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**14. ABSTRACT**
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. Numerous studies over the past 40 years have consistently shown that high levels of the excitatory neurotransmitter, glutamate can damage or kill brain cells. Research has found that TBI causes dangerously high level of glutamate in the brain. We have proposed a new therapy that will reduce brain levels of glutamate by reducing glutamate levels in the blood. This therapy works by enhancing a natural process of converting blood glutamate to an inactive substance. We will test this therapy by injecting rats with natural blood enzymes after they receive a controlled experimental brain injury. We will measure the treatment effects on several important outcome measurements including, blood levels of glutamate, early and delayed brain cell loss, and cognitive performance in a maze. The project is highly relevant to military interests in TBI because it will increase our understanding of the early and delayed effects of exposure to repetitive concussions. The project could also lead to the development of an easily administered treatment of service personnel exposed to TBI.

**15. SUBJECT TERMS**
TBI, glutamate, excitotoxicity, GOT

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## INTRODUCTION:

**The fundamental problem studied** was that to date, strategies to reduce excitotoxicity have focused primarily on blocking glutamate receptors or reducing the release of glutamate from pre-synaptic terminals. The use of glutamate receptor antagonists to treat excitotoxicity comes with several inherent disadvantages. These include problems with drug delivery across the blood-brain barrier (BBB) and an indiscriminant disruption of normal glutamate cell signaling when the antagonist does cross the BBB. Perhaps poor BBB permeability and unwanted side-effects of direct inactivation of neurons is related to the failure to translate previously positive pre-clinical data into viable therapeutic applications in human clinical trials. The recombinant preparation of the enzyme glutamate-oxaloacetate transaminase (rGOT) treatment proposed in this project circumvents both of the problems associated with receptor antagonists because the mechanism of action occurs in the blood; hence, there are no BBB issues. Importantly, the rGOT treatment only targets excessive levels of extracellular glutamate and thus should not disrupt physiologic glutamate cell signaling. Furthermore, rGOT has no interaction with neurons and therefore should not directly affect neuronal function.

**The major objective** of this project is to test the effects of rGOT alone or together with a small concentration of the co-substrate oxaloacetate (OxAc) on multiple aspects of excitotoxicity associated with TBI. The neuroprotective potential of rGOT is based on its ability to cause a rapid reduction of glutamate in the blood due to the catalytic transformation of glutamate to α-ketoglutarate. The role of OxAc is as a necessary co-substrate to further activate rGOT and to contribute to the kinetics of the transformation of glutamate to aspartate and α-ketoglutarate.

**The Specific Aims** of this project were: 1) Determine the effects of TBI on concentrations of glutamate in blood serum and CSF after TBI and to examine an effective dose of rGOT, or the combination rGOT + OxAc to reduce glutamate concentrations after multiple mild or a single moderate TBI in rats, and 2) Determine the effects of the optimal doses of rGOT or the combination of rGOT + OxAc on brain pathology and behavioral outcome after multiple mild or a single moderate TBI in rats.

**The study design** of this project was to measure the time course of changes in glutamate concentration in blood serum after TBI in rats after repetitive mild fluid percussion TBI and after a single moderate TBI. Next, the study quantified the effects of rGOT or OxAc on reducing blood serum and CSF glutamate concentrations after TBI. The design then determined the effects of optimal doses of rGOT or rGOT + OxAc on chronic motor and cognitive behavioral outcome. Finally, the study design evaluated chronic histopathology of neurons.

**The significance** of this project lays in the development a novel treatment for TBI that is effective and void of unwanted side effects. With more than 230,000 cases of TBI reported in service personnel during operations Iraqi Freedom and Enduring Freedom, TBI has become the signature injury in the Mideast war theater. Better understanding of the mechanisms related to post-TBI pathology will identify targets that are essential to the development of new TBI therapies for reducing damage to the brain and improving outcome. 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. Thus, 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:

### Effects of rGOT administration on serum levels of glutamate:

We performed experiments to measure changes in blood serum concentrations of glutamate following tail vein administration of 3.33 ml/kg body weight of 15mM glutamate concentration (a 300 gram rat would receive 1.0 ml of the 15mM glutamate solution). A second group of rats was administered a bolus tail vein injection of rGOT enzyme (130 ug/kg) immediately after administration of 15 mM glutamate.

Intravenous injection of ~ 1.0 ml of 15 mM glutamate increased the blood plasma concentration of glutamate by 23 percent within 30 minutes (Figure 1). Co-administration of rGOT (130 ug/kg) along with 15 mM glutamate completely attenuated the elevation in serum glutamate. These results demonstrate that the glutamate assay can reliably detect changes in blood serum concentrations of glutamate. Importantly, the dosage of rGOT tested (130 ug/kg) was sufficient to completely reduce the elevated serum concentrations of glutamate following tail vein administration of exogenous glutamate.

**Figure 1**

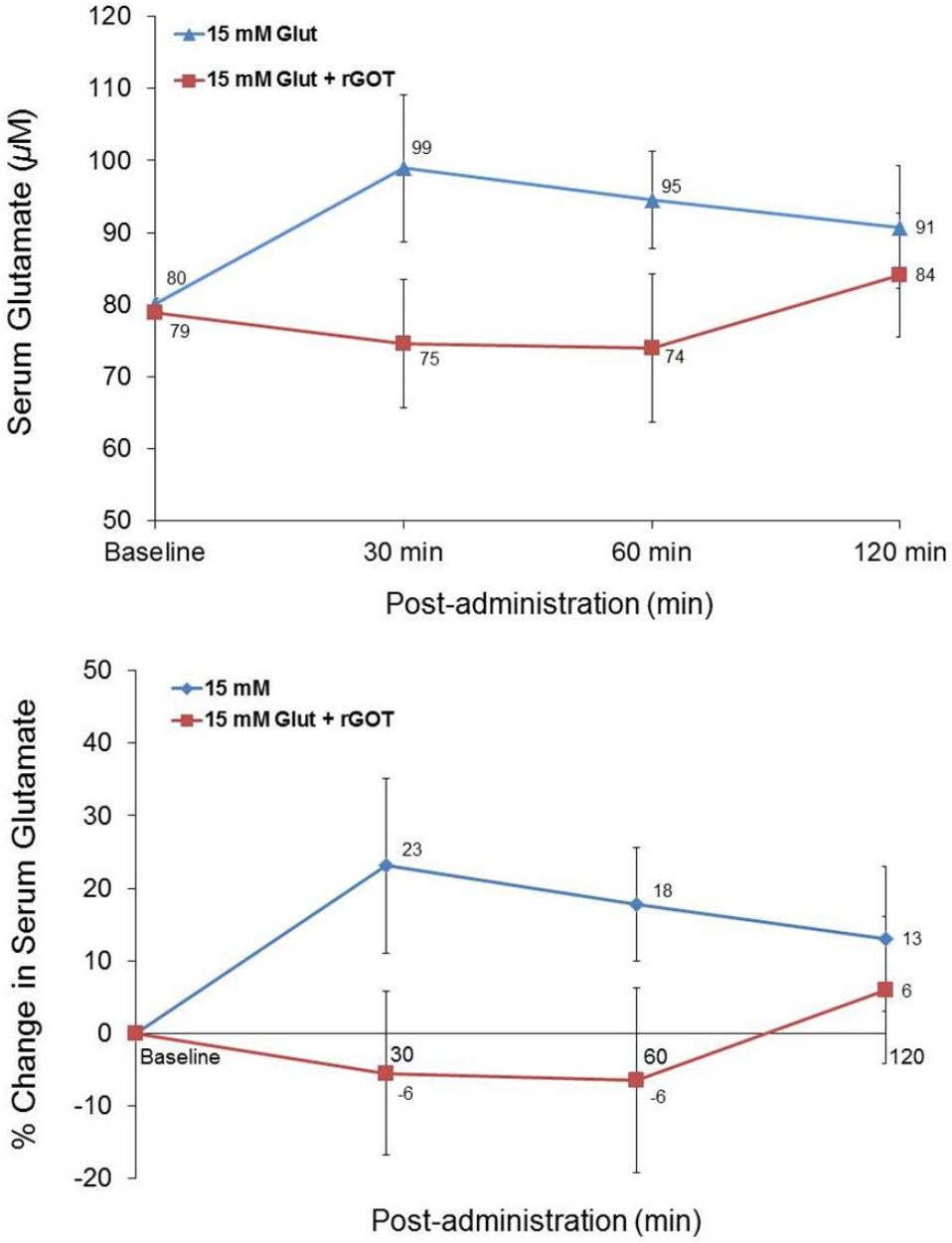



**Figure 1**. Serum glutamate concentrations in adult rats treated with 15 mM exogenous glutamate (n=6) and in combination with rGOT (n=6) (A). Tail vein blood samples were taken at different time points (pre-injection or baseline, 30, 60, 120 min). Animals that received 15 mM Glut showed an increase in serum glutamate concentration at 30 min and it remained elevated at 60 min and 120 min post-administration. Treatment with rGOT (130 µg/kg) immediately after administration of 15 mM Glutamate abolished the increase in serum glutamate level at similar time points. Data from the same experiment expressed as percent change from baseline in the lower graph. All data are mean ± SEM.

