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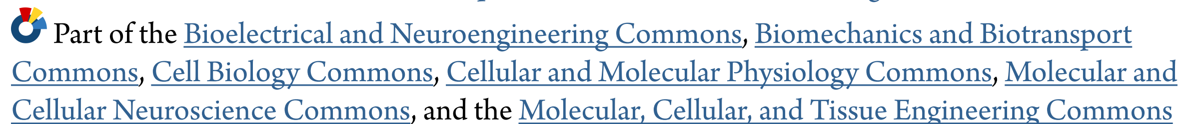
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Role of TRPV4 in Astrocyte Extracellular Matrix Production

Abby Terlouw

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May 2017

1. Introduction

1.1 Traumatic Brain Injury

Traumatic Brain Injury (TBI) is an alteration of brain pathology following damage of the central nervous system (CNS) by an external force [1]. In the United States, approximately 2.8 million Americans sustain a TBI annually with numbers increasing over the last several years. Elderly (≥75 years), children (0-4 years) and young adults (15-24 years) account for the majority of TBI Emergency department visits, hospitalizations, and deaths in the U.S. [2]. Particularly concerning, TBI remains the leading cause of death among infants and children [3-4]. With the prevalence of TBI increasing, research into the mechanics of tissue degeneration and preservation following TBI is warranted. Through increased understanding of cellular response to TBI, targeted therapies and treatment strategies to promote neural function regeneration can be explored.

1.2 The Roles of Astrocytes

In the central nervous system (CNS), glial scar formation often occurs following TBI, and astrocytes are widely believed to contribute to this scar formation. The role of glial scar formation in TBI is not completely understood. However, it is widely believed it performs both positive and negative functions. The barrier created by the glial scar walls off damaged tissue to limit cellular inflammation and degeneration. A secondary effect of this barrier formation is

blocking of axonal regeneration of the area preventing functional regeneration [5-6]. Following TBI and other CNS insults, astrocytes are known to react in a process known as reactive gliosis. Astrocytes are a specialized structure and support cells found in both injured and uninjured CNS responsible for many essential, non-neurotransmission functions [7-8]. Of particular interest to this research is the role astrocytes play in extracellular matrix (ECM) production. While the exact composition of ECM varies among tissue types and hosts, it is generally composed of protein fibers including collagen, glycoproteins, and proteoglycans in a hydrated gel (8). During reactive gliosis, astrocytes congregate around the damaged area and ECM composition is altered (Figure 1) [6]. While the role of astrocytes in ECM production is known, the exact mechanism(s) for this event remain unclear.

