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CONTRACTING ORGANIZATION: University of Kentucky

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14. ABSTRACT
Traumatic Brain Injury (TBI) is a well-established inducer of temporal lobe epilepsy (TLE), a frequently medically intractable and permanent epilepsy syndrome. Unlike many TLE models, which cause global brain injury that do not replicate the human condition, or other TBI models, which do not induce TLE reliably, the controlled cortical impact (CCI) model of posttraumatic epilepsy in mice results in localized cell loss, synaptic reorganization, and development of TLE. Abnormalities in inhibitory neurotransmission are important aspects of TLE in several animal models. Under this award, the CCI model was established in all three collaborating laboratories. Specific parameters of injury associated with epileptogenesis were determined. It was determined that upregulation of the JaK/STAT3 pathway in the injured hippocampus occurs after CCI, which could be blocked by post-injury administration of a JaK/STAT3 inhibitor. Blocking JaK/STAT3 activity did not prevent loss of GABA cells in the injured hippocampus. Inhibitory postsynaptic currents in the dentate gyrus ipsilateral to the injury were reduced in frequency weeks after the injury, recapitulating findings in other models in which aspects of epileptogenesis were attenuated by STAT3 inhibition. These results critically establish model parameters and control measurements, and provide the basis for remaining proposed experiments.

15. SUBJECT TERMS
dentate gyrus, epileptogenesis, GABA(A) receptor, JAK/STAT, posttraumatic epilepsy

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Progress Report Summary #W81XWH-11-1-0502 (July 2011-June 2012)

INTRODUCTION:

This research addresses the FY10 PRMRP topic area of Epilepsy. Traumatic Brain Injury (TBI) is a well-established etiology of temporal lobe epilepsy (TLE), a frequently medically intractable and often progressive epilepsy syndrome. Much evidence indicates that abnormalities in inhibitory neurotransmission are important in TLE. Our overall hypothesis is that Janus Kinase (JaK)/Signal Transducer and Activator of Transcription 3 (STAT3) pathway activation after TBI leads to GABA(A) receptor α1 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. Specifically, long-term decreases in expression of the GABA(A) Receptor α1 subunit gene (*Gabra1)* in the hippocampal dentate gyrus have been shown to occur in the pilocarpine-model of TLE in animals and preventing this repression has been shown to inhibit development of epilepsy in this model. It has been recently established that transcriptional repression of *Gabra1* in the pilocarpine-model of TLE is mediated by inducible cAMP early repressor and phosphorylated CREB, and that ICER transcription is driven by the JaK/STAT signaling cascade. Pharmacological inhibition of the JaK/STAT3 pathway prevents *Gabra1* repression and inhibits progression of epilepsy in the pilocarpine model. Preliminary data suggest that spontaneous seizures also activate the JaK/STAT3 pathway in the pilocarpine model, suggesting this pathway may be involved in the maintenance and progression of TLE. Preliminary evidence that the JaK/STAT3 pathway is activated following TBI in injured hippocampus and cortex after a *diffuse* injury, but the extent and time course of activation, the impact on GABA(A) receptor subunit expression and function, whether this occurs after *focal* injury, and whether it is an important mechanism of post-traumatic epileptogenesis is unknown. Focal injury caused by controlled cortical impact (CCI) has been shown to induce cell loss, synaptic reorganization, and TLE in mice. In order to assess changes in GABA responsiveness and alteration of those changes by Jak/STAT3 inhibition, it was necessary in the initial year of the study to coordinate procedures across three labs, establish that the fundamental changes in JaK/STAT3 activity occurred, and to ensure that the JaK/STAT3 blocker was effective in this model. Specific experiments on critical aspects of GABA function were then initiated in order to address the Specific Aims of the study. 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 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. Establish precise parameters of CCI injury that result in epileptogenesis and markers of the development of epileptic phenotype after 8-12 weeks post-injury
   a. Determine the precise parameters of effective CCI to obtain epileptogenic phenotype. The problem to be overcome: Obtaining epileptogenic phenotype with consistency is necessary in order to establish reliable baseline measurements from electrophysiological studies, but cortical damage after CCI is somewhat variable. CCI injury was performed as described in detail (Hunt et al., 2012; appendix item 1).
   b. Determine the precise spatial *extent* of injury-related epileptogenic changes with respect to distance from impact point, which is critical for determining the hippocampal regions affected by injury. To avoid false negative results in electrophysiological experiments, it was necessary to define the area of the dentate gyrus affected after the injury.

2. Establish that phosphorylation of STAT3 is upregulated and can be inhibited by WP1066 in this model. This includes:
   a. Establish effect and localization of STAT3 phosphorylation after CCI.
   b. Establish effect of WP1066 on STAT3 phosphorylation after CCI.
   c. Establish treatment protocols effective in both laboratories. To ensure consistency of all outcomes across the labs at both University and Kentucky and University of Colorado, these experiments were done in concert and consultation with those performed by the other PIs. Initially, mice responded differently than expected to the WP1066 treatment, resulting in several failed experiments at both research sites. It was determined that details of drug storage and usage were likely culprits and drug ordering, preparation, and storage procedures were modified to address the issues. Once these technical issues were resolved, successful treatment and repeatability was established.

