Investigating the Role of Exosomes in the Development of Neuromuscular Junctions

A Major Qualifying Project Report

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ABSTRACT

The development of the neuromuscular junction involves communication between neuronal and muscle cells as they differentiate. Recently, exosomes have become a popular focus of research on cellular communication and have been found to be involved in the formation of neuromuscular junctions. The goal of this study is to isolate exosomes from a coculture of neuronal and muscle cells, characterize them, and investigate their application towards neuromuscular development alongside a pharmaceutical used to inhibit their biogenesis. This goal was achieved by utilizing an ultracentrifugation protocol to isolate exosomes from a coculture of PC12 and WKO-3M22 cells and characterizing their size using Dynamic Light Scattering. Cultures were then treated with exosomes and the neutral sphingomyelinase inhibitor GW4869 and studied for the degree of neuronal differentiation and neuromuscular junction formation. Sonication and filtration were found to improve sample homogeneity and reduce exosome aggregation following isolation, although further optimizations are necessary. The addition of exosomes or the inhibitor alone did not substantially affect coculture development; however, when added together there was an increase in both the number of neurites per cell, and the development of neuromuscular junctions when compared to control. This observation may be due to altering the ratios of sphingolipids such as ceramide and sphingomyelin, which change during neuronal differentiation. Blocking the conversion of sphingomyelin to ceramide with GW4869 while adding an enriched source of sphingolipids in the form of exosomes may be responsible for enhanced differentiation, presenting an opportunity for future work.

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ACKNOWLEDGEMENTS

First, I would like to express my gratitude to Worcester Polytechnic University for providing me with the opportunity to gain hands on experience by working on a research project in the lab of Professor Suzanne Scarlata. I would like to thank Dr. Scarlata for welcoming me into her lab as first an undergraduate researcher, and later as an MQP student for this project. She has been an outstanding advisor, providing me with guidance, support, and considerable patience as my project developed. I would also like to thank my initial graduate mentor, Dr. Androniqi Qifti, for her guidance, enthusiasm, and teaching me the majority of my biochemical lab skills. Furthermore, I would like to thank the Scarlata lab's graduate students Maddison Rennie and Guanyu Lin for guiding me through my project, and their patience and help with the roadblocks I encountered. I would also like to thank the members of the Gericke lab for helping me use their Dynamic Light Scattering machine, which allowed me to characterize exosome sizes. Finally, I wish to thank my family and friends for always supporting my academic journey.

BACKGROUND

The Neuromuscular Junction

The neuromuscular junction (NMJ) is the point of connection between motor neurons and muscle cells (Fig. 1). It is responsible for linking the neuronal and muscular systems, consisting of a presynaptic motor neuron terminal, a synaptic cleft, and a postsynaptic motor endplate at the muscle cell. When an action potential propagates down a neuron, it triggers an influx of Ca^{2+} that results in the fusion of synaptic vesicles with the neural membrane, releasing the neurotransmitter acetylcholine (ACh) into the synaptic cleft. ACh binds to acetylcholine receptors (AChR) at the motor endplate and triggers an action potential across the muscle membrane. This results in the release of Ca^{2+} ions from the sarcoplasmic reticulum into the muscular sarcoplasm, which triggers muscle contraction (Jimsheleishvili et al., 2022). The formation of the NMJ involves extensive communication between the muscle and neuron mediated by several molecules and pathways. One of the major pathways is mediated by Agrin, a protein secreted by motor neurons, which binds to a receptor Lrp4 that activates MuSK (Li et al., 2018). MuSK activates downstream effects responsible for the clustering and stability of AChR that is needed to create an NMJ. While the Agrin-Lrp4-MuSK pathway is one of the better understood drivers of NMJ formation, the roles of other pathways have recently attracted increased attention. One such mediator of cell signaling that has gained recent prominence are exosomes, which have been suggested as a possible component of NMJ formation.

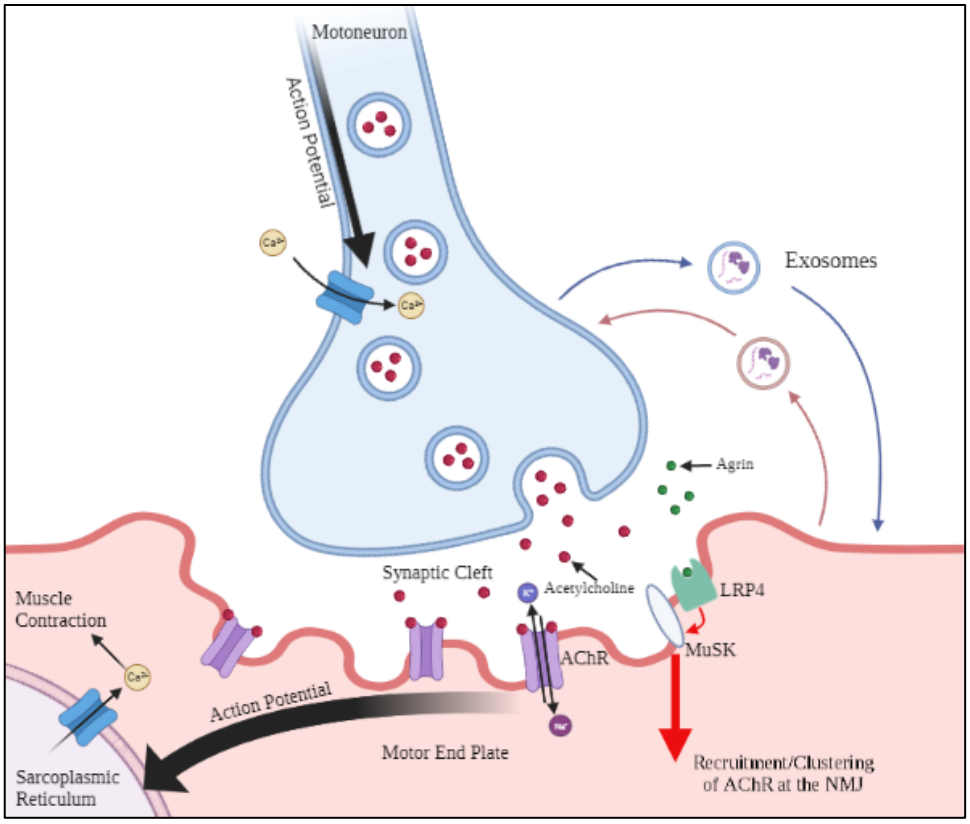

Figure 1. *Simplified model of the NMJ containing an overview of its formation and the process by which a neuron transmits its action potential to muscle contraction.*

