NEURONAL SURVIVAL AFTER TRAUMATIC BRAIN INJURY: 
CARDIOLIPIN-A CRITICAL TARGET

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Traumatic brain injury (TBI) is a serious healthcare problem in the United States, with more than 400,000 individuals hospitalized each year and an estimated annual cost of $25 billion; thus TBI is an enormous socioeconomic burden and has significant public health relevance. TBI leads to both direct mechanical damage and functional disturbance in mitochondria, which are key mechanisms contributing to neuronal death after TBI. Therefore, prevention of mitochondrial damage and/or removal of dysfunctional mitochondria (mitophagy) are promising therapeutic strategies. Indeed, in the in vitro model of mechanical stretch injury, mitophagy was observed as early as 1 h and continued for 24 h; however, neuronal death did not occur until 6 h after the insult. The delayed emergence of neuronal death suggests a possible window of opportunity for targeted therapies. In the current research, we studied the role of cardiolipin (CL), a unique mitochondria inner membrane phospholipid, in neuronal death induced by TBI. Manipulation of neuronal CL levels by knocking down CL synthase (CLS, the rate limiting enzyme in the synthesis of CL) using siRNA technology produced 15% and 46% decrease in CL content at 72 h and at 96 h, respectively, without alteration in mitochondrial morphology or function and CL molecular speciation. CLS/CL deficiency markedly inhibited both mechanical stretch induced mitophagy and neuronal death. Using a model of direct mitochondrial injury (rotenone, complex I inhibitor), we reported that mitophagy resulted in externalization of CL to the mitochondrial outer membrane in the primary neurons and suggested redistribution of
cardiolipin serves as a mitochondrial “eat-me” signal. Using global lipidomics analysis we showed that TBI induced neuronal death was accompanied by oxidative consumption of polyunsaturated CL and accumulation of more than 150 new oxygenated molecular species in CL. By applying the novel brain permeable mitochondria-targeted electron-scavenger-hemigramicidin nitroxide, we fully prevented CL oxygenation in the brain, achieved a substantial reduction in neuronal death both in vitro and in vivo, and markedly reduced behavioral deficits. Taken together, the results from doctoral work explored the role of CL after TBI that represents a novel target for neuro-drug discovery.
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1.0 INTRODUCTION

1.1 TRAUMATIC BRAIN INJURY

TBI remains a serious healthcare problem in the United States, with more than 400,000 individuals hospitalized each year and an estimated annual cost of $25 billion (Langlois et al., 2006). Moreover, TBI is the leading cause of death in infants and children (Langlois et al., 2006), and is an epidemiological risk factor for Alzheimer's disease (AD) and Parkinson's disease (PD) (DeKosky et al., 2010; Johnson et al., 2010; Schofield et al., 1997; Smith et al., 2003). Thus, TBI is a significant public health concern and an enormous socioeconomic burden. TBI results in neuronal death and functional deficits due to both primary and secondary injury mechanisms. Primary injury is the result of immediate mechanical damage that occurs at the time of the insult. Secondary injury, which evolves over a period of hours to days, even months, after the insult, is the result of biochemical, cellular and physiological mechanisms. These mechanisms include inflammation, oxidative stress, ionic imbalance, mitochondrial dysfunction, and excitotoxic damage leading to apoptotic neuronal death and motor and cognitive impairment. Establishing and understanding the mechanisms underlying TBI-induced neuronal death is important for the development of novel therapeutic strategies.

Several complex and interrelated pathways of cell death can occur after TBI in the brain, the relative contribution of each depending upon the duration, degree, and location of and time
after injury, as well as the cell type and developmental stage of the animal. These include necrosis, autophagy, intrinsic and extrinsic caspase-dependent program cell death, and caspase-independent programmed cell death (Arundine et al., 2004; Bittigau et al., 2004; Clark et al., 2008; Raghupathi, 2004; Yakovlev and Faden, 2001). Reactive oxygen species (ROS), which have been implicated as key participants of secondary injury after TBI (Bayir et al., 2006b; Bramlett and Dietrich, 2004; Chan et al., 1995; Kochanek et al., 2008; Lewen et al., 2000; Niizuma et al., 2009; Singh et al., 2006), are generated from a number of sources, including mitochondrial electron transport system, NADPH oxidase, xanthine oxidase, cytochrome p450 system, nitric oxide synthase, and myeloperoxidase. Furthermore, mitochondria-derived ROS plays a central role in the regulation of neuronal death pathway.

Treatment of patients with severe TBI remains as supportive intensive care as there are no specific therapies (Kochanek et al., 2011). Numerous clinical trials (Stoica et al., 2009; Vink and Nimmo, 2009; Xiong et al., 2009) have failed to demonstrate expected improved outcome likely due to complex pathophysiology of the disease process, limited blood brain permeability of the proposed agents and lack of specific targeting.
1.2 CARDIOLIPIN

Cardiolipin (CL) is a dimeric anionic phospholipid with an unusual structure consisting of four acyl side chains in comparison to more typical phospholipids which have two acyl chains (Schlame et al., 2000). Phospholipids are the major components of plasma and intracellular membranes, and phospholipids account for around 25% of the dry weight of the adult rat brain (Sparvero et al., 2010). Phospholipids consist of a glycerol backbone, fatty acid chains, and a phosphoester-connected headgroup. Fatty acids can be released by phospholipase A from the phospholipids and have important roles in cell signaling and metabolism. There are eight most common fatty acid residues in the brain; this gives $8^2 = 64$ possible molecular species for each class. On the other hand, CL has four fatty acid chains which allows for the formation of huge number of possible molecular species (Bayir et al., 2007).

Normally CL is found only in two places in nature: bacterial cell membranes and the inner mitochondrial membrane (IMM) of eukaryotic cells (Iverson et al., 2004; Schlame et al., 2000). In the IMM, CL is distributed between the inner and outer leaflets at a ratio of 60:40 (Hovius et al., 1993; Tyurin et al., 2007). CL is an essential component in many mitochondrial processes, such as electron transport, ADP/ATP translocation, ion permeability, and protein transport (Schlame et al., 2000; Shidoji et al., 1999; Zhang et al., 2002). And the function of many inner mitochondrial membrane respiratory enzymes, such as cytochrome c oxidase, is dependent on their association with CL, and not on other phospholipids (Schagger, 2002). Thus, CL has long been known to affect the stability and catalytic activity of mitochondrial proteins.

Recently, our group reported that CL avidly binds cytochrome c (cyt c), an ancient hemoprotein, partially unfolds the protein, and that the cyt c/CL complex functions as an
oxygenase, catalyzing CL peroxidation, which is essential for the release of pro-apoptotic factors (Kagan et al., 2005). In the cytosol, released cyt c interacts with another anionic phospholipid, phosphatidylserine (PS), and catalyzes its oxidation in a similar catalytic peroxidase oxygenase reaction. Peroxidized PS facilitates its externalization that is essential for the recognition and clearance of apoptotic cells by macrophages (Bayir et al., 2006a). Cyt c has multiple functions in the cells including well-established role as an electron shuttle between complexes III and IV in the mitochondria, antioxidant role as a superoxide scavenger, and the formation of apoptosomes (Bayir et al., 2006a; Kagan et al., 2009a).

Several studies suggest that lipid oxidation markers might be associated with outcome after clinical TBI (Bayir et al., 2002; Hall, 1989; Lewen et al., 2000; Taylor et al., 1999). Lipid peroxidation contributes to the evolution of secondary damage in TBI; however essential information on molecular targets of oxidation is largely unknown (Bayir et al., 2004; Puccio et al., 2009; Singh et al., 2006). Our group reported that two anionic phospholipids–mitochondrial CL and extra-mitochondrial PS – are major targets of TBI-induced oxidation in brain (Bayir et al., 2007). Most importantly, our group has shown lipid peroxidation in pericontusional cortex after TBI was non-random in PND 17 rats; CL is selectively oxidized early after TBI, whereas more abundant brain phospholipids (such as PC and PE) remained non-oxidized at this time point (Bayir et al., 2007). This selectively early oxidation of CL after experimental TBI may represent a therapeutic target.
1.3 AUTOPHAGY AND MITOPHAGY AFTER BRAIN INJURY

Autophagy is the process of catabolism of cellular components, such as the cytosol, organelles and protein aggregates, through their encapsulation by a double-membrane structure known as the autophagosomes (Yang and Klionsky, 2010). Autophagy has long been recognized as an important physiologic adaptation to starvation (Mizushima and Komatsu, 2011); the golden standard for detection of autophagy is ultrastructural evidence of autophagosomes. Covalent attachment of phophatidylethanolamine (PE) to autophagy related protein 8(Atg)/LC3-I (LC3 is the mammalian homologue of the yeast Atg8) at the C-terminal glycine resulting in LC-II, is essential and is one hallmark biochemical feature of autophagy referred to as an “LC3 shift” or “LC3 lipidation” (Mizushima and Levine, 2010). The autophagosomes then engulf large proteins targeted by ubiquitination or organelles such as mitochondria-mitophagy. Mitophagy was first observed in mammals during red blood cell differentiation (Youle and Narendra, 2011), and from current perspective, mitophagy plays important roles in the protective function at the level of mitochondria (Green et al., 2011). Electron microscopy, single cell analysis of LC3 fluorescent puncta, and Western blot analysis of mitochondrial markers are accepted strategies to study mitophagy in neurons (Zhu et al., 2011).

Alteration of autophagy has been observed in many disease conditions of the nervous system. Loss of autophagy in the neuronal system can cause accumulation of toxic proteins and is related with several neurodegenerative diseases (Banerjee et al., 2010; Wong and Cuervo, 2010). Increased autophagy has also been reported in many neuronal disease conditions, such as TBI, stroke, Alzheimer’s disease and critical illness (Clark et al., 2008; Degterev et al., 2005; Lai et al., 2008; Zhu et al., 2005).
Recent work has linked defects in mitophagy to one of the neurodegenerative disease-Parkinson’s disease (PD). Two genes which encode PINK1 and Parkin are mutated in the autosomal recessive PD. Furthermore, PINK1 and Parkin have been found to maintain mitochondrial quality control by regulating mitophagy (Clark et al., 2006; Greene et al., 2003; Kitada et al., 1998; Park et al., 2006; Poole et al., 2008; Valente et al., 2004). However the detailed mechanisms of mitophagy remain unknown.
1.4 MITOCHONDRIAL TARGETED NEUROPROTECTIVE STRATEGY

Mitochondria are the essential source of metabolic energy for the cell. And at the same time damaged mitochondria are important sources of ROS and apoptotic cell death. Thus, autphagic turnover of mitochondria or mitophagy eliminating dysfunctional or damaged mitochondria, can prevent unwarranted cell loss (Green et al., 2011). Many groups have reported impaired mitophagy in different neurodegenerative diseases, such as Huntington disease, PD, and AD (Ayala et al., 2007; Geisler et al., 2010; Wang et al., 2009). Therefore, mitophagy exerts cytoprotective effects by intercepting lethal signals before or at the level of mitochondria.

Based on the new role of CL and cyt c in the mitochondria, inhibition of CL peroxidation, catalyzed by the complex of cyt c and CL, a required stage of apoptosis serves as another mitochondrial level cytoprotective strategy.

Thus promoting mitophagy in specific stage can eliminate dysfunctional or damaged mitochondria; blocking CL peroxidation within mitochondria can inhibit the subsequent apoptotic neuronal death: both represent mitochondria directed neuroprotective strategies.
2.0 SPECIFIC AIMS

The overall goal of this project is to identify the roles of CL in neuronal death after experimental TBI that represent new therapeutic targets for neuro-drug discovery.

The specific aims of the whole project are:

1) To determine the time course of ROS production, lipid peroxidation and biomarkers of apoptosis induced by mechanical stretch in cortical neurons. Mechanical stretch induced cortical neuronal injury is an established in vitro TBI model. We applied several techniques to study the biomarkers of apoptosis and selective peroxidation of anionic phospholipids (CL and PS) in this in vitro TBI model.

2) To investigate mitochondrial anti-apoptotic targeting as a therapeutic strategy for TBI. By applying a novel brain permeable mitochondria-targeted electron-scavenger, XJB-5-131, we tested the neuroprotective effects in both in vivo and in vitro TBI models.

3) To examine the signaling pathway for removal of dysfunctional/damaged mitochondria by rotenone and stretch induced mitochondrial autophagy (mitophagy).
3.0 RESULTS

3.1 MITOCHONDRIAL INJURY AFTER MECHANICAL STRETCH OF CORTICAL NEURONS IN VITRO: BIOMARKERS OF APOPTOSIS AND SELECTIVE PEROXIDATION OF ANIONIC PHOSPHOLIPIDS

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3.1.3 NOTE

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3.1.4 ABSTRACT

Mechanical injury of neurites accompanied by rupture of mitochondrial membranes may lead to immediate non-specific release and spreading of pro-apoptotic factors, activation of proteases, i.e. execution of apoptotic program. In the current work, we studied the time course of the major biomarkers of apoptosis as they are induced by exposure of rat cortical neurons to mechanical stretch. By using transmission electron microscopy, we found that mitochondria in the neurites were damaged early (1 hrs) after mechanical stretch injury whereas somal mitochondria were significantly more resistant and demonstrated structural damage and degenerative mitochondrial changes at a later time point after stretch (12 hrs). We also report that the stretch injury caused immediate activation of reactive oxygen species production followed by selective oxidation of a mitochondria-specific phospholipid, cardiolipin, whose individual peroxidized molecular species have been identified and quantified by electrospray ionization mass spectrometry analysis. Most abundant neuronal phospholipids – phosphatidylcholine, phophatidylethanolamine – did not undergo oxidative modification. Simultaneously, a small-scale release of cytochrome c was observed. Notably, caspase activation and phosphatidylserine externalization - two irreversible apoptotic events designating a point of no return - are substantially delayed and do not occur until 6-12 hrs after the initial impact. The early onset of reactive oxygen species production and cytochrome c release may be relevant to direct stretch-induced damage to mitochondria. The delayed emergence of apoptotic neuronal death after the immediate mechanical damage to mitochondria suggests a possible window of opportunity for targeted therapies.
Key words: cardiolipin; in vitro traumatic brain injury; mitochondria; oxidative lipidomics; oxidative stress
3.1.5 INTRODUCTION

Several interrelated pathways of cell death -necrosis, autophagy and apoptosis- can occur after traumatic brain injury (TBI) (Brophy et al., 2009; Clark et al., 2008b; Liu et al., 2008; Pedersen et al., 2009). The major biological role of apoptosis is to eliminate cells with irreparably damaged DNA to prevent spill-over and penetration of the genotoxic contents into surrounding proliferating cells (Fadeel, 2003). Cell death program includes a series of mitochondrial events culminating in activation of protease (caspase) cascades – a point of no-return in apoptosis (Polster and Fiskum, 2004; Samali et al., 1999; Zhivotovsky et al., 1999). Among early mitochondrial events are the production of reactive oxygen species (ROS), translocation of pro-apoptotic Bcl-2-family proteins Bax and Bak, permeabilization of mitochondrial membrane and release of pro-apoptotic factors into the cytosol (Cardaci et al., 2008; Kroemer et al., 2007; Lahiry et al., 2008; Li et al., 1999; Polster and Fiskum, 2004). This highly coordinated chain of events provides for complete and effective disassembly of intracellular contents – the goal of apoptosis (Fadeel, 2003; Kroemer et al., 2007).

