

# Neuroprotection by Acetyl-L-Carnitine after Traumatic Injury to the Immature Rat Brain

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## Key Words

Controlled cortical impact · Sensorimotor function · Memory · Hippocampus · Acetyl-L-carnitine · Neuroprotection · Novel object recognition

## Abstract

Traumatic brain injury (TBI) is the leading cause of mortality and morbidity in children and is characterized by reduced aerobic cerebral energy metabolism early after injury, possibly due to impaired activity of the pyruvate dehydrogenase complex. Exogenous acetyl-L-carnitine (ALCAR) is metabolized in the brain to acetyl coenzyme A and subsequently enters the tricarboxylic acid cycle. ALCAR administration is neuroprotective in animal models of cerebral ischemia and spinal cord injury, but has not been tested for TBI. This study tested the hypothesis that treatment with ALCAR during the first 24 h following TBI in immature rats improves neurologic outcome and reduces cortical lesion volume. Postnatal day 21–22 male rats were isoflurane anesthetized and used in a controlled cortical impact model of TBI to the left parietal cortex. At 1, 4, 12 and 23 h after injury, rats received ALCAR (100 mg/kg, intraperitoneally) or drug vehicle (normal saline). On days 3–7 after surgery, behavior was assessed using beam walking and novel object recognition tests. On day 7, rats were transcatheterially perfused and brains were harvested for histological assessment of cortical lesion volume, using

stereology. Injured animals displayed a significant increase in foot slips compared to sham-operated rats ( $6 \pm 1$  SEM vs.  $2 \pm 0.2$  on day 3 after trauma;  $n = 7$ ;  $p < 0.05$ ). The ALCAR-treated rats were not different from shams and had fewer foot slips compared to vehicle-treated animals ( $2 \pm 0.4$ ;  $n = 7$ ;  $p < 0.05$ ). The frequency of investigating a novel object for saline-treated TBI animals was reduced compared to shams ( $45 \pm 5\%$  vs.  $65 \pm 10\%$ ;  $n = 7$ ;  $p < 0.05$ ), whereas the frequency of investigation for TBI rats treated with ALCAR was not significantly different from that of shams but significantly higher than that of saline-treated TBI rats ( $68 \pm 7$ ;  $p < 0.05$ ). The left parietal cortical lesion volume, expressed as a percentage of the volume of tissue in the right hemisphere, was significantly smaller in ALCAR-treated than in vehicle-treated TBI rats ( $14 \pm 5\%$  vs.  $28 \pm 6\%$ ;  $p < 0.05$ ). We conclude that treatment with ALCAR during the first 24 h after TBI improves behavioral outcomes and reduces brain lesion volume in immature rats within the first 7 days after injury.

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## Introduction

Traumatic injury to the adult and developing brain is characterized by impairment of cerebral energy metabolism within minutes to hours after the initial injury and therefore prior to most of the subsequent cell death [1, 2].

The molecular basis for the reduced aerobic energy metabolism and associated increase in glycolytic lactate production is not completely elucidated [2–4]. Oxidative modification and inactivation of one or more mitochondrial metabolic enzymes, and possible impairment of the malate-aspartate shuttle function have been reported [4]. Evidence obtained from animal models of both traumatic brain and spinal cord injury and from ischemia/reperfusion paradigms point towards the pyruvate dehydrogenase complex (PDHC) as a critical metabolic enzyme that is particularly sensitive to inhibition within a few hours after the initial injury, by either oxidative modifications or serine phosphorylation [4–15]. Since the overall reaction catalyzed by this complex constitutes the bridge between glycolysis and the first step of the tricarboxylic acid (TCA) cycle, impairment of the aerobic energy metabolism at the level of the PDHC can potentially be overcome by use of alternative fuels that enter the cycle distal to this reaction. Such fuels include ketone bodies, fatty acids and acyl carnitines, which can all be metabolized to form acetyl coenzyme A (CoA), the product of the PDHC reaction and a substrate for the citrate synthase reaction within the TCA cycle [4, 16–22]. Acetyl-L-carnitine (ALCAR) is a naturally occurring metabolic intermediate involved in transmembrane trafficking of acetyl units for both catabolic and anabolic metabolism [23, 24]. ALCAR can be transported across the cell membrane and the mitochondrial inner membrane by carnitine translocases in exchange for free carnitine [24]. Once inside the mitochondrial matrix, ALCAR is converted to acetyl CoA and free carnitine via carnitine acyl transferases [24]. Numerous reports show neuroprotective effects of exogenous ALCAR in a number of neurologic disorders [22, 25–30]. Supraphysiologic levels of exogenously administered ALCAR provide neuroprotection in animal models of neurodegenerative diseases and acute neurologic injury paradigms [22–24, 31]. Posttreatment of adult animals with ALCAR reduces neurologic impairment and/or neuronal death in rodent models of stroke [32–34] and in a canine model of cardiac arrest [21]. ALCAR also reduces spinal cord mitochondrial respiratory impairment and reduces gray matter lesion volume in an adult rodent model of spinal cord injury [6]. There are no published studies, however, that have tested neuroprotection by ALCAR in the injured immature brain, where the pathophysiology is significantly different from that of the mature brain [2, 35–37]. We have recently demonstrated that following systemic administration, the acetyl moiety of  $^{13}\text{C}$ -labeled ALCAR is metabolized for energy via the TCA cycle in astrocytes and neurons within the fore-

brains of 21- to 22-day-old rats [38]. Furthermore,  $^{13}\text{C}$  from ALCAR was incorporated into  $\gamma$ -aminobutyric acid (GABA), glutamate and glutamine, which are formed subsequent to TCA cycle metabolism [38]. In light of the ability of the immature rat brain to utilize ALCAR for both energy metabolism and neurotransmitter formation, this study tested the hypothesis that administration of ALCAR after controlled cortical impact (CCI) traumatic brain injury (TBI) to 21- to 22-day-old rats improves neurologic outcome and reduces brain cell death.

