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Aiming for the Target: Mitochondrial Drug Delivery in Traumatic Brain Injury

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Abstract

Mitochondria are a keystone of neuronal function, serving a dual role as sustainer of life and harbinger of death. While mitochondria are indispensable for energy production, a dysregulated mitochondrial network can spell doom for both neurons and the functions they provide. Traumatic brain injury (TBI) is a complex and biphasic injury, often affecting children and young adults. The primary pathological mechanism of TBI is mechanical, too rapid to be mitigated by anything but prevention. However, the secondary injury of TBI evolves over hours and days after the initial insult providing a window of opportunity for intervention. As a nexus point of both survival and death during this second phase, targeting mitochondrial pathology in TBI has long been an attractive strategy. Often these attempts are mired by efficacy-limiting unintended off-target effects. Specific delivery to and enrichment of therapeutics at their submitochondrial site of action can reduce deleterious effects and increase potency. Mitochondrial drug localization is accomplished using (1) the mitochondrial membrane potential, (2) affinity of a carrier to mitochondria-specific components (e.g. lipids), (3) piggybacking on the cells own mitochondria trafficking systems, or (4) nanoparticle-based approaches. In this review, we briefly consider the mitochondrial delivery strategies and drug targets that illustrate the promise of these mitochondria-specific approaches in the design of TBI pharmacotherapy.

Introduction

Mitochondrial dysfunction is at the core of many neuronal pathologies, serving as a nexus for neurons' ongoing fate decision – survive or succumb. Traumatic brain injury (TBI) and similar acute central nervous system (CNS) injuries (ischemia, stroke) are no exception. TBI

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pathology is biphasic. The first phase centers on the direct physical force on brain tissue, but the second phase includes all the complex reactions of the sterile inflammatory response and discoordinated homeostasis that result from the initial mechanical injury (Prins et al., 2013; Werner and Engelhard, 2007). Ongoing mitochondrial dysregulation during this second phase provides a window of opportunity for intervention and is an attractive target for drug design. The need for effective pharmacotherapy is clear with 275k hospitalizations and 52k TBI-related deaths occurring in the United States annually (Faul et al., 2011). Further, individuals who survive severe TBI often face a lifetime of functional deficit, as well as other significant social and financial costs (Andelic et al., 2009; Coronado et al., 2011; Maas et al., 2008). As we currently lack useful neuroprotective therapies for TBI, new strategies must be considered that maximize the potential of past and prospective drug design efforts.

Development and implementation of rationally designed therapies specifically against TBI mitochondrial pathology have proved challenging. Numerous TBI-relevant and druggable mechanistic targets have been identified within mitochondria, but efforts to address this source of pathology have not translated into clinically effective therapy. Efficacy can be hindered by both the failure to attain adequate intramitochondrial drug concentration and occurrence of off-target effects on extramitochondrial machinery. To illustrate, TBI-induced DNA damage activates Poly(ADP-ribose) Polymerase (PARP1) that facilitates repair of nuclear DNA whereas mitochondrial PARP activation reduces mitochondrial DNA integrity and bioenergetic function (Satchell et al., 2003; Szczesny et al., 2014). Expanded on below, localization of PARP inhibitors to the mitochondria may be more effective than pancellular PARP inhibition. A mitochondrial partitioning strategy could reduce the negative effects of PARP activation in mitochondria while preserving the beneficial effects of PARP1 activation in nuclear DNA repair. Other TBI-relevant therapies might similarly benefit from mitochondrial localization. Generalizable strategies can facilitate highly specific accumulation of a drug in the mitochondria, potentially with submitochondrial specificity. The concept of subcellular site-specific drug partitioning has evolved over the recent past from a laboratory tool to viable therapeutic strategy. Here, “localization” refers to the spatial partitioning of active drug to the subcellular region/organelle relevant to the intended mechanistic site of action, the drug’s “target.” Mitochondrial drug localization can be accomplished using one of four strategies that capitalize upon (1) the mitochondrial membrane potential, (2) affinity to mitochondria-specific membrane lipids, (3) piggybacking on the cells own mitochondria trafficking systems, and (4) other permutations of these approaches that employ nanoparticle-based systems. This review will briefly discuss the potential of several mitochondrial drug localization strategies and describe mitochondrial targets thought to play a critical role in TBI pathology. We use several examples to illustrate how small molecule therapy could benefit from this approach.

Generalizable Methods of Mitochondrial Drug Localization

Mitochondria-localizing laboratory tools have existed for decades, but more recently these strategies have been adapted for clinical use. Mitochondrial drug localization is accomplished using any of four generalizable strategies. Efficient organelle-specific drug partitioning facilitates the colocalization of a therapy with its intended mechanistic target. Different mitochondrial localization methods also hint at the opportunity to target a drug to a

specific submitochondrial region – enrichment of drug specifically in the matrix, inner or outer membrane, or within the intermembrane space. These targeting strategies are not without their own considerations. The partitioning efficiency of these approaches can be negatively influenced by the pathologic state they seek to treat while also altering the pharmacokinetic profile of the core therapeutic compound. Below, we review these four drug localization strategies, illustrated with several examples. Not all the methods to be discussed have been tested in the setting of TBI or acute CNS injury, but each holds promise for therapy design.

