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Combination Drug Therapy of Pioglitazone and D-cycloserine Attenuates Chronic Orofacial Neuropathic Pain and Anxiety by Improving Mitochondrial Function Following Trigeminal Nerve Injury

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Abstract

Objectives—The study aim was to determine how peripheral trigeminal nerve injury affects mitochondrial respiration and to test efficacy of combined treatment with two FDA approved drugs with potential for improving mitochondrial bioenergetics, pain and anxiety related behaviors in a chronic orofacial neuropathic pain mouse model.

Methods—Efficacy of (R)-(+)-4-amino-3-isoxazolidinone (D-cycloserine, DCS), an NMDA antagonist/agonist, and Pioglitazone (PIO), a selective agonist of nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR γ) was investigate in the trigeminal inflammatory compression (TIC) neuropathic nerve injury mouse model. Combined low doses of these drugs (80 mg/kg DCS and 100 mg/kg PIO) were given as a single bolus or daily for 7 days post-TIC to test ability to attenuate neuropathic nociceptive and associated cognitive dependent anxiety behaviors. Additionally, beneficial effects of the DCS/PIO drug combination were explored *ex-vivo* in isolated cortex/brainstem mitochondria at 28 weeks post-TIC.

Results—The DCS/PIO combination not only attenuated orofacial neuropathic pain and anxiety related behaviors associated with trigeminal nerve injury, but it also improved mitochondrial bioenergetics.

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Discussion—The DCS/PIO combination uncoupled mitochondrial respiration in the TIC model to improve cortical mitochondrial dysfunction, as well as reduced nociceptive and anxiety behaviors present in mice with centralized chronic neuropathic nerve injury. Combining these drugs could be a beneficial treatment for patients suffering from depression, anxiety, or other psychological conditions due to their chronic pain status.

Keywords

Trigeminal Inflammatory Compression (TIC) Injury; Mice; 2,4-DNP; Light-Dark Box Place Preference Test; Mechanical Allodynia; Mitochondrial Electron Transport Chain; Mitochondrial Bioenergetics; Bioscience Seahorse XFe24 Flux Analyzer; Respiratory Control Ratio; Clinical Translational

Introduction

Chronic trigeminal neuropathic pain is an orofacial pain condition characterized by chronic aching and burning sensation caused by trigeminal nerve damage. This injury can be due to compression of the trigeminal nerve by an arteriole pulsation or can also be caused by a peripheral nerve injury initiated by facial blunt trauma, endodontics, oral surgery, infections/inflammation, or unknown causes^{1–2}. This type of injury and pain is very difficult to treat so new therapeutics are needed that target key players in the ensuing cellular stress that follows.

Studies have indicated that mitochondrial dysfunction is a major player not only in cell stress, but also in the etiology of inflammatory and chronic neuropathic pain that occurs after peripheral nerve injuries^{3–6}. A study has shown that mitochondrial dysfunction and oxidative stress occur within 24 hours of neuronal injury⁷. However, administering inhibitors to the specific complexes of the mitochondrial electron transport chain (mETC) has been shown to attenuate mechanical allodynia in animals with sciatic nerve injury⁵. Administration of a mild mitochondrial uncoupler such as 2, 4-dinitrophenol (2,4-DNP) protects neuronal components from mitochondrial dysfunction and reactive oxygen species (ROS) production after neuronal injuries in mice^{7,8}. Mild uncouplers decrease the proton concentration gradient in the intermembrane space to allow free flow of H⁺ ions across the mitochondrial membrane into the matrix without crossing through the ATP synthase. During cellular stress, mild mitochondrial uncouplers decrease production of mitochondrial ROS, calcium loading into mitochondria, and subsequent mitochondrial dysfunction⁹. While it has been shown that ROS are produced in the trigeminal nucleus after peripheral nerve injury and that mitochondria-mediated oxidative stress plays a major role in pain transmission¹⁰, further study of mitochondrial dysfunction after peripheral trigeminal nerve injury is warranted. The present study tests the efficacy of combined treatment with two FDA approved drugs with potential for improving mitochondrial bioenergetics, pain and anxiety related behaviors in a chronic orofacial neuropathic pain mouse model.

Pioglitazone (PIO), an FDA approved prescription drug (Actos) in the thiazolidinedione (TZD) class, has hypoglycemic (anti-hyperglycemic, anti-diabetic) action and is used to treat type 2 diabetes. Pioglitazone is a selective agonist of nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR γ). Studies have shown PIO also reduces hypersensitivity in the sciatic nerve injury animal model^{11–14}. Many have theorized that PIO

is acting through PPAR γ to decrease microglial activation and oxidative stress^{14–16}. Recent studies in our laboratory have shown that PPAR γ agonist, pioglitazone (PIO), attenuates mechanical allodynia in the whisker pads of mice after the Trigeminal Inflammatory Compression (TIC) neuropathic nerve injury primarily by a PPAR γ dependent pathway as provided in the companion paper¹⁷. While a single dose of 100 mg/kg of PIO (i.p.) was ineffective, a 300 mg/kg dose of PIO alleviated mechanical allodynia in mice with TIC injury. Our data also suggested that since the analgesic effect occurs within 2 hours, a PPAR γ non-genomic mechanism is a possibility. However, data has shown that PIO is also acting through a PPAR γ independent mechanism on the mitochondria by directly activating mitoNEET to decrease mitochondrial oxidative stress^{18,19}. In the present study, PIO's actions were examined *ex vivo* in isolated mitochondria after chronic peripheral trigeminal nerve injury in support of this mechanism.

