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Award Number: W81XWH-11-1-0501

TITLE: JaK/STAT Inhibition to Prevent Post-Traumatic Epileptogenesis

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REPORT DATE: July 2013

TYPE OF REPORT: Annual

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

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REPORT DOCUMENTATION PAGE			<i>Form Approved</i> <i>OMB No. 0704-0188</i>		
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1. REPORT DATE 31 Jul 2013		2. REPORT TYPE Annual		3. DATES COVERED 1 Jul 2012 - 30 Jun 2013	
4. TITLE AND SUBTITLE JaK/STAT Inhibition to Prevent Post-Traumatic Epileptogenesis				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-11-1-0501	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dr. Amy Brooks – Kayal Dr. Bret Smith E-Mail Amy.Brooks-Kayal@childrenscolorado.org / bnsmit4@email.uky.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS University of Colorado Denver Aurora, CO 80045				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY U.S. Army Medical Research and Materiel 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					
Our proposal tests the hypothesis that JaK/STAT pathway activation after TBI leads to <i>Gabra1</i> repression and is a critical mediator of post-traumatic epileptogenesis and epilepsy progression, and that inhibition of this pathway at the time of TBI and/or after development of post-traumatic epilepsy will inhibit epilepsy development and/or progression after CCI. In the first year of funding, equipment and training necessary to perform the CCI model was obtained at University of Colorado, and CCI was successfully performed in mice at both institutions. Training was completed for all personnel on all necessary molecular, anatomical, electrophysiological and neurophysiological (EEG) techniques, essential baseline data was obtained in the mouse CCI model, and specific outcome measures were established. Moreover, issues with the JAK/STAT3 inhibitor (WP1066) have been mitigated and the drug is now successfully used in all labs. Additional optimization of the WP1066 dosing protocol is underway and is expected to be completed very soon. Two manuscripts and three abstracts related to GABA(A) receptor, JAK/STAT pathway and cell death alterations following TBI were published during the funding period.					
15. SUBJECT TERMS CCI- controlled cortical impact; TBI- traumatic brain injury; <i>Gabra1</i> - GABA(A) Receptor subunit gene; JaK/STAT- Janus Kinase/Signal Transducer and Activator of Transcription					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			USAMRMC
			UU	26	19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION:

Our overall hypothesis is that JaK/STAT pathway activation after TBI leads to GABA(A) receptor $\alpha 1$ subunit gene (*Gabra1*) repression and is a critical mediator of post-traumatic epileptogenesis and epilepsy progression. The JaK/STAT pathway has not been studied in post-traumatic epilepsy, but beyond its role in *Gabra1* regulation, it is known to be an important regulator of neuronal proliferation, survival and gliogenesis, all of which may be important contributors to epileptogenesis. The Specific Aims of our study are:

1. Determine whether activation of the JaK/STAT pathway and downregulation of GABA(A) Receptor $\alpha 1$ subunit gene (*Gabra1*) transcription occur following traumatic brain injury (TBI) and subsequent epileptogenesis. We hypothesize that activation of the JaK/STAT pathway, downregulation of *Gabra1* and subsequent reduction of $\alpha 1$ subunit levels occur in injured hippocampus and cortex following TBI and contribute to posttraumatic epileptogenesis. To test this hypothesis, we will examine activation of the JaK/STAT pathway, and levels of ICER and GABAAR subunits acutely and chronically in the controlled cortical impact (CCI) model in mice before and after development of spontaneous seizures.

2. Determine whether activation of the JaK/STAT pathway and downregulation of *Gabra1* transcription following TBI result in altered inhibitory synaptic neurotransmission in the hippocampus that may contribute to epileptogenesis. We hypothesize that after CCI inhibitory neurotransmission in the dentate gyrus will be altered in a fashion consistent with reduced GABAAR $\alpha 1$ subunit surface expression in association with JaK/STAT pathway activation and *Gabra1* downregulation. To test this hypothesis, we will examine GABAAR-mediated currents in dentate gyrus granule cells using whole-cell patch-clamp recordings in acute hippocampal slices from injured and uninjured mice.

3. Determine whether animals can be rescued from post-traumatic epilepsy development and/or progression via blockade of JaK/STAT pathway activation acutely after controlled cortical impact or chronically after onset of spontaneous seizures. We hypothesize that blockade of the JaK/STAT pathway will inhibit epilepsy development and/or progression after CCI. To address this hypothesis, we will inhibit STAT3 phosphorylation after CCI, either pharmacologically or using virally delivered short hairpin RNA (shRNAs) against JaK2 or STAT3, then use video electroencephalogram (EEG) monitoring to determine whether this treatment prevents or delays epilepsy development and/or progression.

Results of these studies will provide new information regarding the role of the JaK/STAT signaling cascade in regulation of brain inhibition and epileptogenesis after traumatic brain injury, and have the promise of leading to new therapies for the prevention or treatment of post-traumatic epilepsy.

BODY:

Aim I: Performed in laboratory of Dr. Amy Brooks-Kayal at University of Colorado

Task 1: Determine whether activation of the JaK/STAT pathway occurs following traumatic brain injury (TBI) and subsequent epileptogenesis. (Timeframe months 1-12)

1a. Induce TBI using the CCI model in adult CD-1 mice (200 mice- assuming that 25-30% loss due to death or suboptimal injury; Time frame months 1-10).

Status: In progress. CCI has been completed on approximately 320 mice.

1. Establishment of Controlled Cortical Impact (CCI) model as an experimental model of TBI at the University of Colorado, Denver UCD) has included:

- a. Purchase, installation and calibration of CCI apparatus
- b. Testing of CCI apparatus to ensure reproducibility
- c. Training of study personnel in surgical preparation for and performance of CCI injury.

The CCI model was not in use at UCD prior to the start of this project and purchase, setup and training was essential for the completion of all subsequent studies.

1b. Sacrifice mice at 6 and 24 hrs, 7 days and 10 weeks after CCI (150 mice; Timeframe months 1-4).

Status: In progress. Injured mice have been sacrificed at 6 hours, 24 hours, 48 hours, 72 hours, 1 week and 10-16 weeks after injury.

Background necessary for this subtask has also been completed and includes:

1. Training of study personnel in non-traumatic dissection techniques to obtain cortical and hippocampal tissue samples from mouse brains

Accomplishments: Harvested tissue was used for studies of STAT3 phosphorylation at all above time points and for studies of the phosphorylation of JaK2 at 24 hours after injury (see **1c** and **1d** below).

1c. Measure levels of mRNA for JaK1 and 2, STAT1-5 in microdissected subregions of hippocampus (DG, CA1, CA3) and in cortex ipsilateral and contralateral to injury using quantitative reverse transcription polymerase chain reaction (qRT-PCR) (50 mice; Timeframe months 4-7).

Status: In progress

1. Completed training of study personnel in non-traumatic dissection techniques to obtain cortical and hippocampal tissue samples from mouse brains
2. Tested, validated and implemented mRNA protocols for measuring JaK1 and 2 and pSTAT1-5 using quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Accomplishments: Data collection in progress. Planned for end of July 2013.

Supporting Data: None

1d. Measure protein levels of JaK1 and 2, pSTAT1-5 levels using western blotting in homogenates of microdissected brain regions (50 mice; Timeframe months 6-9).

Status: In progress

1. Completed training of study personnel in non-traumatic dissection techniques to obtain cortical and hippocampal tissue samples from mouse brains
2. Optimized and implemented Western blot protocols for protein measurements of JaK1 and 2 and pSTAT1-5 in mouse brain

Based on this work and other work done in the lab, pSTAT3 has been the most reliable indicator of JaK/STAT pathway activation in multiple models of rodent epileptogenesis and, as such, has formed the focus of this investigation. CCI of both moderate (1mm, 3.5 m/s, 400ms) and severe (2mm, 5m/s, 199ms) severity was used for this task. Whole hippocampus was dissected from injured and sham mice at 6 hours, 24 hours, 48 hours, 72 hours, 1 week and 12-16 weeks after CCI. Western blots of protein homogenates from whole hippocampus were probed with pJaK2 (24 hour timepoint), JaK2 (24 hour timepoint), pSTAT3 (all timepoints) and STAT3 (all timepoints) antibodies. Quantification of western blot analysis was performed and pSTAT3 and pJaK2 levels were normalized to STAT3 or JaK2 levels respectively and expressed as a % change compared to shams.

Accomplishments: These studies demonstrated that pJaK2 and pSTAT3 are significantly increased in injured mouse hippocampus after CCI of both moderate and severe severity. In addition, pSTAT3 levels are significantly greater in the more severely injured animals.

These results established that there is an increase in activation of STAT3 following CCI in mice, similar in magnitude and time course to that seen in rat (see related studies, Raible et al., J Neurotrauma, 2012 Sep 4. [Epub ahead of print], PMID: 22827467. Appendix item #1), and suggest that the JaK/STAT pathway may be differentially activated based on injury severity.

Supporting Data: See **Figure 1**.

1e. Assess protein levels and regional/cellular expression of JaK1 and 2, pSTAT1-5 using fluorescent immunohistochemistry with co-staining for cell specific markers (50 mice; Timeframe months 9-12).

