Mitochondrial-based therapeutics for the treatment of spinal cord injury: mitochondrial biogenesis as a potential pharmacological target

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Running Title: Mitochondrial Biogenesis for Spinal Cord Injury Treatment

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Number of Text Pages: 22

Number of Tables: 1

Number of Figures: 3

References: 177

Words in Abstract: 167

Words in Main Text: 5,246

Abbreviations: 5-HT: 5-hydroxytryptamine, serotonin; ADP: adenosine diphosphate; AKT: protein kinase B; ALC: acetyl-L-carnitine; ATP: adenosine triphosphate; ATPSyn β: ATP synthase β; Ca^{2+}: calcium ion; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; CNS: central nervous system; CoA: coenzyme A; COX1: cytochrome *c* oxidase subunit 1; CsA: cyclosporin A; Drp1: dynamin-related protein 1; eNOS: endothelial nitric oxide synthase; ETC: electron transport chain; FDA: Food and Drug Administration; Fis1: mitochondrial fission 1; GSH: glutathione; LP: lipid peroxidation; MB: mitochondrial biogenesis; Mdivi-1: mitochondrial division inhibitor-1; Mfn: mitofusin; MP: methylprednisolone; mPTP: mitochondrial permeability transition pore; mtDNA: mitochondrial DNA; NAC: N-acetylcysteine; NACA: N-acetylcysteinamide; NASCIS: National Acute Spinal Cord Injury Study; ND1: NADH dehydrogenase subunit 1; NDUFS1: NADH:ubiquinone oxidoreductase subunit 1NRF: nuclear respiratory factor; PDH: pyruvate dehydrogenase; PGC-1α: peroxisomal proliferator γ coactivator-1α; PPAR: peroxisome proliferator-activated receptor; ROS: reactive oxygen species; SCI: spinal

cord injury; SIRT1: sirtuin 1; SOD2: superoxide dismutase 2; TBI: traumatic brain injury; TFAM:

mitochondrial transcription factor A; TPP: triphenylphosphonium; UCP2: uncoupling protein 2

Recommended Section: Neuropharmacology

Abstract

Spinal cord injury (SCI) is characterized by an initial trauma followed by a progressive cascade of damage referred to as secondary injury. A hallmark of secondary injury is vascular disruption leading to vasoconstriction and decreased oxygen delivery, directly reducing the ability of mitochondria to maintain homeostasis, leading to loss of ATP-dependent cellular functions, calcium overload, excitotoxicity and oxidative stress, further exacerbating injury. Restoration of mitochondria dysfunction during the acute phases of secondary injury post-SCI represents a potentially effective therapeutic strategy. This review discusses the past and present pharmacological options for the treatment of SCI, as well as current research on mitochondria-targeted approaches. Increased antioxidant activity, inhibition of the mitochondrial permeability transition, alternate energy sources and manipulating mitochondrial morphology are among the strategies under investigation. Unfortunately, many of these tactics address single aspects of mitochondrial dysfunction, ultimately proving largely ineffective. Therefore, this review will also examine the unexplored therapeutic efficacy of pharmacological enhancement of mitochondrial biogenesis, which has the potential to more comprehensively improve mitochondrial function following SCI.

Introduction

There are over 12,000 new cases of spinal cord injury (SCI) in the United States every year, and while active individuals at any age can fall victim, the majority of injuries take place in males younger than 30 (Devivo, 2012). The consequences of spinal cord trauma can range from loss of function to complete paralysis below the injury site. The lack of therapeutics capable of restoring function results in this patient population being dependent upon healthcare support for the remainder of their lifetime. Nevertheless, advancements in medical and surgical care, survivors of SCI generally live long lives after injury, with life expectancy correlating with SCI-induced neurological impairment (Wyndaele and Wyndaele, 2006; Middleton et al., 2012).

Unfortunately, patients with SCI often develop progressive complications in addition to their injury, including cardiovascular disease, gastrointestinal problems, chronic pain and depression (Myers et al., 2007). The resulting cost of care is estimated at greater than $3 million per patient (Devivo, 2012), placing a tremendous burden on patients, caregivers and the healthcare system in general, demonstrating the necessity of continued research into the development of therapeutics for individuals suffering from SCI. Furthermore, the development of more effective ways of maintaining and recovering function post-SCI could allow patients greater levels of both independence and productivity, drastically improving patient outlook.

SCI Pathology

SCI occurs in two phases: primary injury and secondary injury (**Figure 1**). Primary injury refers to the immediate mechanical trauma to the spinal cord, which can be caused by compression, contusion or distension, the most common of which being contusion (Sekhon and Fehlings, 2001; Baptiste and Fehlings, 2006). Complete spinal cord transection can occur, though in these

instances little to no functional recovery has been observed with pharmacological intervention alone; however, combinatorial therapies involving cellular transplantation have shown some promise (Coumans et al., 2001; Fouad et al., 2005). There exists the possibility for pharmacological intervention to aid in recovery following incomplete spinal cord transection, such as that generally observed following contusion, because the remaining intact tissue has the potential for repair (Hall and Springer, 2004). For the purpose of this review, we will focus on incomplete transection.

Within the first minutes to hours following injury, a secondary cascade is initiated, which can last for weeks or months and whose damaging effects are comparative to, if not greater than, that of the initial insult (Tanhoffer et al., 2007; Oyinbo, 2011). Consequences of secondary injury include progressive axon demyelination (Totoiu and Keirstead, 2005), neuronal cell death (Beattie et al., 2002; Anwar et al., 2016), microglia activation and inflammation (Qiao et al., 2010; Qiao et al., 2015), glial scar formation (Shibuya et al., 2009) and mitochondrial dysfunction, all of which contribute to the progressive pathology. Because of the far-reaching effects of secondary injury, pharmacological therapeutics that seek to interrupt or control this stage of injury have the potential to improve neuron survival, allowing functional recovery (Hall and Sullivan, 2004; Oyinbo, 2011).

