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Mitochondrial Damage & Lipid Signaling in Traumatic Brain Injury

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Abstract

Mitochondria are essential for neuronal function because they serve not only to sustain energy and redox homeostasis but also are harbingers of death. A dysregulated mitochondrial network can cascade until function is irreparably lost, dooming cells. TBI is most prevalent in the young and comes at significant personal and societal costs. Traumatic brain injury (TBI) triggers a biphasic and mechanistically heterogenous response and this mechanistic heterogeneity has made the development of standardized treatments challenging. The secondary phase of TBI injury evolves over hours and days after the initial insult, providing a window of opportunity for intervention. However, no FDA approved treatment for neuroprotection after TBI currently exists. With recent advances in detection techniques, there has been increasing recognition of the significance and roles of mitochondrial redox lipid signaling in both acute and chronic central nervous system (CNS) pathologies. Oxidized lipids and their downstream products result from and contribute to TBI pathogenesis. Therapies targeting the mitochondrial lipid composition and redox state show promise in experimental TBI and warrant further exploration. In this review, we provide **1**) an overview for mitochondrial redox homeostasis with emphasis on glutathione metabolism, **2**) the key mechanisms of TBI mitochondrial injury, **3**) the pathways of mitochondria specific

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H.B. and V.E.K designed and conceptualized the review. All authors contributed to writing of the manuscript.

Declaration of Interests

The authors declare no competing interests.

phospholipid cardiolipin oxidation, and **4**) review the mechanisms of mitochondria quality control in TBI with consideration of the roles lipids play in this process.

Keywords

Traumatic brain injury; mitochondria; lipid peroxidation; redox lipidomics; mitochondria quality control; mitophagy

1.0: Introduction

Brain bioenergetics rely on mitochondrial oxidative phosphorylation for energy production. In addition to ATP production, mitochondria are known for their essential contribution to metabolic pathways (e.g, for purines and pyrimidines) as well as for their semi-autonomous role in survival and death signaling, oxidative stress, and intracellular calcium buffering. Aberrant mitochondrial dynamics and metabolism have a central role in the pathogenesis of numerous acute and chronic neurologic diseases. These changes interfere with normal function and stimulate downstream signaling events. Increased electron leakage due to electron transport chain dysregulation and/or aberrant catalytic mechanisms of TCA dehydrogenases such as α-ketoglutarate dehydrogenase (α-KGDH), α-glycerophosphate dehydrogenase (α-GPDH) (Adam-Vizi and Tretter, 2013) results in increased free radical generation, which can overcome endogenous compensatory mechanisms and detrimentally alter DNA, proteins, and lipids. Similarly, a diverse array of pathogenetic mechanisms converge to prompt release of mitochondrially sequestered regulated death and inflammatory factors. Mitochondrial function and dynamic regulation in response to both acute and chronic brain injury are integral to neuronal survival and homeostatic function.

Traumatic brain injury (TBI) is remarkable because of the heterogeneity of neuronal survival, death, and inflammatory signaling mechanisms involved. TBI pathogenesis is biphasic; it entails both a primary mechanical injury and secondary delayed injury mechanisms (Prins et al., 2013). The primary injury is initiated by the acute mechanical disruption of brain tissue and cellular architecture. The mechanical injury damages axons and synapses leading to excitotoxic injury, while tearing of blood vessels results in both hemorrhagic and ischemic injury. Secondary TBI injury is delayed, developing hours-to-weeks later. Both injury phases prompt maladaptive mitochondria-specific lipid oxidation, inflammation, and initiation of regulated death pathways. Each of these processes bears close mechanistic similarities with other acute brain injury pathologies (e.g. ischemia-reperfusion, spinal cord injury, subarachnoid hemorrhage).

Both clinical observations and experimental studies recognize the central role of structural and functional mitochondrial injury in TBI pathogenesis. Mitochondrial dysregulation is a primary driver of neuronal loss and worse clinical outcomes following TBI (Cheng et al., 2012). Therefore, understanding how the heterogenous injury mechanisms of TBI converge on mitochondria and induce a shift from normal to pathological activity can guide rational therapy development. In particular, we illustrate how mitochondria-derived (oxidized) lipid signaling has emerged as an opportunity-rich and exciting area of ongoing TBI research. This review provides: **1**) an overview for mitochondrial redox homeostasis with emphasis on

glutathione metabolism, **2**) the key mechanisms of TBI mitochondrial injury, **3**) the pathways of mitochondrial lipid oxidation, focusing on cardiolipin oxidation, and **4**) an overview of mitochondria quality control mechanisms in TBI with consideration of the role lipids play in these processes.

1.1: Mitochondrial Structure and Redox Function

Mitochondria are complex semi-autonomous organelles. They are unique because they have their own plasmid-like circular genome (mitochondrial DNA, mtDNA) that expresses a small but vital subset of redox proteins (Lagouge and Larsson, 2013). The remaining suite of mitochondrial proteins are nuclearly encoded and must be imported then renatured at their specific submitochondrial destination. The dual mitochondrial membranes are protein rich. The inner (IMM) and outer (OMM) mitochondrial membranes phospholipid (PL)-to-protein ratio are 0.20 and 0.45 (w/w), respectively. In comparison, the plasma membrane has a higher 0.67 PL-to-protein ratio (Horvath and Daum, 2013). Most important, however, is the precise lipid composition and high abundance of oxidizable polyunsaturated acyl chains means mitochondria are a unique and important site for generation of lipid mediators and downstream products.

The electron transport chain (ETC) is the chief source of ATP, but is also a potentially dangerous site of intracellular oxidant production (Willems et al., 2015). The ETC is composed of five protein complexes and the mobile electron carriers, coenzyme Q10 and cytochrome *c* (cyt *c*). ETC Complex I and II receive high energy electrons derived from $NADH/FADH_2$. Electrons are then shuttled through a series of redox centers in Complexes I, III, and IV – a process that culminates in the translocation of protons from the matrix to intermembrane space (IMS). This proton pumping action produces a negative IMM transmembrane potential ($\Delta\psi_m$, −150 to −180 mV) (Kamo et al., 1979). Complex V (F_1/F_0 ATP Synthase) is powered by this electrochemical gradient (i.e. protonmotive force). Proton traversing back across the IMM via Complex V powers a stepwise rotation in the synthase's F_0 c-ring motor, which in turn drives ATP synthesis by the F_1 motor. The establishment and maintenance of $\Delta\psi_m$ are absolutely required for healthy mitochondrial function. Beyond energy production, mitochondrial Ca^{2+} buffering activity and mitochondrial protein import depends on and influences $\Delta\psi_m$ (Hansford and Zorov, 1998; Kulawiak et al., 2013).

Mitochondrial power comes with risk. The ETC is a significant source of intracellular reactive oxygen species (ROS) – such as superoxide anion radicals ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) – and secondary reactive nitrogen species (RNS) (Loschen et al., 1974). Dysregulation of the ETC following TBI or other acute/chronic CNS pathologies can prompt high energy electrons to "jump the track" and react with molecular oxygen (O_2) to form $O_2^{\bullet-}$ (Jastroch et al., 2010). Aside from the respiratory complexes, several dehydrogenases essential for the physiology of neurons can generate $O_2^{\bullet-}$ / H_2O_2 in mitochondria. Chief among them is the TCA cycle's α-ketoglutarate dehydrogenase (α-KGDH), whose E3 subunit generates $O_2^{\bullet-}$ / H_2O_2 at a rate dependent on the $NADH/NAD^+$ ratio (Adam-Vizi and Tretter, 2013). α-KGDH-associated ROS production is enhanced in several naturally occurring E3 subunit mutants (e.g. G194C common in Ashkenazi Jewish and some Arab Muslim populations) (Hong et al., 2003; Shaag et al., 1999). In generating acetyl-CoA for

the TCA cycle, pyruvate dehydrogenase complex (PDHC) activity similarly yields significant amounts of H_2O_2 (Mailloux et al., 2018; O'Brien et al., 2017). Finally, mitochondrial α-glycerophosphate dehydrogenase (α-GPDH, GPD2) is an IMM protein that, together with cytosolic α-GPDH (GPD1), helps shuttle NADH-derived reducing equivalents across the IMM. As mitochondrial Ca^{2+} levels increase, GPD2-associated ROS production increases and normal shuttling function is subsequently inhibited. Regardless of origin, nascent $O_2^{\bullet-}$ is rapidly dismutated to H_2O_2 by nearby mitochondrial manganese superoxide dismutase (MnSOD, SOD2). H_2O_2 is then reduced in mitochondria by the glutathione peroxidase system or diffuse out into cytosol and be eliminated by catalase. Alternatively, H_2O_2 can be utilized by a number of metallo-enzymes, particularly heme-peroxidases, as a source of oxidizing equivalents to feed a variety of oxidation reactions yielding oxidized products, including oxidized lipids with signaling functions (Kanner et al., 1987). Unfortunately, these systems can be overwhelmed under pathological conditions, leading to exacerbation of mitochondrial injury and eventual $\Delta\psi_m$ depletion. Even with brain's reliance on mitochondrial respiration, it is also comparatively sensitive to oxidative insult, due in part to its: **1**) unusually high concentration of molecular oxygen (using ~20% of body O_2 supply), **2**) relatively low catalase expression (10% of the liver), **3**) a high iron/ascorbate ratio that has been shown to be pro-oxidant, and **4**) enrichment of oxidizable polyunsaturated fatty acids (Marklund et al., 1982; Zaleska et al., 1989). Therefore, redundancies exist both in and out of the mitochondria to help keep tight control on cells' redox balance.