Status: Not yet initiated

Accomplishments: Not yet initiated

Supporting Data: None

Task 2: Determine whether upregulation of ICER transcription occurs following traumatic brain injury (TBI) and subsequent epileptogenesis. (Timeframe months 1-12: studies will overlap with Task 1 and be performed in same animals)

2a. Induce TBI using the CCI model in adult CD-1 mice (200 mice [same mice used in Task 1a]; months 1-10)

Status: In progress. See **1a** above.

2b. Sacrifice mice at 6 and 24 hrs, 7 days and 10 weeks after CCI (150 mice [same mice used in Task 1b]; months 1-4)

Status: In progress. Injured mice have been sacrificed at 6 hours, 24 hours, 48 hours, 72 hours, 1 week and 12-16 weeks after injury.

Background necessary for this subtask has also been completed and includes:

1. Training of study personnel in non-traumatic dissection techniques to obtain cortical and hippocampal tissue samples from mouse brains

Accomplishments: Harvested tissue will used for studies of ICER protein and mRNA levels (see **2c** and **2d** below).

2c. Measure levels of mRNA for ICER in brain regions ipsilateral and contralateral to injury using qRT-PCR (50 mice [same 50 mice as used in Task 1c]; months 4-7).

Status: In progress

1. Completed training of study personnel in non-traumatic dissection techniques to obtain cortical and hippocampal tissue samples from mouse brains

2. Tested, validated and implemented mRNA protocols for measuring ICER using quantitative reverse transcription polymerase chain reaction (qRT-PCR) in RNA extracted from mouse brain

Accomplishments: Data collection in progress. Planned for end of July 2013.

Supporting Data: None

2d. Measure protein levels of ICER using western blotting in brain regions (50 mice [same 50 mice as used in Task 1d]; Timeframe months 6-9).

Status: Not yet initiated

Accomplishments: Not yet initiated

Supporting Data: None

2e. Assess protein levels and regional/cellular expression of ICER using fluorescent immunohistochemistry (50 mice [same 50 mice as used in Task 1e]; Timeframe months 9-12).

Status: Not yet initiated

Accomplishments: Not yet initiated

Supporting Data: None

Studies similar to Task 2 have been completed in a related rodent model of posttraumatic epileptogenesis (fluid percussion injury). Poor antibody specificity limited the value of the results of these related studies.

Task 3: Determine whether downregulation of *Gabra1* transcription occurs following traumatic brain injury (TBI) and subsequent epileptogenesis. (Timeframe months 1-12: studies will overlap with Tasks 1 and 2 and be performed in same animals)

3a. Induce TBI using the CCI model in adult CD-1 mice (200 mice- assuming that 25-30% loss due to death or suboptimal injury; months 1-10)

Status: In progress. See **1a** above.

3b. Sacrifice mice at 6 and 24 hrs, 7 days and 10 weeks after CCI (150 mice [same used in Task 1b]; months 1-4)

Status: In progress. Injured mice have been sacrificed at 6 hours, 24 hours, 48 hours, 72 hours, 1 week and 12-16 weeks after injury.

Background necessary for this subtask has also been completed and includes:

1. Training of study personnel in non-traumatic dissection techniques to obtain cortical and hippocampal tissue samples from mouse brains

Accomplishments: Harvested tissue will used for studies of *Gabr* subunit protein and mRNA levels (see **3c** and **3d** below).

3c. Measure levels of mRNA for *Gabr* subunits in brain regions ipsilateral and contralateral to injury using qRT-PCR (50 mice [same mice as used in Task 1c]; months 4-7).

Status: In progress

1. Completed training of study personnel in non-traumatic dissection techniques to obtain cortical and hippocampal tissue samples from mouse brains

2. Tested, validated and implemented mRNA protocols for measuring *Gabr* subunits using quantitative reverse transcription polymerase chain reaction (qRT-PCR) for mouse

Accomplishments: Data collection in progress. Planned for end of July 2013.

Supporting Data: None

3d. Measure protein levels of *Gabr* subunit levels using western blotting in brain regions (50 mice [same mice used in Task 1d]; months 6-9).

Status: In progress

1. Established Controlled Cortical Impact (CCI) model as an experimental model of TBI at the University of Colorado, Denver as described above

2. Tested, validated and implemented protocols for measuring *Gabr* subunits in mouse

3. Initiated experiments for 12, 14, 16 week time points

Western blots were performed using whole hippocampal protein collected from mice 12, 14, 16 weeks after CCI and probed with anti-GABAAR $\alpha 1$, $\alpha 4$, $\gamma 2$, δ , $\alpha 2$, $\alpha 5$, $\beta 2$, $\beta 3$ and β -actin antibodies. GABA_AR subunit levels were normalized to β -actin levels and expressed as a percent change compared to sham-injured animals.

Accomplishments:

1. Demonstrated that GABA_AR subunit protein level $\alpha 1$ is significantly decreased at 12, 14 and 16 weeks after severe CCI. Also, the GABA_AR subunits $\alpha 2$, $\alpha 5$, $\beta 2$, and $\beta 3$ protein levels are not significantly changed in injured hippocampus 12, 14, 16 weeks after severe CCI or moderate CCI in mouse.

Supporting Data: see **Figure 2**

3e. Assess protein levels and regional/cellular expression of *Gabr* subunits using fluorescent immunohistochemistry (50 mice [same mice used in Task 1e]; months 9-12).

Status: Not yet initiated

Accomplishments: Not yet initiated

Supporting Data: None

Aim 2: Performed in laboratory of Dr. Bret N. Smith at University of Kentucky

Determine whether activation of the JaK/STAT pathway and downregulation of *Gabra1* transcription following TBI result in altered inhibitory synaptic neurotransmission in the hippocampus that may contribute to epileptogenesis.

Task 1: Determine whether benzodiazepine modulation of IPSCs in dentate granule cells (DGCs) is altered after CCI and whether this alteration is prevented by inhibiting STAT3 phosphorylation with WP1066. (Timeframe months 1-18).

Task 1a. Induce TBI using CCI model in adult CD-1 mice (200 mice used, 20 sham-injured controls, 80 injured untreated, 20 sham-injured, WP1066-treated controls, 80 injured WP1066-treated; Timeframe months 1-18).

Status: In progress

1. Verified parameters of CCI injury that result in epileptogenesis and markers of the development of epileptic phenotype after 8-12 weeks post-injury. Approximately 30 mice were treated with CCI in the last year, 10 of which were injected with WP-1066. Based on collaborators findings that more severe injury results in greater STAT3 phosphorylation, studies included a few mice with greater injury.

a. The spatial extent of moderate and severe injury-related epileptogenic changes with respect to distance from impact point was initiated.

2. Establish that phosphorylation of STAT3 is upregulated and was inhibited by WP1066 in this model.

a. Establish effect and localization of STAT3 phosphorylation after CCI.

b. Establish effect of WP1066 on STAT3 phosphorylation after CCI.

c. Based on collaborators preliminary results, initiated studies to examine the extent of STAT3 phosphorylation after more severe injury. The results of this comparison will inform future electrophysiological analysis.

Accomplishments:

1. Determined precise parameters of effective CCI (1 mm depth) to obtain epileptogenic phenotype. Phenotype changes are currently being assessed for more severe injury (2 mm), based on preliminary findings from collaborators.

2. Completed determination that CCI increased STAT3 phosphorylation ipsilateral to the injury after 24 hours, but not contralaterally. Western blot analyses of hippocampi from CCI-injured and control mice were concluded after 1 mm depth injury. Both STAT and phosphorylated STAT (pSTAT)

protein expression were compared semi-quantitatively 24 hr after injury (see progress report from year 1). Comparisons were made for hippocampi ipsilateral to the injury, contralateral to the injury, and in sham-operated controls. STAT and pSTAT levels were normalized to those for β -actin. Results indicated that pSTAT ($p < 0.05$), but not STAT ($p > 0.05$), expression was increased in the hippocampus ipsilateral to the injury. We further determined that treatment at 30 and 90 min after CCI (1 mm) with WP1066 (50 mg/kg) inhibits STAT3 phosphorylation in mice. Full analysis was reported (Butler et al., 2012; Boychuk et al., 2012). Conclusion: The biochemical reaction required to perform further analyses of GABA currents is evident after CCI ipsilateral to the 1 mm injury, but not contralaterally. This means that the contralateral dentate gyrus can serve as a control for electrophysiological analyses. Determining that pSTAT3 expression for 2 mm depth is underway.

3. Completed analysis of histopathological features (i.e., MFS and hilar GABA neuron loss) in the dentate gyrus consistent with epileptogenesis. Mossy fiber sprouting analysis was completed in year one (Hunt et al., 2012). Assessment of hilar inhibitory neuron loss was completed in year 2, but preliminary data was included in the previous progress report (Butler et al., 2012; Boychuk et al., 2012). The distribution of inhibitory neuron loss was compared to previous results on mossy fiber sprouting. Significant GABA neuron loss was limited to the injury epicenter and an area extending 800 μ m temporal (ventral) to the injury; areas septal (dorsal) to the injury were unaffected, similar to the distribution of mossy fiber sprouting. Contralaterally and at more ventral levels ipsilaterally, hippocampal pathology was not observed (Butler et al., 2012). Ongoing studies include more severe (2 mm) injury depth. We further determined that treatment with WP1066 does not affect GABA neuron loss. Full analysis of mice from the two injection paradigm (50 mg/kg each), delivered at 30 and 90 min post-injury (1 mm) was completed. The WP1066 treatment did not, however, alter the degree of GABAergic hilar interneuron cell loss ipsilateral to the injury (Butler et al., 2012). Conclusions: The histopathology most likely to be required for studying changes in GABA currents after CCI was associated with hippocampal evulsion, which was observed when using beveled tips (versus rounded tips) to a depth of 1 mm. Treatment with WP1066 showed no evidence of influence on cell proliferation, axon growth, or neuronal survival, Effects of 2 mm depth are in progress.

