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Beta-Amyloid Inhibition of Alpha 7 Nicotinic Acetylcholine Receptors and Factors That Potentially Influence the $A\beta^2$ /nAChR Interaction

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Beta-Amyloid Inhibition of Alpha 7 Nicotinic Acetylcholine Receptors
and Factors That Potentially Influence the A β /nAChR Interaction

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A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of
Master of Science

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ABSTRACT

Beta-Amyloid Inhibition of Alpha 7 Nicotinic Acetylcholine Receptors and Factors That Potentially Influence the A β /nAChR Interaction

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Master of Science

Alzheimer's disease (AD) is a neurodegenerative disorder that manifests in the form of deficiencies in cognitive processes such as memory and learning. The pathological features of AD include hyperphosphorylated tau proteins that form neurofibrillary tangles as well as senile plaques composed primarily of the peptide β -amyloid (A β). When present in high concentrations in the brain, A β inhibits certain subtypes of neuronal nicotinic acetylcholine receptors (nAChRs) in the hippocampus. The effects of A β in the hippocampus have proven to be neurotoxic, resulting in reduced functionality of nAChRs and the subsequent death of neurons in the cholinergic pathway. The early stages of AD are characterized by reduction of nAChR density and by degeneration of the cholinergic neurons that provide input to the hippocampus. Because the hippocampus plays a critical role in memory formation and other cognitive processes, dysfunction in this brain region results in significant cognitive deficiencies. Understanding the interaction between A β and the structurally and functionally diverse nAChR subtypes and possible downstream effects in signaling cascades that might result from that interaction are important steps in comprehending AD pathogenesis. Comprehension of this interaction and factors that might influence it could lead to the development of pharmaceutical agents useful in the treatment of AD.

Keywords: Alzheimer's disease, nAChR, beta-amyloid, Congo Red, antibiotics

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INTRODUCTION

Alzheimer's Disease (AD)

Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive memory loss and other cognitive deficiencies. It is the most common cause of dementia affecting nearly 25 million people worldwide (Conejero-Goldberg, Davies et al. 2008). By 2050 it is expected to affect 13.2 million individuals in the United States alone (Buckingham, Jones et al. 2009).

Although considerable progress in understanding the etiology and pathology of AD has been attained, the mechanisms underlying the pathogenesis of the disorder remain somewhat obscure (Tang, An et al. 2008). However, several pathological features of the disease have been identified. The two major neuropathological hallmarks of AD are deposition of beta-amyloid (A β) plaques and neurofibrillary tangles in various brain regions. In the early stages of AD, these observations are accompanied by selective cholinergic denervation and decreased nicotinic acetylcholine receptor (nAChR) density (Arias, Alés et al. 2004; Ellis, Villemagne et al. 2008; Barrantes, Borroni et al. 2010; Hernandez, Kaye et al. 2010). Studies, both *in vivo* and post mortem, in AD patients indicate cholinergic neuron death as well as a significant reduction in the amount of nAChRs with some subtypes affected more than others. The deficits in learning and memory associated with AD progression reflect this incremental loss of cholinergic neurons and nAChR function/expression in certain brain regions (Wevers, Burghaus et al. 2000; Barrantes, Borroni et al. 2010). In other words, there is a correlation between cholinergic degeneration, nAChR destruction and AD severity (Pettit, Shao et al. 2001; Ellis, Villemagne et al. 2008). Currently, there are no treatments for this neuronal loss.

The neuronal loss characteristic of AD progression selectively affects certain regions of the brain more than others. In studying AD, the brain structure known as the hippocampus is of particular interest due to its role in various cognitive functions. The hippocampus, which is part of the limbic system, is located in the medial temporal lobe of the brain and plays important roles in the consolidation of information from short-term memory to long-term memory as well as spatial navigation and long-term potentiation. Not surprisingly, the selective cholinergic denervation seems to be most severe in the temporal lobes and adjacent limbic and paralimbic areas (Parri, Hernandez et al. 2011). The hippocampus is an early target of the disease-related denervation with the progression of the disease leading to destruction of neurons in the entorhinal cortex and eventually, the neocortex (Buckingham, Jones et al. 2009; Ikonomic, Wecker et al. 2009; Parri, Hernandez et al. 2011).

The pathogenesis of AD as it is observed in the hippocampus is in large part related to the neuronal organization in the hippocampus as well as the distribution of nAChR subtypes therein. Structurally, the hippocampus is composed of two regions, the dentate gyrus (DG) and the Cornu Ammonis (CA). Each of these regions is composed of a main cell layer, the principal cell layer. The nerve cells of the principal cell layer of the DG are the granule cells and of the CA regions, the pyramidal cells. The primary cholinergic input to the hippocampus originates in the basal forebrain, specifically the medial septum-diagonal band complex (MSDB), and innervates inhibitory GABAergic interneurons found within the hippocampus (Albuquerque, Pereira et al. 1998; Sudweeks and Yakel 2000; Parri, Hernandez et al. 2011). Incidentally, post mortem studies have shown that the most vulnerable neurons in patients with AD are the basal forebrain cholinergic neurons (Barrantes, Borroni et al. 2010).

The cholinergic destruction in the hippocampus and resultant cognitive dysfunction seem to be related to nAChR subtype distribution within its neuronal populations. Neuronal populations within the hippocampus have been shown to express multiple nAChR subtypes (Gotti, Zoli et al. 2006). The nAChRs are expressed both presynaptically on the cholinergic neurons where they influence neurotransmitter release, and postsynaptically on the somatodendritic region of GABAergic interneurons on which the cholinergic neurons synapse (Pettit, Shao et al. 2001; Gotti, Moretti et al. 2006; Zolles, Wagner et al. 2009). In normal cognitive processes, these hippocampal nAChRs, in conjunction with cholinergic innervation, modulate GABA transmission and coordinate the synchronous activation of pyramidal cells populations (Albuquerque, Pereira et al. 1997; Gotti, Zoli et al. 2006).

Multiple studies have shown high densities of $\alpha 7$ and $\alpha 4\beta 2$ nAChRs in cholinergic neurons and interneurons in the hippocampus (Hellstrom-Lindahl, Mousavi et al. 1999; Gotti, Zoli et al. 2006). In fact, the most vulnerable neurons targeted for destruction in AD are those expressing high levels of $\alpha 7$ nAChR (Barrantes, Borroni et al. 2010). Because they are commonly recognized as the primary nAChR subtype in the hippocampus, $\alpha 7$ nAChRs have been studied extensively and a small part of their role in the etiology of AD has been elucidated. However, there is still much to be learned concerning the interaction between these $\alpha 7$ subtype receptor and the A β peptide.

Nicotinic Acetylcholine Receptors (nAChRs)

Nicotinic acetylcholine receptors (nAChRs) comprise a class of cys-loop, ligand-gated cationic channels characterized by their ability to be activated by both the endogenous neurotransmitter acetylcholine and the exogenous nicotine molecule (Hogg, Raggenbass et al.

2003; Dajas-Bailador and Wonnacott 2004; Gotti, Zoli et al. 2006). The structure of the channel consists of five protein subunits arranged around a centralized cation-permeable pore that opens in response to the binding of a ligand (Buckingham, Jones et al. 2009; Barrantes, Borroni et al. 2010). The twelve different subunits (α 2-10, β 2-4) identified in mammalian brains can be combined as homo-pentameric (e.g. five α 7 subunits) or as hetero-pentameric (e.g. two α 4 and three β 2 subunits) receptor complexes with each subtype exhibiting distinct pharmacological and biophysical properties (Gotti and Clementi 2004; Zolles, Wagner et al. 2009). Of the neuronal nAChRs subtypes, the homomeric α 7 and heteromeric α 4 β 2 and α 3 β 4 subtypes have been shown to be the most widely expressed in the brain (Buckingham, Jones et al. 2009). For this reason, these subtypes have been extensively characterized, relative to other subtypes.

