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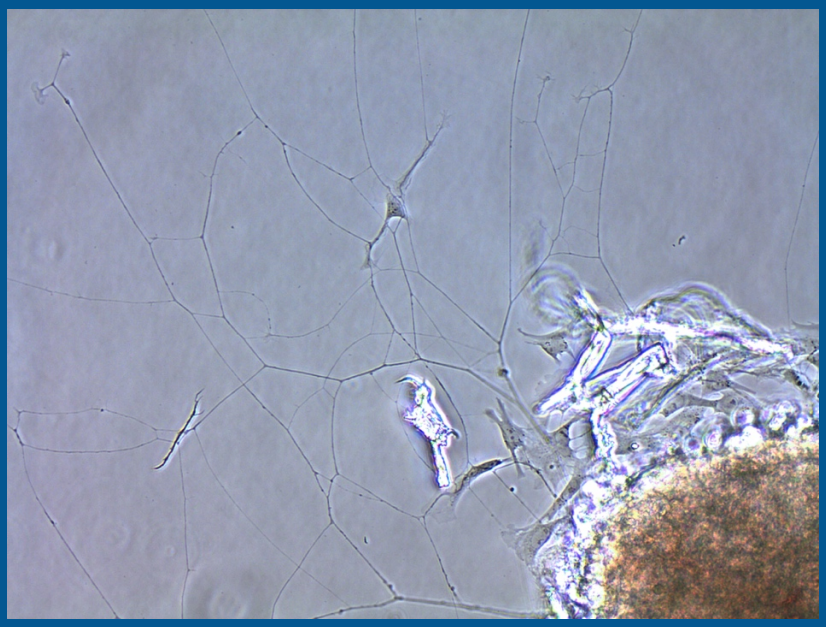

The effects of increasing mechanical pressure on *Gallus gallus* sympathetic neurons as measured by abundance of varicosities formed

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Introduction

Traumatic brain injury (TBI) occurs when a sudden blow to the head results in damage to the brain (Thurman et al., 1999). Each year approximately 2 million Americans suffer a TBI, most commonly caused by motor vehicle crashes, violence, or falls (Smith & Meaney, 2000). The residual effects of a TBI can cause cognitive, emotional, and motor impairments, as well as the financial burden of recovery (Thurman et al., 1999).

Over the last century, scientists have recognized diffuse axonal injury (DAI) as a key element of TBI (Johnson, Stewart, Smith 2013). DAI transpires when neuronal axons over a widespread area are sheared or disconnected by an extreme brain movement inside the skull, leading to the disruption of axonal transport. One major sign of DAI, is the formation of axonal varicosities (Johnson, Stewart, Smith, 2013). When neurons experience a high force of mechanical stress, a channel protein TRPV4 activates an inflow of calcium ions to the cell (Gu et al., 2017). In response to the calcium ions invading the cell a microtubule-binding protein, STOP, is inhibited. The hindrance of the STOP protein results in the destabilization of microtubules thus, disrupting axonal transport (Gu et al., 2017). This sudden disruption in transport leads to clumps or swellings of cellular materials along the axon; these swellings are referred to as varicosities (Gu et al., 2017).

Due to the formation of axonal varicosities, voltage spikes encoded with information may be delayed, redirected, or deleted, decreasing neural functionality (Maia & Kutz, 2014). A study by Tang-Schomer et al. showed evidence for partial interruption of axonal transport by finding dense accumulations of transporter proteins within the varicosities (2012). Voltage spikes travel fastest in axons with large diameters, therefore the presence of obstacles may result in misclassification of encoded information (Maia & Kutz, 2014). Errors in neuronal functionality, due to the presence of varicosities, are directly correlated to behavioral and cognitive deficits.

