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Connexin-43 and Traumatic Brain Injury: A potential target for therapeutic intervention

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ABSTRACT

Traumatic Brain Injury (TBI) is a widespread, degenerative affliction with no current therapeutic interventions. The long-term degradation caused by TBI results from secondary injury cascades that are initiated by primary injury. An early and important step in the damage process is reactive astrogliosis in astrocytes. Astrocytes communicate through gap junctions, which are composed of two connexon hemichannels from the two communicating cells and these connexons themselves are composed of six connexin protein subunits. Connexin 43 (Cx43) is a particularly important connexin to gap junctional communication and could act either to preserve the astrocytes from oxidative stress, or to propagate the damage signals to otherwise healthy cells. The present study investigates the expression of Cx43 in different treatment types in hippocampal samples of Wistar rats in order to elucidate the relationship between Cx43 and secondary injury through Real-Time PCR (qPCR) and cell culture work. While Cx43 has a trend for increased expression in traumatized tissue that is returned to normal levels with an antioxidant treatment, these results are not statistically significant according to a single- sided ANOVA test. Further research is needed to understand the relationship between Cx43 and secondary injury.

INTRODUCTION

The major health issues responsible for injury and death in the United States are typically accompanied by a slew of possible medications and therapies available to the patient. However, traumatic brain injury (TBI), one of the most significant sources of injury and long-term complications occurs in many ways and impacts people in all walks of life. Whether from car accidents, military injuries, recreational accidents, occupational hazards, professional football or high school basketball, TBI is a prominent and pervasive detriment to health and well-being. There are approximately one to two million new incidences of moderate TBI (mTBI) that are documented in the US annually, not including unreported cases (Lovell et al., 2004). Moreover, TBI is a severe affliction throughout the globe with an estimated incidence of 200 per 100,000 people

annually, although this is most likely a significant underestimate given the limitations of a global census (Bryan-Hancock and Harrison, 2011). Moreover, the popularity of high school sports makes mTBI a serious threat to young people through multiple concussions during athletic competition, making young people vulnerable to life-long degenerative afflictions as there is no established pharmaceutical intervention or effective therapy to allay disease progression.

TBI is an overarching term describing both the macroscopic and microscopic physiological processes that occur in response to brain trauma. The initial primary injury is classified in multiple ways based on the size of the affected area, whether or not the skull was penetrated, and the severity of the injury. There are several scales employed to classify the severity of the injury. One diagnostic tool for categorization of injury is the Glasgow Coma Scale, which assesses different responses and functions of the injured patient and translates the results of these various criteria into a numerical score, corresponding to one of three possible ranges representing mild, moderate, and severe TBI (Orange County Neurology Medical Center, n.d.) Severe head injury typically has a Glasgow Coma Scale score less than eight, moderate head injury between nine to twelve and mild injury between thirteen and fifteen (Miller, 2013). Concussions are typically associated with mild TBI. Other classifications of TBI are based on whether or not the damaged tissue is exposed to the local environment and also applied. Focal injuries affect one particular area, whereas diffuse injuries cause more widespread damage. Open TBI refers to injuries in which the skull is penetrated and the wound is exposed, whereas closed TBI involves damage to the brain where the skull remains intact and the brain tissues remain unexposed and separated from the local environment (Heegaard, et al., 2013).

Primary injury, which occurs at the time the trauma is incurred, is considered irreversible; however, the secondary injuries that result from the cytotoxic cascades and destructive biochemical events that occur within cells could potentially be interrupted, possibly limiting the damage typically associated with moderate TBI. The biochemical and cellular events that occur following primary TBI and eventually lead to the damages associated with secondary TBI are complex and heavily researched. Certain events are known to occur following TBI starting with massive, widespread neuronal depolarization and eventually leading to lipid peroxidation, increase in glial-fibrillary acidic protein (GFAP), reactive gliosis, release of excitatory amino acids such as glutamate, higher calcium influx into neurons leading to increased generation of excitatory postsynaptic potentials (EPSPs), nitric oxide (NO) release, and cytotoxicity (Greve, M.W. and B. Zink, 2009). These physiological and biochemical changes initiate the accumulation of damage seen in secondary injury, causing damage to neurons and glial cells and impairing neuronal communication and synaptic transmission, thereby

facilitating a decrease in brain function including impaired learning and memory formation. Three key facets of secondary injury are increased levels of glutamate, free radicals and NO, which constitute a mutually-amplifying triad, referred to as the tripartite synthesis, perpetuating and propagating neuronal damage (Greve, M.W. and B. Zink, 2009). A less emphasized result of TBI is the transient increase in gap junctional coupling that occurs following trauma (Franseva et al., 2002).

