Morphological Effects of Stretch on Actin Cytoskeleton in Co-Cultured Neuronal and Smooth Muscle Cells

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Approved by:

Prof. Suzanne F. Scarlata, Advisor

ABSTRACT

The neuromuscular junction connects neurons to muscle fibers, allowing for brain signals coding involuntary and voluntary movements, to be transmitted to muscle fibers which then contract. In neuromuscular junction formation, the actin cytoskeleton shapes the postsynaptic membrane into folds. Actin filaments help stabilize the structure of the cells and the pre- and postsynaptic terminals by changing shape when tensile forces are at play. The goal of this study is to find how mechanical stress affects the actin cytoskeleton of co-cultured PC12 neuronal cells and WKO-3M22 smooth muscle cells. This will be achieved by cyclically stretching the co-cultured cells on polydimethylsiloxane polymer (PDMS) dishes. The actin cytoskeleton was stained with phalloidin rhodamine and imaged using immunofluorescence microscopy after the cells were fixed. We found that mechanical stress increases the length and number of PC12 neurites and increases the cell body area of WKO-3M22 cells. Understanding how the actin cytoskeleton is affected by mechanical stress will further help us understand how growth pathways play a role in increasing the number of neurites and reshaping the cell body.

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ACKNOWLEDGEMENTS

I would like to thank Worcester Polytechnic Institute for providing me with the opportunity to gain research experience by allowing me to work on a project in Professor Suzanne Scarlata's lab. I would like to thank Professor Scarlata for welcoming me into her lab and for providing me with guidance on my experiments. I would also like to thank Dr. Androniqi Qifti for her support and willingness to walk me through the stretch experiment protocols prior to my experimentations. Furthermore, I would like to thank the graduate students Maddison Rennie and Guanyu Lin for teaching me how to culture cells and use the TIRF microscope and Nikon software to image the cells. I would also like to thank Professor Kristen Billiar for letting me use his PDMS mold and stretch devices. Finally, I would like to thank my family and friends for their support in my academic journey.

BACKGROUND

The Neuromuscular Junction

The neuromuscular junction (NMJ) serves to form a connection between motor neurons and muscle cells. The NMJ consists of a presynaptic neuron terminal, postsynaptic motor endplate at the muscle cell, and a synaptic cleft that is the area between the presynaptic and postsynaptic terminals. When an action potential travels down the neuron, it triggers a voltage-gated calcium channel to open, which results in an influx of Ca^{2+} into the presynaptic terminal. Vesicles then migrate towards the presynaptic terminal and fuse with active zones, releasing acetylcholine (ACh) neurotransmitters into the synaptic cleft. ACh then binds to acetylcholine receptors (AChR) at the junctional folds of a motor endplate. This binding triggers the opening of ACh-gated ion channels, allowing for an influx of Na^{+} into the muscle. The influx of Na^{+} propagates action potential across the muscle membrane, promoting the release of Ca^{2+} from the sarcoplasmic reticulum (SR) into the muscle cell. Ca^{2+} then binds to a calmodulin-myosin light chain kinase to activate the myosin. A cross-bridge between actin and myosin causes thin filaments to slide past thick filaments, causing muscle contraction, which shortens the muscle fibers. Upon Ca^{2+} exiting the muscle cell, the enzyme gets released, inactivating the myosin, and relaxing the muscle (Omar et al., 2022).

Mechanotransduction

Mechanotransduction occurs when mechanical stimuli, such as tension, are transduced into biochemical signals that lead to cellular response. Cells sense mechanical stimuli at the cell membrane, particularly at the extracellular matrix (ECM), through mechanosensitive molecules (Martino et al., 2018). Mechanoreceptors, such as integrins, stretch-activated ion channels and cell surface receptor proteins, respond to such extracellular signals and relay the stimuli into the cell membrane. Integrins form a connection between the ECM and cytoskeleton by associating with a focal adhesion complex that attaches to the F-actin filaments. Stretch-activated ion channels are activated when there is conformational change of the cell membrane and deformation of the actin cytoskeleton that is attached to the channel. Cell-surface receptors such as a G protein-linked receptor may be activated by the deformation of the actin cytoskeleton, initiating intracellular signaling pathways (Romet-Lemonne & Jégou, 2013).