## Methods

### *Brain Injury*

All animal procedures were approved by the University of Maryland Animal Care and Use Committee and are in compliance with the National Institutes of Health guidelines. TBI was induced using an established immature rat model [4]. Male Sprague-Dawley rats were weaned from their dams at postnatal day (PND) 20 and used for experiments on PND 21 or 22. The animals were randomly assigned to 4 groups: CCI + vehicle (normal saline); CCI + ALCAR; sham + vehicle, and sham + ALCAR. At 1, 4, 12 and 23 h after surgery, the animals received 100 mg/kg ALCAR or vehicle (normal saline) by intraperitoneal injection. The rats received maintenance anesthesia (2% isoflurane) via nose cone with 30% oxygen. A rectal probe and heating blanket were used to maintain a rectal temperature of  $37.0 \pm 0.5^\circ\text{C}$ . A left parietal craniotomy was created using a high-speed drill. CCI injury was induced with a 6-mm flat-tipped impactor at a velocity of 5.5 m/s, a depth of 1.5 mm, and a duration of 50 ms. Following the impact, the bone flap was replaced, the craniotomy sealed and the scalp incision closed with sutures. Sham animals were anesthetized and received a craniotomy but no impact. After the surgical procedures had been completed, anesthesia was discontinued.

### *Behavioral Tests*

**Beam Walking.** The rats were tested for their ability to traverse an inclined (35-degree angle) elevated beam (width: 1 cm; length: 100 cm) during postinjury days 3–7; the data were obtained by recording the traverse time and number of foot slips [39].

**Novel Object Recognition.** On day 6 after surgery, animals were habituated in a closed Plexiglas arena (48 cm long, 40 cm wide, 30 cm high) for 1 min as described by Bevins and Besheer [40], and then returned to their home cage. To minimize anxiety, this arena was placed within a 5 times larger arena with dark walls in a dimly lit room. For the exploration phase, which was conducted 1 h after the habituation phase, 2 identical objects were placed equidistantly into the inner arena across from where the rat was placed. The rats were allowed to investigate for 15 min and explore the objects. Exploration was defined as sniffing and/or touching the objects. The rats were returned to their home cages. On postinjury day 7, 24 h after the exploration phase, the rats were again placed within the arena in the absence of objects for 1 min and then returned to their home cages. For the test phase conducted 1 h later, 1 of the familiar objects was replaced with a novel object and the animals were allowed to explore the arena. Rats have a natural predilection for new

items, thus the percent time spent exploring a new object reflects the degree to which they remember the familiar object [41, 42].

### Histology

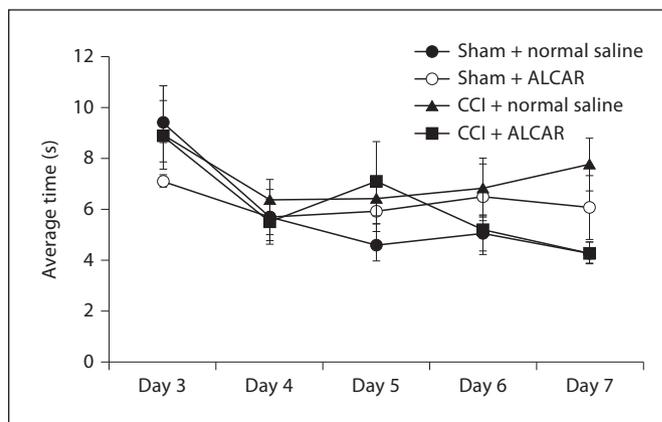
On day 7 after injury or sham surgery and after completion of the behavioral studies, all animals were perfused with 4% paraformaldehyde and brains were harvested. Brains were removed from the skull and transferred into 30% sucrose. Once the brains had sunk to the bottom of the container, they were cut (35  $\mu\text{m}$ ) on a freezing sliding microtome, yielding 24 series per animal, and kept in cryoprotectant ( $-20^\circ\text{C}$ ) until further processing was initiated. Seven sections, 35  $\mu\text{m}$  thick and 840  $\mu\text{m}$  apart, corresponding to the epicenter of the CCI injury, plus 3 sections rostral and 3 sections caudal to the epicenter, were carefully mounted on slides and stained with cresyl violet.

Design-based stereology was performed on the stained sections using a Nikon Eclipse E800 microscope equipped with a MicroBrightField 3-axis, computer-controlled, motorized stage, an Optronics 1-CCD digital video camera and a PC workstation (Intel Core 2 Duo E6850 processor). Quantitative analyses of the entire anterior-posterior extent of the necrotic cortical lesion volume were performed using the Cavalieri method and SterioInvestigator software (MicroBrightField, Wiliston, Vt., USA) by a microscopist blinded to the treatment protocol. For each section analyzed, the Cavalieri estimator probe was utilized to overlay a rectangular lattice of points every 50  $\mu\text{m}$  across the ipsilateral and contralateral cortical areas. The number of points in the lattice that lay within the ipsilateral cortical contusion (tissue lost) and corresponding contralateral section were counted under  $\times 10$  magnification. Borders between healthy and necrotic cortex were generally sharp and easily identified because necrotic tissue was either lost or contained very few viable neurons. Neurons with an intact nucleolus and regular distribution of Nissl bodies throughout their cytoplasm were considered viable, while shrunken neurons with a condensed nucleus and lacking a nucleolus were considered nonviable (fig. 4). Cortical regions surrounding the necrotic divot that were devoid of viable cells were included as part of the lesion volume. Because necrotic tissue is frequently lost with processing, the upper border for ipsilateral lesioned cortex was estimated to reflect the upper border of tissue from the corresponding healthy contralateral cortex (dashed lines in figure 4) from the same slice of tissue. A minimum of 250 points were counted per subject to ensure robustness of the data.