Considering the growing incidence of TBIs, highly concurrent with vehicle crashes, contact sports, and active military personnel, it is important to further investigate the formation of axonal varicosities. A mild TBI may result in a brief state of unconsciousness whereas a more severe TBI may lead to a long-term change in mental state (Maia & Kutz, 2014). To investigate varicosity differences in the severity of a TBI, the current study aims to mechanically stress *Gallus gallus* sympathetic neurons from 10-day old chick embryos at different pressures. In a study by Gu et al., the formation of axonal varicosities were reliably induced at a pressure of 190

mmH2O gravity (2017). An approximation of this pressure will serve as the low experimental condition; Gu et al. reported that no varicosities were seen in axons bearing pressures lower than 190 mmH2O (2017). Medium and high experimental conditions will undergo higher gravitational pressures of mechanical force, an experiment that has yet to be reviewed in the literature. The findings of this study have to potential to reveal the neuronal effects associated with various severities of traumatic force. In the current study, it is hypothesized that neurons mechanically stressed at higher pressures will develop a higher abundance of varicosities compared to the control neurons untreated by mechanical stress.

Materials and Methods

Primary Culture

In this experiment, to prepare for dissection, coverslips were cleaned thoroughly by following *Preparation Day Steps in the Primary Culture of Chick Embryonic Peripheral Neurons 1: Dissection* protocol (Morris, 2015). Dissection of 10-day old chick embryos to culture sympathetic nerve chains and dorsal root ganglia were conducted by following the guidelines of *Preparation Day Steps in the Primary Culture of Chick Embryonic Peripheral Neurons 1: Dissection* protocol (Morris, 2015). Cleaned coverslips were treated with polylysine and laminin during dissections, and Hanks Balanced Salt Solution (HBSS) was used as the dissection medium. For this experiment, mechanical stress was applied to dorsal root ganglia plated on coverslips.

Preparing Pasteur Pipettes

For this experiment, 9" glass Pasteur pipettes were contracted with a flame via Bunsen burner and stretched to construct a tip diameter of ~ 50 μm. The tip diameter measurement was derived from a study by Gu et al., in which varicosity initiation occurred (2017). Using a dissection scope and a stage micrometer, the inner diameter of pipette tips were measured to be ~ 50 μm and the outer diameter was measured as ~ 75 μm. Glass pipettes were placed in a stainless-steel box to autoclave prior to the experiment. Extra pipettes were created as insurance. Pipettes were shared with collaborators Emma Paolella, Morgan Karnes, and Laura Scheidemantel.

Mechanical Pressure Apparatus

The mechanical pressure apparatus was constructed by attaching an autoclaved ~ 50 μm pipette to a 20 mL syringe via Tygon S3 tubing (Gu et al. 2017). A seal was created to connect the syringe to the Tygon tubing by a 10mm cut of aquarium tubing inside the syringe end of the Tygon tubing. Tubing connected to the syringe was thoroughly rinsed with ethanol, and dried by flowing sterile, filtered air through the apparatus. Sterilization was underway 4 hours prior to beginning the experiment. A portable magnetic table was the base of our apparatus. One magnetic ring stand held the syringe with a clamp, another magnetic ring stand possessed a micromanipulator which held the pipette 0.4mm above the coverslip (Gu et al., 2017). These micromanipulator measurements remained constant through all experiments, (y)= .22 cm, (x)= 3.33 cm, (z)= 0.2cm. The syringe cap was used as a valve to control the flow of HBSS. Apparatus was constructed and shared with collaborators Emma Paolella, Morgan Karnes, and Laura Scheidemantel.

Measurement of Mechanical Pressure

For this experiment, ~ 20 mL of HBSS was warmed to 37°C and poured into the apparatus via syringe with the pipette tip facing up. To control the flow, syringe was re-capped and pipette tip was secured into micromanipulator at the correct measurements. Gravitational pressure was identified by the distance (mm) from the pipette tip to the meniscus of HBSS in the syringe. To increase the mechanical pressure, distance between the two components was increased.