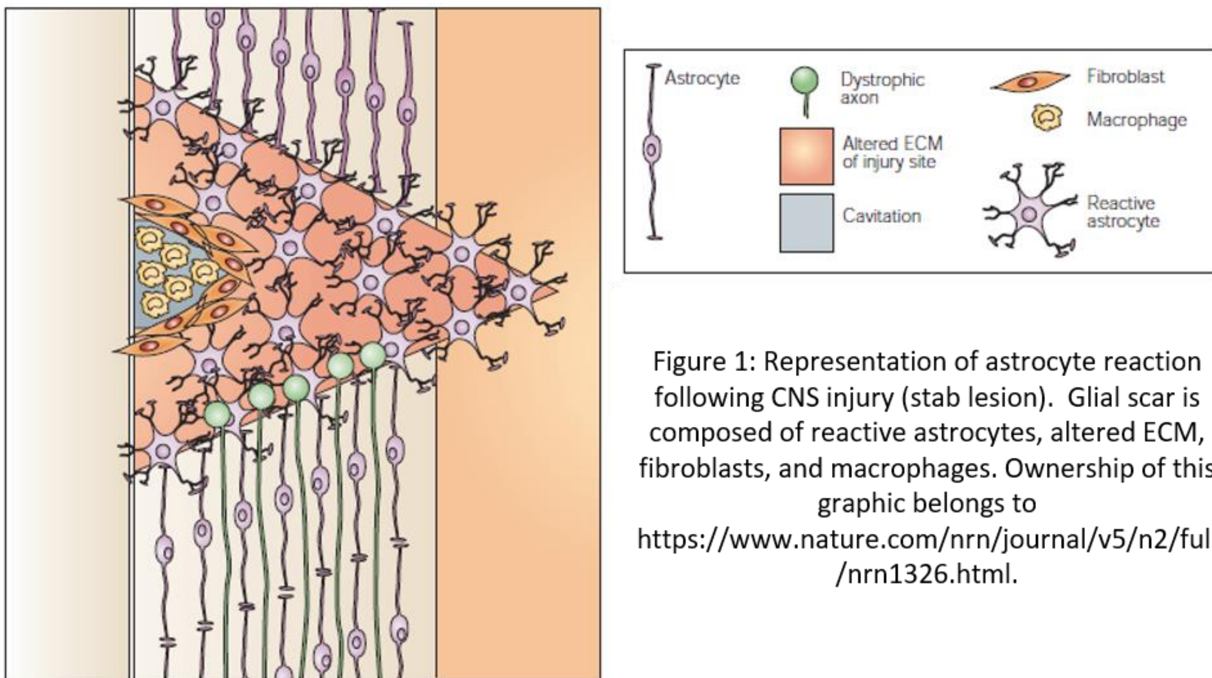

Figure 1: Representation of astrocyte reaction following CNS injury (stab lesion). Glial scar is composed of reactive astrocytes, altered ECM, fibroblasts, and macrophages. Ownership of this graphic belongs to https://www.nature.com/nrn/journal/v5/n2/full/nrn1326.html.

1.3 Transient Receptor Potential Vanilloid 4

One possible component to this mechanism is the activation of transient receptor potential vanilloid 4 (TRPV4). TRPV4 is a non-specific ion channel permeable to calcium ions, and is found in a diverse array of tissues throughout the body. TRPV4 activation is achieved through a variety of stimuli including osmolality and mechanotransduction among others [9-12]. O'Conor et al has shown activation of TRPV4 to increase collagen content in chondrocytes following mechanical loading [10]. Similar mechanical stimuli in TBI may result in similar TRPV4 activation in astrocytes and subsequent increase in collagen levels in glial scar formation. GSK1016790A, a known TRPV4 agonist, and GSK 205, a known TRPV4 antagonist offer one method for TRPV4 activation/inactivation research [10-13]. As a potential activator of astrocyte ECM production, TRPV4 represents a possible link between TBI and glial scar formation. The goal of this study was to compare mouse astrocyte matrix composition and mechanical properties following TRPV4 biochemical activation/inactivation through rheological analysis techniques.

2. Materials and Methods

2.1 Cell Culture

Frozen vials of C8-D1A mouse astrocyte clones were thawed in a 37˚C water bath and added to 7 mL of warmed DMEM supplemented media in a T75 flask. DMEM media was

supplemented with 10% FBS, 1% L-Glutamine, and 1% Penicillin/Streptomycin throughout experiment. Media was replaced every 48 hours with fresh media until 80-90 percent confluency was reached. Cells were then split into two T175 flasks. Media was aspirated, 7 mL trypsin EDTA was added, and flask was incubated until cell detachment was observed. Media (7 mL) was added to neutralized trypsin, and the resulting 14 mL cell solution was collected and centrifuged at 300 G for 10 minutes. Supernatant was aspirated and cell pellet was resuspended in 4 mL media before being evenly distributed between two T175 flasks containing 12 mL warmed media. This process was continued until confluence was reached.

2.2 Gel Mold and Well Preparation

Gel molds were fabricated from 1/8" thick high temperature super-soft silicone rubber sheet (McMaster-Carr) and 22 mm diameter glass coverslips (VWR). Silicone sheeting was cut into circular mold rings with 3/4" outer diameter and 3/8" inner diameter. Silicone rings and coverslips were washed and sonicated in 70% ethanol for 20 minutes. Following sonication, coverslips were gently pressed onto silicone rings forming the molds (Figure 2). Completed molds were then sterilized in an autoclave. All molds were placed coverslip side down into sterile transwell, 6 well plates prior to cell seeding.

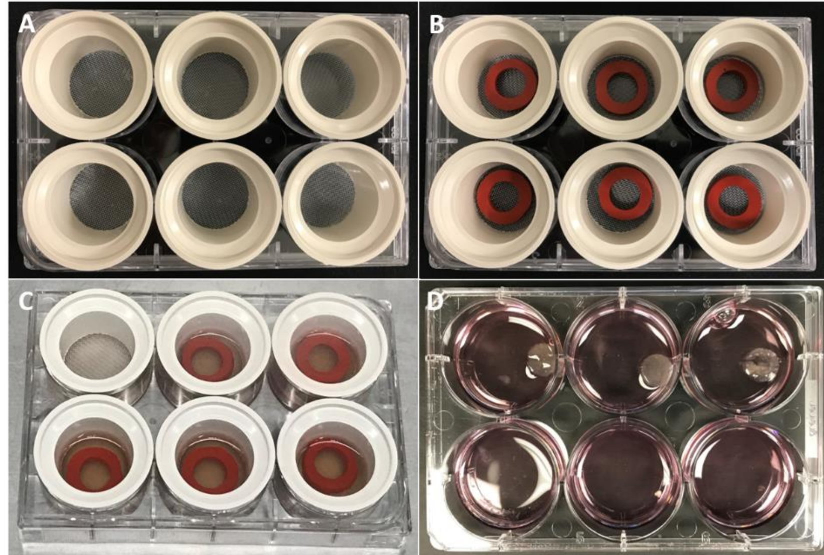

Figure 2: Transwell plates (A) containing silicone molds (B) used to house, grow, and transfer mouse astrocyte gels over the course of experiment. Gels were submerged in supplemented media (C) throughout experiment. Following experiment, gels were removed from molds (D). Gels present only in first three wells.