Role of RhoA Pathway and Actin Cytoskeleton in Mechanotransduction

The actin cytoskeleton plays a central role in mechanotransduction due to its role in generating, transmitting, and undergoing mechanical stress. Alongside the actin cytoskeleton's role, the RhoA signaling pathway regulates the actin cytoskeleton and its response to mechanical stress. To stabilize the generated force, actin-binding proteins regulate the actin cytoskeleton dynamics by controlling its structure and mechanical properties (Ohashi et al., 2017). Upon tension, the LIM kinase (LIMK) activated by the Rho/Rho-associated kinase (ROCK) pathway phosphorylates a severing protein cofilin, preventing cofilin from severing the F-actin fibers. The Rho/ROCK pathway also activates the formin Diaphanous (mDia), promoting F-actin polymerization (Martino et al., 2018).

Experimental Rationale

To view the morphological effects on the actin cytoskeleton in co-cultured cells, mechanotransduction needs to be stimulated by a tensile force such as stretch. It is known that contraction causes the actin fibers to shorten due to Ca^{2+} influx, but researchers want to know the effect of tension on the actin cytoskeleton and its related signaling pathways. Studies have shown that mechanical stress causes a shape change of the cell. Two morphological changes that were observed were that cells become elongated and orient parallel to the direction of the stretch (Du et al., 2022). The length increase results in the reduction of affinity of the F-actin severing protein, cofilin, thus increasing the affinity of myosin II (Li et al., 2020). To study both the NMJ and actin cytoskeleton of cells, we used a model consisting of WKO-3M22 smooth muscle cells and PC12 neuronal cells. PC12 cells have been used in a previous study researching neurite outgrowth (Lin et al., 2020). PC12 cells derive from a pheochromocytoma of the rat adrenal medulla and will develop neurites upon nerve growth factor (NGF) treatment. Vascular smooth muscle WKO-3M22 cells have been previously used in co-culture models with PC12 cells to study the NMJ (Novak, 2022; Skinner, 2021). Previous studies revealed that bradykinin can be used as a stimulating agent to increase the calcium levels in PC12 neuronal cells (Novak, 2022; Qifti et al., 2021). We wanted to see if bradykinin would cause any morphological effects on the actin cytoskeleton and neurite outgrowth, and therefore implemented a chemical stimulation. Our goal is to mechanically stretch and stimulate co-cultured smooth muscle and neuronal cells and

compare it to non-stretched/non-stimulated and non-stretched/stimulated control groups to see how the actin cytoskeleton is affected.

METHODS

Cell Culture

Wistar Kyoto rat PC12 neuronal cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing high glucose complete, 10% inactivated horse serum (HI), 5% fetal bovine serum (FBS), and 1% penicillin streptomycin. Rat smooth muscle WKO-3M22 cells were cultured in DMEM containing high glucose complete, 10% FBS, 1% L-glutamine, 1% penicillin streptomycin, 1% sodium pyruvate, and 5 mL of non-essential amino acids. Both cell lines were incubated at 37 degrees Celsius at 5% CO_2. Cells were washed with PBS prior to being split into new glass bottom dishes. 2 mL of trypsin was used in addition to PBS to help detach WKO-3M22 cells from the dish.

PDMS Plate Preparation

Polydimethylsiloxane polymer (PDMS) plates were made from a mold that allowed the plates to fit into a stretch device from Professor Billiar's lab. The PDMS plates were sterilized in 70% ethanol for 15 minutes. Each plate was coated with 2 mL of a 20 uL of Sulfo-SANPAH solution diluted in 2 mL of HEPES buffer for 15 minutes under UV light. The plates were then washed three consecutive times with PBS for a total of 10 minutes. The plates were then coated with 2 mL of a collagen solution diluted in 0.02M acetic acid and left in the freezer overnight. On day two, the plates were then coated with 2 mL of an antibiotic solution containing 100 ug/mL Gentamycin, 10 ug/mL Ciprofloxacin, 2.5 ug/mL Amphotericin B, and 100 ug/mL penicillin streptomycin, and left in the freezer overnight (Skinner, 2021).