Neurons contain two different pools of mitochondria – one in synaptic terminals, and another in the central soma (Lai et al., 1977). While genotoxic effects and their consequences may represent only a limited risk for post-mitotic neurons, damage to their mitochondria may trigger pro-apoptotic events. In particular, mechanical injury of neurites resulting in rupture of mitochondrial membranes may be associated with elevated risk of apoptosis due to non-specific release of pro-apoptotic factors and activation of proteases (Arundine et al., 2004a; Bayir et al., 2007a; Fiskum, 2000; Slemmer et al., 2008).
Establishing and understanding the mechanisms underlying traumatic brain injury (TBI)-induced neuronal death may be important for developing new therapeutic strategies based on the prevention of the spreading of apoptotic signaling by blocking the upstream events. One of them is recently discovered interactions of a mitochondria-specific phospholipid, cardiolipin (CL), with an intermembrane space protein, cytochrome c (cyt c) (Iverson and Orrenius, 2004; Kagan et al., 2005b; Petrosillo et al., 2006). These two molecules form a complex with peroxidase activity that catalyzes CL peroxidation, facilitates mitochondrial permeability transition and release of pro-apoptotic factors, including cyt c, into the cytosol (Kagan et al., 2005b). Prevention of cyt c release may be achieved by suppression of CL oxidation (Borisenko et al., 2008; Jiang et al., 2008; Kagan et al., 2009a). Experimentally, this may be tested using in vitro impact model of mechanical stretch to neurons where the rupture of mitochondria is not complicated by other cell-cell interactions affecting the pro-apoptotic events.

Here, we studied the time course of the major biomarkers of apoptosis induced by mechanical stretch in cortical neurons. We hypothesized that mechanical stretch injury of neurons is associated with immediate damage to mitochondria in neurites and subsequent triggering of apoptosis accompanied by CL oxidation. We report that the stretch causes immediate damage to neurons that includes CL oxidation and cyt c release. The secondary damage is separated from the former by several hours and is evidenced by caspase activation and phosphatidylserine (PS) externalization. The delayed emergence of apoptotic neuronal death suggests a possible window of opportunity for targeted therapies.
3.1.6 Materials and Methods

3.1.6.1 Reagents.

All chemicals were purchased from Sigma Chemicals (St. Louis, MO) unless otherwise noted.

3.1.6.2 Isolation and culture of cortical neurons

Animal procedures were performed in accordance with the University of Pittsburgh Animal Care and Use Committee. Primary cortical neuronal cultures were prepared from embryonic day 17 rats. Pregnant Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN) were anesthetized with CO₂ and sacrificed via decapitation. Embryos were surgically removed from the adult rat and cortices were isolated separately in ice-cold Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with B-27 (Invitrogen, Carlsbad, CA) and Penicillin-Streptomycin (Invitrogen, Carlsbad, CA). Tissue was then rinsed twice with the same medium and set in 0.25% trypsin with EDTA (Invitrogen, Carlsbad, CA) for 15 min at 37°C. The tissue was again rinsed twice and triturated in ice-cold Neurobasal medium to obtain neuronal cell suspension.

Cells were plated at $2.0 \times 10^6$ cells per ml (3.5 mL/well) onto thin, clear, flexible silicone membranes (Specialty Manufacturing, Saginaw, MI) treated with 0.05% poly-D-lysine hydrobromide (Sigma-Aldrich, St. Louis, MO) for 2 hrs at room temperature (RT), rinsed twice with Hanks' Balanced Salt Solution (Invitrogen, Carlsbad, CA), and allowed to dry under a laminar flow hood for 1 hr. Experiments were performed at 7-9 day, when cultures consist
primarily of neurons (>95% MAP2 immunopositive cells, <5% glial fibrillary acidic protein immunopositive cells). The culture medium was changed every 2 days.

3.1.6.3 *In vitro* TBI model

Trauma was produced using a well-established in vitro model (Lusardi et al., 2004). Briefly, primary neuronal cells cultured on silicone membranes were subjected to a computer controlled quantifiable mechanical insult by displacing the membrane over a hollowed platform. The membranes were stretched with a known pre-tension across the surface of custom designed, stainless steel wells that fit into the cell stretch apparatus. Cultures were subjected to a severe mechanical stretch, consisting of a rapid onset strain pulse [10 s\(^{-1}\) strain rate and 50% membrane deformation]. Severe stretch was chosen in order to simulate a strain field similar to what occurs in animal models of severe TBI. Sterile technique was strictly followed in all procedures. The neuronal cultures were either returned to the incubator for further observation or subjected to further treatment dependent on the experiment after mechanical stretch.

3.1.6.4 Assessment of neuronal viability

Cell viability was assessed using the Alamar blue (Invitrogen, Carlsbad, CA) assay, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, and trypan blue dye exclusion assay. Alamar blue assay is based on conversion of cell permeable non-fluorescent compound resazurin to a fluorescent molecule, resorufin by the reducing environment of living cells (Gartlon et al., 2006; Gil-ad et al., 2001; Shimazawa and Hara, 2006). Cell viability was assessed by the use of 10% resazurin solution for 3 hours at 37\(^\circ\)C, and then cells were examined for fluorescence at 560/590 nm. Fluorescence was expressed as a percentage of that in control
cells (after subtraction of background fluorescence). The MTT solution (5 mg MTT/mL medium) was added to each well 24 h after mechanical stretch at the final concentration of 250 µM and incubated for 3 h. The media was removed, and cells were dissolved in dimethyl sulfoxide (DMSO). Optical density was determined using a spectrophotometer (Spectra MAX 340, Molecular Devices, Sunnyvale, CA) at 550 nm test and 690 nm reference wavelengths. Neurons were stained with trypan blue, and viability was determined using the trypan blue exclusion assay. After incubation with the indicated conditions, neurons were incubated with 0.4% trypan blue in HBSS for 2 min at room temperature. Neurons were observed under the microscope and counted as stained and non-stained cells on a hemacytometer separately, then viable cell ratios were calculated according to the following formula: viable cell ratio (%) = (non-stained cell number/total cell number)*100%.

3.1.6.5 Transmission Electron Microscopy (TEM)

Neurons were fixed with 2.5% glutaraldehyde in phosphate buffered saline (PBS) (pH=7.4) for 2 hrs at RT, then washed and monolayers of neurons were postfixed in 1% OsO₄ and 1% K₃Fe(CN)₆ for 1 hr at 4°C. After three washing with PBS, the monolayers were dehydrated through a graded series of 30% to 100% ethanol then incubated in Polybed 812 epoxy resin (Polysciences, Warrington, PA) for 1h. After several changes of 100% resin (three times for 1hr each), monolayers were polymerized at 37°C overnight with additional hardening at 60°C for 48 hrs. Ultrathin (60 nm) sections were collected on 200 mesh grids and stained with 2% uranyl acetate in 50% methanol for 10 min followed by 1% lead citrate for 7 min. Sections were observed on a JEM 1210 electron microscope (JEOL, Peabody, Japan) at 80 kV.
3.1.6.6 Assessment of mitochondrial superoxide production with MitoSOX

The mitochondrial superoxide production was measured by flow cytometry as described (Ainslie et al., 2008) and [http://bts.ucsf.edu/desai/protocols.html](http://bts.ucsf.edu/desai/protocols.html). Briefly, MitoSOX Red (Invitrogen, Carlsbad, CA) was added to the neuronal cultures at a final concentration of 3μM. The cells were incubated at 37 °C for 5 min to allow loading of MitoSOX Red. The neuronal cultures were then fixed with same volume of 4% paraformaldehyde (in PBS, pH7.4) and incubated at RT for 30 min. The cells were then detached with 0.1% trypsin and then spun down 600 g at 4 °C for 3min. The cells were washed 2 times with PBS and placed in a sterile FACS tube at a concentration of 5–10×10^6 cells per 100 μl. Cells were stored at 4°C for a maximum of 30 min until the samples were analyzed via flow cytometer (Becton-Dickison, Franklin Lakes, NJ, USA). Hoechst 33258 (1 μg/ml; Molecular Probes) was added 1 min before flow cytometer analysis.

3.1.6.7 Mitochondrial cyt c release

Neuronal cells (1×10^7) were washed in PBS then incubated for 3 min in lysis buffer (75 mM NaCl, 8 mM Na₂HPO₄, 1 mM NaH₂PO₄, 1 mM EDTA, and 350 μg/ml digitonin). The lysates were centrifuged at 12,000 g for 1 min, and the supernatant was collected for cytosolic cyt c analysis. The mitochondria-enriched fraction was resuspended in lysis buffer (25 mM HEPES-KOH pH 7.6; 5 mM MgCl₂, 0.5 mM EDTA, 10% Glycerol, 1 mM DTT, 1 mM PMSF). The suspension was then incubated on ice and sonicated twice with 10 seconds each time and 30 second interval. The final mitochondrial lysate was spun at 400,000 g in an ultracentrifuge (Backman, Fullerton, CA) at 4°C for 25 minutes. The supernatant was collected for mitochondrial cyt c analysis. Cyt c was detected with Western blot analysis. In order to evaluate
the outer membrane integrity which might be affected with the use of digitonin in the initial lysis buffer, we evaluated voltage-dependent anion channel (VDAC) and cytochrome c oxidase IV (COX-IV) levels. We found that our protocol with the above lysis buffer did not affect the content of VDAC and COX-IV. Moreover, no significant release of cyt c was observed from control cells.

3.1.6.8 Caspase 3/7 activity

Caspase 3/7 activity was measured using a luminescence Caspase-Glo assay kit obtained from Promega (Madison, WI, USA). Luminescence was determined at baseline and after 1 hr incubation at room temperature using Fusion-α plate reader (Perkline Cop., Boston MA, USA). Caspase 3/7 activity was expressed as the luminescence produced after 1 h incubation per mg of protein.

3.1.6.9 Phosphatidylserine (PS) externalization

Externalization of PS was analyzed by flow cytometry using an annexin V kit (Biovision, Mountain View, CA). Briefly, cells were harvested at the end of incubation, and then stained with annexin V-FITC and propidium iodide (PI) prior to analysis. Ten thousand events were collected on a FACScan flow cytometer equipped with a 488-nm argon ion laser and supplied with the Cell Quest software. Cells that were annexin V-positive and PI-negative were considered as apoptotic cells.
3.1.6.10  Cytotoxicity Detection (Lactate Dehydrogenase [LDH] Release)

The effect of pretreatment with N-acetylcysteine and z-VAD-fmk on cortical neuronal cell injury was quantified by the measurement of LDH at 24 hrs after the insult. An aliquot of bathing media was combined with NADH and pyruvate solutions. LDH activity is proportional to the rate of pyruvate loss, which was assayed by absorbance change by using a microplate reader (Spectra MAX 340, Molecular Devices, Sunnyvale, CA). Blank LDH levels were subtracted from insult LDH values and results normalized to 100% neuronal death caused by 0.5% Triton X-100 exposure.

Cell samples were separated by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membrane by tank blotting method. Nonspecific binding sites were blocked using 5% nonfat milk containing 0.1% Tween-20 in PBS (pH 7.4). Thereafter, membranes were probed overnight at 4°C with mouse monoclonal anti-4-hydroxynonenal antibody (HNE, 1 μg/ml, clone 198960, R&D systems, Minneapolis, MN), or mouse monoclonal anti-cyt c antibody (1:400, clone 7H8.2C12, MAB1800, Millipore, Billerica, MA) diluted in 5% nonfat milk containing 0.1% Tween-20 in PBS (pH 7.4). After three washes with 0.1% Tween-20 in PBS (pH 7.4), immune complexes were labeled for 2 hrs with horseradish peroxidase-conjugated anti-mouse IgG (1:2000, Cell Signaling Technology, Inc. Danvers, MA). After five washes, bound antibodies were visualized by ECL Western Blotting Substrate Kit (Mountain View, CA).
3.1.6.11 **Immunocytochemistry**

Neurons were fixed in 2% paraformaldehyde and then incubated with 5% normal donkey serum and 2% bovine serum albumin in PBS containing 0.2% Triton X-100 for 1 h and then incubated overnight at 4 degree with primary antibody against microtubule-associated protein (MAP2) (1:500, Abcam, Cambridge, MA), followed by , followed by an incubation with donkey anti-mouse IgG Alexa Fluor 488 conjugated secondary antibody (1:1000, Invitrogen, Carlsbad, CA) for 1 h at room temperature. Sections were washed four times in PBS at room temperature in the dark, with the last wash containing 10 μg/ml 4',6-diamidino-2-phenylindole (DAPI). Cells were examined under inverted florescent microscope Leica DM-IL (Leica, Wetzlar, Germany) equipped with a digital Leica DC300 camera (Leica, Wetzlar, Germany).

3.1.6.12 **Lipid analysis**

Total lipids were extracted from neurons by Folch procedure (Folch et al., 1957a). Lipids were separated by 2-dimensional high performance thin layer chromatography (2D-HPTLC) (Rouser et al., 1970). To prevent oxidative modification of phospholipids during separation, plates were treated with methanol containing 1 mM EDTA, 100 uM DTPA prior to separation of phospholipids by 2D-HPTLC. The phospholipids were visualized by exposure to iodine vapors and identified by comparison with authentic phospholipid standards. Lipid phosphorus was determined by a micro-method (Böttcher et al., 1961).
3.1.6.13  **Electrospray ionization mass spectrometry (ESI-MS) analysis of phospholipids**

ESI-MS analysis was performed by direct infusion into a linear ion-trap mass spectrometer LXQ™ with the Xcalibur operating system (Thermo Fisher Scientific, San Jose, CA). Samples collected after 2D-HPTLC separation were evaporated under N₂, re-suspended in chloroform:methanol 1:1 v/v (20 pmol/µL) and directly utilized for acquisition of negative-ion or positive-ion ESI mass spectra at a flow rate of 5 µL/min. The ESI probe was operated at a voltage differential of 3.5-5.0 kV in the negative or positive ion mode. Capillary temperature was maintained at 70 or 150°C. MSⁿ analysis was performed using isolation width of 1 m/z, 5 microscans with maximum injection time 1000 ms. Full-scan ESI–MS analysis in the negative ionization mode was employed for all phospholipids classes. Additionally, MS-spectra were recorded in positive mode for phosphatidylcholine (PC) and sphingomyelin (Sph). Two ion activation techniques were used for MS analysis: collision-induced dissociation (Q=0.25, low mass cut off at 28% of the precursor m/z) and pulsed-Q dissociation technique, with Q=0.7, and no low mass cut off for analysis of low molecular weight fragment ions (Schwartz et al., 2005). Based on MS fragmentation data, chemical structures of lipid molecular species were obtained using ChemDraw and confirmed by comparing with the fragmentation patterns presented in Lipid Map Data Base (www.lipidmaps.org).

3.1.6.14  **Quantification of oxidized molecular species of phospholipids**

Lipid hydroperoxides were determined by fluorescence high performance liquid chromatography (HPLC) of resorufin formed in peroxidase-catalyzed reduction of specific phospholipid hydroperoxides with Amplex Red (N-acetyl-3, 7-dihydroxyphenoxazine,
Molecular Probes Eugene, OR) as previously described (Tyurin et al., 2008). In addition, liquid chromatography (LC)/ESI-MS was utilized for quantitative analysis of oxidized phospholipids as well. LC/MS of phospholipids was performed with Dionex Ultimate™ 3000 HPLC coupled on-line to a linear ion trap mass spectrometer (LXQ Thermo-Fisher, San Jose, CA). The lipids were separated on a normal phase column (Luna 3 μm Silica 100A, 150x2 mm, (Phenomenex)) with flow rate 0.2 mL/min using gradient solvents containing 5 mM CH₃COONH₄ (A - hexane:propanol:water, 43:57:1 (v/v/v) and B - hexane:propanol:water, 43:57:10 (v/v/v).

