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Interactions Between Phospholipase C? and Cyclin-dependent Kinase 18 Contribute to the Neuropathogenesis of Alzheimer's Disease

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Interactions Between Phospholipase Cβ and Cyclin-dependent Kinase 18 Contribute to the Neuropathogenesis of Alzheimer's Disease

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Project Advisor:

Professor Suzanne Scarlata

Abstract

Phospholipase Cβ1 (PLCβ1), a critical component of the mammalian phosphoinositide pathway, may moderate binding between tau, the protein associated with neurodegeneration, and cyclin-dependent kinase 18 (CDK18). Previously published literature suggests that CDK18 phosphorylates tau causing it to aggregate. These aggregates comprise neurofibrillary tangles and contribute to the loss of neurite function that are a hallmark of Alzheimer's Disease. We have previously found that PLCβ1 binds to CDK18 and can inhibit its activity. To investigate PLCβ1's role in tau aggregation, fluorescence lifetime image microscopy (FLIM) and number and brightness (N&B) quantification were used. It was hypothesized that interfering with PLCβ1-CDK18 binding would promote CDK18 phosphorylation of tau and subsequently, tau aggregation. We are using FLIM and N&B analysis to determine the impact of PLCβ1 on tau aggregation.

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Acknowledgements

I would like to express my sincere gratitude to Dr. Suzanne Scarlata for supporting my project morally and financially and for patiently offering advice throughout the duration of these experiments. I have learned an immense amount about pursuing research, and her passion for academia is inspiring. I am grateful for her encouragement and access to high-quality laboratory equipment and reagents used in this project.

I also want to thank Dr. Osama Garwain for training me on countless laboratory techniques and pieces of equipment throughout the past two years. Osama immediately welcomed me to the lab at the beginning of my junior year and took a significant amount of his time to answer my questions and thoroughly explain the background of this research to me. Osama was a truly positive and inspirational mentor to me and I hope to one day pass my knowledge onto others as enthusiastically as he has. He has also contributed data to further this project.

Additionally, I would like to thank Lela, Sid, Androniqi, and Kate, members of the Scarlata lab, for all of their mentorship, patience, and advice throughout this project. Without their encouragement and willingness to help me learn, I would not have been able to become nearly as confident in my research skills and learn as much as I have.

Background

Phospholipase Cβ (PLCβ) is the main effector of the G-protein coupled receptor pathway shown in Figure 1. PLCβ1 has been implicated in transmembrane signaling processes that influence cellular responses ranging from hormone secretion, to immune cell activation, to RNA-induced gene silencing (Balla, 2010). Specifically, PLCβ becomes activated by GTP-bound Gαq and Gβγ subunits, which requires a conformational change in the plasma membrane to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and 1,4,5-triphosphate (IP3) in the G protein-coupled receptor pathway. This hydrolysis stimulates diacylglycerol (DAG) to activate protein kinase C (PKC), and this activation stimulates calcium release into the cell lumen (Scarlata et al, 2015). This release of calcium ions impacts the activity of local enzymes sensitive to calcium and has effects on hormone secretion and activation of immune cells (Balla, 2010). Through this process, interactions between PLCβ1 and cyclin-dependent kinase 16 (CDK16) have been found to inhibit cells from entering the S phase of the cell cycle, thereby reducing their ability to proliferate. This offers potential for therapeutic developments targeting cancers such as neuroblastoma (Garwain et al., 2018). These functions of PLCβ have been studied in knockout mice, and mutations in the enzyme have been proven to impact spatial memory, social behavior, and the ability to filter out redundant information from environmental stimuli (Udawela et al., 2017).

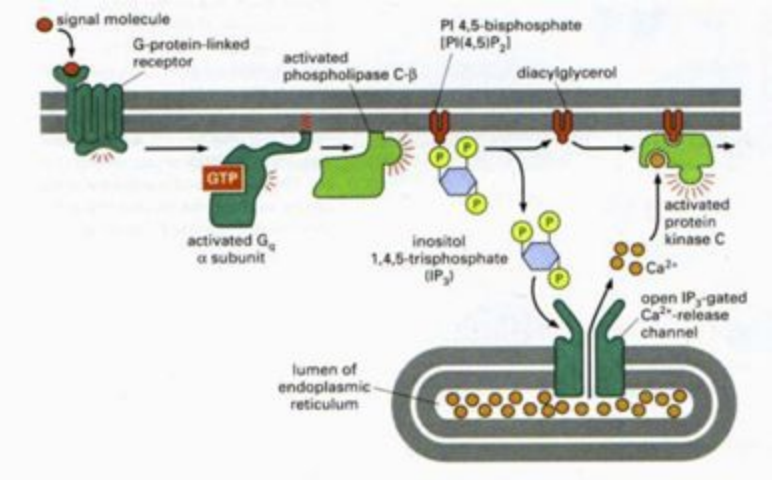


Figure 1: G protein-coupled receptor pathway showing the function of PLCβ1 (SiVaBiochem, n.d.).

In Alzheimer's Disease (AD), synaptic connections among neurons lose their plasticity and therefore become disorganized and dysfunctional (Metaxas & Kempf, 2016). The formation of neurofibrillary tangles (NFTs) contributes to this dysfunction between neuronal synapses, downstream atrophy of the cerebrum, and cognitive decline. Due to this pathogenesis, NFTs are the primary marker of AD (Mak et al., 2018). A higher amount of NFTs is correlated with a more extensive degree of dementia in AD patients due to neuronal loss and dysfunction. These NFTs are composed of hyperphosphorylated monomers of tau protein (shown in Figure 2), as seen in brain samples of AD patients compared to healthy controls. Phosphorylation binds tau proteins causing this accumulation, so the protein kinase and phosphatase processes and members of the signal transduction cascades are research targets to better understand the mechanisms of NFT formation and accumulation. Tau accumulation interferes with microtubule networks within the cellular cytoskeleton and has been shown to cause thinning of tissue in the cortex, which leads to decline in cognition (Brion, 1998); (Mak et al., 2018). Due to tau's extensive role in causing AD, it is an important protein to study in designing therapeutic targets and better understanding the mechanisms of AD.

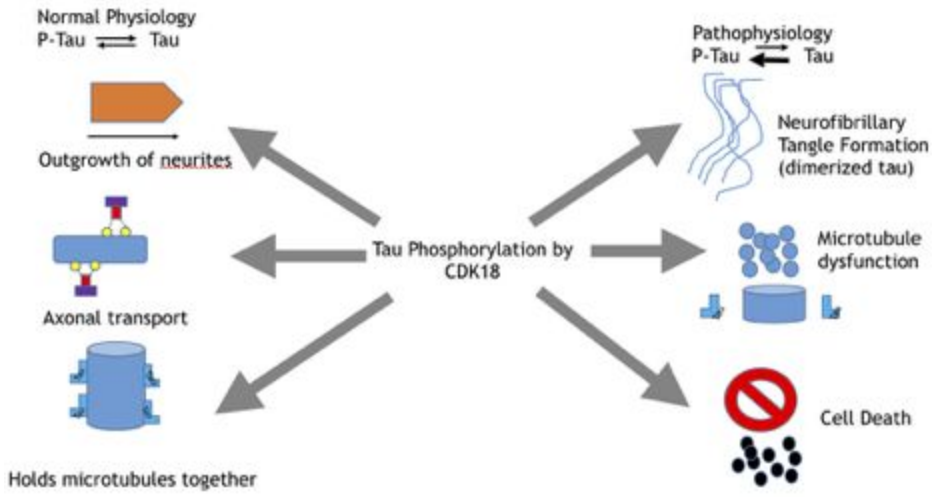


Figure 2: Physiological effects of tau phosphorylation (Adapted from Johnson & Stoothoff, 2004).

AD is characterized by the loss and dysfunction of neurons and synapses, microgliosis, microvascular damage, and the deposition of extracellular amyloid-β proteins, as well as deposits of phosphorylated tau proteins in NFTs. Abnormalities within the cell cycle interfere with cell division and are a neuropathological quality conserved among early-onset familial AD and late-onset sporadic AD (Atwood & Bowen, 2016). According to the mitotic hypothesis of AD, disturbances to the normal cell cycle may promote neurons to undergo apoptosis and lead to neurodegeneration. In a 1996 study, scientists prepared monoclonal antibodies against phospho-epitopes expressed on paired helical filament proteins. Antibodies exhibited immunoreactivity with human neuroblastoma cells in the mitotic phase of the cell cycle, but no reactivity with cells in interphase. Antibody attack was localized to the cytoplasm of these cells. Other antibodies for mitotic phospho-epitopes have shown a strong reaction with NFTs caused by hyperphosphorylated tau proteins in AD, but no reaction in brains of control patients. One possible explanation for this is that mitotic posttranslational mechanisms are activated in AD from the accumulation of NFTs (Vincent & Davies, 1996). Later studies have shown that markers of cell cycle abnormalities may precede the accumulation of plaques and tangles and may account for biochemical, neuropathological, and cognitive changes seen in patients with AD. The formation of new neurons can interfere with previously formed memories and forgetfulness, so any stimulus that causes a cell to re-enter the cell cycle can interfere with the pathways for previously formed memories. When terminally differentiated neurons re-enter the cell cycle, their neurites retract, synapses contract, and neuronal death occurs. Some of the strongest evidence for the mitotic hypothesis of AD came from demonstrations that a significant amount of neurons in the hippocampal pyramidal and basal forebrain neurons, in regions of the brain affected by AD, have completed S phase, evidenced by full or partial replication of DNA. Control brains unaffected by AD in age-matched controls do not show these DNA abnormalities. Then, cells remain polyploid, since mitosis does not progress. A diagram of this cycle is shown in Figure 3.