Supporting Data: Figures 1 & 2 from final progress report, year 1 summarize: 1. Western blot results indicating increased STAT3 phosphorylation after 1 mm CCI ipsilateral to the injury and inhibition of pSTAT3 by systemic WP1066 treatment (50 mg/kg, i.p.) at 30 and 90 min after CCI injury; 2. the hilar GABA neuron loss after CCI and the lack of effect of WP1066 treatment on hilar neuron loss. These data were completed in year 2, but reported in revised progress report for year 1.

Task 1b. Measure effects of zolpidem on IPSCs in DGCs from WP1066-treated and untreated control mice and in mice shortly (i.e., 1-6 weeks) after CCI injury. (100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 1a; Timeframe months 1-9).

Status: In progress

Accomplishments:

1. IPSCs were recorded from granule cells from sham-($n=2$) and CCI-treated ($n=3$) mice (1-6 weeks post-injury). IPSC frequency, amplitude, and decay time constants were measured. Analysis is preliminary and ongoing.
2. Applied $\alpha 1$ -subunit selective benzodiazepine agonist and measured IPSC frequency, amplitude and decay time constant in neurons from control ($n=2$) and CCI-treated ($n=3$) mice. Analysis is

preliminary: benzodiazepine agonist tended to increase time constant; replicates are currently too low to be quantitative.

Supporting Data: None

Task 1c. Measure effects of zolpidem on IPSCs in DGCs from WP1066-treated and untreated control mice and in mice 6-10 weeks after CCI injury. (100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 1a; Timeframe months 4-18)

Status: In progress

1. Establishing precise IPSC parameters in control and CCI-injured mice at 6-10 weeks after injury. This includes:
 - a. Train personnel in recording and analysis techniques.
 - b. Obtain sufficient numbers of recordings to sufficiently identify differences
2. Effects of zolpidem on IPSCs in four treatment groups.
 - a. Obtain DEA license to purchase benzodiazepine agonists
 - b. Use zolpidem or other $\alpha 1$ subunit agonist in recordings from DGCs in slices from four treatment groups; 6-10 weeks postinjury.

Accomplishments:

- 1a. All personnel trained
- 1b. Preliminary data suggest a trend toward longer IPSC decay time constants in CCI-injured mice, 6-10 weeks after injury. These experiments continue and should be completed by August 2013. Experiments on CCI-injured+WP1066-treated mice are scheduled.
2. Effects of $\alpha 1$ subunit-selective agonist on IPSC frequency, amplitude, and decay time constant have been initiated in cells from sham control and CCI-injured mice.

Supporting Data: See **Table 1** below for preliminary assessment of IPSC amplitude, frequency, and time constant for control, CCI-injured, and CCI-injured+WP1066 treated mice.

Task 1d. Perform Timm histological analysis, to detect mossy fiber sprouting in all slices from which recordings are made. (200 mice needed; same mice as in Tasks 1a-c; Timeframe months 1-18).

Status: In progress

Accomplishments:

1. In year 2, approximately 30 mice were treated with CCI, 10 of which were treated with WP1066. Early post-injury experiments are underway; Timm staining has not revealed mossy fiber sprouting in any group at this time-point, as expected.
2. At 6-10 weeks post-injury, Timm staining was observed in CCI-injured, but not sham control mice. CCI-injured+WP1066 treated mice displayed Timm distribution similar to CCI-injured mice at 6-10 weeks post-injury.

Supporting Data: Timm scores for the three groups analyzed: Control= 0.13 ± 0.09 (n=16); CCI-injured= 1.93 ± 0.30 (n=16; $p < 0.05$ vs control); CCI-injured+WP1066= 1.88 ± 0.30 ; (n=16; $p < 0.05$ vs control; $p > 0.05$ versus CCI-injured; ANOVA). Mossy fiber sprouting was not reduced by WP1066 treatment after CCI injury. See **Figure 3** below.

Task 2: Determine if furosemide modulation of IPSCs in DGCs is altered after CCI and if inhibiting STAT3 phosphorylation with WP1066 prevents the alteration. (Timeframe: months 19-36).

Task 2a. Induce TBI using CCI model in adult CD-1 mice (200 mice used, 20 sham-injured controls, 80 injured untreated, 20 sham-injured, WP1066-treated controls, 80 injured WP1066-treated; Timeframe months 19-36).

Status: in progress

Accomplishments: Accomplishments identical to Task 1, 1a.

Supporting Data: Same as Task 1, 1a.

Task 2b. Measure effects of furosemide on IPSCs in DGCs from WP1066-treated and untreated control mice and in mice 1-6 weeks after CCI injury. (100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 2a; Timeframe months 19-27).

Status: In Progress.

Accomplishments: Experiments have been made in DGCs from in 5 mice.

Supporting Data: none

Task 2c. Measure effects of furosemide on IPSCs in DGCs from WP1066-treated and untreated control mice and in mice 6-10 weeks after CCI injury (months 4-18). 100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 2a; Timeframe months 19-36).

Status: In progress

Accomplishments: Experiments have been initiated. IPSC parameters have been established as in Task 1c.

Supporting Data: Table 1.

Task 2d. Perform Timm histological analysis, to detect mossy fiber sprouting in all slices from which recordings are made. (200 mice needed; same mice as in Tasks 2a-c; Timeframe months 19-36).

Status: in progress

Accomplishments: Accomplishments identical to Task 1, 1d.

Supporting Data: Same as Task 1, 1d.

Task 3: Determine if THIP-induced tonic GABA currents in DGCs are altered after CCI and if the alteration is prevented by inhibiting STAT3 phosphorylation with WP1066. (Timeframe months 10-27).

Task 3a. Induce TBI using CCI model in adult CD-1 mice (200 mice used, 20 sham-injured controls, 80 injured untreated, 20 sham-injured, WP1066-treated controls, 80 injured WP1066-treated; Timeframe months 10-27).

Status: in progress

Accomplishments: Accomplishments identical to Task 1, 1a.

Supporting Data: Same as Task 1, 1a.

Task 3b. Measure THIP-induced tonic GABA current in DGCs from WP1066-treated and untreated control mice and in mice 1-6 weeks after CCI injury. (100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 3a; Timeframe months 10-18).

Status: In progress

Accomplishments: 4,5,6,7-Tetrahydroisoxazolo[5,4c]pyridine-3-ol hydrochloride (THIP) currents have been measured in 7 cells from 7 controls and 6 cells in 5 CCI-treated mice.

Supporting Data: Same as Task 1, 1a.

Task 3c. Measure THIP-induced tonic GABA current in DGCs from WP1066-treated and untreated control mice and in mice 6-10 weeks after CCI injury (months 4-18). 100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 3a; Timeframe months 19-27).

Status: in progress

Accomplishments:

1. Determined that THIP-induced tonic GABA current in DGCs 6-10 weeks post-injury are reduced in amplitude relative to sham-operated controls and contralateral DGCs (n=7-14 cells in 7 mice from each group; p<0.05). Based on preliminary results from collaborators in Colorado and on data published recently elsewhere, these experiments were initiated to identify potential functional changes due to altered $\alpha 1$ vs $\alpha 4/\delta$ subunit-containing GABA receptor expression weeks after injury, corresponding to time points where epilepsy is established in this model (i.e., 6-10 weeks post-injury). Granule cells were recorded in ex vivo slices taken from control mice and from slices taken contralateral and ipsilateral to injury site in CCI-injured mice, 6-10 weeks post-injury. Cells were voltage-clamped at 0 mV and THIP (3 μ M) was bath-applied to induce an outward current, ostensibly due to activation of δ subunit-containing GABA receptors (most likely $\alpha 4/\delta$). Bicuculline methiodide (30 μ M) was applied to block all GABA receptors and determine the total available tonic GABA current. Conclusions: Total tonic GABA current in granule cells from control versus CCI-injured mice. THIP-current in cells contralateral to the injury are not different from those in control mice. However, THIP-induced tonic current is reduced by about 50% ipsilateral to the injury in cells from CCI-treated versus controls (p<0.05) or in cells contralateral (p<0.05) to the injury.

2. Recordings were made from 12 granule cells in 7 WP1066-treated CCI-injured animals and tonic GABA and THIP currents were compared to results from control mice and CCI-injured mice. As in other groups, tonic GABA current was unaffected in WP1066-treated CCI-injured mice (p>0.05), as expected. The THIP-current was significantly reduced by about 50% ipsilateral to the injury in cells

from WP1066+CCI-treated versus controls or in cells contralateral to the injury ($p < 0.05$). The THIP current in the WP1066+CCI-injured group was not different from CCI-treated group ($p > 0.05$).