The variability found in nAChR subtypes accounts for their physiological versatility in the body, as nAChRs are widely distributed throughout both the CNS and PNS where they participate in a variety of physiological processes (Gotti and Clementi 2004). In the CNS, the coordination between nAChRs and acetylcholine-producing cholinergic neurons plays a critical role in the modulation of processes such as neurotransmitter release, cell excitability and neuronal integration. Each of these processes influences higher cognitive processes like learning, memory and attention (Pettit, Shao et al. 2001; Gotti, Zoli et al. 2006). While proper functioning of neuronal nAChRs results in the ability to perform cognitive functions and protection from neurodegenerative diseases, cholinergic and receptor dysfunction can contribute to a number of pathologies, including Parkinson's, epilepsy and Alzheimer's disease (Nordberg 2001).

The cation conductance characteristic of nAChRs has more significant implications than simply membrane depolarization. Activated $\alpha 7$ nAChRs are involved in a number of intracellular events via local calcium signaling that initiates second messenger systems (Charpantier, Wiesner et al. 2005). Their participation in a variety of signaling pathways might explain the duality of roles of the receptor as it has been implicated in both neuroprotection and neurotoxicity (Buckingham, Jones et al. 2009). The fate of the cell depends on which pathway is activated, with certain pathways contributing to cell preservation, and others to cell death (Buckingham, Jones et al. 2009). The implications of pathway activation and the effect of A β and nAChRs on those pathways will be discussed in detail.

Beta-Amyloid (A β)

The role of nAChRs in the hippocampus-specific destruction is related in part to its interaction with A β (Liu, Gu et al. 2007). A β has been shown to be neurotoxic, and AD-like symptoms have been seen in mice transgenically overexpressing A β (Buckingham, Jones et al. 2009). Additionally, A β is an inhibitor of postsynaptic nAChRs in the rat hippocampus (Pettit, Shao et al. 2001).

The A β peptide is the product of amyloid precursor protein (APP) cleavage by β and γ -secretases, which results in A β peptides of varying lengths. An increased ratio of A β 42 (42 amino acids long) to A β 40 (40 amino acids long) is associated with AD (Kumar-Singh, Theuns et al. 2006). Individuals possessing genetic mutations in the genes that encode for APP and the APP-processing proteins, such as β and γ -secretases, demonstrate an increased likelihood for development of AD, which is likely attributable to increased production of the A β 42 configuration (Hardy and Selkoe 2002; Glabe 2008).

A β 42, in particular, is a very versatile peptide, possessing the ability to take on monomeric, oligomeric and fibrillar forms under normal physiological conditions (Luheshi, Tartaglia et al. 2007). The form of the amyloid depends upon a number of conditions including solution composition, length of the fragment, peptide concentration, age of the sample, presence of organic solvents, temperature, pH and ionic strength (Snyder, Lador et al. 1994; Schmit, Ghosh et al. 2011). The highly aggregated amyloid fibril, which is characterized by extensive β -sheeting and hydrogen bonding, was at one time considered the most pathogenic form, but more recent studies implicate the soluble oligomeric configuration, citing a strong correlation between overall load of the soluble A β and cognitive decline (Dougherty, Wu et al. 2003; Walsh and Selkoe 2007). Many experimental designs reflect this paradigm shift in favor of the oligomeric A β 42, which has become a popular target in studying the deleterious effects of the amyloid peptide. However, maintaining desired structure of the peptide in an experimental setting has proved difficult and is often times ignored when establishing experimental protocol. The unpredictability of the amyloid protein complicates the reproducibility of any experimental results derived from the use of the molecule. Not only can A β assume monomeric, oligomeric and fibrillar confirmations within solution, but it can also vary its structure significantly within those very generalized structural categories (Glabe 2008).

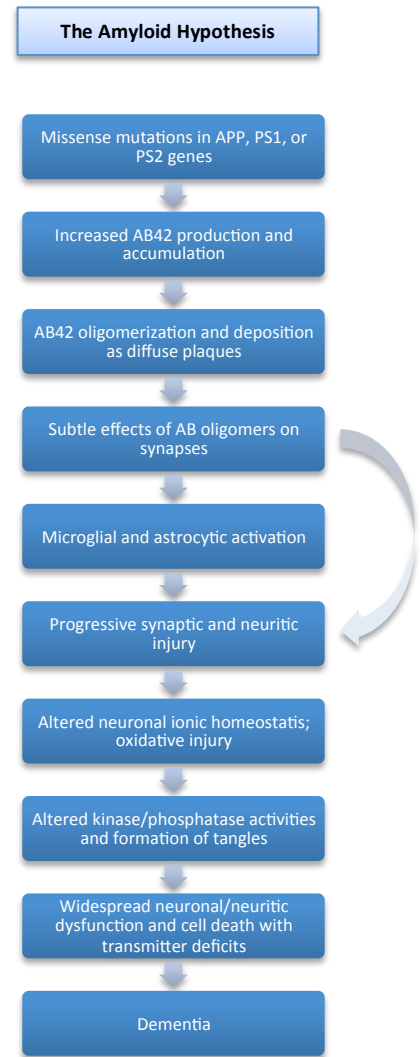


Figure 1: Theoretical cascade of events leading to AD (Hardy and Selkoe 2002).

The structural plasticity and resultant sensitivity to method of preparation and storage observed in A β is a possible explanation for the seemingly paradoxical conclusions that have been drawn up in the literature (Teplow 2006; Buckingham, Jones et al. 2009). It also emphasizes the importance of the standardized handling and storage of *in vitro* A β .

Beta-Amyloid/Nicotinic Acetylcholine Receptor Interaction

Because nAChRs are so functionally and structurally diverse, A β interacts differently with each subtype, making it difficult to predict the implications of those interactions. Some receptor subtypes are more prone to AD-related dysfunction than others. Due to the high density of α 7, α 4 β 2 and α 3 β 2 nAChRs in the hippocampus, characterization of their interaction with A β is of particular importance. The effect of A β on the α 7 subtype has been studied rather extensively, and A β has been shown to have high affinity for α 7 nAChRs (Wang, Lee et al. 2000). In fact, the deposition of A β in the early stages of AD overlaps with α 7 nAChR expression in the basal forebrain cholinergic system (Parri and Dineley 2010). Therefore, the neurons most prone to A β attack are those with large populations of α 7 nAChRs (Li, Wu et al. 2011).

As mentioned before, the nAChR activation is likely upstream to both pathogenic and protective events (Buckingham, Jones et al. 2009; Wang, Stucky et al. 2009). The relationship between second messenger systems, A β , nAChRs and downstream events is quite convoluted but important to investigate in order to understand the complexity of the A β /nAChR interaction in the disease state.

One of the pathways linked to the neuroprotective role of nAChRs is the phosphatidylinositol 3-kinase/v-akt murine thymoma viral oncogene homolog (PI3K/AKT) pathway, a pathway linked to anti-apoptotic cellular activities (Kihara, Shimohama et al. 2001).

Activation of the nAChR by nicotine has been shown to increase phosphorylation of AKT as well as the expression and phosphorylation of B-cell chronic lymphocytic leukemia/lymphoma (BCL2), a downstream component of the PI3K/AKT and known antiapoptotic protein (Kihara, Shimohama et al. 2001; Arias, Alés et al. 2004). The neuroprotective effects of this cascade were inhibited in the presence of nAChR antagonists (Buckingham, Jones et al. 2009). In examining the connection between nAChRs and the PI3K/AKT pathway, a tyrosine kinase known as FYN has been identified as a possible contributor to the nAChR/PI3K relationship. Not only has FYN, a member of the Src-family of kinases (SFKs), been shown to be involved in phosphorylation events in the PI3K pathway, including the phosphorylation of AKT, but it was also shown to be attached to both the nAChR and a subunit of PI3K in fetal rat cortical cell cultures (Kihara, Shimohama et al. 2001; Tang, Feng et al. 2007). Additionally, the Src-family of kinases has been shown to directly interact with the cytoplasmic loop of $\alpha 7$ nAChRs (Charpantier, Wiesner et al. 2005).