Necrotic divot and contralateral cortical areas and volumes were calculated separately by the SterioInvestigator software. The area representing ipsilateral lesioned and contralateral healthy cortex was first calculated per section by multiplying the number of points counted per region by the grid area ( $g^2$ ) associated with each point:  $A = p \cdot g^2$ . The volume of the necrotic divot and contralateral cortical tissue throughout the anterior-posterior extent of the hemispheres was then estimated by multiplying the sum of areas ( $\Sigma A$ ) by the periodicity ( $m$ ) and the mean section thickness ( $t$ ):  $\text{est } (V) = (\Sigma A)mt$ . Finally, the cortical lesion volume in the ipsilateral cortex is expressed as a percentage of the volume of the entire contralateral cortex volume by the equation:  $\%V = (V_{\text{divot}}/V_{\text{contra}}) \times 100$  [43].

### Data Analyses

All data were analyzed using GraphPad prism 5 software. Data from behavioral experiments (beam walking and novel object



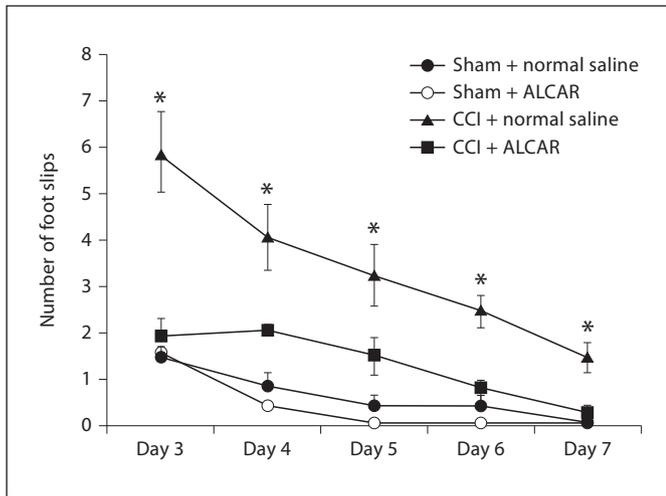
**Fig. 1.** Beam walking traverse time in seconds tested on days 3–7 after sham surgery or CCI followed by treatment with ALCAR or normal saline. Data are presented as means  $\pm$  SEM.  $n = 7$  rats/group.

recognition, NOR) were analyzed using two-way repeated measures analysis of variance (ANOVA) with group (sham or CCI) and treatment (saline or ALCAR) as factors, followed by post hoc testing using the Bonferroni method. Differences in the lesion volumes of CCI + saline and CCI + ALCAR groups were determined using Student's  $t$  test. The level of significance was set at  $p < 0.05$  for all data.

## Results

### Behavioral Outcomes

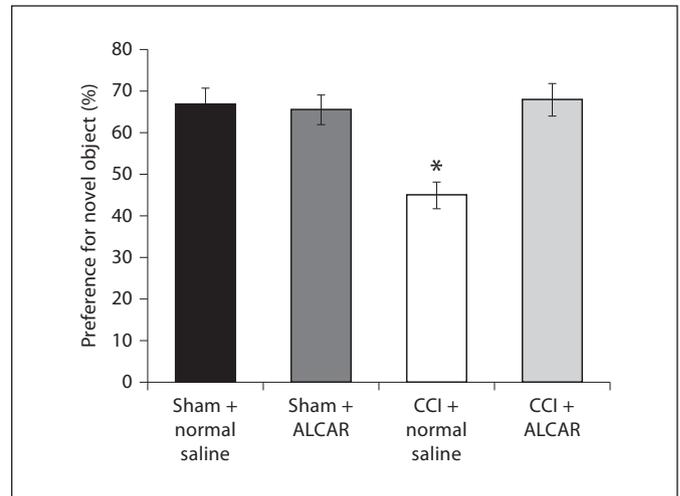
A beam walking test was used to assess the effects of CCI and treatment with ALCAR on the sensorimotor abilities of immature rats. The tests were performed starting at day 3 after CCI or sham surgery and continued for 5 days until day 7 after injury. All groups of sham and injured rats were able to traverse an inclined beam, and there were no significant differences in traverse time between the groups on any of the days studied (fig. 1). The quality of beam walking was assessed by quantifying the number of foot slips, as shown in figure 2. For the foot slip data, a repeated measures two-way ANOVA revealed a group effect [ $F(12, 220) = 3.92$ ;  $p < 0.0001$ ], indicating overall differences between the different treatment groups in the study, as well as a treatment effect [ $F(3, 55) = 24.85$ ;  $p < 0.0001$ ] and group  $\times$  treatment interaction [ $F(12, 220) = 3.92$ ;  $p < 0.0001$ ], indicating significant performance differences between the treatment groups. Since all the groups studied improved over 5 days of beam walking testing, an additional analysis was performed using group and time as factors. In addition to a group



**Fig. 2.** Effect of ALCAR treatment on sensorimotor coordination after CCI. CCI injury resulted in an increased number of foot slips during the beam walking task on days 3–7 after injury (▲) in comparison to sham-operated rats in both vehicle- (●) and ALCAR-treated (○) groups ( $p < 0.001$ ). CCI rats treated with ALCAR during the first 24 h after CCI (■) had significantly fewer foot slips ( $p < 0.005$ ; ANOVA) compared to CCI + saline rats and were not different from the sham groups. Data are presented as means  $\pm$  SEM.  $n = 7$  rats/group. \*  $p < 0.001$ .

effect, it revealed a time effect [ $F(4, 220) = 30.87$ ;  $p < 0.0001$ ], indicating significant overall changes in performance over the duration of the study, and group  $\times$  day interaction [ $F(12, 275) = 2.434$ ;  $p < 0.01$ ], indicating significant performance difference between the groups over time. Post hoc analysis with Bonferroni posttests detected significant differences for days 3–7 between CCI rats treated with saline and both the sham groups and the CCI + ALCAR treatment group ( $p < 0.001$ ). There were no significant differences between the sham + saline and sham + ALCAR groups. The number of foot slips for CCI rats treated with ALCAR was also not significantly different from either sham-operated group.