1.2: Mitochondria & Glutathione Regulation

Members of the glutathione peroxidase (GPX) family are among the most prominent and abundant alternatives to the SOD/catalase system. They function to preserve neuronal mitochondrial function and redox balance. Eight families of human glutathione peroxidases have been identified (Brigelius-Flohé and Maiorino, 2013). GPX1–4 and 6 rely on selenocysteine for their catalytic cycle, while GPX5, 7, and 8 contain standard cysteine. H_2O_2 is the primary oxidizing equivalent for GPX1, 3, 7, and 8. However, GPX1 and 3 can additionally reduce small organic hydroperoxides, including soluble peroxidated free fatty acids. GPX4 is unique in its ability to reduce membrane-associated, PL-esterified peroxidated cholesterols, and polyunsaturated fatty acids (Brigelius-Flohé and Maiorino, 2013). GPX4 is localized throughout the cell, including in mitochondria (m-GPX4), where there is a high preponderance of ROS and oxidizable polyunsaturated PLs.

Unlike catalase, GPX relies on glutathione (γ-glutamylcysteinylglycine, GSH) tripeptide to reduce H_2O_2 or organic peroxides. Mechanistically, GPX4 and other selenocysteine GPXs utilize a two-stage ping-pong mechanism. The first step reduces the target lipid peroxide (L-OOH → L-OH) and oxidizes GPX's selenocysteine residue. In step two, GSH is oxidized to regenerate reduced selenocysteine and active enzyme (Brigelius-Flohé and Maiorino, 2013). Glutathione reductase then converts oxidized GSH (GSSG dimers) back into free GSH using reducing potential derived from NADPH. Alternatively, GSH-mediated ROS detoxification may also proceed non-enzymatically. In this case, GSH reacts with hydroxyl radical, nitric oxide, or superoxide radical (Dringen, 2000) producing the strongly oxidizing thiyl radical ($GS^{\bullet}$). The reactive thiyl radical is a liability for cells. Therefore, this non-enzymatic

mechanism is likely not the primary pathway of GSH-mediated protection, but thiyl radical abundance, measured by electron paramagnetic resonance spectrometry (EPR), can reflect the cellular redox state (Sagristá et al., 2002; Sturgeon et al., 1998).

Synthesis of GSH occurs only in the cytosol and is then imported into most organelles (Griffith and Meister, 1985). GSH is required for many vital functions: **1**) modulating protein s-glutathionylation (Dalle-Donne et al., 2009); **2**) supplying cysteine for protein synthesis (Meister and Anderson, 1983); **3**) DNA synthesis (Suthanthiran et al., 1990); **4**) immune response (Furukawa et al., 1987); **5**) metal ion (iron) storage and homeostasis (Jozefczak et al., 2012); **6**) maintaining protein redox states (e.g. glutaredoxins); and **7**) providing an antioxidant defense (Lu, 2009). As mitochondria are the primary ROS sources within cells, a correspondingly significant proportion of GSH is partitioned to mitochondria (10–15% total cellular GSH) (Griffith and Meister, 1985). Import is accomplished by two IMM membrane anion transporters, the dicarboxylate carrier (DIC, SLC25A10) and the 2-oxoglutarate carrier (OGC, SLC25A11) (Lash, 2006; Meijer et al., 1972). Further, while final GSH synthesis takes place in the cytosol, import of the GSH precursor, cystine (Cys), by the cystine/glutamate antiporter (system Xc^-, SLC7A11) is regulated by cytosolic glutamate (Glu) concentration. A high cytosolic-to-extracellular Glu gradient promotes cystine uptake and downstream GSH synthesis. Cytosolic Glu levels are, in turn, controlled by mitochondrial metabolism and glutaminolysis. Within the mitochondrial matrix, glutamine (Gln) is converted to Glu by glutaminase (GLS). Glu maybe then transaminated into TCA cycle intermediates (e.g. α-ketoglutarate) by glutamate dehydrogenase (GDH) or by the alanine/aspartate transaminases (TAs) (Watford, 2000). Therefore, the levels of cytosolic Glu and Cys are ultimately influenced by the activity of the mitochondrial TCA cycle and specifically, GDH and TA. GSH insufficiency leads to inactivation of GPX4/GSH system and accumulation of peroxidated PLs (PL-OOH). Accumulation of PL-OOHs – particularly peroxidized phosphatidylethanolamines (PEox) – in the setting of GPX4 inhibition (or GSH depletion) results in ferroptotic cell death (Kagan et al., 2017). This was observed following experimental TBI (Kenny et al., 2019). Therapeutically administered glutathione precursors such as *n*-acetylcysteine (NAC) and a more CNS bioavailable version, *n*-acetylcysteine amide (NACA), have demonstrated promise in experimental TBI and spinal cord injury (SCI) (Pandya et al., 2014; Xiong et al., 1999). A small double-blind, placebo controlled clinical study showed that early NAC administration (<24 h) following blast-induced mild TBI was linked to reduced neuropsychological deficit and greater rate of symptom resolution by 7-days post-TBI (Hoffer et al., 2013). Safety and efficacy studies of NAC and NACA in treatment of TBI are ongoing (Clark et al., 2017). In summary, mitochondrial metabolic activity influences both cytosolic Glu and Cys levels and, in turn, regulates GSH production, corresponding GPX activity, and related regulated cell death signaling in TBI.

1.3: TBI Mitochondrial Pathology Overview

Beginning in hours and continuing for weeks, the secondary phase of TBI is marked by excitotoxic, pro-death, and inflammatory responses (Prins et al., 2013). Diffuse depolarization of neurons occurs in the hyperacute stage of injury (first 24 hours after inciting incident). This event results in an unregulated release of excitatory

neurotransmitters, primarily glutamate and aspartate (Fig. 1) (Bullock et al., 1998; Faden et al., 1989). Increased levels of extracellular glutamate can last as long as 4 days with initial glutamate levels (first 24 hours, >20 μmol/L) and concentration positively correlating with worse outcomes (Chamoun et al., 2010). Excitotoxic injury results from overactivation of N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) receptors, and various-gated Na^+ and Ca^{2+} channels, which leads to a swift rise in neuronal cytosolic Ca^{2+} concentration (Arundine and Tymianski, 2004). Increases in cytosolic Ca^{2+} activate and are potentiated by Ca^{2+}-dependent phospholipases and proteases. Examples of the latter include calpains and downstream caspases that induce either necrotic and apoptotic death (Jastroch et al., 2010). If death is not outright, Ca^{2+} is buffered by mitochondrial-associated endoplasmic reticulum and mitochondria using various transporters, including: mitochondrial calcium uniporter (mCU), uncoupling proteins (UCP), H^+/Ca^{2+}, and Na^+/Ca^{2+} exchangers (Williams et al., 2013). Calcium influx is associated with an early transient $\Delta\psi_m$ hyperpolarization that drives increased ROS and RNS production. RNS levels are particularly increased due to the upregulation of inducible nitric oxide synthase early after TBI (Bayır et al., 2005). ROS/RNS modification of nuclear and mitochondrial DNA, lipids, and proteins further exacerbates the injury and interferes with recovery mechanisms (Opii et al., 2007). Therefore, the initial hyperpolarization exacerbates injury and gives way into depolarization. Finally, $\Delta\psi_m$-reliant processes fail and assembly of the mitochondrial permeability transition pore (mPTP) begins.

mPTP formation is the point of no return and when $\Delta\psi_m$ loss becomes irreversible (Szabó and Zoratti, 1991). The exact constituents of the mPTP are still debatable, but three primary suspects have been described (Fig. 1) (Halestrap, 2009). The first component is adenosine nucleotide transporter (ANT), a thiol-containing IMM protein. In its oxidized state, ANT binds cyclophilin D (CyD) and possibly other mitochondrial proteins. It is believed that ANT, CyD, and OMM-localized VDACs form the mPTP complex (Tsujimoto and Shimizu, 2007). ATP synthase subunit c may also participate in the formation of the mPTP conduit (Giorgio et al., 2013; Neginskaya et al., 2019). However, its precise role in the mPTP complex remains controversial; recent studies indicate mPTP formation persist despite mutations in ATP synthase subunits (He et al., 2017). The final mPTP complex creates continuous connection bridging the mitochondrial matrix, IMM and cytosolic space. This permits the release of mitochondrial proteins, such as cyt *c*, SMAC/diablo and apoptosis-inducing factor (AIF), that trigger pathways of caspase-dependent and -independent death (Galluzzi et al., 2009). Further OMM permeabilization occurs secondary to VDAC and other OMM proteins conformational changes induced by BCL-2-associated X protein (BAX) and BCL-2 antagonist or killer (BAK) that lead to the formation of the mitochondrial outer membrane permeabilization (MOMP) channel (Tait and Green, 2010).

