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ABSTRACT

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The exact mechanism and progression of Alzheimer's disease (AD) at present is not fully understood. In patients suffering from AD, damage to the hippocampal region and impairment of learning and memory is present. It is also known that a buildup of β-amyloid plaques occur in AD patients and that β-amyloid interacts with some subtypes of neuronal nicotinic acetylcholine receptors (neuronal nAChRs). These receptors are composed of five subunits. The most prevalent nAChR subunit composition through the brain as a whole is α7. Previous data produced from our lab suggests that α7 nAChRs are also one of the most prevalent subunits expressed by interneurons within the hippocampal region, a part of the brain known to be involved in memory and learning. It is hypothesized that one mechanism through which learning and memory becomes impaired in AD is through the interaction of β-amyloid with these nAChRs. It has previously been established that nanomolar amounts of β-amyloid inhibit the peak currents of α7 nAChRs. However, concentrations of β-amyloid in the picomolar range, in some studies show an activation of α7 nAChRs, while other studies no activation is seen. In this experiment we show that human α7 subunit nAChRs are not activated by β-amyloid$_{42}$ at 1 pM-30 nM concentrations. We also show that short, seven-second applications of β-amyloid interact with the α7 nAChRs to alter the kinetics of the channel, however, the exact mechanism and pattern by which it effects the channel is still unclear.

Keywords: Alzheimer's Disease, β-amyloid, α7 nicotinic acetylcholine receptors

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INTRODUCTION

Alzheimer's Disease

Alzheimer's Disease (AD) is a form of dementia most prevalent in people over the age of 65 (Brookmeyer, Johnson, Ziegler-Graham, & Arrighi, 2007). It is a neurodegenerative disease that leads to decreased cognitive performance, impaired memory, and eventually death (Waldemar et al., 2007). The early stages are rather difficult to diagnose since the symptoms may be subtle or indicative of other disorders. Clinical diagnoses are made from patient histories, cognitive tests, and most recently, the use of advanced medical imaging of the brain (Dougall, Bruggink, & Ebmeier, 2004; Jack et al., 2008). In 2006, there were over 26.6 million people diagnosed with AD worldwide and it is predicted that by 2050, 1 in 85 people globally will be affected (Brookmeyer et al., 2007). The course of AD is unique to each person making it difficult to assess a prognosis for an individual. However, after diagnosis, the mean life expectancy is under seven years with less than three percent of patients surviving more than fourteen years (Molsa, Marttila, & Rinne, 1995). At present, AD is an incurable disease. While the exact mechanisms of development and progression of the disease are currently not fully understood, two hallmarks of AD include damage to the hippocampus and the accumulation of extracellular plaques that contain amyloid proteins (Price, Davis, Morris, & White, 1991).

The hippocampus is located in the medial temporal lobe of the brain and is part of the limbic system. It is known to play a role in learning and memory with acetylcholine being a crucial neurotransmitter for signaling and proper function (Lynch, 2004). Cholinergic input in the hippocampus is delivered via the septum-diagonal band complex which projects to many regions of the hippocampus including the CA1 and CA3 (Frotscher & Leranth, 1985) and has as one of its targets, nicotinic acetylcholine receptors (nAChRs) on hippocampal interneurons

(Jones & Yakel, 1997). Thus, nAChRs in the hippocampus play a critical role in the ability to form memories and in the learning process.

Acetylcholine Receptors

Acetylcholine receptors (AChRs) are broken down into two subgroups; nicotinic AChRs, or muscarinic AChRs (mAChRs), depending on whether nicotine or muscarine can activate the channels in addition to other ligands. NAChRs can be further broken down to either muscle or neuronal nAChRs. Neuronal nAChRs are widely distributed throughout the brain and the peripheral nervous system, and can be found both pre and post synaptically, forming ligand-gated ion channels which increase the permeability of the membrane to cations (Albuquerque, Pereira, Alkondon, & Rogers, 2009).

Each nAChRs is composed of five subunits that form either a homopentamer or heteropentamer ion channel. To date, 17 subunit genes have been identified: 5 of which are classified as muscle-type, 12 are neuronal-type, and only 9 subunits are expressed in the human genome with the remaining subunits being found in the genomes of chicks and rats (Le Novere & Changeux, 1995). The subunits identified thus far for neuronal nicotinic AChRs are: α2 through α10 and β2 through β4 (Albuquerque et al., 2009); with α7, α8, and α9, being the only subunits that can form homopentamer channels (Karlin, 2002). The varying subunit compositions create channels that display different kinetics and ligand binding properties. Thus, a specific agonist or antagonist for one subunit composition will not interact with all subunit configurations in the same manner (Sala et al., 2005).

Overall, the most abundant homopentamer subunit configuration present in the brain is α7 (Figure 1). A high concentration of these subunits is located in the hippocampus, a region

known to play an important role in learning and memory. (Flores, Rogers, Pabreza, Wolfe, & Kellar, 1992; Gopalakrishnan et al., 1996).

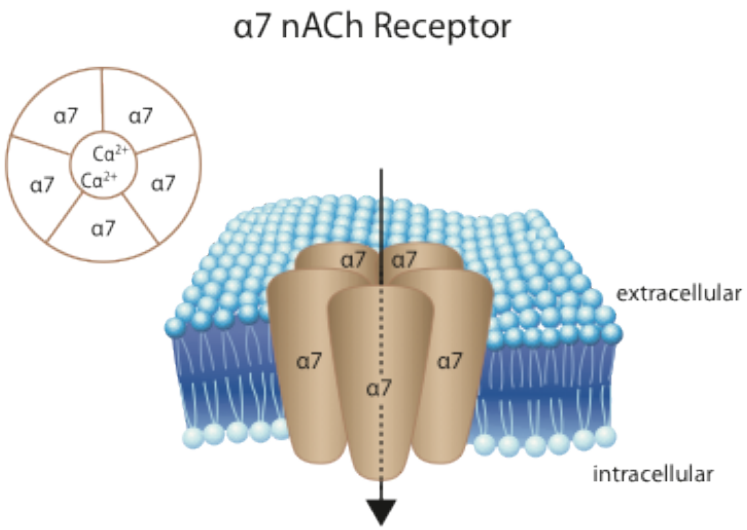

Figure 1 *A homopentameter nAChR composed of five α7 subunits. The subunits assemble together forming a channel where calcium and other positive ions can pass through.*

The hippocampal region of AD patients have consistently shown significant losses in radioligand binding sites corresponding to neuronal nicotinic acetylcholine receptors (nAChRs), implicating a correlation between AD and nAChRs (Nordberg, 2001). In addition to binding ACh, these receptors are also able to interact with β-amyloid (Wang, Lee, Davis, & Shank, 2000).