3. Control studies are ongoing to determine if WP1066 treatment affects non-injured controls.

Supporting Data: See **Figure 4 below** for demonstration of THIP-induced changes in tonic GABA current in sham-operated controls, CCI-injured, and CCI-injured+WP1066, 6-10 weeks post injury.

Task 3d. Perform Timm histological analysis, to detect mossy fiber sprouting in all slices from which recordings are made. (200 mice needed; same mice as in Tasks 3a-c; Timeframe months 10-27).

Status: in progress

Accomplishments: Accomplishments identical to Task 1, 1d.

Supporting Data: Same as Task 1, 1d.

Aim 3: Performed in laboratory of Dr. Amy Brooks-Kayal at University of Colorado

Task 1: Determine whether inhibition of STAT3 phosphorylation after CCI using WP1066 inhibits post-traumatic epilepsy development (PTE) (Timeframe months 12-21)

1a. Induce TBI using the CCI model in adult CD-1 mice and administer WP1066 or vehicle immediately after injury (200 mice- assuming that 30% loss due to death, suboptimal injury, or loss of implanted recording electrodes; months 12-18)

Status: In progress. CCI has been completed on approximately 320 mice.

1b. Sacrifice mice at 6 hrs, 24 hrs and 7 days after CCI to assess molecular and histochemical effects of WP1066 (120 mice [assuming that 25-30% loss due to death, suboptimal injury]; months 12-15)

Status: In progress

1. Established Controlled Cortical Impact (CCI) model as an experimental model of TBI at the University of Colorado, Denver as detailed above
2. Established protocol for WP1066 or vehicle administration

Accomplishments:

1. We performed multiple experiments to identify a dosing paradigm for WP1066 that resulted in inhibition of STAT3 phosphorylation following CCI in mouse, including:
 - a. First we administered WP1066 50 mg/kg ip either 15 min before or 5 min after CCI injury and harvested hippocampal tissue at 6 hrs after CCI. Western blots of protein homogenates from whole hippocampus were then probed with anti-pSTAT3 and STAT3 antibodies. Quantification of Western blot analysis was performed and pSTAT3 levels were normalized to STAT3 levels and expressed as a % change compared to shams. These studies demonstrated that there was no significant difference in pSTAT3 levels at 6 hrs following CCI in injured hippocampus between vehicle treated and WP1066 treated groups (see **Figure 5**).
 - b. We next examined if 15 minute pre-treatment with a WP1066 analogue with enhanced stability (WP117) at varying doses would inhibit phosphorylation of STAT3 in injured hippocampus 1 hour after CCI. Western blots of protein homogenates from whole hippocampus were performed as described above. These studies demonstrated that there was no significant difference in pSTAT3 levels at 1hr following

CCI in injured hippocampus between vehicle treated and WP117 treated groups, and suggested that very early treatment with WP1066 or analogue did not inhibit STAT3 phosphorylation (see **Figure 6**).

c. Next, we administered WP1066 50 mg/kg ip 30 and 90 minute post-CCI and harvested tissue at multiple timepoints after CCI. These studies demonstrated that WP1066 administered at 30 and 90 minutes after CCI partially inhibited STAT3 phosphorylation in injured hippocampus 3, 6 and 24 hours after CCI.

d. Finally, we administered WP1066 75 mg/kg ip 30 and 90 minute post-CCI and harvested tissue at multiple timepoints after CCI. These studies demonstrated that WP1066 at this dose administered at 30 and 90 minutes after CCI more significantly inhibited STAT3 phosphorylation in injured hippocampus 3, 6 and 24 hours after CCI (see **Figure 7**). 30 and 90 minute post-treatment of WP1066 does not inhibit phosphorylation of JaK2 in injured hippocampus 24 hours after severe CCI (see **Figure 8**).

Supporting Data: **Figures 5,6,7,8**

1c. Measure levels of mRNA for JaK/STAT, ICER and *Gabr* subunits in brain regions ipsilateral and contralateral to injury using qRT-PCR (30 mice [subset of mice as in Task 1b]; months 12-15).

Status: In progress

1. Completed training of study personnel in non-traumatic dissection techniques to obtain cortical and hippocampal tissue samples from mouse brains

2. Tested, validated and implemented mRNA protocols for measuring JaK/STAT, ICER and *Gabr* subunits using quantitative rtPCR in mouse

Accomplishments: Data collection in progress

Supporting Data: None

1d. Measure protein levels of JaK/STAT, ICER and *Gabr* subunits using western blotting in brain regions (30 mice [subset of mice as used in Task 1b]; months 15-18).

Status: In progress.

1. Completed training of study personnel in non-traumatic dissection techniques to obtain cortical and hippocampal tissue samples from mouse brains

2. Tested, validated and implemented protocols for measuring JaK/STAT, ICER and *Gabr* subunit levels using western blotting in mouse

Accomplishments:

1. Demonstrated that 30 and 90 minute post-treatment with WP1066 inhibits phosphorylation of STAT3 in injured hippocampus after both moderate and severe CCI at 6, 24, 48 and 72 hours after CCI.

2. Demonstrated that 30 and 90 minute post-treatment with WP1066 rescues changes in alpha1 GABA_AR subunits in injured hippocampus after severe CCI at 24 hours, 48 hours, 72 hours and 12-16 weeks after CCI. We also demonstrated that there are differential decreases in GABA_AR alpha 1 subunit between the CCI-M and CCI-S injured mice at 24 hours and 12, 14, and 16 weeks after injury (**Figure 9**).

Supporting Data: see **Figures 7,9**

1e. Assess protein levels and regional/cellular expression of JaK/STAT, ICER and *Gabr* subunits using fluorescent immunohistochemistry (30 mice [subset of mice used in Task 1b]; months 18-21).

Status: Not yet initiated

Accomplishments: Not yet initiated.

Supporting Data: None

1f. Assess cell injury and neurogenesis using Fluoro-Jade, TUNEL, caspase and nestin staining; (30 mice [same mice used in Task 1e] months 18-21).

Status: Not yet initiated

Accomplishments: Not yet initiated.

Supporting Data: None

1g. Assess glial proliferation using GFAP and ALDH1L1 staining (30 mice [same mice used in Task 1e]; months 18-21).

Status: Not yet initiated

Accomplishments: Not yet initiated.

Supporting Data: None

1h. Implant and video-EEG monitor a subset of mice from 6-10 weeks post injury (80 mice - assuming 30% loss due to death with surgery or loss of implanted recording electrodes; months 16-20)

Status: In progress

1. Established collaborative relationship with University of Colorado Small Animal Neurophysiology core facility, an electrically clean facility on campus for long-term rodent video-EEG monitoring

2. Completed training of study personnel in EEG electrode implantation

3. Optimized EEG electrode implantation protocol for CCI animals. Because the skull and the underlying brain is disrupted during CCI, these animals require a specialized implantation protocol to minimize the impact of the skull and brain disruption on the quality of the collected signal (see **Figure 10**).

4. Implanted 8 animals with moderate CCI, 8 animals with severe CCI and 3 sham animals after injury.

5. Recorded and interpreted the EEGs for 8 animals with moderate CCI, 3 animals with severe CCI (the EEGs for 5 animals are currently under review) and 3 sham animals.

Accomplishments:

1. Demonstrated that later time periods of recording (8-14 weeks) after CCI are more likely to capture evidence of ongoing changes in cortical hyperexcitability in injured animals.

2. Demonstrated that moderately-injured mice subjected to CCI had bursts of spikes on their EEG, indicative of cortical hyperirritability, potentially injury-related. None of the sham-injured animals had similar interictal findings. These studies demonstrate that moderately-injured mice have a propensity for seizures after CCI during the time frame of monitoring. Spike density studies are planned to quantify this hyperexcitability.

3. Demonstrated that severely-injured mice subjected to CCI also had bursts of spikes on their EEG, indicative of cortical hyperirritability, potentially injury-related. None of the sham-injured animals had similar interictal findings. One (of three reviewed) severely-injured animal developed spontaneous, recurrent seizure activity on the EEG. These studies demonstrate that severely-injured mice may develop posttraumatic seizures after CCI during the time frame of monitoring. Spike density studies are also planned to quantify cortical hyperexcitability.

Supporting Data: **Figure 11**

1i. Assess mossy fiber sprouting, cell loss and glial proliferation 10 weeks post injury using Timm and Nissl staining (20 mice [subset of mice used in Task 1h]; months 20-21).

Status: Not yet initiated

Accomplishments: Not yet initiated.

Supporting Data: None

1j. Assess pSTAT3, ICER and *Gabra1* protein levels 10 weeks post injury (20 mice [subset of mice used in Task 1h]; months 20-21).

Status: In progress

1. Completed training of study personnel in dissection techniques as above

2. Tested, validated and implemented protocols for measuring JaK/STAT, ICER and

Gabr subunit levels using western blotting in mouse as above

Accomplishments:

Receptor protein levels assessed at the chronic time point. See **Aim1- Task 3d** above.

Supporting Data: See **Figure 2**.

