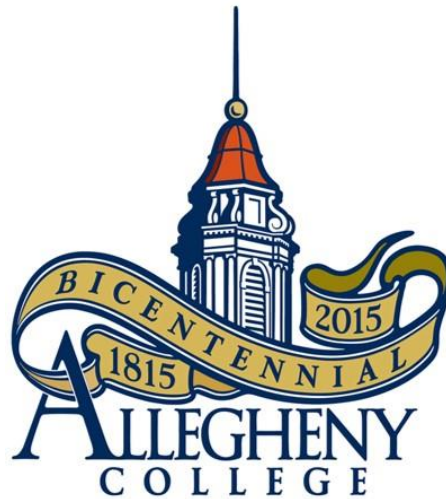


***Testing the specificity of the inhibitory effects of $A\beta_{42}$,
an Alzheimer's related protein, to ion channels Cav 3.2
and Kv 4.2***

By
Lilly Appiah-Agyeman
April 3, 2017



Allegheny College
Department of Neuroscience

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A Senior Comprehensive Project in Partial Fulfillment of the Requirements
for a Bachelor of Science Degree from Allegheny College

***I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor
Code
and to maintain the integrity of both myself and the College community as a whole.***

Pledge

Approval by the Senior Thesis Committee:

Dr. Lauren French, PhD 1st Reader

Dr. Lee Coates, PhD 2nd Reader

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Abstract

Alzheimer's Disease (AD) is a neurodegenerative disorder characterized by the degradation of neurons and synapses in areas of the brain involved with memory. Unfortunately, there is not a known cure for AD, in part because verifying a person's AD can only be done by locating Amyloid beta ($A\beta$) plaque deposits in the brain during postmortem examination. $A\beta_{42}$, the most prevalent isoform of $A\beta$, is linked to AD because it has two hydrophobic amino acids (Isoleucine and Alanine) that give it the ability to aggregate with other $A\beta$ to produce different forms (oligomer, fibril, etc.). These forms can eventually become the $A\beta$ plaques that are readily expressed in AD patients and localized in areas of the brain involved with memory. Presence of extracellular $A\beta$ plaques does not directly align with progression of AD symptoms. Thus, it is important to examine different conformation and locations of $A\beta_{42}$. In addition, although it is not clear the exact neurological pathway $A\beta_{42}$ undergoes to induce symptoms of AD, it is hypothesized that $A\beta_{42}$ is interacting with ion channels. A previous Allegheny College Comprehensive Thesis showed that 0.1 nM of $A\beta_{42}$ inhibited the maximum current of the BK channel hSlo1.1 expressed in oocytes. I hypothesize that the inhibitory effect of the monomeric form of 0.1 nM of $A\beta_{42}$ would be specific to the BK channel hSlo1.1 expressed in *Xenopus* oocytes as compared to Kv 4.2, an A-type voltage-gated potassium channel and Cav3.2, a T-type calcium channel. Two-electrode voltage clamp (TEVC) recordings were conducted on each RNA and water-inject oocyte. A voltage electrode held the oocyte at a voltage from -50mV to +40 mV in increments of +10mV and a current electrode measuring current at each step. The maximum current of Kv4.2 and water-inject oocytes was noted at +40mV while the maximum current of Cav3.2 was noted between -50 mV and -40 mV. Intracellular applications of $A\beta_{42}$ were conducted by applying the $A\beta_{42}$ to the current electrode and allowing the $A\beta_{42}$ to diffuse into the oocyte for 5 minutes before TEVC recordings were conducted. Extracellular applications of $A\beta_{42}$ were conducted by applying $A\beta_{42}$ to the recording bath solution and allowing it to diffuse into the bath before TEVC recordings were conducted. The intracellular and extracellular recordings were analyzed using an independent t- test and matched pair t-test, respectively. It was found that 0.1 nM of $A\beta_{42}$ did not inhibit the maximum current of Kv4.2 and Cav3.2, both intracellularly and extracellularly. These results prove that the inhibitory effects of 0.1nM of $A\beta_{42}$ was specific to hSlo1.1. Although 0.1 μ M of $A\beta_{42}$ did not inhibit the maximum current of Cav3.2 and Kv4.2, these findings are important because it eliminates the possible ion channels that $A\beta_{42}$ affects in the pathway to AD. Therefore, researchers can focus on their time on other ion channels affected by $A\beta_{42}$ to uncover how they relate to AD.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive memory loss. The physical manifestation of this disease is observed through the degradation of brain synapses and neurons in areas of the brain involved with memory and language processing, such as the frontal and temporal lobes (Mattson 2004). Little is known about the precise neurological pathology of AD and as a result there is no known cure for the disease. Thus, humans suffering from this disorder rely on treatments that instead aim to delay the functional decline that occurs with AD. This is cause for concern since this disease kills more people than breast and prostate cancer combined. In 2016 alone, more than 5 million people lived with AD, with an additional incidence rate of 1 new case developing every 66 seconds. As the "Baby Boomers" of the nation are beginning to reach age 65 and older, this number is estimated to triple to about 13.5 million with treatment that will cost the nation 236 billion dollars in 2016 (Alz.org 2016). The need for earlier detection and treatment to better manage such an advancing epidemic is vital and yet, there is no way to clearly diagnose the disease while a patient is alive. This is mostly because memory loss can also stem from other types of illnesses (Mattson 2004).

Currently, the primary indicator that a patient has AD is the presence of excess protein plaques and neurofibrillary tangles examined in the brain postmortem. The intracellular neurofibrillary tangles and extracellular plaques are aggregated forms of tau and amyloid beta ($A\beta$) proteins, respectively. These deposits localize in areas of the brain involved in memory, learning, and emotional behavior. Areas include, but are not limited to, the hippocampus, amygdala, and basal forebrain. These regions tend to have fewer number of synapses because they possess a high density of $A\beta$ plaques (Mattson 2004). Thus, it is believed that $A\beta$ plays a role in AD progression.

A β derives from the proteolysis of the amyloid precursor protein (APP). The proteolytic cleavage of APP at the N and C-terminal by Beta-secretase (BACE) and γ -secretase respectively, produces A β_{40} and A β_{42} (Lazarov et al. 2002). These isomers of A β differ in that A β_{40} is generated within the trans-Golgi network of the Golgi apparatus while A β_{42} is generated in the endoplasmic reticulum (Hartmann et al. 1997). They also differ because A β_{42} has two extra hydrophobic amino acids: Isoleucine and Alanine. Their hydrophobic nature brings about a structural change in A β_{42} where the protein folds in on itself while also causing the protein to have a strong propensity to aggregate with other A β molecules to minimize these amino acids' interaction with an aqueous solution. Thus, A β_{42} can be found not only in its monomeric state (single A β_{42} protein) but also in other forms such as dimers (two monomeric A β_{42} formed together), oligomers (small amounts of monomeric A β_{42} compiled together) and eventually the plaques (sticky buildup of a compilation of different form of A β_{42}) that define AD.

Although A β_{42} plays a critical role in the development of AD, this does not imply that the presence of any concentration of A β_{42} will directly lead to AD. Having healthy amounts of APP has been linked to promoting neuronal survival and strengthening synaptic plasticity (Mattson 1997). A normal, healthy human brain naturally produces low levels of A β_{40} and A β_{42} . These small amounts have been associated with enhancing learning and memory formation. Healthy rats injected with an antibody [(1 μ g a monoclonal antibody, Anti-A β 4G8 (Millipore))] that blocks endogenous A β in their brain (by specifically targeting its amino acid sequence 17-24), before their training, experienced disruption in memory retention during their training as compared to the controls. These rats were specifically trained to learn to associate a context with a mild foot shock, eventually remembering to avoid this context, to avoid shock (an inhibitory avoidance test). In another experiment conducted after this test, rats injected with human A β (1-

42), before their training, experienced better memory retention, as if the amnesia caused by the A β antibody was rescued (Garcis-Osta and Alberini 2009). These findings seem to suggest the presence of the A β already found in their brain is useful in mediating memory formation and enhancement, further backing up the claim that low concentrations of A β can play a positive role in memory.

Higher concentrations of A β_{42} are subsequently linked to AD since A β_{42} has a higher tendency to aggregate with other A β_{42} molecules and form the plaques that are readily attributed to AD. Genetic studies have shown that a mutation at codon 717 of APP leads to the over-accumulation of A β_{42} in the brain (Chartier-Harlin, et al. 1991). Increased levels of A β_{42} for example, have been linked to elevated oxidative stress, a defect present during the early stages of AD. This has been indicated through the reduction of Fe (III) and Cu (II) to Fe (II) and Cu (I) by A β_{42} . The presence of a substantial amount of Fe (II) and Cu (I) in turn drives A β_{42} to capture oxygen and reduces it to hydrogen peroxide. The byproduct of both Fe (II) and Cu (I) and hydrogen peroxide interaction is hydroxyl, a free radical (Huang et al 1999). Radicals, such as hydroxyl, have the ability to destabilize DNA, lipids, and proteins in the brain, as well as cause structural changes and damage to neurons, through the removal of their electron(s).

A β_{42} has also been linked to impairment of long-term potentiation (LTP). LTP is the strengthening of a synaptic pathway activity based on persistent patterns of firing between neurons. LTP plays an important role in memory because increased firing between neurons involved in memory consolidation and formation, strengthens synaptic communication and in turn increases the efficiency of memory consolidation and formation. A β_{42} has been linked to disrupting synaptic connections and LTP (Chapman et al. 1999). Impairment of LTP in mice expressing transgenic human APP (APP₆₉₅SWE transgenic mice) was observed near the dentate

gyrus of their hippocampus. Both the control and APP mice received tetanic stimulation at their perforant pathway to measure their postsynaptic potential response. The excitatory postsynaptic potential spikes (EPSP), membrane depolarization that increases the likelihood that an action potential will occur because of presynaptic firing, was measured extracellularly using field electrodes. Anesthetized APP₆₉₅SWE transgenic mice exhibited smaller field EPSP spikes with smaller measured slopes compared to the field EPSP of the control. (Chapman et al. 1999). This means the mice with APP generated signals that were less likely to stimulate the postsynaptic neuron in the perforant pathway enough to fire an action potential. EPSPs play an important role in LTP, helping to establishing and strengthen pre and post synaptic connection. Although EPSPs do not directly lead to a postsynaptic action potential firing, EPSPs increase signal transmission between the pre and the postsynaptic neurons and strengthens their communication. If the presence of APP reduces EPSPs, it is plausible that the A β ₄₂ produced from them disrupts the neuronal connection needed to enhance synaptic communication.

Discussion of A β ₄₂'s link to AD-related symptoms such as oxidative stress and impairment on long term potentiation serves to further reinforce the idea that A β ₄₂ plays an important role in the development of AD. Although detrimental effects due to the presence of A β ₄₂ in connection to AD has been established and there is a universal understanding that A β ₄₂ found postmortem is a clear indication of AD, symptoms of AD can still develop even before the visible presence of extracellular plaque deposits. That is, progression on the disease does not coincide with the formation of A β ₄₂ plaques. One possible explanation for this delay is the presence of A β ₄₂ found intracellularly.

There is an abundant amount of evidence that suggests intraneuronal A β triggers a pathological cascade leading to AD pathogenesis. As AD mice age, progression of LTP defects

and synaptic dysfunction were apparent with the presence of extracellular A β deposits. One month-old mice did not exhibit deficits in synaptic plasticity. However, by six months, deficits were apparent. In this stage of their AD, extracellular deposits could not be located in the hippocampal region. However, when pyramidal cells located in the hippocampus underwent immunostaining using antibodies that detect A β , there was a strong immunoreactivity response, suggesting that A β was present intracellularly (Oddo et al 2003). In addition, it further established A β 's strong ties to AD. Although it is clear that A β and AD are connected, the exact neurological pathology A β takes that leads to AD is not. One hypothesis is that A β_{42} is interacting with ion channels.

Ion channels are protein pores that span the cell membrane and allow for electrically-charged particles to move in and out of the neuron. The sum of the electrical charge of the ions present within the cell can inhibit the cell or excite it enough to activate an electrical response that propagates along its axon. As the signal travels through the axon and reaches the neuron's end terminal, it can cause the release of chemical messengers called Neurotransmitters (NT) that cells release to communicate and transmit their signal to another neuron. The NT binds to sites on the postsynaptic neuron causing the cycle to continue. Any significant alterations to the way ions flow in the neuron can change a neuronal signaling cascade by either strengthening or weakening the signal being transmitted to the next neuron, or by prohibiting or over exciting such signal. More importantly, it can damage the neuron if there is an overflow or inhibition of the influx or efflux of ions. A β , specifically, has been linked to inhibiting a type of ion channel known as the BK channel.