After preparation, co-cultures of PC12 and WKO-3M22 cells were grown on the PDMS plates. The cell lines were seeded 72 hours prior to the stretch experiment. The plates were washed with PBS prior to seeding the cells. PC12 and WKO-3M22 cells were seeded at $3.00*10^4$ cells/mL and $5.25*10^3$ cells/mL. On day one of cell plating, 2 mL of PC12 DMEM media was added into each plate and let to rest overnight. The next day the media was replaced with differentiation media containing DMEM, 1% penicillin streptomycin, 2% HI, 2% FBS, and NGF at a concentration of 1.5 uL/mL. The cells then differentiated for two days (Skinner, 2021).

CellScale 16-Well Plate Preparation

The preparation for the CellScale plate was the same as the PDMS preparation; however, the amounts for each substance differed. Each well was coated with 250 uL of the Sulfo-SANPAH solution, 250 uL of collagen solution, and 250 uL of the antibiotic solution. The seeding was also the same, but the amount of media was 200 uL.

Stretch Experiments

Professor Billiar's stretch device was used to stretch one PDMS dish. The stretch conditions that were used were uniaxial at a rate of one oscillation per second at 10% stretch. The cells were stretched for 6.5 hours in the y-direction. A CellScale stretching device from Professor Billiar's lab was also used to stretch 16 wells simultaneously using the same conditions stated in the previous sentence.

Actin Staining

Cells adhered to glass bottom imaging dishes were fixed with 3.7% formaldehyde solution in PBS (1:10 dilute 37% stock with PBS) and incubated for 15 minutes. After removing the formaldehyde, the dishes were washed three times using PBS. 2 mL of 0.1% triton-X100 was then added into the dishes for 4 minutes. PBS was used to wash the dishes another three times. For each dish, 5 uL of phalloidin diluted in 200 uL of PBS was added and let sit for 30 minutes at room temperature in the dark. The dishes were then washed with PBS three times. Dishes received 2 mL of PBS and were stored at 4 degrees Celsius.

Neurofilament Marking

After the cells were already stained with rhodamine phalloidin, the cells were permeabilized with 0.2% triton-X100 for 20 minutes at room temperature. After washing the cells with PBS, the cells were then incubated in a blocking solution of PBS containing 5% goat serum, 2% BSA, and 0.1% triton-X100 for one hour. The primary antibody (neurofilament marker RNF 402) was then added at a 1:1000 dilution into the blocking solution and incubated at 4 degrees Celsius overnight. On day two, the cells were washed with PBS, and the secondary antibody (goat anti-mouse IgG) was added at a 1:1000 dilution and incubated at room temperature for one hour protected from light. The cells were then washed with PBS for 10 minutes.

Cell Imaging and Analysis

Cells were imaged under 20x fluorescence microscopy at a 540 nm excitation. The images were captured and manually analyzed using the ImageJ software. Cells were analyzed for the number of neurites, neurite length, neuronal cell body area, and smooth muscle cell body area.

Results

To view the morphological changes of the actin cytoskeleton in co-cultured neuronal PC12 cells and smooth muscle WKO cells from mechanical stretch, the cell lines were cultured individually and then co-cultured two days prior to the experiment. The cells were co-cultured on PDMS plates coated with collagen and antibiotics and differentiated for 48 hours with nerve growth factor prior to the experiment.

Cell Images and Analysis

The cell lines were cultured individually and stained with rhodamine phalloidin to be imaged in the TIRF at 540 nm excitation. The images were taken to get a look at how each cell line is structured in order to accurately count each cell line in the co-cultured dishes. The WKO cells had a less-rounded structure, whereas the PC12 cells were round.