Accomplishments:

1. Determined precise parameters of effective CCI to obtain epileptogenic phenotype, including mossy fiber sprouting and hilar GABA neuron loss. Mice were injured using a variety of tip shapes (round vs beveled) and injury depths (0.5-1 mm). After 6-10 weeks, mice were processed for Timm reactivity and hippocampal damage. The phenotype was most consistently found using injury made with beveled injury tips and at precise 1 mm injury depth calibration (Hunt et al., 2012).
2. Determined that histopathological features (i.e., MFS and hilar GABA neuron loss) in the dentate gyrus consistent with epileptogenesis. Mossy fiber sprouting (Hunt et al., 2012) and hilar inhibitory neuron loss (Butler et al., 2012; Boychuk et al., 2012) were limited to the areas immediately beneath the injury and extending 800 μm ventral to the injury. Cell counts revealed ~40% of somatostatinergic hilar inhibitory neuron loss (Butler et al., 2012). These experiments were performed in male mice expressing EGFP in a subset of hilar interneurons (GIN mice; FVB-Tg(GadGFP)4570Swn/J) to facilitate cell counts. Since this subset of GABA cells expressed EGFP, we also performed Western blot analysis of EGFP levels one week after injury and found EGFP expression to be significantly reduced in the hippocampus ipsilateral to the injury ($p<0.05$). Contralaterally and at more ventral levels ipsilaterally, hippocampal pathology was not observed. Conclusions: The pathology 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.
3. Determined that CCI increased STAT3 phosphorylation ipsilateral to the injury after 24 hours, but not contralaterally. For these studies, Western blot analyses of hippocampi from CCI-injured and control mice were performed. Both STAT and phosphorylated STAT

(pSTAT) protein expression were compared semi-quantitatively 24 hr after injury. 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 (Butler et al., 2012; Boychuk et al., 2012). These experiments were necessary because, although global pSTAT upregulation has been identified after status epilepticus following pilocarpine treatment in rats (Kim et al., 2012) and after diffuse brain injury following fluid percussion in rats (Oliva et al., 2012), its expression had not been previously documented after <u>focal</u> injury with CCI in mice. Conclusion: The biochemical reaction required to perform further analyses of GABA currents is evident after CCI ipsilateral to the injury, but not contralaterally. This means that the contralateral dentate gyrus can serve as a control for electrophysiological analyses. Determining that pSTAT3 expression was increased and that the increase was ipsilateral to the injury allows for testing of all other hypotheses under this award. It was critical, therefore, to establish this baseline measurement.

4. Determined that treatment at 30 and 90 min after CCI with WP1066 (50 mg/kg) inhibits STAT3 phosphorylation in mice, but does not affect GABA neuron loss. Although preliminary data using WP1066 (100 mg/kg, i.p.) in pilocarpine-treated rats indicated that it was able to suppress pSTAT3 upregulation after status epilepticus, this procedure had not previously been perfomed in mice after CCI. Initially, single doses in mice (100 mg/kg) resulted in unacceptably high mortality (approximately 80%). We then assessed the effect of a single dose of 50 mg/kg and found little if any effect on pSTAT3 production. After consulting with technical support at the drug supplier, we modified our storage and use to essentially use the product within a week of shipment and store aliquots at -80 ^{o}C to ensure full potency. In addition, we settled on a two injection paradigm (50 mg/kg each), delivered at 30 and 90 min post-injury. This delivery successfully suppressed pSTAT3 upregulation after injury (Butler et al., 2012; Boychuk et al., 2012). The WP1066 treatment did not, however, reduce the degree of GABAergic hilar interneuron cell loss ipsilateral to the injury (Butler et al., 2012). Conclusions: These data indicated that the inhibition of pSTAT with WP1066 after CCI was effective, but that GABA neuron loss was not prevented by the drug.

<u>Supporting Data</u>: see **Appendix item 1**, **Hunt et al., 2012** for complete description of CCI parameters resulting in MFS and location of damage after injury (Figure 2). See **Appendix items 2 and 3 and Figure 1 below** showing extent of hilar GABA neuron loss after injury in male mice expressing EGFP in a subset of hilar interneurons (GIN mice; FVB-Tg(GadGFP)4570Swn/J). See **Appendix items 2 and 3 and Figure 2 below** depicting Western blot results indicating increased STAT3 phosphorylation after 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 in our hands.

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).
<u>Status</u>: not yet initiated
<u>Accomplishments</u>: none.
<u>Supporting Data</u>: 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)

<u>Status</u>: 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 in recordings from DGCs in slices from four treatment groups

Accomplishments:

1. Personnel (Graduate student Corwin Butler) fully trained to perform recordings;
2. Preliminary recordings of IPSCs from granule cells in control (n=5) and CCI injured (n=7) mice have been conducted, representing approximately half the number necessary to provide quantitative measurements for meaningful comparison.
3. DEA license application procedure in process.

Supporting Data: none.

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. Timm staining parameters for sham control and CCI-injured established;
2. Timm staining for WP1066-treated are in process.

Supporting Data: See **Appendix item 1, Hunt et al., 2012**, figures 1 and 2. Here, the precise location and extent of Timm staining in control and CCI-injured animals is described.

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: not yet initiated

Accomplishments: none.

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: not yet initiated

Accomplishments: none.

Supporting Data: none

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: not initiated
Accomplishments: none
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. Trained Graduate student Corwin Butler to perform all electrophysiological experiments and analyses.
2. Determined that THIP-induced tonic GABA current in DGCs 6-10 weeks post-injury are reduced in amplitude relative to controls and contralateral DGCs (n=5-8 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 $\alpha1$ vs $\alpha4/\delta$ 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 (3μM) was bath-applied to induce an outward current, ostensibly due to activation of $\alpha4/\delta$ subunit-containing GABA receptors. Bicuculline methiodide (30 μM) was applied to block all GABA receptors and determine the total available tonic GABA current. Conclusions: the change in tonic GABA current suggest receptor subunit reorganization. Effects of pSTAT3 blockade on this effect will proceed as planned.
3. WP1066-treated control and CCI-injured animals have been prepared.
   Supporting Data: see **Figure 3 below** for demonstration of THIP-induced changes in tonic GABA current after 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.