The FDA approved drug, (R)-(+)-4-amino-3-isoxazolidinone (D-cycloserine) (DCS), known under the name Seromycin ® (DCS capsules, USP, 250 mg) is a broad spectrum antibiotic used alternatively for tuberculosis. DCS is a derivative of the naturally occurring amino acid serine and acts as a partial agonist at the strychnine insensitive glycine recognition site of the NMDA receptor complex^{20,21}. Binding of DCS to the NMDA receptor enhances glutamate activation and increases calcium influx, thus enhancing excitatory neurotransmission^{22,23}. However, DCS administered in higher doses has been shown to act as an NMDA antagonist to reduce hypersensitivity in sciatic nerve injury in rats²⁴. Clinical trials have shown DCS is effective in extinction of acquired fear when used as an adjuvant to exposure therapy for anxiety disorders (e.g. post-traumatic stress disorder, phobias, obsessive-compulsive disorder)^{25–27}. Only one clinical case study to date has observed anti-allodynic effects of DCS for alleviation of chronic facial pain²⁸. Although DCS may be a prospective new therapy for treatment of orofacial pain, it has not been thoroughly tested in animals nor has a mechanism of action been defined.

In this study, the TIC nerve injury mouse model was employed to investigate the effect of DCS, PIO, and a DCS/PIO low dose combination on the relief of neuropathic nociception and anxiety associated with trigeminal neuropathic pain. The effect of the DCS/PIO combination on isolated brainstem and cortical mitochondria after a peripheral trigeminal nerve injury was also explored.

Materials and Methods

Animals

All experimental procedures were done according to guidelines provided by the National Institute of Health (NIH) regarding the care and use of animals for experimental procedures. Animal protocols were approved by the University of Kentucky's Institutional Animal Care and Use Committee (IACUC). All animals were housed in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and the United States Department of Agriculture (USDA).

All experiments were performed with C57Bl/6 male, wild-type mice (25–35 g, Harlan Laboratories, Indianapolis, IN). Animals were randomly assigned to receive either surgical

procedures to induce the TIC nerve injury model, sham surgical procedures, or to remain in the naïve cohort. Mice were housed in a well-ventilated mouse housing room (21°C) on a reversed 10/14 h dark/light cycle so testing could be performed in their active period. All mice accessed food and water *ad libitum* throughout the studies. Low soy bean content diet normal chow was provided (Teklab 8626, Harlan, Indiana).

Trigeminal Inflammatory Compression (TIC) Injury

Mice were anesthetized with sodium pentobarbital (70 mg/kg, i.p.). The top of the head was shaved, ophthalmic cream applied to their eyes to prevent drying, and mice were placed in a stereotaxic frame. Under standard sterile surgery conditions, a small 15 mm incision was made along the midline of the head. The conjunctiva of the orbit was opened along the top inner corner of the left eye's bony socket with the scalpel tip. Small cotton balls assist blunt nerve isolation and bleeding control in the orbital cavity. The infraorbital nerve was located approximately 5 mm deep in the infraorbital fissure against the maxillary bony. Animals randomly assigned to receive the TIC surgery underwent surgical placement of a 2 mm length of chromic gut suture (6-0), inserted in the infraorbital fissure between the maxillary bone and the infraorbital nerve to restrict nerve movement. This results in mechanical hypersensitivity in the ipsilateral whisker pad due to combined mechanical and chemical inflammatory stimulation of the nerve through nerve compression by the suture and leaching of chromate salt^{29, 30}. Animals assigned to receive sham surgical procedures only received the skin incision without chromic gut suture placement. Naïve animals were also included as a comparison group. All mice were age matched.

Behavioral Assays

All behavioral tests were conducted during the animal's active cycle (zt12-24, i.e. dark phase of the dark/light cycle) during the hours of 8:00 am to 6:00 pm. During testing, either a red-light or a dim lamp illuminated the room. None of the behavioral tests were conducted on the same day. One experimenter was blinded to the drug treatments given for each experiment.

Detecting Mechanical Allodynia on the Whisker Pad with von Frey Fiber Test—

Mechanical threshold of the whisker pad area was tested before and after surgery with a modified up/down method³¹ using a graded series of von Frey fiber filaments (force:0.008 g (size:1.65); 0.02 g (2.36); 0.07 g (2.83); 0.16 g (3.22); 0.4 g (3.61); 1.0 g (4.08); 2.0 g (4.31); 6.0 g (4.74); Stoelting, Wood Dale, IL). One experimenter gently restrained the mouse in their palm (2–5 minutes) to acclimate the animal. A second experimenter then applied the von Frey filaments perpendicularly to the mouse's whisker pad as previously described²⁹. Bilateral mechanical head withdrawal thresholds were determined at baseline and weekly post-surgery. A cohort of naïve control mice were tested for comparisons to sham and TIC nerve injured animals.

After post-injury week 8, efficacy of experimental drugs to reduce mechanical hypersensitivity and chronic pain induced anxiety related behaviors was determined. This time point was chosen because anxiety- and depression-like behaviors can develop 6–8 weeks after injury³². For one-time drug treatments, mechanical threshold was measured at 0,

0.5, 1, 2, 3, and 4 hours post drug injection, with hours 5, 6, 7, and 8 post injection evaluated if drug effect persisted. For daily injections, mechanical thresholds were determined once daily at the same testing time each day. One experimenter was blinded to the drugs given at all times.