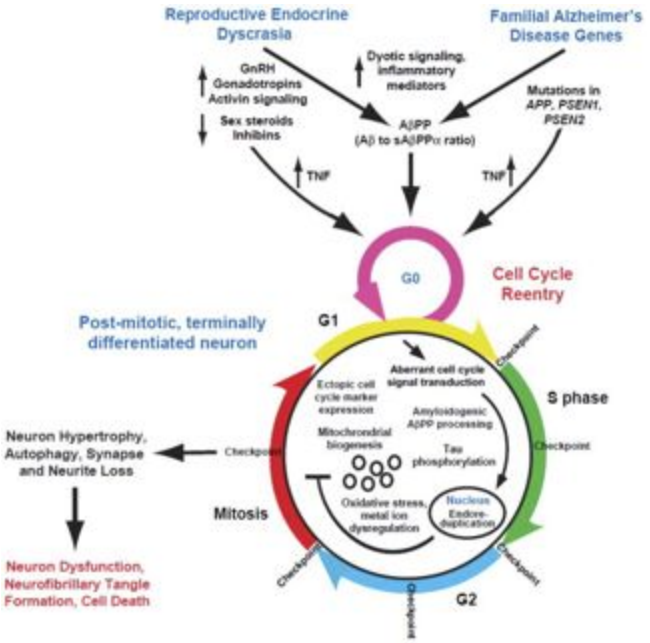


Figure 3: Diagram of the cell cycle, implicated in the mitotic hypothesis of AD (Vincent & Davies, 1996).

CDK18, also known as PCTAIRE3, is a member of the cdc2 protein kinase family. It is measured in elevated levels in the temporal cortex, the primary area of NFT accumulation, of patients with AD compared to healthy controls. In disease patient samples, CDK18 is found to be concentrated within NFTs, and previously published data have suggested that overexpression of CDK18 may indirectly cause the pT231 and pS235 sites on tau to become phosphorylated, which leads to the accumulation of tau proteins into NFTs implicated in cognitive decline (Herskovits & Davies, 2006). An NFT is shown in Figure 4 below.

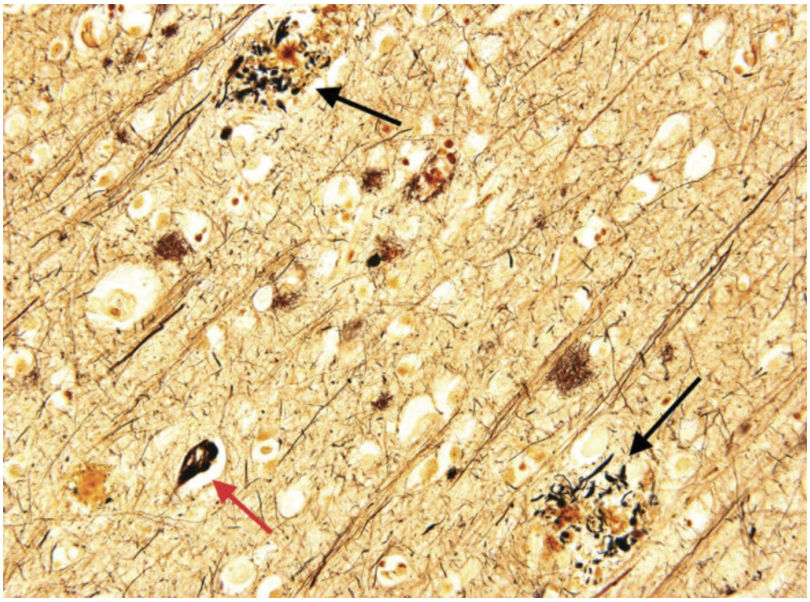


Figure 4: Temporal cortex of Alzheimer's patient with a neurofibrillary tangle shown by the red arrow (Perl, 2010).

Förster Resonance Energy Transfer (FRET) microscopy allows for the study of interactions between cells. This technique measures energy transfer between an excited donor fluorophore and a non-excited acceptor fluorophore by intermolecular dipole-dipole coupling. This allows for the measurement between molecules on the level of angstroms. It is important to choose fluorescent proteins with significantly different, yet overlapping, excitation spectra for efficient energy transfer to occur, which led to the use of mCherry and eGFP labels in the experiments described in this paper. Using FRET, protein movement and interactions within cells can be quantified. FRET microscopy is versatile and can be used in living cells or fixed cells. If there is effective energy transfer, the channel signal from the donor can be reduced and the acceptor channel signal can be intensified to map molecular associations (Sekar & Periasamy, 2003).

For FRET to occur, three conditions must be met. First, the excitation spectrums of the donor and acceptor molecules must overlap to ensure that the observed molecules have compatible energy levels. A graph illustrating this overlap is shown in Figure 5. Second, the

molecules must be within close proximity on the nanometer scale with a radius small enough for energy transfer to occur. This is known as the Förster radius. Lastly, molecules must be appropriately oriented relative to each other.

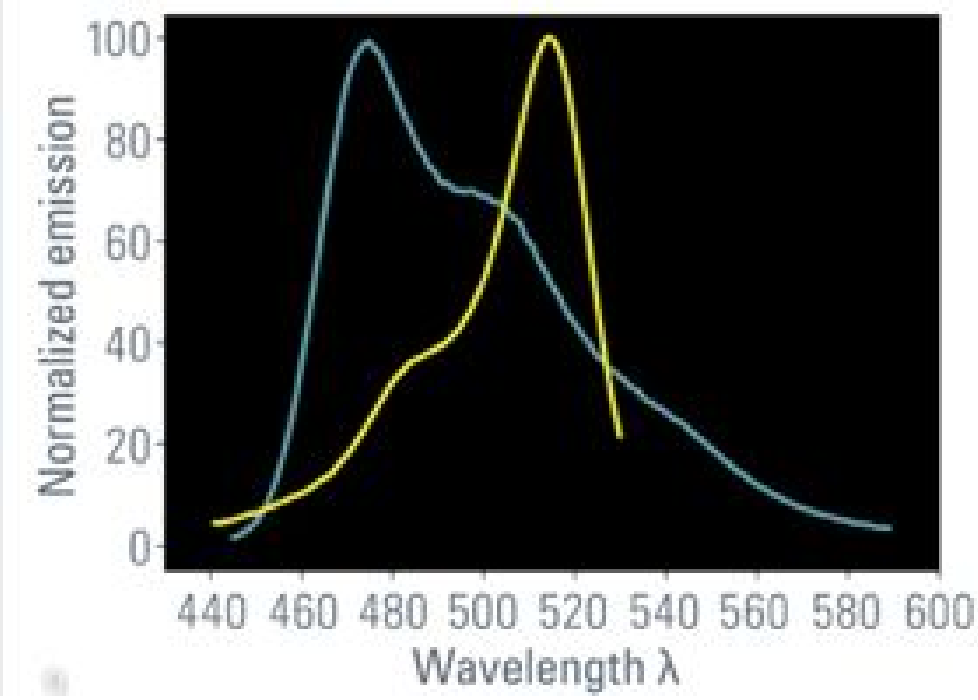


Figure 5: Ideal overlap of excitation spectrums between donor and acceptor molecules in FRET.

Fluorescence Lifetime Imaging Microscopy (FLIM) is one way to measure FRET and is the type of FRET used throughout this project. It accomplishes this lifetime quantification by detecting the amount of time a fluorophore remains excited before it returns to its ground energy state and emits a fluorescence photon. A molecule's time in its excited state (fluorescence lifetime) depends on the molecule's atomic composition and the environment around the fluorophore. Therefore, measuring a molecule's time in its excited state can provide information about its spatial distribution, binding interactions, and biochemical environment. One downside to using FRET as an independent analysis too is that not all donor molecules will transfer their energy to an acceptor molecule. Then, the non-interacting donor molecules will reduce the

accuracy of the results. Therefore, it is important to combine FRET-FLIM imaging with other techniques such as number and brightness analysis.

Measuring the average number and brightness (N&B) of molecules can provide valuable information about protein concentrations, localization, and interactions through biological processes (Papini & Royer, 2018); (Digman et al., 2008). In the aggregation of tau residues implicated in AD, quantifying the number and brightness of molecules involved can provide information about the degree and localization of the interactions. In N&B analysis, the number of particles, N, in the volume specified by the microscope's point spread function, is calculated from two moments of distribution, average intensity ($<F>$), and variance (σ^2) with the equation $N = <F>^2/\sigma^2$. Molecular brightness can be calculated using the equation $B = \sigma^2/ < F >$. The cartoon in Figure 6 below illustrates oligomer measurements using N&B.