KEY RESEARCH ACCOMPLISHMENTS:

- Established precise injury parameters for CCI in mice that yield consistent and reliable outcome measures. All tasks require the CCI model to be established.
- Trained collaborators and lab trainees. This allowed injury procedures and parameters to be successfully repeatable in multiple labs. All tasks for this lab and collaborators require this to be established.
- In mouse, mossy fiber sprouting in the inner molecular layer is regionally and locally enhanced after CCI in a semi-quantitatively measurable manner, paving the way for assessment of STAT3 inhibition on the response. All tasks require that STAT3 inhibition of mossy fiber sprouting be compared to controls.
- In the mouse, phosphorylated STAT3 levels were increased in the hippocampus ipsilateral to the injury 24 hours after CCI. By one week, there was no appreciable activation. All tasks require the establishment of STAT3 phosphorylation to be effective in this model after CCI.
- In mice, 30 and 90 minute post-treatment of WP1066 inhibits phosphorylation of STAT3 in injured hippocampus 24 hours after CCI. All tasks require controls to demonstrate effectiveness of WP1066.
- THIP-activated tonic GABA currents were reduced in granule cells of the dentate gyrus ipsilateral to the injury 6-10 weeks after CCI in mice. Task 3 specifically requires comparison of THIP currents in control and CCI-injured mice to compare with WP1066-treated.

REPORTABLE OUTCOMES: manuscripts, abstracts, presentations

1. Hunt, R.F., Haselhorst, L.A., Sc hoch, K.M., Bach, E.C., Rios-Pilie r, J., Scheff, S.W., Saat man, K.E., and Sm ith, B.N. (2012) Posttraumatic mossy fiber spr outing is related to the degree of cortical damage in three mouse strains. *Epilepsy Res.* 99:167-170. This paper reports on the precise injury parameters that result in epileptogenic phenotype, including mossy fiber sprouting, in mice. In addition, the extent and location of mossy fiber sprouting is documented.
2. Butler CR, Boychuk, JA, Raible, DJ, Frey, L, Brooks-Kayal, AR, and Smith BN (2012) JAK/STAT Activation and GABA neuron loss after focal traumatic brain injury in mice. *Soc. Neurosci. Abs.*, 38:247.03. This abstract reports th at STAT3 phosphorylation is increa sed ipsilateral to the injury after CCI and that system ic treatment with WP1066 inhibits the pSTAT3 upregulation. A subset of hilar GABA neurons a re also lost ip silateral to th e injury. These data w ere presented at the 201 2 Annual Meeting of the Society for Neuroscience in New Orleans, LA in October 2012.
3. Boychuk JA, Butler, CR, Raible, DJ, Frey, L, Brooks-Kayal, AR, and Sm ith BN (2012) Focal traumatic brain damage results in localized GABA neuron loss and JAK/STAT activation early following injury. *Epilepsy Curr*. 13. This abstract re ports that STAT3 phosphor ylation is increased ipsilateral to the injury after CCI and that syst emic treatment with WP 1066 inhibits the pSTAT3 upregulation. A subset of hilar GABA neurons are also lost ipsilateral to the injury. These data will be presented at the 201 2 Annual Meeting of the Am erican Epilepsy Society in San Diego, CA in December 2012.
4. Raible, D, Frey, L, Boychuk, J, Butler CR, Grabenst atter, H, Cruz Del Anges, Y., Russek, S., Smith B, and Brooks-Kayal, A. (2012) Ja K/STAT inhibition to prevent po st-traumatic epileptogenesis. Gordon Research Conference abstract. This abstract reports that TBI in ra ts (FPI) resulted in transcriptional changes in GABA(A) recep tor subunit expression. This data was presented at the Gordon Research Conference on Epilepsy and Neuronal Synchronization in August 2012.

CONCLUSIONS

In the first year of DOD CDMRP funding, the CCI model has been successfully established in mice at both University of Colorado and University of Kentucky, making future experiments feasible. Essential baseline control data has been obtained on optimal techniques for molecular, anatomical, and electrophysiological analysis in the mouse CCI model, and on STAT phosphorylation, mossy fiber sprouting, and cell death alterations following TBI. Specific outcome measures to assess the rigor of the model in individual experiments for molecular, electrophysiological, and anatomical studies have been established. Training has been completed for all personnel on all necessary techniques. Parameters of controls for several experiments have been established. Parameters in CCI-injured mice were also established. Moreover, issues with the JAK/STAT3 inhibitor (WP1066) have been resolved and the drug is now used successfully. Preliminary experiments on IPSC measurements in control and CCI-injured mice are underway. Importantly, experiments on THIP-activated GABA currents indicate that these currents are reduced after 6-10 weeks in dentate granule cells ipsilateral to CCI injury, suggesting receptor subunit reorganization coincident with epileptogenesis. No specific changes in the proposed experiments are suggested.

REFERENCES

Butler CR, Boychuk, JA, Raible, DJ, Frey, L, Brooks-Kayal, AR, and **Smith BN** (2012) JAK/STAT Activation and GABA Neuron Loss After Focal Traumatic Brain Injury in Mice. *Soc. Neurosci. Abs.*, 38:247.03

Boychuk JA, Butler, CR, Raible, DJ, Frey, L, Brooks-Kayal, AR, and **Smith BN** (2012) Focal traumatic brain damage results in localized GABA neuron loss and JAK/STAT activation early following injury. *Epilepsy Curr*. 13:in press.