Membrane Potential-Driven.—Compared to other organelles, mitochondria are uniquely polarized with a trans-inner mitochondrial membrane (IMM) potential ($\Delta\psi_m$) of −150 to −180 mV (Kamo et al., 1979). Lipophilic cations pass easily through the hydrophobic region of lipid bilayers because their positive charge is delocalized over a large area or shielded. As described by the Nernst equation, at 37°C these passively-transported cations accumulate up to 10x per 61.5 mV trans-membrane potential difference. The negative trans-plasma membrane resting potential of most cells aids in initial cytosolic uptake these compounds, with 90–95% of the intracellular cation localizing to the mitochondrial matrix at equilibrium (Liberman et al., 1969). Import efficiency increases in relation to the magnitude of potential difference. Therefore, cation-conjugated compounds possess a minor degree of cell-type selectivity, specifically to excitable cells (e.g. neurons, astrocytes, muscle cells) (Murphy and Smith, 2000; Ransom and Sontheimer, 1992). For neurons with a membrane resting potential of −70 mV, cation-conjugated drug accumulates 5 to 10-fold in cytosol versus extracellular fluid and 100 to 500-fold more in mitochondria versus cytosol at equilibrium. The final quantity of therapeutic compound found within mitochondria *in vivo* varies substantially based on cell type, affected by the properties of the conjugated drug, duration of treatment, cell volume, and number of mitochondria (Reily et al., 2013; Smith et al., 2003). However, explicitly because localization of mitochondrial-targeted cations relies primarily on passive electrochemical-driven transport, disease states associated with significant reduction in plasma membrane or $\Delta\psi_m$ present an unavoidable challenge to the efficiency of this approach.

As the prototypical mitochondria-targeting lipophilic cation, triphenylphosphonium (TPP) and its derivatives have been explored extensively (**Fig 1-A**) (Weissig et al., 1998). Most efforts utilizing TPP-driven drug localization have focused on delivery of lipophilic antioxidants (ubiquinone, tocopherol, quercetin) (Sheu et al., 2006) and superoxide dismutase mimetics (Dhanasekaran et al., 2005). For example, MitoQ is composed of ubiquinone covalently conjugated to TPP by a 10-carbon aliphatic chain linker (Kelso et al., 2001), and it was found to penetrate the blood-brain barrier (BBB) and enrich within neuronal mitochondria (McManus et al., 2011). Preclinical studies found MitoQ was superior compared to unconjugated ubiquinone in reducing indices of oxidative stress and functional defect in models of ischemic or oxidative injury (Adlam et al., 2005). While no studies have reported its clinical effectiveness in TBI, MitoQ was not found to be toxic to humans with prolonged exposure (Orsucci et al., 2011; Smith and Murphy, 2010; Snow et al., 2010). Like other lipophilic cations, TPP's partitioning efficiency relies on an intact $\Delta\psi_m$ (Burns et al., 1995). TBI-induced electron transport chain (ETC) dysregulation often

results in relative $\Delta\psi_m$ dissipation, effectively hampering the matrix localization efficiency of TPP-drug conjugates administered post-injury. MitoQ's potency decreased nearly 25-fold when administered to cells with reduced $\Delta\psi_m$ (Smith and Murphy, 2010). Secondarily, the net movement of TPP and other lipophilic cations across the normally ion-impermeable IMM into the matrix risks "consuming" $\Delta\psi_m$, presenting a dose-dependent risk of inducing or exacerbating ATP synthesis inhibition (Modica-Napolitano et al., 1984; Murphy, 2008). Despite these potential pitfalls, the success of MitoQ and similar agents demonstrates the potential of safe $\Delta\psi_m$-driven approaches. Future drug design efforts should consider this method of delivery for use beyond antioxidants – for any small molecule enzyme inhibitors that could benefit from efficient mitochondrial matrix partitioning.

Affinity Driven.—Certain naturally-occurring antibiotics display strong affinity towards bacterial membranes and, owing to their shared evolutionary history, are similarly attracted to IMM components such as mitochondria-specific phospholipid, cardiolipin (CL) (Ji et al., 2012; Prenner et al., 1999). Unlike lipophilic cations that follow $\Delta\psi_m$ and partition primarily into the matrix, CL-avid compounds are thought to be $\Delta\psi_m$-insensitive and localize to both sides of the IMM. Therefore, IMM or IMS-localized mechanistic targets might be better addressed by this method over traditional cationic strategies. The gramicidin S (GS) derivative, hemigramicidin (Leu-D-Phe-Pro-Val-Orn, "hemi-GS"), and Szeto-Schiller (SS) tripeptides are two prime examples of this localization strategy.

The hemi-GS pentapeptide (**Fig 1-B**) can be linked with a flexible tether to a wide variety of small molecules without compromising the payload's biological activity and ability to interact with mitochondrial proteins (Wipf et al., 2005). Unlike its GS parent, hemi-GS does not permeabilize cell membranes and has no antibacterial activity as it lacks the requisite secondary structure (Escobales et al., 2014). Hemi-GS conjugates are highly effective at concentrating in mitochondria, primarily to the IMS and matrix. One of these conjugates, XJB-5–131 [hemi-GS-4-amino-TEMPO (4-amino-2,2,6,6-tetramethylpiperidine-*N*-oxyl)], was found to be non-toxic, rapidly penetrate the BBB, and enrich in mitochondria over 600-fold compared to cytosol in a $\Delta\psi_m$-independent manner (Ji et al., 2012; Krainz et al., 2016). Hemi-GS is adaptable; varying the length and composition of the hemi-GS to payload linker region can modulate mitochondrial targeting efficiency, payload activity, molecular weight, lipophilicity, and polar surface area (Krainz et al., 2016). Preclinical studies demonstrated that XJB-5–131 is highly effective at reducing injury and preserving behavioral function following TBI and other CNS insults (Ji et al., 2012).

SS peptides are another potential method for affinity-based mitochondrial targeting. This family consists of peptides with <10 amino acid that share an alternating aromatic-cationic motif a 3+ net charge. Basic amino acids, arginine and lysine, provide two positive charges while the free amine of the N-terminus and aminated C-terminus allow for a final 3+ net charge at physiologic pH (Zhao et al., 2003). Experimental evidence suggests that these peptides predominately localize to the IMM (100-fold enrichment) and that despite their cationic nature, only 10–15% of that localization was attributed to a $\Delta\psi_m$-driven mechanism (Szeto, 2008). Like hemi-GS, SS peptides' selectively towards the IMM is reported at least to be partially driven by the presence of CL (Szeto, 2014). These molecules have been

primarily used for their intrinsic antioxidant properties, but several studies have suggested their possible use as a payload delivery system (Cerrato et al., 2015).

