JOURNAL OF NEUROTRAUMA 34:1291–1301 (April 1, 2017)

DOI: 10.1089/neu.2016.4628

Original Articles

Synaptic Mitochondria Sustain More Damage than Non-Synaptic Mitochondria after Traumatic Brain Injury and Are Protected by Cyclosporine A

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Abstract

Currently, there are no Food and Drug Administration (FDA)-approved pharmacotherapies for the treatment of those with traumatic brain injury (TBI). As central mediators of the secondary injury cascade, mitochondria are promising therapeutic targets for prevention of cellular death and dysfunction after TBI. One of the most promising and extensively studied mitochondrial targeted TBI therapies is inhibition of the mitochondrial permeability transition pore (mPTP) by the FDA-approved drug, cyclosporine A (CsA). A number of studies have evaluated the effects of CsA on total brain mitochondria after TBI; however, no study has investigated the effects of CsA on isolated synaptic and non-synaptic mitochondria. Synaptic mitochondria are considered essential for proper neurotransmission and synaptic plasticity, and their dysfunction has been implicated in neurodegeneration. Synaptic and non-synaptic mitochondria have heterogeneous characteristics, but their heterogeneity can be masked in total mitochondrial (synaptic and non-synaptic) preparations. Therefore, it is essential that mitochondria targeted pharmacotherapies, such as CsA, be evaluated in both populations. This is the first study to examine the effects of CsA on isolated synaptic and non-synaptic mitochondria after experimental TBI. We conclude that synaptic mitochondria sustain more damage than non-synaptic mitochondria 24 h after severe controlled cortical impact injury (CCI), and that intraperitoneal administration of CsA (20 mg/kg) 15 min after injury improves synaptic and non-synaptic respiration, with a significant improvement being seen in the more severely impaired synaptic population. As such, CsA remains a promising neuroprotective candidate for the treatment of those with TBI.

Keywords: cyclosporine A; mitochondria; neuroprotection; synaptic and non-synaptic; traumatic brain injury

Introduction

Traumatic brain injury (TBI) represents a significant health crisis. In the United States, there are more than 5 million persons currently living with a disability resulting from a TBI[1] with an associated economic burden of 76.5 billion dollars.[2,3] TBI consists of a primary mechanical injury followed by a secondary injury cascade.[4] Aspects of this cascade include increases in excitotoxic amino acids such as glutamate, increases in intracellular calcium, mitochondrial dysfunction, production of reactive oxygen and nitrogen species (ROS/RNS), initiation and propagation of lipid peroxidation (LP), formation of LP-derived neurotoxic aldehydes, activation of calcium-dependent proteases such as calpain, cytoskeletal degradation, cell death, and neurologic dysfunction.[5–28] The secondary injury cascade should be amenable to therapeutic intervention. Currently, there are no Food and Drug Administration (FDA)-approved pharmacotherapies for the treatment of patients with TBI, however.[12]

Mitochondria play a central role in the secondary injury cascade. Mitochondria are essential regulators of calcium homeostasis[29,30] and buffer increased intracellular calcium after TBI.[8] High levels of mitochondrial calcium, however, lead to mitochondrial dysfunction, including increased generation of ROS/RNS, decreased oxidative phosphorylation and adenosine triphosphate (ATP) production, and induction of the mitochondria permeability transition pore (mPTP).[7,8,15,28,31–36]

The mPTP is a non-selective mega channel located in the inner mitochondrial membrane that is permeable to solutes <1.5 kDa.[28] Opening of the mPTP leads to extrusion of calcium back into the cytosol, mitochondrial swelling, and rupture of the outer mitochondrial membrane.[28,37] Mitochondrial dysfunction contributes to several aspects of the aforementioned injury cascade, including ROS/RNS induction of LP and formation of the LP-derived neurotoxic aldehydes 4-hydroxynonenal and acrolein, which are capable of covalently binding mitochondrial proteins, further exacerbating mitochondrial dysfunction.[7,19,38–42] Additional downstream consequences of mPTP formation include activation of the calcium-dependent protease calpain, cytoskeletal degradation, cell death, and neurologic dysfunction.[7,11–13,15,16,28,38,39,43–49]

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Therefore, as central mediators of the secondary injury cascade, mitochondria are promising therapeutic targets for prevention of cellular death and dysfunction after TBI.

Several therapies targeting mitochondria have been shown to be neuroprotective in experimental models of TBI, including mild uncoupling,[50,51] ketogenic diets,[52,53] increased antioxidant availability,[40,54] and scavenging of neurotoxic aldehydes.[19] One of the most promising and extensively studied mitochondrial targeted TBI therapies, however, is inhibition of mPTP by the FDA-approved immunosuppressant, cyclosporine A (CsA). In experimental TBI, CsA or its non-immunosuppressant analog, NIM811, prevent mitochondrial swelling and axonal pathology,[20,21] maintain mitochondrial membrane potential and decrease production of reactive oxygen species,[33] improve total (synaptic and non-synaptic) mitochondrial respiration,[47] prevent oxidative (synaptic and non-synaptic) mitochondrial damage,[47,55] improve cortical tissue sparing,[56–58] decrease calpain-mediated cytoskeletal degradation and neurodegeneration,[48] and improve motor and cognitive function.[48,55,59,60]

Interestingly, despite the fact that CsA directly targets mitochondria, only a limited number of studies have evaluated the effects of CsA on mitochondria after experimental TBI,[33,47,61] with no studies evaluating the effects of CsA on isolated synaptic and non-synaptic mitochondria. Mitochondria are heterogeneous, consisting of both synaptic and non-synaptic populations. Isolated synaptic mitochondria consist of pre-synaptic mitochondria located within the synaptosome, while isolated non-synaptic mitochondria consist of neuronal (axonal, somal, dendritic) and non-neuronal (glial, vascular, etc.) mitochondria.

To our knowledge, there is currently no method available to further separate the non-synaptic mitochondrial population into non-synaptic neuronal and non-synaptic non-neuronal (e.g., glial) populations. Therefore, whereas synaptic mitochondria consist of pure pre-synaptic neuronal mitochondria, non-synaptic mitochondria are isolated from numerous cell types. Synaptic mitochondria are considered essential for proper neurotransmission and synaptic plasticity,[62–64] processes that are impaired after TBI.[65] Their dysfunction has been implicated in neurodegeneration, as well as degeneration of synapses and neurons absent overt cell death,[66–68] and studies reveal synaptic mitochondria to be more susceptible to dysfunction. Importantly, these two mitochondrial populations show different characteristics both *in vitro*[66,69] and *in vivo*,[10,70] including differential responses to pharmacotherapy.[70]

The heterogeneity of the two populations, however, can be masked in total mitochondrial (synaptic and non-synaptic) preparations, especially because of the high glia to neuron ratio of the cerebral cortex.[71] Therefore, it is essential that mitochondria targeted pharmacotherapies, such as CsA, be evaluated in both populations.

This is the first study to examine the effects of CsA on isolated synaptic and non-synaptic mitochondria after experimental TBI. We hypothesized that synaptic mitochondria would sustain more damage than non-synaptic mitochondria 24 h after severe controlled cortical impact (CCI) injury, and that intraperitoneal administration of CsA (20 mg/kg) 15 min after injury would differentially attenuate injury-induced synaptic and non-synaptic respiratory impairment.