Hunt, R.F., Haselhorst, L.A., Schoch, K.M., Bach, E.C., Rios-Pilier, J., Scheff, S.W., Saatman, K.E., and Smith, B.N. (2012) Posttraumatic mossy fiber sprouting is related to the degree of cortical damage in three mouse strains. *Epilepsy Res.* 99:167-170.

Kim JH, Roberts DS, Hu Y, Lau GC, Brooks-Kayal AR, Farb DH, Russek SJ. (2012) Brain-derived neurotrophic factor uses CREB and Egr3 to regulate NMDA receptor levels in cortical neurons. J Neurochem. 120:210-9.

Oliva, AA, Kang, Y, Sanchez-Molano, J, Furones, C, and Atkins, CM (2012) STAT3 signaling after traumatic brain injury. J. Neurochem. 120:710-720.

APPENDICES

1) Hunt, R.F., Haselhorst, L.A., Schoch, K.M., Bach, E.C., Rios-Pilier, J., Scheff, S.W., Saatman, K.E., and **Smith, B.N.** (2012) Posttraumatic mossy fiber sprouting is related to the degree of cortical damage in three mouse strains. *Epilepsy Res.* 99:167-170.
2) Butler CR, Boychuk, JA, Raible, DJ, Frey, L, Brooks-Kayal, AR, and **Smith BN** (2012) JAK/STAT Activation and GABA Neuron Loss After Focal Traumatic Brain Injury in Mice. *Soc. Neurosci. Abs.*, 38:247.03
3) Boychuk JA, Butler, CR, Raible, DJ, Frey, L, Brooks-Kayal, AR, and **Smith BN** (2012) Focal traumatic brain damage results in localized GABA neuron loss and JAK/STAT activation early following injury. *Epilepsy Curr*. 13:abstract in press.

SUPPORTING DATA:

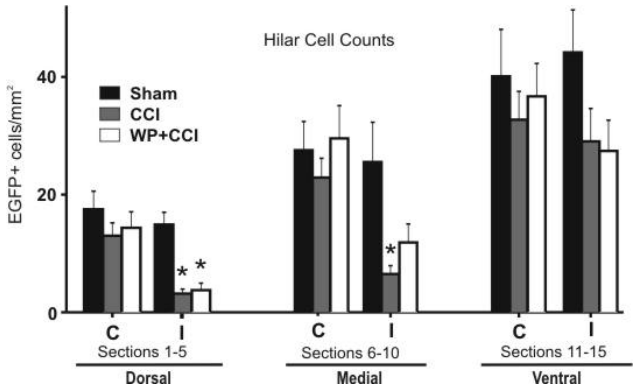

Figure 1: Hilar interneuron counts in Sham, CCI, and CCI with WP1066 treatment. Cell density was reduced in dorsal, but not ventral, hippocampus after CCI. WP1066 treatment did not prevent the cell loss after CCI (ANOVA, Tukey's post hoc; $p<0.05$).

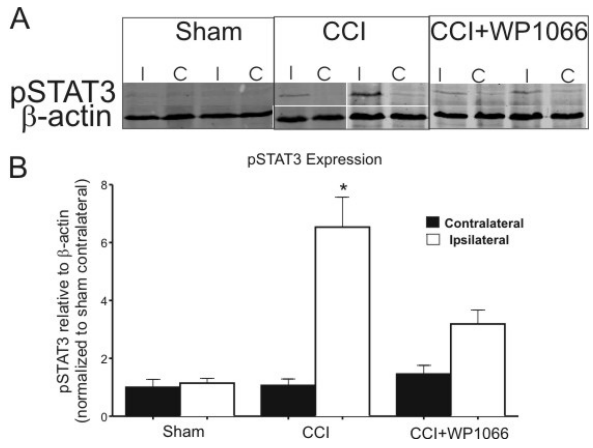

Figure 2: Western blot and representative bar graph of pSTAT3 expression relative to β-actin content in CCI, Sham, and CCI with WP1066 treatment. Expression of pSTAT3 is significantly increased in the ipsilateral hemisphere of CCI hippocampus compared to Sham 24 hr after injury. Expression of pSTAT3 was inhibited after WP1066 treatment relative to CCI ($P<0.05$; ANOVA, Tukey's post hoc), and was similar to levels seen in Shams ($P>0.05$; ANOVA, Tukey's post hoc).

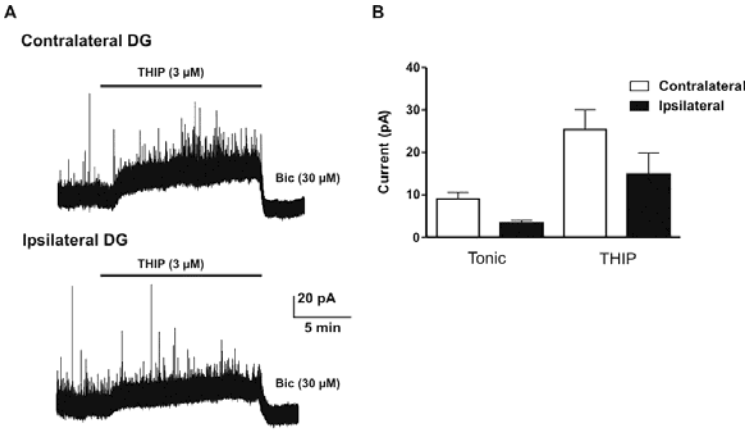

Figure 3. THIP-activated, tonic GABA currents are reduced in dentate granule cells after CCI. A. Representative traces of DGCs located contralateral (upper left) or ipsilateral (lower left) to head injury with CCI. DGCs were voltage clamped at 0 mV (close to the reversal of glutamatergic currents) and recorded in three phases: baseline, THIP (3 μM) and Bicuculline (Bic; 30 μM). **B**. Group data of tonic GABA current and THIP responses of DGCs located either contralateral or ipsilateral to CCI.

