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TITLE:
Novel Mechanism for Reducing Acute and Chronic Neurodegeneration After Traumatic Brain Injury

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CONTRACTING ORGANIZATION:
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14. ABSTRACT

Purpose: The purpose of this project is to develop a radically different strategy to reduce brain glutamate excitotoxicity and treat TBI. We will supplement a natural blood-resident enzymatic system with glutamate-oxaloacetate transaminase (rGOT) and the co-substrate oxalo-acetate (OxAc) with the objective of reducing blood levels of glutamate. This will produce a brain-to-blood gradient of glutamate which will enhance the removal of excess glutamate from the brain.
Scope: We will test this novel and powerful neuroprotective treatment in a rat model of repetitive mild (concussive) TBIs and in a model of a single moderate TBI.
Major Findings: We have:

1. Troubleshooting and refinement of blood serum glutamate assays to reduce variability.
2. Completed statistical analysis of behavioral experiments examining effects of rGOT and rGOT + OxAc on outcome on rotarod and Morris water maze.
3. Measured time course of GOT levels in blood and levels after iv injection of 130ug/kg of rGOT.
4. Completed sectioning of brain tissue and completed 60% of hippocampal neuronal cell counting.
5. Troubleshooting and refinement of CSF extraction resulting in reliable measurement of glutamate in CSF.

15. SUBJECT TERMS

Nothing listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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Introduction:

Traumatic brain injury (TBI) continues to be a major problem and has affected hundreds of thousands of service personnel who have served in the Mideast war theater. Many of these personnel have sustained repeated mild or concussive brain injury and now suffer from long-lasting cognitive and physical symptoms. We have developed a radically different strategy to reduce brain glutamate excitotoxicity and treat TBI. We will supplement a natural blood-resident enzymatic system with glutamate-oxaloacetate transaminase (rGOT) and the co-substrate oxaloacetate (OxAc) with the objective of reducing blood levels of glutamate. This will produce a brain-to-blood gradient of glutamate which will enhance the removal of excess glutamate from the brain. We will test this novel and powerful neuroprotective treatment in a rat model of a single moderate TBI and in a rat model of repetitive mild (concussive) TBIs. Outcome measures include blood and CSF levels of glutamate, acute neuronal degeneration, chronic neuronal cell loss and glial activation. The objective of this project is to evaluate a novel treatment strategy for reducing excessive free glutamate associated with TBI.

This project uses a highly innovative approach to address the long-recognized problem of glutamate excitotoxicity associated with TBI. This novel approach supplements a natural enzymatic system that transforms blood-borne glutamate into α-ketoglutarate. By significantly reducing blood levels of glutamate, a brain-to-blood gradient is produced that enhances the efficiency of Na^+-dependent glutamate transporters located on brain endothelial cells. Thus, excess glutamate in the brain is transported into blood. Compared to the more traditional methods of reducing glutamate excitotoxicity, treatment with rGOT and OxAc circumvents the problems of unwanted side-effect of glutamate antagonists and poor blood-brain barrier penetration associated with receptor antagonist treatments.

Keywords:
Traumatic Brain Injury, Glutamate, GOT enzyme, Oxaloacetate, Fluid percussion, Morris water maze, Rotarod, Behavior

Accomplishments:

What were the major goals of the project?

The major goals of this project are to address the following series of related hypotheses.

- Intravenous administration of rGOT will significantly reduce the concentration of glutamate in blood and subsequently in CSF after TBI
- Treatment with rGOT will reduce functional deficits associated with TBI
- Treatment with rGOT will reduce neural and glial pathology associated with TBI

What was accomplished under these goals?

1. Troubleshooting and refinement of blood serum glutamate assays to reduce variability.
2. Completed statistical analysis of behavioral experiments examining effects of rGOT and rGOT + OxAc on outcome on rotarod and Morris water maze.
3. Measured time course of GOT levels in blood and levels after iv injection of 130ug/kg of rGOT.
4. Completed sectioning of brain tissue and completed 60% of hippocampal neuronal cell counting.
5. Troubleshooting and refinement of CSF extraction resulting in reliable measurement of glutamate in CSF.

Effects of treatment on rotarod motor deficits after moderate TBI:

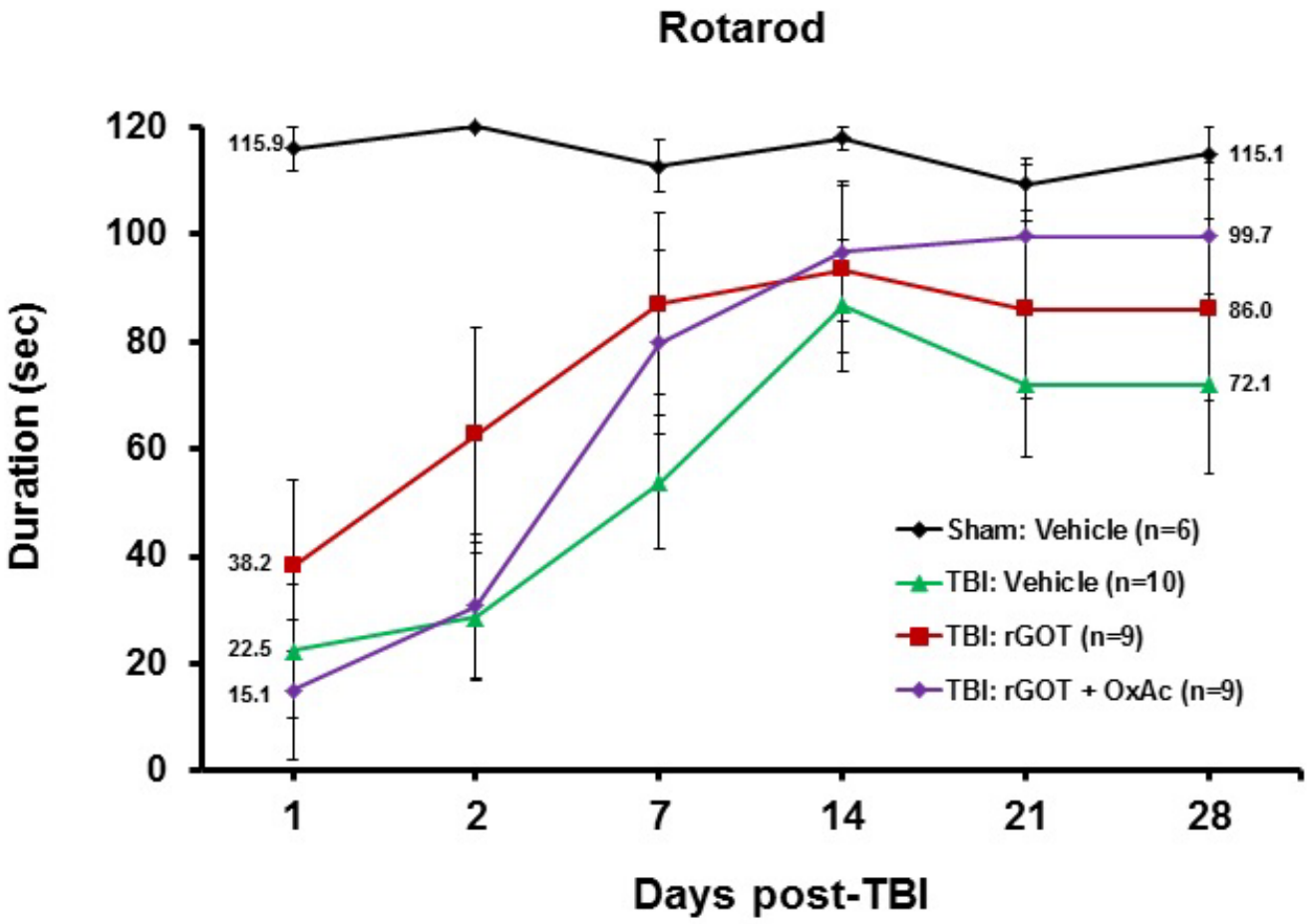
The descriptive characteristics of the four groups (sample size, body weight, injury magnitude, and temperatures) are listed in Table 1. All injury groups received nearly identical levels of fluid percussion injury measured in atmospheres (ATMs). Body weights were not significantly different between groups on the day of injury. Brain temperature, indirectly measured via a needle thermistor placed in the temporalis muscle, and rectal body temperature were all within normal ranges and did not differ between groups.

We have carefully verified all rotarod data entry into SPSS statistical program against individual data sheets for each animal. We have completed the statistical analysis of the verified data. The Sham-TBI group performed consistently near the maximum 120 seconds per trial over the 28 day testing period. TBI caused impairment of performance in sensorimotor functions assessed with the Rotarod test as evident by reduced durations (Figure 1). In general, animals in the three TBI groups showed progressive improvements in motor functions over the 28 day testing period. Repeated measure ANOVA revealed a significant group effect ($p<0.05$) and a significant group X day interaction ($p<0.01$). Post hoc Dunnett's analysis indicated that the performance of the TBI rGOT group was not significantly different from the sham group. The TBI vehicle and the TBI rGOT + OxAc groups were significantly different from the sham group. Thus, the rGOT treatment provided the greatest benefit against motor deficits associated with moderate TBI.

	Weight (g)	ATM	Temporalis Temp.		Rectal Temp.	
			Pre	Post	Pre	Post
Sham (n=6)	333 ± 17	n/a	35.4 ± 0.06	35.3 ± 0.10	36.8 ± 0.41	36.8 ± 0.39
TBI + Vehicle (n=10)	340 ± 21	2.13 ± 0.01	35.8 ± 0.31	35.4 ± 0.31	37.1 ± 0.29	37.1 ± 0.38
TBI + rGOT (n=9)	339 ± 18	2.15 ± 0.01	35.9 ± 0.36	35.5 ± 0.50	37.1 ± 0.39	37.3 ± 0.46
TBI + rGOT + Oxal (n=9)	326 ± 21	2.15 ± 0.02	36.0 ± 0.33	35.5 ± 0.92	37.3 ± 0.35	37.2 ± 0.54

Table 1. Groups, Sample size, Body weight, ATM, Temporalis and Rectal temperatures (means ± SD)