**Effects of moderate TBI on serum levels of glutamate:**

We examined the effects of a moderate fluid percussion TBI in rats on blood serum levels of glutamate. A baseline blood sample was drawn from the tail vein of male Sprague Dawley rats 24 hours prior to moderate fluid percussion TBI. Tail vein blood samples were drawn at 10, 20, 30 and 60 minutes after TBI. Blood was processed for serum and immediately frozen on dry ice for later analysis of glutamate concentration using Amplex Red glutamic acid assay kit (Molecular Probes). Results indicate that moderate TBI (2.14 ATMs) produced no change in serum glutamate after TBI (Figure 2). This indicates that any movement of glutamate from brain interstitial fluid to blood is likely diluted in the large volume of circulating blood (~21 ml/rat). Importantly, rGOT treatment significantly reduced the blood serum concentration of glutamate. This produces an increase in the concentration gradient between brain and blood thereby enhancing the driving force of glutamate from the brain into the blood circulation.

## Figure 2

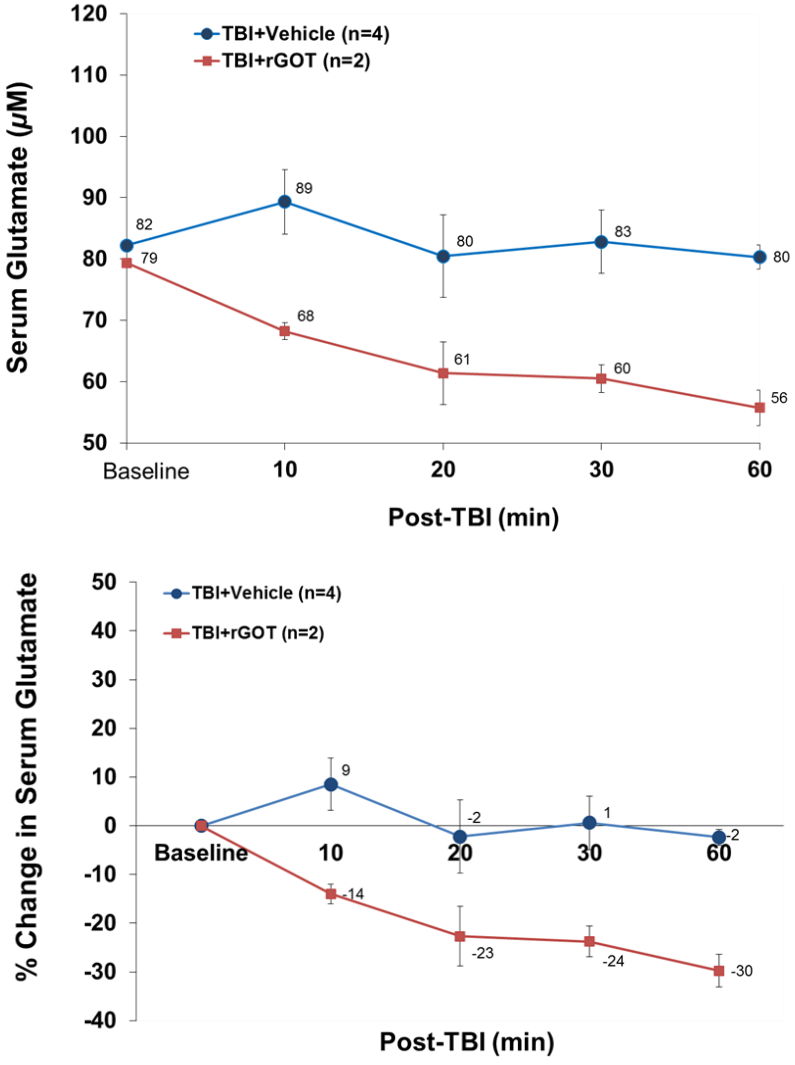



**Figure 2**. Serum glutamate concentrations in adult rats following moderate traumatic brain injury treated with rGOT (130 ug/kg) or saline immediately following TBI. **(A)** Tail vein blood samples

were taken at different time points (baseline, 10, 20, 30, and 60 min). Serum glutamate levels were essentially unchanged by TBI, but rGOT treatment significantly reduced serum glutamate concentrations. Data from the same experiment expressed as percent change from baseline are shown in the lower graph. Treatment with rGOT reduced serum glutamate concentrations by as much as 30 percent at one hour after injection. All data are mean ± SEM.

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

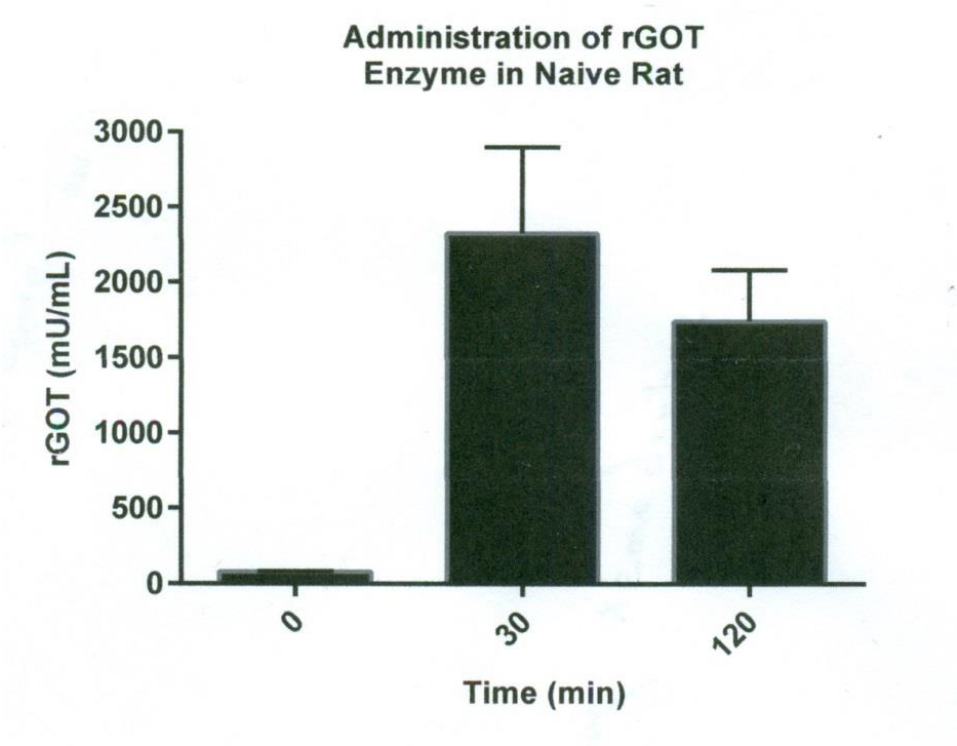
We utilized the UC Davis campus veterinary clinical pathology services to measure GOT (labeled as "aspartate transaminase" in the table 1 below). Baseline blood samples were drawn from the tail vein prior to administration of our standard dose of rGOT (130ug/kg i.v.). Blood samples were subsequently 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 3). 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 1 "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 1**: Raw values of aspartate transaminase (GOT) measurement in blood serum.

**Figure 3**



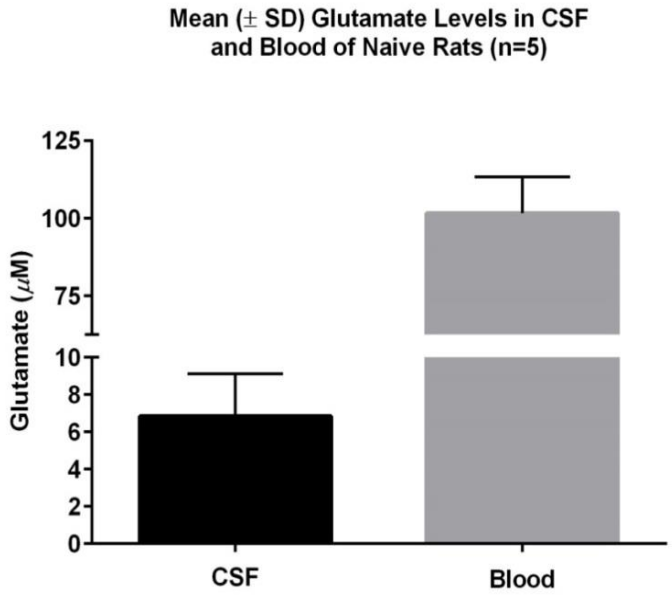

**Measurement of CSF Glutamate Concentration:**

We solved a technical problem of sampling CSF from the cisterna magna and performed analysis of glutamate content in CSF and serum from 5 naïve rats. Table 2 shows individual data from each rat. Figure 4 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 blood (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 2.** Glutamate Levels in CSF and Blood from Naïve Rats.

