Characterization of Neuronal Nicotinic Acetylcholine Receptors and their Positive Allosteric Modulators

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Characterization of Neuronal Nicotinic Acetylcholine Receptors

and Their Positive Allosteric Modulators

Doris Clark Jackson

A dissertation submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
Neuroscience

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ABSTRACT

Characterization of Neuronal Nicotinic Acetylcholine Receptors and Their Positive Allosteric Modulators

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Doctor of Philosophy
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Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that are necessary in memory and cognition. They are pentameric and consist of α and β subunits. They are most commonly heteromeric but, can sometimes be homomeric. nAChRs are activated by many ligands including nicotine (exogenous) and acetylcholine (endogenous).

nAChRs are located on hippocampal interneurons. The interneurons, although sparse, control the synchronous firing of the pyramidal cells. However, the hippocampal interneuron structure and function is quite diverse and not fully characterized. Therefore, we sought to quantify nAChR subunit mRNA levels using real-time PCR of CA1 hippocampal interneurons.

Surprisingly we found that the α3 and β2 mRNA subunits were the highest expressed and highest co-expressed subunits. Additionally, the α4 mRNA subunit was the lowest expressed of the subunits detected. The α4 subunit is one of the most pharmacologically targeted nAChR subunits and is found throughout the rest of the brain at much higher levels than the α3 mRNA subunit. Upon PCR analysis two subpopulations of the α3 and β2 subunits emerged: those that contained 3X more α3 than β2 and those that contained 3X more β2 than α3. Therefore, we hypothesized that two likely α3β2 nAChR stoichiometries are present in hippocampal interneurons. We differentiated their kinetic properties using electrophysiology.

Additionally, like the α4 subunit, the α7 subunit is highly targeted in cognitive therapeutics. Since, the α7 subunit is the most characterized nAChR subunit, there are current efforts to develop allosteric modulators of the α7 subunit. The α7 subunit is found at moderate levels within hippocampal interneurons and remains a valid target. Current treatment options for Alzheimer’s disease, and other dementias are limited and only mildly effective. Therefore, we sought to characterize the effect of 3-furan-2-yl-N-p-tolyl-acrylamide (PAM-2) on α7.

Furthermore, there are no current methods to distinguish the α7 from the α7β2 nAChRs during whole cell electrophysiological recordings. Therefore, we also characterized the PAM-2 effect on α7β2 nAChRs. Our results highlight at least 2 ways PAM-2 can be used to differentiate α7 from the α7β2 during whole-cell recordings.

Keywords: nicotinic acetylcholine receptors (nAChRs), hippocampus, interneurons, electrophysiology, allosteric modulators, real-time PCR
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TABLE OF CONTENTS

TITLE PAGE .................................................................................................................................. i
ABSTRACT .................................................................................................................................... ii
ACKNOWLEDGMENTS ............................................................................................................. iii
TABLE OF CONTENTS ............................................................................................................... iv
LIST OF TABLES ......................................................................................................................... vi
LIST OF FIGURES ...................................................................................................................... vii
LIST OF SYMBOLS AND ABBREVIATIONS .......................................................................... ix
INTRODUCTION .......................................................................................................................... 1
References ........................................................................................................................... 9
CHAPTER 1: Expression of nAChR mRNA in Rat Hippocampal Interneurons ......................... 12
Abstract ............................................................................................................................ 13
Introduction ....................................................................................................................... 14
Methods ..................................................................................................................................... 15
Slice Preparation ............................................................................................................. 15
Cytoplasm Aspiration ................................................................................................. 16
Electrophysiology ........................................................................................................ 16
Primers and Probes ....................................................................................................... 16
RT Reaction .................................................................................................................. 17
Multiplex Reaction and Real-time Quantitative PCR .................................................. 17
Data and Statistical Analysis ....................................................................................... 18
Equation 1: .................................................................................................................. 19
Equation 2: .................................................................................................................. 19
Equation 3: .................................................................................................................. 19
Materials ....................................................................................................................... 20
Results ............................................................................................................................... 20
Discussion ........................................................................................................................... 23
References ............................................................................................................................ 38
CHAPTER 2: The Human Alpha 3 Beta 2 Neuronal Nicotinic Acetylcholine Receptor
Forms Two Distinguishable Subtypes ........................................................................ 40
Abstract ............................................................................................................................ 41
Introduction ....................................................................................................................... 42
LIST OF TABLES

Table 1.1: Summary of Amplicon Lengths (base pairs) and Oligo Sequences of the nAChR Subunit Probes Used in qPCR. ................................................................. 27

Table 1.2: Summary of Oligo Sequences of the nAChR Subunit Primers (+ and -) Used in qPCR ........................................................................................................... 28

Table 1.3: The Proportion of Cells Containing Each nAChR mRNA Subunit (n=93). ................................................................. 29

Table 1.4: The Proportion of Subunit Co-expression for the Highest 8 Subunit Pairs (n=69, the number of cells that contained at least 2 mRNA subunits). .............. 30

Table 1.5: T-test for Pearson Correlation Coefficients for Subunit Co-expression in the stratum oriens and the stratum radiatum ............................................................................... 31

Table 1.6: A Summary of all mRNA Subunit Combinations Observed More Often than Would be Expected (observed/expected>1). ...................................................... 32

Table 1.7: A List of All Observed Subunit mRNA Combinations by Location. ................................................................. 33

Table 2.1: Summary of ACh and Nic Dose-Response Curves. .............................................................................................................. 53

Table 3.1: Pharmacologic Activity of PAM-2 on hα7 and hα7β2 AChRs, hα1β2γ2 and hρ1 GABAARs, and hα1 GlyRs. ...................................................................................... 87

Table 3.2: Kinetics Parameters for ACh in the Absence (Control) and Presence of PAM-2 at the hα7 and hα7β2 AChRs. ..................................................................................... 88

Table 3.3: Molecular Interactions of PAM-2 with Allosteric Sites at the hα7 and hα7β2 Models. ............................................................................................................. 89

Table 3.3: Continued: Molecular Interactions of PAM-2 with Allosteric Sites at the hα7 and hα7β2 Models. ..................................................................................... 90

Table 3.4: RMSD Mean and RMSD Variance Values for PAM-2 Interacting with Different Nicotinic Receptor Subtypes, Including hα7, h(α7)2(β2)3, and h(α7)3(β2)2 nAChRs .............................................................................. 91
LIST OF FIGURES

Figure 1.1: The Cellular Components of Each Region of the Hippocampus ........................................ 7
Figure 1.2: The Cellular Layers Within the CA1 Region of the Hippocampus ..................................... 8
Figure 1.1: Kinetically Diverse Population of nAChRs .................................................................... 34
Figure 1.2: Count of nAChR Subunit Expression. .......................................................................... 35
Figure 1.3: nAChR Subunit Relative Expression by Subunit ........................................................... 36
Figure 1.4: Pearson r Correlations of the Average Fold Expression for 2-way Comparisons......................... 37
Figure 2.1: Ratio of α3 and β2 Expression in Hippocampal Interneurons. ...................................... 54
Figure 2.2: Sample Traces and Likely Stoichiometries. ................................................................. 55
Figure 2.3: IV Plot. ......................................................................................................................... 56
Figure 2.4: Dose-Response Curves ............................................................................................... 57
Figure 2.5: Comparison of Rise Time and Half-Width of ACh Dose-Response Curve ..................... 58
Figure 2.6: Desensitization ......................................................................................................... 59
Figure 3.1: Effect of PAM-2 on (A) Homomeric hα7 and (B) Heteromeric hα7β2 AChRs ...................... 92
Figure 3.2: Change in Properties of Desensitization After a 3 minute PAM-2 Application ........................ 93
Figure 3.3: Effect of 20 µM PAM-2 on Various Kinetic Parameters Obtained from ACh-Induced hα7 and hα7β2 AChR Responses ................................................................. 94
Figure 3.4: Time Dependence of the Change in Peak Amplitude and Current Area of the Respective hα7 (■,□) and hα7β2 (▲,▲) AChR Perfused with 50 µM PAM-2 .............. 96
Figure 3.5: Effect of PAM-2 on Heteromeric hα1β2γ2 (A) and Homomeric hρ1 (B) GABAARs ............ 97
Figure 3.6: Effect of PAM-2 on Recombinant hα1 GlyRs .............................................................. 98
Figure 3.7: Molecular Docking of PAM-2 to the hα7 and h(α7)2(β2)3 Models.......................... 99
LIST OF SYMBOLS AND ABBREVIATIONS