Figure 1



$_{RM}$ANOVA

Group	p = 0.013
Group X Day	p < 0.001

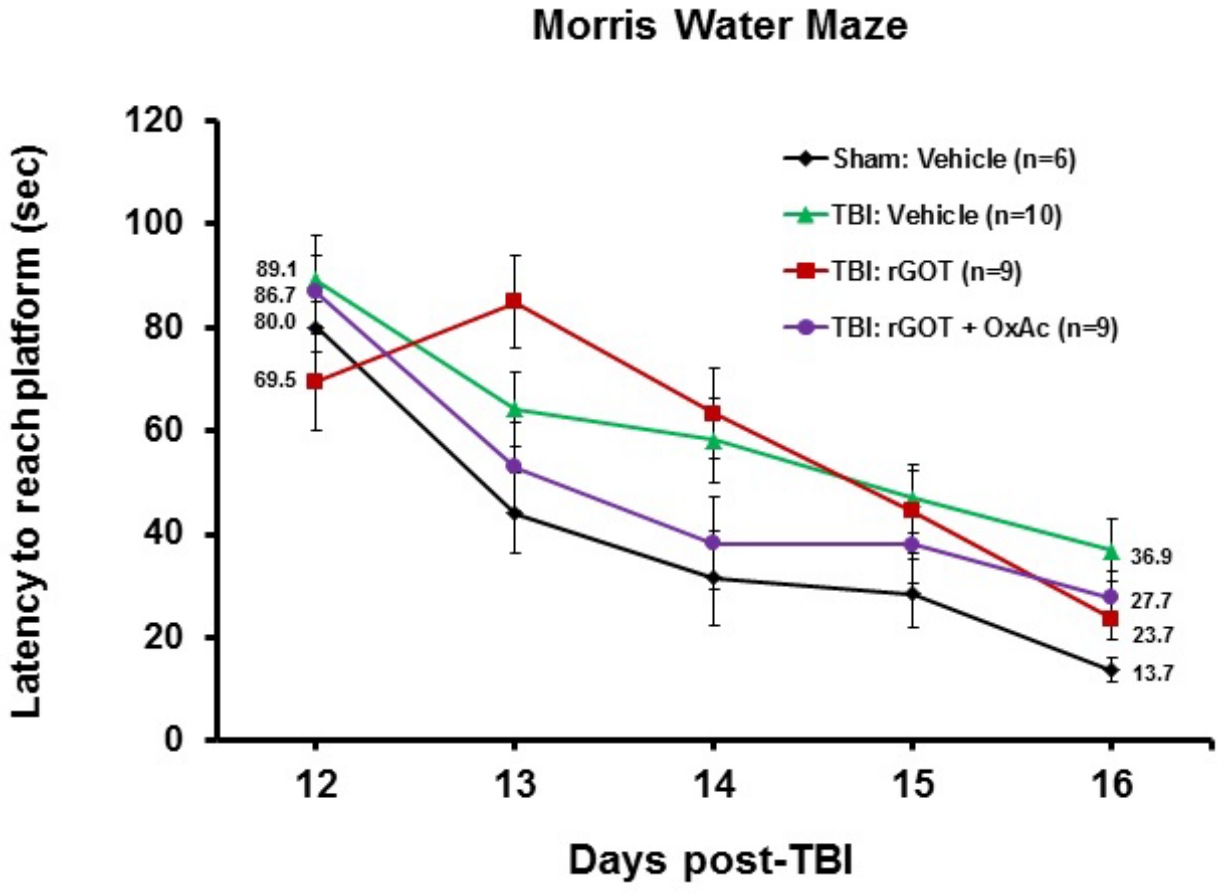
Dunnett's *post hoc*

Sham vs. TBI: vehicle	p = 0.006
Sham vs. TBI: rGOT	p = 0.107
Sham vs. TBI: rGOT+OxAc	p = 0.020

Effects of treatment on MWM cognitive deficits after moderate TBI:

We have carefully verified all MWM data entry into SPSS statistical program against individual data sheets for each animal. We have completed the statistical analysis of the verified data. In general, animals in all four groups showed progressive improvements in cognitive function over days 12 - 16 post-injury. Repeated measure ANOVA revealed a significant group effect ($p<0.05$) and a significant group X day interaction ($p<0.05$). Post hoc Dunnett's analysis indicated that moderate TBI produced a significant spatial memory deficit as evidenced by a significant difference between the TBI + vehicle treated animals when compared to the sham group ($p<0.05$). The performance of the TBI rGOT+OxAc group was not significantly different from the sham group. The TBI rGOT group was significantly different from the sham group. Thus, the GOT + OxAC treatment provided the greatest benefit for reducing cognitive deficits associated with moderate TBI (Figure 2).