Exosomes in the Neuromuscular Junction

Exosomes are a subtype of extracellular vesicles (EVs), that have gained considerable attention in recent years as a form of cell communication and possible therapeutic agent. Compared to other EVs such as microvesicles, the biogenesis and release of exosomes is a more concerted process (Fig. 2). Invagination of the plasma membrane results in the formation of an early endosome, which forms a multivesicular body (MVB) through the endosomal sorting complexes required for transport (ESCRT) pathway. Alternate pathways to generate MVBs are present, such as the ceramide mediated inward budding of endosomes (Teng & Fussenegger, 2020). Once formed, the MVB may receive cargo from various cell sources, and proceed towards degradation or fusion with the cell membrane and subsequent release of exosomes. Exosomes range in size from 50-150nm, and contain a wide array of proteins, DNA, and RNA whose identity may vary depending on their source (Hessvik & Llorente, 2017). Recent research has suggested exosomes are involved in the formation of NMJs, and are capable of transporting membrane-bound proteins such as syt4 between neurons and muscles (Korkut et al., 2013). As they may assist

NMJ development and have the potential to be loaded with cargo, exosomes represent a future therapeutic for conditions that affect NMJs. Indeed, many diseases such as myasthenia gravis, Lambert-Eaton myasthenic syndrome, and botulism affect the formation or function of the NMJ. While evidence shows exosomes may interact with NMJ development, the extent or exact role they play is not yet understood, and the methods used to study exosomes are not standardized. In this study, we adapt the common ultracentrifugation based exosome isolation method for a NMJ model, test a potential exosome biogenesis inhibitor for its effects on NMJ formation, and test if the reintroduction of exosomes may reverse these effects.

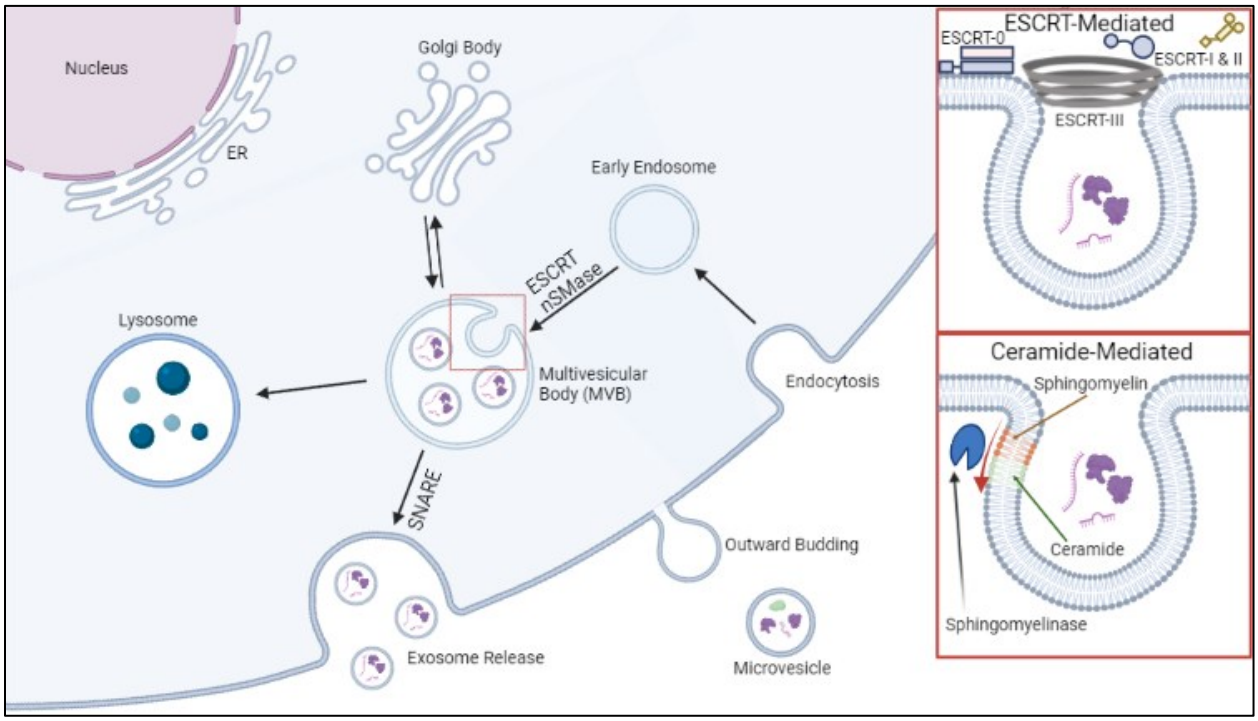

Figure 2. *A simplified model detailing the formation of multivesicular bodies, the biogenesis of exosomes, and their release.*

Experimental Rationale:

In order to study exosomes in the NMJ and the effects of inhibiting their formation, an isolation protocol is crucial. Currently, the majority of researchers isolate exosomes using differential ultracentrifugation, immunoaffinity capture, precipitation, or microfluidics techniques (Li et al., 2017). Of these, ultracentrifugation is often considered the “gold standard” as it offers high purity, yield, and the ability to process large volumes at the expense of long processing times. To study the NMJ, we used a model consisting of PC12 and WKO-3M22 cells. PC12 cells are used

as a neuronal model for in-vitro research and have previously been used in exosome studies (Bahrini et al., 2015). Derived from a pheochromocytoma of a rat adrenal medulla, upon treatment by NGF they will develop neurites/synapses and exhibit neuronal morphology. Vascular smooth muscle WKO cells have previously been cocultured with PC12 cells to serve as a model for studies on the NMJ (Novak, 2022; Skinner, 2021). Therefore, we chose these cell lines as a NMJ model for exosomal studies. The sphingomyelinase inhibitor GW4869 was chosen to test the inhibition of exosome biogenesis on NMJ development. GW4869 is a cell permeable non-competitive inhibitor that has previously been used to inhibit exosome biogenesis in neuronal cells (Dinkins et al., 2014; Men et al., 2019). It functions to inhibit an alternate pathway wherein the ceramide-mediated inward budding of endosomes forms multivesicular bodies as opposed to the ESCRT-mediated pathway. GW4869 was chosen as it is considered to be a common pharmaceutical inhibitor of exosomes, and has been demonstrated to inhibit exosome biogenesis in other cell types as well (Essandoh et al., 2015). The goal of this study was to isolate exosomes from a PC12-WKO co culture via ultracentrifugation, characterize their size and relative amounts, then test the effects of an exosome inhibitor on the development of NMJs and whether or not its effects were reversed by the readdition of exosomes.

METHODS

Culture Plate Preparation

Falcon 150 x 25mm culture dishes (Ref No. 353025) were coated with collagen to facilitate coculture adhesion based on a protocol by a previous MQP (Skinner, 2021). Sulfo-SANPAH (Thermo Fisher Cat No. A35395) was diluted in 50mM HEPES at 10uL of solution per 1mL of HEPES. Each plate was treated with 10mL of solution and placed under UV light for 15 minutes. Plates were washed 3 times with PBS, and 10mL of a collagen solution (0.1mg/mL Collagen in 0.02M Acetic Acid) was added to each plate and left overnight. The following day plates were washed with PBS and plated with cells.

