Molecular mechanisms of mitochondrial dysfunction in regulated necrotic cell death: Implications for stroke and traumatic brain injury

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Abstract

Regulated cell death occurs through several mechanisms. The glutamate/cysteine antiporter (system x_c) is an essential component of ferroptosis, a form of regulated necrosis. This antiporter is responsible for maintaining homeostatic levels of extracellular glutamate. If the function of this system is interrupted or damaged, it results in increased generation of reactive oxygen species and lipid peroxidation. Because traumatic brain injuries and strokes cause release of glutamate through damaged cell membranes and the blood brain barrier, this antiporter is essential. The mechanisms involved in ferroptosis have been studied in cancer cells, however the nuances of the mechanism are still largely unclear in neural cells. In addition to elevated extracellular glutamate, sphingolipids may play a regulatory role in this process. Sphingosine is a key sphingolipid that may trigger ferroptosis. Acid sphingomyelinase (ASM) makes sphingosine by cleaving sphingomyelin. This enzyme is believed to be activated by the increased glutamate, making it of interest in this project. ASM and its relationship to extracellular glutamate and the production of sphingosine makes it likely that it has a regulatory in ferroptosis. The results of the study draw attention to the importance of the cystine/glutamate (system x_c^-) antiporter. When the extracellular concentration of glutamate is elevated, this antiporter cannot supply cystine to produce glutathione, a protective antioxidant. Additionally, acid sphingomyelinase is activated in response to glutathione depletion and results in an increase in sphingosine and ceramide concentrations. The increase in these two bioactive sphingolipids disrupts the mitochondrial respiratory chain and results in oxidative damage and death of oligodendrocytes. The results of this study provide insight into how to protect

oligodendrocytes and other brain cells from secondary injuries following traumatic brain injuries.

Traumatic Brain Injury (TBI)

Traumatic brain injuries (TBIs) have become increasingly common within the past few years and they are one the most reported injuries in the country (Sharma & Lawrence, 2014). This type of injury is defined as, "an impact, penetration or rapid movement of the brain within the skull that results in altered mental state" (Mayumi et al., 2013). There were more than 1.7 million reported TBIs in the United States (Santopietro et. al, 2015). These injuries can be a result of sports related accidents often seen in football, bicycling, or basketball for example. Recent studies on retired professional football players have revealed the long-lasting repercussions of repeated blows to the head. There have been reports of professional football players suffering from chronic traumatic encephalopathy, a neurodegenerative disease believed to be a result of repetitive traumatic brain injuries (Omalu et. al, 2010).

In addition to sports related injuries, automobile accidents and falls are two other major causes of TBIs in the United States. Traumatic brain injuries can lead to deficits in cognitive abilities that last for years after the impact. However, treatment is difficult because no two injuries are identical. Age and lifestyle (not sure about career) can be factors that make individual responses to TBI different across individuals even with similar impacts and changes in neurochemistry and signaling pathways can lead to brain cell death and dysfunction that is unique to each case. For example, there are often changes in the ability to metabolize glucose, as well as significant changes in the concentrations of

potassium, sodium, calcium, and glutamate (Traumatic Brain Injury and Concussion, 2017). The impacts of these injuries are not limited to neurons; oligodendrocytes, for example, also suffer from these changes in neurochemistry (Santopietro et. al, 2015).

Primary Damage – TBI

Primary damage from a traumatic brain injury is defined as damage to the brain tissue at the moment of impact due to physical movement of the brain (Mayumi et al., 2013). This movement often results from a focal injury, a localized injury caused by a moving object coming in contact with the head, or the head was hit on a stationary object (Sharma & Lawrence, 2014). Contusions and hematomas are both examples of focal injuries that can lead to primary cell death. Falls and automobile accidents are responsible for a large portion of focal traumatic brain injuries. These injuries are untreatable because the damaged tissue cannot be reconstructed however, they are often prevented with helmets or seatbelts or another type of safety apparatus. Precautions are taken to prevent focal TBI's because of the potentially severe tissue damage that is untreatable.

Secondary Damage – TBI

Primary damage is often followed by secondary damage that includes changes in neurochemistry and signaling cascades that lead to secondary cell death and can occur up to several months after the impact (Mayumi et al., 2013). An example of a neurochemical change is increased extracellular glutamate. Glutamate is released into the extracellular space and the cerebral spinal fluid because damage to cell membranes and the blood brain barrier. This change alters the homeostatic concentrations necessary for normal neuronal function (Tait, et al., 2014). Glutamate toxicity is also a

result of oxygen deprivation. Glutamate is typically recycled by excitatory amino acid transporters (EAATs), which depend on oxygen to produce the energy required. Glutamate toxicity can also trigger an influx of Ca^{2+} ions as it binds to the NMDA and AMPA receptors (Sattler & Tymianski, 2000). Other causes of secondary damage from TBI include changes in tropic signal transduction, oxidative stress, mitochondrial dysfunction, and inflammation (Mayumi et al., 2013).

Oligodendrocytes

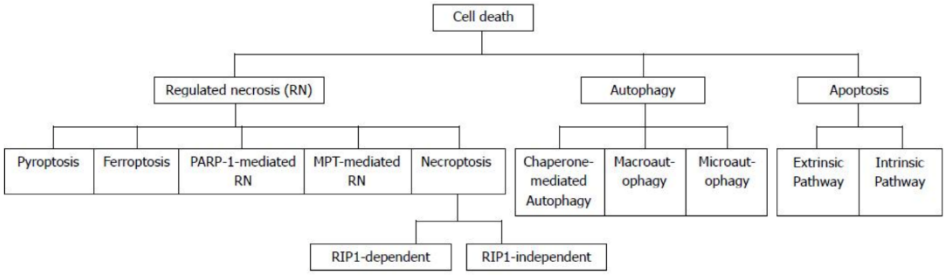
Primary and secondary damage lead to the death of all types of brain cells. Neurons depend on a variety of cells to survive and function normally. Microglia, astrocytes, and oligodendrocytes all help neurons survive. Oligodendrocytes are unique cells responsible for myelinating the axons of neurons in the central nervous system. Myelination is critical for efficient neural signaling as it reduces attenuation and increases the length constant (lambda). Myelin is essential for neurons because it allows action potentials to travel faster and farther because of reduced attenuation of the action potential. This insulating property comes from the high lipid content (70% dry weight) of myelin (Pfeiffer et. al, 1993). The insulation allows for rapid signaling through axons. In a previous study, it has also proposed that oligodendrocytes provide growth factors to neurons (Pfeiffer et. al, 1993). Oligodendrocytes are important for maintaining growth and efficient communication between neurons.