α: alpha nAChR subunit (subtypes include α1-α10)
β: alpha nAChR subunit (subtypes include β1-β4)
γ: gamma nAChR subunit found at the neuro-muscular junction
δ: delta nAChR subunit found at the neuro-muscular junction
ε: epsilon nAChR subunit found at the neuro-muscular junction
ACh: acetylcholine (solubilized)
AChR/nAChR: nicotinic acetylcholine receptor
AD: Alzheimer’s disease
ASD: autism spectrum disorder
ADHD: attention deficit hyperactivity disorder
β-amyloid: a peptide implicated in Alzheimer’s disease
CA1, CA3: sub regions of the hippocampus
CaCl₂: calcium chloride (dihydrate)
CNS: central nervous system
DG: dentate gyrus; sub region of the hippocampus
EC⁵₀, EC⁸⁵, ECₘₐₓ: concentrations on the dose-response curve representing 50%, 85%, and maximum receptor activation
ECD: extracellular domain
GABA: gamma-aminobutyric acid, a neurotransmitter
GlyR: glycine receptor
KCl: potassium chloride
MgCl₂: magnesium chloride
mRNA: messenger ribonucleic acid
NaCl: sodium chloride

Na$_2$HPO$_4$: sodium phosphate

n$_H$: hill slope

Nic: nicotine (solubilized)

OR-2-Ca$^{2+}$: oocyte storage and perfusion solution; also known as Ringer’s solution

PAM-2: 3-furan-2-yl-N-p-tolyl-acrylamide; $\alpha_7^*$ AChR selective positive allosteric modulator

PNS: peripheral nervous system

PTSD: posttraumatic stress disorder

qPCR: real time-polymerase chain reaction

RMSD: root mean square deviation; relates to the distance between two atoms of interacting proteins

TMD: transmembrane domain
INTRODUCTION

Nicotinic acetylcholine receptors (nAChR) are ligand-gated ion channels that are found in the central nervous system (CNS), peripheral nervous system (PNS), and in skeletal muscle at the neuromuscular junction. nAChRs are integral membrane proteins that create cation permeable pores in the extracellular membrane of cells when the neurotransmitter acetylcholine (ACh) binds. In the PNS and skeletal muscle, they are located post-synaptically, in the classic role of a neurotransmitter receptor that awaits signals from the presynaptic cell. In the CNS however, they can be located pre-terminally, pre-synaptically, and post-synaptically. When located post-synaptically, they function as a normal ligand-gated ion channel, changing the membrane potential of excitable cells like neurons and skeletal muscle when activated. However, when located pre-synaptically, they function in a unique role as modulators for the release of other neurotransmitters like glutamate, GABA, serotonin, dopamine, and acetylcholine.

Each nAChR contains 5 subunits making it a pentameric receptor. Different subunit combinations create different functional receptor subtypes. The skeletal muscle nAChR is formed from combinations of the α1, β1, γ, δ and ε subunits. There are 11 known human genes for neuronal nAChR subunits (α2-7, α9-10, and β2-4) (Rubboli et al., 1994, Sargent, 1993). The most common combinations require two α subunits and three β subunits, or three α subunits and two β subunits. Only the α7 and α9 subunits can form homomers. Given the tremendous possible diversity of neuronal nAChR subtypes, many of the potential pentameric combinations are not fully characterized.

Receptors made from different subunit combinations create receptor subtypes that have different physical properties. These include pharmacological properties like agonist and
antagonist affinity, and kinetic properties like activation, inactivation and desensitization rates. Additionally, nAChR subtypes can have different ion permeabilities. All nAChR subtypes are permeable to Na\(^+\) and K\(^+\), but some subtypes like the \(\alpha7\) homomer are also highly permeable to Ca\(^{2+}\), which allows for activation of a variety of intracellular signaling cascades. The hippocampal interneurons we analyzed are GABAergic neurons and release GABA unto the pyramidal cells resulting in inhibition. Since these interneurons synapse with hundreds of pyramidal cells, the interneurons play a vital role in the synchronous firing of the hippocampus.

The hippocampus has an organized firing pattern. In the major hippocampal pathway called the “trisynaptic circuit,” axons enter the hippocampus from the entorhinal cortex and terminate on glutamatergic neurons in the dentate gyrus (DG). The signals then travel from the dentate gyrus to the CA3 region of the hippocampus via the “mossy fibers” (glutamatergic). The third synapse (also glutamatergic) goes from the CA3 region to the CA1 region of the hippocampus (Figure 1) in what are called the “Schaffer collaterals” (Grybko, Sharma, & Vijayaraghavan, 2010). An additional input comes at this stage from the contralateral hippocampus, known as the commissural fibers. This maintains communication between both hemispheres of the hippocampus. Finally, the CA1 region of the hippocampus sends an output signal to the subiculum.

As stated, the pyramidal cells of the hippocampus must fire their output signal synchronously. The nAChRs located in interneurons play a crucial role in the synchronous firing of the hippocampus required for normal cognition. Memory is correlated with theta and gamma frequencies of the pyramidal cells, whereas, attention is correlated with alpha and gamma frequencies (Ward, 2003). It has been shown that desynchronization of this firing can result in a loss of cognition (Hanslmayr, 2012, Mitchell, 2009). Additionally, cognitive diseases like
Alzheimer’s disease are characterized by neurodegeneration of the hippocampus. The neurodegeneration disrupts the interneuron-pyramidal cell relationship.

We analyzed the nAChR subunit mRNAs in the CA1 hippocampal interneurons to elucidate the most likely nAChR subtypes expressed on hippocampal interneurons (circled in Figure 1). Our results could lead to improved nAChR targeted therapies in the treatment of cognitive disorders by identifying the most likely targets on hippocampal interneurons. Specifically our work focused on interneurons of the stratum oriens and stratum radiatum surrounding the CA1 pyramidal neurons (Figure 2) (Graham et al., 2003, Son & Winzer-Serhan, 2006). Using single-cell, real time-PCR (qPCR), we identified the expression of α2, α3, α4, α5, α7, β2, β3, and β4 subunit mRNA in rat hippocampal interneurons. The α6 mRNA subunit was not detected during initial analysis, and therefore, was removed from continued experiments. Statistical analysis of our single-cell qPCR mRNA expression data suggests that the α3 and β2 subunits colocalize more often than any other subunit pair (Sargent, 1993; Wada et al., 1989). However, the characterization of receptors containing the α3β2 subunits is still in its infancy. The current thinking suggests that the most likely stoichiometry is α3(2)β2(3), based mainly on evidence from the muscle nACHR and the α4β2 neuronal nACHR – which is thought to contain two α subunits in its pentameric makeup. However, recent experiments involving other α:β combinations that have been better characterized, like the α4β2 and α2β2 subunits, indicate that the most likely functional receptors would be α3(2)β2(3) or α3(3)β2(2) (Lukas 1988, Moroni et al., 2008, Wu et al., 2006, Zwart et al., 2008). Other stoichiometries are possible, but less likely. In addition, the α3β2 nACHR receptors would likely incorporate additional nACHR subunits such as the α5 subunit – further complicating the native subtypes of the receptor (ChavezNoriega et al., 1997, Wada et al., 1989).
The results of our work quantifying the nAChR subunits mRNAs revealed a highly heterogeneous population of nAChR subunit mRNAs. Furthermore, the α3 subunit is very limited in its expression in other brain regions, possibly making the α3β2 nAChR a valuable therapeutic target for cognitive diseases. Targeting the α3β2 may result in fewer undesired effects while still achieving cognitive improvements. Therefore, we sought to characterize the α3β2 nAChR as a potentially important receptor in hippocampal circuitry.

We characterized this novel receptor—the α3β2—in a simplified cell expression system. We used *Xenopus laevis* oocytes because they are extremely large cells, and when injected with foreign mRNA can produce many channels (i.e., large currents). Additionally, *Xenopus laevis* oocytes do not express native acetylcholine receptors. Also, since these oocytes allow for easier access to the cell surface we can better analyze the ion channel kinetics than neurons in brain slices. The time required for a solution to travel through brain tissue to the channel of interest is not as precise making the interpretation of kinetic data more difficult (Charpantier et al., 2005, ChavezNoriega et al., 1997, Dash, Bhakta, Chang, & Lukas, 2012, Ruud & Henk P. M. Vijverberg Research Institute of Toxicology, 1998).