Cell Culture

PC12 and WKO were seeded onto 100mm x 20mL culture dishes at final concentrations of 3.00*104 cells/mL and 5.25*103 cells/mL respectively. Cells were grown in 20mL PC12 growth media consisting of 10% horse serum, 5% FBS, and 1% pen strep in DMEM until 70% confluency. Cells were then switched to differentiation media consisting of DMEM high glucose without L-glutamine with 1% sodium pyruvate, 1% pen strep, and 5mL non-essential amino acids for 2 days. Cells treated with GW4869 were treated with 100 uL at a final concentration of 20uM. Cells treated with exosomes were treated with 100 uL of a previous isolate, and a further 100uL after the first day. An additional culture was treated with both GW4869 and Exosomes in the same manner. To avoid any effects from adding additional volumes from exosomes suspended in PBS, cultures without exosome treatment were treated with an equivalent amount of PBS. Further, to control for the effects of the DMSO used to reconstitute the GW4869, cells were treated with equivalent amounts. Cells grown for imaging were seeded onto collagen coated MatTek 35mm glass dishes (Part No. P35GC-1.5-14-C) at final concentrations of 3.00*104 cells/mL and 5.25*103 cells/mL respectively. Cells were grown in 2mL PC12 growth media until 70% confluency, then switched to differentiation media for 1-2 days. Cells were treated with GW4869, and exosomes as described previously, although adjusted for 2mL volumes.

Exosome Isolation

Culture media was collected and centrifuged at 1290 rpm for 10 minutes at 4C in a JA-25.50 rotor. The supernatant was collected and centrifuged at 3300 rpm for 10 minutes at 4C. Following this, the supernatant was collected and centrifuged at 12,000 rpm for 30 minutes at 4C. Next, the supernatant was transferred to an ultracentrifuge tube, and centrifuged at 50,000 rpm for 2 hours at 4C in a Beckman 70ti rotor. The supernatant was removed and the pellet was resuspended in 15mL of PBS, then centrifuged again at 50,000 rpm for 2 hours at 4C. Finally, the supernatant was removed and the pellet was resuspended in 3mL of PBS for downstream analysis.

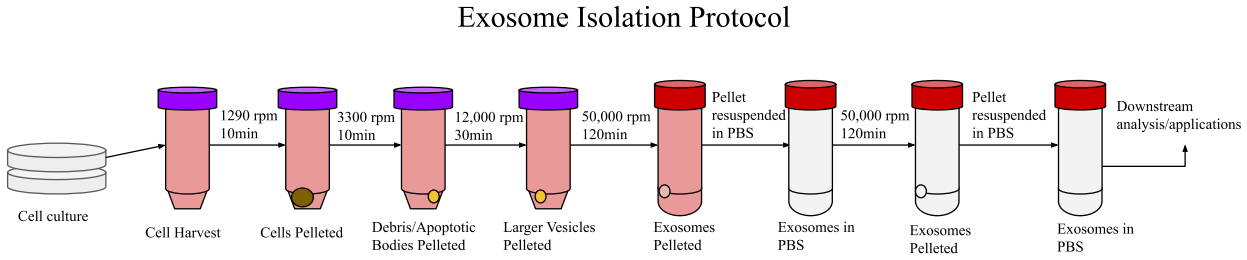

Figure 3. *Exosome isolation workflow using an ultracentrifugation method. Initial centrifugation steps utilized a JA 25.50 rotor, while ultracentrifuge steps were performed with a Beckman 70ti rotor.*

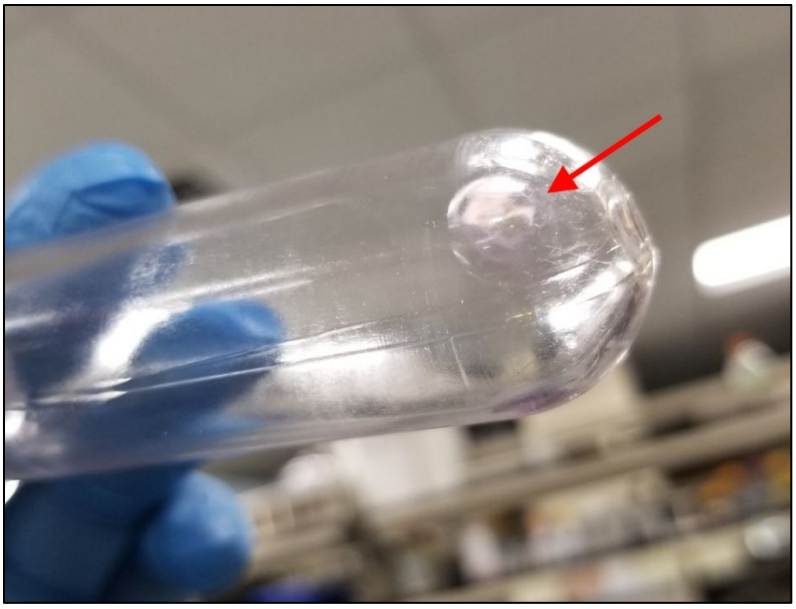

Figure 4. *Translucent exosome pellet following isolation via an ultracentrifugation protocol.*

Exosome Characterization

Size distribution was determined by Dynamic Light Scattering (DLS) based on %intensity. Exosome resuspensions were measured both before and after filtration through a 220 nm syringe filter or sonication for 2 minutes on ice to break up potential exosome aggregates. Exosomes were then lysed by the addition of 500uL of concentrated RIPA lysis buffer to 2.5mL of exosome resuspension. The lysis buffer contained 150mM Tris.HCl pH 7.6, 900mM NaCl, 6% NP-40, 6% sodium deoxycholate, 0.6% SDS, and a Pierce Protease Inhibitor Tablet at 6x concentration (Cat no. A32965). After adding the lysis buffer, samples were incubated at 4C for 30 minutes, then sonicated in an ice bath for 30 seconds. Afterwards they were left on ice for 15 minutes, with 30 seconds of vortexing every 5 minutes. Protein concentration was determined by a BCA assay using the Pierce BCA Protein Assay Kit (Cat no. 23225) according to manufacturer instructions.

Cell Imaging and Analysis

Cells grown for imaging were fixed in a 3.7% formaldehyde in PBS solution for 30 minutes. After washing, cells were solubilized with 0.2% Triton X-100 in PBS for 10 minutes, then blocked for 1 hour (2% BSA, 5% goat serum, 0.1% triton in PBS). Afterwards, cells were washed and stained with Invitrogen™ Rhodamine phalloidin (Cat no. R415) diluted in PBS in a 1:400 ratio for 30 minutes. Cells were kept at 4C in PBS protected from light until imaging. Cells were imaged under 20x fluorescence microscopy at a 540nm excitation. Images were captured and manually analyzed using Fiji, a version of the ImageJ software, and paint.net. Manual analysis was used instead of dedicated neuronal morphology software, as automated analysis has trouble differentiating between neuronal and muscle cells. Cells were analyzed for the number of identified neuronal cells, neurites, and neuromuscular junctions. To maintain a standardized analysis, any “neurite” needed to be greater than or equal to 20 pixels in length, and a neuron was counted only if it possessed at least one neurite. A neuromuscular junction was identified where a neurite of any length was observed to be in contact with a muscle cell.

