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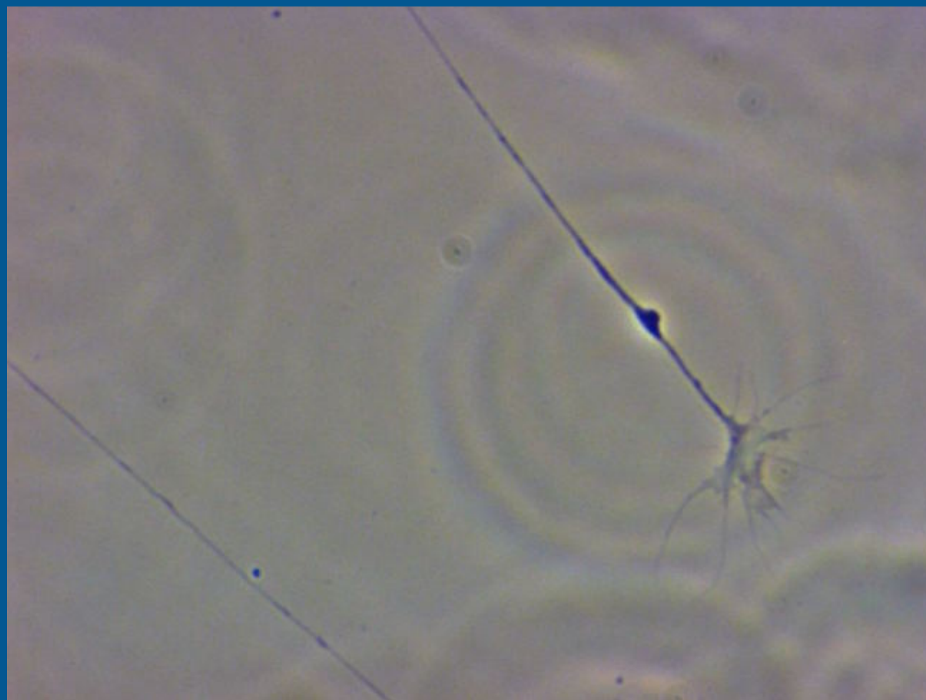

Preliminary study of effect of exposure of neuronal cultures to extracellular 25-35 beta amyloid fragment and K-252a on growth cone dynamics

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Introduction

Alzheimer's Disease (AD) is a neurodegenerative condition that affects function of cognitive processes such as thinking and memory formation. Synaptic plasticity in healthy adult cells is thought to be responsible for learning and memory formation: In patients with AD, plasticity is disrupted and inhibited (Spires-Jones & Hyman, 2014). AD is characterized by neurofibrillary tau-protein tangles and extracellular plaques comprised of deposits of a protein aggregate known as beta amyloid (βA) ("Alzheimer's Disease & Dementia | Alzheimer's Association," n.d.). βA is a derivative of amyloid precursor protein (APP); APP is cleaved by two protein subunits known as beta and gamma secretase to create the amino acid peptide βA (Epis, Marcello, Gardoni, & Di Luca, 2012). βA is known to interrupt synaptic transmission and synaptogenesis (Murphy, LeVine, & III, 2010).

Growth cones are one of the primary initiators of synaptogenesis; in order for neurons to establish connections with other cells, they must first extend projections to neighboring cells (Munno & Syed, 2003). APP affects growth cone phenotype on APP binding matrices including laminin, and is likely to affect axon path finding or outgrowth in vivo. APP is enriched in growth cones, and is likely a crucial component in neural circuitry and axonal growth (Sosa et al., 2013). Actin filled filopodia play a central role in axon guidance as they are the first part of the growth cone that encounters other guidance molecules (Geraldo & Gordon-Weeks, 2009).

