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CONTRACTING ORGANIZATION:	The Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc.
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Posttraumatic headache (PTH) is one of the most common and debilitating symptoms following traumatic brain injury (TBI), and it can resemble the migraine-like phenotype. Although augmentation of the endogenous cannabinoids has been shown to have protective effects in animal models of TBI and migraine, it is unknown whether targeting the endocannabinoid system is also effective in PTH. Using a recently developed closed-head impact model of engineered rotational acceleration (CHIMERA), we found that treatment with the inhibitor of 2-AG hydrolytic enzyme MAGL, MJN110 for 7 days significantly reduced periorbital allodynia and blocked the late development of pain sensitivity to low dose of CGRP in TBI animals. Accumulation of microglia and astrocytes and the production of CGRP in the trigeminal ganglion (TG) and trigeminal nucleus caudalis (TNC) were significantly reduced by MJN110 treatment. Mass spectrometry revealed the reduced production of 2-AG in TG and TNC following the last TBI impact, suggesting a causal role to the development of PTH. This study suggested that augmentation of the endogenous 2-AG by inhibition of its hydrolysis is likely to be a therapeutic strategy for PTH attributed to repetitive mTBI.

15. SUBJECT TERMS

NONE LISTED

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1. INTRODUCTION:

Cannabis has long been used as medicine to alleviate pain, but the psychoactive side effects impede its further development. Since the discovery of the major component of cannabis delta-9-tetrahydrocannabinol and its functional analogues in the body, the interest to the use of cannabis has been increased exponentially. Different from the exogenous cannabinoids, augmentation of the endogenous levels of cannabinoids is likely to avoid the unwanted side effects because the endocannabinoids are upregulated locally, rather than globally. The endocannabinoid system is composed of cannabinoid type 1 (CB1) and type 2 (CB2) receptors, their endogenous ligands anandamide (AEA) and 2-arachidonyl glycerol (2-AG), and the proteins or enzymes for their synthesis, transport and degradation. Although AEA and 2-AG are elevated after brain injury and thought to be protective, the rapid hydrolysis limited their therapeutic effects. Therefore, inhibition of the endocannabinoid degradation should be able to sustain the elevated levels of endocannabinoids and exert their therapeutic effect. Recent studies from our group and others have demonstrated that selective inhibition of either monoacylglycerol lipase (MAGL) or fatty acid amide hydrolysis (FAAH) to increase the brain levels of 2-AG or AEA reduces neuroinflammation and restores motor and cognitive function in the animal models of repetitive mild traumatic brain injury (mTBI). However, it has not been investigated whether modulation of the endocannabinoid system can alleviate posttraumatic headache (PTH), a common and disabling symptom associated with TBI. In this period of study, we utilized a recently developed closed-head impact model of engineered rotational acceleration (CHIMERA) mouse model to study the pathogenesis and treatment of PTH. Our results showed that mice with repetitive mTBI induced by CHIMERA (once a day for 4 consecutive days) demonstrated an enhanced periorbital allodynia at 7 days post-injury and the TBI mice are more vulnerable to the low dose of calcitonin gene related peptide (CGRP)-induced pain at late-time points (30-45 days post-injury). Continued treatment with the MAGL inhibitor MJN110 for 7 days significantly reduced periorbital allodynia and blocked late development of pain sensitivity to low dose of CGRP. Accumulation of microglia and astrocytes and the production of CGRP in the TBI mouse trigeminal ganglion and trigeminal nucleus caudalis were significantly reduced by MJN110 treatment. This study suggested that augmentation of the endocannabinoid 2-AG by inhibition of its hydrolysis is likely to be a therapeutic strategy for PTH attributed to repetitive mTBI.

2. KEYWORDS:

Mild traumatic brain injury, post-traumatic headache, endocannabinoid, monoacylglycerol lipase, 2-AG, trigeminal ganglion, trigeminal nucleus caudalis, CGRP, CHIMERA, mice.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

To determine the role of augmentation of endocannabinoids in the treatment of PTH attributed to mTBI, three specific aims were proposed:

1) To characterize the expression of the endocannabinoid signaling components in the trigeminal pain pathway (including trigeminal ganglion, trigeminal nucleus caudalis, and thalamic relay ventral posterior medialis nucleus) at various time points following repetitive mTBI.

2) To determine the therapeutic effect and the underlying mechanisms of a novel SSCI, LM-4131 in post-traumatic headache following repetitive mTBI.

3) To determine the potential synergy between SSCI and the selective MAGL or FAAH inhibitors in post-traumatic headache following repetitive mTBI.

What was accomplished under these goals?

In this funding period, we have examined the expression of the endocannabinoid signaling components in trigeminal ganglion and trigeminal nucleus caudalis at 7 days after CHIMERA-induced repetitive mild traumatic brain injury (mTBI) using quantitative rtPCR, and no major differences in the mRNA expression were found between sham control and TBI animals. Given that we have recently shown that inhibition of the major endocannabinoid 2-AG hydrolytic enzyme MAGL reduces neuroinflammation, maintains the glutamate and GABA receptors components and improves locomotor function, learning and memory in the repetitive TBI mouse model induced by controlled cortical impact, we hypothesized that treatment with the MAGL inhibitor could alleviate PTH through the cannabinoid receptor mediated mechanisms. The major findings are listed below:

1) Periorbital tactile hypersensitivity following CHIMERA induced mTBI was attenuated by inhibition of the 2-AG hydrolysis

