Examination of the Morphological Changes on PC12 Neurites by NaCl

A Major Qualifying Project Report
Submitted to the faculty of
WORCESTER POLYTECHNIC INSTITUTE
in partial fulfillments for the requirements for the
Degree of Bachelor of Science
in Biochemistry
by

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04/25/2024

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Approved by:

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Prof. Suzanne F. Scarlata, Advisor

Abstract

Nerve cells, or neurons, exist to transmit signals through extensions of their nuclei-containing main cell body known as neurites. Neurons transmit signals through propagation of action potentials which are initiated in response to changes in the electrochemical gradient and potential of the neuronal plasma membrane. Action potentials open specific ion channels which will change the potential of neighboring membranes, thus sending the signal down a chain of neurons until the target organ is reached and the desired response produced. This study examined how changes in the extracellular Na^+ concentration over periods of time affected the length and width of the neurites. The neurites were produced from NGF differentiated PC12 cells and it was concluded that as the concentration and time of NaCl exposure increase, the length and width of neurites decrease. This is likely caused by the hypertonic environment created by the excess Na^+ ions, which induces the cells to maintain osmotic equilibrium through releasing water into the extracellular fluid, thereby decreasing the surface area of the plasma membranes that have contact with the outside environment.

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Acknowledgements

I would like to thank Worcester Polytechnic Institute and the Chemistry and Biochemistry department for the opportunity to complete a project such as this. In particular, I would like to thank Professor Suzanne Scarlata for providing the chance to work with her and her lab on this topic as well as for the advice and guidance throughout the year. Additionally, I would like to thank all the members of the Scarlata lab for welcoming me and creating a fun and supportive learning environment.

I would especially like to thank Ayobami Adeeko for teaching me PC12 cell culture as well as Madison Rennie for teaching me how to use the Zeiss LSM microscope and for all her help troubleshooting at various points and Greta Schmidt for her guidance and assistance. I would also like to thank my fellow undergraduate students completing their MQPs in the lab this year, Bailey Norris, Rafaela Kanil and Kallie Case for their help and encouragement. I am grateful for all the support and encouragement provided by all members of the Scarlata lab throughout the past year and for providing so many opportunities for me to learn and grow.

Introduction

Within eukaryotic organisms it is the responsibility of nerve cells, or neurons, to receive and transmit signals from sensory organs (i.e. eyes, ears) to the central nervous system which contains the brain and spinal cord[1]. A nerve cell consists of a cell body that contains the nucleus and many neurites, or protrusions, that extend from the main cell body[1]. These projections can differentiate into axons, one long extension that conducts electrical signals away from the cell body, and dendrites, smaller branches that provide an increase in surface area to receive signals[1].

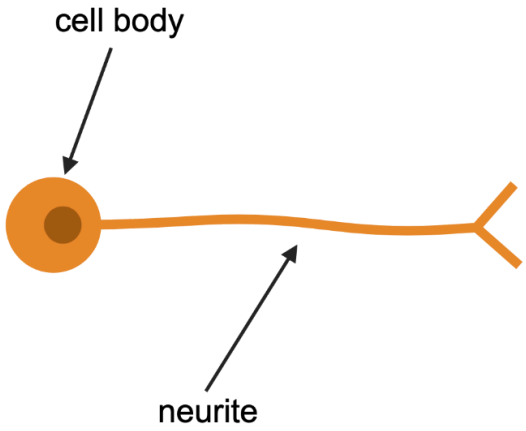

Figure 1: Basic diagram of a neuron *(Figure created with Biorender.com)*

In order to perform their required intracellular and intercellular signaling, nerve cells utilize their membrane potential, which when certain thresholds are exceeded, can trigger action potentials[5]. Action potentials are brief large periods of depolarization and repolarization of neuronal membranes, which travel along a sequence of neuronal membranes until they reach the intended target[5]. The changes that cause action potentials are a direct result of ionic transport across the plasma membrane[7]. As the lipid bilayer that makes up the plasma membrane is an large energy barrier for charged particles, specialized transmembrane proteins, known as ion channels, are responsible for the passage of ions[5,7].