Transporter Driven.—With the exception of 13 core ETC proteins, >99% of mitochondrial proteins are synthesized in the cytosol and transported into the mitochondria through mitochondrial transport machinery (Schmidt et al., 2010). Various linear therapeutic compounds may be similarly delivered by capitalizing upon these intrinsic trafficking systems (**Fig 1-C**). The best described and most common mitochondrial protein trafficking relies on the N-terminal mitochondrial targeting signal peptide (MTP) composed of 10–70 amino acids, while region-specific targeting is controlled by internal stop-transfer sequences (Claros and Vincens, 1996; Stojanovski et al., 2012). The MTP motif forms an amphiphilic helix – one face is enriched with hydrophobic leucine while the opposing side is positively charged (Abe et al., 2000). The translocase of the outer membrane (TOM) complex conducts the pre-protein (protein + MTP) to the outer mitochondrial membrane (OMM) or IMS. IMM or matrix-destined pre-proteins are further processed by the translocase of the inner membrane (TIM) complex in an active, energy- and $\Delta\psi_m$-dependent manner (Wiedemann and Pfanner, 2017). Mitochondrial processing peptidase (MPP) and associated chaperones guide final pre-protein maturation (Gakh et al., 2002). The feasibility of MTP-conjugated drugs has been explored and found to be amenable to mitochondrial localization of most linear biomolecules. Although this method utilizes intrinsic, non-toxic small peptide carriers, unavoidable tradeoffs remain: 1) This transport mechanism uses a narrow (~2 nm) active channel; therefore, the transport is restricted by the size of macromolecules. Larger molecules, such as folded proteins, cannot be delivered into the matrix. 2) Transport is an ATP-dependent, active process. TBI-induced ATP depletion inhibits transport and therapy-delivery. 3) This method competitively inhibits the transport of the essential mitochondrial proteins. 4) Stability and tissue-penetration of the MTS-conjugate may prove challenging.

Few studies exist that use this localization method towards the design of clinical treatment, and no studies have been conducted in TBI. So far, efforts have focused on the delivery of peptides and oligonucleotides. MTP derived from mouse mitochondrial thiolase was shown to successfully deliver a 13-base nucleic acid sequence to mouse myoblast mitochondria (Flierl et al., 2003). Similarly, green fluorescent protein (Zhang et al., 1998), restriction enzyme Sma1 (Tanaka et al., 2002), and the human NADH ubiquinone oxidoreductase subunit 4 (*ND4*) gene (Yu et al., 2012) were all found to localize efficiently under MTS control. The final destination of these composite proteins is the matrix by default. Further refinement of the MTS (or alternative trafficking system) and therapeutic payload may mitigate some drawbacks and facilitate more specific submitochondrial targeting in the future.

Nanoparticle Delivery.—Mitochondriotropic liposomes and nanoparticles (NP) are the syntheses of previously described localization approaches, selectively transporting encapsulated drug into the mitochondria (**Fig 1-D**). However, these particle-based approaches are relatively new compared to previously discussed mitochondrial localization strategies.

Liposomes are self-assembling biodegradable particles (50 to 5,000 nm) composed of phospholipids and cholesterol (Pathak et al., 2015). They are organized into an aqueous core which encapsulate hydrophilic compounds while the outer lipid bilayer can host hydrophobic drugs (Marrache et al., 2013). MITO-Porter liposome demonstrated the mitochondrial targeting potential of liposomes by successfully ferrying cargo to OMM and into the IMS (Yamada et al., 2008). Unlike other cationic liposomes that enter cells primarily via clathrin-mediated endocytosis, MITO-Porter liposomes enter cells due to octa-arginine (R8)-stimulated macropinocytosis. The high positive charge density on the liposomal surface not only stimulates cellular uptake but also aids in efficient escape of intact liposomes from macropinosomes at both neutral and acidic pH. In comparison, other micropinocytosis-simulating motifs (e.g. octalysine, K8) are less efficient at facilitating liposome escape as pH decreases during vesicle maturation (El-Sayed et al., 2008). Free, intact liposomes localize to mitochondria based on the composition of their lipid bilayer, specifically the abundance of fusogenic lipids (especially sphingomyelin and phosphatidic acid) (Yamada et al., 2008). Although mitochondrial targeting of liposomes has been widely used in cancer research, there are currently no reported examples of liposomes that can both penetrate the BBB while simultaneously localizing to the mitochondria. Further, maintaining the structural integrity and preventing lipid hydrolysis is an ongoing challenge to both storage and administration of liposome-based therapies.

Polymeric NP serves as a highly adaptable alternative to liposomes. They too are biocompatible and biodegradable polymers manufactured to specific size, density, and charge to allow modulation of various properties, including BBB penetration and mitochondrial localization. Compared to liposomes where the cargo is burst-released into the mitochondria following membrane fusion, polymeric NP can be designed to precisely modulate the rate of drug release (Kamaly et al., 2013). NP containing hydrophobic polyester blocks demonstrate improved biostability in harsh physiological environments facilitating oral administration and may be more tolerable to variable storage conditions compared to liposomes. For example, polyethylene glycol is a typical hydrophilic block added to NP that (1) increases half-life in plasma by reducing reticuloendothelial system-based clearance and (2) serves as a scaffold for further modification with cationic and other mitochondrial-targeting moieties (Allen and Chonn, 1987; Pathak et al., 2015; Wongrakpanich et al., 2014). Specific to mitochondrial targeting, Marrache et al. observed that NP most efficiently localized to the mitochondria when 80–100 nm in diameter and carrying a zeta potential >22 mV. Indeed, TPP-conjugation augmented NP zeta potential and corresponding mitochondrial accumulation (Marrache and Dhar, 2012). Ghosh et al. found TPP-modified NP loaded with the antioxidant, quercetin, were orally active, penetrated the BBB, and accumulated in mitochondria while decreasing neuronal loss and improving mitochondrial function following cerebral ischemia-reperfusion (Ghosh et al., 2017).