Methods

Animals

Young adult male Sprague-Dawley rats ($n=20$, Harlan, Indianapolis, IN) weighing 300 to 350 g were used for all studies. Animals were allowed food and water *ad libitum* and were housed in the Division of Laboratory Animal Resources of the University of Kentucky Medical Center. All animal and husbandry were conducted in accordance with the University of Kentucky Institutional Animal Care and Use Committee. Animals were randomly assigned to experimental groups: sham ($n=6$), CCI + vehicle ($n=6$), CCI + CsA ($n=8$).

CCI TBI

Animals were initially anesthetized with 4% isoflurane and placed in a stereotaxic frame (David Kopf, Tujunga, CA), where they were maintained at 3% isoflurane for the duration of the procedure. A midline incision was made to expose the skull, and a 6 mm craniotomy was made lateral to the sagittal suture midway between lambda and bregma. The exposed brain with intact dura was injured using a computer controlled pneumatic impactor (TBI 03010; Precision Systems and Instrumentation, Fairfax Station, VA) fitted with a 5 mm beveled tip set to impact at $\sim$3.5 m/sec, 2.2 mm depth, and 500 msec dwell time, as described previously.[19] After injury, Surgicel was placed onto the dura, and an 8 mm plastic disk was affixed with tissue adhesive to close the craniotomy site. Body temperature was monitored and maintained at 37°C with a thermo-regulating heating pad. Sham animals underwent all procedures but did not receive an impact injury.

CsA administration

The CsA concentration chosen was based on previously optimized concentrations for CCI.[33,72] The CCI + CsA group was administered CsA obtained from the University of Kentucky Medical Center Hospital Pharmacy (Perrigo; Minneapolis, MN; 50 mg/mL) 15 min after injury as a single intraperitoneal dose of 20 mg/kg in saline/650 mg cremophor/33.2% (v/v) ethanol diluted in saline to a final concentration of 10 mg/mL. The injection volume was 0.2 mL/100 g of body weight. CCI + vehicle-treated animals received an equivalent volume of saline/cremophor/ethanol 15 min after injury.

Tissue extraction

Animals were euthanized at 24 h using CO_2 anesthetization followed by decapitation, and an 8 mm cortical punch centered over the injury site was collected for analysis of mitochondrial respiration.

Mitochondrial isolation

Mitochondria were isolated as described previously[7,19] with modifications to isolate synaptic and non-synaptic populations.[10,70] Cortical tissue was homogenized in ice-cold isolation buffer (215 mmol/L mannitol, 75 mmol/L sucrose, 0.1% bovine serum albumin, 20 mmol/L HEPES, 1 mmol/L EGTA, pH 7.2) using Potter-Elvejhem homogenizers. Samples were then centrifuged twice at 1400 × g for 3 min at 4°C. Supernatants were collected and spun at 13,000 × g for 10 min at 4°C.

The crude mitochondrial pellet was resuspended and layered onto a discontinuous 7.5% and 10% Ficoll gradient and centrifuged at 100,000 × g for 30 min at 4°C. The non-synaptic mitochondria pellet was resuspended in isolation buffer without EGTA and centrifuged at 10,000 × g for 10 min at 4°C to remove Ficoll and then resuspended to a final concentration of approximately 10 mg/mL in isolation buffer without EGTA.

The synaptosomal layer was removed from the 7.5–10% Ficoll interface, resuspended in isolation buffer and spun at 13,000 × g for 10 min at 4°C to remove Ficoll. The synaptosome pellet was resuspended in isolation buffer, placed into a nitrogen bomb at 1200 psi for 10 min at 4°C to release synaptic mitochondria,[73,74] layered onto a second discontinuous 7.5% and 10% Ficoll gradient, and centrifuged at 100,000 × g for 30 min at 4°C. The synaptic mitochondria pellet was resuspended in isolation buffer without EGTA and centrifuged at 10,000 × g for 10 min at 4°C to remove Ficoll and resuspended in isolation buffer without EGTA. Protein

concentrations were determined with a BCA protein assay kit and measured at absorbance 562 nm with a BioTek Synergy HT plate reader (Winooski, VT). Mitochondria were immediately used for respiratory analysis.

Measurement of mitochondrial respiratory function

Mitochondria respiratory rates were measured using a Clark-type electrode in a continuously stirred, sealed, thermostatically controlled chamber (Oxytherm System, Hansatech Instruments, Norfolk, UK) that was maintained at 37°C. Mitochondria (>30 μg) were placed into a chamber containing 250 μL of KCl respiration buffer (125 mmol/L, 2 mmol/L $MgCl_2$, 2.5 mmol/L KH_2PO_4, 0.1% bovine serum albumin, 20 mmol/L HEPES, pH 7.2). Mitochondria equilibrated for 1 min before complex-I initiation.

Complex-I respiration was initiated with 5 mmol/L pyruvate and 2.5 mmol/L malate, and state II respiration was monitored. Two boluses of 150 μmol/L adenosine diphosphate (ADP) were added to initiate state III respiration. State IV respiration was monitored after 2 μmol/L addition of the ATP synthase inhibitor oligomycin. Maximal state V(I) respiration was initiated by addition of 2 μmol/L of the protonophore FCCP. Complex I was inhibited by addition of 100 nmol/L rotenone. Complex II driven respiration was initiated by addition of 10 mmol/L succinate, and state V(II) was monitored. Respiratory control ratio (RCR) was calculated by dividing the state III respiration rate (second bolus ADP rate) by the state IV respiration rate.[7,50]

Statistical analysis

Statistical analysis was conducted using Prism version 6.0 (Graph Pad, San Diego, CA). Results are reported as mean ± standard deviation. Initial statistical analysis was performed using a two-way analysis of variance (ANOVA), followed by a Tukey *post hoc* analysis when appropriate.

Results

The following details the comparative effects of CCI TBI, with and without early CsA treatment, on the respiratory functional status of non-synaptic versus synaptic mitochondria as measured by changes in oxygen utilization during the various respiratory states. Overall, the results show that, in general, synaptic mitochondria are more susceptible to post-traumatic complex I and II-driven dysfunction within the electron transport chain than non-synaptic mitochondria. Nevertheless, early CsA treatment is able to protect respiration that is linked to neuronal ATP production within the more damaged synaptic mitochondria.

State II: Addition of pyruvate + malate to activate mitochondrial complex I

A two-way ANOVA (injury × population) revealed a significant main effect for injury ($F[1, 20] = 23.75$, $p < 0.0001$) and population ($F[1, 20] = 17.22$, $p = 0.0005$), but not interaction ($F[1, 20] = 0.2018$, $p = 0.6581$). *Post hoc* testing (Tukey) revealed that state II respiration for non-synaptic CCI + vehicle was significantly impaired compared with non-synaptic sham ($p < 0.01$), synaptic CCI + vehicle was significantly impaired compared with synaptic sham ($p < 0.05$), and that the state II respiration rate for synaptic sham was significantly decreased compared with non-synaptic sham ($p < 0.05$) (Fig. 1A).