BK channels are large conductance calcium-activated potassium channels that are activated by calcium influx and membrane depolarization. BK channels are usually localized

near voltage-gated calcium channels. After the firing of an action potential, BK channels are activated to initiate the repolarization and refractory period needed to reset the neuron to fire another action potential as well as deactivate calcium channels. Thus, BK channels play a major role in regulating neuronal excitability. $A\beta_{42}$ disrupts the regulation of this process by inhibiting the channel when a mixture of the oligomeric and monomeric form of $A\beta$ was applied intracellularly (Yamamoto et al. 2011). Whole-cell recordings of pyramidal neurons of AD mice (3xTg mice containing three mutations: APP Swedish, MAPT P301L, and PSEN1 M146V genes) expressing a BK channel exhibited inhibition upon the administration of intracellular $A\beta_{42}$ via a patch pipette. Action potentials were induced and recorded by eliciting a depolarizing current. Spike induced intracellular calcium ions was also recorded. Intraneuronal calcium levels increased and the spike width of the action potential lengthened with the administration of $A\beta_{42}$, as compared to the control. These findings suggest that $A\beta_{42}$ inhibited the BK channel which delayed the calcium channel deactivation and repolarization period (Yamamoto et al. 2011). Excess internal calcium causes an imbalance in neuronal calcium homeostasis which can be damaging to cells and lead to cell death. Neuronal cell death is the main cause of memory loss in AD patients.

Yamamoto et al (2011) only tested the effects of $A\beta_{42}$ applied intracellularly because extracellular applications did not inhibit the BK channel. Yet still, deposits of $A\beta_{42}$ can be found both intracellularly and extracellularly (Oddo et al 2003). A previous Allegheny College Senior Comprehensive Project tested the effects of 0.1 nM of the monomeric form of $A\beta_{42}$, both intracellularly and extracellularly, on the maximum current of the BK channel hSlol.1, expressed in oocytes. Two-electrode voltage clamp recordings (TEVC) were conducted on each oocyte by penetrating two electrodes, one voltage sensing and one current injecting. The voltage electrode

held the hSlo1.1-injected oocyte at a holding potential of -50 mV to +40mV, in increments of +10 mV, while the current electrode measured the current at each voltage step. The maximum current was exhibited at +40 mV. Intracellular application of A β ₄₂ was applied intracellularly by filling the current electrode with A β ₄₂ and allowing it to diffuse into the oocyte. Extracellular A β ₄₂ was applied to the recording bath solution. The results found that intracellular application of 0.1 nM of A β ₄₂ inhibited the maximum current. However, 0.1 nM of extracellular monomeric A β ₄₂ did not inhibit the maximum current (monomeric application could not be statistically analyzed by a matched-pairs t-test due to small sample size [n=1]) (Zuchelkowski 2015).

As profound as these findings are, they also raise the question if A β ₄₂'s inhibitory effects on the maximum current are specific to BK channels. There are many other potassium channels in the brain, making it plausible that A β ₄₂'s inhibition effects can be generalized to other types of potassium channels. Additionally, A β ₄₂'s effects on calcium channels are also not quite understood. AD has been connected to disruption of calcium homeostasis. A β ₄₂ peptide seemed to enhance EAA (Excitatory amino acid receptor agonist, a glutamate receptors agonist)- induced neuronal degeneration by disrupting the neuron's ability to reduce and maintain normal internal calcium levels (Mattson et al 1992). Also as previously stated, with the presence of A β ₄₂ intracellularly, calcium channels were activated for an extended period of time before they were inhibited (Yamamoto et al. 2011). Thus, it is possible that the presence of A β ₄₂ causes the overabundance of calcium ions that disrupts calcium homeostasis. Therefore for my study, I examined the effects of the monomeric form of A β ₄₂ on a voltage-gated potassium channel and a voltage-gated calcium channel.

I hypothesized that the inhibitory effect of the monomeric form of 0.1 nM of A β ₄₂ would be specific to the BK channel hSlo1.1 expressed in *Xenopus* oocytes as compared to Kv4.2,

voltage-gated potassium channel, and Cav3.2, a t-type calcium channel, expressed in oocytes. I administered 0.1 nM of A β ₄₂ both intracellularly and extracellularly on Kv4.2 and Cav3.2-injected oocytes to test which administration of A β ₄₂ caused greater or any channel inhibition.

Methods

DNA Extraction and Purification

Frozen stocks of *E.coli* containing plasmids of either Kv4.2 cDNA, or Cav 3.2 cDNA were stored at -80 °C. Cav 3.2 cDNA was provided to the lab by Dr. Leanne Cribbs of Loyola University and Kv 4.2 was provided by Dr. Robert Bahring of the University of Hamburg.

Overnight bacterial cell cultures in LB Broth were prepared to stimulate enough bacteria growth for plasmid cDNA extraction. Each bacteria culture was scraped and placed into a tube containing 5ml of LB Broth and 5µl of carbenecillin solution. Bacterial cultures were then incubated at a temperature of 37 °C and shaken at a speed of 200 rpm. Bacterial cultures were left overnight for about 16-20 hrs. Afterwards, the plasmid DNA was extracted using the protocol found in Zippy Plasmid™ MiniPrep. To confirm presence of the cDNA, the sample was run through a 1% agarose gel (0.5 g agarose, 50 ml of deionized water, 1ml 50x TAE [tris-acetate-EDTA] buffer), microwaved, until boiling, and cooled before adding 5µL of SYBR safe DNA) to confirm cDNA presence. Nanodrop spectrophotometry analysis was conducted using a Thermo Scientific NanoDrop spectrophotometer to determine concentration of cDNA plasmid. All DNA samples were stored at 4°C.

cDNA Linearization and Purification

Next, the plasmid cDNA was linearized for better accessibility of the channel gene for transcription. Each channel cDNA required a specific enzyme and buffer for DNA linearization. Linearizing enzymes, Af1II and Not1-HF[®], were used to linearize plasmids of Cav3.2, and, Kv 4.2, respectively, according to the manufacturer's protocol. The amount of linearizing enzyme, buffer and sterile, autoclaved MilliQ water added to each individual cDNA solution was

calculated based on the requirement ratio that 10 units of enzyme per 1µg of DNA is added. Combined solution was heated at 37 °C for 2-8 hours to speed up the linearization process. Once again, 1% agarose gel and nanodrop spectroscopy analysis were completed.

Newly, linearized DNA was purified using Zymo Research DNA Clean and Concentrator™, according to the manufacturer's guidelines. 1% agarose gel and nanodrop spectroscopy analysis were conducted.

RNA Synthesis and Cleanup

Newly linearized cDNA was transcribed using an enzyme RNA polymerase. Thermo Fisher Scientific mMessage mMachine™ T7 kit was used to transcribe both Kv4.2 and Cav3.2. 1µg or 6µl of DNA was added to a solution of 2x NTP/CAP, RNA polymerase enzyme, 10x reaction buffer, and sterile, autoclaved water. The solution was heated at 37°C. Afterward, 1% agarose gel electrophoresis and nanodrop spectroscopy analysis were conducted.

Transcribed RNA was purified using Qiagen RNeasy MiniElute cleanup Kit, according to the manufacturer's instructions. This process helps to clean out any enzyme or salts left in RNA solution. Finally, the RNA was run through 1% agarose gel and nanodrop spectroscopy analysis was performed to determine purity and concentration of respectively. All RNA samples are stored at -20° C.

Xenopus laevis Care

Xenopus frogs are housed at 22° C and kept in tanks filled with Amquel-treated water. Each frog was fed a small amount of food pellets, three times a week and fed a small fish (guppy), once a week. Their tanks were cleaned once a week using water and vinegar. In

addition, three times a week, the frogs' health was inspected based on observations made about their skin color and activity.

***Xenopus laevis* Oocyte Extraction**

The protocol for harvesting oocytes was provided by Dr. Lauren French's Lab and approved by the Allegheny College Institutional Animal Care and Use Committee (ARC). Oocytes were extracted from a mature female *Xenopus laevis* via surgery. Female frogs were first anesthetized with a mixture of 300 mg of 3-aminobenzoic acid ethyl ester methanesulfonate salt (MS-222) and 200 mL of tap water filled in a bucket. The frog was left in the anesthesia for about 30 minutes, after which the frog's leg was gently squeezed to ensure the absence of a withdrawal reflex and to confirm that the anesthesia's effects were achieved. Prior to surgery, all surgical equipment (scissors, forceps, curved suture needle, suture scissors, tweezer, rat tooth tweezers) were sterilized with 70% ethanol followed by OR-2 (82.5 mM NaCl, 2 mM KCL, 5 HEPES, 1 L of mega pure H₂O, adjusted pH to 7.5 with NaOH, after autoclaved, 1mL of MgCl₂ and stored at 4°C). During surgery, the frog was placed on its back and a small L-Shaped incision of frog's skin below the midline of its abdomen was made. The skin flap was folded back inward to expose the muscle of the frog. Another incision of the muscle was made to extract the oocytes using sterile forceps. The oocytes were transferred to a 50ml tube containing 20ml of OR-2 to clean off blood on oocytes. The frog's incision was closed with 2-3 single square knot sutures on the muscles and 3-4 to close of the skin. Throughout the entire process, OR-2 was used to clear bloody areas and to prevent drying of the frog's skin. If this was the frog's first surgery, it was placed in a container filled a quarter of the way with tap water. Its head was propped up using paper towels to prevent drowning. After about 5-6 hours, when conscious, the frog was brought back to its tank, according to the Animal Research Committee

(ARC) guidelines. If this was the frog's second surgery, the frog was anesthetized by placing it in the anesthesia for about 4-5 hours or until there is no pulse apparent and disposed of, according to ARC guidelines.

Oocyte Separation and Storage

Oocytes left in OR-2 were transferred to a solution containing 10ml of OR-2 and 20 mg of collagenase and placed in a petri dish where they were gently shaken for 1 hr to ensure separation of clumped oocytes. Then, healthy oocytes were sorted with the aid of a dissection microscope. Health was determined based on the largeness in size, roundness of their shape and richness of its color (yellow on one side and dark brown on the other). 30 oocytes were separated into each small petri dish filled with working solution, making sure the entire oocyte was saturated (2.5 ml of Bovine Serum and 47.5 ml of ND-96). Oocytes were stored in the refrigerator at 17 °C and 18% humidity for a day before RNA injections. The working solution on the oocytes was changed every 24 hours to ensure health of oocytes.

Microinjection of Oocytes

Microelectrodes for injection of channel RNA were pulled using WPI Inc glass capillary tubes and electrode puller (Model p-97, Sutter Instruments Company) set at program 7. Electrode tips were broken to a diameter of about 17-25 μm by puncturing tip through a Kimwipe laid over a breaker. Broken electrodes, forceps, and glass slides were covered in aluminum foil and baked overnight at 180° C, for sterilization.

During injections, the midpoint to end of electrode was filled with sterile, filtered mineral oil and secured tightly in a microinjector (Nanoinject II, Drummond Scientific Company). Oil was ejected through the tip with any excess being carefully wiped away using a Kimwipe. A

sterile glass slide was wrapped in parafilm and placed under microscope where 2 μ L of sterile water, 200 ng/ μ L of Cav3.2, 80-90 ng/ μ L of Kv4.2, or sterile Milli-Q water was pipetted onto the parafilm. The electrode was carefully filled with RNA or water, making sure to avoid air bubbles. Healthy oocytes were arranged in a mesh lined dish filled with working solution or ND-96 (96 mM NaCl, 2 mM KCL, 1.8 mM CaCl₂, 5 mM HEPES, filled to 1L with milliQ water after autoclave, 1M MgCl₂, 1M Na pyruvate, 10mg/ml Gentamycin, stored at 4°C) and were injected with 50 nl of RNA or water. Oocytes were then transferred, in groups of 10, into a labeled petri dish filled with working solution. These oocytes were stored in refrigerator at 17 °C and 18% humidity for about 3-4 days or 5-6 days, respectively, for Kv 4.2 Cav3.2 to express. Working solution was replaced every 24 hours from the time of injection to the time of recording.

TEVC recording

Electrophysiological recordings from either RNA-injected or water-injected oocytes were measured using a two-electrode voltage clamp (TEVC) (Figure 1). The voltage electrode held the membrane potential of the oocyte from -50 mV to +40mV, in increments of 10 mV, while the current electrode measured the current at each voltage step. Axoclamp 900A amplifier, manipulator and pClamp software were used to measure the electrical recordings of the oocytes. Recording electrodes were pulled using electrode puller (Model p-97, Sutter Instruments Company) with program setting at 8.