Non-invasive CHIMERA method was used to induce repetitive mild TBI in male, 10 weeks old, C57BL/6 mice. Briefly, Mice was restrained in the supine position on the CHIMERA device anesthetized with 4.0% isoflurane for induction and 2.0% isoflurane for maintenance. The piston impacted the center on the scalp aligned with bregma on the skull with 0.65J impact energy. Mice received one impact per day for 4 consecutive days. Sham-operated mice received anesthesia and restrain, but no impact. In order to determine if the animals developed periorbital tactile hypersensitivity following CHIMERA, the skin region including the midline area above the eyes was stimulated with different Von Frey filaments (0.2-4.3g). Changes in tactile skin sensitivity were evaluated by the up-down method on days 0, 5 and 7 post-injury. TBI animals were treated with the MAGL inhibitor MJN110 (MJN) at 1.0 mg/kg and 2.5 mg/kg 1 hour after each impact and then for 3 additional days (in total of 7 days). CHIMERA induced repetitive mTBI resulted in a significant reduction in periorbital allodynia compared to the baseline ($p<0.05$) on days 5 and 7 after the last impact. MJN treatment exhibited dose-dependent protective effect on tactile hypersensitivity (Figure 1A). On day 5 and day 7 post-injury,

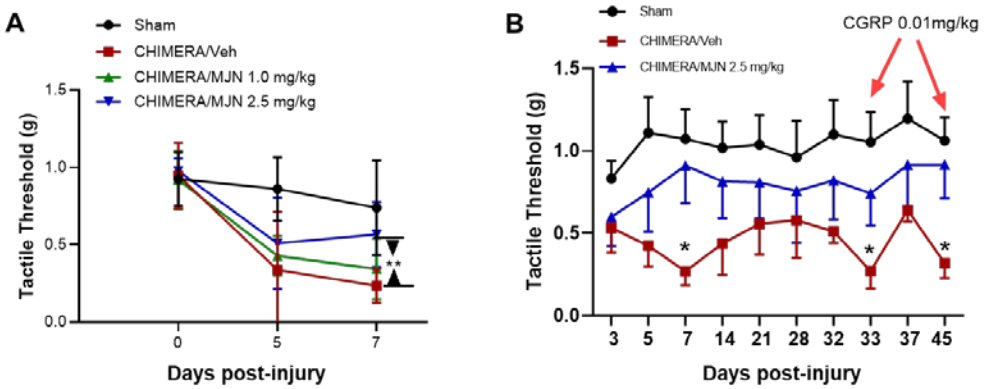

Figure 1. The MAGL inhibitor MJN110 suppressed periorbital allodynia induced by repetitive mTBI and triggered by CGRP. *p<0.05 and **p<0.01 compared between the MJN 2.5 mg/kg and the TBI/vehicle group (N=10/group).

treatment with MJN at 1.0 mg/kg only showed slight elevation of periorbital mechanical threshold compared to the TBI/vehicle. On day 5 post-injury, 2.5 mg/kg MJN treated animals showed moderately improved periorbital allodynia, but at 7 days post-injury, there was a significant reduction in periorbital tactile pain threshold. The mechanical threshold in the 2.5 mg/kg MJN treatment group was significantly greater than that in the TBI/vehicle group ($p<0.01$).

To examine the therapeutic effect of MJN on persistent pain sensitivity, TBI animals were continuously subject to von Frey test weekly until 45 days post-injury. As shown in figure 1B, the TBI animals exhibited strongest hypersensitivity on day 7 post-injury, and the tactile allodynia was gradually reduced at the late time points, even though it was still lower than the sham and MJN treatment groups. To determine whether the TBI animals are more vulnerable to the migraine inducing agents as reported by others, all the animals were given the pain-inducing neurogenic peptide, calcitonin gene related peptide (CGRP) on day 33 and day 45. Two hours following the low dose CGRP treatment (0.01 mg/kg, i.p.), the TBI/vehicle group displayed an enhanced pain sensitivity with the lowest tactile threshold of 0.27 g compared to tactile threshold of 1.05 g in the sham animals. Interestingly, MJN treated group didn't show obvious pain response to the CGRP injection (with the tactile threshold of 0.74 g) similar to the sham control animals. This result suggested that MJN treatment not only ameliorated PTH in the TBI animals but also prevented the animals from being susceptible to the pain stimulus.

2) *Activation of microglia and astrocytes in the trigeminal ganglia and trigeminal nucleus caudalis in CHIMERA mice was ameliorated by the administration of MJN*

TBI mice were subject to 4% PFA transcardiac perfusion and the trigeminal ganglia and trigeminal nucleus caudalis were collected for immunohistochemistry assessment. There was a greatly enhanced Iba1 immunoreactivity in the TBI/vehicle mouse trigeminal ganglia compared to the sham group. Iba1 positively stained cells were found to be in close proximity to neurons. MJN treatment group showed reduced number of Iba1 positive cells (Figure 2A). The quantification of the number of Iba1 positive cells in each group indicated a significant reduction of Iba1 due to the MJN administration (Figure 2C).