3.1.6.15 Statistics

The results are presented as mean ± SD, unless otherwise specified, values from at least three experiments, and statistical analyses were performed by either unpaired Student t-test or one-way ANOVA. The statistical significance of differences was set at P< 0.05.
3.1.7 RESULTS

3.1.7.1 Morphological analysis of neuronal cells

Microscopic examination revealed that the characteristic morphology of control neurons (Fig. 1a, d) with neurites was disrupted by mechanical stretch: an immediate damage to the neurites was evident while the cell bodies remained intact and attached to silastic membranes (Fig. 1b, e). There was no obvious recovery of cells at 24 hrs after injury (Fig. 1c, f). Assessments of cell viability demonstrated that approximately 35% of neuronal cells were dead 24 h after stretch (Fig. 1d).
Figure 1. Viability of cortical neurons after stretch injury.

Representative photomicrographs of control cortical neurons (a, d) and cortical neurons after mechanical stretch, 10 s⁻¹ strain rate and 50% membrane deformation (b, c, e, f). Stretch injury resulted in damage to neuronal processes while the cells’ body remained intact and attached to the substrate, immediately after stretch (b, e), and at 24hrs after stretch (c, f). Green: microtubule associated protein 2 (MAP2) immunostain; Blue: DAPI stain. Quantification of cell viability at 24 hrs after stretch injury using Alamar Blue (g) and MTT (h) assays and trypan blue (i) staining. Data are mean ± SEM, *p < 0.05 vs control, N=6.

TEM evaluation of neurons showed differences in the mitochondrial morphology between sham and stretched neurons (Fig. 2). Mitochondria in both soma and neurites in control neurons appeared normal with clear cristae structure (Fig. 2a). Early after injury (1 hr), the mitochondria in the neurites were swollen and their cristae were distorted, whereas the mitochondria in the soma of neurons appeared similar to those in control neurons (Fig. 2b). However, at later time points (12-24 hrs) after the injury, abnormal mitochondria were observed in both the soma and neurites (Fig. 2c, f, g). Furthermore, dying neurons showed features of apoptosis with intact plasma membrane, fragmented nuclei, chromatin clumping, and cytoplasmic vacuoles as well as necrosis with loss of membrane integrity and cell swelling (Fig. 2 e-g).

3.1.7.2 Mitochondrial ROS production, biomarkers of mitochondrial damage and apoptosis

Analysis of mitochondrial ROS production by mitoSOX using flow cytometry (Fig. 3) revealed a two-phase response to stretch. The initial very rapid production of ROS detectable at
early time points (0.5 and 1 hrs) was followed by a slower, yet significant incremental growth at later time points (up to 24 hrs). One can assume that stretch induced rapid damage of mitochondria in neuritis might be associated with the release of their cyt c. Indeed, there was a substantial (>2.5-fold) release of cyt c from mitochondria into the cytosol already observed at 0.5 hrs after the exposure (Fig. 4a). Cyt c release displayed a two phase response whereby the initial early release reached a plateau at 1 hr. Maximal release of cyt c was observed at 6h and reached a plateau thereafter. Notably, significant amounts of cyt c remained confined within the mitochondrial compartments both during the earlier and later time points after the impact. Two important biomarkers usually associated with post-mitochondrial stages of apoptosis – caspase activation and PS externalization – revealed significant increases after stretch injury. Caspase-3/7 activity was significantly increased only at time points as late as 12 and 24 hrs (Figs. 4b). A slight increase in Annexin V positivity was detected as early as 0.5 h after stretch (Fig. 4c). At 12 and 24 hrs after stretch injury, large increase in the number of Annexin V-positive cells associated with PS externalization was observed (Fig. 4c). PI positivity – corresponding to necrosis – followed the same trend with a lower magnitude. Pre-treatment with z-VAD-fmk improved cell survival as assessed by Annexin V/PI and LDH assays (Fig. 4 d, e).

In order to dissect the primary mechanical stretch induced injury vs secondary oxidative stress-induced damage to neurites, we pre-treated neurons with an antioxidant, N-acetylcysteine. This resulted in a significant decrease in cell death as assessed by PI and Annexin V positivity as well as by LDH viability assay (Fig. 4 d, e). This suggests that both the primary injury to neuritis and secondary oxidative stress-dependent damage contributed to neuronal death after stretch.
Figure 2. Ultrastructural changes induced by mechanical stretch through TEM.

Typical TEM image of control neurons (a) and cortical neurons 1hr (b) and 12 hrs (c) after mechanical stretch. Representative TEM images are presented. Red (a, b) and yellow (b, c) arrows indicate normal and damaged mitochondria, respectively. (d) A representative section of control primary cortical neurons with dispersed chromatin and a clearly defined nucleolus. (e-g) A representative section of mechanically stretched primary cortical neurons showing apoptosis with intact plasma membrane, fragmented nuclei, chromatin clumping, and cytoplasmic vacuoles in 6 hrs (e), 12 hrs (f) and 24 h (g). Enlargement of the rectangular selected areas show the normal (d) or abnormal (f, g) mitochondria after mechanical stretch. Red arrow (e) indicates
apoptotic bodies. Black arrows (g) indicate necrotic neurons with loss of membrane integrity and cell swelling.

Figure 3. Time course of mitochondrial superoxide generation in cortical neurons after stretch injury assessed by flow cytometry.

Insert: histograms demonstrating MitoSOX fluorescence intensities in control neurons and neurons 24 hrs after stretch. Data are mean ± SEM, *p<0.005 vs. control, N=6.
Figure 4. Mechanical stretch-induced apoptosis in cortical neurons.

(a) Release of cyt c after stretch injury in cortical neurons. Typical Western blots (insets) and the densitometric analysis of cyt c in mitochondrial (closed circles) and cytosolic (open circles) fractions of neuronal cells, *p < 0.05 vs control, N=3. Note that release of cyt c from mitochondria occurred as early as 30 min after the insult and peaked at 6 hrs after stretch injury. (b) Changes of caspase 3/7 activity in cortical neurons after stretch injury. Caspase 3/7
activity increased significantly at 12 hrs after stretch. The activity remained high at 24 hrs after injury \( *p < 0.05 \) vs control, \( N=6 \). (c) Flow cytometry analysis of Annexin V and PI responses of cortical neurons to stretch. Early Annexin V-positivity (PS externalization) – indicating apoptosis – was markedly enhanced 6 hrs after stretch. PI positivity – corresponding to necrosis – followed the same trend with a lower magnitude. Data are mean ± SD, \( *p < 0.05 \) vs control, \( N=3 \). Effect of pre-treatment with antioxidant N-acetyl cysteine (NAC, 250 \( \mu M \)) and caspase inhibitor (z-VAD-fmk, 25 \( \mu M \)) on stretch-induced neuronal death assessed by flow cytometry analysis of PS externalization and PI positivity (d) and cytotoxicity (lactate dehydrogenase [LDH] release) relative to Triton exposure (e) at 24h after stretch. Data are mean ± SD, \( *p < 0.05 \) vs respective vehicle group, \( N=4 \).

3.1.7.3 Oxidation of phospholipids in stretched neurons

No significant changes in the phospholipid composition in stretched neurons were found (Fig. 5a). Moreover, all major classes of phospholipids - PC, phosphatidylethanolamine (PE), phosphatidylinositol (PI), PS, and CL – contained polyunsaturated fatty acid residues with 2, 3, 4, 5, and 6 double bonds, the major peroxidation substrates. Notably, a robust oxidation of CL – a mitochondria-specific phospholipid - was clearly detectable 2 hrs after the stretch exposure. A smaller scale oxidation of PS was also observed in stretched neurons 2 hr after injury (Fig. 5b; Fig.6c). No significant oxidation was detectable in most dominant phospholipid classes – PC, PE and PI.
Figure 5. Phospholipid composition and accumulation of phospholipid hydroperoxides in rat cortical neurons after stretch.

(a) Phospholipid composition of control and stretched neurons. Insert: Typical 2D-HPTLC of lipids extracted from rat cortical neurons. Total lipids were extracted and separated by 2D-
HPTLC. (b) Content of phospholipid hydroperoxides in control and stretched neurons. Two hrs after stretch, total lipids were extracted, separated by 2D-HPTLC and phospholipid hydroperoxides (PL-OOH) were determined using AmpleRed protocol. *p<0.05 stretched vs. control neurons. PL, phospholipids; CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; Sph, Sphingomyelin.

To more specifically characterize oxidation of CL and PS, LC-ESI-MS analyses were performed. Typical LC-ESI-MS spectra of CL and PS isolated from primary neurons are presented on Fig. 6a and Fig. 6b, respectively. We found that CL molecular species containing linoleic, arachidonic and docosahexaenoic acids were oxygenated. Moreover, several types of CL oxidation products - with one, two three and four oxygens were accumulated in stretched neurons (Fig. 6c). Among PS molecular species, the ones with oxygenated docosahexaenoic acid were predominantly found in stretched neurons. However, quantitatively, CL underwent a 7.5-fold more robust oxidation than PS. Overall; the results on stretch-induced peroxidation of phospholipids are summarized as a “Hit-map” (Fig. 6d).
Figure 6. Identification of phospholipid molecular species by LC-MS.

(a) Typical LC/ESI-MS spectrum of CL isolated from control neurons. Insert: Major molecular species of CL containing polyunsaturated fatty acids (b) Typical LC/ESI-MS spectrum of PS isolated from control neurons. Insert: Major molecular species of PS containing polyunsaturated fatty acids (c) Accumulation of oxygenated phospholipids in rat cortical neurons 2 hrs after
stretch assessed by LC-MS. Data are mean ± SD, N=3. CL, cardiolipin; PS, phosphatidylserine; PE, phosphatidylethanolamine. (d) Oxidative lipidomics “Hit-map” of stretched neurons.

Interestingly, evaluation of a non-specific product of lipid peroxidation, namely 4-HNE adducts with proteins revealed an early and monotonous growth over 24 hrs (Fig. 7). The 20 kDa band increased at 2h and remained high at 12 and 24 hrs compared to respective control. The increase in 50 kDa band was more delayed and occurred at 12h and remained high at 24h compared to control.

Figure 7. Formation of HNE-protein adducts in P2 fraction isolated from primary neurons after stretch injury.

Using Western blot (a), anti-HNE antibody detected several protein bands with masses ranging from over 50 kD to less than 20 kD in cortical neurons cultured on elastic membranes. Increased
levels of HNE protein adducts were observed after stretch (b). Data are mean ± SD, *p < 0.05 vs respective control, N=3.
3.1.8 DISCUSSION

Derangements in mitochondrial functions have been observed in in vivo models and humans after TBI (Okonkwo and Povlishock, 1999; Robertson, 2004; Singh et al., 2006; Sullivan et al., 1999; Verweij et al., 1997; Xiong et al., 1997). The central role of mitochondria in intrinsic apoptotic pathways makes cells vulnerable to inadvertent initiation and progression of death program upon the rupture of the organelles and release of pro-apoptotic factors. Brain trauma and mechanical stretch injury of neurons may be associated with the breakage of their neurites. It is possible that this will be associated with the mitochondrial mechanical injury and inadvertent triggering of apoptosis. This implies that suppression of mitochondrial events leading to the release of pro-apoptotic factors may limit trauma-associated death of neurons.

Based on intracellular localization, two populations of neuronal mitochondria have been described that include non-synaptic (somal) and synaptic mitochondria (Lai et al., 1977). These two types of mitochondria display different metabolic activities possibly associated with specific features of the phospholipid composition of their membranes (Kiebish et al., 2008a). In particular, levels of CL were found to be lower, whereas levels of ceramide and PS were higher in synaptic versus somal mitochondria (Kiebish et al., 2008a). Because CL molecules contain four fatty acid residues, they may include many different individual molecular species which can be distinguished by MS-analysis (Sparvero et al., 2010). Notably, molecular speciation of CLs in somal and synaptic mitochondria was found to be similar (Kiebish et al., 2008b). The total amount and the diversity of CLs are dependent on the de novo synthesis and remodeling, respectively (Schlame, 2008) (Schlame, 2008). Therefore, it is likely that remodeling of CLs is similar whereas its synthesis is different in synaptic and somal mitochondria of neurons. We
found that mitochondria in the neurites were damaged at early time points after mechanical stretch injury whereas somal mitochondria were significantly more resistant and demonstrated features of structural damage much later after the stretch.

One of the possible reasons for the preferential damage to neurite mitochondria vs soma could be in mechanical strain imposed on neurites versus soma. It is difficult to know the exact strain distribution for neurites and soma we examined in our model; however data from other mechanical cell injury models suggest that the magnitude and orientation of principal strains vary spatially and temporally and depend on cell morphology. It was shown that (Barbee et al., 1994; Kilinc et al., 2008) fluid shear stress injury on cultured primary chick forebrain neurons caused axonal beading and localized cytoskeletal damage. Beads contained accumulated mitochondria and co-localized with focal microtubule disruptions. Laplaca and Thibault used a device that is a parallel disk viscometer applying fluid shear stress with variable onset rate (LaPlaca et al., 1997). They showed that the strain and strain rate for cell bodies and processes of the NT2-N cells (teratoma-derived human neuron like cells) varied widely for a given shear stress stimulus and arrangement. Cells in clumps of four or more had lower average strains than single neurons or neurites. The wide range of strains observed could be due to the variation in local forces, the organization and morphology of the neurons and the spatial orientation and location of the cells and processes on the cell plate. Cell bodies contain less adhesion sites in culture as compared to cell processes, and therefore may be less susceptible to mechanical damage in our model. At the tissue level however other cell types and microvascular environment support forces differently from neurons and have different biochemical responses to traumatic stimuli (LaPlaca et al., 1997; Murphy and Horrocks, 1993). It is also worth mentioning that mitochondria are organized in a network – mitochondrion – connected to other membrane
structures and systems such as endoplasmic reticulum, nucleus and the cytoskeleton in neurons (Kuznetsov et al., 2009; Mironov, 2009) (Kuznetsov et al., 2009; Mironov, 2009). Therefore, any strain will be acting not on isolated mitochondria but on the entire branched system; it is possible that structural specificity of mitochondrion in neuritis is different from that in the somal area – thus contributing to the different response to mechanical strain.

