APPROACHES TO FORMING A WATER-SOLUBLE EXTRACELLULAR DOMAIN FROM *α3* SUBUNITS OF NICOTINIC ACETYLCHOLINE RECEPTORS

An Undergraduate Research Scholars Thesis

by

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Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by
Research Advisor: Dr. Gregg Wells

May 2014

Major: Biochemistry

TABLE OF CONTENTS

ABSTRACT

Approaches to Forming a Water-soluble Extracellular Domain from α3 Subunits of Nicotinic Acetylcholine Receptors. (May 2014)

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Obtaining structural information on the extracellular domain (ECD) of α3 subunits of nicotinic acetylcholine receptors (nAChRs) by x-ray crystallography is desirable for the design of drugs intended to therapeutically target specific nAChRs and treat the various diseases and disorders caused by nAChR complications such as epilepsy, Alzheimer's disease, Parkinson's disease and other disorders of the nervous system. Separating the ECD from the rest of the structure is advantageous because the transmembrane domains make nAChRs difficult to crystallize. In order to explore the possibility of creating a water soluble ECD from the α3 subunits of nAChRs through the proteolysis of the ECD away from the transmembrane domains, the nAChR expression yield of mutant α3 nAChR subunits containing a flexible linker at the ECD/M1 interface was measured. The flexible linker used was an 18 amino acid sequence consisting of the residues alanine-glycine-serine repeated six times (6xAGS) and was designed as a possible flexible environment in which to contain a protease site. The nAChR yield, as measured by yield of [^{3}H]epibatidine binding sites, was much lower for the α3 nAChR subunits containing the flexible linker at the ECD/M1 interface than for the control subunits without the linker.

Therefore, the 6xAGS flexible linker is not the optimum environment in which to contain a protease site in α3 nAChR subunits.

ACKNOWLEDGEMENTS

I would like to thank Dr. Gregg Wells and Alexi Person for their contributions and support in carrying out this project. I would like to thank Shama Dhanani for creating the β2 6xAGS construct that was paired with my α3 6xAGS construct for expression. I would also like to thank Kristen Cloyd for cloning the α3 full length cDNA into the pSP64A plasmid vector to create the starting material used in this project.

CHAPTER I

INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are essential for life. These receptors are integral membrane, ion channels that conduct cations and depolarize a cell when they switch from their closed to open state after a ligand binds the active site (13). They are activated by the neurotransmitter acetylcholine but are also sensitive to activation by nicotine (1). nAChRs are located throughout the central and peripheral nervous system and consist of two major types: neuronal receptors and muscle-type receptors. Neuronal nAChRs are commonly found at five primary locations on the neuron: the cell soma, dendrites, preterminal axon regions, axon terminals, and the myelinated axons (1). The importance of these proteins comes from their ability to transmit signals from neuron to neuron though the release of the neurotransmitter acetylcholine. Nicotinic acetylcholine receptors have been shown to be linked to epilepsy, Alzheimer disease and Parkinson disease and have been implicated in schizophrenia, Tourette's syndrome, anxiety and depression (8).

Understanding the structure of nAChRs is essential for understanding their function and importance. The protein consists of a large, N-terminal extracellular domain which is responsible for ligand binding, four transmembrane domains, a cytoplasmic loop between the third and fourth transmembrane domains, and a Cys-loop made of two cysteine residues with a disulfide bond separated by 13 amino acid residues (13). Each receptor is composed of five subunits. Currently 17 subunits have been identified and named α1-α10, β1-β4, δ, γ and ε (13). Each one of these subunits contains the four transmembrane domains and an N-terminal extracellular

domain. The pentameric assembly of N-terminal extracellular domains work together to form ligand binding sites at interfaces between subunits. The muscle-type nAChRs consist of an α1 and 4 non-α (β,δ, or γ/ε) subunits, while the neuronal nAChRs can be homopentamers or heteropentamers that contain α1-10 or β1-4 in many different combinations (1). The subunits assemble to form a central pore through which cations can pass. This diversity of subunits allows organisms to produce a variety of receptors with specialized functions (1).