**Figure 4**




**Behavioral experiments following moderate TBI:**

Group parameters of body weight, injury magnitude, and temperature are provided in Table 3. The sham-operated rats were not subjected to TBI. Values of the experimental parameters were similar for all treatment groups. Importantly, the magnitude of the injury (ATM) was nearly identical for the three TBI treatment groups. My collaborator, Professor Mirelman, performed extensive dose-response studies in models of excitotoxicity and determined that 130ug/kg rGOT and 15mg/kg of OxAc were optimal for reducing blood levels of glutamate and reducing functional deficits and were subsequently used our experiments.

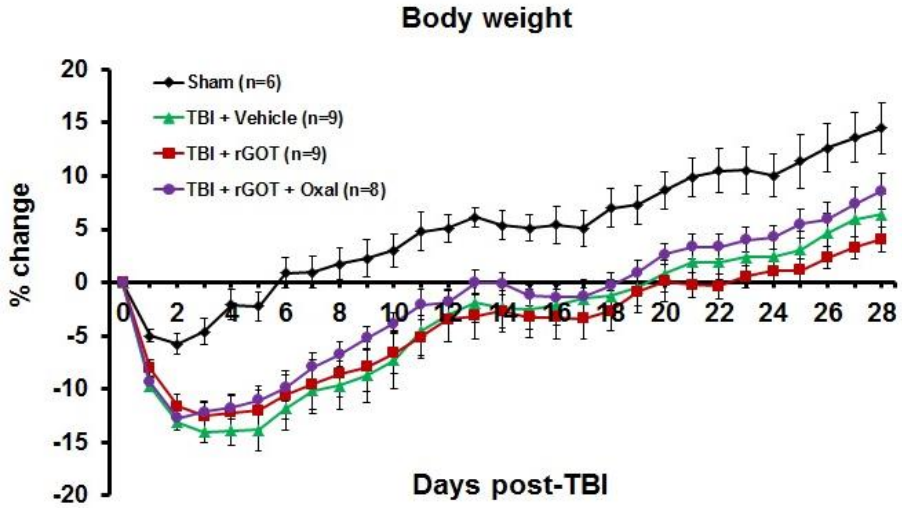
| Groups | Weight (g) | ATM | Temporalis Temp. | | Rectal Temp. | |
|---|---|---|---|---|---|---|
| | | | Pre | Post | Pre | Post |
| Sham (n=6) | 333 ± 17 | n/a | 35.4 ± 0.06 | 35.3 ± 0.10 | 36.8 ± 0.41 | 36.8 ± 0.39 |
| TBI + Vehicle (n=10) | 340 ± 21 | 2.13 ± 0.01 | 35.8 ± 0.31 | 35.4 ± 0.31 | 37.1 ± 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 3**. Groups, Sample size, Body weight, ATM, Temporalis and Rectal temperatures (means ± SD)

**Body Weight Post-TBI**

The average animal body weight (% change) was normalized to the pre-surgery body weight and was calculated as the ratio of the difference between the post-TBI day and pre-TBI body weight over the pre-TBI body weight. Over the course of 28 days following TBI, the mean body weight of the three TBI groups decreased compared to the sham-TBI group (Figure 5). However, there was no significant difference in body weight between TBI groups ($p>0.05$) for any of the days following TBI. The mean maximum body weight loss for the sham and TBI groups were 6% (day 2) and 10-14% (days 3-5), respectively. The mean percent of body weight increased steadily with the sham and TBI groups regaining their pre-surgery body weight at approximately 6 days and 18-20 days post-TBI, respectively.

**Figure 5**



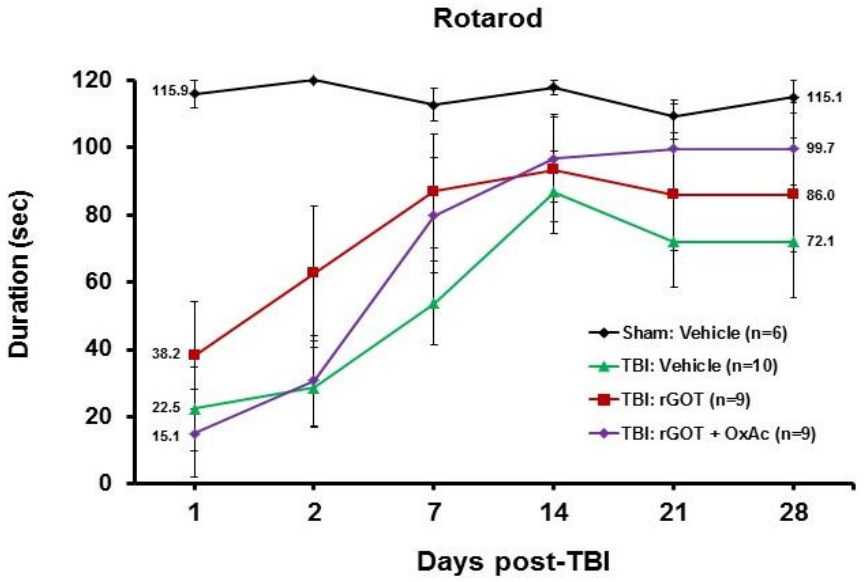

**Effects of treatment on rotarod motor deficits after moderate TBI:**

The descriptive characteristics of the four groups are listed in Table 3. 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.

After carefully verifying all rotarod data entry into SPSS statistical program against individual data sheets for each animal, we 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 6). 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 (130ug/kg) 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.

**Figure 6**

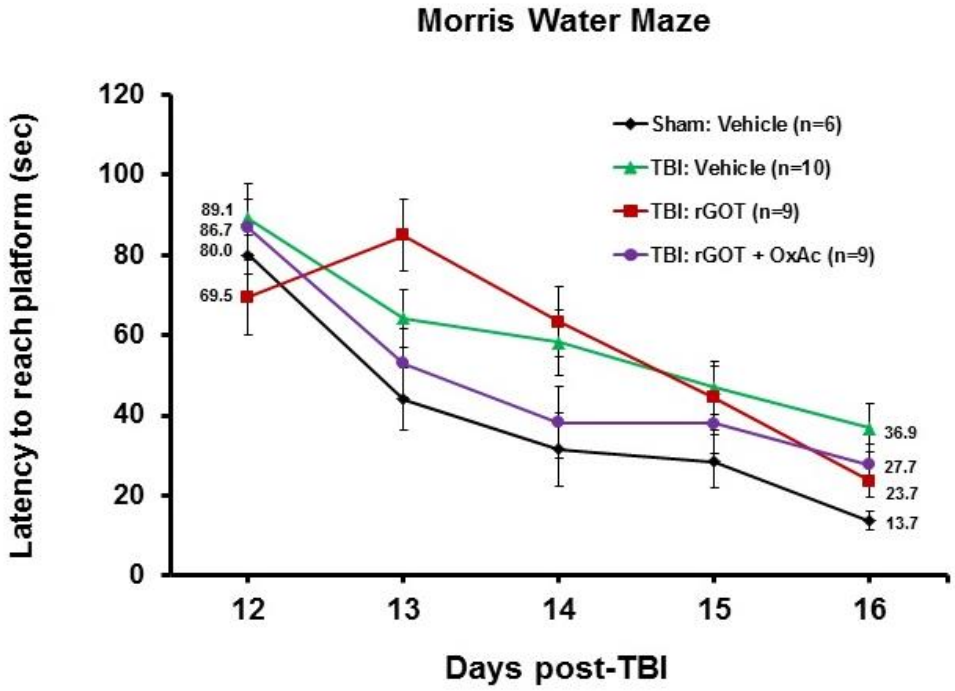



| $_{RM}$**ANOVA** | | **Dunnett's *post hoc*** | |
|---|---|---|---|
| Group | p = 0.013 | Sham vs. TBI: vehicle | p = 0.006 |
| Group X Day | p < 0.001 | Sham vs. TBI: rGOT | p = 0.107 |
| | | Sham vs. TBI: rGOT+OxAc | p = 0.020 |

**Effect of treatment on MWM cognitive deficits after moderate TBI:**

We carefully verified all MWM data entry into SPSS statistical program against individual data sheets for each animal. We 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 7).

