The Role of TMEM97 in Cell Death Following Traumatic Brain Injury

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THE ROLE OF TMEM97 IN CELL DEATH FOLLOWING TRAUMATIC BRAIN INJURY

By

Michael Richard Watson

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the requirements for the degree of
Master of Science

THE ROLE OF TMEM97 IN CELL DEATH FOLLOWING TRAUMATIC BRAIN INJURY

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Herein is described an effort to understand if the intracellular transmembrane protein, TMEM97, participates in cell death cascades following traumatic brain injury (TBI). TMEM97 is an endoplasmic-reticulum (ER) resident protein shown to interact with the lysosomal Niemann-Pick disease type C (NPC) intracellular cholesterol transporter 1 (NPC1) under metabolic sterol stress. To commence experimentation, an in vitro cell stress paradigm was employed to assess how increasing TMEM97 levels affect cell survival following metabolic and sterol stress. To characterize the in vivo expression profile and effects of TMEM97 deletion on cell survival outcome, we generated germ-line TMEM97−/− mice harboring a LacZ reporter gene, and performed sham and controlled cortical impact (CCI) injuries and assessed cell survival by blinded, unbiased stereological assessment. Further, a novel small molecule antagonist of TMEM97, DKR-1677, was systematically administered to sham and CCI injured PLP-GFP mice, and its neuroprotective effect was assessed by quantifying neurons and oligodendrocytes in the injury penumbra and underlying white matter tracts. It was concluded that increased TMEM97 expression resulted in increased cell death in our in vitro paradigm and that TMEM97−/− results in increased survival of neural cell populations that would otherwise express TMEM97 in mice. Additionally, a 7-day intraperitoneal (IP) administration paradigm of DKR-1677 resulted in increased survival trends of both neurons and oligodendrocytes in the cortical penumbra compared to vehicle treatment. In sum, our in vitro and in vivo evidence indicate that TMEM97 signaling is deleterious to cell survival following injury-induced stress.
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Chapter 1: Introduction

1.1 Traumatic Brain Injury

1.1.1 Definition

Traumatic brain injury (TBI) is incurred following a direct mechanical insult and subsuming accelerating/decelerating forces to the cranium and brain tissue\(^1\). The physical insult may be comprised of blunt, penetrating, and shearing forces which impart damage to the brain parenchyma\(^2\). The extent of mechanical damage correlates with injury severity, and it is clinical practice to categorize TBI severity into the categories of mild, moderate, and severe; based on the Glasgow Coma Scale (GCS)\(^3\) which relies on clinical correlates of reticular, midbrain, and cortical function. Neurological damage and deficits sustained from TBI are the result of phenomena known as “primary injury” and “secondary injury”\(^1\).

1.1.2 Epidemiology

TBI continues to be a large contributor to death and neurological impairment across the world\(^4\). A recent systemic meta-analysis of the clinical TBI literature concluded that the pooled incidence rate of TBI across all age and sex ranges was around 349 per 100,000 persons per year equaling 2.6 million cases annually worldwide\(^4\). Most TBIs (>90% of reported cases) fall into the category of mild TBI (closed head concussive) and disproportionately affect men over women (388 per 100,00 for men: 195 per 100,000 for women). It is important to note that this epidemiological difference is more associated with lifestyle and risk-taking behavioral choices rather than being a result of underlying biological susceptibilities\(^5\).

1.1.3 Pathophysiology

TBI pathophysiology is often conceptualized as occurring in two mechanisms: primary and secondary injury\(^2\). The primary injury constitutes the damage sustained directly from the mechanical forces imparted on the skull and underlying neural tissue.
Common consequences of the primary injury (depending on severity) include but are not limited to: epidural, subdural, and intracerebral hemorrhaging\textsuperscript{[6]}: cerebral contusions and lacerations\textsuperscript{[1]}: and traumatic axonal injury (TAI)\textsuperscript{[7]}. The extent and severity of the primary injury often dictate the extent of secondary injury that further damages the brain parenchyma. Complications that lead to secondary injury pathologies include cerebral ischemia which subsequently leads to cerebral hypoxia\textsuperscript{[6]}. Further, leakiness of the blood-brain-barrier (BBB) leads to vasogenic edema causing brain swelling and if untreated will increase intracranial pressure which can eventually lead to brain herniation\textsuperscript{[8]}. In addition to these systemic complications, alterations in the release rate of neurotransmitter pools, particularly the excitatory neurotransmitter glutamate, can cause large scale excitatory neuronal activity which can rapidly lead to excitotoxic cell death\textsuperscript{[9]}. It is important to note that damage sustained from the primary injury is currently believed to be unamenable to the clinician; however, limiting the progression of secondary injury remains the most practical and feasible therapeutic stratagem to improve patient outcome following TBI.

1.1.4 Neural Cell Death

The pathophysiological consequences discussed in Section 1.1.3 can induce cellular mechanisms or signaling cascades that augment neuronal and glial cell death. The most commonly observed mechanisms of TBI-induced cell death are necrosis, apoptosis\textsuperscript{[10]}, and pyroptosis\textsuperscript{[11]}.

Necrosis has classically been known as an “unregulated” process of cell death. In brief, external cues (i.e. pathogens, toxins, inflammation, ischemia) impart cellular swelling (oncosis) which precede necrotic cell death\textsuperscript{[12]}. Once initiated, the following cellular hallmarks that characterize necrotic cell death include membrane blebbing, pyknosis (nuclear shrinkage), karyolysis, and finally cellular lysis\textsuperscript{[13]}.

Apoptotic mechanisms are understood to initiate and execute by two semi-conservative mechanisms: the intrinsic and extrinsic pathways. The intrinsic
(mitochondrial) pathway is largely regulated by the mitochondria, where Bcl-2 holds the unassembled protein channel Bax inactive. BH3-only proteins assist in alleviating Bcl-2 inhibition of Bax allowing for assembly of a functional Bax channel. This channel permeabilizes the mitochondria allowing the mitochondrial resident protein cytochrome c to escape the mitochondria, which then proceeds to interact with APAF-1 which subsequently cleaves procaspase 9. Following procaspase 9 cleavage, the functional apoptosome is formed leading to activation of caspase-3 causing further degradation of cellular proteins and cell death by apoptosis\textsuperscript{12}. The extrinsic (death receptor) pathway eventually converges on the intrinsic mechanism at the level of the mitochondria. Prior to convergence, the external mechanism is initiated when cognate ligands (i.e. TNF-alpha) bind to their receptors causing dimer/trimerization of receptors and recruitment of the adapter protein FADD. These events help form the death-inducing signaling complex (DISC) which aids in the cleavage of procaspase-8 that can then exert effects on the internal pathway or directly activate caspase-3\textsuperscript{12}.