The α3 subunit is mostly expressed in the autonomic nervous system (along with β4), whereas α4 and β2 nAChR subunit expression dominates in the central nervous system (1). The α3β4 nAChR subunits are also the predominant subunits expressed in the autonomic sensory ganglia (5). However, α3 subunits can also be located in the brain, and are found mostly in the visual pathway, pineal gland, cerebellum and the retina (5). The importance of the α3 nAChR subunit was tested by creating α3 genetic knockout mice. These mice experienced impaired growth, increased mortality, enlarged bladders, bladder infections, dribbling urination, urinary stones, extreme mydriasis and lack of pupil contraction in response to light (7). The large amount of bladder problems was probably due to the absence of α3-containing nAChRs in the parasympathetic intramural ganglia of the bladder and the eye problems were from the lack of α3-containing nAChRs in the nerve that controls the contraction and dilution of the pupil (14). These knockout mice showed the major effects of the α3 subunit within the autonomic nervous system.

Obtaining structural information on nAChRs is vital to determine how they function as ion channels and signal transducers, learn their role in physiology, and understand the differences in

receptors within the Cys-loop receptor family (6). This knowledge will help design drugs that will therapeutically target particular types of receptors to cause specific effects and help treat some of the nAChR-related diseases and disorders that are suffered by many. In particular, obtaining structural information on the α3 nAChR subunit will show how the α3 subunit's structure is different from the other subunits and will allow for drugs to be designed to therapeutically target specific types of receptors for effects within the autonomic nervous system.

Some structural information has already been publicized regarding the structure of nAChRs. Electron diffraction of nAChRs was completed from muscle-type nAChRs in helical tubular crystals from *Torpedo marmorata* (an electric fish) at a 4 Å resolution, as well as x-ray crystallography of acetylcholine binding protein (AChBP) at about a 2 Å resolution (13). Structural models have also been developed for cys-loop bacterial receptors from *Gloeobacter violaceus* and *Erwinia chrysanthemi* (9). The problem with existing structural models of nAChR is that they have only partially reproduced ligand binding affinities and subunit assembly properties compared to full length receptors. A more tractable experimental model of full-length nAChR needs to be developed that possesses suitable structural properties of the full receptors.

The ultimate goal of this project is to develop water soluble extracellular domain (ECD) nAChRs for x-ray crystallography. The extracellular domain is defined as the truncated subunits containing the N-terminal sequences including the ligand-binding region (6). This ECD segment should have the same ligand binding affinities and subunit assembly properties as the full length receptor. The transmembrane domains make nAChRs difficult to crystallize, so being able to

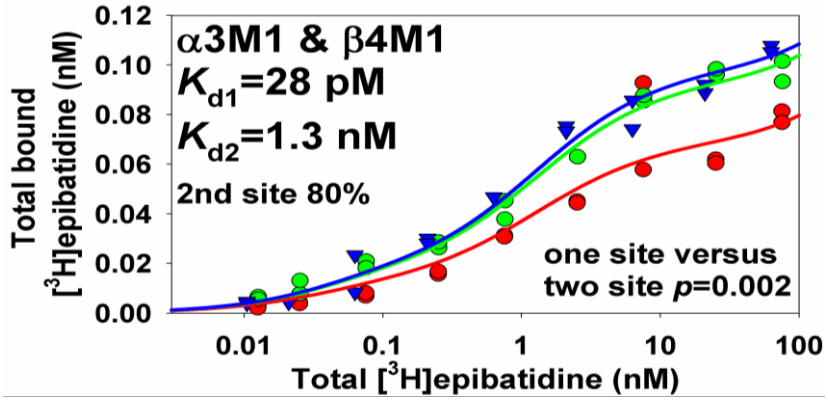
create just the ECD of nAChRs would allow the protein to crystallize more easily (6). The smaller molecular mass of the ECD could also make it a better option for NMR spectroscopy (6).

Past research has shown that the ECD domain cannot be expressed by itself but needs to be expressed in junction with the M1 transmembrane domain (6). However, an epitope tag can be inserted at the M1/ECD junction in the α7 subunit without significantly altering ligand affinity (12). This suggests that a protease site could be inserted at the M1/ECD junction and be used to separate the ECD from the M1 domain after protein expression. The protein expression yields of just the ECD and M1 domains are significantly lower than yields of the full length nAChRs (10). This suggests that the full length nAChR should be expressed before using a protease enzyme to isolate the ECD. It has also been discovered that some amino acid residues at the interface of M1 and the ECD need to be duplicated on both sides of the insert in order to successfully produce the nAChR with an inserted protease site (11). There has also been success in inserting a protease site at the ECD/M1 interface in α7 nAChR (12) and α4β2 nAChR (unpublished). Pursuing this same approach should also yield positive results with the α3 subunit nAChR.