Mitochondrial superoxide production has been implicated in trauma-induced neuronal death (Lewen et al., 2001; Sullivan et al., 1999). Mitochondria targeted hydroethidine (mito-hydroethidine or MitoSOX) became a very popular fluorogenic probe for detecting mitochondrial superoxide radical anion, however its specificity has been recently questioned (Zielonka and Kalyanaraman, 2010). While the reaction between superoxide and mito-hydroethidine generates a specific red fluorescent product, mito-2-hydroxyethidium, another red fluorescent product, mito-ethidium, is also formed, usually at a much higher concentration than 2-hydroxyethidium. Both oxidation products of mito-hydroethidine bind to mitochondrial DNA with a concomitant many-fold (~40-fold) increase in the quantum yield of fluorescence. Thus it is likely that DNA-bound Mito-hydroethidine oxidation product(s) will be the major contributors to the detectable fluorescence response. We utilized flow cytometry for quantitative analysis of MitoSOX data (Figure 3). According to our protocol, MitoSOX (Invitrogen, Carlsbad, CA) was added to the neuronal cultures and incubated for 5 min after which the cells were fixed with 4% paraformaldehyde to stop the reaction. A similar protocol for flow cytometry utilizing 3.7 % paraformaldehyde after incubation of cells with MitoSOX has been used in previous studies (Ainslie et al., 2008). We believe that mitochondrial DNA-bound mito-hydroethidine oxidation products are not readily diffusible from paraformaldehyde treated cells. Further, we did not correct the MitoSOX distribution across plasma and mitochondrial membrane due to a possible
stretch-induced decrease of membrane potentials. Elegant studies by Johnson-Caldwell et al., have shown that a partial depolarization of either membrane could lead to an underestimation of superoxide levels. Clearly such a correction would have enhanced the difference between the control and stretched samples. We cal also add to this, that the activity of intracellular esterases catalyzing the hydolysis of triphenylphosphonium moiety, which targets MitoSOX into mitochondria, might be another factor potentially affecting the fluorescence read-outs. Overall, however, a relatively poor specificity of mito-hydroethidin for superoxide anion rather than possible other deficiencies of the technique (such as effects of paraformaldehyde, dependence on the mitochondrial membrane potential) may be a matter of major concern. Therefore, in the current study, we used other independent measures of oxidative stress – western blotting of HNE-protein adducts and oxidative lipidomics analysis.

Compared to previous studies evaluating intrinsic caspase-mediated apoptosis in primary cortical neurons, the time course of apoptotic events was different in stretched neurons (Stoica et al., 2003; Tyurin et al., 2008). In stretch-induced apoptosis, cyt c release into cytosol is unusually fast and there is a delay in caspase activation. The early release of cyt c can be due to mechanical deformation of the outer mitochondrial membrane with stretch injury and subsequent release of cyt c. There could be several reasons for the observed delay in cyt c release and caspase activation. The initial appearance of small amounts of cyt c triggers a positive feedback loop and further release of cyt c from mitochondria eventually causing caspase activation. In addition, we have shown that during apoptosis released cyt c is scavenged by alpha-synuclein in the cytosol preventing its interaction with APAF-1 thus caspase activation (Bayir et al., 2009). Nevertheless, significant amounts of cyt c remained confined within the mitochondrial compartments at early
and late time points after the injury, possibly indicating its association with less damaged mitochondria in the soma of neurons.

Another mitochondrial event essential for the release of pro-apoptotic factors from mitochondria into the cytosol is robust oxidation of CL (Kagan et al., 2005b; Tyurina et al., 2006). CL oxidation in neurons was detected as early as 2 hr after stretch when caspases were not activated yet mitochondria in neurites were damaged. Studies from several laboratories documented that execution of the mitochondrial stage of apoptosis is accompanied by oxidation of CL (Iverson and Orrenius, 2004; Kagan et al., 2005b; Petrosillo et al., 2006). We discovered that CL represents a selective target of oxidative attack during apoptosis whereby cyt c forms complexes with CL and catalyzes oxidation of the latter (Kagan et al., 2005b; Tyurina et al., 2006). Most importantly, CL oxidation products are essential for the mitochondrial permeability transition and the release of pro-apoptotic factors into the cytosol (Kagan et al., 2005b).

In the stretched neurons, we observed small but significant amount of Annexin V positivity as early as 30 min after injury. This may be due, at least in part, to a transient permeabilization of plasma membrane at damaged sites of processes resulting in possible binding of Annexin V to PS in the inner leaflet of the plasma membrane. Acute plasmalemma damage and permeability has been well documented after TBI both in vivo and in vitro (Geddes-Klein et al., 2006a; Geddes et al., 2003; Whalen et al., 2008). In line with earlier published data, caspase-3 activation is associated with nuclear shrinkage, fragmentation, and apoptotic body formation after mechanical stretch injury (Pike et al., 2000). We found that caspase-3 was significantly activated 12-24 hrs after stretch. Usually, during caspase dependent intrinsic apoptosis, caspase activation is followed by PS oxidation and then PS externalization (Kagan et al., 2005b). We
have shown that cyt c plays an important role in PS oxidation and externalization (Jiang et al., 2004). It is possible that a fraction of the released cyt c interacts with PS resulting in the formation of cyt c/PS complex with peroxidase activity (Kapralov et al., 2007) that induces the accumulation of PS oxidation (PSox) products. PSox are important contributors to externalization of PS on the surface of apoptotic cells and their subsequent clearance (Kagan et al., 2003; Tyurina et al., 2004).

Several interrelated pathways of cell death –necrosis, necroptosis, caspase-dependent and caspase-independent apoptosis– can occur after stretch injury (Stoica et al., 2003). Improvement in cell survival by pre-treatment with a pan-caspase inhibitor, z-VAD-fmk (Pop et al., 2008), suggests that caspase-dependent pathway is playing an important role in stretched-induced neuronal death in our model. However, as mentioned Z-VAD-fmk did not completely attenuate the stretch-induced neuronal death suggesting that other pathways of cell death are also operating after stretch injury. Interestingly, z-VAD-fmk also protected against accumulation of PI+/Annexin V- cells as well as against LDH release from injured neurons thus indicating that caspase-driven mechanisms also participated in the necrotic cell death pathway, i.e. there is a cross-talk between these two cell death pathways. One may assume that incomplete apoptosis and subsequent necrosis are interconnected resulting in the sensitivity of the latter to z-VAD-fmk (Dietz et al., 2007; Suzuki and Koike, 2005). It is also possible that caspase-independent apoptotic mechanisms (e.g., calpain-mediated and AIF-dependent (Cao et al., 2007) and necrosis also interact.

Prevention of CL oxidation may be a preferred target for anti-apoptotic strategies to protect neurons. Indeed, mitochondria-targeted electron scavengers, nitrooxides conjugated with
hemigramicidin S or tri-phenylphosphonium have been shown to effectively safe-guard cells against pro-apoptotic agents (Jiang et al., 2008) in vitro and exert protective effects in vivo after exposure of animals to damaging insults associated with significant accumulation of apoptotic cells (i.e., ionizing irradiation or hemorrhagic shock) (Jiang et al., 2008; Macias et al., 2007). Thus our data invoke reasonable optimism towards potentially useful applications of the mitochondria-targeted inhibitors of CL oxidation as anti-apoptotic neuroprotectors after brain trauma. Indeed, our preliminary experiments demonstrated the usefulness and effectiveness of a hemigramicidin S/nitroxide conjugate, XJB-5-131, to exert significant protection of young rats against TBI induced damage as evidenced by both biochemical and functional assessments (Ji et al., 2010). Corroborating the notion that mitochondrial lipid peroxides are effective therapeutic targets after TBI, Mustafa et al., showed that U-83836E, a selective scavenger of lipid peroxyl radicals, reduced lipid peroxidation and improved bioenergetics in mitochondria after TBI (Mustafa et al., 2010).
3.2 GLOBAL LIPIDOMICS IDENTIFIES CARDIOLIPIN OXIDATION AS A MITOCHONDRIAL TARGET FOR REDOX THERAPY OF ACUTE BRAIN INJURY

In Submission.


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3.2.2  NOTE

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Figure 8 was generated by Drs. Valerian E. Kagan, Hülya Bayır, Andrew Amoscato, Alejandro S. Arias and I.
Figure 9 was generated by Drs. Valerian E. Kagan, Hülya Bayır, and I.
Figure 10 was generated by Drs. Valerian E. Kagan, Hülya Bayır, and Yulia Y. Tyurina.
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3.2.4 ABSTRACT

Brain contains a highly diversified complement of molecular species of a mitochondria-specific phospholipid, cardiolipin (CL), which - due to its polyunsaturation - can readily undergo oxygenation. Here, we used global lipidomics analysis in experimental traumatic brain injury (TBI) and showed that TBI was accompanied by oxidative consumption of polyunsaturated CL and accumulation of more than 150 new oxygenated molecular species in CL. RNAi-based manipulations of CL-synthase and CL levels conferred resistance of primary rat cortical neurons to mechanical stretch - an in vitro model of traumatic neuronal injury. By applying the novel brain permeable mitochondria-targeted electron-scavenger, we fully prevented CL oxygenation in the brain, achieved a substantial reduction in neuronal death both in vitro and in vivo, and markedly reduced behavioral deficits. We conclude that CL oxygenation generates essential neuronal cell death signals and that its prevention by mitochondria-targeted small molecule inhibitors represents a new target for neuro-drug discovery.
Key words: cardiolipin; traumatic brain injury; mitochondria; oxidative lipidomics; controlled cortical impact model; RNA interference; EPR; MALDI-MS; 2D-LC/MS; behavioral outcome
3.2.5 MATERIALS AND METHODS

3.2.5.1 Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. XJB-5-131 was prepared as previously described (Wipf et al., 2005). All surgical, injury, and animal care procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

3.2.5.2 Rat controlled cortical impact model

PND17 male Sprague-Dawley rats were anesthetized with 3.5% isoflurane in O₂. The trachea was intubated with a 14-gauge angiocatheter. A surgical level of anesthesia was maintained with 2% isoflurane in N₂O/O₂ (2:1). Following the insertion of a femoral venous catheter, a craniotomy was made over the left parietal cortex with a dental drill, using the coronal and interparietal sutures as margins. A microprobe (Physitemp Instruments, Clifton, NJ) was inserted through a burr hole into the left frontal cortex to monitor brain temperature, which was maintained at 37 ± 0.5 °C via a heat lamp. A rectal probe monitored core temperature. Following the craniectomy, isoflurane was decreased to 1% and rats were allowed to equilibrate for 30 min. For all studies, a 6 mm metal pneumatically driven impactor tip was used. Velocity of the impact was 4.0 ± 0.2 m/sec, with a penetration depth of 2.5 mm. After the impact, the bone flap was replaced, sealed with dental cement, and the scalp incision was closed. After a 1-h monitoring period, rats were weaned from mechanical ventilation, extubated, and returned to their cages until further study. Sham-control rats underwent the same processes including anesthesia and surgery except that they did not receive the cortical impact. A mortality rate of
≤5% is routinely observed with this protocol by our group (Adelson et al., 1998). All surgical, injury, and animal care procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

3.2.5.3 Primary cortical neuronal culture

Primary cortical neuronal cultures were prepared from embryonic day 16 rats. Pregnant Sprague–Dawley rats (Charles River Lab, CA) were anesthetized with CO₂ and sacrificed via decapitation. Embryos were surgically removed from the adult rat and cortices were isolated separately in ice-cold Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with B-27 (Invitrogen, Carlsbad, CA) and Penicillin-Streptomycin (Invitrogen, Carlsbad, CA). Tissue was then rinsed twice with the same medium and set in 0.25% trypsin with EDTA (Invitrogen, Carlsbad, CA) for 15-20 min at 37 ºC. The tissue was again rinsed twice and triturated in ice-cold Neurobasal medium to yield a neuronal cell suspension. Experiments were performed at day in vitro (DIV) 7-9, when cultures consist primarily of neurons (>95% microtubule-associated protein-2 immunopositive cells, <5% glial fibrillary acidic protein immunopositive cells).

3.2.5.4 Cell viability assays

Cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and lactate dehydrogenase (LDH) release. The MTT solution (5 mg MTT/mL medium) was added to each well 24 h after mechanical stretch at the final concentration of 250 µM and incubated for 3 h. The media was removed, and cells were dissolved in dimethyl sulfoxide (DMSO). Optical density was determined using a spectrophotometer (Spectra MAX 340, Molecular Devices, Sunnyvale, CA) at 550 nm test and
690 nm reference wavelengths. LDH release was measured in duplicate 20 μl supernatant samples. LDH activity is proportional to the rate of pyruvate loss, which was assayed by absorbance change by using a microplate reader (Spectra MAX 340, Molecular Devices, Sunnyvale, CA). Blank LDH levels were subtracted from insult LDH values and results normalized to 100% neuronal death caused by 0.5% Triton X-100 exposure.

### 3.2.5.5 Assessment of mitochondrial superoxide production with MitoSOX

Mitochondrial superoxide production was measured by flow cytometry as described (Mukhopadhyay et al., 2007). Briefly, MitoSOX Red (Invitrogen, Carlsbad, CA) was added to the neuronal cultures with a final concentration of 3 μM at 2 h after stretch. The cells were incubated at 37 °C for 10 min to allow loading of MitoSOX Red. The cells were then detached with 0.1% trypsin followed by centrifugation at 600 g for 3 min at 4 °C. The cells were washed 2 times with PBS and placed in a sterile FACS tube at a concentration of 5–10×10⁶ cells per 100 μl and analyzed by flow cytometry (BD Biosciences, Rutherford, NJ) supported by CellQuest software. Hoechst 33258 (1 μg/mL; Invitrogen, Carlsbad, CA) was added 1 min before flow cytometric analysis. A fluorescence microscope assessment was also performed by incubating neurons with MitoSOX (5 μM) for 5 min at 37 °C at 2 h after stretch. Nuclei were stained with Hoechst 33258 (Invitrogen, Carlsbad, CA). Cells were examined using an inverted fluorescent microscope (Leica DM-IL) equipped with a digital camera (Leica DC300, Wetzlar, Germany).
3.2.5.6 **Assessment of phosphatidylserine (PS) externalization**

Externalization of PS was analyzed by flow cytometry using an Annexin V kit (Biovision, Mountain View, CA). Briefly, cells were harvested at the end of incubation, and then stained with Annexin V-FITC and propidium iodide (PI) prior to analysis. Ten thousand events were collected on a FACScan flow cytometer equipped with a 488-nm argon ion laser and supplied with the Cell Quest software.

3.2.5.7 **Accumulation of cyt c in cytosol after in vitro TBI**

Neuronal cells (1×10⁷) were washed in PBS and lysed by incubating in lysis buffer (75 mM NaCl, 8 mM Na₂HPO₄, 1 mM NaH₂PO₄, 1 mM EDTA, and 350 µg/mL digitonin) for 2 min. The lysates were centrifuged at 10,000 g for 5 min; the supernatant was collected for cytosolic cyt c analysis. Cyt c was detected by western blot analysis using an antibody against cyt c (1:1000 dilutions, BD Pharmingen, San Diego, CA).

3.2.5.8 **XJB-5-131 treatment in PND 17 rats**

Rats were randomized into one of the following groups: naïve, sham plus vehicle, sham plus XJB-5-131, CCI plus vehicle, or CCI plus XJB-5-131. For the XJB-5-131 treatment group, rats were treated with XJB-5-131 (10 mg/kg/dose) administered i.v. at 5 min and i.p. at 24h after CCI, respectively. Rats in the CCI plus vehicle and sham plus vehicle groups received similar treatment, except that XJB-5-131 was replaced with an equal volume of vehicle (50:50 v/v of cremophor el:ethanol dissolved in saline (1:3)).
3.2.5.9 In vitro model of TBI and XJB-5-131 treatment

In vitro TBI was performed as previously described (Geddes-Klein et al., 2006b). In brief, primary cortical neuronal cultures were prepared, cultured on 0.05% poly-D-lysine hydrobromide coated silicone substrate wells, and were subjected to a computer controlled quantifiable mechanical insult by displacing the silicone substrate over a hollowed platform. Severe stretch was chosen in order to simulate a strain field similar to that occurs in the animal model of TBI. Neurons were incubated with XJB-5-131 (concentrations ranging from 1 μM to 25 μM) or vehicle 10 min before mechanical stretch.

