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Beneficial Effects of Sodium or Ethyl Pyruvate after Traumatic Brain Injury in the Rat

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Abstract

Sodium pyruvate (SP) treatment initiated within 5 min post-injury is neuroprotective in a rat model of unilateral cortical contusion injury (CCI). The current studies examined: (1) effects of delayed SP treatments (1000 mg/kg, i.p., at 1, 12 and 24 h), (2) effects of single (1 h) or multiple (1, 12 and 24 h) ethyl pyruvate treatments (EP; at 20 or 40 mg/kg, i.p.), and (3) mechanisms of action for pyruvate effects after CCI. In Experiment 1, both SP and EP treatment(s) significantly reduced the number of dead/dying cells in the ipsilateral hippocampus (dentate hilus+CA3_c and/or CA3_{a-b} regions) at 72 h post-CCI. Pyruvate treatment(s) attenuated CCI-induced reductions of cerebral cytochrome oxidase activity at 72 h, significantly improving activity in peri-contusional cortex after multiple SP or EP treatments. Optical density measures of ipsilateral CD11b immuno-staining were significantly increased 72 h post-CCI, but these measures of microglia activation were not different from sham injury values in SP and EP groups with three post-CCI treatments. In Experiment 2, three treatments (1, 12 and 24 h) of SP (1000 mg/kg) or EP (40 mg/kg) significantly improved recovery of beam-walking and neurological scores in the first three weeks after CCI, and EP treatments significantly improved spatial working memory one week post-CCI. Ipsilateral CA3_b neuronal loss, but not cortical tissue loss, was significantly reduced one month post-CCI with pyruvate treatments begun 1 h post-CCI. Thus, delayed pyruvate treatments after CCI are neuroprotective and improve neurobehavioral recovery; these effects may be mediated by improved metabolism and reduced inflammation.

Keywords

Cell Death; Cytochrome Oxidase; Ethyl Pyruvate; Functional Recovery; Inflammation; Microglia; Neuroprotection; Rats; Sodium Pyruvate; Traumatic Brain Injury

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Sodium or Ethyl Pyruvate Treatment after TBI

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Introduction

We have previously reported a neuroprotective role of sodium pyruvate (SP) treatment in a rat model of cortical contusion injury (CCI) (Fukushima et al., 2009). In those studies a single SP injection (1000 mg/kg, i.p.) within 5 min after CCI was found to significantly increase the brain's extracellular concentration of pyruvate, attenuating the CCI-induced decrease in extracellular glucose and increase in extracellular lactate (Chen et al., 2000; Krishnappa et al., 1999). A single SP treatment was also found to significantly reduce the numbers of dead/dying neurons in the peri-contusional cortex at 6 h post-CCI. However, three injections of SP (at 5, 65 and 125 min) were required in order to obtain a significant reduction of the cortical contusion volume at 2 weeks post-CCI. Similar cell protective effects of exogenous pyruvate, which appear to be mediated through metabolic improvements, have been reported in several animal models of injury to brain or liver (Mongan et al., 2001, 2002, 2003; Suh et al., 2005; Yi et al., 2007).

Apart from their ability to serve as a metabolic substrate (Gonzalez et al., 2005; Zeng et al., 2007), both SP and its aliphatic ester, ethyl pyruvate (EP), are known to have neuroprotective effects that are mediated via other mechanisms. After transient forebrain ischemia induced by bilateral carotid artery occlusion and hypotension, SP almost completely blocked zinc toxicity and prevented cell death in both hippocampus and parietal cortex (Lee et al., 2001). In models of transient or permanent middle cerebral artery occlusion, treatment with either SP or EP suppressed the production of nitric oxide or markers of oxidative damage, inhibited markers of inflammatory responses, significantly reduced neuronal death and infarct volume, and improved behavioral outcomes (Kim et al., 2005; Yi et al., 2007; Yu et al., 2005). Similar beneficial effects and mechanisms of action for EP treatment were reported in an intra-ventricular kainic acid injection model of brain injury (Cho et al., 2006). These therapeutic benefits of pyruvate via anti-oxidant or anti-inflammatory mechanisms have been extensively studied in models of hemorrhagic or septic shock, coronary disease, hepatic failure, renal failure, and bowel disease (Das, 2006; Fink, 2007).

Since potential therapeutic effects of EP treatment(s) and the therapeutic window for SP treatments after traumatic brain injury (TBI) have not been determined, the current studies were conducted to compare and contrast these two pyruvate compounds in our model of CCI. Experiment 1 examined the effects of a single (1 h) or multiple (1, 12 and 24 h) treatments with EP and multiple treatments with SP on hippocampal cell death, regional oxidative capacity, and the regional microglia response to CCI at 72 h post-injury. The first treatment was delayed until 1 h after injury to improve clinical relevance of the treatments, as this therapeutic window has been previously established for SP and EP in rat ischemia models (Lee et al., 2001; Yu et al., 2005). Experiment 2 determined the effect of multiple EP or SP treatments on CCI-induced sensorimotor and cognitive deficits, evaluating potential treatment effects on cortical contusion volume and hippocampal neuron survival at one month post-injury. Our general hypotheses were that pyruvate treatment after CCI would reduce neuronal injury and microglia activation, improve cerebral metabolism, and improve neurological outcomes.

Materials and Methods

All experiments and procedures were approved by the UCLA Chancellor's Committee for Animal Research. Best effort was made to minimize pain and discomfort to the animals.

Subjects and experimental groups

Forty-two adult (65–85 days old at the time of surgery) male Sprague-Dawley rats were used to determine the effects of SP or EP on cell death, metabolism and microglia activation at 72

h post-injury in Experiment 1. The average body weights on the day of surgery did not differ between groups (lowest = 326 ± 12 g; highest = 347 ± 13 g). Animals were randomly assigned to 6 groups (n=7/group). A vehicle control group (CCI-Veh) was injected with Ringer's solution and the SP treatment group (CCI-SP 1000×3) was injected with SP (1000 mg/kg, i.p.), at 1, 12 and 24 h post-CCI. The SP dose was chosen based on prior data indicating this dose was optimal compared to SP at 500 mg/kg after CCI (Fukushima et al., 2009). EP treatment was administered at two dosages (20 mg/kg or 40 mg/kg, i.p.), as either a single injection at 1 h post-CCI (groups CCI-EP 20×1 and CCI-EP 40×1) or via multiple injections at 1, 12 and 24 h after injury (groups CCI-EP 20×3 and CCI-EP 40×3). These doses of EP were selected based on results in prior studies of EP treatment in a rat model of cerebral ischemia (Yu et al., 2005).

Immuno-staining of post-fixed brain tissue sections 72 h post-CCI suggested inhibitory effects of pyruvate treatment(s) on microglia activation. To confirm this effect, an additional 12 rats (75 days old, weighing 334 to 362 g on the day of surgery) were randomized to either sham injury and vehicle injections (Sham-Veh), CCI-Veh, CCI-SP 1000×3 and CCI-EP 40×3 groups (n=3/group). These animals were perfused with paraformaldehyde at 72 h post-surgery to assess microglia activation in fixed tissue sections.

For Experiment 2, twenty-eight additional rats were used to evaluate SP or EP treatments on behavioral outcomes and neuropathology at 1 month post-CCI. These rats were randomly assigned to 4 groups (n=7/group) with either Sham-Veh, CCI-Veh, CCI and SP injections (CCI-SP; 1000 mg/kg, i.p.) or CCI and EP injections (CCI-EP; 40 mg/kg, i.p.). All treatments were administered at 1, 12 and 24 h post-injury. The drugs, dosages and injection times selected for use in this study were based on optimal results obtained in Experiment 1. Animals in Experiment 2 were all 70 to 73 days old on the day of surgery and average body weights did not differ between groups (lowest = 345 ± 11 g; highest = 370 ± 12 g).

