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Calcium and Proteasomal Regulation of ER-to-Golgi Protein Transport in Neurons

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Abstract
Regulatory mechanisms of protein trafficking from the endoplasmic reticulum (ER) are critical to understand since neurodegenerative diseases involve defects in this process leading to chronic ER stress and cell death. This study aimed to better understand the calcium regulatory mechanisms of ER-to-Golgi trafficking in hybrid neuroglioblastoma cells (NG108). Specifically, we asked whether proteasomal degradation of transport machinery was involved in the previously demonstrated upregulation of ER-to-Golgi transport evoked by calcium signaling. Based upon previous literature, we believe that the calcium induced increase in transport is due to the activation of the calcium sensitive regulatory protein apoptosis linked gene 2 (ALG-2) and its interactions with the inhibitory regulatory protein peflin. A potential mechanism is that after calcium induced ALG-2 dissociation from peflin, peflin is degraded by the proteasome and the removal of its inhibitory effects cause the increase in transport. This hypothesis was tested by NG108 transfection with a fluorescent cargo protein engineered to allow inducible, synchronous trafficking from the ER. Cargo transport was completed for 15 minutes and the cells were subsequently fixed and immunolabeled for detection of the Golgi complex. Cells were imaged on a widefield microscope and the images were analyzed to quantitate the transport index, a measure of the rate of cargo transport. Prior to transport cells were treated with the calcium agonists histamine or ATP and/or the proteasome inhibitor MG132. Results indicated that calcium signaling caused an increase in cargo transport, as expected. Importantly, inhibition of the proteasome caused a decrease in cargo transport. However, treatment with both a calcium agonist and the proteasome inhibitor caused an increase compared to treatment with just the proteasome inhibitor. Our new results show that proteasomal activity is required for optimum transport but is not involved in the calcium stimulation of transport per se. This suggests that proteasomal activity and calcium fluctuations function to regulate transport rates independently.

Introduction

Coat-protein II (COPII) coated vesicles are the major mode of protein export from the endoplasmic reticulum (ER) (D'Arcangelo et al., 2013). Approximately one-third of all proteins are exported through this constitutive pathway (Guo et al., 2006). The major components of the COPII machinery are the GTPase, Sar1, the inner coat protein heterodimer of Sec23/24, and the outer coat protein heterodimer of Sec13/31 (D'Arcangelo et. al, 2013). The GTPase, Sar1, completes the first activation step of the machinery. Once cargo is present for transport, Sar1 recruits heterodimers of Sec23/Sec24. These proteins form the inner coat of the COPII machinery which binds to the cargo and fills the vesicle. Once the vesicle has been filled the outer coat of COPII is recruited. The outer coat consists of Sec 13 and Sec 31. This heterodimer is large and has a bent conformation due to the shape of Sec31. This bent conformation induces curvature of the ER membrane and eventually causes budding and scission of the vesicle (D'Arcangelo et. al, 2013).

Two other proteins of relevance are the Penta-EF-hand (PEF) proteins apoptosis linked gene 2 (ALG-2) and Peflin (Maki et al., 2002). These proteins are known to form a heterodimer

that dissociates in a calcium (Ca^{2+})dependent manner (Kitaura et al., 2001, LaCour et al., 2013). Both ALG-2 and Peflin have been associated with the regulation of ER protein export at ER exit sites (ERES) (Rayl et al., 2016, Sargeant et al., 2021). ALG-2 was originally identified, and later characterized, by Shibata et al (2007, 2019) as well as Kanadome et al., 2017. The role of Ca^{2+} and Peflin was not understood until Ca^{2+} dependent effects of peflin were outlined in Sergeant et al., 2021. One hypothesis was that after dissociation from ALG-2, Peflin is degraded by the proteasome through ubiquitination. That would promote formation of ALG-2 homodimers, which promote ER export by stabilizing Sec31A at ER exit sites. While many mechanisms have been proposed of the impact of ubiquitination on ER export, no clear answer has yet emerged from these studies. (Akopian et al., 2022, Gonzalez et al., 2023, Kikuma et al., 2019, Mansur et al., 2023)