Previous work has shown that agonists of nAChRs, like nicotine, can improve cognition in low-doses (Houezec, 1993, Levin, 2002, Sacco, 2004). Conversely, nAChR antagonists impair cognition (Levin, 2002). Additionally, both the α7 and β2 knockout mice show impairment in working memory (Fernandes, 2006, Hoyle, 2006, Levin, 2009). Furthermore, a genome-wide association study, also known as GWAS, shows that the β4 subunit is related to attention function in attention deficit hyperactive disorder. Chapter 3 will highlight a new class of drugs targeting nAChR called positive allosteric modulators. They are not agonists but, do
cause an increase in receptor conductance upon activation. This type of modulation may improve cognition like nAChR agonists.

One of the potential therapeutic implications for understanding the role of neuronal nAChRs in hippocampal interneurons is Alzheimer’s Disease (AD). The neurons that degenerate first in AD are cholinergic neurons that have synapses with the GABAergic interneurons of the hippocampus (Liu et al., 2009). Since the GABAergic interneurons receive signaling from the cholinergic neurons, the GABAergic neurons contain nAChRs and are the population of neurons analyzed in Chapter 1. The International Alzheimer’s Association reports that currently 5 million Americans are living with AD and the number continues to rise. More people die annually of AD than breast cancer and prostate cancer combined, making it the 6th leading cause of death in the United States. The disease impacts the individual as well as the caregivers, the family, and their communities. With years of research dedicated to heart disease and cancer, the disease prevalence of both heart disease and cancer have declined and treatment has improved. The hope is that with continued research in AD we can turn the tide of disease progression and improve treatment options as well.

One of the first signs of AD is memory loss. Unfortunately, when memory loss is first observed, AD has been present for years and has progressed significantly (Mattson, 2004). At this stage of disease, there is little that can be done to halt disease progression or improve quality of life (Corbett, Smith, & Ballard, 2012, Mattson, 2004). Of the treatment options currently available, there is little, if any improvement, for up to six months before improvement becomes negligible.

The most promising method of nAChR targeted therapy in the treatment of cognitive disorders is allosteric modulation. Allosteric modulators bind to a distinct site from the ACh
binding site, and cannot activate the receptor independently. Allosteric modulators require both the binding of the modulator and a ligand to elicit an effect. Allosteric modulation can be positive, negative, or silent. With the binding of a ligand, positive allosteric modulators increase the potentiation, or current, of the electrical signal. Negative allosteric modulators inhibit the electrical signal, but only in the presence of a ligand. Silent allosteric modulators do not potentiate or inhibit the current, but will remove the effect of a positive or negative allosteric modulator.

To further characterize nAChR subtypes, we used positive allosteric modulators to differentiate α7 and α7β2. The most widely characterized nAChR subtype is the homomeric α7. Recent evidence highlights the presence of α7β2 throughout the brain (Moretti, 2014). However, during whole cell, and even single channel recordings, it is extremely difficult to differentiate the subtypes from one another. There is no reliable method in whole cell patch clamp experiments to differentiate using current kinetic analysis or pharmacology. In Chapter 3, we characterize the different effect 3-furan-2-yl-N-p-tolyl-acrylamide (PAM-2) has on α7 and α7β2. Although there are many similar PAM-2 effects on each subtype, we do demonstrate at least 2 ways that PAM-2 can be used to differentiate the α7 from the α7β2.

In conclusion, the following experiments predict possible nAChR subtypes that may be found in hippocampal interneurons and differentiate between subtypes of the α3β2 nAChR. Additionally, PAM-2 may be a promising drug in the treatment of cognitive disorders as it greatly potentiates the conductance of α7* nAChR. Furthermore, we introduce at least 2 ways PAM-2 can be used to differentiate α7 and α7β2 nAChRs.
Figure I.1: The Cellular Components of Each Region of the Hippocampus. Specifically, the pyramidal neurons of the CA3 and CA1 regions and the interneurons of the CA1.
Figure I.2: The Cellular Layers Within the CA1 Region of the Hippocampus.
References


CHAPTER 1: Expression of nAChR mRNA in Rat Hippocampal Interneurons

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Key words: neuronal nicotinic acetylcholine receptors, hippocampus, alpha 3, beta 2, stoichiometry, single cell real-time (q-PCR), mRNA

No conflicts of interest to be reported.

TABLES, FIGURES AND LEGENDS: 4 Figures, 7 Tables (2 included in Methods section)

AUTHOR CONTRIBUTIONS: Conceived and designed experiments: RB SS. Performed the experiments: RB SS. Analyzed the data: DJ ST RB SS. Contributed reagents/materials/analysis tools: SS. Composition of the paper: DJ RB SS.
Abstract

Pyramidal cells are the most populous neurons in the hippocampus, yet they are controlled by a sparse population of inhibitory interneurons. However, the hippocampal interneurons are not well understood and are quite diverse on many fronts. Using qPCR of CA1 hippocampal interneurons, we quantified mRNA subunits expression and detailed possible combinations. We also highlighted differences found between the stratum oriens and the stratum radiatum.

We show that almost all interneurons in the CA1 of the rat hippocampus contain detectable levels of nicotinic acetylcholine receptor (nAChR) mRNA. Our research highlights the complexity of the CA1 nAChR population. Interestingly, the α3 nAChR subunit is one of the highest expressed subunit mRNAs while the α4 is one of the least likely subunits to be detected in the CA1 interneurons. Not as surprisingly, the β2 nAChR subunit is the other highest expressed mRNA. In addition, the subunit co-expression Pearson correlation values highlight key differences between the stratum oriens and the stratum radiatum. Our estimation is that there are approximately 119 different mRNA combinations that are likely in rat CA1 interneurons.

These results provide a new and valid avenue in cognitive therapies by targeting α3 containing nAChRs. Currently, the nAChR targeted cognitive therapies focus on the α4β2 nAChR and the α7 nAChR. This new insight may improve therapies for Alzheimer’s Disease, Autism Spectrum Disorder, Dementia, and Attention-Deficit Hyperactivity Disorder.
Introduction

Understanding the scope of expression of nicotinic acetylcholine receptors (nAChRs) within the interneurons of the hippocampus would lend great insight for cognitive therapies used in Alzheimer’s Disease (AD), Attention-Deficit Hyperactivity Disorder (ADHD), Autism Spectrum Disorder (ASD), and Post-Traumatic Stress Disorder (PTSD). The GABAergic interneurons are controlled by nAChRs found on their dendrites, soma, and on the axon terminal (Jones, 1997). These GABAergic neurons control the synchronous firing of the highly populated pyramidal neurons (Cobb, 2005). Therefore, modulation of the nAChRs contributes to the regulation of glutamatergic pyramidal neurons (Ji, 2000).

When considering our electrophysiological results, we suspect that the nAChR assembly is made of various combinations of eight subunits: $\alpha_2$-$\alpha_5$, $\alpha_7$, and $\beta_2$-$\beta_4$. The nAChRs in hippocampal interneurons are in a key position to modulate cognitive functions because interneurons play a major role in coordinating hippocampal activity, reviewed in Freund (1996). Accordingly, nAChRs have been implicated in diseases affecting cognition, of which Alzheimer’s disease is the most principal (Gray, 1996).

Compositional variations of nAChR subunits within the hippocampus have been shown to mediate nicotinic receptor kinetics (Sudweeks, 2000) and enable plasticity in interneuron function (Alkondon, 2001). Of the neuronal nAChR subunits, $\alpha_7$ has been studied most extensively. Use of subunit-specific antagonists to $\alpha_7$ homomeric receptors (either $\alpha$-bungarotoxin or methyllycaconitine) has provided an important tool in isolating contributions of this subunit in receptor kinetics, physiology, and disease. High-affinity binding, for example, has been shown for $\alpha_7$ to $\beta$-amyloid, a peptide implicated in Alzheimer’s disease (Wang, 2000), which binding results in the blockage of current flow through the nAChR (Pettit, 2001). There
are many functional roles (and resultant diseases or disorders) attributed to the α7 nAChR (within and outside of the hippocampus) including depression, cognition, gastric cancer, and memory (Zhang, 2016, Chen, 2015).