Previous research examined how TBIs impacted oligodendrocyte and oligodendrocyte progenitor cell function. Dent et al. (2015) found that following a traumatic brain injury, there was a significant increase in oligodendrocyte cell death, and an increase in oligodendrocyte progenitor cells. The conduction block means that the

signal is too weak for the post synaptic neuron to reach threshold and fire another action potential (Smith et. al, 1979). This is an example of how changes in the neurochemistry and neurochemical pathways following a TBI can have several effects on a variety of cell types as well as neuronal circuits.

Mechanisms of Cell Death

Cell death during secondary injuries can be broken into two general categories often informally referred to as regulated and accidental. Accidental cell death occurs through independent signaling or communication that triggers a particular process or cascade that ultimately ends in cell death. This process has no regulation and occurs at a variety of times and under a wide array of environments. Regulated cell death differs from accidental death in that there are specific signaling molecules or cascades that trigger cell death (Tait et. al, 2014).



This diagram highlights the types of cell death seen in humans. They all have distinct characteristics. Ferroptosis, the focus of this study, is a distinct type of regulated necrosis

Initially apoptosis was assumed to be the only form of regulated cell death, however recent findings have uncovered a regulated form of necrosis which shows that there is much to learn about cell death as whole. Necroptosis, regulated necrosis, and apoptosis are separate processes and they are characterized uniquely. Morphologically, apoptosis involves reduction of nuclear volume, retraction of pseudopods, and chromatin condensation. Necroptosis is recognizable because the plasma membrane

ruptures and the organelles swell. A particular form of regulated necrosis is ferroptosis (Yang & Stockwell, 2016). Ferroptosis is distinct from apoptosis and necrosis and results from the accumulation of iron-dependent lipid peroxides that lead to cell death.

Ferroptosis is a distinct type of regulated necrotic cell death. Morphologically, cells that die from this process tend to have deformed mitochondria (small, reduced cristae, ruptured outer membrane) (Xie et al., 2016). Mitochondria are responsible for producing adenosine triphosphate and normally functioning mitochondria will produce low amounts of reactive oxygen species. This type of damage to the mitochondria is rarely seen in apoptosis and necroptosis. When the respiratory chain in the mitochondria gets inhibited following a traumatic brain injury, high amounts of these species are produced, potentially triggering cell death.

In addition to the morphological distinction of the damaged mitochondria, regulatory molecules that induce apoptosis, necroptosis, and ferroptosis are all different. For example, erastin is a molecule that only induces cell death through ferroptosis and BcI-2 proteins trigger apoptosis (Xie et al., 2016). Each of these types of cell death also has distinct immune features. Apoptosis often has an anti-inflammatory response, necroptosis triggers a pro-inflammatory response in some cases and an anti-inflammatory response in others and ferroptosis illicit only pro-inflammatory response (Xie et al., 2016). This is a brief example of how each of these types of cell death is different, and it highlights the importance of focusing on one particular type, ferroptosis.

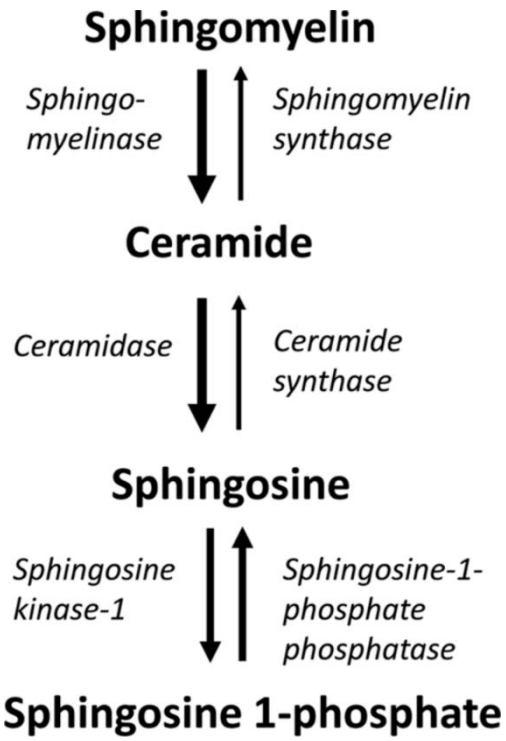
Ferroptosis requires that iron ions be present in the cell. When these iron ions are metabolized, lethal reactive oxygen species and lipid peroxidation products are formed. Ferroptosis can also be induced through dysregulation of the cystine/glutamate

(system x_c^-) antiporter (Yu et al., 2017). his system typically functions to transport intracellular glutamine out of the cell and extracellular cystine into the cell. The movement of these amino acids is important in order to create an important antioxidant, glutathione (GSH). In addition to reduced cystine, synthesis of glutathione requires two additional amino acids: glutamate, and glycine (Yu et al. 2017). This molecule helps prevent lethal reactive oxygen species from damaging or killing the cell.

As brain cells die following a traumatic brain injury, glutamate is released into the extracellular space as cell membranes are damaged, altering the concentration gradient that system x_c^- depends on to function properly (Stoica & Faden, 2010). As this gradient is shifted from its natural equilibrium, the antiporter cannot bring cystine into the cell. Without cystine, the cell is unable to produce glutathione, so damage through oxidation is likely. Essentially, when cystine uptake is inhibited, the cell is unable to adequately defend against reactive oxygen species. Without this defense, cell death continues after the initial trauma and results in secondary injuries. Ferroptosis, if it can be prevented, could potentially reduce the lasting cognitive damage that is caused by traumatic brain injuries (Conrad et al., 2016). If the mechanisms of ferroptosis can be accurately prescribed, drugs and treatments may be able to protect the brain from additional degeneration and cell death.