Immunohistochemical evaluation of GFAP and Iba1 was also performed in the TNC, a second order neurons in the trigeminal pain pathway (Figure 2B). Increased GFAP immunoreactivity in the TNC was found in the TBI/vehicle animals compared to the sham. There were greatly reduced GFAP cells in the MJN treated groups. Quantification of the number of Iba1 and GFAP positive cells in the TNC revealed a significant increase in the cell numbers

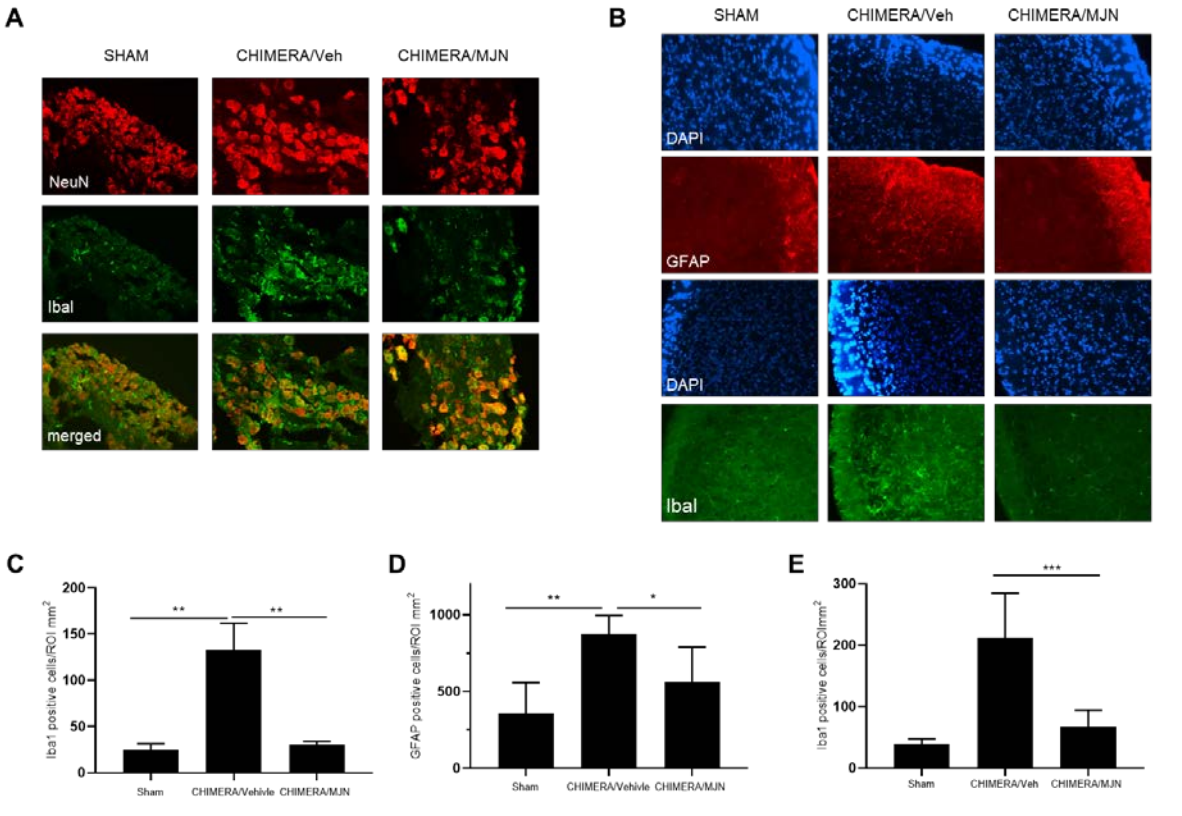

Figure 2. Treatment with MJN110 reduced glial cell accumulation in trigeminal ganglion (TG; A) and trigeminal nucleus caudalis (TNC; B) at 7 days post-TBI. Iba1 stains microglia/macrophages, GFAP stains astrocytes.*p<0.05, **p<0.01 and ***p<0.001 (n=6/group).

in the TBI/vehicle group, with the means of Iba1 and GFAP positive cells are 132 cells/mm^2 and 873 cells/mm^2, respectively. The means of Iba1 and GFAP positive cells in the sham control are 24 cells/mm^2 and 357 cells/mm^2. The increased Ibal1 and GFAP positive cells in the TBI group were brought down to the 30 cells/mm^2 and 560 cells/mm^2 by MJN (Figure 2D and 2E) suggesting that MJN treatment significantly attenuated neuroinflammation in the pain transduction pathway.

3) *MJN110 attenuated the expression of calcitonin gene related peptides (CGRP) in trigeminal ganglia and trigeminal nucleus caudalis of mTBI mice*

mTBI injury resulted in an increased expression of CGRP in the TG by 5 folds and in the TG by 2 folds over the shame control at 7 days post-injury (Figure 3). In the in the TG, most CGRP were expressed in neurons, while in the TNC, the expression of CGRP were seen along the superficial layer of lamina close to the trigeminal tract (Figure 3A). MJN exerted the same effect on the increased CGRP expression as it did to the activation of microglial and astrocytes in TG and TNC. A greatly reduced CGRP immunoreactivity in TG and TNC was found in the TBI animals treated with MJN, as indicated by the quantification of the positive CGRP stained cells in each experimental group (Figure 3B and 3C).