The NOR test was used to assess the effects of CCI and treatment with ALCAR on recognition memory. The rats were familiarized with the objects on day 6 after injury and their preference for novel objects was tested on day 7 after injury; the results of these tests are shown in figure 3. Both sham groups exhibited a strong preference for the novel object with  $66.8 \pm 4.0\%$  (sham + saline) and  $65.5 \pm 3.5\%$  (sham + ALCAR) of the test period spent exploring the novel object. The percent time that sham rats spent exploring the novel object is comparable to values reported for normal adult and juvenile rats [40, 44,



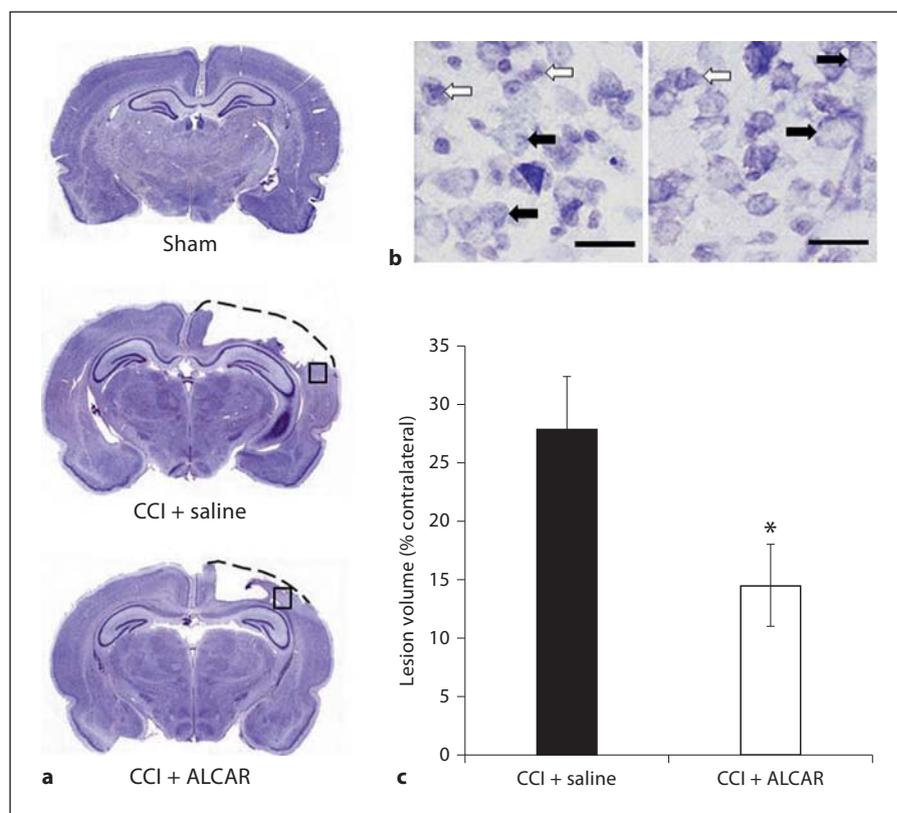
**Fig. 3.** Effect of ALCAR treatment on NOR test performance 7 days after CCI injury. CCI rats treated with saline had significantly impaired 24-hour delay NOR task performance ( $p < 0.001$ ) compared to both sham groups and the CCI + ALCAR rats. The performance of CCI + ALCAR rats was significantly better than that of CCI + saline rats and was not different from that of either sham group. Values are expressed as percentage of time spent exploring the novel object (means  $\pm$  SEM).  $n = 7$  rats/group. \*  $p < 0.001$ .

45]. The CCI rats treated with ALCAR also exhibited a significant preference for the novel object ( $67.9 \pm 3.9\%$ ) and did not differ from either sham group in this respect. In contrast, rats subjected to CCI + saline treatment spent significantly less time exploring the novel object ( $44.9 \pm 3.1\%$ ;  $p < 0.001$ ) than the CCI + ALCAR and the sham groups. The amount of time CCI + saline rats spent exploring the novel object was similar to a random chance performance of 50%. A repeated measures ANOVA yielded a significant group effect [ $F(1, 24) = 7.17$ ;  $p = 0.013$ ], indicating overall differences between the treatment groups in the study, as well as a treatment effect [ $F(1, 24) = 8.96$ ;  $p < 0.01$ ] and group  $\times$  treatment interaction [ $F(1, 24) = 21.77$ ;  $p < 0.005$ ]. Post hoc analysis using the Bonferroni posttest detected a significant difference ( $p < 0.001$ ) between the CCI + normal saline group and the other groups studied (sham + normal saline, sham + ALCAR and CCI + ALCAR).

#### Cortical Lesion Volume

The CCI model resulted in a moderate degree of TBI, defined by extensive unilateral loss of cortical brain tissue without gross damage to underlying structures including the hippocampus [43]. At day 7 after injury, 27.8

**Fig. 4.** Effect of ALCAR treatment on lesion volume after CCI. **a** Histological analysis of brain sections (cresyl violet staining) at day 7 after injury shows that the CCI + ALCAR rats had less tissue loss than the CCI + saline rats. Dashed lines approximate upper border of necrotic lesion. **b** Higher magnification shows the presence of both injured (shrunken neurons with condensed nucleus and lacking nucleolus; white arrows) and noninjured neurons (nucleolus intact with regular distribution of Nissl bodies throughout cytoplasm; black arrows) in cortical regions of saline- (left panel) and ALCAR-treated (right panel) animals following CCI. Scale bar = 25  $\mu$ m. **c** Quantified lesion volume using stereologic analysis measured at day 7 after CCI + ALCAR versus CCI + saline. Data are presented as means  $\pm$  SEM of the percent lesion volume.  $n = 7$  rats/group. \*  $p < 0.05$ .