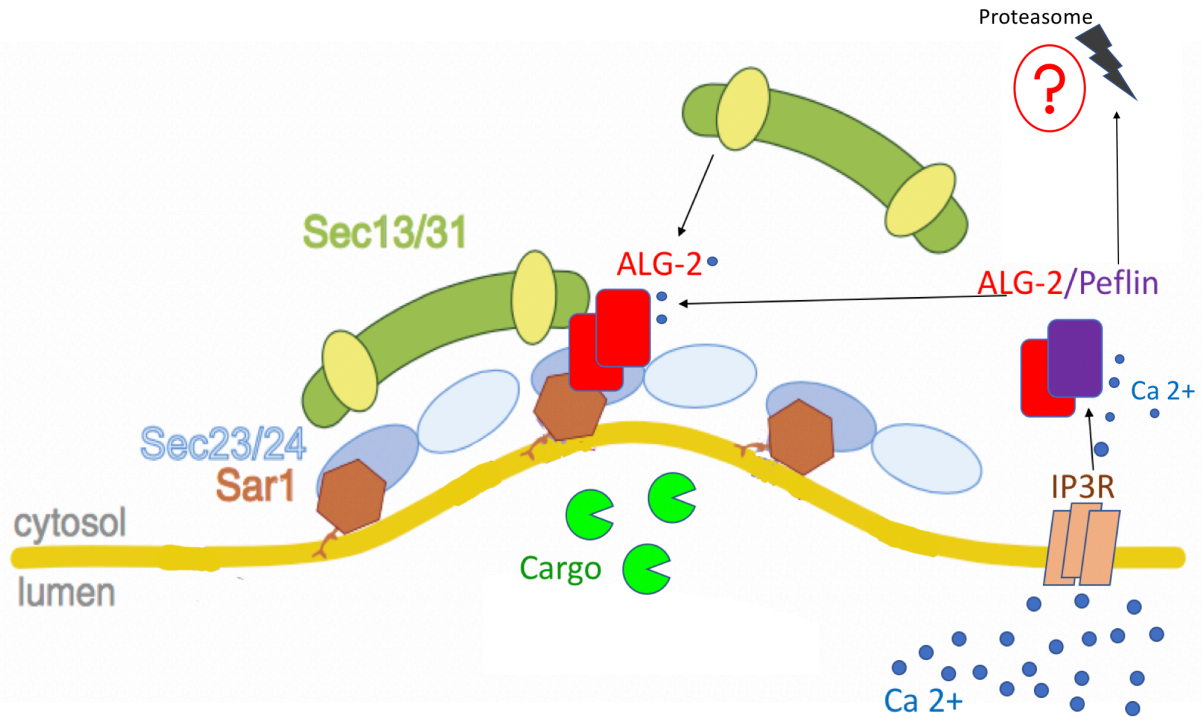

Figure 1: Diagram of COPII coat and ERES adapted from D'Arcangelo et. al, 2013. Sar1, a GTPase, recruits the inner coat of COPII composed of heterodimers of Sec23 and Sec24. The outer coat of COPII composed of Sec13 and Sec31 is bound next and is stabilized by the homodimer of ALG-2. ALG-2 and Peflin, which are both Ca^{2+} sensitive PEF proteins, normally exist as a heterodimer. When Inositol triphosphate (IP3, not shown) binds to the inositol triphosphate receptor (IP3R), Ca^{2+} is released from the ER to the cytosol. This Ca^{2+} then binds to the ALG-2/Peflin heterodimer causing dissociation and the formation of the ALG-2 homodimer, which aids in the recruitment of Sec13/31. The shape of the Sec13/31 heterodimer is what forces the membrane of the ER at an ERES to begin to bubble and form a vesicle that will be transported to the Golgi.

The function of Ca^{2+} regulation in intracellular trafficking has been examined multiple times. The largest compiled work of understanding today is Sargeant & Hay (2022) which reviews the mechanisms by which Ca^{2+} fluctuates ER export. They outline that Ca^{2+}, which is normally at very low levels in the cytosol, can be increased through uptake of Ca^{2+} from the extracellular environment or through release from intracellular stores, such as the ER. When cytosolic Ca^{2+} levels increase this causes the dissociation of the ALG-2/Peflin heterodimer. This changes the rate of ER export in one of two ways. If Peflin is present at high levels the rate of ER export is decreased (Rayl et al., 2016). If ALG-2 is present at high levels then the transport rate is increased. This is because Sec31 possesses a binding motif for ALG-2. This makes ALG-2 capable of stabilizing Sec31, and thereby the entire outer coat, at ERES. Sec31 has a long, bent structure which is thought to be a part of the process of vesicle scission at ERES (Sargeant & Hay 2022, Shibata et al., 2019).