β-amyloid

β-amyloid, the main constituent of the amyloid plaques present in AD patients, is a transmembrane protein that is formed by several successive cleavages of the amyloid precursor

protein (APP). Cleavage of APP by α-secretase disrupts the β-amyloid region, preventing the release of β-amyloid. However, β-secretase cleavage followed by γ-secretase cleavage releases the β-amyloid protein (Figure 2) (Kametani, 2008; Selkoe, 1998). This protein can be 36-43 amino acids long, with 40 or 42 being the most common isoforms.

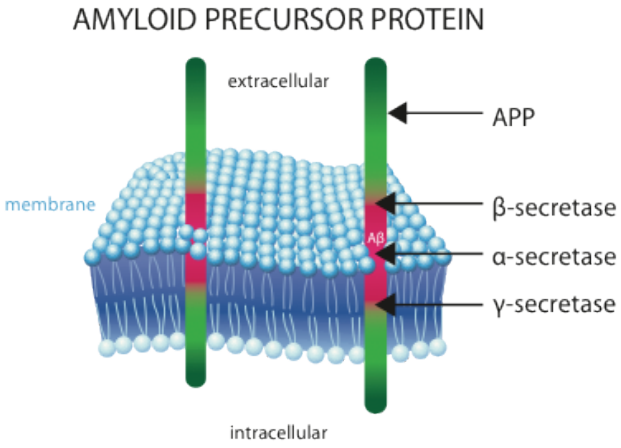

Figure 2 *β-amyloid, a transmembrane protein, is released when APP is cleaved by β and γ-secretases. Cleavage of APP by α-secretase prevents the release of β-amyloid.*

β-amyloid$_{40}$ is the main constituent of the β-amyloid population in normal individuals, however, in AD the balance between β-amyloid$_{40}$ and β-amyloid$_{42}$ shifts so that β-amyloid$_{42}$ becomes the most predominate form (Kuo et al., 1996; Schupf et al., 2010). The production of β-amyloid is thought to follow a circadian rhythm, possibly explaining why early onset AD is associated with chronic sleep deprivation (Kang et al., 2009). The estimated levels of endogenously produced β-amyloid in healthy rodents are in the picomolar range (Cirrito et al., 2003).

Along with being present in an increased amount in AD patients, β-amyloid$_{42}$ has also been shown to be toxic to neurons (Lambert et al., 1998). Furthermore, studies have shown that

mice transgenically engineered to over express β-amyloid display symptoms similar to those seen in AD (Hsiao et al., 1996; van Groen, Kiliaan, & Kadish, 2006). Under physiological conditions, β-amyloid proteins have been found to aggregate. Three different forms of β-amyloid have been identified: monomers, oligomers, and fibrils. These forms of β-amyloid display differing degrees of cell toxicity (Dahlgren et al., 2002).

Originally, due to studies that showed solutions containing β-amyloid in the fibrillar form induced neuronal cell death, it was thought that amyloid fibrils played the biggest role in development of AD (Lorenzo & Yankner, 1994). However, that hypothesis was later challenged as studies began to emerge showing that there was little to no correlation between the amount of fibrillar β-amyloid build up and the progression of neurological deficits. Due to an increase in evidence suggesting a direct correlation between the levels of oligomers and the progression of AD, it is now widely thought that oligomers are the most toxic form of β-amyloid (Gong et al., 2003).

β-amyloid Concentrations and α7 nAChR interactions

Recently studies have shown that different concentrations of β-amyloid alter the kinetics of α7 nAChRs in different ways. While several studies have demonstrated antagonistic results at a nanomolar (or higher) concentrations of β-amyloid, (Grassi et al., 2003; Liu, Kawai, & Berg, 2001; Pettit, Shao, & Yakel, 2001) reports of the effects of picomolar concentrations of β-amyloid are not quite as consistent. Some studies report that picomolar concentration of β-amyloid inhibit the activation of α7 nAChRs (Grassi et al., 2003; Lamb, Melton, & Yakel, 2005; Pym et al., 2005), while other studies report direct activation of nAChRs by β-amyloid in the picomolar range (Dineley, Bell, Bui, & Sweatt, 2002; Puzzo et al., 2008; Wu, Khan, & Nichols,

2007). When activation of α7 channels is reported, it is also unclear if activation by β-amyloid occurs repetitively or only on the first exposure to β-amyloid. The discrepancy in these findings could possibly be due to different forms of β-amyloid (monomers, oligomers, or fibrils), whether the β-amyloid used is human or rodent β-amyloid, or differences between human subunit genes and rodent subunit genes.

SPECIFIC AIMS

In this study we use human α7 subunit nAChRs expressed in Xenopus oocytes to do the following:

1. Determine if picomolar concentrations of β-amyloid will activate α7 nAChRs. If they do then we will establish a dose response curve. Acetylcholine will be used to activate the channels to ensure they are working properly.
2. If no direct activation is seen then we will determine the effects of β-amyloid on the kinetics of the channel in response to ACh. This will be done by administering two applications of ACh for seven seconds each. Next, β-amyloid will be applied for seven seconds and the two applications of ACh will be applied again. We will then evaluate the peak amplitude, rise time, decay time, and decay tau, comparing the first peak pre β-amyloid and the first peak post β-amyloid. We will evaluate the same comparisons for the second peaks.

METHODS

Plasmid DNA Preparation

Human α7 (origene# SC124074-20) subunit genes in the pCMV6-XL4 plasmid were purchased from Origene Technologies, Rockville MD. All plasmids were transformed into

chemically competent One Shot® e.coli cells (Invitrogen , Carlsbad CA) according to the protocol supplied by the manufacturer. Plasmid isolation and purification were performed using the HiSpeed® plasmid purification kit by QIAGEN Inc. (Valencia CA).