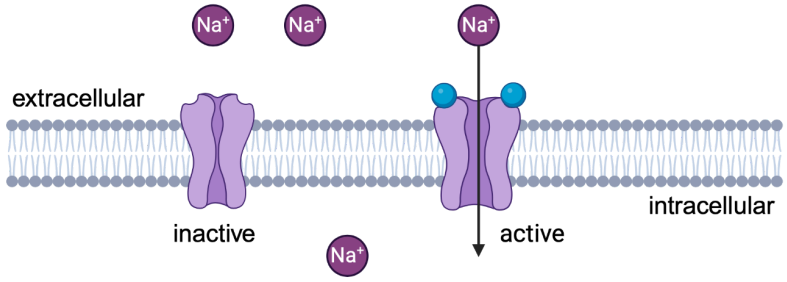

Figure 2: Diagram of ligand-gated ion channels in both an active and inactive state *(Figure created with Biorender.com)*

Ion channels are highly selective due to the function of their selectivity filters, a sequence of amino acids found in the channel that will stabilize only one specific ion[7]. There are two main types of ion channels which function in nerve cells - ligand-gated and voltage-gated[5]. Ligand-gated channels open when a specific neurotransmitter, such as acetylcholine or glutamate binds to an appropriate receptor allowing the movement of select ions across the plasma membrane[1]. In contrast, voltage-gated ion channels open in response to changes in the membrane potential[5]. The membrane potential can be changed depending on the differences in ion concentration between the extracellular and intracellular fluid[,7].

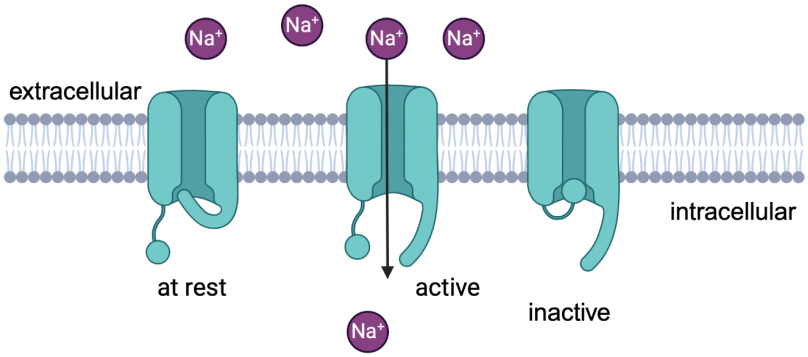

Figure 3: Diagram of voltage-gated ion channels at rest, in an active and an inactive state *(Figure created with Biorender.com)*

During an action potential, the binding of neurotransmitters to specific receptors causes the Na^+ channels to open, allowing Na^+ ions to spontaneously flow across the membrane into the cell[7].

This causes a change in membrane potential and allows the voltage-gated Na^+ channels to open, causing a local depolarization in the membrane[7]. This subsequently induces the voltage-gated K^+ channels to open which results in a K^+ ion efflux[7]. Before the Na^+ and K^+ ions can reach equilibrium, the channels spontaneously close, causing depolarization of the membrane[7]. However, this depolarization induces the voltage-gated Na^+ channels on a neighboring neuron to open, repeating the process and transmitting the signal in one direction along a sequence of nerve cells[7].