Pyroptotic cell death is an inflammatory form of regulated necrotic cell death that is mediated by inflammasome assembly, caspase-1 signaling, and gasdermin pore formation\textsuperscript{14}. In short, molecular moieties like pathogen/damage-associated molecular patterns (PAMPs/DAMPs) bind their cognate toll-like and nod-like receptors which cause polymerization of pro-caspase-1/ ASC aggregates forming a multi-protein assembly: the inflammasome. Once assembled, procaspase-1 is cleaved to caspase-1 which then induces a positive feedback cycle that increases pro-inflammatory cytokines such as IL-1\textbeta\textsuperscript{12}.
1.1.5 **Mouse Model of Controlled Cortical Impact (CCI) Injury**

There are numerous experimental paradigms to study traumatic brain injury in rodents; these include but are not limited to: fluid percussion injury (FPI), controlled-cortical impact (CCI) injury, penetrating ballistic-like brain injury (PBBI), multiple weight-drop paradigms, and blast injury\[^{15}\]. Each model comes with its strengths and weaknesses and impart various forces that attempt to mimic the types of forces observed in human TBI patients\[^{16}\]. In these studies, CCI injury was implemented as it is highly reproducible, provides a low mortality rate, produces a rather well-defined injury penumbra, and can be graded to damage or preserve the underlying white matter tracts and hippocampus as desired\[^{17}\]. The CCI injury device operates by firing an air-compressed beveled piston at the desired speed, depth, and dwell time to the mouse brain.

1.2 **Cholesterol Metabolism in the Adult Central Nervous System**

1.2.1 **Cholesterol Synthesis**

While all CNS cell types possess the ability to synthesize cholesterol *de novo* from acetyl-CoA, during adult homeostasis the bulk of CNS cholesterol is synthesized in the ER of astrocytes via the Kandutsch-Russell pathway\[^{18}\]. In brief, when cellular sterol levels are low, the ER resident insulin-induced gene 1 (Insig-1), sterol response element binding protein (SREBP), and SREBP cleavage activating protein (SCAP) heterotrimer dissociates. This allows for trafficking of the SREBP/SCAP complex to the Golgi apparatus, where SCAP will cleave the N-terminus of SREBP, allowing the liberated SREBP N-terminus to translocate to the nucleus and act as a basic helix-loop-helix (bHLH) transcription factor, causing transcription of genes involved in the synthesis and trafficking of cholesterol\[^{19}\]. To anabolize cholesterol, there is believed to be at least 20 enzymes and roughly 34 biochemical steps to complete its synthesis\[^{20}\], a full description of which is beyond the scope of this document.
1.2.2 Cholesterol Trafficking

Once synthesized, cholesterol is packaged into apolipoprotein-E (Apo-E) rich low-density lipoprotein (LDL) particles which are excreted by exocytosis\[^{18}\]. These sterol rich Apo-E LDL particles are then endocytosed by neurons and these cholesterol rich endosomes begin to slowly acidify into mature lysosomes. Lysosomes house many sterol transfer proteins including steroidogenic acute regulatory (StAR) proteins, oxysterol receptor proteins (ORP) as well as the lysosomal membrane bound Niemann-Pick Disease Type C Intracellular Cholesterol Transporter 1 (NPC1) and lumenal Niemann-Pick Disease Type C Intracellular Cholesterol Transporter 2 (NPC2) proteins\[^{21}\]. Mature lysosomes are trafficked to many cellular membranes including the ER, Golgi apparatus, mitochondria, and plasma membranes where they can deliver their internalized sterol cargo.

1.2.3 Cholesterol Aberrations Following TBI

Assessments on ventricular cerebrospinal fluid (CSF) after TBI in human patients has shown that the number of Apo-E particles decreases, yet interestingly the composition of these particles were found to be denser in cholesterol composition\[^{22}\], suggesting altered intercellular cholesterol trafficking. Following experimental TBI in rodents and other forms of neuronal injury, alterations in lipid and sterol moiety balance become pronounced, namely increased abundance of cholesterol ester and oxysterol metabolites at the level of the whole cortex\[^{23, 24}\]. While useful and highly informative, these studies lack a cell-specific assessment of the lipid profiles, making it hard to infer cell-type lipid responses to TBI.

It has been shown that following rodent TBI, lysosomal-mediated autophagic flux is impaired in neurons\[^{25}\], leading to dysregulation of the cellular lipidome, and increased likelihood of cell death\[^{26}\]. While the immediate effects of TBI on neuronal lysosomal sterol abundance are currently unclear, build-up of lysosomal cholesterol species is commonly
observed in many neurodegenerative diseases\textsuperscript{[21]}. The molecular variables leading to this awry sterol metabolism remain unidentified and still largely unexplored, underlying the need to study the causative factors.

1.3 Transmembrane Protein 97 (TMEM97)

1.3.1 Description

TMEM97 is an 18-21 kDa molecular weight transmembrane protein. Mutagenic studies have revealed a C-terminal “KRKKK” ER retention signal, causing TMEM97’s ER localization during cellular homeostasis\textsuperscript{[27]}. While the exact molecular function of TMEM97 is still under investigation, studies implicate it in the binding of hydrophobic motifs\textsuperscript{[28]} as well as modulating intracellular sterol metabolism\textsuperscript{[29]}. Further, TMEM97 has been identified as a sterol response element binding protein (SREBP) response gene, transcribed when cells require the synthesis and trafficking of cholesterol\textsuperscript{[30]}. Interestingly, when clonal cell lines have been subjected to sterol depletion stress, TMEM97 localization alters from predominantly ER bound to localized in diffuse intracellular puncta which largely co-localize with lysosomal markers as well as trace expression on the plasma membrane\textsuperscript{[30]}.