1k. Assess ICER and *Gabra1* mRNA levels 10 weeks post injury (20 mice [subset of mice used in Task 1h]; months 20-21).

Status: Not yet initiated

Accomplishments: Not yet initiated.

Supporting Data: None

Task 2: Determine whether inhibition of STAT3 phosphorylation after CCI using shRNAs inhibits PTE development (Timeframe months 21-29)

2a. Induce TBI using the CCI model in adult CD-1 mice 1 week after administration of shRNAs for JaK2 or STAT3 or scrambled shRNAs (400 mice- assuming that 30% loss due to death, suboptimal injury, or loss of implanted recording electrodes; months 21-26)

2b. Sacrifice mice at 6 hrs, 24 hrs and 7 days after CCI to assess molecular and histochemical effects of shRNAs (240 mice [assuming that 25-30% loss due to death, suboptimal injury]; months 21-24)

2c. Measure levels of mRNA for JaK/STAT, ICER and *Gabr* subunits in brain regions ipsilateral and contralateral to injury using qRT-PCR (60 mice [subset of mice as in Task 2b]; months 21-24).

2d. Measure protein levels of JaK/STAT, ICER and *Gabr* subunit levels using western blotting in brain regions (60 mice [subset of mice as used in Task 2b]; months 22-25).

2e. Assess protein levels and regional/cellular expression of JaK/STAT, ICER and *Gabr* subunits using fluorescent immunohistochemistry (60 mice [subset of mice used in Task 2b]; months 24-27).

2f. Assess cell injury and neurogenesis using Fluoro-Jade, TUNEL, caspase and nestin staining; (60 mice [same mice used in Task 2e] months 24-27).

2g. Assess glial proliferation using GFAP and ALDH1L1 staining (60 mice [same mice used in Task 2e]; months 24-27).

2h. Implant and video-EEG monitor a subset of mice from 6-10 weeks post injury (160 mice - assuming 30% loss due to death with surgery or loss of implanted recording electrodes; months 22-28)

2i. Assess mossy fiber sprouting, cell loss and glial proliferation 10 weeks post injury using Timm and Nissl staining (40 mice [subset of mice used in Task 2h]; months 24-26).

2j. Assess pSTAT3, ICER and *Gabra1* protein levels 10 weeks post injury (40 mice [subset of mice used in Task 2h]; months 25-28).

2k. Assess ICER and *Gabra1* mRNA levels 10 weeks post injury (40 mice [subset of mice used in Task 2h]; months 27-29).

Status: Not yet initiated.

Accomplishments: Not yet initiated.

Supporting Data: None

The overall goal of the shRNA studies in Task 2 is to examine the effects of modulation of JaK/STAT pathway activation on epileptogenesis after experimental TBI using a second independent method. However, extensive background work we have performed in the past year in preparation for the planned studies has shown that the vector we proposed to use for shRNA introduction (lentivirus) is selective for neural progenitor and glial cells only, making the lentiviral delivery of shRNAs using currently available vectors, less useful for our planned epileptogenesis studies that are focused on changes in neuronal excitability. These findings suggest that the shRNA studies, as planned, are not feasible for the proposed studies and will not add significantly to the body of findings generated during this project. Instead, our lab has executed additional experiments with a more severe injury to take the place of the above task that are also designed to vary the severity of the experimental TBI (in the opposite direction, of course) and assess for alterations in JaK/STAT activation.

Task 3: Determine whether inhibition of STAT3 phosphorylation with WP1066 in animals with PTE reduces seizure frequency and/or inhibits PTE progression (Timeframe months 30-36)

3a. Induce TBI using the CCI model in adult CD-1 mice (50 mice- assuming 25-30% loss due to death or suboptimal injury; months 30-31)

Status: In progress. See **Aim 1-1a** above.

3b. Implant with subdural electrodes and video-EEG monitor mice from 6-8 weeks post injury as baseline (40 mice [assuming 30% loss due to death with surgery or loss of implanted recording electrodes]; months 32-34)

Status: In progress. See **Aim 3- 1h** above.

Accomplishments: Data collection in progress.

Supporting Data: **Figure 11.**

3c. Administer WP1066 50-100 mg/kg or vehicle daily for 2 weeks and continue video-EEG monitoring (30 mice; months 32-35)

Status: In progress

Accomplishments: Data collection in progress.

Supporting Data: None.

3d. Sacrifice animals and assess protein and mRNA levels for Gabr subunits and perform histological assessment of cell counts and mossy fiber sprouting (30 mice [same mice used in 3c]; months 34-36)

Status: In progress

1. Completed training of study personnel in dissection techniques as above

2. Tested, validated and implemented protocols for measuring JaK/STAT, ICER and *Gabr* subunit levels using western blotting in mouse as above

Accomplishments:

Receptor protein levels assessed at the chronic time point. See **Aim1- Task 3d** above.

Supporting Data: See **Figure 2.**

An additional aspect of any intervention to prevent the development of epilepsy after TBI is to ensure that the intervention itself does not hamper recovery from the injury. Our lab has used two behavioral tests-

an accelerating rotorod test and a novel object recognition test- to assess motor and memory function pre-injury and at 3 days and 7 days post-injury in injured animals and plan to compare these results to those from WP1066-treated animals.

Accomplishments:

Injured animals reliably display injury-related declines in function on both tests, with the more severely injured animals performing worse than the moderately injured animals.

Supporting Data: See **Figures 12 and 13.**

KEY RESEARCH ACCOMPLISHMENTS:

1. Demonstrated that JaK2 and pSTAT3 are significantly increased in injured mouse hippocampus after CCI of both moderate and severe severity. In addition, pSTAT3 levels are significantly greater in the more severely injured animals suggesting that the JaK/STAT pathway may be differentially activated based on injury severity, and may potentially contribute to the differential sensitivity to post-traumatic epilepsy seen in humans with varying severities of TBI.
2. Demonstrated that GABA_AR subunit protein level $\alpha 1$ is significantly decreased at 12, 14 and 16 weeks after severe CCI. Also, the GABA_AR subunits $\alpha 2$, $\alpha 5$, $\beta 2$, and $\beta 3$ protein levels are not significantly changed in injured hippocampus 12, 14, 16 weeks after severe CCI.
3. We demonstrated that treatment with WP1066 75 mg/kg ip at 30 and 90 minute post-CCI inhibits phosphorylation of STAT3 in injured hippocampus 3, 6 and 24 hours after CCI. This establishes feasibility of using WP1066 to examine the effects of inhibition of pSTAT3 on epilepsy development after CCI in mouse.
4. Demonstrated that 30 and 90 minute post-treatment with WP1066 rescues changes in alpha1 GABA_AR subunits in injured hippocampus after severe CCI at 24 hours, 48 hours, 72 hours and 12-16 weeks after CCI. We also demonstrated that there are differential decreases in GABA_AR alpha 1 subunit between the CCI-M and CCI-S injured mice at 24 hours and 12, 14, and 16 weeks after injury.
5. Demonstrated that mossy fiber sprouting in the inner molecular layer is regionally and locally enhanced after CCI in a semi-quantitatively measurable manner. STAT3 inhibition did not affect mossy fiber sprouting 6-10 weeks post-injury.
6. Demonstrated selective hilar GABA interneuron loss near the injury site after CCI-injury and that STAT3 inhibition did not alter the cell loss.
7. Demonstrated that total tonic GABA currents were not significantly altered in granule cells of the dentate gyrus ipsilateral to the injury 6-10 weeks after CCI in mice. However, THIP-induced tonic currents were reduced ipsilateral to the injury by 6-10 weeks after CCI-injury. Inhibition of STAT3 with WP1066 at the time of CCI-injury did not reinstate the THIP-activated tonic current ipsilateral to the injury.
8. Demonstrated that moderately-injured mice subjected to CCI had bursts of spikes on their EEG, indicative of cortical hyperirritability, potentially injury-related. None of the sham-injured animals had similar interictal findings. These studies demonstrate that moderately-injured mice have a propensity for seizures after CCI during the time frame of monitoring.
9. Demonstrated that severely-injured mice subjected to CCI also had bursts of spikes on their EEG, indicative of cortical hyperirritability, potentially injury-related. None of the sham-injured animals had similar interictal findings. One (of three reviewed) severely-injured animal developed spontaneous, recurrent seizure activity on the EEG. These studies demonstrate that severely-injured mice develop posttraumatic seizures after CCI during the time frame of monitoring.

10. Injured animals reliably display injury-related declines in cognitive function on behavioral testing, with the more severely injured animals performing worse than the moderately injured animals.