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SHORT COMMUNICATION

Posttraumatic mossy fiber sprouting is related to the degree of cortical damage in three mouse strains

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Epileptogenesis;
Epilepsy model;
Seizure;
Traumatic brain injury

Summary Controlled cortical impact injury was used to examine relationships between focal posttraumatic cortical damage and mossy fiber sprouting (MFS) in the dentate gyrus in three mouse strains. Posttraumatic MFS was more robust when cortical injury impinged upon the hippocampus, versus contusions restricted to neocortex, and was qualitatively similar among CD-1, C57BL/6, and FVB/N background strains. Impact parameters influencing injury severity may be critical in reproducing epilepsy-related changes in neurotrauma models.

Introduction

Mossy fiber sprouting (MFS) into the inner molecular layer of the dentate gyrus is a consistent marker of the epileptic dentate gyrus after traumatic brain injury (TBI) in humans (Swartz et al., 2006) and animals (Kharatishvili et al., 2006; Hunt et al., 2009, 2010, 2011). MFS is generally more robust after severe versus mild TBI (Santhakumar et al., 2000; Kharatishvili et al., 2006, 2007; Hunt et al., 2009), but responses in posttraumatic animals can be highly variable. We recently described the development of posttraumatic epilepsy (PTE) and localized, robust MFS and synaptic reorganization 6—12 weeks after controlled cortical impact (CCI) injury in mice (Hunt et al., 2009, 2010). However, background strain may influence cellular events and seizure thresholds in mice after TBI (Chrzaszcz et al., 2010). Other studies detected only mild mossy fiber reorganization in posttraumatic mice at similar time points after injury (Hanell et al., 2010). These findings could be due to considerable technical differences among laboratories or high variability in the degree of cortical damage in individual animals. Tissue responses produced after CCI injury depend greatly on external injury parameters (i.e., impact depth

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and velocity, impactor shape and size, and number of craniotomies) (Mao et al., 2010; Pleasant et al., 2011). While CCI is increasingly used to model epilepsy-related changes after TBI, the parameters of focal cortical damage necessary to consistently reproduce MFS in posttraumatic animals is unknown.

Methods

All procedures were approved by the University of Kentucky Animal Care and Use Committee and adhered to NIH guidelines for the care and use of laboratory animals. Six- to ten-week-old CD-1 (Harlan), C57BL/6 (The Jackson Laboratory), or FVB/N (The Jackson Laboratory) mice were subjected to a unilateral cortical contusion by CCI injury as previously described (Hunt et al., 2009, 2010, 2011). We chose these strains because they display different cellular responses in status epilepticus models (Schauwecker and Steward, 1997), and/or are often used in transgenic studies. Severe brain injury was delivered using an electronically controlled, pneumatically driven impactor fitted with a stainless steel tip 3 mm in diameter (Precision Systems and Instrumentation, Fairfax, VA) to compress the cortex to a depth of 1.0 mm (or, for C57BL/6 mice only, 1.2 mm), at 3.5 m/s and 400—500 ms duration. Mice were injured with two differently shaped impactor tips, beveled or rounded, to achieve a variable degree of cortical damage. Impactor shape is an important determinant for CCI-induced cortical damage (Mao et al., 2010; Pleasant et al., 2011) and has been a common variation among studies examining epilepsy-related changes after CCI. A subset of injured CD-1 mice was monitored for injury-induced behavioral seizures during a 90 min interval beginning 90 min post-injury. Seizure severity was scored from 1 to 5, according to a modified Racine scale (Hunt et al., 2009, 2010).

Mice were perfused with 0.37% sodium sulfide in 0.1 M $NaHPO_4$ followed by 4% paraformaldehyde in 0.15 M phosphate buffer. Brains were cryoprotected with 30% sucrose in 0.01 M phosphate-buffered saline; 20 μm coronal brain sections were cut on a cryostat and collected at 400 μm intervals. Timm's and Nissl staining was performed as previously described to visualize mossy fibers and cell bodies (Shibley and Smith, 2002) ipsilateral and contralateral to the injury. Timm's scores were plotted with respect to the distance of each section from the injury epicenter, which was qualitatively defined as the section with the most extensive cortical damage. Scores for sprouting were assigned from 0 to 3 based on the rating scale of Tauck and Nadler (1985). If Timm's staining between the blades of the granule cell layer was variable, an averaged score was used (e.g., if the lower blade was scored 1 while the upper blade was scored 2, the section was given an overall grade of 1.5). MFS was defined as at least one section with a Timm's score >1 (Hunt et al., 2009, 2010, 2011).

Data were analyzed using Microsoft Excel and Instat3 programs. Numerical data are presented as the mean ± SD. The nonparametric Chi square or Kruskal—Wallis test with Dunn's post hoc tests were used to analyze Timm score differences between groups. Mann—Whitney *U* was used to examine differences between pairs. Significance was set at $P < 0.05$.