Surgery and cortical contusion injury

Surgery to induce CCI has been previously described in detail (Fukushima et al., 2009; Taylor et al., 2008). In brief, general anesthesia was induced in a chamber with 4% isoflurane in oxygen and after animals were secured on a stereotaxic frame, isoflurane was reduced and maintained at 2% during surgery. Core body temperature was monitored continuously by a rectal probe and set to maintain the body temperature at 36.4°C with a thermostatically controlled heating pad. A 6 mm diameter circular craniotomy, centered -3 mm from Bregma and 3.5 mm lateral (left) from the midline, was made using a high-speed drill under a surgical microscope. CCI was produced using a pneumatically-driven (20 psi; range of 2.21-2.40, mean of 2.32 m/sec velocity) dual-stroke piston with a 5 mm diameter flat-tipped impactor which compressed the exposed dura mater and underlying brain to a depth of 2 mm for 250 msec (Fukushima et al., 2009; Sutton et al., 1993). Sham injury controls underwent similar anesthetic and surgical interventions, including craniotomy, without CCI application. After injury the scalp was sutured closed, bupivacaine (0.1-0.14 mg/kg, s.c.) was injected around the incision site, and rats were placed in a heated recovery cage (36.0 to 38.0°C) until ambulatory.

Drug preparation

The SP (P2256: Sigma Aldrich, Saint Louis, MO, USA) was dissolved in sterile water at a concentration of 400 mg/ml. The EP (E47808: Sigma Aldrich) was mixed in Ringer's solution containing concentrations (mmol/L) of Na^+ (130), K^+ (4), Ca^{++} (2.7) and Cl^- (139) ions, with the EP concentration adjusted (to 8 or 16 mg/ml) to enable equivalent injection volumes for low (20 mg/kg) and high (40 mg/kg) dose treatments. All SP and EP doses were prepared just before each injection. Vehicle-treated rats received injection(s) of an equivalent volume of Ringer's solution.

Histopathological evaluations (72 h)

Rats (n=42) sacrificed at 72 h after CCI were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused via the ascending aorta with 400 mL of cold (4 °C) 0.1 M phosphate buffer saline (PBS; pH 7.4) containing 10% sucrose and 1% sodium nitrate. After perfusion the brains were rapidly extracted, flash frozen at -55 °C in 2-methylbutane, and stored in a -70 °C freezer until sectioning. Brains were sectioned in the coronal plane at 20 µm thickness on a cryostat (Model CM3050: Leica Microsystems GmbH, Wetzlar, Germany), and 3 series of sections were mounted on gelatinized microscope slides, saving 2 adjacent sections every 500 µm for each series. Slides/sections intended for cytochrome oxidase histochemistry were stored at -70 °C and all other slides were stored at room temperature until staining.

Twelve rats used only for immunohistochemistry at 72 h after CCI were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused with PBS followed with 400 mL of 4% paraformaldehyde in 0.1 M PBS. Brains were extracted and post-fixed for one h in the paraformaldehyde solution (4 °C) and then cryoprotected in graded sucrose solutions (10%, 20% and 30% in PBS, pH 7.4, 24 h each) at 4 °C. Brains were frozen and sectioned (40 µm) on a sliding microtome (American Optical, Model 860, Buffalo, NY, USA) saving 2 adjacent sections every 500 µm in an anti-freeze solution (4.36 g Na₂HPO₄ and 1.25 g NaH₂PO₄ in 320 mL of H₂O, 240 mL of ethylene glycol and 240 mL of glycerol). These sections were maintained at -20 °C until processed for immunohistochemistry. Anatomical nomenclature and anterior-posterior (mm from Bregma) location of structures analyzed on histological sections are consistent with those of Paxinos and Watson (1986).

Fluoro-Jade B staining and analysis—Tissue sections were fixed in 10% formalin overnight before staining with Fluoro-Jade B (FJB; 2FJB: Histo-Chem Inc., Jefferson, AR, USA) and 4',6-diamidino-2-phenylindol dihydrochloride (DAPI; D9542: Sigma Aldrich). Final concentrations of dyes were 0.0004% for FJB and 0.0002% for DAPI (Schmued and Hopkins, 2000). After staining the sections were dried on a slide warmer, cleared in Citrisolv® (Fisher Scientific, Fair Lawn, NJ, USA), and cover-slipped using Cytoseal 60® (Richard Allan Scientific, Kalamazoo, MI, USA).

Dead/dying (FJB-positive) neurons in the hippocampus were counted using an epifluorescent microscope (480 nm excitation; Model DMRE: Leica Microsystems GmbH) interfaced with a computer running Stereo Investigator software (version 3.0: MicroBrightField Inc., Colchester, VT, USA). FJB-positive cells in the CA3_{a-b} pyramidal cell layer and FJB-positive cells contained within the dentate hilus+CA3_c [including polymorphic neurons of the dentate gyrus (PoDG) and pyramidal cells of the CA3_c lying within the dentate granule cell layers; see Fig. 1A] were counted at 3 different levels (-2.8, -3.3 and -3.8 mm from Bregma). Counts were made in each region after placing two-dimensional contours over each region as guided by DAPI-positive cell staining (340 nm excitation light). Only those cells with a neuronal morphology and clearly stained initial axon segment were counted. Cell counts in each region were averaged across sections for each animal and the final group data are presented as the average number of FJB-positive cells/mm² in each region counted.

Cytochrome oxidase histochemistry—Cytochrome oxidase histochemistry was used to assess regional oxidative capacity in brain tissue sections, using methods similar to those previously reported (Hovda et al., 1991). In brief, tissue sections were batch stained for 45 min in a 37 °C dark oven by submersion in a solution containing 150 mg of cytochrome c (C2506: Sigma Aldrich) and 300 mg of 3',3'-diaminobenzidine tetrahydrochloride (DAB; D8001: Sigma Aldrich) in 500 mL of 0.1 M phosphate buffer (PB, pH 7.4). Staining reactions were stopped by 3×5 min rinses in PB and sections were air dried over several days. The dried sections were

dehydrated in ethanol, cleared in Citrisolv[®], and cover-slipped using Cytoseal 60[®]. After cover-slips had dried to slides and both the slides and cover-slips were cleaned thoroughly, images of all sections were digitally captured (8 bit, 600 dpi) on a flat-bed scanner.

Optical density measures of the cytochrome oxidase staining intensity were obtained bilaterally (averaging 5-20 readings/hemisphere) from different regions of interest using ImageJ software (version 1.38: National Institutes of Health, Bethesda, MD, USA). The regions assessed included: (1) frontal cortex (+2.7 to +1.7 mm from Bregma), (2) peri-contusional cortex (parietal and temporal regions from +1.2 to -4.3 mm, excluding obviously contused tissues), (3) caudate nucleus (+2.2 to +1.2 mm), (4) dorsal hippocampus (-2.3 to -3.8 mm), (5) ventral thalamic nuclei (portions of the ventral medial, lateral, posteromedial and posterolateral nuclei, -2.3 to -3.3 mm), (6) dorsal lateral geniculate nucleus (DLG; -4.3 to -4.8 mm), and (7) medial geniculate nucleus (MG; -4.8 to -5.8 mm). After confirming that optical density measures of the contralateral (right) hemisphere regions of interest did not differ significantly between groups, the degree of cytochrome oxidase staining asymmetry in each region was calculated using the formula: $[(\text{left} - \text{right})/(\text{left} + \text{right})] \times 100$. These asymmetry values, where negative values reflect decreases in oxidative metabolism ipsilateral to injury, control for within subject variability as well as for variation in the reaction intensity during the *in vitro* incubation procedure (Hovda et al., 1991).