REPORTABLE OUTCOMES (Year 2): manuscripts, abstracts, presentations

D Raible, L Frey, J Boychuk, C Butler, H Grabenstatter Y Cruz Del Angel, S Russek, B Smith and A Brooks-Kayal. JaK/STAT inhibition to prevent posttraumatic epilepsy. Poster Presentation. Rocky Mountain Regional Neuroscience Group Annual Meeting, UC AMC. 2013

D Raible, L Frey, J Boychuk, C Butler, H Grabenstatter Y Cruz Del Angel, S Russek, B Smith and A Brooks-Kayal. JaK/STAT inhibition to prevent posttraumatic epilepsy. Poster Presentation. CTSA national pre-doctoral meeting. 2013

D Raible, L Frey, J Boychuk, C Butler, H Grabenstatter Y Cruz Del Angel, S Russek, B Smith and A Brooks-Kayal. JaK/STAT inhibition to prevent posttraumatic epilepsy. Poster Presentation. Department of Neurology Research Retreat. 2013

D Raible, L Frey, J Boychuk, C Butler, H Grabenstatter Y Cruz Del Angel, S Russek, B Smith and A Brooks-Kayal. JaK/STAT inhibition to prevent posttraumatic epilepsy. Oral Presentation. Rocky Mountain Regional Neuroscience Group Annual Meeting. 2013

Butler, CB, Boychuk, JA, Smith, BN. Inhibitory signaling to dentate granule cells following traumatic brain injury. *Soc. Neurosci. Abs.*, 39:in press. This abstract describes results of tonic GABA modulation by THIP in control and CCI-injured mice.

CONCLUSIONS:

In year 2 of DOD CDMRP funding, the CCI model has been successfully used in mice at both University of Colorado and University of Kentucky, along with molecular, anatomical, electrophysiological and neurophysiological (EEG) techniques, to examine GABA(A) receptor expression, JAK/STAT pathway activation, and post-traumatic seizure development following TBI. We have demonstrated that JaK2 and pSTAT3 are significantly increased in injured mouse hippocampus after CCI of both moderate and severe severity, and that pSTAT3 levels are significantly greater in the more severely injured animals suggesting that the JaK/STAT pathway may be differentially activated based on injury severity, and may potentially contribute to the differential sensitivity to post-traumatic epilepsy seen in humans with varying severities of TBI. We have further shown that protein levels of GABA_AR α 1 subunit, but not other GABA_AR subunits, are significantly decreased at multiple chronic timepoints after severe CCI. We have also demonstrated that treatment with WP1066 75 mg/kg ip at 30 and 90 minute post-CCI inhibits phosphorylation of STAT3 in injured hippocampus 3- 24 hours after CCI, and that this treatment prevents CCI-induced reductions in alpha1 GABA_AR subunits in injured hippocampus after severe CCI at all acute and chronic timepoints. Select GABA neuron loss is seen shortly after injury, and mossy fiber sprouting develops after several weeks post-injury, neither of which outcome was altered in WP1066-treated mice. Total tonic GABA currents in granule cells are unaffected by the injury. However, contrary to some other models, THIP-activated tonic GABA currents are reduced in granule cells ipsilateral to the injury, suggesting a reduction in δ GABA

receptor subunits (possibly $\alpha 4/\delta$ -containing), as hypothesized. Treatment with WP1066 did not reinstate the reduction in THIP-activated current, suggesting that the decrease in δ -subunit containing GABA receptors after injury is not affected by STAT3 inhibition. Previously, Raible et al (2012) showed that $\alpha 4$ -subunits are decreased one week after fluid percussion injury. Often, $\alpha 4$ -subunits pair with δ -subunits. The reduction in THIP-activated current is consistent with this finding. It is also consistent with a lack of influence of STAT3 phosphorylation on δ -subunit expression. Using continuous intracranial video EEG, we have shown that both moderately- and severely-injured mice subjected to CCI also had bursts of spikes on their EEG, indicative of cortical hyperirritability, potentially injury-related, but that only severely-injured animal developed spontaneous, recurrent seizure activity on the EEG. Finally, we found that injured animals reliably display injury-related declines in cognitive function on behavioral testing that varied proportionally to injury severity. These findings suggest that changes in GABA_AR $\alpha 1$ subunit expression after TBI may contribute to development of post-traumatic hyperexcitability and seizures and that the JaK/STAT signaling pathway is a critical regulator of these changes. Studies in Year 3 of this award will further clarify this association by examining the effects of JaK/STAT inhibition after CCI on subsequent development of spontaneous seizures and cognitive dysfunction.

Appendices

D Raible, L Frey, J Boychuk, C Butler, H Grabenstatter Y Cruz Del Angel, S Russek, B Smith and A Brooks-Kayal. JaK/STAT inhibition to prevent posttraumatic epilepsy. Abstract- American Epilepsy Society Annual meeting. December 2013

D Raible, L Frey, J Boychuk, C Butler, H Grabenstatter Y Cruz Del Angel, S Russek, B Smith and A Brooks-Kayal. JaK/STAT inhibition to prevent posttraumatic epilepsy. Oral Presentation. Abstract- Rocky Mountain Regional Neuroscience Group Annual Meeting. 2013

SUPPORTING DATA:

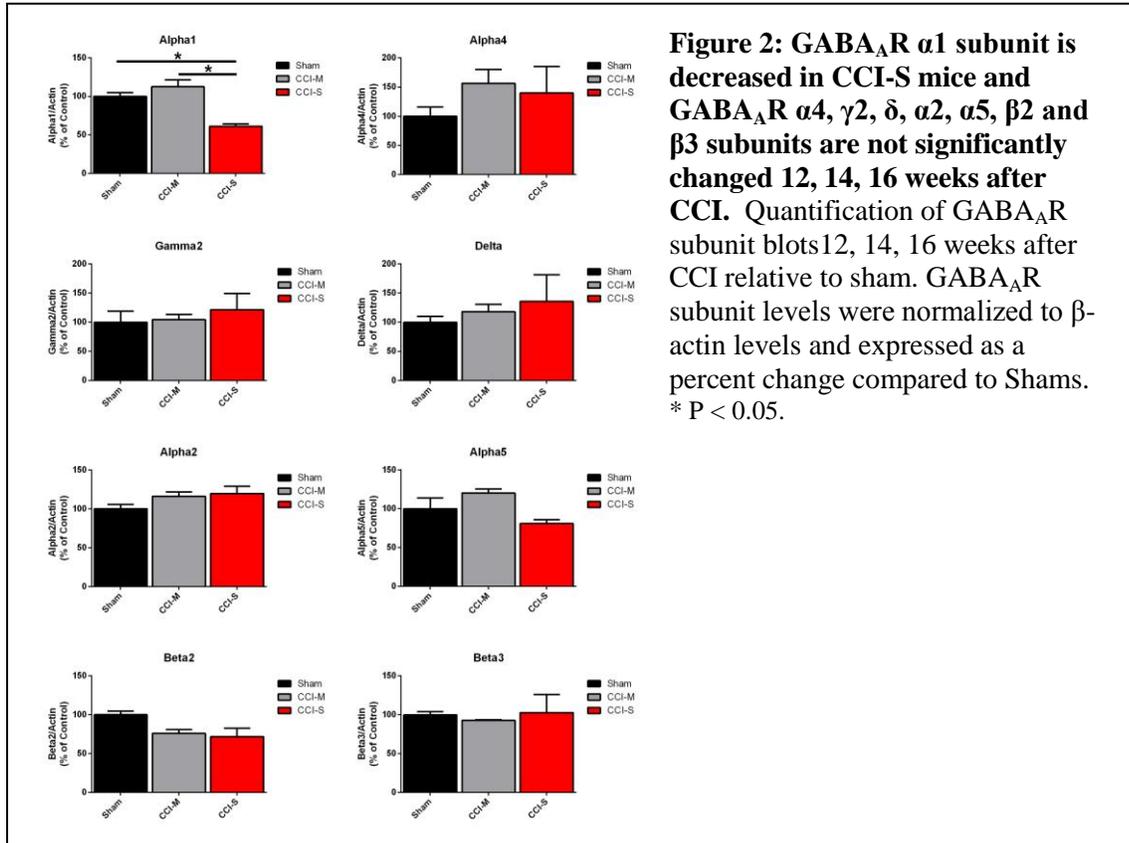
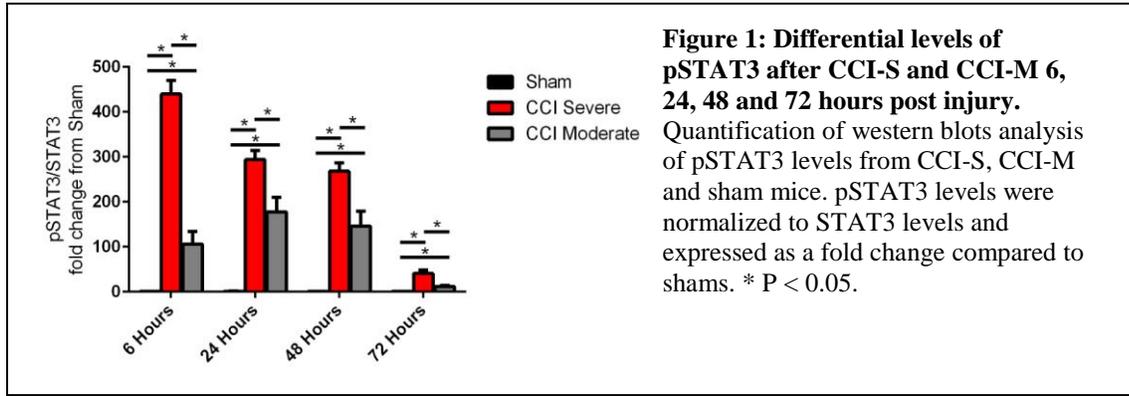
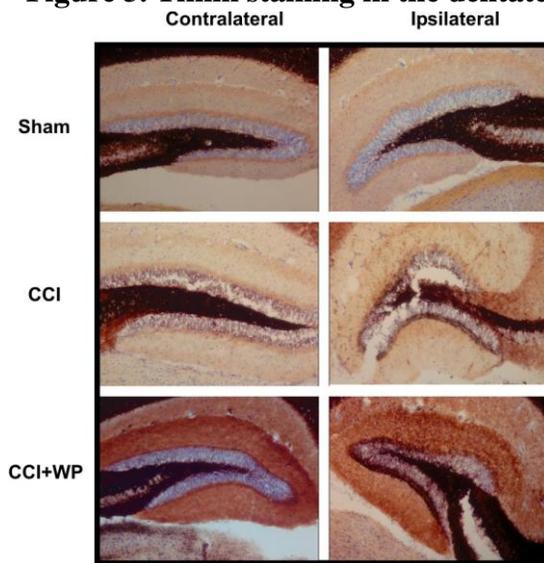