RESULTS

Two major aims were established for this study; first, the development of an ultracentrifugation protocol to isolate exosomes from a neuromuscular coculture model and investigate their properties. Second, to investigate the effects of using exosomes on neuromuscular development, and whether their addition would reverse any effects from a nSMase inhibitor.

Dynamic Light Scattering

The size distribution of isolated exosomes was measured using Dynamic Light Scattering (DLS). A peak ranging from 100-140 nm was observed following exosome resuspension. A larger peak was observed ranging from 2000-6000 nm which was believed to be either debris, air bubbles, or exosome aggregates.

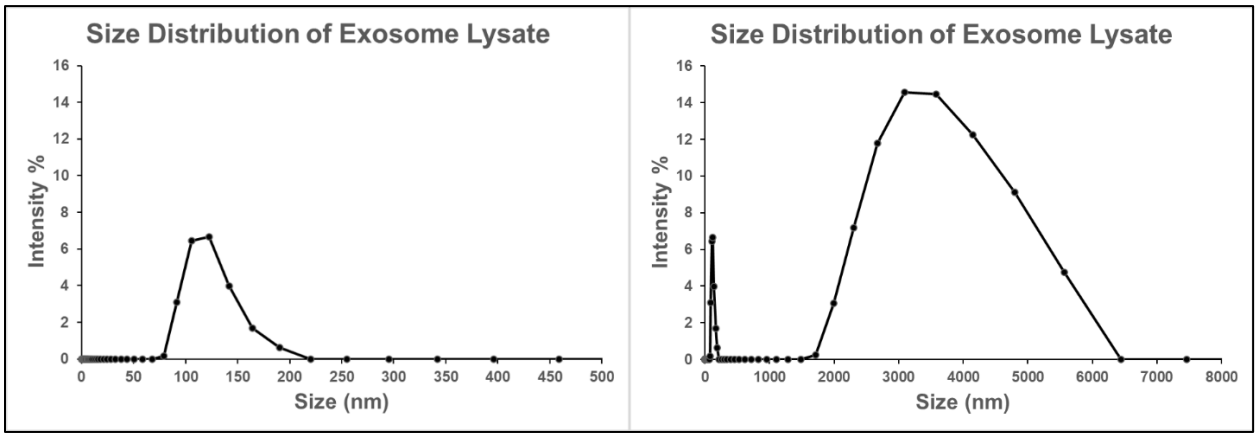

Figure 5. *DLS results following exosome isolation and resuspension showing the full size distribution (right), and sizes up to 500 nm (left).*

To test this hypothesis, the exosome isolate was subjected to either filtration or sonication. Following either filtration or sonication, there was a significant reduction in sizes above 2000 nm. However, the relative activity of sizes in excess of typical exosome ranges (>150 nm) was increased, and following sonication there was a small peak around 30 nm suggesting exosome lysis.

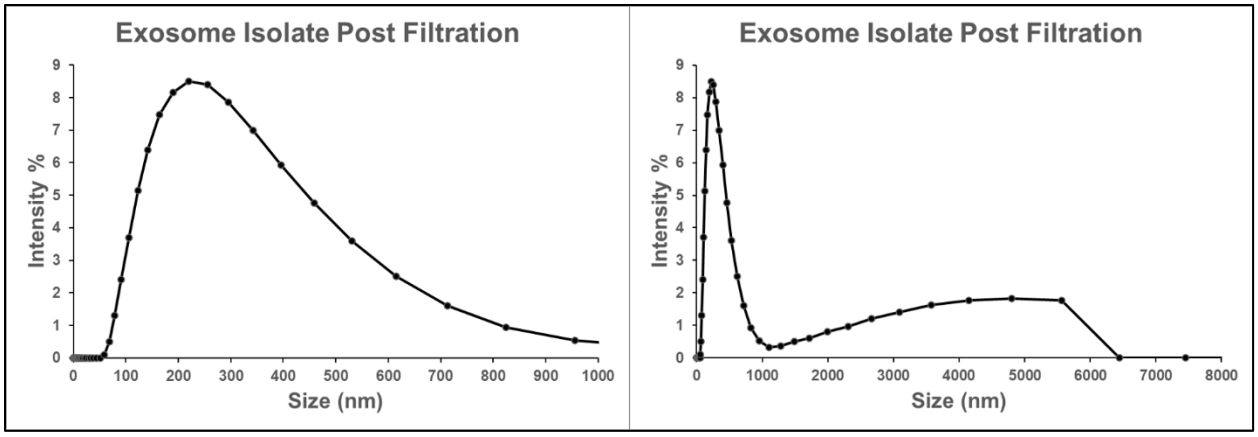

Figure 6. *DLS results following exosome isolation, resuspension, and filtration through a 220 nm syringe filter. The full size distribution is displayed (right), and sizes up to 1000 nm (left).*

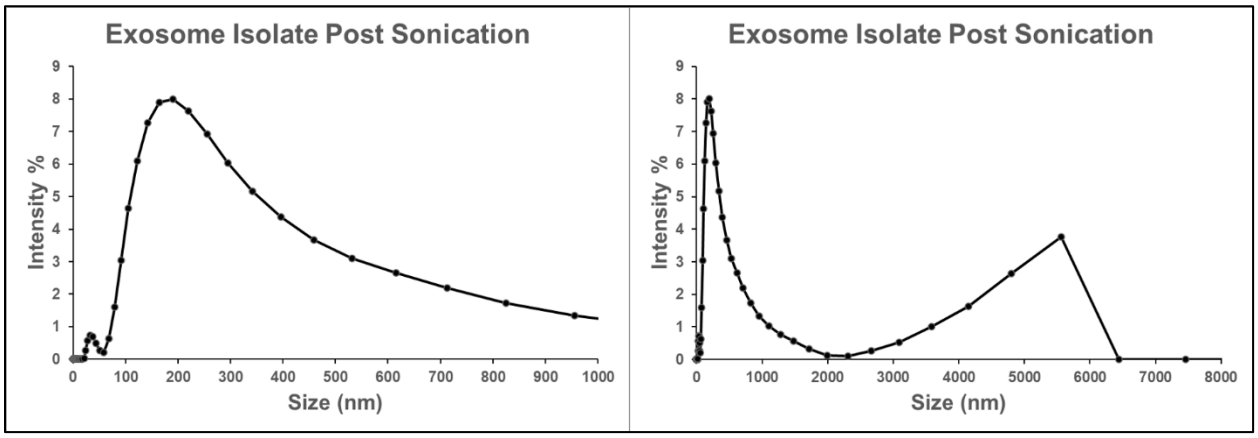

Figure 7. *DLS results following exosome isolation, resuspension, and sonication for two minutes on ice. The full size distribution is displayed (right), and sizes up to 1000 nm (left).*

Exosome protein concentration results

Protein concentration was measured via a BCA assay from exosome isolates to compare the relative levels of exosomes from cells grown in the presence of added exosomes, nSMase inhibition, and nSMase inhibition with re-added exosomes from a previous isolation. Protein concentration measurements showed an insignificant difference between the conditions except for nSMase treatment, which produced a slight drop in concentration. The inhibitor treatment resulted in a concentration of around 0.123mg/mL as opposed to 0.130-0.133 mg/mL shown in other conditions, suggesting a 5-8% decrease in relative exosome levels.