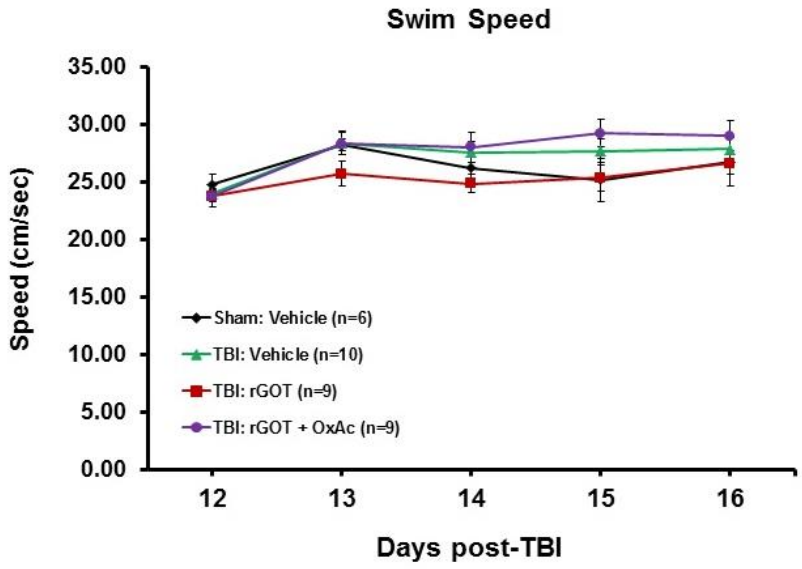
**Figure 7**




| $_{RM}$**ANOVA** | | **Dunnett's *post hoc*** | |
|---|---|---|---|
| Group | p = 0.024 | Sham vs. TBI: vehicle | p = 0.015 |
| Group X Day | p = 0.034 | Sham vs. TBI: rGOT | p = 0.034 |
| | | Sham vs. TBI: rGOT+OxAc | p = 0.37 |

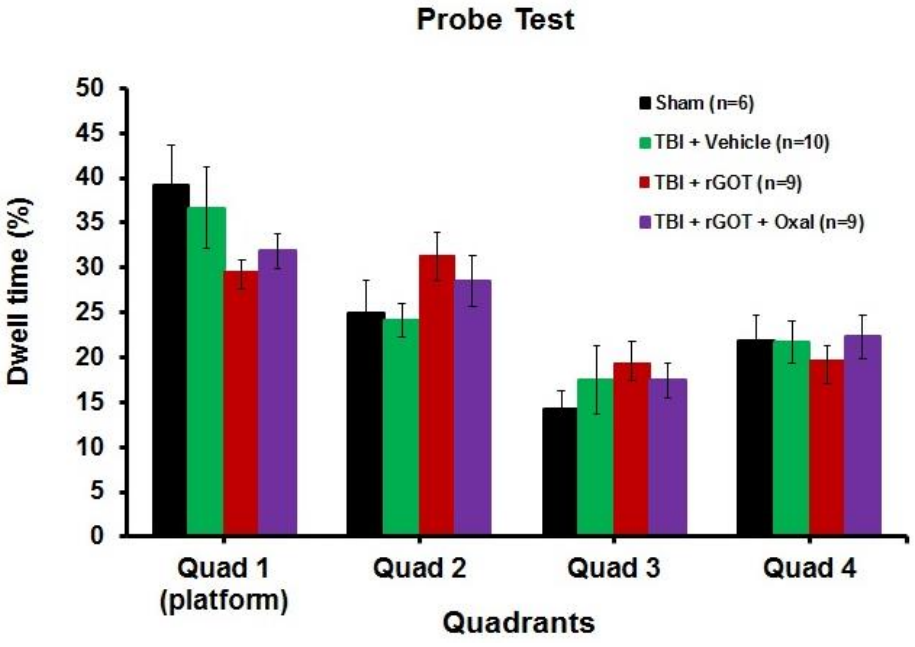
The average swim speed did not differ significantly between groups (Figure 8) indicating that interpretation of cognitive deficits was not confounded by motor deficits.

**Figure 8**



The probe trials (60 sec) of the Morris water maze were performed seven days following the final test trial on day 5 of the spatial learning task. The data showed no cognitive deficits existed in any of the groups in regards to the time spent in the target quadrant (Figure 9).

**Figure 9**

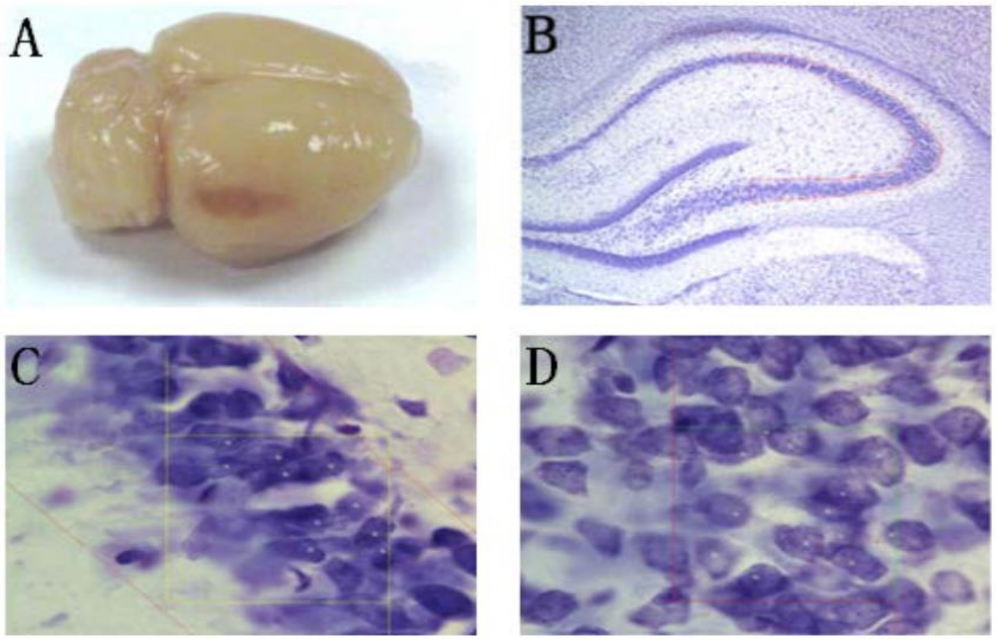


**Effects of rGOT and rGOT + OxAc on chronic histology following moderate TBI:**

The region of interest for measurement of surviving neuronal cells encompassed the dorsal stratum pyramidale of the hippocampus CA2 and CA3 (Figure 10B). 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.

An investigator uninformed of the group assignment performed pyramidal neurons counts of the CA2 and CA3 hippocampal fields. 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 10B). 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 10C,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 10**




**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)**

We performed ANOVA followed by post hoc Tukey HSD on the groups listed in Table 4. The overall ANOVA was significant $F(3,27)=17.92$, $p<0.001$ indicating differences between groups. The post hoc Tukey test indicated that TBI with vehicle treatment produced a significant loss of ~ 21,600 CA2/3 pyramidal neurons compared to sham injury. Treatment with rGOT alone did not affect neuronal cell counts compared to the TBI vehicle-treated group. There was a trend for the rGOT + Oxaloacetate -treated group to have an increased number of neurons (~7,000) compared to the TBI Vehicle group ($p=0.089$) (Figure 11).

| Groups | Weight (g) | ATM |
|---|---|---|
| Sham (n=5) | 333 ± 17 | n/a |
| TBI + Vehicle (n=10) | 340 ± 21 | 2.13 ± 0.01 |
| TBI + rGOT (n=8) | 339 ± 18 | 2.15 ± 0.01 |
| TBI + rGOT + Oxal (n=8) | 326 ± 21 | 2.15 ± 0.02 |