Experimental and Control

For these experiments, three primary tissue cultures of plated ganglia were attained for two control and three experimental condition groups. All cultures were plated in growth medium on the same day and stored in an incubator at 37°C. This study was carried out over the course of two days. There was two control groups of untreated neurons; one culture was imaged 24 hours after plating before the low condition pressure was applied and the second culture was imaged 48 hours after plating before the medium and high condition pressures were applied. The low condition of applied pressure was measured as 190 mmH2O gravity, recommended by a previous study to induce the formation of varicosities (Gu et al., 2017). Mechanical pressure was exerted on the low condition ganglia 24 hours after being plated. The medium condition pressure was measured as 223 mmH2O gravity, a 33mm increase in pressure from the low group. The high condition pressure was measured as 278 mmH2O gravity, a 55mm increase from the low group. Mechanical force was applied to high and medium condition cultures 48 hours after being plated. Micromanipulator measurements remained constant for all conditions as gravity pulled HBSS through the sterile pipette onto ganglia. Flow of HBSS was continuous for ~ 1 minute within each condition. Three trials were conducted following this protocol. The application of mechanical pressure on low condition ganglia was performed with Emma Paolella, Morgan Karnes, and Laura Scheidemantel. Experimental group trials were completed independently.

Microscopy and Imaging

A Nikon Eclipse TS100 Inverted Phase Contrast microscope was used on 40X, phase 2 magnification to image a specific ganglion prior to and after the application of mechanical pressure. The Idea Camera, model #27.2-3.1MP, acquired all images and connected to the SPOT software 5.3 on an Apple iMac computer, so that images were displayed on the desktop. During the application of mechanical pressure onto cells, a Nikon Dissection Stereo microscope, with an Idea Camera attached, was used to visualize the location of flow above the ganglion, and to confirm the stable flow of HBSS. The camera was connected to an Apple iMac computer and images were viewed through SPOT.

For each group, ganglia of interest was focused on and images of various axons around the perimeter of the ganglia were collected. During imaging, all dishes were kept at 37°C with an electrical space heater and the temperature was monitored by a digital thermometer taped to the stage of the microscope. All images were taken in the ICUC lab and 1145 lab at Wheaton College, MA and saved to ICUC server.

Image Analysis

For the purpose of quantification, one image was selected from each of the groups to count the abundance of varicosities. The images chosen for analysis had the highest

concentration of axons in their group after applied mechanical pressure. For analysis consistency, an axon was defined as a uniform caliber extension off ganglia and a varicosity was defined as a swelling 150% the width of its adjacent axon width. Image analysis was conducted using ImageJ version 1.52a. Varicosities were analyzed and counted per axon length, as well as combined for a total abundance count for the image. For each axon a ratio was computed and the number of varicosities per micron was calculated. All ratios for each group were averaged together for a single data value. The number of axons as well as the average axon length for each group was also computed. All measurements were converted to microns at the proper 40x magnification scale using a conversion factor. Axons or swellings that appeared indistinguishable, or regions with many interconnected areas were not used for analysis.

Results

The minimal presence of axonal varicosities can be observed in both control group images, as seen in figure 1. Comparatively, the increased presence of varicosities is clearly detected in all experimental groups that experienced applied mechanical force, shown in figure 2. Using the measurement of average number of varicosities per 1μm, abundance of varicosities was measured in both controls and low, medium, and high-pressure conditions. Figure 5 yields as the pressure exerted onto neurons is increased, so is the abundance of varicosities. A one-way ANOVA statistical test was conducted to determine statistical significance between groups. The test revealed that there was a significant difference between abundance of varicosities within groups based on the pressure exerted.