Table 1. IPSC frequency, amplitude and decay time constant in control, CCI-injured and CCI-injured+WP1066 treated mice.

sIPSCs	Sham		CCI				CCI+WP1066			
	Baseline	THIP	Contralateral		Ipsilateral		Contralateral		Ipsilateral	
			Baseline	THIP	Baseline	THIP	Baseline	THIP	baseline	THIP
Frequency(Hz)	1.41±0.09	0.79±0.16	1.30±0.26	82±0.08	0.80±0.12	0.68±0.08	1.33±0.34	0.74±0.35	0.72±0.09	0.69±0.07
Peak Amplitude(pA)	20.36±1.92	21.02±1.20	20.89±1.88	22.11±3.05	22.85±3.02	20.93±2.24	21.72±2.58	23.34±3.36	18.71±1.57	21.22±1.78
10-90% Rise Time(ms)	2.23±0.10	2.29±0.09	2.29±0.18	2.24±0.44	2.47±0.11	2.53±0.18	2.30±0.13	2.33±0.09	2.45±0.18	2.42±0.12
Decay Time Constant(ms)	14.06±3.40	13.28±1.86	15.33±3.26	14.17±2.02	17.04±3.56	16.03±1.63	14.10±0.67	13.35±0.88	16.97±1.49	15.70±2.05

Synaptic responses were assessed at baseline and at steady-state during 4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridine-3-ol hydrochloride (THIP; 3 μ M) application. Sample sizes are sham injury (n=7), CCI injury (Contralateral: n=7, Ipsilateral: n=7) or CCI injury with acute WP1066 treatment (Contralateral: n=5, Ipsilateral: n=7). Data shown as mean \pm SEM.

Figure 3. Timm staining in the dentate gyrus from sham control, CCI-injured, and CCI-injured+WP1066 treated mice.



Images of the dentate gyrus contralateral (left images) and ipsilateral (right), beneath the skull opening in sham-operated controls, CCI-injured, or CCI-injured+WP1066-treated mice after ~8 weeks post-injury. Contralateral images are from equivalent hippocampal levels contralateral to the skull opening. Mossy fiber sprouting into the inner molecular layer is evident ipsilateral to the injury in CCI-injured and CCI-injured+WP1066-treated mice and is similar to that seen previously to be associated with synaptic reorganization.

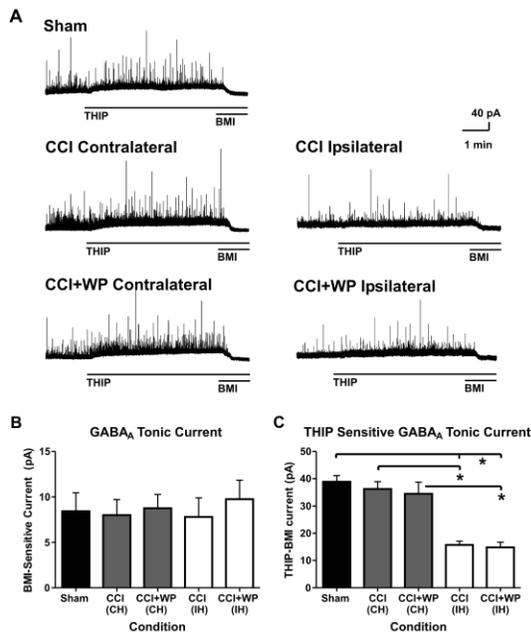


Figure 4. Reduced THIP-sensitive tonic GABA_A currents in dentate granule cells (DGCs) located ipsilateral to controlled cortical impact (CCI) during 6-13 weeks post-injury. **A.** Representative traces of tonic GABA_A currents in DGCs after sham injury (upper left; n=7), CCI injury (middle row; Contralateral: n=7, Ipsilateral: n=7) or CCI injury with acute WP1066 treatment (bottom row; Contralateral: n=5, Ipsilateral: n=7). DGCs were voltage clamped at 0 mV (near reversal potential of glutamatergic currents) and recorded in three phases: baseline, THIP (3 μM; Sigma, USA) and Bicuculline Methiodide (BMI; 30 μM; Tocris, USA). **B.** Group data of tonic GABA_A currents measured as the

change in steady-state holding current values of baseline to BMI application (Baseline I_{Hold} – BMI I_{Hold}). **C.** Group data of THIP-sensitive tonic GABA_A currents measured as the change in steady-state holding current values of THIP application to bicuculline application (THIP I_{Hold} – BMI I_{Hold}). Given the recording parameters here, an increase in tonic GABA_A receptor-mediated currents using THIP produced an outward shift whereas blockade of GABA_A receptors with BMI produced an inward shift in the holding current. Data shown as mean ± SEM and n= # of cells.

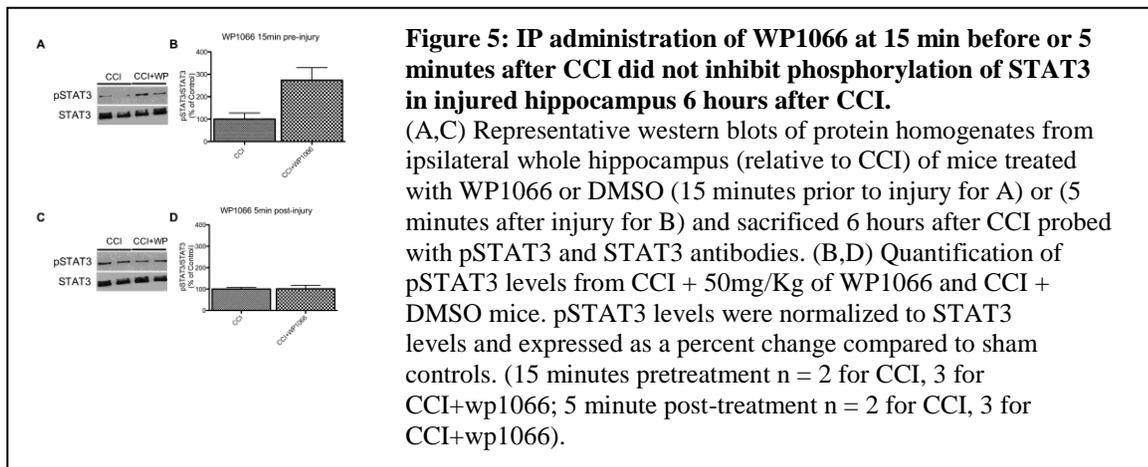


Figure 5: IP administration of WP1066 at 15 min before or 5 minutes after CCI did not inhibit phosphorylation of STAT3 in injured hippocampus 6 hours after CCI.

(A,C) Representative western blots of protein homogenates from ipsilateral whole hippocampus (relative to CCI) of mice treated with WP1066 or DMSO (15 minutes prior to injury for A) or (5 minutes after injury for B) and sacrificed 6 hours after CCI probed with pSTAT3 and STAT3 antibodies. (B,D) Quantification of pSTAT3 levels from CCI + 50mg/Kg of WP1066 and CCI + DMSO mice. pSTAT3 levels were normalized to STAT3 levels and expressed as a percent change compared to sham controls. (15 minutes pretreatment n = 2 for CCI, 3 for CCI+wp1066; 5 minute post-treatment n = 2 for CCI, 3 for CCI+wp1066).

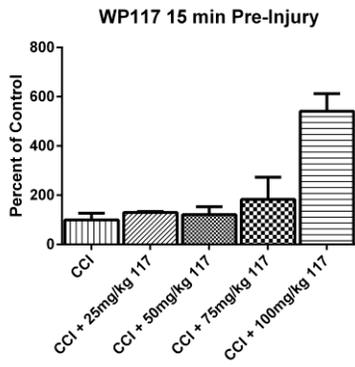


Figure 6: 15 minute pre-treatment IP administration of WP1066 analog (WP117) at varying doses did not inhibit phosphorylation of STAT3 in injured hippocampus 1 hour after CCI. Quantification of pSTAT3 levels from CCI + 25mg/kg WP117, CCI + 50mg/kg WP117, CCI + 75mg/kg WP117, CCI + 100 mg/kg WP117 and CCI + DMSO mice. n =6 for CCI, n = 4 for CCI + 25mg/kg WP117; n = 7 for CCI + 50mg/kg WP117; n = 2 for CCI + 75mg/kg WP117; n = 3 for CCI + 100mg/kg WP117.

