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CONTRACTING ORGANIZATION: University of Texas MD Anderson Cancer Center

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13. SUPPLEMENTARY NOTES

The lack of effective treatments reflects the fact that the mechanisms underlying neuropathic pain are incompletely understood. Therefore, gaining further insight into the mechanisms that maintain neuropathic pain is expected to reveal new therapeutic targets for disease-modifying treatment. Studies have shown that astrocytes, immune-like cells in the spinal cord and brain, contribute to the maintenance of neuropathic pain by releasing signals that activate neurons in pain pathways. However, it is not known how astrocytes remain activated during neuropathic pain. We have new evidence to suggest that antibodies are generated against cell components that are released by damaged neurons after peripheral nerve injury. In turn, the antibodies signal at receptors expressed by astrocytes. Using a rat model of neuropathic pain that mimics nerve injury to limbs (e.g., due to blasts or high velocity projectiles), this proposal aims to characterize the antibodies that are generated after peripheral nerve injury. It also aims to identify the signaling pathways that are engaged in astrocytes upon recognition of the antibodies, and to learn how they maintain pain signaling at neurons in pain pathways.

15. SUBJECT TERMS:
NONE LISTED

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1. INTRODUCTION

Nearly half of all Service members sustain a traumatic injury to the nervous system during combat, which can devolve into a type of chronic pain known as neuropathic pain. Neuropathic pain is exceedingly difficult to treat as the available drugs are only minimally effective. Recent studies have shown that astrocytes, immune-like glial cells in the spinal cord and brain, contribute to the maintenance of neuropathic pain when activated by releasing signals that promote neuronal activity in pain pathways. However, it is not known how astrocytes remain activated during neuropathic pain. We have new evidence to suggest that antibodies are generated against cell components that are released by damaged neurons after peripheral nerve injury. In turn, the antibodies signal at receptors expressed by astrocytes in the spinal cord. Using rodent models of neuropathic pain that mimics nerve injury to limbs (e.g., due to blasts or high velocity projectiles), this proposal aims to characterize the antibodies that are generated after peripheral nerve injury. It also aims to identify the signaling pathways that are engaged in astrocytes upon recognition of the antibodies, and to learn how they maintain pain signaling at neurons in pain pathways. Preliminary evidence suggests that sustained mediator production by astrocytes is facilitated by activation of FcgammaR subtype IIa (FcγRIIa) via autoimmune complexes comprising immunoglobulin G (IgG) autoantibodies and autoantigens, resulting in production of pronociceptive gliotransmitters and inflammatory mediators. Aim 1 seeks to identify the autoimmune complexes that contribute to neuropathic pain via FcγRIIa activation. Aim 2 is designed to identify the short-onset FcγRIIa signaling pathway that contributes to neuropathic pain activation. Aim 3 is to identify the long-onset FcγIIa signaling mechanisms that contribute to neuropathic pain activation. The short-term impact of these activities will be to add to our scientific understanding of how astrocytes remain activated and therefore maintain neuropathic pain after peripheral nerve injury — a current knowledge gap. The long-term impact is that the identified signaling axis is expected to offer new, non-opioid pharmacological targets for neuropathic pain.

2. KEYWORDS

FcgammaR, neuropathic pain, astrocyte, spinal cord, immunoglobulin G, autoantibodies, neuroinflammation, gliotransmitter, Syk, P2X7R, NFkappaB

3. ACCOMPLISHMENTS

What were the major goals of the project?

Compliance-Specific Tasks:

Task: Ethics approval for animal work	**Months**	**MDACC**	**UCSD**
Subtask 1: Obtain approval from Institutional Animal Care and Use Committee (IACUC), and ACURO	1-4	Dr. Grace	
Milestone Achieved: Animal protocols are approved	4		

Research-Specific Tasks:

Specific Aim 1: Identify autoimmune complexes that contribute to neuropathic pain via FcγRIIa activation			
Subtask 1: Identify and quantify the temporal profile of autoantigens, autoantibodies, and their cellular source in the spinal column and draining tissues after peripheral nerve injury.	4-9	Dr. Grace	Dr. Svensson
Milestone Achieved: Identification of auto-antigens and auto-antibodies generated after peripheral nerve injury (Objective 1).	9		
Subtask 2: Purify autoantigens and autoantibodies from donor rodents and create deglycosylated autoantibodies and Fab fragments.	9-20	Dr. Grace	Dr. Svensson
Subtask 3: Test the extent to which autoimmune complexes activate cells from the central nervous system *in vitro* in an FcγR-dependent manner.	20-23	Dr. Grace	

Milestone Achieved: Identify the extent to which identified autoantigen-antibody immune complexes signal via FcγRIIa in vitro (Objective 2).	23		
Subtask 4: Test the extent to which autoimmune complexes induce FcγR-dependent nociceptive hypersensitivity *in vivo*.	20-24	Dr. Grace	
Subtask 5: Test the extent to which antibody generation is necessary for the initiation and maintenance of neuropathic pain.	4-9	Dr. Grace	
Milestone Achieved: Identify the extent to which pain is dependent on autoantigen-antibody immune complexes in vivo (Objective 3).	24		
Specific Aim 2. Identify the short-onset FcγRIIa signaling pathway that contributes to neuropathic pain activation			
Subtask 1: Test the extent to which FcγRIIa activation induces release of gliotransmitters via a Syk → P2X7 → $[Ca^{2+}]_i$ pathway *in vitro.*	4-8	Dr. Grace	
Milestone Achieved: Identify the short-onset FcγRIIa signaling pathway in vitro and in vivo (Objective 4).	8		
Subtask 2: Test the extent to which Syk is phosphorylated and associates with FcγRIIa *in vitro*.	9-13	Dr. Grace	
Subtask 3: Test the extent to which Syk is phosphorylated over time after CCI.	10-14	Dr. Grace	
Subtask 4: Test the extent to which Syk activation after CCI is dependent on FcγRIIa.	15-18	Dr. Grace	
Milestone Achieved: Identify the physical association between FcγRIIa and Syk in vitro and in vivo (Objective 5).	18		
Subtask 5: Test the extent to which induction of gliotransmitters via the FcγRIIa → Syk → P2X7 → $[Ca^{2+}]_i$ pathway contributes to nociceptive hypersensitivity.	25-31	Dr. Grace	
Milestone Achieved: Test the extent to which the short-onset FcγRIIa signaling pathway contributes to neuropathic pain in vivo (Objective 6).	26		
Specific Aim 3. Identify the long-onset FcγRIIa signaling mechanisms that contribute to neuropathic pain.			
Subtask 1: Test the extent to which FcγRIIa activation induces release of proinflammatory mediators via a FcγRIIa → Syk → NFκB pathway *in vitro.*	14-18	Dr. Grace	
Milestone Achieved: Identify the long-duration FcγRIIa signaling pathway in vitro (Objective 7).	18		
Subtask 2: Test the extent to which CCI induces release of proinflammatory mediators from astrocytes via a FcγRIIa → Syk → NFκB pathway *in vivo.*	24-30	Dr. Grace	
Milestone(s) Achieved: Identify the long-duration FcγRIIa signaling pathway in vivo (Objective 8).	30		
Subtask 3: Test the extent to which activation of astrocytes via a FcγRIIa → Syk → NFκB pathway is sufficient for pain.	30-33	Dr. Grace	
Subtask 4: Test the extent to activation of FcγRIIa → Syk → NFκB pathway is necessary for CCI pain.	32-36	Dr. Grace	
Milestone(s) Achieved: Test the extent to which the long-duration FcγRIIa signaling pathway contributes to neuropathic pain in vivo (Objective 9).	36		

What was accomplished under these goals?