1.3.2 TMEM97 as the Gene Which Encodes the Sigma 2 Receptor

Recently, our collaborators set out to clone the gene which encodes the sigma 2 receptor and found that it was synonymous with the already cloned gene TMEM97\textsuperscript{[31]}. It has been known since its discovery\textsuperscript{[32]} that the sigma 2 receptor displayed rich CNS expression and that particular ligands which bound it exerted opioid-type effects, however its molecular identity remained uncertain since its initial description\textsuperscript{[33]}. The identification of this gene as TMEM97 provides a novel opportunity to probe its function using modern molecular biology and biochemical techniques.
1.4 Niemann-Pick Disease Type C Intracellular Cholesterol Transporter 1 (NPC1)

1.4.1 Description

The NPC1 protein is known to be a lysosomal resident, 13-transmembrane pass cholesterol transporter. While sterol transport can be bidirectional, NPC1 predominantly effluxes cholesterol from the lysosome to target cellular organelles. NPC1 obtains lysosomal cholesterol from the lumenal transfer protein NPC2, with an energetic bias towards subsequent sterol egress from NPC1. Mutations in this channel may lead to Niemann-Pick Disease Type C, a lysosomal storage disorder where large cholesterol deposits remain confined in lysosomes, which leads to progressive lipid build-ups in the spleen, liver, and brains of affected individuals which cause systemic complications, neurodegeneration, and eventually death.

1.4.2 Reported Interactions with TMEM97

Co-immunoprecipitation techniques in vitro have revealed that TMEM97 localization to lysosomes is most likely mediated through interactions with the lysosomal 13-transmembrane pass NPC1 protein, which fluxes sterol species from lysosomes to target cell membranes. Further, it has been shown that silencing TMEM97 reverses lysosomal cholesterol storage in patient-derived mutant NPC1 fibroblasts, suggesting that TMEM97 interactions with NPC1 may limit NPC1’s ability to egress sterol species. We propose that following TBI, TMEM97/NPC1 interactions cause lysosomal sterol retention, attributing to impaired autophagic and lysosomal flux, and ultimately worsened cell survival outcome.
Chapter 2: TMEM97 Expression Levels Correlate with Cell Survival Outcome

2.1 Overview

Despite years of pre-clinical research and subsequent TBI phase III clinical trials, all proposed therapeutics have failed to substantially reduce secondary injury in humans\[^{[36]}\], highlighting the need to test novel agents which modulate processes important to cellular function and survival following TBI. It has recently been shown that synthetic small molecule ligands possessing high affinity for TMEM97, recently identified as the gene which encodes the sigma 2 receptor\[^{[31]}\], are neuroprotective in both \textit{C. elegans}\[^{[37]}\] and murine\[^{[38]}\] models of neurodegeneration. TMEM97 is a sterol regulatory element binding protein (SREBP) response gene that functionally interacts with the NPC1 protein responsible for sterol egress from lysosomes to numerous cellular membranes\[^{[27, 30]}\].

We hypothesize that TMEM97 interactions with NPC1 following brain injury limit sterol egress from lysosomes, leading to sequestration in lysosomes. Further, we hypothesize that dysregulated intracellular sterol trafficking is one of the factors contributing to secondary cell death cascades and that disrupting TMEM97/NPC1 interactions will be beneficial to cell survival following TBI. We set out to address these questions by first establishing an \textit{in vitro} model of cellular stress to assess how increasing TMEM97 protein levels alters cell survival outcome, as well as if our small molecule antagonist DKR-1677, could exert pro-survival effects. We next developed a germ-line TMEM97 knock-out animal harboring LacZ as a reporter gene (with assistance of the UC Davies KOMP facilities) to assess how TMEM97\(^{-/-}\) alters cell survival following CCI injury in mice. Finally, we administered the TMEM97 antagonist DKR-1677 \textit{in vivo} to assess its potential to elicit pro-survival effects in neurons and oligodendrocytes following CCI injury in mice. In brief, our studies indicate that TMEM97 signaling following cell stress is detrimental, and that antagonizing TMEM97 with DKR-1677 can partially ameliorate this effect.
2.2 Results

2.2.1 Over-expression of TMEM97 increases cell death in HEK293T cells

We first determined whether SW stress alone in HEK293T cells occurs independently of cellular cholesterol alterations (Fig 2.1B). We used the cholesterol specific probe, Filipin III, and quantified the average cholesterol levels per cell in various stress conditions. We found that SW alone does not significantly alter cellular cholesterol levels. However, SW plus our β-cyclodextrin mediated sterol alterations successfully changed cellular cholesterol levels. Figure 2.1A shows that 24 hours of SW lead to ~20% reduction in HEK293T cell viability, which was independent of the average change in free cholesterol levels (Fig. 2.1B). We noted that administration of 2%w/v empty 2-Hydroxypropyl-β-cyclodextrin (HPβCD) (a sterol sequestering paradigm) lead to reduced cellular cholesterol levels (Fig. 2.1B) and reduced cell survival following SW stress (Fig. 2.1A). Interestingly, administration of 5 μM cholesterol to stressed cells using pre-loaded methyl-β-cyclodextrin (MβCD) delivery increased both sterol levels and cell viability (Fig. 2.1A, B), suggesting that providing cells with cholesterol under metabolic sterol stress can benefit cell survival. Interestingly, if high levels of cholesterol were delivered (i.e. >25 μM) there was a worsening of cell viability, demonstrating that too much cholesterol can be detrimental to cell survival (Fig. 2.1A). We next examined the effect of TMEM97 and NPC2 over-expression in stressed HEK293T cells. Over-expression of TMEM97 lead to enhanced cholesterol retention (~2-fold, Fig. 2.1D) and a dose-dependent reduction in cell viability (Fig. 2.1C). NPC2 over-expression had no effect on cholesterol levels or cell viability. These findings support our hypotheses and rationale for examining TMEM97’s effect on cholesterol trafficking and cell survival in neurons after TBI.
Fig 2.1. Cholesterol homeostasis is important for HEK293T cell survival. Serum withdraw (SW) stress leads to decreased HEK293T cell viability (A) independent of cellular sterol levels (B). HPβCD reduced cellular sterol levels and cell viability, while MβCD restores cholesterol levels and cell viability (A, B). Dose-dependent over-expression of TMEM97 but not NPC2 further reduces cell viability through increasing cholesterol retention 2-fold in cells (C, D). N=2 assays n=6-9 replicate wells. *p <0.05; **p<0.01; ***p<0.001. One-way ANOVA w/ Tukey’s multiple comparisons.
2.2.2 Antagonizing TMEM97 with small molecule DKR-1677 increases cell survival in HEK293T cells

We initially screened DKR-1677 in our established HEK293T cell stress model to assess its relative safety and efficacy in increasing cell survival. We noted that in the absence of TMEM97 over-expression (Empty Vector [Ev] transfection), vehicle treatment (DMSO), DKR-1677, and Siramesine (a known TMEM97 agonist) had no detrimental effect on cell survival (Fig 2.2). Interestingly, when TMEM97 was over-expressed we observed DKR-1677 to improve and Siramesine to worsen cell survival outcome compared to vehicle treatment (Fig 2.2).