Role of Sphingolipids

Sphingolipids are a type of fatty acid containing sphingosine, a long chain amino alcohol, or one of its derivatives. Sphingosine and ceramide, bioactive sphingolipids, are believed to play a regulatory role in cell death following traumatic brain injuries. These molecules are an integral structural component of cell membranes and they are synthesized through one of four different pathways. Each pathway produces ceramide, an essential metabolite in sphingolipid production. Another relevant pathway is the de novo pathway of sphingolipid synthesis, sphingokinases phosphorylate ceramide, sphingosine, and dihydrosphingosine to make more complex sphingolipids (Novgorodov & Gudz, 2009). The recycling of exogenous ceramide pathway uses short chain ceramides converted to sphingosine by ceramidase. The sphingosine is then converted into ceramide or sphingosine-1-phosphate with ceramide synthase and sphingosine kinase, respectively (Novgorodov & Gudz, 2009). The salvage pathway converts complex sphingolipids, sphingosine, sphingomyelin, and glucosylceramide, to ceramide with a variety of enzymes. Sphingomyelin is a molecule found in the myelin sheath on neural axons, and there is a final pathway that



The pathway in which sphingomyelin is converted to ceramide, sphingosine, and the less harmful sphingosine 1-phosphate

interconverts sphingomyelin and ceramide. The enzymes in this pathway are sphingomyelin synthase and acid sphingomyelinase. The substrates, products, and enzymes of each pathway are shown in diagram above.

Acid sphingomyelinase is an essential enzyme in the hydrolysis of sphingomyelin into ceramide and sphingosine. It does this by catalyzing the cleavage of the phosphorylcholine linkage (Chudakova et. al, 2008). This enzyme is also important in maintaining the structural integrity of cell membranes by recycling and replacing sphingolipids and as a signaling molecule for other biological processes.

In addition to glutamate, sphingolipids may play a role in the regulation of ferroptosis. Previous work has found that sphingosine and ceramide are both important in the other types of regulated cell death (apoptosis and necroptosis). It is possible that sphingosine or another sphingolipid has a regulatory role in ferroptosis. Research has shown that following a traumatic brain injury, there is significant accumulation of sphingosine in the mitochondria that inhibits proper function (Novgorodov et. al, 2014).

Accumulation of sphingosine in the mitochondria can lead to dysfunction in the respiratory chain. Mitochondria contain a respiratory chain containing four different complexes (Novgorodov & Gudz, 2009). Glutamate induced oxygen consumption is an indication of the efficiency of complexes I, III, and IV and succinate induced oxygen consumption is an indication of the efficiency of complexes II, III, and IV and the activity of cytochrome oxidase (COX). These data show that sphingosine accumulation in the mitochondria following a TBI reduces the activity of COX (Novgorodov, & Gudz, 2009).

While traumatic brain injuries are the focus of this experiment, the results may provide insight into how to protect brain cells following strokes or ischemia/reperfusion

injuries. They may do this because ferroptosis is likely involved in those injuries as a result of sphingolipid accumulation (acid sphingomyelinase) and glutamate toxicity. Induced ferroptosis has also been shown to be able to target and treat cancer. There have been several studies exploring this technique as a potential treatment. The data show that some types of tumor cells may be susceptible to this treatment, while other types are not. The discovery of the process is relatively new but it may significantly change how a variety of injuries and diseases are treated and how secondary injuries following a TBI may be reduced.

The objective of this study is to understand the mechanisms of cell death following traumatic brain injuries. Although a variety of types of cell death exist, ferroptosis is the process of interest. The role of system x_c^- as well as acid sphingomyelinase and sphingolipids will be examined using oligodendrocytes to better understand how secondary injuries caused by elevated extracellular glutamate, glutathione depletion, and bioactive sphingolipid signaling. It is possible that the findings could be used to develop drugs that target a particular component of this signaling cascade.

Materials/Methods:

Animals:

Female time-pregnant Sprague-Dawley rates from Charles River Laboratories in Wilmington, MA were allowed to acclimate for one week before experimentation began. Acid sphingomyelinase deficient mice were obtained from the animal core facility at the Medical University of South Carolina. All experimental protocols were approved by the

Institutional Animal Care and Use Committee at the Medical University of South Carolina and followed guidelines set by the National Institute of Health.

Reagents:

DMEM/F12 and FBS were obtained from GIBCO (Thermo Fisher, Waltham, Massachusetts).

Protease inhibitor cocktail and Phosphostop phosphatase inhibitor cocktail were obtained from Roche Applied Science (Indianapolis, Indiana). Reclast, Z-VAD-fmk, and epoxyquinone G109 were obtained from Enzo Biochem (Farmingdale, New York). Fumonisin B1, myriocin, and GKT137831 were obtained from Cayman Chemical (Ann Arbor, Michigan). 2',7'-dicholordihyfrofluorescein diacetate (H2-DCF), 4-acetamido-TEMPO, and MitoSox Red were obtained from Thermo Fisher. C10-bisphosphonate (C10-BPA) was obtained from Avanti Polar Lipids (Alabaster, Alabama). Necrostatin-1 and Necrostatin-1s were obtained from BioVision (Milpitas, California). LOXBlock-1 was obtained from ChemBridge Corporation (San Diego, California). GSK-872 and IDO were obtained from EMD Millipore (Billerica, Massachusetts). NIM-811 was provided by Novartis (Cambridge, Massachusetts). LCL-521 was provided by Lipidomics Core facility at the Medical University of South Carolina. All other reagents were obtained from Sigma-Aldrich (St. Louis Missouri).

Antibodies

Rabbit monoclonal anti-receptor-interacting protein kinase (RIPK)1, anti-RIPK-3, anti-caspase-8, anti-LC3, anti-beclin-1 antibodies, and rabbit polyclonal anti-p62 antibodies were obtained from Cell Signaling Technology (Danvers, Massachusetts). Mouse monoclonal anti-beta-actin antibodies were obtained from Sigma-Aldrich. Mouse

monoclonal anti-RIPK1 was obtained from R&D Systems (Minneapolis, Minnesota). Rabbit polyclonal anti-acid sphingomyelinase antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, California). Rabbit polyclonal anti-mixed-lineage kinase domain-like antibody was obtained from Thermo Fisher. Secondary horseradish peroxidase conjugated antibodies were obtained from Jackson ImmunoResearch (West Grove Pennsylvania).

Cell Culture

Cell cultures were prepared by dissociating the cortices of rat and 2-day-old mouse pups. The dissociated cortices were plated on poly-L-lysine coated flasks and allowed to grow for ten days. They were grown in DMEM/F12 medium with 10% FBS at 37°C and 5% CO_2. After ten days oligodendrocytes were separated by first using a shake off process followed by centrifugation at 1000g for 5 minutes. The oligodendrocytes were plated in cell culture dishes or 96 well plates using the same DMEM/F12 medium with 10% FBS and allowed to attach for 24 hours.

