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14. ABSTRACT
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Report Title

Final Report: A Neuroprotective and Anti-inflammatory Approach to Traumatic Brain Injury

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Scientific Progress

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Technology Transfer

patent development of injectable alpha-linolenic acid as a therapy against TBI.

DARPA Progress Report

December 26, 2016

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Title: A neuroprotective and anti-inflammatory approach to traumatic brain injury

Our group began this project on July 1, 2015. No work on this project was performed prior to July 1, 2015.

This is the final report for this project.

Alpha-linolenic Acid treatment reduces the contusion and prevents the development of anxiety-like behavior induced by a mild traumatic brain injury in rats

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Abstract

Approximately, 1.7 million Americans suffer a TBI annually and TBI is a major cause of death and disability. The majority of the TBI cases are of the mild type and while most patients recover completely from mild TBI (mTBI) about 10% result in persistent symptoms and some result in lifelong disability. Anxiety disorders are the second most common diagnosis post-TBI. Of note, TBI-induced anxiety disorders are difficult to treat and remain a chronic condition suggesting that new therapies are needed. Previous work from our laboratory demonstrated that a mild TBI induced an anxiety-like phenotype, a key feature of the human condition, associated with loss of GABAergic interneurons and hyperexcitability in the basolateral amygdala (BLA) in rodents seven and thirty days after CCI. We now confirm that animals display significantly increased anxiety-like behavior thirty days after a mild controlled cortical impact (CCI) injury. The anxiety-like behavior was associated with a significant loss of GABAergic interneurons and significant reductions in the frequency and amplitude of spontaneous and miniature $GABA_A$-receptor mediated inhibitory postsynaptic currents (IPSCs) in the BLA. Significantly, subchronic treatment with alpha-linolenic acid (ALA) after CCI prevents the development of anxiety-like behavior, the loss of GABAergic interneurons, hyperexcitability in the BLA and reduces the impact injury. Taken together, administration of ALA after CCI is a potent therapy against the neuropathology and pathophysiological effects of mTBI in the BLA.

Introduction

Traumatic brain injury (TBI) is a major public health concern in the United States and results in more than five million deaths per year across the globe. There are approximately 1.8 million brain injuries annually [1] and the costs to society are approximately 140 billion dollars annually [2]. Ninety percent of those who sustain a TBI may be classified as mild, in that patients do not display overt morphological or functional abnormalities immediately upon injury [3-4]. Most patients recover fully from mild TBI (mTBI), however for a significant fraction (10-15%), there are persistent cognitive, behavioral, and emotional sequelae [5-10]. A major concern is the prevalence of anxiety disorders after mTBI.

Anxiety disorders are the second most common disorders after depression post-TBI [11-13]. Recent reports indicate that about one in five individuals sustaining mTBI are at risk for developing anxiety disorders [14], and, particularly in the military, mTBI significantly increases the risk of developing posttraumatic stress disorder (PTSD) [15]. Twelve TBI studies demonstrated that 29% of almost 1200 individuals in these studies received a diagnosis of anxiety [16]. Post-traumatic stress disorder defined as individuals exhibiting heightened anxiety, avoidance, and re-experience of the initiating trauma is associated with TBI [16-17]. Among anxiety disorders, general anxiety disorders and PTSD are most common post-TBI; PTSD did not resolve very well over time and general anxiety disorders became a chronic condition [18]. While clinical TBI profiles are variable, mild and moderate TBI have been frequently localized to medial temporal lobe regions including the amygdala, and are associated with long-term neuropsychiatric symptoms. New brain targets involved in anxiety need to be discovered and alterations in network mechanisms within and surrounding those brain targets need to be

identified and delineated in order to translate novel therapies to prevent or remedy post-TBI anxiety disorders.

The amygdala is a limbic structure deep within the temporal lobe that is involved in processing emotion and regulating behavioral and physiological responses to stressors [9; 19]. Amygdalar hyperactivity has been observed in the majority of functional neuroimaging studies investigating anxiety disorders [20]. Significantly, bilateral amygdalar hyperactivity has been observed in U.S. soldiers after blast-induced TBI [21]. Thus, amygdalar dysfunction after a TBI, and in particular, neuronal hyperexcitability and hyperactivity in the basolateral nucleus of the amygdala (BLA), may be a key feature in the pathology of anxiety disorders, including PTSD, in TBI victims. Although it is well established that anxiety disorders are more prevalent after mTBI, only a few studies have begun to identify the functional and morphological alterations that take place in the amygdala underlying hyperexcitability after mTBI. Targeting the pathways involved in amygdalar hyperexcitability will lead to new more efficacious drugs are needed to treat TBI

The nutraceutical, α-linolenic acid (ALA), is an essential omega-3 polyunsaturated fatty acid found in vegetable oils, seeds, nuts, vegetables, poultry and egg yolk; soybean and canola oil, offer good sources of ALA [22]. The current dietary recommendation of ALA is 2.2 grams/day [23].