Beta amyloid is also known to induce tau phosphorylation and the creation of neurofibrillary tau tangles. βA peptide has been shown to activate several protein kinases which exaggerate the phosphorylation of tau and induce cell loss by disrupting the neuronal cytoskeleton in primary neuronal cultures (Zheng, Bastianetto, Mennicken, Ma, & Kar, 2002). The kinase PKN is identified as a direct cause of the phosphorylation of human tau protein both in vitro and in vivo. PKN has been found in degenerative neurites with senile plaques (Kawamata et al., 1998). K-252a, a protein kinase inhibitor, is a neuro-protective compound that facilitates neurite outgrowth and maintenance (Tapley, Lamballe, & Barbacid, 1992). K-252a in small concentrations has been shown to increase filopodia behavior in growth cones (Cheng, Mao, & Rehder, 2000).

Both βA and protein kinase inhibitor K-252a have documented effects on phosphorylation of tau and βA plaques. In the present study, βA 25-35 is investigated in two

experimental conditions as measured by the activity of filopodia, an illustration of growth cone dynamics. Growth cone dynamics were assayed by the appearance of existing filopodia and the appearance of new filopodia. I propose that βA 25-35 will inhibit growth cone dynamics in peripheral neurons. In addition, I propose that exogenous K-252a will demonstrate neuro-protective effects (increase growth cone dynamics) in the presence of βA 25-35.

Materials and Methods

Materials

Amyloid Beta Protein Fragment 25-35, Cat No. A4559-1 MG purchased from Sigma. Amyloid Beta Protein Fragment 35-25, Cat No. A2201- 250UG purchased from Sigma. Protein kinase inhibitor K252-a, Cat No. sc-200517 purchased from Santa Cruz. Nikon Eclipse E200 inverted microscope at 40x magnification with Sony Digital Interface Camera DFW-X700. BTV software used, version 6.0b1, on a Macintosh computer- "Capricorn" in the ICUC at Wheaton College in Norton, MA.

Preparation of Growth Medium

Neurons were grown and maintained in a modified Leibowitz L-15 medium "F+ medium" (fibroblast medium plus nerve growth factor (NGF)). Medium created by 100 mL L-15, 2 mM glutamine, 0.6% glucose, 10% fetal bovine serum, 100U,μg/ml pen/strep, and 50ng/ml NGF. Methods outlined in "Neurobiology/Bio 324 – Problem Set 1" (Morris 2017). Addition of pen/strep will protect against any bacterial contamination in cell culture ("Penicillin-Streptomycin, Pen Strep Solution (10,000 U/mL) | Sigma-Aldrich," n.d.).

Coverslip Preparation and Treatment, Dissection, and Cell Culture

Pre-cleaned glass coverslips were treated with poly-lysine and laminin for proper cell adhesion. 10-day chick *Gallus gallus* embryos were dissected as approved by IACUC Wheaton through sterile surgical protocol derived from "Primary Culture of Chick Embryonic Peripheral Neurons" (Morris 2015a). Peripheral dorsal root ganglion cells and sympathetic nerve chains were isolated for the experiment and transferred onto treated cover slips for observation (2015a). Cells incubated at 37°C. All data were collected using 2-day old neurons observed from extracted dorsal root ganglia. Cells were exposed to treatment at age 2 days and image data sets were collected 24 hours following treatment using a chip chamber technique from Morris (2015a).

Stock Solutions and Exposure

To find volume of DMSO needed to create stock solutions, the following formula was used: Molarity = (mass solid/molecular weight of solid)/volume. Target molarity for working solutions of both reverse and forward beta amyloid fragments were derived from an experimental procedure found successful by Yang et al. (1998). Target molarity for working solutions of K-252a was adopted from a procedure found in Roux et al. (2002).

Control Stock and Working Solutions – βA 35-25

To create control stock solution, 94 μL DMSO was added to 250 μg βA 35-25 to create concentration of 2.5mM. 10 μL of 2.5 mM βA 35-25 stock solution was added to 1mL of F+ growth medium applied directly to cells to create control working solution with a concentration of 25 μM. Reverse fragment βA 35-25 was used to control for forward fragment βA 25-35 as it contains all amino acids in reverse order.

Experimental Condition 1 - Stock and Working Solutions – βA 25-35

To create first experimental condition, 377 μL DMSO was added to 1 mg βA 25-35 to create concentration of 2.5mM. 10 μL of 2.5 mM βA 25-35 stock solution was added to 1mL of

F+ growth medium applied directly to cells to create first experimental condition with a concentration of 25 μM.