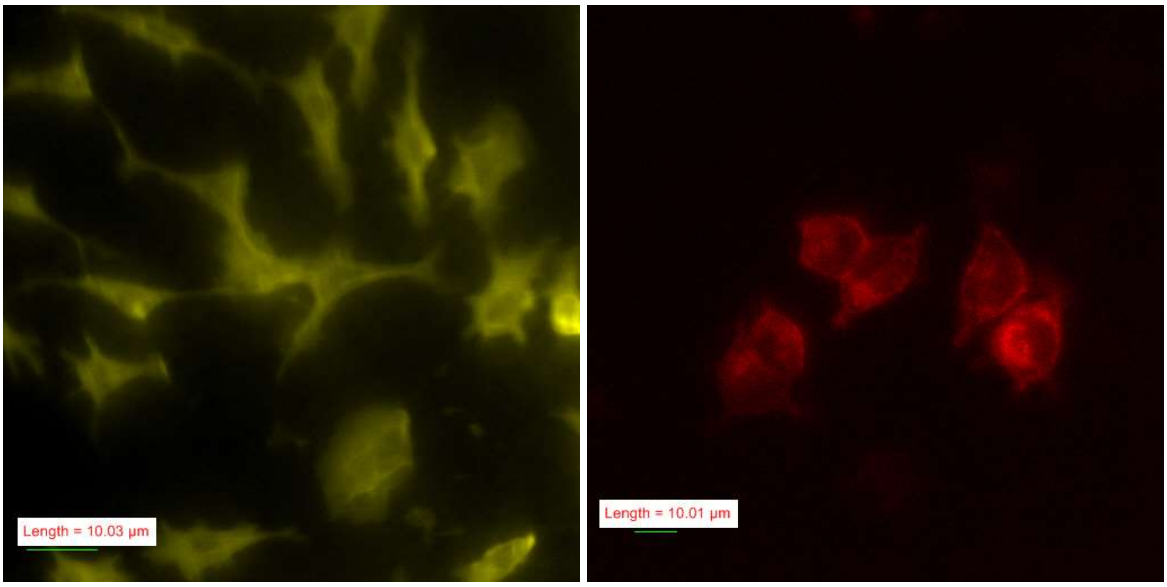

Figure 1. *Images of WKO-3M22 (left) and PC12 (right) cell lines individually cultured. These images were used to view the morphology of each cell line to use as a reference in identifying each cell in the co-cultured dishes. Neuronal cells have a more rounded structure, whereas muscle cells are less rounded.*

Co-cultured PC12 and WKO cells were not stretched and used as a control to compare results with a stretched co-culture. In both the control and stretch groups, cells were fixed with rhodamine phalloidin and imaged in the TIRF microscope at 540 nm excitation. The cells were also stained with a neurofilament marker (RNF 402) primary antibody and a goat anti-mouse IgG secondary antibody but there were no noticeable markings of neurofilaments on the images using this stain. The cells were then manually analyzed using the ImageJ software to measure and count the number of neurites per neuronal cell and measure the cell body areas of the WKO cells and PC12 cells, as well as the number of NMJs per neuronal cell.