The paucity of other highly subunit-specific antagonists of the nAChR has impeded progress in characterizing the roles of other subunit combinations to the extent of α7. Until more subunit-specific agents are discovered, other means for characterizing the individual nAChR subunits must be utilized. On-going studies to characterize the extent of nAChR subunit co-expression within individual neurons may identify trends shared by functionally distinct networks or subpopulations of neurons (Jones, 1999). In addition, the identification of co-expression trends between nAChR subunits and other neurochemical markers may facilitate more comprehensive characterization of the nAChR and of neuronal subpopulations. Therefore, we sought to differentiate and classify hippocampal interneuron expression of neuronal nAChR subunit mRNA through single-cell qPCR.

Methods

Slice Preparation

All CA1 hippocampal slice and interneuron aspiration experiments were prepared and carried out as described previously (Sudweeks, 2000). In brief, to obtain the interneurons, coronal brain slices (either 300 or 350 μm thick) were made from 8 to 23 day old Wistar rats using a Vibratome 1000-Plus. The slices were cut in ice-cold oxygenated (95% O₂, 5% CO₂) artificial cerebrospinal fluid and placed in room-temperature oxygenated ACSF for at least 30 minutes prior to placing in microscope recording chamber. IUCAC approval was obtained for all animal experiments.
Cytoplasm Aspiration

An upright microscope with infrared light was used to individual hippocampal interneurons from the Amun’s horn (CA1) *stratum oriens* and *stratum radiatum* were visually identified as neurons outside the pyramidal cell layer. Interneurons were then aspirated into a standard whole-cell patch-clamp pipette containing 5 μL Intracellular Fluid as described previously (Sudweeks, 2000).

Electrophysiology

A whole-cell recording configuration of the interneuron was obtained in voltage-clamp mode prior to cytoplasm aspiration. Interneuron membrane potentials were held at -70mV. Where recordings were performed, responses were induced by the gravity flow application of 1 mM ACh for 1 second, using an Axon 200. Whole-cell recordings were filtered at 1 kHz, and sampled at 100 Hz, using the pCLAMP Clampex software (version 7.01.31, Axon Instruments). Acetylcholine (ACh) was applied using an electronically triggered valve (General Valve Co., Fairfield, NJ, USA) connected to a synthetic quartz tube (inner diameter of 320 μm, Polymicro Technologies Inc., Phoenix, AZ, USA) placed approximately 90—120 μm from the cell body. The flow rate for the ACh application from the gravity flow perfusion system was 250 μl min. A washout period of at least 3 min was included between subsequent recordings.

Primers and Probes

The primers and probes were designed using either Vector NTI version 7.0 (Invitrogen) or Primer Express version 2.0 (ABI Prism) software.
**RT Reaction**

A cDNA library representing each CA1 hippocampal interneuron was made by running a reverse transcription reaction using BIORAD iScript cDNA Synthesis Kit with a final volume of 10 μl.

**Multiplex Reaction and Real-time Quantitative PCR**

A multiplex PCR reaction was run (15 cycles) for each aspirated interneuron using all neuronal nAChR primers and the primer for 18s rRNA (see Table 1 and Table 2) with a final volume of 75 μl. The α6 subunit was not examined because initial experiments showed no detection of this subunit in any hippocampal interneurons examined. The multiplex reaction was run using Platinum® Taq DNA Polymerase and PCR nucleotides (10mM). A second round of PCR was run (60 cycles) for each specific target (18s, α2–α5, α7, β2, β3, β4) using an ABI 7000 Sequence Detection System utilizing BIORAD iTaq Supermix with ROX. Cycle threshold values for each target were compared to the reference gene 18s for analysis (more in Real-Time Analysis).

Standard curves (efficiency tests) for each cDNA target were developed by running 60-cycle real-time quantitative PCR assays on positive controls (rat whole-brain homogenate) for six known concentrations (100, 33.3, 10, 3.33, 1, 0.333 ng cDNA/μL). Two types of negative controls were run as well. First, we aspirated extracellular fluid and tested for the presence of nAChR mRNA. Any subunit that had a cycle threshold greater than the mRNA detected in the extracellular fluid was omitted from that interneuron’s profile. The second negative control was to simply run the quantitative PCR assay with no cellular components. This was used to detect any mRNA contaminants.
To correct the standard curves for efficiency so all mRNAs were equally efficient, upstream (primer +) and downstream (primer –) primer concentrations were adjusted to optimize amplification. The efficiency of the amplification reaction is calculated using the slope of the log(concentration) vs. CT plot. The formula for PCR efficiency $= 10^{-\frac{1}{slopec}} - 1$ (Bustin, 2004). Reaction efficiencies were run in triplicate and the amplification efficiencies were compared using an ANOVA to determine if there were significant differences between any of the primer/probe sets (18s, $\alpha_2-\alpha_5$, $\alpha_7$, $\beta_2$, $\beta_3$, $\beta_4$).

To calculate primer efficiencies, triplicate reactions of each cDNA target were averaged and a linear regression equation was calculated (SLOPE function, Microsoft Excel) of the CT values corresponding to the six known concentrations (100, 33.3, 10, 3.33, 1, 0.333 ng cDNA/μL) in the standard curve primer efficiency tests. The PCR efficiency was then determined by incorporating the slope of the linear equation using the formula described above (see Multiplex Reaction and Real-time Quantitative PCR) (Burgon 2005).

**Data and Statistical Analysis**

Following the real-time quantitative PCR on hippocampal interneurons, raw fluorescence ($\Delta Rn$) values across 60 cycles were curve-fit using a Boltzmann Sigmoidal function with an output of either 2000 or 4000 data points in the new curve using GraphPad v. 4.0 software. The second derivative graph for the curve-fit data was then determined, also using Graphpad. The cycle threshold (CT) value used was the cycle number of the maximum second derivative value of the fluorescence.

For comparison of expression levels between cDNA targets, fold expression values from the triplicate CT averages were calculated as compared to the lowest level of cDNA detection using the $2^{-\Delta\Delta Ct}$ method described by Livak (2001). Significance between relative levels of
mRNA expression was calculated by comparing mean fold expression values using a Mann-Whitney test (calculated using InStat ver. 3.05). Blinding was not used because it is not an appropriate approach to mRNA expression analysis or qPCR.

For the qPCR analysis, the proportion of neurons expressing a particular mRNA is equal to the number of observed positive neurons divided by the total number of neurons analyzed for that mRNA transcript. The standard error of the proportion (S.E.P.) was calculated for each mRNA transcript examined using the following formula (Moore, 1995):

\[
\text{Equation 1:} \quad \text{S.E.P.} = \sqrt{\frac{p(1-p)}{n}}
\]

where \( p \) = the proportion of neurons in the population with detectable expression, and \( n \) = the number of neurons in that population.

To test for significant expression of each mRNA when compared to the background qPCR calculated false positive proportion, and to compare the mRNA expression of the two populations with functional nAChR responses, we used a \( z \)-test for comparing two sample proportions. The formula is (Moore and McCabe, 1995):

\[
\text{Equation 2:} \quad z = \frac{\hat{p}_1 - \hat{p}_2}{\sqrt{\hat{p}(1-\hat{p}) \left( \frac{1}{n_1} + \frac{1}{n_2} \right)}}
\]

where \( \hat{p}_x \) is the proportion of cells with detected expression in each population, \( n_x \) is the number of neurones examined in that population, and \( \hat{p} \) (no subscript) is the pooled estimate of \( \hat{p}_1 \) and \( \hat{p}_2 \).

The calculation for \( \hat{p} \) is (Moore and McCabe, 1995):

\[
\text{Equation 3:} \quad \hat{p} = \frac{\text{Total count of successes in both samples}}{\text{Total count of observations in both samples combined}}
\]
Each $z$-value was compared to a normal curve using the Excel NORMSDIST(absolute value of $z$) function, which gives the probability ($p$) that the two proportions are equal. The proportions were considered significantly different when $p \leq 0.01$.

**Materials**

Artificial cerebrospinal fluid: 124 NaCl, 2 KCl, 1 Na$_2$PO$_4$, 26 NaHCO$_3$, 11 Glucose, 2 CaCl$_2$, 1 MgSO$_4$ (in mM): Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103 USA.