![Graph showing cell survival](image)

**Fig 2.2. DKR-1677 improves survival of TMEM97 expressing HEK293T cells.** TMEM97 synthetic ligands DKR-1677 and Siramesine exert no effects on cell survival in the absence of TMEM97 over-expression. When TMEM97 is over-expressed DKR-1677 improves and Siramesine worsens cell survival outcome compared to vehicle controls N=2-5 assays n=6-30 replicate wells. *p <0.05; ***p<0.001. One-Way ANOVA w/ Tukey’s multiple comparisons.
2.2.3 Validation of TMEM97\textsuperscript{\textminus/+} mice harboring a LacZ reporter gene

Following these \textit{in vitro} findings, we wanted to have a biological system where we could assess the effects of the presence and/or absence of TMEM97 \textit{in vivo}. We utilized the expertise of the Knockout Mouse Project (KOMP) Repository at UC Davies, to create TMEM97\textsuperscript{\textminus/+} mice\textsuperscript{[39]}. We chose a germ-line TMEM97 knock-out model with a β-Galactosidase (LacZ) reporter gene placed under the endogenous TMEM97 promoter, as there are no verified/reliable TMEM97 antibodies to probe for detection in mouse tissue. The KOMP repository sent confirmed heterozygous mutants (TMEM97\textsuperscript{\textastar/+}) which were then mated in house to obtain homozygous mutants (TMEM97\textsuperscript{\textminus/+}). Figure 2.3 depicts our validation of our TMEM97\textsuperscript{\textminus/+} animals, evident by the absence of detected transcript by Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR).

![Graph](image.png)

\textbf{Fig 2.3. Confirmation of TMEM97\textsuperscript{\textminus/+} mice.} qRT-PCR analysis of TMEM97 KO and TMEM97 Heterozygous animals. Data points graphed are technical replicates. N=2 n=6. No statistical analyses were performed on this data.
2.2.4 Characterization of TMEM97 expression in the adult mouse brain

We next wanted to determine the expression profile of TMEM97 in the adult mouse brain. Using a modified LacZ staining protocol to stain mouse tissue in situ which implemented 5-bromo-4-chloro-3-indoly-l-β-D-galactopyranoside (X-Gal) and Nitroblue Tetrazolium (NBT) reagents [40], we detected LacZ as our experimental proxy for TMEM97 expression. We chose to initially stain sagittal sections of TMEM97+/− animals in sham and 6 hour CCI injured conditions (Fig 2.4 A-C, D-F) to get a global appreciation of TMEM97 brain expression before and following our injury paradigm. We noted very intense staining in anatomical regions largely populated by neurons (i.e. hippocampal pyramidal layer and dentate gyrus, granular layer of the cerebellum) as well as staining throughout all layers of the neocortex. Interestingly, we observed a marked decrease in LacZ staining intensity in the injury epicenter and surrounding injury penumbra (Fig 2.4 B vs 2.4E). We argue (supported by data that will be presented in sections 2.2.5 and 2.2.7) that this decrease in expression is the result of increased death in cells expressing TMEM97, and not a decrease in the protein expression levels. To bolster our inclinations of a largely neuronal expression profile, co-immunostaining was performed with the neuronal markers NeuN and Map2 showing co-localization with LacZ in the pyramidal layer (Fig 2.4H) and overlying cortex (Fig 2.4I, J). It is important to explicitly state: we do not claim that TMEM97 is expressed only in neurons. We merely claim that a substantial portion of LacZ+ cells in our model system co-express with neuronal markers. Immunohistochemical stains with the astrocytic marker GFAP, for example, revealed trace amounts of co-localization with LacZ in the cortex (not-shown). However, we conclude there is a predominantly neuronal expression of TMEM97 in the brains of our transgenic mice. This justifies our focus on neuronal analyses discussed in Section 2.5 (Future Directions).
2.2.5 TMEM97<sup>−/−</sup> increases cell survival following CCI injury in mice

Noting how TMEM97 over-expression induced increased cell death in our in vitro cell stress model (Fig 2.1C), we hypothesized that genetic deletion of TMEM97 would increase cell survival following TBI in mice. We began our analysis looking at an acute injury time point (1 dpi), and subjected TMEM97<sup>+/−</sup> and TMEM97<sup>−/−</sup> mice to either sham or CCI injury conditions and performed blinded stereological assessment of the cortical penumbra (Fig 2.5). It is important to note the population that was counted were only LacZ<sup>+</sup> cells, and owing to reasons outlined in section 2.2.4, this does not necessarily represent a purely neuronal population. That caveat stated, we did observe a statistically significant increase in the number of LacZ<sup>+</sup> cells in CCI injured TMEM97<sup>−/−</sup> mice compared to TMEM97<sup>+/−</sup> mice. These results suggest that TMEM97 expression in cells resident in an injury milieu are more susceptible to undergoing cell death following TBI.
2.2.6 Antagonizing TMEM97 with small molecule DKR-1677 increases neuronal and oligodendroglial survival following CCI injury in mice