Contribution of mitochondrial dysfunction to secondary injury after TBI

Neurons are dependent on mitochondria to supply ATP through oxidative phosphorylation (OxPhos), but they also rely on this organelle for both survival and death signaling, biosynthetic function (e.g. heme synthesis, iron-sulfur cluster synthesis), and other bioenergetic roles to survive and thrive (Hall et al., 2012). This system is highly coordinated;

the overwhelming majority of the >1000 mitochondrial-localized proteins are encoded by the nuclear genome compared to the 13 protein coding genes provided by mitochondrial DNA (mtDNA). Accordingly, even small perturbations to mitochondrial coordination and function have potentially devastating consequences across the neurovascular unit and are central in TBI secondary injury. While endogenous mechanisms work towards repair or elimination of dysfunctional mitochondria, restorative forces are embattled by competing pro-death processes on multiple fronts – defeat is common, victory is pyrrhic. Mitochondrial drug localization might unlock a therapy's true potential, but everything relies on choosing clinically effective targets and understanding when an intervention can provide maximum benefit both in isolation and as part of a multimodal treatment strategy. Below we provide a brief primer outlining selected TBI-relevant mitochondrial pathological mechanisms and their temporal relationship that aids in the design and use of mitochondrial localizing therapies (Fig 2). However, these pathways of mitochondrial dysfunction operate in a wide range of neurologic disease states making what will be discussed applicable to more than TBI.

Prevention is the only way to stop primary mechanical TBI injury and the initial phases of secondary injury. The hyperacute injury is characterized by widespread neuronal depolarization resulting in a torrent of excitatory neurotransmitters (glutamate, aspartate). Resultant hyperactivation of N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and various voltage-gated Na^+ and Ca^{2+} channels drastically increases cytosolic Ca^{2+} (Arundine and Tymianski, 2004; Meldrum and Garthwaite, 1990). Protracted Ca^{2+} overload results in death through activation of catabolic proteases (e.g. calpains, caspases) (Saatman et al., 2010). Therefore, along with the smooth endoplasmic reticulum, mitochondria buffer excess cytosolic Ca^{2+} by the electrogenic activity of mitochondrial calcium uniporter (mCU), uncoupling proteins (UCP), H^+/Ca^{2+} and Na^+/Ca^{2+} exchangers, and mitochondrial-associated endoplasmic reticulum (Duchen, 2000; Raturi and Simmen, 2013; Waldeck-Weiermair et al., 2010; Williams et al., 2013). However, accumulation of mitochondrial Ca^{2+} increases oxidant production and comes at the long-term expense of ATP generation by reducing $\Delta\psi_m$ (**Fig 2-A**). With no window for intervention, the start of this process is currently unavoidable.

As a consequence of the mechanical injury and excitotoxity, initial significant bursts of mitochondrially generated reactive oxygen (ROS) and nitrogen (RNS) species occur too rapidly for intervention. However, ROS production remains high for several days following injury – making it an extremely popular therapeutic target. Oxidant production increases rapidly following TBI in parallel with the early Ca^{2+}-facilitated hyperpolarization of the mitochondrial membrane, while longitudinal RNS production is fueled by the upregulation of inducible NO synthase (**Fig 2-B**) (Bayir et al., 2005). Antioxidant stress response elements in mitochondria can compensate for basal ROS production, but TBI overwhelms defensive reserves and potentiates mitochondrial dysfunction. Reactive oxidant production begins with the partial reduction of oxygen by a single electron, producing superoxide that is rapidly converted into stable hydrogen peroxide (H_2O_2) by mitochondrial manganese superoxide dismutase (MnSOD) (Anthonymuthu et al., 2016; Bayir et al., 2007). While superoxide's effects are spatially constrained, it can interact with nitric oxide (NO) to

rapidly form lethal peroxynitrite radicals (Gray and Carmichael, 1992; Kissner et al., 2003). Oxidative and nitrative stress leads to modification of nuclear and mitochondrial DNA, proteins, and lipids – disrupting physiologic function, potentiating injury, and interfering with beneficial injury resolution mechanisms (Bayir et al., 2007; Singh et al., 2006).

When the initial lines of mitochondrial defense are broken, neurons turn to mitophagy – selective autophagy of mitochondria – to remove liabilities, consolidate functional units, and free resources for eventual reconstruction (**Fig 2-C**). Under physiologic conditions, the occasional rogue mitochondria are fused with their healthy comrades to restore function. Unsalvageable mitochondria are set adrift by mitochondrial fission and degraded by mitophagy. These processes are controlled dynamically; 1) fusion is guided primarily by mitofusins (MFN1, MFN2) and the cardiolipin (CL)-binder Dominant Optic Atrophy 1 (OPA1) (Song et al., 2009), while 2) fission is mediated by Dynamin-related protein 1 (DRP1) and its multiple upstream recruitment mediators (Fis1, Mff) (Loson et al., 2013). TBI-induced damage is recognized early (<24 h pos-tinjury) by PTEN-induced kinase 1 (PINK1) and Parkin whose accumulation on the OMM facilitates the recruitment of autophagy protein LC3 (Chu et al., 2013). Simultaneous phospholipid scramblase 3 (PLS3)-mediated externalization of CL helps anchor LC3 to damaged mitochondrial units. The utility of mitophagy modulators appears particularly context- and time-dependent. Dysregulation of this system – too much or too little at a given time – exacerbates injury and makes for a difficult therapeutic target (Galluzzi et al., 2016). For example, promotion of autophagy/mitophagy following spinal cord injury promoted axonal regeneration and motor recovery (Wang et al., 2014), while other studies demonstrated autophagy inhibition could reduce brain edema and behavioral function following controlled cortical impact (Cui et al., 2015). When these repair and recycle processes fail to properly compensate for ongoing mitochondrial dysfunction, death becomes the only escape.