To assess drug effect, state II respiratory rates for CCI + vehicle and CCI + CsA were calculated as a percentage of the sham respiratory rate. A two-way ANOVA (treatment × population) revealed a significant main effect for treatment ($F[1, 24] = 11.07$, $p = 0.0028$), but not for population ($F[1, 24] = 0.06332$, $p = 0.8035$) or interaction ($F[1, 24] = 3.182$, $p = 0.0871$). While CsA improved state II respiration in both injured non-synaptic and injured synaptic mitochondria, *post hoc* testing (Tukey) revealed that this effect was only significant in the synaptic population ($p < 0.01$) (Fig. 1B).

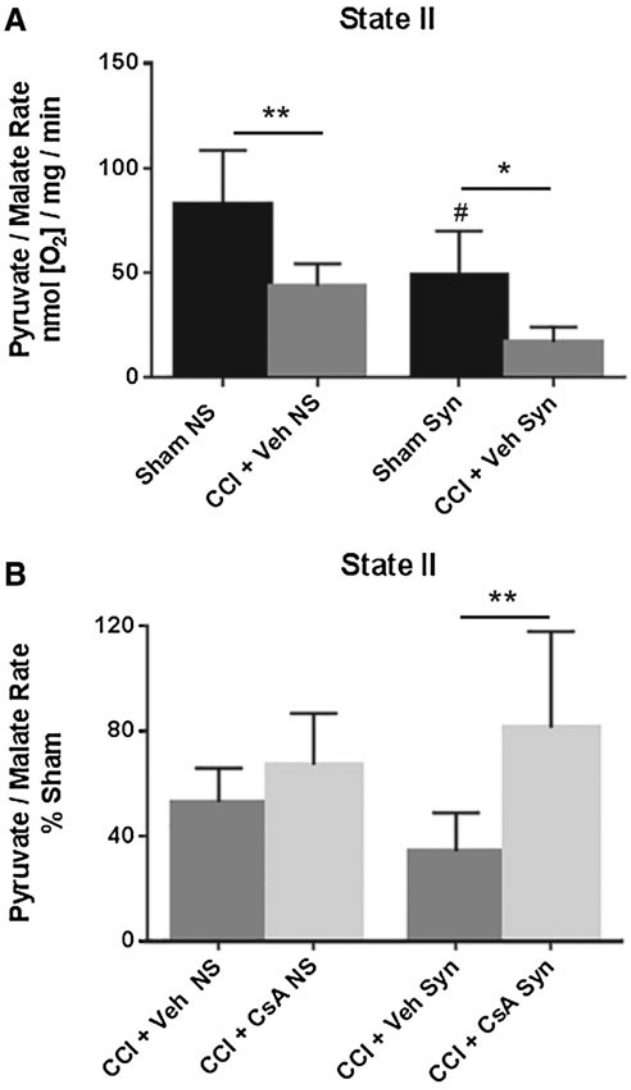

FIG. 1. (**A**) Effect of injury on non-synaptic and synaptic mitochondria for state II respiration (pyruvate/malate) 24 h after severe controlled cortical impact (CCI). (**B**) Effect of early post-injury (15 min) intraperitoneal administration of cyclosporine A (CsA) (20 mg/kg) on non-synaptic and synaptic mitochondria for state II respiration (pyruvate/malate) 24 h after severe CCI, calculated as % sham. Sham NS, sham non-synaptic ($n = 6$), Sham Syn, sham synaptic ($n = 6$), CCI + Veh NS, CCI + vehicle non-synaptic ($n = 6$), CCI + Veh Syn, CCI + vehicle synaptic ($n = 6$), CCI + CsA NS, CCI + CsA non-synaptic ($n = 8$), CCI + CsA Syn, CCI + CsA synaptic ($n = 8$); values, mean ± standard deviation; two-way analysis of variance followed by Tukey *post hoc*; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\#}p < 0.05$ compared with non-synaptic sham.

State III: Addition of ADP to activate complex V ATP production

A two-way ANOVA (injury × population) revealed a significant main effect for injury ($F[1, 20] = 31.10$, $p < 0.0001$) and population ($F[1, 20] = 12.39$, $p = 0.0022$), but not interaction ($F[1, 20] = .0075$,

$p = 0.9318$). *Post hoc* testing (Tukey) revealed that state III respiration for non-synaptic CCI + vehicle was significantly impaired compared with non-synaptic sham ($p < 0.01$), and synaptic CCI + vehicle was significantly impaired compared with synaptic sham ($p < 0.01$) (Fig. 2A).

To assess drug effect, state III respiratory rates for CCI + vehicle and CCI + CsA were calculated as a percentage of the sham respiratory rate. A two-way ANOVA revealed a significant main effect for treatment (F [1, 24] = 10.45, $p = 0.0036$), and population (F [1, 24] = 4.847, $p = 0.0375$), but not interaction (F [1, 24] = 0.5409, $p = 0.4692$). While CsA improved state III respiration in both injured non-synaptic and injured synaptic mitochondria, *post hoc* testing (Tukey) revealed this effect was only significant in the synaptic population ($p < 0.05$) (Fig. 2B).