**Table 4**. Histology Groups, Sample size, Body weight, ATM (means ± SD)

**Figure 11**

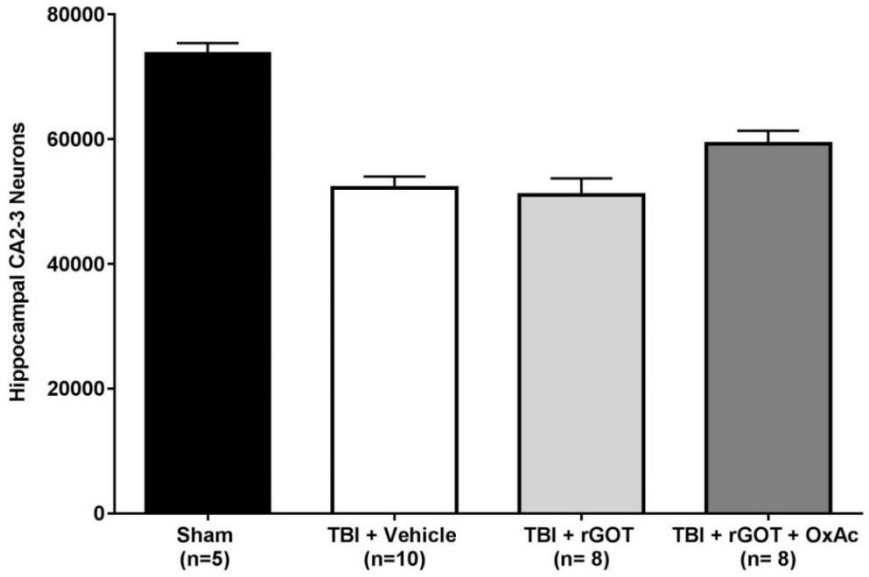



**Figure 11:** CA 2/3 neuronal cell counts performed at day 14 post-injury. TBI produced a significant loss of neurons. Treatment with rGOT + OxAc produced a trend for increased survival of neurons compared to the TBI vehicle group. Data are means +SEM.

**Analysis of blood serum levels of GOT enzyme:**

We performed analysis of GOT-1 enzyme levels in blood serum with our moderate and multiple mild TBI paradigms.

Blood samples were obtained via tail vein prior to moderate TBI and at 24 hours after TBI. For multiple mild TBIs, tail vein blood samples were taken prior to the first bilateral mild TBI, 1 hour post TBI and 24 hour post TBI (which was just prior to the second bilateral TBI), one hour post the second bilateral TBI and finally at 24 hours post the second bilateral TBI. Blood samples were coagulated and centrifuged to produce serum samples. Samples were analyzed at the UC Davis Comparative Pathology Laboratory using a Roche Diagnostics Cobas Integra 400 Plus clinical chemistry analyzer.

The multiple mild bilateral TBIs: Rats were mounted in a stereotaxic frame, a scalp incision made along the midline, two 4.8-mm diameter craniectomies were performed on the right and left parietal bone (centered at -4.5 mm Bregma and right and left lateral 3.0 mm). A rigid plastic injury tube (modified Leur-loc needle hub, 2.6-mm inside diameter) was secured to each of the craniectomies with cyanoacrylate adhesive. Care was taken to leave the exposed dura intact. Two skull screws (2.1 mm diameter, 6.0 mm length) were placed into burr holes, 1 mm rostral to Bregma and 1 mm caudal to Lambda.

A fluid percussion pulse of 1.25 ATM to create a mild TBI was delivered to the right hemisphere after disconnection from the ventilator and then repeated on the contralateral side one minute later. During each percussion pulse, the contralateral non-pulsed injury tube was plugged to prevent leakage of saline. Immediately after the bilateral TBIs were induced, the rat was ventilated with a 2:1 nitrous oxide/oxygen mixture. The two injury tubes were then plugged, and the scalp was sutured. The repetitive aspect of the injury was performed twenty-four hours later.

Results show that TBI produced an increase in endogenous serum GOT measured at 1 hour after a single moderate TBI (Figure 12). This was also observed after a single bilateral mild TBI (Figure 12). In our multiple bilateral mild TBI paradigm, GOT levels in blood serum were elevated after the first bilateral TBI at 1 hour and then returned to baseline at 24 hours and was again elevated to a similar level at 1 hour after the second bilateral mild TBI - returning to baseline 24 hours later (Figure 13). We speculate that this elevation after TBI (in the absence of rGOT treatment) may be due to systemic effects of the brain injury on organ function, notably liver function where GOT is produced. Acute sympathetic nervous system activation may contribute to elicit both inflammation and immunodepression. This may be occurring through multiple pathways including (1) tissue chemokines producing inflammation in peripheral organs, (2) activation of monocytes and IL-10, (3) HPA activation. Interestingly, this increased GOT level response is similar in mild and moderate TBI and was observed after the first and second bilateral mild TBI events.

**Figure 12**

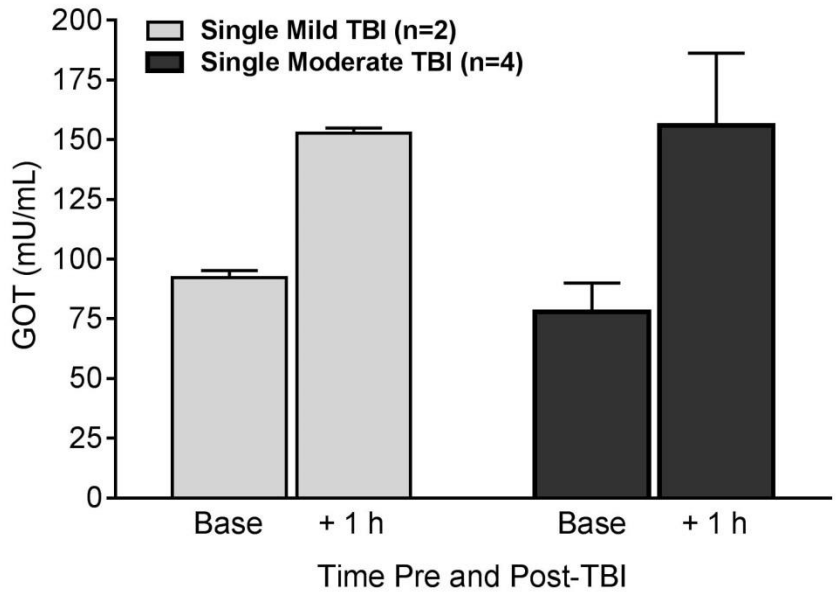



**Figure 12.** Endogenous blood serum levels of GOT enzyme increase 1 hour after a single mild bilateral TBI or after a single moderate lateral TBI.

**Figure 13**

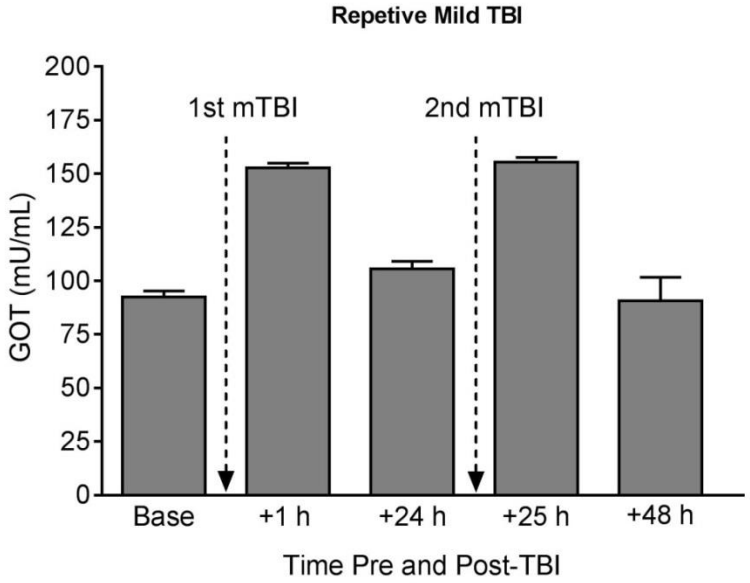



**Figure 13.** Endogenous blood serum levels of GOT enzyme increase 1 hour after mild bilateral TBI and return to baseline by 24 hours after TBI (n=4). This pattern is nearly identical after a second mild bilateral TBI.

It is important to note that the beneficial effects of administering exogenous rGOT enzyme greatly increases the serum levels of GOT (Figure 14) far in excess (15-fold difference) of the elevation we have shown in Figure 12 due to the TBI alone.