Gap junctions are critical facilitators of intercellular signaling formed by the interaction of two different cells' connexons, which are hemichannel proteins embedded in the cells' plasma membranes. These connexon hemichannels are each composed of six smaller connexin (Cx) protein subunits. Connexin proteins have a short half-life of approximately one to three hours, demonstrated from in vitro tissue cultures (Solan and Lampe, 2007). This rapid recycling indicates that connexin expression is a tightly regulated and adaptable process capable of undergoing rapid alterations in response to cellular changes. Connexin proteins are synthesized in the rough endoplasmic reticulum (RER), passed through the trans-Golgi complex, and finally trafficked via vesicles to the plasma membrane. Oligomerization of the six connexin subunits into one connexon hemichannel is believed to begin in the ER as the protein is synthesized, and end in the Golgi apparatus, where further modifications are completed (Lampe and Lau, 2004).

During this process, the identity of the connexon is established through its connexin composition. Twenty different connexin isoforms are known to exist in the human genome, classified by their molecular weight and their genetic relatedness. Dysfunction and dysregulation of connexins has been implicated in a variety of diseases including… (Wei et al., 2014). Any of these connexins can be recruited during oligomerization to form connexons, giving rise to profound potential for diversity among connexons.

Obstruction of the glial network, and consequential neuronal signaling dysfunction, is considered to be a major contributor in the accumulation of long-term damage from mTBI (Prochnow et. al, 2014). It is likely that gap junctional communication allows for the transmission of damage signals produced through the secondary injury of TBI or, alternatively, allows for the rapid distribution of these signals throughout the glial network to prevent individual afflicted cells from harboring lethal levels of these damaging agents. Either way, changes in gap junctional communication are likely to play an important role in secondary injury from mTBI.

Due to the vast array of possible connexon conformations, there are connexon classifications based on composition. Connexons consisting of six identical connexin subunits are called homomeric hemichannels, whereas connexons consisting of different connexin subunits are called heteromeric hemichannels. Different connexin

isoforms have varying abundances in various cell types, suggesting functional relevance in their respective cell type (Wei et al., 2004). Further contributing to the diverse repertoire of gap junctional communication is the composition of the gap junctions themselves. While gap junctions are composed of two different connexon hemichannels, they do not have to be composed of identical connexons. A gap junction is homotypic if the connexon hemichannels contributed by both interacting cells are identical, and heterotypic if they are not. The possibility for diversity at multiple levels in gap junction formation allows for an abundant resource for gap junction communication, and different biological functions among differently structured gap junctions. Permeability, weight, selectivity for ions and electrical conductance are all factors that diverge widely in channels formed by different connexin subunits (Nagy and Rash, 2000).

Connexin-43 (Cx43) is the most widely-expressed connexin in the body, having important roles in development and important roles in specific tissues and cell types because, unlike many other connexin isoforms, it not only is expressed throughout development, but also continues to be expressed into adulthood (Dbouk, et al., 2009; Chang and Balice-Gordon, 2000). Cx43 is particularly known for its importance in astrocytes, one of the four glial cell types found in the brain. Astrocytes play a critical role in maintaining homeostasis to support healthy neuronal function by encasing regions of axons and whole synapses and acting as insulation and protection from the surrounding fluctuating ionic environment, allowing for different ionic conditions to exist in close proximity and also help maintain stable levels of extracellular potassium ions.

The vast coupling of astrocytes in the brain forms a glial syncytia, or network, allowing for uniformity of cytoplasmic composition, rapid signaling, and widespread distribution of ions and metabolites. Astrocytic elements with gap junctions tend to localize near neuronal structures, surrounding synapses, along the unmyelinated Nodes of Ranvier of axons, and near glomeruli; these gap junctions universally contain Cx43, although the Cx43 concentration in astrocytic gap junctions is not uniform and is more localized at points of close interaction between astrocytes and neuronal structures (Nagy and Rash, 2000). Most connexins are expressed in glial cells, implicating connexins as important players in proper maintenance and functioning of the glial network. Astrocytes are also important from their role in influencing the blood brain barrier (BBB) through the interactions between their end-feet projections and the capillaries of the BBB. Astrocytes respond to TBI by becoming reactive and undergoing astrogliosis, which ultimately leads to formation of a glial scar. Typically, astrocytic projections do not overlap with one another; however, reactive astrocytes increase their synthesis of glial fibrillary acidic protein (GFAP) – commonly used to stain astrocytes

in vitro – and vimentin, two intermediate filaments that help develop the cytoskeletal scaffolding needed for the projections to grow and extend outward, overlapping with neighboring astrocytes. The consequential glial scar formed provides a physical barrier to prevent further interaction between injured and healthy neurons, and a chemical barrier due to its growth-inhibiting secretions. This has both detrimental and beneficial effects. Formation of the glial scar is needed to help maintain and repair the integrity of the BBB; however, it prevents neurons from growing and regenerating properly. Axon projections attempt to extend only to be blocked by the network of overlapping astrocytes that form a homogenous barricade via their gap junctional connections. Cx43's interactions with cytoskeletal proteins and subunits affect the scaffolding and shape of the cell, as changes in cell shape have been observed following mTBI. In regard to cell arrangement, a monolayer of cells has been demonstrated to be more effective for neuroprotection and better for cell survival.