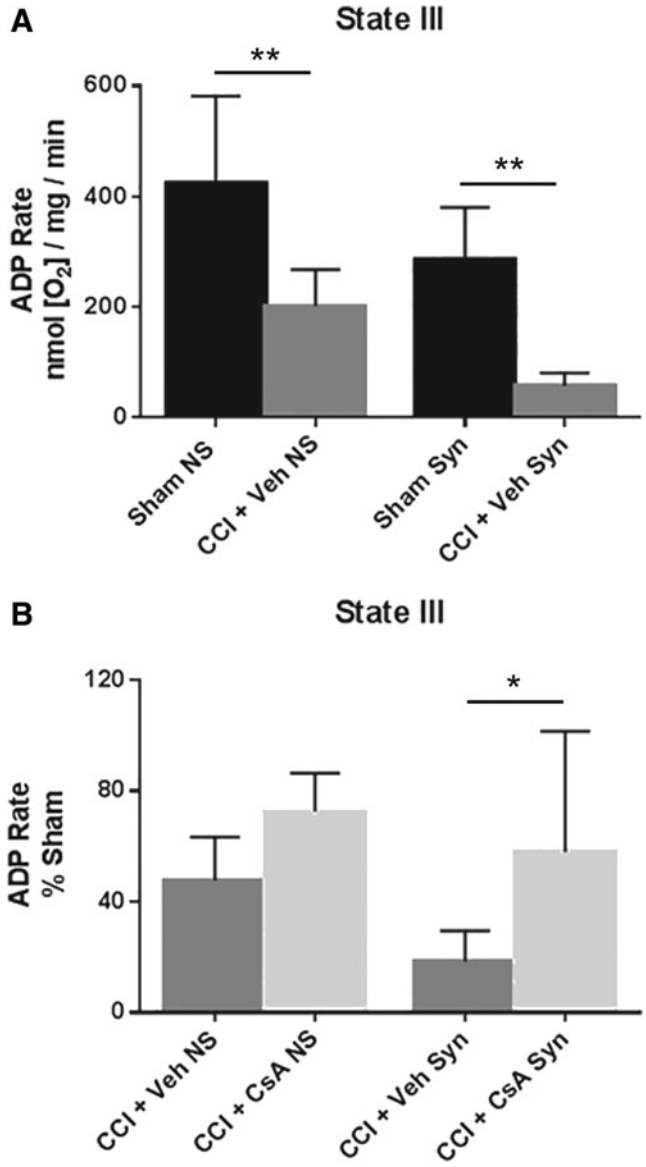

FIG. 2. (**A**) Effect of injury on non-synaptic and synaptic mitochondria for state III respiration (pyruvate/malate/adenosine diphosphate [ADP]) 24 h after severe controlled cortical impact (CCI). (**B**) Effect of early post-injury (15 min) intraperitoneal administration of cyclosporine A (CsA) (20 mg/kg) on non-synaptic and synaptic mitochondria for state III respiration (pyruvate/malate/ADP) 24 h after severe CCI, calculated as % sham. Sham NS, sham non-synaptic ($n = 6$), Sham Syn, sham synaptic ($n = 6$); CCI + Veh NS, CCI + vehicle non-synaptic ($n = 6$); CCI + Veh Syn, CCI + vehicle synaptic ($n = 6$); CCI + CsA NS, CCI + CsA non-synaptic ($n = 8$); CCI + CsA Syn, CCI + CsA synaptic ($n = 8$); values = mean ± standard deviation; two-way analysis of variance followed by Tukey *post hoc*; *$p < 0.05$, **$p < 0.01$.

State IV: Addition of oligomycin to inhibit complex V ATP production

A two-way ANOVA (injury × population) revealed a significant main effect for injury (F [1, 20] = 27.77, p < 0.0001) and population (F [1, 20] = 8.680, $p = .0080$), but not interaction (F [1, 20] = .0276, $p = 0.8698$). *Post hoc* testing (Tukey) revealed that state IV respiration for non-synaptic CCI + vehicle was significantly impaired compared with non-synaptic sham ($p < 0.01$), and synaptic CCI + vehicle was significantly impaired compared with synaptic sham ($p < 0.01$) (Fig. 3A).

To assess drug effect, state IV respiratory rates for CCI + vehicle and CCI + CsA were calculated as a percentage of the sham respiratory rate. A two-way ANOVA revealed a significant main effect for treatment (F [1, 24] = 11.91, $p = 0.0021$), but not population (F [1, 24] = 1.964, $p = 0.1738$) or interaction (F [1, 24] = 0.0046, $p = 0.9465$). While CsA improved state IV respiration in both injured non-synaptic and injured synaptic mitochondria, *post hoc* testing (Tukey) revealed these effects were not significant (Fig. 3B).

RCR (state III/state IV): Difference in oxygen utilization between activation and inhibition of ATP production

A two-way ANOVA (injury × population) revealed a significant main effect for injury (F [1, 20] = 26.02, $p < 0.0001$), population (F [1, 20] = 12.23, $p = 0.0023$), and interaction (F [1, 20] = 5.418, $p = 0.0305$). *Post hoc* testing (Tukey) revealed that RCR for synaptic CCI + vehicle was significantly impaired compared with synaptic sham ($p < 0.001$) and that synaptic CCI + vehicle was significantly impaired compared with non-synaptic CCI + vehicle ($p < 0.01$) (Fig. 4A]. To assess drug effect, RCR for CCI + vehicle and CCI + CsA was calculated as a percentage of sham RCR. A two-way ANOVA revealed a significant main effect for treatment (F [1, 24) = 7.092, $p = 0.0136$) and population (F [1, 24] = 9.680, $p = 0.0048$), but not interaction (F [1, 24] = 3.437, $p = 0.0761$). *Post hoc* testing (Tukey) revealed that CsA treatment significantly improved RCR in the injured synaptic population ($p < 0.05$) and that the RCR for synaptic CCI + vehicle is significantly impaired compared with the RCR for non-synaptic CCI + vehicle ($p < 0.05$) (Fig. 4B).

State V(I): Maximal complex I-driven respiration after addition of the protonophore FCCP

A two-way ANOVA (injury × population) revealed a significant main effect for injury (F [1, 20] = 37.82, $p < 0.0001$) and population (F [1, 20] = 9.556, $p = 0.0058$), but not interaction (F [1, 20] = 0.2492, $p = 0.6231$]. *Post hoc* testing (Tukey) revealed that state V(I) respiration for non-synaptic CCI + vehicle was significantly impaired compared with non-synaptic sham ($p < 0.001$), and synaptic CCI + vehicle was significantly impaired compared with synaptic sham ($p < 0.01$) (Fig. 5A).

To assess drug effect, state V(I) respiratory rates for CCI + vehicle and CCI + CsA were calculated as a percentage of the sham respiratory rate. A two-way ANOVA revealed a significant main effect for treatment (F [1, 24] = 14.25, $p = 0.0009$), but not population (F [1, 24] = 3.580, $p = 0.0706$) or interaction (F [1, 24] = 0.0084,

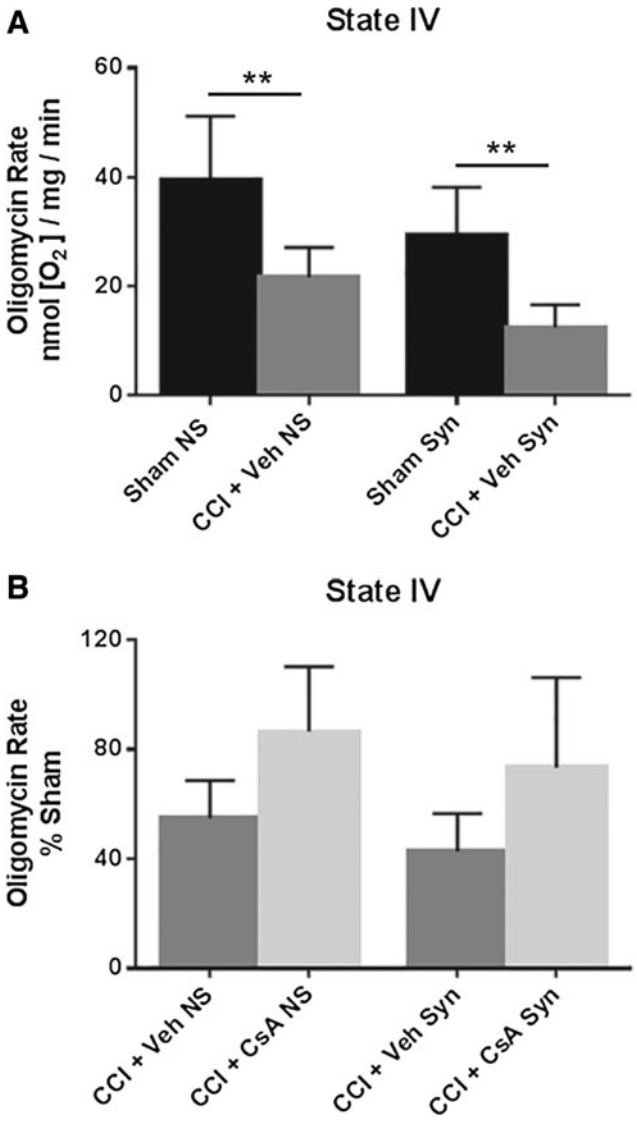

FIG. 3. (**A**) Effect of injury on non-synaptic and synaptic mitochondria for state IV respiration (oligomycin) 24 h after severe controlled cortical impact (CCI). (**B**) Effect of early post-injury (15 min) intraperitoneal administration of cyclosporine A (CsA) (20 mg/kg), on non-synaptic and synaptic mitochondria for state IV respiration (oligomycin) 24 h after severe CCI, calculated as % sham. Sham NS, sham non-synaptic ($n=6$); Sham Syn, sham synaptic ($n=6$); CCI + Veh NS, CCI + vehicle non-synaptic ($n=6$); CCI + Veh Syn, CCI + vehicle synaptic ($n=6$); CCI + CsA NS, CCI + CsA non-synaptic ($n=8$); CCI + CsA Syn, CCI + CsA synaptic ($n=8$); values = mean ± standard deviation; two-way analysis of variance followed by Tukey *post hoc*; **p < 0.01.