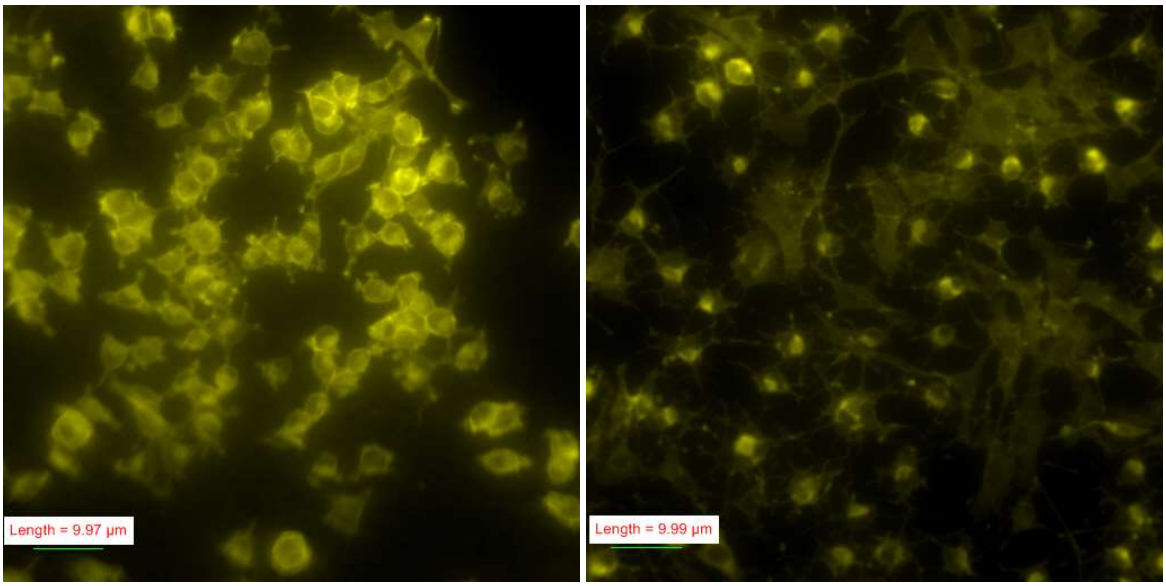

Figure 2. *Co-cultured WKO-3M22 and PC12 cells in no-stretch control (left) and stretch (right) groups. The PC12 cells in both images appear brighter under actin staining, particularly towards the edge of the cell. As number, cell ratio, and density impacts differentiation, the images were chosen to resemble similar characteristics for analysis.*

We found that the number of neurites per neuronal cell was higher in the stretch group compared to the control ($p < 0.001$). The neurite length also increased after being stretched, showing a significant difference between the control and stretch groups ($p < 0.001$). When analyzing the cell body area of each cell line, it appeared that stretch had no effect on the area of PC12 cells ($p > 0.10$) but increased the area of the WKO muscle cells ($p < 0.005$). The number of NMJ per neuronal cell had also increased after stretch and was significantly higher than the control ($p < 0.001$).

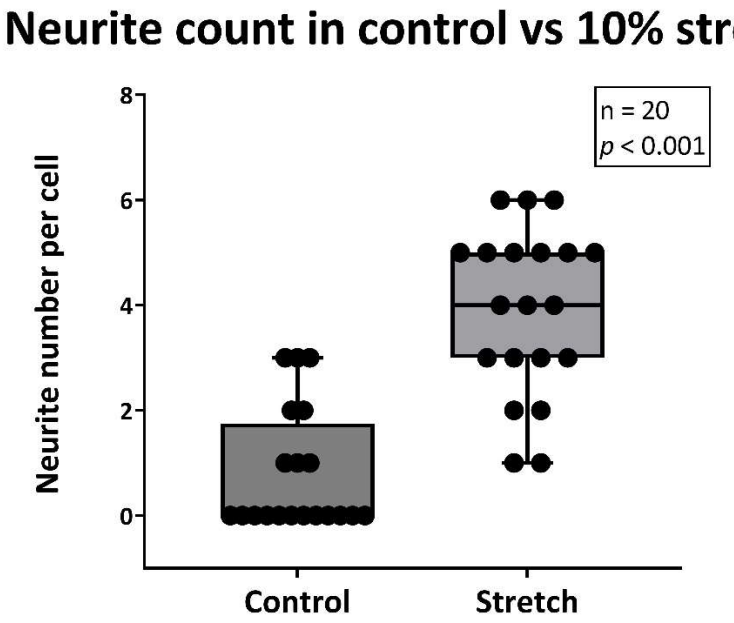

Figure 3. *The number of neurites per neuronal cell from 10% stretch compared to the control group. Both the control and stretch groups differentiated for 2 days. The stretch group was stretched for 6.5 hours in the y-direction. Mechanical stretch produced a higher number of neurites per cell compared to the control.*