RNAi

To down regulate expression of acid sphingomyelinase silencing RNA from GE Healthcare/Dharmacon (Rockford, Illinois). Four sequences were used to target different regions of the gene. A Nucleofector electroporation system was used to transfect the oligodendrocytes with the silencing RNA. Instructions from Amaxa Biosystems were used to carry out the transfection. Cells ($6X10^6$) were mixed with 100ul of Nucleofector reagent and 1ul of the silencing RNA were used.

Cell Survival Assay

Lactate dehydrogenase based CytoTox-ONE homogenous membrane integrity assay was used to measure cell death. Fluorescence was measured at 590nm emission with 560nm excitation in a microscopic plate reader, Synergy H1 from BioTek (Winooski, Vermont).

Enzyme Activity Assays

Caspase activity assay: Activity of executioner caspase 3/7 was measured with an Apo-One kit from Promega and the kit instructions were followed. Following cleavage of Z-DEVD-Rodamine-110 by caspase 3/7, fluorescence was measured at 590nm emission and560nm excitation on the microplate reader

Acid sphingomyelinase and neutral sphingomyelinase activity: Activity of these enzymes was determined with an assay kit from Echelon Biosciences. The same kit was used for both enzymes. The difference was that neutral sphingomyelinase used a reaction mixture that contained 100mM Tris, instead of 100mM sodium acetate and 100mM of Mg^{2+}.

Cell Respiration

Oligodendrocytes were plated on a 96 well plate (3×10^5 cells per well). DMEM/F12 medium was used and the cells were maintained for 24 hours in humidified 5% CO_2 and 95% air at 37°C. Oligodendrocytes were treated with glutamate for 6 hours and then the medium was replaced with DPBS and supplemented with 10mM glucose. Oxygen consumption rate was measured with a Seahorse Bioscience XF-96 extracellular flux analyzer and rates were calculated with Seahorse associated software.

Mitochondria Isolation

Mitochondria were isolated using hypotonic swelling.

Confocal Microscopy

Oligodendrocytes in HBSS were loaded with 200nM TMRM or 5uM of MitoSox Red. Once loaded, the cells were washed and incubated with 50nM TMRM of 1ul of MitoSox Red to ensure equal distribution of the fluorophore. They were then imaged with a Zeiss LSM 510 NLO inverted laser scanning confocal/multiphoton microscope. Fluorescence of TMRM was detected at 560nm and 580nm for MitoSox Red.

Reactive Oxygen Species Generation

After the oligodendrocytes were treated with glutamate and other inhibitors/compounds, they were washed three times with DPBS and incubated with 2uM of H2-DCF diacetate in DPBS with 10mM of glucose at 37°C. Reactive oxygen species generation was measured using H2-DCF oxidation. Fluorescence imaging was used to detect oxidized H2-DCF (520nm). Mitochondrial reactive oxygen species generation was assessed with MitoSox Red. The oligodendrocytes were incubated in DPBS with 5ul of MitoSox Red for 30 minutes at 37°C and then washed with DPBS. Fluorescence was measured at 510nm with the Synergy H1 microplate reader.

Glutathione Content

A glutathione fluorometric assay kit from BioVision was used to determine glutathione content.

Lipid Peroxidation Measurements

Oligodendrocytes were treated with glutamate and other test compounds and allowed to incubate for 18 hours with BODIPY 581/591 C11. This compound indicates lipid peroxidation by shifting the emission peak from 581/591nm to 488/510nm. The ratio is

used to determine the degree of lipid peroxidation. The products were then measured with a malondialdehyde assay kit from Sigma Aldrich.

Immunoprecipitation

Cell lysates were precleared in buffer A (0.15M NaCl, 0.5mM EDTA, 0.5% Igepal CA-360, protease and phosphate inhibitor, 0.05M Tris (pH 7.5) and 0.2% BSA) by incubation with IgG-conjugated magnetic beads for 1 hour. Following the incubation, antibodies were added and incubated overnight at 4°C. The antibody antigen complexes were captured with Dynabeads and washed twice with buffer A and twice with TBS. The precipitates were eluted by boiling in SDS-sample buffer.

Western Blot

Proteins were separated by 4-15% SDS-PAGE, blotted to PVDF membrane, blocked with 5% nonfat dry milk from Bio-Rad (Hercules, California) or 5% BSA in TBS-T buffer (10mM Tris, 150mM NaCl, and 0.2% Tween-20 at pH 8.0). Appropriate primary antibodies were then used and immunoreactive bands were visualized with a SuperSignal West Dura substrate from Thermo Fisher.

Sphingolipid Analysis

Sphingolipid content was analyzed using mass spectrometry. The lipids were extracted using 0.5mg of mitochondria or cell lysate added to 2ml of ethyl acetate/isopropanol/ water in a 60:30:10% ratio. Internal standards were added and the sample was subsequently dried with nitrogen. After drying, 100ul of methanol was used to reconstituted the dried sample and analyses was done using a Thermo Fisher TSQ Quantum triple quadruple mass spectrometer.

Statistics

Analysis was done using a one-way ANOVA or Student's *t*-test when appropriate. Statistical significance was recognized when $P < 0.05$.

Results:

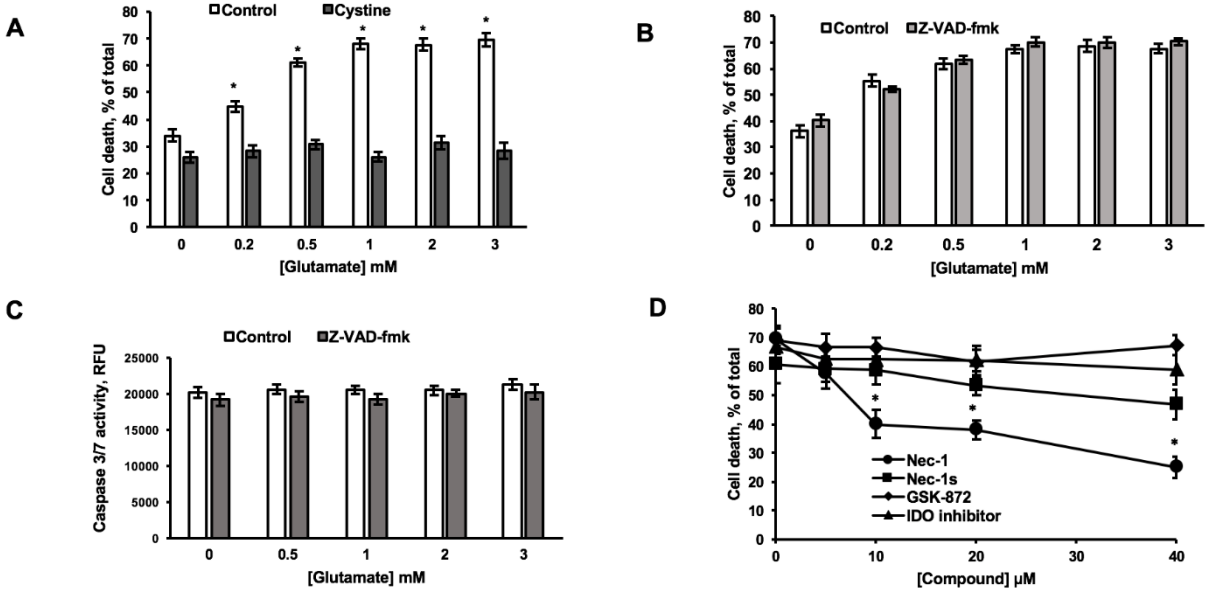


Figure 1: Cystine attenuated oligodendrocyte death. Z-VAD-fmk had no impact on oligodendrocyte survival or caspase 3/7 activity. Necrostatin-1 significantly reduced oligodendrocyte death.

The glutamate/cysteine antiporter system x_c is disrupted when extracellular glutamate is elevated. Figure 1A shows that when extracellular glutamate is elevated, the addition of cysteine effectively reduces oligodendrocyte survival. Z-VAD-fmk shown in Figure 1B/C is a is a caspase inhibitor and it had no significant effect on augmenting oligodendrocyte survival in these conditions. This indicates that caspase 3/7 plays no significant role in oligodendrocyte death. Figure 1D clearly shows that necrostatin-1s, a necroptosis inhibitor, reduced oligodendrocyte death when extracellular glutamate is elevated. In addition to necrostatin-1s, necrostatin-1, necrostatin-1s, GSK-872, and IDO inhibitor were also used. These compounds are also necroptosis inhibitors, however there was no significant decrease in oligodendrocyte death when they were used. The results show that death is likely not caused by the caspase dependent apoptotic

pathway or necroptosis. The reduction in oligodendrocyte cell death caused by necrostatin-1s may indicate that a unique pathway is activated by the extracellular glutamate. It necroptosis was occurring through the known pathway; the other inhibitors should have had a similar effect.

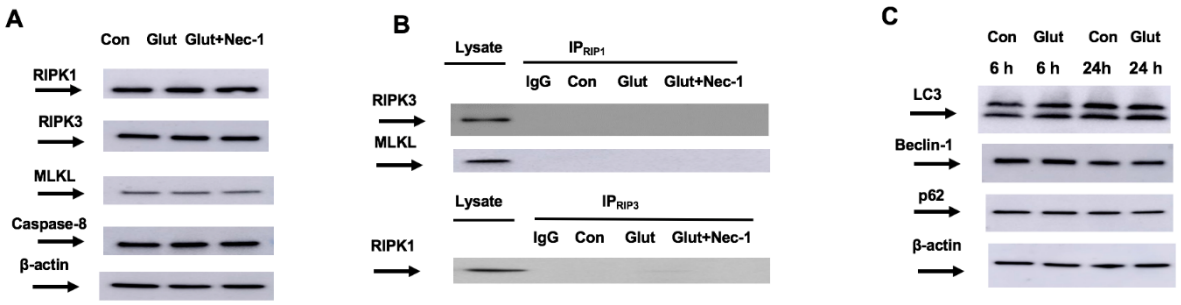


Figure 2: Expression of RIPK1, RIPK3, and MLKL was not changed in response to glutamate or necrostatin-1. No changes were seen in the expression of proteins indicative of autophagy and no activation of RIPK3 or RIPK1 pathways.

To further clarify, immunoprecipitation and western blot was used to characterize how elevated extracellular glutamate effected the expression of necroptosis signaling pathway meditators RIPK1, RIPK3, and MLKL. Figure 2A shows that the expression of RIPK1, RIPK3, and MLKL was seen in oligodendrocytes and the degree of expression did not change in response to necrostatin-1. This indicates that under these conditions, oligodendrocytes are not activating the necroptosis pathway. Additionally, Figure 2B used immunoprecipitation to visualize necroptosis complex formation. The results indicate that this machinery did not change in response to glutamate. Figure 2C examined proteins (LC3, Beclin-1, and p62) expressed during autophagy and involved in the signaling pathway. There results show no change over a 24h period and indicate

that autophagy is not activated when extracellular glutamate is elevated. These results clarify the speculation that a unique type of cell death and signaling pathway is involved.

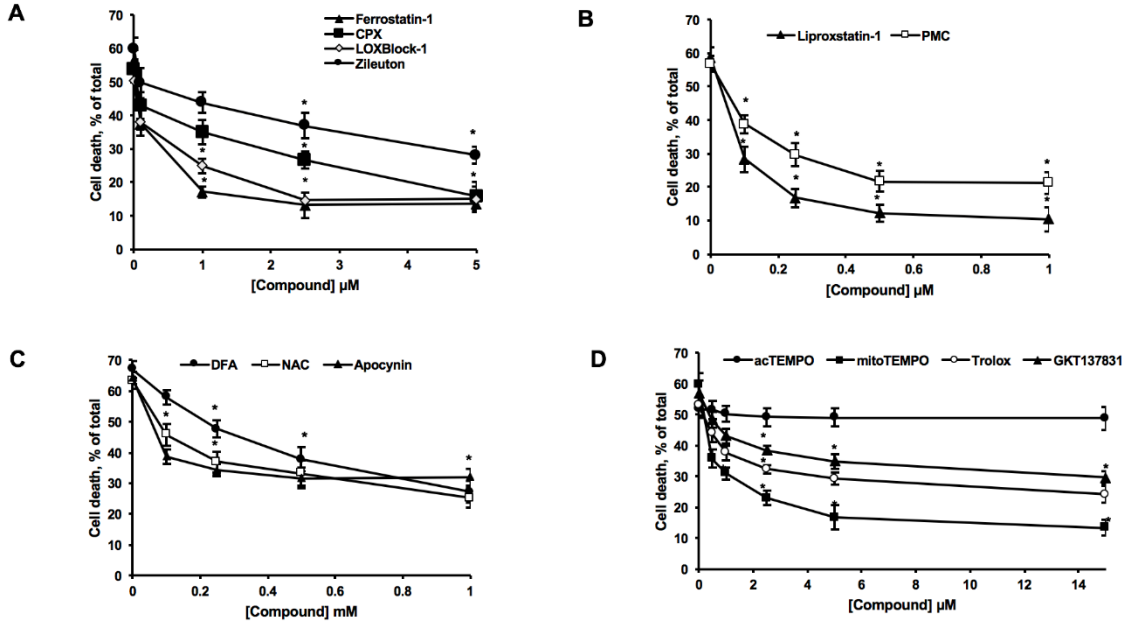


Figure 3: Addition of iron chelators, antioxidants, and pharmacological inhibitors attenuated oligodendrocyte death.