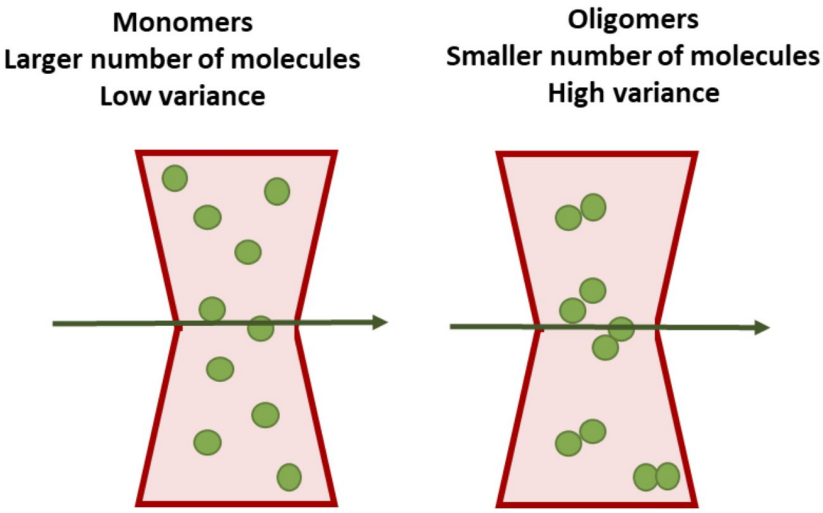


Figure 6: How particle number can give information about oligomer formation in N&B.

Project Purpose

The purpose of this project was to use FRET-FLIM and N&B to investigate the interactions between PLCβ1, CDK18, and tau proteins to elucidate the mechanism of neurofibrillary tangle development and accumulation in neuronal cells. Various transfections were carried out to study how PLCβ1-CDK18 interactions modulated tau accumulation. Tau transfection was carried out using two subunits, tau-10 and tau-11, which, when phosphorylated, combine to complete the eGFP molecule. Therefore, tau aggregation could be visualized and quantified by measuring eGFP fluorescence.

Materials and Methods

Carbachol: The cellular calcium signaling pathway was stimulated using 10 mM carbachol from Sigma.

Cell culture: PC12 cells were seeded onto 2cm poly-D-lysine coated glass covered culture dish from Mat-Tek and kept in Dulbecco Modified Eagle Medium (DMEM) composed of 10% Fobium Fetal Bovine Serum (FBC) and 5% penicillin/streptomycin antibiotic. Cells were split using trypsin for detachment.

Cell type: PC12 cells are derived from a pheochromocytoma of the rat adrenal medulla and can be differentiated into cells similar to neurons. In these experiments, PC12 cells were used as models to investigate protein interactions. PC12 cells were purchased from American Tissue Cell Culture (ATCC) and cultured in DMEM (Corning) with 10% horse serum from PAA (Ontario, Canada), 5% FBS (Atlanta Biological, Atlanta, GA), and 1% penicillin/streptomycin. Cells were incubated in 37 °C, 5% CO2, and 95% humidity.

FRET/FLIM, N&B: Cells were imaged using the ISS Alba FCS/FLIM microscope to detect energy transfer yielding information about protein interactions. Cells were imaged 72 hours post-transfection.

Plasmid Midi-Prep: eGFP-tauBiFC (Bimolecular Fluorescence Complementation) pair constituents, mCherry-CDK18, and unlabeled Gq were prepared using the Qiagen Endo-free Plasmid Maxi-Prep kit and protocols.

Plasmid transfections: eGFP-tau10/eGFP-tau11 (part of a bimolecular fluorescence complementation pair from Addgene, Plasmid #71433 & 71434), mCherry-CDK (Plasmid #23710 from Addgene), and unlabeled Gq (Plasmid #24502 from Addgene) were transfected into PC12 cells using Lipofectamine 3000 (Invitrogen, Inc.). Downregulation of PLCβ1 was completed by transfecting siRNAs specific to PLCβ1 into the dish using Lipofectamine 3000 and

then co-transfecting mCherry-CDK18 and eGFP-tau10, and eGFP-tau11 24 hours later using antibiotic-free media. Carbachol was added in specified trials to increase calcium signaling and activate Gq.

Recombinant Human Beta-Nerve Growth Factor: Cell neurite growth was stimulated using NFG obtained from NovoProtein

Ro 3306: Cyclin-dependent kinase (Cdk1) inhibitor Abcam (ab141491) was a gift from Dr. Osama Garwain at UMass Medical School used to arrest and synchronized cells in the G2/M phase of the cell cycle.

*siRNA(PLC*β1): PC12 cell PLCβ1 expression was decreased due to siRNA obtained from Dharmacon.

Thymidine: 10 mM thymidine from Sigma was added to media for 16 hours, three days in a row to arrest and synchronize cells in the G1/S phase of the cell cycle.

Results

This series of experiments was designed to measure how interactions between PLCβ1 and CDK18 affect tau polymerization within unsynchronized and synchronized (in G2/M) PC12 cells. Since phosphorylation of the eGFP-labeled tau BiFC by CDK18 was expected to cause aggregation, and therefore, eGFP expression, FRET-FLIM was used to measure the proximity between mCherry-CDK18 and aggregated tau labeled with eGFP. Analysis suggests that mCherry-CDK18 binds PLCβ1 overexpressed in PC12 cells to limit tau polymerization compared to the control condition lacking mCherry-CDK18.

The initial trials were completed by transfecting PC12 cells with mCherry-CDK18 and the tau Bi-fluorescence complementation pair (BiFC), which had an average lifetime of 2.015 +/- 0.089 ns (n= 9, standard error 0.02967). A control transfection with the tau BiFC complex and no mCherry-CDK18 showed an average lifetime of 2.032 +/- 0.043 ns (n= 7, standard error = 0.01625). These data showed that the control, as anticipated, had a higher lifetime than the experimental condition; however this lifetime was only minimally higher. It was observed that cells in these two conditions had the NFT fluorescence mainly expressed around the periphery, with lack of expression around the nucleus. This is shown in Figure 7 below.

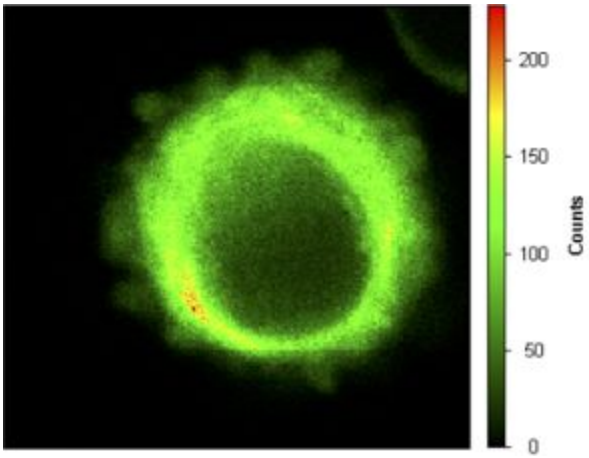


Figure 7 : Fluorescence expressed in a band around the nucleus of an undifferentiated PC12 cell.

When cells were transfected with unlabeled Gq, mCherry-CDK18, and eGFP-tau BiFC complex and then stimulated with carbachol (to activate the constituents of the calcium-signaling pathway including Gq), the average fluorescence lifetime after 72 hours was 2.151 +/- 0.068 (n= 9, standard error 0.022667). Cells in the control condition with mCherry-CDK18, eGFP-tau BiFC complex stimulated with carbachol had an average lifetime of SD 2.126 +/- 0.078 (n= 9, 0.026). Carbachol was expected to activate the calcium signaling pathway within cells to attract PLCβ1 to Gq, freeing CDK18 to phosphorylate tau subunits and cause aggregation. Since the control lifetime was shorter than that of cells transfected with Gq, it seems that Gq actually prevented CDK-tau binding compared to the control. This suggests that binding between CDK18 and tau is transient and minimal after 72 hours. The longer lifetime suggests that CDK bound tau to a lesser extent when Gq was transfected, since fluorescent transfer speed between molecules is inversely proportional to distance between cells. When unlabeled Gq, mCherry-CDK18, and only one tau subunit were transfected, the lifetime increased to SD 2.945 +/- 0.181 ns (n= 5, 0.08095), indicating lack of CDK-tau binding in the control condition. This dramatic increase in lifetime was expected, since only one unit of the BiFC tau complex was used in the transfection, so binding, and subsequently phosphorylation, would occur to a lesser extent and prevent tau aggregation. When cells were differentiated with nerve growth factor (NGF) and co-transfected with mCherry-CDK18 and both subunits of the tau BiFC complex, the average lifetime was 1.90 +/- 0.014 ns (n= 2, standard error 0.009899). These results were likely due to background, baseline fluorescence and were not indicative of tau interactions. In this trial, there was limited growth of neurites, perhaps because the NGF concentration was too low. In addition, two days after NGF was added to the dish, cells were only minimally differentiated, and most of them had died. When cells were transfected with siRNA to downregulate PLCβ1 expression, aggregated clumps were visualized within cells, making accurate data collection difficult. SiRNA specific to PLCβ was expected to increase CDK-tau binding and lower lifetime, since decreased PLCβ1 concentration should reduce binding to mCherry-CDK, freeing CDK to phosphorylate the components of the tau complex and cause aggregation.The measured lifetime for cells transfected with siRNA was 2.087 +/- 0.041 (n=5, standard error 0.01834).

Trials with Gq transfection, which binds PLCβ1 in the G protein signaling pathway, did not have the expected impact on tau phosphorylation. It was hypothesized that Gq would free CDK18 from PLCβ1 to increase phosphorylation and lower the lifetime of eGFP-tau, but this effect was not observed, likely due to the transience of CDK18-tau interactions. When Gq was co-transfected with mCherry-CDK18 and eGFP-BiFC tau complex, the lifetime increased from 2.015 ns (without Gq), to 2.151 ns with the Gq addition. This could be due to Gq expression failing; however, there is still a significant difference between cells‘ lifetimes with and without Gq transfection. Lifetimes are compared across conditions in Figure 11.