Currently, there is no effective therapeutic intervention available to allay the secondary injury associated with TBI. Current research seeks to understand the events involved in propagating this damage in hopes of finding a point in the cytotoxic cascade to target and interrupt the accumulation of damage. Many important facets of secondary injury have been described, but scarce research has been conducted on the role of gap junctional coupling in transmitting these damaging signals, given its apparent importance. There is a need for understanding the role of connexin components in the gap junctional communication associated with TBI; particularly, connexin-specific regulators need to be identified and connexin-specific contributions to TBI must be established. Additionally, the relative abundance of the different connexin isoforms in different regions of the brain must be quantified definitively and mapped out and relative biological function of each connexin isoform established, including the nature and consequences of Cx43's interactions with myriad proteins.

Among the research conducted on this topic, various studies have reported a damaging effect of Cx43-gap junctions through its spread of damage signals to otherwise healthy neighboring cells in the glial syncytium (Frantseva et al., 2002). Moreover, Cx43 deletion in astrocytes of mouse brain resulted in milder injury and less ATP release from damaged cells, a significant factor in secondary injury accumulation, indicating that Cx43 presence begets cellular damage. Tissues bordering spinal cord injury (SCI) sites demonstrated upregulated Cx43 (Huang, 2012). Notably, Cx43-knockout mice exhibited less severe reactive astrogliosis following TBI and failed to develop Cx43 plaques like their wild-type counterparts. This study concluded that gap junctions destructively spread damage signals following trauma (Huang et. al, 2012). Cx43-gap junctional channels are exceptionally permeable to ATP,

ADP, and AMP (Lampe & Lau, 2004), making them potential regulators of changes in cellular metabolism following injury.

Other studies have reported contrasting results supporting the neuroprotective roles of such channels. One study concluded that reactive astrogliosis was a neuroprotective process from their findings that ablation of reactive astrocytes following controlled cortical impact (CCI) trauma exacerbated injury, suggesting that its net effect was protective in mTBI through limiting inflammation and preventing damage to neural tissue. Interestingly, there was no net effect observed following severe trauma, indicating that there is a threshold for the usefulness of this possibly neuroprotective process (Myer et al., 2006).

Regardless of their contradictory findings, these studies tend to agree that gap junctional coupling has an elusive, yet seemingly critical role in attenuating or exacerbating neuronal apoptosis following mTBI due to an apparent, transient increase in gap junctional coupling following TBI (Frantseva et al., 2002). The notable correlation between changes in the phosphorylation of Cx43 and changes in gap junctional communication suggest that Cx43 has particular importance in the post-TBI gap junctional communication processes, and also makes sense due to its high permeability for ATP, ADP, and AMP. Phosphorylation mediates Cx43 after trauma, and Cx43 throughout normal brain tissue is overwhelmingly phosphorylated. Dephosphorylated Cx43 causes changes in gap junctional intercellular communication (GJIC) and reported changes in the conduction of the gap junction channel that may all be caused by increased neural activity. Given that over 90% of homologous astrocytic gap junctions contain Cx43 on both faces of the connection (Nagy & Rash, 2000), it is possible that homotypic Cx43-composed gap junctions are responsible for the patterns observed in gap junctional communication post-TBI, as an increase in levels of Cx43 is observed, accompanied by a structural modification that changes exposed epitopes. However, this change is not observed in cases of severe trauma and extensive neuronal damage; paradoxically, the Cx43 levels are actually decreased in these regions of severe damage. There is a need for understanding the relative levels of Cx43 and phosphorylated-Cx43 expressed in normal tissue and traumatized tissue, identifying specific channel blockers, and identifying the nature of the damage signals spread throughout the glial syncytium - IP3 a suggested candidate due to its alleged involvement in cell death as well as the increase in IP3-mediated signaling post-TBI (Frantseva et al., 2002).

Cx43 has the secondary classification of gap junction alpha-1 (Gja-1), also the name of the gene that encodes the Cx43 protein. Gja-1 refers to the similarities of Cx43 with other Cx protein sequences, and also indicates that Cx43 was the first alpha Cx discovered, explaining why it has the number one. Cx43 is known to have a short half- life, allowing for its expression to be tightly regulated and rapidly altered (Dbouk, et al.,

2009 and Lampe and Lau, 2004). Cx43 is a potential key player in secondary injury post- TBI, although its role is evidently unclear and highly debated due to its contradictory neuroprotective and also neurotoxic effects. Knockout mice have been used in multiple studies to assess the relationship between Cx43 and the extent of neuronal cell death.

GFAP-positive astrocytes were found to be more abundant in Cx43+/+ mice than Cx43+/- mice (Ohsumi, A. et al, 2000). These findings indicate that a deficit in Cx43 expression could possibly disrupt the accumulation of GFAP and somehow dampen reactive gliosis. Furthermore, the extent of neuronal cell damage was significantly reduced in Cx43 knockout mice (Franseva et al., 2002). The present study hypothesizes that the presence of Cx43 in secondary injury processes occurs early on in reactive astrocytes and is a precursor for the changes observed in cellular metabolites and interactions seen later in secondary injury. Since Cx43 directly associates with intermediate filament proteins, and has been shown to undergo phosphorylation changes post-TBI that ultimately have some effect on gap junctional communication (GJC) in the glial syncytium, it is likely that the structural changes and consequential functional changes that occur and rearrange Cx43's exposed epitopes allow for the upregulation of GFAP and vimentin proteins and consequentially usher in astrogliosis. Due to the relationship between the phosphorylation of Cx43 and its structural changes, as well as Cx43's interaction with intermediate filament proteins involved in astrogliosis, it is possible that changes in Cx43 phosphorylation states lead to the structural changes that occur in intermediate filaments of reactive astrocytes. Given the Catch-22 effects of glial scar formation and reactive astrocytes, it makes sense that changes in Cx43 would produce contradictory neuroprotective and neurotoxic effects experimentally.