Task: Ethics approval for animal work
Subtask 1: Obtain approval from Institutional Animal Care and Use Committee (IACUC), and ACURO
Status: Completed

IACUC and ACURO approval of protocols related to all proposed experiments were obtained.

Specific Aim 1: Identify autoimmune complexes that contribute to neuropathic pain via FcγRIIa activation
Subtask 1: Identify and quantify the temporal profile of autoantigens, autoantibodies, and their cellular source in the spinal column and draining tissues after peripheral nerve injury.
Status: In progress

The aim of this subtask is to measure how autoantigens, autoantibodies, and autoantibody receptor expression at the dorsal root ganglia (DRG), spinal cord, and lymph nodes change over time following peripheral nerve injury. Toward the completion of this subtask, 42 male and female rats underwent unilateral chronic constriction injury (CCI), a model of neuropathic pain in which mechanical allodynia develops over the course of days and remains heightened for several weeks before resolving to baseline nociceptive sensitivity by day 70. As seen in the schematic of the experimental timeline presented in **Figure 1A**, rats that received CCI were perfused at multiple timepoints after injury: day 3, day 7, day 10, day 14, day 21, day 35, and day 70 (n = 6 per timepoint). Eight naïve rats were used as controls (i.e. day 0). After euthanasia and perfusion with ice-cold saline, the following tissues were collected: spinal cord dorsal horn lumbar 4-5 (L4-5) ipsilateral and contralateral to CCI; spinal cord dorsal horn cervical 3-4 (C3-4) ipsilateral to CCI; and DRG L4-5 ipsilateral and contralateral to CCI (**Figure 1B**).

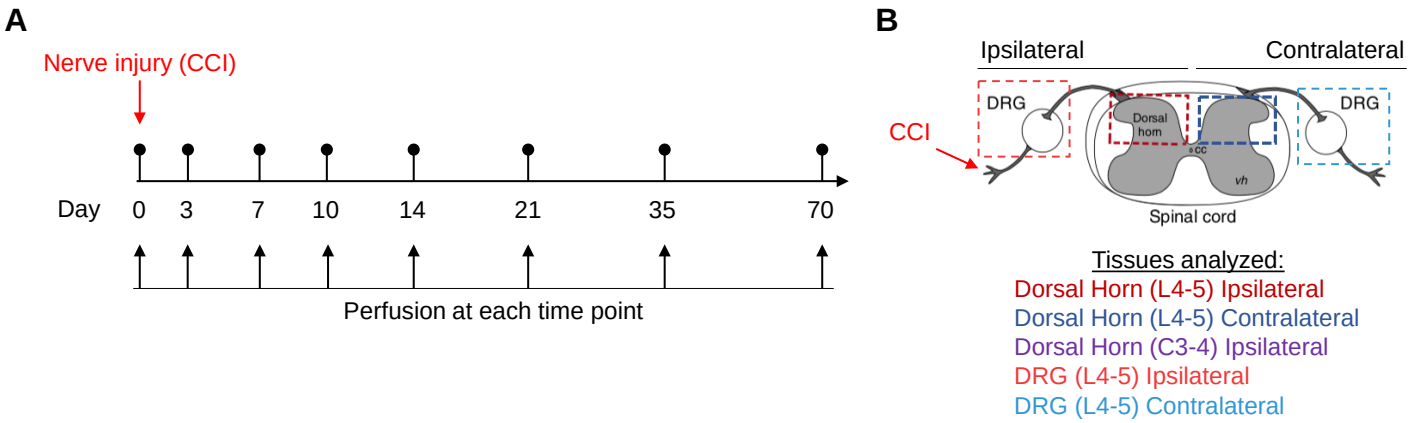

Figure 1: Experimental design. **A.** Subjects were adult male and female Sprague-Dawley rats (10 weeks old upon arrival). Under isoflurane anesthesia, rats received unilateral chronic constriction injury (CCI) of the sciatic nerve (four 4-0 chromic gut sutures). As a comparison for baseline expression, naïve rats were used as controls (i.e. day 0). n = 8 (5 males and 3 females) for day 0; n = 6 (3 males and 3 females) for all other timepoints. **B.** Diagram of tissues collected. DRG and lumbar dorsal horns were isolated ipsilateral and contralateral to nerve injury. Ipsilateral cervical dorsal horn was also collected to evaluate transcriptional changes at a site distal from injury. Image adapted from Häring et al., 2018, *Nature Neuroscience,* PMID: 29686262.

One objective of this subtask is to quantify gene expression of FcγRIIa in these tissues. Based on the preliminary data gathered for this proposal suggesting that FcγRIIa expression on spinal astrocytes contributes to neuropathic pain, we hypothesized that FcγRIIa expression would increase in the spinal cord after CCI. To address this hypothesis, we extracted RNA from tissues using the TRIzol phenol-chloroform method, reverse transcribed cDNA from purified RNA (Bio-Rad iScript gDNA Clear cDNA Synthesis kit), and used this cDNA to perform real-time quantitative PCR. As seen in **Figure 2**, we report that *Fcgr2a* transcription increases selectively in ipsilateral dorsal horn L4-5 on days 10 and 14 after injury, at a time

when nociceptive sensitization following CCI reaches maximum expression. There is no change in *Fcgr2a* compared to naïve controls in contralateral dorsal horn at each measured timepoint, nor is any change in *Fcgr2a* observed in ipsilateral dorsal horn C3-4 at a site distal from injury. We also did not observe any changes in *Fcgr2a* expression in ipsilateral or contralateral DRG L4-5 following CCI. We further confirmed that markers for astrocyte activation (*Gfap*) (**Figure 3**) and microglial activation (*Cd11b*, also known as *Itgam*) (**Figure 4**) were elevated in the ipsilateral lumbar dorsal horn after CCI. These data indicate that *Fcgr2a* transcription is elevated following nerve injury only in ipsilateral dorsal horn, suggesting that injury-induced changes in FcγRIIa is restricted to cells within the spinal cord. These results support the underlying hypothesis that astrocytes can contribute to pain following peripheral nerve injury through activation of FcγRIIa in ipsilateral spinal cord.