Just what role FYN plays in the apoptosis/antiapoptosis scheme is unclear as different studies report very different findings. Mutant $\alpha 7$ nAChRs lacking cytoplasmic loop tyrosine residues were more active than wild-type receptors and insensitive to kinase or phosphatase inhibition. Tyrosine kinase inhibition by genistein decreased $\alpha 7$ nAChR phosphorylation and strongly increased acetylcholine-evoked currents, whereas tyrosine phosphatase inhibition by pervanadate produced the opposite effect (Charpantier, Wiesner et al. 2005). Whether or not this phosphorylation/dephosphorylation dynamic results in increased surface receptors is disputed. However, the evidence seems to suggest that the changes in nAChR responsiveness are not due to increased/decreased receptor number, but rather to conformational changes in

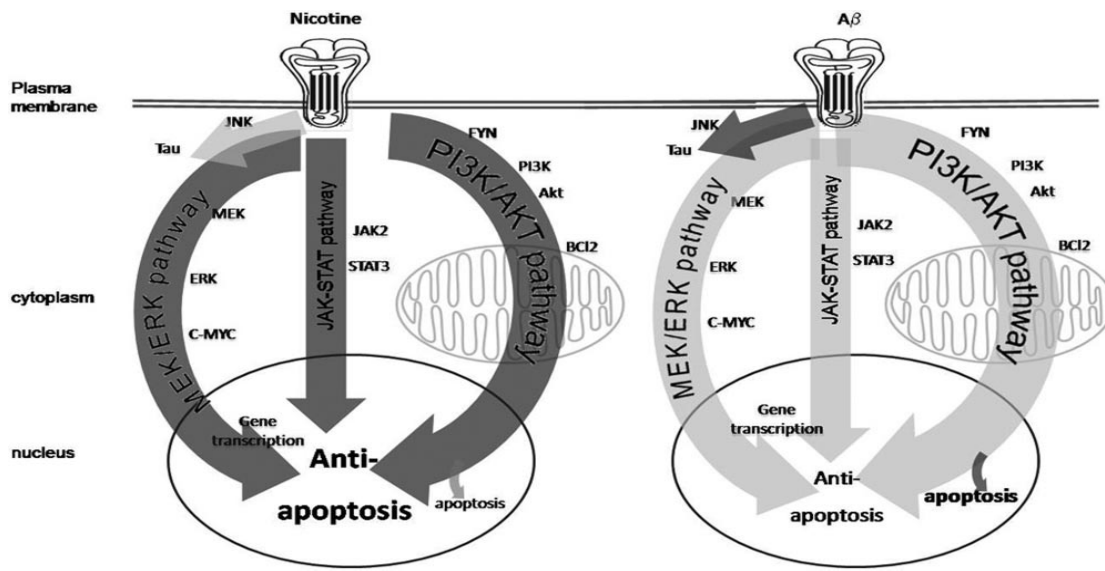


Figure 2: Model of the various pathways activated by nAChR agonists, nicotine or A β and how those pathways are involved in determining cell fate (Buckingham, Jones et al. 2009).

the receptor as a result of FYN activity (Charpantier, Wiesner et al. 2005; Cho, Song et al. 2005).

But what seems to remain a consistent assertion is the idea that the phosphorylation state of the receptor has implications in the functionality and survivability of the cell.

The actions of FYN are significant because, while it appears to play a neuroprotective role under normal physiological conditions, it has also been shown to be involved in cell dysfunction when activated by A β , an idea supported by the finding that A β 42 induces tau protein phosphorylation through its interaction with α 7 nAChRs and FYN (Wang, Li et al. 2003; Buckingham, Jones et al. 2009). The idea of a toxic relationship between A β and nAChRs was further confirmed by the finding that A β -induced lethality in SH-SY5Y cells was reduced when α 7 was rendered inert by RNAi (Buckingham, Jones et al. 2009). Aside from abnormal activation of FYN, the neurotoxic effects of A β activation of nAChRs have been linked to the MAPK/ERK-2 and MAPK/JNK pathways, which are also involved in tau hyperphosphorylation and apoptotic processes (Buckingham, Jones et al. 2009). Which pathway is activated by A β and the pathway's

effect on cell viability is dependent upon the concentration of A β as well as the length of A β exposure (Bell, O'Riordan et al. 2004; Buckingham, Jones et al. 2009).

The nicotinic acetylcholine receptor's participation in these second messenger systems is important as it introduces yet another mode whereby the receptor can interact with molecules like A β . Because nAChRs are known participate in cognitive processes in the hippocampus and have been implicated in AD pathogenesis due to their interaction with A β , nAChRs have become targets of pharmacological intervention in the treatment of AD (Wevers, Burghaus et al. 2000; Pettit, Shao et al. 2001).

Congo Red

Just how A β interacts with nAChRs is largely dependent on the amyloid structure.

Changes in that structure can drastically affect

experimental results. Establishing amyloid structure is of the utmost importance in standardizing experimental results. There are several accepted and common methods for detecting and quantifying the level of A β fibrillation *in vitro*, including circular dichroism spectroscopy, Thioflavin T (ThT) fluorescence, Congo Red binding, transmission electron microscopy (TEM), and Fourier transform infrared spectroscopy (FTIR) (Nilsson 2004). One of those methods, the Congo Red assay, has proved to be a cheap and effective method for quantifying aggregation due to the availability of the reagents. Congo Red, a secondary diazo dye, has been a commonly implemented tool for identifying amyloid fibrils in tissues post mortem (Nilsson 2004; Wu, Scott et al. 2012), but because of its ability to bind to the secondary β -sheet structure of the β -amyloid and the resultant change in absorbance spectrum of the dye,

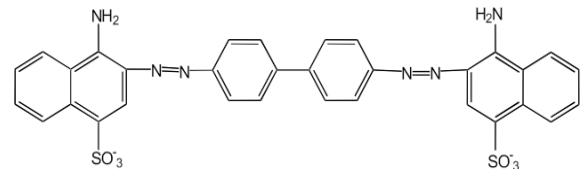


Figure 3: Chemical structure of Congo Red (Nilsson 2004).

Congo Red has been implemented as a means of identifying amyloid aggregation *in vitro* (Klunk, Jacob et al. 1999; Nilsson 2004). How the Congo Red binds to the amyloid fibrils is not exactly known, but with newer imaging techniques, the interaction between the two molecules is slowly becoming clearer. A series of intermolecular forces and binding sites are likely to participate in the CR/A β fibril interactions, including hydrogen bonding, ionic interactions, hydrophobic interactions and steric relationships (Nilsson 2004). When bound to amyloid fibrils, the CR molecules lie parallel to the fibril axis, resulting in torsional constriction and a shift in UV absorbance (Wu, Scott et al. 2012). The resultant shift in in absorbance maximum, from 490 nm to 540 nm, observed when amyloid samples are added to CR can be translated into concentration of CR bound to A β through a series of mathematical calculations. Calculating the amount of bound CR by observing maximal spectral shift at 540 nm, allows for the relative quantification of amyloid fibrils. Because the red shift in absorption maximum is only seen when CR binds to amyloid fibrils and not the soluble monomeric or oligomeric isoforms, an increase in CR bound to A β indicates an increase in fribriation (Klunk, Jacob et al. 1999). Lack of shift in spectral absorbance indicates the absence of amyloid fibrils.

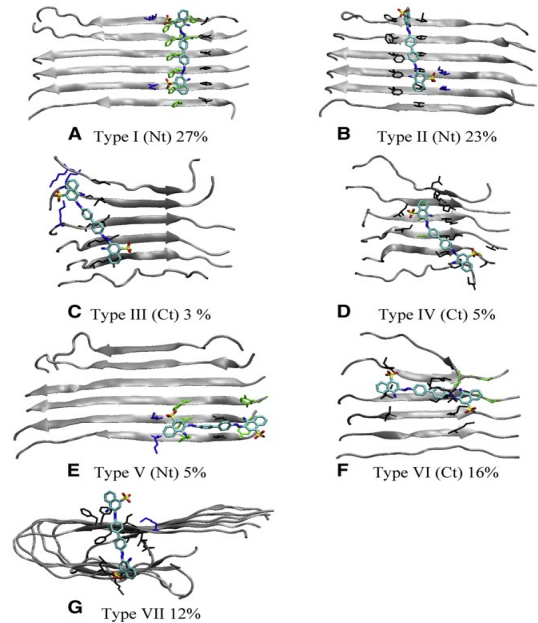


Figure 4: Seven proposed A β -CR binding sites (A-G) observed from binding simulations. Abundance is noted (Wu, Scott et al. 2012).