All primers, all probes, Vector NTI version 7.0, Platinum® Taq DNA Polymerase, PCR nucleotides: Invitrogen/Thermo Fisher Scientific, 168 Third Avenue, Waltham, MA USA 02451 USA.

Vibratome 1000-Plus: Pelco, Ted Pella, Inc., P.O. Box 492477, Redding, CA 96049-2477 USA.

Nikon E600-FN Microscope: A.G. Heinz, 20291 Valencia Circle, Lake Forest, CA 92630-8155 USA.

Borosilicate capillaries: Harvard Apparatus, 84 October Hill Road, Holliston, Massachusetts 01746 USA.

Intracellular Fluid: 10 MgCl$_2$, 0.1 CaCl$_2$, 1 EGTA, 10 HEPES, 135 K-Gluconate, and 2 Na-ATP (in mM): Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103 USA.

Multiclamp 700A: Axon Instruments/Molecular Devices, 1311 Orleans Drive, Sunnyvale, CA 94089 USA.

ABI 7000 Sequence Detection System and Primer Express version 2.0, ABI Prism: Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404 USA.

iScript cDNA Synthesis Kit and iTaq Supermix with ROX: BIORAD, 2000 Alfred Nobel Drive, Hercules, California 94547 USA.

GraphPad v. 4.0 and GraphPad Instat v. 3.05: GraphPad Software, Inc., 7825 Fay Avenue, Suite 230, La Jolla, CA 92037 USA.

Microsoft Excel: Microsoft Building 92, 15010 NE 36th St, WA 98052-6399 USA.

**Results**

Our results highlight the heterogeneity of nAChR mRNAs in the hippocampal interneurons as demonstrated by the variety of kinetic responses during whole-cell electrophysiology and ACh applications (Figure 1). We sought to dissect the population of nAChR subtypes by through single cell real-time-PCR (qPCR) of the *stratum oriens* and *stratum radiatum*. Of the 93 cells analyzed, 83, or 89.2%, expressed detectable nAChR mRNA. Figure 2 quantifies the number of mRNA subunits found in each cell. We found that each cell had an
average of 3.8 (± 0.22 S.E.M.) mRNA subunits detected with a slight kurtosis and skewness to the left.

The simplified summary of expression is given in Table 3 which reports the proportion of expression of the number of cells in which at least one subunit was expressed divided by the total number of cells expressing at least one nAChR mRNA subunit (n=83). Somewhat surprisingly, the α3 subunit was one of the most common subunits expressed, found in 54% of the interneurons examined. In addition, the α4 subunit had the lowest expression of the subunits analyzed (27%). Figure 3 shows the relative expression of each subunit as histograms normalized to the median expression level for all nAChR subunits detected. This result highlights the high level of expression and the larger number of cells that express the α3, β2, and α5 mRNA subunits. The α4 histogram reiterates the small number of cells expressing α4, but adds that the relative expression levels are low as well when compared to the average fold expression (Figure 3).

To extend the proportion analysis further, we calculated the most commonly co-expressed pairs of subunits. The two subunits that co-express at the highest rate are the α3 and β2 (n=69, cells expressing at least 2 subunits) (Table 4).

A Mann-Whitney test of all subunits examined showed that only the α3 had a significantly different relative expression in the stratum oriens compared to the stratum radiatum, with the stratum oriens having overall larger values of expression (p =0.0165, α3 stratum oriens, mean±SEM = 3.47±1.11, α3 stratum radiatum, mean±SEM = 1.24±0.21). The α2 subunit and α4 subunit had p-values that were not quite significant, but a larger sample size may reveal the true trend (α2 p=0.0603, stratum oriens larger; α4 p=0.0945, stratum radiatum larger).
In addition, we calculated the Pearson r values for subunit co-expression regardless of location in the stratum oriens or stratum radiatum (Figure 4.A). Our results show that all of the β mRNA subunits are highly correlated with each other. Also, the α5 and α3 fold expression levels are moderately correlated with the β2 mRNA subunit (Figure 4.A). The α5 mRNA subunit is also moderately correlated with the β4 subunit (Figure 4.A). Lastly, no other subunits appear to be correlated regarding mRNA expression levels.

We then assessed all the Pearson r values dependent on area. The stratum radiatum results show a wide range in correlation values (Figure 4.B). The range is similar to the stratum oriens; however, the stratum oriens appears to have two groupings (those not correlated and those with very strong correlations, Figure 4.B). Therefore, we tested the Pearson r correlation values for significance across the stratum oriens and stratum radiatum. Table 5 summarizes our results. We only tested values greater than 0.9 in either the stratum oriens or stratum radiatum. Surprisingly, all our tests show significance p-values (p<0.05, two-tailed).

In addition to 2-way subunit combinations, we also analyzed all 3-way, 4-way, 5-way, 6-way, 7-way, and 8-way combinations that were observed. The expected values were calculated by simply multiplying the portion of expression for each subunit in the combination (values from Table 3). As an example, the expected proportion co-expressing α2 + α3 would be 0.37 X 0.54 = 0.2, or 20%. The actual proportion of cells observed with both α2 + α3 was 0.23. Therefore, the Observed/Expected value is 1.1, very close to what would be expected. A z-test of two proportions indicated that 63 different combinations appeared more often than expected (significant if p<0.01) (Table 6).

Table 7 lists all the mRNA subunit combinations we observed, as well as their location in either the stratum oriens or stratum radiatum. The two most common combinations were
α3α4α5α7β2 and α3α5β2β3β4. The former occurred 4 times solely in the *stratum radiatum* while the later appear 2 times in the *stratum radiatum* and 2 times in the *stratum oriens*.

Our analysis revealed 59 combinations of mRNA subunit expression in the 93 cells tested. Of the 93 cells, 10 cells did not have detectable levels of expression and 42 cells had a unique combination of mRNA subunits. Using the coverage estimation calculation provided by Chao and Lee (1992), we estimated that we had 49.4% coverage.

Estimated sample coverage = 1 - (# single observations / # of total observations)

= 1 - (42/83)

= 0.494

This implies that our data collection only accounted for about one-half of all the possible mRNA subunit combinations expressed in CA1 interneurons. Chao and Lee also provide a way to calculate the estimated number of possible combinations.

= (# of combinations observed) / coverage

= 59 / 0.494

=119.4

Therefore, there are likely at least 119 different mRNA subunit combinations that could be found in the CA1 of the rat hippocampus.

Discussion

Our results are not able to completely differentiate nAChR mRNA subunit expression between the *stratum oriens* and the *stratum radiatum*. However, they do bring to light some interesting conclusions. Namely, the high rate of expression of the α3 mRNA subunit and the overall diversity with an estimated 119 different combinations of mRNA subunit expression.
We would like to add to these results and explore changes in subunit concentrations during development (Alkondon, 2007, Gahring, 2005, Rogers, 1998, Winzer-Serhan, 2005). The role of nAChRs in development may be quite diverse considering that nAChRs are found on glial cells, pyramidal cells, and neurons (Didier, 1995). Therefore, we feel it imperative that future studies should focus on understanding early development of the hippocampus. This could improve treatment of ADHD or early interventional therapies for ASD. Likewise, understanding normal aging as opposed to dementia or AD may improve targeted therapies since some nAChR subtypes have been shown to be sensitive to β-amyloid (Pettit, 2001, Wang, 2000).

Previous studies reporting quantitative mRNA expression between strata radiatum and oriens found higher proportion of interneurons expressing α2 in the stratum oriens and a higher proportion of interneurons expressing α5, β2, and β4 in the stratum radiatum (Sudweeks 2000, Khiroug, 2004). This study, however, indicated no significant difference in the detection of these subunits between the two strata (stratum radiatum: n=45 samples each ran in triplicate, stratum oriens: n=38 samples each ran in triplicate). Yet, we did find a significant difference in α3 expression between the two strata. These discrepancies may have resulted in differences in the number of PCR cycles performed. In the study mentioned above, sixty-five cycles were run on their target transcripts, while we measured amplification across seventy-five cycles of PCR. Running a higher number of amplification cycles may have afforded us a more sensitive analysis of the target mRNAs. Additionally, with the high level of diversity demonstrated in the hippocampal interneurons, it should not be surprising that different samples would highlight different results.