According to data previously collected by Dr. Osama Garwain, shown in Figure 8, tau aggregates were significantly brighter, by a factor of almost 8, in neuronal cells with siRNA to down-regulate PLCβ1 expression. Compared to the wild type neuronal cells expressing normal levels of PLCβ1, this increased brightness in the control condition indicates increased tau polymerization. These data suggest that PLCβ1 indirectly limits aggregation of tau. One likely explanation for this is that PLCβ binds CDK18 naturally in cells, however when PLCβ expression decreased, CDK18 is free to bind and phosphorylate tau so that the tauBiFC complex dimerizes and expresses eGFP. The two conditions in Figure 9 compare brightness of tau in neuronal cells with wild-type PLCβ1 expression compared with silenced PLCβ1. The silenced condition shows increased brightness, which is congruous with the data in Figure 8 which as previously mentioned, shows increases in tau brightness when PLCβ1 was silenced. The down-regulation of this protein limits the amount, preventing CDK18 from binding and enabling it to bind and phosphorylate tau.

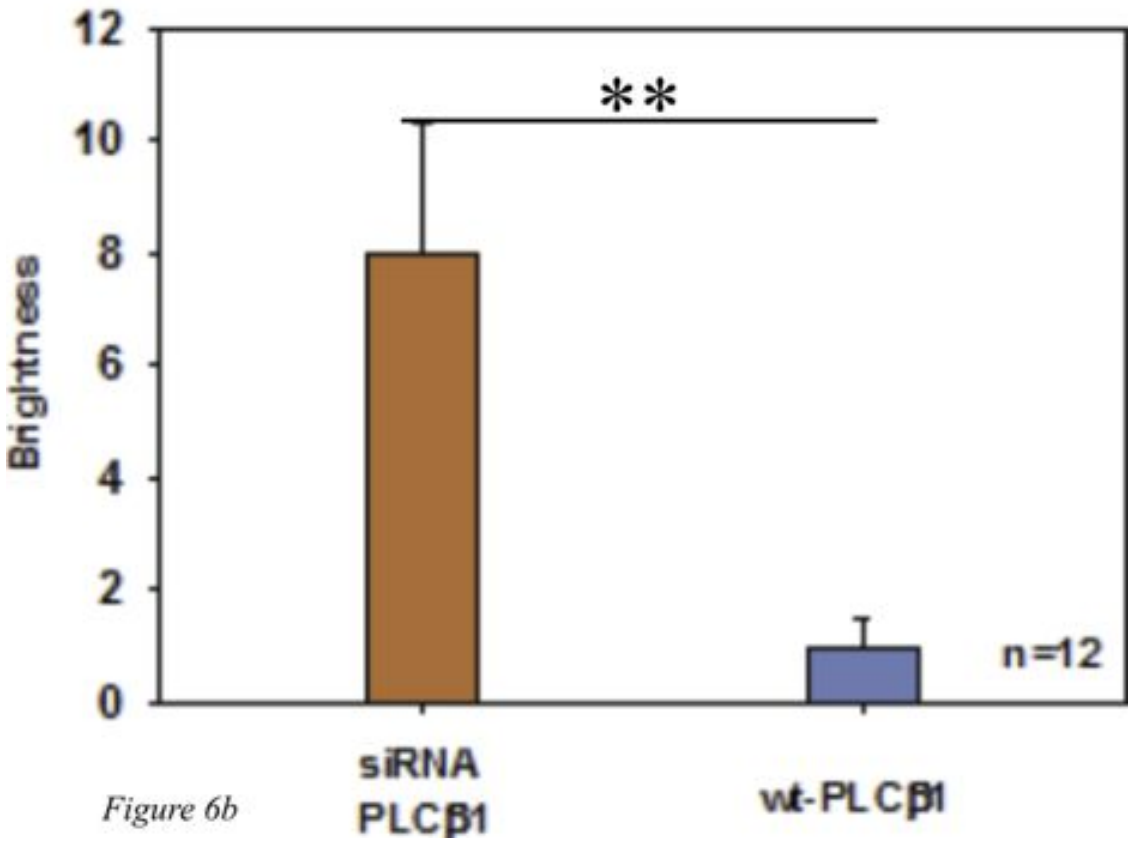


Figure 8: Tau brightness when PLCβ1 was silenced in neuronal cells, compared to the control condition with normal PLCβ1 expression.

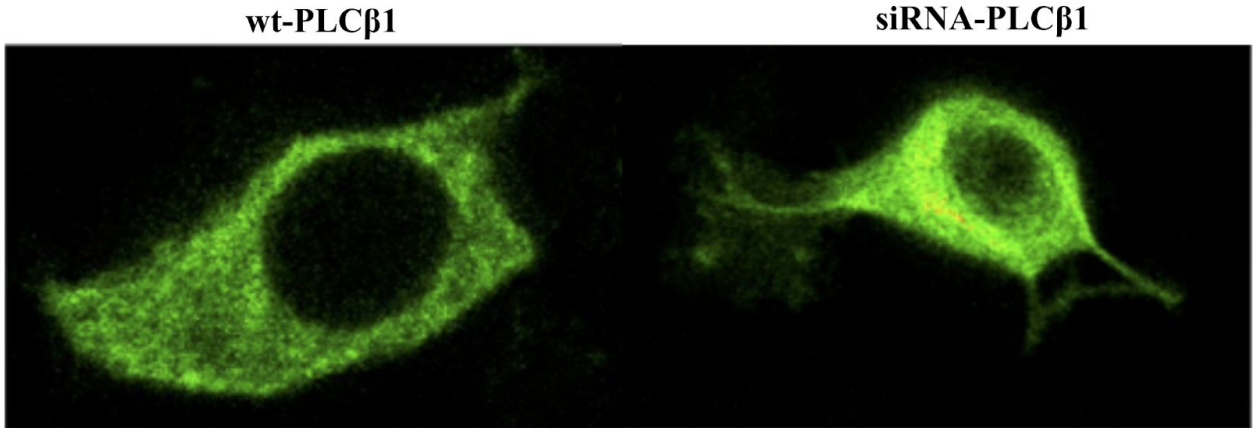


Figure 9: Brightness of eGFP-tau in a cell expressing the wild type levels of PLCβ1 (left) compared with a cell transfected with RNA silencing PLCβ1.

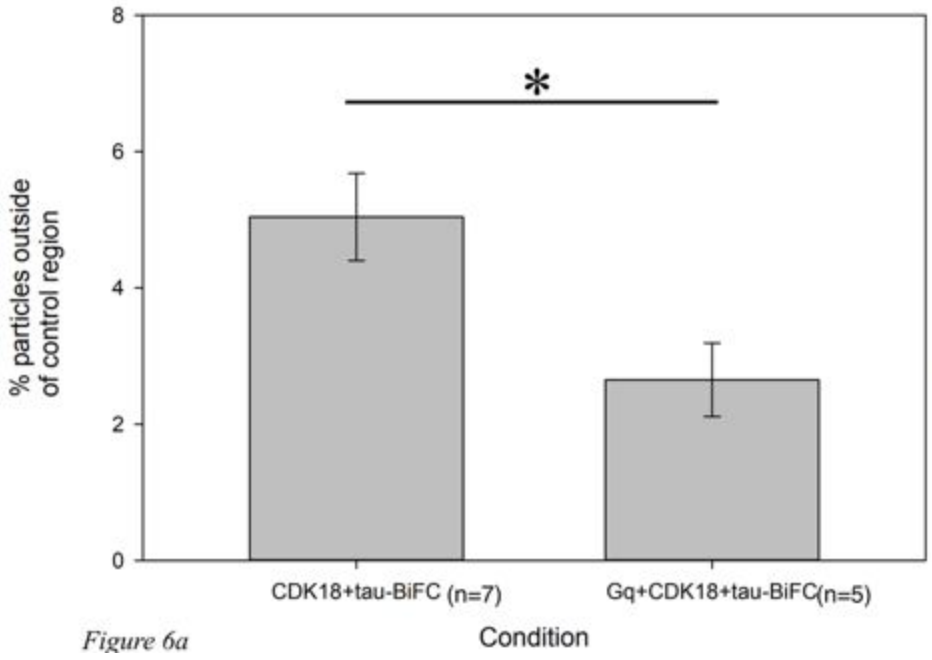


Figure 10: N&B analysis of particles in the control region of neuronal cells in the control and experimental conditions.

As hypothesized, when neuronal cells were transfected with Gαq, a constituent of the phosphoinositide pathway which dissociates its alpha subunit to bind PLC, more particles were localized to the body of the cell, indicating higher levels of tau aggregation compared to the control condition without Gαq, shown in Figure 10. These results also support the hypothesis that PLCβ1 must be freed from CDK18 to allow CDK18 to phosphorylate and subsequently dimerize tau. Since the alpha subunit of Gq dissociates to bind PLCβ when the G-protein coupled receptor cascade is activated, PLCβ will be unable to bind CDK18. CDK18's ability to bind and phosphorylate tau leads to more particles in the selected region of the optimal control cell compared with particles outside the cells. Results were statistically significant, suggesting that the G-protein signaling cascade impacts tau phosphorylation and aggregation.