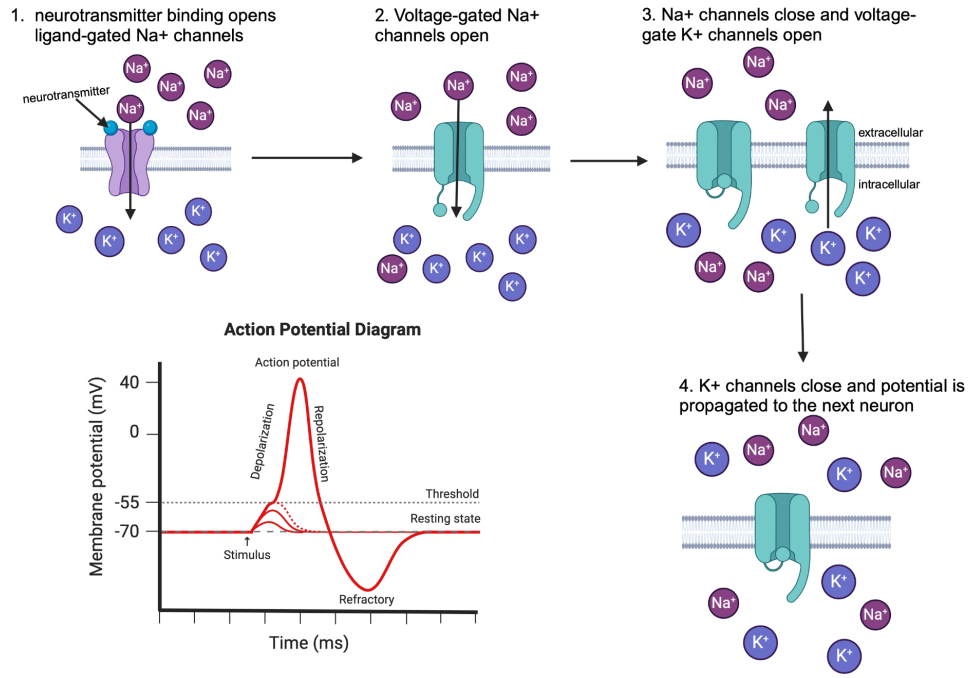

Figure 4: Diagram of the steps of an action potential on ion channels as well as a graph of how membrane potential changes over the course of the milliseconds it takes for an action potential to propagate *(Figure created with Biorender.com)*

As a consequence of the action potential, the ionic gradients of nerve cells are partially dissipated and are restored by the action of the Na^+-K^+ ATPase pump[4]. The movement of Na^+ ions, in particular, is interesting as it can be coupled to the movement of Ca^{2+} and K^+ ions[3]. Additionally the activity of the Na^+-K^+ pump is also responsible for the electrical excitability of the neuronal

membranes, emphasizing the importance of the Na^+ ion electrochemical gradient in the signaling mechanisms of neurons[7].

The purpose of this study was to observe the effects of the addition of extracellular NaCl of various concentrations at various times of exposure on the membranes of neurons as well as their connections to each other. It has previously been determined that the Na^+ ion channels of neurons are more susceptible to changes in the extracellular concentration of Na^+ ions than other ion channels such as potassium[2]. Therefore, changes in extracellular Na^+ was the focus of this study. To do this, a stock of PC12 cells were maintained and differentiated with nerve growth factor (NGF) for 48 hours before exposure to NaCl.

Methodology

PC12 Cell Maintenance and Differentiation

A stock of PC12 cells were maintained in 10 cm culture plates with 10mL of complete media. The complete media contains 500mL DMEM, 10% horse serum HI, 5% FBS and 1% pen strep. The cells were split as needed around 65% confluency to prevent overgrowth. Additionally, when needed, cells were split into 2 cm glass bottom imaging dishes and allowed to grow in complete media for 24 hours prior to differentiation. After this time, 2mL of the differentiation media containing 500mL DMEM, 1% horse serum HI and 1% pen strep that had been mixed with 10µL of nerve growth factor (NGF) was added to each glass bottom dish.

NaCl Stress and Fixation

After the cells were allowed to differentiate for 48 hours, each glass bottom was exposed to a solution of NaCl ranging in concentration from 300mM to 600mM for a time frame ranging from 1 minute to 10 minutes. After the stress, each dish was washed three times with PBS before incubation in a 3.7% PFA solution for 10 minutes to affix the cells to their plates. Once fixation was complete, the cells were stored in 1mL PBS in the cold room until staining and imaging.

Laurdan Staining and Imaging

Prior to imaging, the cells were incubated with 20µL of Laurdan and 1mL of non sterile PBS for 15 minutes. As Laurdan is light sensitive the dishes were covered with aluminum foil during this time. After 15 minutes, the cells were washed three times with PBS before a new 1mL of PBS was placed in the dish. The cells were then imaged using the Zeiss LSM microscope with the Argon/2 and the HeNe1 lasers and the Plan-Apochromat 63x/1.4 Oil DIC objective. The resulting images were collected and analyzed using ImageJ software.

Results

Differentiated PC12 cells were exposed to NaCl in various concentrations and for different periods of time over the course of this experiment. The differences in neurite length and width were then compared to the control dishes that contained PC12 cells that were differentiated with NGF but not exposed to NaCl.