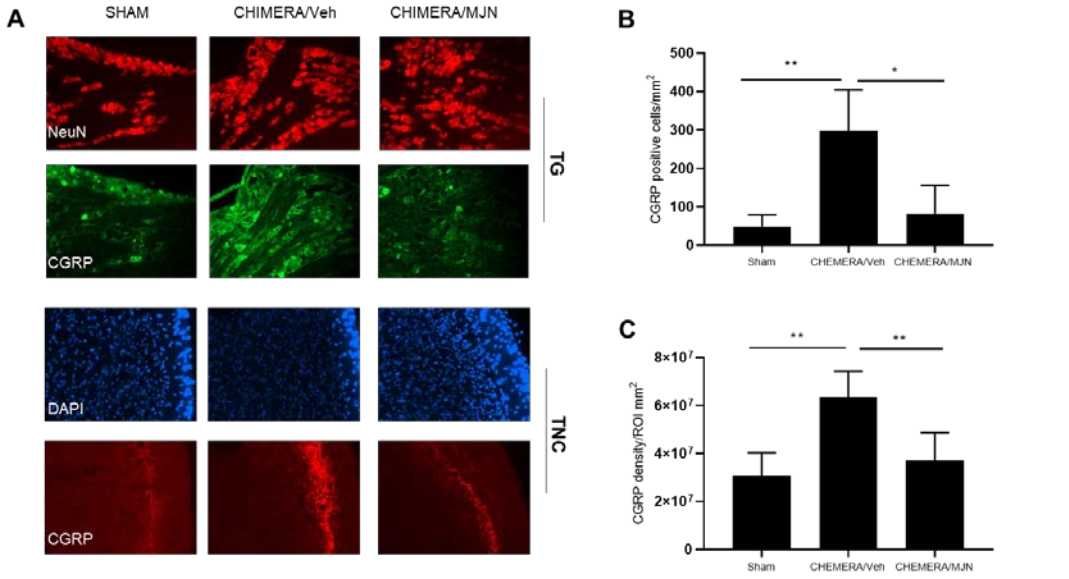

Figure 3. Treatment with MJN110 attenuated the expression of CGRP in trigeminal ganglion (TG) and trigeminal nucleus caudalis (TNC) at 7 days post-TBI. *p<0.05 and **p<0.01 (n=6/group).

Consistent with the results in immunohistochemistry, the production of CGRP (assessed by ELISA) in the TG 7 days post-injury was significantly increased and attenuated in the MJN treated animals (Figure 4A). Similarly, the increased mRNA expression of CGRP receptors, the calcitonin-receptor like receptor (CALCR1) and the receptor activity modifying protein 1 (RAMP1) in the TG was also suppressed in the MJN treated group (Figure 4B and 4C).

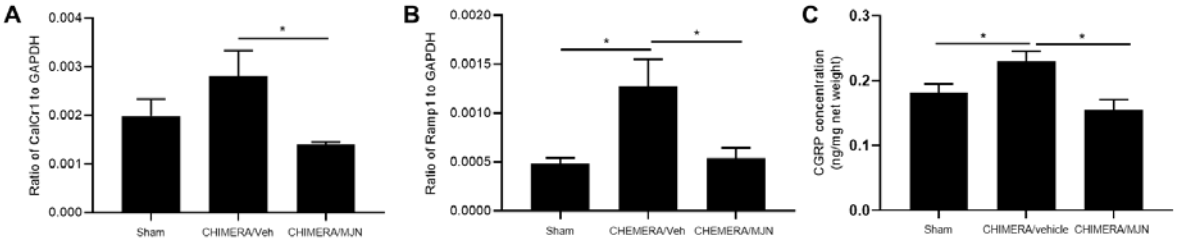

Figure 4. MJN110 treatment suppressed the mRNA expression of Calcr1 (A) and Ramp1 (B) and the production of CGRP (C) in trigeminal ganglion (TG) at 7 days post-TBI. *p<0.05 (n=10/group).

4) *The suppressive effect of MJN110 on TBI induced periorbital allodynia was dependent on the cannabinoid signaling pathway*

In order to determine whether the therapeutic effect of MJN treatment on PTH relies on the cannabinoid signaling, the mTBI mice were co-administrated with MJN and the cannabinoid type 1 (CB1) receptor antagonist AM281 or the cannabinoid type 2 (CB2) receptor antagonist AM630. Von Frey test was performed on the periorbital region of mice on days 6 and 14 post-TBI. A significantly reduced tactile threshold was seen

in the TBI/vehicle group when compared to the sham group on both days. Treatment of MJN with AM281 or AM630 partially reversed the improved periorbital allodynia by MJN alone treatment (Figure 5). On day 6, TBI animals treated with MJN with the CB1 or the CB2 receptor antagonists had moderately higher tactile threshold compared to the TBI/vehicle group, whereas the MJN alone treated TBI animals had a remarkable higher tactile threshold compared to the vehicle group. On day 14 post-injury, we found that co-administration of AM281 or AM630 with MJN greatly reversed the MJN therapeutic effect. Animals treated with MJN and AM281 or AM630 had a similar tactile threshold to the TBI/vehicle group. The tactile thresholds were 0.64g in the MJN and AM281 group, 0.68 g in the MJN and AM630 group and 0.67 g in the TBI/vehicle group. Compared to 1.06 g tactile threshold in the MJN alone treatment group, the addition of CB1 antagonist 281 significantly increased periorbital hypersensitivity compared to the MJN treatment alone.