The IMS houses several important pro-apoptotic factors whose cytosolic release can trigger caspase-dependent and caspase-independent cell death (Galluzzi et al., 2009). Beyond its role in mitophagy, CL serves an additional function in apoptotic signaling. $\Delta\psi_m$ disruption and Ca^{2+}-mediated transporter activation, including the action of PLS3, result in mobilization of CL (Chu et al., 2013; Kagan et al., 2016; Schug and Gottlieb, 2009). When in the presence of H_2O_2 and absence of LC3 binding, CL interacts with partially unfolded cytochrome (cyt) c forming a H_2O_2-fueled CL-specific peroxidase complex (Hanske et al., 2012; Kagan et al., 2005). Oxidized CL (CLox) can no longer interact with cyt c, which is released into the cytosol promoting apoptosis (Kagan et al., 2005). Further hydrolysis of CLox by calcium-independent phospholipase A2 (e.g. iPLA2γ) generates a suite of potentially pro-inflammatory and immunomodulatory oxidized free fatty acids (FFAox) (**Fig 2-D**) (Tyurina et al., 2014). While inflammation can provide benefit through removal of cellular debris (via macrophage, microglia recruitment) and promotion of injury resolution, inappropriate immune activity exacerbates injury. The role and net benefit or harm of mitochondrial-derived FFAox in TBI progression is unexplored.

Early increases in $\Delta\psi_m$ induce ROS production, but eventually potential-dissipating influences, like that of fatty-acid-dependent UCP, prevail. While $\Delta\psi_m$ reduction by UCP activation is effective in reducing lesion volume and preserving neurologic function after

TBI (Pandya et al., 2007), concurrent mitochondrial outer membrane permeabilization (MOMP) progresses neurons towards inescapable death. MOMP results from Bax/Bak-mediated conformational changes in voltage-dependent anion channels (VDACs) (Chipuk et al., 2006; Kalkavan and Green, 2018; Kim et al., 2003). These OMM pores further dissipate $\Delta\psi_m$ and, along with the action of CLox, prompt the release of pro-apoptotic factors including cyt c, Smac/Diablo, HtrA2/Omi, EndoG, and apoptosis inducing factor (AIF) from the IMS (Galluzzi et al., 2009). Mitochondrial Ca^{2+} accumulation and $\Delta\psi_m$ alteration drive the assembly of the mitochondrial permeability transition pore (mPTP) complex – consisting of cyclophilin D (CyD) in the matrix, adenosine nucleotide transporter (ANT) at the IMM, and VDAC at the outer mitochondrial membrane (OMM) (Tsujimoto and Shimizu, 2007). Specifically, oxidation of ANT's key thiol group induces conformational changes permitting CyD binding (McStay et al., 2002). The ANT-CyD complex likely binds VDAC, bringing the mitochondrial membranes in close proximity and creating a continuous flow between matrix, IMS, and cytosol. Other proteins including peripheral ATP synthase, benzodiazepine receptor, hexokinase, creatine kinase, and members of the Bcl-2 family (Bax, Bak) have been associated with mPTP regulation though their roles are less clear (Bernardi, 2013; Karch and Molkentin, 2014). Assembly of the MOMP and mPTP is followed by osmotic swelling and lysis of mitochondria; the loss of the mitochondria's bioenergetic function and release of pro-death factors assures neuronal demise (**Fig 2-E**) (Kitsis and Molkentin, 2010).

Utility of Mitochondrial Targeting in Existing and Prospective Therapies

Few specific mitochondria localizing therapies have been explored in TBI. An extensive array of various untargeted compounds and their effects on mitochondrial pathology in experimental TBI and related acute brain injuries have been reviewed elsewhere (Xiong et al., 2009; Yonutas et al., 2016). Many preclinical successes fail during clinical translation. A combinatorial treatment strategy, simultaneously targeting multiple pathways of cellular and mitochondrial dysfunction, may ultimately be needed to benefit patients with TBI. However, finding and testing compatible therapeutic combinations will be challenging. The likelihood of success can be increased by improving the specificity and efficacy of each component of a mono- or poly-mechanism targeted treatment. Below, we present four recognized TBI therapeutic targets to discuss the potential utility of specific drug localization to mitochondria. Therapies against mitochondriagenerated oxidants or to provide alternative energy sources have been well described over the past decade and continue to improve. Therefore, here we will focus consideration on several enzyme inhibitors that can benefit from future application of mitochondria partitioning strategies.

Electron and Oxidant Scavenging.

The most recognized use of mitochondrial drug localization for clinical use is focused on the elimination of mitochondria-derived oxidants. The local concentrations of endogenous antioxidants are typically controlled by the same mechanisms regulating their spatial distribution (Shull et al., 1991), but this system is ill-equipped to tackle rapid TBI-induced mitochondrial oxidant production increases. Mitochondrial oxidative stress can interfere with key energy production complexes, including pyruvate dehydrogenase (PDH), exacerbating ATP depletion while further limiting repair capacity. Energy supplementation