Together, the formation of MOMP and mPTP conclusively deplete $\Delta\psi_m$ and eventually lead to osmotic swelling then lysis of mitochondria (Kalkavan and Green, 2018; Kitsis and Molkentin, 2010). Consequently, IMS proteins are released into the cytosol. Interactions between the released cyt *c*, apoptotic protease activating factor (Apaf-1) and ATP yields apoptosomes that active caspase 3 and downstream caspases (Ola et al., 2011). In summary, the combined inhibition of mitochondrial bioenergetic function leads to GSH depletion and

ferroptotic cell death while also contributing to the irreversible release of pro-apoptotic proteins that ensures the neuronal death (Kitsis and Molkentin, 2010).

2.0: Mitochondrial Lipid Redox Signaling

2.1: CNS Pathology & Lipids Overview

Abnormalities in lipid abundance and metabolism are observed in numerous acute and chronic neurological diseases. Brain characteristically is enriched with oxidizable polyunsaturated lipids and overall possesses a high lipid-to-protein ratio (O'Brien and Sampson, 1965). The advent of high fidelity liquid chromatography tandem mass spectrometry (LC-MS/MS) lipidomics methods has aided the recognition of signaling roles lipids play in the CNS (Anthonymuthu et al., 2018). With these advances, the extent and consequences of PL oxidation in CNS insults have begun to emerge. For example, analysis of CSF PLs from ten TBI patients showed a distinct difference in the speciation, time course, and abundance of PL between those that survived vs succumbed to their injuries (Pasvogel et al., 2010). Similarly, levels of polyunsaturated free fatty acids, namely arachidonic, docosahexaenoic, and linoleic acids, were found to be increased in CSF of TBI patients compared to healthy controls (Pilitsis et al., 2003). In acute cerebral ischemia, membrane PL alterations are among the earliest pathological events observed (Bazan and Rodriguez de Turco, 1980). Plasma levels of the global lipid peroxidation marker, F2-isoprostane (F2-isoP), correlates with the infarct growth in stroke (Lorenzano et al., 2018). Even chronic conditions such as Alzheimer's disease (AD) display altered levels of PL, ceramides, and sulfatides within the brain, CSF, and blood (Kosicek and Hecimovic, 2013). The following sections will focus on the contributions of mitochondrial PL signaling and injury in neurological diseases, including TBI.

2.2: Mitochondrial Lipid Composition

Every organelle has its own unique lipid signature that is essential for its structure and function (van Meer et al., 2008). Like other organelles, mitochondrial membranes are largely composed of phospholipids (PL) with the following unique characteristics: **1**) mitochondria are almost devoid of sphingolipids, phosphatidylserine (PS), and sterols; **2**) they have a high protein-to-phospholipid ratio compared to other organelles; **3**) the mitochondria-specific PL, cardiolipin (CL), represents 15–20% of all mitochondrial lipids; **4**) mitochondrial PLs are enriched with unsaturated and oxidizable fatty acyl chains (Daum, 1985). Mitochondrial membrane PLs are asymmetrically distributed across the OMM and IMM (Daum and Vance, 1997). In particular, CL and phosphatidylinositol (PI) are significantly enriched within the inner leaflet (matrix side) of the IMM, whereas phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are randomly distributed across the IMM and OMM (Horvath and Daum, 2013). Lipid exchange between mitochondria and smooth endoplasmic reticulum (sER) is critical for import and overall maintenance of mitochondrial lipid composition (Tatsuta et al., 2014). For example, PS transferred from sER to mitochondria is rapidly decarboxylated to PE in mitochondria. Mitochondria contain synthetic machinery to produce four major classes of glycerophospholipids, namely: CL, PE, phosphatidic acid (PA), and phosphatidylglycerol (PG) (Flis and Daum, 2013; Vance and Vance, 2004).

CL is the one true mitochondria-specific lipid class. Canonical mitochondrial CL synthesis involves two key phases: 1) *de novo* CL synthesis via cardiolipin synthase that produces hetero-acylated, nascent-CL; and 2) secondary remodeling steps carried out by phospholipid-lysophospholipid transacylases, such as Tafazzin, that convert hetero-acylated CLs to homo-acylated, mature CL (Schlame, 2008). The identity of CL's new acyl chain depends on both the transacylase specificity and PUFA abundance. Apart from the cerebellum, the brain expresses relatively little Tafazzin and has a correspondingly heterogenous CL population vs most other tissues (e.g. heart). Complete loss-of-function mutations in the gene which encodes Tafazzin (TAZ) results in a rare X-linked disorder called Barth Syndrome (BTHS). Barth syndrome is best known as a cardiac disease but has been acknowledged as a multi-syndrome disorder, including the CNS causing various cognitive deficits. Mice that contain mutated/knockdown TAZ have decreased CL – most prevalent in long-chain polyunsaturated fatty acids (PUFA) (22:6, 22:5 and 22:4). Further, loss of intact CL was matched by accumulation of monolyso-CL (mCL) (19-fold increase observed in whole brains from TAZ knockdown vs wild-type mice), which were enriched in short-chain saturated 16:0/16:1 and 18:0/18:1 fatty acids (Cole et al., 2018). The result was CNS mitochondria from these mice showed an increased state I respiration, attenuated spare capacity, and increased ROS generation. While motor function is retained, defects in memory retention in line with hippocampal degeneration are observed (Cole et al., 2018). These findings demonstrate how proper CL speciation and remodeling is critical for normal brain mitochondrial function and memory.

Though the results from many studies linked neurological diseases with altered mitochondrial lipid regulation, the precise roles of mitochondrial lipids and their oxidation products long remained unclear (Nakahara et al., 1991). The recent advent of high-fidelity liquid chromatography-tandem mass spectrometry (LC-MS/MS) lipidomics approaches has allowed for study of low abundance but important mitochondrial signaling lipids, like cardiolipin. These new approaches have provided a wealth of information on mitochondria-specific lipid injury during neurological diseases.

2.3: Linking CNS Injury & Mitochondrial Lipid Perturbations

Both acute and chronic CNS disorders commonly lead to increased ROS/RNS levels in mitochondria (Salim, 2017). This, combined with abundance of highly oxidizable polyunsaturated acyl chain-containing PLs, creates an ideal environment for lipid peroxidation in mitochondria (Vladimirov et al., 1980). Throughout the cell and in mitochondria, polyunsaturated PL peroxidation proceeds either non-enzymatically through a transition metal ion (iron/copper)-initiated free radical reaction or enzymatically catalyzed by cytochrome *c*, cyclooxygenases (COX) and lipoxygenases (LOX). Compared to the free radical mechanisms, enzymatic oxidation generates specific, structurally-defined PL peroxidation products that serve as specific downstream signals (Niki, 2009). If not rapidly reduced by the GPX4/GSH system, PL-OOH species break down non-enzymatically to yield a suite of small reactive electrophiles. These reactive electrophiles are a liability, and unabated, detrimentally alkylate DNA (nuclear & mitochondrial) and key nucleophilic protein residues.