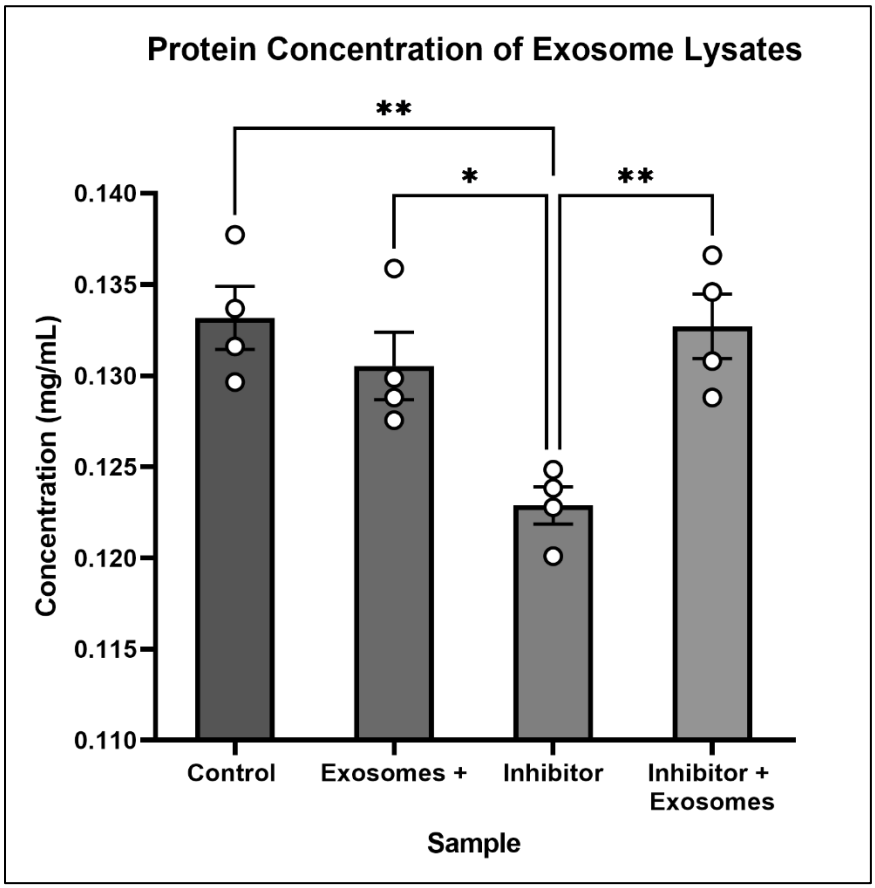

Figure 8. *Protein concentration of 3mL of exosome isolates post lysis from cocultures treated with Exosomes, GW4869 nSMase inhibitor, or both.*

Cell Images and Analysis

Cells were fixed, stained with rhodamine phalloidin, and imaged in the TIRF at a 540 nm excitation. Cells were manually analyzed for the number of neuronal cells, neurites, and

neuromuscular junctions. Focusing on the number of neurites per neuron, and the number of neuromuscular junctions per neuron, we found similar results for the control, added exosomes, and inhibitor conditions; however, the inhibitor with added exosomes had a noticeably higher ratio of neurites to neurons (Fig. 10). This observation was not as distinct when considering the ratio of neuromuscular junctions per neuron, due to high variability in the data (Fig. 11).

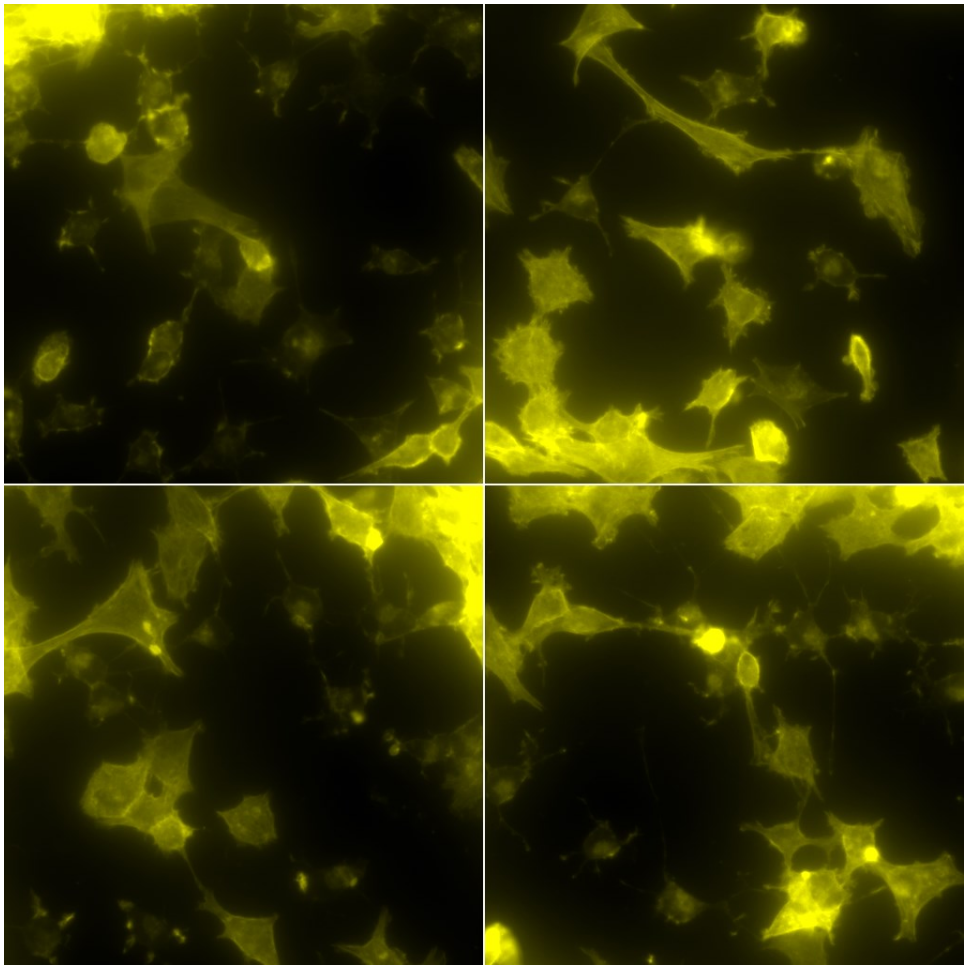

Figure 9. *Cocultured cells under different conditions following 2 days of differentiation. Conditions are (in clockwise order): control, added exosomes, added exosomes and treatment with GW4869, and GW4869 treatment alone. Muscle cells appear brighter under actin staining and have a less rounded morphology. As number, density, and ratio of cells impacts differentiation, images were chosen to match each other for these factors as closely as possible.*