Results

Gross damage 8—12 wks after CCI consisted of a cortical cavity 3 mm in diameter extending through the thickness of the neocortex at the injury epicenter, located midway between lambda and bregma, 5 mm lateral to midline. In all mice injured with a rounded-tip impactor, the cortical cavity at the injury site was restricted to the neocortex ($n = 5$ CD-1; $n = 9$ FVB; $n = 12$ C57BL/6). In most mice injured with a beveled tip, a variably sized cavity extended into the hippocampus (260—1070 μm^3), accompanied by hippocampal distortion extending 300—1600 μm from the injury epicenter ($n = 18$ of 20 CD-1; $n = 18$ of 23 FVB; $n = 7$ of 10 C57BL/6). These results are consistent with recent studies demonstrating greater hippocampal damage after injuries administered using beveled versus rounded-tip impactors (Mao et al., 2010; Pleasant et al., 2011).

MFS was detected ipsilateral to the injury in all three strains of posttraumatic mice. In contrast, none of the hippocampi contralateral to the injury had abnormal mossy fiber organization (i.e., all Timm scores were ≤1). The degree of hippocampal distortion and pattern of MFS were variable (Fig. 1). The most robust Timm's staining was always found within 800 μm of the injury epicenter toward the ventral pole (Fig. 2).

All mice, regardless of strain, in which the cortical cavity impinged upon the hippocampus had MFS into the inner molecular layer ipsilateral to the injury (CD-1, $n = 18$ of 18; FVB, $n = 18$ of 18; C57BL/6, $n = 7$ of 7). In mice where the cavity was restricted to the neocortex, MFS was observed ipsilateral to the injury in 57% of CD-1 ($n = 4$ of 7), 29% of FVB ($n = 4$ of 14), and 27% of C57BL/6 ($n = 4$ of 15) mice, with no detectable difference between strains ($X^2 = 2.241$, d.f. = 2, $P = 0.33$). We evaluated ''peak'' Timm scores in sections that were 400 μm ventral to the injury epicenter to examine whether damage to the hippocampus was associated with greater MFS. For this analysis, we compared hippocampi ipsilateral to the injury, in mice with and without a cavity into the hippocampus, with contralateral hippocampi. A Kruskal—Wallis test detected a significant difference in Timm score ranges among groups for each strain (CD-1: $H_{(2, 49)} = 38.58$, $P < 0.001$; FVB: $H_{(2, 61)} = 47.21$, $P < 0.001$; C57BL/6: $H_{(2, 43)} = 24.79$, $P < 0.001$; Fig. 2E). Post hoc analysis revealed that ipsilateral hippocampi had higher Timm scores than contralateral hippocampi for all strains, regardless of the extent of cortical damage. However, Timm scores were greater in mice in which the cortical cavity included portions of the hippocampus versus mice in which the cavity was restricted to the neocortex, regardless of strain. No difference was detected in the time post-TBI in which MFS was evaluated between mice with (9.86 ± 1.1 wks) and without (9.22 ± 0.8 wks) a cavity into the hippocampus for any strain ($P > 0.05$).

The development of MFS after pilocarpine administration in mice relates to seizure number induced during status epilepticus (Shibley and Smith, 2002). Therefore, we evaluated whether Timm's scores were greater in CD-1 mice that displayed immediate injury-induced behavioral seizures versus mice that did not have seizures. Five mice displayed immediate seizures after TBI (one to four seizures/mouse; category 2—5) and had an average Timm's score of 2.2 ± 0.27. Mice that did not have immediate seizures had an average Timm's score of 2.2 ± 0.53 ($n = 20$; $P > 0.05$). Immediate seizures after TBI did not predict the development of posttraumatic MFS.

Discussion

Our finding that MFS is increased in mice with posttraumatic hippocampal cavitation is consistent with previous