Given that genetic deletion of TMEM97 increased cell survival we tested whether antagonizing TMEM97 with the small molecule DKR-1677 ($K_i=5.1\text{nM}$, $t_{1/2}=1.2\text{h}$) would elicit pro-survival effects. To assess this, we examined whether treatment with DKR-1677, initiated at 1 hour post injury (hpi), improved neuron and oligodendrocyte survival following CCI injury. DKR-1677 (3 mg/kg/day, intraperitoneal [IP]) was administered once per day for 7 days followed by blinded stereological assessment of cortical neurons in the injury penumbra as well as oligodendrocytes in the cortex, corpus callosum, and external capsule of mice expressing green fluorescent protein (GFP) under the proteolipid (PLP) oligodendrocyte promoter (i.e. PLP-GFP mice). We observed a significant reduction in
cortical neurons identified by anti-NeuN immunoreactivity (Fig 2.6A) as well as a trend toward reduced numbers of cortical GFP-positive oligodendrocytes in vehicle treated CCI injured mice (Fig 2.6B). However, we observed no statistically significant differences between sham and CCI injured mice treated with 3 mg/kg/day DKR-1677 (Fig 2.6A and B). We observed a similar effect with oligodendrocytes residing in all three white matter regions quantified, in which the external capsule residing below the injury epicenter showed a significant reduction in vehicle-treated mice (Fig 2.6C). Representative low magnification confocal images show differences in tissue sparing between vehicle (Fig 2.6G) and DKR-1677 (Fig 2.6J) treated CCI-injured mice as compared to vehicle-treated sham controls (Fig 2.6D); however, it should be noted that tissue sparing was not an outcome measure quantified in these studies. Higher magnification images of the penumbra and corpus callosum reveal differences in neuron (Figs 2.6E, H, and K) and oligodendrocyte (Figs 2.6F, I, and L) cell density mediated by DKR-1677 treatment. Taken together, our findings suggest DKR-1677 elicits pro-survival effects in both neurons and oligodendrocytes after CCI injury.
2.2.7 mRNA transcript analysis of TMEM97 and NPC1 following TBI

Throughout our analyses on our TMEM97 transgenic animals, we repeatedly observed a marked decrease in LacZ expression density in the injury epicenter and surrounding penumbra (Fig 2.4E). To get an indication as to whether this is a true regulatory decrease, or whether it is merely the result of penumbral cell death (Fig 2.5), we performed a timecourse qRT-PCR analysis for both TMEM97 as well as NPC1 (the putative interacting partner of TMEM97). We subsequently subjected WT mice to either sham or CCI injuries and collected the cortex and hippocampus for analysis at 6 hours, 1 day, and 3 days post injury. While more animals are needed to complete this study, it appears that in both the cortex and hippocampus we observe no statistically significant
differences in the transcript levels of either TMEM97 or NPC1. This supports the possibility that the decreased LacZ+ expression detected in the cortical penumbra is the result of increased cell death and not a result of transcriptomic down-regulation.

Fig 2.7. qRT-PCR analysis of TMEM97 (A, C) and NPC1 (B, D) expression levels in the cortex and hippocampus following sham and CCI injury in WT mice. Data plotted are the average of technical triplicates N=3-6 mice/group. No statistically significant differences detected. Kruskal-Wallis test by ranks.
2.3 Discussion

These studies set out to examine what role, if any, TMEM97 plays in cell death cascades following metabolic stress, and more importantly cellular stress incurred following TBI. Further, we wanted to assess whether the small molecule modulator of TMEM97, DKR-1677, is a promising pre-clinical therapeutic to reduce TBI pathology. While further experimentation is warranted, we feel that taken together we have amassed a body of evidence that shows a correlation between TMEM97 protein expression and worsened cell survival outcome following cellular damage and TBI.

We appreciate that serum withdrawal (SW) stress may differ from cell stress after TBI; however, establishing cell stress paradigms is often important for gaining insight into protein function. SW is a well-established model to induce cell stress in vitro, as is the implementation of cyclodextrin compounds to alter cholesterol content; however, incorporating both methodologies in the same experiment is, to our knowledge, novel. What prompted this synthesis of techniques were findings from Bartz and colleagues who discovered that cholesterol depletion in HeLa cells using HPβCD caused a drastic increase in the abundance of TMEM97 transcript levels. We reasoned that this was a cellular response to sterol stress, and that if TMEM97 were to be pre-maturely over-expressed prior to SW stress and sterol depletion, that cell survival would be improved. Interestingly, we repeatedly observed the opposite trend. TMEM97 over-expression in our in vitro platform repeatedly worsened cell survival outcome compared to empty vector controls (Figure 2.1C and Figure 2.2).

Building on these findings, we probed the potential of synthetic TMEM97 ligand DKR-1677 to alter the observed increase in cell death caused by TMEM97 over-expression. As a positive control, we included the known TMEM97 synthetic agonist Siramesine, shown to induce cell death in various cancer and clonal cell lines that express TMEM97 at high levels. As expected, Siramesine decreased cell survival in
TMEM97 over-expressing HEK293T cells (Figure 2.2). Interestingly, the TMEM97 modulator DKR-1677 increased cell survival of TMEM97 over-expressing cells compared to vehicle control (Figure 2.2); it is on these grounds that we consider DKR-1677 a TMEM97 antagonist.

These *in vitro* findings peaked our desire to develop an *in vivo* system to assess the effects of the presence or absence of TMEM97 following TBI. Owing to their ease of genetic modification and commercially available resources (KOMP Repository at UC Davies) we chose to develop a TMEM97−/− mouse line. It is important to note that there are no published or reliable murine TMEM97 antibodies to date; being the case, we chose to develop these mice harboring LacZ as a reporter gene driven by the endogenous TMEM97 promoter. By implementing an established *in situ* staining protocol[40], we could detect and quantify cells that transcribe (or, would transcribe in the case of TMEM97+) TMEM97. The brain expression profile of TMEM97 (Fig 2.4) mirrors the known mRNA *in situ* hybridization profile known to date (Allen Brain Atlas), revealing dense expression in the hippocampal pyramidal cell layers and granular layer of the cerebellum, as well as diffuse expression in the neocortex. Interestingly, the mRNA *in situ* expression profile of NPC1 (the putative interacting partner of TMEM97) presents a very similar brain expression pattern (Allen Brain Atlas).

Once our animals were developed and validated (Fig 2.3) we performed a stereological assessment comparing sham and CCI injured TMEM97+/− and TMEM97−/− mice at 1 day following injury. We noted a statistically significant increase in the number of surviving LacZ+ cells in the CCI injured TMEM97−/− mice compared to TMEM97+/− mice (Fig 2.5). These results bolstered our *in vitro* findings. If over-expression of TMEM97 increases the likelihood of cell death following stress, it stands to reason that removing TMEM97 during cell stress would subsequently improve cell survival outcome. One caveat of this study (hinted at earlier, yet re-iterated here) is that the cell populations were
quantified based on being LacZ+ (ipso facto: TMEM97 expressing). While we have provided evidence that a substantial proportion of these cells are neurons, we by no means are stating that this metric represents purely neuronal cell survival, and acknowledge that this population of cells may be heterogeneous in nature.