Many human studies have been conducted to ascertain the beneficial effects of ALA and emphasize the possible role of ALA deficiency in the diet that may increase one's risk for certain neurodegenerative disorders [24-28]. In animal studies, ALA is a highly efficacious therapy

either as a pretreatment or post-treatment against several models of neurodegenerative disorders including status epilepticus, spinal cord injury and stroke [29-34; 26; 24; 35-39]. However, the therapeutic efficacy of ALA in a model of TBI is obscure. One study showed an anti-inflammatory effect of ALA in the controlled cortical impact model of TBI with improved functional outcome [40].

We have recently discovered that, in an experimental model of mTBI, mild controlled cortical impact (mCCI), there is a reduction in $GABA_A$ receptor-mediated inhibitory synaptic transmission in the BLA, a loss of GABAergic interneurons, and a decrease in the surface expression of $GABA_A$ receptors [41]. These results suggest that a reduced inhibitory tonus in the amygdala could significantly contribute to the hyperexcitability and associated emotional deficits observed in mTBI patients. This finding is an important guide to developing novel therapeutic approaches for mTBI, in that it directs efforts towards protecting the brain against loss of GABAergic neuronal function, and the resultant reduced inhibitory tonus, that occur after TBI. Here, we used the CCI model of mild TBI to determine the therapeutic efficacy of ALA on the impact injury, the number of GABAergic interneurons and inhibitory tonus in the BLA and on anxiety-like behavior.

Experimental Procedures

Ethics Statement

All animal experiments were conducted following the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council) and were in accordance with the guidelines and approved by the Uniformed Services University of

the Health Sciences Institutional Animal Care and Use Committees (IACUC). All efforts were made to minimize the number of animals used and any pain or distress associated with these experiments.

Animals

Experiments were performed using 5-6 week old male, Sprague–Dawley rats (Taconic Farms, Germantown, NY, USA). Rats were pair-housed on arrival and acclimated for 3 days. A total of sixty rats were used for the study. One day prior to and after the surgery animals were individually housed in cages in an environmentally controlled room (20-23°C, ~44% humidity, 12-h light/12-h dark cycle [350-400 lux], lights on at 6:00 am), with food (Harlan Teklad Global Diet 2018, 18% protein rodent diet; Harlan Laboratories; Indianapolis, IN) and water available ad libitum. Cages were cleaned twice a week and animal handling was minimized to reduce animal stress [42].

Controlled Cortical Impact Injury

Groups of rats were subjected to either a unilateral cortical contusion using the previously established protocol of the controlled cortical impact (CCI) model of the traumatic brain injury [43-44] or sham. All animals were anesthetized with Isoflurane + 100% Oxygen (5.0 %, induction; 2.0-2.5%, maintenance). Isoflurane was delivered using a precision vaporizer via a rodent anesthesia machine. Induction occurred in an appropriately-sized clear viewing chamber. Following induction, rodents' heads were shaved and placed in the stereotaxic frame and isoflurane administration maintained via nose cone. Waste anesthetic gases were passively scavenged using a charcoal filter. Lack of paw-pinch reflex was used to ensure adequate depth

of anesthesia prior to beginning the CCI procedure. Core body temperature of the animals was maintained at 36-37°C using a heating pad and D.C. Temperature Control System (FHC, Bowdoin, ME). Ophthalmic ointment was applied to the eyes to prevent dryness.

The skin was cleaned with betadine and alcohol pads and the skull exposed using a small surgical incision along the sagittal suture of the frontal and parietal bones. After retracting the skin, a 4.0 mm in diameter craniotomy (3.0 mm lateral to the midline and 4.0 mm posterior to the bregma was performed over the left temporal-parietal cortex) using a hand-held trephine. After the bone cap was removed, and the dura mater exposed, a single contusion was delivered onto the surface of the dura over the temporal-parietal cortex with a 3 mm diameter impact tip and tip penetration depth of 2 mm, contact velocity of 3.5 m/sec and dwell time of 200 ms. These parameters have been shown to produce a contusion injury in the underlying cortex due to the impact of the piston but no loss of principal neurons in the BLA and hippocampus [44]. Following CCI, the skullcap was placed back and fixed using bone wax (Ethicon, Sommerville, NJ). The incision was closed with absorbable sutures (Stoelting, IL), and the animal placed in the cage on the water-heated pad for recovery. Sham animals were subjected to the identical procedure with the exception that they did not receive a contusion. All animals received subcutaneously buprenorphine (0.03-0.05 mg/kg) for pain alleviation and sterile Ringer's solution (5 mL) was injected subcutaneously to reduce dehydration after surgery.

Treatment groups

Animals were initially divided into four study groups: sham-alpha-linolenic acid (ALA) [sham-ALA], sham-vehicle (0.05%) [sham-veh], CCI-veh and CCI-ALA.

The sham-ALA and CCI-ALA animals were administered alpha-linolenic acid subcutaneously (1500 nmol/kg, Nu-Chek, Elysian, MN) 30 min, 3 days and 7 days after CCI or sham. Control groups was injected subcutaneously with vehicle (0.05% ethanol) at 30 min, 3 days and 7 days after CCI or sham. Animals were weighed prior to every injection to ensure that the proper ALA dose was injected subcutaneously; the identical volume of vehicle was injected into control animals. A needle size ranging from 22 to 25 gauge was used and the injected volume was 0.25-0.35 ml.