$\pm 4.5\%$  of the total cortical volume in the left (ipsilateral) cerebral hemisphere (compared to the contralateral side) was lost in CCI + saline rats. In contrast, the CCI animals treated with ALCAR had significantly less cortical lesion volume lost ( $14.5 \pm 3.5\%$ ) than the CCI + saline group ( $p < 0.04$ ;  $t$  test), as shown in figure 4.

## Discussion

The overall findings of this study are that treatment with ALCAR after moderate TBI in immature rats from CCI reduced both the neurologic impairment and the cortical lesion volume that occurred within 7 days after injury. ALCAR was administered 4 times intraperitoneally at a dose of 100 mg/kg at 1, 4, 12 and 23 h after the CCI [46]. The dosing schedule by Aureli et al. [46] was used since it led to a complete recovery of brain ATP levels at 2–48 h after injury in a rat stroke model. Previous findings in our laboratory also demonstrated neuroprotection with 100 mg/kg ALCAR in an adult rat stroke model and in an adult canine cardiac arrest model [21, 33]. Although the current study is the first study to dem-

onstrate neuroprotection by ALCAR in any TBI model, ALCAR administered intraperitoneally at 300 mg/kg also reduced lesion volume and improved the respiration of isolated mitochondria in a rat spinal cord injury model [6]. No study has examined dose-response relationships, thus it remains unknown whether greater neuroprotection would be observed in the rat CCI model with doses higher than 100 mg/kg or whether lower doses would also be effective. The present study and the two other studies employing rat models all used 2 or more doses of ALCAR [6, 33, 46], whereas the canine study employed 1 early intravenous bolus administration [21]. It is not known how many doses of ALCAR are optimal for neuroprotection after TBI. Importantly, all of the rodent studies demonstrated neuroprotection by ALCAR treatment administered 1 h after injury, which is the clinically relevant time of treatment for human TBI. As the safety profile of ALCAR is extremely good [47, 48], it could be readily translated for use in clinical trials.

The finding that significant improvement was observed in two discrete behavioral tests supports the conclusion that ALCAR is neuroprotective after TBI. Beam walking is a well-established method to assess sensori-

motor function in rats and mice [39], and is used to assess impairment after cortical focal [49–51] and global [39] injury. This method is widely used in assessing neurologic insult and/or recovery after TBI [49]. The number of foot slips on the beam walking test was significantly higher in the CCI + saline rats compared to the sham groups. This finding is consistent with other studies that reported increased numbers of foot slips in injured rodents following brain trauma [49, 51]. Our results show that treatment with ALCAR during the first 24 h after CCI led to improved neurologic outcome, as determined by a significantly decreased number of foot slips compared to the CCI + saline group. Appelberg et al. [49] reported that feeding a ketogenic diet after CCI injury to juvenile rats led to improved beam walking performance and spatial memory.

The performance of all animal groups in our study improved over time, as has been reported by other investigators [49]. The mechanism of this motor improvement is not completely understood [52]. One possibility is that motor recovery is regulated by modulating cerebellar input since isolated direct cerebellar injury results in impaired beam walking [52–54]. In the present study, no differences were detected in the time required to traverse the beam, consistent with reports from other groups that TBI injury did not affect traverse time for beam walking in juvenile (PND 35) and adult rats (PND 75) [49].

Hippocampal neuronal death typically accompanies the overlying cortical lesion in the rat CCI model of TBI [43]. Although the hippocampus remains anatomically intact, secondary injury to the hippocampus leads to impaired function, which can be assessed by behavioral tasks including learning and recognition memory [55]. The NOR test, which involves memory of a familiar object as well as detection and encoding of a novel object, has been extensively used to study cognitive function in rodents [41]. It is particularly useful for studying memory in immature rodents since object recognition is well studied in many species including humans [55]. At day 7 after TBI, the rats were 28–29 days old and developmentally capable of exhibiting novel object preference up to 24 h after familiarization [44]. In our experiments, TBI caused significant impairment of NOR 7 days after TBI; however, this deficit was not observed in the CCI rats treated with ALCAR during the first 24 h after TBI. In addition to improving neurologic outcome, treatment with ALCAR after injury significantly reduced the cortical lesion volume 7 days after CCI. The lesion volume in CCI + ALCAR rats was approximately 50% smaller than the lesion volume in the CCI + saline rats. Lesion volume

is a highly objective outcome measure, based on the rigorous and unbiased stereologic approach used. Future experiments will determine the efficacy of ALCAR in sparing selective neuronal death in the penumbral hippocampal region. Although Prins et al. [56] reported that a ketogenic diet decreased the lesion volume by 58% in 35-day-old rats 7 days after TBI, treatment with the ketogenic diet had no effect on the 17-day-old rats tested.

The primary limitation of this study is that only short-term behavioral and histologic results were obtained. Future studies will be necessary to determine if treatment with ALCAR also improves long-term neurologic outcome and reduces cortical lesions and delayed cell death in the penumbra several months after injury. Another limitation is that only male animals were used, and therefore the results cannot be extrapolated to females.

The mechanism of neuroprotection by ALCAR is not well understood. Based on what is known about the biochemical mechanisms of secondary injury due to TBI and the neurochemical effects of ALCAR, it is likely that ALCAR provides neuroprotection by improving cerebral energy metabolism and, therefore, lessens the chance of necrotic cell death caused by metabolic failure. Indeed, Patel et al. [6] demonstrated that treatment with ALCAR ameliorated mitochondrial dysfunction after contusion spinal cord injury. Since cerebral oxidative glucose metabolism is impaired after TBI in the immature brain [4], at several levels including the PDHC, alternate fuels that can bring substrates directly to the TCA cycle (after the block at the PDHC) are likely to provide therapeutic benefit. Our group and others have shown that the maximal activity and/or immunoreactivity of PDHC subunits is reduced within the first few hours after both global cerebral ischemia and TBI [5–8, 10–14]. ALCAR can supply acetyl CoA as a substrate for brain energy metabolism, and can both improve brain ATP levels and decrease brain lactate after injury [21, 30, 46]. Furthermore, administration of 3-hydroxybutyrate, another substrate that enters the metabolism at the level of acetyl CoA, improves ATP production after TBI in adult rats [57].