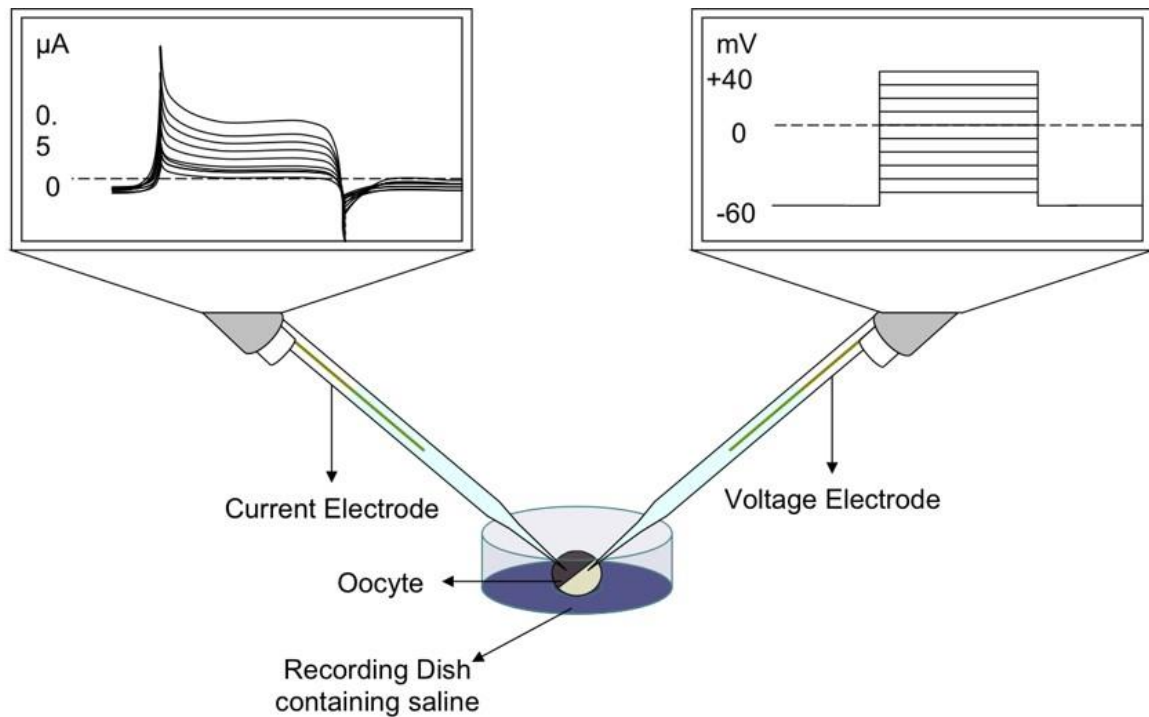


Figure 1. TEVC Recording of an oocyte. TEVC is an electrophysiological technique where the membrane potential (mV) is artificially held at a certain voltage and the current (mA) induced (in oocyte) is recorded. The voltage electrode held the voltage at a hyperpolarizing potential of -50mV to a depolarizing potential at +40 mV in increments of +10Mv. The current electrode measure the current of the oocyte at each voltages step. Both electrodes are filled with 3 M KCL and the recording solution saline is made of 1x ND-96 recording solution. (Louderback 2009)

On recording days, recording electrodes were filled $\frac{1}{2}$ of the way from the tip to the midpoint to the electrode with 3M KCL. The current and voltage electrodes were each lowered into a dish filled with 1x ND-96 recording solution (100 mL 10x ND96, 900 mL H₂O) and resistance was determined. The ideal resistance fell between 0.5 and 5 MΩ. Using a dissection microscope, each electrode tip was lowered slightly enough to punctured the oocyte for recording, being careful not to damage the tip or the oocyte.

The initial membrane potential of the oocyte measured by both electrodes were recorded. A resting membrane potential equal to or more hyperpolarized than -20mV was considered viable for recording. The oocyte was held at a starting holding potential that fell between the two resting membrane potentials. Afterwards, the set holding potential was hyperpolarized to -60mV.

Using the transcient_pro protocol from the pClamp software, the oocyte was depolarized from -50mV to +40 mV in increments of +10mV while the current at each step was recorded. The maximum current exhibited was recorded.

Amyloid-Beta Monomerization

Beta-Amyloid treated with HFIP was resuspended and sonicated with 1% of NH₄OH and either 3 M KCL or 1x ND-96 to a concentration of 1mg/ml A β ₄₂ (or 221.588 μ M A β ₄₂), according to protocol provided by © rPeptide. The buffer added next was based on the median used for either intercellular and extracellular recordings. For example, 1 vial of A β ₄₂ contained 3 M KCL, because for intracellular recordings, A β ₄₂ was applied to the current electrode which was filled with 3 M KCL. In another vial, the A β ₄₂ was resuspended with 1x ND-96 because A β ₄₂ was applied in the recording bath solution which is made up of 1x ND-96.

The total volume of A β ₄₂ obtained from sonication and resuspension was aliquoted into individual tubes of amounts ranging from 10 μ l, 20 μ l, 50 μ l and 100 μ l and kept frozen at -20 °C in microcentrifuge tubes. On TEVC recording days, frozen concentrations of A β ₄₂, depending on type of recording being conducted, was diluted with either 1x ND-96 recording solution for extracellular application or 3M KCL for intercellular application. Each frozen tube of A β ₄₂ was used once because multiple freeze-thaw cycles could cause aggregation.

Intracellular and Extracellular application of A β ₄₂

For intracellular application of A β ₄₂, the A β ₄₂ was diluted first from 221.58 μ M to 1 μ M and then to 0.1 nM using 3 M KCL. This A β ₄₂ solution was used to fill the current electrode. The A β ₄₂ was allowed to diffuse into each RNA-injected or water-inject oocyte before TEVC recordings, using transcient_pro, was conducted. TEVC recordings of oocytes with application

of A β ₄₂ and without A β ₄₂ were done on different oocytes because changing the current electrode from one without A β ₄₂ to one with A β ₄₂ could affect the health and survival of the oocytes since it would have to endure being impaled multiple times by the electrodes for re-recordings.

Unlike intracellular recordings, TEVC recordings for extracellular application of A β ₄₂ were conducted to the same oocyte before and after extracellular application of A β ₄₂ since health of oocyte was not significantly affected by multiple recordings. The 221.58 μ M concentration of A β ₄₂ was first diluted to 1 μ M and then to 0.1 μ M of A β ₄₂ using 1x ND-96. To achieve a concentration of 0.1 nM of A β ₄₂, 1 μ L of 0.1 μ M was added to 999 μ L of the 1x ND 96 recording bath solution and allowed to diffuse for 5 mins.

Data Analysis

The current at each voltage step, from -50mV to +40mV, was average for all RNA-injected and water-injected oocytes. The average maximum current for water-injected oocytes without any application of A β ₄₂ was subtracted from the current at each voltage step for each RNA-injected oocyte and averaged. For extracellular experiments, all the currents were then normalized to the average maximum current of the oocyte before application of extracellular A β ₄₂ because each TEVC recording of the current before and after extracellular application of A β ₄₂ was done on the same oocyte. This was accomplished by dividing the current at each voltage step for an oocyte, both with and without extracellular application of A β ₄₂, by the average maximum current of the same oocyte without extracellular A β ₄₂. Afterward, the current across each voltage step was averaged. Therefore, when normalized, a current of 1 indicated the maximum current and a current less than 1 indicates a current less than the maximum current while a current greater than indicates a current that exceeds the maximum current. Intracellular application, however, could not be normalized because TEVC recording of the intracellular

application with and without $A\beta_{42}$ was done on different oocytes. All extracellular data was statistically analyzed using a matched paired t test while all intracellular data was statistically analyzed using an independent t-test.

Results

The results below are from a previous study conducted by Zuchelkowski. I am using these results as a comparison for my results. Zuchelkowski tested 0.1 nM of A β both intracellularly and extracellularly on hSlo1.1, expressed in oocytes. The results showed that the maximum current was inhibited by intracellular monomer A β (28.4% reduction relative to baseline, $t=-3.064$, $p=0.00394$), indicated by Figure 2 but not significantly by extracellular monomer A β (monomeric application could not be statistically analyzed by a matched-pairs t -test due to small sample size [$n=1$]), indicated by Figure 3.

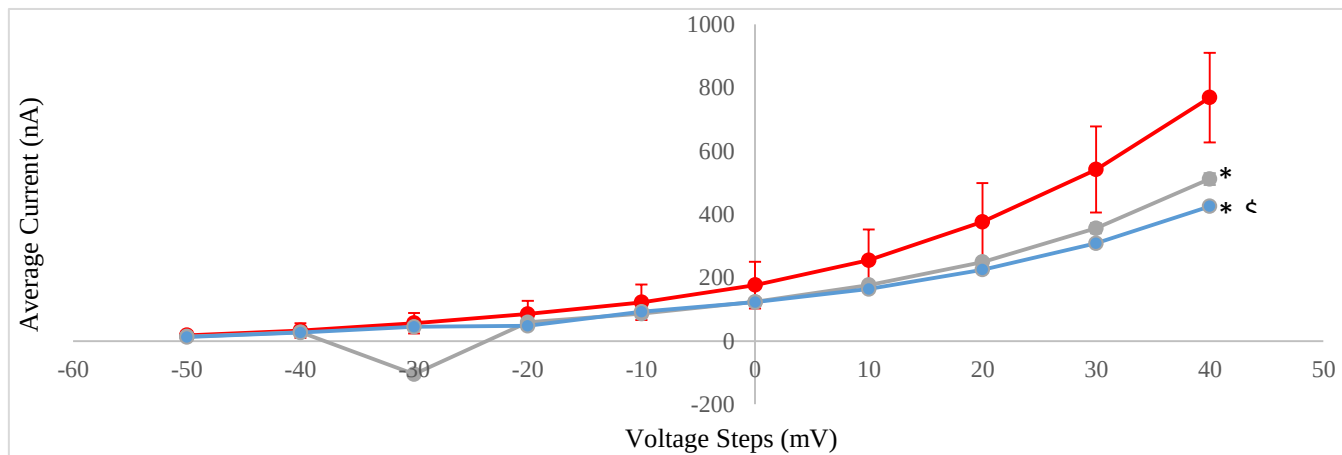


Figure 2. Average BK current vs Voltage plot from hSlo1.1-injected oocytes exposed to intracellular A β 42 The current at each voltage step was averaged across each oocyte. The red line represents the oocyte with no A β ₄₂ ($n=13$), the gray represents the BK channel with intracellular monomer A β ($n=5$), and the blue line represents the endogenous current affected by intracellular fibril ($n=12$). * indicates statistical significance at $p<0.01$ relative to “BK+No A β and \$ indicates statistical significance at $p<0.05$ relative to “BK+Monomer”. Error Bars represent standard deviation. (Zuchelkowski 2015)

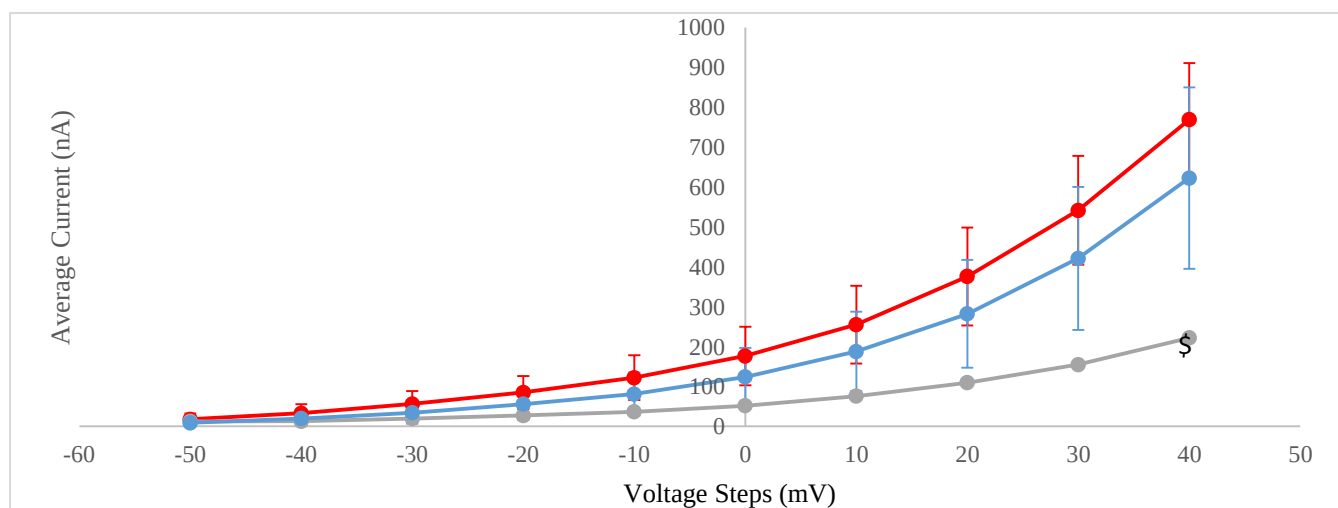


Figure 3: Average BK current vs voltage plot from hSlo1.1-injected oocytes exposed to extracellular A β 42. Current at each voltage step was averaged across oocytes in each group. The red line represents BK current with no A β ₄₂ ($n=13$), blue line represents BK current affected by extracellular fibril ($n=8$) and the gray line represents BK current affected by extracellular monomer. \$ indicated that data for monomer application was not statistically analyzed due to small sample size ($n=1$). Error bars represent standard deviation. (Zuchelkowski 2015)

I tested that same concentration, 0.1nM of $A\beta_{42}$, both intracellularly and extracellularly, on Cav3.2 and Kv4.2 expressed in oocytes to find out if this concentration would also inhibit the maximum current, as compared to hSlo1.1. When this concentration did not appear to significantly inhibit the maximum current, I tested the effects of 0.1 μ M of $A\beta_{42}$ on Cav3.2 and Kv4.2 to find out if a higher concentration made a significant difference in my findings.