Confluent cells were removed from T175 flasks as previously described in 2.1 and counted using a hemocytometer. Following counting, desired cell volume was centrifuged at 300 G for 10 minutes, media was aspirated, and cells were resuspended in cold Matrigel (Corning). Cell/Matrigel suspension was seeded into molds at a concentration of 500,000 cells per mold (300 µL Matrigel). Plates were incubated at 37˚C for 30 minutes until gelation occurred. 5 mL media was then added to each well ensuring molds were completely submerged. Gels were incubated for 48 hours prior to first treatment. Media was replaced every 48 hours until experiment completion.

2.3 Astrocyte Sample Treatment

Astrocyte seeded gels were exposed of to one of three treatments – no treatment control, TRPV4 agonist, or TRPV4 antagonist – over the course of three weeks with six gels per treatment group. Control treatment media contained normal supplements with added trace amounts of DMSO consistent with DMSO concentrations in alternate treatments. TRPV4 agonist media contained 1µM GSK1016790A (Sigma-Aldrich). TRPV4 antagonist media contained 100µM GSK205 (EMD Millipore). Treatments were diluted to desired concentration through a series of dilution steps. Treatment media was added to six well plates and transwells containing astrocyte seeded gels were transferred to wells containing respective treatment media. Gels were submerged and incubated for 30 minutes at which time transwells and gels were returned to original plate containing fresh, non-treated media. This process was repeated every 48 hours over the course of three weeks.

2.4 Astrocyte Viability Testing

Cell viability was testing using Molecular Probes™ Invitrogen detection technologies Live/Dead® Viability/Cytotoxicity Kit for mammalian cells. Reagent stock solutions were warmed to room temperature. 20 µL EthD-1 stock solution was added to 10 µL sterile PBS and vortexed to ensure proper mixing. 5 µL calcien-AM stock solution was added to the 10 µL EthD-1 solution and vortexed to ensure proper mixing. Astrocyte seeded gels in molds were prepared prior to the assay with two rinses of sterile PBS. 150 µL prepared reagent solution was added to prepared

cells and incubated, while covered, at room temperature for 45 minutes. One gel per treatment group was tested. Following incubation, cover slips were placed over top of molds, and cells were visualized and imaged under fluorescence microscope at 4X magnification.

2.5 Gel Rheological Testing

Following treatments, gel mechanical properties were tested using a Discovery HR-2 rheometer with 10 mm parallel plate attachments (Figure 3A). Gels were removed from molds just prior to treatment by first removing the coverslip then excising gels from silicone molds using a 15-mm biopsy punch. The rheometer inertia, friction, and rotational mapping were calibrated. Gels were then positioned on the bottom rheometer plate warmed to 37˚C, and the plate gap was set to 1500 µm. This gap and temperature were used for all samples. Drops of 1X PBS solution were placed around sample to prevent dehydration during testing (Figure 3B). A frequency sweep from 0.1 - 100 hertz was performed on a log scale with five points per decade and at a strain of 10%. Frequency, storage modulus, and loss modulus data was collected for each sample. Rheometer plates were cleaned with 70% ethanol and allowed to dry between tests. After test completion, each sample was stored in a 1.5 mL microcentrifuge tube at -80˚C for additional testing out of the scope of this project.