Next, we wanted to assess the efficacy of DKR-1677 (shown to increase cell survival in vitro) to elicit neural survival in vivo following CCI injury. Our treatment paradigm, of 3 mg/kg/day starting at 1 hpi and administered once per day for 7 days IP, showed modest improvements in neuronal and oligodendrocyte survival in the cortex (and oligodendrocytes in the underlying white matter tracts); however, these improvements did not reach the threshold of statistical significance.

Having recourse again to Figure 2.4B and E, we wondered whether the decrease in staining density was merely the result of cell death, or whether this represented a transcriptomic and/or proteomic down regulation of TMEM97. Owing to our lack of validated antibodies, we chose to address this question at the transcript level. We performed a timecourse qRT-PCR analysis of TMEM97, as well as NPC1, on dissected cortical and hippocampal tissue at 6hr, 1 day, and 3 days following sham and CCI injury. While more animals need to be completed, a preliminary non-parametric analysis indicates there is no statistically significant increase or decrease in either TMEM97 or NPC1 levels. This notion bolsters our confidence in claiming that the observed decrease in LacZ expression is the result of cell death.

2.4 Conclusion

We conclude that the presence of TMEM97 in an injured/stressed milieu (both in vitro and in vivo) increases the probability of cell death, albeit the mechanism of this cell death remains an open-ended question. We also conclude that antagonizing TMEM97 with DKR-1677 possesses pro-survival potential. We stress again that this is the first study assessing the role TMEM97 following neurotrauma in a genetic and molecular biological
context (and not solely utilizing pharmacology). We find the novelty of these findings exciting; however, it brings along the caveat that there is no body of data/literature to hold our findings against as a comparative metric. It is our hope that these findings as well as our planned future directions will excite the TBI field and encourage other investigators to pursue this line of inquiry.

2.5 Future Directions

For each experiment performed, we offer a further experiment/s that would probe deeper into the phenomena that we are most interested in: what is the function of TMEM97 that is causing increased neural cell death following TBI.

The in vitro studies performed in this work were conducted in HEK293T cells. While a useful biological system, one could argue that this is not the best biomimetic in vitro platform to address our question; to which we would agree. Future studies will be harvesting primary hippocampal neurons from WT and TMEM97\(^{-/-}\) E18 embryos and performing similar metabolic stress assays. We are confident that any results obtained in those studies will be more indicative of neuronal signaling than what would be occurring in transfected HEK293T cells.

Our in vivo assessment completed to date on the effect of genetic deletion of TMEM97 on cell survival has only been performed at one time point (1 dpi). Future studies will look at longer timepoints (i.e. 7 dpi) and will also be coupled with a motor learning assessment (Rotarod) to confirm if the increased number of cells detected correlate with improved motor learning. We additionally will be performing a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay on TMEM97\(^{+/+}\) vs TMEM97\(^{-/-}\) animals to test if there is a corresponding increase in cell death in the TMEM97\(^{+/+}\) compared to TMEM97\(^{-/-}\) mice.

Further, while TMEM97 has been implicated in numerous studies in cholesterol metabolism and/or trafficking, none of our studies performed address this question. Future
studies will be performing a lipidomic assessment utilizing gas chromatography mass spectrometry (GC-MS) and liquid chromatography tandem MS (LC-MS/MS) on the whole cortex, isolated cortical neurons, and on neuronal organelles following sham and CCI injured conditions to assess how injury and how TMEM97 deletion perturb the distribution and abundance of cholesterol and its metabolites in neurons.

While our IP administration schema showed some neuroprotective trends, one could argue that while the mean neuron and oligodendrocyte densities are increased in our CCI DKR-1516 groups compared to CCI Vehicle, these differences were not statistically significant, which is less than desirable for translational potential. While possessing tight binding affinity for TMEM97 (Kᵯ=5.1nM), DKR-1677 possesses a relatively short biological half-life ~1.2h. Further there is rich expression of TMEM97 in other steroidogenic organs (i.e. liver and intestines) and for these reasons we argue that the delivery system implemented (IP) was sub-optimal to reach the brain parenchyma. Future studies will incorporate an osmotic pump delivery system, allowing for a constant release of DKR-1677 directly to the injured brain.

2.6 Future Directions Preliminarily Underway

I have begun two of the studies proposed in the previous section: rotarod motor analysis and cortical lipidomic assessment. As one might infer from the section heading, given the provisional nature of the data, we are tentative to draw conclusions from these data, and instead use them to offer insight and suggestions.

Following training on the rotarod apparatus prior to surgery, we subjected TMEM97⁺/⁺ and TMEM97⁻/⁻ mice to sham and CCI injury and assessed motor performance at 3, 5, and 7 dpi (Figure 2.8). Both sham groups (TMEM97⁺/⁺ and TMEM97⁻/⁻) performed statistically better than the CCI injured groups, indicating our CCI injury paradigm successfully induces motor deficits [main effect(day)= F(3,93)= 26.63, p<0.001: main effect(animal group)= F(3,31)= 8.78, p<0.001]. In comparing the TMEM97⁺/⁺ and
TMEM97−/− CCI injured groups, we observe the TMEM97 animals to perform better at all time points than TMEM97+/− mice, with this difference being statistically significant by 7 dpi.

We have also commenced our lipidomic analyses. We set out to determine how CCI injury (alone and in conjunction with TMEM97+/−) alters the cortical lipid metabolite profile. To do so, following 1 dpi, we implemented a modified methyl-tert-butyl-ether (MTBE) lipid extraction protocol[45] to efficiently isolate cortical lipids from one WT and TMEM97−/− sham and CCI injured animal. Once lipids were extracted, they were desiccated and stored at -80°C until analysis. For quantification, lipids were re-suspended in chloroform and subjected to LC- MS/MS by the mass spectrometry core here at the

![Time on Rotarod](image)

**Fig 2.8. TMEM97−/− benefits motor learning following CCI Injury.** Both TMEM97−/− and TMEM97+/− sham groups performed significantly better than CCI Injured groups at +3, +5, and +7 dpi (significance not shown). Animal group interaction(day): F(9,93) = 22.71: p<0.001.***p<0.001 vs TMEM97+/− Sham; *p<0.05, **p<0.001 vs TMEM97−/− Sham: #p<0.05 vs Het CCI. N=9 mice/group. Two-way ANOVA repeated measures w/ Bonferroni's multiple comparisons.
University of Miami. We stress again that this sample size is far below what is required for statistical analyses; however, we have shown the data obtained to date (Figure 2.9).