Over twenty-five mechanisms of secondary injury following SCI have been identified, as well as temporal association of their occurrences, ranging from seconds (acute) to years (chronic) post-injury (Oyinbo, 2011). There are multiple reviews, to which the reader is directed for a more thorough discussion of secondary injury (Tator and Fehlings, 1991; Anderson and Hall, 1993; Hall and Springer, 2004; Rowland et al., 2008). In brief, the initial primary trauma results in mechanical disruption of spinal cord vasculature, leading to vasoconstriction and contributing to hemorrhage, edema, hypoperfusion and ischemia (Baptiste and Fehlings, 2006; Graumann et al., 2011).

Ischemia is considered a key mechanism of secondary injury, with the degree of functional loss being proportional to the degree of ischemia post-injury (Tator and Fehlings, 1991). While angiogenesis does take place following SCI, the emerging vessels are often leaky, and therefore do not allow for the necessary delivery of nutrients or removal of waste within the injured spinal cord (Kundi et al., 2013). Furthermore, the subsequent local decrease in oxygen delivery directly reduces the ability of mitochondria to maintain homeostatic function (Graumann et al., 2011; Kundi et al., 2013).

Mitochondria Following SCI

Mitochondria are double-membraned organelles that, through oxidative phosphorylation, produce the majority of adenosine triphosphate (ATP) for the cell. The outer mitochondrial membrane is a phospholipid bilayer containing voltage-dependent anion channels that, when open, allow the passage of small molecules including ions, ATP and adenosine diphosphate (ADP) (Lemasters and Holmuhamedov, 2006; McEwen et al., 2011). The more complex inner membrane, while freely permeable to oxygen, water and carbon dioxide, contains numerous tightly controlled channels, which regulate the electron transport chain (ETC) to maintain the necessary electrochemical gradient ($\Delta\psi$) for ATP synthesis (Saraste, 1999; Kinnally et al., 2011). Various reactive oxygen species (ROS; e.g. superoxide anion, hydrogen peroxide and hydroxyl radicals) can be formed when electrons leak from the ETC and combine with O_2 in the mitochondrial matrix. Under control circumstances, endogenous antioxidant systems protect from ROS-induced toxicity (Candas and Li, 2014); however, disruption of the ETC under pathological conditions can cause not only an energy deficit due to loss of ATP synthesis, but also an increase in ROS production (Turrens, 2003) beyond the neutralizing capabilities of antioxidant systems.

Recently, the understanding of the role of mitochondria within the central nervous system (CNS) has shifted from merely energy suppliers to essential contributors to both neural homeostasis and neurodegeneration (Dubinsky, 2005). Mitochondrial dysfunction following SCI has been suggested to be crucial for the proliferation of secondary injury and subsequent neuronal cell death (Sullivan et al., 2007). Neurons depend upon stringent and efficient ATP-dependent regulation of various ions across the plasma membrane to maintain electrical homeostasis, and to readily accommodate action potential conduction and the release/uptake of neurotransmitters. Additionally, neurons have limited capacity to buffer oxidative stress (Adibhatla and Hatcher, 2010).

Given these large energy requirements and limited antioxidant defenses, neurons rely heavily on mitochondrial metabolism and ATP production, and are susceptible to compromised mitochondria (Uttara et al., 2009; Moskowitz et al., 2010; Wang and Michaelis, 2010); even small mitochondrial defects can cause functional consequences and eventual pathology within the CNS (Dubinsky, 2005). Loss of mitochondrial function, such as that observed with secondary injury following SCI, results in the loss of ATP and inactivation of ATP-dependent ion pumps required for regulation of ion concentrations, as well as reuptake of the excitatory neurotransmitter glutamate. This dysfunction ultimately leads to excitotoxicity, calcium overload and the eventual initiation of cell death cascades, all of which are hallmarks of SCI and further exacerbate injury in this self-propagating cycle (Choi and Rothman, 1990; Rowland et al., 2008; Oyinbo, 2011).

An early secondary event following SCI is depolarization and opening of voltage-dependent ion channels, leading to the release of neurotransmitters, including glutamate. Glutamate binds to glutamate receptors, opens corresponding ion channels, and results in accumulation of intracellular Ca^{2+} (Hall and Springer, 2004). Such ionic shifts can persist for days in injured tissue following

SCI (Young and Koreh, 1986; Demediuk et al., 1990; LoPachin et al., 1999). Under control conditions, mitochondria can sequester and retain exogenous Ca^{2+} via an electrogenic carrier that facilitates transport across the inner membrane. Once in the mitochondrial matrix, Ca^{2+} is stored in the form of inactive precipitates, which are eventually slowly released back into the cytosol (Crompton, 1999; Starkov, 2010). When accumulated above a certain threshold, however, Ca^{2+} will trigger the opening of the mitochondrial permeability transition pore (mPTP, **Figure 2**).

The opening of the mPTP results in the loss of $\Delta\psi$ leading to the cessation of ATP synthesis and has been linked to necrosis and apoptosis following brain injury, neurodegenerative disorders and SCI (Hirsch et al., 1998; Lemasters et al., 1998; Crompton, 1999; Friberg and Wieloch, 2002; Norenberg and Rao, 2007; Bezprozvanny, 2009; Pivovarova and Andrews, 2010). Additionally, opening of the pore allows molecules and water into the mitochondria, causing the matrix to swell as it equilibrates with the cytosol and enlarging the inner membrane until the outer membrane ruptures, releasing accumulated Ca^{2+}, ROS and pro-apoptotic proteins, such as cytochrome *c*, into the cytosol, and promoting cell death (Sesso et al., 2004; McEwen et al., 2011). Importantly, Sullivan et al. (2004a) demonstrated that spinal cord mitochondria have a reduced Ca^{2+} threshold for opening of the mPTP than that of mitochondria isolated from the brain, further indicating the necessity of restoring mitochondrial homeostasis following SCI.