using neuron-metabolizable alternative biofuels (ketones, acetyl-L-carnitine) permit glycolysis-independent acetyl-CoA production for TCA cycle input while also supporting GSH synthesis (Scholpa and Schnellmann, 2017). Alternatively, GSH precursors modified to improve BBB penetration and bioavailability have shown great promise to improve functional outcome following CNS trauma. Large-scale preclinical studies demonstrated that aminated NAC (NACA) could not only successful cross BBB, but also chelate copper (a potential source of non-enzymatic ROS production) and scavenge free radicals ultimately preserving mitochondrial bioenergetics (e.g. aminated N-acetylcysteine, NACA) (Pandya et al., 2014; Patel et al., 2014). However, despite the direct contribution of oxidative stress in TBI secondary injury pathogenesis, the untargeted delivery of antioxidants has shown no or limited efficacy in clinical trials (Razmkon et al., 2011). Part of the problem may arise from the extremely rapid induction of oxidant injury that begins immediately following the primary mechanical injury. Unavoidable delays in access to definitive care might limit the clinical utility of anti-ROS interventions. Untargeted antioxidants may fail to achieve sufficient mitochondrial concentrations (Murphy and Smith, 2000). Conversely, untargeted supplementation may lead to high concentrations of extramitochondrial antioxidants, in sufficient quantities to interfere with physiologic extramitochondrial ROS signaling and the pro-survival roles provided (Cochemé and Murphy, 2010). As the reaction rate of primary free radicals is mostly diffusion-controlled, the quenching efficiency of free radical scavengers is affected by their physical proximity to the free radical source (Halliwell et al., 1992). Compared to a pancellular treatment, mitochondrial targeting offers explicit proximity-based advantage, improving specificity and potency. Recently, nitroxides possessing stable nitroxyl radicals, such as 4-amino TEMPO and carboxy-Proxyl, have been shown to be particularly amenable to specific mitochondrial targeting and electron scavenging owing to their unique self-renewing redox cycling mechanism. These properties make mitochondria-targeted nitroxides particularly promising candidates for TBI-related ROS/RNS suppression compared to past attempts (e.g. MitoQ, ebselen, MnSOD mimetic) (Fink et al., 2007). We found that XJB-5–131 administration early after experimental TBI virtually abolished CL oxidation and reduced lesion volume. Neurobehavioral testing revealed the XJB5–131-treated group recovered motor and cognitive function faster, and the improvements were enduring, measured up to a month following injury (Ji et al., 2012).

Targeting mPTP Opening.

Few have attempted to localize enzymatic inhibitors to mitochondria, but the benefits of such an approach should be further explored. Arguably one of the most effective drugs aimed at mitigation of mitochondrial dysfunction following TBI is cyclosporin A (CsA). CsA binds CyD in the mitochondrial matrix disrupting its interaction with IMM-localized ANT, thus inhibiting mPTP formation. CsA administration can improve $\Delta\psi_m$ and reduce intramitochondrial Ca^{2+} and oxidant production following TBI (Broekemeier et al., 1989; Yonutas et al., 2016). These mitochondria changes were paralleled by a reduction in neuronal death and lesion volume, along with corresponding improvements in cognitive function, synaptic plasticity, and BBB integrity (Kilbaugh et al., 2011; Okonkwo and Povlishock, 1999; Riess et al., 2001; Scheff and Sullivan, 1999; Sullivan et al., 2000). While early administration of CsA provides maximum benefit (Sullivan et al., 2011), significant protective effects have been observed up to 24 hours post-TBI (Sullivan et al., 2000). Phase

III trials are still needed, but preliminary studies of CsA in clinical TBI show measurable improvements of functional outcome, cerebral metabolism, and cerebral perfusion (Hatton et al., 2008; Mazzeo et al., 2008). CsA is typically well tolerated but carries a notable risk of serious immune, cardiovascular, renal, and hepatic toxicities (Empey et al., 2006; Hatton et al., 2008; Mazzeo et al., 2009; Mazzeo et al., 2006). Further, it poorly penetrates the BBB and requires high systemic dosing to be detectable in cerebrospinal fluid (Brophy et al., 2013; Okonkwo et al., 2003).

Critically, there are several theoretical benefits to targeting and trapping CsA in the mitochondria with its TBI-relevant target, CyD. First, the most common intended use of CsA its for is ability to suppress T-cell activation via inhibition of calcineurin (Li et al., 2011; Pundir et al., 2017). Unfortunately, hospital-acquired infections are frequent occurrences following moderate and severe TBI – especially in patients with a history of chronic illness, multiple traumas, or recent surgery (Kourbeti et al., 2011; Schirmer-Mikalsen et al., 2013) – while TBI itself is linked to significant early (<24 h) and protracted (>4 d) decreases in circulating T lymphocytes (Mrakovcic-Sutic et al., 2010). By partitioning CsA to the mitochondrial compartment – safely sequestered from cytosolic/nuclear-localized calcineurin – the consequences of CsA-associated immunosuppression might be reduced. CsA analogs without off-target inhibition of calcineurin (NIM-811) are currently being tested (Mbye et al., 2009; Mbye et al., 2008; Readnower et al., 2011). However, use of mitochondrial targeting strategies may accomplish the same goal more readily in terms of development time and expense. Finally, the choice of localization strategy might affect overall efficacy. NP-based mitochondrial targeting strategies might aid CsA's poor BBB penetration (McManus et al., 2011), while lipophilic cation or IMM-affinity driven localization techniques can maximize CsA accumulation at its intended site of action.

Targeting CL Oxidation.

Preventing the release of the pro-apoptotic signals from the mitochondria is yet another potential target in TBI. The majority of efforts focus on directly preventing the release of cyt c into the cytosol. Mitochondrial-targeted, imidazole conjugated stearic acid (TPP-ISA) and oleic acid (TPP-IOA) were shown to inhibit apoptosis following radiation injury (Atkinson et al., 2011). The imidazole moiety present in these fatty acids serve as a coordinating ligand for cyt c's heme, thereby preventing its peroxidase activity when bound to CL (Jiang et al., 2014). This can reduce CLox-derived FFAox release, along with their associated inflammatory effects. Alternative strategies have attempted to convert the oxidizable CL pool into non-oxidizable CL that are resistant to cyt c-mediated oxidation and apoptosis. The formation of non-oxidizable CL with mitochondrial-targeted oleic acid supplementation was shown to suppress actinomycin D-induced apoptosis (Tyurina et al., 2012).