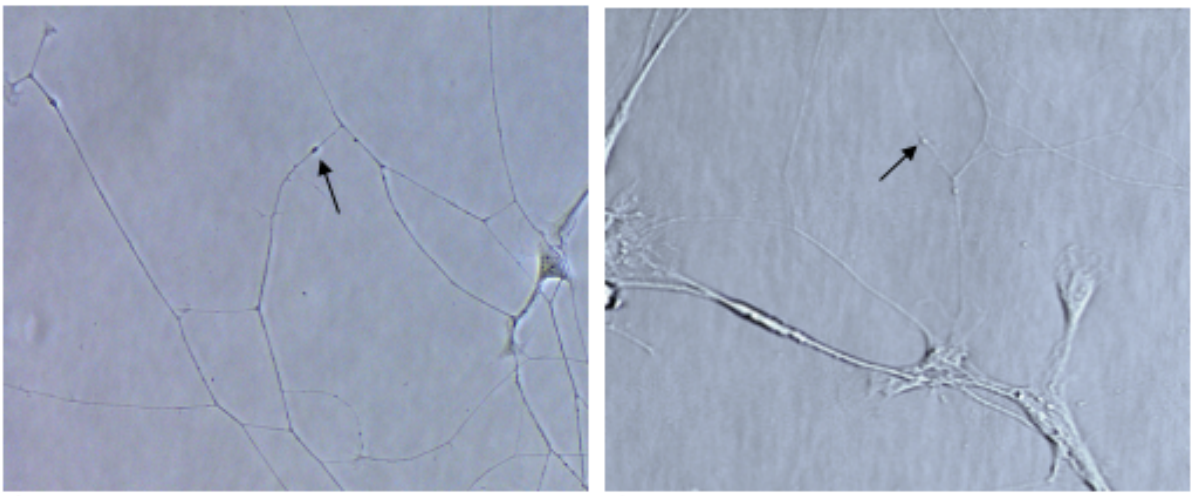

Figure 1: Axonal varicosities in *Gallus gallus* neurons untreated with mechanical force. **a)** This 40x transmitted light image was taken 24 hours after plating prior to application of force, represents control 1 condition. **b)** This 40x transmitted light image was taken 48 hours after plating before force was applied, represents control 2 condition. Varicosities are indicated by the black arrows. Image **a** was generated in collaboration with Emma Paolella, Morgan Karnes, and Laura Schiedemantel. The above images are 140μm.