Antibiotics

Various factors affecting the nAChR/A β interaction have been discussed. Another potential factor that could play a role in the interaction is that of antibiotics. Antibiotics, specifically gentamicin and streptomycin, are commonly used in oocyte-incubating solutions as a means to maintain oocyte health. The use of antibiotics, like these, which are classified as aminoglycosides, has been linked to side effects such as ototoxicity, nephrotoxicity and neuromuscular block (Amici, Eusebi et al. 2005). It has been suggested that these side effects might be related to the interaction between aminoglycosidic antibiotics and nAChRs. Indeed, extended exposure to aminoglycosidic antibiotics such as gentamicin and streptomycin result in a decreased responsiveness to ACh by $\alpha 7$ nAChRs expressed in *Xenopus* oocytes, as evidenced by drops in peak response amplitude, as well as an increased susceptibility to desensitization. These effects were not easily reversible (Amici, Eusebi et al. 2005). A similar effect is seen in $\alpha 9$ nAChRs expressed in *Xenopus* oocytes when exposed to various antibiotics, including but not limited to aminoglycosides (Rothlin, Katz et al. 2000).

The reasons behind these effects are not well understood, but it has been suggested that aminoglycosides destabilize the open nAChR pore by blocking Ca^{2+} binding sites located in the extracellular region (Galzi, Bertrand et al. 1996; Rothlin, Katz et al. 2000; Amici, Eusebi et al. 2005). This theory is supported by the fact that increasing extracellular Ca^{2+} concentrations attenuates the inhibitory effects of the antibiotics (Rothlin, Katz et al. 2000). An alternate theory proposes that the decrease in response to ACh is voltage dependent and might be attributable to endogenous, Ca^{2+} -activated Cl^- channels (Amici, Eusebi et al. 2005). The fact that antibiotics affect the kinetics of $\alpha 7$ nAChRs so significantly complicates the matter even more as

it potentially adds another variable to the nAChR/A β equation. If both antibiotics and A β directly interact with the receptor, might their co-localization affect the A β /nAChR dynamic?

SPECIFIC AIMS

In this study we utilize human $\alpha 7$ nicotinic acetylcholine receptors expressed in *Xenopus laevis* oocytes to do the following:

1. Determine effect of A β on the kinetics of nAChRs.
2. Once the relationship between A β exposure and nAChR inhibition is established, examine the effect length of A β exposure has as it relates to the inhibition in ACh-activated nAChRs.
3. Examine different variables that might affect the interaction between A β and nAChRs, such as antibiotics and A β conformation.
4. Standardize our preparation of A β in order to enable accurate reproduction of experimental results by performing assays shown in the literature to effectively characterize and quantify A β form.

MATERIALS AND METHODS

Chemicals and Solutions

Oocyte Recording Solution

OR-2 solution with Ca^{2+} and theophylline (82 mM NaCl, 2.5 mM KCl, 1 mM Na_2HPO_4 , 5 mM HEPES, 1 mM CaCl_2 , 1 mM MgCl_2 anhydrous, 500 mM theophylline anhydrous) was made by dissolving prepared salt solute in distilled water and adjusted to pH 7.5 with NaOH. OR-2 with Ca^{2+} and theophylline was supplemented with a combination of streptomycin (50 $\mu\text{g}/\text{mL}$) and penicillin (50 units/mL) (Invitrogen).

Beta-amyloid Preparation

A β 42 and A β 42 scramble (AnaSpec, Inc., Fremont, CA) were prepared from lyophilized powder stored at -20 degrees C and reconstituted in either distilled water or OR-2 with Ca^{2+} to a concentration of 1 mg/mL and briefly vortexed. The reconstituted peptide was diluted to a final stock concentration of either 10 μM , 1 μM , 500 nM or 100 nM and stored at 80 degrees Celsius. Immediately prior to administration of A β 42, stock concentrations were thawed and diluted to 30 nM for use in experimentation. Unused stock aliquots were refrozen after thawing to prevent self-aggregation.

Congo Red Preparation

Congo Red (AMRESCO, Solon OH) was prepared at a stock concentration of 10 mM in OR-2 with Ca^{2+} (pH 7.5 with NaOH) and stored at room temperature in order to prevent formation of micelles. CR was filtered a 0.2 μm cellulose acetate sterile syringe filter immediately prior to use.

Acetylcholine

Acetylcholine solutions were prepared fresh each week from powdered ACh (Sigma, St. Louis MO) in OR-2 with Ca^{2+} (pH 7.5) at a stock concentration of 10 mM. Stock concentration was diluted to the desired concentration prior to application.

RNA Preparation

Human $\alpha 7$ (Origene # SC124074-20) subunit genes in the pCMV6-XL4 plasmid (Origene Technologies, Rockville MD) were transformed in One Shot[®] E. coli chemically competent 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 the $\alpha 7$ genes were linearized with Xba1 according to the protocol recommended by manufacturer. The mRNA was then transcribed, capped on the 5' end and PolyA tail was added. LiCl purification was performed using the mMessage mMachine[®] T7 Ultra Kit (Ambion, Carlsbad CA) according to the protocol provided by the manufacturer. The $\alpha 7$ mRNA was resuspended in TE Buffer (Bioexpress, Layton UT), aliquoted and stored at -80 degrees Celsius until used.

Frog Care and Oocyte Preparation

Female *Xenopus laevis* frogs (Xenopus 1, Inc. Dexter MI) were housed under 12-h day/night cycle at temperatures maintained from 17-19 degrees Celsius. Surgeries were performed once a week under sterile conditions and in accordance with the IACUC protocol. Prior to surgery frogs were anesthetized by immersion in a solution (12mM NaCl, 0.134 mM KCl, 0.181 CaCl_2 , 0.476 mM NaHCO_3 , 5mM HEPES, adjusted to pH 7.5 with NaOH) containing

0.1% ethylmetaaminobenzoate (MS-222; Sigma, St. Louis MO) for 15 minutes until unresponsive. Ovary sacs were removed and placed in a Ca^{2+} -free OR-2 solution (89.5 mM NaCl, 2 mM KCl, 1 mM MgCl_2 anhydrous, 5 mM HEPES, adjusted to pH 7.5 with NaOH), and manually separated with forceps. The follicular layer was removed using two different methods. Oocytes were either defolliculated by treatment with collagenase A (Sigma, St. Louis MO) for 2 hours on a tilting table at 17-19 degrees Celsius or manually defolliculated with fine-tip forceps under a microscope at 12x magnification. The cells were then rinsed with the Ca^{2+} -free OR-2 solution and subsequently stored in either an OR-2 solution with Ca^{2+} and theophylline or the same solution supplemented with Pen Strep (Invitrogen) at 14-17 degrees Celsius for a minimum of 24 hours prior to injection.

Additional oocytes were purchased for injection and recording from EcoCyte Bioscience (Austin TX). Oocytes were shipped in Barth's solution (88 mM NaCl, 1 mM KCl, .33 mM $\text{Ca}(\text{NO}_3)_2 \times 4\text{H}_2\text{O}$, .41 mM $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, .82 mM $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 5 mM Tris-HCl, 2.4 mM NaHCO_3) supplemented with Pen Strep (100 $\mu\text{g}/\text{mL}$).

RNA Expression in *Xenopus* Oocytes

A nanojet II automatic nanoliter injector (Drummond Scientific, Broomall PA) was used to inject human $\alpha 7$ mRNA into oocytes. Each oocyte was injected with 50.6 nL of mRNA for a total concentration of 75.9 ng of $\alpha 7$ mRNA per oocyte. Following injection the oocytes were either stored in an OR-2 Ca^{2+} with theophylline solution with or without Pen Strep at 14-17 degrees Celsius. Solution was changed daily, and recordings were obtained 3-5 days following injections.