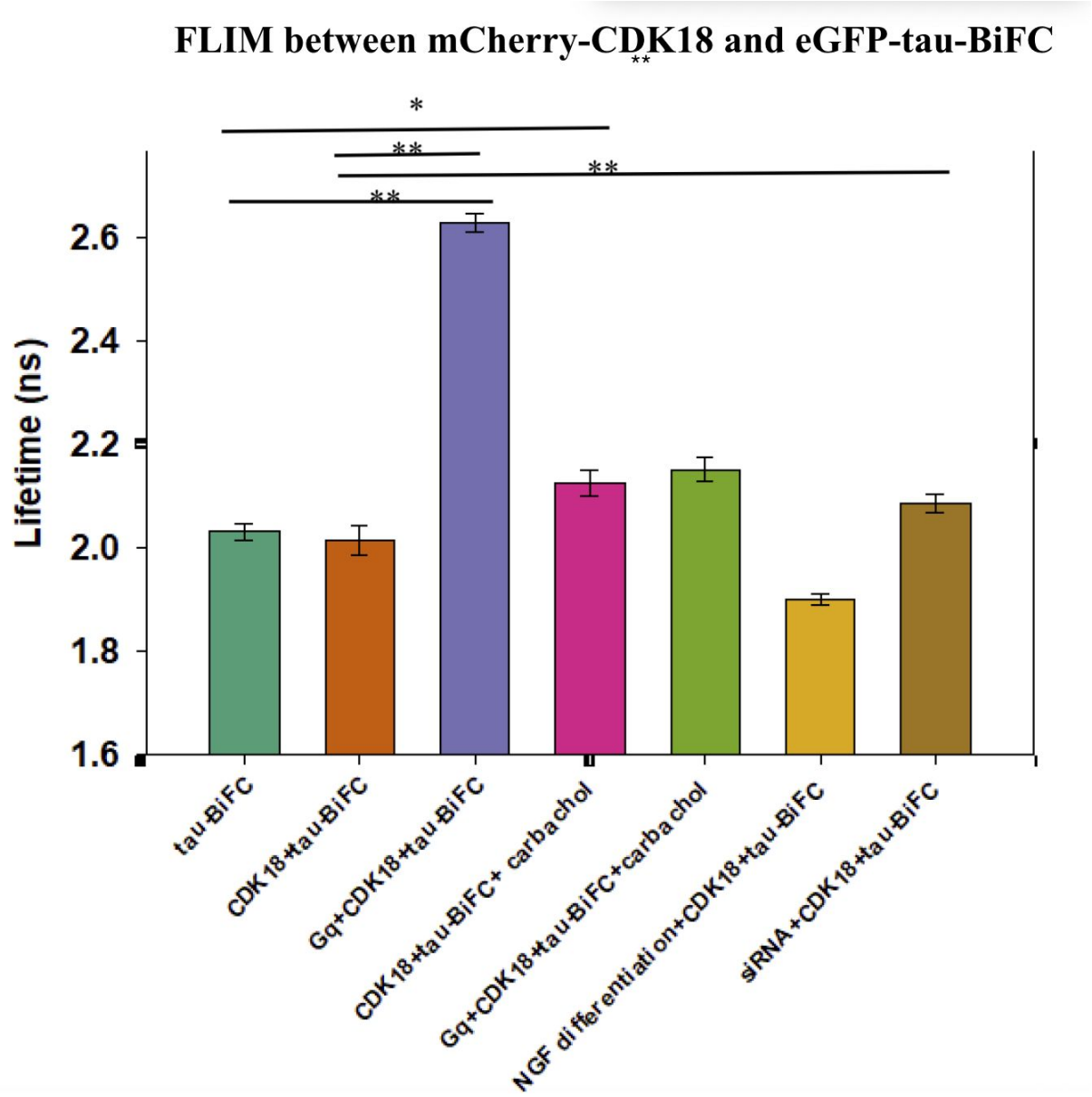


Figure 11 shows fluorescence lifetime measurements of tau 72 hours post transfection to assess binding interactions between mCherry-CDK18 and eGFP-tau.

As shown in figure 11, fluorescent lifetimes of the eGFP donor molecule labeling tau aggregates varied between conditions, but were overall generally consistent. The major change in lifetime occurred when Gq, CDK18, and tau-BiFC were transfected into neuronal cells,

indicating that Gq decreased binding between CDK18 and tau-BiFC. Lifetime measures associations between cells through fluorescent interactions. If cells are farther apart in their biochemical environment, then the fluorescent donor and acceptor molecule have a longer lifetime, taking more time to exchange energy. When proteins are close together, the fluorescent lifetime decreases due to faster transfer of energy, across a shorter distance. Interactions between CDK18 and tau are assumed to be transient, and since cells were imaged 72 hours post transfection, there should be consistencies in lifetime values. Lifetimes indicate minimal binding between CDK18 and tau at this point; however lifetimes may be artificially low due to homotransfer of fluorescence between tau molecules. Since the tauBiFC pair is composed of two subunits of tau which must combine to fluoresce, the two molecules may interact and interfere with accurate lifetime measurements. Lifetime changes between the condition with tauBiFC alone and Gq, CDK18, and tauBiFC were significant, as were changes between CDK18 and tauBiFC compared with silenced PLCβ1 and the condition with Gq, CDK18, and tauBiFC. Lifetime changes between tauBiFC alone and the condition with CDK18, tauBiFC, and carbachol stimulation were also significant. Carbachol was expected to stimulate the g-protein coupled receptor signaling cascade to free the Gαq subunit of Gq to bind PLCβ1, freeing CDK18 to phosphorylate tau. Since Gq was not transfected into this condition, carbachol may have stimulated the normal levels of Gq in the cell, but Gq was not overexpressed.

Figure 12 below compares lifetime phasor plots of eGFP-PLCβ1 alone and eGFP-PLCβ1 transfected with mCherry-CDK18. In the presence of mCherry-CDK18, points move inside the phasor arc, indicating FRET. Since the donor molecule's lifetime decreased, interactions between PLCβ1 and CDK18 increased in the mitotic phase of cell division. These increased interactions in the mitotic phase of cell division can also be seen in Figure 13, which compares FLIM lifetimes of PLCβ1 and CDK18 in synchronized and unsynchronized PC12 cells. Cells synchronized in the G2/M phase of the cell cycle transfected with PLCβ1 and CDK18 showed an average lifetime of approximately 2.38 ns, compared with the PLCβ1-only control (2.67 ns), the mCherry fluorophore control (2.57 ns) and the unsynchronized cells transfected with PLCβ1 and CDK18 (2.56 ns). The results were significant, showing that there is increased binding between

β1 and CDK18 in the mitotic phase of cell division, and supporting the Mitotic Hypothesis of AD.

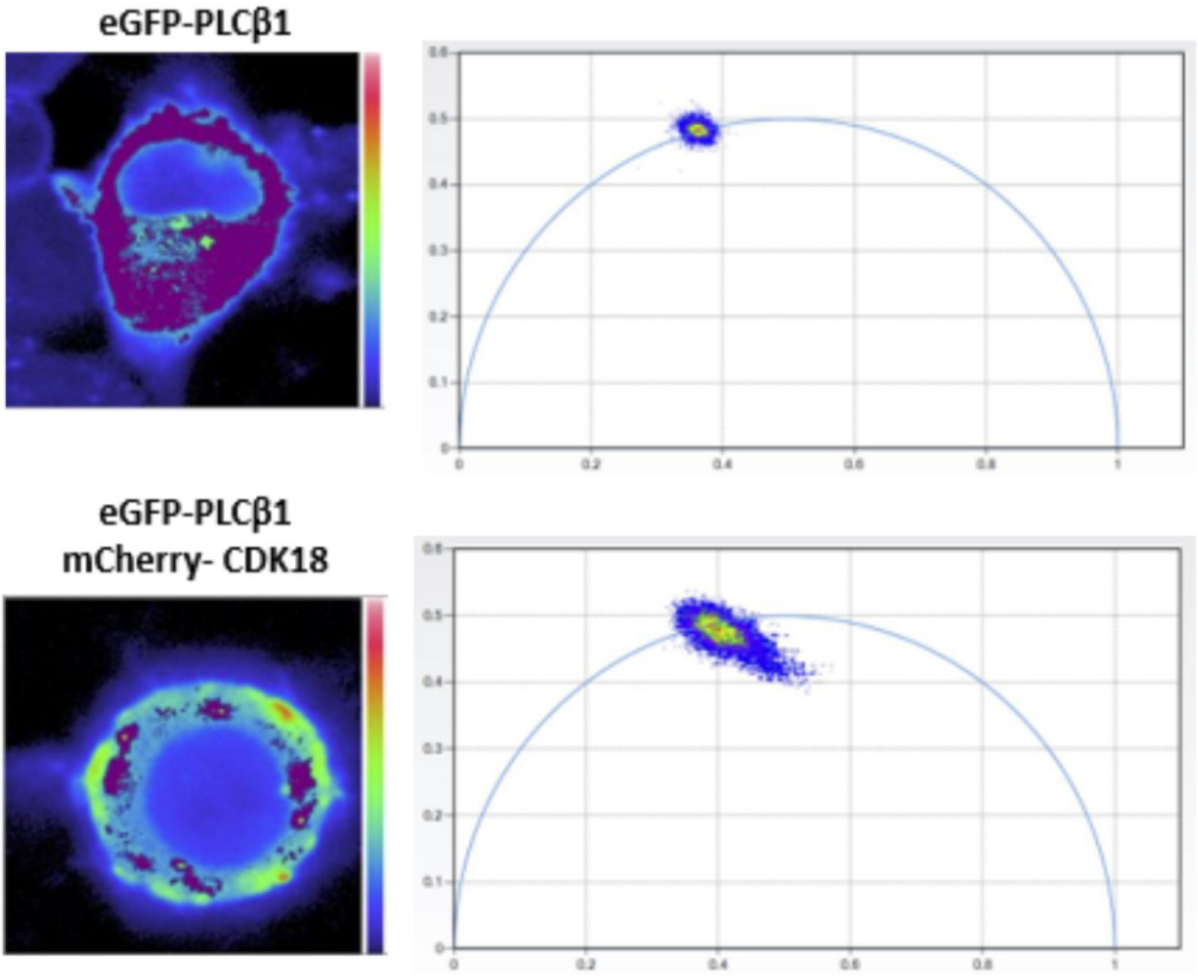


Figure 12: Phasor arc lifetimes of a PC12 cell transfected with only eGFP-PLCβ1 (top) compared with a cell transfected with PLCβ1 and CDK18 (bottom).

