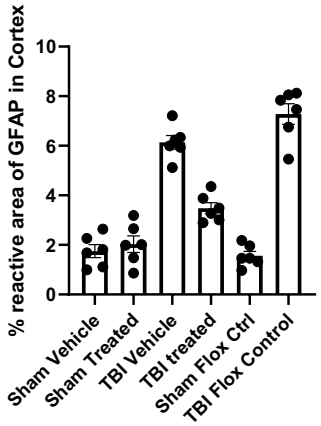
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	
Sham Veh vs. Sham Treated	0.2975	-1.706 to 2.301	No	ns	.997	A-B
Sham Veh vs. TBI Veh	-4.191	-6.195 to -2.187	Yes	***	<.001	A-C
Sham Veh vs. TBI Treated	-1.772	-3.776 to 0.2312	No	ns	.105	A-D
Sham Veh vs. WT Sham	0.2202	-1.783 to 2.224	No	ns	>.999	A-E
Sham Veh vs. WT TBI	-3.752	-5.755 to -1.748	Yes	***	<.001	A-F
Sham Treated vs. TBI Veh	-4.489	-6.492 to -2.485	Yes	***	<.001	B-C
Sham Treated vs. TBI Treated	-2.070	-4.074 to -0.06621	Yes	*	.040	B-D
Sham Treated vs. WT Sham	-0.07724	-2.081 to 1.926	No	ns	>.999	B-E
Sham Treated vs. WT TBI	-4.049	-6.053 to -2.045	Yes	***	<.001	B-F
TBI Veh vs. TBI Treated	2.419	0.4150 to 4.422	Yes	*	.012	C-D
TBI Veh vs. WT Sham	4.411	2.408 to 6.415	Yes	***	<.001	C-E
TBI Veh vs. WT TBI	0.4394	-1.564 to 2.443	No	ns	.983	C-F
TBI Treated vs. WT Sham	1.993	-0.01102 to 3.996	No	ns	.052	D-E
TBI Treated vs. WT TBI	-1.979	-3.983 to 0.02441	No	ns	.054	D-F
WT Sham vs. WT TBI	-3.972	-5.976 to -1.968	Yes	***	<.001	E-F



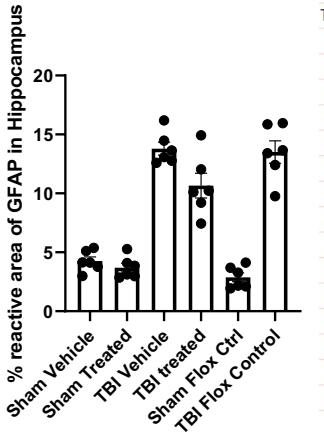


Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	
Sham Veh vs. Sham Treated	0.6348	-1.640 to 2.910	No	ns	.956	A-B
Sham Veh vs. TBI Veh	-7.659	-9.933 to -5.384	Yes	***	<.001	A-C
Sham Veh vs. TBI Treated	-3.696	-5.971 to -1.422	Yes	***	<.001	A-D
Sham Veh vs. WT Sham	0.01625	-2.258 to 2.291	No	ns	>.999	A-E
Sham Veh vs. WT TBI	-6.472	-8.747 to -4.197	Yes	***	<.001	A-F
Sham Treated vs. TBI Veh	-8.293	-10.57 to -6.019	Yes	***	<.001	B-C
Sham Treated vs. TBI Treated	-4.331	-6.606 to -2.056	Yes	***	<.001	B-D
Sham Treated vs. WT Sham	-0.6186	-2.893 to 1.656	No	ns	.960	B-E
Sham Treated vs. WT TBI	-7.107	-9.382 to -4.832	Yes	***	<.001	B-F
TBI Veh vs. TBI Treated	3.962	1.687 to 6.237	Yes	***	<.001	C-D
TBI Veh vs. WT Sham	7.675	5.400 to 9.949	Yes	***	<.001	C-E
TBI Veh vs. WT TBI	1.186	-1.088 to 3.461	No	ns	.613	C-F
TBI Treated vs. WT Sham	3.713	1.438 to 5.987	Yes	***	<.001	D-E
TBI Treated vs. WT TBI	-2.776	-5.050 to -0.5010	Yes	**	.010	D-F
WT Sham vs. WT TBI	-6.488	-8.763 to -4.214	Yes	***	<.001	E-F

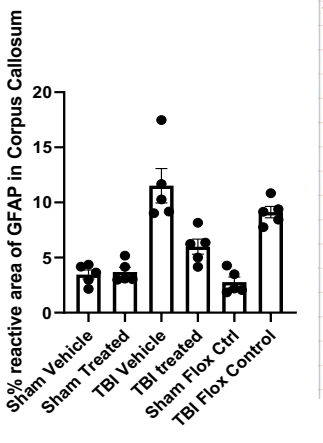
4.44. GFAP - Astroglial activation (IHC) in PTEN$^{fl/fl}$/CX3CR1^{cre+} mice after r-mTBI/r-sham injury
Data from mice fed on tamoxifen for 14days, at 2.5months post-injury timepoint.



Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Sham Vehicle vs. Sham Treated	-0.2799	-1.539 to 0.9791	No	ns	.983
Sham Vehicle vs. TBI Vehicle	-4.388	-5.647 to -3.129	Yes	***	<.001
Sham Vehicle vs. TBI treated	-1.733	-2.992 to -0.4743	Yes	**	.003
Sham Vehicle vs. Sham Flox Ctrl	0.1910	-1.068 to 1.450	No	ns	.997
Sham Vehicle vs. TBI Flox Control	-5.531	-6.790 to -4.272	Yes	***	<.001
Sham Treated vs. TBI Vehicle	-4.108	-5.367 to -2.849	Yes	***	<.001
Sham Treated vs. TBI treated	-1.453	-2.712 to -0.1945	Yes	*	.016
Sham Treated vs. Sham Flox Ctrl	0.4708	-0.7881 to 1.730	No	ns	.862
Sham Treated vs. TBI Flox Control	-5.251	-6.510 to -3.992	Yes	***	<.001
TBI Vehicle vs. TBI treated	2.655	1.396 to 3.914	Yes	***	<.001
TBI Vehicle vs. Sham Flox Ctrl	4.579	3.320 to 5.838	Yes	***	<.001
TBI Vehicle vs. TBI Flox Control	-1.143	-2.402 to 0.1161	No	ns	.092
TBI treated vs. Sham Flox Ctrl	1.924	0.6653 to 3.183	Yes	***	<.001
TBI treated vs. TBI Flox Control	-3.798	-5.057 to -2.539	Yes	***	<.001
Sham Flox Ctrl vs. TBI Flox Control	-5.722	-6.981 to -4.463	Yes	***	<.001

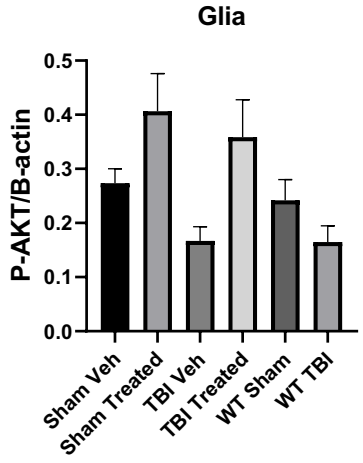


Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Sham Vehicle vs. Sham Treated	0.5567	-2.341 to 3.454	No	ns	.991
Sham Vehicle vs. TBI Vehicle	-9.538	-12.44 to -6.641	Yes	***	<.001
Sham Vehicle vs. TBI treated	-6.405	-9.303 to -3.508	Yes	***	<.001
Sham Vehicle vs. Sham Flox Ctrl	1.372	-1.525 to 4.269	No	ns	.703
Sham Vehicle vs. TBI Flox Control	-9.253	-12.15 to -6.356	Yes	***	<.001
Sham Treated vs. TBI Vehicle	-10.09	-12.99 to -7.197	Yes	***	<.001
Sham Treated vs. TBI treated	-6.962	-9.859 to -4.065	Yes	***	<.001
Sham Treated vs. Sham Flox Ctrl	0.8152	-2.082 to 3.712	No	ns	.954
Sham Treated vs. TBI Flox Control	-9.810	-12.71 to -6.912	Yes	***	<.001
TBI Vehicle vs. TBI treated	3.133	0.2355 to 6.030	Yes	*	.028
TBI Vehicle vs. Sham Flox Ctrl	10.91	8.013 to 13.81	Yes	***	<.001
TBI Vehicle vs. TBI Flox Control	0.2852	-2.612 to 3.182	No	ns	>.999
TBI treated vs. Sham Flox Ctrl	7.777	4.880 to 10.67	Yes	***	<.001
TBI treated vs. TBI Flox Control	-2.848	-5.745 to 0.04968	No	ns	.056
Sham Flox Ctrl vs. TBI Flox Control	-10.62	-13.52 to -7.727	Yes	***	<.001