A consequence of the persistent ion shift during secondary injury is increased ROS. ROS are normal byproducts of mitochondrial function, but Ca^{2+} overload increases production in the CNS (Lewen and Hillered, 1998; Sullivan et al., 2004b). SCI induces a detrimental self-proliferating cycle of increased ROS production, leading to oxidative damage and additional ROS production, until pathological levels are eventually reached. A particularly detrimental consequence of ROS is the formation of the powerful oxidant peroxynitrite (Violi et al., 1999). Development of

peroxynitrite is increased following injury due to the increased concentration of superoxide and Ca^{2+}-induced activation of nitric oxide synthase within the mitochondria (Bringold et al., 2000). Peroxynitrite can also trigger cell membrane lipid peroxidation (LP), protein carbonylation and tyrosine nitration, damaging and impairing mitochondria and altering neuronal function post-SCI (Violi et al., 1999; Sullivan et al., 2007; Hall et al., 2016).

LP results in additional free radicals, which propagate damage (Hall et al., 2016). LP can occur in blood vessels and neurons, not only impairing neuronal and vascular integrity, but also promoting ischemia, and further contributing to secondary neuronal injury (Hall and Springer, 2004). Targeting reestablishment of mitochondrial homeostasis prior to damaging levels of ROS formation could potentially attenuate secondary injury following SCI. Temporal analysis revealed altered mitochondrial morphology beginning 2 h post-SCI, with increases in markers of oxidative damage beginning approximately 8 h after injury and continuing until at least 24 h post-SCI (Sullivan et al., 2007; Jia et al., 2016). These data reveal the potential existence of an 8 h window for therapeutic intervention to regain mitochondrial homeostasis following SCI.

Current Treatment for SCI – NASCIS and Methylprenisolone

Based on their ability to reduce peritumoral brain edema in tumor patients, glucocorticoid steroids, including methylprednisolone (MP), were primarily used to treat SCI in 1960s and 1970s, with the assumption that they would also reduce post-SCI edema (Reulen et al., 1973). The National Acute Spinal Cord Injury Study (NASCIS I), a clinical trial performed in the early 1980s, found that the benefits of steroid treatment were limited to none, with increased risk of infection, a known side effect of glucocorticoid dosing, being observed with high-dose treatments (Bracken et al., 1984; Bracken et al., 1985). Based on these findings, a general consensus within the neuroscience

JPET #244806

community was reached concluding that the use of steroids after SCI was simultaneously risky and unhelpful (Hall and Springer, 2004).

NASCIS II, which took place in the 1990s following enhanced knowledge into the mechanism of post-SCI LP (Hall and Braughler, 1981; Anderson et al., 1982; Young and Flamm, 1982; Hall et al., 1984), revealed that patients functionally benefited from treatment with high-dose MP (30 mg/kg i.v bolus plus hourly 5.4 mg/kg for 23 h), presumably via LP inhibition, lessening injury progression, as long as dosing was initiated within 8 h post-injury (Bracken et al., 1990; Bracken et al., 1992; Bracken and Holford, 1993). Based on these data, the standard of care for the treatment of SCI became the systemic administration of MP for 24 h (Rabchevsky et al., 2011). In the late 1990s, the NASCIS III clinical trial evaluated MP using the same dosing regimen used in NASCIS II, extended MP doses (48 h) and a third treatment consisting of one 30 mg/kg MP bolus followed by 48 h administration of tirilazad, a non-glucocorticoid steroid (Braughler et al., 1988; Hall et al., 1994; Bracken et al., 1997; Bracken et al., 1998). In general, all three treatment groups produced comparable degrees of recovery when initiated within 3 h post-SCI. When initiated between 3 and 8 h post-injury, 48 h MP was the most effective, yet also had the highest incidence of glucocorticoid-related side effects (Bracken et al., 1997; Bracken et al., 1998).

There are many potential side-effects of high doses of MP, including increased risk of GI bleeding, deep vein thrombosis, pneumonia, septic shock and delayed wound healing (Evaniew et al., 2015), which can offset the neuroprotective effects of MP, compromising functional outcome and even survival. Additionally, treatment initiation past the 8 h window can actually exacerbate injury and decrease recovery compared to no treatment (Bracken and Holford, 1993). Glucocorticoid-induced neurotoxicity has also been observed in certain neuronal populations, such as the hippocampus (Sapolsky, 1985; McIntosh and Sapolsky, 1996).

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In the decade following the NASCIS trials, multiple highly critical reviews of the studies surfaced criticizing the lack of functional assessment, lack of placebo groups, the safety of high-dose MP and small effect sizes in only a subpopulation of patients (Coleman et al., 2000; Hurlbert, 2000; Short et al., 2000; Evaniew et al., 2015). In 2013, the "Guidelines for the Management of Acute Cervical Spine and Spinal Cord Injuries" downgraded data obtained from NASCIS trials from Class I ("well-executed") to Class III ("unhelpful for establishing quality"), and no longer recommends the use of MP for the treatment of acute SCI, stating that the evidence supporting beneficial effects was inconsistent and likely due to random chance (Walters et al., 2013). As such, treatment with MP following SCI is now up to the discretion of the attending physician (Rabchevsky et al., 2011).

Mitochondrial-Based Treatment

Despite promising treatments in animal models of SCI, there remains no meaningful therapy for the treatment of SCI in humans. Secondary injury is a complex cascade of events that initiates many additional pathologies; therefore, therapeutics targeting specific downstream events following SCI may prove merely palliative and ultimately non-efficacious. Based on temporal data presented by Sullivan et al. (2007), restoration of mitochondrial function shortly after injury may be a more comprehensive approach for the treatment of SCI (McEwen et al., 2011; Rabchevsky et al., 2011).