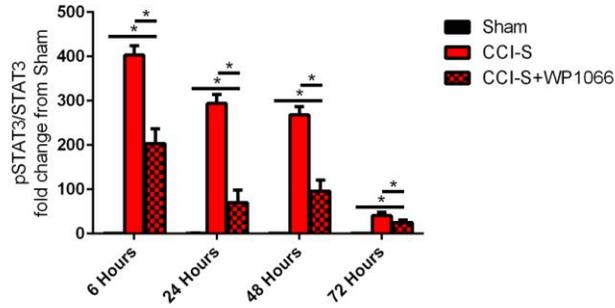


Figure 7: 30 and 90 minute post-treatment of WP1066 does inhibit phosphorylation of STAT3 in injured hippocampus 6, 24, 48, 72 hours after severe CCI (CCI-S) Quantification of pSTAT3 levels from CCI + 75 mg/kg of WP1066 30 and 90 minutes post-treatment, CCI + DMSO, or Sham injured animals sacrificed 6, 24, 48 or 72 hours after injury. pSTAT3 levels were normalized to STAT3 levels and expressed as a fold change compared to

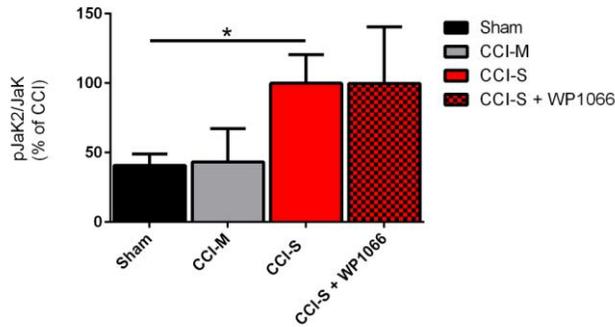
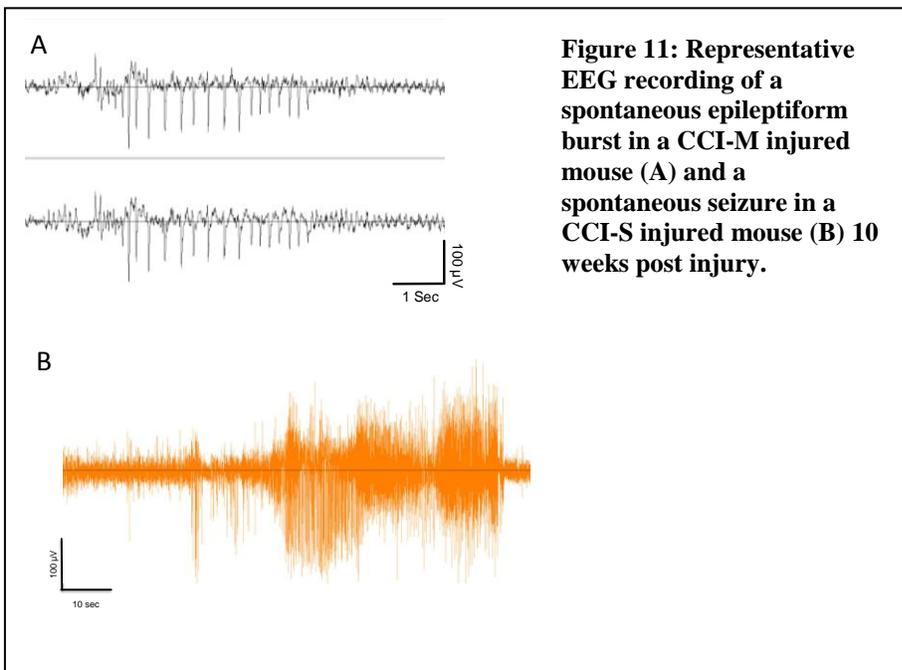
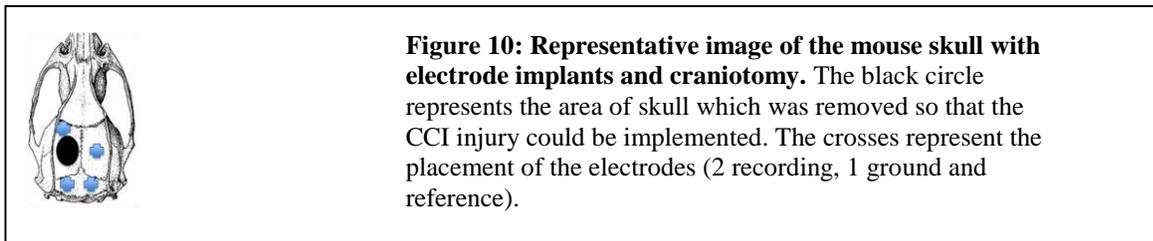
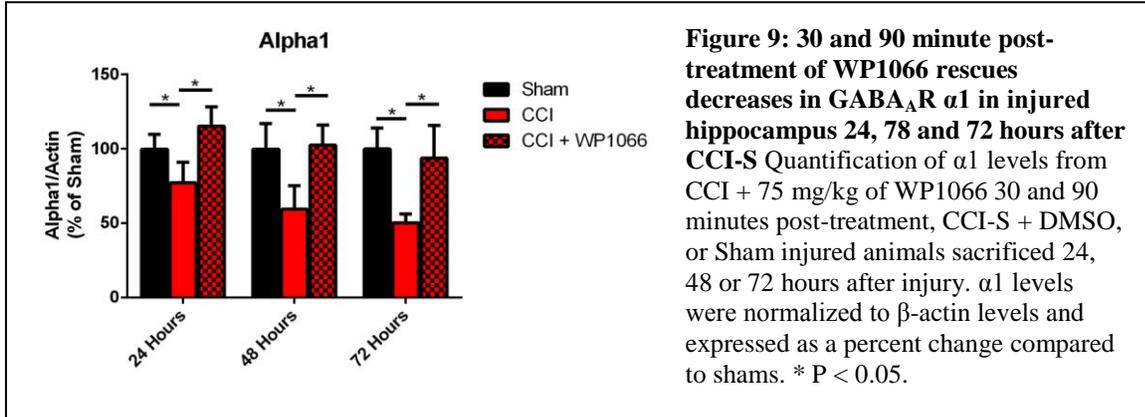


Figure 8: 30 and 90 minute post-treatment of WP1066 does not inhibit phosphorylation of JaK2 in injured hippocampus 24 hours after CCI-S. Quantification of pJaK2 levels from CCI + 75 mg/kg of WP1066 30 and 90 minutes post-injury. All animals sacrificed 24 hours after injury. pJaK2 levels were normalized to JaK2 levels and expressed as a percent change compared to shams. *P < 0.05.



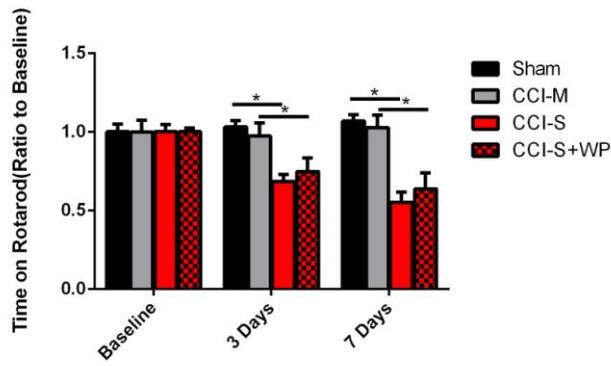


Figure 12: Rotarod performance was significantly decreased 3 and 7 days post injury in severe CCI and severe CCI + WP mice. Mice were placed on the rotarod and the time the mouse spends on the rotarod was recorded. The rotarod started at 4 RPM and increased to 40 RPM in 300 seconds.

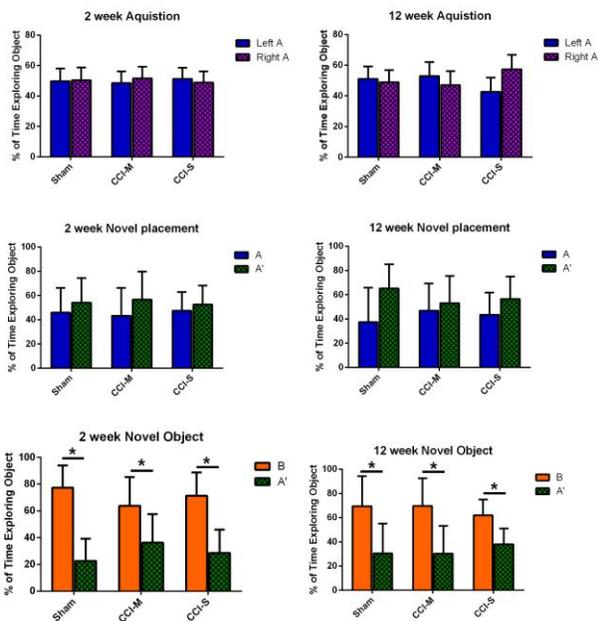


Figure 13: Both moderate and severe CCI animals were able to identify the new object during the last phase of the Novel Object and Placement testing.