Targeting mt-PARP.

PARP1 serves a pivotal role in base excision repair of damaged nuclear DNA by recruiting and modifying histones, topoisomerases, and DNA polymerases (Dantzer et al., 2006), including damage resulting from TBI. However, PARP1-mediated accumulation of poly(ADP-ribose) polymers following severe cytologic injury results in regulated cell death (Fatokun et al., 2014). While PARP inhibitors showed strong benefit in experimental TBI,

they failed to translate clinically (Stoica et al., 2014). Recently, the role of mitochondria-localized PARP (mt-PARP) has been the subject of study (Masmoudi et al., 2006; Masmoudi and Mandel, 1987). mt-PARP activation is thought to be responsible for the TBI-induced differential PARylation of a wide range of mission-critical mitochondrial proteins (Lai et al., 2008). Several of these modifications have been observed before significant nuclear PARP activation – stimulated by recognition of mtDNA injury and Protein Kinase A (PKA)-mediated phosphorylation (Brunyanszki et al., 2014; Brunyanszki et al., 2016).

Injury-induced mt-PARP consequences are divided into two categories, NAD^+ consumption and PARylation of mitochondrial proteins. First, as limited mitochondrial NAD^+ stores are consumed by mtPARP, ATP production and NAD^+/NADH-dependent reactions are inhibited (Stein and Imai, 2012). Failure to maintain $\Delta\psi_m$ follows as cytosolic NAD^+ cannot directly or rapidly replenish the mitochondrial pool. Instead, NAD^+ must be resynthesized from precursors in an ATP-consuming process – undesirable at a time where ATP production is already faltering. Second, PARylation of AIF, ETC complexes, and mtDNA repair enzymes negatively affect their catalytic activity and stimulate regulated cell death pathways (Fatokun et al., 2014; Zhou et al., 2006). For example, in contrast to nuclear PAR signaling, mt-PARP activity is linked to reduced mtDNA integrity through inhibition of the mitochondria-specific DNA repair proteins, endo/exonuclease G, and polymerase-γ (Szczesny et al., 2014; Van Houten et al., 2016). These effects collectively potentiate mitochondrial dysfunction and increase mt-PARP activation in a vicious cycle. Therefore, clinically effective PARP inhibitors might be those that are mitochondrially targeted – maximizing mitochondrial benefit and minimizing nuclear harm.

Conclusion

TBI pathology is complex and often heterogeneous. Convergent pathways throughout affected neurons ensure they meet an unfortunate end. Mitochondria are the common denominator, centrally contributing to energetic failure, the release of pro-death signals, and inflammation. This sets mitochondria apart as a prime TBI pharmacotherapy target. Past attempts to intervene in mitochondrial pathology have stumbled due to efficacy-limiting effects on extra-mitochondrial pathways, poor accumulation of drug at its mechanistic target, and other unintended consequences. However, specific localization of a drug to its mechanistic target in the mitochondria might alleviate some of these concerns. Localization can be accomplished by taking advantage of the mitochondrial matrix potential, affinity towards the IMM, intrinsic trafficking machinery, or nanoparticles. Each of these methods is not without potential downsides, and our understanding of their safety and utility in humans is lacking. These methods have a proven track record in a diverse range of disease models *in vitro* and *in vivo*, and they can be readily adapted to accommodate their payload. Subcellular drug targeting to mitochondria or other organelles will become an increasingly important consideration in future drug design. This approach offers an opportunity to revive past pharmacological failures and design novel neuroprotective therapies.

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Terms/Abbreviations:

ANT
Adenosine Nucleotide Transporter

AMPA
α-Amino-3-Hydroxy-5-Methyl-4-Isoxazoleproprionic acid

BBB
Blood Brain Barrier

Ca^{2+}
Calcium

iPLA2γ
Calcium-independent Phospholipase A2

CL
Cardiolipin

mitochondrial targeting moiety
Carrier

CBF
Cerebral Blood Flow

CNS
Central Nervous System

CSF
Cerebrospinal Fluid

cyt c
Cytochrome c

CyD
Cyclophilin D

CsA
Cyclosporin A

DNP
2,4-dinitrophenol

OPA1
Dominant Optic Atrophy 1

DRP1
Dynamin-related Protein 1

ETC
Electron Transport Chain

GSH
Glutathione

GPx
Glutathione Peroxidase

GS
Gramicidin S

hemi-GS
Hemigramicidin

H_2O_2
Hydrogen Peroxide

IMM
Inner Mitochondrial Membrane

IMS
Intermembrane Space

mtHsp
LC3, Mitochondrial Heat Shot Protein

mPTP
Mitochondrial Permeability Transition Pore

mtPARP
Mitochondrial Poly(ADP-ribose) Polymerase

MPP
Mitochondria Processing Peptide

$\Delta\psi_m$
Mitochondrial Membrane Potential

MnSOD
Mitochondrial Manganese Superoxide Dismutase

MTP
Mitochondrial Targeting Signal Peptide

MFN
Mitofusin

NP
Nanoparticles

NO
Nitric Oxide

NMDA
N-methyl-D-aspartate

OMM
Outer Mitochondrial Membrane

CLox
Oxidized Cardiolipin

FFAox
Oxidized Free Fatty Acids

active drug attached to carrier
Parkin, Payload

PARP1
Poly(ADP-ribose) Polymerase

PINK1
PTEN-induced kinase 1

RNS
Reactive Nitrogen Species

ROS
Reactive Oxygen Species

SS
Szeto-Schiller

drug's binding/interaction partner
Target

delivering a drug to a specific subcellular location, interchangeable with "localization"
Targeting