**Figure 14**

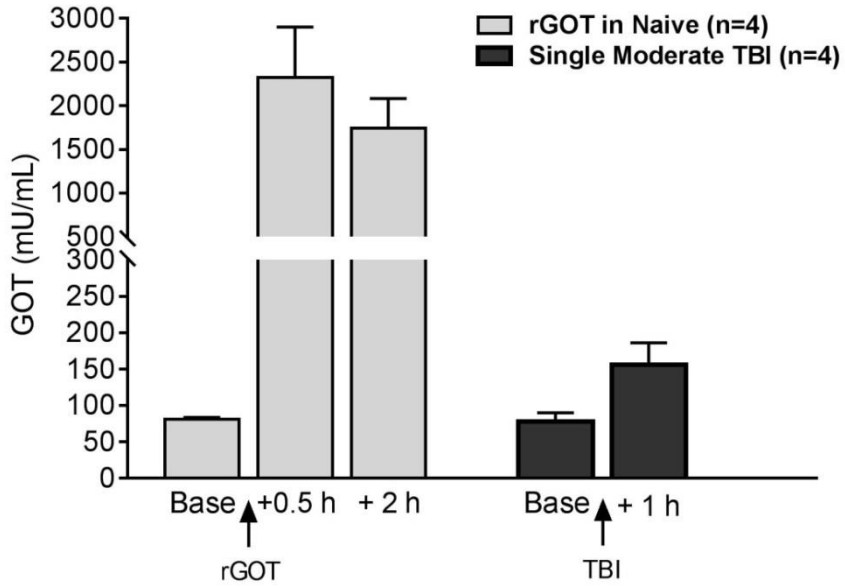



**Figure 14.** Comparison of blood serum levels of GOT enzyme after i.v. administration of exogenous rGOT (130ug/kg) enzyme (light grey bars) and after moderate TBI (black bars). Note that the elevation on blood serum levels of GOT enzyme are 15-fold higher after the iv injection of rGOT versus the blood serum levels of GOT at 1 hour after TBI.

Administration of rGOT 5 minutes after each mild TBI increased the serum concentrations 20-fold (Figure 15).

**Figure 15**

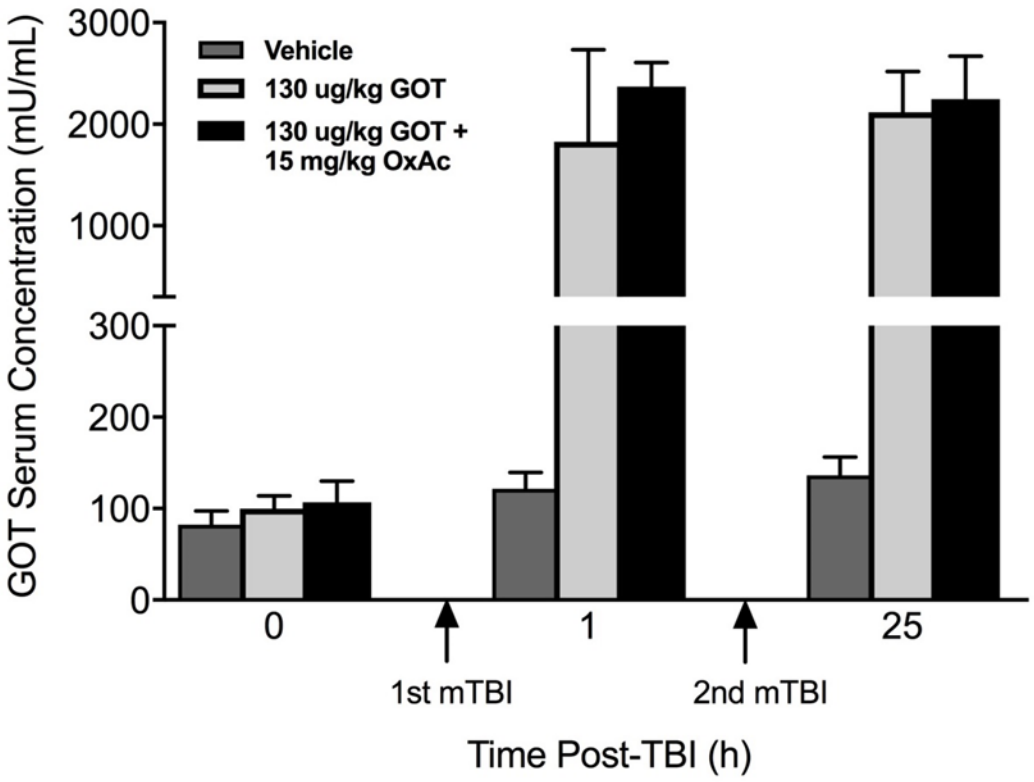



**Figure 15.** Blood serum levels of GOT enzyme after i.v. administration of exogenous rGOT enzyme after bilateral mild TBIs. Note that the consistent elevation on blood serum levels of GOT enzyme are 20-fold higher after the iv injection of rGOT was injected 5 minutes after each mTB).

**Pharmacokinetic results:**

Pharmacokinetic results show that administration of 130ug/kg of rGOT elevates blood serum levels for at least 5 hours after injection and return to baseline values by 24 hours post-injection (Figure 16). We also note that endogenous serum GOT measured at 1 hour after multiple bilateral mild TBIs is somewhat elevated after each pair of TBIs (see Vehicle-treated group in Figure 15). GOT levels in blood serum were elevated after the first bilateral TBI at 1 hour and then returned to baseline at 24 hours and was again elevated to a similar level at 1 hour after the second bilateral mild TBI - returning to baseline 24 hours later (Figure 16).

It is important to note that the beneficial effects of administering exogenous rGOT enzyme (as we have previously reported) greatly increases the serum levels of GOT far in excess (20-fold difference) of the elevation we have shown in Figure 14 due to the TBI alone.

**Figure 16**

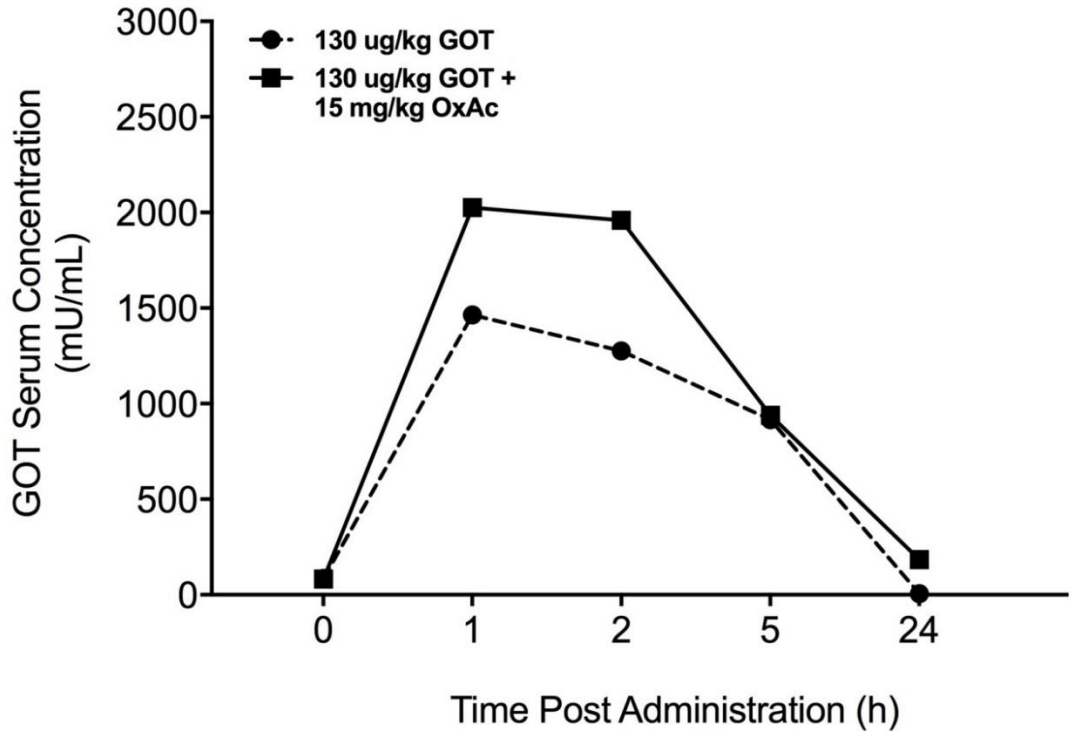



**Figure 16.** Pharmacokinetics of blood serum levels of GOT after i.v. administration of rGOT. Time zero represents pre-injection baseline value of endogenous GOT enzyme. GOT emzyme levels remain significantly elevated for up to 5 hours post-injection.

**Effects of treatment on cognitive performance after multiple mild TBIs:**

The multiple mild bilateral TBIs: Rats were mounted in a stereotaxic frame, a scalp incision made along the midline, two 4.8-mm diameter craniectomies were performed on the right and left parietal bone (centered at -4.5 mm Bregma and right and left lateral 3.0 mm). A rigid plastic injury tube (modified Leur-loc needle hub, 2.6-mm inside diameter) was secured to each of the craniectomies with cyanoacrylate adhesive. Care was taken to leave the exposed dura intact. Two skull screws (2.1 mm diameter, 6.0 mm length) were placed into burr holes, 1 mm rostral to Bregma and 1 mm caudal to Lambda.