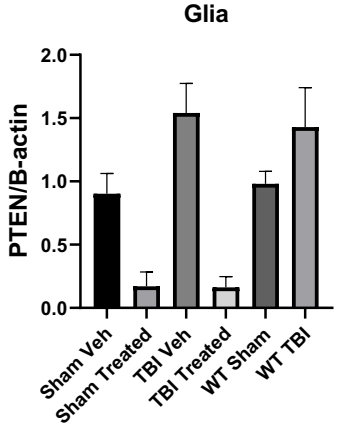


Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	
Sham Vehicle vs. Sham Treated	-0.2338	-3.687 to 3.219	No	ns	>.999	A-B
Sham Vehicle vs. TBI Vehicle	-8.061	-11.51 to -4.608	Yes	***	<.001	A-C
Sham Vehicle vs. TBI treated	-2.527	-5.980 to 0.9256	No	ns	.247	A-D
Sham Vehicle vs. Sham Flox Ctrl	0.6896	-2.763 to 4.142	No	ns	.989	A-E
Sham Vehicle vs. TBI Flox Control	-5.660	-9.112 to -2.207	Yes	***	<.001	A-F
Sham Treated vs. TBI Vehicle	-7.827	-11.28 to -4.374	Yes	***	<.001	B-C
Sham Treated vs. TBI treated	-2.293	-5.746 to 1.159	No	ns	.343	B-D
Sham Treated vs. Sham Flox Ctrl	0.9234	-2.529 to 4.376	No	ns	.960	B-E
Sham Treated vs. TBI Flox Control	-5.426	-8.879 to -1.973	Yes	***	<.001	B-F
TBI Vehicle vs. TBI treated	5.533	2.081 to 8.986	Yes	***	<.001	C-D
TBI Vehicle vs. Sham Flox Ctrl	8.750	5.297 to 12.20	Yes	***	<.001	C-E
TBI Vehicle vs. TBI Flox Control	2.401	-1.052 to 5.854	No	ns	.297	C-F
TBI treated vs. Sham Flox Ctrl	3.217	-0.2360 to 6.670	No	ns	.078	D-E
TBI treated vs. TBI Flox Control	-3.132	-6.585 to 0.3204	No	ns	.091	D-F
Sham Flox Ctrl vs. TBI Flox Control	-6.349	-9.802 to -2.896	Yes	***	<.001	E-F

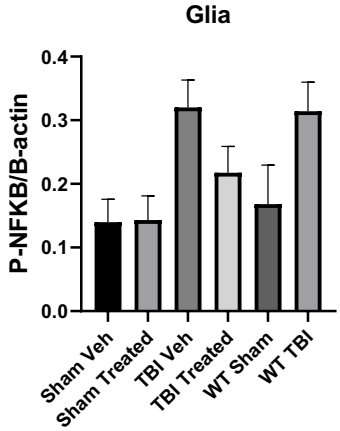
4.4.5. Biochemistry of CD11b+ microglial lysates in PTEN$^{fl/fl}$/CX3CR1^{cre+} mice after r-mTBI/r-sham injury. Data from mice fed on tamoxifen for 14days, at 2.5months post-injury timepoint.



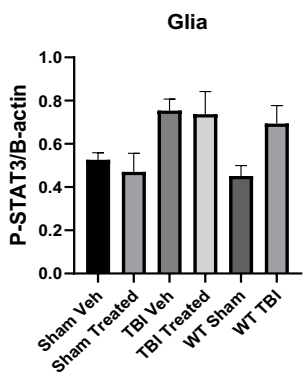
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Sham Veh vs. Sham Treated	-0.1331	-0.2392 to -0.02693	Yes	**	.010
Sham Veh vs. TBI Veh	0.1064	0.0002712 to 0.2126	Yes	*	.049
Sham Veh vs. TBI Treated	-0.08527	-0.1914 to 0.02088	No	ns	.161
Sham Veh vs. WT Sham	0.03142	-0.07472 to 0.1376	No	ns	.930
Sham Veh vs. WT TBI	0.1087	0.002511 to 0.2148	Yes	*	.043
Sham Treated vs. TBI Veh	0.2395	0.1333 to 0.3456	Yes	***	<.001
Sham Treated vs. TBI Treated	0.04781	-0.05833 to 0.1540	No	ns	.709
Sham Treated vs. WT Sham	0.1645	0.05836 to 0.2706	Yes	**	.001
Sham Treated vs. WT TBI	0.2417	0.1356 to 0.3479	Yes	***	<.001
TBI Veh vs. TBI Treated	-0.1917	-0.2978 to -0.08554	Yes	***	<.001
TBI Veh vs. WT Sham	-0.07499	-0.1811 to 0.03115	No	ns	.266
TBI Veh vs. WT TBI	0.002240	-0.1039 to 0.1084	No	ns	>.999
TBI Treated vs. WT Sham	0.1167	0.01054 to 0.2228	Yes	*	.026
TBI Treated vs. WT TBI	0.1939	0.08778 to 0.3001	Yes	***	<.001
WT Sham vs. WT TBI	0.07723	-0.02891 to 0.1834	No	ns	.239



Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Sham Veh vs. Sham Treated	0.7320	0.3147 to 1.149	Yes	***	<.001
Sham Veh vs. TBI Veh	-0.6372	-1.055 to -0.2199	Yes	**	.002
Sham Veh vs. TBI Treated	0.7398	0.3225 to 1.157	Yes	***	<.001
Sham Veh vs. WT Sham	-0.07785	-0.4952 to 0.3395	No	ns	.990
Sham Veh vs. WT TBI	-0.5256	-0.9429 to -0.1082	Yes	**	.009
Sham Treated vs. TBI Veh	-1.369	-1.787 to -0.9519	Yes	***	<.001
Sham Treated vs. TBI Treated	0.007825	-0.4095 to 0.4252	No	ns	>.999
Sham Treated vs. WT Sham	-0.8098	-1.227 to -0.3925	Yes	***	<.001
Sham Treated vs. WT TBI	-1.258	-1.675 to -0.8402	Yes	***	<.001
TBI Veh vs. TBI Treated	1.377	0.9597 to 1.794	Yes	***	<.001
TBI Veh vs. WT Sham	0.5594	0.1420 to 0.9767	Yes	**	.005
TBI Veh vs. WT TBI	0.1116	-0.3057 to 0.5290	No	ns	.954
TBI Treated vs. WT Sham	-0.8177	-1.235 to -0.4003	Yes	***	<.001
TBI Treated vs. WT TBI	-1.265	-1.683 to -0.8481	Yes	***	<.001
WT Sham vs. WT TBI	-0.4477	-0.8651 to -0.03039	Yes	*	.031



Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Sham Veh vs. Sham Treated	-0.003245	-0.1043 to 0.09780	No	ns	>.999
Sham Veh vs. TBI Veh	-0.1802	-0.2812 to -0.07915	Yes	***	<.001
Sham Veh vs. TBI Treated	-0.07741	-0.1785 to 0.02363	No	ns	.196
Sham Veh vs. WT Sham	-0.02830	-0.1293 to 0.07274	No	ns	.944
Sham Veh vs. WT TBI	-0.1742	-0.2753 to -0.07318	Yes	***	<.001
Sham Treated vs. TBI Veh	-0.1770	-0.2780 to -0.07591	Yes	***	<.001
Sham Treated vs. TBI Treated	-0.07417	-0.1752 to 0.02688	No	ns	.232
Sham Treated vs. WT Sham	-0.02506	-0.1261 to 0.07599	No	ns	.966
Sham Treated vs. WT TBI	-0.1710	-0.2720 to -0.06994	Yes	***	<.001
TBI Veh vs. TBI Treated	0.1028	0.001738 to 0.2038	Yes	*	.045
TBI Veh vs. WT Sham	0.1519	0.05085 to 0.2529	Yes	**	.002
TBI Veh vs. WT TBI	0.005967	-0.09508 to 0.1070	No	ns	>.999
TBI Treated vs. WT Sham	0.04911	-0.05193 to 0.1502	No	ns	.642
TBI Treated vs. WT TBI	-0.09682	-0.1979 to 0.004229	No	ns	.065
WT Sham vs. WT TBI	-0.1459	-0.2470 to -0.04488	Yes	**	.003



Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Sham Veh vs. Sham Treated	0.05632	-0.1063 to 0.2189	No	ns	.875
Sham Veh vs. TBI Veh	-0.2276	-0.3902 to -0.06498	Yes	**	.004
Sham Veh vs. TBI Treated	-0.2107	-0.3733 to -0.04807	Yes	**	.007
Sham Veh vs. WT Sham	0.07508	-0.08753 to 0.2377	No	ns	.688
Sham Veh vs. WT TBI	-0.1672	-0.3298 to -0.004617	Yes	*	.042
Sham Treated vs. TBI Veh	-0.2839	-0.4465 to -0.1213	Yes	***	<.001
Sham Treated vs. TBI Treated	-0.2670	-0.4296 to -0.1044	Yes	***	<.001
Sham Treated vs. WT Sham	0.01876	-0.1438 to 0.1814	No	ns	.999
Sham Treated vs. WT TBI	-0.2235	-0.3862 to -0.06093	Yes	**	.004
TBI Veh vs. TBI Treated	0.01691	-0.1457 to 0.1795	No	ns	>.999
TBI Veh vs. WT Sham	0.3027	0.1401 to 0.4653	Yes	***	<.001
TBI Veh vs. WT TBI	0.06037	-0.1022 to 0.2230	No	ns	.841
TBI Treated vs. WT Sham	0.2858	0.1231 to 0.4484	Yes	***	<.001
TBI Treated vs. WT TBI	0.04345	-0.1192 to 0.2061	No	ns	.954
WT Sham vs. WT TBI	-0.2423	-0.4049 to -0.07969	Yes	**	.002

5. Long term study with Pioglitazone (125 and 500mg/kg chow) and Zileuton (50 and 200mg/L). Mice were injured at 3 months and received treatment after approx. 3months until 6 months. Behavior and brain/isolated glia biochemistry analyses were conducted on these mice.

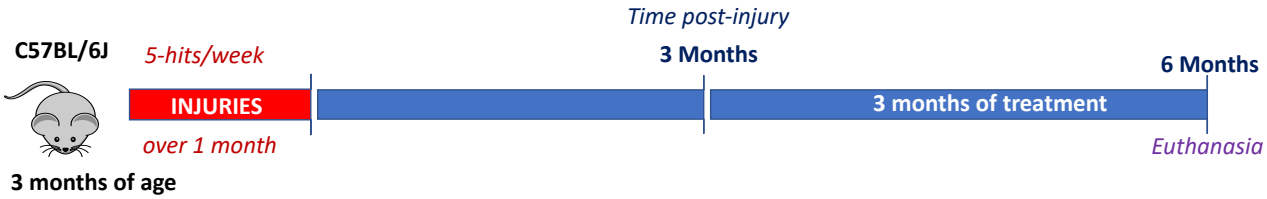
Animals: We purchased 10-week-old C57BL/6 male mice from Jackson Laboratories (Bar Harbor, ME) and housed them in standard cages under a 12-h light/12-h dark schedule at ambient temperature. All procedures were performed in accordance with Office of Laboratory Animal Welfare guidelines under a protocol approved by the Roskamp Institute Institutional Animal Care and Use Committee. Animals were randomly assigned to the different groups ($n = 12$ per group).

Injuries: Closed-head injuries were conducted using our previously established model involving a 1-mm depth impact delivered to the closed skull using a 5-mm flat tip electromagnetic stereotaxic impactor (Leica Biosystems) at a velocity of 5 m/sec with a dwell time of 200 msec. Mice were anesthetized using 1.5 L/min of oxygen and 3% isoflurane and maintained under anesthesia through a nose cone throughout the injury procedure. Injuries were administered at 3 mos of age, 5 times a wk over one month.

Barnes Maze: Cognitive function was evaluated using the Barnes maze (BM). Ethovision XT (Noldus, Wageningen, NL) was used to track and record the movement of each animal. Mice were given 90 sec to locate and enter the target box, and they were required to remain in the target box for 30 sec prior to retrieval, regardless of success. For a period of 6 days, 4 trials were given per day, with mice starting from one of 4 cardinal points on each trial. On the 7th day a single probe trial lasting 60 sec was performed with the mouse starting from the center of the maze and the target box removed. An Ethovision XT system was used to continually record the position of the mouse and measured the distance from the target box 30 times per second for the duration of each trial. The sum of that value was expressed as cumulative distance from target hole. Escape latency measured the time taken for the mouse to enter in the box

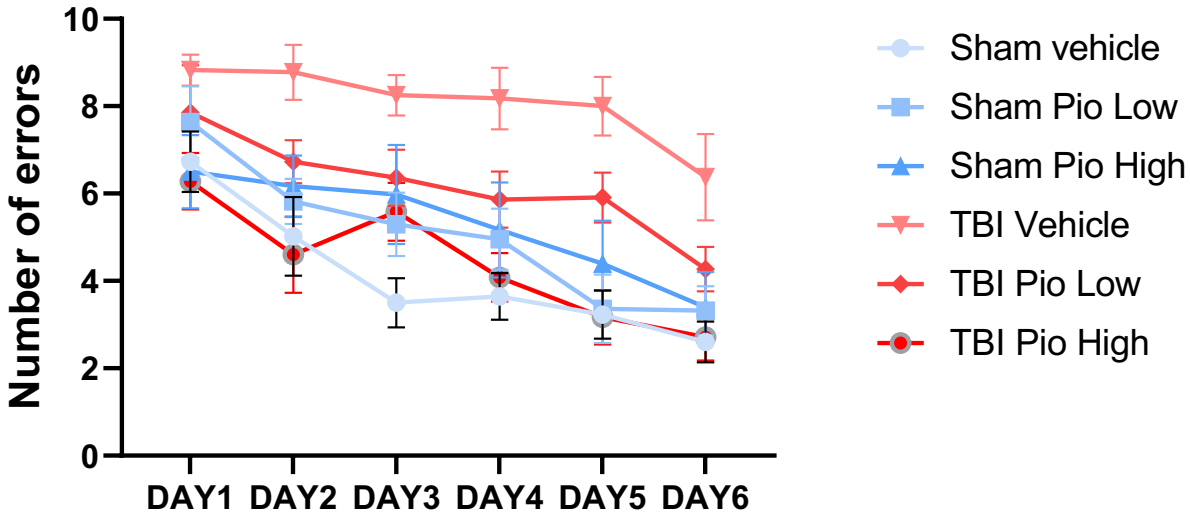
Analyses: All ANOVA experiments were followed by pairwise comparisons with correction for multiple comparisons using the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli (BKY) to control the false discovery rate. ANOVA was performed using Graph Pad prism version 7.0 statistical software (La Jolla, CA). Mauchley test was used to examine the assumption of sphericity before ANOVA analyses. If the sphericity assumption was violated, the Geisser-Greenhouse correction to the degrees of freedom and p values was reported.

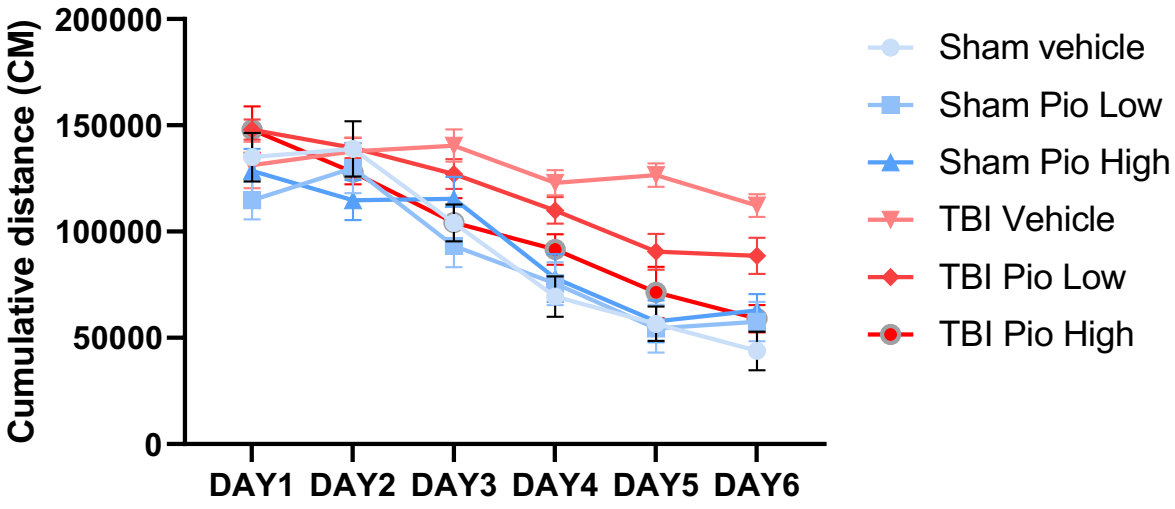
Experimental paradigm for long-term treatment study