Immunohistochemistry for microglia—Microglia responses at 72 h after CCI were assessed using a monoclonal antibody against CD11b (MCA275R: Serotec Ltd., Oxford, UK). The pre-mounted tissue sections (20 μ m) were fixed in 10% formalin in PBS for 10 min. These sections and the paraformaldehyde-fixed (40 μ m, free floating sections) were rinsed 2 $\times$ 5 min in 0.05 M Tris buffered saline (TBS) and the endogenous peroxidase activity was quenched by immersing sections in 1% hydrogen peroxide solution in TBS for 5 min. After rinsing in TBS/TX (0.25% Tween 20 in TBS) for 3 $\times$ 5 min, sections were blocked with 2.5% normal horse serum for 20 min at room temperature. Primary antibody was diluted at 1:500 by TBS/TX with 2.5% normal horse serum and sections were incubated for 24 h at 4 °C. After rinsing 3 $\times$ 5min in TBS, anti-mouse immunoglobulin ImmPRESS reagent (MP-7402: Vector Laboratories Inc., Burlingame, CA, USA) was applied to the sections and incubated for 30 min at room temperature. Sections were rinsed in TBS 3 $\times$ 5min and the primary antibody was visualized by exposing sections for 60 sec in 0.5% DAB in TBS with 0.4% nickel chloride and 0.01% hydrogen peroxide. Subsequent to the DAB reaction the sections were rinsed in TBS, and free floating sections were then mounted onto gelatinized slides. After air drying at room temperature all sections were dehydrated in ethanol, cleared in xylene, and cover-slipped with Cytoseal 60[®]. After cover-slips had dried to slides and both the slides and cover-slips were cleaned thoroughly, images of sections were digitally captured by a flat-bed scanner at 8 bit, 600 dpi.

To quantify the extent of activation of CD11b-positive cells, optical density measures of microglia staining intensity (5 reading per hemisphere) were obtained bilaterally from three different levels of the frontal cortex (+1.2, +0.7 and +0.2 mm relative to Bregma), the peri-contusional temporal cortex (-2.8, -3.3 and -3.8 mm), and the dorsal hippocampus (-2.8, -3.3 and -3.8 mm) using the ImageJ software. Background measures of immunoreactivity were obtained from the internal capsule in all animals, and all regional optical densities were normalized to this remote, unaffected, white matter region using the formula: $[(\text{regional intensity} - \text{remote intensity})/\text{remote intensity}] \times 100$ (Chen et al., 2000).

Behavioral training and post-operative testing

Rats used in behavioral studies were gentled by daily handling (15 min/day) for 1 week. During the next week prior to surgery they were trained over 5 days on a beam-walking task (Sutton

and Feeney, 1992) and examined for sensorimotor neurological functions on the last 2 days of beam training. All tests were scored/rated by two investigators, at least one of whom was blinded to the drug treatment conditions, and in cases of rating discrepancies the average of both scores was used.

Neurological examinations—Three exams to assess forelimb or hind limb sensorimotor functions were administered and used to construct a composite neuroscore for each animal, where the best possible test day score was 0 (completely normal response) and the worst possible score was 6 (completely abnormal response), after summing the 3 daily scores. These exams consisted of: (A) Contraflexion: the animal is held by the base of the tail, rapidly picked up, and the angle formed between the left and right forelimbs is recorded (average of 2 trials/test day). Scores assigned were 0 (both limbs extend toward table, $<30^\circ$ angle), 1 ($30\text{--}90^\circ$ angle formed due to forelimb contralateral to injury flexing back toward ventral body surface) and 2 ($>90^\circ$ angle between forelimbs). (B) Hind limb Extension: the latency (60 sec maximum) for the animal to retract the left or right hind limb to its normal posture under the body after the hind paw is pulled laterally and posterior is recorded for the right and left limb (average of 2 trials/limb/test day). Scores assigned were 0 (immediate retraction), 1 (2–30 sec) and 2 (≥ 30 sec). (C) Tactile Placing: reflexive lifting and placement of the left or right forepaw onto a table surface after lightly touching the dorsum of the forepaw on the vertical edge of the table is tested (5 trials/limb/test day). Scores assigned were 0 (5/5 placements), 1 (1–4/5 placements) and 2 (0/5 placements). In addition to the pre-surgical exams, neurological examinations were administered on post-operative day (POD) 1, 5, 9, 16 and 23. The average daily neurological scores were calculated for each treatment group.

Beam-walking—Skilled locomotor function was assessed using a beam-walk task, where rats were trained to traverse a 140 cm long, 1.7 cm wide, elevated (36 cm) wooden beam and enter a dark goal box (20 cm wide, 25 cm long and 17 cm high). A 60 W light source positioned over the starting point of the beam provided the only illumination in the room during test sessions, and white noise (50–60 dB) and “prodding” (index finger tapping of the tail) were used to encourage locomotion if rats stopped walking. With exception of the first pre-surgical testing day, animals were given a single beam-walking trial on assigned testing days. Beam-walking performance was rated using a 7-point rating scale, similar to those previously published (Feeney et al., 1982; Sutton and Feeney, 1992). Time to transverse the beam and enter the dark box was also recorded, and a trial was ended if the animal had not entered the goal within 60 sec. Post-surgical beam-walk testing was performed every other day, starting on POD 1 (1 h after the 24 h drug treatment) and continuing until POD 13.

Plus maze memory test—Spatial working memory was tested on either POD 7 or 8 using a four-arm plus maze as described previously (McNay et al., 2000; Taylor et al., 2008). The maze (made of black Plexiglas, with 12 cm high walls) contained an open central space (25-cm diameter) from which four symmetrical arms (10 cm wide $\times$ 25 cm long) radiate at 45° angles. On the day of testing, rats were placed into the center of the maze facing an open arm and were allowed to explore freely for 20 min. Rats will normally spontaneously alternate between all four arms of the maze during exploration periods, using spatial working memory to retain knowledge of previously entered arms of the maze. The number of arm entries and sequence of entries into each arm (denoted A, B, C and D) are recorded. An entry is counted only when both hind limbs cross a line between the center of the maze and the entrance to the arm. Memory performance was measured using the percent four/five alternation criterion (McNay et al., 2000). An alternation was counted when the rat visited each arm of the maze within a span of five arm choices. For example, one alternation is counted if entries 1–5 included entry into arms A, B, C and D, another alternation is counted if entries 2–6 included entry into all four arms, and so on for all subsequent spans of five arm choices. No alternation is counted

if the animal does not enter all four arms within five sequential arm choices. The total possible number of alternations in this test is equal to the total number of arm entries minus four. The rats' percent four/five alternation (performance) score is calculated as: $[\text{number of alternations} / (\text{total number of alternations} - 4)] \times 100$. Chance performance on this task is 45-50%.

Histology (1 month)

Rats used for behavioral testing were sacrificed at POD 29 or 30, as described above, with both a PBS flush and perfusion with 4% paraformaldehyde. Cryoprotected brains were sectioned at 20 μm thickness on a cryostat, saving 2 adjacent sections every 500 μm ; these sections were processed for standard thionin staining.