3.2.5.10 Cardiolipin synthase and cytochrome c RNA interference

Small interfering RNA (siRNA) targeting rat cardiolipin synthase, cyt c, and pooled scrambled control siRNA were purchased commercially (Life Technologies, Carlsbad, CA). Primary neurons were transfected on day 4 with 45 nmol of cardiolipin synthase or cyt c or control siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The efficacy of cardiolipin synthase and cyt c knockdown was assessed by western blot using an antibody against cardiolipin synthase (customized antibody made by Invitrogen, Carlsbad, CA) and cyt c (BD Pharmingen, San Diego, CA). Experiments were performed 72 h after transfection.

3.2.5.11 EPR-based analysis of partitioning and distribution of nitroxides in cultured neurons and in CSF

Primary cortical neurons (DIV 7, 5x10^6 cells/well) were incubated with 10 μM XJB-5-131 for 15 min at 37 °C. At the end of incubation, cells were washed twice with serum-free medium. Mitochondria-enriched fractions were obtained using a mitochondria isolation kit.
(Pierce, Rockford, IL). CSF was collected from the cisterna magna of naïve PND 17 rats 30 min after i.v. injection of XJB-5-131 (10 mg/kg). Each cellular fraction (whole cell pellet, mitochondria, and cytoplasm) or CSF was mixed with DMSO (1:1 v/v) or 1.5 mM of potassium ferricyanide to convert nitroxides to EPR-detectable radical forms. An aliquot of 70 μl of the cellular fraction or CSF was loaded into Teflon tubing (0.8 mm internal diameter) (Alpha Wire Corp., Elizabeth, NJ) which was folded in half and placed into an open ESR quartz tube (inner diameter of 3.0 mm). EPR measurements were made in triplicate using a JEOL-RE1X EPR spectrometer (Jeol, Tokyo, Japan) using the following conditions: 334.7 mT center field, 5 mT sweep width, 0.079 mT field modulation, 20 mW microwave power, 0.1 s time constant, and 2 min scan time at 22.5°C. Utilizing signal magnitude and isolated volumes, the amount and concentration of XJB-5-131 were calculated for each sample.

3.2.5.12 In vivo EPR imaging of distribution of nitroxides in the rat brain

L-Band EPR spectroscopy and micro computerized tomography (CT) were applied for the co-imaging of XJB 5-131 spin label in brain of naïve PND 17 rats. After i.v. injection of XJB-5-131 (50 mg/kg) or vehicle into PND 17 rats, head of animals were imaged every 15 min using ELEXSYS E540 L-Band spectrometer and 23mm resonator (Bruker Biospin GmbH, Germany), assuring a high filling factor (sensitivity) for small animals. For observation of spin label spatial distribution in brain tissue the images were obtained in the presence of two capillaries filled with 2.5 and 5 μl of 10 mM 3-carboxy-proxyl solution. Acquisition of EPR data were accomplished using following settings: modulation amplitude = 2 G; modulation frequency = 100 kHz; microwave power = 40 mW; conversion time = 30 ms; image field of view = 25 mm; acquired angles = 31, gradient = 24 G/cm. Finally we proceed with creation of 2D EPR by
employing of following strategies: zero order of baseline correction; FT deconvolution using a gaussian window with a width of 0.15 mm; filtered back projection.

3.2.5.13 Imaging of nitroxides in rat brain by matrix assisted laser desorption ionization (MALDI) MS.

Tissue samples were prepared as described previously (Koizumi et al., 2010). Briefly, rats were sacrificed at 30 min after 10mg/kg single i.v. injection of XJB5-131. The brains were dissected and immediately flash frozen in liquid nitrogen. Subsequently, tissues were cut without fixation or embedding. Minimal OCT (Sakura FineTek USA, Torrance CA) was used only to attach the tissue to the cryostat block, and at no time did the blade touch the OCT. Coronal sections were cut at -19 °C at 10 μm thickness with a cryostat (Leica Microsystems in Wetzlar, Germany), and placed directly onto indium-tin oxide coated MALDI slides (Delta Technologies, Stillwater MN). Prepared slides were immediately vacuum-dried for at least one hour, and stored at −20 °C until matrix application and subsequent MS imaging analysis. The adjacent brain section, also 10 μm in thickness, was counter stained with Hematoxylin-and-Eosin (H&E). Tissue slices were incubated with phospholipase-C (PL-C) at 3.3 milli-units of activity per microliter) for 20 min at room temperature to remove interference from cationic phospholipid signals. The PL-C solution was then carefully pipetted off, followed by two washes with 0.5 M lithium acetate and overnight vacuum drying. As such, XJB was subsequently detected as its lithium adduct at 966 m/z. The MALDI matrix 2,5-dihydroxybenzoic acid (DHB, 98%+ grade) was purchased from Sigma-Aldrich (St. Louis, MO) and was freshly dissolved (55mg/mL in 70% methanol/0.02% trifluoroacetic acid) prior to use. The matrix solution was applied with a commercial airbrush (1 sec burst every 30 sec, with 15
min of spraying followed by 15 min of vacuum drying, total of 90 bursts per slide). MS Imaging was done in reflector-positive mode using an Autoflex MALDI TOF/TOF (Bruker Daltonics, Billerica, MA) with a Smartbeam laser focused to approximately 50 μm. The raster scan was performed automatically at a data point interval of 200 μm with a random walk within each area in order to gain the most signal possible. This yielded approximately 1750 data points for analysis by FlexImaging 3.0 software (Bruker Daltonics, Billerica, MA).

3.2.5.14 Assessment of CL oxidation by 2-dimensional liquid chromatography/mass spectrometry (2D-LCMS)

Total lipids were extracted using the Folch procedure (Folch et al., 1957b) from crude brain mitochondrial fraction. Lipids were analyzed in the first dimension by normal phase chromatography on a Luna silica column (2.0 mm (i.d.) x 15 cm, Phenomenex, Inc.). Solvents consisted of A: chloroform: methanol: 30% ammonium hydroxide (80:19.5:0.5, v/v/v) and B: chloroform: methanol: water: 30% ammonium hydroxide (60:34:5.5:0.5, v/v/v/v). Flow was maintained at 0.2 mL/min and a linear gradient of 0-100% B in 30 min was run. Eighty percent of the flow was diverted to a fraction collector while the remainder was utilized for MS analysis. Individual phospholipid classes from the first dimension chromatography were subsequently analyzed in a second dimension system. For CL, a C8 column was used (5 micron, 4.6 mm x 15 cm, Phenomenex, Inc.) with an isocratic solvent system consisting of 2-propanol: water: triethylamine: acetic acid (45:5:0.25:0.25). Flow was maintained at 0.4 mL/min and analysis was performed on a Waters Premier Q-TOF mass spectrometer. For the remaining phospholipids and for quantitative purposes for CL, lipids were analyzed on an ion trap instrument (LCQ-Duo, Thermo-Finnigan, Inc.) using a C18 column (4.6 mm x 15 cm,
Phenomenex, Inc.) with a solvent system consisting of A: acetonitrile: water: triethylamine:acetic acid (45:5:0.25:0.25) and B: 2-propanol:water:triethylamine:acetic acid (45:5:0.25:0.25) using a linear gradient from 50-80% B in 15 min and ramping to 100% B by 30 min, modified from a previously described system (Kim et al., 2011; Minkler and Hoppel, 2010). Flow was maintained at 0.4ml/min. Instrument conditions for the Waters Premier Q-TOF mass spectrometer were as follows: capillary voltage, 2.85 kV, negative mode; source temperature, 100 °C; desolvation gas, 400 L/h; sampling cone, 60 V; extraction cone: 4.5 V; ion guide, 3.0 V. Tuning was optimized for all lipids across the scan range. Instrument conditions for the ion trap analysis were as follows: capillary temperature, 250 °C; spray voltage, 4.5 kV; sheath gas, 30. All other tuning parameters were optimized for each individual lipid class.

3.2.5.15 Neurocognitive outcome assessment

Neurocognitive outcome was assessed by an observer blinded to experimental group. Motor function was evaluated using the beam balance and inclined plane tests on days 1–5 after CCI or sham injury (Adelson et al., 1997; Kline et al., 2004). Motor training consisted of providing three trials one day before injury. For the beam balance task, each rat was placed on a suspended, narrow wooden beam (1.5 cm wide), and the latency that the rat remained on the beam was measured up to a maximum of 60 sec. For the inclined plane task, each rat was placed on a flat board at an initial angle of 45°. The angle of the plane was then increased in 5° increments to a maximum of 85°. The steepest angle at which the rat could maintain its position on the board for 10 sec was recorded. Each rat performed 3 trials at each angle with a 5 sec inter-trial interval. A water maze task was utilized to assess spatial learning. Briefly, the maze consisted of a plastic pool (180 cm diameter; 60 cm high) filled with water (26 ± 1 °C) to a depth
of 28 cm and situated in a room with salient visual cues that remained constant throughout the study. The platform was a clear Plexiglas stand (10 cm diameter, 26 cm high) that was positioned 26 cm from the maze wall in the SW quadrant and held constant throughout the study for each animal. Acquisition of spatial learning began on post-operative day 11 and consisted of providing a block of four daily trials for five consecutive days (days 11–15) to locate the platform when it was submerged 2 cm below the water surface. For each daily block of trials the rats were placed in the pool facing the wall at each of the four possible start locations (North, East, South, and West) in a randomized manner. Each trial lasted until either the rat climbed onto the platform or 120 s had elapsed, whichever occurred first. Rats that failed to locate the goal within the designated time were manually guided to it. All animals remained on the platform for 30 sec before being placed in a heated incubator between trials (4 min inter-trial interval). The data were obtained using a spontaneous motor activity recording & tracking (SMART) system (San Diego Instruments, San Diego, CA).

### 3.2.5.16 Caspase 3/7 activity assay

Caspase 3/7 activity was measured using Caspase GloTM 3/7 assay kit (Promega, Madison, WI). Briefly, ipsilateral rat cortical or neuronal homogenates (around 20 µg protein) were mixed with 50 µL of Caspase-GloTM reagent and incubated at room temperature for 1 h. Caspase-3 activity was expressed as the luminescence produced within 1 h of incubation at 25 °C using an ML1000 luminescence plate reader (Dynamech Labs, Horsham, PA). Protein concentrations were determined using the BioRad assay (Bio-Rad Laboratories, Inc., Hercules, CA).
3.2.5.17 **Fluorescence assay of brain thiols**

Brain thiols – GSH and PSH were estimated in ipsilateral cortical homogenates using ThioGloTM-1 as described previously with minimal modifications (Tyurin et al., 2000). GSH concentrations were determined by addition of GSH peroxidase and hydrogen peroxide to the brain homogenates, and the resultant fluorescence response was subtracted from the fluorescence response of the same samples without addition of GSH peroxidase and hydrogen peroxide (Sigma, St. Louis, MO). Levels of protein thiols were determined as an additional fluorescence response after addition of 4 mM SDS to the same sample. A Shimadzu spectrophotometer RF-5301PC (Shimadzu, Kyoto, Japan) was used to detect fluorescence using excitation and emission wavelengths of 388 nm and 500 nm, respectively.

3.2.5.18 **Histological Assessment**

Rats were anesthetized and transcardially perfused with 50 mL ice-cold heparinized saline followed by 50 mL 10% paraformaldehyde (in phosphate-buffered saline, pH 7.4). Brains were removed and further post-fixed in 10% paraformaldehyde for at least 72 h. The resulting paraffin blocks were sequentially sectioned coronally at 5 µm. Fluoro-Jade C staining of brain sections was used to identify degenerating neurons. Briefly, 5-µm coronal brain sections were deparaffinized in a series of xylene, immersed twice in 100% ethanol (EtOH) and 1% sodium hydroxide (in 80% EtOH) for 90 s, and in then 70% EtOH for 30 s. Slides were then placed on a shaker in 0.06% potassium permanganate for 10 min and washed in distilled water before immersion in a 0.006% working solution of Fluoro-Jade C (Chemicon, Temecula, CA) with 4',6-diamidino-2-phenylindole (Sigma, St Louis, MO) for 30 min. Fluoro-Jade C positive neurons within the peri-contusional area were counted by CellProfilerTM software (Broad Institute, MA).
3.2.5.19 Statistical Analysis

Statistical analyses of functional outcome were performed using Statview 5.0.1 software (Abacus Concepts, Inc., Berkeley, CA). The motor and cognitive data were analyzed by repeated-measures analysis of variance (rANOVA). When the overall ANOVA revealed a significant effect, the data were further analyzed with the Bonferroni/Dunn post-hoc test to determine specific group differences. The behavioral data are presented as the mean ± s.e.m. and are considered significant when corresponding P values are less than 0.05 or as determined by the Bonferroni/Dunn statistic after adjusting for multiple comparisons. All other data (non-behavioral) were expressed as mean ± s.d. Data involving primary cortical neurons were obtained from at least three independent experiments. Statistical comparisons between groups were performed by Student’s t-test. Differences were considered statistically significant when P value was less than 0.05 and are indicated in figure legends.
Every year in the United States alone, an estimated 1.7 million people sustain acute brain injury from trauma and of those, 52,000 die and 85,000 suffer from long term disabilities (Faul, 2010; Thurman et al., 1999). This includes injuries from many etiologies such as road traffic accidents, falls, assaults, and sports concussion. Also, the RAND Corporation estimates that over 400,000 US soldiers have suffered TBI in Operation Iraqi Freedom and Operation Enduring Freedom (RAND, June 26, 2011). A specific therapy for TBI does not yet exist and standard treatment remains supportive in nature. Like many forms of acute brain injury, TBI involves primary injury that is felt to be refractory to treatment and secondary injury, characterized by a cascade of biochemical and cellular events contributing to neuronal damage over time (Ghajar, 2000). Secondary injury involves multiple pathways of neuronal death which represent viable therapeutic targets (Maas et al., 2010; Stoica and Faden, 2010).

Complexity of brain functions requires sophisticated coordination of highly diversified operational signals. Numerous small molecule-signals formed from a relatively limited number of metabolic precursors are accountable for the precision and effectiveness of the brain’s operational language. Among these are polyunsaturated lipids capable of undergoing enzymatic oxidation and other metabolic conversions yielding oxygenated signaling entities - from eicosanoids, prostanoids and resolvins to cannabinoids and neuroprotectins. There is substantial archaeological evidence that exploitation of abundant food resource enriched with polyunsaturated lipids was essential for sustaining the comparatively large size, the apparent unique complexity and high level of interconnectivity in the modern human brain and provided
the advantage in multi-generational brain development, thus making possible the advent of H. sapiens (Broadhurst et al., 2002). The demand for diverse polyunsaturated lipid precursors is so large that mitochondria-specific CL is represented in the brain—but not in other tissues—by hundreds of polyunsaturated individual molecular species (Kiebish et al., 2008b). Our previous work demonstrated that in fatally injured cells, oxidation of polyunsaturated CL species by cytochrome c (cyt c) generates death signals essential for mitochondrial permeability transition and release of death factors into the cytosol (Kagan et al., 2005a).