Many pharmacological agents that have proven beneficial for the treatment of SCI *in vivo* affect mitochondria or mitochondrial function to some extent. For example, the antibiotic minocycline was found to have neuroprotective effects and induce behavioral and cellular recovery following SCI in rats (Wells et al., 2003; Teng et al., 2004; Sonmez et al., 2013; Aras et al., 2015; Ahmad et al., 2016). Included in the spectrum of effects of minocycline is mitochondrial stabilization,

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inhibition of the release of cytochrome *c* and antioxidant activity (Wells et al., 2003; Casha et al., 2012; Aras et al., 2015). Additionally, lithium treatment has been reported to stimulate mitochondrial respiration in human brain tissue and enhance neuronal regeneration after SCI *in vivo* (Yick et al., 2004; Maurer et al., 2009). Unfortunately, however, both of these treatments proved ineffective during phase II clinical trials (Casha et al., 2012; Yang et al., 2012). Restoration of mitochondrial function post-SCI remains a popular therapeutic strategy and can be targeted directly via several different mechanisms, including inhibition of the mPTP, the use of alternate energy sources, enhanced antioxidant activity and altered mitochondrial morphology.

Inhibition of the mPTP

As stated previously, opening of the mPTP contributes to several pathological events that take place during secondary injury. Therefore, targeting components of the mPTP to inhibit the mitochondrial permeability transition following SCI may have therapeutic benefits (**Figure 2**). The immunosuppressant cyclosporin A (CsA) binds to and inhibits the mPTP and has been associated with enhanced mitochondrial function and decreased cell death in the CNS (Waldmeier et al., 2003; Basso et al., 2005; Kim et al., 2014). Particularly, studies have demonstrated that CsA has neuroprotective properties in models of traumatic brain injury (TBI) and stroke (Matsumoto et al., 1999; Scheff and Sullivan, 1999; Sullivan et al., 1999; Sullivan et al., 2000; Uchino et al., 2002).

Unfortunately, assessments of the neuroprotective effects of CsA following SCI have proven inconclusive and inconsistent (Ibarra et al., 1996a; Ibarra et al., 1996b; Rabchevsky et al., 2001; Ibarra et al., 2003; McMahon et al., 2009). The differences in efficacy of CsA between TBI and SCI may be attributed to fundamental differences in spinal cord and cortical mitochondria (Sullivan et al., 2004a). Regardless of any positive results, however, CsA is highly toxic, making

it less than ideal as a therapeutic (Caramelo et al., 2004; Schenk et al., 2010; Rabchevsky et al., 2011; Szalowska et al., 2015). NIM811 is an analog of CsA that also inhibits the mPTP and is much less toxic and lacks immunosuppressive properties (Waldmeier et al., 2002). Very few studies have been performed regarding the therapeutic potential of NIM811 in the CNS, with even fewer investigating SCI (Waldmeier et al., 2002; McEwen et al., 2007; Ravikumar et al., 2007; Mbye et al., 2008; Mbye et al., 2009). The data obtained from these limited studies, however, suggest NIM811-induced neuroprotection post-SCI (McEwen et al., 2007; Ravikumar et al., 2007) and strongly indicate that the therapeutic efficacy of NIM811 deserves further investigation.

Alternate Energy Sources – "Biofuels"

Following SCI, several mitochondrial enzymes are inactivated due to oxidative damage. Of these is pyruvate dehydrogenase (PDH), a critical enzyme in the generation of acetyl coenzyme A (CoA) (McEwen et al., 2011). Acetyl-CoA is necessary for the citric acid cycle and the production of NADH and $FADH_2$, electron donors for the ETC. Because of this PDH deficit, introduction of alternate energy sources ("biofuels") could potentially alleviate mitochondrial dysfunction post-SCI (**Figure 2**).

Acetyl-L-carnitine (ALC) is an endogenous component of the inner mitochondrial membrane that readily crosses the blood-brain barrier and provides acetyl groups to facilitate the synthesis of acetyl-CoA, thereby bypassing the need for PDH (Pettegrew et al., 2000; McEwen et al., 2011). ALC also increases the production of glutathione (GSH), giving it a bipartite effect, further increasing its therapeutic appeal (Pettegrew et al., 2000; Karalija et al., 2012). ALC has been shown to have beneficial effects for a number of neurodegenerative diseases including Parkinson's disease, Alzheimer's disease and multiple sclerosis (Puca et al., 1990; Pettegrew et al., 2000; Tomassini et al., 2004). Interestingly, Karalija et al. (2012) demonstrated that chronic ALC

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administration reduces neuronal degeneration following SCI in rats. Furthermore, Patel et al. (2010; 2012) found that treatment with ALC post-SCI maintained mitochondrial function, improved functional recovery and protected both white and gray matter within the spinal cord from further injury. ALC administration was also shown reduce the number of damaged mitochondria, improve mitochondrial membrane potential and decrease SCI-induced apoptosis in rats (Zhang et al., 2015). These studies, while few in number, suggest the potential for ALC as a therapeutic treatment for SCI.

Antioxidant Approaches

The consequences of ROS formation and oxidative damage following SCI are well-characterized and were briefly discussed above. For a more comprehensive description, the reader is directed to a review by Hall et al. (2011), and for a more thorough review on antioxidant-based therapeutics for the treatment of SCI, see Bains et al. (2012). Pharmacological intervention of oxidative damage post-SCI (**Figure 2**) can occur via several different mechanisms, both direct and indirect. Indirect mechanisms include preventing the formation of ROS and ROS scavenging; direct mechanisms include halting LP propagation or scavenging LP-induced free radicals (Hall, 2011; Bains and Hall, 2012). One significant limitation of the aforementioned indirect mechanisms is a short therapeutic window. Multiple studies have reported near instantaneous increases in ROS production following SCI (Liu et al., 1998; Bao and Liu, 2004; Liu et al., 2004; Xiong et al., 2007), meaning pharmacological agents would need to be administered immediately to ensure that they are able to act and interfere with the initial "burst" of free radical production that occurs following SCI (Hall, 2011; Bains and Hall, 2012; Hall et al., 2016).