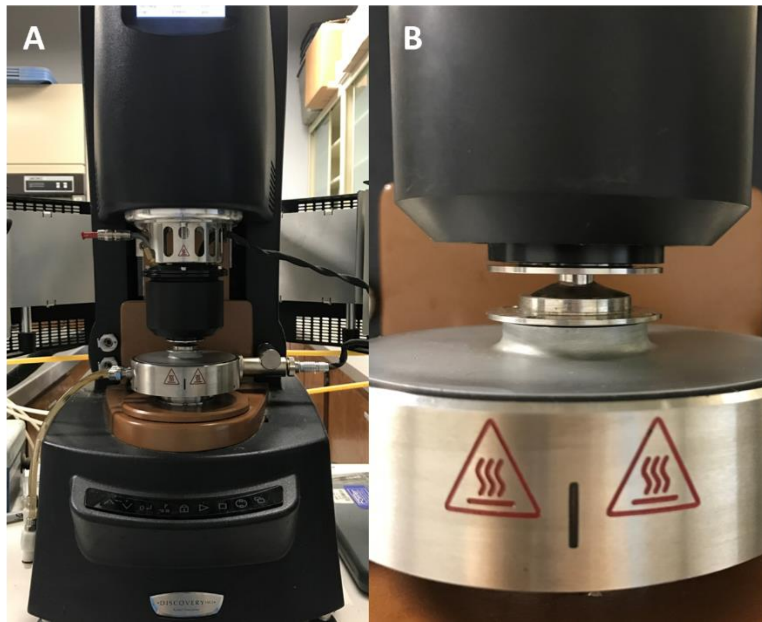

Figure 3: Discovery HR-2 rheometer with 10 mm parallel plate attachments and heated base (A), and gel sample between 1500 µm plate gap surrounded by 1X PBS (B).

2.6 Statistical Analysis

Treatment groups were compared using a oneway ANOVA and Tukey-Kramer HSD. Acceptance criteria for statistical significance required p values less than 0.05. Storage modulus (G') and loss modulus (G'') data at 1 hertz were used for comparison as this frequency most closely resembles physiological frequencies. All graphs represent mean values with standard error bars.

3. Results

Cell viability testing was performed prior to rheological testing to ensure cells had survived during three-week treatment period. Each treatment group sample showed similar levels of viable cell density (qualitative) with no staining of dead cells visible (Figure 4). Cells appeared to be dispersed across all planes within the gel.

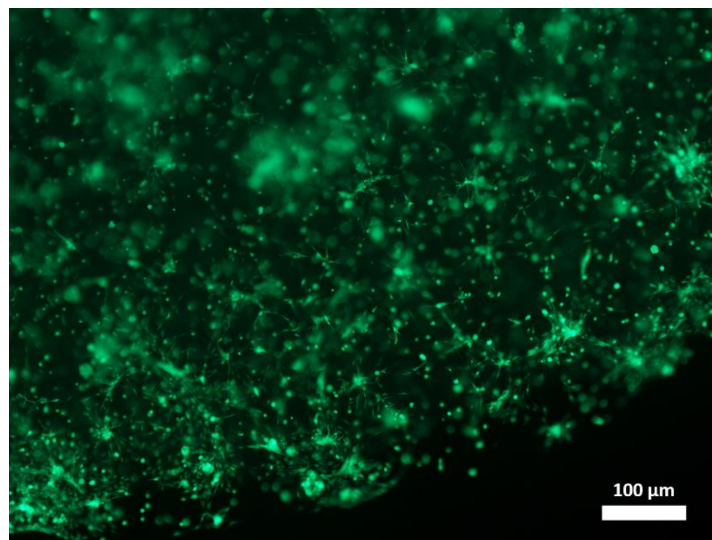

Figure 4: EthD-1 staining of astrocyte seeded gel post treatments at 4X magnification. Dark region (lower left) corresponds to inner edge of silicone mold. Calcien-AM staining (not imaged) was negligible.

Rheological testing data showed exponential increased in storage modulus with increased frequency with similar trends across all treatment groups. Loss modulus showed no discernable trend across frequency sweep (Figure 5).