While lipid peroxidation has been long associated with acute brain injury, its specific role in mediating damaging pathways and signaling cascades is not well understood (Chan and Fishman, 1980; Verweij et al., 2000). We reasoned that aberrant CL peroxidation may be an important pathogenic pathway in acute brain injury. Using a newly developed 2-dimensional liquid chromatography mass spectrometry (2D-LC-MS) protocol, we performed global lipidomics analysis of phospholipids, which revealed almost 190 individual molecular species of CL in normal brain, of which only 10 were oxygenated. Notably, experimental TBI - controlled cortical impact (CCI) - induced oxidation of majority of polyunsaturated molecular species of CL; the number of non-oxidized CL species decreased to ~100, while that of oxygenated species increased to 166. Quantitatively, the content of oxidized CL species increased 20-fold at the expense of decreased amounts of non-oxidized CL (Fig. 8a, 8c). This oxidation effect was specific to CL, as other, more abundant polyunsaturated phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), remained non-oxidized. The scale of these CCI-induced oxidative changes in CL far exceeded the detectable but much less pronounced decreases of the levels of glutathione (GSH) and protein sulfhydryls (PSH) (Fig.9), thus again emphasizing the specificity of CL oxidation.
To gain further insight into the role of CL and its oxidation in CCI induced damage, we utilized an in vitro TBI model of mechanical stretch injury in rat primary cortical neurons. Mechanical stretch triggered robust and selective peroxidation of CL (Fig. 10) but not PC or PE, which comprise 49% and 30%, respectively, of total phospholipids in primary neurons. Stretch also decreased neuronal viability assessed by LDH and MTT assays and induced caspase 3/7 activation (Fig. 11a, 11b and Fig. 12). We further utilized an RNAi approach to manipulate the content of CL in neurons by knocking-down CL-synthase (Fig. 13a), the key-enzyme of de novo CL biosynthesis, and assessed their responses to stretch. The CL content decreased to 44% of its level in parental cells (Fig. 13b), whereas the ATP content did not change (Fig. 13c). Notably, characteristic markers of stretch-induced cell death – elevated caspase 3/7 activity, release of cyt c from mitochondria into the cytosol, increased Annexin V-positivity, and release of LDH– were all markedly attenuated in CL-deficient cells (Fig. 11a-d). Our previous work demonstrated that cell death-associated CL oxidation was catalyzed by peroxidase activity of the cyt c/CL complex (Kagan et al., 2005a). With this in mind, we generated cyt c-deficient primary neuronal cells (Fig. 13d) and examined their vulnerability to stretch-induced damage compared to control cells. Sensitivity of cyt c-deficient neurons to stretch was lower than that of parental cells as evidenced by caspase 3/7 activity, cyt c release, Annexin V positivity, and cell viability (Fig. 11a-d). Taken together, these in vitro results are compatible with our hypothesis that CL oxidation, possibly catalyzed by cyt c, was involved in TBI induced damage of neurons.
Figure 8. Assessment of molecular species of CL and its oxidation products by 2D-LCMS after TBI.
(a) Typical spectra of CL obtained from brain cortex. Upper panel shows the presence of multiple non-oxidized (8-10 major clusters of mass ions shown in blue) and few oxidized (shown in red, CLOx) clusters of CL in naïve rat. Left upper insert: 1st dimension chromatographic separation of phospholipids in lipid extracts of the ipsilateral cortex. CL eluted with the 10–12 min retention time window. Right inserts: 2nd dimension chromatographic separation of non-oxidized and oxidized CL. The latter eluted during the 5-6 min retention time window. Middle panel demonstrates non-oxidized (blue) and the appearance of numerous oxidized (red) CL species after TBI. Lower panel illustrates the effect of XJB-5-131 administration after TBI on the profile of non-oxidized (blue) and oxidized (red) CL species. (b) Quantification of CL oxidation by 2D-LCMS. Increased content of CLOx at 3 h after CCI and its attenuation by XJB-5-131. *P < 0.05 vs. naïve and XJB-5-131; error bars, standard deviation, n = 4. (c) Evaluation of the number of non-oxidized and oxidized molecular species of CL in the brain.

Figure 9. Quantification of reduced glutathione (GSH, a) and protein sulphydryl (PSH, b) levels in the ipsilateral cortex after CCI.
XJB-5-131 (red bars) attenuated CCI-induced GSH and PSH depletion in PND 17 rats (blue bars). *P < 0.01 vs. naïve and sham controls, CCI 3h, and CCI (3h and 24h) + XJB-5-131; error bars, standard deviation; n = 4.
Figure 10. Increase in the contents of oxidized cardiolipin (CLox), phosphatidylcholine (PCox) and phosphatidylethanolamine (PEox) in rat cortical neurons 2 hours after stretch assessed by LC-MS.

Data are presented as pmol of oxidized phospholipid per nmol of phospholipid; means ± standard deviation; n = 4.
Figure 11. Response of CL or cyt c deficient neurons to *in vitro* TBI.

Quantification of cytotoxicity (lactate dehydrogenase (LDH) release) relative to Triton exposure (a), caspase 3/7 activity (b), cyt c release from mitochondria into cytosol (c), annexin V (d) and propidium iodide (PI) positivity (e) in rat cortical neurons transfected with Cardiolipin synthase (CS) or Cytochrome c (CYC) or scrambled control (SC) siRNA after mechanical stretch. Rat
cortical neurons were transfected 72 h before mechanical stretch and measurements were obtained at 24 h after stretch injury. C: control normal neurons; N: non-transfected neurons. * P < 0.01 vs. N and SC; error bars, standard deviation; n = 4. Stretch induced PI positivity did not change in CL and cyt c deficient neurons (P>0.05).

Figure 12. Effects of XJB-5-131 (1-25 μM) on primary rat cortical neurons in culture exposed to in vitro trauma by mechanical stretch. Vehicle or XJB-5-131 was added to the medium 10 min before stretch. a. Percent cytotoxicity (lactate dehydrogenase (LDH) release relative to Triton exposure (corrected for background LDH). b. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as a percentage of normal control conditions. Mechanical stretch induced approximately 35% neuronal death at 24 hours assessed by MTT and LDH. A marked reduction in neuronal death was produced by 5, 10, and 25 μM XJB-5-131 treatments. *P < 0.01 vs. stretch only neurons; error bars, standard deviation; data are from three independent experiments and include 8-11 wells for each condition.
Figure 13. Generation of cardiolipin (CL) and cytochrome c (cyt c) deficient neurons.

a. Primary rat neurons were transfected with CL-synthase (CS) siRNA or a scrambled control (SC) siRNA. Insert: Representative western blot showing effective knockdown of CS at 48h and 72h after siRNA treatment.

b. Quantification of total CL content in control and CS knock down neurons by electrospray ionization MS (ESI-MS). Transfection with CS produced ~15% (72 h)
and 56% (96 h) decrease in CL content, and did not change the mitochondrial member markers (as assessed by cytochrome c oxidase IV (COX IV) and transporter outer membrane 40 (TOM 40)).

c. Assessment of ATP levels in control and CS deficient neurons. CL deficiency did not change cellular ATP levels.

d. Primary rat neurons were transfected with Cytochrome c (CYC) or SC siRNA. Insert: Representative western blot showing effective knock down of cyt c at 48 h and 72 h after siRNA treatment. N, nontransfected neurons. *P < 0.01 vs. N and SC; error bars, standard deviation; n = 4.

Because mutation or suppression of CL synthesis and cyt c is therapeutically impractical, we hypothesized that inhibition of CL peroxidation could attenuate neuronal death and brain injury. This might be achieved by targeting electron scavengers which will prevent formation of H₂O₂ - the fuel for cyt c/CL peroxidase - in the mitochondria. Therefore, we designed novel mitochondria-targeted nitroxide pay-loads conjugated to selective transporters into mitochondria (Wipf et al., 2005). Stable nitroxide radicals combine electron and free radical scavenging actions with superoxide dismutase (SOD)-mimicking and recycling capacities. One of these nitroxides, Tempol, has been shown to improve motor function after experimental TBI; however, high millimolar concentrations were required to attain this improvement. We reasoned that these novel mito-nitroxides may represent effective therapeutic agents for brain injury, if they i) accumulate in mitochondria and prevent oxidative damage; ii) penetrate into the central nervous system (CNS) and accumulate in the brain tissue, and iii) display low toxicity and high neuroprotective potential. To this end, we used XJB-5-131 (Fig. 14) – a conjugate of 4-amino TEMPO and a chemically modified segment of a bacterial membrane targeting antibiotic, Gramicidin S (GS) – that effectively delivered the nitroxide into mitochondria. The characteristic triplet electron paramagnetic resonance (EPR) signal of GS-nitroxide was detected almost exclusively in the mitochondrial fraction of primary cortical neurons treated with XJB-5-131 (Fig. 15a), without affecting cell viability (Fig. 12).
Figure 14. Structure of the hemigramicidin S-peptidyl 4-amino TEMPO conjugate, XJB-5-131.
Figure 15. Analysis of distribution of XJB-5-131 in neurons and brain.

(a) XJB-5-131 (10 μM) partitions into mitochondria in primary cortical neurons. Recovered nitroxide radicals in whole cells, mitochondria, and cytosol fractions were suspended in phosphate buffered saline in the presence or absence of 1.5 mM ferricyanide (K$_3$Fe(CN)$_6$). Insert: representative EPR spectra of XJB-5-131 in different fractions of neurons in the presence of ferricyanide. *P < 0.05 vs. without ferricyanide; error bars, standard deviation; n = 4. (b) EPR-based analysis of XJB-5-131 in CSF in naïve rats. Naïve rats were given XJB-5-131 intravenously (i.v. 10 mg/kg). CSF was obtained at 30 min after injection. A typical signal of ascorbate radical (top spectra) is detected by EPR analysis in the absence of oxidant, ferricyanide. After addition of ferricyanide, a typical nitroxide signal of XJB-5-131 (bottom spectra) is detected. (c) Imaging of XJB-5-131 in the brain of naïve rats by L-Band EPR spectroscopy. For optimal positioning of the head, micro-CT was utilized (upper panel). Right upper panel shows an outline of the CT image. Lower panels demonstrate typical EPR images of distribution of XJB-5-131 in the brain obtained at 5 min and 25 min after its i.v. injection (50 mg/kg). The images were obtained in the presence of two nitroxide radical standards (2.5 and 5 μL of 10 mM 3-carboxy-proxyl solution) placed in proximal portions of capillary tubes (red arrows). (d) Distribution of XJB-5-131 in rat brain assessed by mass spectrometry imaging (MSI) and
corresponding Hematoxylin-and-Eosin (H&E) staining of the frozen section (upper panel). Naïve rats were given XJB-5-131 i.v. (10 mg/kg) and frozen brain sections were obtained at 30 min after injection. Sections were treated with phospholipase-C and lithium acetate in order to selectively remove highly abundant phospholipids (which would otherwise mask the signal of XJB-5-131). As such XJB-5-131 was detected as its lithium adduct at 966 m/z in positive mode TOF/TOF MSI with 2,5-dihydroxybenzoic acid (DHB) matrix. The white scale bar is 2 mm. The pixels are a heat map with red being the highest intensity. MSI was performed at 200 micron spatial resolution. Determination of the ratio of XJB-5-131 in its original radical (nitroxide) form and in the reduced hydroxylamine form by LC/MS (lower panel). Nearly all XJB-5-131 was converted into the reduced hydroxylamine form in the brain.

To evaluate CNS penetration of XJB-5-131, we measured the drug concentration in cerebrospinal fluid (CSF) by EPR spectroscopy. Typical nitroxide signals were detected in CSF when XJB-5-131 (10 mg/kg) was administered intravenously (i.v.) to naïve post-natal day 17 (PND17) rats (Fig. 15b). By employing micro computerized tomography (CT) and L-Band in vivo EPR imaging, we documented a time- and dose-dependent distribution of XJB-5-131 in naïve PND 17 rat brains after i.v. (Fig. 15c) injection. We further utilized mass spectrometric imaging and confirmed the presence of XJB-5-131 in the brain tissue after a single i.v. dose (Fig. 15d). Finally, direct quantification of XJB-5-131 in the brain tissue by LC-MS also demonstrated its accumulation to a level of 16.5 ± 4.3 pmole/gram of tissue (mean ± s.d.) at 3 h after a single 10 mg/kg i.v. injection.

We further examined whether XJB-5-131 would inhibit TBI-induced CL oxidation. Strikingly, CL oxidation was almost completely blocked by XJB-5-131 administered 10 min after TBI (Fig. 8b, c). The number of non-oxygenated and oxygenated species was comparable to those in control brain. Quantitatively, the amounts of non-oxidized and oxidized CLs remained also similar to those in the non-traumatized brain (Fig. 8c). In addition, XJB-5-131
protected brain thiols - GSH and PSH – oxidized by TBI (Bayir et al., 2002; Bayir et al., 2007b) (Fig. 9).

Based on the ability of XJB-5-131 to protect against CL oxidation, we evaluated its neuroprotective potential in TBI and performed neurobehavioral testing on PND 17 rats subjected to CCI injury. We conducted beam-balance and inclined platform, and Morris water maze tests, which are sensitive to motor and cognitive function/dysfunction after TBI (Hamm et al., 1992; Kline et al., 2004) on post-injury days 1-5 and 11-15, respectively. Balancing ability or inclined platform performance did not differ among groups prior to surgery (Fig. 16a, b). However, after surgery, we observed a significant improvement in motor deficits on both tasks on days 3 to 5 in the sham and CCI + XJB-5-131 groups compared to the CCI + vehicle group (Fig. 16a, b). Although all groups began at a similar level in the water maze, both the CCI + XJB-5-131 and sham groups performed progressively better vs. the CCI + vehicle group over the course of training (P < 0.001) (Fig. 16c). Swim speed did not differ among groups (P = 0.12), indicating that water maze performance was not influenced by differences in swimming ability or motivation. To evaluate the potency of XJB-5-131 in preventing neuronal death, we employed Fluoro-Jade C staining (Fig. 16d), commonly used to detect degenerating neurons regardless of the specific cell death pathway (Schmued et al., 2005). The number of Fluoro-Jade C positive cells increased at 24 h vs. 3 h after TBI and naïve/sham. Fluoro-Jade C positivity was observed predominantly in the pericontusional area and was significantly attenuated by treatment with XJB-5-131.