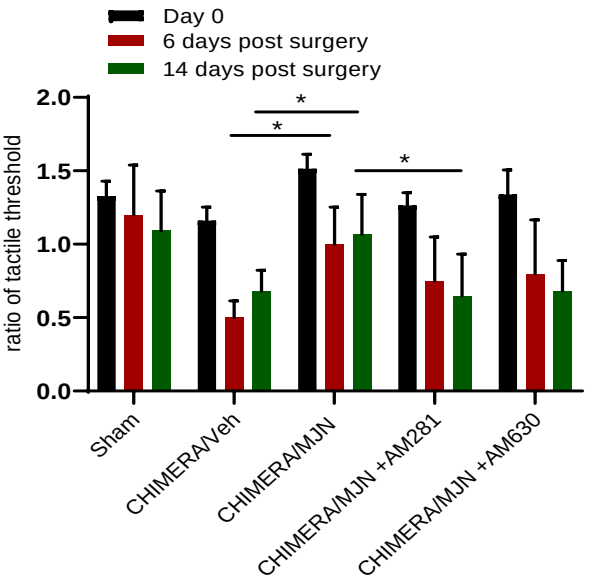

Figure 5. The suppressive effect of MJN110 on periorbital allodynia is mediated by cannabinoid receptor activation. *p<0.05 (n=7/group).

5) Anti-inflammatory effect by increasing endogenous 2-AG in TG and TNC of the TBI mouse was counteracted by the combined treatment of MJN with the cannabinoid receptor antagonists

To determine whether the anti-inflammatory effects of MJN were also mediated by the cannabinoid receptors, the expression of Iba1 and GFAP in TG and TNC was compared in the MJN treatment groups with and without the CB1 or CB2 receptor antagonist. Compared to the sham, there was a strong Iba1 immunoreactivity in the TG and TNC regions in the TBI/vehicle group, which was greatly weakened by the MJN treatment (Figure 6). In TG, inhibition of microglia activation by MJN was reversed by co-administration of the CB1 or CB2 receptor antagonists, despite the statistical significance was only observed in the AM281 and MJN co-treatment group (Figure 6B). In the TNC region, the inhibitory effect of MJN on microglia activation seemed to be mainly dependent on the CB2 receptor activation (Figure 6C). The same phenomenon was also observed in the GFAP immunostaining (Figure 7). Quantification of the GFAP positive staining in the TNC region showed that animals treated with the combination of MJN and the CB1 or the CB2 receptor antagonist possessed a

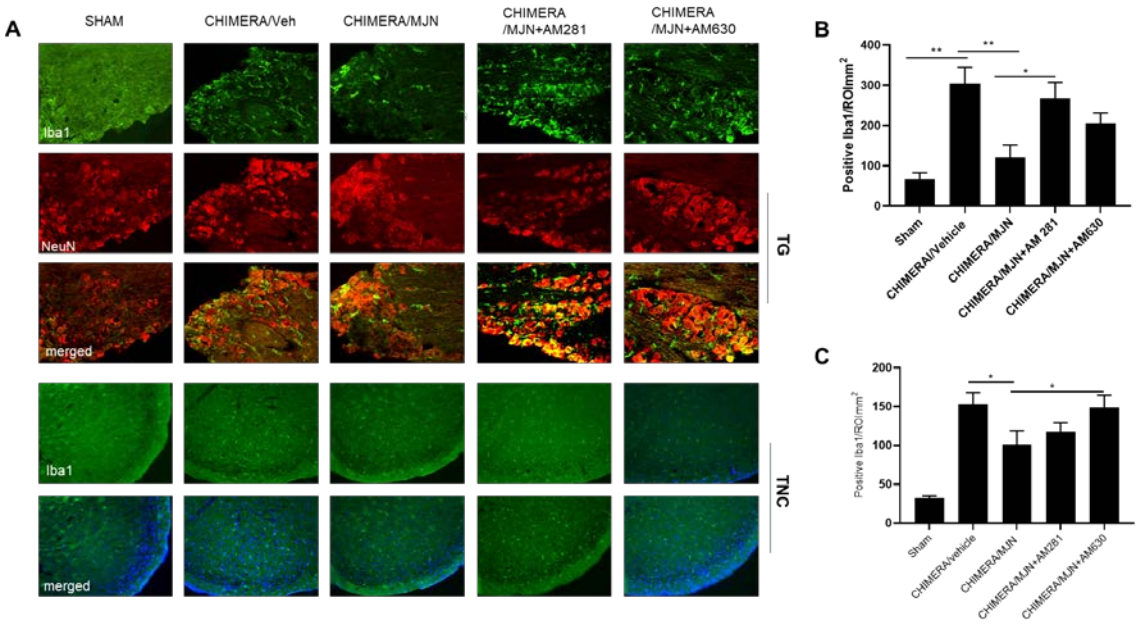

Figure 6. The inhibitory effect of MJN110 on microglia accumulation in TG and TNC is dependent on CB1 and CB2 cannabinoid receptor activation. *p<0.05 and **p<0.01 (n=7/group).

significantly greater GFAP staining than that in the MJN alone treatment group, and was almost the same to that in the TBI/vehicle group (Figure 7).

Similarly, the reduced expression of CGRP in TG and TNC of the mTBI mice by MJN was also reversed by co-administration of the CB1 or CB2 receptor antagonist (Figure 8A). Quantification of the intensity or the number of positively stained CGRP in the TG region showed that CB2 seemed to be more responsible to regulate the CGRP expression, although addition of CB1 antagonist also reversed the MJN inhibitory effect on CGRP expression (Figure 8B). In the TNC region, the TBI animals treated with MJN and AM281, but not with MJN and AM630 had significantly increased CGRP intensity compared to that in the MJN alone (Figure 8C).