Thionin-stained sections were viewed using bright field illumination, and surviving hippocampal pyramidal neurons within two CA3_b subsectors and the PoDG were counted in 3 tissue sections for each rat (-2.8, -3.3 and -3.8 from Bregma). Contours for the PoDG included all tissue lying between the inner/outer blades of the dentate granule cells with exception of the CA3_c pyramidal cell layer, and those for the CA3 consisted of two rectangular counting grids (200 $\times$ 400 μm ; W $\times$ H) centered over pyramidal cells at the medial and lateral aspects of the CA3_b sector (see Fig. 1A). Cell density counts for each region (medial CA3_b, lateral CA3_b and PoDG) were averaged for each animal and expressed as number of cells/ mm^2 prior to calculating group averages.

The non-damaged cortical mantle lying dorsal to the rhinal sulcus and corpus callosum was measured bilaterally in 14 thionin-stained sections (every 500 μm) between +1.7 and -4.8 mm from Bregma using ImageJ software. Cortical tissue volume in each hemisphere was calculated and, after verifying no significant group differences in the right/contralateral hemisphere, percent tissue volume loss in the left/injured cortex was calculated $[100 - ((\text{Left/Right}) \times 100)]$, as previously described (Fukushima et al., 2009; Taylor et al., 2008).

Statistical analysis

Group data are expressed as the mean $\pm$ standard error of the mean (SEM). The parametric (histology, plus maze) data were analyzed by one-way analysis of variance followed by Tukey's post-hoc test or the Dunnett post-hoc test for multiple comparisons. Ordinal scaled (beam, neuroscore) data were analyzed using the Kruskal-wallis test followed by the Steel-Dwass or Steel's test (Hollander and Wolfe, 1999). Statistical significance was accepted with a 2-tailed $p < 0.05$. Statistical analysis was performed using SPSS software (version 17: SPSS Inc., Chicago, IL, USA) and Ekuseru-Toukei 2008 software (Social Survey Research Information Co. Ltd., Tokyo, Japan).

Results

Fluoro-Jade B cell counts

Numbers of dead/dying cells (FJB-positive cells with neuronal profiles) in the CA3_{a-b} ipsilateral to CCI at 72 h after injury were significantly reduced by either SP or EP treatment (s) (Fig. 1B). Compared to the CCI-Veh group, FJB-positive neurons were significantly ($p < 0.05$) reduced in the CCI-SP 1000 $\times$ 3 group. EP treatment(s) begun 1 h after CCI also significantly reduced dead/dying cells in the ipsilateral CA3, in a dose-dependent fashion, compared to CCI-Veh controls. FJB-positive neuronal profiles after a single low dose of EP (CCI-EP 20 $\times$ 1; $p < 0.05$ vs. CCI-Veh) were most similar to numbers seen after SP treatments, and the optimal neuroprotection in CA3_{a-b} was found in the CCI-EP 40 $\times$ 3 group ($p < 0.001$ vs. CCI-Veh).

The pyruvate treatment effects on dead/dying cells in the ipsilateral hilus+CA3_c mirrored those found in the CA3_{a-b} (Fig. 1C), albeit the extent of reduced numbers of FJB-positive hilar +CA3_c neurons observed in the CCI-SP 1000×3 and CCI-EP 20×1 groups were not significant compared to CCI-Veh. However, both the CCI-EP 40×1 and CCI-EP 20×3 groups had significantly ($p<0.05$) fewer FJB-positive cells in the hilus+CA3_c than did CCI-Veh controls. Dose-dependent EP effects observed for the CA3_{a-b} persisted in the hilus+CA3_c, where the greatest reduction in FJB-positive neuronal profiles was observed in the CCI-EP 40×3 group ($p<0.01$ vs. CCI-Veh).

Cerebral metabolism

Since the optical density readings in the right/contralateral hemisphere did not differ between groups in any region of interest, regional asymmetry scores were calculated for cytochrome oxidase staining intensity, where negative values reflect the depression of activity in the left compared to right hemisphere (Hovda et al., 1991). These asymmetry scores showed that, by 72 h after CCI, there was a generalized and widespread reduction in cytochrome oxidase activity in all ipsilateral regions measured (Table 1). The greatest reductions in activity were observed in the peri-contusional (parietal and temporal) cortex, the dorsal hippocampus, and the thalamic nuclei of the CCI-Veh control animals. With few exceptions (e.g. frontal cortex of CCI-EP 20×1 and CCI-EP 20×3 groups, hippocampus of CCI-SP 1000×3, CCI-EP 20×1 and CCI-EP 20×3 groups, and the DLG of CCI-SP 1000×3 and CCI-EP 20×3 groups), treatment(s) with pyruvate attenuated the widespread reduction of cytochrome oxidase activity after CCI. This attenuation of the asymmetry scores was significant in the peri-contusional cortex for all of the multiple pyruvate treatment conditions compared to the CCI-Veh group (CCI-SP 1000×3, $p<0.05$; CCI-EP 20×3, $p<0.05$; CCI-EP 40×3, $p<0.005$).

Inflammatory response to CCI

Light microscopy analyses revealed that all CD11b-positive cells were lightly stained and retained the ramified processes that are characteristic for resident microglia in the cortex and dorsal hippocampus (Fig. 2A) of the Sham-Veh rats. In contrast, large numbers of activated microglia were present throughout the cortex and the dorsal hippocampus ipsilateral to injury in the CCI-Veh group by 72 h post-injury. These CD11b-positive cells had a round cytoplasm with short and thickened cell processes, characteristic of ameboid microglia (Fig. 2B). The morphology of many CD11b-positive cells in both cortex and hippocampus of the SP- or EP-treated rats were consistent with that of activated microglia, as observed in the CCI-Veh group (Fig. 2C and 2D). However, there appeared to be fewer activated microglia and the density of immuno-staining appeared to be lower in the pyruvate-treated groups compared to the CCI-Veh group.

Although the morphological changes in microglia ipsilateral to CCI were examined in all rats surviving for 72 h, and appeared to show less activation (ameboid characteristics) in all pyruvate-treated groups, these changes were most notable when multiple injections were administered. Based on our FJB and cytochrome oxidase staining results for SP and EP, our initial optical density analyses of CD11b changes in pre-mounted (20 μ m) and post-fixed (10% formalin) tissue sections were restricted to the groups with CCI and multiple treatment conditions ($n=7$ /group). The optical density readings in the right/contralateral hemisphere did not differ between groups in any region of interest. Readings for CD11b staining intensity in the left hemisphere indicated that microglia activation in the ipsilateral temporal cortex was significantly reduced in the CCI-SP 1000×3 (15.7 ± 3.3 ; $p<0.01$) and CCI-EP 40×3 (16.9 ± 2.4 ; $p<0.05$) groups compared to CCI-Veh controls (30.2 ± 1.9). In the dorsal hippocampus ipsilateral to injury, optical density values were reduced from 15.9 ± 1.0 in the CCI-Veh group to 13.1 ± 2.0 and 9.3 ± 2.1 in the CCI-SP 1000×3 and CCI-EP 40×3 groups, respectively, and these differences were not significant.

We repeated the CD11b immunohistochemistry in animals perfused with paraformaldehyde 72 h post-injury (n=3/group). Results of the optical density analysis of CD11b-positive cells in the frontal cortex, temporal cortex, and dorsal hippocampus ipsilateral to injury for the four groups are shown in Table 2. In all of the regions examined, the CCI-Veh group had a significantly ($p<0.05$) higher density of CD11b immuno-staining than was observed in the Sham-Veh group. The microglia staining density in the SP- and EP-treated groups was reduced in all ipsilateral regions compared to the CCI-Veh group, and neither pyruvate-treated group was statistical different from the Sham-Veh group. The largest attenuation of microglia activation was observed in the dorsal hippocampus of the CCI-EP group (treated at 1, 12 and 24 h post-injury), where the CD11b staining density was significantly ($p<0.05$) lower than that of the CCI-Veh group.