One breakdown product of PL oxidation, 4-hydroxynonenal (4-HNE), is particularly well documented as increased in both acute and chronic CNS pathologies (Liu et al., 2011; Zhong and Yin, 2015). 4-HNE is a potent protein-modifying electrophile that inhibits mitochondrial respiration (Vaishnav et al., 2010). Studies in controlled cortical impact (CCI) and contusion spinal cord injury (SCI) showed increased mitochondrial 4-HNE beginning within 3 hours and lasting at least 72h after injury (Carrico et al., 2009; Singh et al., 2013; Sullivan et al., 2007). Likewise, modification of the ETC complex V, ATP synthase α subunit by 4-HNE in the entorhinal cortex of Braak stages I/II Alzheimer's (AD) patients suggests the involvement of mitochondrial lipid peroxidation in AD neurofibril formation (Siegel et al., 2007; Terni et al., 2010). In Parkinson disease (PD), 4-HNE has been detected in Lewy bodies. Increased accumulation of 4-HNE alkylation products is linked to development of mitochondrial dysfunction in parkin-deficient mice (Palacino et al., 2004). Similar observations have been made in the Huntington disease models as well (Lee et al., 2011). Although 4-HNE is a good marker of *global* lipid peroxidation, it is nonspecific and cannot provide conclusive information about the identity of its upstream parent oxidized lipid. This is an important consideration because understanding the identity of the intact parent lipid can reveal the localization, drug targeting, and potential signaling roles of oxidized lipids in both pathologic and reparative processes.

2.4: Enzymatic Cardiolipin Oxidation in TBI

The specific signaling roles of oxidized mitochondrial lipids in CNS pathology have been well demonstrated for CL. Alterations in CL metabolism show the best link between neuronal injury and druggable enzymatic mitochondrial lipid oxidation (Fig. 2). Selective oxidation of brain CL was observed in TBI models starting from 3h post-injury (Bayir et al., 2007; Chao et al., 2018). Upon injury, the asymmetric distribution of CL collapses and cytochrome *c* (cyt *c*) can interact and bind CL (Chao et al., 2019). The initial interaction is via the electrostatic interface of the positively charged lysines in cyt *c* and two negatively charged phosphates of CL (Kagan et al., 2009; Kooijman et al., 2017). This interaction leads to a considerable disruption of the native hexa-coordinated compact structure of the protein. The notable conformational effects are: **1**) opening of the heme crevice; **2**) decreased hydrophobic core volume; **3**) disruption of the heme iron (Fe) to Met80 bond; **4**) distal movement of Trp59 from the heme leading to change in the protein tertiary structure; **5**) generation of a mix of penta-coordinated and His33/His26 hexa-coordinated heme (Droghetti et al., 2006); **6**) decreased Fe(II)/Fe(III) couple redox potential that is ~400 mV more negative than native cyt *c*, meaning it can no longer shuttle electrons for the ETC (Barker et al., 1996; Di Marino et al., 1987); and **7**) acquisition of peroxidase activity. Breaking of the Met80-Fe bond further permits access of substrate into the newly formed peroxidase active site. The newfound peroxidase activity relies on several residues, including His26, His33, Arg38, and Arg91 that aid in the H-bonding and proton shuttling (McClelland et al., 2014), as well as four tyrosine and a tryptophan residue that serve as radial intermediates in the peroxidase catalytic cycle (Tsong, 1976). Heme-containing peroxidases can oxidize substrates using different inorganic (e.g. H_2O_2) or, more rarely, organic peroxides (e.g. lipid hydroperoxides). The Cyt *c*/CL complex has superior lipid hydroperoxidase activity compared to its H_2O_2 peroxidase activity. It has an affinity for anionic lipid substrates, including oxidized CL (CL-OOH, CLox). In turn, CLox is a good

source of oxidizing equivalents for membrane-associated cyt *c*/CL (Kagan et al., 2009). CL is most enriched near ETC Complex I, III, and IV – potent sources of pathology-induced H_2O_2 production. Therefore, the proximity, abundance, and electrostatic affinity of CL to the newly formed cyt *c*/CL peroxidase enzyme makes CL a preferred lipid peroxidation substrate (Fig. 2) (Ardail et al., 1990). CL peroxidation by cyt *c* in the presence of mitochondrially-generated H_2O_2 prompts pro-apoptotic mitochondrial permeabilization and cytosolic cyt *c* release (Kagan et al., 2005). Alternatively, the liberation of CL's oxidized acyl chains through the action of phospholipases (e.g. iPLA2γ) yields a suite of pro- and anti-inflammatory signaling molecules.

2.5: Inflammatory & Pro-coagulant Roles of Cardiolipin in TBI

Apart from signaling apoptosis, CLox is also involved in the generation of various lipid mediators (Fig. 2). Hundreds of unique oxidized cardiolipins species are produced by the cyt *c*/CL complex after TBI (Ji et al., 2012). They are then hydrolyzed by mitochondrial calcium-independent phospholipase A2 (iPLA2γ) (Tyurina et al., 2014). Hydrolysis of CLox following experimental TBI produces a plethora of well-characterized inflammatory mediators such as hydroxyoctadecadienoic acids (HODEs), hydroperoxyoctadecadienoic acids (HpODEs), and hydroxyeicasotetraenoic acids (ETEs) (Fig. 2) (Liu et al., 2017). These mediators are known for regulating pro- and anti-inflammatory responses (Dennis and Norris, 2015). For example, 9-HODE, 12-HETE are involved in the pro-inflammatory response such as the recruitment of leukocytes (Vangaveti et al., 2010). In contrast, 13-HODE, 15-HETE, 14,15-epoxyeicasatrienoic acid (EpETrE) and 11,12-EpETrE are known to promote anti-inflammatory responses (Roman, 2002). Levels of these pro and anti-inflammatory signals increase by 4h after CCI, fitting well with the timeline of CL oxidation (Anthonymuthu et al., 2017). Treatments targeting CL oxidation or CL hydrolysis attenuate neurodegeneration and improve neurocognitive outcome after CCI (Chao et al., 2018; Ji et al., 2012). These data suggest that both oxidation and hydrolysis of CL play important roles in neuronal injury response to TBI.

Brain-derived CLs are released into systemic circulation acutely following TBI (Anthonymuthu et al., 2019a). Some of the released brain CLs are likely present in the form of brain-derived microparticles (Zhao et al., 2016). However, the specific identities and oxidation states of various CLs in these microparticles has not been analytically verified (Fig. 2). Surface exposure of anionic phospholipids, (oxidized) PS and CL, promotes phagocytosis of cells and particles by macrophages and microglia (Balasubramanian et al., 2015). Additionally, CLs and PS present in microparticles are potent anti-coagulation factors contributing to trauma-associated coagulopathy (Midura et al., 2015; Tian et al., 2015). Coagulopathy is common in TBI patients (Stein and Smith, 2004), and it is associated with poor outcome (Sun et al., 2011). Thus, prevention of coagulopathy may be a potential therapeutic target in TBI. In line with this, induction of phagocytotic clearance of brain-derived microparticles by apoptotic cell-scavenge factor lactadherin was shown to prevent TBI-Induced coagulopathy and improve outcome in rodents (Zhou et al., 2018b). In most cases, this will lead to an anti-inflammatory phenotype, but it has also been suggested that clearance of brain-derived microparticles by microglia produces a secondary pro-inflammatory response following intracerebral injury (Kumar et al., 2017). With regards to

extracellular CL, it causes immune-paralysis of innate immune cells by preventing the formation of TLr4-Md2 hetero-oligomeric complex required for activation of macrophages/microglia to produce and release cytokines (Balasubramanian et al., 2015).

2.6: Release of Cardiolipin in Systemic Circulation

TBI is accompanied by a reduction of CL content in the contusional and peri-contusional regions (Chao et al., 2018; Sparvero et al., 2016). Despite mounting evidence suggesting the release of CL into the systemic circulation, identification of CL in plasma was not possible until recently. Technical limitations prevented accurate identification and speciation of low abundance CL products circulating in plasma following CNS injury. Exploiting the power of mass spectrometry-based lipidomics approach; we recently developed a sensitive LC-MS/MS method for the identification of circulating CL. The brain expresses a uniquely diverse CL profile, rich in long-chain PUFA, due to its relatively low CL remodeling activity. In comparison, other tissues express a relatively homogenous and lower molecular weight CL population, dominantly composed of tetralinoleic CL (TLCL). Therefore, the appearance of brain-type CL in plasma early after TBI was hypothesized to correlate with brain injury severity. Moreover, the approach may provide benefit in cases of poly-trauma where brain-type CL (and associated CNS injury) can be distinguished from CL released from non-CNS tissue. We found increased plasma brain-type CL content after clinical or experimental cardiac arrest-induced ischemic brain injury and CCI in rats (Anthonymuthu et al., 2019a). In animal models of global cerebral ischemia and trauma, the release of brain-specific CL into systemic circulation correlated with the loss of brain CL. Plasma concentration of brain-type CL sampled within 6 hours post-cardiac arrest correlated with the brain injury severity and long-term outcome in a cohort of 41 patients (Anthonymuthu et al., 2019b). Therefore, detection and quantification of plasma CL levels have potential utility as an early marker of traumatic (or ischemic) brain injury severity and therapeutic response.