Alpha-tocopherol is a naturally occurring form of vitamin E, which can scavenge lipid peroxyl radicals and has been shown to improve recovery and decrease LP following SCI (Anderson et al.,

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1988; Bozbuga et al., 1998; Al Jadid et al., 2009; Morsy et al., 2010; Morsy and Bashir, 2013).

Unfortunately, this process is 1:1 and after scavenging, the radical form of vitamin E is produced, which has no antioxidant properties. Furthermore, it has been suggested that high-dose supplementation of vitamin E, such as that which would be necessary to decrease baseline LP levels in humans (Roberts et al., 2007), can increase mortality (Miller et al., 2005) and, as such, should be avoided.

N-acetylcysteineamide (NACA), a membrane permeable FDA-approved thiol-containing variant of the GSH precursor N-acetylcysteine (NAC), was observed to enhance GSH content, improving mitochondrial bioenergetics and correlating to functional recovery in rat models of both TBI and SCI when administered 15-30 min post-injury (Pandya et al., 2014; Patel et al., 2014). While these results are undoubtedly encouraging, additional studies need to be performed to assess the therapeutic window for treatment initiation, particularly considering NAC was previously found ineffective in rats if not given within 1 h after TBI (Xiong et al., 1999).

Spin trap molecules, such as the free radical scavengers tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl) and Neu2000 (2-hydroxy-5-[2,3,5,6-tetrafluoro-4-trifluoro-methyl-benzylamino]-benzoic acid), have produced inconsistent results following SCI, in part due to their lack of targeted mitochondrial selectivity (Patel et al., 2009; Xiong et al., 2009; Springer et al., 2010; McEwen et al., 2011; Visavadiya et al., 2013). Biomolecules consisting of antioxidants covalently bonded to mitochondrial targeting compounds, such as triphenylphosphonium cation (TPP) have been generated to combat this limitation (Murphy, 1997; Murphy, 2001; Murphy and Smith, 2007); however, the efficacy of these compounds has not yet been tested in SCI.

Fission and Fusion

Mitochondria change form and function to meet requirements of the cell, and as such, they are both highly controlled and dynamic. Alterations in size and number of mitochondria are regulated by the coordination of fission, the division of single mitochondria into multiple daughter mitochondria, and fusion, the formation of a single mitochondrion from previously independent structures (Scott and Youle, 2010). In physiological conditions, mitochondria are constantly undergoing balanced fission and fusion. Because mitochondria cannot be formed *de novo*, fission is necessary for cell division; however, fission and fusion are also consistently observed in many non-dividing cells, partially due to the necessity of replacing or removing damaged mitochondrial components. Furthermore, mutations in fission and fusion regulatory genes are associated with various pathologies, indicating the importance of normal mitochondrial dynamics (Zuchner et al., 2004; Ranieri et al., 2012).

Recently, it has been shown that SCI alters fission and fusion, contributing to mitochondrial dysfunction. Cao et al. (2013) observed a biphasic response in mitochondrial morphology within the first 24 h following SCI in rats. At 3-6 h post-SCI, spinal cord neuronal mitochondrial were larger and fewer in number, correlating with increased expression of fusion proteins mitofusin (Mfn) 1 and 2, and decreased expression of the primary mammalian fission-related proteins mitochondrial fission 1 (Fis1) and dynamin-related protein 1 (Drp1). By 12-24 h after injury, however, the opposite pattern was observed. Temporal analysis of mitochondrial morphology following SCI by Jia et al. (2016) similarly revealed larger mitochondria and increased Mfn1 expression at early time points, peaking by 8 h post-SCI, then decreasing by 24 h, while Drp1 expression was diminished as early as 2 h after injury, then gradually increased by 24 h.

Mitochondrial fission and fusion are closely related to not only morphology, but also cellular function and apoptosis, in that mitochondrial fusion is thought to inhibit apoptosis, while fission

is thought to promote it (Jia et al., 2016). Additionally, studies have indicated that spinal cord cell death is abundantly due to apoptosis after injury, as opposed to a direct effect of the trauma (Liu et al., 1997). It was observed that as spinal cord Drp1 increased within the first 24 h after injury, mitochondrial membrane potential decreased, and cytochrome *c* release and caspase-3 expression increased, culminating in apoptosis (Jia et al., 2016). These data indicate that fusion and fission are integral to early and late stages of acute SCI, respectively (Cao et al., 2013; Jia et al., 2016) and suggest that therapeutic intervention targeting fusion/fission prior to this switch could prove beneficial post-SCI.

Mitochondrial division inhibitor-1 (Mdivi-1, **Figure 2**), a selective Drp1 inhibitor, has proven beneficial in *in vivo* models of various CNS and non-CNS pathologies, including TBI (Wu et al., 2016), amyotrophic lateral sclerosis (Luo et al., 2013), stroke (Zhang et al., 2013; Cui et al., 2016), acute kidney injury (Tang et al., 2013) and myocardial infarction (Ding et al., 2017). Despite these data, only two studies have thus far investigated the effect of Mdivi-1 on SCI (Li et al., 2015; Liu et al., 2015). Li et al. (2015) observed that treatment with Mdivi-1 prior to SCI in rats increased ATP and mitochondrial membrane potential, and decreased caspase-3 release and the number of apoptotic cells by 72 h post-injury . These effects correlated with improved locomotor function in the treated group. Similarly, Liu et al. (2015) observed neuroprotective effects of Mdivi-1, both in cultured spinal cord neurons exposed to glutamate and following ischemic/reperfusion SCI in rats. Mdivi-1 treatment resulted in increased endogenous antioxidant activity, decreased ROS and decreased cytochrome *c* release *in vitro*, as well as improved locomotor function *in vivo* (Liu et al., 2015). While these data are promising, one study used a pretreatment method, while the other began treatment at the initiation of injury; therefore, additional work is necessary to assess the therapeutic efficacy of Mdivi-1 after SCI.

