1. Introduction
Mitochondrial dysfunction and oxidative damage are prominent features of acute spinal cord injury (SCI). Mitochondria are known to play a pivotal role in neuronal survival and death following injury. Several SCI have a well-documented increase in excitotoxicity that leads to increased mitochondrial CA3 and ultimately cell death. N-acetylcycteine (NAC), a well-known, cost-saving third-generation antioxidant, facilitates the glutathione (GSH) biosynthesis by reducing the oxidative stress to cytoplasm, supplying sulfhydryl (-SH) groups required by endogenous GSH antioxidant function, and enhancing glutathione-S-transferase activity. Additionally, NAC scavenges free radicals via its nucleophilic reactions with ROS. However, bioavailability of NAC is very low because it is rapidly metabolized in liver to form a concomitant of physiologically inactive NAC-glycine, which is a negatively charged compound. Recently, amidic NAC, N-acetylcysteine amide (NACA), was synthesized. By virtue of a neutral (carboxy) group, NACA is more lipophilic and facilitates both blood-brain barrier and cell membrane permeability. Another potential mechanism by which NACA increases GSH pool is the exchange of sulfhydryl group with oxidized glutathione (GSSG). Moreover, NACA has also been shown to scavenges free radical, choline copper, and augments experimental animal models of diabetes mellitus and multiple sclerosis. Our hypothesis predicts that NACA will increase GSH levels and reduce mitochondrial oxidative stress following SCI, thereby improving histopathological and functional recovery.

2. Potential Mechanism of NACA Actions
Pharmacological and in vitro studies have revealed that NACA (1) increases the intracellular GSH levels, (2) scavenges free radicals, (3) augments the activities of antioxidant enzymes, and (4) stimulates the mitochondrial biogenesis following SCI. 

3. Materials and Methods

Spinal Cord Preparation: Adult female 7-8 weeks old Sprague-Dawley cord sections at the upper thoracic level (T8-L2) were obtained from an in-house transgenic (TRPV1-CFP; TRPV1-GFP) or wild-type female population (TRPV1-GFP) at the University of Kentucky, Lexington. Cords were divided into two sets with one set immediately frozen in liquid nitrogen and stored for later analysis and the other set fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for histological characterization. Paraffin-embedded sections were cut at 5 µm and stained with cresyl violet for histological evaluation.

Mitochondrial-Prescriptive Animals were decapitated at 24 h following injury and tissue samples were obtained from the remaining thoracic levels (T8-L2). Tissue samples were homogenized in 4% PFA and stored at −20°C until analysis.

Measurement of Mitochondrial Function: Mitochondrial function was assessed using the Seahorse Bioscience XFe24 Analyzer (Seahorse Biosciences, North Billerica, MA) to analyze the respiration rates of isolated mitochondria. Mitochondrial O2 consumption, ATP production, and ROS production were measured as described previously.

4. Effects of NACA (Bolus) on Mitochondrial Oxygen Consumption after SCI

Bares represent the oxygen consumption rates (OCR) in terms of mitochondrial oxygen consumption. Panel A-B shows that SCI (Vehicle) significantly decreases ADP phosphorylation rates (Black bar) and State 3:4 (State 3 minus State 4) respiratory control ratio (RCR) compared to Vehicle. NACA treatment with NACA at 75, 150 or 300 mg/kg significantly increased mitochondrial respiration in a dose-dependent manner. Conversely, NACA treatment at 600 mg/kg was not effective in restoring respiration. For example, (Panel B) and non-SCI (Panel C), mitochondria, NACA treatment rate at 300 mg/kg showed significant increase in mitochondrial oxygen consumption compared to Vehicle. NACA treatment rate at 150 mg/kg showed significant increase in mitochondrial oxygen consumption compared to Vehicle. NACA treatment rate at 300 mg/kg showed significant increase in mitochondrial oxygen consumption compared to Vehicle.

5. Effects of NACA (Bolus) on Activities of Mitochondrial Enzymes after SCI

Bares represent the activities of mitochondrial enzyme complexes in terms of mitochondrial oxygen consumption. For example, (Panel A) and non-SCI (Panel C), mitochondria, NACA treatment rate at 300 mg/kg showed significant increase in mitochondrial oxygen consumption compared to Vehicle.