The characteristics of the Ca^{2+} fluctuations also change the ways in which transport is regulated. As outlined in Sargeant & Hay, 2022, continuous Ca^{2+} increases have a different effect of ER to Golgi transport than discontinuous Ca^{2+} pulses. They also propose that another regulator must be present but do not propose what this regulator is or its mechanisms. This work of identifying the roles and regulators of the COPII coat by Ca^{2+} has largely been completed in epithelial cell lines thus far.

Additional COPII studies have been confounded by the fact that the COPII coat was thought to form 50 nm vesicles but these small vesicles cannot explain the transport of large proteins such as procollagen, which is 200nm long and is COPII dependent. Malis et al. (2022) and Shomron et al. (2021) discuss a modified conceptual arrangement of the COPII machinery in which they outline that the COPII coat forms 50 nm vesicles at ERES but quickly uncovers the vesicles leaving room for expansion while COPII forms a “collar” which regulates protein intake to these vesicles. This does not require a major modification of the understanding of the functions and mechanistic changes of the COPII coat and ER to Golgi transport.

Halperin et al., 2019, outlined that mutations in the critical COPII outer coat component Sec31 cause a neurological syndrome. This has spurred the investigation of the constitutive secretory pathway in neuronal cell lines as well as the implication of the constitutive secretory pathway in neurological diseases. They outline two cases of severe developmental disorders, which resulted in fatalities by the age of 4, for two human cases. They found that the mutation may have been linked to inbreeding and measured brain volumes with MRI and further investigated the mutation in *Drosophila melanogaster*, a common neurological model organism. Alpha-synuclein, a pathogenic element of the neurodegenerative Parkinson’s disease, is known to block ER-to-Golgi traffic, a further link of COPII to neurological syndromes (Cooper et al., 2006). Additionally, protein secretion rates have been shown to be increased in various cancers and the elucidation of this pathway will help to further our understanding of these diseases and increase our ability to treat them in the future. The further study of ALG-2, Peflin, and the Ca^{2+} regulation of ER to Golgi transport is important for further research and the eventual implications in human pathologies.

Previously the COPII coat and its regulators, the calcium (Ca^{2+}) sensitive proteins ALG-2 and Peflin, have been studied in epithelial cell lines (NRK cells) but not in neuronal cell lines. This study investigates the Ca^{2+} and proteasomal regulation of transport in NG108 cells. NG108 cells are a hybrid cell line of mouse neuroblastoma cells and rat glioma cells. Both of these cell lines are derived from neurological cancers. NG108 cells exhibit qualities typical of neuronal cells but, due to their growth capabilities, can be cultured in lab conditions.

ALG-2 is a known binding partner of the Sec13/Sec31 heterodimer and has been shown to stabilize the complex at ERES and increase the secretion rate of cargo proteins in epithelial cells. The first goal of this study was to confirm that this phenomenon is consistent between neuronal cells and epithelial cells. To conduct these studies we used fluorescently tagged cargo proteins which were transfected into cultured NG108 neurons. These GFP-tagged cargos are transport-inducible. This means that they only undergo intracellular transport in the right conditions. By using a specific 15 minute transport step the rate of transport can be measured by fluorescence microscopy and a brightness-based assay of Golgi and ER brightness. This allows for a comparison of conditions between the cells and different drug treatments. By administering adenosine triphosphate (ATP) and histamine extracellularly various intracellular cascades can be initiated which induce the release of Ca^{2+} from stores within the ER causing an increase in the level of intracellular Ca^{2+}, which normally is at very low levels. Additionally this study investigated the role of Peflin degradation by the proteasome in ER-to-Golgi transport. The proteasome inhibitor MG132 was administered to the cells and the same transport assay was performed on these cells.