Mitochondrial Biogenesis

The current available therapeutics are not sufficient to effectively treat SCI. In fact, as of 2013, there was no recommended pharmacological intervention after injury (Walters et al., 2013). While there have been promising preliminary studies investigating the efficacy of NACA, NIM811, Mdivi-1 and mitochondrial-targeted antioxidants, there remains a great deal of work to be done with these compounds and, as is often the case, there is no guarantee that the results observed in animals will translate to humans. Hall et al. (2016) suggested that combinatorial therapies could address the obvious deficit in treatment, as pursuing a single facet of the mitochondrial dysfunction-induced damage that occurs post-SCI may not be enough to produce effective neuroprotection. An alternate method of targeting multiple aspects of mitochondrial function that has not yet been effectively explored for the treatment of SCI is pharmacological enhancement of mitochondrial biogenesis (MB).

Regulation of MB

MB is a transcriptional program that can be defined as the repair, growth and/or division of pre-existing mitochondria (Ventura-Clapier et al., 2008). This process involves an intricate network of several transcriptional pathways for both nuclear- and mitochondrial DNA-encoded genes, many of which are outlined in **Figure 3**. MB is governed by the "master regulator" peroxisomal proliferator γ coactivator-1α (PGC-1α), which controls the expression of this network (Kelly and Scarpulla, 2004; Ventura-Clapier et al., 2008). PGC-1α interacts with and co-activates several transcription factors, including nuclear respiratory factors 1 and 2 (NRF1 and 2) and peroxisome proliferator-activated receptors (PPARs), resulting in the transcription of nuclear-encoded subunits of the ETC, including ATP synthase β (ATPSyn β) and NADH:ubiquinone oxidoreductase subunit 1 (NDUFS1), antioxidant proteins such as superoxide dismutase 2 (SOD2), as well as other

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mitochondrial genes, including uncoupling protein 2 (UCP2) and mitochondrial transcription factor A (TFAM). Following its transcription and translation, TFAM translocates into the mitochondrial matrix where it stimulates mitochondrial DNA (mtDNA) replication and the transcription of mitochondrial-encoded genes, including, for example, cytochrome *c* oxidase subunit 1 (COX1) and NADH dehydrogenase subunit 1 (ND1) (Ventura-Clapier et al., 2008). Nuclear-encoded proteins are then transferred into the mitochondria, where nuclear- and mitochondrial-encoded subunits of the ETC are assembled.

Pharmacological agents can augment MB through interaction with the various pathways that regulate this process. For example, agonism of G protein-coupled serotonin (5-hydroxytryptamine, 5-HT) and β-adrenergic receptors can activate the AKT/eNOS/cGMP pathway (Wills et al., 2012; Garrett et al., 2014), enhancing MB. Additionally, nitric oxide donors can stimulate cGMP activation and phosphodiesterase inhibitors can prevent the hydrolyzation of cGMP and cAMP (Cameron et al., 2016; Whitaker et al., 2016). Furthermore, resveratrol, a polyphenol that stimulates MB through activation of sirtuin 1 (SIRT1), which catalyzes deacetylation of PGC-1α, is currently being investigated for the treatment of various neurodegenerative disorders, including Alzheimer's and Huntington's disease (Kim et al., 2007; Ho et al., 2010). While distinct, these pathways all converge on activation of PGC-1α, leading to increased MB (Fujisawa et al., 2009; Dumont et al., 2012). Fortunately, multiple pharmacological agents that induce MB are already approved by the FDA for the treatment of various pathologies (**Table 1**). Therefore, attaining approval for the use of these drugs for the treatment of SCI could be an expeditious process.

MB and SCI

Multiple diseases and injuries, including those of the CNS, are accompanied by mitochondrial dysfunction, often including diminished MB. For example, Alzheimer's, Parkinson's and

Huntington's diseases are all characterized by decreased PGC-1α, decreased expression of oxidative phosphorylation proteins and, in many cases, decreased MB (Hirai et al., 2001; Chaturvedi et al., 2009; Kim et al., 2010; Coskun et al., 2012). Furthermore, ischemic injury, such as that which occurs with SCI, is also followed by reduced oxidative phosphorylation proteins, as well as decreased PGC-1α and TFAM (Whitaker et al., 2016). Given this dysfunction and loss of mitochondrial proteins, pharmacological enhancement of MB, and subsequently mitochondrial gene expression, for the treatment of numerous disorders has gained interest. For reviews on MB for the treatment of various diseases, including pathologies of the CNS, the reader is directed to Whitaker et al. (2016) and Cameron et al. (2016).

Currently no studies have investigated the effect of pharmacological activation of MB on functional recovery following SCI, though published data suggest potential therapeutic efficacy. Hu et al. (2015; 2016) recently reported that not only is PGC-1α expression decreased in the spinal cord after contusive SCI in rats, but also spinal lentiviral overexpression of PGC-1α immediately after injury attenuates neuronal cell death and promotes functional recovery, suggestive of the potential benefit of pharmacologically increasing PGC-1α and MB following injury. In support of this idea, treatment of mice subjected to renal ischemia/reperfusion with mitochondrial biogenic compounds 24 h after post-insult, when injury was maximal, increased PGC-1α expression and restored mitochondrial and renal function (Garrett et al., 2014; Jesinkey et al., 2014a). These data indicate the need for further exploration into the therapeutic efficacy of pharmacologically augmenting MB following SCI.