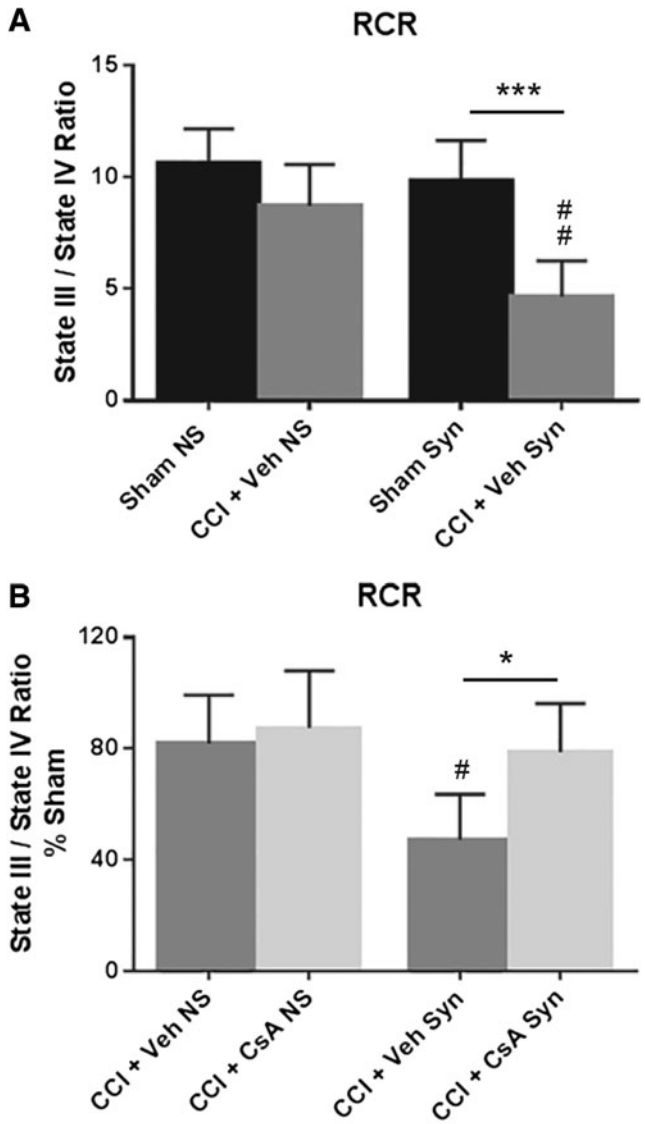

FIG. 4. (**A**) Effect of injury on non-synaptic and synaptic mitochondria for respiratory control ratio (RCR) (state III/state IV) 24 h after severe controlled cortical impact (CCI). (**B**) Effect of early post-injury (15 min) intraperitoneal administration of cyclosporine A (CsA) (20 mg/kg), on non-synaptic and synaptic mitochondria for RCR (state III/state IV) 24 h after severe CCI, calculated as % sham. Sham NS, sham non-synaptic ($n=6$); Sham Syn, sham synaptic ($n=6$); CCI + Veh NS, CCI + vehicle non-synaptic ($n=6$); CCI + Veh Syn, CCI + vehicle synaptic ($n=6$); CCI + CsA NS, CCI + CsA non-synaptic ($n=8$); CCI + CsA Syn, CCI + CsA synaptic ($n=8$); values = mean ± standard deviation; two-way analysis of variance followed by Tukey *post hoc*; *$p<0.05$, **$p<0.001$, $^{\#}p<0.05$ vs non-synaptic vehicle, $^{\#\#}p<0.01$ vs. non-synaptic vehicle.

$p=0.9276$). While CsA improved state V(I) respiration in both injured non-synaptic and injured synaptic mitochondria, *post hoc* testing (Tukey) revealed neither effect was statistically significant (Fig. 5B).

State V(II): Succinate-sctivated complex II-driven respiration after inhibition of complex I with rotenone

A two-way ANOVA (injury × population) revealed a significant main effect for injury ($F\ [1, 20]=23.97$, $p<0.0001$) and population ($F\ [1, 20]=15.37=0.0008$), but not for interaction ($F\ [1, 20]=0.3787$, $p=0.5452$). *Post hoc* testing (Tukey) revealed that state V(II) respiration for non-synaptic CCI + vehicle was significantly impaired compared with non-synaptic sham ($p<0.01$), synaptic CCI + vehicle was significantly impaired compared with synaptic sham ($p<0.05$), and that the state V(II) respiration rate for synaptic sham was significantly decreased compared with non-synaptic sham ($p<0.05$) (Fig. 6A).

To assess drug effect, state V(II) respiratory rates for CCI + vehicle and CCI + CsA were calculated as a percentage of the sham