2.7: Therapeutic Targeting of Mitochondrial Phospholipid Oxidation

The various pathologic effects of mitochondrial PL oxidation make therapeutic targeting of these pathways an exciting avenue for TBI research. By virtue of their lipophilic nature, many of the antioxidants are effective in preventing mitochondrial PL peroxidation without specific mitochondrial targeting (Maiti et al., 2018). However, previous TBI studies showed non-targeted global antioxidants administration (e.g. vitamin E, CoQ_{10}, or nitroxyl radicals) provided little to no benefit on survival or neurocognitive function. This failure was likely, in part, due to lack of outcome specificity (i.e. understanding the source/localization/identity of oxidized PL), poor localization of therapeutic compound to the pathogenic target site, and/or deleterious extramitochondrial off-target and side-effects (Lamade et al., 2019). These deficiencies have been addressed by **1**) LC-MS/MS based lipidomics have allowed deeper understanding of TBI's effects on mitochondrial lipids and their redox states, and **2**) by the use of specific mitochondria-targeted antioxidants and enzyme inhibitors that overcome localization concerns. Mitochondrially-targeted therapeutics have shown preclinical success in both ischemic and traumatic brain injury. They are composed of two key components: **1**) a mitochondrial targeting/binding moiety; and **2**) an antioxidant, electron scavenger, or enzyme inhibitor moiety. Lipophilic but positively charged molecules, such as triphenyphosphonium ion (TPP^+), are drawn to mitochondria's highly negative $\Delta\psi_m$ matrix.

Based on the Nernst equation, passively transported cations accumulate 10x per 61 mV trans-membrane potential difference at 37°C. For neurons with a membrane resting potential of −70 mV, cation-conjugated drug accumulates 5 to 10-fold in cytosol vs extracellular fluid and 100 to 500-fold more in mitochondria vs cytosol at equilibrium. Two key examples of $\Delta\psi_m$-driven mitochondria-localizing drugs that showed promise in TBI are Mito-Q (TPP-CoQ_{10}) and SS-31 (Zhu et al., 2018). Treatment of mice subjected to a weight drop model of TBI with Mito-Q was shown to improve neurological deficits, alleviate brain edema and inhibit cortical neuronal apoptosis. This improvement was associated with the reduction in levels of nonspecific lipid peroxidation end-product (e.g. malondialdehyde) and greater mitochondrial membrane integrity. These together point towards likely inhibition of mitochondrial lipid peroxidation by Mito-Q (Zhou et al., 2018a). The results of the Mito-Q clinical trials in Parkinson's Disease have shown Mito-Q to be nontoxic, even at high concentrations (Snow et al., 2010). The drawback of $\Delta\psi_m$-reliant strategies is that pathology (i.e. TBI) can significantly dissipate $\Delta\psi_m$, causing a significant reduction in drug localization efficiency. Moreover, movement of TPP^+ and other cations into the matrix necessarily dissipates $\Delta\psi_m$, conceivably exacerbating injury at high concentrations. In contrast, development of $\Delta\psi_m$-independent mitochondrial localization strategies can minimize these concerns while improving chances for consistent, efficacious therapeutic effects. This alternate strategy takes advantage of a targeting moiety's affinity towards mitochondria-specific/enriched proteins or lipids. For example, the short peptide, hemigramicidin (HS, a derivative of gramicidin S), enriches in mitochondria >100-fold. This effect is likely propelled by HS's affinity to IMM-localized CL rather than $\Delta\psi_m$.

One particularly promising example of this alternative strategy is the mitochondria-targeted nitroxide is XJB-5–131. It is comprised of a hemigramicidin (HS) peptide conjugated to TEMPO. The HS-TEMPO conjugate can cross the intact blood-brain barrier while TEMPO by itself cannot (Ji et al., 2012). XJB-5–131 has been shown to attenuate lesion volume and improve behavioral deficits following experimental TBI. Mechanistically, XJB-5–131 and the structurally similar JP4–039 act as electron scavengers to prevent the formation of $O_2^{\bullet-}$ and H_2O_2. In the context of mitochondrial lipid peroxidation, reduced mitochondrial ROS limits substrate for the cyt *c*/CL peroxidase complex resulting attenuation of CLox generation and CL loss. Alternative strategies that decrease CL oxidation include mitochondrial-targeted imidazole-conjugated fatty acids that inhibit the peroxidase activity of the cyt *c*/CL complex (Kagan et al., 2015); or mitochondria-targeted oleic or stearic acids that promote remodeling of the CL lipidome into a less oxidizable form rich in mono-unsaturated fatty acids. The latter remodeling approach has shown potential in various disease states where mitochondrial generation of ROS is a key in the pathophysiology of the disease (Atkinson et al., 2011; Jiang et al., 2014; Tyurina et al., 2012). Further development and clinical translation of mitochondria-targeted therapies will require detailed studies of unique pharmacodynamic and pharmacokinetic properties of these compounds.

3.0: Mitochondrial Quality Control

3.1: Mitochondrial Fate Decisions

Mitochondrial injury following TBI produces a litany of pro-survival and death effects, discussed previously. In reaction, the mitochondrial network is dynamically regulated to preserve functional mitochondrion units while removing potential liabilities. To maintain homeostasis, the mitochondrial quality control system (MQC) governs the opposing pathways of mitochondrial fission and fusion. Broadly, fusion is a consolidating force. It enables salvage of intact, functional mitochondrial components to increase cristae density and maximize bioenergetic function necessary for post-injury survival and eventual recovery. Conversely, fission has two divergent roles: removal of damaged, dangerous mitochondrion or proliferation and expansion of the mitochondrial network (Youle and van der Bliek, 2012). Mitophagy is responsible for the ultimate recycling of fissioned, dysfunctional mitochondria. An organelle-specific example of autophagy, mitophagy orchestrates the bulk turnover of mitochondria through an autophagosomal-lysosomal mechanism. Mitophagy provides potential benefits to injured neurons by reducing the mitochondrial contribution to vicious redox cycles, including pathways governed by oxidative lipid signaling (Chao et al., 2019; Diskin et al., 2005). However, there is a delicate balance between mitophagy's beneficial effects vs harm – the latter harm stems from potentially overzealous elimination of pro-survival mitochondrial functional capacity. MQC system dysfunction contributes to multiple acute and chronic brain pathologies, including in traumatic and ischemic brain injury (Lipinski et al., 2015; Sarkar et al., 2014).

3.2: Mitochondrial Fusion

Mitochondrial fusion is a coordinated process involving the discriminative union of inner and outer mitochondrial membranes, as well as the contents of the intermembrane space and matrix. Once initiated, mitochondrial merging can proceed rapidly over several minutes (Jakobs et al., 2003; Twig et al., 2008), but the process maintains sufficient fidelity to ensure compartment-specific transfer of mitochondrial DNA, proteins, lipids, and metabolites. This autonomous mechanism can proceed in the absence of active nuclear instructions or *de novo* translation (Pernas and Scorrano, 2016). Fusion pathways are fundamentally conserved in eukaryotes (Okamoto and Shaw, 2005). Loss of fusion function is incompatible with life and leads to embryonic lethality in mice (Chen et al., 2003). Fusion is also a selective process with most mitochondrial contacts not resulting in union (Twig et al., 2008). Suitability of fusion candidates is dictated by $\Delta\psi_m$ with depolarized mitochondria being unlikely to fuse compared to their more functional counterparts. Several studies have demonstrated that uncoupling drugs not only increase mitochondrial fragmentation (fission) but also arrest fusion (Ishihara et al., 2003; Malka et al., 2005). This selective mechanism may help ensure that only healthy mitochondrial fragments are reconsolidated after TBI (Wu et al., 2016) and other acute/chronic neurodegenerative conditions (Knott et al., 2008).