Figure 2



RMANOVA

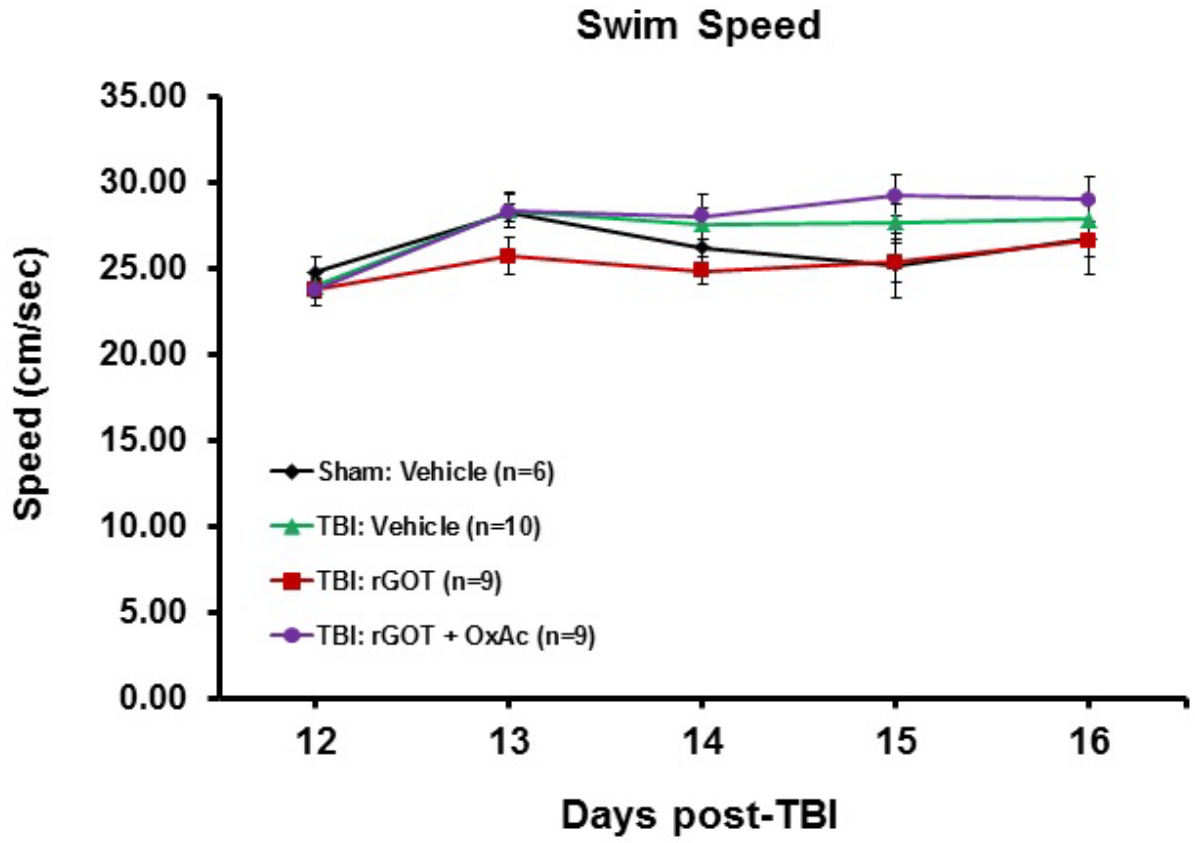
Group	p = 0.024
Group X Day	p = 0.034

Dunnett's *post hoc*

Sham vs. TBI: vehicle	p = 0.015
Sham vs. TBI: rGOT	p = 0.034
Sham vs. TBI: rGOT+OxAc	p = 0.37

The average swim speed did not differ significantly between groups (Figure 3) indicating that interpretation of cognitive deficits was not confounded by motor deficits affecting swim performance.

Figure 3



Effects of rGOT and rGOT + OxAc on chronic histology following moderate TBI:

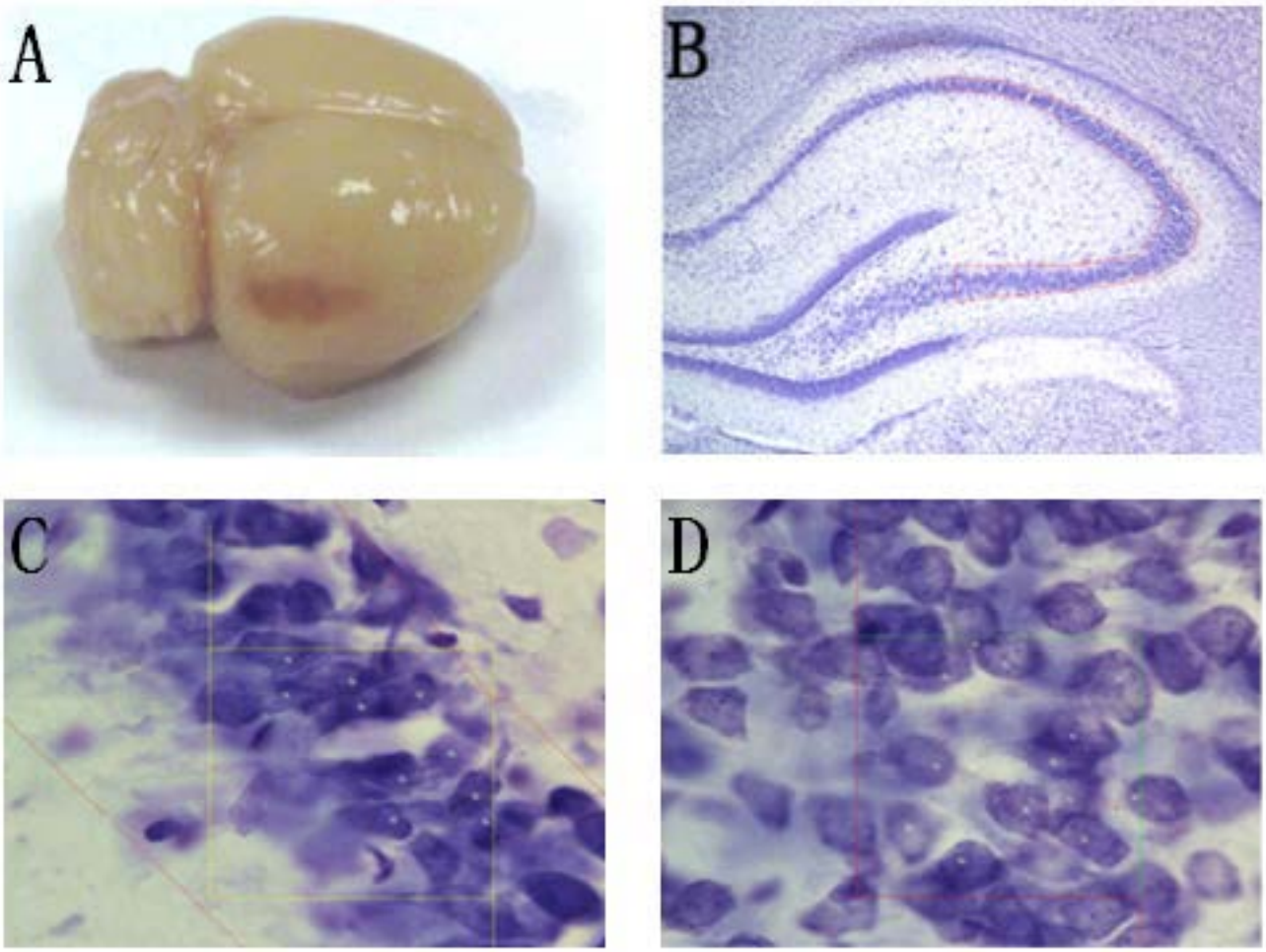
We are making strong progress on this task. We have sectioned and mounted all tissue for histological staining for the four groups listed in Table 1. We have completed staining of the tissue with cresyl violet and are now in the process of completing stereological cell counts of surviving neurons in the hippocampus.