Ferroptosis was another possible form of cell death that may occur in response to glutamate. Several pharmacological inhibitors were used under these conditions and oligodendrocyte death was measured. Figure 3A/B show that ferrostatin-1, liproxstatin-1 zileuton, and LOXBlock-1 all significantly reduced oligodendrocyte death. In addition to the previously mentioned inhibitors, iron chelators were used because for ferroptosis to occur, iron must be present. The chelators CPX and deferoxamine (DFA) seen in Figure 3C show that cell death is augmented when iron is unavailable. Another distinguishing characteristic of ferroptosis is the production of reactive oxygen species, lipid oxidation products, and reduced glutathione production. Antioxidants were used (N-acetylcysteine [NAC], trolox, and 2,2,5,7,8-pentamethyl-6-chromanol [PMC]) and effectively reduced oligodendrocyte death (Figure 3B/D). NADPH oxidase inhibitors GKT137831 and apocynin also augmented oligodendrocyte death (Figure 3C/D). These results indicate

that elevated extracellular glutamate caused oligodendrocyte death by activating the ferroptosis signaling pathway.

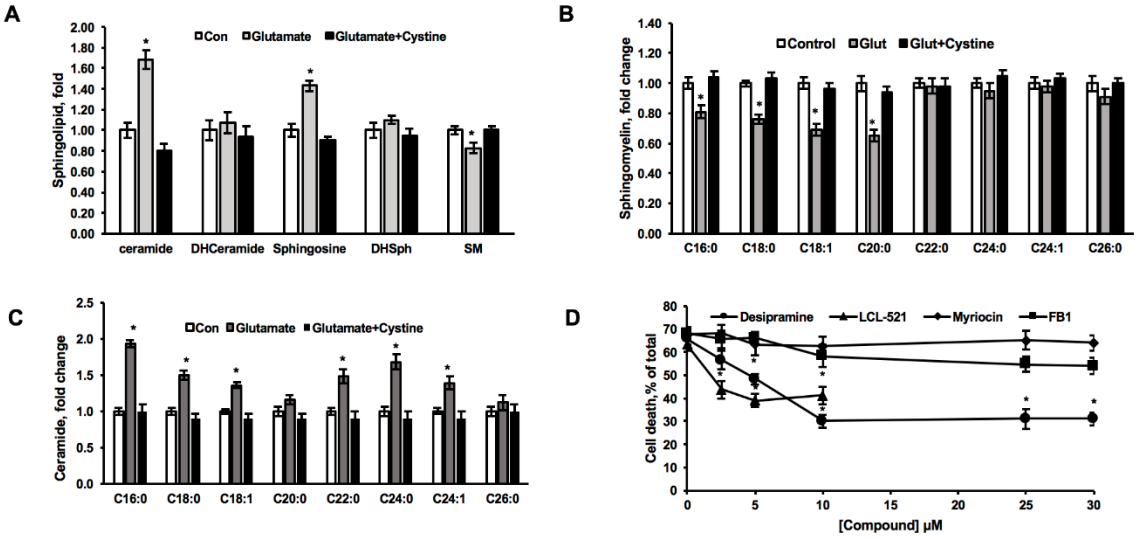


Figure 4: Change in sphingolipid concentrations in response to glutamate. Acid sphingomyelinase inhibitors (Desipramine and LCL-521) attenuated oligodendrocyte death.

Sphingolipids have been implicated in regulating autophagy and apoptosis, making them a possible component of ferroptosis activation and signaling. Figure 4A shows that when extracellular glutamate is elevated above homeostatic levels, concentrations of sphingosine and ceramide increase while the concentration decreased. This indicates activation of sphingomyelin hydrolysis during high glutamate conditions. Additionally, the concentrations of dihydroceramide dihydrosphingosine were not altered. These sphingolipids are characteristic of sphingolipid synthesis and because they remained unchanged, it can be inferred that the sphingolipid hydrolysis is the pathway of interest. Figured 4B/C show changes in the concentration of sphingosines and ceramides with varying acyl chain lengths. Figure 4A indicates activation of the sphingomyelin hydrolysis pathway, specific inhibitors desipramine, LCL-521 were used to verify this. Desipramine, an indirect acid sphingomyelinase

inhibitor, significantly reduced oligodendrocyte death, confirming that assertion that the hydrolysis pathway is involved in glutamate induced oligodendrocyte death (Figure 4D).