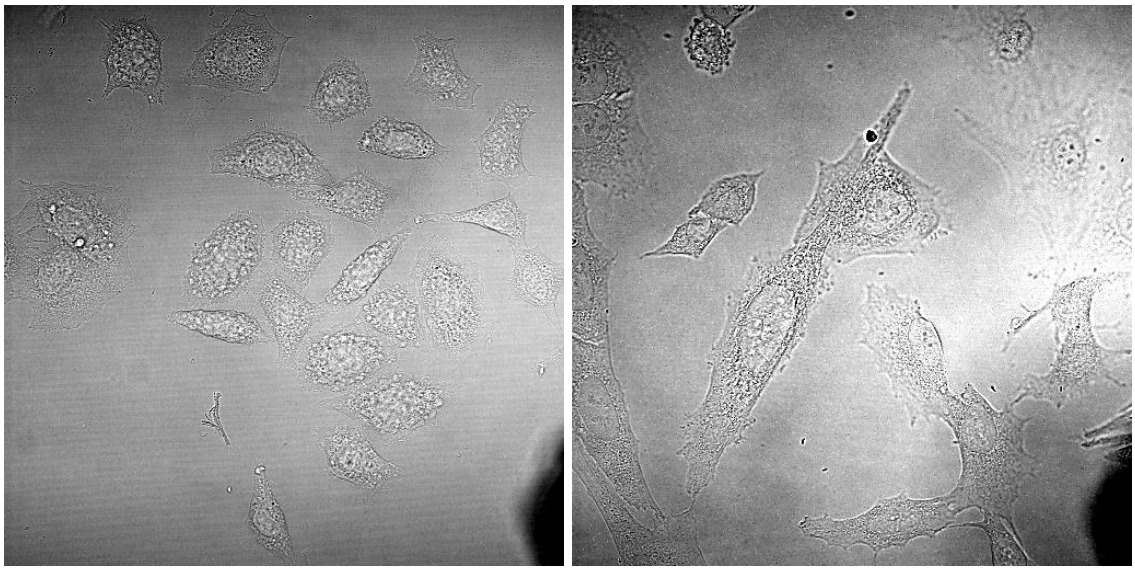

Figure 5: Images of cells from control dishes that had no exposure to NaCl

In comparison to the controls, the following figures illustrate the effects of the different stress conditions on differentiated PC12 cells, increasing in intensity.

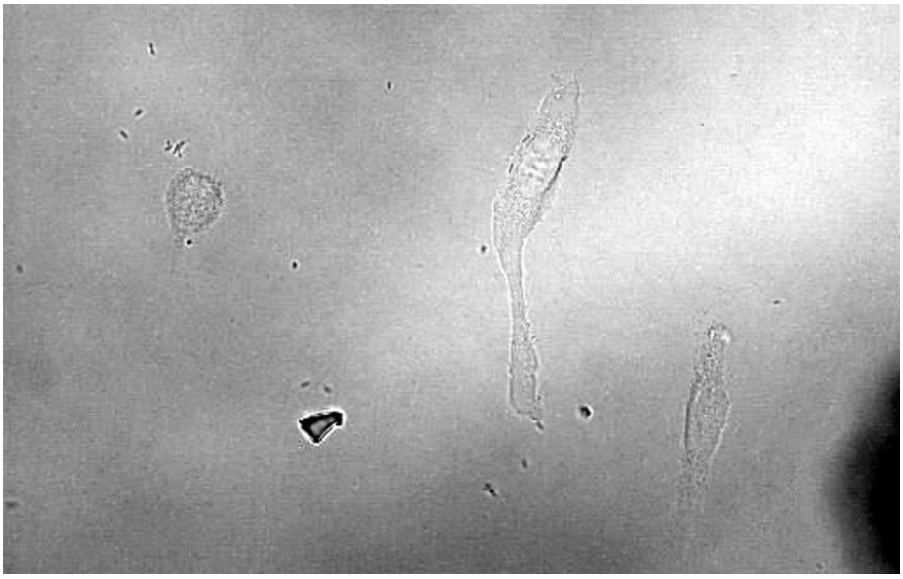

Figure 6: Image of differentiated PC12 cells exposed to 300mM NaCl for 1 minute

After stress for 1 minute at a concentration of 300mM, the time of exposure was increased to 3 minutes.