At the center of both fusion and fission pathways are several highly conserved GTPases. These 'large GTPases' are distinguished from "small regulatory" GTPase (e.g. Ras-like regulatory GTPases or α-subunits of heterotrimeric G-proteins) not only by their size, but also, by their **1**) oligomerization-dependent GTPase activation, **2**) low GTP-binding

affinities, **3**) self-oligomerization capacity, and **4**) affinity for lipid membranes (Praefcke and McMahon, 2004). The three critical GTPases of mitochondrial fusion are the Mitofusins (MFN1 and MFN2) and dominant optic atrophy 1 (OPA1). MFN1 and 2 regulate the first key steps of OMM fusion. As fusion-destined mitochondria approach, OMM-anchored MFNs dimerize and bridge the (~20 nm) gap between membranes. Subsequent GTP binding and hydrolysis induces a conformation change in the MFNs, bringing the outer membranes into closer proximity (Fig. 3A) (Cohen and Tareste, 2018). The lipid bilayers must then be integrated. This is again facilitated by MFNs, specifically the HR1 (alternatively, HB1) and HR2 domains. These domains were shown to facilitate the fusion of OMM-like PC liposomes *in vitro* and were required for mitochondrial fusion in cells (Daste et al., 2018). Once brought in proximity by MFNs' GTPase activity, HR1 helps disrupt the OMM stability to allow integration of the two mitochondria. OMM regions containing lipid packing defects are most susceptible to HR1. These regions contain increased content of cone-shaped lipids, such as PE, which aid HR1 disrupt the bilayer and induce joining. Other bulky mitochondrial lipids may also play a role in fusion, such as PA and CL. Interestingly, while most CL is IMM-localized, TBI induces phospholipid scramblase 3 (PLS3)-mediated CL translocation to the OMM where it has been implicated in mitophagy, discussed below. Moreover, CL content is enriched at OMM-IMM contact sites (Paradies et al., 2014), a proposed nidus for final membrane fusion (Fritz et al., 2001). Changes in OMM lipid composition due to TBI-induced PL loss or oxidation (which changes lipid geometry and packing efficiency) likely serve as an additional regulatory checkpoint in fusion.

Relatively less information is known about the coordination of IMM fusion beyond the role of OPA1, which is present in eight isoforms (Del Dotto et al., 2018). Deletion of OPA1 **1**) decreases mitochondrial network connectivity (increased fragmentation), **2**) leads to disorganization of cristae membranes (Frezza et al., 2006), and **3**) overall decreases mitochondrial function ($\Delta\psi_m$) (Di Pietro et al., 2017). Conversely, overexpression confers protection in neurodegenerative disease models (Ramonet et al., 2013). OPA1 is localized to the IMM, directed by a conventional $\Delta\psi_m$-dependent N-terminal mitochondrial targeting sequence and affinity for IMM-localized CL (Liu and Chan, 2017). At the IMM, it is renatured into its membrane-bound long isoform (L-OPA1). Further proteolytic processing may occur, which produces the soluble short OPA1 isoform (S-OPA1). Only the L-OPA1 isoform is required for stress-induced mitochondrial fusion (Song et al., 2007). In contrast, both the S- and L-OPA1 appear to contribute to cristae organization in unstressed conditions at a 1:1 ratio (Ishihara et al., 2006). Stress-induced (decreased $\Delta\psi_m$ and reduced ATP levels) activation of OPA1 proteases (i.e. OMA1, YME1), enhances S-OPA1 production and depletes L-OPA1 (Alavi and Fuhrmann, 2013). Upregulation and multimerization of S-OPA1 further inhibits mitochondrial fusion and promotes OMM permeabilization. Therefore, the fusion activity is reduced in unhealthy mitochondria – including acutely after TBI (Fischer et al., 2016). Indeed, persistent depolarization and energetic failure simulate the core fission GTPase, dynamin-related protein-1 (DRP1), and eventual mitophagy.

3.3: Fission and Mitophagy

Fission pathways cause mitochondrion to fragment. In unstressed cells, fission supports mitochondrial proliferation and regionalization. This enables both the proper and persistent

distribution of healthy mitochondria across the cell, including after injury. For example, following diffuse axonal injury, newly created mitochondria are segregated and then transported through repaired axons to sites of need (boutons, terminal synapses). Conversely, under active stress conditions, fission enables the mitophagy-mediated elimination of dangerous, dysfunctional mitochondrial components. It may also promote cytosolic release of pro-apoptotic factors (Estaquier and Arnoult, 2007). The large GTPase, DRP1, drives fission by mechanically constricting and severing both the OMM and IMM (Fig. 3B). Fission is regulated at both the level of DRP1 activity (Taguchi et al., 2007) and its docking with mitochondria. DRP1 lacks a membrane-binding domain and is recruited to the mitochondrial membrane with assistance by several adaptor proteins: mitochondrial fission 1 (Fis1), mitochondrial fission factor (MFF), and mitochondrial elongation factors 1 & 2 (MIEF1, MIEF2). Their precise mechanisms and relative contributions to DRP1-mediated fission remains unsettled (Otera et al., 2010; Samangouei et al., 2018; Zhao et al., 2011). More recent studies suggest the critical role of OMM lipid composition in anchoring and activation of both DRP1 and its adaptors (Macdonald et al., 2014). Notably, increased OMM CL content has been linked to increased fission activity (Bustillo-Zabalbeitia et al., 2014; Francy et al., 2017). As discussed previously, CL is externalized from the IMM to OMM with TBI (Chao et al., 2019; Chu et al., 2013; Ji et al., 2012). Conversely, increased PA abundance suppresses DRP1-driven fission (Adachi et al., 2016; Kameoka et al., 2018). Once membrane-bound and active, electron microscopy studies of yeast demonstrated that DRP1 forms large spiral structures (average 60 nm radius) (Mears et al., 2011). These DRP1monomers oligomerize into constricting filaments, encircling the mitochondria and breaking it apart (Bui and Shaw, 2013).

Newly generated mitochondrial fragments are then distributed or eliminated. Elimination takes place via mitophagy – the selective autophagic removal of mitochondria. Several different autophagic mechanisms have been described (Glick et al., 2010). The common denominator of these mechanisms is microtube-associated A1/1B-light chain 3 (LC3). More specifically, LC3 can be modified into its active LC3-II form following association with various lipids, including PE. LC3 binding to the OMM mediates the final recognition and targeting of mitochondria to the autophagosome. Mitophagy can proceed via ubiquitin (Ub)-dependent or -independent pathways (Fig. 3C).

First, the prototypical Ub-dependent pathway relies on the binding and activities of phosphatase and tensin homologue (PTEN)-induced punitive kinase 1 (PINK1) and Parkin. In functional mitochondria with normal $\Delta\psi_m$, PINK1 is transported to the IMM where it is proteolytically degraded (Sekine and Youle, 2018). However, under stress conditions (including TBI), membrane potential dissipation inhibits PINK1 import and promotes PINK1 stabilization on the OMM. Subsequent OMM-localized PINK1 and other factors (e.g. BECN1) (Choubey et al., 2014) promote Parkin recruitment and activation (Aguirre et al., 2017). The E3 ubiquitin (Ub)-ligase, Parkin, and PINK1 together phospho-poly-ubiquitinate a range of OMM proteins (Harper et al., 2018; Shiba-Fukushima et al., 2014) in a feed-forward fashion (Ordureau et al., 2014). While not required for mitophagy, fission and DRP1 may serve to help restrict Parkin activity to dysfunctional mitochondrial subdomains (Burman et al., 2017). Conversely, Parkin-mediated mitofusin ubiquitination prevents the reintegration of segregated mitochondria while ubiquitination of Miro (Rho-

GTPase, serving as an OMM-cytoskeleton anchor) inhibits mitochondrial trafficking (Shlevkov et al., 2016). Other E3 ubiquitin ligases also contribute to Parkin-independent pro-mitophagic OMM ubiquitination in a cell- and/or tissue-dependent manner (Palikaras et al., 2018; Szargel et al., 2016). Ultimately, phospho-poly-Ub OMM proteins are recognized by the central autophagic protein, LC3, with assistance by several adaptors (OPTN, NDP52, p62, TAX1Bp1, and NBR1) (Wong and Holzbaur, 2014). Finally, LC3 mediates autophagosome formation and final execution of mitophagy (Tanida et al., 2008).