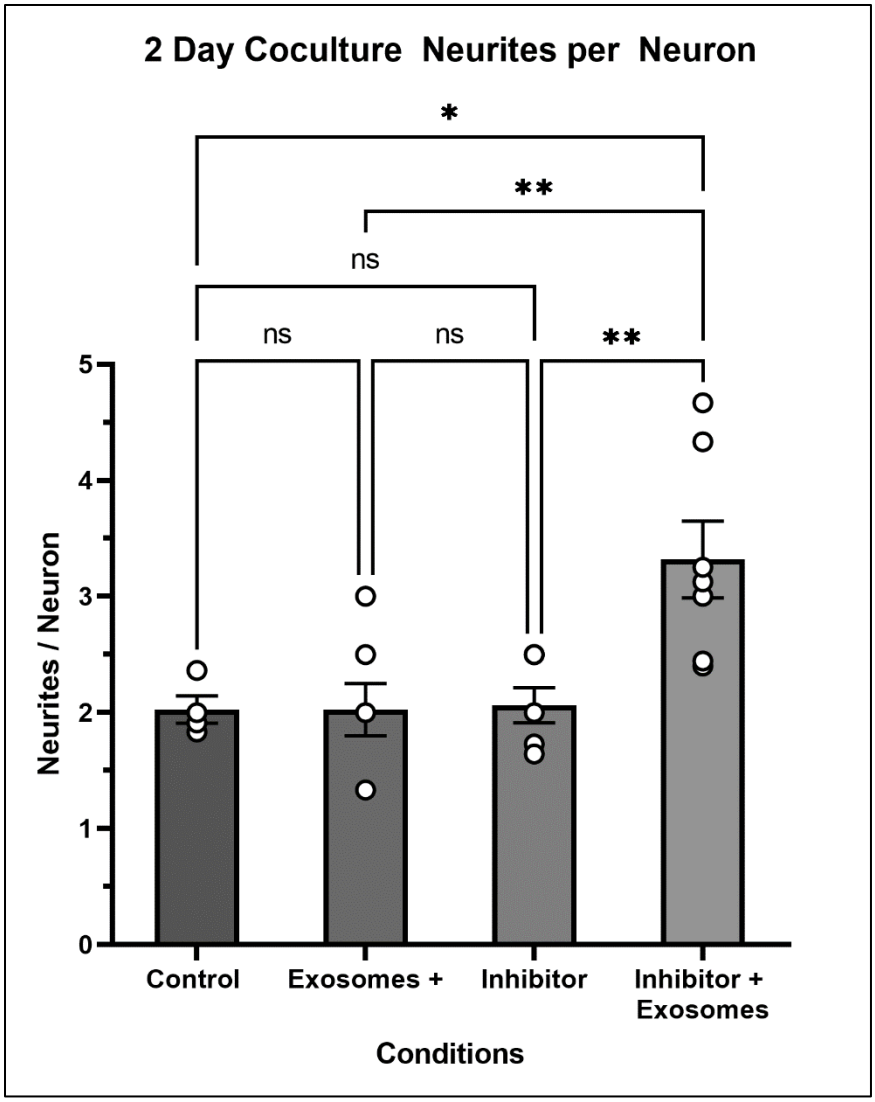

Figure 10. *The ratio of neurites to neurons for conditions after 2 days of differentiation. Added exosomes, and treatment with the GW4869 nSMase inhibitor did not produce any noticeable change in the ratio of neurites compared to control. However, treatment with both resulted in a higher degree of differentiation.*

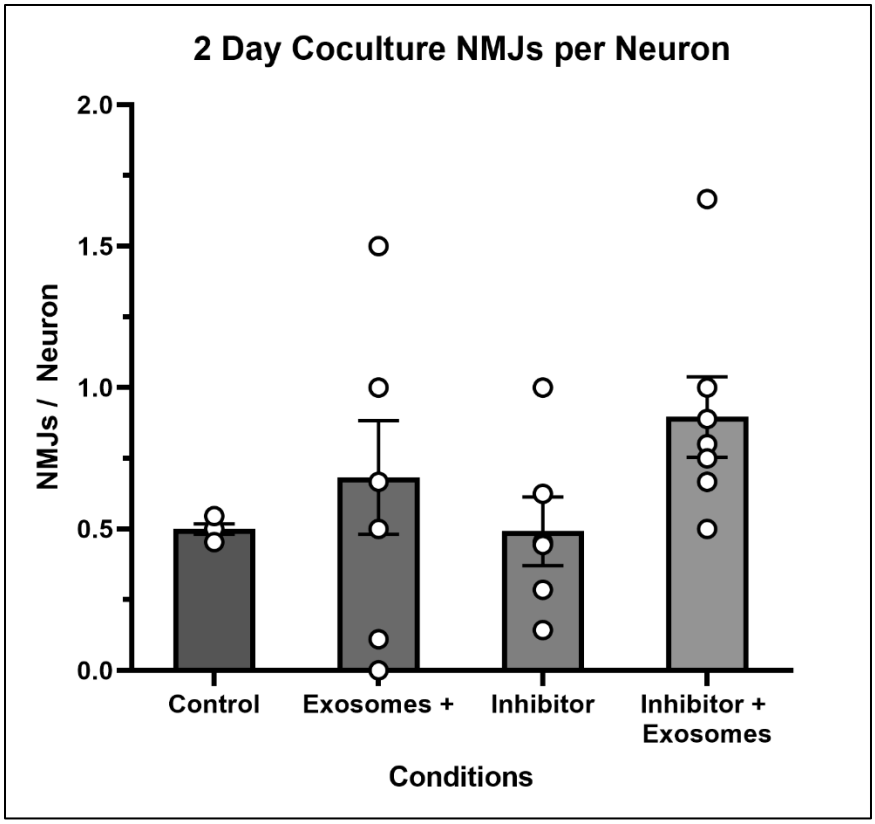

Figure 11. *The ratio of neuromuscular junctions to neurons for conditions after 2 days of differentiation. When cells were treated with both GW4869 nSMase inhibitor and exosomes, there were slightly more NMJs per neuron compared to the control or GW4869 treatment alone. The range in data points from exosomes added to cells is too high to draw any significance from.*

DISCUSSION

Exosomes have recently garnered the attention of researchers for their role in a variety of diseases and cell behaviors. Recent developments have begun to unravel the role exosomes play in the formation of neuromuscular junctions which requires intense communication between neuronal and muscle cells. However, the specific role and extent that exosomes play in NMJ formation is not yet known. The present study was conducted to develop and test an ultracentrifugation method for exosome isolation and study. Furthermore, the study aimed to investigate the effects of exosomes and a common pharmaceutical exosome inhibitor on the development of a neuromuscular coculture.