Two Compartment Light-Dark Box Place Preference Test—The light-dark box used to assess anxiety related behaviors consisted of two equally-sized chambers ($11 \times 19 \times 12$ cm each), one illuminated and one darkened. The 5×5 cm connecting doorway allowed mice to move freely between chambers³³. Immediately after exposure to a mild acoustic startle disturbance (2 min), mice were placed in the dark side of the box facing away from the light chamber. Animals remained in the light-dark preference box for a total of 10 minutes. Quantified behaviors were (1) time spent in the light chamber, (2) number of transitions between chambers, (3) number of rearing events, (4) entry latency into the light chamber, and (5) latency of first re-entry (transition) back into the dark chamber. The light-dark test was conducted in post-operative weeks 1, 4, and 8.

Mitochondrial Isolation Assays

Isolated mitochondrial assays were performed as previously described^{9,34–36}.

Cortex and Brainstem Mitochondria Sample Preparation—Mitochondrial bioenergetics were assessed at a chronic time point in week 28 post TIC injury. Mice were euthanized with CO₂ and rapidly decapitated. The excised brains were quickly placed on an ice cold dissecting plate (4°C) and mitochondria from cortex and brainstem regions isolated using a Ficoll based purification method as reported previously³⁶. The cerebral cortex and medullary trigeminal dorsal horn quadrant were homogenized in 2 ml of ice cold (4°C) mitochondrial isolation buffer (MIB) containing (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20mM HEPES, 1mM EGTA, pH 7.2)^{8,34}. Homogenates were centrifuged twice at $1300 \times G$ for 3 minutes at 4°C. The supernatants were removed and centrifuged at $13,000 \times G$ for 10 minutes at 4°C. The mitochondrial/synaptosomal pellets were burst in compressed nitrogen containing cell disruption chamber (1200 psi for 10 minutes at 4°C). Mitochondrial pellets were placed on top of a discontinuous Ficoll gradient (7.5% Ficoll layered over 10% Ficoll solution) and centrifuged at $100,000 \times G$ for 30 min at 4°C. The pellets were re-suspended in EGTA-free MIB and centrifuged at $10,000 \times G$ for 10 minutes at 4°C. The final pellet was re-suspended in EGTA-free MIB for a final concentration of 10 mg/ml. A BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) was used according to manufacturer's instructions to determine the protein concentrations by measuring absorbance at 562 nm wave length in a Biotek Synergy HT plate reader (BioTek, Winooski, VT, USA).

Bioscience Seahorse XF^e24 Flux Analyzer Assay—The Seahorse XF^e24 Flux Analyzer (Agilent Technologies, Santa Clara, CA, USA) was used to measure mitochondrial bioenergetics in isolated mitochondrial preparations as previously described^{34,35}. The stock mitochondrial substrates and inhibitors were prepared (500 mM pyruvate, 250 mM malate, 30 mM ADP, 1 mg/ml oligomycin-A, 1 mM FCCP, 1 mM rotenone, and 1 M succinate, pH 7.2, and stored at -20°C). The 24 well dual-analyte sensor cartridge was hydrated for 24 h

and kept overnight in a non-CO₂ incubator at 37°C. Sensor cartridge ports A to D were loaded with the appropriate mitochondrial substrates or inhibitors, and sequentially injected into the assay plate. All mitochondrial working stocks were prepared in mitochondrial respiration buffer (MRB) (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 2 mM MgCl₂, 2.5 mM KH₂PO₄, pH of 7.2) and stored at 4°C. The substrates/inhibitors were loaded for each port (500 µl RB volume) and placed into the Seahorse XF24 Flux Analyzer for automated calibration.

Brain mitochondrial samples (10 µg) of both TIC and naïve mice were analyzed on a single experimental plate. After being re-suspended in MRB, mitochondrial samples and MRB controls (50 µl each) were added into experimental wells. The XF24 plate was centrifuged for 4 minutes at 3,000 rpm at room temperature. To half of the mitochondrial samples from TIC and naïve mice, 450 µl of MRB (37°C) was gently added for a final volume of 500 µl per well. DCS (50 nM) and PIO (50 nM) in 450 µl MRB (37°C) were added to the other half of the mitochondrial samples. Mitochondrial respiration was assessed with the Seahorse XF²⁴ flux analyzer.

Appropriate substrates/inhibitors were added into the wells and oxygen consumption rate (OCR) recorded for each well³⁵. Substrates/inhibitors were added sequentially from port A to port D of the sensor cartridge into experimental wells: Port A- 50 µl (mixture of pyruvate, malate and ADP), Port- B 55 µl (Oligomycin A), Port C- 60 µl (FCCP), and Port D- 65 µl (rotenone and succinate). Pandya and colleagues have previously described the detailed method and interpretation of mitochondrial bioenergetics parameters for experimental procedure^{36,37}. The OCR rate was measured in the presence of substrates (5 mM pyruvate, 2.5 mM malate, 1 mM ADP) and reported as State III response (Port A). The State III response is a good indicator of mitochondrial health by providing a complex I driven ADP phosphorylation rate and ATP synthesis. The OCR rates in the presence of 1 µM oligomycin A addition indicate the State IV response (Port B). The oligomycin A addition inhibits complex V (ATP synthase) action thereby measuring proton/electron leak during the State IV condition. The OCR ratios of State III/State IV responses measure the Respiratory Control Ratio (RCR) which is used to determine how well coupled electron transport is for ATP production. A RCR greater than 5 is reported for healthy mitochondrial respiration, whereas uncoupled electron transport or mitochondrial dysfunction led to decreased RCR responses.

Drug Preparation

D-cycloserine (DCS), an NMDA agonist/antagonist, was soluble in saline (0.9% NaCl, 10 mg/ml). Pioglitazone (PIO), PPAR γ agonist, was mixed in saline, vortexed (30 sec), and dissolved in the sonicator (20 min). The 2,4-Dinitrophenol (2,4-DNP) was dissolved in 10% DMSO solution in saline and vortexed.