In order to determine how to maximize protein expression of the α3 nAChR subunit containing a protease site at the ECD/M1 junction, a nAChR mutant containing a non-native insert at the ECD/M1 junction was created. This non-native insert is a flexible linker domain consisting of the amino acids alanine-glycine-serine repeated six times (6xAGS). This linker could act as a flexible environment in which to insert the protease site in order to allow the protein more freedom to form its tertiary structure. This extra freedom and flexibility could make up for the decrease in protein expression that is seen when just the protease site is inserted into the protein.

I tested the ability of the α3 subunit to tolerate the 6xAGS linker at the ECD/M1 junction by evaluating the protein expression levels and measuring the yield of [^{3}H]epibatidine binding sites as an indicator of the production of α3 nAChR.

Epibatidine is a compound which binds with high affinity to α3 nAChR. Its ability to bind to the agonist binding site of α3 nAChR makes it a valuable tool for validating the structure of that site in structural models. When epibatidine is tagged with radioactive isotope tritium to form [^{3}H]epibatidine, its binding can be detected with a liquid scintillation counter (2). In our ligand binding assay, our mutated protein product is incubated with [^{3}H]epibatidine. The amount of [^{3}H]epibatidine that remains bound to the receptor is used to calculate the yield of [^{3}H]epibatidine binding sites. The yield of [^{3}H]epibatidine binding sites allows conclusions to be drawn about the successful formation of the receptor's active site (2). Studies of the full length α3β4 nAChR and M1-containing α3β4 nAChR and their ability to bind [^{3}H]epibatidine confirms that each receptor has two sites for binding [^{3}H]epibatidine molecules (Fig. 1) (10). This two-site model will be taken into account when the yield of [^{3}H]epibatidine binding sites is determined for the mutant α3 nAChR subunit containing the flexible linker.



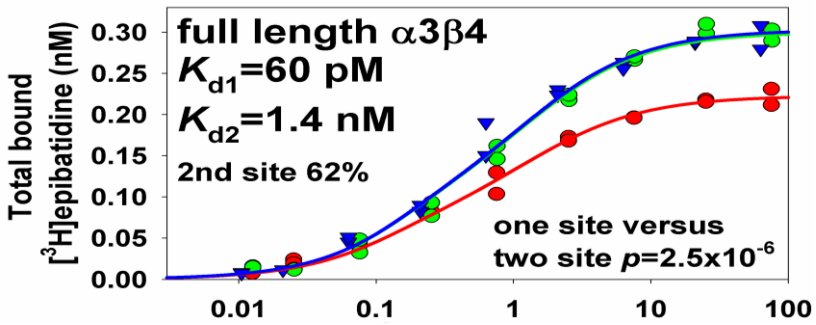


Figure 1. [3H]epibatidine binding curves of full length and ECD α3β4 nAChR and M1 and ECD α3β4 nAChR. Data supports the presence of two sites in full length and ECD α3β4 nAChR.

In this project, I created a mutant α3 nAChR subunit containing a flexible 6xAGS linker at the ECD/M1 junction and evaluated its expression levels in *Xenopus laevis* oocytes as well as evaluated its ability to bind [^{3}H]epibatidine by calculating the yield of [^{3}H]epibatidine binding sites. This information will allow us to determine if the flexible linker 6xAGS would be a good environment in which to contain a protease site in the α3 nAChR subunit and possibly other nAChR subunits.