Plasmids containing α7 genes were linearized by restriction digest with SacI (New England BioLabs). The conditions were followed according to the suggested protocol from New England BioLabs. The mRNA was then transcribed, capped on the 5' end, a poly(A) tail was added, and LiCL purification was performed using the mMessage mMachine® T7 Ultra Kit (Ambion , Carlsbad CA) according to the protocol provided. RNA was re-suspended in TE Buffer (Bioexpress, Layton UT), aliquoted, and stored at -80 degrees Celsius until used.

Xenopus Oocyte Isolation

Oocytes were harvested from female *Xenopus laevis* frogs purchased from Xenopus 1, Inc. (Dexter MI). The frogs were housed in an environmental chamber with a 12-hour day/night cycle with the temperature was maintained at 17-19 degrees Celsius. The frogs were fed either beef heart or liver bi-weekly.

Surgeries were performed once a week under sterile conditions and in accordance with the IACUC protocol. Frogs were anesthetized by submersion in a solution (12 mM NaCl, 0.134 mM KCl, 0.181 mM $CaCl_2$, 0.476 mM $NaHCO_3$, 5mM Hepes) containing 0.1% ethylmetaaminobenzoate (MS-222; Sigma, St. Louis MO) adjusted to pH 7.5 with NaOH, for at least 15 minutes and until notably unresponsive. Each frog underwent a maximum of six surgeries with at least six weeks between each surgery. After the sixth surgery the frog was sacrificed.

Ovary sacs were placed in a calcium free OR-2 solution (89.5 mM NaCl, 2mM KCl, 1mM $MgCl_2$ anhydrous, 5mM Hepes, adjusted to pH 7.5 with NaOH) and manually separated

with forceps. Oocytes were then defollicuated by treatment with collagenase A (Sigma, St. Louis MO). The oocytes were incubated for 2-2.5 hours on a tilting table at 17-19 degrees Celsius. The cells were then rinsed and stored in an OR-2 solution with Ca^{2+} (82 mM NaCl, 2.5 mM KCl, 1 mM Na_2HPO_4, 5 mM Hepes, 1 mM CaCl, 1 mM $MgCl_2$ anhydrous, adjusted to ph 7.5 with NaOH) at 14-17 degrees Celsius for a minimum of 24 hours prior to injections.

RNA Expression in *Xenopus* Oocytes

Human α7 mRNA was injected into oocytes using a nanoject II automatic nanoliter injector (Drummond Scientific, Broomall PA). Each oocyte was injected with 50.6 nL of mRNA for a total concentration of 75.9 ng α7 mRNA per oocyte. The oocytes remained in a solution of OR-2 with Ca^{2+} at 14-17 degrees Celsius. The solution was changed daily and recordings were obtained 3-5 days later.

Two-electrode Voltage Clamp Electrophysiology

The kinetics of the ion channels were measured by two-electrode voltage clamp electrophysiology using a standard Geneclamp 500B amplifier (Axon Instruments, Sunnyvale CA) and a Digidata 1322A digitizer (Axon Instruments, Sunnyvale CA). Traces were recorded using Clampex 9.2 software (Axon Instruments, Sunnyvale CA) and analyzed on ClampFit 9.2 (Axon Instruments, Sunnyvale CA). Electrodes containing 3 M KCl and having a resistance of 1 to 10 mΩ were made from 1.5mm borosilicate glass capillaries (Harvard Apparatus, Kent, England) pulled on a Flaming/Brown micropipette puller model P-97 (Sutter Instrument, Novato CA). Holding potentials were clamped at -60 mV. Traces were sampled at 5 KHz and filtered at 1000 Hz.

Oocytes were continuously perfused with OR-2 with Ca^{2+} at 15-18 mL/minute. The oocytes were stabilized on a perfusion platform (model RC-1Z Warner Instruments, Hamden CT). Acetylcholine solutions (10 μM, 33 μM, 100 μM, 333 μM, 100 μM) were prepared fresh each week from powdered ACh (Sigma, St. Louis MO) in OR-2 with Ca^{2+}. Administration was controlled by an automated perfusion system (Valvelink 8 Automate Scientific Inc., Berkley CA). A β-amyloid scrambled peptide was used as a control to ensure that any interactions observed were specific to the β-amyloid$_{42}$ peptide. The scramble peptide is composed of the same amino acids that make up β-amyloid$_{42}$, but they are arranged in a different sequence than the β-amyloid$_{42}$ peptide.

β-amyloid$_{42}$ and β-amyloid scrambled (AnaSpec Inc., Fremont CA) were prepared from powder and were dissolved in OR2 with Ca^{2+} for a final concentration of 1 pM, 30 pM, 300pM, 1 nm, and 30 nm. β-amyloid dilutions were made fresh and kept at room temperature. ACh, β-amyloid, and β-amyloid scrambled were applied for seven seconds. All electrophysiological experiments were performed at room temperature.

A control valve containing OR2 with Ca^{2+} was used to ensure there were no perfusion artifacts as a result of switching valves. The programs used are as follows:

The program to obtain the dose-response curve with ACh was as follows:

5 seconds OR-2 with Ca^{2+}
7 seconds control valve OR-2 with Ca^{2+}
10 seconds OR-2 with Ca^{2+}
7 seconds Ach (1000 μM, 333 μM, 100 μM, 33 μM, or 10μM)
91 seconds wash with OR-2 with Ca^{2+}

The program for paired peak perfusion of ACh was as follows:

5 seconds OR-2 with Ca^{2+}
7 seconds ACh 333 μM
7 seconds OR-2 with Ca^{2+}
7 seconds ACh 333 μM
91 seconds OR-2 with Ca^{2+}

The program for paired peak perfusion with β-amyloid and β-amyloid scramble was as follows:

5 seconds OR-2 with Ca^{2+}
7 seconds β-amyloid or β-amyloid scramble (1 pM, 30 pM, 300 pM, 1 nM, and 30 nM)
7 seconds OR-2 with Ca^{2+}
7 seconds ACh 333 μM
7 seconds OR-2 with Ca^{2+}
7 seconds ACh 333 μM
80 seconds OR-2 with Ca^{2+}

The programs included five repetitions (sweeps) of the above protocols to measure reproducibility of the responses measured.