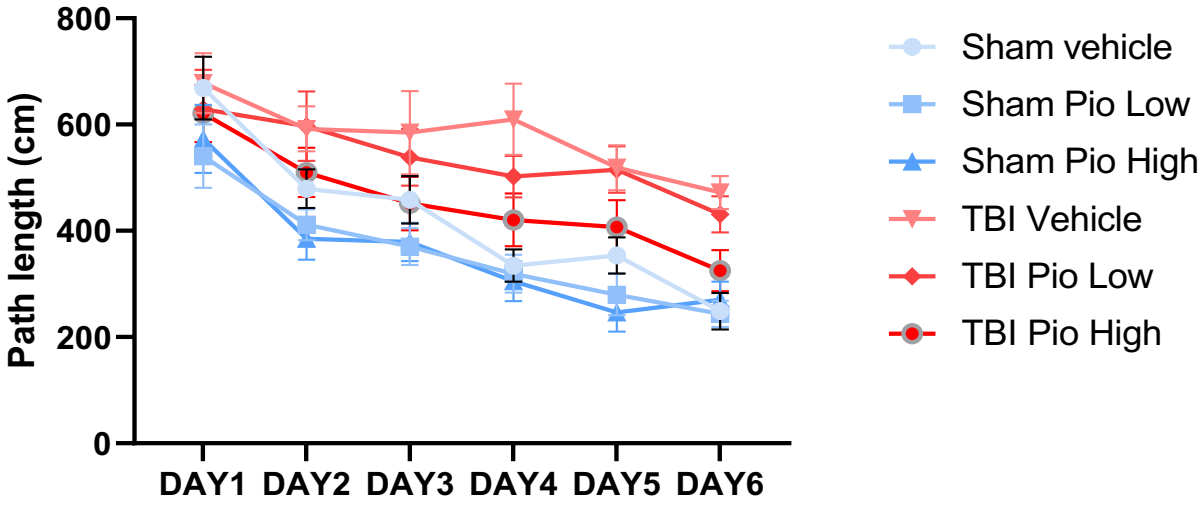


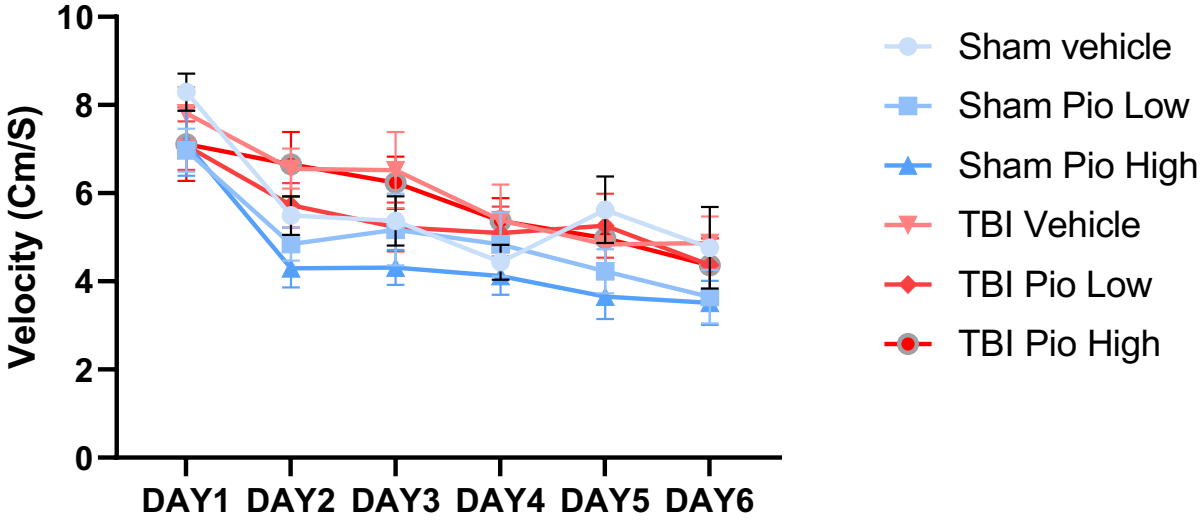
5.1. Barnes maze acquisition and probe trial data to assess learning and memory

5.1.1. Acquisition and Probe trial data

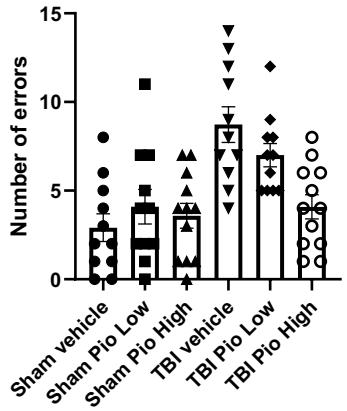




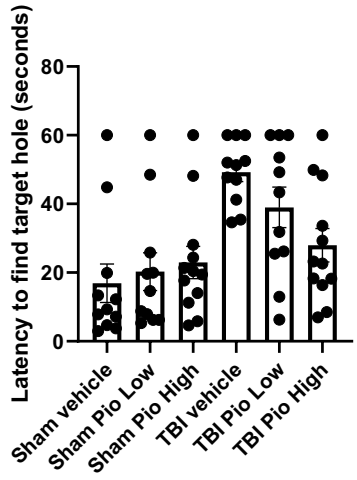

800
600
400
200
0
Path length (cm)
DAY1 DAY2 DAY3 DAY4 DAY5 DAY6
Sham vehicle
Sham Pio Low
Sham Pio High
TBI Vehicle
TBI Pio Low
TBI Pio High


10
8
6
4
2
0
Velocity (Cm/S)
DAY1 DAY2 DAY3 DAY4 DAY5 DAY6
Sham vehicle
Sham Pio Low
Sham Pio High
TBI Vehicle
TBI Pio Low
TBI Pio High

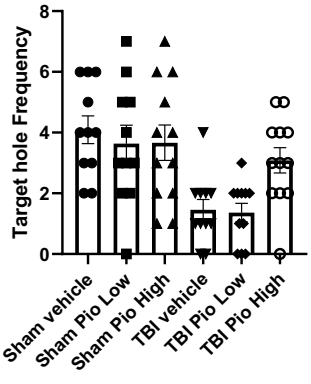
5.1.2. Probe trial data



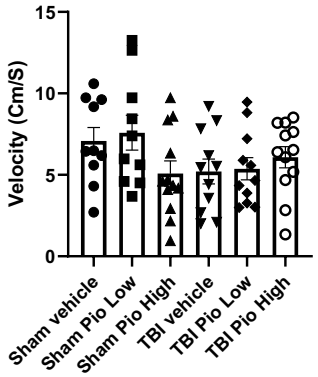
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	
Sham vehicle vs. Sham Pio Low	-1.182	-4.589 to 2.226	No	ns	.910	A-B
Sham vehicle vs. Sham Pio High	-0.6742	-4.010 to 2.661	No	ns	.991	A-C
Sham vehicle vs. TBI vehicle	-5.818	-9.226 to -2.411	Yes	***	<.001	A-D
Sham vehicle vs. TBI Pio Low	-4.091	-7.498 to -0.6835	Yes	**	.010	A-E
Sham vehicle vs. TBI Pio High	-1.174	-4.510 to 2.161	No	ns	.904	A-F
Sham Pio Low vs. Sham Pio High	0.5076	-2.828 to 3.843	No	ns	.998	B-C
Sham Pio Low vs. TBI vehicle	-4.636	-8.044 to -1.229	Yes	**	.002	B-D
Sham Pio Low vs. TBI Pio Low	-2.909	-6.317 to 0.4983	No	ns	.137	B-E
Sham Pio Low vs. TBI Pio High	0.007576	-3.328 to 3.343	No	ns	>.999	B-F
Sham Pio High vs. TBI vehicle	-5.144	-8.480 to -1.808	Yes	***	<.001	C-D
Sham Pio High vs. TBI Pio Low	-3.417	-6.752 to -0.08097	Yes	*	.042	C-E
Sham Pio High vs. TBI Pio High	-0.5000	-3.762 to 2.762	No	ns	.998	C-F
TBI vehicle vs. TBI Pio Low	1.727	-1.680 to 5.135	No	ns	.671	D-E
TBI vehicle vs. TBI Pio High	4.644	1.308 to 7.980	Yes	**	.002	D-F
TBI Pio Low vs. TBI Pio High	2.917	-0.4190 to 6.252	No	ns	.120	E-F



Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Sham vehicle vs. Sham Pio Low	-3.359	-24.43 to 17.71	No	ns	.997
Sham vehicle vs. Sham Pio High	-6.027	-26.65 to 14.60	No	ns	.955
Sham vehicle vs. TBI vehicle	-32.35	-53.41 to -11.28	Yes	***	<.001
Sham vehicle vs. TBI Pio Low	-22.08	-43.15 to -1.011	Yes	*	.035
Sham vehicle vs. TBI Pio High	-11.04	-31.66 to 9.587	No	ns	.619
Sham Pio Low vs. Sham Pio High	-2.668	-23.29 to 17.96	No	ns	.999
Sham Pio Low vs. TBI vehicle	-28.99	-50.06 to -7.921	Yes	**	.002
Sham Pio Low vs. TBI Pio Low	-18.72	-39.79 to 2.347	No	ns	.109
Sham Pio Low vs. TBI Pio High	-7.678	-28.30 to 12.95	No	ns	.882
Sham Pio High vs. TBI vehicle	-26.32	-46.94 to -5.696	Yes	**	.005
Sham Pio High vs. TBI Pio Low	-16.05	-36.68 to 4.572	No	ns	.214
Sham Pio High vs. TBI Pio High	-5.010	-25.18 to 15.16	No	ns	.977
TBI vehicle vs. TBI Pio Low	10.27	-10.80 to 31.34	No	ns	.707
TBI vehicle vs. TBI Pio High	21.31	0.6866 to 41.93	Yes	*	.039
TBI Pio Low vs. TBI Pio High	11.04	-9.582 to 31.67	No	ns	.618



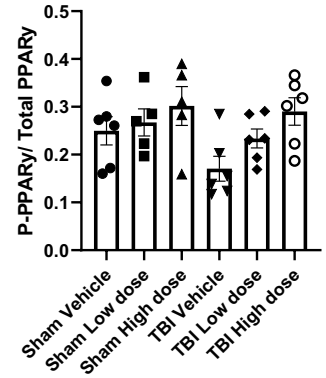
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	
Sham vehicle vs. Sham Pio Low	0.4545	-1.524 to 2.433	No	ns	.984	A-B
Sham vehicle vs. Sham Pio High	0.4242	-1.512 to 2.361	No	ns	.987	A-C
Sham vehicle vs. TBI vehicle	2.636	0.6582 to 4.615	Yes	**	.003	A-D
Sham vehicle vs. TBI Pio Low	2.727	0.7491 to 4.705	Yes	**	.002	A-E
Sham vehicle vs. TBI Pio High	1.008	-0.9290 to 2.944	No	ns	.647	A-F
Sham Pio Low vs. Sham Pio High	-0.03030	-1.967 to 1.906	No	ns	>.999	B-C
Sham Pio Low vs. TBI vehicle	2.182	0.2036 to 4.160	Yes	*	.022	B-D
Sham Pio Low vs. TBI Pio Low	2.273	0.2945 to 4.251	Yes	*	.015	B-E
Sham Pio Low vs. TBI Pio High	0.5530	-1.384 to 2.490	No	ns	.959	B-F
Sham Pio High vs. TBI vehicle	2.212	0.2756 to 4.149	Yes	*	.016	C-D
Sham Pio High vs. TBI Pio Low	2.303	0.3665 to 4.240	Yes	*	.011	C-E
Sham Pio High vs. TBI Pio High	0.5833	-1.311 to 2.477	No	ns	.944	C-F
TBI vehicle vs. TBI Pio Low	0.09091	-1.887 to 2.069	No	ns	>.999	D-E
TBI vehicle vs. TBI Pio High	-1.629	-3.565 to 0.3078	No	ns	.148	D-F
TBI Pio Low vs. TBI Pio High	-1.720	-3.656 to 0.2169	No	ns	.110	E-F



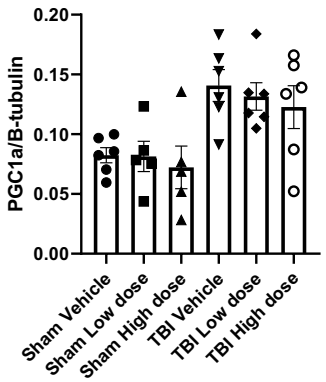
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	
Sham vehicle vs. Sham Pio Low	-0.5029	-3.959 to 2.954	No	ns	.998	A-B
Sham vehicle vs. Sham Pio High	2.000	-1.310 to 5.309	No	ns	.487	A-C
Sham vehicle vs. TBI vehicle	1.876	-1.501 to 5.253	No	ns	.579	A-D
Sham vehicle vs. TBI Pio Low	1.709	-1.668 to 5.086	No	ns	.672	A-E
Sham vehicle vs. TBI Pio High	0.9993	-2.310 to 4.309	No	ns	.948	A-F
Sham Pio Low vs. Sham Pio High	2.503	-0.8068 to 5.812	No	ns	.241	B-C
Sham Pio Low vs. TBI vehicle	2.379	-0.9980 to 5.756	No	ns	.315	B-D
Sham Pio Low vs. TBI Pio Low	2.212	-1.165 to 5.589	No	ns	.396	B-E
Sham Pio Low vs. TBI Pio High	1.502	-1.807 to 4.812	No	ns	.764	B-F
Sham Pio High vs. TBI vehicle	-0.1234	-3.350 to 3.103	No	ns	>.999	C-D
Sham Pio High vs. TBI Pio Low	-0.2908	-3.517 to 2.935	No	ns	>.999	C-E
Sham Pio High vs. TBI Pio High	-1.000	-4.156 to 2.155	No	ns	.936	C-F
TBI vehicle vs. TBI Pio Low	-0.1674	-3.463 to 3.128	No	ns	>.999	D-E
TBI vehicle vs. TBI Pio High	-0.8769	-4.103 to 2.349	No	ns	.966	D-F
TBI Pio Low vs. TBI Pio High	-0.7095	-3.936 to 2.517	No	ns	.987	E-F

5.2. Biochemistry

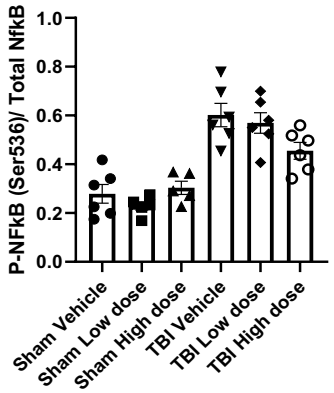
5.2.1. Hippocampus analyses



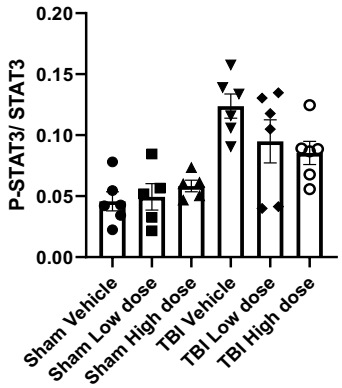
Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli	Mean Diff.	Discovery?	q value	Individual P Value	
Sham Vehicle vs. Sham Low dose	-0.01761	No	.671	.676	A-B
Sham Vehicle vs. Sham High dose	-0.05188	No	.380	.223	A-C
Sham Vehicle vs. TBI Vehicle	0.07937	No	.189	.055	A-D
Sham Vehicle vs. TBI Low dose	0.01607	No	.671	.689	A-E
Sham Vehicle vs. TBI High dose	-0.04030	No	.483	.319	A-F
Sham Low dose vs. Sham High dose	-0.03427	No	.542	.437	B-C
Sham Low dose vs. TBI Vehicle	0.09698	No	.124	.027	B-D
Sham Low dose vs. TBI Low dose	0.03367	No	.542	.425	B-E
Sham Low dose vs. TBI High dose	-0.02269	No	.671	.590	B-F
Sham High dose vs. TBI Vehicle	0.1313	Yes	.037	.004	C-D
Sham High dose vs. TBI Low dose	0.06795	No	.277	.114	C-E
Sham High dose vs. TBI High dose	0.01159	No	.712	.783	C-F
TBI Vehicle vs. TBI Low dose	-0.06330	No	.277	.122	D-E
TBI Vehicle vs. TBI High dose	-0.1197	Yes	.037	.005	D-F
TBI Low dose vs. TBI High dose	-0.05636	No	.325	.167	E-F