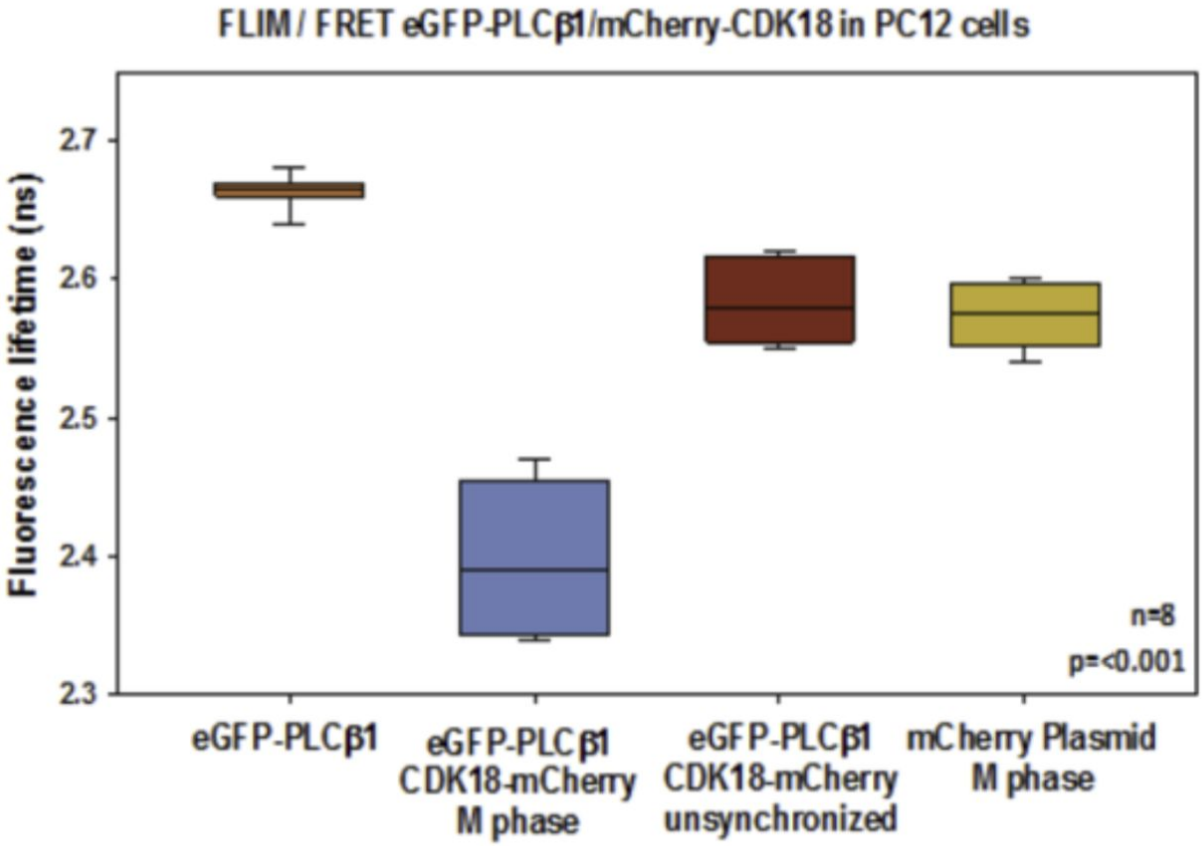
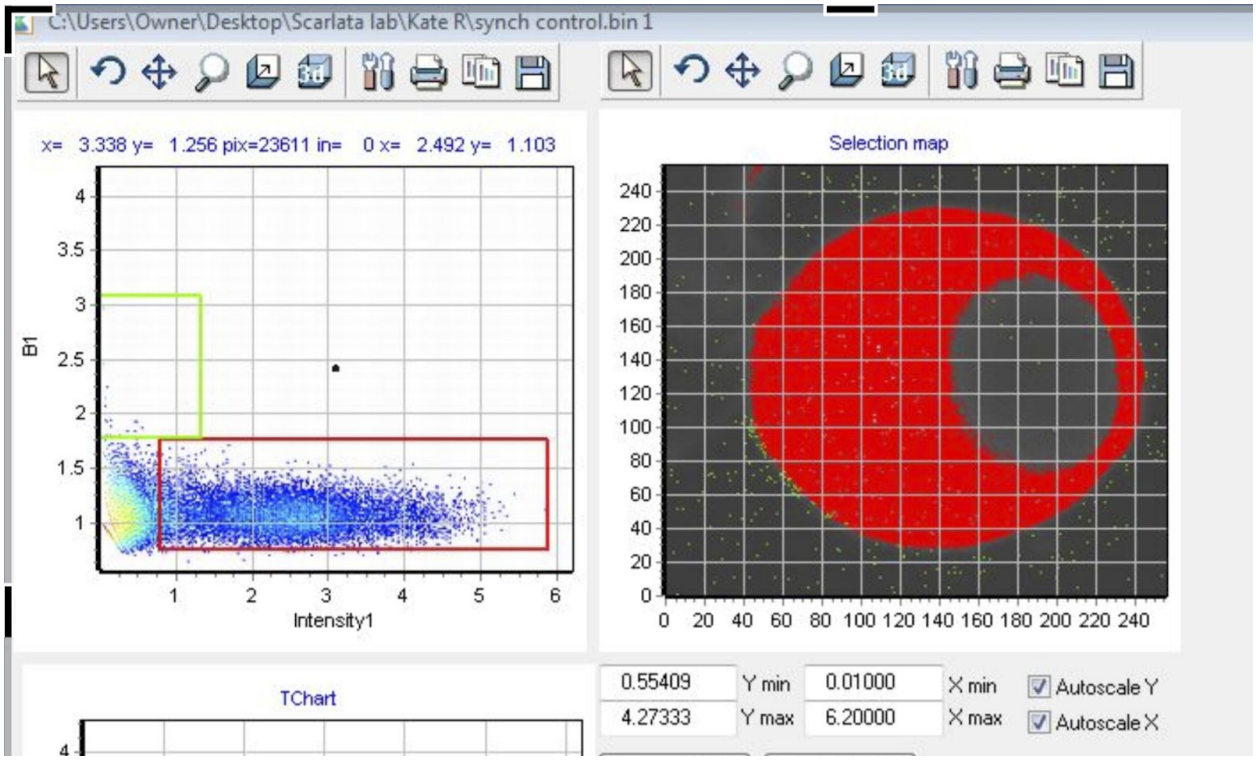