Data Analysis and Statistical Procedures

The acetylcholine dose-response curve was fit with a sigmoidal curve using Prism 4.0, GraphPad software (San Diego CA). InStat ver. 3.05, GraphPad software (San Diego CA) was used to perform repeated measures ANOVA, Tukey-Kramer post-hic testing, and two-tailed T-test statistical analysis. The peak amplitude for each oocyte in response to a 333 μM ACh application was used to normalize the data to a 100% response for each oocyte. Repeated measures ANOVA analysis was used to compare the peak amplitude, rise time, decay time and tau decay for the first paired peak pre β-amyloid with the first paired peak post β-amyloid application. The same statistical analysis was use to compare the second paired peak pre β-amyloid to the second paired peak post β-amyloid. The Tukey-Kramer post-hoc analysis was also used to compare these categories when relevant. Statistical significance was determined by $p<0.05$.

RESULTS

Acetylcholine Dose-Response Curve

To ensure that naïve oocytes (oocytes prior to α7 mRNA injections) were not responsive to acetylcholine applications we applied 333 μM acetylcholine to an uninjected (naïve oocyte) (Figure 3). No response was observed. When acetylcholine is applied to an oocyte injected with α7 mRNA, a downward peak is produced, showing activation of the α7 nAChR (Figure 4).

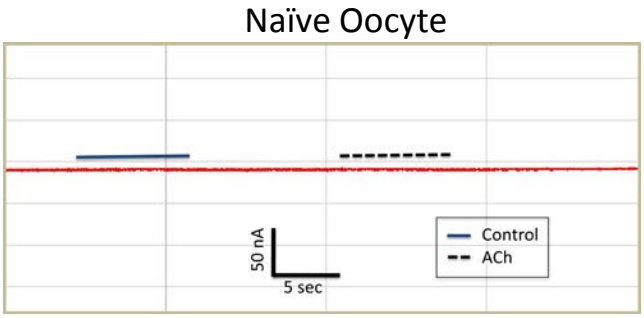

Figure 3 *A control valve with OR-2 shows that there are no artifacts resulting from changing profusion valves. A naïve oocyte shows no response when 333 μM acetylcholine is applied.*

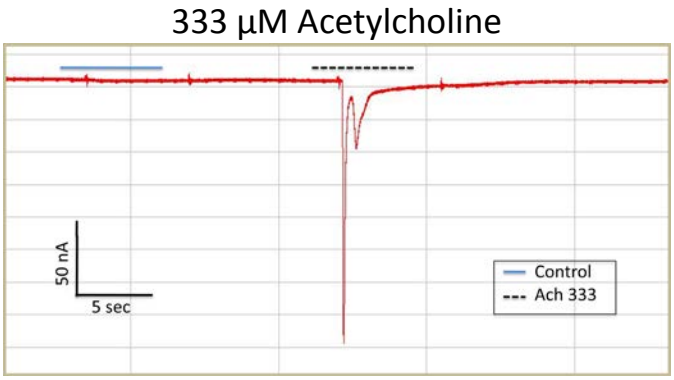

Figure 4 *An oocyte injected with α7 mRNA demonstrates a response to 333 μM acetylcholine.*

Acetylcholine was applied to oocytes expressing α7 nAChRs for seven seconds at concentrations of 1000 μM, 333 μM, 100 μM, 33 μM, or 10μM. Data was collected at each concentration with five sweeps of the acetylcholine application program. All data points were normalized to the average peak response of the 333 μM ACh concentration. The peak amplitudes were measured at each concentration and used to make a dose-response curve (Figure 5). The EC50 obtained is 85 μM (r^2 = .9378, Hill coefficient = 2.518) and fits within the previously established range of the EC value for α7, verifying that the expression of α7 nAChRs display normal characteristics for this subtype configuration (Briggs & McKenna, 1996; Houlihan et al., 2001; Papke, Dwoskin, & Crooks, 2007).

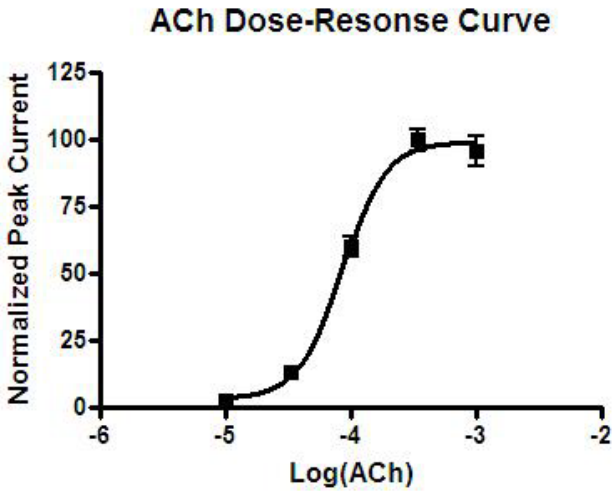

Figure 5 *Acetylcholine dose-response curve on α7 nAChRs, using acetylcholine concentrations of 1000 μM, 333 μM, 100 μM, 33 μM, and 10μM. The EC50 = 85 μM.*

Picomolar Concentrations of β-amyloid on α7 nAChRs

Occasionally, when changing the valves applying solutions, a pressure artifact would occur. To test for these pressure artifacts, a control program was run on each oocyte where OR-2 solution was applied, then the valve was switched to a different valve that still administered OR-2. Any changes resulting from a valve switch could then be detected.

Oocytes were perfused with β-amyloid for seven seconds at 1 pM, 30 pM, 300 pM, 1,000 pM, and 30,000 pM, after which acetylcholine was applied for seven seconds. The test application of β-amyloid was followed by two applications of acetylcholine to ensure that the α7 nAChRs were responsive to a known agonist. Data was collected for five sweeps of the program at each concentration. None of the concentrations of β-amyloid showed significant activation of α7 nAChRs, therefore no dose-response curve for β-amyloid was obtainable. Figure 6 shows the lowest concentration of β-amyloid (1 pM) and figure 7 shows the highest concentration of β-amyloid (30,000 pM).