Considering that only the α3 mRNA subunit had statistically different expression levels in the stratum oriens and stratum radiatum we found the results in Figure 4 and Table 5 quite
surprising. We expected the correlations between regions to not be statistically different considering the expression levels were not different (except α3). This may suggest different functional roles of the stratum oriens and stratum radiatum. Figure 4.B showing more polarized groupings of the Pearson r values in the stratum oriens could suggest a more selective expression of subtypes than the stratum radiatum which shows a broader range its Pearson r values. This could indicate a difference in the functional properties of nAChRs in each strata.

One interesting result is that of those interneurons that expressed only 1 subunit the majority of those interneurons expressed the α7 mRNA. Since the α7 subunit can form a homomer those results are expected. However, there were a few interneurons with sole detection of β2, β4, or the α5 mRNA subunit. However, none of the other subunits investigated here form homomeric channels. The results are likely the result of the snapshot view of mRNA levels at any one time in the interneuron. mRNA levels likely rise and fall in response to cellular communication.

The high levels of co-expression of α3 and β2 were somewhat surprising because the α3 has only been shown to be in the medial habenula and the sympathetic ganglion (Yeh, 2001). However, our results suggest that that the α3 subunit has a significant role in hippocampal interneurons. Since the α3 and β2 subunits had the highest overall expression and the highest co-expression rate, it highlights the importance of further characterizing the α3β2 nAChR (Jackson, 2017).

We suggest that the α3β2, and even the α3β4 nAChRs, may play a more important role in the hippocampus than the α4β2 nAChRs. Our results also suggest that α3 containing cells frequently expressed α5 mRNA as well. This may suggest a likely incorporation of α5 subunits into α3 containing nAChRs. Not as surprising were the relatively abundant α7 mRNA
expression results. They appear to be fairly average in the relative expression levels and the proportion of expression. This validates the use of α7 targeted therapies. Considering that α3 is found in very few regions of the brain and α7 has a more ubiquitous expression, α3 targeted therapies may prove effective (Vernallis, 1993, Ullian, 1997, Lindstrom, 1996, Yeh, 2001).

Future studies may include reconstituting the significant combinations of nAChR subunit co-expression (Table 4) into cellular expression systems such as *Xenopus laevis* oocytes. Studies using expression systems benefit from the finer controllability of diffusion rates and drug application concentrations versus interneurons within the brain slice. Findings from electrophysiological recordings in response to pharmacological application can then be used to elucidate the characteristics and contributions of the significant nAChR subunit co-expression combinations identified in this study (Table 4). These studies would then help us understand the role of nAChRs in modulating hippocampal function. Further pursuing these results may identify networks or subpopulations in the hippocampus and throughout the CNS affected in the pathogenesis of neurodegenerative diseases and ultimately give rise to the discovery of treatments that prevent or abate these disorders. The results in this study are a gateway to the characterization of the diverse range of hippocampal nicotinic receptor subtypes.
Table 1.1: Summary of Amplicon Lengths (base pairs) and Oligo Sequences of the nAChR Subunit Probes Used in qPCR.

<table>
<thead>
<tr>
<th>Real-time PCR probes</th>
<th>Length</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>nAChR β2 rat</td>
<td>77 bp</td>
<td>CCCAGCCAAGCCCTGCACCTGAT</td>
</tr>
<tr>
<td>nAChR β3 rat</td>
<td>145 bp</td>
<td>AAGGACCCCATGGACCGCTTCT</td>
</tr>
<tr>
<td>nAChR β4 rat</td>
<td>72 bp</td>
<td>CTGGTCAGGGTCCCTCATCCCAG</td>
</tr>
<tr>
<td>nAChR α2 rat</td>
<td>103 bp</td>
<td>CTCCATGGCTCCCCGATCTGAA</td>
</tr>
<tr>
<td>nAChR α3 rat</td>
<td>68 bp</td>
<td>TTGAACCTGCTCCCAGGGTCATG</td>
</tr>
<tr>
<td>nAChR α4 rat</td>
<td>356 bp</td>
<td>TGGGTGAAGCAGGAGTGGCAGGA</td>
</tr>
<tr>
<td>nAChR α5 rat</td>
<td>77 bp</td>
<td>TGTCTTTGCCATCAAATCCACACCCACC</td>
</tr>
<tr>
<td>nAChR α7 rat</td>
<td>214 bp</td>
<td>CAAGAGCTCCTGCTACATTGACGTTGC</td>
</tr>
<tr>
<td>18s rRNA rat</td>
<td>133 bp</td>
<td>TGGAGCGATTTGTCTGGTTAATTCCGATAAC</td>
</tr>
</tbody>
</table>
Table 1.2: Summary of Oligo Sequences of the nAChR Subunit Primers (+ and -) Used in qPCR.

<table>
<thead>
<tr>
<th>Real-time PCR primers</th>
<th>+ Primer</th>
<th>- Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>nAChR β2 rat</td>
<td>CTGCGGCTGACCCATGTAC</td>
<td>TGGGCTCAGCTCGGAAAG</td>
</tr>
<tr>
<td>nAChR β3 rat</td>
<td>CCCGAGATGGCTTTGCAT</td>
<td>GGAAAGCGGACCAGAACTCTTTTC</td>
</tr>
<tr>
<td>nAChR β4 rat</td>
<td>CGTCCCGGTCTTGAAGTCA</td>
<td>CAGTATCAGCTGTGGCCAAGTG</td>
</tr>
<tr>
<td>nAChR α2 rat</td>
<td>TGCCCAGGTGGCTGATG</td>
<td>CATGTTAGTCTCTAGCCAATGG TATGA</td>
</tr>
<tr>
<td>nAChR α3 rat</td>
<td>TTGGGTCAAGGCGGTGTG</td>
<td>TCACCCTGGTCGGCCTAGT</td>
</tr>
<tr>
<td>nAChR α4 rat</td>
<td>CCAGATGATGACAACCAACG</td>
<td>CCACACGGCTATGAATGCTC</td>
</tr>
<tr>
<td>nAChR α5 rat</td>
<td>GATTTTCGTGACCCTATCCAT TATG</td>
<td>GCGTTGTGTTGGAGGAAGA</td>
</tr>
<tr>
<td>nAChR α7 rat</td>
<td>TTGCCAGTATCTCCCTCCAG</td>
<td>CTTCTCATTCCTTTTGCCAG</td>
</tr>
<tr>
<td>18s rRNA rat</td>
<td>GTGCATGGCCGGTTCTAGTT G</td>
<td>GCCACTTGTCCTCTAAGAAGTG</td>
</tr>
</tbody>
</table>
Table 1.3: The Proportion of Cells Containing Each nAChR mRNA Subunit (n=93). Ten of the 93 interneurons tested did not contain detectable levels of any nAChR mRNA subunit analyzed.

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>α3</td>
<td>0.54</td>
</tr>
<tr>
<td>β2</td>
<td>0.54</td>
</tr>
<tr>
<td>α5</td>
<td>0.43</td>
</tr>
<tr>
<td>α7</td>
<td>0.42</td>
</tr>
<tr>
<td>β4</td>
<td>0.42</td>
</tr>
<tr>
<td>β3</td>
<td>0.40</td>
</tr>
<tr>
<td>α2</td>
<td>0.37</td>
</tr>
<tr>
<td>α4</td>
<td>0.27</td>
</tr>
</tbody>
</table>
Table 1.4: The Proportion of Subunit Co-expression for the Highest 8 Subunit Pairs (n=69, the number of cells that contained at least 2 mRNA subunits).

<table>
<thead>
<tr>
<th>Percentage</th>
<th>Subunit Co-expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>52%</td>
<td>α3 and β2</td>
</tr>
<tr>
<td>49%</td>
<td>α3 and α5</td>
</tr>
<tr>
<td>44%</td>
<td>α7 and β2</td>
</tr>
<tr>
<td>42%</td>
<td>α5 and β2</td>
</tr>
<tr>
<td>41%</td>
<td>α3 and β4</td>
</tr>
<tr>
<td>41%</td>
<td>β2 and β4</td>
</tr>
<tr>
<td>39%</td>
<td>α3 and β3</td>
</tr>
<tr>
<td>38%</td>
<td>β2 and β3</td>
</tr>
</tbody>
</table>
Table 1.5: T-test for Pearson Correlation Coefficients for Subunit Co-expression in the *stratum oriens* and the *stratum radiatum*. The selected comparisons were done because at least one location (*oriens* or *radiatum*) had a Pearson correlation coefficient >0.90. All regions analyzed showed significance in at the 0.05 level using a two-tailed t-test.