Behavioral Analysis

Open Field Test. Anxiety-like behavior was assessed in all groups of rats using an open field apparatus (40 x 40 x 20 cm clear Plexiglas arena) [45-46] during 2 sessions before the CCI and one session at 30 days after the CCI. Animals were acclimated to the apparatus in the first session. The second session provided the baseline measurements before CCI surgery. The third session was performed at 30 days after the surgery to test for the long-term effects of mTBI on locomotor activity and anxiety-like behavior, and assess the efficacy of ALA treatment. On the test day, rats were placed in the center of the open field and their activity was recorded for 20 min, using an Accuscan Electronics infrared photocell system (Accuscan Instruments Inc., Columbus, OH). Data were automatically collected and transmitted to a computer equipped with "Fusion" software (from Accuscan Electronics, Columbus, OH), which analyzed Locomotion (distance traveled in cm), Total Movement Time, Vertical Activity, and Time Spent in the Center of the open field. Anxiety behavior was measured as the ratio of the time spent in the center over the total movement time and expressed as a percentage of the total movement time, as previously described [46].

Contusion analysis and Immunohistochemistry

Fixation and Tissue Processing. CCI and sham animals were deeply anesthetized by intraperitoneal injection of Fatal Plus (65-75 mg/kg, i.p.) ten days after CCI and 30 days after CCI following completion of the behavior experiments. Once adequate anesthesia was achieved (lack of response to toe pinch, slow, even respirations), the rats were perfused with phosphate buffered saline (PBS, 200 ml) followed by 4% paraformaldehyde (250 ml). After adequate perfusion, the brains were removed and post-fixed in 4% paraformaldehyde overnight at 4°C, then transferred to a solution of 30% sucrose in PBS until brains were saturated with sucrose. Thereafter, brains were frozen with dry ice and stored at -80°C until sectioning. Sectioning was performed as previously described [47-48]. A 1-in-5 series of sections containing the rostrocaudal extent of the amygdala were cut at 40 μm thickness on a sliding microtome (Leica Microsystems SM2000R). 1-in-5 series of free-floating sections were collected from the cryoprotectant solution, and washed three times for 10 min each. Slices were mounted on a slide, air-dried overnight and processed for Nissl staining with cresyl violet.

Volumetric Analysis of Cortical Contusion. Volumetric analysis was performed 10 and 30 days after CCI. Nissl-stained sections containing the hippocampal formation (sections were 400 μm apart) were used to estimate the volume of the cortical contusion based on the previously described Cavalieri principle using stereological analysis [49]. Sections were viewed with a Zeiss Axioplan 2ie fluorescent microscope with a motorized stage (Oberkochen, Germany), interfaced with a computer, running StereoInvestigator 9.0 (MicroBrightField, Williston, VT). The cortical contusion was identified on slide-mounted sections under a 2.5x objective and traced using the Stereo Investigator 9.0. The volume was calculated by using the stereological probe called Cavalieri Estimator. An overlay of a rectangular lattice with a grid size of 50 μm

was placed over cortical contusion tracings, and each point marked was counted to estimate the volume. For each animal, the coefficient of error (CE) was calculated to assure sufficient accuracy of the estimate (CE < 0.05). The analysis of the contusion was performed by a person who was blinded to the treatment groups.

GAD-67 Immunohistochemistry. To label GAD-67 immunoreactive neurons, 1-in-5 series of free-floating sections were collected from the cryoprotectant solution, washed in 0.1 M PBS three times for 10 min each, and incubated in a blocking solution containing 10% normal goat serum (Millipore Bioscience Research Reagents, Temecula, CA) and 0.5% Triton X-100 in PBS for 1 hour at room temperature. The sections were then incubated in a solution containing mouse anti-GAD-67 serum (1:1000, MAB5406; Millipore Bioscience Research Reagents), 5% normal goat serum, 0.3% Triton X-100, and 1% bovine serum albumin overnight at 4°C. After rinsing in 0.1% Triton X-100 in PBS three times for 10 min each, the sections were incubated with Cy3-conjugated goat anti-mouse antibody (1:1000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and 0.0001% 4,6-diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich) in PBS for 1 hour at room temperature. After a final rinse in PBS for 10 min, sections were mounted on slides, air-dried for at least 30 min, and slip-covered with ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA).