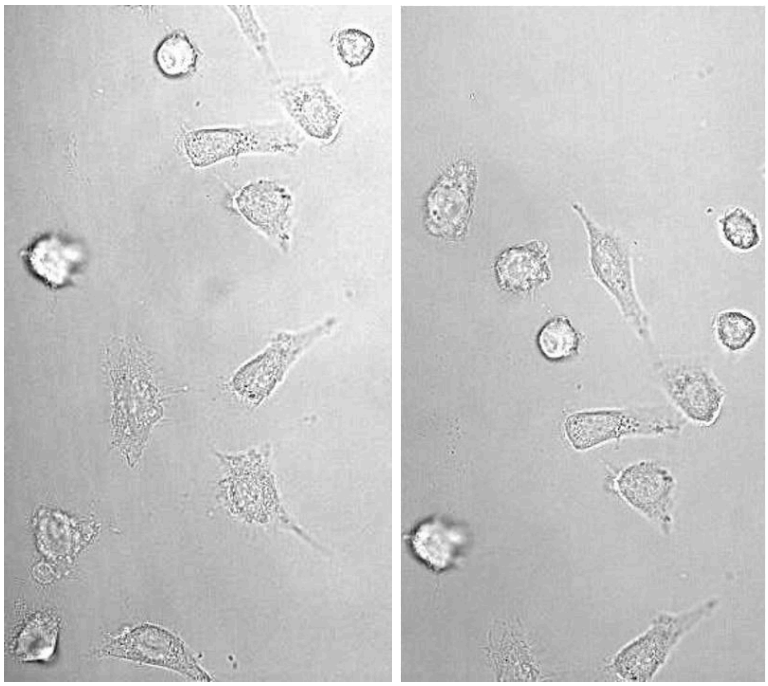

Figure 7: Images of differentiated PC12 cells exposed to 300mM NaCl for 3 minutes

As seen in Figures 6 and 7, the neurites of the PC12 cells were in various stages of retraction after NaCl exposure. Some completely retracted while others were only partially retracted. In subsequent tests, the concentration of NaCl was doubled as the time of exposure continued to increase.

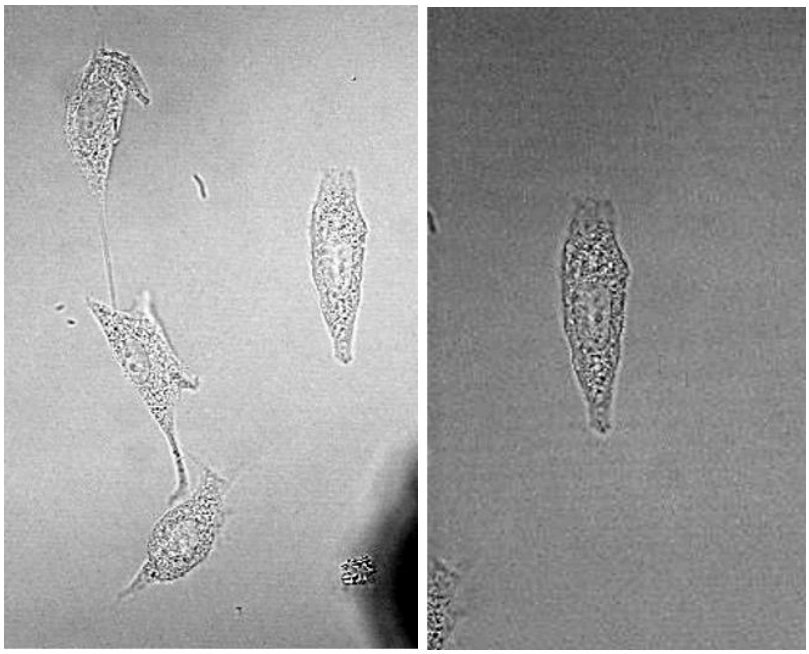

Figure 8: Images of differentiated PC12 cells exposed to 600mM NaCl for 5 minutes