It is important to note that the maximum current was exhibited at different holding potentials. For Kv 4.2 and water-injected oocytes, the maximum current were exhibited at a depolarizing holding potential of +40 mV. For Cav3.2-injected oocytes, maximum current fluctuated at a hyperpolarizing potential to either -50 mV or -40 mV. This is because the positive influx of Ca^{2+} ions for Cav3.2 was competing with the negative influx of Cl^- ions calcium-activated chloride channel of the oocyte.

Over the course of the experiment, a total average of 2,400 oocytes were sorted and injected with either RNA or water. Out of this, a total of 44 and 46 viable extracellular and intracellular recordings were collected for Kv4.2 and Cav3.2 respectively. For the control, (Megapure water-injected) a total of 52 viable recordings were collected.

All extracellular $A\beta_{42}$ application data were normalized to the maximum current of the RNA or water-injected oocyte without application of $A\beta_{42}$. The current of each oocyte at each voltage step, -50mV to +40mV, before and after application of $A\beta$ was divided by the maximum current of oocyte before application of $A\beta_{42}$.

Control

The endogenous calcium-activated chloride-channels (water injected) had an average maximum current of 339.8 nA displayed at a holding potential of +40mV. An example of its current trace can be seen in Figure 4. The upward deflection of each current trace reflects the inward movement of negative Cl^- ion into the cell when the channel is activated.

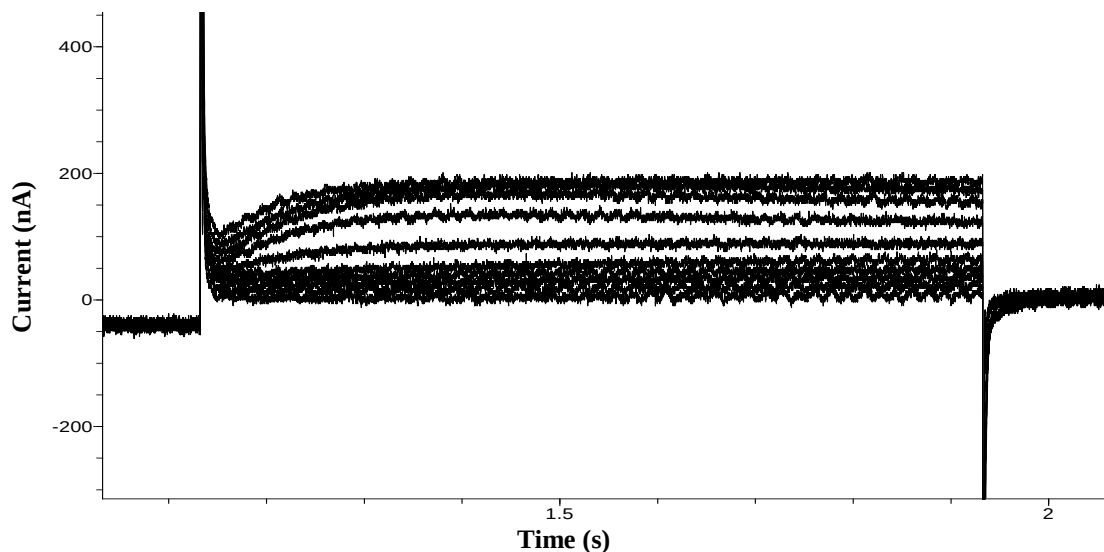


Figure 4. Current vs Time trace of a water-injected oocyte. Each horizontal line represents the outward current of the calcium-activated chloride channel at a given voltage step. The oocyte was held from -50mV to +40 mV in increments of 10mV. The lowest trace represents the current exhibited at a holding potential of -50mV while the highest trace is the current exhibited when held at +40mV.

Intracellular application of 0.1nM of A β ₄₂ did not significantly inhibit the maximum current, at +40 mV, of water-inject oocytes (df=29.2306, t=0.984638, p=0.3329). Figure 5 supports this conclusion.

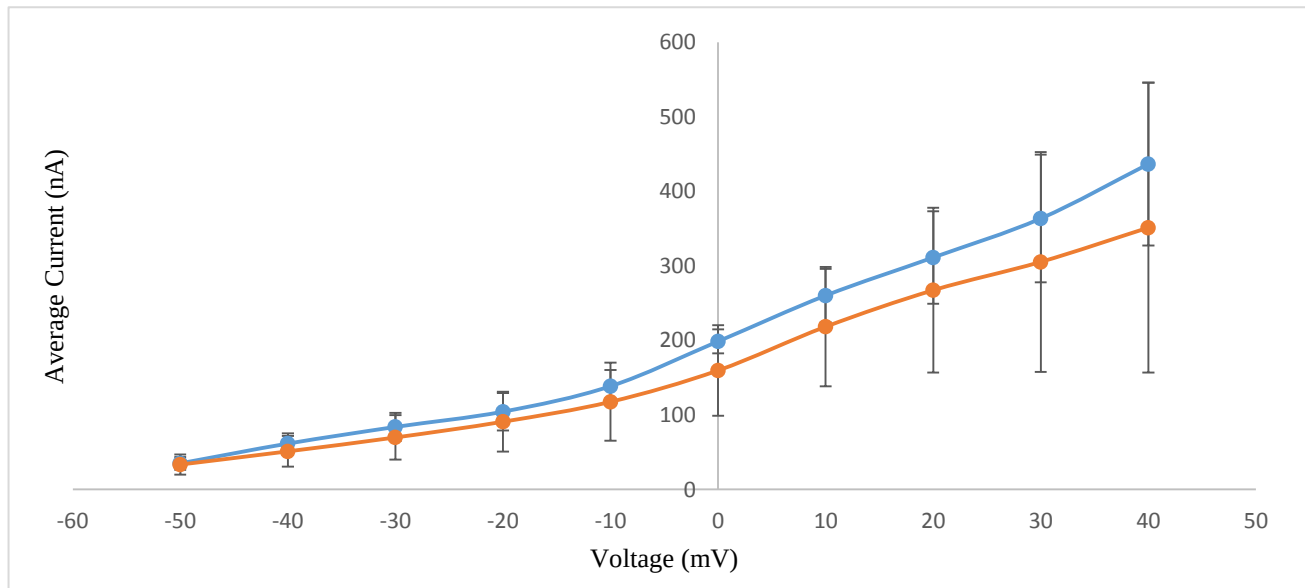


Figure 5. Average current vs voltage graph from water-injected oocytes exposed to intracellular 0.1 nM A β ₄₂. The current for each water-injected oocyte at each voltage step, from -50mV to +40mV, was averaged. The blue line represents the average current without intracellular application of 0.1 nM A β (n=5). The orange line represents the average current with intracellular application of 0.1 nM A β (n=18)). Error bars are the standard deviation.

At a higher concentration of A β ₄₂, 0.1 μ M, the endogenous maximum current was still not significantly inhibited by intracellular application (p=0.2461, t=-1.22083, df=11.72844). This can be seen in Figure 6.

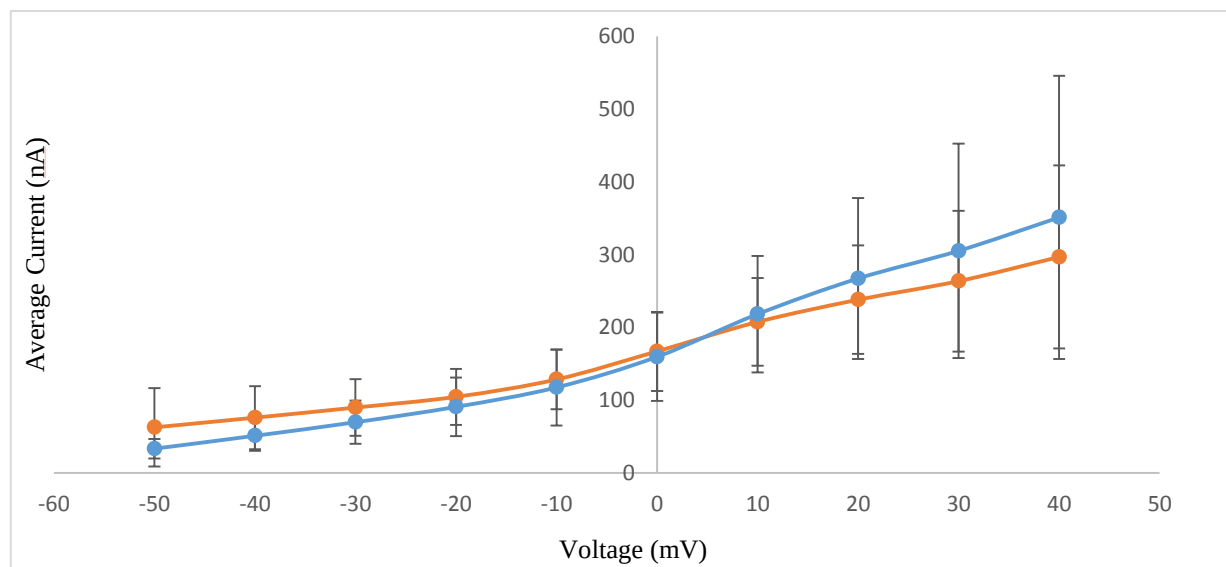


Figure 6. Average endogenous vs voltage graph for oocytes exposed to intracellular 0.1 μ M A β ₄₂. The current for each water-injected oocyte at each voltage step, from -50mV to +40mV, was averaged. The blue line represents the average current without intracellular application of 0.1 μ M A β (n=18). The orange line represents the average current with intracellular application of 0.1 μ M A β (n=16)). Error bars are the standard deviation.

Like the results of intracellular application of 0.1 μM of $\text{A}\beta_{42}$, both extracellular application of 0.1nM and 0.1 μM of $\text{A}\beta_{42}$ did not significantly inhibit the endogenous current ((df=2 t=0.218671 p=0.8472; df=9 p=0.4068 t=-0.87025). Figure 7 supports the conclusion that extracellular application of 0.1nM $\text{A}\beta_{42}$ did not inhibit the current while Figure 8 supports the conclusion that the endogenous current was not inhibited by extracellular 0.1 μM of $\text{A}\beta_{42}$.

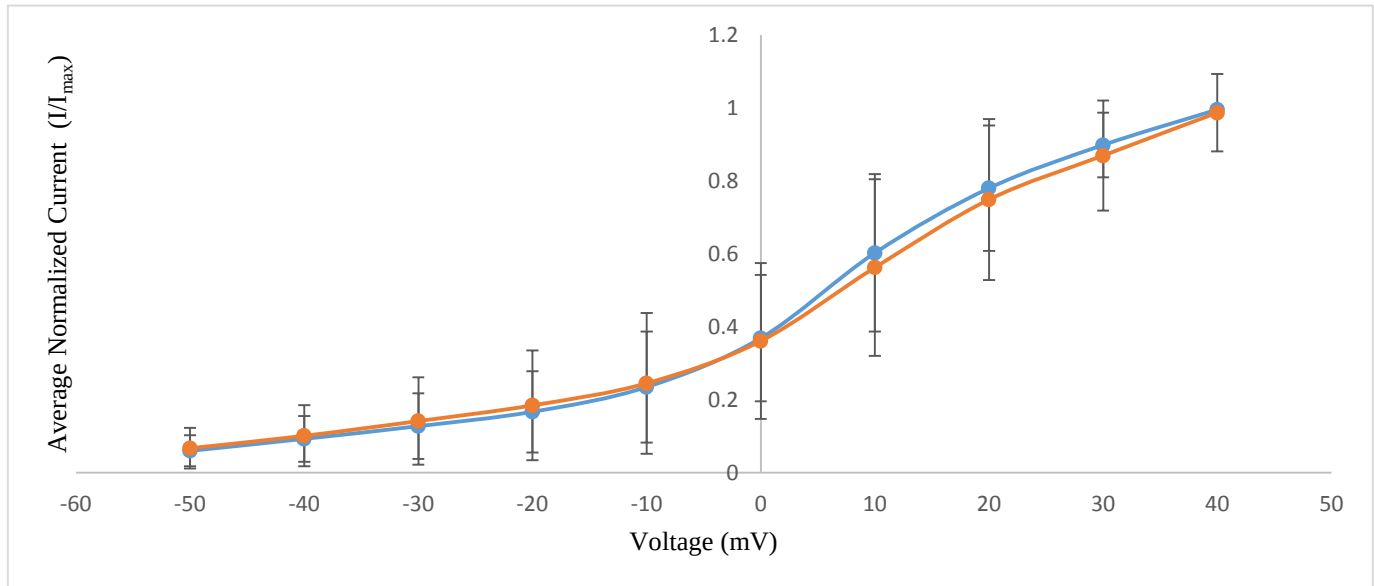


Figure 7. Average Normalized endogenous current vs voltage graph for water-injected oocytes exposed to extracellular 0.1 nM $\text{A}\beta_{42}$. The current for each water-injected oocyte at each voltage step, from -50mV to +40mV, before and after application of $\text{A}\beta$ was normalized to the average maximum current, at +40mV, of the same oocyte before application of 0.1 nM of $\text{A}\beta$. The current for each water-injected oocyte at each voltage step from -50mV to +40mV was averaged. The blue line represents the average current without application of extracellular 0.1 nM of $\text{A}\beta$ (n=3). The orange line represents the average current with application of extracellular 0.1 nM of $\text{A}\beta$ (n=3). Error bars represent the standard deviation.