Neurobehavioral deficits after CCI

All rats achieved beam-walk ratings of 7 (beam traversal with no more than 2 foot slips) prior to surgery (Fig. 3), and rats in the Sham-Veh group maintained this level of performance throughout the post-operative testing period. All groups with CCI showed significant ($p's<0.01$) beam-walking deficits compared to Sham-Veh controls up to POD 11. Equivalent impairments (average beam ratings of 2.9-3.0, signifying ability to cross the beam without the right/contralateral limbs aiding in forward locomotion) occurred on POD 1 for rats in the CCI-Veh, CCI-SP and CCI-EP groups. Gradual improvement in beam-walking ability (spontaneous recovery) was observed over sequential testing days in the CCI-Veh group. Mean beam-walk ratings in pyruvate-treated groups began to improve above those of vehicle-treated counterparts by POD 3, and the beam-walking ability of both the CCI-SP and CCI-EP groups was significantly better than that of the CCI-Veh group on POD 7 ($p's<0.05$) and POD 9 ($p's<0.05$). By POD 13, the beam-walking ability of rats in each of the CCI groups had recovered to a level that was no longer significantly different from the Sham-Veh group.

All rats exhibited normal responses on the tactile placing, hindlimb extension and contraflexion tests prior to surgery, with the composite neurological scores averaging to 0 in all groups (Fig. 4). The Sham-Veh group exhibited stable (near 0), normal neurological performance throughout the post-operative examination period. Neurological scores deteriorated significantly ($p's<0.01$ vs. Sham-Veh) after CCI, with equivalent worsening of sensorimotor neurological function occurring in the CCI-Veh, CCI-SP and CCI-EP groups on POD 1 (within 30-45 min of the 24 h treatment). Both the SP and EP treatment groups showed improved neurological functions relative to CCI-Veh over the post-injury testing period, and the CCI-SP and CCI-EP groups did not differ significantly on any testing day. In the CCI-SP group, composite neurological scores were significantly lower ($p<0.05$) than scores in the CCI-Veh group on POD 9, 16 and 23. Scores for the CCI-EP group, although lower than those for vehicle-treated rats from POD 9 onward, only reached statistical significance compared to the CCI-Veh group on POD 23 ($p<0.05$). All three injury groups showed persistent sensorimotor neurological abnormalities, demonstrated by significantly higher composite neuroscores (CCI-Veh, $p<0.01$; CCI-SP, $p<0.05$; CCI-EP, $p<0.05$) compared to the Sham-Veh group on POD 23.

In the plus-maze test of working memory, data from 5 of the CCI (2 CCI-Veh, 1 CCI-SP and 2 CCI-EP) rats was excluded from the analysis due to insufficient movement/arm entries (<15 arm entries during the 20 min test period). Average numbers of arm entries were 37.4 ± 3.9 , 38.7 ± 3.8 , 34.3 ± 3.8 and 31.9 ± 3.1 for animals remaining in the Sham-Veh, CCI-Veh, CCI-SP and CCI-EP groups, respectively, and were not significantly different among the groups. As measured using the percent four/five alternation scores (Fig. 5), cognitive abilities in the CCI-Veh group were significantly impaired (approximately chance level) at one week post-injury compared to the Sham-Veh group ($p<0.01$). In contrast to the sensorimotor benefits seen with

SP treatments, working memory impairments in the CCI-SP group were not significantly different from those found in CCI-Veh controls. However, the percent four/five alternation scores were significantly improved ($p < 0.05$) in the CCI-EP group as compared to the CCI-Veh group, and the EP-treated group did not differ from Sham-Veh controls.

Chronic histopathology after CCI

Area measures of the left cortical mantle for the groups in Experiment 2 are illustrated in Fig. 6A. It can be seen that the CCI groups had substantial loss of cortical tissue compared to Sham-Veh controls, with the most profound damage occurring posterior to Bregma (-1.3 to -4.8mm). Statistical analysis indicated significant effects of Group ($p < 0.001$) and anterior-posterior tissue section level ($p < 0.001$). Although tissue loss in the injured hemisphere was slightly reduced in sections incorporating anterior portions of the forelimb and hind limb motor cortex (0.2 to -0.8mm) of the CCI-SP and CCI-EP groups, no significant effect of Treatment was found in comparisons between the three CCI groups. As anticipated, the tissue volumes of the right/contralateral cortex did not differ significantly between the sham injury and CCI groups at one month post-surgery (average volume of all CCI rats being 95.6% of the right cortical volume in the Sham-Veh group). The left parietal CCI resulted in a significant percent tissue volume loss in the injured cortex compared to the Sham-Veh group ($p < 0.001$). As is shown in Fig. 6B, the percent tissue volume loss in the left/ipsilateral cortex was $40.0 \pm 5.2\%$ in the CCI-Veh group. Cortical tissue volume loss was slightly less in the CCI-SP ($35.0 \pm 4.1\%$) and CCI-EP ($34.2 \pm 5.3\%$) groups, but these reductions were not significant compared to the CCI-Veh group.

The ipsilateral PoDG neuron counts in thionin-stained tissue at 1 month after surgery in the CCI-Veh group (101.4 ± 9.2 cells/mm²) were significantly ($p < 0.01$) lower than counts in the Sham-Veh group (161.7 ± 8.9). More surviving PoDG neurons were observed in CCI-SP (128.8 ± 12.7) and CCI-EP (115.9 ± 11.2) groups than in the CCI-Veh group, but these increases did not reach statistical significance. Similar trends were seen in the ipsilateral CA3_b cell density counts at 1 month post-surgery. Surviving pyramidal neurons in the lateral CA3_b were significantly ($p < 0.001$) reduced in the dorsal hippocampus ipsilateral to CCI (CCI-Veh: 236.7 ± 36.3 , CCI-SP: 279.2 ± 12.4 , CCI-EP: 279.2 ± 24.1) compared to the Sham-Veh group (397.5 ± 14.2), but did not differ between the drug treatment conditions. Surviving pyramidal neurons in the medial CA3_b were also decreased significantly ($p < 0.001$) in the CCI-Veh group (265.0 ± 14.5) compared to the Sham-Veh group (415.0 ± 12.6). However, both SP and EP treatments significantly ($p < 0.05$) increased the number of surviving neurons in the medial CA3_b in the ipsilateral hippocampus (CCI-SP: 320 ± 11.7 , CCI-EP: 311.7 ± 16.4) compared to the CCI-Veh group.