CHAPTER II

METHODS

Design of DNA Plasmids

The cDNA of the human α3 and β2 nAChR subunits were cloned into the pSP64A Poly(A) vector (Promega). Primers were designed that contained the genes encoding the new 6xAGS flexible linker and also the IRRL residues which occur naturally right before the M1 domain. The primers were designed to insert the linker between the M1 domain and the extracellular domain. They were then synthesized by Invitrogen. PCR was performed with Accuprime PFX DNA Polymerase (Invitrogen) to insert the 6xAGS sequence into the α3 subunit coding sequence. The DNA was amplified by bacterial transformation in One Shot® OmniMAX™ 2 T1 Chemically Competent *E.coli* (Invitrogen) and then purified and isolated with QIAfilter® Plasmid Midi Prep Kit from Qiagen. Sequencing was performed by Lone Star Labs to confirm the presence of the 6xAGS insert. The plasmid DNA containing the α3 Full and IRRL 6xAGS sequence was linearized using AseI restriction digest (New England Biolabs) and the correct linear DNA production was confirmed by gel electrophoresis. RNA production from the linearized DNA was performed using the Ambion mMessage mMachine® SP6 Kit. The RNA product was visualized and quantified by gel electrophoresis.

Protein Expression in *Xenopus* Oocytes

Oocytes from *Xenopus laevis* frogs were prepared using collagenase type 1 (Invitrogen) following standard procedures (4). Protein expression of the nAChR constructs was achieved by

injecting a 40 nl aliquot containing 20 ng of cRNA of a pair of nAChR subunits into the cytoplasm of each oocyte. This process was repeated for each pair of subunit constructs being studied. The oocytes were then incubated at 18°C for 3 days in 50% Leibovitz's L-15 medium (Invitrogen) in 10 mM HEPES, pH 7.5, containing 10 units/ml penicillin, 10 µg/ml streptomycin, and 43 µg/ml gentamicin. After incubation, the oocytes were homogenized by hand in an ice-cold buffer (Buffer A: in mM: 50 sodium phosphate, 50 NaCl, 5 EDTA, 5 EGTA, 5 benzamidine, 15 iodoacetamide, pH 7.5) and then centrifuged for 30 minutes at 14,500 x *g* at 4°C. The pellet was solubilized in a buffer (Buffer B: Buffer A with 2% w/v Triton X-100) with gentle agitation for 2 hours at 4°C. The mixture was then centrifuged again (30 min, 14,500 x *g*, 4°C). The membrane fraction was the fraction that was extracted by the detergent, and it was used for all of the immunoblotting and ligand binding assays.

Immunoblotting

The samples were denatured by heating at 65°C for 5 minutes in 1% SDS and 15 mM dithiothreitol and separated by SDS-PAGE on a 12.5% acrylamide gel. The proteins were then transferred to an Immun-Blot™ polyvinylidene difluoride (PVDF) membrane (BioRad) using a GENIE® electrophoretic transfer device (Idea Scientific) for 30 minutes. Before probing, the membrane was blocked over the weekend at 4°C with 5% powdered milk in PBS buffer and 0.05% Tween®20. The primary antibody, anti-HA antibody (Roche), was diluted 1:5000 in blocking solution and incubated with the membrane overnight at 4°C. The membrane was washed a minimum of three times for one hour each wash with PBS and 0.05% Tween. Goat anti-mouse (Pierce) secondary antibody was diluted 1:5000 in blocking buffer and incubated with the membrane overnight at 4°C. The membrane was washed again for a minimum of three

times for one hour each wash with PBS and 0.05% Tween. The proteins on the membrane were then visualized on BioMax ML film (Eastman Kodak Co.) with SuperSignal West Dura Extended Duration Substrate (Pierce) according to the manufacturer's instructions.