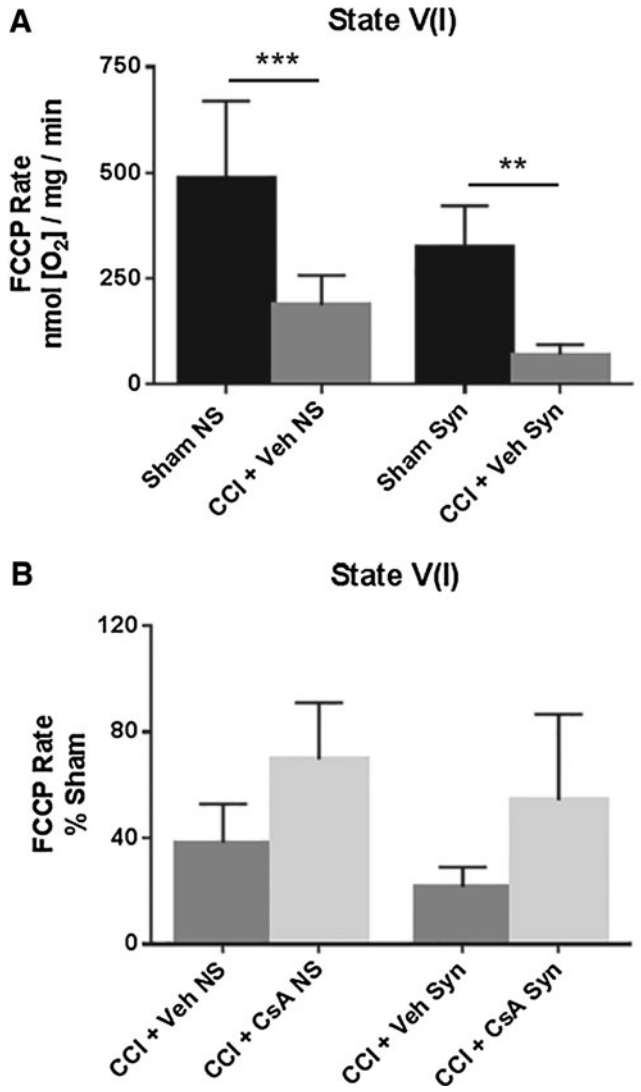

FIG. 5. (**A**) Effect of injury on non-synaptic and synaptic mitochondria for state V(I) respiration (FCCP) 24 h after severe controlled cortical impact (CCI). (**B**) Effect of early post-injury (15 min) intraperitoneal administration of cyclosporine A (CsA) (20 mg/kg) on non-synaptic and synaptic mitochondria for state V(I) respiration (FCCP) 24 h after severe CCI, calculated as % sham. Sham NS, sham non-synaptic ($n=6$); Sham Syn, sham synaptic ($n=6$); CCI + Veh NS, CCI + vehicle non-synaptic ($n=6$); CCI + Veh Syn, CCI + vehicle synaptic ($n=6$); CCI + CsA NS, CCI + CsA non-synaptic ($n=8$); CCI + CsA Syn, CCI + CsA synaptic ($n=8$); values = mean ± standard deviation; two-way analysis of variance followed by Tukey *post hoc*; $^{**}p<0.01$, $^{***}p<0.001$.

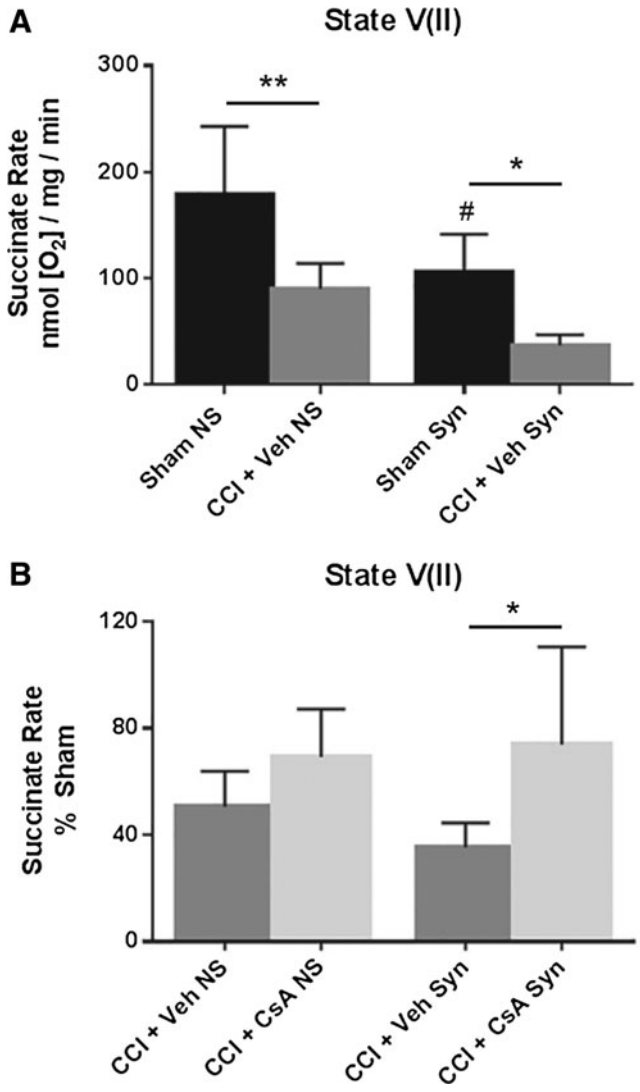

FIG. 6. (**A**) Effect of injury on non-synaptic and synaptic mitochondria for state V(II) respiration (rotenone/succinate) 24 h after severe controlled cortical impact (CCI). (**B**) Effect of early post-injury (15 min) intraperitoneal administration of CsA (20 mg/kg) on non-synaptic and synaptic mitochondria for state V(II) respiration (rotenone/succinate) 24 h after severe CCI, calculated as % sham. Sham NS, sham non-synaptic ($n=6$); Sham Syn, sham synaptic ($n=6$); CCI + Veh NS, CCI + vehicle non-synaptic ($n=6$); CCI + Veh Syn, CCI + vehicle synaptic ($n=6$); CCI + CsA NS, CCI + CsA non-synaptic ($n=8$); CCI + CsA Syn, CCI + CsA synaptic ($n=8$); values = mean ± standard deviation; two-way analysis of variance followed by Tukey *post hoc*; $^{*}p<0.05$, $^{**}p<0.01$, #p < 0.05 vs. non-synaptic sham.

respiratory rate. A two-way ANOVA revealed a significant main effect for treatment ($F\ [1, 24]=10.45$, $p=0.0035$), but not for population ($F\ [1, 24]=0.3533$, $p=0.5578$) or interaction ($F\ [1, 24]=1.272$, $p=0.2706$). While CsA improved state V(II) respiration in both injured non-synaptic and injured synaptic mitochondria, *post hoc* testing (Tukey) revealed this effect was only significant in the synaptic population ($p<0.05$) (Fig. 6B).

Discussion

These results indicate that synaptic mitochondria sustain more damage than non-synaptic mitochondria 24 h after severe CCI and that intraperitoneal administration of CsA (20 mg/kg) 15 min after injury improves synaptic and non-synaptic respiration (Fig. 7), with a significant improvement being seen in the more severely impaired synaptic population.

CsA is an FDA-approved immunosuppressant, used clinically to prevent organ rejection after transplant through a mechanism that involves inhibition of calcineurin and T-cell activation.[75] CsA, however, also has the ability to bind the mitochondrial matrix protein cyclophilin D,[76,77] which inhibits its interaction with the adenine nucleotide transporter, an inner mitochondrial membrane