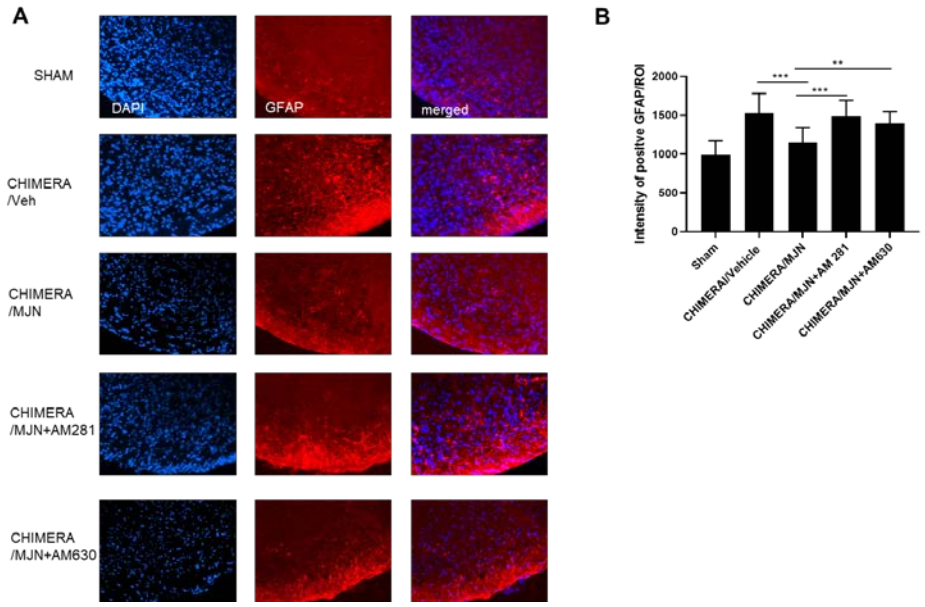

Figure 7. The inhibitory effect of MJN110 on astrocyte accumulation in TNC is dependent on CB1 and CB2 cannabinoid receptor activation. **p<0.05 and ***p<0.01 (n=7/group).

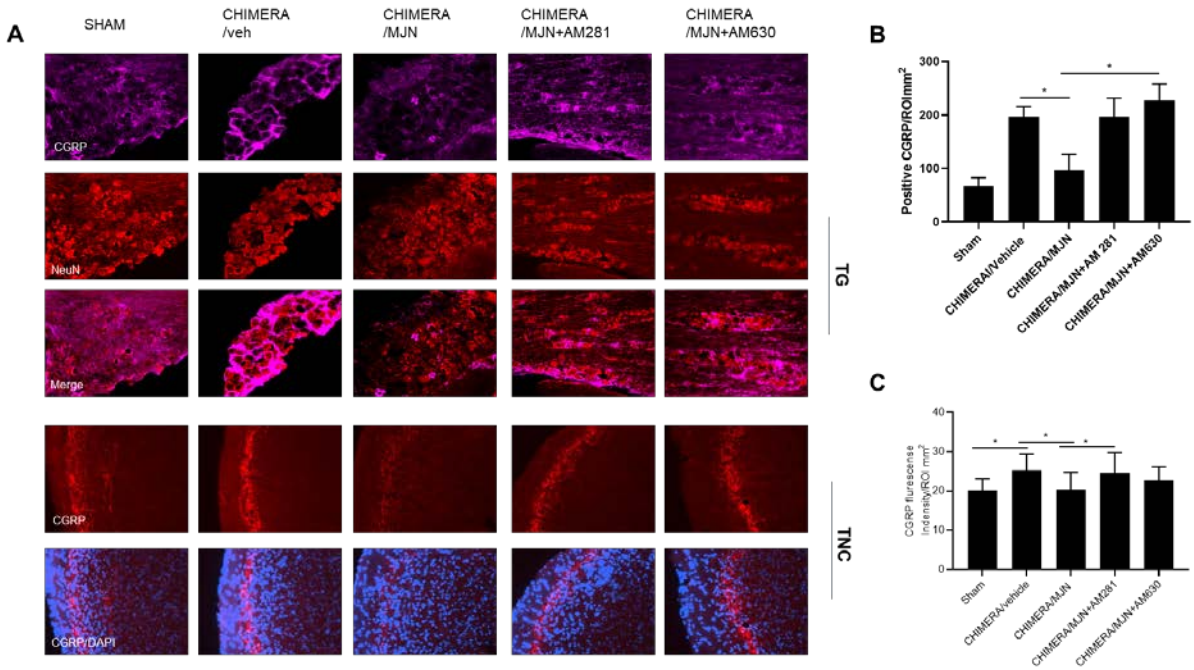

Figure 8. The suppressive effect of MJN110 on CGRP expression in TG and TNC is dependent on CB1 and CB2 cannabinoid receptor activation. *p<0.05 (n=7/group).

6) *Augmentation of 2-AG in the trigeminal system is crucial to ameliorate TBI induced posttraumatic headache*

To further determine the role of 2-AG in the posttraumatic headache, the TBI animals was given MJN110, the 2-AG synthetic enzyme DAGL-α inhibitor DO34, and combination of these two drugs. Von Frey test was employed to evaluate pain hypersensitivity in the periorbital region of the TBI mouse brain. Similar to the above findings, MJN treatment significantly alleviated periorbital pain sensitivity at 7 days post-TBI, the therapeutic effect of MJN was reversed by co-administration of DO34, the specific inhibitor to block the 2-AG synthesis. Consistently, treatment with DO34 itself did not significantly affect the periorbital pain sensitivity (Figure 9).