Electrophysiology

Recordings of oocyte response to ACh and A β were measured via two-electrode voltage clamp (TEVC) 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 ClampFlit 9.2 (Axon Instruments, Sunnyvale CA).

Electrodes containing 3M KCl and having a resistance between 1 and 10 M Ω were made from 1.5 mm 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 -60mV unless otherwise stated, and traces were sampled at 5 KHz and filtered at 1000 Hz.

Oocytes were stabilized on a perfusion platform (model RC-1Z Warner Instruments, Hamden CT) and continuously perfused with OR-2 with Ca²⁺ at 15-18 mL/minute. Administration of OR-2 with Ca²⁺, ACh and A β was controlled by an automated perfusion system (Valvelink 8 Automate Scientific Inc., Berkeley CA). All electrophysiological experiments were performed at room temperature. A β was also applied using the same perfusion system with subsequent exposure to either the 333 μ M or 1000 μ M concentrations of ACh. The length of exposure to ACh and A β varied as the experiment dictated.

Unless otherwise stated, recording protocol consisted of five 2-minute sweeps. The sequence of solution administrations within those sweeps was as follows (cell clamped at -60 mV):

1. 5 sec OR-2 with Ca²⁺

2. 7 sec OR-2 with Ca^{2+} from control valve
3. 10 sec OR-2 with Ca^{2+}
4. 7 sec ACh (333 μM unless otherwise indicated)
5. 91 sec OR-2 with Ca^{2+} washout

The above sequence of events was preceded by 30 sec, 1 min, 2 min, 3 min, 4 min or 5 min perfusion with $\text{A}\beta$ where indicated.

Congo Red Spectroscopic Assay

Stock solution of 10 mM CR was filtered and diluted in OR-2 with Ca^{2+} to a concentration of 50 μM . UV-Vis spectrophotometer was zeroed between 400 and 700 nm at room temperature with a 1.5 μL sample of OR-2 with Ca^{2+} . A 5 μL sample of OR-2 with Ca^{2+} was added to 5 μL of the 50 μM CR solution and briefly triturated by pipetting the solution up and down. A 1.5 μL sample of the CR/OR-2 solution was scanned between 400 and 700 nm and the spectrum recorded. The same process was repeated with 5 μL of $\text{A}\beta_{42}$ sample added to 5 μL of 50 μM CR and allowed to incubate at room temperature for 30 min after which the spectrum was recorded. In order to insure that no residual CR/ $\text{A}\beta$ solution was transferred between measurements, the absorbance of the OR-2 solution used as a blank was run between each sample. The Congo Red spectrum was analyzed in order to detect the presence or absence of a maximal spectral difference at 540 nm.

Absorbance Assay (280 nm)

An absorbance assay was run using A280 program (version 3.5.1) on a NanoDrop ND-1000 spectrophotometer (Wilmington DE). The spectrophotometer was zeroed with 2 μL OR-2

buffer solution, and the absorbance of 2 μL of the peptide solution was measured at 280 nm. $\text{A}\beta_{42}$ peptide concentrations were calculated using the molar extinction coefficient (ϵ) for tyrosine (tyr; $1280 \text{ M}^{-1}\text{cm}^{-1}$).

Data Analysis

InStat ver. 3.05, GraphPad software (San Diego CA) was used to perform repeated measures ANOVA statistical analysis. Statistical significance was determined by $p < 0.05$.

RESULTS

IV Plot

In order to verify expression and characterize the nAChR expressed in the *Xenopus* oocytes, an I/V plot was obtained by clamping the membrane potential at -60 mV, -40 mV, -20 mV, 0, 20 mV and 40 mV and measuring the respective currents upon application of 333 μM ACh. The I/V plot was found to be consistent with that of an $\alpha 7$ nAChR as described in the literature (Fig. 5a).

Acetylcholine Dose Response Curve

Naïve oocytes, not injected with $\alpha 7$ mRNA, were exposed to ACh according to the protocol previously outlined. Naïve oocytes not expressing the receptor exhibited no ACh-induced depolarization (figure not shown).

We did, however, see the characteristic ACh-induced response in oocytes injected with $\alpha 7$ mRNA with the downward current indicative of the influx of Ca^{2+} ions into the cell via the open cation pore. To further characterize the receptor subtype, a dose-response curve was obtained by exposing the cell to 10 μM , 33 μM , 100 μM , 333 μM and 1000 μM ACh as previously described. Peak amplitudes were measured at each concentration and normalized to the average peak response to 1000 μM ACh. An EC_{50} of 57 μM

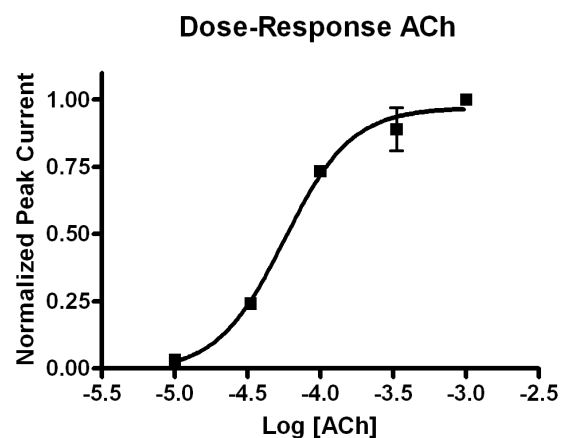
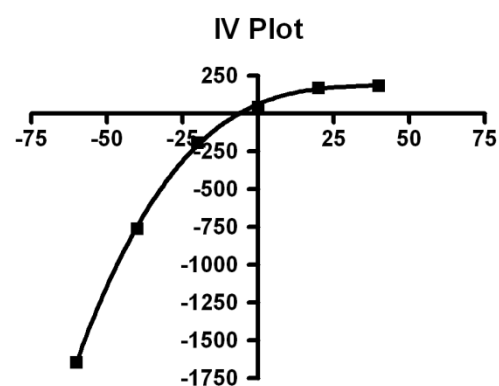


Figure 5a: The I/V plot for $\alpha 7$ nAChRs expressed in *Xenopus* oocytes. The reversal potential for the channels was calculated to be -6 mV.

Figure 5b: The dose response curve to ACh of $\alpha 7$ nAChRs expressed in *Xenopus laevis* oocytes. The EC_{50} is 57 μM ($r^2=0.9721$, Hill coefficient=1.889).

($r^2=.9721$, Hill coefficient=1.889) was observed based on the dose-response curve (Fig. 5b). This represents normal values for the $\alpha 7$ nAChR subtype and further confirms the expression of the receptor (Briggs and McKenna 1996).

A β Inhibition of nAChRs as a Function of Exposure Time

Previously in our lab, it was determined that A β -induced inhibition of nAChRs was most significant and consistently observed with extended applications (5 minutes) of 30 nM A β .

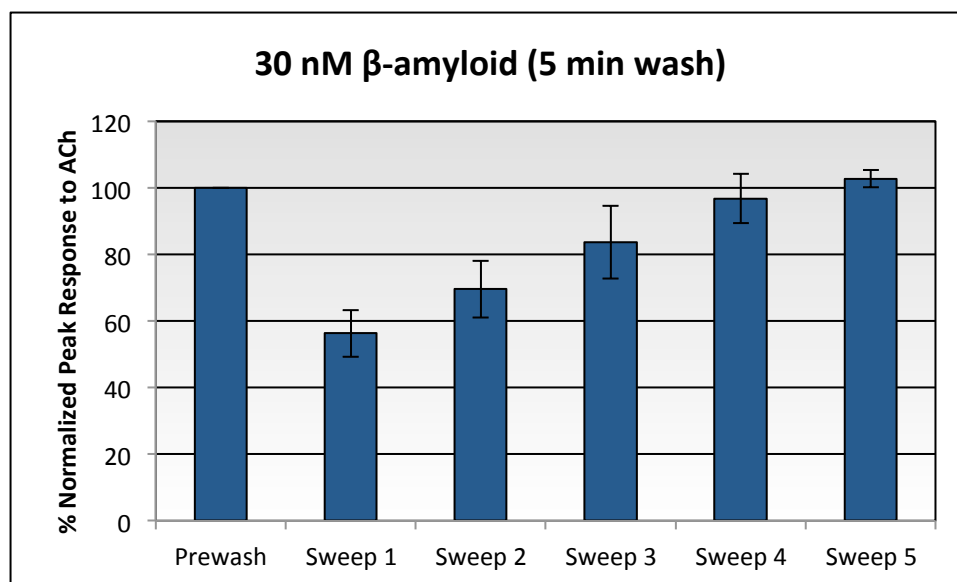


Figure 6: A β -induced inhibition of nAChRs by 30 nM A β and subsequent recovery of full ACh response following washout.