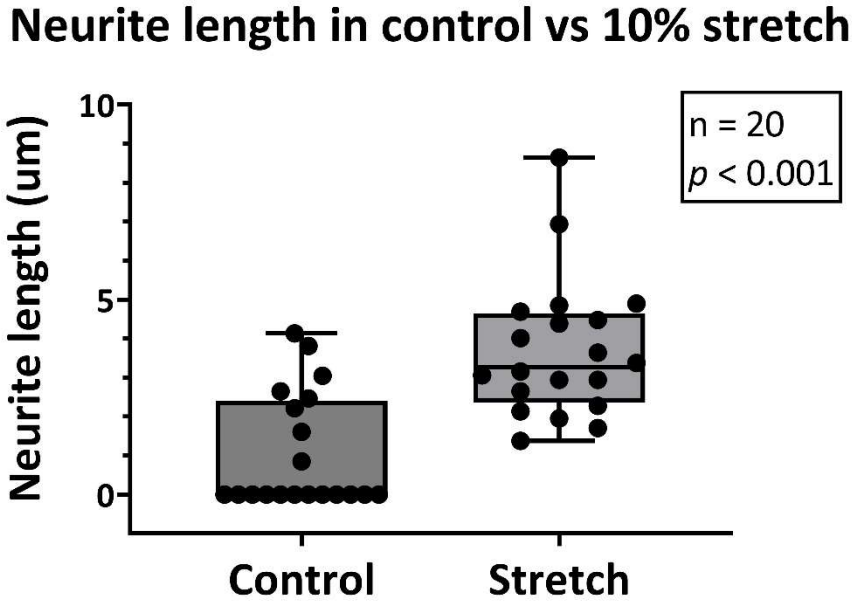

Figure 4. *The neurite length from 10% stretch compared to the control group. Both the control and stretch groups differentiated for 2 days. The stretch group was stretched for 6.5 hours in the y-direction. Mechanical stretch produced an increase in neurite length in the stretch group compared to the control.*

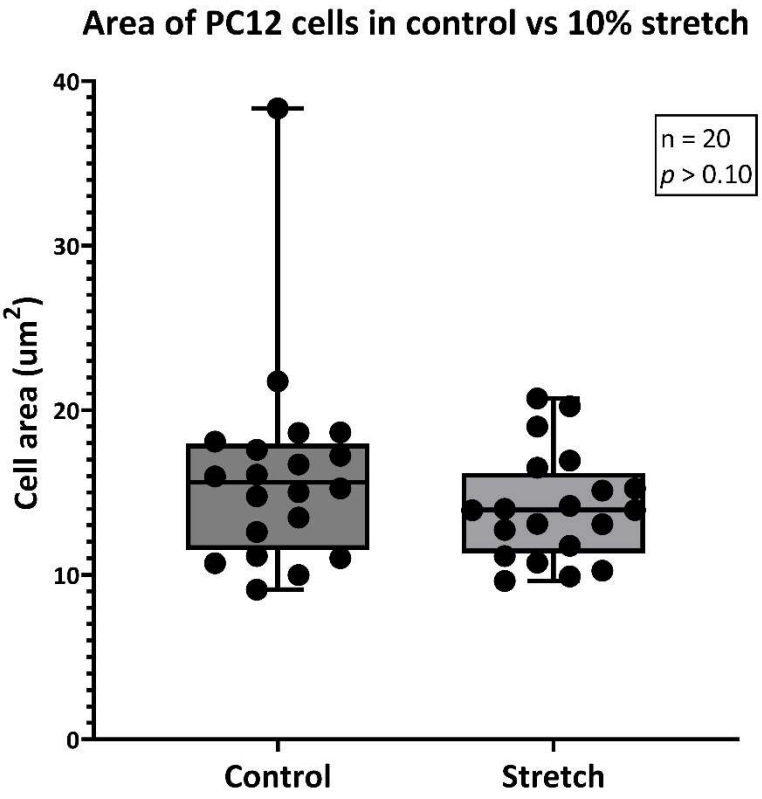

Figure 5. *Cell body area of PC12 neuronal cells. Both the control and stretch groups differentiated for 2 days. The stretch group was stretched for 6.5 hours in the y-direction. Mechanical stretch did not produce any significant change in the cell body area of PC12 cells in the stretch group compared to the control.*