**Fig 2.9. Cortical LC-MS/MS lipidomic analysis suggest global lipid aberrations 1 day following CCI Injury.** Panels A-D all represent the same data set, merely presented different ways. A) Area under the curve. B) Normalized percent of lipid per sample. C) Normalized to the picomolar abundance of internal control lipid. D) Heat Map depicting the percent change (RED= increase, BLUE= decrease). Note the large lipid accumulations following CCI injury in WT conditions (Column 2) and how these accumulations are less severe when compared to TMEM97−/− CCI injured animals (Column 3). N=1 mouse/ group, analyzed in triplicate (positive and negative ion mode).
3.1 HEK293T Cell Survival Assay

HEK293T cells were maintained, split at 80-90% confluency (up to 20 passages) and passaged in Falcon polystyrene plates in 10% fetal bovine serum (FBS) and Dulbecco’s modified Eagle’s medium (DMEM) (Gibco no. 11965-092) in 0.4% penicillin/streptomycin. To begin the assay, HEK293T cells were initially seeded in 24 well plates (0.5 mL media/well) at a density of 80,000 cells per well. Following 24h, cells were Lipofectamine transfected (Polypus jetPRIME®) with plasmids of interest [1X= .125μg plasmid DNA/ Well, 2X=.25μg plasmid DNA/ Well, 4X= 0.5μg plasmid DNA/ Well] (TMEM97=pCMV3-mTmem97-HA, Sino Biological, no. MG53817-CY: EV= pcDNA3.1, Addgene no. V790-20: NPC2= pCMV3-NPC2-HA, Sino Biological, no. HG13341-CY) and incubated for 24h. The media was replaced with fresh 10% FBS DMEM for another 24h. To induce metabolic stress, control media was replaced with serum depleted media (serum withdrawal-SW-) and incubated for 24 h in the presence DKR1677 or vehicle (1% DMSO) control. When sterol concentrations were modulated with cyclodextrin compounds, reagents were added to the media 3 hours prior to assessing cell survival. For sterol sequestration, cell media was brought to 2% (2-Hydroxypropyl)- β- cyclodextrin (Sigma- Aldrich, no. 332607) w/v. For sterol delivery, cell media was brought to the respective cholesterol concentrations using a cholesterol: methyl- β- cyclodextrin complex (Cholesterol-Water Soluble, Sigma-Aldrich, no. C4951). To determine cell survival, cells were mechanically removed from the plates, and a suspension was formed by gentle pipetting. A 50:50 solution of cell suspension: Trypan Blue was then mixed and 10μL of the mixture was loaded into dual-chamber cell counting slides (Bio-Rad, no. 1450003). Trypan Blue uptake followed by automated cell counting (Bio-Rad TC20 Automated Cell Counter) determined the cell survival percentage.
3.2 Detection and Quantification of HEK293T Cellular Cholesterol Levels

To visualize cholesterol levels in HEK293T cells we used the cholesterol specific polyene probe, Filipin III, as a proxy for cholesterol levels. Briefly, HEK293T cells were fixed with cold 4% PFA for 10 minutes. This solution was removed and cells were incubated in a 1.5 mg/ml solution of Glycine to quench auto-fluorescence. Next a .15mg/ml solution of Filipin III complex (Sigma F9765) in 10% Fetal Bovine Serum (FBS) PBS was used to probe Free Cholesterol for 2h at room temperature. Next, to stain for cell nuclei, a .75X solution of nuclear marker RedDot1 (Biotium no. 40060) was incubated at room temperature for 30 minutes. Following 3 washes in PBS, cholesterol levels per cell were detected using the Cellomics ArrayScan VTI Live HCS Reader (at the Miami Project to Cure Paralysis High Content Screening Core) in a non-biased automated fluorescent scanner.

3.3 Transgenic Animals

Male WT, TMEM97<sup>+/−</sup>, TMEM97<sup>−/−</sup>, and PLP-EGFP transgenic mice were bred on a C57Bl/6J background. TMEM97 transgenic mice were purchased as heterozygotes from the KOMP Repository at UC Davies and mated to obtain homozygous knockouts. All animal procedures were performed using male mice between the age of 2~4 months. Animals were kept under normal 12 h light/dark cycle conditions, and all procedures were approved by the University of Miami Animal Care and Use Committee (IACUC). Following surgical procedures, animals were housed singly without environmental enrichment.

3.4 Sham and CCI Surgeries

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) via intraperitoneal (IP) injection and placed on a heating pad to maintain body temperature. The animal’s head was shaved and then placed on a stereotaxic frame where an incision
was made through the skin, exposing the skull. For CCI injured mice, a ∼5 mm diameter craniectomy was made over the right parietal cortex (bregma: −2.0 mm; lateral −2.5 mm), leaving the dura intact. Mice were then subjected to a moderate CCI injury with a piston velocity of 4.0 m/s and depth of 0.55 mm using an eCCI-6.0 device (Custom Design & Fabrication, Virginia Commonwealth, VA, United States), which contains a pressurized stainless steel beveled piston to deliver the impact. Sham controls underwent an identical surgical procedure with the absence of the craniectomy and injury. The incision was closed using 4-0 REDISILK black braided silk non-absorbable sutures (Ethicon, Inc., Piscataway, NJ, United States), and mice were placed in a clean, single housed cage on a heating pad. For hydration and analgesia, animals were administered 1 mL of a 100:1, lactated ringer: 0.3 mg/mL buprenorphine, solution subcutaneously 1 h post injury (hpi) as well as 2 and 3 days post injury (dpi).

3.5 Tissue Processing

At the desired time point following injury, mice were anesthetized with a ketamine/xylazine cocktail and transcardially perfused with 0.01 M PBS (pH 7.4) followed by 4% paraformaldehyde (PFA, pH 7.4). Brains were harvested and post-fixed overnight in 4% PFA at 4°C. The following day, brains were transferred to 30% sucrose in PBS. Brains were then placed in molds, mounted in a 60: 40 solution of Tissue-Tek Cryo-OCT (Fisher Scientific, United States): 30% Sucrose PBS, and serially cryo-sectioned at 30 μm thickness.