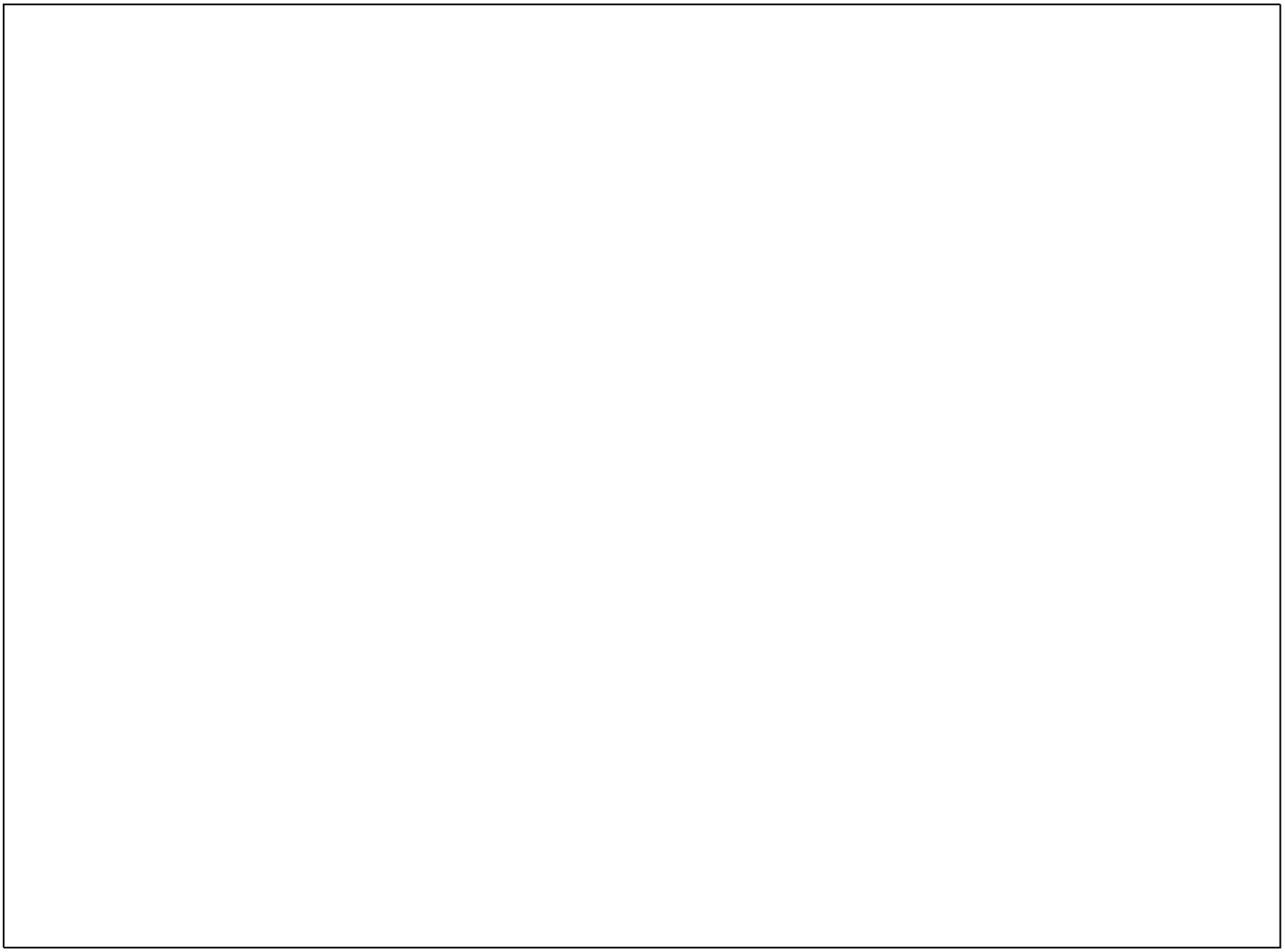

Figure 2: CCI nerve injury increases *Fcgr2a* in ipsilateral lumbar spinal dorsal horn at a time corresponding with maximal allodynia. **A.** *Fcgr2a* expression in dorsal horn L4-5 ipsilateral to CCI upregulates after injury, reaching peak expression on days 10 and 14 after injury, before resolving to baseline expression. *Fcgr2a* expression does not change after CCI in (**B**) contralateral dorsal horn L4-5, (**C**) ipsilateral dorsal horn C3-4, (**D**) ipsilateral DRG L4-5, and (**E**) contralateral DRG L4-5. Relative gene expression against the housekeeping gene *Gapdh* was determined using the $2^{-\Delta\Delta Ct}$ method. $n = 8$ (5 males and 3 females) for day 0; $n = 6$ (3 males and 3 females) for all other timepoints. All samples were included unless there was a failure of quality control (failure of sample to amplify or housekeeping gene amplification deviation ±2 SD from mean). Data are mean ± SEM, with bar charts below displaying individual sample data. Kruskal-Wallis and Dunn's post hoc tests; $*p < 0.05$; $**p < 0.01$ compared to day 0.