Ultracentrifugation serves as a robust platform for exosome isolation, as relatively large sample volumes may be processed with high yields. However, one drawback from ultracentrifugation is the difficulty in resuspending the exosome pellet, and the aggregation of exosomes due to high g-forces and long spin times. The initial exosome isolate had a high size activity in excess of 2000 nm, which questioned the purity of the sample preparation. To account for this, the sample was tested with both sonication, and filtration steps. Both steps substantially reduced the activity of larger sizes; however, a broadening of the “exosome peak” was observed to include activity from intermediate sizes (300-1000 nm). Some plausible explanations may account for this observation. In the case of sonication, the larger aggregates may have been only partially broken apart, leaving smaller aggregates present. During filtration, forces may have been sufficient to push small aggregates through pores, leading to heterogeneity. Studies using transmission electron microscopy would best support the aforementioned explanations and confirm if the larger sized peaks are indeed due to aggregation of exosomes. Nevertheless, to account for aggregation in exosome samples obtained with ultracentrifugation, either sonication or filtration offer potential solutions, albeit with limitations. Filtration is less likely to damage the structural integrity of exosomes, although a loss of yield would be expected due to exosomes remaining in the membrane. Sonication may avoid excessive losses, but can damage or lyse exosomes. Some evidence of lysing was observed following sonication, evidenced by size activity below the exosome range.

Beyond isolating exosomes, this project aimed to investigate their relationship in the formation of the neuromuscular junction. By adding additional exosomes or inhibiting their biogenesis, their role in NMJ development could be investigated. The inhibitor chosen was GW4869, which inhibits nSMase and therefore the hydrolysis of sphingomyelin to ceramide. It was chosen as it is a cell permeable pharmaceutical agent commonly used to inhibit the ceramide mediated biogenesis of exosomes. The exosomes added were previously isolated from cocultured cells. We hypothesized that following treatment with GW4869, adding exosomes would reverse any effects the treatment had, as exosomes would be restored to the media. First, the amount of exosomes generated by cells in each condition was compared by measuring the protein concentration of exosome isolations. As protein concentration scales with the amount exosomes in a purified fraction, relative comparisons can be made. We found that the inhibitor only slightly reduced exosome generation as opposed to stopping biogenesis. Although this may be due to adding an insufficient quantity of inhibitor, it is not surprising as the ceramide mediated pathway for exosome biogenesis is not the canonical ESCRT pathway. While GW4869 is a commonly used exosome inhibitor, its effectiveness is not consistent across cells.

An interesting observation was seen when a coculture was treated with both exosomes and the inhibitory agent. Either condition on their own did not significantly change morphological parameters such as neurites per neuron or the number of NMJs per neuron; however, treatment with both resulted in a noticeable increase in the differentiation of neurons, and a slight increase in the number of NMJs per neuron. This was an unexpected result, but a plausible explanation may lie with possible changes of the levels of sphingomyelin and ceramide, which could affect differentiation. Sphingomyelin and ceramide are lipids which are present in the cell membrane, and whose relative amounts are known to change during neuronal differentiation. A study showed that during the initial days of differentiation in PC12 cells, there is around 300% the normal levels of sphingomyelin in cell membranes, and that this increase may play a role in neurite outgrowth as it is a structural component of membranes (Kagan et al., 2022). Similarly, the researchers found that changes in ceramide levels are also involved in differentiation. Ceramide may be generated from the hydrolysis of sphingomyelin as previously described, but may also be synthesized de novo in cells (McCluskey et al., 2022). Therefore, while the nSMase

inhibitor blocks the conversion of sphingomyelin to ceramide in the cell membrane, it will not affect the synthesis of new ceramide. As the cell has the ability to still produce ceramide, treatment with the inhibitor alone may not have produced dramatic effects on differentiation compared to the control. Exosomes are known to be highly enriched in sphingolipids such as ceramide and sphingomyelin (Verderio et al., 2018). As such, treatment of cells with exosomes would be expected to introduce a large amount of sphingolipids to cells, which would be expected to enhance differentiation. However, nSMase activity may be metabolizing much of the extra sphingomyelin to ceramide, and thus limiting the utility of adding exosomes on their own. When both the exosomes and the inhibitor are added, cells are being given large amounts of sphingomyelin and ceramide but are not able to effectively break down the extra sphingomyelin. This mechanism may help fuel the additional neurite growth observed. A simple diagram is presented outlining the proposed model for the aforementioned observations (Fig. 12).