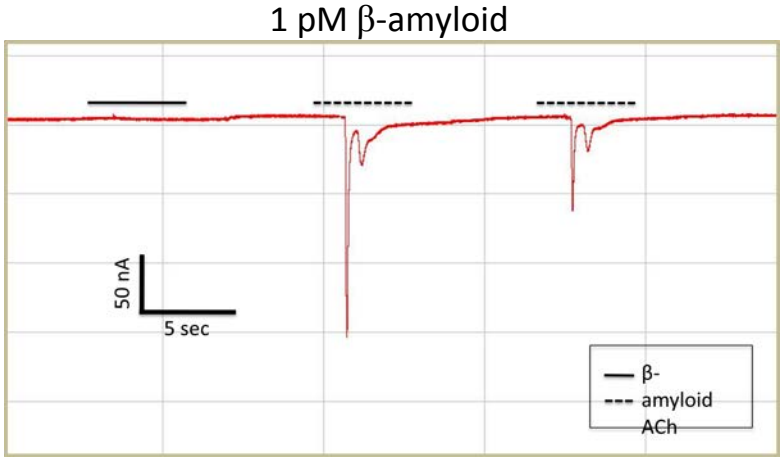

Figure 6 *Application of 1 pM β-amyloid elicits no response on α7 nAChRs. The oocyte was responsive to acetylcholine, indicating that the α7 nAChRs were functioning properly.*

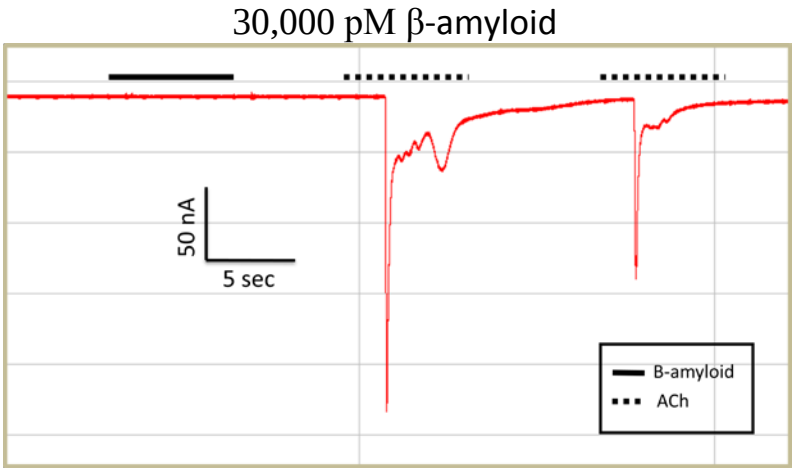

Figure 7 *Application of 30,000 pM β-amyloid elicits no response on α7 nAChRs. The oocyte was responsive to acetylcholine, indicating that the α7 nAChRs were functioning properly.*

Seven-second Wash of β-amyloid

Since no activating effects were observed with β-amyloid by itself, we wanted to see if β-amyloid would alter the activation or desensitization kinetics of the α7 ion channels in response to activation by the normal ligand, ACh. We first ran five sweeps of a paired peak program in which 2 doses of acetylcholine were administered seven seconds apart. Then we ran five sweeps of the β-amyloid program, which administered β-amyloid for seven seconds, washed the oocyte for 7 seconds with OR-2, then applied two seven-second applications of acetylcholine. The peak amplitudes, rise times, decay times, and decay taus were compared between the first paired peak before β-amyloid was applied and the first paired peak after application. The same comparisons were made on the second paired peak pre β-amyloid application to the second paired peak post β-amyloid application (Table 1). A fresh oocyte was used at each concentration and data was collected for five sweeps of each program.

At 30 pM β-amyloid, the peak amplitudes of the first peaks were decreased by approximately 25 percent (Figure 8). At 300 pM β-amyloid, the peak amplitudes of the first

peaks were increased (Figure 9). All other concentrations showed no relative changes in peak amplitude when comparing the first peaks.

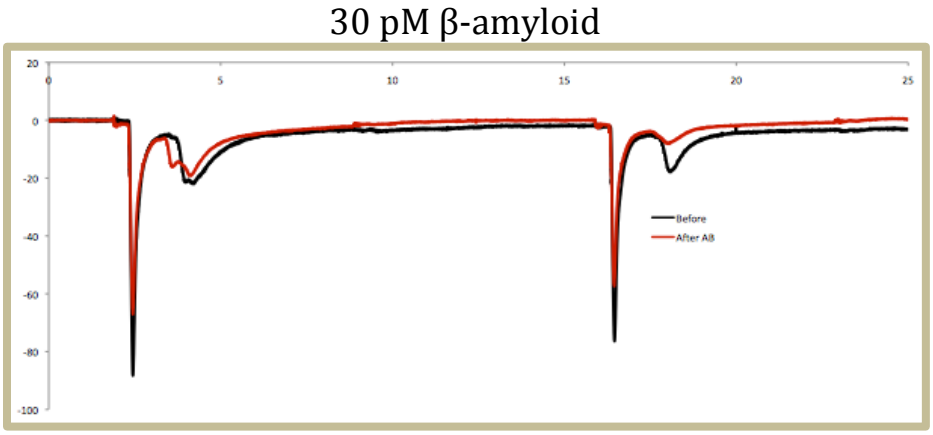

Figure 8 *Two seven-second applications of 333 µM acetylcholine result in downward peaks (black trace). A seven second application of 30 pM β-amyloid was applied to the oocyte, followed by a seven-second wash with OR-2, and then two seven second applications of 333 µM acetylcholine. The two seven-second applications of 333 µM acetylcholine applied after the β-amyloid (red trace) show a decrease in peak amplitude by approximately 25%.*

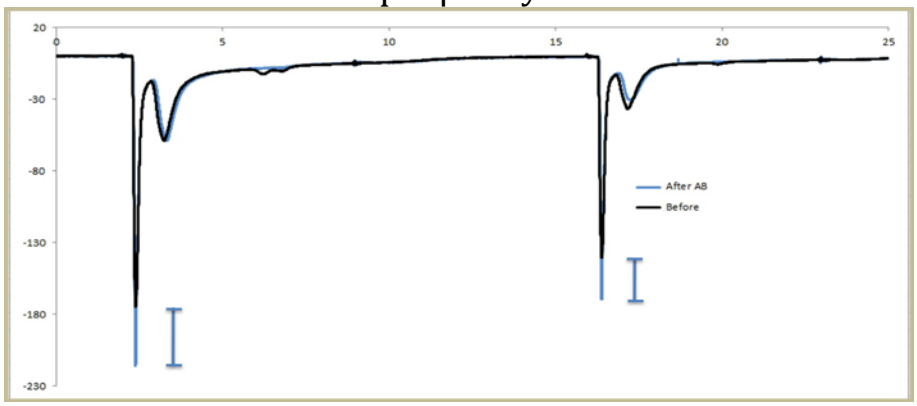

Figure 9 *Two seven-second applications of 333 µM acetylcholine result in downward peaks (black trace). A seven second application of 300 pM β-amyloid was applied to the oocyte, followed by a seven-second wash with OR-2, and then two seven second applications of 333 µM acetylcholine. The two seven-second applications of 333 µM acetylcholine applied after the β-amyloid (red trace) show an increase in peak amplitude.*