Experimental Condition 2 - Stock Solution and Working Solutions – βA 25-35 + K-252a

To create second experimental condition, 214 μL DMSO was added to 100 μg K-252a to create 1mM stock solution. 2 μL of 1mM stock was diluted in 198 μL DMSO to create step-wise working solution of 10 μM. 10 μL of 10 μM K-252a was added directly to cells in 1 mL of growth medium to create final working solution of 100 μM. 10 μL of 25 μM βA 25-35 was added directly to cells in 1 mL growth medium in addition to 10 μL K-252a to create second level of experimental treatment.

Data Collection and Analysis

Data were collected as images captured every 30 seconds for a 15 minute period using BTV software on a MacIntosh computer paired with an microscope with camera attached. Cultures were kept at a constant temperature of 37° C using an external heater throughout observation. Data were analyzed by observing numbers of filopodia of one growth cone in consecutive image frames for control and experimental conditions. Filopodia were defined by several criteria. Filopodia were uniform caliber, thread-like projections emerging from the edge of a growth cone. Filopodia may or may not persist over two image frames. A single filopodium had a single tip, and branched filopodia were counted as multiple filopodia by counting their multiple tips. Filopodia were considered "new" if they appeared in a frame in which they did not exist previously.

Data were analyzed by reporting the average filopodia count per image for each condition using pooled data. Averages of new filopodia from frame to frame for each condition were also determined to better understand growth cone dynamics. New filopodia appeared in a frame that had not appeared in a previous frame and consisted for at least two frames. Filopodia were counted with the naked eye on a MacIntosh application "Preview". Significance was determined by using an ANOVA test on Macintosh application "Minitab Express".

My collaborators for this experiment were Nate Awkerman, Ryan McKeon, Lena O'Flynn, Omar Raouf, Jiali Zhu, Jintan Shao, and Luis Lazo. All collaborators used reverse beta amyloid fragment 35-25 for control condition. K-252a reagent was shared with Nate Awkerman to study effects of kinase inhibitor in the presence of forward beta amyloid fragment.

Results

Figures 1, 2 and 3 depict time-lapse filopodia changes in growth cones of neuronal cultures in control (reverse beta amyloid fragment 35-25), experimental condition 1 (forward beta amyloid 25-35) and experimental condition 2 (forward beta amyloid 25-35 + K-252a), respectively. Figure 4 represents the average amount of filopodia per frame for each condition. The control condition had the most increase in average number of filopodia throughout the time lapse. Experimental condition 1 had the fewest filopodia visible in the frame on average. Figure 5 reports the average number of new filopodia visible from frame to frame in each condition. The control condition had the most new filopodia from frame to frame. Experimental condition 1 had the fewest new filopodia visible from frame to frame.