The relative levels of Cx43 phosphorylation have been shown to have significant correlation with injury processes. Phosphorylation of Cx43 is conferred by the extracellular-signal regulated kinase (ERK) pathway, and this causes a significant reduction in the permeability of these gap junctions (Ohsumi, A, et al., 2010; Prochnow, N., 2011). Given that a relationship exists between the phosphorylated state of Cx43 (p-Cx43), the permeability of certain gap junctions, and post-injury processes, it seems that Cx43 is a key player in secondary injury. Moreover, experimentally induced expression of Cx43 provides neuroprotection, but apparently not through gap junctional communication, as this affect is still observed in the presence of gap junctional blockers (Butkevich, et al., 2004). Levels of p-Cx43 are in flux, with varying levels observable at different times post-injury and an apparent peak of p-Cx43 levels at approximately six hours post-injury (Ohsumi, et al., 2010). Astrocytes of the hippocampus demonstrated an increase in p-Cx43 following mTBI, which induced ERK-regulated

phosphorylation and led to an increase in Cx43 expression in these cells apparently facilitating GJC among astrocytes of the hippocampus (Ohsumi et al., 2004). Because the hippocampus is the region of the brain primarily responsible for formation and storage of memories, damage to the hippocampus results in learning and memory deficits. It seems that Cx43 expression following mTBI is highly conditional upon the cellular and environmental factors the cell is experiencing, demonstrating a dependency on time, location and the state of neighboring cell types. It may be that hemichannels composed of Cx43, as well as the gap junctional coupling that occurs among them, have both damaging and neuroprotective effects post-injury that dominate at different times and in different circumstances.

The debate over whether reactive astrocytes are damaging or neuroprotective is an argument between the "Good Samaritan" and the "Bystander Effect" hypotheses. There is neuronal cell loss observed after TBI, and also glial cell loss, including astrocytes; however, viable glial cells respond to this damage by overcompensating, leading to reactive gliosis. Cx43 is the connexin isoform most strongly associated with astrocytes and, in traumatized astrocytes, may allow for propagation of the harmful biochemical and messengers that lead to further secondary tissue damage in what is commonly referred to as the 'Bystander Effect,' 'kiss-of-death,' or cellular 'fratricide.' Cx43-composed gap junctions of astrocytes cause an observable increase in calcium overload, oxidative stress, and metabolic dysfunction (Ohsumi et al., 2004). Alternative to this destructive, damage- signal-propagation effect is the idea of the 'Good Samaritan,' a neuroprotective strategy possibly employed by the glial network in the spirit of 'share-the-load,' to prevent individually damaged cells from reaching a critical load of harmful cytotoxins by distributing these agents among otherwise healthy neighboring cells to prevent any one cell from becoming overwhelmed. The neuroprotective effect is supported by the contrasting observation of astrocytic Cx43-gap junctions as scavengers of damaging cytotoxic agents (Ohsumi et al., 2004). It is possible that the reactive astrocytes are both Good Samaritans and Bystander Killers at different points in the secondary injury process.

The present study investigates the role of Cx43 in the secondary incidence of mTBI to reveal a potential correlation between Cx43 expression and the biochemical events responsible for the secondary injury associated with mTBI in order to shed light on the role of this specific protein subunit and its importance as a therapeutic target. Supported by the work of Franseva, et al., which calls for connexin-specific assessment of impact on neuronal cell death possible through the use of specific anti- connexin antibodies, the present study assesses the extent of fluctuation in Cx43 expression in response to different treatments in astrocyte cell culture. Moreover, Frantseva, et al. maintain that the levels of Cx43 synthesized and expressed in normal subjects is

unestablished, identifying a need for this knowledge. The present study seeks to somewhat address this gap in knowledge via comparative analysis of Cx43 expression in normal hippocampal tissue samples from a Wistar rat (sham), Wistar rats subjected to moderate TBI and treated with saline (control), and Wistar rats subjected to moderate TBI and treated with gamma-glutamylcysteine ethyl ester (GCEE), an antioxidant. GCEE is an important antioxidant due to its ability to increase production of glutathione (GSH), which is the most prominent antioxidant in the body and helps to combat oxidative stress and reduce reactive oxygen species (ROS) that are largely responsible for oxidative stress. The structure of GCEE is depicted in Figure 16 and the structure of GSH is depicted in Figure 18. Comparative analysis will be conducted through quantification of Cx43 expression in the hippocampus through RNA extraction, reverse transcription, and subsequent qPCR analysis of twenty different hippocampal rat brain samples from three different experimental populations: sham, TBI + saline, and TBI + GCEE.