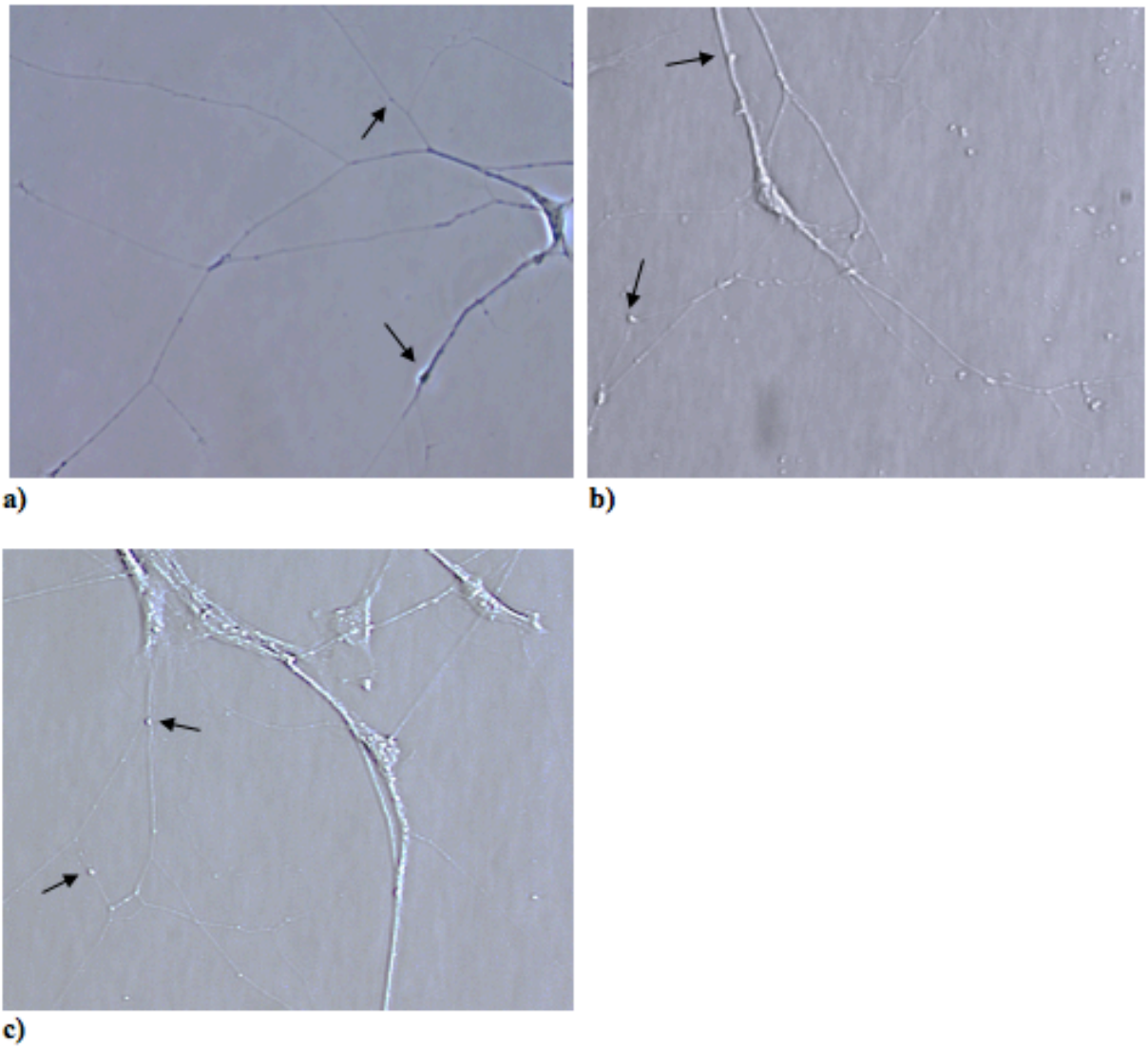

Figure 2: 40x transmitted light images of axonal varicosities in *Gallus gallus* neurons treated by the application of increasing mechanical pressures. a) The presence of varicosities following the application of low gravitational pressure. **b)** The presence of varicosities following application of the medium gravitational pressure. **c)** The presence of varicosities following the application of a high gravitational pressure. All varicosities are indicted by the black arrows. Image **a** was generated in collaboration with Emma Paolella, Morgan Karnes, and Laura Schiedemantel.