Stereological Quantification. Design-based stereology was performed to quantify the total number of neurons in Nissl-stained sections and interneurons in GAD-67-immunostained sections in the BLA thirty days (30) after CCI. Sections were viewed with a Zeiss Axioplan 2ie fluorescent microscope (Oberkochen, Germany) with a motorized stage, interfaced with a computer running StereoInvestigator 8.0 software (MicroBrightField, Williston, VT). The BLA

was identified on slide-mounted sections and delineated for each slide of each animal, under a 2.5x objective, based on the atlas of Paxinos and Watson [50]. All sampling was done under a 63x oil immersion objective. Nissl-stained neurons were distinguished from glia cells by their larger size and pale nuclei surrounded by darkly stained cytoplasm containing Nissl bodies. The GAD-67-positive neurons were identified and counted in real time by their bright fluorescent precipitate within their cell body and axonal process, and by their relatively pale nuclei. The total number of Nissl-stained and GAD-67-immunostained neurons was estimated by using the optical fractionator probe, and, along with the coefficient of error (CE), was calculated by using the StereoInvestigator 9.0 software (MicroBrightField). The CE was calculated based on the equation of Gundersen et al., (m = 1; [51]).

For Nissl-stained neurons in the BLA, 1-in-5 series of sections were analyzed (six sections on average). The counting frame was 35 X 35 μm, the counting grid was 190 X 190 μm, and the dissector height was 12 μm. Nuclei were counted when the cell body came into focus within the dissector, which was placed 2 μm below the section surface. Section thickness was manually measured at every counting site, and the average mounted section thickness was 16.5 μm. The average number of neurons per rat counted was 263.61 ± 6.6, and the average CE was 0.06 for Gunderson equation.

For GABAergic interneurons immuno-labeled for GAD-67 in the BLA, 1-in-5 series of sections were analyzed (on average six sections). The counting frame was 60 X 60 μm, the counting grid was 100 X 100 μm, and the dissector height was 20 μm. Nuclei were counted when the top of the nucleus came into focus within the dissector, which was placed 2 μm below the section surface. Section thickness was manually measured at every fifth counting site, and the

average mounted section thickness was 24.3 μm. The average number of GAD-67 positive neurons per rat counted was 236.7 ± 14.2 and the average CE was 0.07 for Gunderson equation.

Amygdala Slice Electrophysiology

Electrophysiological experiments. The rats were anesthetized with isoflurane and then decapitated at either ten (10) or thirty (30) days after CCI or sham. Coronal brain slices (400 μm thick) containing the amygdala (-2.64 to -3.36 from bregma) were cut using a vibratome (Leica VT1200 S; Leica Microsystems, Buffalo Grove, IL) in ice-cold cutting solution consisting of (in mM): 115 sucrose, 70 N-methyl-D-glucamine (NMDG), 1 KCl, 2 $CaCl_2$, 4 $MgCl_2$, 1.25 NaH_2PO_4, 30 $NaHCO_3$, and 25 D-glucose. The slices were transferred to a holding chamber, at room temperature, in a bath solution containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 21$NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$, and 25 D-glucose. Recording solution (artificial cerebrospinal fluid; ACSF) was the same as the holding bath solution. All solutions were saturated with 95% O2-5% CO_2 to achieve a pH near 7.4. For whole cell recordings, the slice chamber (0.7-ml capacity) had continuously flowing ACSF (8 ml/min) at temperature of 32 to33°C. The osmolarity of external solution was about 330 mOsm/kg.

For whole cell recordings, neurons were visualized under infrared light using Nomarski optics of an upright microscope (Axioskop 2; Zeiss, Thornwood, NY) through a x40 water-immersion objective, equipped with a CCD-100 camera (Dage-MTI, Michigan City, IN). The patch electrodes had resistances of 3.5– 4.5 MΩ when filled with the internal solution (in mM): 60 $CsCH_3SO_3$, 60 KCH_3SO_3, 10 KCl, 10 EGTA, 10 HEPES, 5 Mg-ATP, 0.3 Na_3GTP (pH 7.2), about 290 mOsm/kg. When $GABA_A$Rs-mediated sIPSCs were recorded, the internal chloride

concentration was 1 mM, and osmolarity was adjusted to about 300 mOsm/kg with potassium gluconate. We used KCl bridge electrode holders (ALA Scientific Instruments, Farmingdale, NY), which provide stable offset potentials and make the concentration of Cl^- in the pipette solution irrelevant (the Ag/AgCl wires are in constant contact with 2 M KCl). Tight-seal (about 1 GW and higher) whole cell recordings were obtained from the cell body of pyramidal-shaped neurons in the BLA region, which were identified on the basis of their electrophysiological properties [52-53]. Access resistance (15–24MΩ) was regularly monitored during recordings, and cells were rejected if the resistance changed by about 15% during the experiment.

Ionic currents and action potentials were amplified and filtered (1 kHz) using the Axopatch 200B amplifier (Axon Instruments, Foster City, CA) with a four-pole, low-pass Bessel filter, digitally sampled (up to 2 kHz) using the pClamp 10.2 software (Molecular Devices, Sunnyvale, CA), and further analyzed using the Mini Analysis Program (Synaptosoft, Fort Lee, NJ) and Origin (OriginLab, Northampton, MA). The charge transferred by postsynaptic currents was calculated as area under sIPSCs (in pico-Coulombs; pC) using the Mini60 software by Synaptosoft.

Drugs used were as follows: D-AP5 (50μM), an N-methyl-D-aspartate receptor antagonist, SCH 50911(10μM), a $GABA_B$ receptor antagonist, and LY 341495 (3μM), a metabotropic glutamate group II/III receptor antagonist (all purchased from Tocris Bioscience, Ellisville, MO).