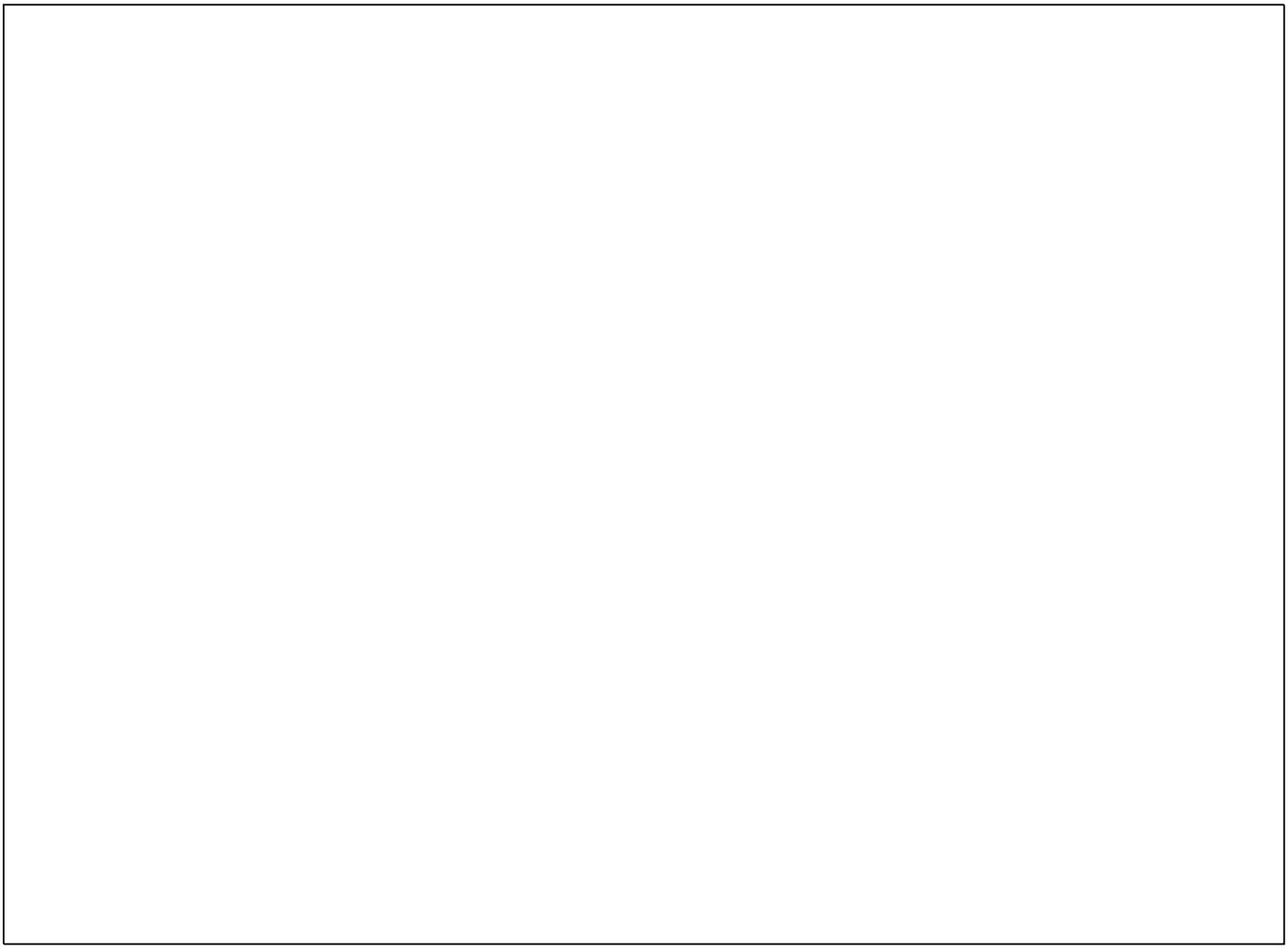

Figure 3: CCI nerve injury increases *Gfap* in ipsilateral lumbar spinal dorsal horn. *Gfap* expression was quantified in (**A**) the ipsilateral dorsal horn L4-5, (**B**) contralateral dorsal horn L4-5, (**C**) ipsilateral dorsal horn C3-4, (**D**) ipsilateral DRG L4-5, and (**E**) contralateral DRG L4-5. Relative gene expression against the housekeeping gene *Gapdh* was determined using the $2^{-\Delta\Delta Ct}$ method. $n = 8$ (5 males and 3 females) for day 0; $n = 6$ (3 males and 3 females) for all other timepoints. All samples were included unless there was a failure of quality control (failure of sample to amplify or housekeeping gene amplification ±2 SD from mean). Data are mean ± SEM, with bar charts below displaying individual sample data. Kruskal-Wallis and Dunn's post hoc tests; *$p < 0.05$; ***$p < 0.001$ compared to day 0.