Studies have also demonstrated a positive correlation between PGC-1α and angiogenesis (Arany et al., 2008; Chinsomboon et al., 2009; Saint-Geniez et al., 2013), a necessary occurrence for effective treatment of SCI pathology. Therefore, therapeutics targeting reestablishment of

mitochondrial homeostasis through increased MB represent a hitherto unexploited mechanism for alleviating several facets of secondary injury progression and improving functional and vascular recovery, as well as neuronal survival following SCI.

Conclusions

Targeting mitochondria for the treatment of SCI is not a novel idea. Mitochondrial dysfunction is a well-characterized consequence of secondary injury following SCI and many promising experimental therapeutics enhance mitochondrial function, though generally through prevention via pretreatment or decreased injury through early administration following insult. Unfortunately, many of these agents remain to be assessed in humans, and of those that have been, none have proven successful for the treatment of SCI. A plausible explanation for this is that in general, these compounds target singular facets of mitochondrial dysfunction, which may not be enough to successfully improve patient outcome. Alternative approaches enhancing several, if not all, aspects of mitochondrial function could prove more efficacious in accelerating recovery of SCI function. Combinatorial therapies, such as pharmacologically increasing antioxidant activity and decreasing mitochondrial fission simultaneously, could address multiple aspects of mitochondrial dysfunction following SCI. Such strategies, however, would undoubtedly require a great deal of refinement and consideration of multiple factors, including drug-drug interactions. Conversely, pharmacological augmentation of MB has the potential to more efficiently address this deficit. Therefore, the efficacy of mitochondrial biogenic compounds should be investigated for the therapeutic treatment of SCI.

Acknowledgements

None.

Author Contributions

Wrote or contributed to the writing of the manuscript: Scholpa and Schnellmann.

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JPET #244806

Footnotes

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Legends for Figures

Figure 1. Spinal cord injury (SCI) pathology. The extent of damage following SCI is a combination of the initial trauma and secondary injury. The primary injury induces damage to the vasculature of the spinal cord, reducing local oxygen delivery, which decreases mitochondrial function and ATP synthesis, and increases ROS production. In addition to mitochondrial dysfunction, hallmarks of secondary injury following SCI include neuronal cell death, axon demyelination and severing, microglia activation and glial scar formation. ATP: adenosine triphosphate, ROS: reactive oxygen species.

Figure 2. Mechanisms to target mitochondrial homeostasis for the treatment of SCI. Following SCI, cellular Ca^{2+} influx results in the opening of the mPTP and loss of the electrochemical gradient ($\Delta\psi$) necessary for ATP synthesis. mPTP opening also allows water and other molecules to move into the mitochondrial matrix, causing the matrix to swell and the outer membrane to rupture, releasing ROS, Ca^{2+} and pro-apoptotic proteins such as cytochrome *c*, into the cytosol. Cyclosporine A (CsA) and its analog NIM811 act by binding to and inhibiting opening of the mPTP, preventing mitochondrial dysfunction. Biofuels, such as acetyl-L-carnitine (ALC) serve as alternate energy sources, allowing the citric acid cycle to continue despite the oxidative damage-induced inactivation of PDH. Antioxidants neutralize the activity of ROS through various mechanisms, contributing to enhanced mitochondrial function. Evidence indicates that mitochondrial fission is initiated shortly after injury, contributing to SCI-induced neuronal apoptosis. Compounds such as mitochondrial division inhibitor-1 (Mdivi-1), which inhibit Drp1, a major protein in mammalian mitochondrial fission, thereby prevent fission and decreased mitochondrial function. ATP: adenosine triphosphate, Ca^{2+} : calcium ion, CsA: cyclosporin A,

Drp-1: dynamin-related protein 1, e$^-$:electron, H$^+$: hydrogen ion, H$_2$O: water, mPTP: mitochondrial permeability transition pore, PDH: pyruvate dehydrogenase, ROS: reactive oxygen species.

Figure 3. Regulation of mitochondrial biogenesis (MB). MB is a highly regulated cellular process that involves an array of diverse pathways. Various pharmacological agents can augment MB by targeting different aspects of these pathways, including agonism of G protein-coupled receptors, increased AMPK and cGMP, enhanced SIRT1-mediated PGC-1α deacetylation, and activation of co-activators that interact with PGC-1 α, all culminating in increased expression of mitochondrial genes and ultimately MB. 5-HTRs: 5-hydroxytryptamine receptors, Ac: acetyl group, ADP: adenosine diphosphate, AKT: protein kinase B, AMP: adenosine monophosphate, AMPK: adenosine monophosphate-activated kinase, ATP: adenosine triphosphate, ATPSyn β: ATP Synthase β, β2ARs: β$_2$ adrenergic receptors, cAMP: cyclic adenosine monophosphate, cGMP: cyclic guanosine monophosphate, CREB: cAMP response element binding, CoQ: coenzyme Q, COX1: cytochrome *c* oxidase subunit 1, Cyt *c*: cytochrome *c*, e$^-$: electron, eNOS: endothelial nitric oxide synthase, GCs: glucocorticoids, GMP: guanosine monophosphate, mtDNA: mitochondrial DNA, ND1: NADH dehydrogenase subunit 1, NDUFS1: NADH:ubiquinone oxidoreductase subunit 1, NO: nitric oxide, NRFs: nuclear respiratory factors, O$_2^{\bullet-}$: superoxide, P$_i$: inorganic phosphate, PDE: phosphodiesterase, PGC-1α: peroxisome proliferator-activated receptor-γ coactivator 1-α, PI3K: phosphoinositide-3 kinase, PPARs: peroxisome proliferator-activated receptors, sGC: soluble guanylate cyclase, SIRT1: sirtuin 1, SOD2: superoxide dismutase 2, TFAM: mitochondrial transcription factor A, UCP2: uncoupling protein 2.

Tables

Table 1. Partial list of FDA-approved mitochondrially biogenic agents.