Whole-cell patch-clamp recordings in the rat brain slices containing amygdala.

$GABA_A$Rs-mediated sIPSCs were recorded at holding potential (V_h) of +30 mV in the presence of D-AP5 (50μM); SCH50911(10μM); LY341495 (3μM). After a BLA pyramidal cell was patch-clamped, the holding potential was switched from conventional -70 mV to +30 mV. The cell was left to equilibrate with the new V_h for about 4 min in drug-free bath solution (ACSF) and then another 4 min in antagonists-containing bath solution. Spontaneous IPSCs were recorded during 4 min after that. Charge transferred by sIPSCs was estimated for 40s-long time-window. At V_h = +30 mV all outward currents (sIPSCs) were carried via $GABA_A$ channels and were blocked by 20μM bicuculline ($GABA_A$Rs antagonist; not demonstrated).

Statistical Analysis

Statistical values are presented as means ± standard error (SE) of the mean. Results from sham animals that received vehicle were compared with results of sham animals that received Alpha-Linolenic Acid using the independent-t test. Since there were no significant differences in the results between the sham-veh and sham-ALA animals, the animals were grouped and the results compared with each CCI-treated group. Results were compared using one-way ANOVA followed by the Least Significant Difference (LSD) post-hoc test. Results were considered statistically significant when the p value was < 0.05. Sample sizes (n) refer to the number of rats, except for the electrophysiology results where sample size (n) refers to the number of neurons for the whole cell experiments.

Results

Treatment with α-linolenic acid reduces the cortical contusion volume after mTBI

Sham animals showed a cortical volume of 1.95 ± mm^3 (n=8) measured at the site of impact (3 mm extent, left hemisphere). Animals that received treatment with α-linolenic acid (CCI-ALA group) showed a statistically significant reduction in the cortical contusion volume measured 10 days after CCI (6.18 ± 1.9 mm^3, n=6) compared to animals that received vehicle (CCI-veh group: 19.87 ± 3.5 mm^3, n=6, p=0.009). Similar results were found thirty days after CCI (CCI-veh group: 21.89 ± 2.1 mm^3, n=6 versus CCI-ALA group: 7.5 ± 1.8 mm^3, n=6; p=0.0008). Both CCI groups showed a significant increase in the lesion volume measured at the site of impact compared with sham animals (1.55 ± 0.5 mm^3, n=8; p<0,001) (Figure 1).

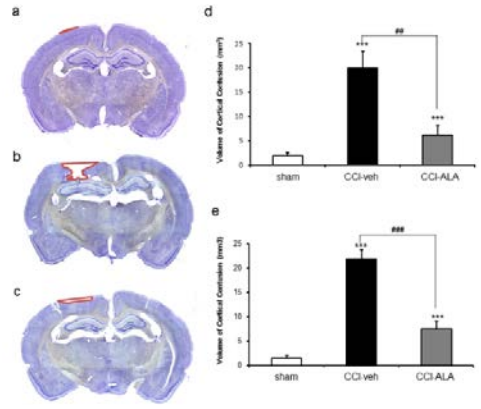

Figure 1. Treatment with α-Linolenic acid at 30 min, 3 days and 7 days after CCI reduces the volume of the cortical contusion quantified 10 and 30 days after CCI. Representative photomicrographs of a coronal brain slice showing the tracing of sham (a) controlled cortical impact-vehicle (CCI-veh) (b) and controlled cortical impact treated with α-linolenic acid (CCI-ALA) (c) animals. Top bar graph (d) shows average and standard errors of cortical contusion volume 10 days after CCI (n = 6-8 group), and bottom bar graph (e) shows average and standard errors of cortical contusion volume 30 days after CCI (n=6- 8 group). ***p<0.001 compared with sham; ## p<0.01 and ### p< 0.001 compared with CCI-ALA.

Treatment with α-linolenic acid prevents the long-term increased anxiety-like behavior

Significant differences in the percent time spent in the center of the open field were found between CCI-veh rats and both sham and CCI-ALA animals (p=0.005; Figure 2A) thirty days after the surgery. CCI-veh rats spent significantly less time in the center of the open field (9.9 ± 1% of the total movement time) compared to CCI-ALA animals (15.9 ± 1.9% of the total movement time; p=0.01) or to sham rats (17.1 ± 1.8% of the total movement time; p=0.002). CCI-veh animals did not differ significantly from CCI-ALA or sham animals in distance traveled (2,536.3 ± 178.7cm for sham animals, 3,020.9 ± 238.9 cm for CCI-veh animals and 2,435.2 ± 107.5 cm for CCI-ALA animals) [Figure 2B], vertical activity (136.9 ± 8.8 beam crosses for sham animals, 138.9 ± 8.5 beam crosses for CCI animals and 131.7 ± 6.4 beam crosses for CCI-ALA animals) [Figure 2C], or movement time (802.1 ± 24.5 sec for sham animals, 869.5 ± 17.9 sec for CCI animals and 826.7 ± 15.7 sec for CCI-ALA animals) [Figure 2D].