MATERIALS & METHODS

Expression levels for Cx43 in different regions of the hippocampus are quantified in the present through methods of RNA extraction, reverse transcription, and real-time PCR (qPCR), and treatments on cultured astrocytes alongside immunohistochemistry. RNA kit, and primers for qPCR were all purchased from Life Technologies (Carlsbad, CA).

RNA Extraction Protocol:

- 100mg per sample were individually weighed from brain tissue samples stored in the -80ºC and transferred on ice to separate 1.5mL tubes containing approximately 1mL each of RNAlater reagent (Life technologies, Carlsbad, CA) and stored for approximately 30 minutes.
- Brain tissue was removed from 1.5mL tubes and transferred to individual Lysing Matrix D tubes containing 1mL each of Trizol.
- The tissue was lysed in a FastPrep-24 machine (NP Biomedicals, Newport, CA) for six cycles at 45 seconds per cycle. In order to prevent degradation of the tissue from excessive heating, samples were iced for one minute between each cycle.
- The sample mixture was then pipetted from the Lysing matrix D tubes, without transferring any beads from the Lysing Matrix D tubes, to new 1.5mL tubes, which were subsequently shaken in an incubator at room temperature for approximately five minutes.
- Tubes were centrifuged at 12,000 x g at 4ºC for ten minutes to separate the layers
- Liquid-liquid extraction was performed, pipetting the upper

aqueous layer, without any material from the lower layer, into a new 1.5mL tube for each sample.
- Approximately 200µL of chloroform was added and subsequently shaken for fifteen seconds by hand, then incubated for 2-3 minutes at room temperature.
- Tubes were centrifuged again at 12,000 x g at 4ºC for fifteen minutes, and the upper aqueous layer was transferred into a new tube, avoiding the transfer of any material from the lower layers.
- Approximately 300µL of cold isopropanol (stored at approximately -20ºC for at least twenty minutes) was then added to each tube and inverted five times by hand and subsequently stored for thirty minutes at -20ºC.
- Tubes were again centrifuged at 12,000 x g at 4ºC for approximately ten minutes, and supernatant was pipetted off and discarded, leaving pellets which were subsequently washed with approximately 1mL of cold 75% ethanol (mixed one mL nuclease-free water for every three mL absolute ethanol).
- Small, white pellets were then vortexed back into solution and subsequently centrifuged at 7,500 x g at 4ºC for ten minutes.
- Ethanol was pipetted off and discarded and the pellet was subsequently air dried at room temperature for five minutes, although pellets were monitored to avoid complete desiccation.
- Pellet was then resuspended in 100µL of nuclease-free water and stored in the - 80ºC freezer for future use

NanoDrop Protocol:
- RNA samples were retrieved from the -80ºC freezer and thawed on ice to prevent degradation of RNA.
- Once thawed, RNA samples were analyzed using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). The NanoDrop's sensor was first cleaned by running a blank sample of nuclease-free water.
- Each sample was briefly vortexed before a drop was pipetted onto the NanoDrop's sensor, and analysis was run using the NanoDrop's software technology; the Nanodrop sensor was cleaned by wiping with a KimWipe between each sample run.
- NanoDrop analyses of all RNA samples' concentration was compiled (ng/µL) and purity was assessed (260/280 abs). A spreadsheet of these results was produced and checked to make sure samples were pure by looking at the 260/280 abs. A 260/280 sample readout with a ratio of two or less indicates a purer sample.
- RNA samples were returned to the -80ºC freezer after use and stored for later analysis.

Gel Protocol:

- FlashGel™ System (Lonza Rockland, Inc., Rockland ME) for RNA separation and analysis was used to verify the presence of RNA and not DNA, since the NanoDrop cannot make this distinction. The Flashgel™ System detects RNA quantities <10ng per band.
- RNA samples were removed from the -80ºC freezer and thawed on ice to prevent degradation from heat.
- Once thawed, each RNA sample was prepared in a separate tube with FlashGel™ Loading Dye (Lonza Rockland, Inc., Rockland ME 04841) so that each tube contained a 5µL load with a concentration of less than or equal to 200ng of RNA per band; for these wells, 2µL RNA were mixed by pipetting with 3µL Loading Dye. These prepared loads were stored on ice until ready for gel loading.
- Flashgel™ RNA Cassette was opened and flooded with nuclease-free water using a pipette. Water was subsequently absorbed with a KimWipe by tilting the cassette and allowing the water to drain out of the corner. Wells were not directly blotted or handled.
- Flashgel™ RNA Cassette was loaded onto dock as shown in Figure 5. Dock was subsequently connected to power supply with red and black cables.
- Once connected, power supply was turned on and voltage was set to 225mV. Top was snapped on to dock as shown in Figure 6, and gel was run for at least eight minutes without interruption.
- After eight minutes, voltage was turned off and leads were disconnected from power supply to remove cassette.
- Gel was then allowed to sit for approximately ten minutes and subsequently exposed and photographed, as shown in Figure 7.
- RNA was returned to the -80ºC freezer for storage until further use.