Drug Administration

The PIO and DNP were administered intraperitoneally (i.p.) at a volume of 10 ml/kg. For single and combined low dose DCS/PIO (80 mg/kg DCS and 100mg/kg PIO) treatments, DCS was administered subcutaneously (s.c.) at a volume of 5 ml/kg/site.

Drug Treatments

Drug treatments were done as four experiments in separate cohorts of animal. All groups received baseline behavioral testing to assess mechanical head withdrawal response thresholds before and after sham or TIC surgery (Fig. 1).

Initially, mice with TIC injury were given a DCS injection (s.c.) (40, 60, 80, 100, 160, and 320 mg/kg in 150 μ l/mouse) in ascending sequence with a one week interval between each treatment. These doses were chosen based on previous published studies^{26,28,38,39}. All drugs were administered at least 8 weeks after TIC injury surgery since anxiety-like behaviors reportedly do not develop before 6–8 weeks in pain models²⁹. The goal was to establish a dose response curve to evaluate efficacy of DCS by establishing the mechanical threshold and observing anti-anxiety effects. Mechanical sensitivity was assessed on the whisker pad every hour for 4 h.

Experiment 1: Monotherapy testing and dose response profiling of DCS for allodynia and anxiety behaviors

After post-surgery week 8, mice received daily s.c. injections of a low dose of DCS (40, 60, or 80 mg/kg) for 7 days. Mechanical head withdrawal thresholds were determined daily 2.5–3 hours after injection. On the 7th day of drug treatment, anxiety related behaviors were determined in mice receiving the 80 mg/kg dose using the light/dark place preference box. Vehicle control mice with either TIC or sham injury received a daily s.c. injection of normal saline (150 μ l/ mouse).

Experiment 2: Combination therapy (DCS/PIO) testing for allodynia and anxiety behaviors (One time injection)

In a separate cohort of animals in post TIC injury week 8, mice were injected with either PIO (100 mg/kg, i.p.), DCS (80 mg/kg s.c.), or combined PIO (100 mg/kg i.p.) /DCS (80 mg/kg s.c.). The reported LD50 of PIO given systemically in a mouse ranges from 181 mg/kg-1200 mg/kg (United States Pharmacopeial Convention, 2013).

Vehicle control mice with TIC received normal saline (200 μ l, i.p.). Mechanical sensitivity on the whisker pad was evaluated post injection (0, 0.5, 1, 2, 3, 4, 5, and 6 hr).

Experiment 3: Combination therapy (DCS/PIO) testing for allodynia and anxiety behaviors (One time injection followed by 6 days DCS monotherapy)

In weeks 8–10 after TIC nerve injury, a separate cohort of mice with TIC or sham nerve injury were treated once daily with DCS (80 mg/kg, s.c.) for 7 days. On the last treatment day, an additional one-time bolus of PIO (100 mg/kg, i.p.) was given just after the DCS treatment. The vehicle control mice with TIC injury or sham injury received daily saline injections (150 μ l, s.c.). Mechanical head withdrawal thresholds were measured daily at 2.5–3 hours after injection. Anxiety related behaviors were measured on the last treatment day using the light/dark box.

Experiment 4: Monotherapy testing of mitochondrial uncoupler 2,4-DNP for allodynia behavior

At least 8 weeks after TIC injury, a separate cohort of mice were given either 2,4, DNP (5 mg/kg in 10% DMSO in 0.9% saline, 150 μ l, i.p.) or vehicle (10% DMSO in 0.9% saline, 150 μ l). The 2,4-DNP used in the same dose reduces neuronal cell death in the hippocampus, cortical mitochondrial reactive oxygen species (ROS) production, and improves cognitive outcome in a traumatic brain injury model in rats⁸. Mechanical head withdrawal thresholds were determined (0, 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 hr).

Statistical Analysis

GraphPad Prism 6 software package (GraphPad Software, Inc. La Jolla, CA, USA) was used for statistical analysis and graphing of data from all behavioral tests, drug administrations, and mitochondrial assays. Data are shown as mean $\pm$ standard error (SEM). Data were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test or by unpaired, two-tailed Student's t-test. A p 0.05 was considered significant for all tests. Using JMP Pro (version 12.1.0), a power analysis for 80% was conducted after the study for each experiment.

Results

Unilateral Whisker Pad Mechanical Allodynia Developed in Mice with TIC Injury

Baseline whisker pad mechanical thresholds were similar for mice with TIC injury and mice with sham surgery (3.84 ± 0.35 g vs. 3.47 ± 0.00 g). One week post TIC, the mechanical threshold was significantly decreased ipsilaterally compared to the contralateral side (0.37 ± 0.39 g vs. 3.45 ± 0.04 g; $p < 0.0001$) (Figure 1) indicating development of mechanical allodynia which persisted until the animals were euthanized. The ipsilateral mechanical threshold was significantly decreased compared to sham mice ($p < 0.0001$) where the average threshold remained unchanged side to side (3.41 ± 0.02 g and 3.43 ± 0.05 g).

D-Cycloserine Attenuated Mechanical Allodynia But Did Not Relieve Anxiety Behaviors Associated with Hypersensitivity

Single doses of DCS were given to mice with TIC injury to determine a dose response curve (Figure 2A). Single DCS doses (40 mg/kg, 60 mg/kg, 80 mg/kg, 100 mg/kg, 160 mg/kg, and 320 mg/kg, s.c.) were given in ascending sequence with a one week interval between each treatment. Only the higher DCS doses, 160 mg/kg and 320 mg/kg, were effective in alleviating whisker pad mechanical allodynia in mice with TIC injury. At 3 – 4 hour post injection, there was a statistically significant mechanical threshold increase for the 160 mg/kg dose treatment group (3 hr: 0.51 ± 0.13 g; 4 hr: 0.43 ± 0.12 g) compared to the saline treatment group (3 hr: 0.00 ± 0.00 g; 4 hr: 0.01 ± 0.00 g; $p < 0.0001$). The mechanical threshold of the 320 mg/kg dose group was significantly elevated at 3 hour post injection (0.17 ± 0.11 g) compared to the vehicle control group (0.00 ± 0.00 g; $p < 0.0001$; $n = 4-8$).