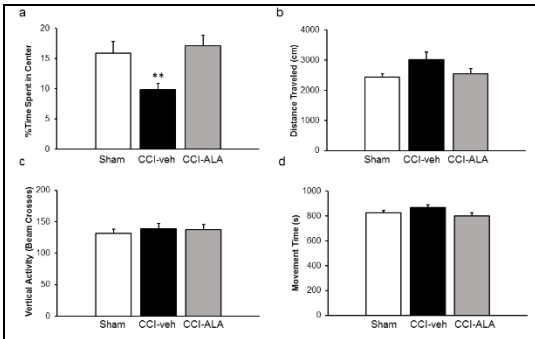

Figure 2. Treatment with Alpha Linolenic Acid 30 min, 3 days and 7 days after Mild Traumatic Brain Injury prevents long term increased anxiety like behavior in the open filed test. (a) CCI-veh (n=18) rats spent significantly less % time in the center of the open field 30 days after the injury compared to CCI-ALA (n= 18) and sham (n=18) rats. No significant differences were found between sham, CCI-veh and CCI-ALA rats in distance traveled (b), vertical activity (c), and movement time (d). **p<0.01 compared with the other groups.

Treatment with α-linolenic acid prevents the loss of GABAergic inhibitory interneurons in the BLA after mild TBI.

Estimation of the total number of interneurons in the BLA, using an unbiased stereological method to quantify GAD-67 immunoreactive cells, showed a significant loss of GABAergic interneurons thirty days after the injury in CCI-veh animals compared to CCI-ALA or sham animals (Table 1; Figure 3A). There was no significant difference between numbers of GAD-67 positive interneurons in sham and CCI-ALA animals.

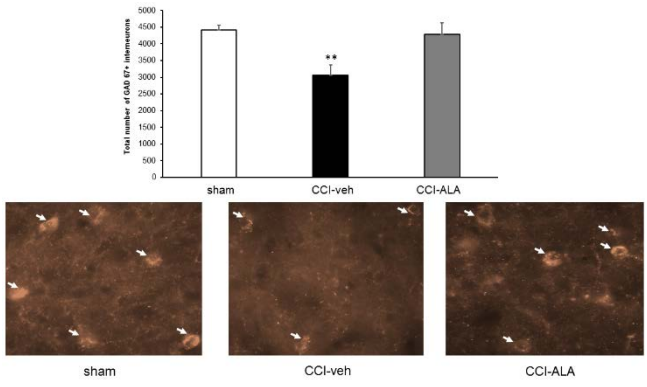

Figure 3. Treatment with Alpha Linolenic Acid 30 min, 3 days and 7 days after mTBI prevents loss of GABAergic inhibitory interneurons in the BLA. (Top) Group data showing the mean and standard error of the stereologically estimated total number of GAD-67 positive cells in the BLA 30 days after the injury. Only CCI-veh rats show a significant reduction in GAD-67 positive cells compared to sham or CCI-ALA rats (n=4-5 per group). (Bottom) Representative photomicrograph of GAD-67 immunohistochemically stained GABAergic interneurons in the BLA of sham, CCI-veh and CCI-ALA rats 30 days after the injury. Total magnification is 63X, scale bar 50µm. **p<0.01 compared with other groups.

Table 1. Total Number of GAD 67 Immunoreactive Interneurons 30 Days After mTBI

Group	# of Cells ± SEM
sham	4290.5 ± 337.1
CCI-veh	3060.3 ± 308.8**
CCI-ALA	4423.8 ± 141.1

**p<0.01

The loss of GABAergic interneurons was not due to an overall neuronal loss since estimation of the total number of neurons in the BLA, using an unbiased stereological method in Nissl-stained

sections, revealed that there was no significant reduction in the total number of neurons thirty days after the injury in CCI-veh animals compared to sham or CCI-ALA animals (Table 2; Figure 4).

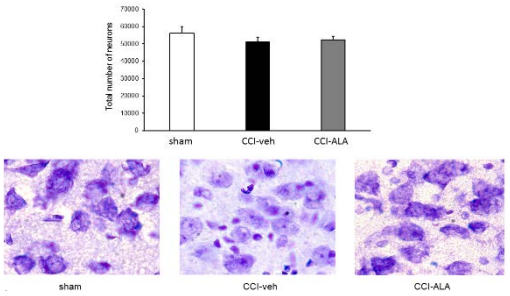

Figure 4. Mild CCI does not cause a significant loss of neurons in the BLA 30 days after injury. (Top) Group data (mean ± SE; n = 4-5 for each group) of stereological estimation of the total number of Nissl-stained neurons in the BLA. (Bottom) Representative photomicrographs from Nissl-stained sections showing BLA cells from sham, CCI-veh, and CCI-ALA animals, respectively. Total magnification is 630X; scale bar, 50 µm.