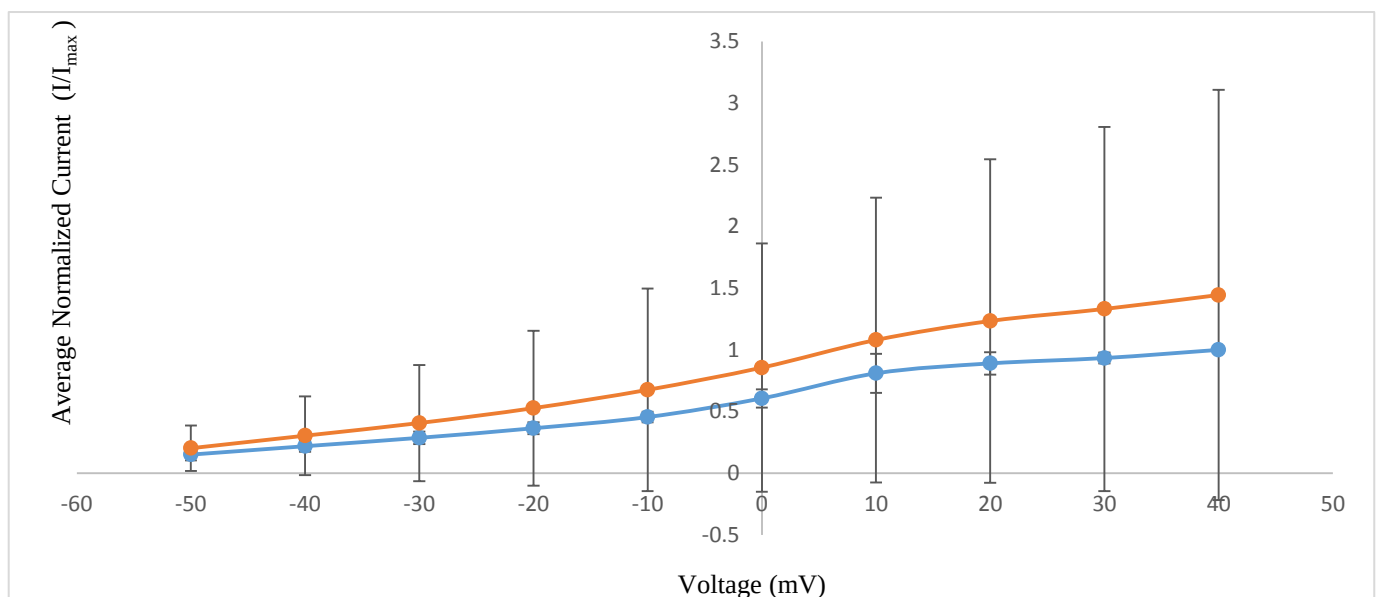


Figure 8. Average Normalized endogenous current vs voltage graph for water-injected oocyte exposed to extracellular 0.1 μM $\text{A}\beta_{42}$. The current for each water-injected oocyte at each voltage step, from -50mV to +40mV, before and after application of $\text{A}\beta$ was normalized to the average maximum current, at +40mV, of the same oocyte before application of 0.1 μM of $\text{A}\beta$. The current for each water-injected oocyte at each voltage step from -50mV to +40mV was averaged. The blue line represents the average current without application of extracellular 0.1 μM of $\text{A}\beta$ (n=10). The orange line represents the average current with application of extracellular 0.1 μM $\text{A}\beta$ (n=10). Error bars represent the standard deviation.

Kv 4.2

The average maximum current for Kv 4.2 was +14,699.62 nA at +40mV. Expression was noted when the channel displayed the unique upper ward, curved current shapes, seen in Figure 9, and had a maximum current that was 500nA or greater. Since Kv4.2 is a voltage-gated potassium channel, the upward deflection of the current trace reflects the outward movement of positive K⁺ ions leaving the cell when the channel is activated.

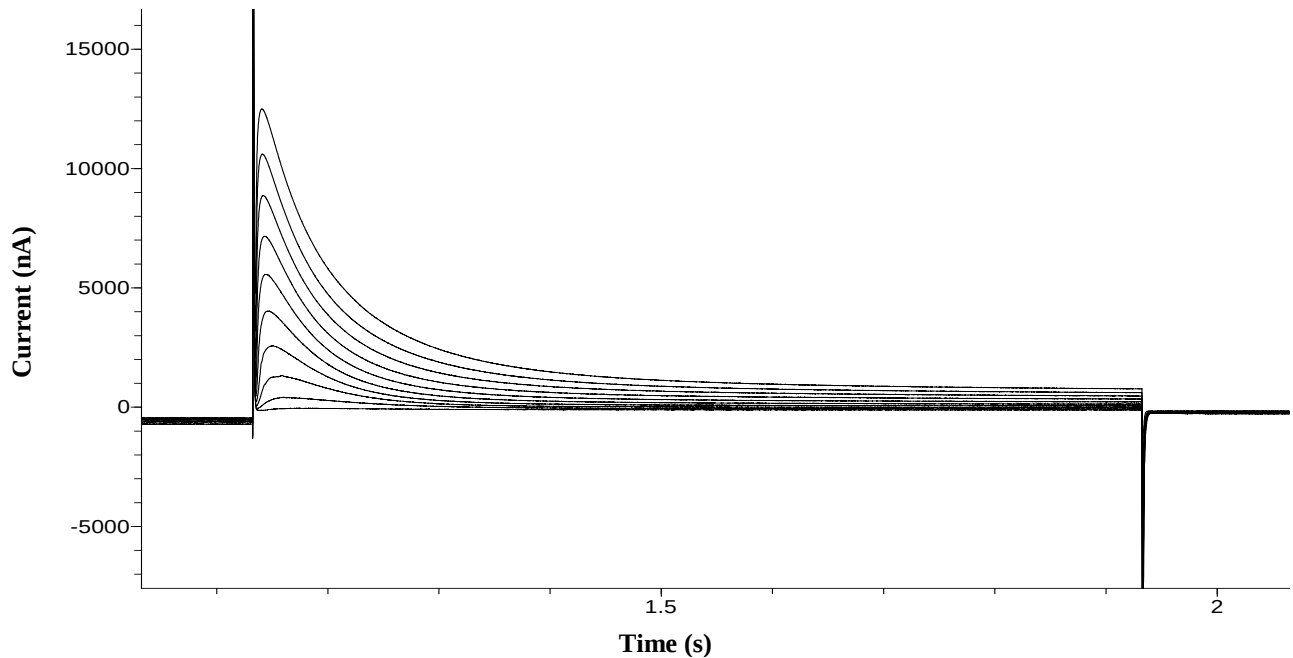


Figure 9. Current vs Time trace of a Kv4.2-injected oocyte. Each horizontal line represents the outward moving current of the Kv 4.2 voltage gated potassium channel at a given voltage step. The oocyte was held from -50mV to +40 mV in increments of 10mV. The lowest trace represents the current exhibited when the holding potential was at -50mV while the highest trace is the current exhibited when held at +40mV.

Similar to water-inject oocytes, intracellular application of 0.1nM of A β ₄₂ did not significantly inhibit its maximum current ($t=-1.41603$, $df=2.478194$, $p=0.2694$) when compared to maximum current of the Kv4.2 without intracellular application of 1nM of A β ₄₂.

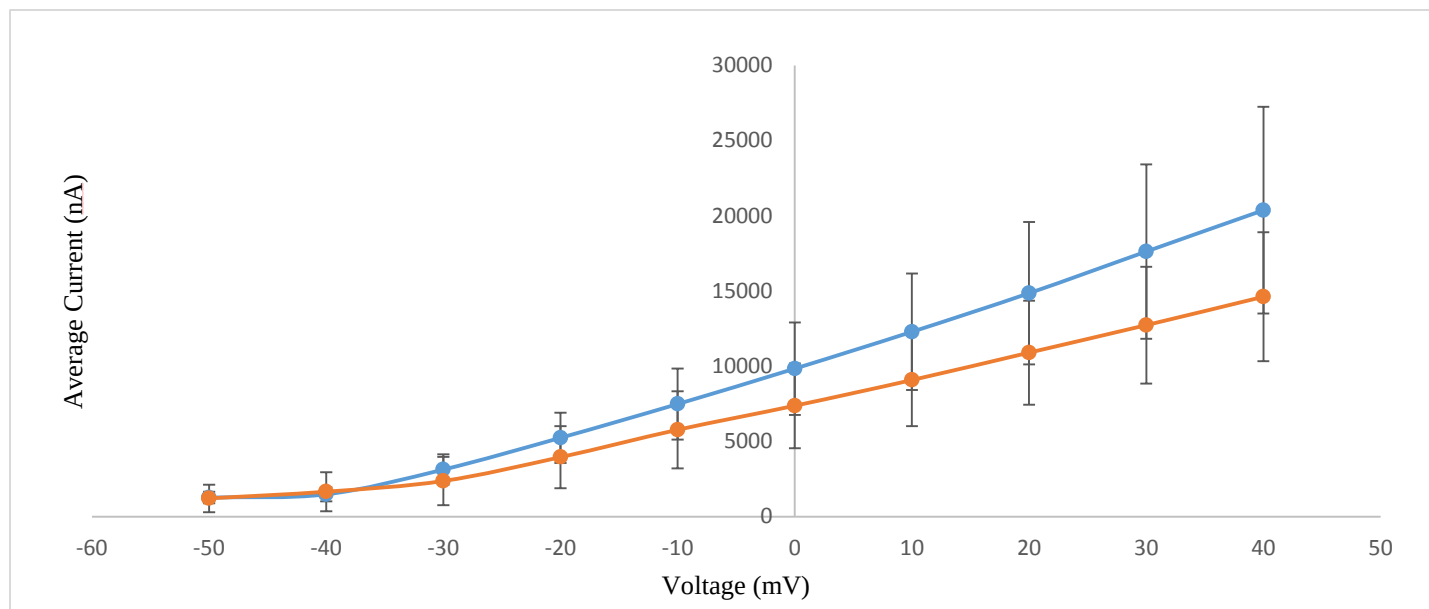


Figure 10. Average Current vs voltage graph for Kv4.2-injected oocytes exposed to intracellular 0.1 nM Aβ₄₂. The current for each Kv4.2-injected oocyte at each voltage step, from -50mV to +40mV, was averaged. The blue line represents the average current without intracellular application of 0.1 nM Aβ (n=12). The orange line represents the average current with intracellular application of 0.1 nM Aβ (n=3)). Error bars are the standard deviation.

At a higher concentration of Aβ₄₂, 0.1μM, applied intracellularly still did not inhibit the maximum current (t=-1.41603, df=2.478194, p=0.2694), as supported by Figure 11.

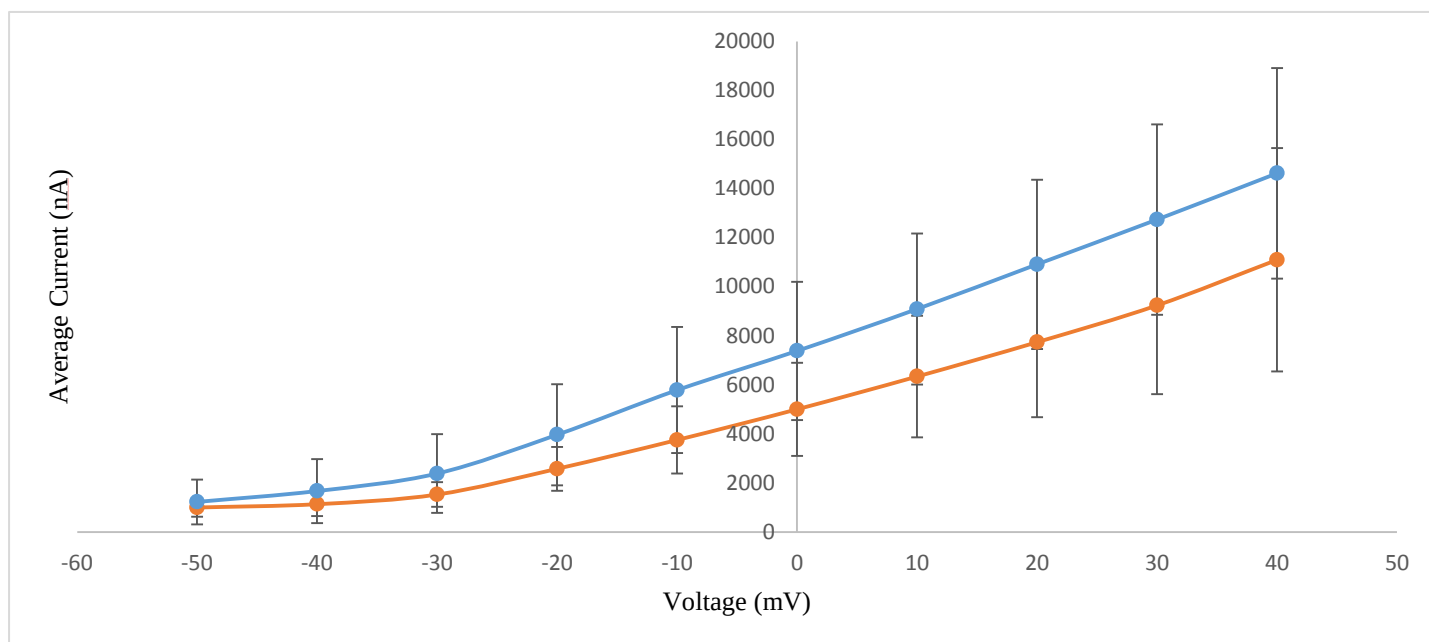


Figure 11. Average Current vs voltage graph for Kv4.2 injected oocytes exposed to intracellular 0.1 μM Aβ₄₂. The current for each Kv4.2-injected oocyte at each voltage step, from -50mV to +40mV, was averaged. The blue line represents the average current without intracellular application of 0.1 μM Aβ (n=12). The orange line represents the average current with intracellular application of 0.1 μM Aβ (n=7)). Error bars are the standard deviation.