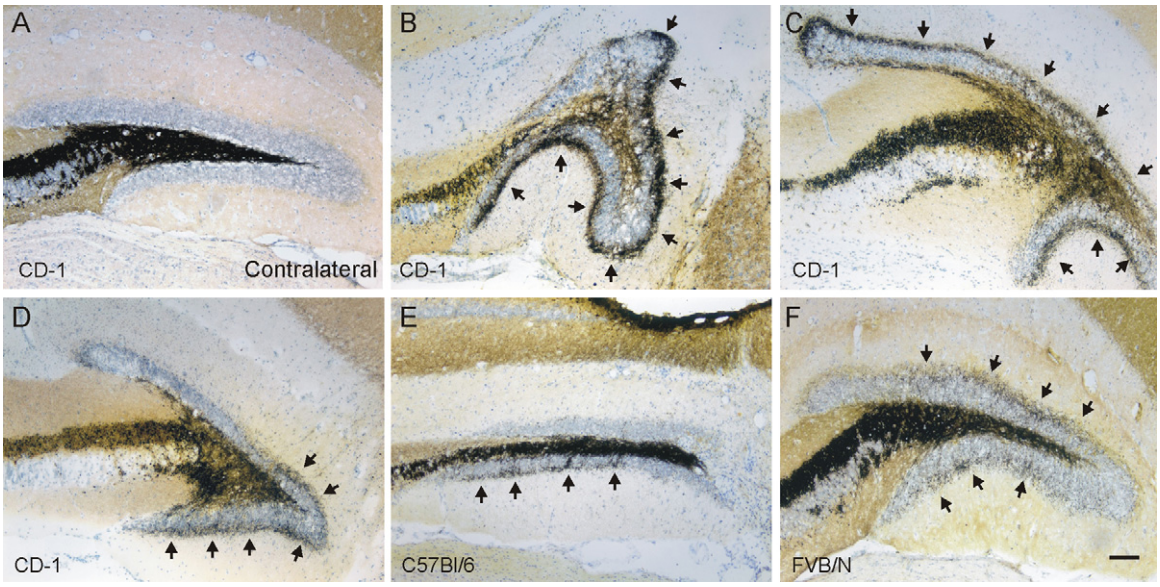

Figure 1 MFS after cortical contusion injury is not uniform. Example Timm's and Nissl stained sections of the dentate gyrus 8—12 wk after CCI injury. (A) Representative image of hippocampus contralateral to the injury shows the absence of mossy fiber sprouting in the inner molecular layer (Timm score = 0). (B—F) Representative images of Timm's staining in the ipsilateral dentate gyrus near the injury epicenter. Note that the pattern of MFS and distortion of the granule cell layer is different in each section. MFS into the inner molecular layer is indicated by arrows. (B—D) Sections obtained from CD-1 mice. Timm scores for these sections are B, 2; C, 3; D, 2. E. Section from a C57BL/6 mouse (Timm score = 1.5). F. Section from an FVB mouse (Timm score = 2). Scale bar is 100 μm.

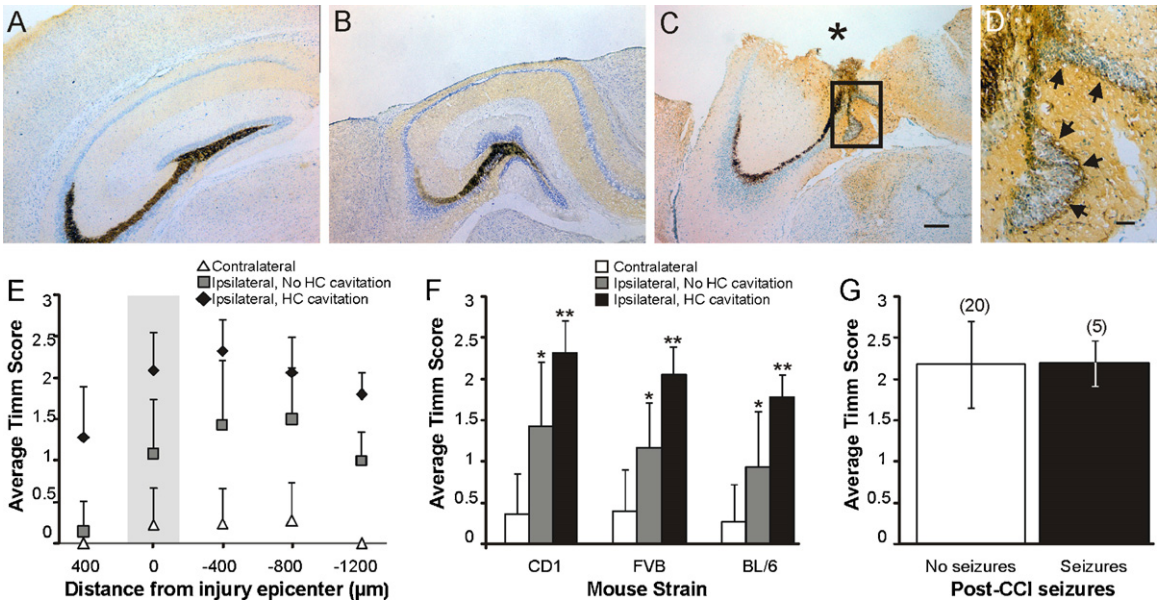

Figure 2 Timm scores are greater in mice with cortical cavitation that enters the hippocampus (HC). (A) Image of Timm's and Nissl stained contralateral dentate gyrus. (B) Image of Timm's and Nissl stained ipsilateral dentate gyrus at the injury epicenter. Note that the cortical cavity does not include portions of the hippocampus. (C) Image of Timm's and Nissl stained ipsilateral dentate gyrus at the injury epicenter in an animal where the cortical cavity extended into the hippocampus (asterisk). The contralateral hippocampus from this mouse is shown in (A). (D) Enlarged image of the boxed area in (C) shows MFS into the inner molecular layer (arrows). Scale bar is 200 μm in (A—C) and 50 μm in (D); sections from CD-1 mice shown in (A—D). (E) In CD-1 mice, average Timm score in relation to the distance from the injury epicenter (zero on the *x*-axis), septal (400 μm) to temporal (−1200 μm). (F) Average ''peak'' Timm score at −400 μm for each group in CD-1, FVB, and C57BL/6 (BL/6) mice. Asterisk indicates significant difference from slices contralateral to the injury. Double asterisk indicates significant difference from both contralateral hippocampi and ipsilateral hippocampi without hippocampal cavity. (G) Timm scores 8—12 weeks after injury are not greater in mice observed to have behavioral seizures in the first 90 min post-TBI, versus mice in which seizures were not observed. Number of mice in each category is shown in parentheses.

reports describing greater spontaneous seizure incidence after severe CCI using a beveled impactor (36—40%; Hunt et al., 2009, 2010) versus rounded-tip impactors (9—13%; Bolkvadze et al., 2009; Statler et al., 2009). Hippocampal damage was more likely with beveled tips. Injuries without hippocampal cavitation resulted in less prevalent MFS, despite similar impact depth. In addition to impact parameters, rodent species, animal age, or injury location, might also affect MFS and seizure incidence after CCI injury.