The hypotheses tested were as follows: 1) increases in cytosolic Ca^{2+} by extracellular treatment with ATP and Histamine will cause an increase in cargo transport due to the increased recruitment of ALG-2 and reduced peflin recruitment to ERES; 2) inhibition of the proteasome with MG132 will inhibit peflin turnover, increasing peflin at ERES and inhibit cargo transport; 3) treatment with both MG132 and ATP or Histamine will still cause a decrease in cargo transport, because the higher levels of peflin caused by proteasome inhibition will still inhibit transport at ERES, blocking the Ca^{2+} regulation. As will be outlined below, the first 2 predictions were supported by the data, whereas the third was not. Instead, the data favor a model in which Ca^{2+} regulation of transport and proteasomal regulation of transport operate independently of each other.

Methods and Materials

For transport experiments NG108 cells were grown in DMEM with 4.5 g/L glucose containing 10% fetal calf serum on poly-L-Lysine treated glass coverslips at 37°C. After 2-3 days of normal growth at 37°C, the cells were transfected with 1 μg of DNA coding for the cargo and 3 μL of polyjet per well. Cells were allowed to recover 24 h before transport experiments. For experiments using temperature sensitive vesicular stomatitis virus glycoprotein (VSV-G_{ts}-GFP) the cells were placed in a 41°C incubator post transfection. Experiments with FM4-linked VSV-G_{tm} (FM4-VSV-G_{tm}-GFP) remained at 37°C.

After 24 hours of cargo expression, transport was performed. To induce transport, VSV-G_{ts}-GFP transfected cells were placed in pre-equilibrated medium at 32°C in a 32°C incubator for 15 minutes. FM4-VSV-G_{tm}-GFP-transfected cells were placed in medium containing 500 nM D/D-solubilizer. The cells were fixed by dropping coverslips directly into

fixative for the zero transport condition or transferred to fixative (4% paraformaldehyde, 0.1 M sodium phosphate pH 7.0) after the 15 minutes of transport were completed. Conditions with drug treatments had ATP (1 μM), histamine (100 μM), or MG132 (500 nM) added to the medium 2.5 hours before transport as well as to the transport wells. For the washout conditions in Figure 2, cells were exposed to the drug for 2.5 hours and then rinsed 3x with PBS before returning to drug free medium for 3.5 hours before transport. After being fixed the coverslips were neutralized with 0.1 M glycine and were then permeabilized with the detergent Triton-x100. After permeabilization the cells were immuno-labeled using anti-mannosidase II antibodies and an anti-rabbit CY-3 secondary antibody. Coverslips were then sealed with nail polish for image collection to be completed within one week

Images were collected in a consistent manner with regard to cell morphology, protein expression levels and exposure. A single wide-field image plane was collected for each color channel (GFP and mannosidase II) for each field of cells randomly encountered. Slides were analyzed using a 60x objective on a widefield microscope with a Cool LED illumination unit, PCO Panda camera, automated using the Micro-Manager software.

Images were analyzed using ImageJ open source image software with automation by a custom script that randomized the images and blindly presented them to the user to avoid bias. The user identified transfected cells and subcellular regions while the script extracted the fluorescence values for the extracellular background, Golgi intensity and diffuse cytoplasmic ER intensity. Transport index was calculated for each individual cell as (Golgi maximum - background) / (ER mean - background), and all extracted parameters were written to an output file along with a cell number and image title so that the data was traceable. Graphs were created in the Kaleidagraph software. Statistical analysis was also performed on the data in the Kaleidagraph software using the student t-test analysis. P values less than 0.05 were considered statistically significant.

Results

Ca^{2+} releasing agents cause increases in ER-to-Golgi transport in NG108s

Previous studies in epithelial cells showed that Ca^{2+} releasing agents could cause an increase in cargo transport. To examine this phenomenon the Ca^{2+} releasing compounds histamine (100μM) and ATP (1μM) were added to the extracellular medium of the NG108 cells for 2.5 hours before the transport protocol. After completion of transport, when compared to control conditions without the drugs, these cells exhibited higher rates of protein transport (Fig. 2, column 2 vs. 3 and 5). Additionally when the cells were treated with a 2.5 hour exposure to the drugs followed by a washout and return to normal medium without the drugs for another 3.5 hours the transport rates transport was significantly decreased when compared to their respective drug conditions, demonstrating that the effects are reversible (Fig. 2 column 2 vs. 4 and 6). This experiment confirms that NG108 cells' ER export rates are regulated by Ca^{2+}. This also suggests that the ALG-2 and peflin interaction mechanisms maintain a consistent regulation between epithelia and neuronal cells.