Anatomical Regions of Interest and Stereological Cell Counting Methods

The region of interest for measurement of surviving neuronal cells encompassed the dorsal stratum pyramidale of the hippocampus CA2 and CA3 (Figure 4B). Systematic random sampling techniques were used for selecting tissue sections for staining and stereological analysis. Every fifth section was sampled starting at a section randomly determined from the first through fifth rostral-most sections. The tissue sections were then mounted onto gelatin-coated slides and stained with the Nissl stain, cresyl violet.

Pyramidal neurons counts of the CA2 and CA3 hippocampal fields were performed by an investigator uninformed of the group assignment. Sections were examined on a microscope with a motorized stage using computer software (Stereo Investigator). The region of interest was outlined under 4X magnification (Figure 4B). Criterion for counting pyramidal neurons required visualization of the nucleus of morphologically distinct cell bodies. Neuronal cell counting was performed with a 100X oil objective (Figure 4C,D). The total number of neurons in the region of interest was quantified using optical fractionator stereological methods. The spacing of the optical dissectors produced an average area sampling fraction (ASF) of 0.030. The guard height was set at 0.40 μM producing a tissue sampling fraction (TSF) of 0.70. Target cells in every fifth section were counted producing a section sampling fraction (SSF) of 0.20.

Figure 4

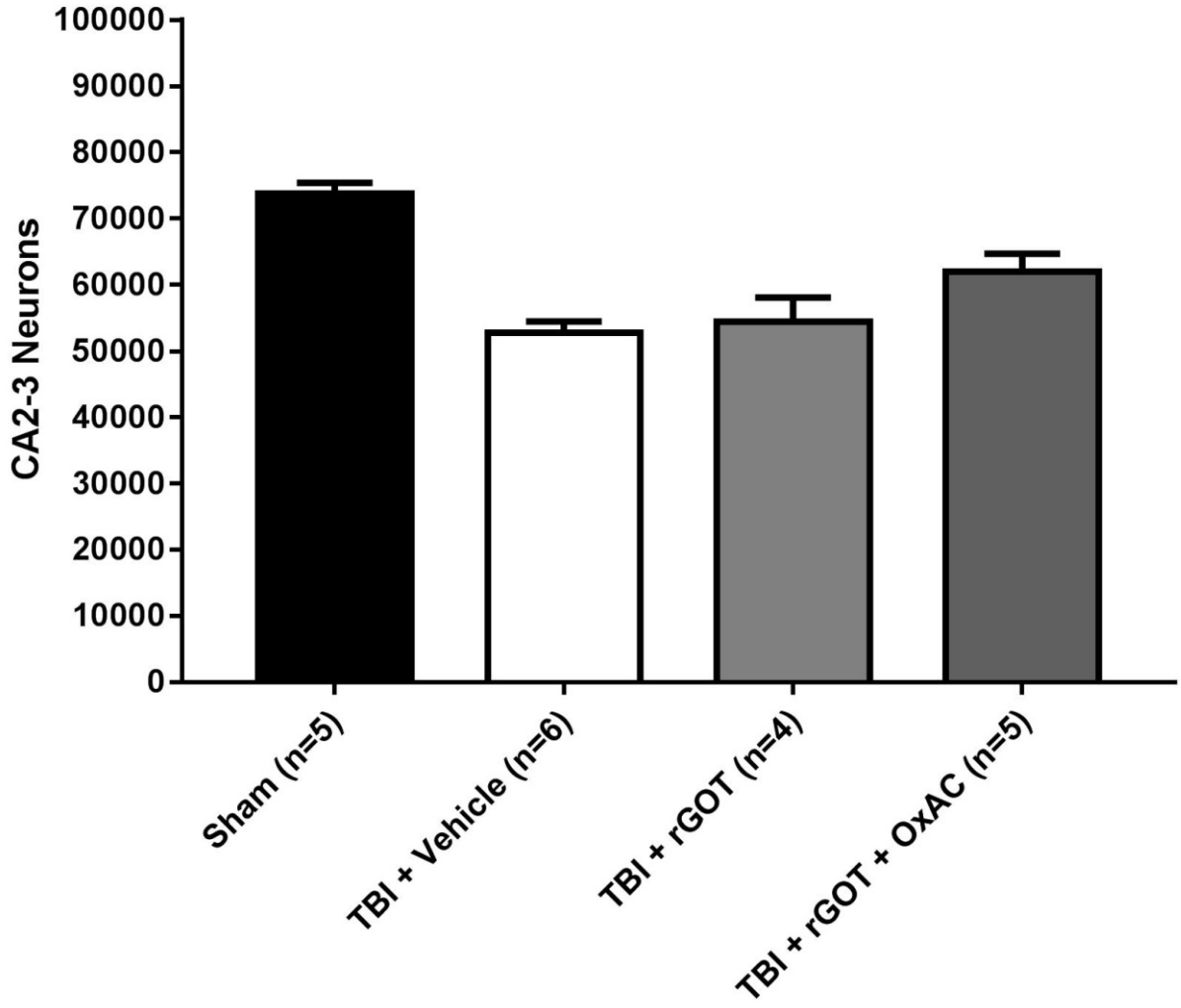


A: Gross pathology. Note typical area of infarction in the ipsilateral parietal cortex
B: Coronal section of ipsilateral hippocampus stained with cresyl violet (4X magnification)
C: Representative section from the ipsilateral CA2 (100X oil)
D: Representative section from the ipsilateral CA3 (100X oil)

Interim Results