Maximum inhibition was seen with the first sweep, followed by gradual recovery of the peak amplitude by sweep 5, indicating that the A β -induced inhibition is reversible (Fig.6). Based on these findings, a concentration of 30 nM A β was used in all amyloid/nAChR experiments.

While we have determined the dose-dependency of the A β inhibition in previous experiments, the timing of the block has yet to be closely examined in our lab. Because the A β inhibition of the receptor possibly occurs by mechanisms besides simple competition at a binding

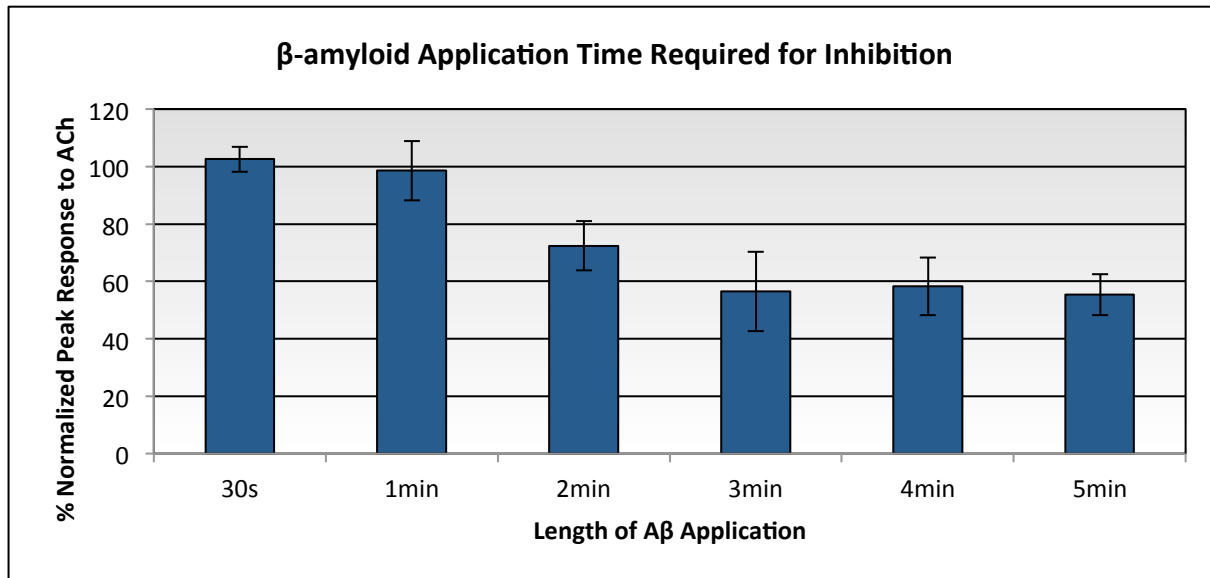


Figure 7: Time-dependent inhibition of nAChRs by Aβ. Two minute application was required for onset of Aβ-induced inhibition

site, we decided to examine the time required to induce the Aβ block. Aβ perfusion was administered for 30 sec, 1 min, 2 min, 3 min, 4 min and 5 min, and peak amplitudes were compared as a function of length of Aβ exposure (Fig.7). Inhibition was not seen in the oocytes exposed for just 30 sec or even 1 min. The inhibitory effect was not observed until at least a 2 min Aβ wash was applied, at which point, the peak response amplitude was reduced by 30%. A maximum inhibition of almost 50% was observed as a result of the 3 min Aβ wash. This data is consistent with the idea that the Aβ-induced inhibition on nAChRs is a function of the initiation of signaling events within the cell and agrees with the findings that activation of the JNK/MAPK and ERK/MAPK pathways is time-dependent (Bell, O'Riordan et al. 2004; Buckingham, Jones et al. 2009).

Possible Reduction of Aβ Inhibition in Presence of Antibiotics

Due to the unpredictability of Aβ, reproducing data has proven to be challenging at times. To enhance the health of our oocytes, we began to supplement the OR-2 with Ca²⁺

solution with antibiotics. While the addition of antibiotics to our holding solutions seemed to improve oocyte health, it also seemed to coincide with a sudden inability to elicit the same amyloid-induced inhibition we had seen consistently in our experiments (Fig.8b).

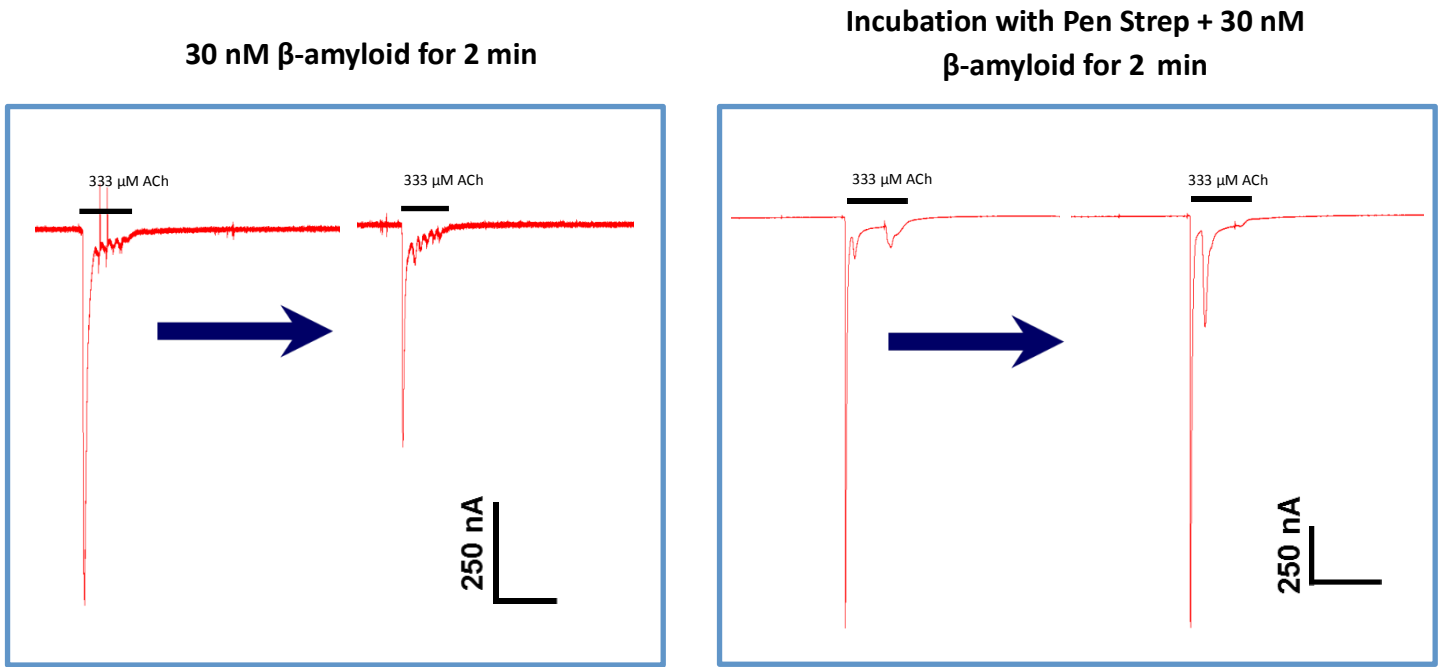


Figure 8a: Typical Aβ-induced inhibition of ACh current.