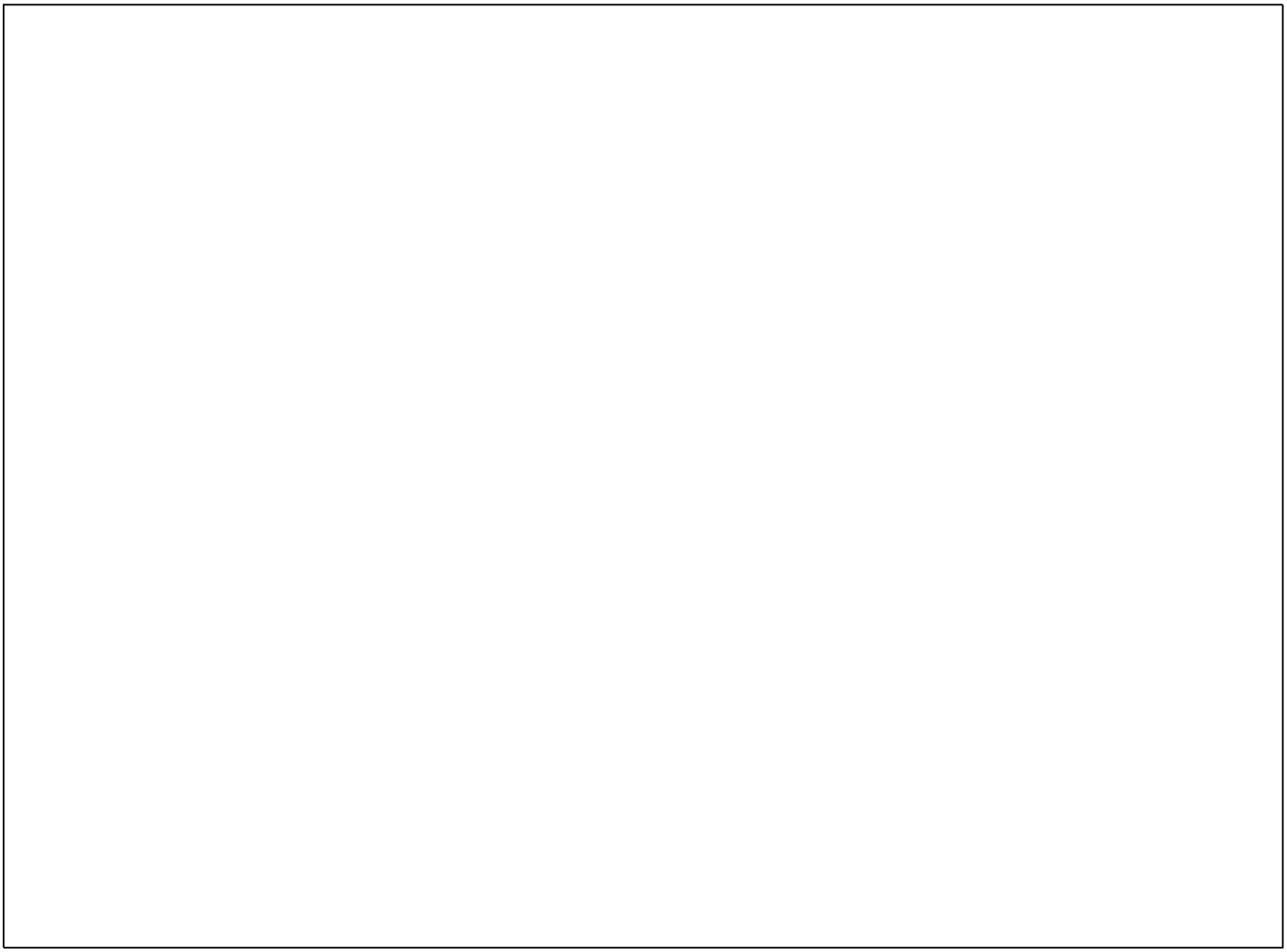

Figure 4: CCI nerve injury increases *Cd11b* in ipsilateral lumbar spinal dorsal horn. *Cd11b* expression was quantified in (**A**) the ipsilateral dorsal horn L4-5, (**B**) contralateral dorsal horn L4-5, (**C**) ipsilateral dorsal horn C3-4, (**D**) ipsilateral DRG L4-5, and (**E**) contralateral DRG L4-5. Relative gene expression against the housekeeping gene *Gapdh* was determined using the $2^{-\Delta\Delta Ct}$ method. $n = 8$ (5 males and 3 females) for day 0; $n = 6$ (3 males and 3 females) for all other timepoints. All samples were included unless there was a failure of quality control (failure of sample to amplify or housekeeping gene amplification ±2 SD from mean). Data are mean ± SEM, with bar charts below displaying individual sample data. Kruskal-Wallis and Dunn's post hoc tests; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ compared to day 0.

To validate and expand upon this dataset, we have performed a similar timecourse experiment in mice in which meninges and draining organs were also collected for processing. A total of 224 male and female C57BL/6J mice underwent CCI surgery and were perfused at multiple timepoints after injury: day 0 (naïve controls), day 1, day 3, day 7, day 10, day 14, day 28, and day 56 ($n = 28$ per timepoint, 14 males and 14 females). After euthanasia and perfusion with ice-cold saline, the following tissues were collected: spinal cord dorsal horn lumbar 4-5 (L4-5) ipsilateral and contralateral to CCI; spinal cord dorsal horn cervical 3-4 (C3-4) ipsilateral to CCI; DRG L4-5 ipsilateral and contralateral to CCI; inguinal and axillary lymph nodes ipsilateral and contralateral to CCI; and spinal cord meninges at L4-5. Tissues from 6 to 8 mice per timepoint will be used to quantify transcriptional changes by PCR, measure IgG and B cell distribution in meninges in lymph nodes, and to quantify antibody and antigen protein levels in these tissues by Western blot. All tissues have been collected and are either stored at –80°C or have been post-fixed in 4% paraformaldehyde and cryopreserved in 30% sucrose at 4°C. These data are currently being collected and the analysis is in progress.

We hypothesize that post-translational modifications to proteins are a source of autoantibody generation following nerve injury. Peptidylarginine deiminase (PAD) enzymes convert arginine to citrulline amino acid residue (i.e. citrullination), and this post-translational modification can result in the generation of immunogenic epitopes. To investigate if protein citrullination occurs in the spinal cord following peripheral nerve injury, we performed sham or CCI surgery on male and female rats and extracted ipsilateral dorsal horn L4-5 14 days after injury. Tissue samples were sent to collaborators at the Mass Spectrometry Proteomics Core at Baylor College of Medicine to perform post-translational modification analysis of the citrullination proteome by liquid chromatography–mass spectrometry. The data from these samples has been collected and the analysis is currently ongoing.

Subtask 4: Test the extent to which autoimmune complexes induce FcγR-dependent nociceptive hypersensitivity *in vivo*.
Status: In progress

The objective of this subtask is to determine if autoimmune complexes signaling at FcγRIIa expressed by spinal cord astrocytes is causal to the development of nociceptive hypersensitivity. We sought to develop a method for selective inhibition of FcγRIIa expression in spinal astrocytes to demonstrate a causal role for astrocytic FcγRIIa in nociceptive hypersensitivity. To achieve cell type-specific inhibition of FcγRIIa restricted entirely to spinal cord astrocytes, we leveraged CRISPR-Cas9 gene editing to generate adeno-associated virus (AAV) serotype 5 expressing *Staphylococcus aureus* Cas9 and single guide RNA (sgRNA) targeting *Fcgr2a* under the promotion of GFAP (*Cas9-Fcgr2a*). Transfection of spinal cord tissue following intrathecal injection of *Cas9-Fcgr2a* AAV can therefore selectively silence *Fcgr2a* in $GFAP^+$ spinal astrocytes.

We first sought to test if FcγRIIa in spinal astrocytes is necessary for the development of nociceptive hypersensitivity following nerve injury. The experimental timeline can be seen in **Figure 5A.** Rats received intrathecal injection of AAV and were given 14 days to allow for virus to express. CCI was performed as described above. Von Frey testing of the ipsilateral hindpaw was performed at baseline before CCI surgery, and on days 3, 7, 10, and 14. As seen in **Figure 5B**, we find that intrathecal treatment with *Cas9-Fcgr2a* prevents the development of mechanical allodynia. These results indicate that FcγRIIa signaling from spinal cord astrocytes are likely causal in the development of nociceptive hypersensitivity following nerve injury.