Reverse Transcription Reaction Protocol:

- RNA was removed from the -80ºC freezer and thawed on ice to prevent degradation.
- Reverse Transcription is used to convert the RNA samples into cDNA and was performed using a high-capacity cDNA kit (Applied Biosystems, Foster City, CA, Part No. 4368814); cDNA kit components were thawed on ice until ready for use.
- 2X RT Master Mix was prepared on ice, first by calculating the volume of each kit component needed for the required number of reactions, plus 10% of that number to account for any errors or spills during transfer. Calculations were done for 31 samples, so component volumes needed were calculated for 34.1 samples

in order to provide enough excess in case of error, based on initial sample size. Calculations were based by multiplying the sample number by the volumes listed in Table 1.
- The 2X Master Mix was mixed in a 1.5mL tube on ice then mixed gently via pipette
- 10µL 2X master mix was then pipetted into 31 individually, labeled 0.2mL PCR tubes for each reaction
- 10µL RNA sample was subsequently pipetted into corresponding tube and pipetted up and down two times gently to mix. Tubes were then sealed.
- 2mL tubes with their caps cut off were used as nests for centrifugation. The 0.2mL reaction tubes were place in the 2mL nested tubes centrifuge and centrifuged for approximately 15 seconds on pulse in order to eliminate any possible air bubbles.
- Tubes were placed on ice until ready for loading into the thermal cycler
- Thermal cycler was programmed according to Table 4, which is also depicted in Figure 8.

PCR Protocol:
- Real-Time PCR (qPCR) analyzes relative mRNA concentrations of target genes expressed in samples, reflecting expression levels of target genes. To run the reaction, Universal Master Mix (Applied Biosystems, Carlsbad, CA) is used, which includes Taqman primers and probes, as well as PCR reagents. The cDNA made by reverse transcription of extracted mRNA is used to run the reaction.
- Subsequently, reaction is run with both experimental assay gap junction alpha-1 (Gja1, 87 amplicon length, Life Technologies, Carlsbad, CA) as well as a control, glyceraldehyde-3-phosphate dehydrogenase (Gapdh, 174 amplicon length, Life Technologies, Carlsbad, CA), which should be equally expressed across all cells since it is a “housekeeping gene” responsible for standard cellular metabolism events. Half of the prepared reaction plate is experimental and half the prepared reaction plate is control, with each sample run twice (once on each side of the reaction plate)
- 1µL of 20X TaqMan gene expression assay solution, 10µL 2X gene expression master mix, and 8µL nuclease-free water were used per reaction well. Before beginning, the amount of each reagent needed was calculated based on the desired number of reactions and these volumes were subsequently mixed in two different reaction tubes, using Gapdh as the assay reagent for one of the tubes, and Gja1 for the other.
- Gja1 assay solution was used as the experimental assay, and

glyceraldehyde-3- phosphate dehydrogenase was used as a control.
- Assays were mixed by pipetting up and down twice and then vortexed briefly.
- 1µL cDNA sample was subsequently added to each designated well and a standard TaqMan PCR program was immediately performed.
- The ΔΔCT is run with desired number of well plates, "TaqMan reagents" and "standard" (2 hour) time.
- Plate layout must be assigned with "Plate set-up," then "define targets and samples." Samples are defined by highlighting wells and assigning them with either Gapdh control or with Gja1 experimental.
- The plate is lined up to reflect the plate layout already assigned, and then the run is started. The PCR reaction takes approximately two hours per reaction plate.

Astrocyte Cell Culture Protocol:

Astrocytes were stored in liquid nitrogen tank until ready for use to preserve viability (Invitrogen, Carlsbad, CA). Astrocytes were thawed in a 37º water bath for two minutes, swirling vial occasionally to ensure complete melting of ice. All live cell culture work was done under a flow hood to prevent contamination. UV-light in hood was turned on prior to beginning and allowed to warm-up. Astrocytic media was prepared (85% DMEM + 15% FBS) by removing 88.5mL DMEM from a 500mL DMEM bottle and replacing it with 88.5mL FBS (Invitrogen, Carlsbad, CA). Under the flow hood, the astrocytes were transferred from their vial to a new 15mL tube. The vial was then rinsed with 1mL of the prepared medium, dropwise, and this solution was subsequently added to the 15mL tube, making a total volume of 2mL of cells. 8mL of complete medium were then added to the tube to make a total of 10mL in the tube. This tube was centrifuged for five minutes at 250 x g and then the supernatant was decanted under the flow hood. 2mL medium were added to this 15mL tube and the astrocytes were resuspended by pipetting up and down. This solution was then pipetted onto a plate in a circular pattern, to ensure distribution of the cells and spread them out across the plate. Plated cells were observed under a microscope in order to ensure that they were spread evenly and not crowding each other. The plate containing the cells was then incubated at 37ºC, 5% CO2, 90% humidity. Media was replaced approximately every 4 -5 days and a cell viability (MTT) assay was done to test for cell stress. Four different populations of astrocytes were compared: normal/untreated, treatment to reduce Cx43 expression (octanol) (Pekny and Nilsson, 2005), hydrogen peroxide (H2O2) treatment to induce oxidative stress, and antioxidant treatment (750 uM GCEE). The effects of Cx43 on neuronal damage was

subsequently assessed through DCF and MTT assays (relative oxidative stress levels and cell viability, respectively) of all four astrocytic populations.