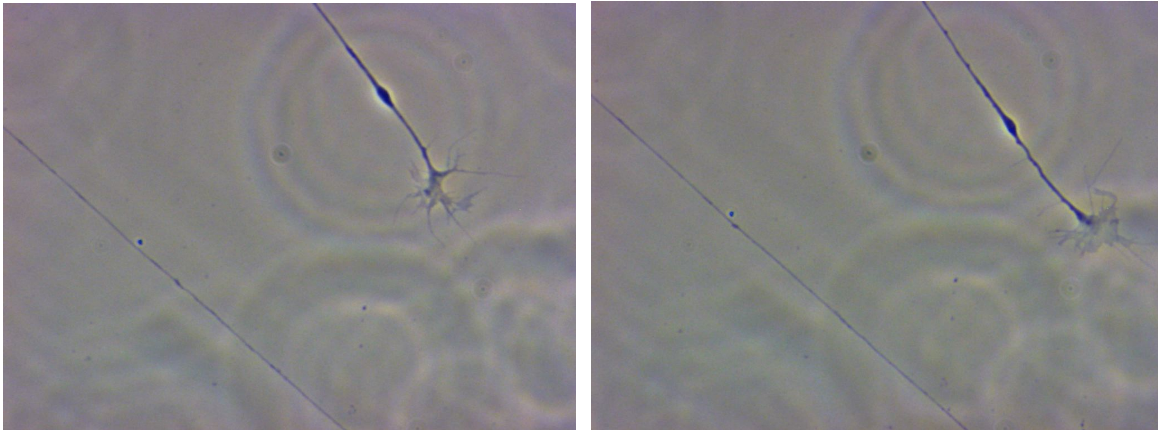
A B

Figure 1. Growth cones of 2-Day neurons extracted from dorsal root ganglia from reverse beta amyloid fragment 35-25 (control condition). Image A was captured at 30 seconds and image B captured at 19 minutes. Number of filopodia increased from Image A to Image B.

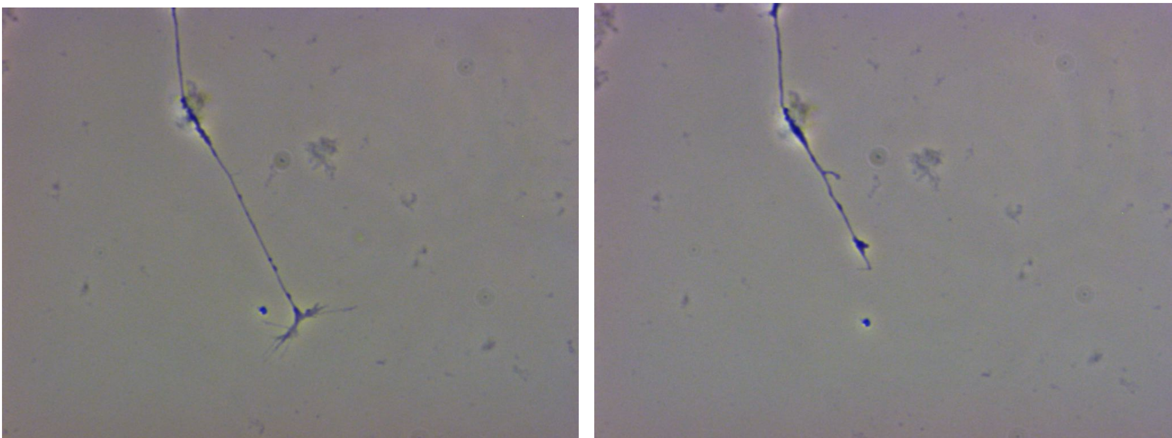
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Figure 2. Growth cones of 2-day neurons extracted from dorsal root ganglia from forward beta amyloid 25-35 fragment (experimental condition 1). Image A at was captured at 30 seconds and image B was captured at 17.5 minutes. Number of filopodia decreased from Image A to Image B.