As shown in Figure 2B, a repeated daily 80 mg/kg dose of DCS attenuated ipsilateral whisker pad mechanical allodynia of TIC injury mice on the 6th day of injection (1.10 ± 0.62 g; $n = 4-7$). Interestingly, the 40 mg/kg and 60 mg/kg doses of DCS had no effect on mice

with TIC injury, but the whisker pad of sham mice that received the 40 mg/kg dose of DCS became hyposensitive to the von Frey fiber stimuli bilaterally (0.37 ± 0.36 g) as indicated by the open squares in Figure 2B. The mechanical threshold of the sham mice with DCS 40 mg/kg dose was significantly different from shams receiving the 60 mg/kg dose (3.76 ± 0.23 g) and the 80 mg/kg dose treatment (3.43 ± 0.05 g; $p < 0.001$; $n = 4-7$).

Previous studies have reported that DCS is involved in alleviating anxiety-related disorders²⁵⁻²⁷. The light-dark box preference test was used to determine if the 7-day DCS treatment would attenuate the anxiety-like behaviors that developed in mice with TIC injury. Figure 2C depicts the time of light side occupancy of the mice with TIC injury injected with saline (235.30 ± 55.80 sec) vs. mice with TIC + DCS (271.30 ± 65.16 sec; $p = 0.69$; unpaired t test; $n = 5$). Although there is no statistically significant difference in the responses of DCS treated mice compared to the saline treated, the DCS treated mice did spend substantially more time in the light side. This indicated that DCS had minimal effect at the dose tested and might be more effective in relieving the anxiety-like behaviors in a higher dose range.

Attenuation of Mechanical Allodynia and Anxiety with Combined Ineffective Low Doses of D-cycloserine and Pioglitazone

Figure 3A depicts the one-time drug dose combination of 100 mg/kg (i.p.) of PIO and 80 mg/kg (s.c.) of DCS compared to these same doses given alone in mice with TIC injury. The mechanical threshold increased to 0.94 ± 0.34 g (50%) in mice with TIC injury treated when treated with the PIO + DCS drug combination. This was a significant increase in mechanical threshold compared to the saline treated animals with TIC (0.00 ± 0.00 g; 3 hours post injection $p < 0.001$, $n = 5-9$) (Figure 3B). The effect peaked at 2-3 hours and persisted for 4 hours.

Seven consecutive day treatment with DCS (80 mg/kg, s.c.) was repeated with the addition of a 100 mg/kg (i.p.) bolus of PIO given on the 7th day. The drug combination had an attenuating effect on mechanical allodynia on the 7th day of DCS treatment compared to saline treatment in mice with TIC nerve injury. Two hours after the injection, the mice treated with the drug combination (DCS + PIO) spent a significantly greater amount of time in the light side of the place preference box compared to saline treated mice indicating reduced anxiety (drug: 221.0 ± 52.33 sec vs saline: 75.28 ± 36.65 sec; $p < 0.05$) (Figure 3C).

Taken together, these data demonstrate that the low dose PIO and DCS combination had a potentiating effect in both reducing mechanical hypersensitivity and in reversing anxiety-like behaviors associated with the TIC injury.

2.4-DNP Attenuated Whisker Pad Mechanical Allodynia in Mice with TIC Injury

At week 8 post injury, a single dose injection of the mitochondrial uncoupler 2,4-DNP (5 mg/kg, i.p.) effectively attenuated whisker pad mechanical allodynia in mice with TIC injury (Figure 4). The effect started after 2 hours (1.03 ± 0.15 g) and persisted for 5 hours (3hr: 1.64 ± 0.21 g; 4hr: 1.17 ± 0.09 g; 5hr: 0.83 ± 0.07 g). This decrease in mechanical allodynia was statistically significant compared to mice with TIC injury given vehicle (10% DMSO) (0.02 ± 0.00 g; * $p < 0.05$, **** $p < 0.0001$).

Cortical Mitochondria Had a Decreased Respiratory Control Ratio (RCR) in Mice with TIC Injury Indicating Cortical Mitochondrial Dysfunction

The mitochondrial isolation assay and Seahorse XF^e24 analyzer was used to determine the mitochondrial oxygen consumption of isolated cortical mitochondria in the mice with TIC injury. The State III oxygen consumption rate (OCR) of the mitochondria from mice with TIC injury (382.40 ± 10.47) was significantly increased compared to the State III OCR of the mitochondria from naïve animals (253.9 ± 11.99 ; $p < 0.001$) (Figure 5A). The State IV OCR was increased in mitochondria from mice with TIC injury compared to the mitochondria from naïve controls (TIC: 86.78 ± 1.93 vs. 47.14 ± 8.54 ; $p < .05$) (Figure 5B). The Respiratory Control Ratio (RCR), defined as $RCR = \text{State III}/\text{State IV}$, significantly decreased in mitochondria from mice with TIC injury compared to the RCR in mitochondria of naïve controls (TIC: 4.41 ± 0.07 vs. naïve: 6.64 ± 1.01 ; $p < 0.05$) (Figure 5C). These results indicate mitochondrial dysfunction occurred in the mice with TIC injury since the RCR is less than 5.