<table>
<thead>
<tr>
<th>subunit</th>
<th>subunit</th>
<th>Stratum oriens</th>
<th>Stratum radiatum</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>α3</td>
<td>α5</td>
<td>0.961</td>
<td>0.361</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>α3</td>
<td>β4</td>
<td>0.987</td>
<td>0.937</td>
<td>0.00527</td>
</tr>
<tr>
<td>α5</td>
<td>β2</td>
<td>0.996</td>
<td>0.053</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>α5</td>
<td>β3</td>
<td>0.986</td>
<td>0.858</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>α5</td>
<td>β4</td>
<td>0.926</td>
<td>0.118</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>β2</td>
<td>β3</td>
<td>0.989</td>
<td>0.428</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>β2</td>
<td>β4</td>
<td>0.944</td>
<td>0.759</td>
<td>0.00829</td>
</tr>
<tr>
<td>β3</td>
<td>β4</td>
<td>0.938</td>
<td>0.565</td>
<td>0.00022</td>
</tr>
</tbody>
</table>
Table 1.6: A Summary of all mRNA Subunit Combinations Observed More Often than Would be Expected (observed/expected>1). Expected values were calculated by multiplying the proportion values from Table 3.

<table>
<thead>
<tr>
<th>Combination</th>
<th>Observed/Expected</th>
<th>Combination</th>
<th>Observed/Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>α3+α5</td>
<td>1.6</td>
<td>α4+α5+α7+β2</td>
<td>4.1</td>
</tr>
<tr>
<td>α3+α4+α7</td>
<td>2.7</td>
<td>α4+α7+β2+β3</td>
<td>4.5</td>
</tr>
<tr>
<td>α3+α5+β2</td>
<td>2.2</td>
<td>α4+α7+β2+β4</td>
<td>3.4</td>
</tr>
<tr>
<td>α3+α5+β3</td>
<td>2.3</td>
<td>α4+α7+β3+β4</td>
<td>4.0</td>
</tr>
<tr>
<td>α3+α4+β4</td>
<td>2.3</td>
<td>α4+β2+β3+β4</td>
<td>3.1</td>
</tr>
<tr>
<td>α3+β2+β3</td>
<td>2.1</td>
<td>α5+α7+β2+β3</td>
<td>3.3</td>
</tr>
<tr>
<td>α3+β2+β4</td>
<td>2.0</td>
<td>α5+α7+β2+β4</td>
<td>2.6</td>
</tr>
<tr>
<td>α3+β3+β4</td>
<td>2.4</td>
<td>α5+α7+β3+β4</td>
<td>3.2</td>
</tr>
<tr>
<td>α4+α7+β2</td>
<td>3.0</td>
<td>α5+β2+β3+β4</td>
<td>3.9</td>
</tr>
<tr>
<td>β2+β3+β4</td>
<td>2.3</td>
<td>α7+β2+β3+β4</td>
<td>3.4</td>
</tr>
<tr>
<td>α2+α3+α5+β3</td>
<td>2.9</td>
<td>α2+α3+α7+β2+β3</td>
<td>4.9</td>
</tr>
<tr>
<td>α2+α3+α7+β2</td>
<td>2.4</td>
<td>α3+α4+α5+α7+β2</td>
<td>7.7</td>
</tr>
<tr>
<td>α2+α3+β2+β3</td>
<td>2.6</td>
<td>α3+α4+α7+β2+β3</td>
<td>6.6</td>
</tr>
<tr>
<td>α2+α4+α7+β2</td>
<td>3.9</td>
<td>α3+α4+α7+β2+β4</td>
<td>6.3</td>
</tr>
<tr>
<td>α2+α7+β2+β3</td>
<td>3.0</td>
<td>α3+α4+α7+β3+β4</td>
<td>7.4</td>
</tr>
<tr>
<td>α3+α4+α5+α7</td>
<td>4.1</td>
<td>α3+α4+β2+β3+β4</td>
<td>5.8</td>
</tr>
<tr>
<td>α3+α4+α5+β2</td>
<td>3.9</td>
<td>α3+α5+α7+β2+β3</td>
<td>5.7</td>
</tr>
<tr>
<td>α3+α4+α7+β2</td>
<td>4.6</td>
<td>α3+α5+α7+β2+β4</td>
<td>4.4</td>
</tr>
<tr>
<td>α3+α4+α7+β3</td>
<td>3.6</td>
<td>α3+α5+α7+β3+β4</td>
<td>6.0</td>
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Table 1.7: A List of All Observed Subunit mRNA Combinations by Location.

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Figure 1.1: Kinetically Diverse Population of nAChRs. This figure is a representation of the diversity of responses while patching onto rat hippocampal interneurons. The arrow indicates a 1 second, 1 mM ACh application. Patches were made on the neuron cell bodies.
Figure 1.2: Count of nAChR Subunit Expression. A histogram reporting the number of nAChR mRNA subunits detected using qPCR (n=83). The average number of subunits per cell was 3.8 ± 0.22 (mean±SEM) with slight kurtosis and skewness to the left.
Figure 1.3: nAChR Subunit Relative Expression by Subunit. All nAChR mRNA subunits have variable expression levels as indicated by the histograms. Relative expression levels were binned with 1 being the mean fold expression of the cells.
Figure 1.4: Pearson r Correlations of the Average Fold Expression for 2-way Comparisons. A. Correlations of combined data from both the stratum oriens and stratum radiatum. B. Correlations of both the stratum oriens and stratum radiatum. The stratum radiatum has a greater variability in Pearson r values while the stratum oriens has Pearson r values centered near zero and a separate group with values greater than 0.90. Noted correlations listed from smallest to largest.
References


CHAPTER 2: The Human alpha 3 beta 2 Neuronal Nicotinic Acetylcholine Receptor Forms Two Distinguishable Subtypes

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Key words: neuronal nicotinic acetylcholine receptor (nAChR), alpha 3, beta 2, stoichiometry, ligand-gated ion channel, cognition, electrophysiology, desensitization, nicotine, acetylcholine (ACh), *Xenopus laevis* oocyte, dose-response curve

No conflicts of interest to be reported.
TABLES, FIGURES AND LEGENDS: 6 Figures, 1 Table
AUTHOR CONTRIBUTIONS: Conceived and design experiments: DJ SS. Performed experiments: DJ MH. Analyzed the data: DJ SS. Contributed reagents/materials/analysis tools: SS. Wrote the paper: DJ MH SS.
Abstract

The hippocampal interneuron population is diverse yet almost always contains nicotinic acetylcholine receptors (nAChRs). The nAChRs regulate the release of GABA on to glutamatergic pyramidal cells. The highest co-expressed nAChR subunits in the Amun’s horn (CA1) of the rat hippocampal interneurons are the α3 and β2 subunits yet the α3β2 subtype(s) has not been extensively characterized.

We expressed the human α3β2 nAChRs in *Xenopus laevis* oocytes to differentiate possible subtypes using two-electrode whole cell voltage clamp. We can distinguish at least two subtypes based on their acetylcholine (ACh) and nicotine affinities. Regarding acetylcholine, injecting 5X more β2 mRNA gives an EC50=12 μM±1.7, while injecting 5X more α3 mRNA gives an EC50=264±1.6 μM. Additionally, these mRNA ratios yields significant differences in rise time, half width, and ACh desensitization (p<0.05). The desensitization after 30 seconds of ACh exposure revealed the clearest kinetic differences between subtypes.

In summary, the human α3β2 neuronal nAChR subunits can form at least two different and functional subtypes that can easily be distinguished with long term ACh applications. These hippocampal subtypes could provide new drug targets for cognitive therapies for diseases such as Alzheimer’s Disease (AD), Autism Spectrum Disorder (ASD), and Attention Deficit Hyperactivity Disorder (ADHD).
Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are important in the synchronous firing of the hippocampus (Grybko, 2010, Ji, 2000). These receptors are found in the interneurons of the hippocampus (Cobb, 2005, Winzer-Serhan, 2005, Jones, 1997). Hippocampal interneurons are relatively sparse compared to pyramidal cells and glial cells, but are necessary in cognition as evidenced by the cognitive drugs that target nAChRs (Dannenberg, 2015, Bezaire, 2016). Overall, the nAChR population in the interneurons is extremely heterogeneous and not fully characterized (Sudweeks, 2000, Szabó, 2014, Jackson, 2017). Patch clamp recordings of the rat hippocampus reveal a large diversity of electrophysiological properties indicating a large diversity of receptors subtypes (Figure 1). Rates of subunit co-expression in the rat hippocampus indicate that the $\alpha_3$ and $\beta_2$ subunits are the highest co-expressed subunits within the stratum oriens and stratum radiatum of the CA1 (Amun’s horn) (Jackson, 2017). Yet, the $\alpha_3\beta_2$ nAChR subtypes have not been differentiated.