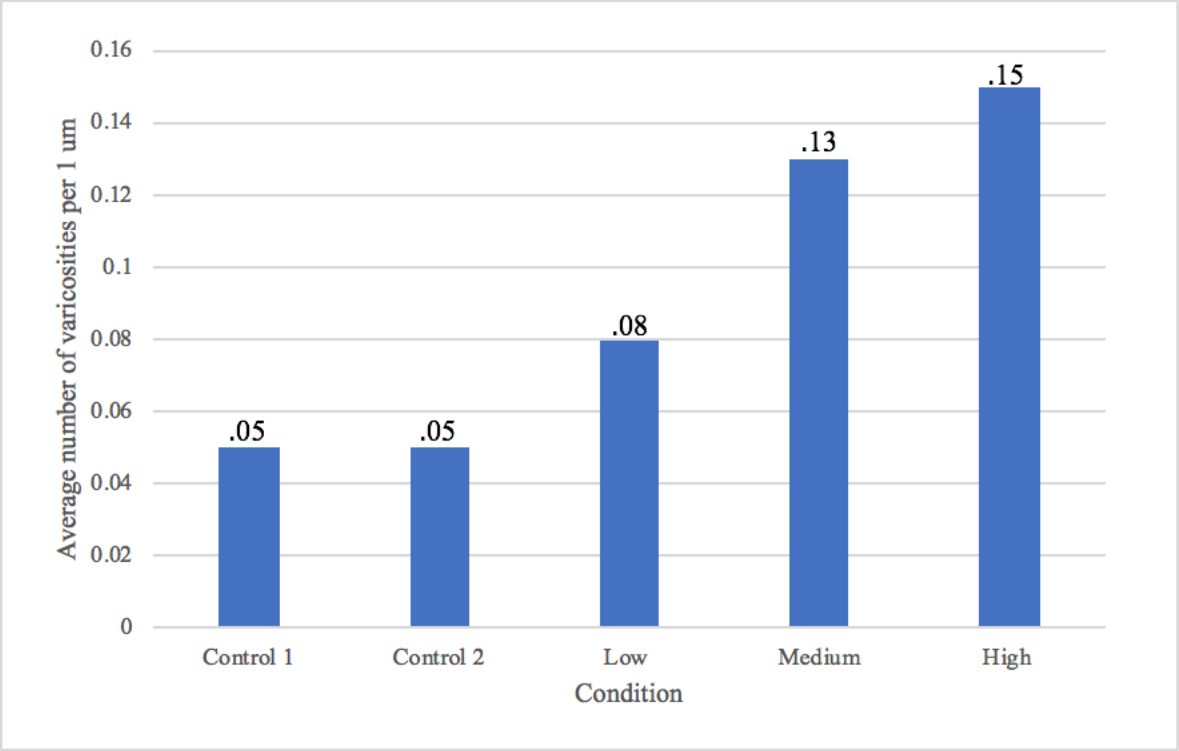

Figure 3: Compares the average number of varicosities per 1 μm after applying mechanical force within each group. This indicates that increasing gravitational pressure leads to an increase of varicosity density. Data are derived from measurements of the abundance of varicosities per 1 μm in axons from the two control condition cultures and three experimental condition cultures. There was a significant effect of amount of pressure on presence of varicosities at the $p<0.05$ level for five conditions [$F(4,127) = 6.36$, $p = 0.0001$].

Discussion & Conclusion

In the current study, it was hypothesized that *Gallus gallus* sympathetic neurons mechanically stressed at higher pressures will develop a higher abundance of varicosities than the neurons untreated by mechanical stress. Based on these preliminary results, the proposed hypothesis was supported. The data demonstrates that as the pressure exerted onto neurons increases, the number of varicosities per 1μm also increases. In accordance with the previously published literature by Gu et al., the results of the low condition showed varicosities as a consequence of applied mechanical pressure (2017). The medium condition showed a rapid acceleration in the density of varicosities per l μm compared to the low condition, indicating a greater disruption in axonal transport. The high condition showed a less extreme increase in the density of varicosities per 1μm.

A major risk of this experiment was increasing the pressure exerted on neurons. The goal was to increase pressure enough to demonstrate various severities of a TBI, without reaching the pressure at which the axons are completely blown away or disconnected. Disconnection of axons due to mechanical stress is referred to as primary axotomy, and occurs in more severe cases of

TBI. (Christman et al., 1994). Whereas the disruption of axonal transport, also known as the formation of varicosities, is referred to as secondary axotomy, commonly prevalent in acute cases of TBI (Christman et al., 1994). The results provide support that secondary axotomy occurred, but it is possible the selected pressures were not high enough to achieve primary axotomy. One future direction for this study is to investigate the pressure at which primary axotomy occurs. One feature of axon disconnection is the formation of axonal bulbs on the proximal and distal ends of disconnected neurons (Smith et al., 1999). So, data collected on the formation of axonal bulbs could help determine the exact moment of primary axotomy.

One limitation to this study was the low condition experienced pressure 24 hours after plating whereas the medium and high conditions experienced pressure 48 hours after plating. This poses as a challenge because axons form natural varicosities as they age (Shepard & Raastad, 2003). Research shows that varicosities, also referred to as synaptic boutons, form evenly spaced along aging axons to increase neural connectivity (Shepard & Raastad, 2003). To account for the natural varicosities that formed within 48 hours of plating, I included two control unperturbed groups; one at 24 hours and one at 48 hours. The density of varicosities per 1μm for both control groups was the same, 0.05 varicosities. The inclusion of two control groups eliminates the possibility of aging as a rationale for the increase of varicosity density seen in the results. Another limitation of this study was variability in determining varicosities. Distinguishing the boundaries of a varicosity in comparison with its axon was difficult, but since this challenge presented itself in analysis of all groups, there is no evidence of bias.

To refine this experiment, all conditions should experience pressure on the same time schedule. Doing so will sidestep the additional analysis necessary to add another control group to the data set. Also, to obtain more conclusive and specific data, starting at the low pressure of 190 mmH2O, increase the pressure by 5mm each trial. As shown in the results section, the medium condition experienced a 61.5% increase in varicosity density from the low condition. Having incremental pressure trials will help pinpoint which pressure causes this intense spike of varicosities seen in the data.

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