A fluid percussion pulse of 1.25 ATM to create a mild TBI was delivered to the right hemisphere after disconnection from the ventilator and then repeated on the contralateral side one minute later. During each percussion pulse, the contralateral non-pulsed injury tube was plugged to prevent leakage of saline. Immediately after the bilateral TBIs were induced, the rat was ventilated with a 2:1 nitrous oxide/oxygen mixture. The two injury tubes were then plugged, and the scalp was sutured. The repetitive aspect of the injury was performed twenty-four hours later.

We evaluated the behavioral effects of rGOT treatment in our model of multiple mild bilateral TBIs. The magnitudes of TBIs were very consistent between groups and between first and second set of TBIs (Figure 17). Body weight profiles over days between groups were not significantly different (Figure 18).

**Figure 17**

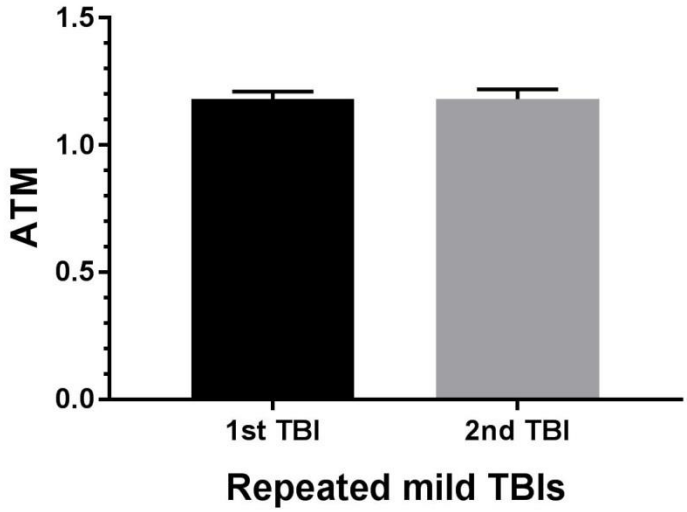



**Figure 17**: TBI magnitude collapsed across groups. Fluid percussion TBIs were very consistent in magnitude between the first set of TBIs and the second set of TBIs 24 hours later.

**Figure 18**

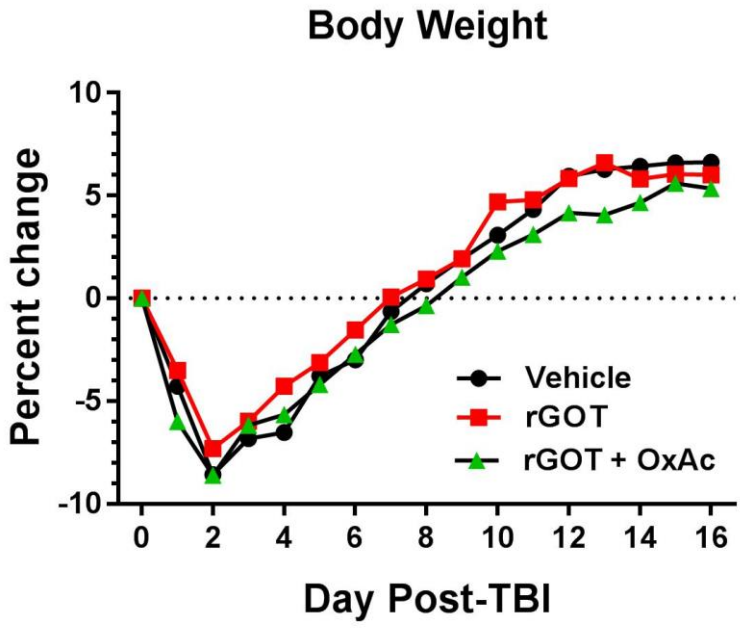



**Figure 18**. Body Weight percent change from baseline (Day 0). Rats lost weight for two days post-TBI and then steadily gained weight for the duration of the experiment. All three groups had similar changes in body weight that were not significantly different from each other. Mean baseline weights for each group: Vehicle = 318g, rGOT = 318g. rGOT + OxAc = 325g

Rats were evaluated for cognitive performance on the Morris water maze on days 11-15 post-TBI.

Hidden platform trials were conducted on 5 consecutive days. They consisted of a series of acquisition trials in which a hidden (submerged) platform (transparent circular escape platform, 12cm diameter, was placed in a fixed position in the tank 2 cm below the water surface. The hidden platform was located in a different quadrant from the previous day's visible platform. Each hidden platform trial was started by placing the rat in the water close to, and facing the wall of the tank in one of the four cardinal start locations. The starting quadrant was changed randomly for each trial so that the rat is forced to learn the spatial location of the escape platform, rather than simply a swimming direction or response (ie. right vs. left). Rats were allowed 120 sec to find and mount the escape platform. If they did not find the platform, they were placed on it. They were left on the platform for 30 sec before being removed from the maze for the next trial, which begins 4 min later. Rats received 4 trials/day over 5 consecutive days. Data from all trials were recorded using a video tracking system (Poly-Track, San Diego Instruments). Time to find and mount the submerged platform and distance swum were recorded. Performance for each day was the mean latency of four trials to find the platform.

Repeated measures ANOVA with the Sham and 3 TBI groups revealed a significant main effect of Group [F(3. 39)=8.48, p<0.001] with post hoc Dunnett test revealing significant deficits in all 3 TBI groups (p<0.01) compared to the Sham control group. Thus, multiple mild bilateral TBIs produced significant spatial cognitive deficits (Figure 19).

Repeated measure ANOVA restricted to the 3 TBI groups revealed no significant main effect of Group [F(2. 26)=0.412, p=<0.667]. Thus, treatment with rGOT or treatment with rGOT + OxAc did not affect cognitive spatial performance (Figure 19).

**Figure 19**

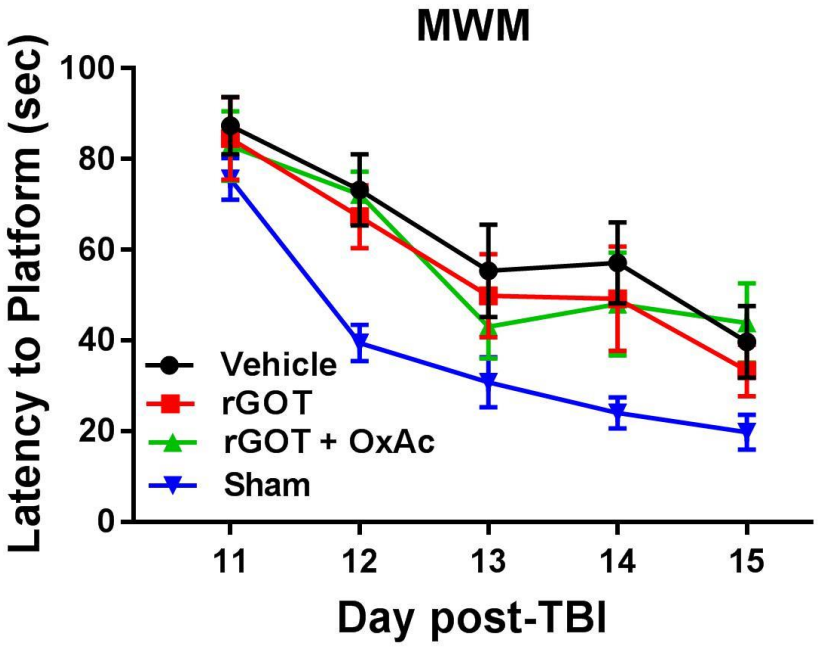



**Figure 19.** Morris water maze acquisition analysis.

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The probe trial consisted of a final hidden platform trial to measure memory retention was performed 7 days after the completion of the acquisition trials. A 60-sec "probe" trial was performed in which the escape platform is removed. The time spent in each quadrant pool was measured. Retention of memory for platform location was inferred by the length of time spent in the quadrant that previously contained the hidden platform.

One way ANOVA revealed no difference between groups [$F(2,26)=1.33$, $p=0.282$] in time spent in the target zone (Quadrant 1). Thus, treatment with rGOT or treatment with rGOT + OxAc did not affect spatial memory retention.