Table 2. Total Number of Neurons 30 Days After mTBI

Group	# of Cells ± SEM
sham	56214.3 ± 3616.5
CCI-veh	51182 ± 2584.9
CCI-ALA	52245 ± 1986.6

Effect of α-linolenic acid on altered $GABA_A$-mediated spontaneous IPSCs of the principle neurons in the BLA after mTBI

Whole-cell recordings were obtained from BLA neurons that were identified on the basis of their size, pyramidal-like shape, firing patterns in response to depolarizing current pulses in the current-clamp mode, and the presence of a current activated by hyperpolarizing voltage-steps (I_h), in the voltage clamp mode. Depolarizing current injections generated variable patterns of accommodating spiking. Four 1 s-long hyperpolarizing pulses starting from V_{hold} -70 to -80 mV and ending with -110 mV elicited nonlinear I_h current in principal neurons (data not shown).

To determine whether treatment with ALA prevented the reduction in inhibitory synaptic transmission observed after mTBI [41], we recorded spontaneous $GABA_A$ receptor-mediated IPSCs (sIPSCs) from principal neurons in the BLA in the presence of D-AP5, SCH50911, and LY 3414953 at a holding potential of +30 mV. We examined the mean charge transferred area under sIPSC. Thirty days after mTBI, the mean charge transferred by $GABA_A$Rs-mediated IPSCs in CCI-veh animals (270 ± 30 pC; n = 8) was significantly decreased compared to sham (400 ± 40 pC; n = 15, p=0.023) and CCI-ALA animals (490 ± 90 pC; n = 9, p=0.031). Sham and CCI-ALA animals did not differ in the amount of charge transferred by sIPSCs thirty days after CCI (Figure 5). Since no significant difference was found between CCI-ALA and sham animals at thirty days, we also examined the mean charge transferred by $GABA_A$Rs-mediated IPSCs ten days after mTBI. Ten days after mTBI, the mean charge transferred by $GABA_A$Rs-mediated IPSCs in CCI-veh animals (314 ± 27 pC; n = 9) was significantly decreased compared to sham (471 ± 25 pC; n = 13, p=0.032) and CCI-ALA animals (497 ± 67 pC; n = 7, p=0.032). As observed in the thirty day group of animals, sham and CCI-ALA

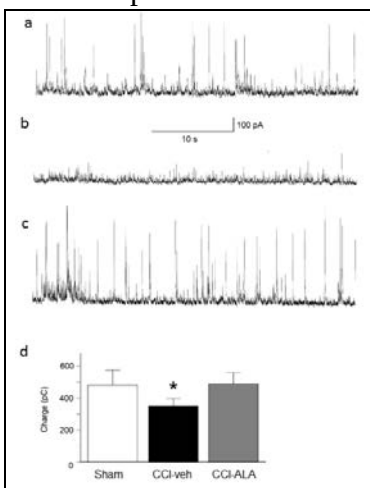

Figure 5. Effect of ALA on sIPSCs of the principle neurons in the BLA 30 days after mTBI. (a) sIPSCs recorded from BLA pyramidal neuron from sham male rat. (b) sIPSCs recorded from BLA pyramidal neuron from CCI-veh male rat. (c) sIPSCs recorded from BLA pyramidal neuron from CCI-ALA male rat. a-c: holding potential + 30 mV. (d) Ordinate axis: charge transferred by sIPSCs measured in pico-Coulombs (pC). Abscissa: columns illustrating the amount of charge transferred by sIPSCs during 40 s at Vh = +30 mV for different animals in different conditions. There was a significant reduction in amount of charge transferred by sIPSCs in CCI-veh rats 30 days after the injury. * $p < 0.05$ compared with other groups.

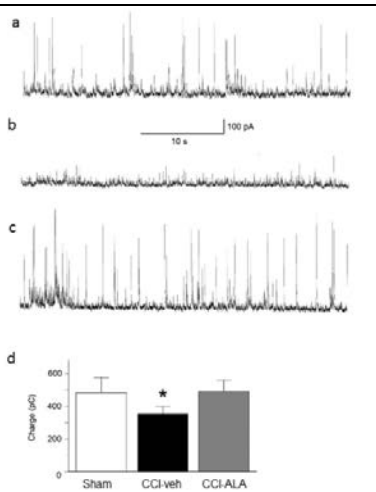

Figure 6. Effect of ALA on sIPSCs of the principle neurons in the BLA 10 days after mTBI. (a) sIPSCs recorded from BLA pyramidal neuron from sham male rat. (b) sIPSCs recorded from BLA pyramidal neuron from CCI-veh male rat. (c) sIPSCs recorded from BLA pyramidal neuron from CCI-ALA male rat. A, B, C: holding potential + 30 mV. (d) Ordinate axis: charge transferred by sIPSCs measured in pico-Coulombs (pC). Abscissa: columns illustrating the amount of charge transferred by sIPSCs during 40 s at Vh = +30 mV for different animals in different conditions. There was a significant reduction in amount of charge transferred by sIPSCs in CCI-veh rats 30 days after the injury. * $p < 0.05$ compared with other groups.

animals did not differ in the amount of charge transferred by $GABA_AR$-mediated IPSCs ten days after CCI (Figure 6).