Figure 13: FLIM lifetimes showing interactions between PLCβ1 and CDK18 in synchronized and unsynchronized PC12 cells.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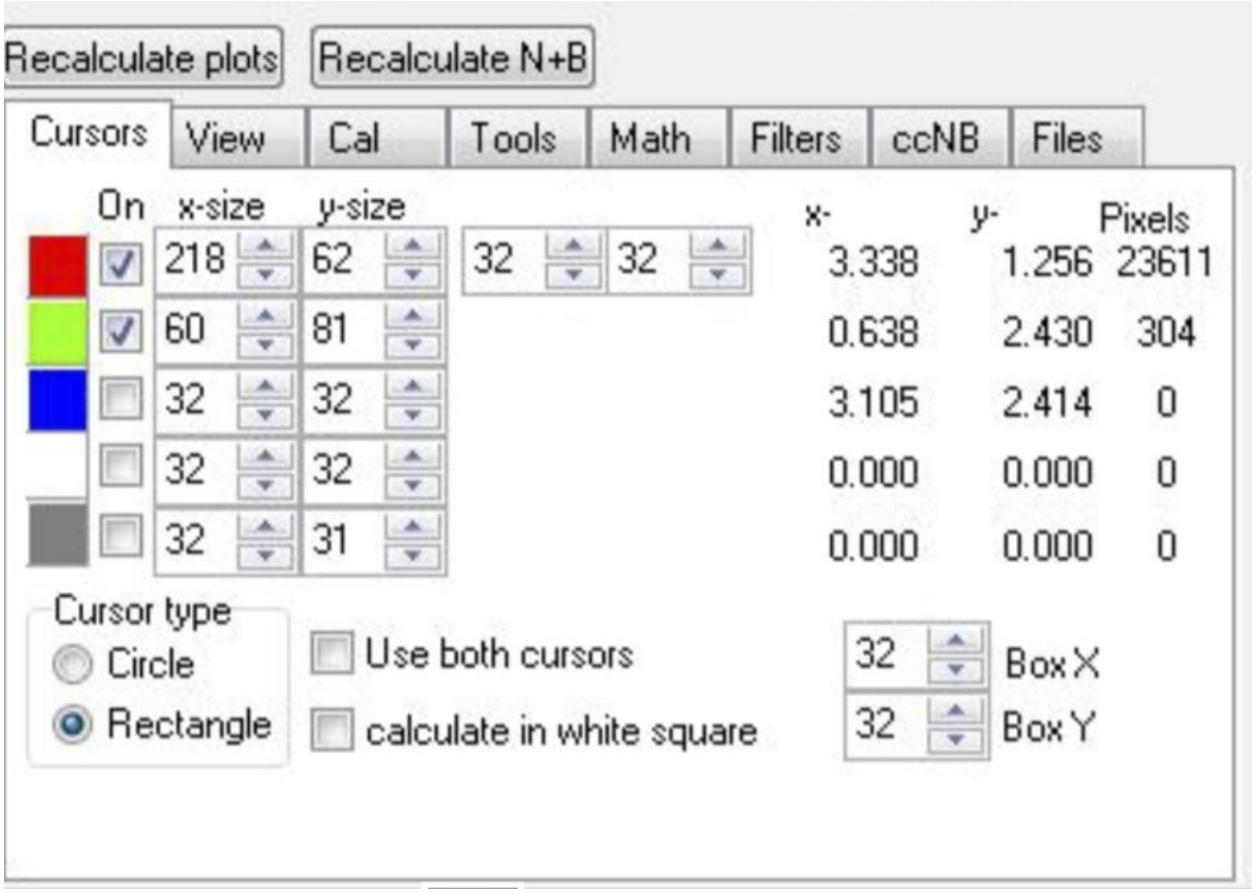
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32
31
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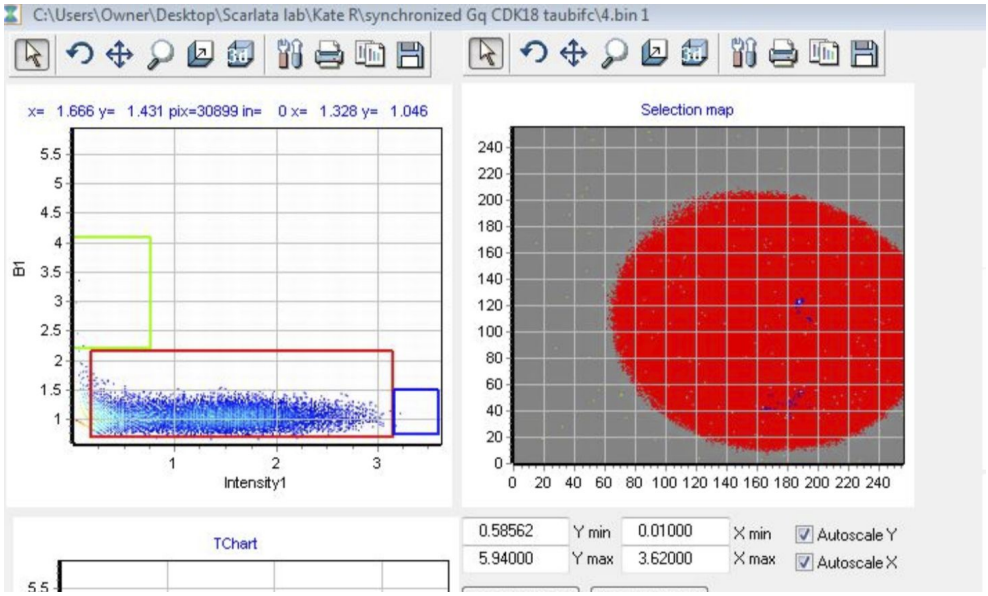
0.000
0
Cursor type
Circle
Rectangle
Use both cursors
calculate in white square
32
Box X
32
Box Y

Figure 14: Main analysis screen looking at pixel localization and brightness during the control N&B analysis of synchronized PC12 cells.



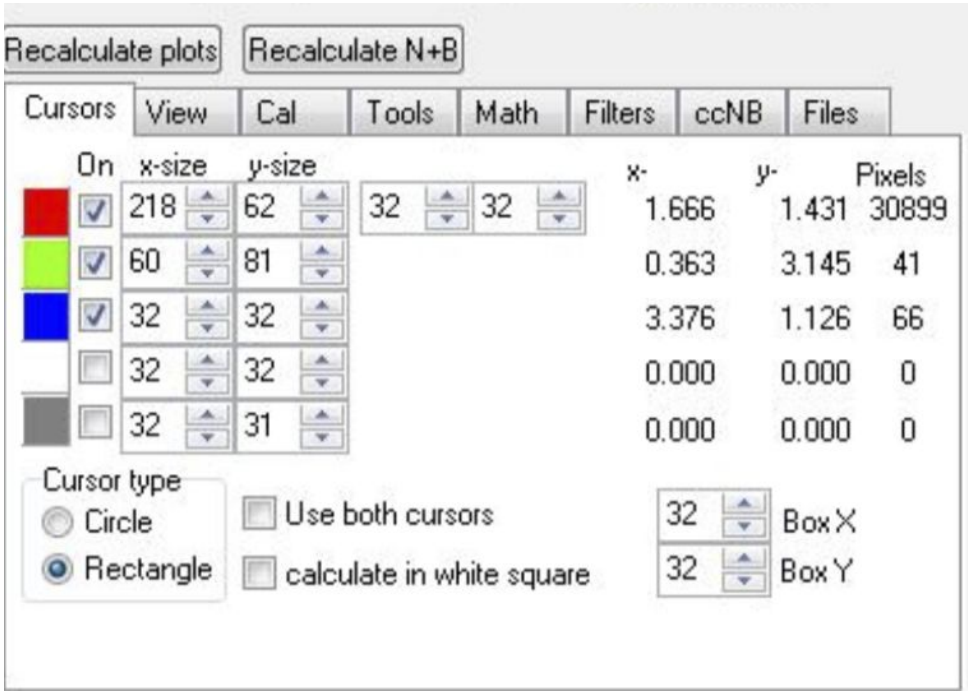


Figure 15: Main analysis screen looking at pixel localization and brightness during the experimental condition N&B analysis of synchronized PC12 cells.

Figures 14 and 15 above show the main N&B analysis screen to assess the percentage of pixels outside of the control region of PC12 cells synchronized in the G2/M phase of the cell cycle. A lower percentage of pixels outside of the control region indicates increased tau aggregation, and increased intensity indicates more fluorescent pixels from phosphorylated tau. The graph in Figure 17 shows that surprisingly, when cells were synchronized, Gq addition caused decreased particle localization compared with the control of tau-BiFC and CDK18. Figure 14 shows that eGFP pixels in the control had a higher intensity than pixels in the experimental condition, indicating increased aggregation in the control compared to the experimental. Many of the cells were only dimly expressing fluorescence in these trials. Figure 16 shows that synchronized cells transfected with Gq trended toward decreased brightness, but results were not significant (P=0.06).

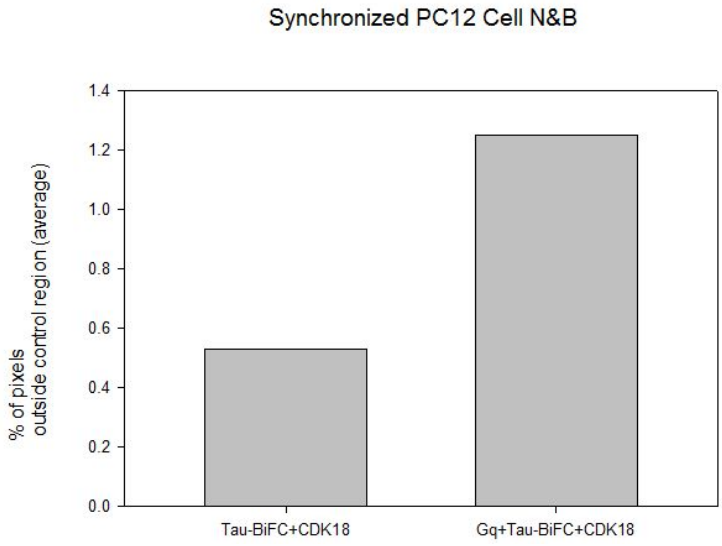


Figure 16: N&B analysis of synchronized PC12 cells.