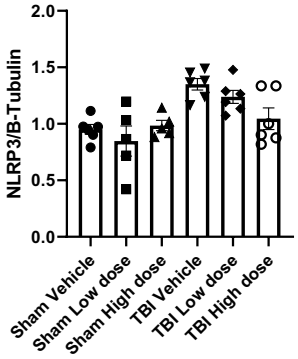
Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli	Mean Diff.	Discovery?	q value	Individual P Value	
Sham Vehicle vs. Sham Low dose	0.001001	No	.538	.960	A-B
Sham Vehicle vs. Sham High dose	0.01020	No	.396	.610	A-C
Sham Vehicle vs. TBI Vehicle	-0.05828	Yes	.012	.004	A-D
Sham Vehicle vs. TBI Low dose	-0.04917	Yes	.020	.014	A-E
Sham Vehicle vs. TBI High dose	-0.04023	Yes	.043	.042	A-F
Sham Low dose vs. Sham High dose	0.009195	No	.396	.659	B-C
Sham Low dose vs. TBI Vehicle	-0.05928	Yes	.012	.006	B-D
Sham Low dose vs. TBI Low dose	-0.05017	Yes	.020	.017	B-E
Sham Low dose vs. TBI High dose	-0.04123	Yes	.043	.046	B-F
Sham High dose vs. TBI Vehicle	-0.06847	Yes	.012	.002	C-D
Sham High dose vs. TBI Low dose	-0.05937	Yes	.012	.006	C-E
Sham High dose vs. TBI High dose	-0.05042	Yes	.020	.016	C-F
TBI Vehicle vs. TBI Low dose	0.009103	No	.396	.633	D-E
TBI Vehicle vs. TBI High dose	0.01805	No	.291	.346	D-F
TBI Low dose vs. TBI High dose	0.008944	No	.396	.639	E-F



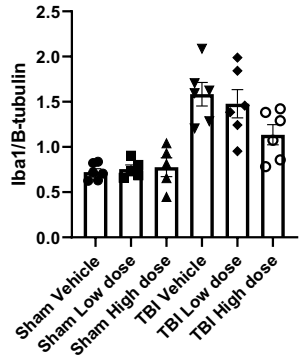
Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli	Mean Diff.	Discovery?	q value	Individual P Value	
Sham Vehicle vs. Sham Low dose	0.04974	No	.148	.366	A-B
Sham Vehicle vs. Sham High dose	-0.02474	No	.228	.651	A-C
Sham Vehicle vs. TBI Vehicle	-0.3229	Yes	<.001	<.001	A-D
Sham Vehicle vs. TBI Low dose	-0.2907	Yes	<.001	<.001	A-E
Sham Vehicle vs. TBI High dose	-0.1762	Yes	.001	.002	A-F
Sham Low dose vs. Sham High dose	-0.07448	No	.087	.198	B-C
Sham Low dose vs. TBI Vehicle	-0.3727	Yes	<.001	<.001	B-D
Sham Low dose vs. TBI Low dose	-0.3404	Yes	<.001	<.001	B-E
Sham Low dose vs. TBI High dose	-0.2260	Yes	<.001	<.001	B-F
Sham High dose vs. TBI Vehicle	-0.2982	Yes	<.001	<.001	C-D
Sham High dose vs. TBI Low dose	-0.2659	Yes	<.001	<.001	C-E
Sham High dose vs. TBI High dose	-0.1515	Yes	.005	.009	C-F
TBI Vehicle vs. TBI Low dose	0.03229	No	.201	.537	D-E
TBI Vehicle vs. TBI High dose	0.1467	Yes	.005	.008	D-F
TBI Low dose vs. TBI High dose	0.1144	Yes	.017	.035	E-F



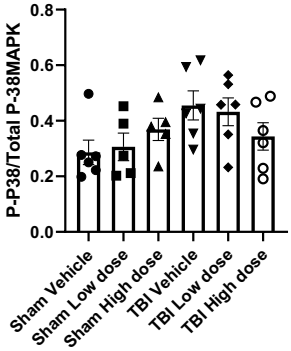
Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli	Mean Diff.	Discovery?	q value	Individual P Value	
Sham Vehicle vs. Sham Low dose	-0.003737	No	.458	.818	A-B
Sham Vehicle vs. Sham High dose	-0.01263	No	.307	.438	A-C
Sham Vehicle vs. TBI Vehicle	-0.07813	Yes	<.001	<.001	A-D
Sham Vehicle vs. TBI Low dose	-0.04915	Yes	.007	.003	A-E
Sham Vehicle vs. TBI High dose	-0.03971	Yes	.021	.015	A-F
Sham Low dose vs. Sham High dose	-0.008892	No	.360	.600	B-C
Sham Low dose vs. TBI Vehicle	-0.07439	Yes	<.001	<.001	B-D
Sham Low dose vs. TBI Low dose	-0.04541	Yes	.014	.009	B-E
Sham Low dose vs. TBI High dose	-0.03598	Yes	.031	.033	B-F
Sham High dose vs. TBI Vehicle	-0.06550	Yes	<.001	<.001	C-D
Sham High dose vs. TBI Low dose	-0.03652	Yes	.031	.031	C-E
Sham High dose vs. TBI High dose	-0.02708	No	.078	.103	C-F
TBI Vehicle vs. TBI Low dose	0.02898	No	.058	.069	D-E
TBI Vehicle vs. TBI High dose	0.03842	Yes	.022	.018	D-F
TBI Low dose vs. TBI High dose	0.009437	No	.350	.542	E-F



Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli	Mean Diff.	Discovery?	q value	Individual P Value	
Sham Vehicle vs. Sham Low dose	0.1032	No	.269	.351	A-B
Sham Vehicle vs. Sham High dose	-0.03387	No	.477	.758	A-C
Sham Vehicle vs. TBI Vehicle	-0.3995	Yes	.003	<.001	A-D
Sham Vehicle vs. TBI Low dose	-0.2880	Yes	.015	.010	A-E
Sham Vehicle vs. TBI High dose	-0.09455	No	.269	.370	A-F
Sham Low dose vs. Sham High dose	-0.1371	No	.225	.238	B-C
Sham Low dose vs. TBI Vehicle	-0.5027	Yes	<.001	<.001	B-D
Sham Low dose vs. TBI Low dose	-0.3912	Yes	.004	.001	B-E
Sham Low dose vs. TBI High dose	-0.1978	No	.084	.080	B-F
Sham High dose vs. TBI Vehicle	-0.3657	Yes	.005	.002	C-D
Sham High dose vs. TBI Low dose	-0.2541	Yes	.036	.027	C-E
Sham High dose vs. TBI High dose	-0.06068	No	.393	.582	C-F
TBI Vehicle vs. TBI Low dose	0.1115	No	.251	.292	D-E
TBI Vehicle vs. TBI High dose	0.3050	Yes	.012	.007	D-F
TBI Low dose vs. TBI High dose	0.1934	No	.084	.073	E-F

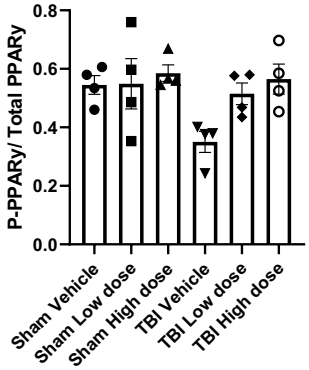


Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli	Mean Diff.	Discovery?	q value	Individual P Value
Sham Vehicle vs. Sham Low dose	-0.03357	No	.250	.833
Sham Vehicle vs. Sham High dose	-0.05443	No	.237	.733
Sham Vehicle vs. TBI Vehicle	-0.8610	Yes	<.001	<.001
Sham Vehicle vs. TBI Low dose	-0.7561	Yes	<.001	<.001
Sham Vehicle vs. TBI High dose	-0.4125	Yes	.006	.011
Sham Low dose vs. Sham High dose	-0.02086	No	.252	.900
Sham Low dose vs. TBI Vehicle	-0.8274	Yes	<.001	<.001
Sham Low dose vs. TBI Low dose	-0.7225	Yes	<.001	<.001
Sham Low dose vs. TBI High dose	-0.3789	Yes	.011	.023
Sham High dose vs. TBI Vehicle	-0.8066	Yes	<.001	<.001
Sham High dose vs. TBI Low dose	-0.7016	Yes	<.001	<.001
Sham High dose vs. TBI High dose	-0.3581	Yes	.012	.031
TBI Vehicle vs. TBI Low dose	0.1049	No	.172	.492
TBI Vehicle vs. TBI High dose	0.4485	Yes	.004	.006
TBI Low dose vs. TBI High dose	0.3436	Yes	.012	.030