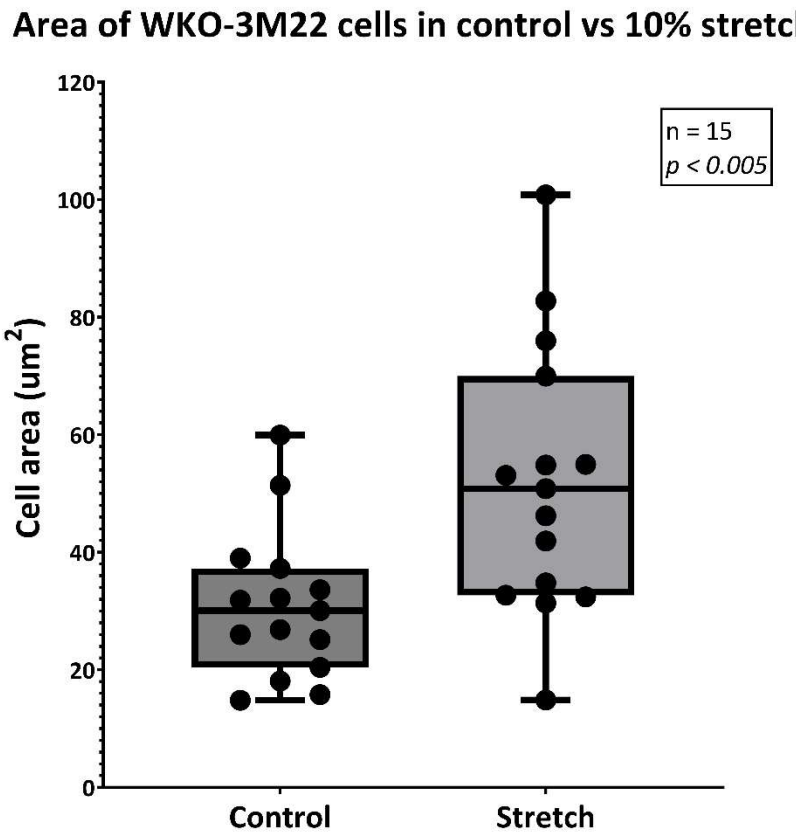

Figure 6. *Cell body area of WKO-3M22 smooth muscle cells. Both the control and stretch groups differentiated for 2 days. The stretch group was stretched for 6.5 hours in the y-direction. Mechanical stretch produced an increase in cell body area of WKO-3M22 cells in the stretch group compared to the control.*