**Figure 20**

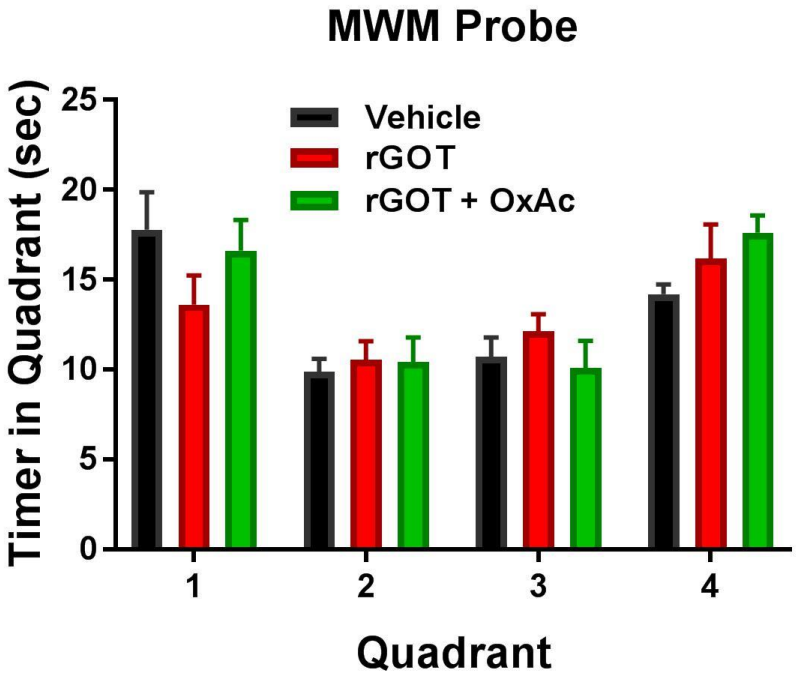


**Figure 20**. Morris water maze probe trial analysis. The target quadrant where the escape platform was located during the acquisition trials was located in Quadrant 1.

## Histological Results of Multiple Mild TBIs:

Rats were euthanized 24h after TBI by deep sodium pentobarbital anesthesia (100mg/kg,ip) followed by transcardial perfusion with 100ml of 0.1M sodium phosphate buffer(PB) (pH 7.4) followed by 350ml of 4% paraformaldehyde (pH7.4). Brains were removed and post-fixed for1h in 4% paraformaldehyde at 4 degrees C. Then brains were cryoprotected in 10% sucrose solution for 1day followed by 2 days in a 30% sucrose solution, frozen on powdered dry ice, and 45 mm coronal sections were cut on a sliding microtome (American Optical, Model 860). Every serial section starting at -2.12mm Bregma and ending at -4.80mm Bregma was saved in 24-well cell culture plates. Systematic random sampling techniques were used for selecting tissue sections for staining and stereological analysis.

Brains of animals subjected to multiple mild TBIs were harvested and sectioned. Six sections from each animal were mounted on microscope slides and stained with cresyl violet dye for detection of cells. We completed stereological cell counting procedures on a subset of animals in the study. There was no significant differences between treatment groups for left [$F(2,11)=0.23$, $p=0.80$] and right [$F(2,11)=1.97$, $p=0.20$] hemispheres. Note that the analysis is underpowered due to small sample size analyzed, However, the rGOT + OxAc group tended to have larger numbers of surviving cells compared to the vehicle group (Figure 21).

**Figure 21**

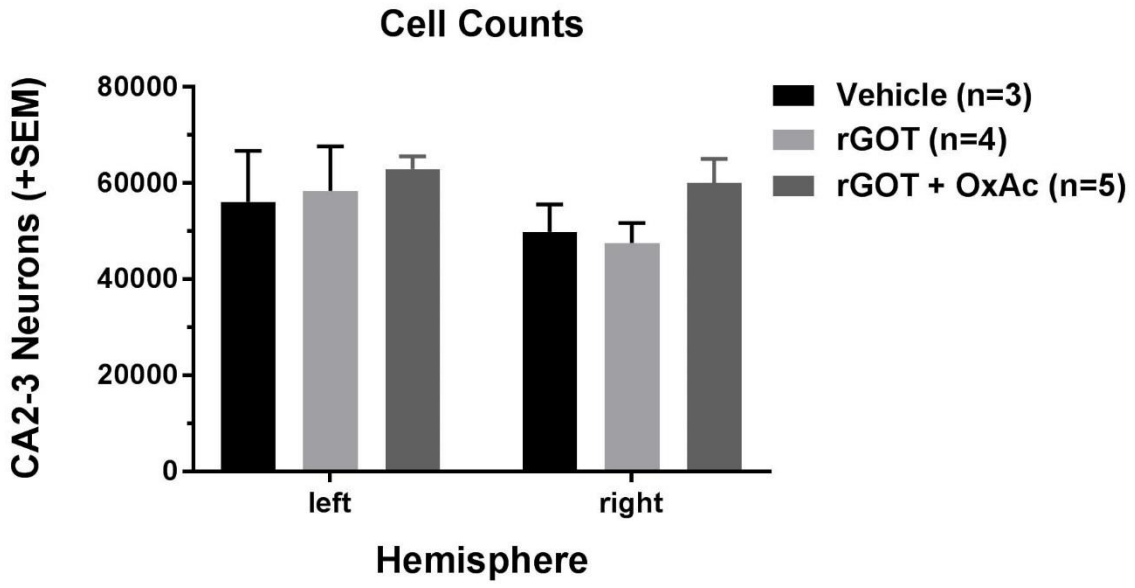


**Figure 21.** Within this subset of animals from each of the groups, there is no significant differences between groups, although there was trend for the rGOT + OxAc treatment group to have larger number of surviving neurons in the right hemishphere.

The data shown above comprises the work completed as of the end data of the project. A second no-cost extension was denied and thus, all laboratory work ceased as of June 30, 2018.

## Impact:

The findings from this project indicate a potential for rGOT treatment to positively impact outcome after TBI.

We demonstrated that a single iv administration of rGOT significantly elevates serum GOT concentrations for at least 5 hours and reduces serum levels of glutamate measured after moderate fluid percussion TBI in the rat. These findings are congruent with our hypothesis that elevating blood levels of GOT enzyme will lower blood levels of glutamate. This would produce a concentration gradient between brain and blood that would favor the transport of excess glutamate from the brain parenchyma.

We demonstrated that rGOT treatment or rGOT + OxAc treatment produced no adverse side effects, indicating that this treatment is safe. Treatments reduced motor and cognitive deficits associated with moderate magnitude fluid percussion TBI in rats. Treatment with rGOT or rGOT + OxAc was generally similar in effectiveness, indicating that OxAc is not a rate-limiting factor in the enzymatic reaction of converting glutamate to α-ketoglutarate. Treatment with rGOT + OxAc produce a trend for reducing neuronal cell loss in the hippocampus.

The results of the repeated bilateral mild TBI experiments, as with the moderate TBI study, did not produce adverse side effects. However, behavioral outcome deficits were not affected by treatment with either rGOT or rGOT + OxAc. Due to the small sample size the effects of treatments on neuronal cell death were inconclusive. However, there was an indication of a possible trend for rGOT + OxAc to enhance neuronal cell survival in the hippocampus.

The results indicate that rGOT treatment would be effective in more severe TBI rather than mild or repeated mild TBI. This is congruent with the higher degree of glutamate release and subsequent excitotoxicity associated with more severe magnitudes of TBI.

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.

## Changes/Problems:

Year 2: We experienced 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.

Year 3: We experienced a slowdown in performance due technician Ken Van having to take extended family leave to care for his critically ill infant. These issues resolved and I subsequently requested and was granted a one-year no-cost extension of the project.

Year 4 (no-cost extension): Progress has slowed due to the resignation of Ken Van who took a new position as a research scientist at a biotech company on November 17, 2017.

## Products:

I am currently writing a manuscript to submit for publication that will incorporate the data presented in this final report.

## Participants & Other Collaborating Organizations:

**UC Davis participants:**

| | |
|---|---|
| Name: | Bruce Lyeth, PhD |
| Project Role: | Principle Investigator |
| Researcher Identifier: | 252972781 (UC Davis ID) |
| Contribution to the project: | Dr. Lyeth supervised the project and wrote all reports |

| | |
|---|---|
| Name: | Gene Gurkoff |
| Project Role: | Postdoctoral fellow |
| Contribution to the project: | Dr. Gurkoff performed calibration of the sensors |

| | |
|---|---|
| Name: | Ken Van, MS |
| Project Role: | Technician |
| Contribution to the project: | Mr. Van performed the surgeries and histology |

| | |
|---|---|
| Name: | Emily Doisy, BS |
| Project Role: | Staff Research Associate |
| Researcher Identifier: | 897554960 (UC Davis ID) |
| 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. |

**Weizmann Institute of Science participant:**

| | |
|---|---|
| Name: | David Mirelman, PhD |
| Project Role: | Consultant |
| Contribution to the project: | Professor Mirelman synthesized and provide the rGOT used in this project. He visited Dr. Lyeth once per year to consult on experimental design and interpretation of results and plan for future studies. |

## Special Reporting Requirements:

None

## Appendices:

None