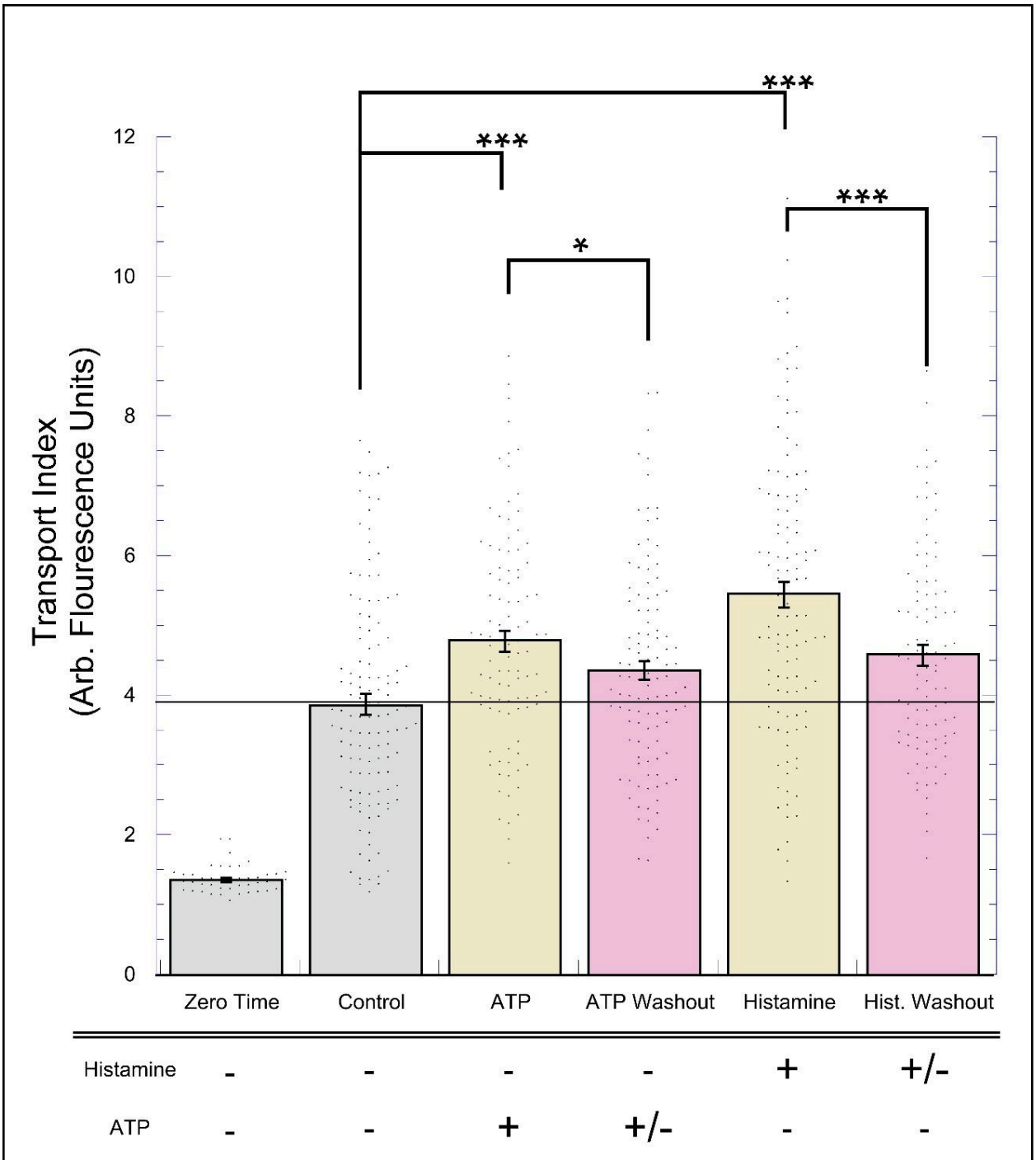

Figure 2: ATP and histamine cause increased cargo transport: Zero Time (41 °C), 15 minute transport control, 2.5 hour treatment with 1μM ATP followed by 15 minute transport, 2.5 hour treatment with 1μM ATP followed by 3.5 hour wash followed by 15 minute transport, 2.5 hour treatment with 100μM histamine followed by 15 minute transport, and 2.5 hour treatment with 100μM histamine followed by 3.5 hour wash followed by 15 minute transport. Asterisks indicate significance, all colored columns are significantly higher than the control. (*** = p <.0005, * = p <.05 student t-test)