TIM
Translocase of the Inner Membrane

TOM
Translocase of the Outer Membrane

TBI
Traumatic Brian injury

TPP
Triphenylphosphonium

UCP
Uncoupling Proteins

VDAC
Voltage-dependent Anion Channel

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- TBI prompts mitochondrial dysfunction; no clinically effective therapies exist
- Generalizable strategies allow specific drug targeting of mitochondrial pathology
- Colocalization therapy with its target may improve potency and specificity
- Mitochondrial-localization can improve new and old therapies in TBI

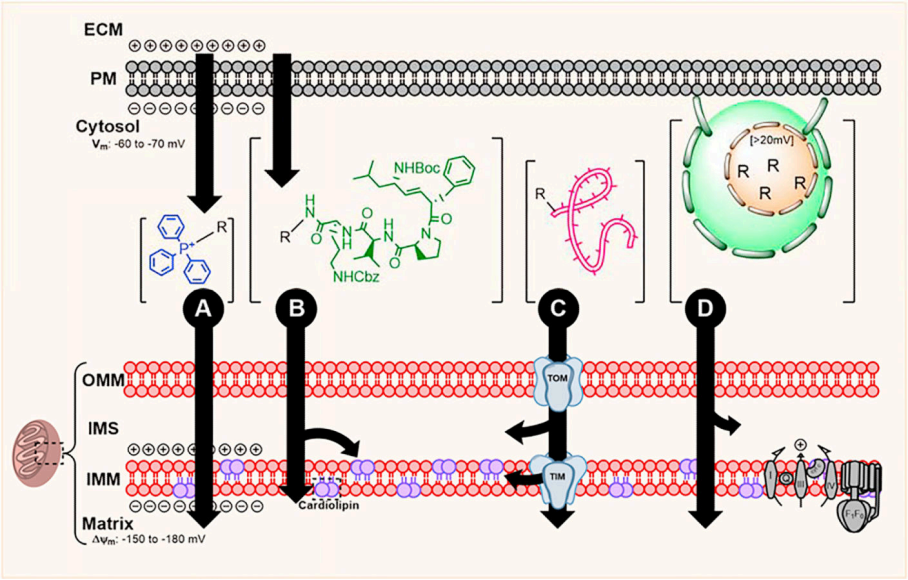

Fig. 1.

Methods of mitochondrial drug localization. (A) Membrane potential-driven localization uses a lipophilic cation, TPP. (B) Affinity-based localization uses the distinct composition of mitochondria to partition drug, hemi-gramicidin binds IMM-specific cardiolipin. (C) Intrinsic mitochondria protein trafficking systems, such as the TIM/TOM system, can deliver linear molecules. (D) Nanoparticles can be modified for specific mitochondrial localization and membrane fusion.

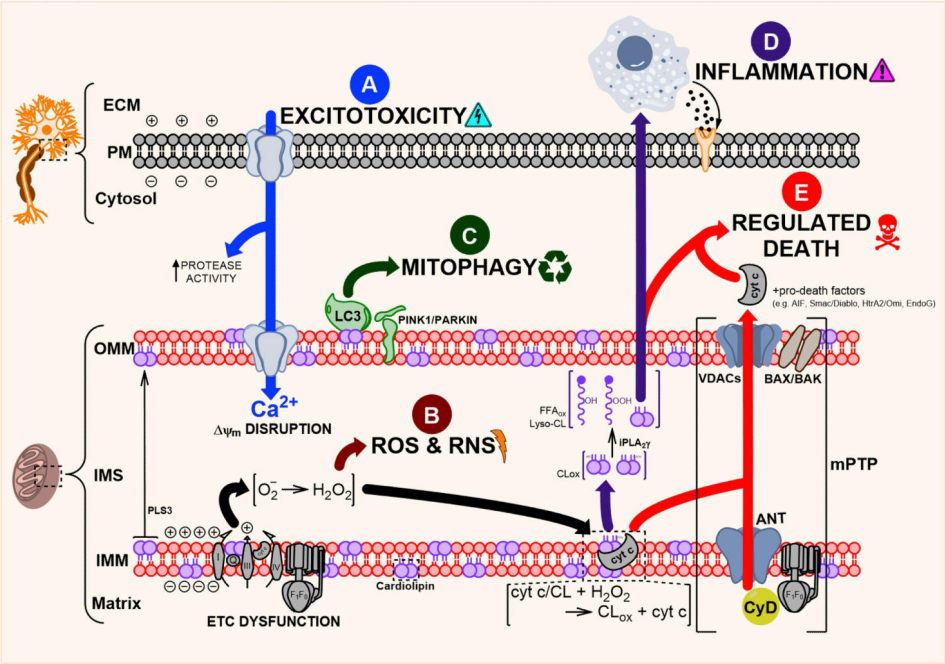

Fig. 2.

Mitochondria-focused mechanisms of neuronal injury following TBI. (A) Widespread neuronal depolarization results in the unregulated release of excitatory neurotransmitters and activation of their corresponding ion channels. Increased cytosolic Ca2þ is buffered, in part, by the mitochondria, disrupting Djm. (B) The ensuing ETC dysfunction causes protracted increases in ROS & RNS production. Injury becomes self-perpetuating as endogenous oxidant stress response systems are overwhelmed. (C) Damaged and nonfunctional mitochondria are actively eliminated through mitophagy. This system maintains a delicate balance between removing unsalvageable mitochondria and consolidating functional units. (D) Mitochondrial lipids, especially CL, are important sources of oxidized free fatty acid inflammatory mediators. CL is oxidized by the specific cyt c/CL peroxidase, and its oxidized acyl chains are liberated through the action of iPLA2g. (E) Permeabilization of the mitochondrial membranes and oxidation of CL prompts the release of pro-death factors e ultimately leading to neuronal death.