Discussion

In the past decade there has been increasing evidence for a therapeutic role of pyruvate in neurological disorders. Previous work using the unilateral CCI model of experimental TBI in rats (Fukushima et al., 2009) found that administration of exogenous SP at doses of 500 or 1000 mg/kg (4.5 or 9.0 mmol/kg, respectively) significantly reduced numbers of dead/dying neurons in the ipsilateral cortex at 6 h post-injury, but a single treatment at these doses given 5 min post-injury did not alter cortical tissue volume loss at two weeks after CCI. While neuroprotection afforded by 500 or 1000 mg/kg of SP at 6 h did not differ significantly the higher dose was slightly more effective, thus 1000 mg/kg of SP was administered at 5, 65 and 125 min post-injury and found to significantly reduce cortical tissue loss at two weeks. One goal of the current studies was to determine if the therapeutic window for SP treatments after experimental TBI, including time from injury to treatment onset and time between treatments, could be extended. The current results show that SP treatments (1000 mg/kg), given at 1, 12

and 24 h after CCI, significantly reduced neuronal injury in the ipsilateral hippocampus (CA3_{a-b}) at 72 h and one month, and significantly improved sensorimotor neurobehavioral recovery assessed over the first two to three weeks post-injury. However, working memory impairments assessed at one week and cortical tissue volume loss one month post-injury were not significantly altered by these delayed SP treatments. The current behavioral results are compatible with the report that a 30 min infusion of SP (~500 mg/kg) begun 1 h after closed head injury in the rat significantly improves neurobehavioral recovery (Zlotnik et al., 2008). The available data from TBI models are also congruent with reports that *in vivo* administration of exogenous SP can reduce brain or spinal cord damage and/or neurological deficits in a variety of injury models (Choi et al., 2010; Gonzalez-Falcon et al., 2003; Kim et al., 2005, 2007, 2010; Lee et al., 2001; Mongan et al., 2001, 2003; Park et al., 2007; Ryu et al., 2003, 2004, 2006; Sharma et al., 2008; Suh et al., 2005; Wang et al., 2009b; Yi et al., 2007; Yoo et al., 2004).

Despite the positive effects reported for SP in a variety of neurological disorders and preclinical models of critical illnesses (cf. Fink, 2007; Kao and Fink, 2010), there have been recurring concerns about the therapeutic use of SP due to its instability in aqueous solutions. These problems can be largely overcome if solutions are mixed fresh, as in the current study, but spontaneous rapid dimerization to pyruvate hydrate and a condensation reaction forming parapyruvate (Montgomery and Webb, 1956) limits the storage, and hence clinical applicability, of SP solutions. EP (ethyl 2-oxopropanoate) was developed as a more stable and lipophilic derivative (ethyl ester) of pyruvic acid, and this electrically neutral compound can penetrate biological membranes to enter cells without monocarboxylate transporters needed for pyruvate cellular transport (cf. Fink, 2007; Kao and Fink, 2010, and references therein; Tokumaru et al., 2009). Initial *in vitro* and *in vivo* studies (Sappington et al., 2003; Sims et al., 2001; Varma et al., 1998) found that EP was more effective than treatment with equimolar concentrations of SP for amelioration of structural or functional injury to cells subjected to inflammatory or redox-mediated stressors. Comparisons of equimolar SP and EP in the central nervous system are sparse, and have only been conducted using *in vitro* injury models. In neuronal cultures subjected to oxygen glucose deprivation or hydrogen peroxide exposure both SP and EP at 5 mmol were found to reduce cell death, while EP but not SP (at 5 to 20 mmol concentrations) was found to improve microglia cell viability and reduce inflammatory markers in cultured microglia exposed to lipopolysaccharide (Kim et al., 2005). In a cortical slice model of hydrogen peroxide exposure Zeng et al. (2007) reported that 20 mmol EP was more effective than 20 mmol SP for reduction of apoptosis and prevention of ATP and N-acetylaspartate reductions. *In vivo* studies within the experimental neurology literature have indicated that EP, at doses of 20 or 40 mg/kg (0.17 or 0.34 mmol/kg), is at least as neuroprotective as SP at doses of 500 to 1000 mg/kg, with EP having a much wider therapeutic window (Kim et al., 2005; Lee et al., 2001; Yu et al., 2005).

A second goal of the current studies was to determine if EP can also confer beneficial effects after CCI injury. The results of Experiment 1 indicate that a single low dose of EP (20 mg/kg) given 1 h post-CCI was equivalent to three SP treatments (1000 mg/kg at 1, 12 and 24 h) for reduction of neuronal injury in the ipsilateral hippocampus (CA3_{a-b}) at 72 h. A single high dose (40 mg/kg) of EP, as well as three injections of either low or high dose EP at 1, 12 and 24 h, significantly reduced neuronal damage in both the CA3_{a-b} and hilus+CA3_c regions of the ipsilateral hippocampus at 72 h after CCI, with optimal neuroprotection being observed in the CCI-EP 40×3 treatment group. In Experiment 2 multiple EP treatments (40 mg/kg at 1, 12 and 24 h) significantly reduced working memory deficits one week post-CCI, improved sensorimotor neurobehavioral recovery during the first two to three weeks, and reduced ipsilateral CA3 neuronal cell death (but not the loss of cortical tissue volume) at one month after injury. These results indicate that administration of a cumulative dose of 120 mg/kg of EP is as beneficial as 3000 mg/kg of SP administered within 24 h of CCI, albeit the EP treatments were superior to SP with regard to improvement of cognitive outcome. Overall,

these observed benefits of EP treatments after TBI are consistent with *in vivo* neuroprotective effects reported for EP in experimental brain injury induced by cerebral ischemia (Kim et al., 2005, 2010; Yu et al., 2005), kainic acid (Cho et al., 2006), spinal cord ischemia (Wang et al., 2009a), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Choi et al., 2010) and perinatal hypoxia-ischemia (Shen et al., 2010).

Experimental TBI has been previously shown to reduce the capacity for oxygen consumption in the mitochondrial electron transport chain, with decreased activity of complex I as well as decreased activity and expression of cytochrome *c* oxidase (complex IV) subunits (Dai et al., 2009; Harris et al., 2001; Hovda et al., 1991; Huttemann et al., 2008; Opii et al., 2007). Therefore, in Experiment 1 we employed cytochrome oxidase histochemistry to determine the effects of SP or EP treatments on mitochondrial metabolism. The cytochrome oxidase complex is the terminal enzyme of the mitochondrial respiratory chain, which transfers electrons from cytochrome *c* to molecular oxygen, which is subsequently reduced to water. Cytochrome oxidase works in conjunction with nicotinamide adenine dinucleotide (NADH) dehydrogenase and the *bc1* complex to generate the mitochondrial membrane potential, which is utilized by ATP synthase to produce adenosine triphosphate (ATP). Thus, if the activity of cytochrome oxidase is decreased, a build up of electrons in the electron transport chain could increase production of reactive oxygen species and the synthesis of ATP would be decreased (Huttemann et al., 2008; Opii et al., 2007). Results of Experiment 1 show that CCI leads to widespread ipsilateral reductions in cytochrome oxidase activity at 72 h after injury. Multiple treatments of SP (1000 mg/kg) or EP (20 mg/kg) produced equivalent and significant attenuation of this decrease in the peri-contusional cortex. Multiple treatments with the higher dose of EP (40 mg/kg), while not significantly different from the CCI-SP 1000×3 or the CCI-EP 20×3 group produced the largest magnitude of increased cytochrome oxidase activity in the peri-contusional cortex. Our results are compatible with data showing pyruvate enhancement of cytochrome oxidase activity in human brain tissue (Verwer et al., 2002) and with multiple *in vitro* and *in vivo* studies indicating that pyruvate serves as a metabolic anti-oxidant or free radical scavenger (Chen et al., 2010; Kim et al., 2008; O'Donnell-Tormey et al., 1987; Ryu et al., 2006; Tokumaru et al., 2009; Wang et al., 2009a; Zeng et al., 2007). Cellular metabolism of exogenous pyruvate can improve mitochondrial redox states (NAD/NADH or lactate/pyruvate ratio) to facilitate oxidative phosphorylation (Lee et al., 2004; Mongan et al., 2001; Scholz et al., 1995; Sharma et al., 2003) and can also improve the glutathione redox cycle (NADP/NADPH) which will help offset oxidative stress (Alvarez et al., 2003; Kashiwagi et al., 1997; Mongan et al., 2002). It was recently reported that SP (1000 mg/kg, i.p.), administered at 15 min after surgery and then every 24 h, attenuated a TBI-induced decrease in hippocampal and plasma pyruvate dehydrogenase (PDH) levels and increased PDH E1- α subunit immuno-staining in the hippocampus (Sharma et al., 2009). This latter report is consistent with prior reports that SP treatment can stimulate PDH activity, its own oxidation, and increase ATP levels in cortex or liver after hemorrhagic shock or in astrocytes exposed to *in vitro* ischemia (Mongan et al., 2003; Sharma et al., 2003).