The DCS/PIO drug combination added *ex vivo* to the mitochondrial respiration buffer (MRB) did not significantly change State III or State IV OCR in the TIC group. However, the drug combination did significantly increase the RCR of the cortical mitochondria of mice with TIC (cortex: 6.00 ± 0.71) compared to untreated mitochondria of mice with TIC (cortex: 4.407 ± 0.07 , $p < 0.5$) (Figure 5C). The drug combination significantly decreased the RCR of the cortical mitochondria of naïve mice (4.08 ± 0.21) compared to untreated cortical mitochondria from naïve controls (6.64 ± 1.008 ; $p < 0.5$) (Figure 5C).

Drug Combination Treatment Increased Respiratory Control Ratio in Brainstem Mitochondria of Mice with TIC Injury

Isolated brainstem mitochondria of TIC injury mice were analyzed along with mitochondria from age matched naïve mice. The OCR of State III and State IV of the mitochondria from mice with TIC injury (State III: 502.10 ± 48.26 ; State IV: 70.44 ± 9.43) was not significantly different from mitochondria of naïve mice (State III: 513.90 ± 27.81 ; State IV: 85.87 ± 9.17) (Figure 6A & 6B). The RCR of the mitochondria from TIC injury mice (7.43 ± 0.34) was not significantly different compared to the mitochondria from naïve mice (6.24 ± 0.35) (Figure 6C). However, with the two drug combination, the only significant change observed was that the mitochondria of the drug treated mice with TIC injury had an increased RCR compared to the mitochondria from the untreated mice with TIC injury (treated: 9.00 ± 0.59 vs. untreated: 7.43 ± 0.34 , $p < 0.05$) (Figure 6C).

Discussion

Although previous studies have determined that the PPAR γ receptor agonist, PIO, attenuates hypersensitivity induced by neuronal injury^{12–14}, the present study is the first to find low dose drug combination of DCS and PIO effectively reduced trigeminal neuropathic hypersensitivity and anxiety measures associated with this orofacial TIC injury mouse model of neuropathic pain. This suggests the drug combination has a potentiating effect in higher order brain regions with ability to attenuate the anxiety-related behaviors associated with the neuropathic pain^{40–43}. Furthermore, the low dose DCS/PIO combination produced

no overt side effects observable in the mice. There are no negative interactions reported for PIO and DCS in the literature. Therefore, based our experimental results with this lower dose drug combination, it would be interesting to test treatment of patients who suffer from depression, anxiety, or other psychological conditions due to chronic pain.

Clinical trials have been conducted with DCS for alleviation of chronic back pain, anxiety/stress disorders, and fear related to pain^{25–27}. In one study successful attenuation of chronic orofacial pain is reported in which the patient received transcranial stimulation in combination with DCS treatment²⁸. While chronic treatment with the 80 mg/kg dose of DCS for 7 days partially attenuated mechanical allodynia on the whisker pads of the mice with TIC nerve injury on the 6th day, it did not improve the anxiety-like behavior associated with TIC nerve injury in the light-dark box preference test.

DCS is known to have an affinity for a specific glycine binding site on the NMDA receptor. Previous studies have shown that at lower doses, DCS will act as a partial agonist on the NMDA receptor to produce hypersensitivity, but at higher doses, DCS can act as a partial antagonist of NMDA^{38,39}. Similarly, in the present study the hyposensitivity induced by low dose DCS (60 mg/kg) in sham operated control animals suggests DCS acts as an agonist at low doses, and acts as an antagonist at high doses (160, 320 mg/kg) in the mice with TIC injury for alleviation of chronic pain. As explanation for the dual effect, Mony and colleagues speculated that many ligands in high doses desensitize the NMDA receptor⁴⁴.

Alternatively, DCS and PIO could be acting independently through both the NMDA receptor and PPAR γ , as another explanation for the potentiated effect. In fact, some studies have supported a role for PIO's action through PPAR γ independent pathways directly on the mitochondrial protein mitoNEET. The mitoNEET is vital for mitochondrial respiration and has been demonstrated to directly alter mitochondrial oxidative phosphorylation^{18,19}. An NMDA-like receptor has been identified on the mitochondrial membrane⁴⁵. This evokes speculation as to whether the DCS/PIO combination is alleviating trigeminal pain by altering the mitochondrial function bringing these mechanisms into balance.

In the present study the mild mitochondrial uncoupler, 2,4-DNP significantly attenuated the mechanical allodynia by uncoupling of mitochondrial respiration in the mice with TIC injury. It should be noted that the mechanical allodynia effectively reduced by the mitochondrial uncoupling therapy had been chronically maintained for 8 weeks following TIC injury. The effectiveness of 2,4-DNP in increasing mechanical threshold induced by TIC nerve injury is supportive of the rationale that mitochondrial dysfunction is a causal factor in chronic neuropathic pain.

Further, this study included *ex vivo* mitochondrial differentiation assays to support the *in vivo* studies. In addition to the evidence provided by the 2,4-DNP uncoupler supporting a pivotal role for mitochondria in the chronic pain state, the *ex vivo* studies conducted determined cortical and brainstem involvement in the mitochondrial dysfunction. The mitochondrial isolation assays determined that State III and State IV OCR increased in cortical mitochondria in mice with TIC nerve injury, but OCR did not change in brainstem mitochondria. The RCR decreased in the isolated cortical mitochondria after TIC without

the treatment compared to naïve mice. While the RCR of cortical mitochondria from naïve mice decreased when treated with the DCS/PIO drug combination, the treatment in mice with TIC injury increased RCR not only in cortical mitochondria but also in the brainstem mitochondria.