3.6 Detection of LacZ in Mouse Tissue

LacZ transgenic animals were sacrificed and tissue was processed as described above. Following sectioning and mounting on gelatin subbed slides, sections were washed in 0.1M potassium phosphate buffer (PB). Next, tissues were incubated 3x15 minutes in Rinse Solution (0.02% IGEPAL CA-630, 0.01% sodium deoxycholate, 1.25 mM EDTA,
2mM MgCl₂, 0.1M PB). Tissues were then incubated in Staining Solution-1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Gold-Bio no. X4281), 0.4 mg/mL nitroblue tetrazolium (NBT, Thermo-Scientific no. 34035) in rinse solution- for 6 hours in a dark humidified chamber at 37°C. Following incubation tissues were washed in PB and then underwent successive dehydration incubations -1 minute each- (Ethanol 50%, 70%, 95%, 95%, 100%, 100%, 100%: Xylene, Xylene, Xylene). Following clearing in Xylene, tissue was cover slipped in Surgipath Sub-X toluene based mounting medium (Leica Biosystems no. 3801740).

3.7 RNA Extraction and qRT-PCR Analysis

Hippocampal and cortical tissues from sham-treated and CCI-injured WT, TMEM97+/−, and TMEM97−/− mice were prepared for qRT-PCR analysis using the Direct-zol RNA Mini-Prep Kit (R2050, Zymo Research) and real time (RT) reactions using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™ no.4368814) for RNA extraction, purification, and cDNA synthesis according to the manufacturer’s instructions. The absence of reverse transcriptase (i.e., no RT) was used as a negative control for all qRT-PCR analyses. Samples were run on a QuantStudio 3 Thermocycler (Applied Biosystems™) using TaqMan assays for Tmem97 (Mm01608791_g1, Applied Biosystems™) or Npc1 (Mm00435300_m1, Applied Biosystems™). Glycerinaldehyde 3-phosphate dehydrogenase, Gapdh (Mm99999915_g1, Applied Biosystems™) was used as an internal control gene. ΔCt was calculated by subtracting the corresponding GAPDH Ct from each sample of interest Ct, and ΔΔCt was calculated by subtracting the average ΔCt of calibrator samples from the ΔCt of experimental samples. Data were presented as $2^{\Delta\Delta Ct}$ expression. All samples were run in technical triplicate.
3.8 Compound Formulation and Administration of DKR-1677 in vivo

DKR-1677 was dissolved in DMSO (10 mg/mL) and diluted to the desired concentration (3mg/kg/day) with a final formulation of 10% DMSO (Fischer Scientific), 10% Kolliphor (Sigma-Aldrich), and 80% D5W (5% dextrose in water, pH 7.2). The compound was administered to mice by IP injection starting at 1 hpi and continued once per day for seven days.

3.9 Immunohistochemistry for cell quantification

Tissue was thawed at room temperature and OCT was removed following washing in PBS. Tissue was permeabilized for 1h at room temperature in 0.4% Triton-X PBS. Tissue was then blocked for 1h in a 3% serum solution of the species that the secondary antibody of interest was generated in. Primary antibody incubations occurred O/N at 4°C, neurons were detected by immunoreactivity with anti- NeuN antibodies (1:300 Cell Signaling no. 24307) as well as anti-MAP2 (1:500 Sigma-Aldrich no. M1406) Primary antibody staining was then detected following incubation with an Alexa Fluor® 594 conjugated secondary antibody (1:500 Life Technologies no. A21207 & A21203) for 2 hours at RT. Following 3 PBS washes, tissue was cover-slipped in Fluoro-Gel with Tris Buffer (Electron Microscopy Sciences, no. 17985).

3.10 Stereological quantification

Cell quantification was blinded and performed using Micro Bright-Field Stereo Investigator software implementing the Optical Fractionator Probe (3 sections per animal, 25 sections spaced apart) (MBF Bioscience, Williston, VT, United States). The medial cortex, lateral cortex, corpus callosum, external capsule below injury epicenter, and lateral external capsule were contoured under 4× magnification. The counting frame for all contours was 75:75 μm with an SRS Grid Layout of 175:175 μm. Once contoured, the cell counts were performed at 63× in immersion oil. Counts with a Gundersen Coefficient of
error value of <0.1 were deemed reliable and included in the study.

3.11 Rotarod Motor Assessment

To habituate the animals to the Rotarod Apparatus (Pan Lab Harvard Apparatus no. 76-0770), at 3 and 2 days prior to surgery mice underwent 4 trials of training which consisted of being placed on a constantly moving rod (4 rpm) until they have remained facing in the proper direction (forward) for 1 minute. At 1 day prior to surgery and at 3, 5, and 7 dpi, animals were subjected to 4 test trials which consisted of being placed on the accelerating rod (ramp 4-40 rpm over a ramp time of 390 seconds). Data points graphed represent the average time in seconds the mice spent on the rod of the 4 trials conducted each day.

3.12 Cortical Lipid Extraction and Analysis with LC-MS/MS

Following perfusion with PBS, the cortex of sham and CCI injured animals was harvested, weighed, and placed in a 5mL glass threaded centrifuge tube in 900 µL of 0.01% Butylated Hydroxytoluene (BHT, Sigma-Aldrich no. W218405) in methanol (Methanol Optima, Fisher Scientific no. A456-500). The tissue was mechanically homogenized with a glass pestle and 3000 µL of tert-butyl methyl ether (MTBE, Sigma-Aldrich no. 34875) was added, a Teflon lined cap added to the tube, and the sample allowed to rock O/N at 4°C. The following day phase-separation was induced by addition of 750 µL of .15M Ammonium Acetate and allowed to rock for 1 hour. The samples were then centrifuged for 10 mins at 1,000g at 4°C. The upper organic fraction was collected and an additional extraction (maintaining the same 20:6:5 ratio of MTBE: methanol: ammonium acetate) was performed to ensure maximum lipid yield. The samples were then desiccated in a speed-vac and stored at -80°C until time for mass spectrometry. The Mass Spectrometry Core at the University of Miami Miller School of Medicine performed
the shotgun lipidomics utilizing the Q Exactive Hybrid Quadrupole Orbitrap Mass Spectrometer, implementing both positive and negative ionizing sources.
References