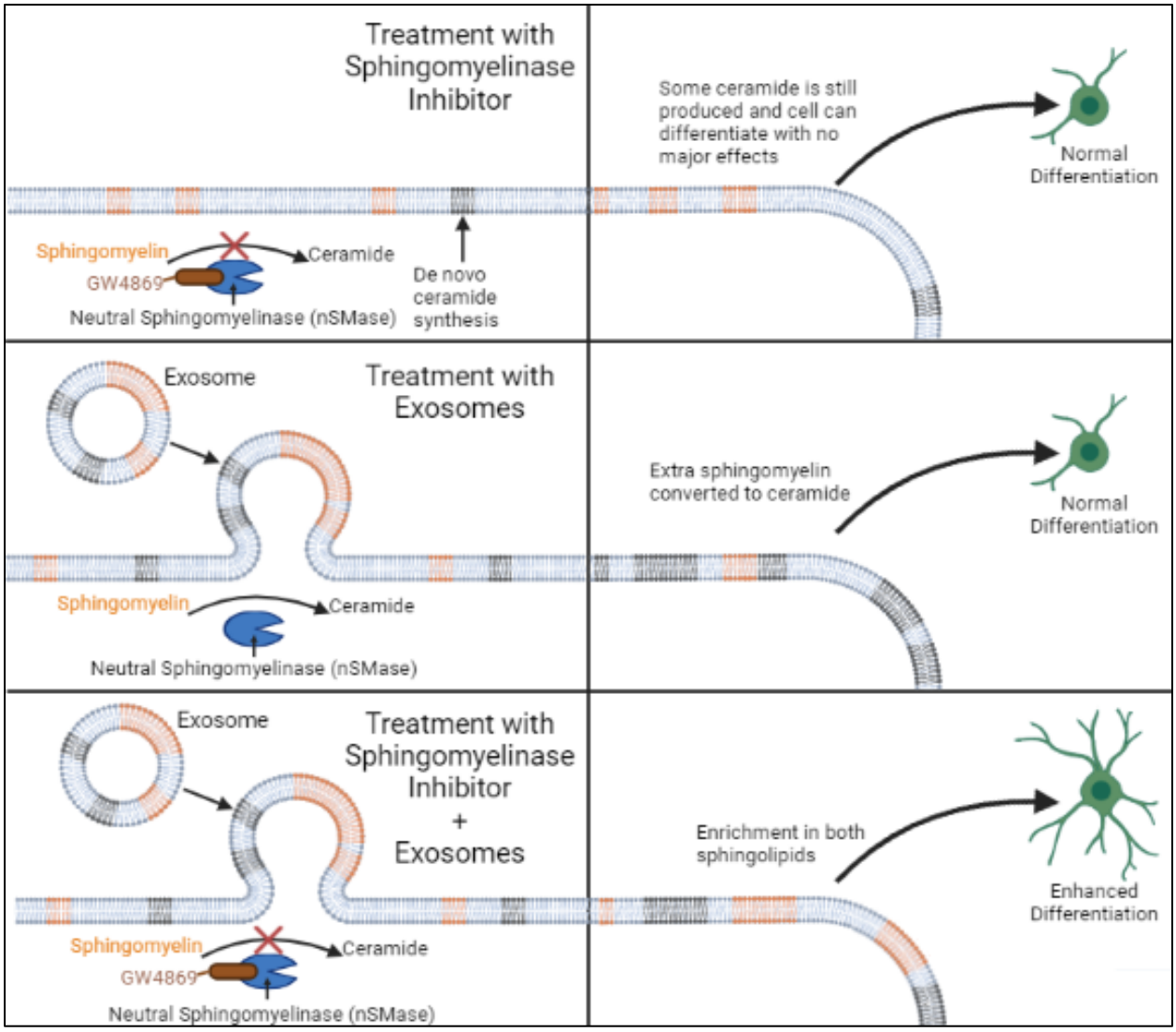

Figure 12. *Proposed model for the observed effects on neuronal development based on hypothesized changes in sphingomyelin and ceramide. Treatment with exosomes or a sphingomyelinase inhibitor either alone or together may alter the amount of sphingolipids leading to changes in neuronal differentiation.*

The presence of muscle cells in coculture may also present an additional confounding variable acting by some unknown mechanism. Further study would be needed to add or withdraw support to this hypothesis; investigating the lipid content of cellular membranes under the tested conditions may help elucidate the roles played by sphingomyelin and ceramide.

CONCLUSION

We demonstrated the isolation and size characterization of exosomes, and an application towards neuromuscular coculture. Our results show that exosomes isolated by ultracentrifugation are prone to aggregation, and that sonication or filtration is a viable option to break apart aggregates. Further study to optimize dispersion of exosome aggregates without damaging their shape or function would improve exosome studies and potential uses. In addition, the application of exosomes and the effects of the nSMase inhibitor GW4869 was observed on co-cultured cells. Treatment with GW4869 slightly inhibited exosome generation but did not significantly affect neuronal differentiation and NMJ formation. The addition of exosomes did not significantly alter these morphological parameters, but when added with the inhibitor improved neuronal differentiation. This may be due to changing sphingolipid amounts in cellular membranes, which is an avenue for future work to study. These studies may include characterizing lipid contents of exosomes derived from neuromuscular coculture, and the change of lipid contents in cocultures following GW4869 and exosome treatment. Furthermore, investigating the specific protein or mRNA content of exosomes from a neuromuscular co culture may help guide their therapeutic uses.

REFERENCES

Bahrini, I., Song, J.-hoon, Diez, D., & Hanayama, R. (2015). Neuronal exosomes facilitate synaptic pruning by up-regulating complement factors in microglia. *Scientific Reports*, *5*(1). https://doi.org/10.1038/srep07989

Dinkins, M. B., Dasgupta, S., Wang, G., Zhu, G., & Bieberich, E. (2014). Exosome reduction in vivo is associated with lower amyloid plaque load in the 5XFAD mouse model of alzheimer's disease. *Neurobiology of Aging*, *35*(8), 1792–1800. https://doi.org/10.1016/j.neurobiolaging.2014.02.012

Essandoh, K., Yang, L., Wang, X., Huang, W., Qin, D., Hao, J., Wang, Y., Zingarelli, B., Peng, T., & Fan, G.-C. (2015). Blockade of exosome generation with GW4869 dampens the sepsis-induced inflammation and cardiac dysfunction. *Biochimica Et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1852*(11), 2362–2371. https://doi.org/10.1016/j.bbadis.2015.08.010

Hessvik, N. P., & Llorente, A. (2017). Current knowledge on exosome biogenesis and release. *Cellular and Molecular Life Sciences*, *75*(2), 193–208. https://doi.org/10.1007/s00018-017-2595-9

Jimsheleishvili, S., Marwaha, K., Sherman, Al. (2022). *Physiology, Neuromuscular Transmission.* https://www.ncbi.nlm.nih.gov/books/NBK541133/

Kagan, T., Stoyanova, G., Lockshin, R. A., & Zakeri, Z. (2022). Ceramide from sphingomyelin hydrolysis induces neuronal differentiation, whereas de novo ceramide synthesis and sphingomyelin hydrolysis initiate apoptosis after NGF withdrawal in PC12 cells. *Cell Communication and Signaling*, *20*(1). https://doi.org/10.1186/s12964-021-00767-2

Korkut, C., Li, Y., Koles, K., Brewer, C., Ashley, J., Yoshihara, M., & Budnik, V. (2013). Regulation of postsynaptic retrograde signaling by presynaptic exosome release. *Neuron*, *77*(6), 1039–1046. https://doi.org/10.1016/j.neuron.2013.01.013

Li, L., Xiong, W.-C., & Mei, L. (2018). Neuromuscular junction formation, aging, and disorders. *Annual Review of Physiology*, *80*(1), 159–188. https://doi.org/10.1146/annurev-physiol-022516-034255

Li, P., Kaslan, M., Lee, S. H., Yao, J., & Gao, Z. (2017). Progress in exosome isolation techniques. *Theranostics*, *7*(3), 789–804. https://doi.org/10.7150/thno.18133

McCluskey, G., Donaghy, C., Morrison, K. E., McConville, J., Duddy, W., & Duguez, S. (2022). The role of sphingomyelin and ceramide in motor neuron diseases. *Journal of Personalized Medicine*, *12*(9), 1418. https://doi.org/10.3390/jpm12091418

Men, Y., Yelick, J., Jin, S., Tian, Y., Chiang, M. S., Higashimori, H., Brown, E., Jarvis, R., & Yang, Y. (2019). Exosome reporter mice reveal the involvement of exosomes in mediating neuron to astroglia communication in the CNS. *Nature Communications*, *10*(1). https://doi.org/10.1038/s41467-019-11534-w

Novak, J. (2022). *Understanding How Mechanical Stress Affects Calcium Release in Co-Cultured Neuronal and Smooth Muscle Cells.* Worcester Polytechnic Institute.

Skinner, C. (2021). *The Effects of Stretch on Intracellular Ca^{2+} Signaling in Cocultured Neuronal and Smooth Muscle Cells*. Worcester Polytechnic Institute.

Teng, F., & Fussenegger, M. (2020). Shedding light on extracellular vesicle biogenesis and bioengineering. *Advanced Science*, *8*(1), 2003505. https://doi.org/10.1002/advs.202003505

Verderio, C., Gabrielli, M., & Giussani, P. (2018). Role of sphingolipids in the Biogenesis and biological activity of extracellular vesicles. *Journal of Lipid Research*, *59*(8), 1325–1340. https://doi.org/10.1194/jlr.r083915