1st Paired Peak vs. 1st Paired Peak Post β-amyloid Application

Peak Amplitude		
β-amyloid Concentration	**Peak Amplitude Post β-amyloid application**	**P value**
1 pM	No Statistical Difference	P >0.05
30 pM	Decreased	P <0.001
300 pM	Increased	P <0.01
1000 pM	No Statistical Difference	P >0.05
30000 pM	No Statistical Difference	P >0.05

Rise Time		
β-amyloid Concentration	**Rise Time Post β-amyloid application**	**P value**
1 pM	No Statistical Difference	P >0.05
30 pM	No Statistical Difference	P = 0.5293
300 pM	Increased	P <0.05
1000 pM	No Statistical Difference	P = 0.8751
30000 pM	No Statistical Difference	P = 0.4461

Decay Time		
β-amyloid Concentration	**Decay Time Post β-amyloid application**	**P value**
1 pM	No Statistical Difference	P = 0.9026
30 pM	Slower	P <0.05
300 pM	Faster	P <0.01
1000 pM	N/A	N/A
30000 pM	N/A	N/A

Decay Tau		
β-amyloid Concentration	**Decay Tau Post β-amyloid application**	**P value**
1 pM	Faster	P <0.01
30 pM	Slower	P <0.01
300 pM	No Statistical Difference	P = 0.1252
1000 pM	No Statistical Difference	P = 0.1504
30000 pM	No Statistical Difference	P >0.05

2nd Paired Peak vs. 2nd Paired Peak Post β-amyloid Application

Peak Amplitude		
β-amyloid Concentration	**Peak Amplitude Post β-amyloid application**	**P value**
1 pM	No Statistical Difference	P >0.05
30 pM	Decreased	P <0.001
300 pM	Increased	P <0.01
1000 pM	No Statistical Difference	P >0.05
30000 pM	Decreased	P <0.05

Rise Time		
β-amyloid Concentration	**Rise Time Post β-amyloid application**	**P value**
1 pM	No Statistical Difference	P >0.05
30 pM	No Statistical Difference	P = 0.5293
300 pM	Increased	P <0.01
1000 pM	No Statistical Difference	P = 0.8751
30000 pM	No Statistical Difference	P = 0.4461

Decay Time		
β-amyloid Concentration	**Decay Time Post β-amyloid application**	**P value**
1 pM	No Statistical Difference	P = 0.9026
30 pM	No Statistical Difference	P >0.05
300 pM	No Statistical Difference	P >0.05
1000 pM	No Statistical Difference	N/A
30000 pM	No Statistical Difference	P = 0.8528*

Decay Tau		
β-amyloid Concentration	**Decay Tau Post β-amyloid application**	**P value**
1 pM	Increased	P <0.05
30 pM	No Statistical Difference	P >0.05
300 pM	No Statistical Difference	P = 0.1252
1000 pM	No Statistical Difference	P = 0.1504
30000 pM	No Statistical Difference	P >0.05

Table 1 *Summary table of statistical analysis using a repeated measures ANOVA. Peak amplitude, rise time, decay time, and decay tau were calculated comparing the first peak pre β-amyloid to the first peak post β-amyloid application. The second peak pre β-amyloid compared to the second peak post β-amyloid application was also evaluated.* ** T-test used to calculate the P value.*

Long wash of β-amyloid

Since variable inhibiting effects on acetylcholine induced peaks were observed on α7 nAChRs when β-amyloid was applied for seven seconds, we tried increasing the wash times to see if a longer application of β-amyloid in the picomolar range would cause any effects. Five sweeps of a normal application of 300 μM acetylcholine was applied to determine the baseline maximal ACh response, the oocyte was then washed with β-amyloid for 2-5 minutes, and another application of acetylcholine was applied. The post β-amyloid wash application of

acetylcholine was administered five times, with a wash time of 2 mins with OR-2 with Ca^{2+} between each application. At 30,000 pM β-amyloid there appears to be inhibition of the peak amplitude, with recovery occurring after each wash with OR-2 with Ca^{2+} (Figure 11). However, at all other concentrations, no significant inhibition is observed. A β-amyloid scrambled peptide was used as a control to ensure that any interactions observed were specific to the β-amyloid$_{42}$ peptide (Figure 10).

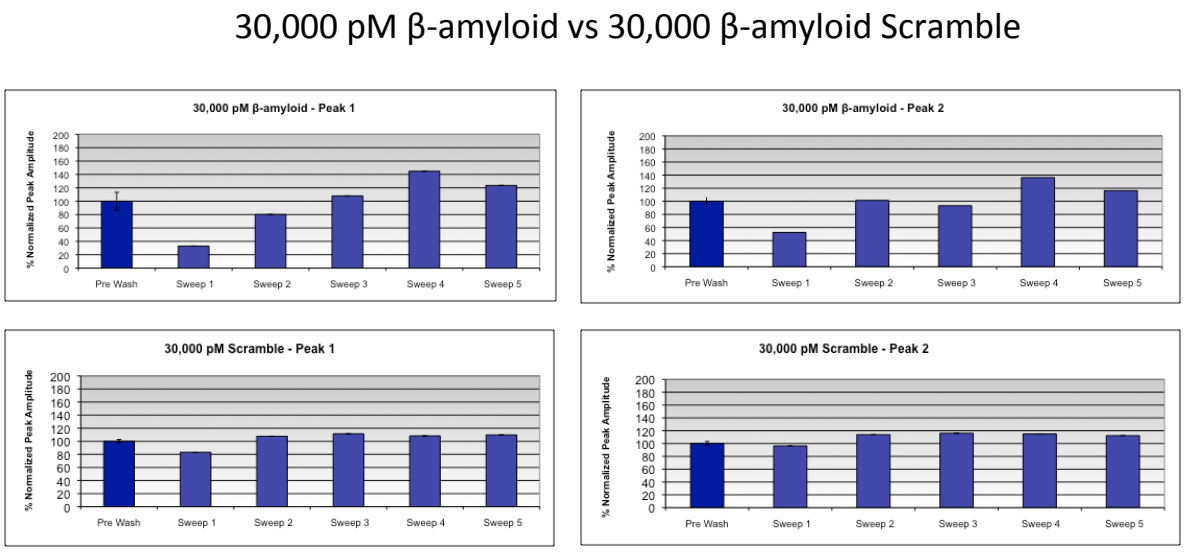

Figure 10 *The top two charts show the percent block of peak amplitude after 30,000 pM β-amyloid is applied for five minutes. During each sweep a seven-second application of 333 µM acetylcholine is administered, followed by 2 minutes of washing with OR-2. The lower two charts show the same experiment using 30,000 pM (30 nM) β-amyloid scramble peptide instead of β-amyloid.*