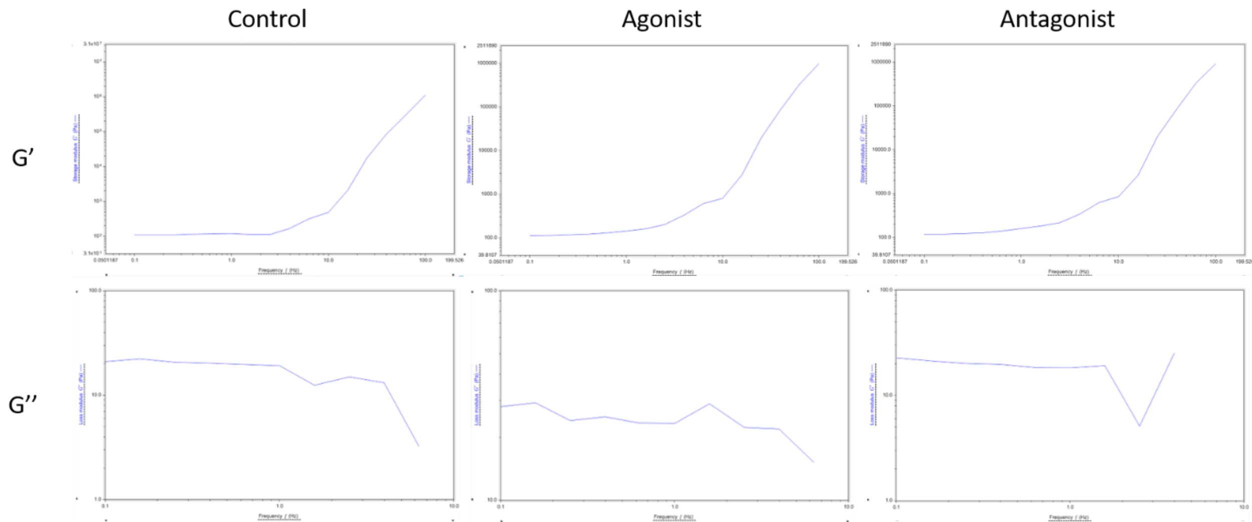

Figure 5: Sample representation of storage modulus and loss modulus frequency sweeps from 0.1-100 hertz for control, agonist, and antagonist treatments

At one hertz, storage modulus mean values of control, agonist, and antagonist treatment groups were 147.503 Pa, 184.468 Pa, and 176.845 Pa respectively. Agonist storage modulus was significantly higher as compared to control (p=0.0422). While not statistically significant (p=0.1139), antagonist treatment showed an increase in storage modulus as compared to control, as well (Figure 6A). Loss modulus mean values of control, agonist, and antagonist treatment groups at one hertz were 17.686 Pa, 22.685 Pa, and 19.108 Pa respectively. Loss modulus data was found to not be statistically different across all groups (Figure 6B)

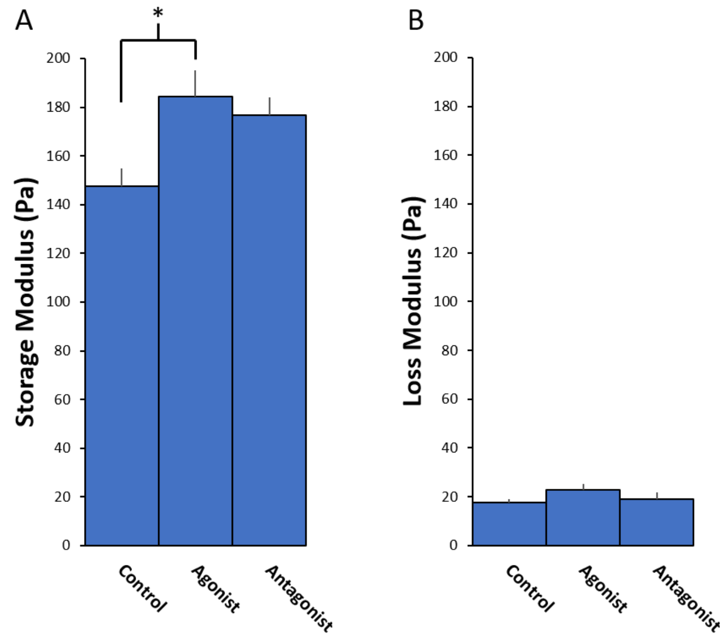

Figure 6: Mean storage modulus (A) and loss modulus values for control, agonist, and antagonist samples at 1 Hz in parallel plate rheometer frequency sweep. Storage modulus was found to have increase significantly (*) in agonist treated samples as compared to control.

4. Discussion and Conclusion

Matrigel in silicone and glass molds provided a suitable growth environment for mouse astrocytes throughout treatments as demonstrated by negligible cell death after three weeks. Further cell quantification methods are required to determine magnitude of cell growth during this period. Previous research has confirmed TRPV4 gene expression in these cells.

Storage modulus data corresponds to elastic properties of the samples while loss modulus data corresponds to viscous properties of the samples. Storage modulus data was significantly higher than loss modulus data showing the astrocyte seeded gels possessed mostly elastic properties. When the agonist protein, 10 µM GSK 101, was introduced, treated samples displayed

a higher mean storage modulus value as compared to control samples. There are many possible sources for this change, however one explanation would be an increase in collagen deposition as collagen displays primarily elastic properties [11]. This would suggest that activation of TRPV4 plays a role in astrocyte ECM production, specifically collagen production. The increase in collagen levels will be confirmed in subsequent research including hydroxyproline assay studies of samples. As the mean value storage modulus data also increased for the antagonist treatment group, although not significantly, there likely exists a more complex relationship between TRPV4 activation and astrocyte ECM production that warrants further studies and analysis.

5. Acknowledgements and References

5.1 Acknowledgements

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