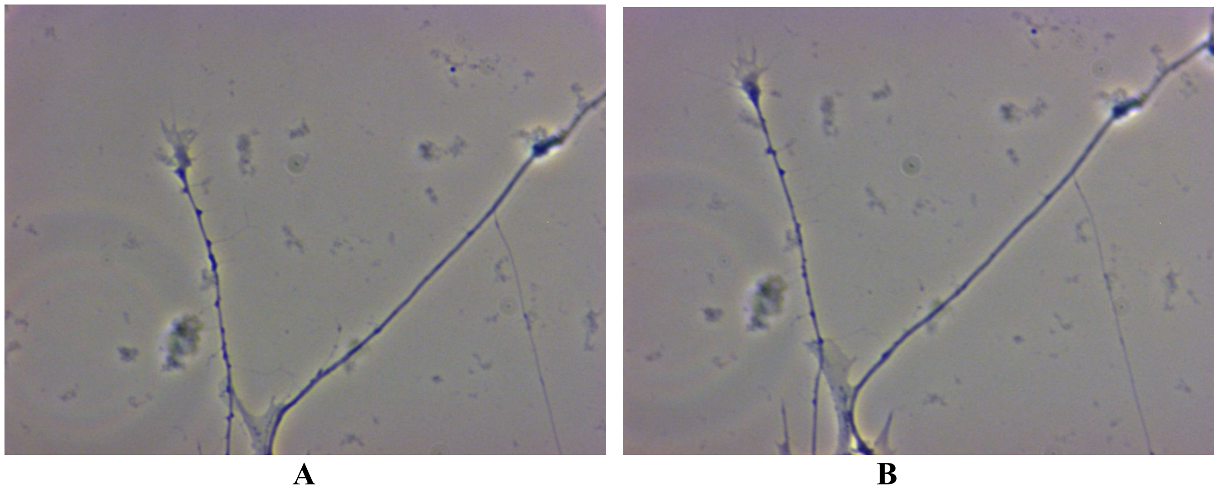

Figure 3. Growth cones of 2-day neurons extracted from dorsal root ganglia from forward beta amyloid fragment 25-35 plus K-252a (experimental condition 2). Image A was captured at 30 seconds and image B was captured at 14 minutes. Number of filopodia increased from Image A to Image B.

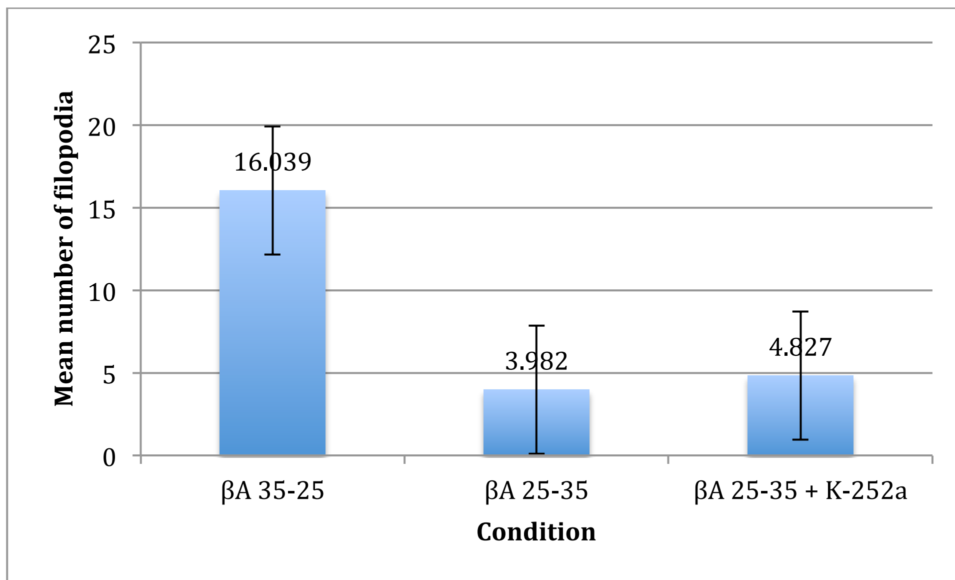

Figure 4. Pooled averages of filopodia count per frame. Data are derived from analysis of 818 filopodia observed in 51 image frames of one growth cone in one trial for the control condition. Experimental condition 1 data are derived from 219 filopodia observed in 55 image frames of one growth cone in one trial. Experimental condition 2 data are derived from 140 filopodia observed in 29 image frames of one growth cone in one trial ($p<0.0001$).