Ligand Binding Assay

Immulon 4 HBX plastic microwells (Thermo Labsystems) for solid phase binding assays were coated with the primary antibody, 35 mAb, diluted in 10 mM CAPSO to a concentration of 4 μg/ml of CAPSO and incubated for 3 days. Monoclonal antibody 35 was used as the primary antibody because it selectively binds α3 subunits of nAChR (3). Then the wells were washed with NaN_3-PBS. After the wash, the wells were blocked with 3% bovine serum albumin, fraction V (EM Science), in PBS (in mM: 137 NaCl, 2.7 KCl, 1.4 KH_2PO_4 10 Na_2HPO_4), incubated for 2 hours and washed with wash buffer. A volume of detergent extract containing the equivalent of 0.5 to 2 injected oocytes was added to each mAb-coated microwell. The microwells were incubated overnight at 4°C and then washed with ice cold buffer B. In order to measure [^{3}H]epibatidine binding, an aliquot, of an amount determined by the constructs, of (±)-[^{3}H]epibatidine (PerkinElmer Life Sciences; specific activity: 55.8 Ci/mmol) in ice-cold buffer B was added to each microwell and incubated overnight at 4°C. After incubation, the microwells were washed with ice-cold buffer B and stripped with a solution of 5% SDS and 25 mM dithiothreitol in buffer B. A liquid scintillation counter was used to measure the amount of [^{3}H]epibatidine bound for each sample. Each data point was the average of duplicate measurements. Yield was calculated as the amount of [^{3}H]epibatidine binding sites per oocyte. The yield of [^{3}H]epibatidine binding sites for each subunit combination was defined as the amount of bound [^{3}H]epibatidine with a total [^{3}H]epibatidine concentration of 1 nM. The

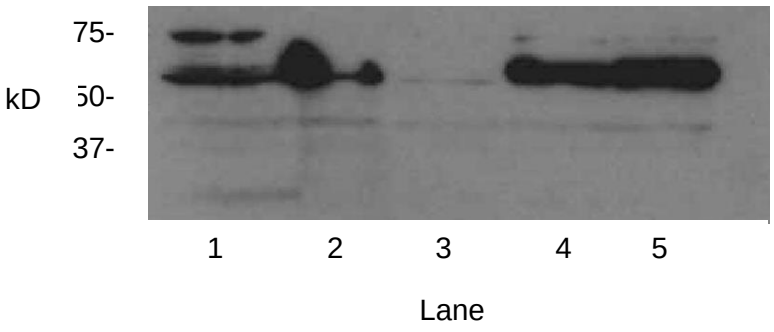
controls used were α3β2 or α3β4 nAChRs. Nonspecific binding was measured from uninjected oocytes.

CHAPTER III

RESULTS

Immunoblotting

The mutated α3 nAChR subunit containing the 6xAGS flexible linker was injected into *Xenopus laevis* oocytes for protein expression in combination with a mutated β2 nAChR subunit containing the same flexible linker at the ECD/M1 junction and an HA epitope tag. A positive control, α4 full with a FLAG epitope tag paired with β2 full with a HA epitope tag, and a negative control, α3 full paired with β4 full, were also injected into the *Xenopus laevis* oocytes. The expressed proteins were purified and separated by SDS-PAGE on a 12.5% acrylamide gel. Protein expression of the mutant β2 nAChR subunit was confirmed by immunoblotting with anti-HA antibody, which binds the HA epitope tag that was cloned into the N-terminus of the β2 sequence. Visualization of the blot revealed that the β2 subunit protein containing the 6xAGS flexible linker segment was expressed at a level similar to the control β2 subunit which did not contain the flexible linker (Fig. 2). Uninjected oocytes were used as a control.



Lane:	Construct:
1	Uninjected Control
2	Positive Control: α4 Full 3xFlag/β2Full 3xHA
3	Negative Control: α3 Full/ β2 Full
4	Sample 1: α3 Full 6xAGS/ β2 Full 6xAGS 3xHA
5	Sample 2: α3 Full 6xAGS/ β2 Full 6xAGS 3xHA

Figure 2. Immunoblotting. Probing with the anti-HA antibody revealed an adequate protein expression yield of nAChR β2 subunits containing the 6xAGS flexible linker.

Ligand Binding Assay

The ligand binding assay measured the yield of [^{3}H]epibatidine binding sites on the expressed α3/β2 subunit pair containing the flexible linker. Yield of [^{3}H]epibatidine binding sites was calculated by separating the receptors of interest from the oocyte lysate using microwells coated with the α3-specific mAb 35 antibody, and then incubating the bound, epitope-tagged subunits with [^{3}H]epibatidine overnight. The amount of [^{3}H]epibatidine bound to the receptors was measured using liquid scintillation counting and was measured in disintegrations per minute [DPM]. From these data the yield of [^{3}H]epibatidine binding sites per oocyte was calculated using the specific activity of [^{3}H]epibatidine adjusted for radioactive decay and a 1.0 nM concentration of [^{3}H]epibatidine. This concentration was sufficient to saturate binding sites with native-like structure. α3/β4 full and α3/β2 full pairs without the flexible linker were used as controls along with uninjected oocytes. The assay was performed on the protein collected from two separate injections, because the oocytes can often vary in their protein expression levels. In the first injection, a very low yield of [^{3}H]epibatidine binding sites was seen for the α3/β2

subunit pair containing the 6xAGS flexible linker compared to the controls without the linker. The second injection showed practically no yield of [^{3}H]epibatidine binding sites (Fig. 3).