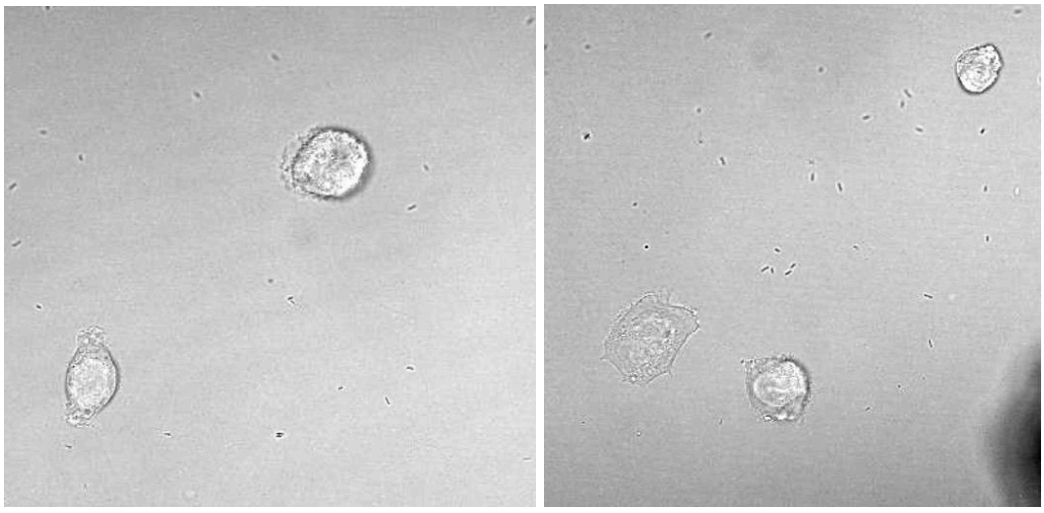

Figure 9: Images of differentiated PC12 cells exposed to 600mM NaCl for 10 minutes

After images were acquired for each condition, a graph comparing average neurite length and width for each condition were generated.

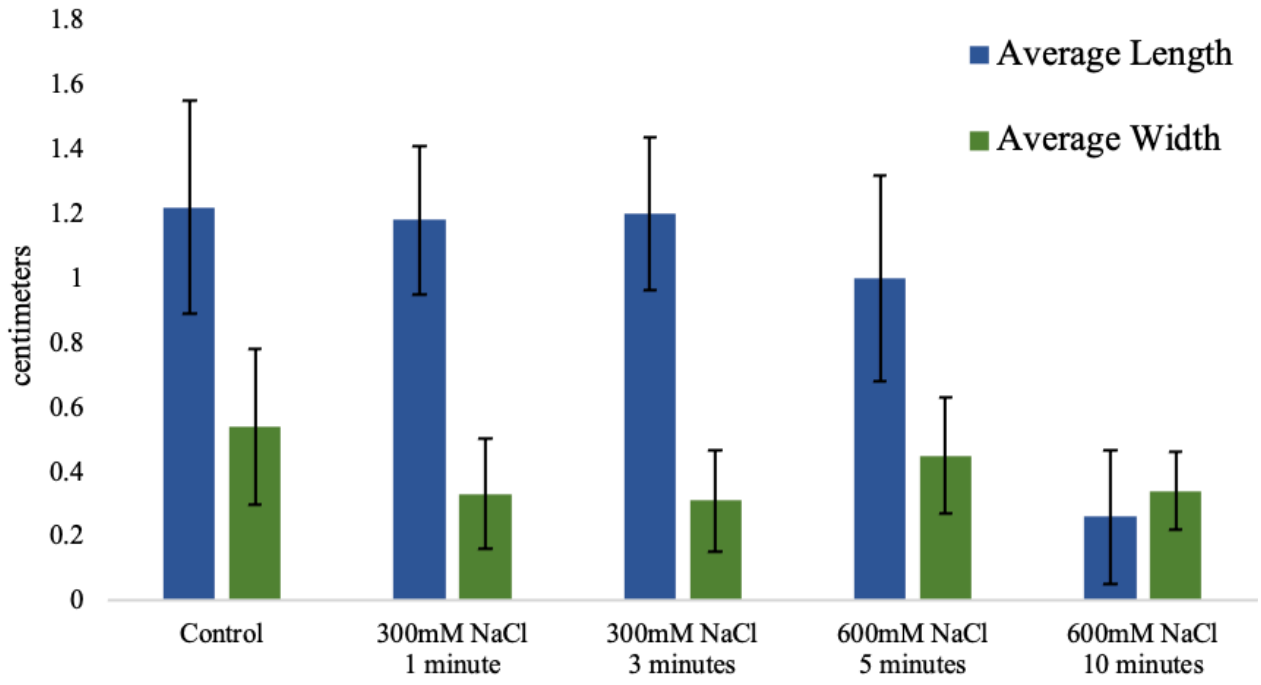

Figure 10: Graph of average neurite length and width for each condition including the control and error bars