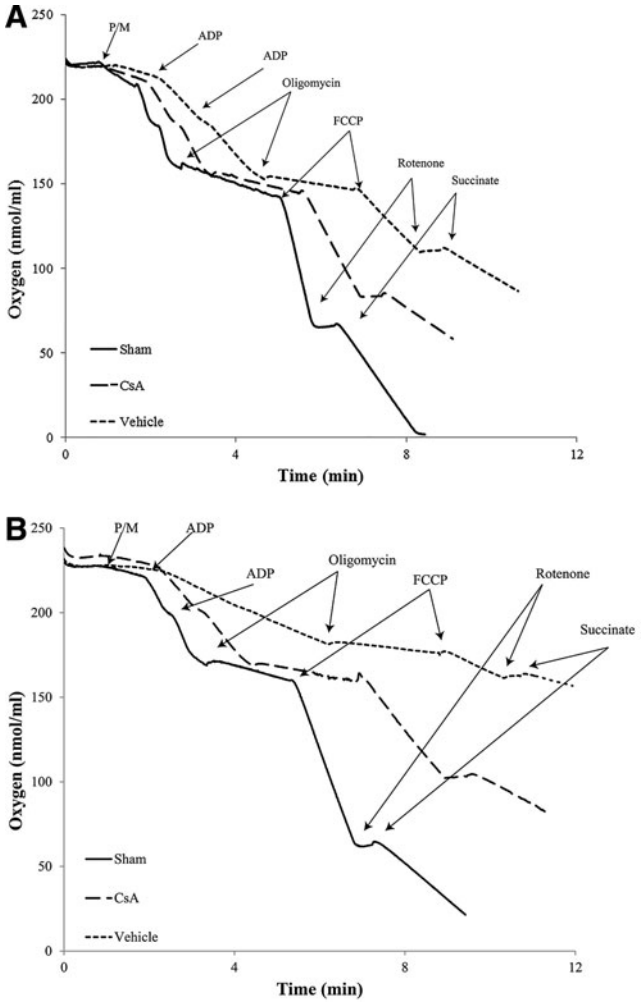

FIG. 7. Representative oxymetric traces indicating rates of oxygen consumption of sham, vehicle, and cyclosporine A (CsA) (20 mg/kg, intraperitoneally, 15 min post-injury) for (**A**) non-synaptic and (**B**) synaptic mitochondria isolated from ipsilateral cortex 24 h after severe controlled cortical impact (CCI). Purified mitochondrial protein (>30 μg) was suspended in respiration buffer (125 mmol/L KCl, 2 mmol/L $MgCl_2$, 2.5 mmol/L KH_2PO_4, 0.1% BSA, 20 mmol/L HEPES, pH 7.2) in a final volume of 250 μl, and oxygen consumption rates were measured using a Clark-type oxygen electrode in the presence of 5 mmol/L pyruvate and 2.5 mmol/L malate (state II), two boluses of 150 μmol/L ATP (state III), 2 μmol/L oligomycin (state IV), 2 μmol/L FCCP (state VI), and 100 nmol/L rotenone and 10 mmol/L succinate (state VII). Sham, sham; CsA, CCI + CsA; vehicle, CCI + vehicle; ADP, adenosine diphosphate.

protein, thus preventing formation of mPTP.[78] It is through inhibition of mPTP that CsA exerts its neuroprotective effects after TBI.

NIM811, a non-immunosuppressive analog of CsA unable to bind calcinuerin, but capable of binding cyclophilin D and inhibiting mPTP,[79] maintains the neuroprotective effects of CsA, improving mitochondrial respiration, decreasing oxidative damage, decreasing calpain-mediated cytoskeletal degradation and neurodegeneration, and improving motor and cognitive function after experimental TBI.[47,48,55]

Under normal physiologic conditions CsA is minimally blood–brain barrier penetrable.[80] After TBI, however, CsA is able to enter the CNS because of blood–brain barrier breakdown,[81] and although concerns have been raised regarding CsA and neurotoxicity,[82–89] two phase II clinical trials show CsA to be safe for use in patients with severe TBI.[90,91]

Despite the fact that the neuroprotective effects of CsA rely on direct protection of mitochondria,[28,47,48,58] many experimental TBI studies using CsA have focused on outcome measures downstream of mitochondrial dysfunction, such as cortical lesion volume,[56–58,72] axonal pathology,[20,21,48,92,93] behavior,[48,59] and synaptic plasticity.[94] Although important work does confirm the ability of CsA to protect mitochondria after injury, those studies focused on acute injury,[33] microscopic structure analysis,[21] or more indirect measures of mitochondrial function such as brain oxygen consumption[59] and whole brain ATP levels.[95] Only two studies have assessed mitochondrial bioenergetics via oxygen consumption rates, one of which did not extend beyond 12 h and was conducted in mice,[47] and one of which used juvenile animals.[61] Importantly, none of these mitochondria-focused studies evaluated isolated synaptic and non-synaptic populations.

While it should be noted that Sullivan and coworkers[33] showed that CsA maintains mitochondrial membrane potential in isolated non-synaptic mitochondria and intact synaptosomes acutely (30–60 min) after CCI, this is the first study to examine the effects of CsA on isolated and purified synaptic and non-synaptic mitochondria after experimental TBI. As such, CsA was administered 15 min post-injury. Early administration allows for the greatest chance of neuroprotection and allows for contextualization of future therapeutic window studies. The 20 mg/kg intraperitoneal dosage used is considered optimal and has been used extensively in previous experimental TBI studies.[33,47,48,57,58,61,72,93,94]

The CsA used in this study is solubilized in a vehicle containing cremophor. Toxicity concerns have been raised in regard to cremophor, and reported side effects include hypersensitivity reactions, hyperlipidemia, erythrocyte aggregation, and peripheral neuropathy, but are usually associated with intravenous infusions or high cremophor concentrations.[96] Several studies have shown that cremophor has the ability to impair mitochondrial function. These studies, however, were performed in mitochondria isolated from heart muscle, skeletal muscle, and kidney tissue, and cremophor was either administered chronically or *in vitro*.[97–99]

Interestingly, chronic administration of cremophor or CsA + cremophor impairs heart and skeletal muscle mitochondrial similarly, suggesting that any cremophor toxicity is still observable when administered along with CsA. The safety of low doses of CsA + cremophor, such as the dose used in this study (20 mg/kg intraperitoneally), is well supported in the literature, however. For example, administration of CsA + cremophor to sham animals has no effect on long-term potentiation,[94] motor or cognitive function,[60] or brain metabolism,[95] although chronic administration can result in weight loss.[60]

Although it is feasible that administration of vehicle or CsA to sham animals could alter basal mitochondrial respiration, evaluating the protective effect of CsA after injury in comparison with sham animals not receiving vehicle or drug is the most clinically appropriate assessment. Therefore, neither vehicle nor CsA was administered to sham animals for this study.

The respiratory function of isolated synaptic and non-synaptic mitochondria was assessed 24 h after injury. Total (synaptic and non-synaptic) mitochondrial respiratory dysfunction peaks between 12 and 24 h after severe CCI in the rat, [8] with several studies confirming mitochondrial respiratory dysfunction 24 h after injury.[8,50,100]

In sham animals, synaptic mitochondria showed decreased respiratory rates compared with non-synaptic mitochondria, decreases

that were only significant in state II and state V(II). Previous studies have also shown that healthy synaptic mitochondria have decreased respiration rates compared with healthy non-synaptic mitochondria,[10,101] as well as decreased pyruvate dehydrogenase activity,[102] the enzyme responsible for generating the nicotinamide adenine dinucleotide that feeds into complex I of the electron transport chain. Because non-synaptic mitochondria contain mitochondria from both neurons and glia, it is possible that the higher respiration rates found in non-synaptic mitochondria are because of astrocytic mitochondria; mitochondria isolated from cultured astrocytes have higher respiration rates than mitochondria isolated from cultured neurons as well as higher levels of electron transport chain subunits.[74]

RCR (state III/state IV) is considered one of the best general measures of mitochondrial function and health and represents the ability of mitochondria to couple oxidation of substrates with generation of ATP via ADP phosphorylation under minimal proton leak. Therefore, healthy mitochondria will have a high RCR, while damaged mitochondria will have a low RCR.[103] In this study, the RCR for synaptic mitochondria was significantly reduced 24 h after injury compared with sham, while the RCR for non-synaptic mitochondria was not. The RCR for injured synaptic mitochondria was also significantly reduced compared with the RCR for injured non-synaptic mitochondria. Interestingly, statistical analysis revealed a significant interaction between injury and population for this measure, indicating that synaptic mitochondria are indeed more susceptible to injury.