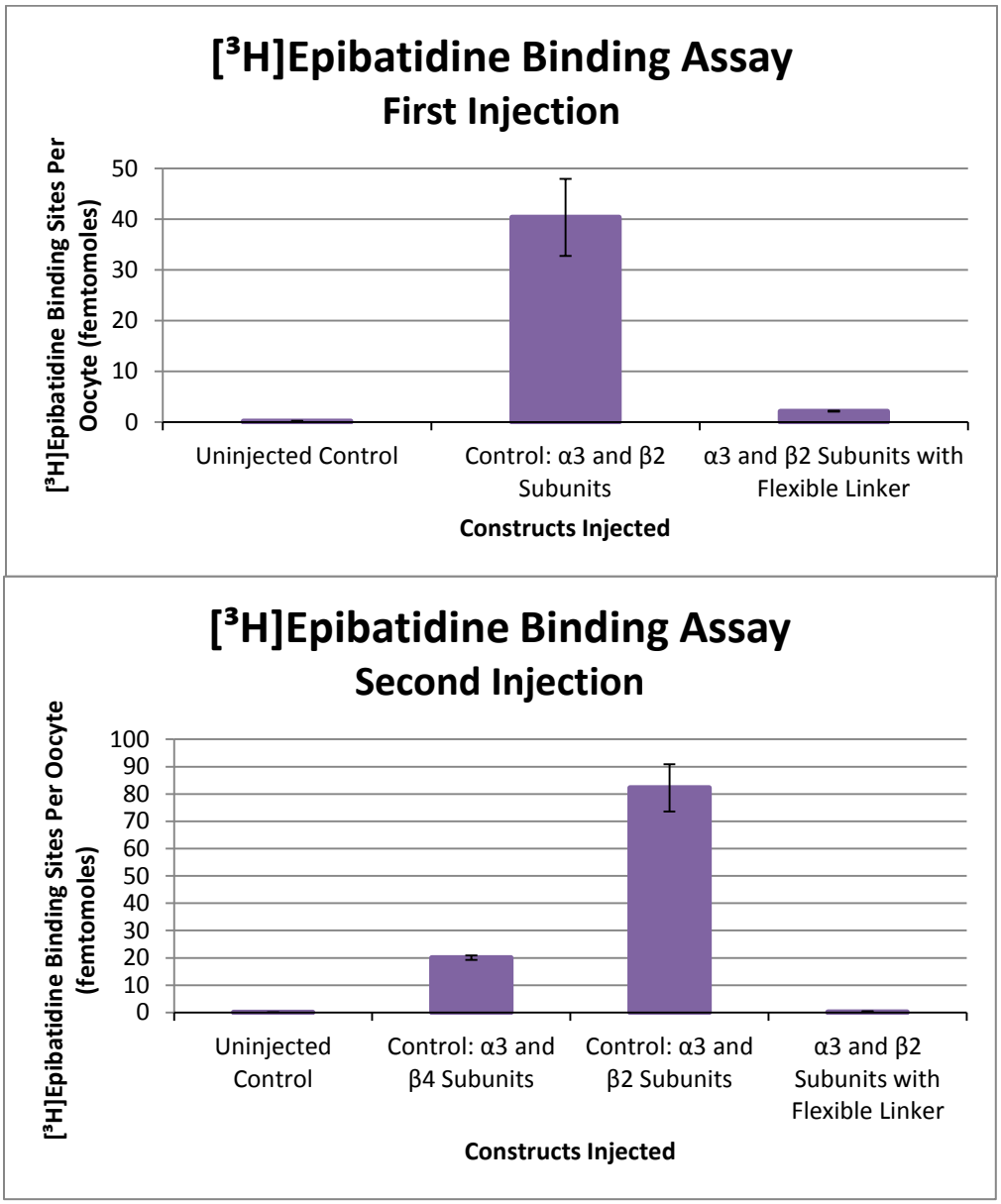


Figure 3. Epibatidine binding assay. Very low yield of [^{3}H]epibatidine binding sites was seen when the α3full 6xAGS was paired with the β2full 3xHA 6xAGS subunit as compared to the control subunits without the flexible linker. Uninjected oocytes were used as a control.