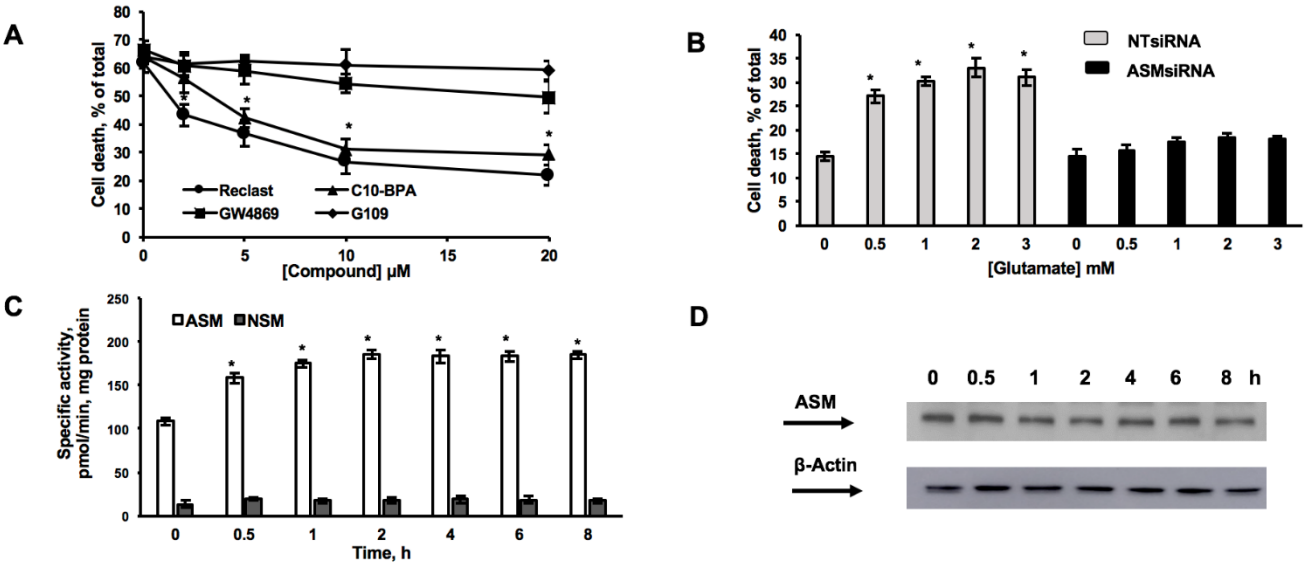


Figure 5: Inhibition of acid sphingomyelinase reduced oligodendrocyte death. Additionally, knocking down acid sphingomyelinase had the same effect.

Indirect inhibition of acid sphingomyelinase with despiramine showed decreased oligodendrocyte death and led to the use of direct inhibitors and silencing RNA. Figure 5A shows significant reduction in oligodendrocyte cell death when treated with Reclast and C10-BPA. Additionally, silencing RNA was used to knockdown expression of SMPD1, the gene responsible for acid sphingomyelinase production reduced oligodendrocyte death. Inhibitors and knocking down neutral sphingomyelinase had no effect on oligodendrocyte survival. Figure 5D shows no change in expression of acid sphingomyelinase and it can be inferred that post translational modification or activation occurs when extracellular glutamate is elevated.

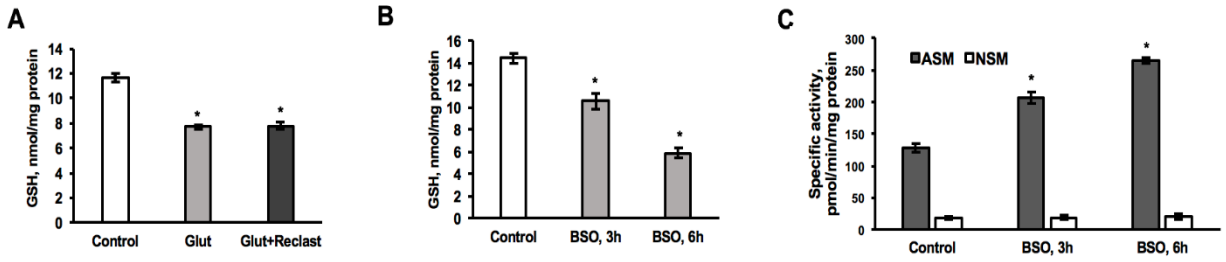


Figure 6: Low concentrations of glutathione (GSH) result from elevated extracellular glutamate. Low GSH also activates acid sphingomyelinase.

Elevated extracellular glutamate has been shown to disrupt the function of glutamate/cysteine antiporter system x_c and one result of this is reduced glutathione production. Glutathione is an essential antioxidant that offers oligodendrocytes and other cell types protection against oxidative molecules. Figure 6A shows that the concentration of GSH is reduced when glutamate is high, and not when reclast (acid sphingomyelinase inhibitor) is introduced. Figures 6B/C show the addition of BSO, an inhibitor of glutathione production. The addition of BSO shows reduced glutathione concentration in oligodendrocytes resulting in augmented acid sphingomyelinase activity. Additionally, the BSO had no significant impact on neutral sphingomyelinase activity. These results indicate that low glutathione activates sphingomyelinase as part of the sphingomyelin hydrolysis pathway.

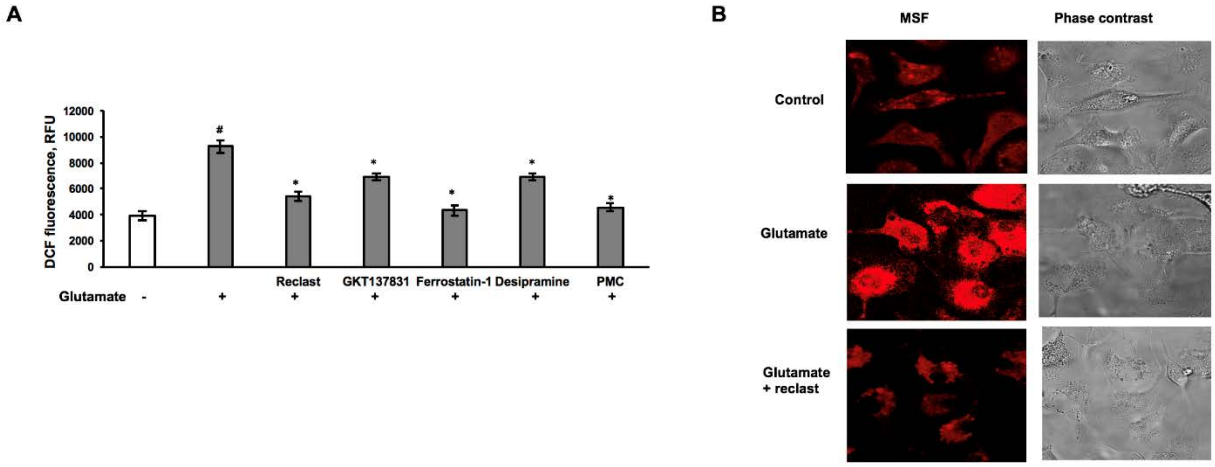


Figure 7: Addition of reclast, GKT137831, ferrostatin-1, desipramine, and PMC all significantly reduced reactive oxygen species production when glutamate was high. Part B is an example using reclast and the image and it clearly shows the change in fluorescence

Reactive oxygen species generation was quantified with fluorescence. H2-DCF which reacts with these species and produces DCF, a fluorescent compound. Using microscopy, the fluorescence was quantified when various compounds were applied. Reactive oxygen species production was reduced by ferrostatin-1 and PMC which are both inhibitors of ferroptosis. GKT137831, a NOX inhibitor had a similar effect. Reclast and desipramine, inhibitors of acid sphingomyelinase also significantly reduced reactive oxygen species generation under these conditions. Figure 7B is a fluorescence image that clearly indicates an increase in generation when extracellular glutamate is high, and that can be reduced with the addition of Reclast, for example.