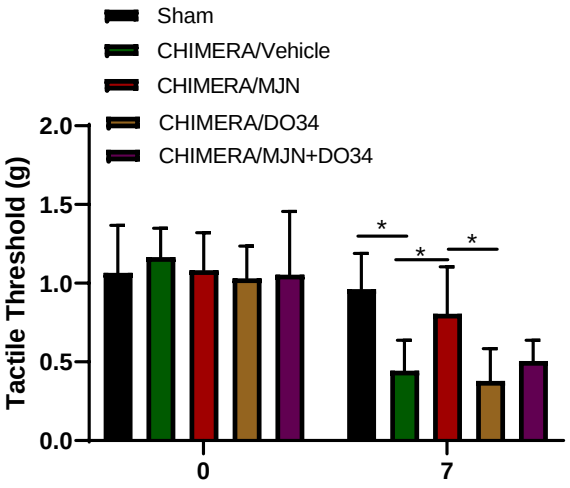

Figure 9. The inhibitory effect of MJN110 on periorbital allodynia of TBI mice was blocked by co-administration of the inhibitor of 2-AG synthesis.*p<0.05 (N=7/group).

To measure the endogenous levels of 2-AG in TG, TNC and PAG that are crucial sites for pain modulation, animals treated with MJN, DO34 and the drug combination were euthanized following 4 days repetitive TBI and drug treatment and the fresh TG, TNC and PAG were collected for the measurement of 2-AG, AEA and arachidonic acid (AA) using mass spectrometry. Treatment with MJN110 at 2.5 mg/kg significantly increased the 2-AG levels in all the regions examined (Figure 10A, 10D, 10G).

Interestingly, the 2-AG levels in the TBI animals were significantly reduced compared to the sham control in TG and TNC (Figure 10A and 10D). The 2-AG levels in PAG seemed to be also reduced, despite the significant difference was not observed (Figure 10G). Notably, the 2-AG levels were dramatically reduced in the DO34 treated animals in all these regions. Since AA and its metabolites prostaglandins can induce pain, we also measured the levels of AA in these regions. We found a more than 5-fold

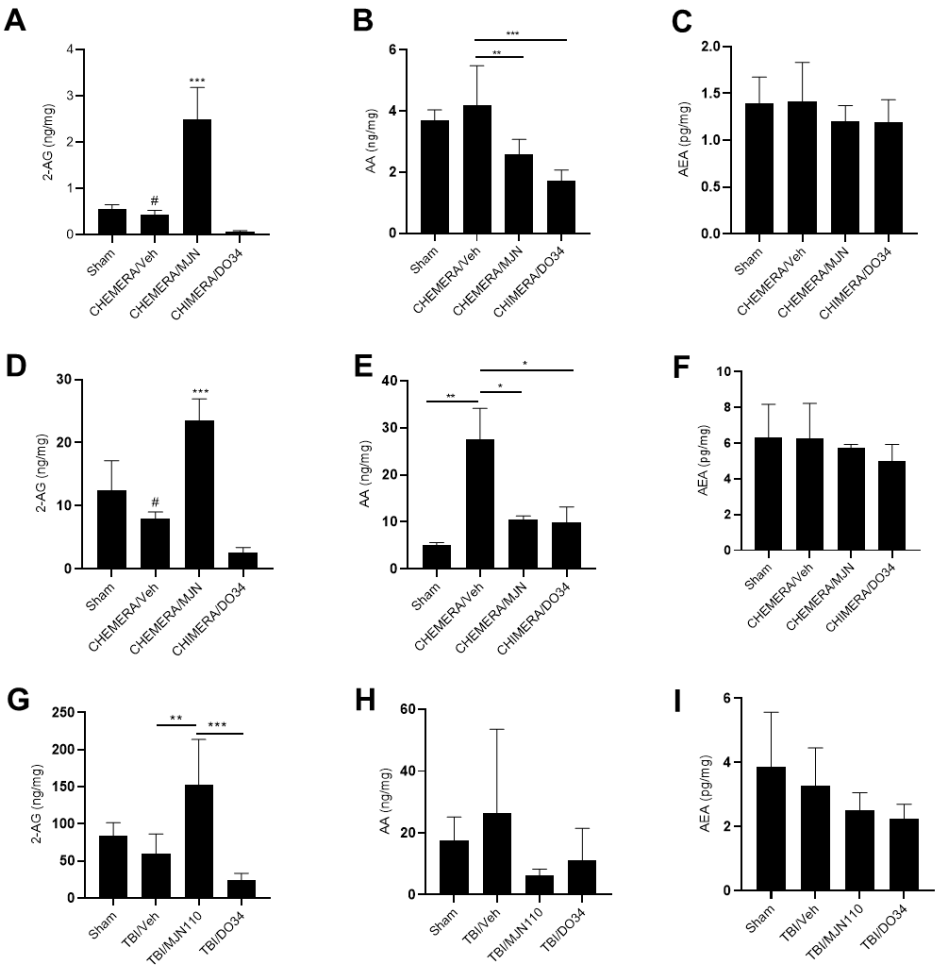

Figure 10. Treatment with MJN110 and DO34 on the 2-AG, AEA and AA levels in TG (A-C), TNC (D-F) and PAG (G-I) of TBI mice. #p<0.05 compared to sham and TBI/DO34 groups; ***p<0.001 compared to all the other groups in A and D; *p<0.05, **p<0.01 and ***p<0.001 in B, E and G (n=6/group).