Second, ubiquitin-independent mitophagy involves direct interaction of LC3 (LC3-II) and OMM proteins or CL. Several OMM proteins possess an LC3-interacting region (LIR) motif. These may directly mediate LC3 mitophagy (Hanna et al., 2012; Liu et al., 2012) while also promoting Parkin-dependent mitophagy (Gao et al., 2015; Yoo and Jung, 2018). Several have been characterized, including in their contributions to TBI-relevant hypoxic injury. They are BNIP3, NIX (Ma et al., 2019), PHB2 (Wei et al., 2017), and FUNDC1 (Chen et al., 2014; Zhou et al., 2017). However, the precise regulation of LC3's interaction with these targets is not fully understood. Like its roles in both fusion and fission, CL also serves to mediate LC3-mediated mitophagy (Chu et al., 2014). Externalization of the CL from the inner leaflet of the IMM to the surface of the OMM functions as a mitophageal signal recognized by the LC3. Two translocations occur during externalization of CL from inner leaflet of IMM to the outer leaflet of the OMM. The first one is from the inner leaflet to the outer leaflet of IMM catalyzed by PLS3. The second one is from the outer leaflet of IMM to the inner leaflet of the OMM. The hexameric intermembrane space protein, NDPK-D (or NM23-H4), is responsible from translocation of CL from IMM to the OMM (Fig. 2) (Kagan et al., 2016). A R90D NDPK-D mutant that does not bind CL is inactive in promoting mitophagy. Furthermore, mitophagy-inducing CL-transfer activity of NDPK-D is closely associated with the dynamin-like GTPase OPA1, implicating fission-fusion dynamics in mitophagy regulation. Mitochondrial depolarization secondary to TBI prompts phospholipid scramblase 3 (PLS3) activation and externalization of CL from the IMM inner leaflet to the outer leaflet of the OMM. Externalized CL interacts with the terminal helices of LC3 and aids recruitment of the autophagosome (Chao et al., 2019). Knockdown of PLS3 or inhibition of CL synthesis inhibits TBI-induced mitophagy (Chao et al., 2019). The diverse suite and interactions of receptor and adaptor molecules participating in mitochondrial quality control and mitophagy highlights the fine control necessary to maintain cellular homeostasis.

3.4: Pharmacologic Targeting Mitophagy in TBI

The mitochondrial quality control system and mitophagy are indispensable for homeostasis and restoration of function following TBI. Over- or underutilization of these systems may worsen outcomes. A delicate balance is required, making development of clinical therapies targeting mitophagy, and more generally, autophagy, challenging. Further confounding clinical translation, the protective or harmful effects of mitophagy-targeted therapies may be highly dependent on injury severity and time course. Specific and non-toxic mitophagy-inducers have not been well described. Melatonin-mediated upregulation of mitophagy was associated with protection following subarachnoid hemorrhage (Cao et al., 2017; Wang et al., 2018), commonly seen with TBI. Conversely, inhibition of fission and subsequent

mitophagy via the small molecule DRP1 inhibitor, mitochondrial division inhibitor 1 (Mdivi1), worsened outcomes and increased neuronal injury following CCI (Niu et al., 2019). Other studies showed contradictory results, suggesting Mdivi1-mediated mitophagy inhibition preserved $\Delta\psi_m$ and blood-brain barrier function after TBI (Wu et al., 2018). More generally, autophagy inducers and inhibitors have had mixed results following TBI (Zhang and Wang, 2018). Development of specific small molecule inhibitors of the key players in fusion, fission, and mitophagy is required to better understand the extent, timing, and therapeutic potential of mitophagy modulation after TBI.

4.1: Conclusions

The secondary injury of TBI persists chronically even after the amelioration of the initial acute phase. The consequences of secondary TBI poses a heavy individual and societal burden. Despite the great need, no effective therapies exist. Restoration of mitochondrial function after TBI is necessary to preserve cellular energy production and redox homeostasis. However, preserving mitochondrial function following TBI is challenging due to interaction between the multiple converging pathways that each cause and feed-forward into dysfunction. A combination of several agents that comprehensively cover the complex mechanism may be the optimal but pharmacologically complicated route. The role of mitochondrial redox lipid signaling in development of TBI secondary injury has begun to emerge in recent years. Early accumulation of CLox after CCI is followed by hydrolytic reactions, which produce monolyso-CLs and PUFAox signaling intermediates (Chao et al., 2018). Mitochondrial CLox is directly implicated in triggering and execution of apoptosis (Kagan et al., 2009). It is likely that mitochondria control other programs of regulated neuronal death via indirect but essential metabolic mechanisms. For example, excessive production of H_2O_2 by mitochondria ETC and dehydrogenases creates excessively high peroxidase tone by turning on a variety of GSH-dependent peroxidases, which ends up depleting intracellular GSH. The GSH insufficiency causes inactivation of the GPX4/GSH system inducing alternative cell death mechanisms beyond apoptosis, like ferroptotsis. The direct role of mitochondrial-localized ferroptotic PE oxidation, as well as opposing anti-ferroptotic mechanisms (i.e. ferroptosis suppressor protein 1, FSP1), in TBI-induced neuronal death are emerging and need systematic exploration. Drugs that specifically target lipid localization or redox state have shown optimistic outcomes in experimental models, but translation to human subjects is necessary to demonstrate therapeutic efficacy. Similarly, therapies targeting MQC systems – fusion, fission, mitophagy – have shown promise but lack of clarity around injury time course and severity has produced contradictory results. Further exploration of the molecular and cellular mechanism of mitochondria lipid signaling and complimentary MCQ pathways in secondary injury is warranted.

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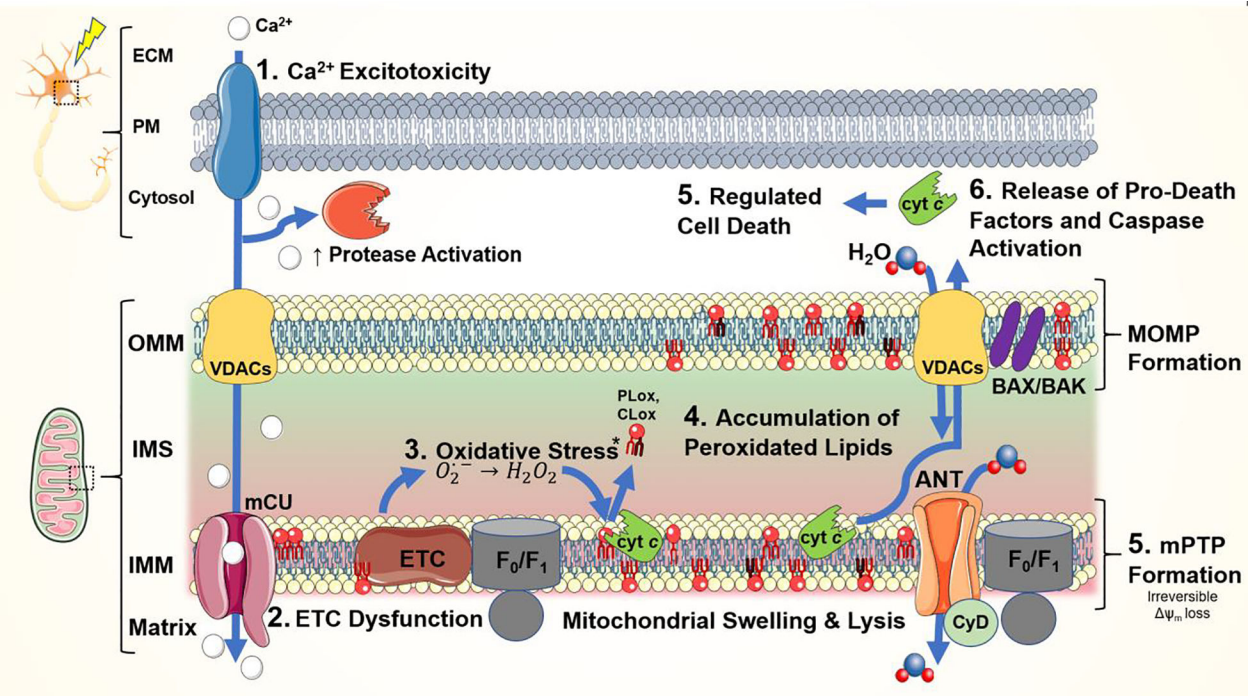

Figure 1. Mitochondria-focused mechanisms of neuronal injury following TBI.