Proteasome inhibition causes a decrease in transport but does not render the export system insensitive to Ca^{2+} regulation

To examine the regulation of transport by the proteasome, cells were treated with 2.5 hr exposure to histamine (100µM), MG132 (500nM), or both, prior to the same transport protocol.

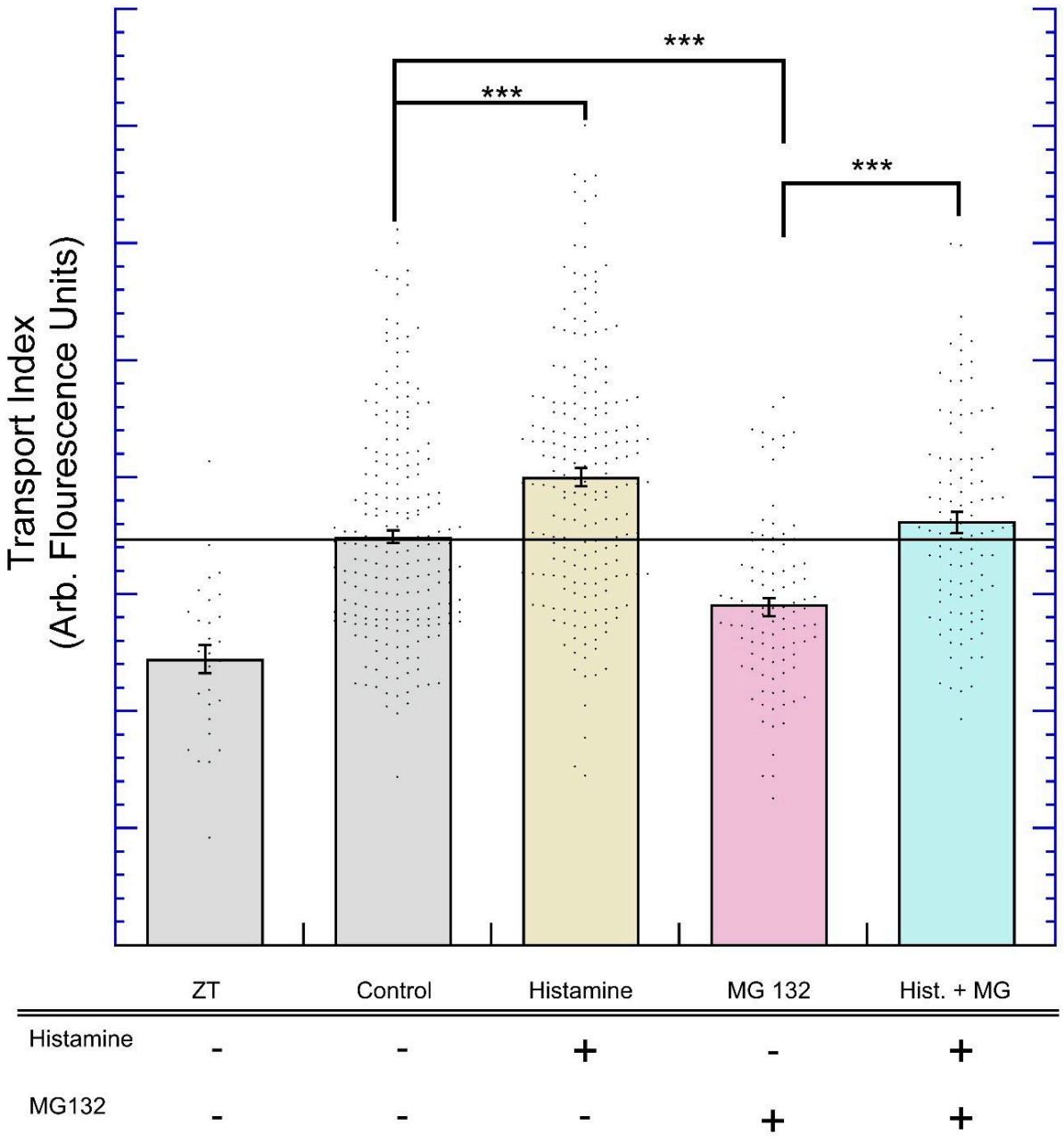

Figure 3: Proteasome inhibition decreases transport but does not prevent Ca^{2+} regulation of transport: Zero Time, control transport, 2.5 hour treatment with 100µM histamine followed by transport, 2.5 hour treatment with 500nM MG132 followed by transport, 2.5 hour treatment with 100µM histamine and 500nM MG132 followed by transport. Asterisk indicates significance compared to control using a student-t test (*** = $p < .0005$)

Treatment with MG132 significantly decreased the rate of protein transport (Fig. 3 columns 2 vs. 4). Treatment with histamine again increased the rate of protein transport relative to the control (fig. 3 columns 2 vs. 3). Interestingly, treatment with both histamine and MG132 caused a significantly increased rate of protein transport compared to treatment with only MG132, showing that the system is still sensitive to regulation by Ca^{2+} release. Treatment with both drugs was not significantly changed compared to the control (fig. 3 columns 2 vs. 5).

Conclusion

This study used transport analysis of neuronal cells to confirm that cytosolic Ca^{2+} increases, caused by the administration of ATP and Histamine, cause an increase in the rate of ER-to-Golgi cargo transport. The proposed mechanism for this effect is the action of the protein ALG-2 and its stabilizing effect on the COPII outer coat protein Sec31 at ERES.

This study also used transport analysis to demonstrate that inhibition of the proteasome by MG132 causes a decrease in the rate of ER-to-Golgi cargo transport. This effect could be due to an elevated presence of Peflin, a negative regulator of ER-to-Golgi transport known to undergo rapid proteasome-dependent turnover (Kitaura et al., 2007). A novel discovery of this study includes that the inhibition of transport, caused by the inhibition of the proteasome and the subsequent increase in Peflin, did not nullify the Ca^{2+} induced increases of transport. This novel data supports the conclusion that ALG-2 and peflin function to regulate transport independently of the function of the proteasome. Free peflin binds to ALG-2 which stops ALG-2 from stabilizing Sec13/31 at ERES and