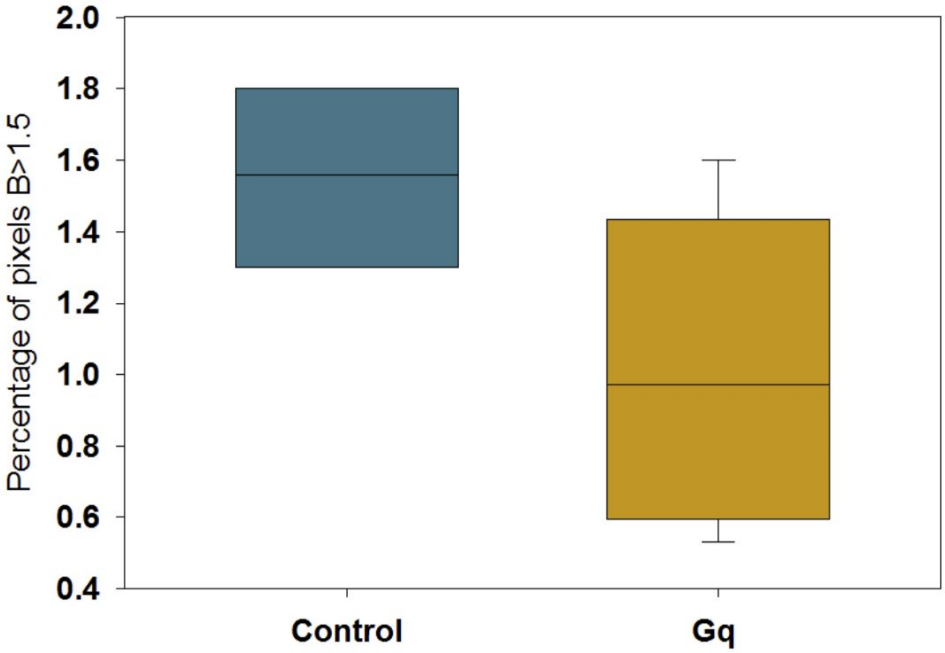


Figure 17: N&B analysis of brightness in synchronized PC12 cells.

Discussion

In the experiments detailed above, tau aggregation as a result of PLCβ1-CDK18 interactions was assessed using FLIM and N&B. Prior literature has shown that CDK18 is found concentrated in NFTs in the brains of patients with AD. It is thought that CDK18 contributes to the neuropathogenesis of AD by phosphorylating the pT231 and pS235 sites on tau, causing it to aggregate. This leads to symptoms of cognitive decline seen in AD, such as problems with learning and memory. Results showed that silencing PLCβ1 increased the intensity and localization of tau brightness, indicating that interactions between PLCβ1 and CDK18 play an important role in the neuropathogenesis of AD. When the alpha subunit of the Gq heterotrimeric protein in the G-protein coupled receptor cascade was overexpressed in unsynchronized PC12 cells, tau localization increased, suggesting that Gq bound PLCβ1, freeing CDK18 to phosphorylate tau and cause localization and fluorescence. Figure 9 shows the increased fluorescence of a PC12 cell when PLCβ1 was downregulated by siRNA. Since tau was transfected into cells in separate subunits (the BiFC pair), expression of eGFP meant that the tau subunits were phosphorylated, causing them to aggregate into NFTs and complete the eGFP fluorophore, allowing for fluorescence.

Although FLIM showed limited interactions between the eGFP-tau donor molecule and the mCherry-CDK18 acceptor molecule, these lifetimes do not lend much information to the neuropathogenic mechanism of tau. CDK18 is thought to only transiently bind tau to induce phosphorylation, and FLIM images were taken 72 hours post-transfection. Therefore, information about binding interactions was limited. More FLIM measurements should be taken in cells synchronized to the mitotic phase of the cell cycle to determine whether tau aggregation increased. Since tau-BiFC is composed of two subunits expressing eGFP when combined, it is possible that lifetimes were artificially deflated due to fluorescence of tau molecules. FLIM was meant to assess interactions between the donor eGFP molecule and the acceptor mCherry molecule; however tau aggregation may have interfered with these results. A prior PLC pulldown had shown 0.0025% tau spectrum in synchronized PC12 cells, suggesting low

interactions between tau and PLCβ1. Therefore, future experiments should continue to focus on interactions between CDK18 and tau, rather than PLCβ1 and tau.

When PC12 cells were synchronized to test the influence of Gq on tau aggregation, limited data could be collected due to problems with the microscope software and then subsequently, problems with the cells during the synchronization. The triple thymidine block which must be done before cells arrest in G2/M is harsh and caused many cells to die in both conditions, both times the experiment was executed. Cells were difficult to image due to their low transfection efficiency and dim expression of eGFP-tau. Although results from the synchronized cell experiment suggest that control cells were more localized to the control region than the cells transfected with Gq, CDK18, and tau-BiFC, more trials are needed to confirm these results. Synchronized cells showed that brightness in the Gq condition trended toward a decreased compared to the control condition ($P=0.06$), likely because the thymidine block was so harsh and media was changed 8 hours after the plasmid transfection, limiting time for cells to uptake and express plasmid DNA. Cells may have either been too unhealthy to continue to be metabolically active and express fluorescence of tau, or the transfection only had limited efficacy in the first place. More trials are needed with optimization for synchronization to determine accurate differences in aggregation. In unsynchronized cells, however, the overexpression of Gq clearly caused tau to localize, decreasing the percentage of pixels outside of the control region using N&B. These data suggest that CDK18 does phosphorylate tau, since the alpha subunit of Gq bound PLCβ, freeing CDK18 to interact with tau.

These results suggest that CDK18 may be therapeutic target for Alzheimer's Disease. If a pharmacological compound can cross the blood brain barrier and bind the pT231 and pS235 sites on tau, phosphorylation by CDK18 may be inhibited, preventing NFT accumulation. More testing is needed to look at other proteins involved in NFT accumulation. These results suggest protein interactions which may play a significant role in Alzheimer's Disease, and future research should further elucidate the mechanism of tau phosphorylation.

References

Atwood, Craig S and Richard L Bowen. "A Unified Hypothesis of Early- and Late-Onset Alzheimer's Disease Pathogenesis" *Journal of Alzheimer's disease : JAD* vol. 47,1 (2015): 33-47.

Balla, T. (2010). Putting G protein–coupled receptor-mediated activation of phospholipase C in the limelight. *The Journal of General Physiology*, *135*(2), 77–80. http://doi.org/10.1085/jgp.200910396

Brion, J.P. (1998). Neurofibrillary tangles and Alzheimer's Disease. *European Journal of Neu rology, 40(3),* 130-40.

Dana, H., Chalbatani, G.M., Mahmoodzadeh, H., Karimloo, R., Rezaiean, O., Moradzadeh, A., & Gharagouzlo, E. (2017). Molecular Mechanisms and Biological Functions of siRNA. *International Journal of Biomedical Science : IJBS*, *13*(2), 48–57.

Digman, M. A., Dalal, R., Horwitz, A. F., & Gratton, E. (2007). Mapping the number of mole cules and brightness in the laser scanning microscope. *Biophysical journal*, *94*(6), 2320-32.

Garwain, O., Valla, K., & Scarlata, S. (2018). Phospholipase Cβ1 regulates proliferation of neu ronal cells. FASEB J. 32(5):2891-2898. doi: 10.1096/fj.201701284R. Epub 2018 Jan 12. PubMed PMID: 29401590; PubMed Central PMCID: PMC5901387.

Herskovits, A.Z., & Davies, P. (2006). The regulation of tau phosphorylation by CDK18: implications for the pathogenesis for Alzheimer's Disease. *Neurobiology Disorders*, *23(2)*, 398-408. Doi: 10.1016/j.nbd.2006.04.004

Mak, E., Bethlehem, R., Romero-Garcia, R., Cervenka, S., Rittman, T., Gabel, S., Suren dranathan, A., Bevan-Jones, R. W., Passamonti, L., Vázquez Rodríguez, P., Su, L.,

Arnold, R., Williams, G. B., Hong, Y. T., Fryer, T. D., Aigbirhio, F. I., Rowe, J. B., & O'Brien, J. T. (2018). In vivo coupling of tau pathology and cortical thinning in Alzheimer's disease. *Alzheimer's & dementia (Amsterdam, Netherlands)*, *10*, 678-687. doi:10.1016/j.dadm.2018.08.005

Metaxas, A., & Kempf, S. J. (2016). Neurofibrillary tangles in Alzheimer's disease: elucidation of the molecular mechanism by immunohistochemistry and tau protein phospho-pro teomics. *Neural regeneration research*, *11*(10), 1579-1581.

Papini, C., & Royer, C. A. (2018). Scanning number and brightness yields absolute protein con centrations in live cells: a crucial parameter controlling functional bio-molecular interac tion networks. *Biophysical reviews*, *10*(1), 87-96.

Perl, D.P. (2010). Neuropathology of Alzheimer's Disease. *The Mount Sinai Journal of Medi cine*. Doi: 10.1002/msj.20157

Scarlata, S., Singla, A., & Garwain, O. (2018). Phospholipase Cβ interacts with cytosolic partners to regulate cell proliferation. *Adv Biol Regul,* 67:7-12. doi: 10.1016/j.jbior. 2017.09.004. Epub 2017 Sep 9. Review. PubMed PMID: 28919329; PubMed Central PMCID: PMC5807145.

Sekar, R. B., & Periasamy, A. (2003). Fluorescence resonance energy transfer (FRET) mi croscopy imaging of live cell protein localizations. *The Journal of cell biology*, *160*(5), 629-33.

Udawela, M., Scarr, E., Boer, S., Um, J.Y., McOmish, C., Felder, C.C., Thomas, E.A., & Dean, B. (2017). Isoform Specific Differences in Phospholipase C Beta 1 Expression in the Prefrontal Cortex in Schizophrenia and Suicide. *NPJ Schizophrenia,* 3(19). PMC. Web. 3 Sept. 2018. https://doi.org/10.1038/s41537-017-0020-x

Unit -III: IP3 and DAG Pathway. (n.d.). Retrieved October 1, 2018, from http://www.sivabio.50webs.com/ip3.htm

Vincent I, Rosado M, Davies P. Mitotic mechanisms in Alzheimer's disease? J Cell Biol. 1996 Feb;132(3):413-25. PubMed PMID: 8636218; PubMed Central PMCID: PMC2120731.