Extracellular application of 0.1 nM of $A\beta_{42}$ did not appear to inhibit the maximum current at a concentration of 0.1 nM ($p=0.219$ $df=2$ $t=1.768818$), as seen in Figure 12.

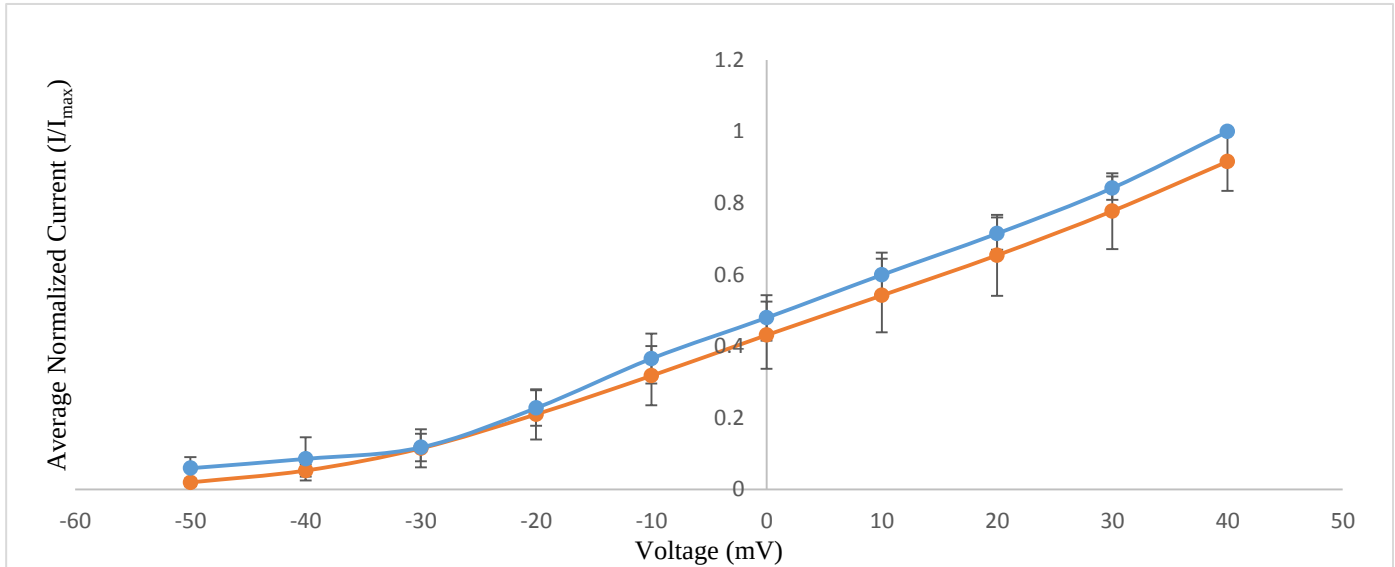


Figure 12. Average Normalized current vs voltage graph for Kv4.2- injected oocytes exposed to extracellular 0.1 nM $A\beta_{42}$. The current for each Kv4.2-injected oocyte at each voltage step, from -50mV to +40mV, before and after application of $A\beta$ was normalized to the average maximum current, at +40mV, of the same oocyte before application of 0.1 nM of $A\beta$. The current for each Kv4.2-injected oocyte at each voltage step from -50mV to +40mV was averaged. The blue line represents the average current without application of extracellular 0.1 nM of $A\beta$ ($n=4$). The orange line represents the average current with application of extracellular 0.1 nM $A\beta$ ($n=4$). Error bars represent the standard deviation.

However, at a higher concentration of 0.1 μ M of $A\beta_{42}$, extracellular application did significantly inhibit the maximum current ($p=0.0328$ $df=7$ $t=2.652837$). Although, this is not clearly exhibited in Figure 13, this conclusion is statistically supported by a matched paired t test. It is possible that outliers in the data could have affected the maximum current. Out of the 8 of the extracellular recordings collected on Kv4.2-injected oocytes, 5 recordings showed that current outputs were not significantly inhibited while 3 recording showed that current outputs were significantly inhibited by $A\beta_{42}$.

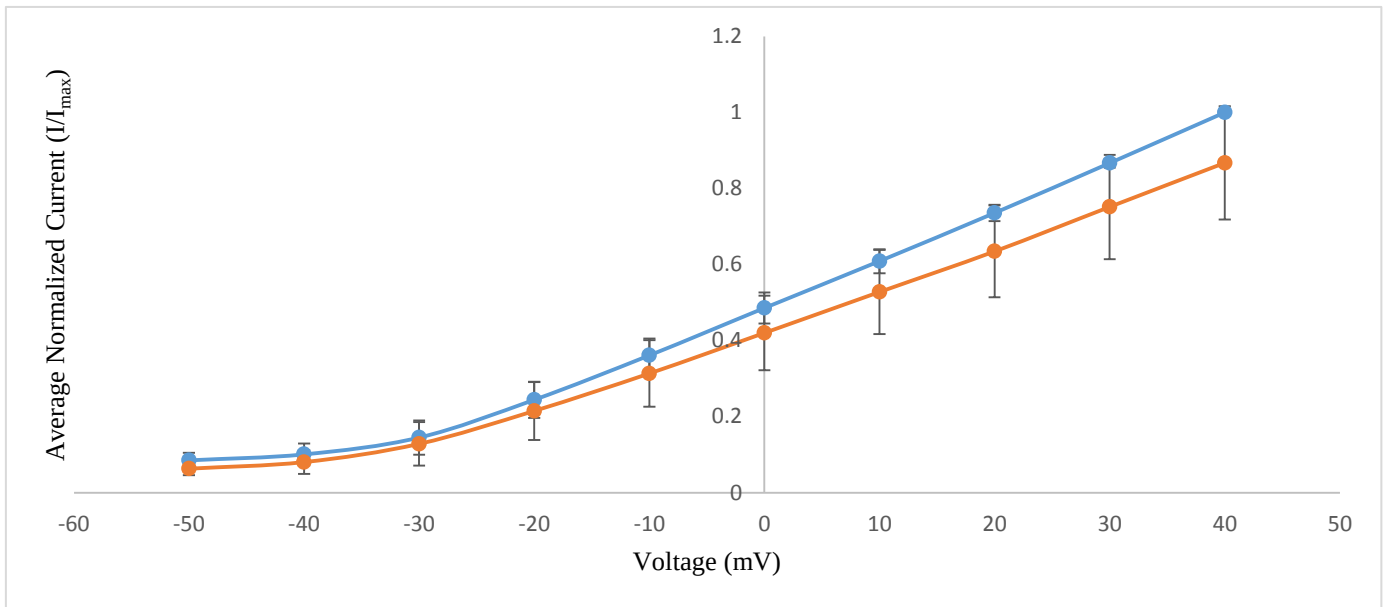


Figure 13. Average Normalized current vs voltage graph for Kv4.2 injected oocytes exposed to extracellular 0.1 μM A β 42. The current for each Kv4.2-injected oocyte at each voltage step, from -50mV to +40mV, before and after application of A β was normalized to the average maximum current, at +40mV, of the same oocyte before application of 0.1 μM of A β . The current for each Kv4.2-injected oocyte at each voltage step from -50mV to +40mV was averaged. The blue line represents the current without application of extracellular 0.1 μM of A β (n=10). The orange line represents the average current with extracellular 0.1 μM A β (n=10). Error bars represent the standard deviation.

Cav3.2

Unlike Kv4.2 and hSlo1.1, Cav3.2 expression is characterized by inward movement of positive ions because it is a calcium channel that upon activation, causes influx of Ca^{2+} ions. The maximum current is therefore exhibited at hyperpolarizing voltages. The maximum current for each oocyte was either exhibited at -50 mV or -40mV with a maximum average current of -3366.82 mV. Expression of this channel is indicated by an inward positive current of -200nA or lower and its unique downward current shape, as indicated by Figure 14. The downward deflection of each current trace reflects the inward movement of positive Ca^{2+} ion.

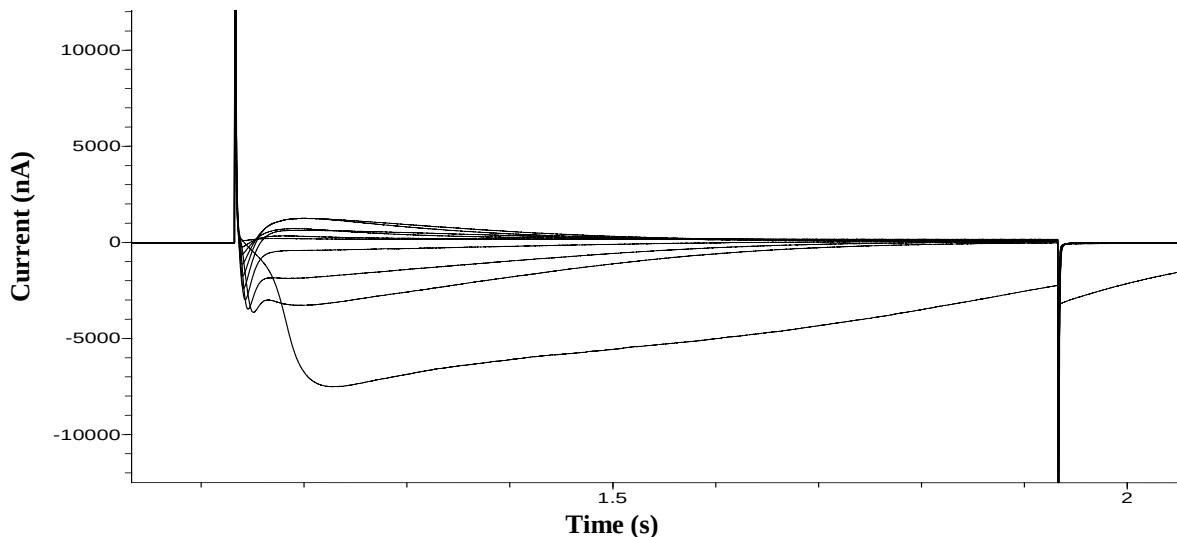


Figure 14. Current vs Time trace of a Cav3.2-injected oocyte. Each horizontal line represents the inward moving current of a Cav3.2 calcium channel at a given voltage step. The oocyte was held from -50mV to +40 mV in increments of 10mV. The lowest trace represents the current exhibited when the holding potential was at -50mV while the highest trace is the current exhibited when held at +40mV. The max current is exhibited at either -50mV or -40mV.

As seen in Figure 15, 0.1nM application of A β ₄₂ did not inhibit the Cav3.2 current ($t=-0.03665$ $df=11.9996$ $p=0.9714$). In addition, a 0.1 μ M intracellular application did not significantly affect the current when compared to recordings without application of intracellular A β ₄₂ ($df=29.2306$, $t=0.984638$, $p=0.3329$). Figure 16 supports these findings.