Discussion

The current study shows that CCI results in a significant contusion (lesion) volume ten and thirty days after CCI in the absence of treatment. Importantly, administration of ALA by subcutaneous injection at 30 min, 3 days and 7 days after CCI results in a significant reduction in contusion (lesion) volume. Although the definition of mild TBI in humans excludes brain injury by imaging studies, moderate and severe TBI can and do sustain lesions in the brain [54]. The brain injury results in the loss of tissue and neurological dysfunction that depends upon the brain region affected by the injury. Secondary injury cascades initiated by the mechanical injury result in the activation of caspase-dependent and independent mechanisms leading to neuronal and non-neuronal cell death [55]. Moreover, TBI triggers a neuroinflammatory response that involves activation of microglia and astrocytes leading to the release and synthesis of pro-inflammatory cytokines [56]. It is possible that the pleiotropic properties of ALA are involved in the significant reduction of tissue loss from the impact injury over the thirty day period [33].

Anxiety is the second most common neuropsychiatric disorder following TBI and of the anxiety disorders, general anxiety disorders are most common [11-13]. Clinical data show amygdalar hyperactivity in the majority of functional neuroimaging studies investigating anxiety disorders [20]. These results formed the fundamental basis to determine the basic pathophysiological mechanisms in the BLA that are involved in the observed amygdalar hyperactivity observed in humans after TBI.

Previous work from our laboratory demonstrated that there is an internalization of $GABA_A$ receptors as well as loss of GABAergic neurons in the BLA leading to a decrease in inhibitory transmission; there is increased function of the α-7 containing nicotinic receptors, one week after mTBI, a possible compensatory mechanism for the GABAergic hypofunction observed after CCI. Unaltered cholinergic drive in the hippocampus indicates that this compensation is specific to the amygdala. Moreover, CCI-induced mTBI resulted in an increase in anxiety-like behavior, as tested by the open field test, seven and thirty days after CCI [41; 57].

We confirmed in this study that the anxiety-like behavior, measured by the open field test, lasts for at least thirty days after CCI. There were no differences in the distance traveled, vertical activity or movement time across CCI-induced and sham animals in the open field task indicating that the significant reduced time in the center was a specific effect of the brain injury in the absence of treatment. We now show that the increase in anxiety-like behavior is associated with and may be the result of a significant loss of GABAergic inhibitory neurons as well as a significant reduction in $GABA_A$ receptor-mediated inhibitory synaptic transmission and increased excitability in the BLA thirty days after CCI in the absence of treatment; there was no significant loss of principal neurons in the BLA. These results suggest that a single mechanical brain injury to the cortex can exert detrimental effects in the BLA, a structure deep in the temporal lobe. Due to the proximity to bony protrusions, the amygdala is easily injured as the brain ricochets inside the skull after concussive impact. Importantly, we did not observe a significant reduction in the number of GABAergic interneurons or a reduction in GABAergic synaptic transmission in the BLA at thirty days following administration of ALA at 30 min, 3 days and 7 days after CCI. Because prior work showed a reduction in GABAergic synaptic

transmission in the BLA at seven days, we determined the electrophysiological properties of groups of sham and CCI-induced animals three days after ALA treatment, ten days after CCI. Results showed a significant reduction in the GABAergic synaptic transmission in the BLA in the group of animals subjected to CCI in the absence of treatment. In contrast, there was no significant difference in the GABAergic synaptic transmission in the BLA between sham and the group of CCI-induced animals injected with ALA at 30 min, 3 days and 7 days after CCI. These results suggest that ALA prevents the significant reduction in the GABAergic synaptic transmission observed at thirty days. These results also suggest that the selective vulnerability of the inhibitory interneurons in the BLA leading to hyperexcitability may contribute significantly to the increase in anxiety-like behavior because administration of ALA after CCI prevented the loss of inhibitory interneurons, the reduction in GABAergic synaptic transmission and the anxiety-like behavior thirty days after CCI. The ALA treatment results substantiate the conclusions that the increase in anxiety-like behavior is due to the significant loss of GABAergic interneurons and reduced GABAergic synaptic transmission in the BLA.

Traumatic brain injury is responsible for over five million deaths annually throughout the world and is a leading cause of disability and death especially in young adult males [1; 58]. TBI is often bilateral and affects limbic structures such as the amygdala and hippocampus, frontotemporal and basal ganglia resulting in behavioral, emotional, and cognitive impairment. Long-term consequences include neurological and neuropsychiatric disorders; anxiety disorders rank second only after depression. The most common diagnosis within the anxiety disorders is general anxiety disorders. While treatments for these long-term consequences are available, no formal clinical trials have been conducted in those that develop these disorders post-TBI [59-60;

18; 12]. In addition, general anxiety disorders develop into chronic conditions within the mild to moderate group of those individuals that sustain a traumatic brain injury [12]. These findings suggest that newer more effective therapies are needed for post-TBI anxiety disorders. ALA, a nutraceutical with pleiotropic properties and a natural product that has a very wide safety margin, should be further developed as a therapy to reduce brain injuries and prevent the long-term anxiety disorders that develop after TBI.

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