Discussion

The results of this study highlight the importance of mitochondrial dysfunction caused by traumatic brain injuries and strokes. Following these injuries, significant damage to brain tissue can occur because of the drastic change in the extracellular environment. Specifically, the results of the study draw attention to the importance of the cystine/glutamate (system x_c^-) antiporter. When the extracellular concentration of glutamate is elevated, this antiporter cannot supply cystine to produce glutathione, a protective antioxidant. Additionally, acid sphingomyelinase is activated as a result of insufficient glutathione and it likely triggers a signaling cascade the ultimately leads to disrupts mitochondrial function and a buildup of reactive oxygen species. These changes cause cell death and can occur long after the initial impact or injury.

The data also suggests that the death occurs via a distinct pathway, different than previously studied types of cell death such as apoptosis or autophagy. This interpretation is supported through the use of pharmacological inhibitors. These molecules did not reduce oligodendrocyte death and support the interpretation that this is a novel process. In addition to inhibitors, iron chelators and antioxidants were used to investigate whether ferroptosis may be responsible for oligodendrocyte demise. These were chosen because iron is essential in ferroptosis, and when is becomes unavailable cells are unable to carry out this process and survive. Antioxidants were used based on the fact that the glutathione concentrations are reduced and as a result the oligodendrocytes become vulnerable to oxidative damage. We noticed that antioxidants were able to reduce oligodendrocyte death which supports this assertion.

Acid sphingomyelinase activity was also altered under these conditions. The results show that following a traumatic brain injury, it is likely that acid sphingomyelinase increased the production of sphingosine, a product of sphingomyelin hydrolysis. We believe that elevated sphingosine may disrupt mitochondrial function and lead to oxidative damage and death in oligodendrocytes and it is believed that this occurs by disrupting the mitochondrial respiratory chain.

These results may be beneficial for individuals who have suffered from a traumatic brain injury by reducing the severity of secondary injuries caused by the altered cellular environment in the brain. As mentioned previously, traumatic brain injuries are common around the world and can occur from a variety of activities. Suspicions and preliminary data suggest that activities that don’t involve direct impact to the head such as horseback riding or jet skiing, can result in damage to the brain. If this

is shown to be accurate, many more individuals may benefit from these findings than previously thought. With more people potentially suffering from these injuries, the need for treatment will rise, likely resulting in higher healthcare costs. Because of this, a way to protect the brain is essential.

It is possible that further research may identify a point in the signaling cascade that could be inhibited or regulated with specific drugs. A potential target could be acid sphingomyelinase because we are confident that sphingosine plays a significant role in oligodendrocyte death. If the glutathione dependent activation of this enzyme could be inhibited or reduced, there would be significantly less sphingosine molecules available to disrupt mitochondrial dysfunction. This may be challenging because acid sphingomyelinase is responsible for other processes and inhibiting it could have detrimental side effects.

Drug researchers and developers may want to focus a portion of their resources on developing drugs that could replicate the augmented oligodendrocyte death seen in this study however, desipramine, a drug used in this study, is a commercially available drug often prescribed for nerve pain and depression and we saw a significant decrease in oligodendrocyte death because it reduced acid sphingomyelinase activity. Additional pharmaceutical drugs have been shown to have the same effect on acid sphingomyelinase as desipramine. These drugs are referred to as tricyclic antidepressants and one in particular is amitriptyline. This drug was first made available in 1961 as an antidepressant and it has since been shown to act as an inhibitor of acid sphingomyelinase activity, similar to desipramine (Beckman et al., 2014). These drugs

effectively inhibit acid sphingomyelinase activity, however they should be used with caution because of potential side effects.

Another study focused on the drug fluoxetine, initially developed as a selective serotonin reuptake inhibitor, and its protective attributes seen in the brains of individuals who have suffered an ischemic stroke. While the cause of the damage to the brain is not a result of an impact, the internal environment following the stroke is similar to the environment following a traumatic brain injury so it is a logical assertion that individuals suffering from traumatic brain injuries may benefit the same way. The results of the randomized placebo controlled study show that fluoxetine significantly enhanced motor recovery and modulated brain plasticity when given to patients immediately following the stroke (Chollet et al., 2011). These findings support the assertion that existing antidepressants may be beneficial and promote a neural protection by modulating enzymes and pathways in the brain.

This poses interesting questions about the potential for drug repositioning and additional uses for existing drugs. Because it has already been through clinical trials and been approved for safety, one may speculate that it may be beneficial to prescribe it to individuals who have sustained a traumatic brain injury despite the fact that they may not require an antidepressant.

With recent research on football players showing extremely high rates of brain damage, it is reasonable to assume that these findings could contribute to future research into how to prevent or treat this damage. The study found that out of 202 deceased NFL football players, 87% of the players had chronic traumatic encephalopathy (Mez, Daneshvar, Kiernan, 2017). This neurodegenerative disease is a

result of repeated blows to the head of an extended period of time. Much still has to be learned about the mechanisms of chronic traumatic encephalopathy, however it is likely that acid sphingomyelinase is involved because it results from repeated traumatic brain injuries. While the nature of chronic traumatic encephalopathy is slightly different than a traumatic brain injury, it may be effective to prescribe NFL players existing antidepressants as a preemptive measure to reduce risk of developing chronic traumatic encephalopathy by protecting the brain from additional cell death.

Traumatic brain injuries are prevalent and research on how to reduce the severity of secondary injuries is an important topic. This study provided significant evidence about the mechanism of regulated cell death when extracellular glutamate is elevated following the impact. System x_c^- is essential and when its function is disrupted, there is a lack of glutathione to protect the cell. This is believed to then trigger the hydrolysis of sphingomyelin and the products, ceramide and sphingosine, disrupt the function of the mitochondrial respiratory chain. More research should be done on acid sphingomyelinase to better understand how manipulating its activity in the brain could impact other processes. These results are an important step in developing a way to combat the secondary injuries of traumatic brain injuries.

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