With approximately 60% of tissue counting complete, our interim analysis indicates that the TBI + vehicle group (52,827) had a significant loss of pyramidal neurons compared to sham controls (73,790). Cell counts in the rGOT (54,477) and rGOT + OxAc (62,035) treatment groups are trending to be higher than the TBI + Vehicle group (Figure 5), indicating that treatment with rGOT + OxAc appears promising to reduce neuronal cell loss. Statistical analysis will be performed upon completion of the cell counts in the first quarter of year 3.

Figure 5



Time course of serum levels of GOT following iv administration of rGOT:

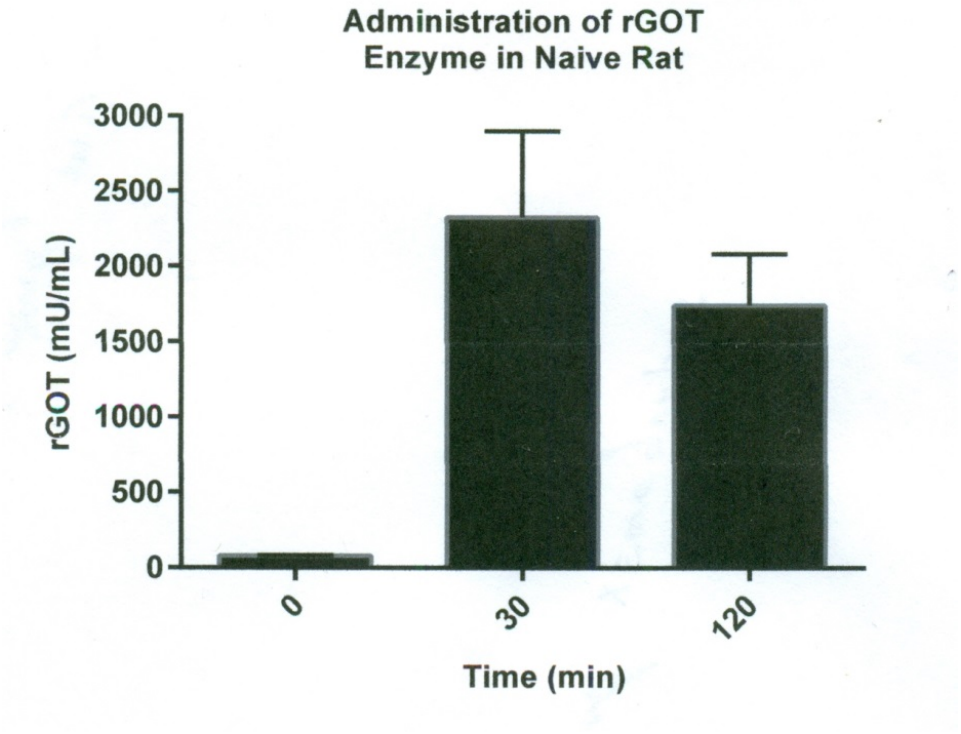
We have utilized the UC Davis campus veterinary clinical pathology services to measure GOT (labeled as “aspartate transaminase” in the table 2 below). Baseline blood samples were drawn from the tail vein prior to administration of our standard dose of rGOT (130ug/kg i.v.). Blood samples were subsequently drawn at 30 minutes and 120 minutes after rGOT injection. Endogenous blood levels of GOT were very consistent (~80 U/L). Blood levels of GOT were highly elevated at 30 minutes and 120 minutes after injection (Figure 6). GOT levels were extremely high in our stock rGOT and required considerable dilution (750:1) to fall below the maximum detection range of the analyzer (see Table 2 “Stock GOT”).

These results demonstrate the potency of our rGOT stock and that we achieve consistently high levels of rGOT in the blood for up to 2 hours post injection.

Animal ID	Timepoint	Aspartate Transaminase U/L	Hemolysis
1-B	Baseline without GOT	80.2	none
1-30	30 min after GOT	1626.6	none
1-120	120 min after GOT	1263.9	none
2-B	Baseline without GOT	84.2	none
2-30	30 min after GOT	2608.2	none
2-120	120 min after GOT	2035.2	none
3-B	Baseline without GOT	82.0	none
3-30	30 min after GOT	2117.2	none
3-120	120 min after GOT	1752.8	none
4-B	Baseline without GOT	78.6	none
4-30	30 min after GOT	2948.2	none
4-120	120 min after GOT	1921.5	none
Stock GOT	N/A	373800.0	none
Short sample; result obtained by dilution.			
Value obtained by manual dilution (x750 dilution factor)			

Table 2: Raw values of aspartate transaminase (GOT) measurement in blood serum.