Figure 8b: Incubation of the oocytes in Pen Strep solution prevents 30 nM Aβ blockade of receptors.

To test whether or not the antibiotic interfered with the Aβ/ACh interaction, we began to store a portion of our oocytes in antibiotic-free solution to see if the amyloid inhibition of nAChRs might be restored. Indeed, upon removal of the antibiotic from the OR-2 storage solution, the Aβ inhibition was once again observed as expected (Fig.8a). Unfortunately, this was never confirmed on oocytes from the same surgery but was observed in oocytes from different batches exposed to the same Aβ preparation. Attempts to reproduce this data in oocytes from the same surgery continually failed due to different variables that are being addressed by standardizing Aβ preparation and handling.

A280 Absorbance Assay

To address the variability between our A β preparations, we performed a series of assays designed to characterize and quantify the composition of peptides in our A β solution. First, we employed the A280 absorbance assay to verify that the lyophilized peptide had indeed been solubilized and was present in our solution preparations. Detecting peptide concentrations using the A280 absorption technique requires the presence of tyrosine (Tyr, Y) and/or tryptophan (Trp, W) residues, which, due to their aromaticity, absorb UV light at 280 nm. Because A β contains a tyrosine residue, and the molar extinction constant of Tyr ($1280 \text{ M}^{-1}\text{cm}^{-1}$) is known, Beer's law can be applied to calculate peptide molarity using the following calculation where c represents the concentration of the sample in mg/mL, ϵ represents the molar extinction constant and A_{280} the UV absorption of the sample at 280 nm:

$$c = A_{280} / \epsilon$$

In order to verify the presence and quantity of A β peptide in solution, this A280 technique was applied to two different fresh preparations of the amyloid peptide reconstituted in two different solvents (dH₂O and OR-2 with Ca²⁺) and diluted to varying concentrations (10 μM , 1 μM and 500 nM).

Reconstitution of desiccated peptide varies from lab to lab, with some labs dissolving it directly in buffer solution, others in distilled water or basic solutions such as ammonium hydroxide. Up to this point we have favored the reconstitution in buffer solution with mixed results. To test

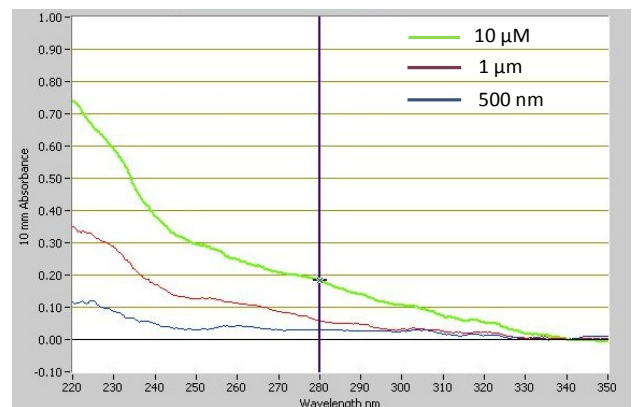


Figure 9: UV absorbances at 280 nm for 10 μM , 1 μM and 500 nM preparations of A β solution.

the ability of the peptide to dissolve in water relative to buffer solutions, we measured the concentration of peptides in solution and compared that to our theoretical concentrations. Additionally, some labs recommend storing the A β at stock concentrations in the μ M range, while others recommend concentrations less than 1 μ M due to the tendency of the peptide to self-aggregate at higher concentrations. To test which theoretical concentration favors actual A β concentration similar to what we expect percent yield for several different concentrations of A β peptide solution was calculated and compared. Additionally, to test the impact of temperature on the A β composition, the preparations were stored either at room temperature or -80 degrees Celsius.

Previous preparations of A β stored at stock concentrations of 500 nM in a -80 degree Celsius freezer showed no absorption at 280 nm (data not shown). The fresh preparation of A β did, however, exhibit absorption at 280 nm, albeit very minimal (fig.9). Calculated

concentrations revealed peptide molarities far below what was expected from the reconstitution of amyloid peptide, with the 10 μ M A β solution producing the lowest percent yield (Fig.10).

There are several possible explanations for the unexpectedly

low yield which will be addressed in the discussion section. One possible explanation that merits discussion is the aggregation of the amyloid into fibrils, which can skew the A280

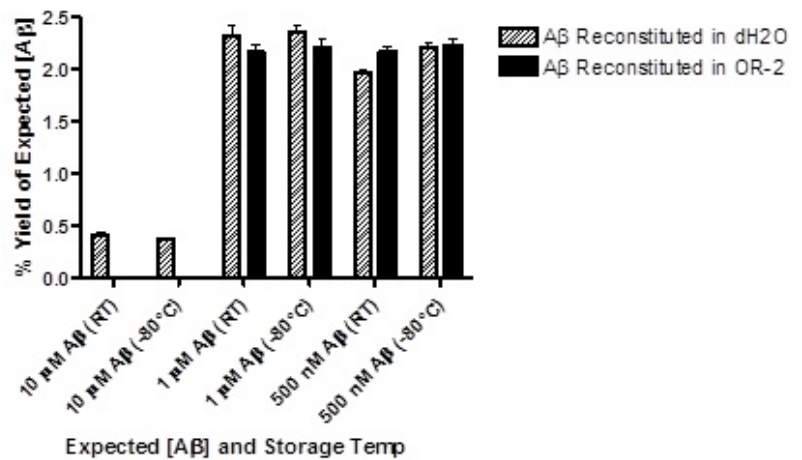


Figure 10: Percent yield of expected A β concentration as a function of solvent, temperature and A β concentration.

absorbance reading, as the assay is designed to measure just the soluble forms of the peptide. The more fibrils formed, the lower the absorbance one might expect to see. This might explain why the percent yield in the higher 10 μ A β solution yielded such low actual soluble amyloid concentration of the lyophilized peptide. Additionally, variations in salt content of the solvent, in this case OR-2 with Ca²⁺, can affect the ability of the lyophilized peptide to fully dissolve in the solvent due to ionic interactions.

Congo Red Assay

One variable that has been shown to affect both absorbance at 280 nm as well as the interaction between A β and nAChRs is the level of A β aggregation in solution. To test the propensity for aggregation in our preparations and to see if aggregation might be the reason behind the lower than expected A280, we tested our samples for spectral shifts that indicated amyloid fibrillation. We attempted to induce aggregation in by incubating them at room temperature for two days, a method that had been shown to increase fibrillation. However, upon repeated analysis using the Congo Red assay, none of the samples significantly exhibited the spectral shift at 540 nm indicative of the presence of A β aggregation. A false negative is a possibility when examining sample at such low concentrations.

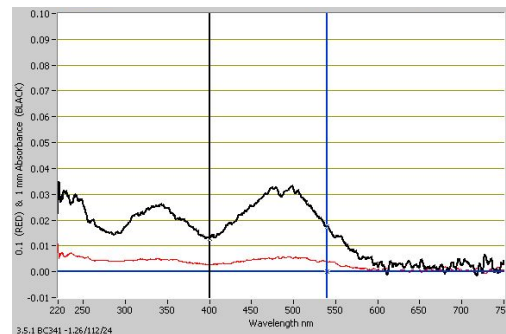


Figure 11: CR+OR-2 control, exhibiting peak absorbance between 490 and 500 nm.

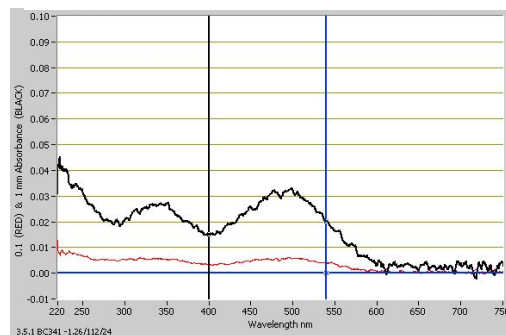


Figure 12: CR+100uM A β exhibited a similar absorbance peak between 490 and 500 nm, indicating the absence of a spectral shift and amyloid fibril formation.