Ca^{2+} causes ALG-2 and peflin to dissociate. When Ca^{2+} causes this dissociation peflin is likely poly-ubiquitinated, but proteasome-dependent degradation of peflin is not required for the increased transport. Important to note is that this investigation did not directly measure peflin levels or poly-ubiquitination, which will be important measurements to confirm the findings and mechanism proposed here.

This investigation helps fill a gap in the knowledge of the ER-to-Golgi transport pathway regulation. The COPII coat and its components have been well understood for years (D'Arcangelo et al., 2013) but its regulation remains poorly understood. Though previous research in this laboratory has outlined some calcium regulatory mechanisms in epithelial cell lines, few experiments until this one investigated calcium transport regulation in neuronal cells. This examination of the effects of Ca^{2+} fluctuations help elucidate some of the regulation of the secretory pathway in neuronal cells. Additionally this study sheds light on the possible mechanisms by which Peflin negatively regulates transport.

To validate the results found here, confirmation with multiple trials and larger sample sizes is necessary. Additionally, future research should confirm the mechanisms by which these changes take place through colocalization assays of ALG-2 and the COPII coat with Ca^{2+} fluctuations. Quantitative analysis of Peflin levels also needs to be completed to confirm that MG132 inhibition of the proteasome does or does not cause an increase in the level of peflin present in the cell. Western blot analysis will provide insight to this. The interaction of Peflin and other proteins is yet to be understood and requires further investigation as Peflin has no known

binding partners other than ALG-2. The interactions of peflin could be further examined through co-immunoprecipitation or Surface Plasmon Resonance Spectroscopy (SPR). Additionally, the mechanisms by which Peflin decreases ER-to-Golgi transport requires more molecular definition.

These future research objectives, combined with the transport data outlined here, further our understanding of the mechanisms and regulation of ER-to-Golgi transport. This investigation may help guide future research in cell culture investigations of the constitutive protein trafficking pathway. Additionally, future neurodegeneration treatments could be based on the results shown here.

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