Experiment 1 also evaluated the microglia response to CCI and treatments of either SP or EP, as activation of these cells is one of the first responses of the brain to injury or inflammatory challenge. Subsequent to activation, resident astrocytes and microglia can exacerbate tissue damage by secretion of soluble cytokines and chemokines, activating several cell death pathways and enhancing accumulation of blood-derived neutrophils and leukocytes in the injured brain (Lucas et al., 2006; Morganti-Kossmann et al., 2007). The current results show that microglia activation was significantly increased at 72 h after CCI in rats treated with Ringer's vehicle compared to sham injury controls. Optical densities of microglia immuno-staining in the dorsal hippocampus and frontal or temporal cortex ipsilateral to CCI for animals receiving multiple SP (1000 mg/kg) or EP (40 mg/kg) treatments from 1 to 24 h post-injury were increased, but not significantly, compared to values in sham injury controls. Although

the CCI-EP group did not differ significantly from the CCI-SP treated animals, only the former group exhibited significantly decreased activation of microglia in the hippocampus compared to the CCI-Veh controls. These results are consistent with the strong anti-inflammatory properties reported for SP (see review by Das, 2006) or EP (see reviews by Fink, 2007; Kao and Fink, 2010) in several models of disease as well as with reported anti-inflammatory effects of these compounds using *in vitro* and *in vivo* models of brain injury (Cho et al., 2006; Kim et al., 2005, 2008; Park et al., 2007; Ryu et al., 2004, 2006; Sharma et al., 2003, 2008, 2009; Shen et al., 2010; Wang et al., 2009a, 2009b; Yu et al., 2005).

The current studies indicate that while SP and EP produce similar neuroprotection, metabolic and anti-inflammatory effects in an experimental TBI model, EP is more potent on a molar basis. As is discussed in the recent review by Kao and Fink (2010) multiple studies have previously reported that EP is 10- to 100-fold more potent than pyruvate. This increased potency for EP was suggested to be related to the lipophilic properties of EP. Others have speculated that actions of EP derive solely from its intracellular deliver, where intracellular esterase can cleave EP into ethanol and pyruvate (Zeng et al., 2007). These authors reported that 20 mmol EP and SP produced identical tricarboxylic acid metabolites, as detected using nuclear magnetic resonance spectroscopy, in rat brain tissue slices exposed to hydrogen peroxide stress. In a recent metabolic imaging study, Hurd and colleagues (2010) report that total carbon delivery to brain after EP administration is significantly greater than carbon deliver by pyruvate, with preferential uptake of EP and EP-hydrate being observed. Some authors have proposed that ethanol derived from EP contributes to its anti-inflammatory actions (Sharma and Mongan, 2010), based on a report of ethanol effects in a rat model of hemorrhagic shock (Phelan et al., 2002). However, an equal dose of ethanol did not exert the beneficial effects of EP for protection of rodent lens tissue from oxidative damage (Varma et al., 1998), and Mizutani et al. (2010) reported that 5 mmol EP, but not equimolar concentrations of pyruvate, ethanol or pyruvate plus ethanol, significantly inhibited tumor necrosis factor-induced activation of NF- κ B signaling pathways in alveolar epithelial cells. Although we do not know of any *in vivo* studies that have directly compared equal doses of ethanol versus EP, future studies will be needed to address this potential mechanism for EP-mediated effects. Although ethanol concentrations at doses of EP used in the current and many prior reports are likely to be relatively low, it is worth noting that an intraperitoneal ethanol dose of 1 g/kg 40 min prior to CCI significantly reduced the severity of beam walking deficits and the one month cortical lesion volume (Kelly et al., 1997). This same dose of ethanol attenuated CCI-induced hyperglycolysis and reductions in cerebral blood flow immediately post-injury and increased cerebral metabolic rates of glucose and cerebral blood flow at 24 and 72 h post-injury (Kelly et al., 2000). These effects were proposed to be related to ethanol effects on N-methyl-D-aspartate receptor-mediated excitotoxicity and/or to the vasodilatory effects of ethanol and its major metabolite (acetate).

In conclusion, the neuroprotective effects found for SP or EP treatments begun one h post-TBI in our CCI model are likely due to multiple interrelated effects of these compounds. The current results indicate that SP or EP treatments may increase activity of cytochrome oxidase to improve oxidative phosphorylation and reduce oxidative stress, and these treatments also reduce the cerebral inflammatory response to TBI. Although there were some indications that EP might impart greater early-stage neuroprotection (72 h) or cognitive improvement (one week) than does SP after CCI, the early-stage metabolic or inflammatory effects and the one month histology outcomes for the two treatments did not differ. Further studies with either of these pyruvate formulations appears warranted in order to optimize treatment dosage, intervals between treatments, and/or treatment durations to achieve more complete neurological recovery and histological preservation as well as to further characterize the pharmacology and mechanisms of pyruvate-induced neuroprotection after TBI.

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List of Abbreviations

ATP	adenosine triphosphate
CCI	cortical contusion injury
DAB	3',3-diaminobenzidine tetrahydrochloride
DAPI	4',6-diamidino-2-phenylindol dihydrochloride
DLG	dorsal lateral geniculate nucleus
EP	ethyl pyruvate
FJB	Fluoro-Jade B
i.p	intraperitoneal
MG	medial geniculate nucleus
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NF-kB	nuclear factor-kappaB

PB	phosphate buffer
PBS	phosphate-buffered saline
PDH	pyruvate dehydrogenase
POD	post-operative day
PoDG	polymorphic neurons of the dentate gyrus
SEM	standard error of the mean
s.c.	subcutaneous
SP	sodium pyruvate
TBI	traumatic brain injury
TBS	Tris buffered saline
TBS/TX	0.25% Tween 20 in Tris buffered saline
Veh	vehicle (Ringer's solution)

Fig. 1.

Panel A illustrates the contoured areas used for counts of Fluoro-Jade B (FJB)-positive neurons in the CA3_{a-b} and hilus+CA3_c (combined PoDG plus CA3_c regions). Also shown in panel A are the contour sites used for counts of lateral and medial CA3_b neurons and PoDG neurons (excluding the CA3_c) at one month post-surgery. Panels B and C illustrate data (mean $\pm$ SEM) for FJB-positive cells in the left/ipsilateral CA3_{a-b} (B) and hilus+CA3_c (C) at 72 h after cortical contusion injury (CCI) and treatment with either vehicle (Veh), sodium (SP) or ethyl pyruvate (EP). CCI groups (n=7/group) denoted in the legend box and panels B and C were treated post-injury as follows: CCI-Veh = Ringer's at 1, 12 and 24 h; CCI-SP 1000x3 = SP (1000 mg/kg) at 1, 12 and 24 h; CCI-EP 20x1 = EP (20 mg/kg) at 1 h; CCI-EP 40x1 = EP (40 mg/kg) at 1 h; CCI-EP 20x3 = EP (20 mg/kg) at 1, 12 and 24 h; CCI-EP 40x3 = EP (40 mg/kg) at 1, 12 and 24 h. Both SP and EP decreased the number of dead/dying hippocampal neurons, with dose-dependent neuroprotection apparent for the EP treatments. *: p<0.05, **: p<0.01, ***: p<0.001 compared to CCI-Veh group.