Our group recently demonstrated that [2-<sup>13</sup>C]ALCAR (with <sup>13</sup>C present in the acetyl moiety) is utilized for energy and neurotransmitter synthesis in the brains of normal, 21- to 22-day-old rats [38]. The formation of <sup>13</sup>C-labeled glutamate, glutamine and GABA, which are all metabolites generated from TCA cycle intermediates, demonstrated for the first time that exogenously administered ALCAR is used by the brain for energy metabolism [38]. Future studies will determine if the oxidative metabolism of <sup>13</sup>C-labeled ALCAR in the brain is in-

creased after TBI, relative to the metabolism observed in sham rats. The high rate of metabolism of ALCAR via the pyruvate recycling pathway in the immature brain may provide additional neuroprotection [38]. Other possible mechanisms of neuroprotection by ALCAR include the use of the acetyl moiety for synthesis of the inhibitory neurotransmitter GABA [38], general anti-inflammatory actions of exogenously administered carnitine [58], the reduction of oxidative stress [30, 59–65] and the ability of ALCAR to activate the Nrf2 pathway of inducing anti-

oxidant gene expression [26, 27, 66]. The possibility that there are multiple mechanisms responsible for neuroprotection by ALCAR supports its use in additional preclinical studies and for potential clinical trials.

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### References

- Xu Y, McArthur DL, Alger JR, Etchepare M, Hovda DA, Glenn TC, Huang S, Dinov I, Vespa PM: Early nonischemic oxidative metabolic dysfunction leads to chronic brain atrophy in traumatic brain injury. *J Cereb Blood Flow Metab* 2010;30:883–894.
- Robertson CL, Scafidi S, McKenna MC, Fiskum G: Mitochondrial mechanisms of cell death and neuroprotection in pediatric ischemic and traumatic brain injury. *Exp Neurol* 2009;218:371–380.
- Verweij BH, Amelink GJ, Muizelaar JP: Current concepts of cerebral oxygen transport and energy metabolism after severe traumatic brain injury. *Prog Brain Res* 2007;161:111–124.
- Scafidi S, O'Brien J, Hopkins I, Robertson C, Fiskum G, McKenna M: Delayed cerebral oxidative glucose metabolism after traumatic brain injury in young rats. *J Neurochem* 2009;109(suppl 1):189–197.
- Vaishnav RA, Singh IN, Miller DM, Hall ED: Lipid peroxidation-derived reactive aldehydes directly and differentially impair spinal cord and brain mitochondrial function. *J Neurotrauma* 2010;27:1311–1320.
- Patel SP, Sullivan PG, Lyttle TS, Rabchevsky AG: Acetyl-L-carnitine ameliorates mitochondrial dysfunction following contusion spinal cord injury. *J Neurochem* 2010;114:291–301.
- Sharma P, Benford B, Li ZZ, Ling GS: Role of pyruvate dehydrogenase complex in traumatic brain injury and measurement of pyruvate dehydrogenase enzyme by dipstick test. *J Emerg Trauma Shock* 2009;2:67–72.
- Opii WO, Nukala VN, Sultana R, Pandya JD, Merchant ML, Klein JB, Sullivan PG, Butterfield DA: Proteomic identification of oxidized mitochondrial proteins following experimental traumatic brain injury. *J Neurotrauma* 2007;24:772–789.
- Thomale UW, Griebenow M, Mautes A, Beyrer TF, Dohse NK, Stroop R, Sakowitz OW, Unterberg AW: Heterogeneous regional and temporal energetic impairment following controlled cortical impact injury in rats. *Neurol Res* 2007;29:594–603.
- Robertson CL, Saraswati M, Fiskum G: Mitochondrial dysfunction early after traumatic brain injury in immature rats. *J Neurochem* 2007;101:1248–1257.
- Richards EM, Rosenthal RE, Kristian T, Fiskum G: Postischemic hyperoxia reduces hippocampal pyruvate dehydrogenase activity. *Free Radical Biol Med* 2006;40:1960–1970.
- Vereczki V, Martin E, Rosenthal RE, Hof PR, Hoffman GE, Fiskum G: Normoxic resuscitation after cardiac arrest protects against hippocampal oxidative stress, metabolic dysfunction, and neuronal death. *J Cereb Blood Flow Metab* 2005;26:821–835.
- Bogaert YE, Rosenthal RE, Fiskum G: Postischemic inhibition of cerebral cortex pyruvate dehydrogenase. *Free Radic Biol Med* 1994;16:811–820.
- Bogaert YE, Sheu KF, Hof PR, Brown AM, Blass JP, Rosenthal RE, Fiskum G: Neuronal subclass-selective loss of pyruvate dehydrogenase immunoreactivity following canine cardiac arrest and resuscitation. *Exp Neurol* 2000;161:115–126.
- Xing G, Ren M, Watson WA, O'Neil JT, Verma A: Traumatic brain injury-induced expression and phosphorylation of pyruvate dehydrogenase: a mechanism of dysregulated glucose metabolism. *Neurosci Lett* 2009;454:38–42.
- Prins ML: Cerebral metabolic adaptation and ketone metabolism after brain injury. *J Cereb Blood Flow Metab* 2008;28:1–16.
- Faria MH, Muniz LR, Vasconcelos PR: Ketone bodies metabolism during ischemic and reperfusion brain injuries following bilateral occlusion of common carotid arteries in rats. *Acta Cir Bras* 2007;22:125–129.
- Koppaka SS, Puchowicz, LaManna JC, Gatica JE: Effect of alternate energy substrates on mammalian brain metabolism during ischemic events. *Adv Exp Med Biol* 2008;614:361–370.
- Dardzinski BJ, Smith SL, Towfighi J, Williams GD, Vannucci RC, Smith MB: Increased plasma  $\beta$ -hydroxybutyrate, preserved cerebral energy metabolism, and amelioration of brain damage during neonatal hypoxia ischemia with dexamethasone pretreatment. *Pediatr Res* 2000;48:248–255.
- Massieu L, Haces ML, Montiel T, Hernandez-Fonseca K: Acetoacetate protects hippocampal neurons against glutamate-mediated neuronal damage during glycolysis inhibition. *Neuroscience* 2003;120:365–378.
- Rosenthal RE, Williams R, Bogaert YE, Getson PR, Fiskum G: Prevention of postischemic canine neurological injury through potentiation of brain energy metabolism by acetyl-L-carnitine. *Stroke* 1992;23:1312–1317.
- Zanelli SA, Solenski NJ, Rosenthal RE, Fiskum G: Mechanisms of ischemic neuroprotection by acetyl-L-carnitine. *Ann NY Acad Sci* 2005;1053:153–161.
- Ames BN, Liu J: Delaying the mitochondrial decay of aging with acetylcarnitine. *Ann NY Acad Sci* 2004;1033:108–116.
- Jones LL, McDonald DA, Borum PR: Acylcarnitines: role in brain. *Prog Lipid Res* 2010;49:61–75.
- Virmani MA, Caso V, Spadoni A, Rossi S, Russo F, Gaetani F: The action of acetyl-L-carnitine on the neurotoxicity evoked by amyloid fragments and peroxide on primary rat cortical neurones. *Ann NY Acad Sci* 2001;939:162–178.
- Calabrese V, Mancuso C, Pennisi G, Calafato S, Bellia F, Guiffrida Stella AM, Schapira T, Dinkova Kostova AT, Rizzarelli E: Cellular stress response: a novel target for chemoprevention and nutritional neuroprotection in aging, neurodegenerative disorders and longevity. *Neurochem Res* 2008;33:2444–2471.
- Calabrese V, Ravagna A, Colombrita C, Scapagnini G, Guagliano E, Calvani M, Butterfield DA, Guiffrida Stella AM: Acetylcarnitine induces heme oxygenase in rat astrocytes and protects against oxidative stress: involvement of the transcription factor Nrf2. *J Neurosci Res* 2005;79:509–521.