increase in the levels of AA in the TNC, which was significantly reduced by MJN treatment (Figure 10E). Although there was no difference in the AA levels between the sham and TBI animals in the TG region, the AA level was significantly reduced by MJN treatment (Figure 10D). The AA levels in the PAG region were no differences in all the treatment groups possibly due to the large variations (Figure 11H). In addition to 2-AG and AA, we also measured the endocannabinoid AEA contents in those regions. No difference was found among all the animal groups (Figure 10C, 10F and 10I). These results indicated that MJN110 and DO34 selectively affected the 2-AG, but not the AEA metabolism.

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

Nothing to Report.

How were the results disseminated to communities of interest?

Some results have been presented in the Gordon Research Conference and the relevant work has been published in the peer reviewed journals.

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

1) We will determine whether our CHIMERA-induced TBI mouse model is vulnerable to bright-light stress induced pain sensitivity and examine the role of MJN110 treatment on mechanical allodynia in both periorbital and hind paw regions.

2) Our preliminary results showed that treatment with the substrate-selective COX-2 inhibitor (SSCI) LM-4131 suppressed inflammatory response in the TG and TNC and reduced periorbital allodynia at 7 and 14 days post-TBI. In the following up study, we will further characterize the therapeutic effect of LM-4131 in this repetitive TBI mouse model and determine whether the suppressive effect of LM-4131 on PTH is mediated by cannabinoid receptor dependent mechanisms.

3) To investigate the role of boosting endocannabinoid AEA in the treatment of PTH in this TBI model system.

4) To determine the molecular changes in the TG and TNC regions at different time points post-TBI using RNAseq and Mass Spectrometry.

4. IMPACT:

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

The studies from our group and others have previously shown that augmentation of endogenous levels of 2-AG by inhibition of its hydrolysis suppressed neuroinflammation, maintained synaptic neurotransmission and restored motor performance, learning and memory function in the animal models of traumatic brain injury (TBI). In this study, we found that inhibition of the 2-AG hydrolytic enzyme MAGL with MJN110 alleviated PTH associated with repetitive mTBI. The therapeutic effect of MJN110 is likely due to its inhibition of glial activation, CGRP production and the arachidonic acid metabolism. Activation of CB1 and CB2 receptors might be attributable to the protective effect of MJN110. Our results suggest that inhibitors of 2-AG hydrolysis can be used as a therapeutic strategy for the treatment of PTH.

What was the impact on other disciplines?
Nothing to report.

What was the impact on technology transfer?
Nothing to report.

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

5. CHANGES/PROBLEMS:

Although we did not experience major problems, the small amount of tissues available in TG, TNC and other brain regions seem to be a limiting factor to perform multiple assays. Therefore, more animals might be required for detailed analysis.

Actual or anticipated problems or delays and action or plans to resolve them
Nothing to report.

Changes that had a significant impact on expenditures
Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report.

Significant changes in use or care of human subjects
Nothing to report.

Significant changes in use of biohazards and/or select agents
Nothing to report.

6. PRODUCTS:

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

1. Langlois L, Selvaraj P, Simmons S, Gouty S, Zhang Y,* and Nugent F*. Repetitive mild traumatic brain injury induces persistent alterations in spontaneous synaptic activity of hippocampal CA1 pyramidal neurons. IBRO Neuroscience Reports 2022; 12, 157-162. *Co-corresponding author.
2. Selvaraj P, Tanaka M, Wen J, and Zhang Y. The Novel Monoacylglycerol Lipase Inhibitor MJN110 Suppresses Neuroinflammation, Normalizes Synaptic Composition and Improves Behavioral Performance in the Repetitive Traumatic Brain Injury Mouse Model. Cells 2021; 10, 3454.
3. Wen J, Tanaka M, and Zhang Y. Therapeutic effect of inhibition of the 2-AG hydrolytic enzyme monoacylglycerol lipase on posttraumatic headache in CHIMERA induced mTBI mouse model. Cannabinoid Function in the Central Nervous System Gordon Research Conference (GRC), Nov. 7-12, Ventura, CA.

Journal Publications
Nothing to report.

Books or other non-periodical, one-time publications
Nothing to report.

Other publications, conference papers, and presentations
Nothing to report.

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

Technologies or techniques
Nothing to report.

Inventions, patents applications, and/or licenses
Nothing to report.

Other products
Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name	Yumin Zhang
Project Role	PI
Nearest person month worked	3.6
Contribution to Project	Oversees entire project
Funding Support	Full time federal employee

Name	Joseph McCabe
Project Role	Co-Investigator
Nearest person month worked	1.2
Contribution to Project	Assisting in TBI modeling and behavioral experiments
Funding Support	Full time federal employee

Name	Irwin Lucki
Project Role	Co-Investigator
Nearest person month worked	.60
Contribution to Project	Oversees rat behavioral core at USUHS
Funding Support	Full time federal employee

Name	Sean Moran
Project Role	Collaborator
Nearest person month worked	.60
Contribution to Project	Assist in measurements within mouse brain tissues
Funding Support	Full time federal employee

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

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

8. SPECIAL REPORTING REQUIREMENTS:

QUAD CHARTS: See attached

9. APPENDICES:

See attached