Figure 6



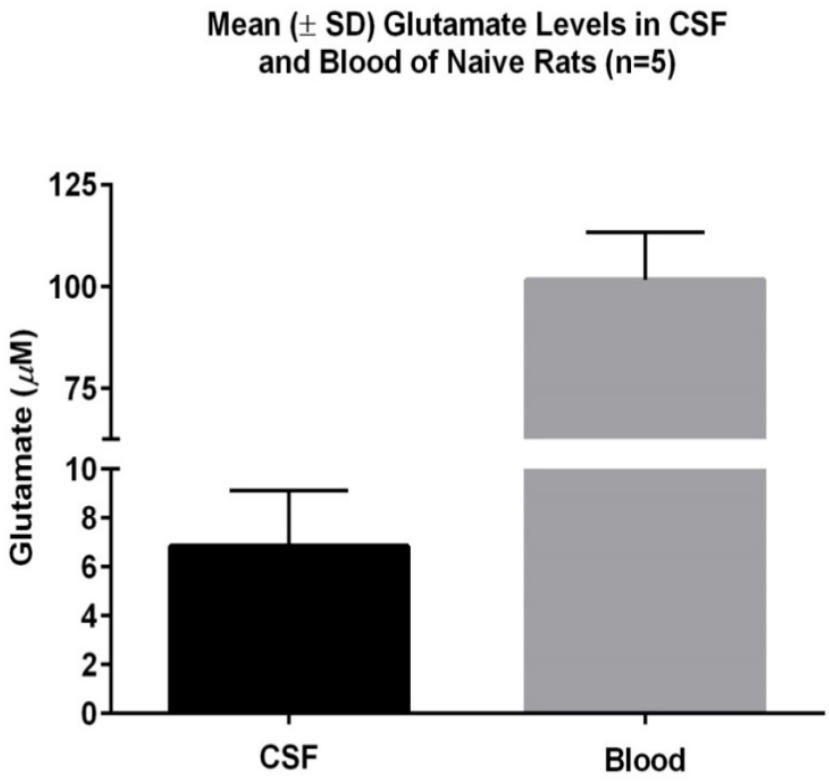
Measurement of CSF Glutamate Concentration.

We have solved our technical problems of sampling CSF from the cisterna magna and have now performed analysis of glutamate content in CSF and serum from 5 naïve rats. Table 3 shows individual data from each rat. Figure 7 shows average results of five naïve animals in which CSF and blood serum were collected and analyzed with our colorimetric glutamate analysis kit. As predicted, we detected much lower levels of glutamate in the CSF as compared to plasma. CSF glutamate levels are now similar to those reported in the literature.

Treatment Group	Weight (g)	Glutamate Levels in CSF (µM)	Glutamate Levels in CSF (µM)
Naïve- rat #1	323	5.78	116
Naïve- rat #1	352	3.66	93.6
Naïve- rat #1	343	9.67	91.9
Naïve- rat #1	355	7.08	93.8
Naïve- rat #1	327	8.03	112.7
Mean ± SD	340 ± 14.5	6.84 ± 2.28	102 ± 11.7

Table 3. Glutamate Levels in CSF and Blood from Naïve Rats.

Figure 7


Mean (± SD) Glutamate Levels in CSF
and Blood of Naive Rats (n=5)
Glutamate (μM)
125
100
75
10
8
6
4
2
0
CSF
Blood

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

Nothing to Report

How were the results disseminated to communities of interest?

Preliminary results were presented in an oral session at the 2015 MHSRS Symposium.

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

During quarter one of year three, we will proceed to complete the studies of single moderate TBI. Now that we have solved problems of CSF sampling and reliability of blood serum glutamate assays, we expect to rapidly proceed into studies of multiple mild TBIs.

Reportable Outcomes:

A scientific manuscript is under preparation that will include the behavioral and histological results from the moderate TBI study data in this annual report.

Impact:

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

The results thus far on this project have a potentially high impact on the acute treatment of moderate TBI. The significant reduction in motor and cognitive deficits using a treatment that does not have to penetrate into the brain parenchyma is quite remarkable. The rGOT treatment targeting glutamate excitotoxicity is especially noteworthy since so far there are no indications of toxic or adverse effects.

What was the impact on other disciplines?

The positive results of the rGOT treatment can have an impact on other neurological disorders that are mediated by glutamate excitotoxicity such as stroke and epilepsy.

What was the impact on technology transfer?

The rGOT technology devised by collaborator Dr. Mirelman at the Weizmann Institute may have a substantial impact upon future treatments of TBI and other conditions involving glutamate excitotoxicity.

What was the impact on society beyond science and technology?

Nothing to Report

Changes/Problems

We were experiencing problems in consistently extracting a sufficient volume of CSF from the cisterna magna multiple times from the same rat. This was especially notable for TBI rats in which elevated intracranial pressure hindered CSF extraction likely due to reduced CSF flow dynamics and reduced volume of the cisterna magna. We have spent considerable time addressing this issue and have now refined our techniques such that we now can consistently withdraw adequate CSF sample sizes from the cisterna magna in TBI rats.

Products:

Nothing to Report

Participants & Other Collaborating Organizations:

What individuals have worked on the project?