- 28 Fiskum G, Liu Y, Bogaert YE, Rosenthal RE: Acetyl-L-carnitine stimulates cerebral oxidative metabolism and inhibits protein oxidation following cardiac arrest in dogs; in Kriegelstein J, Oberpichler-Schwenk H (eds): *Pharmacology of Cerebral Ischemia*. Stuttgart, Wissenschaftliche Verlagsgesellschaft, 1992, pp 487–491.
- 29 Ishii T, Shimpo Y, Matsuoka Y, Kinoshita K: Anti-apoptotic effect of acetyl-L-carnitine and L-carnitine in primary cultured neurons. *Jpn J Pharmacol* 2000;83:119–124.
- 30 Al-Majed AA, Sayed-Ahmed MM, Al-Omar FA, Al-Yahya AA, Aleisa AM, Al-Shabanah OA: Carnitine esters prevent oxidative stress damage and energy depletion following transient forebrain ischaemia in the rat hippocampus. *Clin Exp Pharmacol Physiol* 2006;33:725–733.
- 31 Pettegrew JW, Panchalingam K, Withers G, McKeag D, Strychor S: Changes in brain energy and phospholipid metabolism during development and aging in the Fischer 344 rat. *J Neuropathol Exp Neurol* 1990;49:237–249.
- 32 Shuaib A, Waqaar T, Wishart T, Kanthan R, Howlett W: Acetyl-L-carnitine attenuates neuronal damage in gerbils with transient forebrain ischemia only when given before the insult. *Neurochem Res* 1995;20:1021–1025.
- 33 Lolic MM, Fiskum G, Rosenthal RE: Neuroprotective effects of acetyl-L-carnitine after stroke in rats. *Ann Emerg Med* 1997;29:758–765.
- 34 Jalal FY, Bohlke M, Maher TJ: Acetyl-L-carnitine reduces the infarct size and striatal glutamate outflow following focal cerebral ischemia in rats. *Ann NY Acad Sci* 2010;1199:95–104.
- 35 Hagberg H, Mallard C, Rousset CI, Wang X: Apoptotic mechanisms in the immature brain: involvement of mitochondria. *J Child Neurol* 2009;24:1141–1146.
- 36 Bayir H, Kochanek PM, Kagan VE: Oxidative stress in immature brain after traumatic brain injury. *Dev Neurosci* 2006;28:420–431.
- 37 Bauer R, Fritz H: Pathophysiology of traumatic injury in the developing brain: an introduction and short update. *Exp Toxicol Pathol* 2004;56:65–73.
- 38 Scafidi S, Fiskum G, Lindauer SL, Bamford P, Shi D, Hopkins I, McKenna MC: Metabolism of acetyl-L-carnitine for energy and neurotransmitter synthesis in the immature rat brain. *J Neurochem* 2010;114:820–831.
- 39 Carter RJ, Morton J, Dunnett SB: Motor coordination and balance in rodents. *Curr Protoc Neurosci* 2001;chapt 8:unit 8.12.
- 40 Bevins RA, Besheer J: Object recognition in rats and mice: a one-trial non-matching-to-sample learning task to study 'recognition memory'. *Nat Protoc* 2006;1:1306–1311.
- 41 Ennaceur A: One-trial object recognition in rats and mice: methodological and theoretical issues. *Behav Brain Res* 2010;215:244–254.
- 42 Ennaceur A, Delacour J: A new one-trial test for neurobiological studies of memory in rats. 1. Behavioral data. *Behav Brain Res* 1988;31:47–59.
- 43 Hall ED, Bryant YD, Cho W, Sullivan PG: Evolution of post-traumatic neurodegeneration after controlled cortical impact traumatic brain injury in mice and rats as assessed by the de Olmos silver and fluorojade staining methods. *J Neurotrauma* 2008;25:235–247.
- 44 Reger ML, Hovda DA, Giza CC: Ontogeny of rat recognition memory measured by the novel object recognition task. *Dev Psychobiol* 2009;51:672–678.
- 45 Broadbent NJ, Squire LR, Clark RE: Spatial memory, recognition memory, and the hippocampus. *Proc Natl Acad Sci USA* 2004;101:14515–14520.
- 46 Aureli T, Micheli A, di Cocco ME, Ghirardi O, Giuliani A, Ramacci MT, Conti F: Effect of acetyl-L-carnitine on recovery of brain phosphorus metabolites and lactic acid level during reperfusion after cerebral ischemia in the rat: study by <sup>13</sup>P- and <sup>1</sup>H-NMR spectroscopy. *Brain Res* 1994;643:92–99.
- 47 Parnetti L, Gaiti A, Mecocci P, Cadini D, Senin U: Pharmacokinetics of IV and oral acetyl-L-carnitine in a multiple dose regimen in patients with senile dementia of Alzheimer type. *Eur J Clin Pharmacol* 1992;42:89–93.