6. Effects of NACA (Continuous) on Mitochondrial Glutathione Content after SCI

Bares represent the oxygen consumption rates (OCR) in terms of mitochondrial oxygen consumption. Panel A-B shows that SCI (Vehicle) significantly decreases ADP phosphorylation rates (Black bar) and State 3:4 (State 3 minus State 4) respiratory control ratio (RCR) compared to Vehicle. NACA treatment with NACA at 75, 150 or 300 mg/kg significantly increased mitochondrial respiration in a dose-dependent manner. Conversely, NACA treatment at 600 mg/kg was not effective in restoring respiration. For example, (Panel B) and non-SCI (Panel C), mitochondria, NACA treatment rate at 300 mg/kg showed significant increase in mitochondrial oxygen consumption compared to Vehicle. NACA treatment rate at 150 mg/kg showed significant increase in mitochondrial oxygen consumption compared to Vehicle. NACA treatment rate at 300 mg/kg showed significant increase in mitochondrial oxygen consumption compared to Vehicle.

7. Effects of NACA (Continuous) on Mitochondrial Glutathione Content after SCI

Bares represent the oxygen consumption rates (OCR) in terms of mitochondrial oxygen consumption. Panel A-B shows that SCI (Vehicle) significantly decreases ADP phosphorylation rates (Black bar) and State 3:4 (State 3 minus State 4) respiratory control ratio (RCR) compared to Vehicle. NACA treatment with NACA at 75, 150 or 300 mg/kg significantly increased mitochondrial respiration in a dose-dependent manner. Conversely, NACA treatment at 600 mg/kg was not effective in restoring respiration. For example, (Panel B) and non-SCI (Panel C), mitochondria, NACA treatment rate at 300 mg/kg showed significant increase in mitochondrial oxygen consumption compared to Vehicle. NACA treatment rate at 150 mg/kg showed significant increase in mitochondrial oxygen consumption compared to Vehicle. NACA treatment rate at 300 mg/kg showed significant increase in mitochondrial oxygen consumption compared to Vehicle.

8. Effects of NACA (Continuous) on Hindlimb Motor Recovery

Bares represent the oxygen consumption rates (OCR) in terms of mitochondrial oxygen consumption. Panel A-B shows that SCI (Vehicle) significantly decreases ADP phosphorylation rates (Black bar) and State 3:4 (State 3 minus State 4) respiratory control ratio (RCR) compared to Vehicle. NACA treatment with NACA at 75, 150 or 300 mg/kg significantly increased mitochondrial respiration in a dose-dependent manner. Conversely, NACA treatment at 600 mg/kg was not effective in restoring respiration. For example, (Panel B) and non-SCI (Panel C), mitochondria, NACA treatment rate at 300 mg/kg showed significant increase in mitochondrial oxygen consumption compared to Vehicle. NACA treatment rate at 150 mg/kg showed significant increase in mitochondrial oxygen consumption compared to Vehicle. NACA treatment rate at 300 mg/kg showed significant increase in mitochondrial oxygen consumption compared to Vehicle.

10. Summary of Results

Biochemical Assessments
• SCI resulted in significantly compromised mitochondrial bioenergetics in terms of respiratory and enzyme activities in all three popolations of spinal cord tissues.
• Acute treatment with NACA (bolus 75, 150 and 300mg/kg) significantly improved total mitochondrial oxidative phosphorylation in a dose-dependent manner during 24 hrs; 600mg/kg NACA was not effective.
• Alternatively, only the 300mg/kg NACA dosage showed improved activities and respiratory rates for cytochrome and non-cytochrome mitochondrial bioenergetics during 24 hrs.
• SCI resulted in significant depletion of glutathione after 24 hrs but continuous treatment with 300mg/kg NACA maintained normal levels. 
• Unexpectedly, continuous treatment with NACA showed insignificant trends for increased total mitochondrial respiratory, likely due to pharmacokinetics.

Behavioral Assessments
• Despite pharmacokinetic caveat, BBB and Kinnian analyses showed that prolonged, continuous treatment with NACA significantly improved hind limb locomotor recovery.
• Correlative histological quantification revealed that NACA treatment reduced lesion volume and increased tissue sparing at injury epicenter.

Overall, preservation of mitochondrial function with acute and prolonged NACA treatment is associated with improved long-term hindlimb functional recovery and increased tissue sparing following severe contusion SCI.

Acknowledgments
We thank to Dr. David Muggan and Mr. Johnny Morehouse (U. Louisville) for help with kinematic gait analysis.

This study was supported by grants from NIH/NINDS R01NS096303 (AGR & PS), the Craig H. Neilsen Foundation R101015 (AGR), NIH/NINDS 2P30NS012160 (CC4-UK).

References