To explore the mechanism of neuroprotection by XJB-5-131, we utilized LC-MS to quantify its initial radical form and the reduced, hydroxylamine form. The two likely
mechanisms of XJB-5-131’s action are either as a SOD-mimetic - catalyzing the dismutation of superoxide radicals into H\textsubscript{2}O\textsubscript{2} - or as an electron scavenger - preventing one-electron reduction of molecular oxygen to superoxide. In the former case, XJB-5-131 would retain its radical state; while in the latter case, XJB-5-131 would be reduced to hydroxylamine. We found that nearly all XJB-5-131 (91.7 ± 14.5%) was converted into the hydroxylamine form (Fig. 15d). Given that the hemigramicidin S-derived alkene peptide isostere sequence in XJB-5-131 is responsible for its predominant localization in mitochondria, we conclude that its action was mainly through scavenging of electrons leaking from discoordinated electron carriers, and to a lesser extent through involvement of SOD-like activity.
Figure 16. Assessments of neurobehavioral and histological outcome in PND 17 rats treated with XJB-5-131 after TBI.
(a) Ability of rats to remain (seconds) on the beam balance apparatus before and after CCI or sham injury. A repeated measures ANOVA revealed a significant group ($F_{2,15} = 14.452$, $P = 0.0003$), day ($F_{5,75} = 47.631$, $P < 0.0001$), and group by day interaction effect ($F_{10,75} = 2.186$, $P=0.028$). Bonferroni post hoc analyses revealed that the CCI + XJB group performed significantly better than the CCI + vehicle group (*$P = 0.001$; error bars, standard error; $n = 7-10$). (b) Maximum angle (degrees) for rats to remain on an inclined platform. A repeated measures ANOVA revealed significant group ($F_{2,15} = 13.164$, $P =0.0005$) and day ($F_{5,75} = 18.085$, $P < 0.0001$) effects, as well as a significant group by day interaction ($F_{10,75} = 2.902$, $P=0.004$). Bonferroni post hoc analyses revealed that the TBI + XJB group performed significantly better than the TBI + vehicle group (*$P < 0.001$; error bars, standard error; $n = 7-10$). (c) Latency (seconds) for rats to locate a hidden (submerged) platform on post-TBI days 11-15. A repeated measures ANOVA revealed significant group ($F_{2,15} = 19.753$, $P < 0.0001$) and day ($F_{4,120} = 22.126$, $P<0.0001$) differences, as well as a significant group × day interaction ($F_{8,120} = 2.437$, $P = 0.018$). Bonferroni post hoc analyses revealed that the TBI + XJB group performed significantly better than the TBI + vehicle group ($P <0.001$; error bars, standard error; $n = 7-10$) and did not differ from the sham controls ($P = 0.08$). (d) Assessment of neurodegeneration by Fluoro-Jade C (FJC) staining. Neurodegeneration observed in the pericontusional area at 24 h after CCI was attenuated by XJB-5-131. *$P < 0.05$ vs. naïve and sham controls, CCI 3h + Vehicle, and CCI + XJB-5-131; error bars, standard deviation; $n = 4$.

To better define possible cell death pathways responsible for neuronal degeneration and the effect of XJB-5-131 in vivo, we examined caspase 3/7 activity. Ipsilateral cortical caspase-3/7 activity was increased at 24 h (but not at 3 h) after CCI (Fig. 17). This effect was attenuated by XJB-5-131, pointing to its anti-apoptotic mechanism of action. Similarly, treatment with XJB-5-131 (5, 10, and 25 μM) attenuated stretch-induced neuronal death (Fig. 12) via inhibition of mitochondrial superoxide production (Fig. 18a) and stretch-induced cytochrome c release into the cytosol (Fig. 18b), suggesting that mitochondrial damage was involved in neuronal cell death.
Figure 17. Quantification of caspase 3/7 activity in the ipsilateral cortex.
XJB-5-131 attenuated CCI-induced increase in caspase 3/7 activity in PND 17 rats. *P < 0.01 vs. naïve and sham controls, CCI 3h + Vehicle, and CCI + XJB-5-131; error bars, standard deviation; n = 4.

Figure 18. Biochemical effects of XJB-5-131 after *in vitro* TBI.

**a.** Assessment of mitochondrial superoxide generation in primary cortical neurons after mechanical stretch. Left panel shows quantitative analysis of mitochondrial superoxide generation by flow cytometry. Mechanical stretch injury resulted in increased superoxide formation in neuronal mitochondria at 2 hours after stretch injury compared with control. XJB-5-131 attenuated stretch induced mitochondrial superoxide generation. Right panel shows fluorescence microscope assessment of MitoSOX (red) staining in neurons. Co-staining of nuclei with Hoechst 33342 (blue).

**b.** Quantification of cytochrome c (cyt c) release from mitochondria.
into cytosol after mechanical stretch. Right insert: representative western blots showing control, stretch + vehicle, and stretch + XJB-5-131. XJB-5-131 attenuated stretch-induced cyt c release into the cytosol. *P < 0.01 vs. control and stretch + XJB-5-131; #P < 0.05 vs. control; error bars, standard deviation; n = 4.

The central role of oxidative stress in acute brain injury has been implicated for decades (Chan and Fishman, 1980; Siesjo, 1981; Wei et al., 1981) and prompted some of the earliest randomized controlled trials in the field of TBI (Muizelaar et al., 1993). However, limitations with specificity, potency, blood-brain barrier and/or cell penetration with conventional antioxidant approaches may have contributed to these past treatment failures (Lo et al., 2001). The use of the mitochondria-targeted nitroxides offers considerable therapeutic advantages compared to strategies used in the past, in terms of getting to the source of oxidative stress at an early point with the highest specificity and the least toxicity, and possibly limiting deleterious effects on oxidative signaling in other brain lipids.

It is known that the immature brain has compromised antioxidant defenses compared to adults (Fan et al., 2003), implying that the beneficial effects of XJB-5-131 could potentially be magnified in the developing brain. Given the enormous burden of TBI in pediatrics, our use of a developmental TBI model may be of additional clinical relevance. germane to our findings, considerable mitochondrial dysfunction with altered bioenergetics (Scheff and Sullivan, 1999), increased oxidative stress markers (Singh et al., 2007), membrane permeabilization (Alessandri et al., 2002) and release of proapoptotic factors (Lewen et al., 2001) have been demonstrated both in experimental models and in humans after severe TBI. There is accumulating evidence in support of the role of TBI as a trigger for a number of neurodegenerative diseases such as dementia (DeKosky et al., 2010), Amyotrophic lateral sclerosis, and Parkinson’s disease (McKee et al.,
2010). Given the role of oxidative stress across the field of brain injury, our work suggests the possibility that CL peroxidation may be characteristic of other types of acute brain injury, such as that seen in stroke and cardiac arrest. Therefore, this mechanism and the linked new therapeutic approach are relevant to other forms CNS injury.

In summary, this work identified selective peroxidation of CL as an important pathogenic mechanism for TBI. The peroxidation process yields a high diversity of oxygenated CL products that may be required to activate the neuronal death program. It is likely that CL peroxidation is not an aftermath of cell death but rather is causative to it, thus offering an opportunity for a targeted therapy. Indeed, we found that mitochondrial delivery of small molecule inhibitors – electron scavengers – lead to prevention of both accumulation of a large number of CL oxidation products as well as brain damage.
3.3 CARDIOLIPIN IS EXTERNALIZED IN RESPONSE TO MITOCHONDRIAL INJURY IN NEURONS

In preparation
3.3.1 ABSTRACT

Mitochondrial dysfunction is a key contributor to neuronal death in different CNS disease conditions. Thus, timely elimination of impaired mitochondria via selective mitochondrial autophagy (mitophagy) could be an effective strategy for prevention of neuronal death. In order to investigate mitophagy, we evaluated the temporal course of mitochondrial damage with a focus on mitophagy using three different insults: mechanical stretch injury, rotenone and staurosporine exposure in primary cortical neurons. Because cardiolipin (CL), a mitochondria-specific phospholipid, plays an important role in the maintenance of mitochondrial functions, we also explored possible effects of manipulation of CL content on mitophagy after mechanical stretch in primary cortical neurons. Mitophagy was assessed by LC 3 shift, electron microscopy (EM), degradation of mitochondrial markers (Cytochrome c oxidase (COX IV), MnSOD, and TOM 40), and co-localization of GFP-LC3 and mitochondria. Mitophagy occurred at an early time point prior to neuronal death (assessed by caspase 3/7 activity and phosphatidylserine externalization) after the insult. Decrease of CL levels by knocking down CL synthase (CLS, the rate limiting enzyme in the synthesis of CL) using siRNA technology markedly inhibited mitophagy induced by mechanical stretch, rotenone and staurosporine in neurons. Normally CL is prevented from interaction with cytosol due to its asymmetric distribution to the inner mitochondrial membrane (IMM). However, outer mitochondrial membrane (OMM) fraction exhibited increased CL levels (assessed by mass spectrometry) relative to the IMM after rotenone exposure. Furthermore, phospholipid scramblase-3 (PLSCR-3) – a protein implicated in translocation of CL to OMM-- underwent phosphorylation during mitophagic process. Taken together, our data indicate that CL and CLS play an important role in mitophagy and various
insults that lead to phosphorylation of PLSCR-3 and exposure of CL to the OMM elicit mitophagy in primary neurons.
Key words: cardiolipin; in vitro traumatic brain injury; rotenone; staurosporine; mitochondria; lipidomics; LC 3; RNA interference; phospholipid scramblase-3
3.3.2 NOTE

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Figure 19 was generated by Drs. Valerian E. Kagan, Hülya Bayır, Jiangfei Jiang, and I.
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Figure 21 was generated by Drs. Valerian E. Kagan, Hülya Bayır, Andrew Amoscato, Jiangfei Jiang, and I.
Figure 22 was generated by Drs. Valerian E. Kagan, Hülya Bayır, Jiangfei Jiang, and I.
Figure 23 was generated by Drs. Valerian E. Kagan, Hülya Bayır, and I.
3.3.4 MATERIALS AND METHODS

3.3.4.1 Primary cortical neuronal culture

Primary cortical neuronal cultures were prepared from embryonic day 16 rats. Pregnant Sprague–Dawley rats (Charles River Lab, CA) were anesthetized with CO2 and sacrificed via decapitation. Embryos were surgically removed from the adult rat and cortices were isolated separately in ice-cold Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with B-27 (Invitrogen, Carlsbad, CA) and Penicillin-Streptomycin (Invitrogen, Carlsbad, CA). Tissue was then rinsed twice with the same medium and set in 0.25% trypsin with EDTA (Invitrogen, Carlsbad, CA) for 15-20 min at 37 ºC. The tissue was again rinsed twice and triturated in ice-cold Neurobasal medium to yield a neuronal cell suspension. Experiments were performed at day in vitro (DIV) 7-9, when cultures consist primarily of neurons (>95% microtubule-associated protein-2 immunopositive cells, <5% glial fibrillary acidic protein immunopositive cells).

3.3.4.2 Mechanical stretch and rotenone treatments in the neurons

In brief, primary cortical neuronal cultures were prepared, cultured on 0.05% poly-D-lysine hydrobromide (Sigma, MI, USA) coated silicone substrate wells, and were subjected to a computer controlled quantifiable mechanical insult by displacing the silicone substrate over a hollowed platform. Severe stretch was chosen in order to simulate a strain field similar to that occurs in the animal model of TBI. For rotenone treatments, neurons were cultured in poly-D-lysine coated 10 cm dish or 6-well plates. Different doses of rotenone were added to neurons on DIV7-8; DMSO (Sigma, MI, USA) was used as vehicle to dissolve rotenone.
3.3.4.3 Cortical neuronal transfection

Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions with modifications. Briefly, a ratio of 0.8–1.0 µg of total DNA/1 µl lipofectamine pre-incubated in Optimem was combined with transfection media (MEM pH 7.4, supplemented with 2% glutamax, 20 mm HEPES, 33 mm glucose, 1 mm Na-pyruvate) on DIV 5. Cells were incubated at 37°C in a non-CO2 incubator for 6 h, and then media were replaced with normal Neurobasal media (Invitrogen, Carlsbad, CA) with B27 supplements (Invitrogen, Carlsbad, CA). Cells were transfected with plasmids expressing mtDsRed2 (Clontech, MA, USA), GFP-tagged LC3 (a gift from Dr Tamotsu Yoshimori, Research Institute of Microbial Diseases, Osaka University, Japan) and/or empty control vector plasmid. Stretch, rotenone or DMSO-vehicle control treatments were performed 72 hrs following transfection.

3.3.4.4 Imaging and quantification methods

All immunocytochemical images were captured using an Olympus Fluoview 1000 confocal microscope (×60 oil immersion lens, NA: 1.42) at room temperature. Images of individual cells were taken as z-stacks (0.5 µm slices, 640 × 640 pixel resolution, 20 µs/pixel) encompassing the entire depth of the cell, using sequential laser imaging and Kalman filter correction. Cells were imaged randomly from across each cover slip, and 10–30 cells/cover slip was imaged. Images were blinded to condition, and cells were analyzed throughout each z-plane using Olympus Fluoview Viewer software.
3.3.4.5 Isolation of mitochondria and separation of inner and outer mitochondrial membrane (IMM and OMM)

Primary neuron cultures (DIV 7-9) were treated with rotenone (125 and 250 µM, Sigma, MI, USA) for 1 or 2 hrs. At the end of treatment, mitochondria were isolated from neuron using Percoll density gradient centrifugation (Sims and Anderson, 2008). The inner and outer mitochondrial membrane fractions were obtained by incubating the mitochondria (10 mg/mL) with digitonin (w/w, 1:0.6). The samples were then centrifuged at 12,000 g; the pellets and supernatants were collected as inner and outer mitochondrial membrane fractions, respectively. Protein samples were then subjected to 12-15% SDS–PAGE gels and transferred to polyvinylidene fluoride using a BioRad Tank Transfer apparatus. Western blots of the gels were then probed using rabbit-anti-LC3 (1:1000; Cell signaling, MA, USA), rabbit-anti- cytochrome C oxidase subunit IV (1:1000; Cell signaling, MA, USA), mouse-anti-TOM 40 (1:1000; Santa Cruz, CA, USA), rabbit-anti-MnSOD (1:1000; Cell signaling), mouse-anti-actin or rabbit-anti-actin (1:2000, Sigma, MI, USA). Appropriate secondary antibodies were applied before developing.

3.3.4.6 Transmission Electron Microscopy (TEM)

Neurons were fixed with 2.5% glutaraldehyde in phosphate buffered saline (PBS) (pH=7.4) for 2 hrs at RT, then washed and monolayers of neurons were postfixed in 1% OsO4 and 1% K3Fe(CN)6 for 1 hr at 4°C. After three washing with PBS, the monolayers were dehydrated through a graded series of 30% to 100% ethanol then incubated in Polybed 812 epoxy resin (Polysciences, Warrington, PA) for 1h. After several changes of 100% resin (three times for 1hr each), monolayers were polymerized at 37°C overnight with additional hardening.
at 60°C for 48 hrs. Ultrathin (60 nm) sections were collected on 200 mesh grids and stained with 2% uranyl acetate in 50% methanol for 10 min followed by 1% lead citrate for 7 min. Sections were observed on a JEM 1210 electron microscope (JEOL, Peabody, Japan) at 80 kV.

### 3.3.4.7 Caspase 3/7 activity

Caspase 3/7 activity was measured using a luminescence Caspase-Glo assay kit obtained from Promega (Madison, WI, USA). Luminescence was determined at baseline and after 1 hr incubation at room temperature using Fusion-α plate reader (Perkline Cop., Boston MA, USA). Caspase 3/7 activity was expressed as the luminescence produced after 1 h incubation per mg of protein.

### 3.3.4.8 Phosphatidylserine (PS) externalization

Externalization of PS was analyzed by flow cytometry using an annexin V kit (Biovision, Mountain View, CA). Briefly, cells were harvested at the end of incubation, and then stained with annexin V-FITC and propidium iodide (PI) prior to analysis. Ten thousand events were collected on a FACScan flow cytometer equipped with a 488-nm argon ion laser and supplied with the Cell Quest software. Cells that were annexin V-positive and PI-negative were considered as apoptotic cells.