Fig. 2.

Immuno-staining of CD11b-positive microglia in the ipsilateral dorsal hippocampus (lateral CA3 region), 72 h after sham or cortical contusion injury (CCI) and treatments (at 1, 12 and 24 h) of either vehicle (Veh), sodium (SP, 1000 mg/kg) or ethyl pyruvate (EP, 40 mg/kg). In the Sham-Veh group CD11b-positive cells exhibited morphology characteristic of resident microglia (A), whereas staining characteristics of activated microglia were observed in the CCI-Veh group (B). CD11b-positive cells of the CCI-SP (C) or CCI-EP (D) treatment groups showed some morphology characteristic of stimulated or activated microglia, but the density of immuno-staining was reduced compared to the CCI-Veh group (see Table 2). Inserts in each panel are higher magnification images of cells within that panel. Scale bars: panels = 100 μ m; inserts = 20 μ m.

Fig. 3. Mean ($\pm$ SEM) beam-walk ratings for rats ($n=7$ /group) after sham or cortical contusion injury (CCI) and treatments (at 1, 12 and 24 h) of either vehicle (Veh), sodium (SP, 1000 mg/kg) or ethyl pyruvate (EP, 40 mg/kg). The initial beam-walking deficits induced by CCI were equivalent in all groups. The recovery of beam-walking ability in the CCI-SP and CCI-EP groups was significantly improved compared to the spontaneous recovery rate seen in CCI-Veh controls. *: $p<0.05$ compared to CCI-Veh group.

Fig. 4.

Mean ($\pm$ SEM) neurological scores of rats ($n=7$ /group) with sham or cortical contusion injury (CCI) and treatments (at 1, 12 and 24 h) of either vehicle (Veh), sodium (SP, 1000 mg/kg) or ethyl pyruvate (EP, 40 mg/kg). Equivalent worsening of the combined neuroscore occurred in all CCI groups early after injury. Both the CCI-SP and CCI-EP groups exhibited significantly better recovery from these neurological deficits than did vehicle-treated rats with CCI. *: $p<0.05$ compared to CCI-Veh group.

Fig. 5.

Percent four/five alternation scores (mean ± SEM) for groups evaluated in the plus maze one week after sham (n=7) or cortical contusion injury (CCI) and treatments (at 1, 12 and 24 h) of either vehicle (Veh, n=5), sodium (SP, 1000 mg/kg, n=6) or ethyl pyruvate (EP, 40 mg/kg, n=5). Significant working memory deficits were observed for the CCI-Veh ($p<0.01$) and the CCI-SP ($p<0.05$) groups compared to Sham-Veh controls. The EP treatments after CCI significantly improved the percent four/five alternation scores. *: $p<0.05$ compared to CCI-Veh group.

Fig. 6.

Panel A shows the mean ($\pm$ SEM) area of the left cortical mantle at 500 μ m intervals (1.7 to -4.8 mm relative to Bregma) at one month after sham ($n=7$) or cortical contusion injury (CCI) and treatments (at 1, 12 and 24 h) of either vehicle (Veh, $n=7$), sodium (SP, 1000 mg/kg, $n=7$) or ethyl pyruvate (EP, 40 mg/kg, $n=7$). All CCI groups had significant loss of tissue area compared to Sham-Veh controls ($p<0.001$), but no significant difference was observed between the CCI groups. Panel B illustrates the percent tissue volume loss (mean $\pm$ SEM) in the left/injured cortex [$100 - ((\text{Left/Right}) \times 100)$]. All CCI groups showed significant volume loss compared to the Sham-Veh group ($p<0.001$). Both the CCI-SP and CCI-EP groups showed slightly less volume loss than the CCI-Veh group, but these reductions were not significant.

Table 1

Regional asymmetry scores of cytochrome oxidase histochemistry.

Mean $\pm$ SEM asymmetry in optical density of regional cytochrome oxidase activity using the formula: [(Left–Right)/(left+Right)] $\times$ 100. Optical density was read 72 h following cortical contusion injury (CCI) in rats (n=7/group) treated with vehicle (Veh), sodium (SP) or ethyl pyruvate (EP). The numbers for group designations represent the treatment dose (mg/kg) $\times$ number of injections (1 or 3).

	CCI-Veh	CCl-SP 1000 $\times$ 3	CCI-EP 20 $\times$ 1	CCI-EP 40 $\times$ 1	CCI-EP 20 $\times$ 3	CCI-EP 40 $\times$ 3
Frontal Cortex	-2.64 $\pm$ 0.99	-1.31 $\pm$ 0.79	-2.80 $\pm$ 1.61	-1.16 $\pm$ 0.76	-2.61 $\pm$ 0.62	-0.98 $\pm$ 0.52
Peri-contusional Cortex	-5.60 $\pm$ 1.28	-3.14 $\pm$ 0.57 *	-3.40 $\pm$ 0.53	-3.63 $\pm$ 0.64	-3.14 $\pm$ 1.19 *	-1.49 $\pm$ 0.58 **
Caudate	-1.91 $\pm$ 0.49	-1.43 $\pm$ 0.30	-1.44 $\pm$ 0.56	-0.22 $\pm$ 0.59	-1.36 $\pm$ 0.43	-1.11 $\pm$ 0.64
Hippocampus	-3.32 $\pm$ 0.77	-4.70 $\pm$ 1.38	-4.74 $\pm$ 1.34	-1.67 $\pm$ 0.46	-3.68 $\pm$ 0.75	-2.57 $\pm$ 0.54
Ventral Thalamus	-3.91 $\pm$ 1.19	-2.91 $\pm$ 0.71	-2.81 $\pm$ 0.46	-1.16 $\pm$ 0.40	-3.35 $\pm$ 0.46	-2.38 $\pm$ 0.75
DLG	-4.98 $\pm$ 0.90	-5.88 $\pm$ 1.37	-3.60 $\pm$ 0.41	-2.26 $\pm$ 0.98	-5.45 $\pm$ 0.37	-2.85 $\pm$ 1.20
MG	-3.95 $\pm$ 1.47	-1.71 $\pm$ 0.93	-0.33 $\pm$ 1.67	-1.36 $\pm$ 0.83	-2.54 $\pm$ 0.81	-1.00 $\pm$ 0.79

* p<0.05

** p<0.005 compared to CCI-Veh group.

Table 2

Regional optical density values for CD11b immunohistochemistry.

Mean $\pm$ SEM optical density of CD11b immuno-stained sections of paraffinaldehyde-perfused animals as a measure of microglia activation. Optical density was read 72 h following sham or cortical contusion injury (CCI) in rats (n=3/group) treated at 1, 12 and 24 h with vehicle (Veh), sodium (SP, 1000 mg/kg) or ethyl pyruvate (EP, 40 mg/kg).

	Frontal cortex	Temporal cortex	Hippocampus
Sham-Veh	18.0 $\pm$ 0.7	23.8 $\pm$ 1.0	29.7 $\pm$ 1.9
CCI-Veh	56.9 $\pm$ 11.1 *	64.6 $\pm$ 12.2 *	92.0 $\pm$ 12.5 **
CCI-SP	46.0 $\pm$ 6.7	51.7 $\pm$ 6.4	66.8 $\pm$ 11.0
CCI-EP	40.1 $\pm$ 1.3	49.2 $\pm$ 2.1	41.9 $\pm$ 1.4 #

* p<0.05

** p<0.01 compared to Sham-Veh group.

p<0.05 compared to CCI-Veh group.