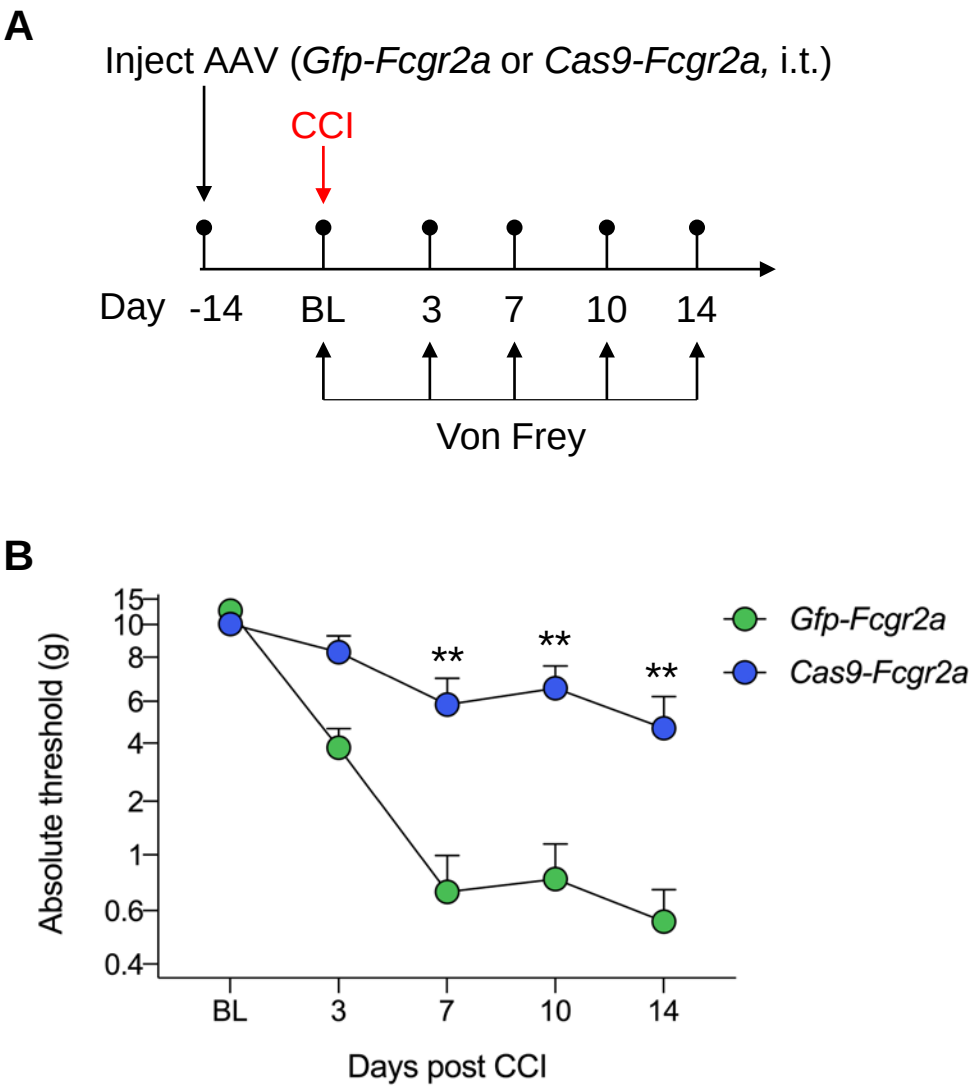

Figure 5. CRISPR-Cas9 gene knockout of *Fcgr2a* from spinal cord astrocytes prevents the development of mechanical allodynia following nerve injury. **A.** Timeline and design of the experiment. Rats (Sprague-Dawley males, 10 weeks old upon arrival) received intrathecal (i.t.) injections of adeno-associated virus (AAV) 14 days before CCI surgery. To knockout *Fcgr2a* selectively from GFAP^{+} astrocytes, AAV5 constructs contained saCas9 for gene editing and sgRNA targeting *Fcgr2a* (*Cas9-Fcgr2a*) under the promotion of *Gfap*. Control virus contained green fluorescent protein (*Gfp*) and *Fcgr2a* sgRNA (*Gfp-Fcgr2a*) under the promotion of *Gfap*. Rat received viral injections (i.t.) by polyethylene catheter (10 µL, 5x10$^{\wedge 9}$ - 5x10$^{\wedge 10}$ Vg). After 14 days, rats underwent CCI surgery. **B.** Withdrawal thresholds to mechanical stimulation of the ipsilateral hindpaw were measured by von Frey filaments at baseline (BL) and on days 3, 7, 10, and 14 after CCI. n = 7 (*Gfp-Fcgr2a* control group); n = 9 (*Cas9-Fcgr2a* group). Data are mean ± SEM. Two-way ANOVA and Sidak's post hoc test; **$p < 0.01$.

Subtask 5: Test the extent to which antibody generation is necessary for the initiation and maintenance of neuropathic pain.
Status: In progress

The aim of this subtask is to determine if the production of antibodies are necessary for the initiation and maintenance of neuropathic pain. Mature B cells are essential for the production and release of antibodies, so we sought a pharmacological method to broadly disrupt the release of antibodies at the spinal cord. B-cell lymphoma 6 (BCL6) is a transcriptional master regulator for germinal center immune cells, including B cells, T follicular helper cells, and T follicular regulatory cells, and a small molecule inhibitor of BCL6

(compound 79-6) can prevent the production of antibodies in mice. Therefore, we delivered a BCL6 inhibitor (i.t.) or vehicle to male mice once every two days for 14 days (**Figure 6A**). On the day of the first drug treatment, mice underwent CCI or sham control surgery. Von Frey testing of the ipsilateral hindpaw was performed at baseline before CCI surgery, and on days 3, 7, 10, and 14. As seen in **Figure 6B**, we find that CCI-treated mice with vehicle treatment develop mechanical allodynia to the von Frey filaments, while spinal delivery of BCL6 inhibitor prevents the development of nociceptive hypersensitivity. These data support the premise that antibody production and release in the spinal cord is necessary in the development of neuropathic pain.