RESULTS

RNA extraction was performed on five separate occasions, as listed in Table 2. Extractions were among hippocampal samples with different treatments at different time points including sham, TBI + saline at 30 minutes, TBI + saline at 60 minutes, TBI + GCEE at 30 minutes, and TBI + GCEE at 60 minutes. The samples are listed according to treatment classification in Table 3. NanoDrop results indicated RNA samples extracted were pure and of good quality, having a 260/280 abs of approximately less than or equal to two, as depicted in Figure 1. All three FlashGels™ run indicated that uncontaminated RNA was present in all samples extracted, as shown in Figures 2, 3 and 4.

Extracted mRNA was successfully converted to cDNA through reverse transcription and subsequently analyzed through qPCR. In order to conserve use of resources, only twenty samples were run: six sham, six TBI + GCEE at 30, and eight TBI + saline at 30. Samples used are listed in Figures 13 and 14. Two qPCR plates were run and their corresponding amplification plots are depicted in Figures 9 and 10. A schematic of the two plates' set-up and the samples run can be seen in Figures 13 and 14. Fold-change calculations were done to determine the normalized gene expression of Gja1 amongst the three different treatment populations. Expression in the sham population was set to one, as a standard for comparison. The fold change of the saline-treated samples was 1.412 compared to the sham control. The fold change of the GCEE-treated samples was 0.903 compared to the sham control. A single-sided ANOVA statistical analysis was performed with the ΔCT values of the qPCR for each treatment, and is depicted in Figure 12. A single-sided ANOVA was practical for statistical analysis of these results because it compares variation with in situations where there are more than two treatment types, or populations. A single-sided ANOVA compares the variation between samples of one treatment type to the variation between the differences observed in the different treatment types and indicates whether or not there is a significant change observed between different treatment types relative to the variation between samples in one treatment type. Despite the trend observed, the results of the ANOVA indicated that this trend was not statistically significant.

Cell culture work was delayed because the cells died during a 13-day period of heavy snow that made the cells inaccessible and therefore media could not be replaced. This experiment is a future direction and will completed during the spring and summer 2015.

CONCLUSIONS/DISCUSSION

Despite the lack of statistical significance from the results of the qPCR research, a noteworthy trend does exist that should be investigated further, and the implications of such a trend are discussed here. Normalized fold-change of gene expression for GJA1 demonstrated an increase from sham levels in the mTBI with administered saline 30 minutes post-injury. However, this increased level of GJA-1 expression returned back to below sham levels for the mTBI with administered GCEE 30 minutes post-injury. If this trend is reflective of a statistically significant phenomena, these results would indicate that the expression of GJA-1, and consequentially the amount of Cx43 protein translated, correlates with oxidative stress. The mTBI with saline model indicates that there is more Cx43 protein translated when oxidative stress is introduced. With the addition of the GCEE antioxidant, which substantially relieves oxidative stress, there could be less of a need for Cx43, causing GJA1 to be less expressed. These observations would likely support the 'Good Samaritan' view of Cx43's role, as this oxidative-stress-dependent presence would likely be compensatory. The increased expression of GJA1 in the mTBI with saline treatment could support either the 'Good Samaritan' or 'Bystander Effect' contentions, but the decrease in GJA1 expression observed after administering antioxidant more likely lends itself to the 'Good Samaritan' hypothesis. A possible explanation for this phenomenon is the synthesis of the most prominent natural antioxidant employed by the body to combat oxidative stress: glutathione (GSH). GSH is composed of three amino acids, glutamate, glycine and cysteine. Because of the significantly lower concentration of cysteine in the brain as compared to glycine and glutamate, cysteine is the limiting factor for GSH synthesis (Drake, et al., 2002). GSH is available at greater levels in astrocytes than in neurons, and is the most pronounced way that astrocytes are able to protect neurons from oxidative stress. For co-cultured systems containing both neurons and astrocytes, the neurons were more susceptible to damage from oxidative stress if the astrocytes' gap junctional communication was inhibited (Sáez, 2003). Astrocytic protection of neurons is crippled when GSH levels are decreased in the astrocytes, but when GSH is available astrocytes are able to protect approximately twenty neurons per astrocyte against damage from oxidative stress, particularly from the reactive oxidative species , hydrogen peroxide (Dringen, et al., 2000). GSH synthesis requires an interplay between astrocytes and neurons. Cysteine availability limits GSH production in neurons alone and determines their GSH levels. However, when astrocytes are present, GSH levels increase in neurons, meaning that astrocytes provide and essential cysteine- precursor that the neurons are then able to use to fuel GSH production to combat oxidative stress. This nature of this precursor has been experimentally identified as CysGly