### 3.3.4.9 Assessment of phosphorylation of phospholipid scramblase 3 (PLSCR-3) by immunoprecipitation

After different doses and duration of exposure to rotenone, neuronal pellet was collected and it was used for immunoprecipitation (IP) utilizing Thermo Scientific Pierce Classic IP Kit
Briefly, 200 μg proteins was incubated with polyclonal anti-PKC δ (Santa Cruz Biotechnology, CA, USA) or anti-PLSCR3 (Abcam, MA, USA) antibody conjugated to protein A beads according to the manufacturer’s instructions (Thermo, Rockford, IL USA). Proteins associated with antibodies were eluted with the electrophoresis sample buffer, resolved by 12% polyacrylamide gel electrophoresis and analyzed by Western blot.

3.3.4.10 Electrospray ionization mass spectrometry (ESI-MS) analysis of phospholipids

ESI-MS analysis was performed by direct infusion into a linear ion-trap mass spectrometer LXQ™ with the Xcalibur operating system (Thermo Fisher Scientific, San Jose, CA). Samples collected after 2D-HPTLC separation were evaporated under N₂, re-suspended in chloroform:methanol 1:1 v/v (20 pmol/µL) and directly utilized for acquisition of negative-ion or positive-ion ESI mass spectra at a flow rate of 5 µL/min. The ESI probe was operated at a voltage differential of 3.5-5.0 kV in the negative or positive ion mode. Capillary temperature was maintained at 70 or 150°C. MSⁿ analysis was performed using isolation width of 1 m/z, 5 microscans with maximum injection time 1000 ms. Full-scan ESI–MS analysis in the negative ionization mode was employed for all phospholipids classes. Additionally, MS-spectra were recorded in positive mode for phosphatidylcholine (PC) and sphingomyelin (Sph). Two ion activation techniques were used for MS analysis: collision-induced dissociation (Q=0.25, low mass cut off at 28% of the precursor m/z) and pulsed-Q dissociation technique, with Q=0.7, and no low mass cut off for analysis of low molecular weight fragment ions (Schwartz et al., 2005). Based on MS fragmentation data, chemical structures of lipid molecular species were obtained using ChemDraw and confirmed by comparing with the fragmentation patterns presented in
Lipid Map Data Base (www.lipidmaps.org). For quantitative analysis of CL, samples were mixed with internal standard (1,1′,2,2′-tetramyristoyl-cardiolipin, Avanti Polar Lipids Inc., Alabaster, AL).

3.3.4.11 Statistics

Significance was determined using one-way ANOVA or t-test, where appropriate, followed by post-hoc analysis (Student's t-tests with Bonferroni correction or Newman–Keuls test) using alpha = 0.05.
3.3.5 RESULTS AND DISCUSSION

Neurons depend on mitochondria due to their high energy demand; however injured or dysfunctional mitochondria could release death factors that threaten the integrity of the entire neuron. As a result, neurons have developed mechanisms to sequester and degrade dysfunctional mitochondria via mitophagy. Mitophagy is a programmed pathway whereby dysfunctional or damaged mitochondria are selectively degraded by autophagic process (Youle and Narendra, 2011). It is different from bulk autophagy, which degrades cytosolic contents, including proteins and many types of organelles (Yang and Klionsky, 2010). Numerous models have been proposed for studying selective clearance of damaged or dysfunctional mitochondria since the term “mitophagy” was coined by Lemasters and colleagues in 2005 (Lemasters, 2005). However the mechanisms underlying selective clearance of damaged mitochondria by mitophagy remain largely unknown.

Neurons are extremely sensitive to mitochondrial damage due to their post-mitotic state, oxidative metabolism, and synaptic function (Galluzzi et al., 2009). Indeed, mitochondrial dysfunction is a key factor contributing to neuronal death in numerous CNS disease conditions, such as Parkinson’ Disease (PD), Alzheimer’s Disease (AD), Huntington’s Disease (HD), and traumatic and ischemic brain injury (Lin and Beal, 2006). Therefore, removal of dysfunctional mitochondria by mitophagy is a promising therapeutic strategy for prevention of neuronal death (Green et al., 2011). CL, an anionic phospholipid, is not found in any other organelle but mitochondria in mammalian cells including neurons. Furthermore, CL is normally prevented from interaction with cytosol due to its asymmetric distribution to the inner mitochondrial membrane (IMM) (Osman et al., 2011). We investigated the hypothesis that during mitophagy,
CL is externalized to the outer mitochondrial membrane (OMM) and acts as an essential signal for elimination of damage mitochondria by mitophagy. Here, we show that collapse of CL asymmetry and its externalization to the OMM are essential for clearance of dysfunctional mitochondria by mitophagy induced by sub-lethal injury in primary cortical neurons.

We induced mitophagy in primary rat cortical neurons using three different insults: i) complex I inhibitor-rotenone; ii) mechanical stretch injury; and iii) protein kinase inhibitor-staurosporine (Fig. 19) and focused our attention to sub-lethal dose and time points (Fig.4, Fig. 19a). Rotenone exposure (250 nM, 2hrs) caused increased colocalization of mitochondria with GFP-LC3 (“mitophagosomes”) (Fig. 19e) and loss of mitochondrial proteins from each of the major mitochondrial compartments (OMM, IMM, and matrix) without neuronal death. Furthermore, degradation of mitochondrial proteins was reversed by the treatment with bafilomycin A-1, which prevents autophagosome-lysosome fusion (Klionsky et al., 2008) (Fig. 19c, d). These data indicate that degradation of mitochondria was likely through autophagosome-lysosome system. Remarkably, CLS/CL deficiency inhibited rotenone induced mitophagy at this sub-lethal dose and time point (Fig. 22). Similar results were obtained for staurosporine exposure (Fig. 22).

As described in our initial study, mechanical stretch can cause immediate and direct damage to the mitochondria. Mitophagy was observed as early as 1h and continued for 24h after injury as assessed by LC-3 shift, degradation of mitochondrial proteins, and demonstration of autophagosome containing mitochondria by EM, and GFP-LC3 co-localization with mitochondria (Fig. 19 f-h). Neuronal cell death did not occur until 6-12 h after injury (Fig.4).
Similar to rotenone exposure, stretch-induced mitophagy was inhibited in the CLS/CL deficient neurons (Fig. 20).
e

f

Normal  Stretch, 1 h

No. of GFP-LC3 dots co-localized with mitochondria per neuron

h
Figure 19. Rotenone and mechanical stretch induced apoptosis and mitophagy in primary rat cortical neuron.

(a) Caspase-3/7 activation. Primary neuronal cultures (DIV 7-9) were treated with rotenone (125, 250, and 500 nM) for indicated period. (b) Externalization of phosphatidylserine by flow cytometry using an annexin V/PI kit. * p<0.05 vs non-treated neurons. (c) Mitochondrial components degradation indicating rotenone induced mitophagy in primary rat cortical neurons by western blotting. Primary neuronal cultures (DIV 7-9) were treated with rotenone (125 and 250 µM) for indicated time (1 or 2 hours). Protein extracts were prepared using RIPA buffer (30 min on ice). Effect of bafilomycin A1 on rotenone induced mitochondrial components degradation by western blotting. (d) Colocalization of GFP-LC3 with mitochondria using confocal microscopy. Neurons (DIV 5) were transfected with plasmid expressing mtDsRed2 and GFP-LC3 and/or CLS siRNA. After 48 hours, neurons were treated with rotenone for indicated time. (e-g) Mechanical stretch induced mitophagy as evidenced by degradation of mitochondrial components, colocalization of GFP-LC3 with mitochondria, and TEM.
Figure 20. Isolation mitochondria and IMM/OMM fractions from primary cortical neurons.

Primary neuron cultures (DIV 7-9) were exposure to rotenone (125 and 250 µM) for 2 hours. At the end of the exposure, mitochondria were isolated from neurons using Percoll density gradient centrifugation. The inner and outer mitochondrial membrane fractions were obtained by incubating the mitochondria (10 mg/mL) with digitonin (w/w, 1:0.6). The samples were then centrifuged at 12,000 g, the pellets and supernatants were collected as IMM and OMM fractions respectively.

In order to study the redistribution of CL from IMM to OMM during mitophagy, we measured levels of CL by mass spectrometry in the OMM and IMM fractions after isolation of mitochondria from neurons exposed to rotenone (Fig. 21). The OMM fraction exhibited increased CL levels relative to the IMM after rotenone exposure (Fig. 21). Similar results were obtained for staurosporine exposure. Thus, CL externalization and mitophagy occur prior to commitment to cell death in response to injury.
Figure 21. Determination of CL asymmetry in mitochondria isolated from primary rat cortical neurons using LC/MS.

Primary neuron cultures (7-9 days in vitro) were treated with rotenone (125 and 250 µM) for 2 hours. At the end of treatment, mitochondria were isolated from neurons using Percoll density gradient centrifugation. The inner and outer mitochondrial membrane fractions were obtained by incubating the mitochondria (10 mg/mL) with digitonin (w/w, 1:0.6). The samples were then centrifuged at 12 000 g, the pellets and supernatants were collected as inner and outer mitochondrial membrane fractions respectively. Lipid extracts were prepared using Folch procedure, and then subjected to LC/MS analysis. For quantitative analysis of CL, samples were mixed with internal standard (1,1′,2,2′-tetramyristoyl-cardiolipin, Avanti Polar Lipids Inc., Alabaster, AL). The CL content was normalized to the protein content of the mitochondrial outer membrane markers translocases of the outer membrane 40 (TOM40).
Figure 22. Effect of cardiolipin synthase deficiency on rotenone and STS-induced mitophagy in primary rat cortical neurons.

Primary neurons were transfected on day 4 with 45 nM of cardiolipin synthase (CLS) or scrambled non-targeting siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 72-h post-transfection incubation, neurons were exposure to rotenone (125, 250 nM) for 2 hours. (a, c) Rotenone and mechanical stretch induced degradation of mitochondrial components. (b, d) Co-localization of GFP-LC3 with mitochondria by confocal microscopy. (e, f) STS induced mitophagy was attenuated in CLS/CL deficient neurons as assessed by LC-3 shift and quantification of GFP-LC3 with mitochondria by confocal microscopy.

Next, we investigated a potential candidate protein responsible for the movement CL from IMM to OMM since this pathway may represent a promising drug discovery strategy to promote mitophagy. Several proteins, including phospholipid scramblase-3 (PLSCR-3), nucleoside diphophate kinase D (NDPK-D), creatine kinase (mtCK), and tBID, have been proposed to play a role in CL distribution in mitochondria (Schlattner et al., 2009). The collapse of CL asymmetry and its accumulation in the OMM has been reported to be initiated by the PLSCR-3 via Thr21 phosphorylation by PKC-σ (Liu et al., 2003). Using immunoprecipitation (with anti-PLSCR-3) and western blotting (Phospho-Threonine antibody), we showed that PLSCR-3 phosphorylation occurred in neurons after rotenone and staurosporine exposure (Fig.23).
Figure 23. Phosphorylation of PLSCR-3 in neurons after rotenone and STS exposure.
The phosphorylation of PLSCR-3 in rotenone and staurosporine exposed primary neurons was
determined by immunoprecipitation (anti-PLSCR-3) and western blot (Phospho-Threonine
antibody).

Mitophagy, selective degradation of dysfunctional or damaged mitochondria by
autophagic elimination, has been studied in the yeast (Kanki and Klionsky, 2010; Schweers et
al., 2007), in the red blood cell maturation (Aerbajinai et al., 2003; Kundu et al., 2008;
Mortensen et al., 2010; Sandoval et al., 2008; Schweers et al., 2007) and in CNS disease
conditions (Clark et al., 2006; Greene et al., 2003; Kitada et al., 1998; Poole et al., 2008; Tanaka
et al., 2010; Valente et al., 2004). However, the exact mechanism how the damaged or
dysfunctional mitochondria are recognized by the autophagic machinery remains unknown. One
can envision that removal of dysfunctional or damaged mitochondria by mitophagy could be a
pro-survival pathway when neurons are faced with intracellular or extracellular stress.
Externalization of PS, a cytosolic anionic phospholipid, to the cellular membrane is an essential step for recognition and clearance of apoptotic cells by macrophages during phagocytosis. Our data suggest that externalization of CL performs a similar function during mitophagy: collapse of CL asymmetry and its externalization to the OMM are essential for clearance of dysfunctional mitochondria by mitophagy.

Whether CL and its externalization is essential for mitophagy in other cell types remains to be determined. Our studies did not address whether cyt c plays a role for CL externalization and how externalized CL is recognized by the autophagic machinery. Interestingly, the essential autophagy protein LC3 contains basic surface patches (Sugawara et al., 2004), which are conserved in all vertebrate isoforms and unique phospholipid structure of CL includes a compact, negatively charged head group. It is possible that LC3, which mediates both autophagosome formation and cargo recognition (Johansen and Lamark, 2011) contains CL binding sites important for recruitment of mitochondria into autophagosomes. Further studies are required to investigate the interaction of LC3 with CL and its role in neuronal mitophagy.

In summary, CL and CLS play an important role in mitophagy and various injuries (mechanical stretch, rotenone or staurosporine exposure) that lead to phosphorylation of PLSCR-3 and exposure of CL to the OMM elicit mitophagy in primary neurons.
4.0 SUMMARY AND FUTURE DIRECTIONS

In the present study, the role of cardiolipin, CL, a mitochondrial specific phospholipid, during neuronal injury was explored. In the first part, we developed an in vitro TBI model, mechanical stretch injury in primary cortical neurons. We determined the time course of ROS production, lipid peroxidation, and major biomarkers of apoptosis induced by mechanical stretch, and reported that stretch injury caused selective early oxidation of CL, which lead to maximal release of cyt c and caspase activation and PS externalization. This delayed emergence of apoptotic neuronal death after the immediate mechanical damage to mitochondria and early oxidation of CL suggest a possible window of opportunity for targeted therapies. In the second part of this thesis, we investigated mitochondrial anti-apoptotic targeting as a therapeutic strategy for TBI. We applied a new technology, 2D-LC/MS, and established that selective peroxidation of CL was as an important pathogenic mechanism for CCI- an in vivo TBI model. We showed that mitochondrial delivery of an electron scavenger -XJB-5-131 – lead to prevention of accumulation of CL oxidation products, achieved a substantial reduction in neuronal death both in vitro and in vivo, and markedly reduced behavioral deficits observed after TBI. Thus, CL oxygenation represents a new target for neuro-drug discovery. In this work, we employed a pediatric TBI model. It is known that the immature brain has compromised antioxidant defenses compared to adults (Bayir et al., 2006b), implying that the beneficial effects of XJB-5-131 could potentially be magnified in the developing brain. Given the enormous burden of TBI in
pediatrics, our use of a developmental TBI model may be of additional clinical relevance. There is accumulating evidence in support of the role of TBI as a trigger for a number of neurodegenerative diseases such as dementia (DeKosky et al., 2010), Amyotrophic lateral sclerosis, and Parkinson’s disease (Bazarian et al., 2009; McKee et al., 2010). Given the role of oxidative stress across the field of brain injury, our work suggests the possibility that CL peroxidation may be characteristic of other types of acute brain injury, such as that seen in stroke and cardiac arrest. Therefore, this mechanism and the linked new therapeutic approach are relevant to other forms CNS injury. Finally, we examined the signaling pathway for removal of dysfunctional/damaged mitochondria by mitochondrial autophagy or mitophagy. We demonstrated that CL deficiency in neurons markedly inhibit mitophagy induced by three different insults, and collapse of CL asymmetry and its externalization to the OMM likely functions as an “eat-me” signal for recognition of dysfunctional mitochondria and their clearance by mitophagy. Taken together, CL externalization and oxygenation after brain injury are new targets for neuroprotective drug discovery.


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