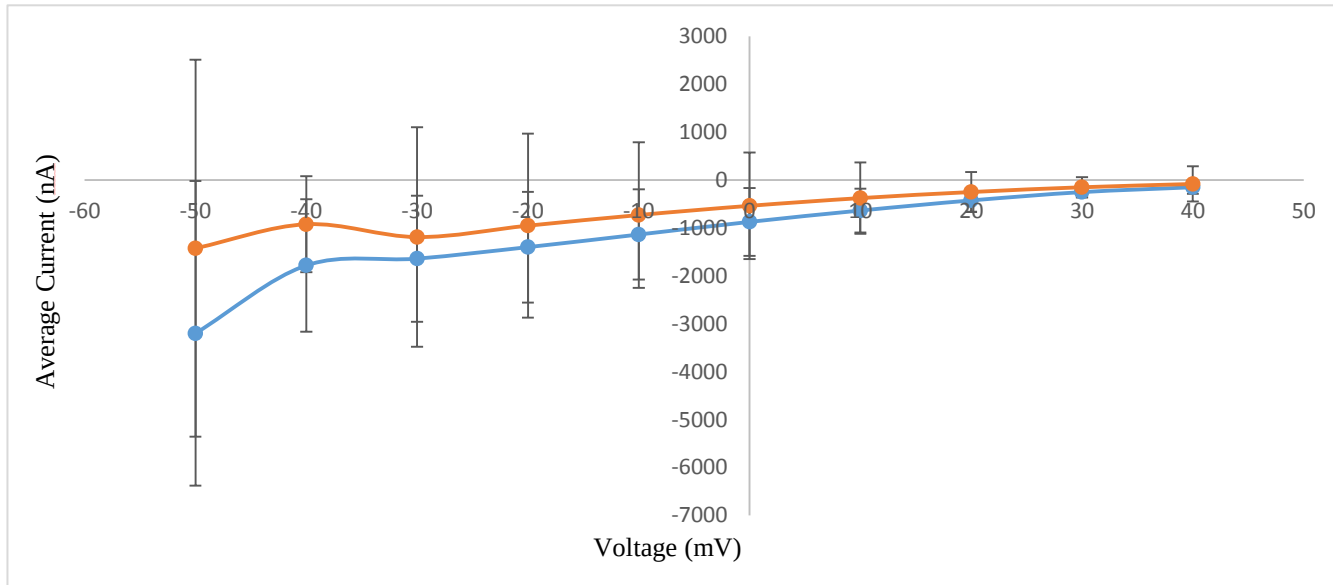


Figure 15. Average Current vs voltage graph for Cav3.2 injected oocytes exposed to intracellular 0.1 nM A β ₄₂. The current for each Cav3.2-injected oocyte at each voltage step, from -50mV to +40mV, was averaged. The blue line represents the average current without intracellular application of 0.1 nM A β (n=4). The orange line represents the average current with intracellular application of 0.1 nM A β (n=15). Error bars are the standard deviation.

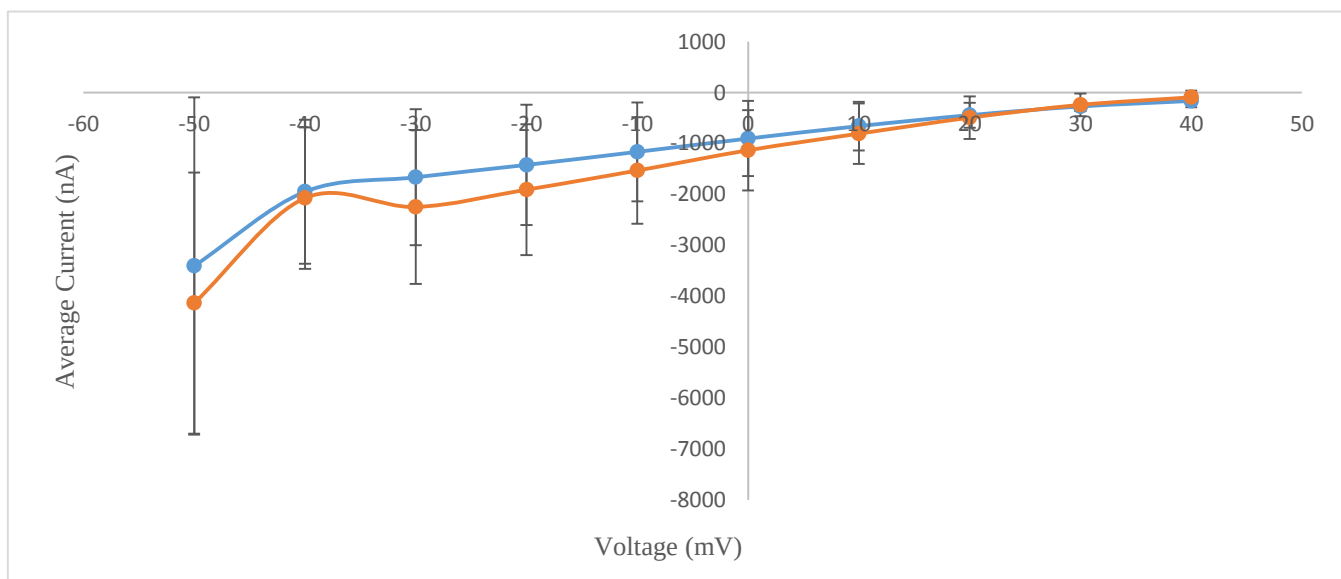


Figure 16. Average Current vs voltage graph for Cav3.2 injected oocytes exposed to intracellular 0.1 μ M A β ₄₂. The current for each Cav3.2-injected oocyte at each voltage step, from -50mV to +40mV, was averaged. The blue line represents the average current without intracellular application of 0.1 μ M A β (n=15). The orange line represents the average current with intracellular application of 0.1 μ M A β (n=13). Error bars are the standard deviation.

Similar to intracellular application of A β , both 0.1 μ M and 0.1 nM extracellular application of A β did not appear to greatly manipulate the current ($t = -1.79709$ $p = 0.1467$ $df = 4$; $t = -1.30771$ $df = 6$ $p = 0.2388$), supported by Figure 17 and 18.

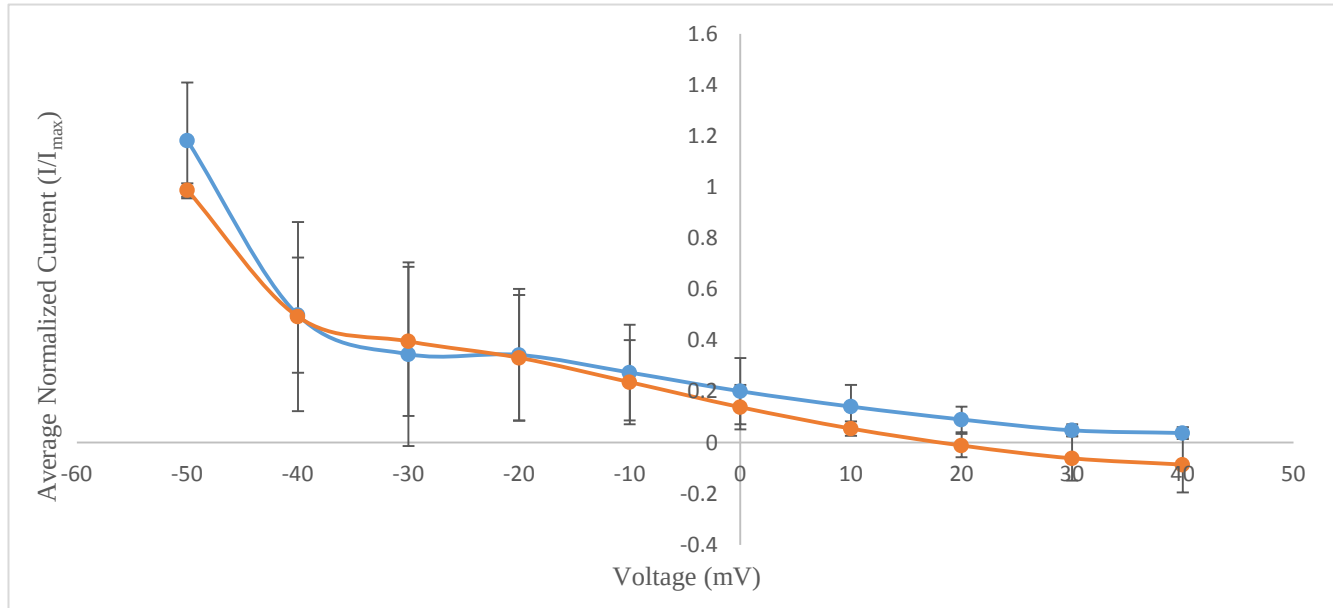


Figure 17. Average Normalized Cav3.2 injected current vs voltage graph for oocytes exposed to extracellular 0.1 nM A β 42. The current for each Cav3.2-injected oocyte at each voltage step, from -50mV to +40mV, before and after application of A β was normalized to the average maximum current, at -50mV or -40mV, of the same oocyte before application of 0.1 μ M of A β . The current for each Cav3.2-injected oocyte at each voltage step from -50mV to +40mV was averaged. The blue line represents the current without application of extracellular 0.1 nM of A β ($n = 10$). The orange line represents the average current with extracellular 0.1 nM A β ($n = 10$). Error bars represent the standard deviation.

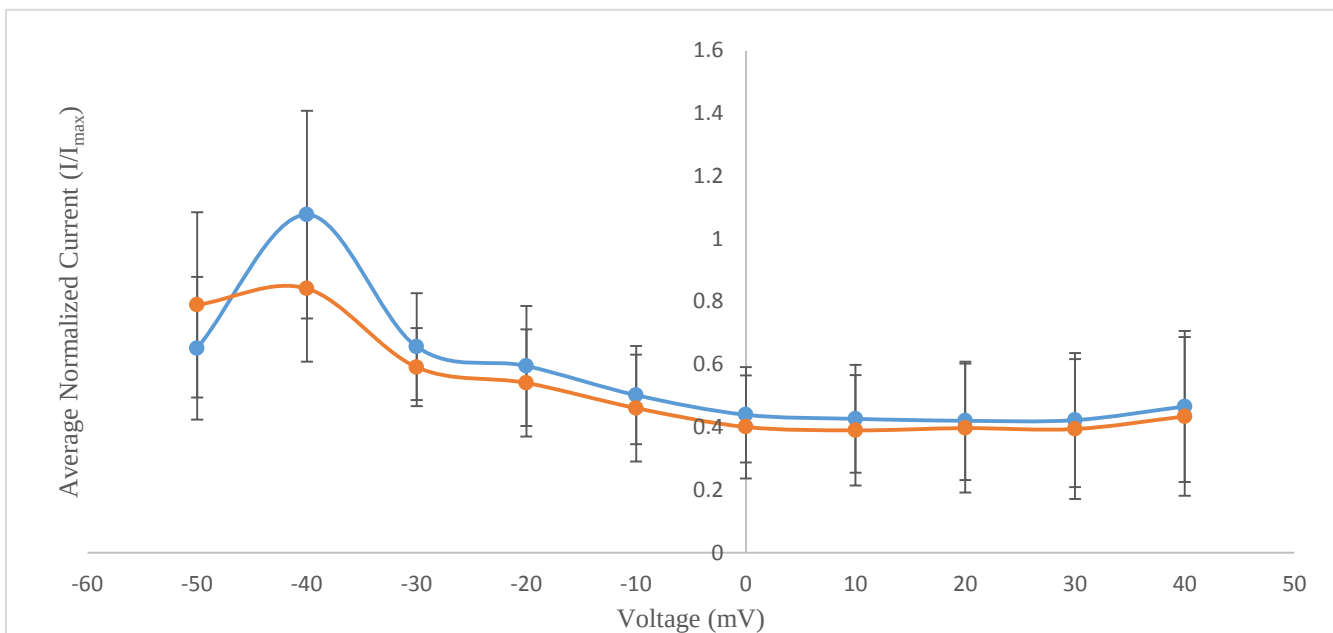


Figure 18. Average Normalized current vs voltage graph for Cav3.2 injected oocytes exposed to extracellular 0.1 μ M A β 42. The current for each Ca $^{2+}$ -injected oocyte at each voltage step, from -50mV to +40mV, before and after application of A β was normalized to the average maximum current, at -50mV or -40mV, of the same oocyte before application of 0.1 μ M of A β . The current for each Cav3.2-injected oocyte at each voltage step from -50mV to +40mV was averaged. The blue line represents the current without application of extracellular 0.1 μ M of A β ($n = 8$). The orange line represents the average current with extracellular 0.1 μ M A β ($n = 8$). Error bars represent the standard deviation.

Summary of Results

The findings from the Cav3.2, Kv4.2 and water-injected oocytes suggest that at a concentration of 0.1 nM of A β ₄₂, A β ₄₂ applications both extracellularly and intracellularly did not significantly inhibit the maximum current. Therefore, inhibition of the maximum current due to intracellular application of 0.1 nM A β ₄₂ is specific to hSlo1.1. At a higher application of 0.1 μ M A β ₄₂, extracellular application of 0.1 nM of A β to Kv4.2 was the only application of 0.1 μ M A β ₄₂ that inhibited the current. However, statistical findings could have been affected by outliers found within the data. This summary is described in Table 1.