The significant decreases the RCR of mice with TIC compared to naïve mice are supportive of an adaptive mechanism that is occurring in the cortical mitochondrial of the nerve injured animals. Since mitochondria were analyzed 28 weeks post injury after completion of all the behavioral studies, it is sufficient to say that in a chronic state the increased State III respiration is needed to provide sufficient ATP production for brain function. The State III respiration increase is supportive of increased electron leak that then leads to increased ROS production. While State III indicates complex I driven ADP phosphorylation and general mitochondrial oxidation, State IV is a sufficient indicator of electron leak^{46,47}. Further studies are needed to confirm that increased mitochondrial ROS is generated in mice with TIC injury. Since the respiratory control ratio (RCR) (State III/State IV) was less than 5 in the cortical mitochondria of the mice with TIC injury, this would support the idea that the electron transport chain complexes are not very well coupled to the production of ATP, an indication of mitochondrial dysfunction⁴⁷⁻⁴⁹.

The drug combination (DCS/PIO) increased RCR in the TIC injured cortical and brainstem mitochondria, but decreased RCR in the cortical mitochondria of the naïve mice. While the DCS/PIO combination improved RCR suggesting ability to improve mitochondrial dysfunction after injury, the drug combination is not beneficial in naïve mice. The decreased RCR specifically in cortical mitochondria of the naïve drug treated group compared to naïve group is due to higher uncoupling of mitochondrial respiration, which supports higher brain region specific drug effects on mitochondrial function.

Thus, along with the behavioral improvement provided by the combined treatment, the mitochondrial dysfunction evidence suggests that mitochondria are essential targets with potential for improving behavioral consequences of chronic pain. Although mitochondrial dysfunction has previously been shown to be responsible for maintaining chronic pain after nerve injury³⁻⁶, the present data extend this information indicating mitochondrial dysfunction is maintained through a chronic 28 week post nerve injury time point in this trigeminal neuropathic pain model. Treatment with the DCS + PIO drug combination provided improved or restored mitochondrial dysfunction.

Limitations

A limitation of this study is that only one dose was used in the drug combination test (80 mg/kg DCS + 100 mg/kg PIO). Future studies should be conducted at different dose combinations to determine if lower doses of both drugs would elicit a similar or more effective anti-allodynic effect. In this study, the mice with TIC were only given 7 days of DCS treatment and on the 7th day received a bolus of PIO. It would also be interesting to determine if simultaneously feeding both DCS and PIO chronically would resulted in a greater increase in mechanical threshold. A future isobolographic study would need to determine an effective chronic treatment dose of combined PIO. Once determined, low dose

PIO and DCS might be given together chronically. Based on the data from the study in which the one-time low dose combination of DCS/PIO attenuated mechanical allodynia, speculation is that chronic treatment of even lower doses will elicit an effect over time.

However, questions would arise as to the mechanism: 1) Are the drugs acting through their receptors (DCS→NMDAR and PIO→PPAR γ), 2) Are they acting through receptor independent pathways (mitochondrial actions), or more likely 3) Are they acting through both receptor dependent and independent pathways? The underlying mechanism for the effects of the DCS and PIO combination is unknown and needs to be further investigated. Future studies could confirm the extent of DCS action through protein-dependent, NMDA-like receptors in mitochondria. Determination should be made of the potential involvement of mitoNEET with PIO, or whether improved mitochondrial bioenergetics are occurring through protein-independent mechanisms. Future studies looking at specific mETC complex activity, in particular the redox state of complex I could be beneficial in uncovering the mechanism⁵⁰. The current study observed the receptor independent actions of the drug combinations and effects on improving mitochondrial function after the TIC injury. Another limitation of this study is that only State III, State IV, and the RCR were conducted for mitochondrial dysfunction in the Seahorse assay. Alternatively, future studies could determine specifically if complex I is dysfunctional by measuring the NADH/NAD⁺ ratio before and after nerve injury. Since complex I is important for State III and State IV electron transport respiration, this beneficial experiment would determine if the mitochondrial dysfunction that is observed is due to alteration of the complex I redox state^{35,36}. Furthermore, the activity of the remaining complexes in the mETC could be observed in future studies using the Oxytherm method to determine if one or more complex is responsible for the mitochondrial dysfunction that occurs after TIC injury in the mitochondrial cortex^{7,51}. Since there are no changes observed in the calcium dynamics of the isolated mitochondria (cortex and brainstem), measuring total oxidative stress could also provide explanation for the mitochondrial dysfunction observed. By measuring reactive oxygen/nitrogen species using spectrophotometric assays along with the H₂O₂ production⁷, we could not only determine if oxidative stress is occurring in the mitochondria, but what particular oxidative stress species is responsible. Taken together with the experiments listed above, a mechanism for mitochondrial dysfunction could be deduced.

Future studies could determine more specifically how the DCS/PIO drug combination is acting on the mitochondria to reverse dysfunction, whether from the mechanisms listed above or another mechanism. Further study could also analyze if there is even greater reversal of cortical mitochondrial dysfunction with chronic DCS/PIO combination treatment. The mitochondrial dysfunction detected in cortical mitochondria but not in the brainstem could be due to the large area of cortex dedicated to the trigeminal somatotopic map as compared to the trigeminal dorsal horn which is a small portion of the brainstem. Nevertheless, the drug combination was acting to increase RCR to ADP phosphorylation at both brain levels. This was paralleled by improvement in both pain and anxiety related behavioral measures.

The data suggest the DCS\PIO combination might be of value in the clinic for patients suffering from continuous trigeminal neuropathic pain particularly since many chronic pain

patients also develop anxiety. While 2,4-DNP was removed from the market due to increased fatality rates, the low ineffective doses DCS and PIO given in combination alleviated hypersensitivity without any observed side effects in the mice.