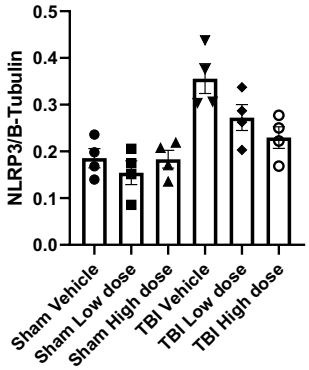


Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli	Mean Diff.	Discovery?	q value	Individual P Value	
Sham Vehicle vs. Sham Low dose	-0.01992	No	.816	.777	A-E
Sham Vehicle vs. Sham High dose	-0.08311	No	.477	.242	A-C
Sham Vehicle vs. TBI Vehicle	-0.1692	No	.214	.017	A-D
Sham Vehicle vs. TBI Low dose	-0.1464	No	.214	.036	A-E
Sham Vehicle vs. TBI High dose	-0.05743	No	.564	.394	A-F
Sham Low dose vs. Sham High dose	-0.06320	No	.564	.392	B-C
Sham Low dose vs. TBI Vehicle	-0.1492	No	.214	.041	B-D
Sham Low dose vs. TBI Low dose	-0.1264	No	.314	.080	B-E
Sham Low dose vs. TBI High dose	-0.03752	No	.780	.594	B-F
Sham High dose vs. TBI Vehicle	-0.08604	No	.477	.226	C-D
Sham High dose vs. TBI Low dose	-0.06324	No	.564	.371	C-E
Sham High dose vs. TBI High dose	0.02568	No	.816	.715	C-F
TBI Vehicle vs. TBI Low dose	0.02280	No	.816	.734	D-E
TBI Vehicle vs. TBI High dose	0.1117	No	.325	.103	D-F
TBI Low dose vs. TBI High dose	0.08892	No	.477	.191	E-F

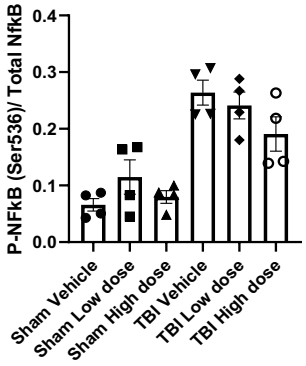
5.2.2. Isolated CD11b specific analyses



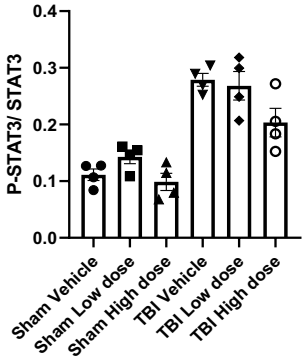
Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli	Mean Diff.	Discovery?	q value	Individual P Value	
Sham Vehicle vs. Sham Low dose	-0.003763	No	.737	.958	A-E
Sham Vehicle vs. Sham High dose	-0.04003	No	.676	.573	A-C
Sham Vehicle vs. TBI Vehicle	0.1948	Yes	.035	.012	A-D
Sham Vehicle vs. TBI Low dose	0.03014	No	.676	.671	A-E
Sham Vehicle vs. TBI High dose	-0.01988	No	.676	.779	A-F
Sham Low dose vs. Sham High dose	-0.03627	No	.676	.609	B-C
Sham Low dose vs. TBI Vehicle	0.1985	Yes	.035	.011	B-D
Sham Low dose vs. TBI Low dose	0.03390	No	.676	.633	B-E
Sham Low dose vs. TBI High dose	-0.01611	No	.676	.820	B-F
Sham High dose vs. TBI Vehicle	0.2348	Yes	.035	.003	C-D
Sham High dose vs. TBI Low dose	0.07017	No	.630	.327	C-E
Sham High dose vs. TBI High dose	0.02015	No	.676	.776	C-F
TBI Vehicle vs. TBI Low dose	-0.1646	No	.068	.030	D-E
TBI Vehicle vs. TBI High dose	-0.2146	Yes	.035	.006	D-F
TBI Low dose vs. TBI High dose	-0.05002	No	.676	.482	E-F



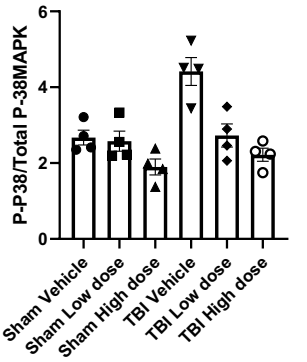
Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli	Mean Diff.	Discovery?	q value	Individual P Value
Sham Vehicle vs. Sham Low dose	0.03113	No	.316	.392
Sham Vehicle vs. Sham High dose	0.002217	No	.666	.951
Sham Vehicle vs. TBI Vehicle	-0.1702	Yes	<.001	<.001
Sham Vehicle vs. TBI Low dose	-0.08691	Yes	.037	.025
Sham Vehicle vs. TBI High dose	-0.04408	No	.213	.230
Sham Low dose vs. Sham High dose	-0.02891	No	.319	.426
Sham Low dose vs. TBI Vehicle	-0.2014	Yes	<.001	<.001
Sham Low dose vs. TBI Low dose	-0.1180	Yes	.008	.004
Sham Low dose vs. TBI High dose	-0.07521	No	.056	.048
Sham High dose vs. TBI Vehicle	-0.1725	Yes	<.001	<.001
Sham High dose vs. TBI Low dose	-0.08913	Yes	.037	.022
Sham High dose vs. TBI High dose	-0.04630	No	.213	.208
TBI Vehicle vs. TBI Low dose	0.08333	Yes	.040	.030
TBI Vehicle vs. TBI High dose	0.1262	Yes	.006	.002
TBI Low dose vs. TBI High dose	0.04283	No	.213	.243



Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli	Mean Diff.	Discovery?	q value	Individual P Value
Sham Vehicle vs. Sham Low dose	-0.04893	No	.090	.147
Sham Vehicle vs. Sham High dose	-0.01391	No	.329	.672
Sham Vehicle vs. TBI Vehicle	-0.1980	Yes	<.001	<.001
Sham Vehicle vs. TBI Low dose	-0.1753	Yes	<.001	<.001
Sham Vehicle vs. TBI High dose	-0.1251	Yes	.001	.001
Sham Low dose vs. Sham High dose	0.03502	No	.165	.292
Sham Low dose vs. TBI Vehicle	-0.1491	Yes	<.001	<.001
Sham Low dose vs. TBI Low dose	-0.1263	Yes	.001	.001
Sham Low dose vs. TBI High dose	-0.07617	Yes	.024	.030
Sham High dose vs. TBI Vehicle	-0.1841	Yes	<.001	<.001
Sham High dose vs. TBI Low dose	-0.1614	Yes	<.001	<.001
Sham High dose vs. TBI High dose	-0.1112	Yes	.003	.003
TBI Vehicle vs. TBI Low dose	0.02277	No	.257	.490
TBI Vehicle vs. TBI High dose	0.07294	Yes	.027	.037
TBI Low dose vs. TBI High dose	0.05017	No	.090	.138



Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli	Mean Diff.	Discovery?	q value	Individual P Value
Sham Vehicle vs. Sham Low dose	-0.03140	No	.073	.225
Sham Vehicle vs. Sham High dose	0.01256	No	.186	.621
Sham Vehicle vs. TBI Vehicle	-0.1676	Yes	<.001	<.001
Sham Vehicle vs. TBI Low dose	-0.1570	Yes	<.001	<.001
Sham Vehicle vs. TBI High dose	-0.09201	Yes	<.001	.002
Sham Low dose vs. Sham High dose	0.04396	Yes	.033	.096
Sham Low dose vs. TBI Vehicle	-0.1362	Yes	<.001	<.001
Sham Low dose vs. TBI Low dose	-0.1256	Yes	<.001	<.001
Sham Low dose vs. TBI High dose	-0.06061	Yes	.010	.026
Sham High dose vs. TBI Vehicle	-0.1802	Yes	<.001	<.001
Sham High dose vs. TBI Low dose	-0.1695	Yes	<.001	<.001
Sham High dose vs. TBI High dose	-0.1046	Yes	<.001	<.001
TBI Vehicle vs. TBI Low dose	0.01064	No	.189	.675
TBI Vehicle vs. TBI High dose	0.07561	Yes	.003	.007
TBI Low dose vs. TBI High dose	0.06497	Yes	.008	.018



Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli	Mean Diff.	Discovery?	q value	Individual P Value
Sham Vehicle vs. Sham Low dose	0.09454	No	.601	.801
Sham Vehicle vs. Sham High dose	0.7753	No	.076	.051
Sham Vehicle vs. TBI Vehicle	-1.745	Yes	<.001	<.001
Sham Vehicle vs. TBI Low dose	-0.05520	No	.618	.883
Sham Vehicle vs. TBI High dose	0.4531	No	.249	.237
Sham Low dose vs. Sham High dose	0.6807	No	.108	.082
Sham Low dose vs. TBI Vehicle	-1.839	Yes	<.001	<.001
Sham Low dose vs. TBI Low dose	-0.1497	No	.558	.691
Sham Low dose vs. TBI High dose	0.3585	No	.330	.346
Sham High dose vs. TBI Vehicle	-2.520	Yes	<.001	<.001
Sham High dose vs. TBI Low dose	-0.8305	No	.066	.038
Sham High dose vs. TBI High dose	-0.3222	No	.346	.395
TBI Vehicle vs. TBI Low dose	1.689	Yes	<.001	<.001
TBI Vehicle vs. TBI High dose	2.198	Yes	<.001	<.001
TBI Low dose vs. TBI High dose	0.5083	No	.218	.187

Conclusion

- We reveal that Pioglitazone at high dose (500mg/kg in chow) demonstrates efficacy in our r-mTBI model, restores learning and memory deficits and dampens inflammation.
- We also reveal that PTEN deletion in microglia demonstrates efficacy in our r-mTBI model, significantly dampening inflammation.
- PPARg and PTEN could serve as a novel target against inflammation and microglial activation following r-mTBI
- Future work will be needed to determine whether Pioglitazone mediated its effects primarily through PPARg or independent of PPARg (i.e. other off-target influence).
- Future work will also explore novel time windows of opportunity to intervene.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to Report

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

NOTHING TO REPORT

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and

research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to Report

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to Report

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

N/A

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:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report (In progress, two manuscripts planned for submission in Jan-March 2022)

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

  Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Neurotrauma meeting 2019 (Poster) – Andrew pearson, Fiona Crawford, Joseph Ojo. Zileuton reduces neuroinflammation and glial pathology following repetitive mild TBI by inhibiting proinflammatory bioactive lipid synthesis.*

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report (roskampinstitute.org website will highlight the results of our findings once our two manuscripts planned for early 2022 have been accepted for publication).

- **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**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *1234567*
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name: *Fiona Crawford (no change)*
Project Role: *PI*
Researcher Identifier (e.g. ORCID ID): *N/A*
Nearest person month worked: *1.2*
Contribution to Project: *Dr Crawford directs all aspects of this project and provides supervision on the overall approach and data interpretation for the experiments outlined in this application. In particular, she will interact with all of the team members listed on this application and provide full oversight as they implement the work proposed in this application, including regular communication with the expert consultants.*

Name: *Joseph Ojo (no change)*
Project Role: *Co-PI*
Researcher Identifier (e.g. ORCID ID): *N/A*
Nearest person month worked: *2.4*
Contribution to Project: *Dr. Ojo works alongside Dr. Crawford in directing all aspects of this project and providing supervision on mTBI animal modeling, histopathological analyses and data interpretation. He is responsible for overseeing all aspects of animal manipulation and ensuring that the projects are executed in a timely fashion. He will also perform histopathological assessments in both humans and animal models as described in the proposal.*

Name: *Andrew Pearson (no change)*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *N/A*
Nearest person month worked: *12*
Contribution to Project: *A. Pearson will be involved in animal handling and surgical procedures, as well as histopathological characterization and molecular analyses, such as western blotting and ELISA. She will assist in all surgical procedures (primarily years 1 and 2) and conduct histopathological analyses with the supervision of Dr. Ojo (primarily Years 2 & 3).*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

Please find the quad chart attached on the next page.

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Please find the quad chart attached on the next page.

Novel Therapeutic Target Identification through analysis of Convergent AD and TBI Pathogenic Mechanisms

Log Number: AZ160110

W81XWH-17-1-0638

PI: Drs Fiona Crawford / Joseph Ojo **Org:** Roskamp Institute, Sarasota, FL **Award Amount:** $800,000

Study Aim(s)

Aim 1: Validation of potential targets for therapeutic intervention in the pathogenic TBI-AD interrelationship - eicosanoid signaling, PI3-kinase/Akt /PTEN/mTOR/insulin signaling and RXR/PPAR pathway.

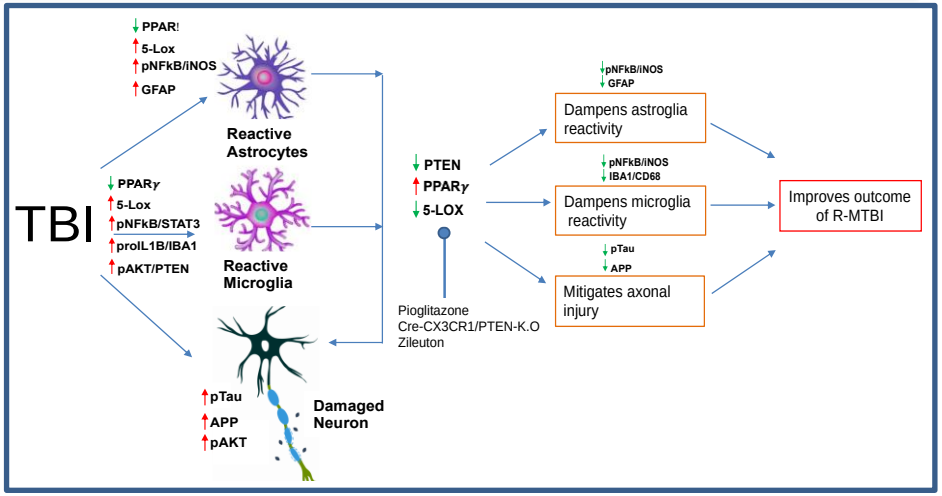
Aim 2: Chronic evaluation of the efficacy of three potential therapeutics against the pathogenic TBI-AD interrelationship

Approach

i) Evaluate target engagement and efficacy in recovering normal molecular profiles in TBI mice using compounds that modulate the TBI and AD coincident molecular changes in a short-term (2wks) treatment Paradigm

ii) In vivo validation of therapeutic targets in mice with TBI using chronic intervention (6-month treatment regimen) with the top three performing compounds for three different targets from Aim 1 to evaluate the long-term neurobehavioral, neuropathological and biochemical outcomes

iii) Selection of downstream convergent tractable targets between the three different compounds for further exploration.



Accomplishment: Demonstrated PPARg and PTEN as novel anti-inflammatory targets to mitigate against the negative consequences of r-mTBI.

Timeline and Cost

Activities CY	18	19	20/21
MAJOR TASK ONE OR AIM 1			
MAJOR TASK TWO OR AIM 2			
Estimated Direct Budget (500K)	**$180K**	**$150K**	**$170K**

Last updated: (September 2017)

Goals/Milestones

CY17 Goal

☑ Obtain regulatory approval to begin animal studies

☑ Validate identified targets in mouse and human TBI/AD brains

CY18 Goals

☑ Initiate short treatment study to evaluate target engagement and efficacy

CY19 Goals

☑ Evaluate and select Top performing therapeutics for use in Aim 2

☑ Initiated chronic treatment study with the Top 2 performing compounds

CY20 Goal

☑ Completed chronic treatment study and evaluated the neurobehavioral, neuropathological and biochemical outcomes

Comments/Challenges/Issues/Concerns

- 6-8 Mos behind schedule due to lag in time for ACURO review/COVID-19
- An eight month extension was requested and granted.

Budget Expenditure to Date

Projected Expenditure: $798,949.00 Actual Expenditure: $798,949.00