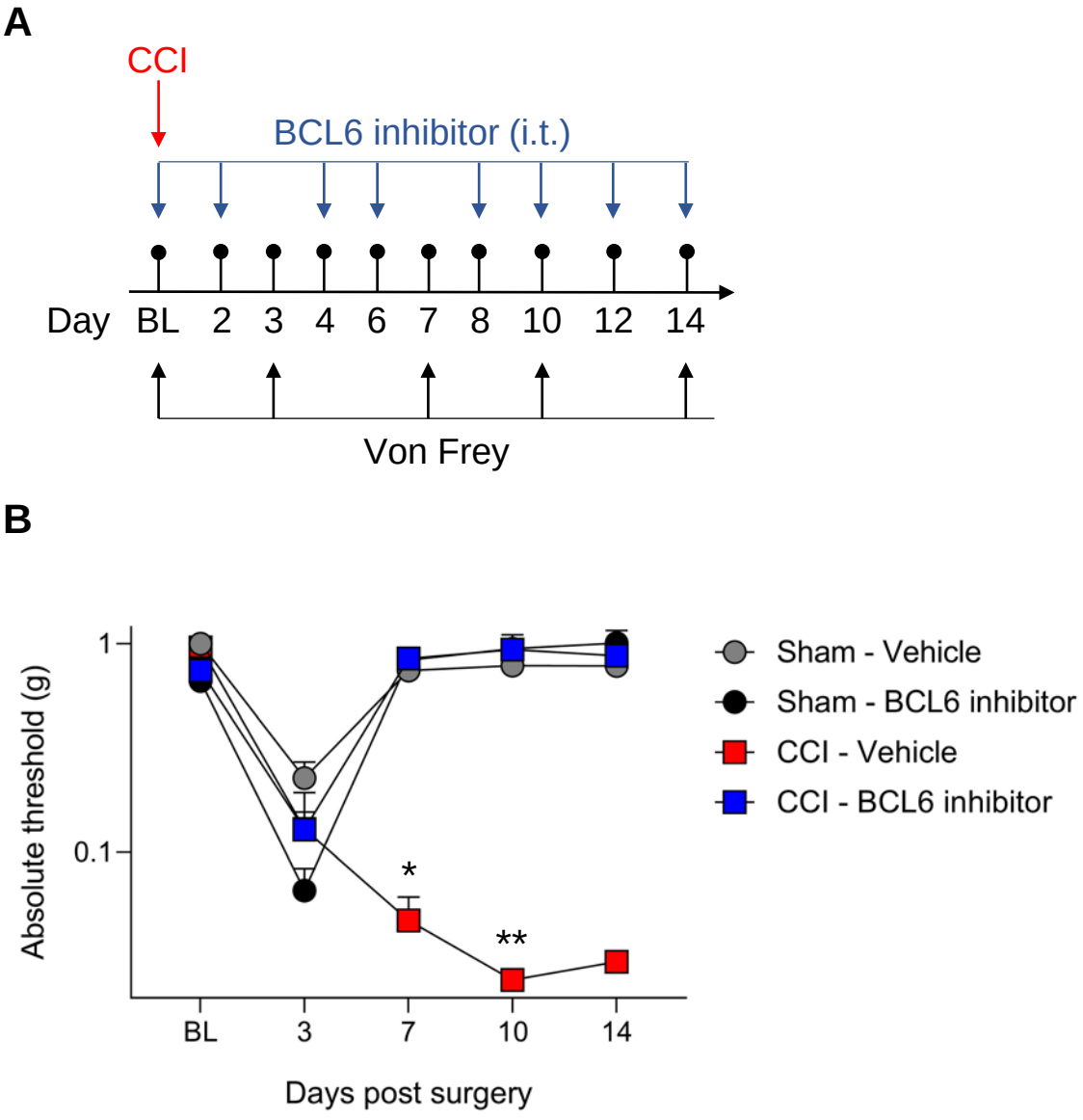

Figure 6. Inhibiting antibody production by BCL6 inhibitor prevents the development of neuropathic pain. **A.** Timeline and design of the experiment. Mice (C57BL/6J males, 10 weeks old upon arrival) received intrathecal (i.t.) injections of vehicle or BCL6 inhibitor once every two days. On the day of initial treatment, mice received sham or CCI surgery. **B.** Withdrawal thresholds to mechanical stimulation of the ipsilateral hindpaw were measured by von Frey filaments at baseline (BL) and on days 3, 7, 10, and 14 after CCI. $n = 3$ (Sham - Vehicle); $n = 3$ (Sham – BCL6 inhibitor); $n = 6$ (CCI - Vehicle); $n = 6$ (CCI – BCL6 inhibitor). Data are mean ± SEM. Two-way ANOVA and Dunnett's post hoc test; $^{*}p < 0.05$; $^{**}p < 0.01$ compared to Sham - Vehicle.

Specific Aim 3. Identify the long-onset FcγRIIa signaling mechanisms that contribute to neuropathic pain.

Subtask 1: Test the extent to which FcγRIIa activation induces release of proinflammatory mediators via a FcγRIIa → Syk → NFκB pathway *in vitro.*
Status: In progress

The aim of this subtask is to interrogate if FcγRIIa activation leads to proinflammatory cytokine and chemokine production from astrocytes through a FcγRIIa → Syk → NFκB signaling pathway. To address this, we have been purifying and culturing primary rat astrocytes through immunopanning to perform *in vitro* manipulations. We have completed experiments in which changes in proinflammatory cytokine and chemokine gene transcription were measured following activation of FcγRs by immunoglobulin G immune complex (IgG-IC). Postnatal rat astrocytes were purified by immunopanning and cultured in 96-well plates for 7 days. Cells were pre-treated with inhibitors for Syk (R406) or NFκB (PDTC, ammonium pyrrolidinedithiocarbamate) before application of IgG-IC (normal mouse IgG as antigen and affinity-purified rat anti-mouse IgG as antibody) to activate FcγRs. Cells were lysed and RNA was extracted after 4 hours, and PCR was performed as described above. As seen in **Figure 7**, IgG-IC application to astrocytes significantly increases transcription of proinflammatory cytokines and chemokines, including *Tnf*, *Il1b*, *Cxcl1*, and *Ccl2*. Pharmacological inhibition of Syk or NFκB blocked the increased transcription of *Tnf* and *Il1b* in response to IgG-IC. In contrast, both *Cxcl1* and *Ccl2* transcription was blocked by Syk inhibition but not NFκB. These results suggest that proinflammatory cytokines and chemokines induced by activation of FcγRIIa by IgG-IC results are downstream of Syk signaling, while the cytokines TNF and IL-1β require NFκB signaling in astrocytes.