Conclusion

This study demonstrated that the combination of low dose DCS/PIO attenuated not only the whisker pad mechanical hypersensitivity indicative of orofacial neuropathic pain, but also alleviated the anxiety behaviors associated with the TIC injury through receptor independent mechanisms. These mechanisms were supported by the finding that the DCS/PIO combination corrected the cortical mitochondrial imbalance that we identified as dysfunctional in the mice with TIC injury.

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Figure 1. TIC Injury Induced Unilateral Whisker Pad Mechanical Allodynia

The 50% mechanical threshold (in gram force) was measured bilaterally on the whisker pads of the mice with TIC injury and the sham mice. The mechanical threshold was decreased on the ipsilateral whisker pad of mice with TIC injury within one week post injury. The mechanical threshold of contralateral whisker pad was unaffected by the surgery. The mechanical threshold of the ipsilateral and contralateral whisker pads of the sham or naïve mice also did not change. TIC n=9; TIC (ipsilateral) vs. TIC (contralateral) **** $p < 0.0001$; two-way ANOVA, Bonferroni post hoc test.

Figure 2. DCS Attenuated Whisker Pad Mechanical Allodynia, but Does Not Reverse Anxiety-Like Behavior in the Mice with TIC Injury

(A) Dose response curve for DCS showed that higher doses of DCS (160 mg/kg and 320 mg/kg) are effective at alleviating the mechanical allodynia on the whisker pad of the mice with TIC injury (n=6). (B) When mice were given a 7-day treatment of lower doses of DCS (s.c.), only the 80 mg/kg dose elevated the mechanical threshold in the mice with TIC injury (n=8). (C) On day 7, two hours post-injection, there was no difference in the light side occupancy times for the mice with TIC injury treated with either the vehicle or with 80

mg/kg dose of DCS in the light-dark box preference test (n=5). A and B: t-test * $p<0.05$, *** $p<0.01$; **** $p<0.0001$; two-way ANOVA, Bonferroni post hoc test. C: Unpaired t-test, n.s.

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Figure 3. Combined DCS and PIO Attenuated Whisker Pad Mechanical Allodynia and Anxiety-Like Behavior in Mice with TIC Injury

(A) One-time dose of DCS (80 mg/kg) + PIO (100 mg/kg) elevated mechanical threshold on the whisker pad of the mice with TIC injury. This was significantly different from the effect of a single dose of either DCS (80 mg/kg) or PIO (100 mg/kg) only (n=6–8). The mechanical threshold after the drug combination was also significantly different from the threshold of the vehicle treated group indicating reduced hypersensitivity. (B) A bolus of PIO (100 mg/kg) was given on the last day of the 7 consecutive day DCS (80 mg/kg)

treatment course. The whisker pad mechanical threshold was significantly increased in the mice with TIC injury compared to the vehicle treated mice with TIC injury (n=5). (C) Two hours after injection of the PIO bolus on day 7, the drug and the vehicle treated mice with TIC injury were tested with the light-dark box place preference test (n=8). The drug treated group spent significantly more time in the lighted chamber compared to the vehicle treated group A and B: * $p<0.05$, *** $p<0.001$; **** $p<0.0001$; two-way ANOVA, Bonferroni post hoc test. C: * $p<0.05$; unpaired t-test.

Figure 4. A Mild Mitochondrial Uncoupler Attenuated the Whisker Pad Mechanical Allodynia in the Mice with TIC Injury

The 2,4-DNP (5 mg/kg) significantly increased the 50% mechanical threshold of the whisker pad of mice with TIC injury compared to vehicle treated mice. This effect started at one hour, peaked at 3 hour, and persisted for 5 hours post injection (n=8). * $p < 0.05$, **** $p < 0.0001$; two-way ANOVA, Bonferroni post hoc test.

Figure 5. Isolated Cortical Mitochondria from Mice with TIC Injury Had Increased State III and State IV OCR, but Decreased RCR That was Reversed with Combined DCS + PIO
 (A) Cortical mitochondria isolated from mice with TIC injury had a significantly increased oxygen consumption rate (OCR) for State III compared to naïve mice. (B) Cortical mitochondria isolated from mice with TIC injury had an increased State IV respiration compared to naïve mice. (C) The respiratory control ratio (RCR = State III/State IV)) decreased in the cortical mitochondria of mice with TIC injury. However, the drug combination of DCS and PIO decreased the OCR in State IV, and therefore increased the

RCR. (n=8/group) * $p<0.05$, ** $p<0.01$; two-way ANOVA, Bonferroni post hoc test; # $p<0.05$, unpaired t-test

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Figure 6. Combined DCS + PIO Increased the Brainstem Mitochondrial RCR in Mice With TIC Injury

(A) For isolated brainstem mitochondria there was no significant difference in the oxygen consumption rate (OCR) of State III for mice with TIC injury compared to naïve mice. However, due to the *ex vivo* drug combination treatment (50 nM DCS+ 50 nM PIO), the OCR for State III increased in brainstem mitochondria from mice with TIC injury compared to treated brainstem mitochondria from naïve mice. (B) There were no significant differences in the oxygen consumption rate (OCR) of State IV. (C) While the respiratory

control ratio (RCR) did not change significantly in the isolated brainstem mitochondria from the mice with TIC injury compared to naïve mice, the RCR of the brainstem mitochondria from the mice with TIC injury was significantly increased compared to treated mitochondria from naïve mice. (n=4/group) * $p<0.05$; two- way ANOVA, Bonferroni post hoc test