CHAPTER IV

CONCLUSION

We studied the effects of inserting a long flexible linker between the extracellular domain (ECD) and the first transmembrane domain on the ability of the α3 and β2 subunits of nicotinic acetylcholine receptors (nAChRs) to bind the ligand [^{3}H]epibatidine and monoclonal anti-nicotinic acetylcholine receptor antibody 35 in order to evaluate the ability of the linker to be used as a flexible environment in which to place a protease site. After expression of the mutated α3 subunits in combination with similarly mutated β2 nAChR subunits, the proteins were purified, separated on a SDS-PAGE gel, transferred to a membrane and probed with anti-HA antibody to detect the presence of the β2 subunits, which contained a HA epitope tag. As compared to the control, there was adequate expression of the β2 subunits. From the immunoblotting results it can be concluded that the 6xAGS flexible linker does not affect the ability of the protein, without consideration of tertiary structure, to be expressed and survive in the cell.

Epibatidine binds with a high affinity to neuronal nicotinic receptors and when it is tagged with radioactive isotope tritium its binding can be detected with a liquid scintillation counter (2). When the amount of [^{3}H]epibatidine binding sites was measured for the mutated α3 nAChR subunits containing the 6xAGS flexible linker in combination with the β2 subunit containing the same linker it was found to be significantly lower than for the controls. The first oocyte injection showed some [^{3}H]epibatidine binding, but it was almost 20 times less than the control. The second oocyte injection had even worse yield of [^{3}H]epibatidine binding sites and was barely

detectable. This low yield of [^{3}H]epibatidine binding sites for the mutated α3 and β2 nAChR subunits containing the 6xAGS flexible linker suggests that the protein is not forming its correct secondary and tertiary structure. Without the correct protein structure, the active site is not properly formed and the [^{3}H]epibatidine is not able to bind. It can be concluded that the specific design of the flexible linker inserted between the ECD and the M1 domain negatively influences the protein's ability to fold into its proper structure and is therefore a poor environment in which to place a protease site.

The results of this study raised many questions on why this flexible linker destroyed the ability of the protein to form properly. One possibility is that the region between the ECD and the M1 domain is crucial for forming the secondary and tertiary structure and adding the insert there disrupted some important interactions. Another possibility is that the length of this 6xAGS flexible linker was too long to allow the ECD and the active site to form properly.

A new hypothesis was raised that we hope will answer some of these questions. We hypothesize that the length of an insertion at the extracellular domain/M1 interface affects yield of [^{3}H]epibatidine binding sites of α3-containing nAChRs. To test this new hypothesis we have developed two new mutants containing inserts at the ECD/M1 interface in α3 subunit nAChRs. The first mutant contains a short sequence of only five amino acids. Testing this short sequence will reveal if the size of the insert influences protein expression and ligand binding. The second mutant contains a TEV protease site at the ECD/M1 junction. This TEV protease site is the minimal length necessary for site-specific proteolysis and could be used to liberate the extracellular domain after the protein has been expressed. The shorter length of these inserts at

the ECD/M1 junction will hopefully lead to increased yield of [^{3}H]epibatidine binding sites for α3-containing nAChRs.

Successful expression and binding of α3 nAChR subunits containing a protease site at the ECD/M1 junction would allow the same techniques to be applied to the other nAChR subunits. Use of the protease site to separate the ECD from the rest of the receptor would allow the study of how to best crystallize the ECD molecule to begin. Successful crystallization would allow the ECD and most importantly the ligand binding sites of the different subunits of nicotinic acetylcholine receptors to be studied. This valuable information could be used to enhance therapeutic drug design for these nicotinic acetylcholine receptors and possibly other related Cys-loop receptors and help treat the detrimental effects of neurological disorders.

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