Drug	Approved to Treat	Mechanism	MB References
Atomoxetine	ADHD	SNRI	(Jesinkey et al., 2014b)
Cilostazol	claudication	PDE4 inhibitor	(Chen et al., 2016)
Fluoxetine	MDD, OCD	SSRI	(da Silva et al., 2015)
Formoterol	COPD, asthma	β_2AR agonist	(Wills et al., 2012)
Metformin	Type II diabetes	AMPK activator	(Kristensen et al., 2013)
Metoprolol	hypertension	β_1-AR blocker	(Sharma et al., 2008)
Riociguat	pulmonary hypertension	sGC stimulator	(Cameron et al., 2016)
Rosiglitazone	Type II diabetes	PPARγ agonist	(Strum et al., 2007)
Sildenafil	erectile dysfunction	PDE5 inhibitor	(Whitaker et al., 2013)

ADHD: attention-deficit/hyperactive disorder; AMPK: adenosine monophosphate-activated kinase; β_2AR: β_2-adrenergic receptor; COPD: chronic obstructive pulmonary disease; MDD: major depressive disorder; OCD: obsessive-compulsive disorder; PDE4: phosphodiesterase-4; PDE5: phosphodiesterase-5; PPARγ: peroxisome proliferator-activated receptor γ; sGC: soluble guanylate cyclase; SNRI: serotonin and norepinephrine reuptake inhibitor; SSRI: selective serotonin reuptake inhibitor

Figures

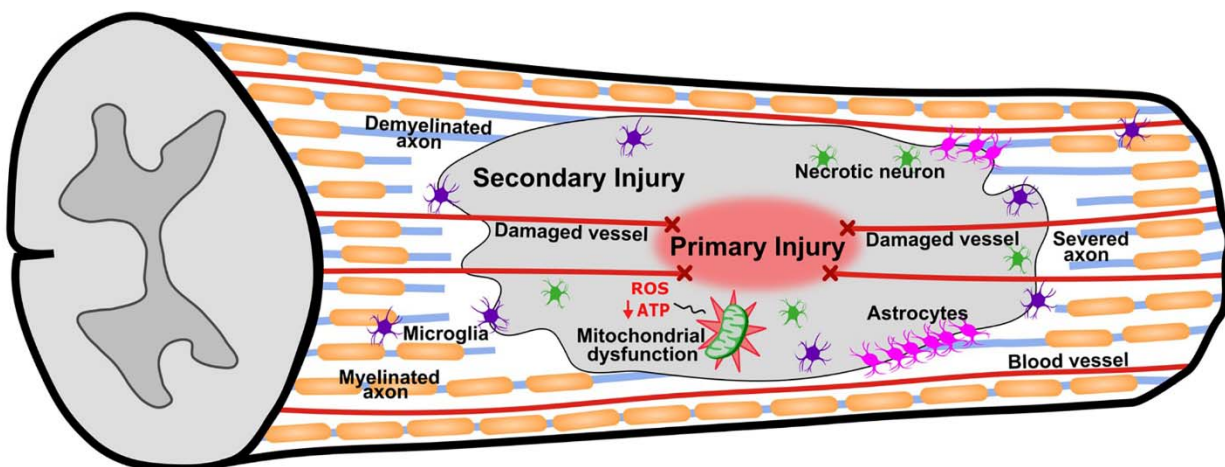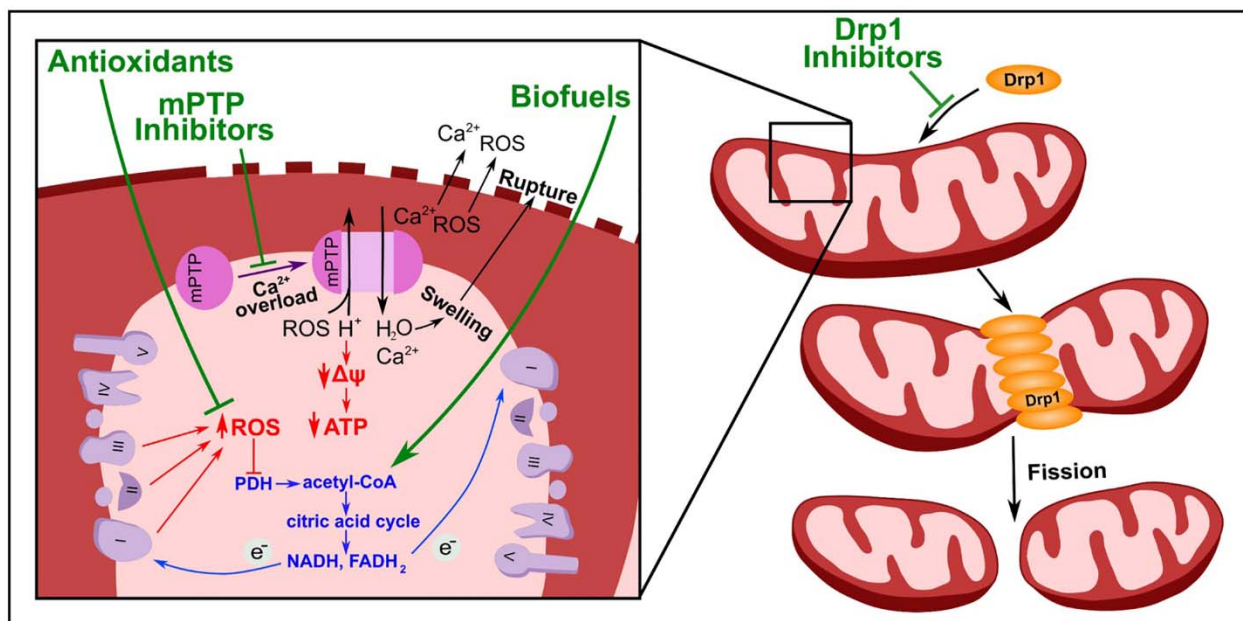## Figure 1

Figure 2

Figure 3