Table 1: Summary of Aβ₄₂ intracellular and extracellular effects on inhibiting the maximum average current				
	hSlo1.1	Cav 3.2	Kv4.2	Water
	Was there inhibition of maximum current?	Was there inhibition of maximum current?	Was there inhibition of maximum current?	Was there inhibition of maximum current?
Intracellular 0.1nM	Yes	No	No	No
Intracellular 0.1μM	N/A*	No	No	No
Extracellular 0.1nM	N/A [€]	No	No	No
Extracellular 0.1 μM	N/A*	No	Yes [°]	No

Note: * indicates that Zuchelkowski previous Allegheny College Thesis did not test application of 0.1 μ M of A β ₄₂ on hSlo1.1. € indicates that the extracellular application of 0.1 nM of A β ₄₂ could not be statistically analyzed by a matched-pairs t-test due to small sample size [n=1]. ° indicates that outliers found in the data could have skewed the average maximum current and therefore affect the statistical findings found with a matched

Discussion

The focus of my study was to investigate whether the monomeric form of 0.1 nM of A β ₄₂ would significantly inhibit the maximum current of Kv4.2 and Cav3.2 as it did hSlo1.1 in a previous Allegheny College Senior Comprehensive Project. Zuchelkowski tested the effects of intracellular 0.1nM of A β ₄₂ on hSlo1.1. He found that intracellular A β ₄₂ significantly inhibited the maximum current of the BK channel (Figure 2). The intracellular recording technique he used was adapted from Yamamoto et al (2011). This finding is important because it showed that the maximum current could be observed without compromising the health of the oocyte (Zuchelkowski 2015). Because this method produced viable results, it allowed me to confidently use this technique knowing that conclusions made about A β ₄₂'s intracellular effects on Kv.42, Cav 3.2, and water were reliable. For my experiment, I administered 0.1 nM of A β ₄₂ to water, Kv4.2, and Cav3.2-injected oocytes. When inhibition was not noted at this concentration of hSlo1.1, I increased the application of A β ₄₂ to 0.1 μ M.

Intracellular and extracellular A β ₄₂ monomer at 0.1 nM and 0.1 μ M did not inhibit the maximum current of water-injected oocytes (Figure 5, Figure 6, Figure 7, Figure 8, Table 1). This finding proved significant because it goes against the channel hypothesis theory of AD, which proposes that A β ₄₂ are damaging and killing neurons by forming ion channels. These proposed channels created by A β are voltage-independent, generally large and allow for ions such as Ca²⁺ to flow through. Zn²⁺ and Al³⁺ can also block this channel (Kagan et al 2002). However, intracellular and extracellular recordings of A β ₄₂ did not indicate the presence of another ion channel because the current output without A β ₄₂ was similar to the current output with A β ₄₂ (Figure 5, Figure 6, Figure 7, Figure 8, Table 1). Nonetheless, an argument could be made that allowing the A β ₄₂ to diffuse either intracellularly or extracellularly, for 5 minutes, was

not a sufficient amount of time for the A β ₄₂ to form a channel. Therefore, a future study could test whether allowing A β ₄₂ to diffuse into the cell for a longer period of time would have an effect on the current, assuming that this does not affect the health to the oocyte. In addition, Zn²⁺ and Al³⁺ could be administered to test if administration negatively affects the current.

For Kv4.2, Figure 10 and Figure 11 show that intracellular A β ₄₂ monomer at 0.1 nM and 0.1 μ M did not inhibit the current. The fact that 0.1 nM of A β does not significantly inhibit Kv4.2's current specifically, was a tremendous find because out of all my experimental groups, Kv4.2 is most similar to hSlo1.1, since they are both voltage-gated potassium channels. The only difference is that Kv4.2 is a rapidly, inactivating A-type potassium channel that upon activation, K⁺ ions can leave the cell for a short period of time before the channel is quickly inactivated once again. hSlo1.1, on the other hand, is a voltage-gated and Ca²⁺ activating potassium channel (An et al 2000). These findings prove that A β 's effects are specific to hSlo1.1 and further proves that inhibition of one type of channel by A β ₄₂ cannot be generalized to similar channels.

Extracellular A β ₄₂ monomer at 0.1 nM also did not inhibit the maximum current of Kv4.2 (Figure 12). However, extracellular A β ₄₂ monomer at 0.1 μ M inhibited the maximum current based on statistical findings observed using a match paired t- test (Figure 13). It is possible that the mean of the average maximum current was skewed due to outliers found within the data. Of the 8 recordings conducted with application of extracellular 0.1 μ M of A β ₄₂, 3 showed significant inhibition while 5 showed minimal inhibition. Since it, ultimately, is not particularly clear how 0.1 μ M A β is affecting the Kv4.2 current, a dose-dependent application of A β ranging from 0.1 nM to 0.1 μ M could be tested to observe if application of A β ₄₂ inhibited the Kv4.2, and if so, at what concentration.

Figure 15,16, 17, and 18 demonstrations that the current of Cav3.2 was not inhibited by both intracellular and extracellular applications of 0.1nM of A β ₄₂ and 0.1 μ M of A β ₄₂. Therefore, it can be concluded that A β ₄₂ does not affect the Cav3.2. A future study could test a higher concentration of A β ₄₂ to further supports these findings. However, by increasing the concentration of A β ₄₂, A β ₄₂ could start to cause non-specific effects that indirectly led to the inhibition of the current.

Even though A β ₄₂ did not specifically inhibit Cav3.2 channel, it is important to note that A β ₄₂ can still affect the flow of Ca²⁺ ion, disrupting Ca²⁺ homeostasis. Yamamoto et al. 2011 found that inhibiting the BK channel current through intracellular application of A β ₄₂, via a patch pipette, caused the increase of intraneuronal calcium levels. Therefore, by indirectly inhibiting the BK channel, the deactivation and repolarization phase of the calcium channel was delayed, affecting the normal flow of Ca²⁺ ions. Furthermore, A β can disrupt intracellular calcium homeostasis by increasing the Ca²⁺ ion levels by activating the NMDA receptor subunit, GluN2B, expressed in cultured rat cortical cell (Ferreira et al 2012). Disruptions to Ca²⁺ homeostasis, such as this, can eventually lead to calcium-induced apoptosis which connects back to AD because memory loss occurs in-part because of neuronal cell death (Nakagawa 2000). Such findings emphasize the fact that even though A β may not be directly affecting Cav 3.2, it still can indirectly and directly affect calcium levels through other mechanisms.

The monomeric form of A β ₄₂ was used throughout this experiment both intracellularly and extracellularly. The A β was resuspended and sonicated with 1% of NH₄OH and either 3 M KCL or 1x ND-96, based on the manufacturer's protocol provided by ©rPeptide. Because the A β ₄₂ could be aliquoted to different amounts, it allowed for recording to be conducted over a period of time as opposed to only for one use. However, because the A β could only be

resuspended in either ND-96 or KCL, two vials of A β were needed in order to conduct both intracellular and extracellular recordings. Purchase of these A β vials, \$350 per vial, were expensive and required additional funds provided by the class of 1939 Senior Research Fund. Such a high cost emphasizes that as effective as these techniques are, they are also an expensive one to fund and replicate. For this reason, there is a need for a cost-effective way to synthesize and purify the A β_{42} invitro. Each frozen stock of monomeric A β_{42} underwent a freeze/ thaw cycle before it was diluted for recording. Barghon et al (2005) found that the monomeric form of A β aggregated to fibril form after it had been incubated in 0.1% NH₄OH for 1 day. The manufacturers treated the A β_{42} with HFIP to ensure that the A β stayed in its monomeric form but did not test the effects of the monomeric state after a freeze-thaw cycle, so there was not a proper technique to ensure that the monomeric A β tested was purely in its monomeric state for each recording. This further proves that a technique that would allow for vitro transcription of A β would be effective for these experiments to comeback aggregation that ultimately cannot be prevented. If A β could be synthesized the same day as each recording period, this could ensure that the optimal monomeric form of A β is used.

For intracellular application of A β_{42} , application was done based off of a technique adapted by Yamamoto et al (2011). Although, it is proven to effectively inhibit the current without compromising the oocytes' health, this method also has its limitations. For example, intracellular recordings of an oocyte with and without A β can only be done on different oocytes. This leaves room for outliers in the current data that is collected because the currents recorded can range, greatly, which in turn can skew the data. For example, the maximum current of a Cav3.2-injected oocyte can vary from -400 mV to -4000 mV, meaning that the average intracellular current outputs could only be compared to one another and not normalized. On the

contrary, extracellular application of A β ₄₂ were successfully able to be completed on the same oocyte. However, this technique is also limiting because this method does not include a proper wash-out technique which I was unable to perform. This technique would mean that after A β is applied to the recording bath, the solution could be replaced with fresh solution, that does not contain A β , to test whether the current would revert back to baseline (maximum current before extracellular application of A β ₄₂). Therefore, there is not a proper way to determine if inhibition is occurring based off A β application techniques or because of the A β itself. A future study could test different washout techniques. Although, improvement can be made to both methods, these methods, nonetheless, still proved to be effective as seen by my ability to collect viable intracellular and extracellular results.

All ion channels examined in my data were expressed in oocytes by injecting the RNA into the oocyte and allowing for expression of the ion channel in *Xenopus* oocytes. This technique has been proven to be an effective way to study ion channels in a controlled, in vivo environment (Goldin 1991). The oocytes' large size and ability to express ion channels makes it a good model for studying ion channels. However, the disadvantage of using oocytes is that each oocyte has a calcium-activated chloride channel that upon activation hyperpolarizes the cell by Cl⁻ ions influx. This means that during each of my TEVC recordings, activation of either Cav3.2 and Kv4.2 also activated this chloride channel. This specifically affected, Cav3.2 current recordings because the current from the influx of Ca²⁺ ions is competing with the influx of Cl⁻ ions causing the maximum current of Cav3.2 to fluctuate between -50 mV and -40mV. I took the influx of Cl⁻ ion into account through water subtractions to ensure that I was just analyzing the current output of the RNA. This highlights the need to collect water injected-data and conduct water subtractions on all RNA intracellular and extracellular data.

As effective as the oocytes were in expressing each RNA ion channel, survival rate and expression of the RNA in each oocyte, from the injection period to the recording period (3-4 days for Kv4.2 and 5-6 days for Cav3.2), was low and greatly varied due to several factors. These factors included the type of injection needle used (size of tip, how sterile the injection tip is, etc), the time lapse between injection and recording, the purity of the RNA injected, the ability of the oocyte to survive TEVC recording which includes holding different voltage steps and the overall health of oocytes produced by a given *Xenopus laevis*. Because of all these factors, my sample size for each application both intracellularly and extracellular ranged from 3 (the lowest) to 12 (highest). Although, this range seems relatively small, for this particular type of experiment, because of the many factors that can affect the oocyte survival rate, this sample size is relatively decent. In Zuchelkowski's Allegheny College Senior Comprehensive Project, he was able to draw conclusion from a sample size that ranged from 13 oocytes to 3 oocytes (Zuchelkowski 2015). Therefore, my ability to replicate both intracellular and extracellular methods and successfully collect a decent sample size, the lowest 3 and the highest 12, for each oocyte strengthens the idea that this model could be used in further research to study AD.

Another goal of this study, was to compare which application, extracellularly or intracellularly, of 0.1nM of A β ₄₂ would cause the greatest or any channel inhibition. Since at 0.1nM A β ₄₂, inhibition of the maximum current was not exhibited for Kv4.2 and Cav3.2, intracellular 0.1 nM A β ₄₂ monomer of hSlo1.1 exhibited the greatest channel inhibition. At a higher concentration, 0.1 μ M of A β , Kv4.2 was the only channel that was inhibited. However, outliers in the data suggest that further research must be done on Kv4.2 before conclusions about A β 's effects on the current can be made. In addition, intracellular application of A β on hSlo1.1 was the only application that produced the channel inhibition which supports the idea that in

even before the presence of A β extracellularly in AD, A β is first affecting the neurons intracellularly. Oddo et al (2003) found that AD-related defects, such as LTP defects and synaptic dysfunction were exhibited intraneuronally in mice before the presence of extracellular deposits of A β was exhibited.

Since intracellular application of 0.1nM of A β significantly inhibited the maximum current of hSlo1.1, studies should to further examine hSlo1.1's role in AD. For example, Williams et al. (2004) found that the inhibition of the BK channel can possibly affect neurons' ability to sense oxygen. The membrane complex of BK channels was speculated to include an oxygen sensor. Antibodies that tag the BK ion channel complex identified Hemoxygenase-2 (HO-2). To further examine the possibility that HO-2 was indeed part of the complex, a knockdown of HO-2 expression was administered and a reduction in channel activity developed as a result. On the other hand, carbon monoxide administration, a product of HO-2 activity rescued this loss. If an oxygen sensor is indeed present, inhibition of BK channel would cause the inability for neurons to detect oxygen supplies which would also, in turn, damage neurons. The connection to AD becomes apparent because such extensive alterations to neuronal activity can lead to extensive neuronal damages and neuronal cell death as well.

Although Cav3.2 was not inhibited by 0.1 μ M of A β and Kv4.2 and Cav3.2 were not inhibited by 0.1 nM of A β , these findings are still important. The exact neuronal pathology that A β_{42} takes in the brain that eventually leads to AD is still not known. Researchers and drug companies investing how ion channels are significantly affected by A β_{42} can benefit from the understanding of how A β interacts with different ion channel because this knowledge can help narrow the type of ion channels they study. Therefore, if A β does not affect a specific channel, they can draw conclusion that A β 's effects are not significant to this specific channel and allow

for them to invest their time on conducting research on other types. Therefore, they can better aliquot their time, energy, and money. Therefore, these results add to the body of knowledge about A β can eventually lead to AD. The 5 million people living with this disease rely on this type of research because although there is not a cure for AD, research can aid in the creation of better medication that can delay the onset of AD as well as improve the quality of life for AD patients (Alz.org 2016).

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