(Dringen et al., 2000). Because glutamine is released from astrocytes, and CysGly is produced extracellularly through the action of an astrocytic ectoenzyme, that reacts with GSH so that these components can be uptaken by neurons and subsequently used to produce GSH within the neurons, astrocytes furnish all three necessary ingredients for GSH production to neurons (Dringen, et al., 2000). This interaction is depicted in Figure 15. If the trend observed in the RNA work of this study is statistically significant, it could be explained by this phenomenon, as the Cx43 increase observed from TBI would aid diffusion of GSH, glutamine, and ATP out of the astrocyte at a greater rate through Cx43 hemichannels, allowing for the raw materials for GSH synthesis to be uptaken by the neurons. The decrease in Cx43 expression in the TBI with GCEE treatment administered that may be reflective of a significant trend would also make sense when considered in this context, because GCEE would provide the raw materials needed for neuronal uptake to fuel GSH production, bypassing the need for an increase in Cx43 hemichannels in astrocytes since the diffusion of these reactants out of astrocytes would be unnecessary since the reactants would already be provided by the GCEE.

The metabolism of glutathione is depicted in Figure 17. GSH synthesis requires ATP for two different steps, making ATP a limiting factor as well depending on the antioxidant demands of the brain. In a time of injury, when oxidative stress poses a serious threat to the health of the brain, GSH production increases in order to help preserve the brain's tissues and functioning. Studies have demonstrated that a major component of the release of ATP from astrocytes that occurs following injury is through opening of Cx43 hemichannels because ATP can diffuse through Cx43 hemichannels and Cx43-composed gap junctions (Huang, et al., 2012). In this way, a plausible trend for GJA1 upregulation could be reflective of the brain's attempt at self-preservation by increasing the amount of Cx43 so that Cx43 hemichannels or gap junctions can be constructed to allow for the more rapid and widespread distribution of ATP throughout astrocytes as well as increased ability for astrocytes to provide the necessary reactants to neurons to facilitate neuronal production of GSH and prevent neuronal damage from oxidative stress. Cx43 hemichannels do not preferentially allow the passage of GSH, but would help increase the rate of GSH synthesis in neurons by providing the raw reactant needed. Additionally, the increased ATP release through Cx43 gap junctions and hemichannels affects the formation of astrocytic stress fibers, as well as the morphological changes that occur post-trauma, like cellular flattening (Lin, et al., 2003), which helps facilitate reactive astrogliosis processes.

Aside from facilitating passage of signaling molecules, secondary messengers, ATP, and ions, gap junctions also allow for the exchange of small interfering RNA (siRNA) between cells (Dbouk, et al., 2009), which

could allow it to regulate gene expression for certain proteins. Moreover, Cx43 has been postulated as a regulator of gene expression through recruitment of transcription factors (Giepmans,B. N G., 2004). Therefore, the effects of Cx43 on gene expression of both protective and damaging factors should be investigated further.

In the future, this research should be re-done with a greater sample size per treatment type in order to determine whether or not the observed trend is statistically significant. Moreover, it would be helpful to investigate any possible relationship between p-Cx43 and treatment type. Phosphorylation is a means of Cx43 regulation (Lampe and Lau, 2004) and p-Cx43 is mostly concentrated in brain tissue six-hours post injury (Ohsumi, et al., 2010), indicating that the phosphorylation state of Cx43 plays a role in post-traumatic processes, and it would be interesting to investigate whether or not reducing Cx43 expression or administering an antioxidant (i.e. GCEE) would have any effect on the ratio of unphosphorylated-Cx43 and p-Cx43. Another future direction of this research would be to assess the possible structural changes of the astrocyte projections resulting from intermediate filament activity under different treatment conditions, and observe any potential influence of Cx43 on intermediate filament behavior or morphology to ultimately assess if Cx43 plays a role in the morphological changes of astrocytes during reactive astrogliosis that lead to glial scar formation and plaques. Astrocytes upregulate their intermediate filament production following trauma, facilitating the process of reactive astrogliosis and have been a strong reason some researchers consider astrocytes to be predominantly protective following injury (Pekny and Nilsson, 2005; Dbouk et al., 2009). Moreover, mice with a predisposition to lower levels of Cx43 expression were demonstrated to have less rapid upregulation of GFAP following trauma, slowing the process of reactive astrogliosis (Ohsumi, et al. 2010).

Additionally, drebrin (developmentally-regulated brain protein) – a protein that binds to actin, an important intermediate filament - is a newly-recognized binding partner of Cx43's carboxy-terminal tail and has been implicated as a key player for maintenance of gap junctional communication, responding to changes in cellular signaling, drebrin-deficient astrocytes had lower levels of p-Cx43 and caused a decline in the permeability of gap junctions (Butkevich, et al., 2004). Because Cx43 has direct interactions with multiple intermediate filaments, which is related to the phosphorylation state of Cx43, it would be good to research this relationship's response to different treatment types including one that induces oxidative stress (i.e. H2O2) and one that reduces Cx43 expression (i.e. octanol).

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