Percent block of Peak Amplitude when β-amyloid is applied for 2-5 minutes

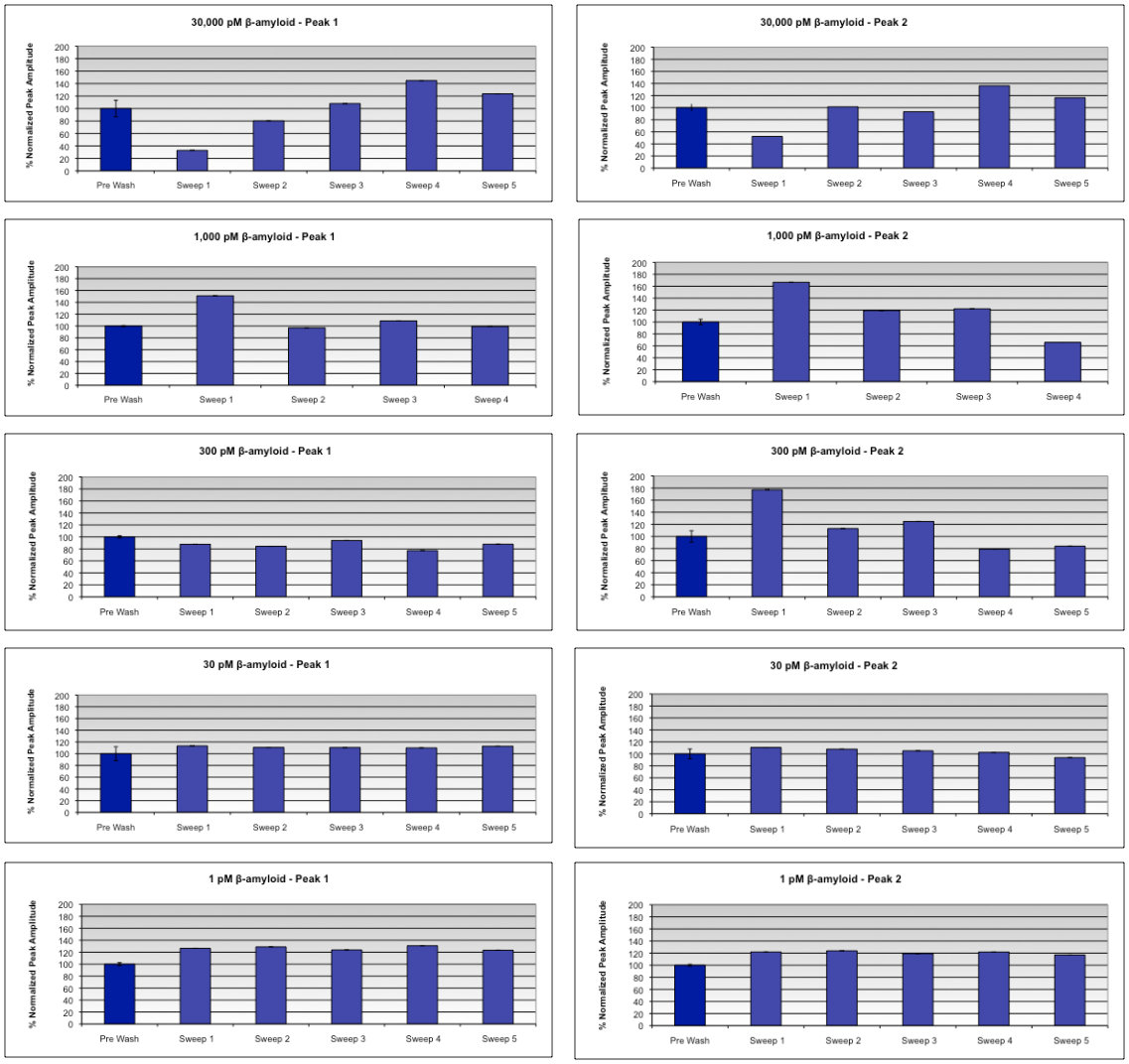

Figure 11 *The pre wash is the peak amplitude for the average of five acetylcholine activated peaks. Then β-amyloid was applied (three minutes for 1,000 pM, all other concentrations β-amyloid was applied for five minutes). Sweep one was measured right after β-amyloid application. During each sweep a seven-second application of 333 μM acetylcholine is administered, followed by 2 minutes of washing with OR-2. Two minutes of washing with OR-2 occurred between each successive sweep. Application of 30,000 pM β-amyloid blocks the peak amplitude, which washes out over time, with complete recovery of the peak amplitude after six minutes of washing.*

Percent Block of Peak Amplitude by β-amyloid

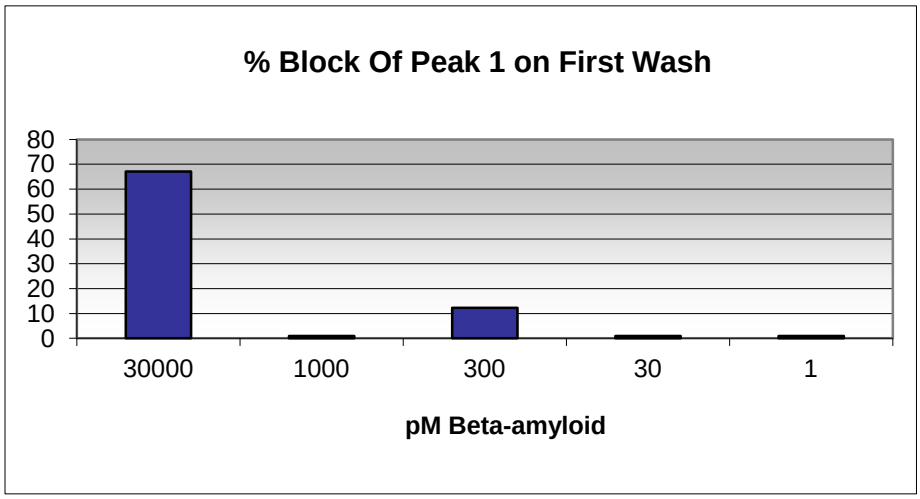

Figure 12 *Applications of 30,000 and 300 pM β-amyloid, for five minutes, are the only concentrations that show inhibition of the peak amplitude when acetylcholine is administered.*