Importantly, the decrease in synaptic RCR was significantly attenuated by CsA administration. While RCR represents an overall measure of mitochondrial function, its value is affected by multiple aspects of oxidative phosphorylation.[103] Therefore, in addition to RCR we assessed individual states of respiration to identify specific aspects of bioenergetic impairment.

State II respiration is measured after addition of the complex-I substrates pyruvate and malate, but before ADP addition, and represents a slow state of respiration. Severe CCI significantly reduced state II respiration for both synaptic and non-synaptic mitochondria 24 h after injury. Decreases in state II respiration are consistent with the fact that pyruvate dehydrogenase, the enzyme linking glycolysis with the citric acid cycle, is known to have decreased activity after TBI.[104,105] While CsA was able to improve injury induced decreases in state II respiration in both populations, the improvements only reached statistical significance in the synaptic population.

State III respiration is measured after addition of ADP, allowing coupling of oxidative phosphorylation, and is considered one of the most important states of respiration to measure after injury, because decreases in state III respiration are indicative of defects in complex I-driven substrate oxidation and/or ATP turnover.[103] Severe CCI significantly reduced state III respiration for both synaptic and non-synaptic mitochondria 24 h after injury. While CsA was able to improve injury-induced decreases in state III respiration in both populations, the improvements only reached statistical significance in the synaptic population. Importantly, the ability of CsA to significantly improve state III synaptic respiration is a major contributory factor to the ability of CsA to also significantly improve synaptic RCR (state III/IV) after injury.

State IV is measured after addition of the ATP synthase inhibitor oligomycin, which returns the mitochondria to a basal state of respiration. Severe CCI significantly reduced state IV respiration for both synaptic and non-synaptic mitochondria 24 h after injury. While CsA was able to improve injury-induced decreases in state IV respiration in both populations, the improvements were not statistically significant.

State V(I) respiration is measured after addition of the protonophore FCCP, which uncouples substrate oxidation from ATP production, and is used to assess maximal respiration, with decreases in state V(I) respiration being indicative of defects in complex-I driven substrate oxidation.[103] Severe CCI significantly reduced state V(I) respiration for both synaptic and non-synaptic mitochondria 24 h after injury. While CsA was able to improve injury-induced decreases in state V(I) respiration in both populations, the improvements were not statistically significant.

State V(II) respiration is assessed after addition of the complex-I inhibitor rotenone and the complex-II substrate succinate. Therefore, decreases in state V(II) respiration are indicative of defects in complex-II driven respiration. Severe CCI significantly reduced state V(II) respiration for both synaptic and non-synaptic mitochondria 24 h after injury. While, CsA improved injury-induced decreases in complex-II driven respiration in both populations, the improvements only reached statistical significance in the synaptic population.

In summary, synaptic mitochondria sustain more damage 24 h after severe CCI than non-synaptic mitochondria, as best evidenced by the changes in RCR. This is in agreement with previous work indicating that synaptic mitochondria are more susceptible to dysfunction. For example, synaptic mitochondria are more susceptible to damage 3 h after moderate CCI compared with non-synaptic mitochondria, an effect that is compounded by aging.[10] In addition, *in vitro,* healthy synaptic mitochondria have a decreased ability to buffer calcium before undergoing permeability transition compared with non-synaptic mitochondria.[66]

Several hypotheses have been offered to explain the increased vulnerability of synaptic mitochondria compared with non-synaptic mitochondria, including high concentrations of cyclophilin D, leading to increased susceptibility toward calcium-induced permeability transition,[69] as well as increased exposure of synaptic mitochondria to oxidative damage.[66,69] Because non-synaptic mitochondria contain both neuronal and non-neuronal cell types, however, and mitochondria isolated from neurons and glia have differing properties,[69,74] part of the increased susceptibility of synaptic mitochondria to injury may be because synaptic mitochondria represent of a more purely neuronal population,[66,69,106] rather than their specific subcellular localization to the pre-synaptic terminal. It is likely, however, that both cell type and subcellular localization contribute to synaptic mitochondria vulnerability.

These results also indicate that intraperitoneal administration of CsA (20 mg/kg) 15 min after injury attenuates respiratory dysfunction in both populations 24 h after severe CCI, with a statistically significant improvement being seen in the synaptic population. Although, there was a significant decrease in each respiration state after injury for the non-synaptic mitochondria, the non-synaptic RCR was unaffected by injury, indicating no overall bioenergetics dysfunction.[103] It is therefore likely that the improvements CsA had on the non-synaptic respiration states were only non-significant because of a lack of robust injury effect in this population, and that CsA would offer significant improvements at time points later than 24 h when non-synaptic mitochondria are more significantly impaired.

The fact that CsA is able to significantly protect synaptic mitochondria after injury is impressive, because synaptic mitochondria do not always respond as favorably to pharmaceutical intervention as the non-synaptic population. For example, following spinal cord injury, the catalytic peroxynitrite inhibitor, tempol, is only effective in non-synaptic mitochondria, while the mitochondrial uncoupler 2–4 DNP works in both populations, but has a shorter therapeutic window in the synaptic mitochondria.[70]

Future studies will investigate whether there is, indeed, a difference in the therapeutic window for CsA between synaptic and non-synaptic mitochondria. If so, studies may explain the fact that while CsA is able to improve cortical tissue sparing when administration is delayed up to 8 h, it is significantly more protective when administered within 3 h of injury.[56] Further, the ability of CsA to protect synaptic neuronal mitochondrial respiration is made more impressive by the knowledge that there are higher concentrations of the CsA target protein cyclophilin D in synaptic mitochondria,[66] gamma-aminobutyric acid-ergic interneurons,[106] and cultured neurons when compared with cultured astrocytes.[69]

In fact, *in vitro* healthy synaptic mitochondria require higher concentrations of CsA to prevent calcium-induced permeability transition.[69] Future studies will therefore evaluate the ability of CsA to attenuate calcium-induced permeability transition in both populations after injury, as well as the ability of CsA to retain its synaptic neuroprotective effect at time points beyond 24 h.

Conclusion

This study confirms that synaptic mitochondria are more vulnerable than non-synaptic mitochondria after experimental TBI, and therefore emphasizes the need for further characterization of synaptic and non-synaptic mitochondria after experimental TBI, including the contribution each population makes to TBI pathology, as well as the response each population has to mitochondrial-directed pharmacotherapies.

While the pathology of TBI is complex and factors other than mitochondrial dysfunction contribute to downstream processes such as cytoskeletal degradation, neurodegeneration, and neurologic impairment, it is likely that successful protection of the more vulnerable synaptic population greatly contributes to inhibition of these downstream processes. Because this study confirms the ability of CsA to significantly improve synaptic respiration after injury, CsA remains a promising neuroprotective candidate for the treatment of those with TBI.

Acknowledgments

This work was supported by 5R01 NS083405, 5R01 NS084857, 5P30 NS051220, 1F30 NS096876, and funding from the Kentucky Spinal Cord & Head Injury Research Trust (KSCHIRT).

Author Disclosure Statement

No competing financial interests exist.

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