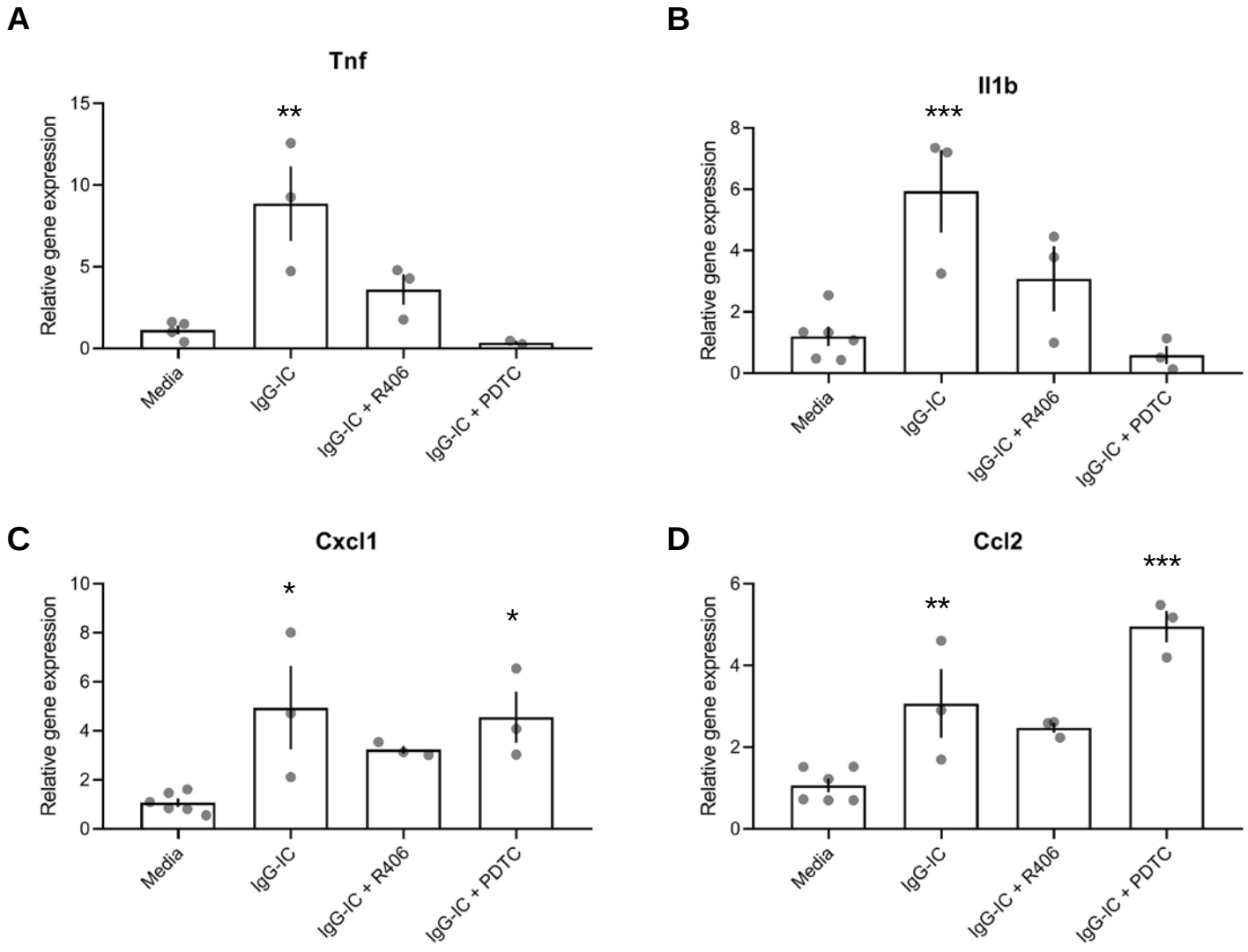

Figure 7. Proinflammatory cytokines and chemokine gene transcription in astrocytes following activation of FcγR by IgG-IC requires Syk, and TNF and IL-1β require NFκB. Primary rat astrocytes were purified by immunopanning. IgG-IC (30 μg/mL) was applied to wells which were pre-treated with media, Syk inhibitor R406 (0.3 μM), or NFκB inhibitor PDTC (30 μM). After 4 h RNA was extracted for real-time qPCR. $n = 3$ to 6 replicates. One-way ANOVA and Dunnett's post hoc test; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ compared to Media.

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

The research personnel listed on this project have had extensive training and professional development opportunities. These include the technical training the methods used in this project, like the von Frey test, CCI surgeries, intrathecal delivery of virus, plasmid construction, necropsy, PCR, and astrocyte immunopanning. Professional development opportunities include poster presentations at conferences (Centre for Nanoscale Biophotonics annual meeting), seminars (Macquarie University, Sydney, Australia) and oral presentations at departmental lab meetings.

How were the results disseminated to communities of interest?

The results from the experiments have been presented at an international conference hosted by the Centre for Nanoscale Biophotonics in Clare Valley outside of Adelaide, South Australia in October 2019. Results from these experiments were also presented at an invited seminar for the Department of Molecular Sciences at Macquarie University in Sydney, Australia, in October 2019.

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

Our goal is to complete the following subtasks that are currently in progress:

Specific Aim 1: Identify autoimmune complexes that contribute to neuropathic pain via FcγRIIa activation
Subtask 1: Identify and quantify the temporal profile of autoantigens, autoantibodies, and their cellular source in the spinal column and draining tissues after peripheral nerve injury.
Subtask 4: Test the extent to which autoimmune complexes induce FcγR-dependent nociceptive hypersensitivity *in vivo*.
Subtask 5: Test the extent to which antibody generation is necessary for the initiation and maintenance of neuropathic pain.
Specific Aim 3. Identify the long-onset FcγRIIa signaling mechanisms that contribute to neuropathic pain.
Subtask 1: Test the extent to which FcγRIIa activation induces release of proinflammatory mediators via a FcγRIIa → Syk → NFκB pathway *in vitro.*

We plan to complete additional experiments that include characterizing how autoimmune complexes activate cells from the central nervous system *in vitro* in an FcγR-dependent manner, by employing the CRISPR-Cas9 gene editing system to purified rodent cells. These experiments will work toward accomplishing goals outlined in Specific Aim 1, Subtask 3. Additional experiments toward the completion of Specific Aim 1 include determining if intrathecal immune complex delivery produces nociceptive hypersensitivity in an FcγR-dependent manner through conditional gene editing of astrocyte FcγRIIa. Furthermore, we plan to use more selective methods for B cell depletion (through delivery of a murine anti-CD20 monoclonal antibody) to determine if antibody generation from B cells is required for neuropathic pain behaviors following CCI.

4. IMPACT

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

Nothing to report.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS

Experimental work was halted due to the COVID-19 shutdown of research operations at MD Anderson between March 22 and June 15. Since June, we have been operating at 50% capacity through shiftwork, which is expected to continue through September. A research assistant also left the lab during the shutdown, and a new one was hired, starting in September. However, extensive training will be required. These hurdles have dramatically reduced our progress towards to the project goals. However, personnel working remotely have continued data analysis, experimental planning and preparation of a review article.

6. PRODUCTS

Journal publications

Lacagnina MJ, Heijnen CJ, Watkins LR, Grace PM. Autoimmune mechanisms of chronic pain. (invited review for PAIN REPORTS in preparation).

Other publications, conference papers, and presentations.

Presentations at invited seminars and conferences

Lacagnina MJ. (2019). Astrocyte Fc gamma receptor signaling in spinal cord as a novel mechanism for neuropathic pain. Centre for Nanoscale BioPhotonics Annual Conference, Adelaide, Australia.

Lacagnina MJ. (2019). Astrocyte Fc gamma receptor signaling in spinal cord as a novel mechanism for neuropathic pain. Invited Seminar, Department of Molecular Sciences, Macquarie University, Sydney, Australia.

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

Nothing to report.

Other products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Peter Grace
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	orcid.org/0000-0002-8999-1220
Nearest person month worked:	3.6 months
Contribution to Project:	Dr. Grace was responsible for overall project management, oversight of all experimental designs and execution, and presentation of the results at national forums. Dr. Grace also trained personnel on experimental procedures.

Name:	Michael Lacagnina
Project Role:	Postdoctoral Researcher
Researcher Identifier (e.g. ORCID ID):	orcid.org/0000-0003-2468-2467
Nearest person month worked:	9.6
Contribution to Project:	Dr. Lacagnina performed experiments, including drug preparations, injections, animal surgeries, behavioral assessments, tissue collection, cell culturing, immunohistochemistry, RNA and protein assays, data collection and analysis. Managerial responsibilities include procuring materials required for experiments, training personnel, and overseeing work of research assistants.

Name:	Andrew Alfaro
Project Role:	Research Assistant I
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	9.6
Contribution to Project:	Mr. Alfaro performed experiments, including drug preparations, injections, animal surgeries, behavioral assessments, tissue collection, and RNA and protein assays.

Name:	Sabina Lorca
Project Role:	Research Assistant II

Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	3
Contribution to Project:	Ms. Lorca performed experiments, including drug preparations, injections, animal surgeries, and tissue collection. Managerial responsibilities included procuring materials required for experiments.

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

Yes, see Support document, attached.

What other organizations were involved as partners?
No

8. SPECIAL REPORTING REQUIREMENTS

Collaborative Awards:
N/A