DISCUSSION

While several studies have demonstrated antagonistic results (block of peak current for a7 nAChRs) at a nanomolar (or higher) concentration of β-amyloid, the effects of picomolar concentrations are not quite as consistent. Some studies report that picomolar concentrations of β-amyloid inhibit the activation of α7 nAChRs, while other studies report direct activation of nAChRs by β-amyloid alone. The estimated normal levels of endogenously produced β-amyloid are in the picomolar range (Cirrito et al., 2003), while those of Alzheimer's patients are much higher. Understanding the interactions between β-amyloid and nAChRs at picomolar concentrations versus nanomolar (or higher) concentrations can help us better understand the differences in functioning that occur between normal individuals and Alzheimer's patients.

In this study we show that picomolar concentrations (1 pM, 30 pM, 300 pM, 1,000 pM, and 30,000 pM) of β-amyloid do not directly activate human α7 nAChRs. This is contrary to a

previous study by Dineley et. al. which reported that picomolar concentrations of β-amyloid activated α7 nAChRs. The discrepancy in these findings could be a result of several different factors.

Three different confirmations of β-amyloid exist: monomers, oligomers, and fibrils. The conditions under which β-amyloid is prepared and stored affect the form it takes. It is possible that the different confirmations will interact with the α7 nAChRs differently. In our study we prepared the β-amyloid fresh from powder by mixing it with OR-2 solution. One of the ways to change from monomers to oligomers is to let the β-amyloid incubate for a few hours. Since our experiments started early in the morning and lasted until the evening, we wanted to rule out the possibility of the β-amyloid clustering with time and taking on a different form. Thus, possibly interacting with the α7 nAChRs differently. To ensure that the interactions remained consistent through out the day, we ran the same experiment (same protocol and same concentration of β-amyloid) at the beginning of the day and at the end of the day. We then compared the peak amplitude, rise time, and decay time. No statistical differences were observed between the two experiments. We weren't able to characterize which confirmation was most predominant, but we were able to rule out the possibility of the interaction of β-amyloid changing through out the day.

Some other differences in β-amyloid types are human versus rat, or the length of the peptide sequence. In our experiments we used human β-amyloid, 42 amino acids long. Differences could also be produced depending on the species of the α7receptors used, rat versus human. We used human α7 mRNA, Dineley et al. used rat α7 mRNA. In addition, the application time of β-amyloid could produce different results.

We demonstrated that at a seven-second application of β-amyloid, 30 pM will inhibit the peak amplitude of an ACh induced response and 300 pM will increase the peak amplitude of α7

nAChRs. However, at a longer (five minute) application of β-amyloid, 300 pM slightly inhibited the peak amplitude. Inhibition is also seen at the 30,000 pM concentration. This inhibition washes out with time, with full recovery occurring at six to eight minutes of wash time at 15-18 mL/minute.

At picomolar concentrations of β-amyloid, we do observe interactions with the α7 nAChRs that significantly alter the kinetics of the channels when they are activated by the endogenous ligand, ACh. However the exact pattern of the interactions remains unclear at this time since some concentrations showed an increase in current and other concentrations showed a decrease. More experiments will have to be preformed to more fully characterize the interactions that occur. As an example, performing the seven-second application of β-amyloid followed directly by an application of acetylcholine, instead of administering a seven-second wash between the two, may provide more clear data. Or perhaps co-application of β-amyloid with ACh would provide more consistent findings. Our profusion rate of 15-18 mL/minute is rather fast compared to previous reports and could account for some of the differences we observed in our experiments compared to those already published, since the higher perfusion would be expected to wash off any ligands more quickly, perhaps minimizing the effects seen.

Also, we'd like to repeat the experiments where we used a five-minute application of β-amyloid in order to obtain enough data points so that statistical analysis can be performed. Repeating this experiment will also help us to better evaluate the wash out times and how quickly we are able to see recovery.

This data is clinically relevant to the human population because the endogenous levels in a healthy individual of β-amyloid are estimated to be in the picomolar range. During the disease state of Alzheimer's Disease these levels increase into the nanomolar or higher range. At these

higher concentrations, it has been shown that β-amyloid inhibits activation of α7 nAChRs. Understanding how the nAChRs interact with β-amyloid at normal levels will help us to better understand the change in functioning that occurs in the brain between a healthy individual and an individual with Alzheimer's disease. A more clear understanding of the changes that occur in the development of Alzheimer's disease will help us to better know what types of treatments to investigate.

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EDUCATION

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RESEARCH EXPERIENCE

Thesis Project ***Provo, UT 2009-2011***
Brigham Young University
"The Effects of β-amyloid on α7 Nicotinic Acetylcholine Receptors Expressed in Xenopus Oocytes."
Techniques included two-electrode voltage clamp electrophysiology, plasmid preparation, and expression of genes in Xenopus oocytes.

Research &Development Research Assistant ***Salt Lake City, UT 2008-2009***
Idaho Technologies
Developed and optimized new PCR protocols for various instruments as part of the research and development sample preparation group.

Research Internship ***Madrid, Spain 2008***
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Evaluated the role of microRNAs in regulating gene expression in lymphomas. Techniques included PCR, sequence analysis, designing primers, transfection, restriction enzyme digestion, cloning, luciferase assay, western blot, and RNA/DNA extraction from tissue samples.

Research Assistant ***Provo, UT 2006-2007***
Brigham Young University, Dr. O'Neil's Cancer Research Lab
Correlated with another student to design and carry out a project to compare the intracellular quantity and activity of TK. Trained new lab members, assigned teams weekly assays, and data compilation. Research techniques included flow cytometry, Bradford assay, radioassay, tissue culture, ELISA, and preparation of media and reagents. Radiation certified.

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