The relatively low seizure incidence in PTE models suggests the need for surrogate biomarkers. We found that the degree of neocortical damage might be a less useful predictor of posttraumatic MFS than is hippocampal cavitation; all mice with hippocampal cavitation developed sprouting. Why MFS occurs is controversial. Among potential triggers include hilar or hippocampal cell loss, neurogenesis, and growth factor overexpression, all of which occur after CCI. Correlation of MFS with these parameters may be useful for identifying other key features of posttraumatic epileptogenesis. While MFS is qualitatively related to epileptogenesis, it does not correlate quantitatively with seizure frequency or severity in temporal lobe epilepsy models (Buckmaster and Dudek, 1997). MFS ipsilateral to TBI might be related to posttraumatic EEG spike activity (Kharatishvili et al., 2007); MRI markers have been used to evaluate brain damage after brain injury in rodents (Kharatishvili et al., 2007, 2009; Onyszchuk et al., 2007). Perhaps the presence of hippocampal damage could serve as a biomarker for animals with the highest probability for developing epilepsy.

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JAK/STAT Activation and GABA Neuron Loss After Focal Traumatic Brain Injury in Mice

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Traumatic brain injury (TBI) is among the most common causes of acquired temporal lobe epilepsy (TLE). The latent period after head injury and prior to the expression of seizures includes plasticity events that support epileptogenesis, including cell loss and synaptic reorganization in the dentate gyrus. A murine model of TBI using controlled cortical impact (CCI) was used to examine aspects of hilar GABA systems days after injury and the effect of JAK/STAT inhibition on those systems. Western blots were used to assess expression levels of pStat3 as an indicator of JAK/STAT activation in CD-1 mice at 24 hours after CCI or in sham-operated controls. The effectiveness of the Stat3 inhibitor WP1066 (EMD Millipore; 50mg/kg; i.p.; 30 and 90 min post-CCI) on blocking JAK/STAT activation was also tested. The number of surviving GABAergic hilar interneurons was assessed in mice that express GFP in a subset of inhibitory neurons (GIN mice; FVB-Tg(GadGFP)4570Swn/J) 48-72 hours after CCI or in controls. Preliminary results show that CCI results in a dramatic increase in pStat3 protein expression within the injured hemisphere. Low expression of pStat3 was detected in the uninjured hemisphere after CCI, equivalent to that detected in sham-operated controls. Administration of the WP1066 after CCI reduced pStat3 expression to levels similar to those observed in control mice**.** Hilar inhibitory interneurons were reduced in number ipsilateral to the injury in the dorsal hippocampus. Ongoing studies plan to assess the effects of WP1066 on GABAergic hilar interneuron cell loss and how inhibitory synaptic transmission is affected in dentate granule cells during these early time-points following CCI.

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Focal traumatic brain damage results in localized GABA neuron loss and JAK/STAT activation early following injury

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Rationale

Traumatic brain injury (TBI) is a leading cause of acquired temporal lobe epilepsy (TLE), yet the mechanisms underlying posttraumatic epileptogenesis are not known. TBI is associated with cell loss and changes in cellular and synaptic signaling in cortical structures. Activation of the JAK/STAT3 pathway has been implicated as a participant in the reactive plasticity associated with epileptogenesis. Here, a murine model of TBI that results in spontaneous seizures was used to examine aspects of hippocampal GABA network modification shortly after brain injury. We tested the hypothesis that regional JAK/STAT3 pathway modulation and hilar GABAergic interneuron loss occurs shortly after focal brain injury.

Methods

Severe controlled cortical impact (CCI; impact depth= 1.0 mm) was administered to male mice at 6-8 weeks of age. 24 hours post- injury, hippocampi from male CD-1 mice were isolated and processed for western blot analysis of STAT3 and pSTAT3 proteins, with the latter used as a marker of JAK/STAT3 activation. The effectiveness of the STAT3 inhibitor WP1066 (EMD Millipore; 30 and 90 min post-CCI; 50mg/kg; i.p.) on blocking JAK/STAT3 activation was also tested. Hilar GABA cell loss 2-3 days following CCI was examined in male mice expressing GFP in a subset of hilar interneurons (GIN mice; FVB-Tg(GadGFP)4570Swn/J). Coronal sections (30 μm) were sampled in a 1 in 5 series. GFP-positive cell counts and hilus area were analyzed using a laser scanning confocal microscope (Zeiss, LSM 5 LIVE).

Results

At 24 hours following CCI an increase in hippocampal pSTAT3 protein expression ipsilateral to the injury was observed, relative to either the contralateral hemisphere or Sham-operated controls. Both Sham-operated controls and the contralateral hemisphere of injured animals exhibited low levels of hippocampal pSTAT3 protein expression. Administration of the STAT3 inhibitor WP1066 inhibited pSTAT3 protein expression in the hippocampus ipsilateral to the injury, but had little effect on the contralateral hemisphere of injured animals or Sham operated controls. Quantification of GFP positive cells from GIN mice revealed a decrease in the number of hilar interneurons within dorsal hippocampus ipsilateral to the injury relative to the contralateral hemisphere or to Sham-

operated controls. Preliminary data indicate that administration of WP1066 did not inhibit hilar GABA cell loss ipsilateral to the injury.

Conclusions

Early time-points following brain injury with CCI are associated with changes in hippocampal GABA networks. These changes are prominent in the hippocampus ipsilateral to the injury and include a loss of hilar GABAergic interneurons as well as activation of the JAK/STAT signaling pathway. Previous studies have associated both of these changes with alterations in GABA signaling associated with TLE. Alterations in hippocampal GABA neuron function following head injury may support the eventual expression of spontaneous seizures in posttraumatic epilepsy.