1) Widespread neuronal depolarization results in the unregulated release of excitatory neurotransmitters and the activation of their corresponding ion channels. Increased cytosolic Ca^{2+} is buffered, in part, by the mitochondria leading to disruption of the mitochondrial membrane potential ($\Delta\psi_m$). Ensuing **2**) electron transport chain (ETC) dysfunction causes protracted increases in **3**) ROS & RNS production. Injury becomes self-perpetuating as endogenous oxidant stress response systems are overwhelmed. **4**) Mitochondrial lipids, especially cardiolipin (CL), are important sources of oxidized free fatty acid inflammatory mediators. Both non-enzymatic and cyt *c*/CL peroxidase-mediated enzymatic CL peroxidation occurs in the setting of high ROS levels. **5**) Permeabilization of the mitochondrial membranes and oxidation of CL prompts the release of pro-death factors – ultimately leading to neuronal death. (OMM, outer mitochondrial membrane; IMS, intermembrane space; IMM, inner mitochondrial membrane; ANT, adenine nucleotide translocator; BAK/BAX, Bcl-2-associated X protein; CyD, cyclophilin D; F_0/F_1, ATP synthase; mCU, mitochondrial calcium uniporter, VDAC, voltage-dependent anion channel)

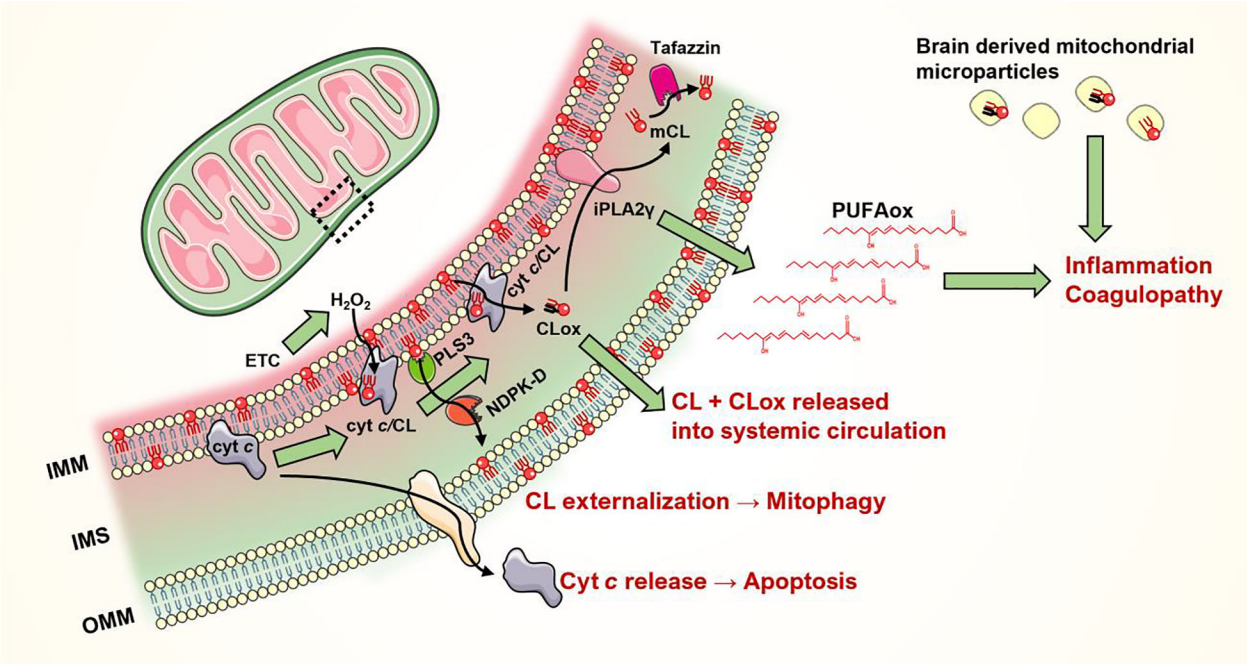

Figure 2. Mitochondrial CL signaling in brain injury.

The inner leaflet of mitochondrial inner membrane (IMM) is enriched in cardiolipins (CL). Upon injury, CLs are translocated to the outer leaflet of IMM, where it forms a complex with cytochrome *c*. The cytochrome *c*/cardiolipin (cyt *c*/CL) complex is a potent peroxidase that oxidizes the polyunsaturated acyl chains of CL (or other anionic lipids, to a lesser extent). Oxidation of CL (CLox) leads to the translocation of cyt *c* to the cytoplasm and subsequent apoptosis. IMM-localized non-oxidized CL are translocated to the outer membrane by the phospholipid scramblase 3 (PLS3) and mitochondrial nucleoside diphosphate kinase (NDPK-D). Externalized CL serve as a “eat me” signal for mitophagy. CLs previously oxidized by the cyt *c*/CL complex undergo hydrolysis by calcium-independent phospholipase A2 - gamma (iPLA2γ) to release oxidized polyunsaturated fatty acids (PUFAox). These PUFAox serve as lipid mediators that regulate inflammation and can lead to coagulopathy. Intact tetraacylated CLs can be regenerated from monolyso-CL (mCL) by phospholipid-lysophospholipid transacylases (e.g. tafazzin). CLs present on the brain-derived mitochondrial microparticles also regulates the injury-related inflammation and coagulation.

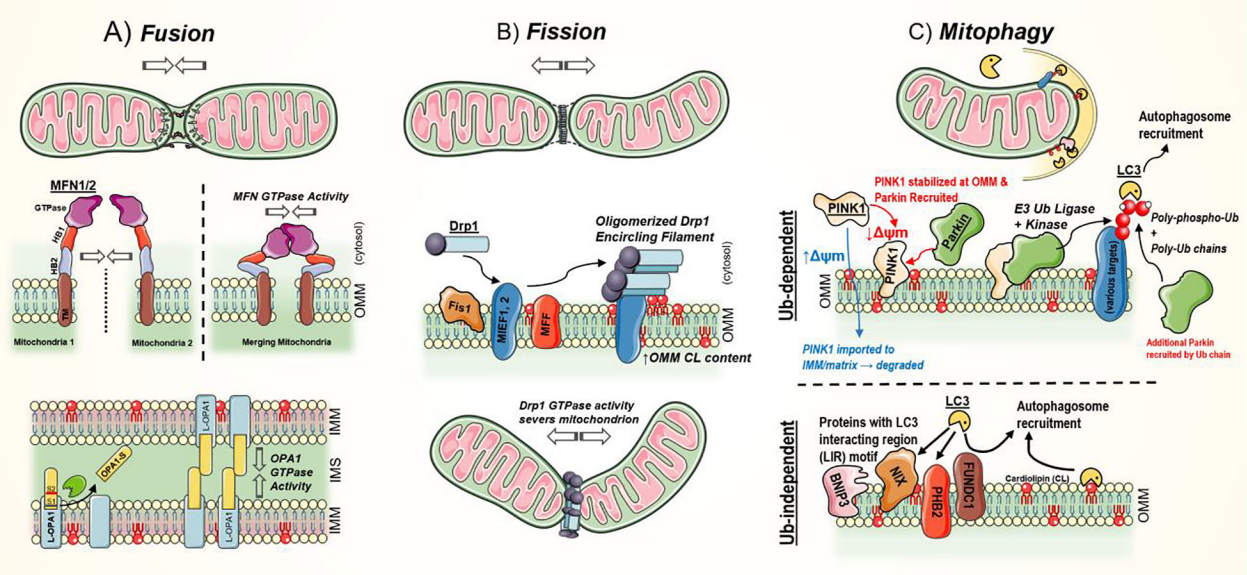

Figure 3. Mitochondrial quality control system and mitophagy.

A) Mitochondrial fusion leads to the joining of two mitochondrion. As fusion-destined mitochondria approach, outer mitochondrial membrane (OMM)-anchored mitofusins 1 and 2 (MFN1/2) dimerize and bridge the (~20 nm) gap between membranes. Subsequent MFN GTPase activity pulls the two organelles into proximity. The HB domains then participate in membrane destabilization and integration. This process is most common at sites of lipid packing detects – due to presence of bulky or conical lipids, like cardiolipin. **B**) Mitochondrial fusion leads to increased network fragmentation. The large GTPase, dynamin-related protein 1 (Drp1), drives fission by mechanically constricting and severing both the OMM and inner mitochondrial membrane (IMM). Soluble Drp1 is initially recruited to the OMM with assistance by OMM-bound adaptors: mitochondrial fission 1 (Fis1), mitochondrial fission factor (MFF), and mitochondrial elongation factors 1 & 2 (MIEF1, MIEF2). Increased OMM cardiolipin and phosphatidic acid content are linked to efficient fission activity. **C**) Mitophagy is a selective autophagy of mitochondria. Broadly, it can proceed in a ubiquitin (Ub)-dependent and -independent manner. In the Ub-dependent pathway, phosphatase and tensin homologue (PTEN)-induced punitive kinase 1 (PINK1) accumulates in the OMM when mitochondrial membrane potential ($\Delta\psi_m$) is depleted. PINK1 recruits Parkin, an E3 Ub ligase. The PINK1/Parkin complex then proceeds to poly-phospho-ubiquitinate a range of mitochondrial proteins (e.g. translocase of the OMM, Miro, MFNs) and itself in feed-forward mechanism. By direct engagement or assistance of several adaptor proteins (OPTN, NDP52, p62, TAX1Bp1, and NBR1), LC3 (LC3-II) binds these poly-Ub and poly-phospho-ubiquitinated proteins, leading to autophagosomal engulfment. Ub-independent mechanisms similarly proceed in an LC3-dependent manner. However, in this case, LC3 directly engages various OMM protein (contain an LC3 interacting motif, e.g. BNIP3, NIX, PHB2, and FUNDC1) or select lipids, like cardiolipin (CL). This leads to autophagosome recruitment.