Name:	Bruce Lyeth, PhD
Project Role:	Principle Investigator
Researcher Identifier:	252972781 (UC Davis ID)
Nearest person month:	4
Contribution to the project:	Dr. Lyeth performed the fluid percussion TBIs and supervised the conduct of the project.
Funding Support:	1R01NS089901, NIH (PI: Liu, Dazhi) 20% effort; 1R43DA041760-01, NIH (PI: Fitzpatrick, B.) 7% effort

Name:	Ken Van, MS
Project Role:	Staff Research Associate
Researcher Identifier:	613144013 (UC Davis ID)
Nearest person month:	10
Contribution to the project:	Mr. Van performed the surgeries, the blood draws, glutamate assays, and behavioral testing.
Funding Support:	No other source

Name:	Gene Gurkoff, PhD
Project Role:	Assistant Researcher
Researcher Identifier:	727993875 (UC Davis ID)
Nearest person month:	1
Contribution to the project:	Dr. Gurkoff assisted with the serum glutamate assays.
Funding Support:	No other source

Name:	Emily Doisy, BS
Project Role:	Staff Research Associate
Researcher Identifier:	897554960 (UC Davis ID)
Nearest person month:	1
Contribution to the project:	Ms. Doisy is the Laboratory Manager & Safety Officer. She managed ordering of supplies and managed safety training and concerns in the laboratory. She also assisted with the serum glutamate assays.
Funding Support:	No other source

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

PI Lyeth is now a co-investigator on NIH 1R01NS089901 (20% effort) and a consultant on NIH 1R43DA041760-01 (7% effort),

What other organizations were involved as partners?

Collaborator, Dr. David Mirelman of the Weizmann Institute was instrumental in providing rGOT for this project. He also participated in monthly discussion via Skype and with annual visits to UC Davis to discuss scientific progress and problems.

Special Reporting Requirements:

Collaborative Awards:

Not applicable

Quad Chart:

Updated quad chart is attached.

Appendices:

None

Novel Mechanism for Reducing Acute and Chronic Neurodegeneration after TBI

Log number: PT120075

Award Number: W81XWH-14-1-0195

PI: Bruce Lyeth, Ph.D. **Org: University of California, Davis** **Award Amount : $763,916**

DMRDP

Study Aims

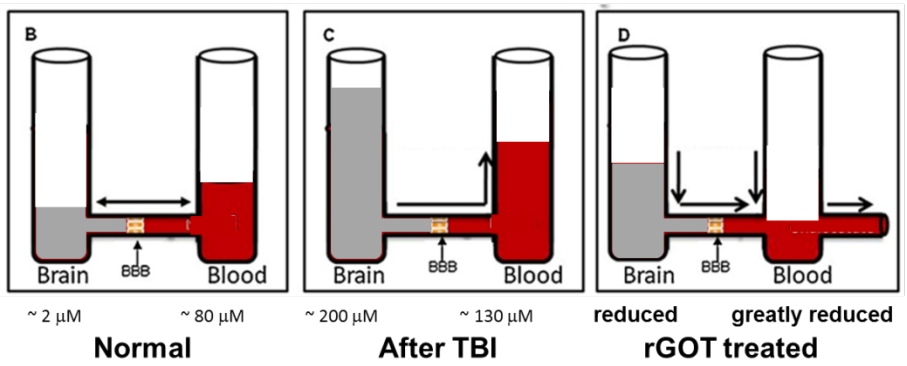
- **Specific Aim 1:** Determine the effects of TBI on glutamate levels in serum and CSF after TBI and determine the effects of rGOT on glutamate after multiple mild or a single moderate TBI in rats.
- **Specific Aim 2:** Determine the effects of the optimal doses of rGOT on acute and chronic brain pathology and behavioral outcome after TBI in rats.

Approach

An intravenous administration of the enzyme, glutamate oxaloacetate transaminase (GOT) that converts glutamate into α-ketoglutarate will be evaluated for reducing excessive free glutamate associated with TBI. We will examine the mechanism of action of GOT on blood and CSF levels of glutamate and the therapeutic potential of GOT to reduce cellular and behavioral pathology associated with TBI. These objectives are addressed using two clinically relevant models of experimental TBI in the rat.

Mechanism of glutamate diffusion between blood and brain:
rGOT treatment reduces blood glutamate, creating a diffusion gradient that scavenges glutamate from the brain.

Estimated Glutamate Concentration



Timeline and Cost

Activities FY	14	15	16
Aim 1a: Determine optimal rGOT dosing for reducing blood and brain levels of glutamate.	■		
Aim 1b: Determine effects of rGOT on chronic pathology and behavior after repeated mild TBI			■
Aim 2: Determine effects of rGOT on pathology and behavior after moderate TBI		■	
Estimated Budget ($K in total costs)	254	254	254

Updated: 29 JUL 2016

Goals/Milestones

CY14 Goal – Experiment ramp-up & determine optimal dosing

- ☑ Optimize experimental methods and glutamate assay
- ☑ Determine optimal rGOT dosing for reducing glutamate in blood

CY15 Goals – Evaluate treatment effects on moderate TBI

- ☑ Determine rGOT effects on brain pathology after moderate TBI
- ☑ Determine rGOT effects on behavior after moderate TBI

CY16 Goal – Evaluate treatment effects on repeated mild TBI

- ☐ Determine rGOT effects on behavior after mild TBI
- ☐ Determine rGOT effects on brain pathology after mild TBI

Comments/Challenges/Issues/Concerns

- No changes in timeline.
- spending off by <1 quarter due to early start in spending.

Budget Expenditure to Date

Projected Expenditure: $508,000

Actual Expenditure: $537,563