Figure 10 illustrates that as the stress conditions increased in intensity, the average neurite length and width generally decreased.

Discussion

As demonstrated in Figure 10, as the time of NaCl exposure increased, on average the length and width of neurites decreased. This could be due to the neurons releasing water as the extracellular Na^+ ion concentration increases. To reestablish osmotic equilibrium with their surroundings, the cells will release water from their cytoplasm into the extracellular space, causing their membranes to shrink and their neurites to retract thereby decreasing the overall surface area maintaining contact with the hypertonic solution. When the neurites retract far enough, the cells are no longer connected to each other and action potentials cannot be propagated across them.

However in Figures 6 and 7, after a 300mM NaCl stress some of the neurites partially retracted while others completely retracted. These two populations could have been the result of higher concentrations of Na^+ ions building up in certain areas of the plate. As demonstrated in Figure 9, all the neurites had retracted after a 10 minute exposure of a NaCl concentration of 600mM - suggesting that the more Na^+ present, the more likely it is that neurites will retract. This build up could have been caused by some of the cells uptaking Na^+ in an attempt to reestablish the necessary electrochemical gradient and then subsequently releasing them back into the extracellular fluid. This release would possibly be triggered by the cells attempting to communicate with each other or pairing the release of Na^+ with an energetically unfavorable transport of ions or proteins across the neuronal membrane. Then, as the Na^+ ions build up in certain areas of the extracellular fluid, the cells closest will experience more pressure to reestablish osmotic equilibrium and will therefore retract their neurites completely.

Additionally, when the extracellular Na^+ concentration increases it can cause depolarization of the neuronal membranes. Since neurons maintain a strict concentration gradient of sodium ions, only allowing them into the cell under specific circumstances through specific channels, this depolarization can make action potentials easier to induce. If the right conditions are met, the voltage-gated sodium channels can be induced to open, which would allow additional influx of Na^+ ions. If the action potential continues, an efflux of K^+ ions would follow as well though without the neurite connections, the chain won't be able to continue to a subsequent neuron.

Furthermore, to prevent too much sodium from accumulating inside of the cells, the Na^+/K^+-ATPase pump is most likely activated. This will help maintain the ionic equilibrium of the membrane electrochemical gradient through reimporting the K^+ ions while removing the Na+ ions back into the extracellular fluid. The Na^+/K^+ pump can also help maintain the excitability of the plasma membrane, either increasing the likelihood of an action potential or regenerating the resting membrane potential in the aftermath.

This study examined several different conditions but further experimentation and more conditions would need to be tested to make any definitive conclusions about neuron activity and how increasing the extracellular concentration of sodium ions affects intercellular signaling. Next steps would include examining neurite recovery after NaCl stress as well as introducing neurotransmitters to be able to visualize the process of action potentials under these stress conditions.

Conclusion

The protrusions of nerve cells, generally known as neurites, help propagate electrochemical signals between cells. These signals, known as action potentials, generate a specific response in a target organ and are reliant upon the careful maintenance of ionic gradients by ion channels, which only allow passage of specific ions. In particular, the Na^+ gradient is necessary to generate action potentials as the influx of these ions depolarizes the plasma membrane, thereby initiating the cascade that results in a targeted response in cells outside of the central nervous system. This study examined the neurites of NGF differentiated PC12 cells and was able to observe that as the time of exposure and concentration of the extracellular NaCl increased, the average neurite length decreased. This is most likely caused by the cell's decreased surface area as water is released into the extracellular fluid in order to maintain osmotic equilibrium. However, while this study examined multiple different conditions, more tests including exploring neurite recovery and the introduction of neurotransmitters would need to be run to make any definitive conclusions about neuron activity.

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