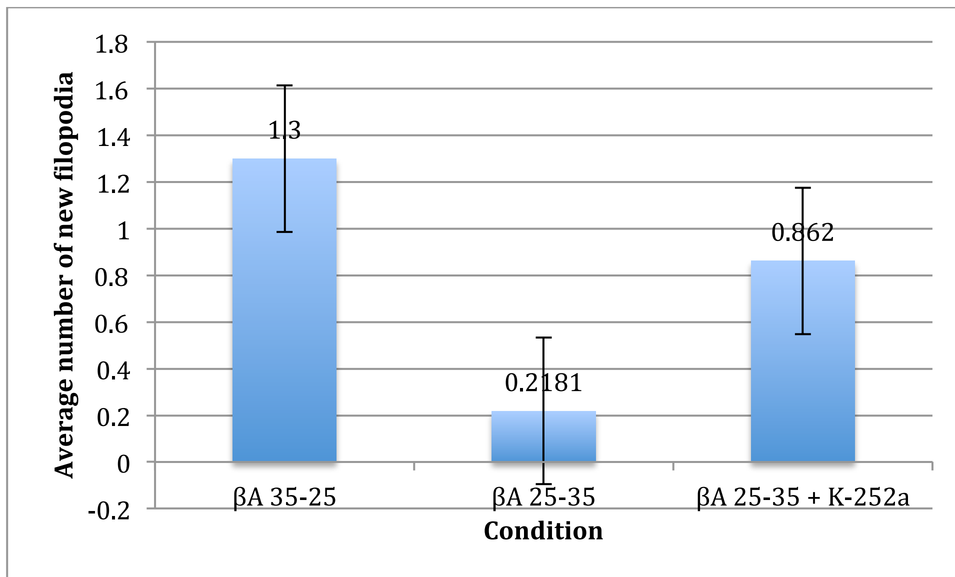

Figure 5. Average number of new filopodia per frame. Data are derived from analysis of 65 new filopodia observed in 50 image frames of one growth cone in one trial for control condition. Experimental condition 1 data are derived from analysis of 12 new filopodia observed in 55 image frames of one growth cone in one trial. Experimental condition 2 data are derived from analysis of 25 filopodia observed in 29 image frames of one growth cone in one trial (p=0.0003).

Discussion and Conclusions

The trends in data of the current study suggest that βA 25-35 may inhibit growth cone dynamics, and protein kinase inhibitor K-252a may induce growth cone dynamics on average number of filopodia present and emergence of new filopodia. However, the hypothesis of the current study cannot be statistically supported.

Cultures treated with βA 25-35 may inhibit growth cone dynamics as compared to the control condition. Average number of filopodia report an encouraging trend toward seeking a significant difference between the control condition (βA 35-25) and experimental groups 1 and 2 (Figure 4). Though the reported p value encourages statistically significant differences of the arboreal nature of the growth cones measured in the three conditions, overlapping error bars between experimental conditions 1 and 2 and very small sample size restrict stating a statistically significant difference between these groups.

Similarly, average number of new filopodia display an encouraging trend for the current hypothesis (Figure 5). These data are limited to accepting statistical significance for similar reasons (Figure 5). There is no error bar overlap between experimental groups 1 and 2, which may indicate that protein kinase inhibitor K-252a has had some positive impact on growth cone dynamics as measured in average of new filopodia (Figure 5). The data of the current study are encouraging for identifying βA 25-35 as an inhibitor of growth cone dynamics and the neuro-protective potential of protein kinase inhibitor K-252a, but many more trials with larger sample sizes are needed to indicate this hypothesis as properly supported.

βA is known to interrupt synaptic transmission and synaptogenesis (Murphy, LeVine, & III, 2010). Forward fragment βA 25-35, as perhaps supported in the current study, has a inhibitory role in growth cone dynamics and thus an inhibitory role in synaptogenesis, as growth

cones are the primary initiators of synaptogenesis (Munno & Syed, 2003). From the trends of the present study it can be supported that beta amyloid is partially responsible for decline in synaptogenesis (forming new connections) indicative of Alzheimers Disease, and that potential treatments such as protein kinase inhibitor K-252a are available to counteract βA and the formation of beta amyloid plaques.

In order to refine this experiment, it would be beneficial to use larger sample sizes to have increased assurance of statistical tests. One limitation of the study was the availability of research on protein kinase K-252a. As found in Cheng et al., K-252a produces neuro-protective effects found in small concentrations (2000). Cheng et al. used nanomolar concentrations to treat neuronal cultures which was beyond the scope of the current study. Instead, methods were adapted using a serial dilution of reagent K-252a at a micromolar concentration. Effects of beta amyloid 25-35 with nanomolar concentrations of K-252a would be an interesting direction for future studies. Multiple trials for the current study were not conducted due to time and budget constraints. For future experiments, one could experiment with multiple micromolar concentrations of beta amyloid paired with nanomolar concentration of protein kinase inhibitor K-252a and observe effects of growth cone dynamics.

I have abided by the Wheaton College Honor Code in this work.
Signed: Cassandra Kennie

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