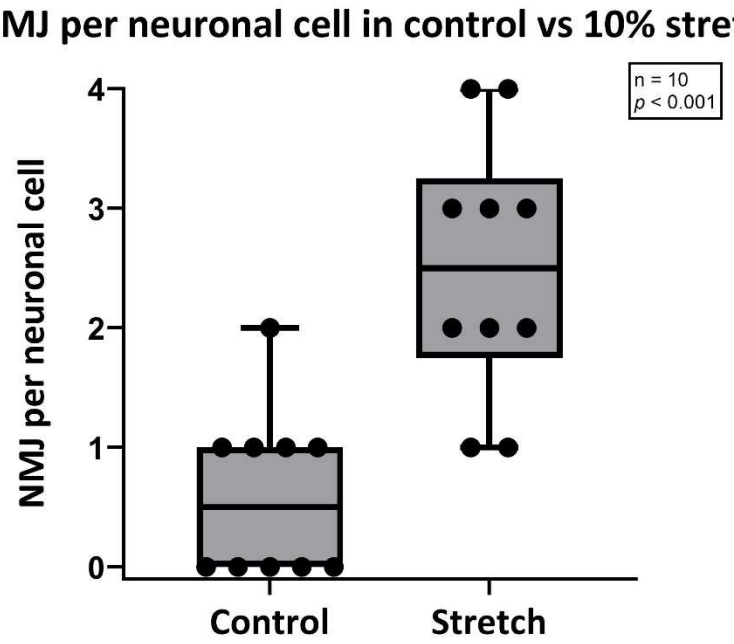

Figure 7. *Neuromuscular junctions per neuronal cell. Both the control and stretch groups differentiated for 2 days. The stretch group was stretched for 6.5 hours in the y-direction. Mechanical stretch produced an increase in neuromuscular junctions between the neuronal cells and smooth muscle cells in the stretch group compared to the control.*

Discussion

In this study, our goal was to identify the morphological effects of actin cytoskeleton caused by mechanical stretch and stimulation. Prior to experimentation, we had wanted to set up multiple conditions and planned to see how stimulation from bradykinin would affect the actin cytoskeleton of co-cultured cells. We thought that using a 16-well CellScale plate would be more efficient in simultaneously performing multiple experiments for stretch only and bradykinin stimulation after stretch. However, after stretching the cells for 6.5 hours and fixing the cells on the CellScale plate, there were no longer cells attached to the wells. This may have occurred due to the possibility of the cells not fully adhering to the bottom of the wells because of the plate's material, which may have needed an additional coating of either fibronectin or poly-d-lysine. Another possible reason is that there was rust on the stretch device that had fallen into the wells during the removal of the plate prior to fixing the cells with paraformaldehyde. Due to this technical issue, we were unable to use this plate for imaging, and therefore could not compare stretched and stimulated co-cultured cells to the control and stretch groups. We had also wanted to only stimulate the co-cultured cells with bradykinin but were unable to due to the PC12 cells not differentiating properly.

Stretch conditions were applied to the co-cultured cells to compare how stretch affected the morphology compared to the no stretch control. The number of neurites per cell and neurite length increased after stretch, which may have been caused by the distance between the neurons and other neurons or muscle cells. This means that the neurons would have had to extend their neurites to connect with neighboring neurons or muscle cells. The cell body area of neuronal PC12 cells did not change after stretch, which means that there was a possibility that the PC12 cells divided. Cells tend to maintain a constant size, so the PC12 cells would have divided to keep a constant area. The cell body area of smooth muscle WKO cells increased after stretch; the RhoA signaling pathway may have been playing the role in stabilizing the actin cytoskeleton by phosphorylating cofilin to prevent it from severing the actin filaments. Rather than shortening the length of the actin filaments, the actin filaments most likely elongated to relieve the cell from the tensile force.

Conclusion

We viewed the morphology of the actin cytoskeleton in co-cultured cells induced by mechanical stretch. Our results show that mechanical stretch induced neurite growth, increased NMJs per neuron, and increased the cell body area of smooth muscle cells. In addition to the results, stretch may be initiating growth pathways that allow for the neurite growth. To test this hypothesis, further studies could be geared towards looking at regulatory proteins in signaling pathways, such as p38 or RhoA. Because RhoA manages the activation of LIMK to inhibit cofilin, it may serve as a base to figure out how much of a role RhoA has at stabilizing the actin cytoskeleton. An approach could be to inhibit the RhoA protein partially and completely to compare it to control group with functional RhoA. Another possible protein to study is profilin, which binds monomeric G-actin to F-actin and contributes to actin polymerization; the inhibition of profilin may also show how it regulates the actin cytoskeleton. Upon inhibiting these proteins, the co-cultured cells can then be stretched using the same conditions used in our experiment. Finding out how signaling pathways react to mechanical stimuli may lead to better understanding of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) (Hensel & Claus, 2017).

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