- 48 Youle M, Osio M: A double-blind, parallel-group, placebo-controlled, multicentre study of acetyl-L-carnitine in the symptomatic treatment of antiretroviral toxic neuropathy in patients with HIV-1 infection. *HIV Med* 2007;8:241–250.
- 49 Appelberg KS, Hovda DA, Prins ML: The effects of a ketogenic diet on behavioral outcome after controlled cortical impact injury in the juvenile and adult rat. *J Neurotrauma* 2009;26:497–506.
- 50 Fox GB, Faden AI: Traumatic brain injury causes delayed motor and cognitive impairment in a mutant mouse strain known to exhibit delayed Wallerian degeneration. *J Neurosci Res* 1998;53:718–727.
- 51 Fox GB, Fan L, LeVasseur RA, Faden AI: Effect of traumatic brain injury on mouse spatial and nonspatial learning in the Barnes circular maze. *J Neurotrauma* 1998;15:1037–1046.
- 52 Boyeson MG, Callister TR, Cavazos JE: Biochemical and behavioral effects of a sensorimotor cortex injury in rats pretreated with the noradrenergic neurotoxin DSP-4. *Behav Neurosci* 1992;106:964–973.
- 53 Allen GV, Chase T: Induction of heat shock proteins and motor function deficits after focal cerebellar injury. *Neuroscience* 2001;102:603–614.
- 54 Williams AJ, Ling GS, Tortella FC: Severity level and injury track determine outcome following a penetrating ballistic-like brain injury in the rat. *Neurosci Lett* 2006;408:183–188.
- 55 Squire LR, Wixted JT, Clark RE: Recognition memory and the medial temporal lobe: a new perspective. *Nat Rev Neurosci* 2007;8:872–883.
- 56 Prins ML, Fujima LS, Hovda DA: Age-dependent reduction of cortical contusion volume by ketones after traumatic brain injury. *J Neurosci Res* 2005;82:413–420.
- 57 Prins ML, Lee SM, Fujima LS, Hovda DA: Increased cerebral uptake and oxidation of exogenous BHB improves ATP following traumatic brain injury in adult rats. *J Neurochem* 2004;90:666–672.
- 58 Winter BK, Fiskum G, Gallo LL: Effects of L-carnitine on serum triglyceride and cytokine levels in rat models of cachexia and septic shock. *Br J Cancer* 1995;72:1173–1179.
- 59 Poon HF, Calabrese V, Calvani M, Butterfield DA: Proteomics analyses of specific protein oxidation and protein expression in aged rat brain and its modulation by L-acetylcarnitine: insights into the mechanisms of action of this proposed therapeutic agent for CNS disorders associated with oxidative stress. *Antioxid Redox Signal* 2006;8:381–394.
- 60 Ferraresi R, Troiano L, Roat E, Nemes E, Lugli E, Nasi M, Pinti M, Calvani M, Iannucelli M, Cossarizza A: Protective effect of acetyl-L-carnitine against oxidative stress induced by antiretroviral drugs. *FEBS Lett* 2006;580:6612–6616.
- 61 Shen W, Liu K, Tian C, Yang L, Li X, Ren J, Packer L, Head E, Sharman E, Liu J: Protective effects of R- $\alpha$ -lipoic acid and acetyl-L-carnitine in MIN6 and isolated rat islet cells chronically exposed to oleic acid. *J Cell Biochem* 2008;104:1232–1243.
- 62 Liu Y, Rosenthal RE, Starke-Reed P, Fiskum G: Inhibition of postcardiac arrest brain protein oxidation by acetyl-L-carnitine. *Free Radic Biol Med* 1993;15:667–670.
- 63 Liu J, Head E, Kuratsune H, Cotman CW, Ames BN: Comparison of the effects of L-carnitine and acetyl-L-carnitine on carnitine levels, ambulatory activity, and oxidative stress biomarkers in the brain of old rats. *Ann NY Acad Sci* 2004;1033:117–131.
- 64 Loots DT, Mienie LJ, Bergh JJ, van der Schyf CJ: Acetyl-L-carnitine prevents total body hydroxyl free radical and uric acid production induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the rat. *Life Sci* 2004;75:1243–1253.
- 65 Yasui F, Matsugo S, Ishiashi M, Kajita T, Ezashi Y, Oomura Y, Kojo S, Sasaki K: Effects of chronic acetyl-L-carnitine treatment on brain lipid hydroperoxide level and passive avoidance learning in senescence-accelerated mice. *Neurosci Lett* 2002;334:177–180.
- 66 Barhwal K, Hota SK, Jain V, Prasad D, Singh SB, Ilavazhagan G: Acetyl-L-carnitine (ALCAR) prevents hypobaric hypoxia-induced spatial memory impairment through extracellular related kinase-mediated nuclear factor erythroid 2-related factor 2 phosphorylation. *Neuroscience* 2009;161:501–514.