DISCUSSION

While important advancements continue to be made in Alzheimer's research, consolidating and making sense of all of the available data regarding the proposed interaction between nAChRs, A β is a monumental task. Many factors affect the dynamics of this system, and much of the discrepancies contained within the literature point to lack of standardization of amyloid handling. Much of this is due to the simple lack of understanding of the A β molecule itself, but additional measures could be taken to standardize procedure as much as possible.

The instability of A β in solution is affected by numerous factors that must be controlled in an experimental setting. One of those factors, that I feel we should address in our lab is the impact of solvent composition on A β solubility and aggregation. The inconsistencies in our results and the results of other labs could simply be attributed to lack of understanding of the solvent/solute relationship. Comprehending that relationship is of particular importance in establishing consistent and repeatable results (Snyder, Ladror et al. 1994; Nilsson 2004). Any possible variable that destabilizes the desired oligomeric isoform of the molecule should be examined and addressed.

Aside from the effect that our solvent might have on A β solubility, other explanations exist that might shed light on the obstacles faced in our experiments. Despite the results indicated by the Congo Red assay, much of the difficulty we are experiencing points to the aggregation of fibrils in our A β solution. The lower than anticipated concentrations of A β seen in both the distilled water and OR-2 is concerning and may be due to several different factors. Aside from experimenter error, peptide impurities and solvent composition, another factor not already mentioned is the ability of the NanoDrop spectrophotometer to measure peptides or

fibrils at such low concentrations. In fact, a higher than recommended ratio of CR to peptide solution had to be used in the Congo Red Assay due to the inability of the equipment to clearly record absorbance at CR concentrations at or below 5 μ M. Even though the Congo Red assay indicated a lack of fibrillation, various evidence points to it as a possible explanation for the lack of reproducibility in our experimentation. The lower than recommended concentration of A β used in the Congo Red Assay should definitely be considered, and the possibility of fibril formation within our preparations should not be eliminated based on our results. With that said, considering the low concentrations that we use in our A β protocol, the presence of A β fibrils in our solutions might be better detected by alternative methods such as circular dichroism or fluorescence assays.

With that said, despite the difficulty we've experienced in repeating results, the questions that arise from those results are valid and compelling and merit additional examination. The effect of antibiotics on the A β /nAChR dynamic could have far-reaching implications, including pharmacological applications. If the unpredictable nature of the A β could be controlled, there is a number of interesting research avenues that could be pursued with regards to antibiotics.

However, possibly the most promising avenue in researching the role of A β in cytotoxicity is that of signaling cascades. Of particular interest is the tyrosine kinase, FYN, whose exact role in neuroprotection or neurodegeneration is not well understood but seems to be an important factor in the A β /nAChR relationship. Examining that role within the parameters of our capabilities and resources and experimental design is a compelling possibility.

The literature indicates the significance of the phosphorylation state of the receptor in its ability to function optimally. The possibility that FYN and other SFKs are responsible for the phosphorylation state of $\alpha 7$ nAChRs, and that changes in FYN and SFK activity are seen in the presence of $\alpha 7$ nAChR agonists as well as in the presence of $A\beta$, implicates FYN as a possible downstream molecule involved in the pathogenicity of $A\beta$. The potential role of a second messenger cascade is substantiated by our experimental data which indicates a delay in the response of $\alpha 7$ nAChRs to $A\beta$. The idea that the $A\beta$ effect is not immediate is consistent with the hypothesis that the inhibition of nAChRs is more than just a simple, direct physical blockade of the ion pore, but that the inhibition might be attributable to alterations in second messenger signaling cascades feeding back onto the nAChR. It seems feasible that $A\beta$ could initiate phosphorylation of the $\alpha 7$ nAChR by FYN or another SFK, thus reducing its responsiveness to ACh. A reduction in responsiveness to its agonists could very well represent the initial stages of the cholinergic dysfunction characteristic of AD. The deleterious effects of altering signaling cascades might also explain the various symptoms exhibited by Alzheimer's sufferers, including memory loss, long-term potentiation (LTP) inhibition, irritability, etc. A focus of our continuing research should be the changes seen in the signaling cascade, specifically the phosphorylation state of the $\alpha 7$ receptor, due to nAChR/ $A\beta$ interaction. Moving forward we could easily apply some of the methods employed by Charpantier to our experimental protocol as we seek to examine the specific mechanisms behind the $A\beta$ -induced inhibition of $\alpha 7$ nAChRs. But once again, our ability to further our research depends upon our ability to control the $A\beta$ peptide.

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CURRICULUM VITAE

Christopher Jacobsen

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Objective

Completion of Master's degree

Work Experience

DAT Prep Instructor

Sept 2012-present Kaplan Test Prep, Provo, UT
Currently training to teach both GRE and DAT prep courses

Research Assistant

April 2012-present Brigham Young University, Provo, UT
Currently managing Dr. Sterling Sudweeks' research project
Assign and organize responsibilities of undergraduate students
Presented and co-authored poster at Neuroscience 2012 conference

Laboratory Assistant

Jan 2004-Apr 2005, Jan 2012-Apr 2012 Brigham Young University, Provo, UT
Lab assistant for Advanced Physiology Lab
Set up experiments and exercises in advanced physiology including computer simulations of muscle function, endocrine disease, and human physiology
Taught students physiological principles and how to properly interpret data from scientific experimentation

Teacher's Assistant

Aug 2010-Dec 2011 Brigham Young University, Provo, UT
Pathophysiology teacher's assistant
Helped students apply their understanding of various scientific disciplines (e.g. physiology, microbiology, etc.) to case study analysis

Assistant Office Manager

Aug 2008-Aug 2010 Family Legacy Dental, Orem, UT
Discussed dental treatment options with patients
Coordinated dental insurance benefits
Translated for Spanish-speaking patients

Education

Brigham Young University, Provo, UT

Aug 2010-present
MS student Physiology and Developmental Biology
3.95 GPA

Southern Illinois School of Dental Medicine, Alton, IL

Aug 2005-Oct 2007

- Completed two years of dental education

Brigham Young University, Provo, UT

Aug 1998-Apr 2005

- B.S., Zoology
- 3.51 GPA
- Member of the BYU Predental Club

Awards & Scholarships

Brigham Young University, Provo, UT

Aug 2010-present

- Department of Physiology and Developmental Biology partial tuition scholarship

Aug 2010-Apr 2011, Aug 2011-Dec 2011

- Teaching assistantship in Pathophysiology and Advanced Physiology Lab courses

April 2011-Aug 2011, Jan 2012-June 2012, Aug 2012-Dec 2012, Apr 2013-June 2013

- Research assistantship in Dr. Sterling Sudweeks' lab

Research Interests

Aug 2010-present

I am currently involved in researching the interactions between B-amyloid proteins and various nicotinic receptor subtypes typically found in the hippocampus of the brain. I am responsible for preparing mRNA and injecting it into frog oocytes. Following expression, I characterize the receptors expressed via two electrode voltage clamping (TEVC).

Aug 2004-Apr 2005

I was able to participate in research revolving around the effect of a high sodium diet on the periventricular nucleus (PVN) of rats. I was primarily responsible for creating rat brain slices using the Cryostat and slide preparation.

Volunteer Experience

- 2 year LDS mission in Carlsbad, CA (Spanish speaking)
- Volunteer tutor at BYU
 - Currently tutor fellow students in chemistry, biology, physiology
- Utah Special Olympics volunteer
 - Served as committee head for fundraising and opening ceremonies for Utah's summer games
- Illinois Special Olympics volunteer
 - Helped organize dental exams for the participants
- Volunteer Spanish translator for Give Kids a Smile at SIUE SDM
- Volunteer judge for the 2012 Central Utah Science and Engineering Fair (CUSEF)

**Extracurricular
Activities**

- Officer in BYU's Neuroscience Club
- Intramural sports (basketball, football, softball, water polo and volleyball)
- Avid reader
- Avid runner
 - Completed a 180-mile, 24-hour relay race with a twelve member team

Miscellaneous

- Officer in BYU's Neuroscience Club
- Fluent in Spanish
- CRLA level 1 certified tutor