Cognitive therapeutics targeting nAChRs in the hippocampus are only mildly effective and often target the $\alpha_7$ containing nAChRs (Wallace, 2011, Aracava, 2005, Martin, 2004, Leiser, 2009, Soderman, 2011, Thomsen, 2010). Other nAChR-targeted therapies often target the $\alpha_4\beta_2$ nAChR (Rode, 2012, Timmerman, 2012, Sater, 2009). Both the $\alpha_7$ homomer and $\alpha_4\beta_2$ heteromer are found throughout the brain. However, the $\alpha_4$ subunit is expressed at relatively low levels among the CA1 hippocampal interneurons and may be one reason why cognitive therapies targeting the $\alpha_4\beta_2$ are only mildly effective (Jackson, 2017). The $\alpha_7$ is often found pre-synaptically and helps regulate neurotransmitter release (Liu, 2003). Due to the high co-expression level of $\alpha_3\beta_2$ and possible different location within the neuron we feel that $\alpha_3\beta_2$ subtypes should be fully investigated as possible drug targets. The $\alpha_3\beta_2$ nAChR has been
somewhat characterized (Chavez-Noriega, 1997, Wang, 1998, Chavez-Noriega, 2000, Wu, 2008, Wang, 2015), but the distinction between two different α3β2 subtypes has not been explored. Like the α4β2 and the α2β2 nAChRs, we believe that there are at least 2 stoichiometries of the α3β2 nAChR (Papke, 1989, Ussing, 2013, Zwart, 1998, Khiroug, 2004, Dash, 2014). We are excited about the possible therapeutic targets that α3β2 nAChRs could serve as for various diseases affecting the hippocampus such as Alzheimer’s disease (AD), autism spectrum disorder (ASD), ethanol and/or nicotine addiction, and attention deficit hyperactivity disorder (ADHD) (Kamens, 2009). Therefore, we sought to characterize the kinetic properties of the α3β2 nAChRs, and differentiate possible subtypes.

Methods

Plasmids containing human α3 (hα3) and human β2 (hβ2) genes were transformed into One Shot® E. coli chemically competent cells, and plasmid DNA was then isolated and purified using the HiSpeed® plasmid purification kit. Plasmids containing the hα3 and hβ2 genes were linearized by restriction digest with SacI. The mRNA was then transcribed, capped on the 5’ end, a poly(A) tail was added (optional), and LiCL purification was performed using the mMMessage mMMachine® T7 Ultra Kit according to the protocol provided. RNA was re-suspended in Tris-EDTA Buffer, mixed in various ratios, aliquoted, and stored at -20°C. For some nAChR subtypes, the poly(A) tail appears to increase expression yield. However, we had similar results with or without the poly(A) tail addition.

Ratios of hα3 (1.0 µg/µL) and β2 (1.0 µg/µL) AChR subunit mRNAs were injected into Xenopus laevis oocytes. Naive Xenopus laevis oocytes are not responsive to ACh, but when injected with the appropriate mRNA they are also able to express a high yield of nAChR protein. The ratios of α3:β2 1:5 and α3:β2 5:1 were used to increase the likelihood of expression of each
probable stoichiometry (α3β2 and α3β2 respectively) (Nelson, 2003, Moroni, 2006). Each oocyte was injected with 50.6 nL of mRNA for a total of 50.6 ng of mRNA per oocyte. The injection needles and recording needles were made of borosilicate glass. Following injection, the oocytes were stored in Ringer’s solution (OR-2-Ca²⁺-pen-strep) at 17-19°C for 6-9 days on a rocker (pH~7.5). This solution consists of (in mM unless noted): 82 NaCl, 2.5 KCl, 1 Na₂HPO₄, 5 HEPES, 1 CaCl₂, 1 MgCl₂, 0.5 theophylline, 100,000 units penicillin, and 10 mg streptomycin. After 9 days any oocytes not recorded on were stored at 4°C to stop further maturation of the cell until the oocyte was used for recordings.

Recordings of the cell’s electrical activity were obtained through two-electrode voltage clamp. Traces were recorded using Clampex 9.2 software and analyzed on ClampFit 9.2. Various concentrations of Acetylcholine (ACh) and Nicotine (Nic) were suspended in OR-2 solution and were perfused (1 psi) over the oocyte every 90 seconds and the cell’s responses were recorded (ACh concentrations between 100 nM and 100 mM; Nic concentrations between 1 mM and 1.5 M). Except for the desensitization tests, ACh was perfused for 3 seconds every 90 seconds and nicotine was perfused for 1 sec every 90 seconds. To measure the degree of desensitization, the EC₈₅ (effective concentration 85% of maximum response) for each subtype was applied for 60 seconds with a 2 minute wash between applications (replicates of 3). We did not blind our experiments since the protocols for ACh and nicotine were different. In order to analyze the response, it was necessary to know the ligand and the exact concentrations applied.

For voltage clamp recordings, the oocytes were clamped at -60 mV while solutions were perfused at 17 ml·min⁻¹. The IV plot was generated by clamping the oocyte at -90, -75, -60, -45, -30, -15, 0 (all in mV), and then we performed the same procedures as the voltage clamp recordings. All ligands were dissolved in OR-2-Ca²⁺ without theophylline, streptomycin, and
penicillin. Solutions were perfused using an 8-valve (pinch), computer controlled, pressurized perfusion system. Oocytes were impaled with microelectrodes filled with 3 M KCl and a resistance between 0.1-2 MΩ. Clampex 9.2 was used to run the electrophysiology protocols through a GeneClamp 500B amplifier and Digidata 1322A digitizer. Data was sampled at 5 kHz and filtered at 2 kHz. Peak amplitudes ranged from 3 nA to 200 nA. The range in peaks was dependent on each oocytes protein expression level as well as the concentration of acetylcholine used to generate the response.

All recordings were normalized to the maximums ($E_{\text{MAX}}$) for each subtype. This was necessary since each oocyte expressed a different number of channels based on health, efficiency, and incubation time. Upon collection of the recordings, we used Clampfit to analyze multiple parameters for each recording including rise time, half-width (width of the peak at 50% of the peak amplitude), and desensitization. For our analysis, we compared the 1:5 and 5:1 results at the $E_{\text{C50}}$ (10 µM, 333 µM respectively) and the $E_{\text{C85}}$ (333 µM, 10 mM respectively). However, for desensitization we only compared the $E_{\text{C85}}$. Analysis of the steady state currents were compared at 30 seconds during the 60 second ACh application.

Data and Statistical Analysis

Analysis of previously collected qPCR data (Jackson, 2017) was extended to determine if there was more than one stoichiometry of the hα3 and the hβ2 nAChR. Microsoft Excel (2013) was used to analyze the data for all figures except Figure 2 and Figure 4. Specifically, the “=AVERAGE()” and “=STDEV()/(SQRT(COUNT()))” were used to calculate the mean and standard error of the mean. These values were then used to generate Figure 1 using “Insert Bar Graph” (Excel, 2013). Likewise, Figure 5A-B and Figure 6 were generated using the same calculations and tools. Also, the approximate $E_{\text{C50}}$ and approximate $E_{\text{C85}}$ (effective
concentration 85% of maximum response) were used as the points of comparison for the kinetic parameters (rise time and half-width). The data points for Figure 3 were also generated using the formula for mean and standard error of the mean. However, a scatterplot fitted with a linear trendline is more appropriate to present this data than a bar graph. Only the first 5 points of Figure 3 were fitted for the trendline because the data shows a strong inwardly rectifying channel. Fitting the line linearly better estimates the reversal potential of an inwardly rectifying channel. Figure 4 was generated using GraphPad Prism v. 4. The data was fit using the “sigmoidal dose-response (variable slope)” tool. The hill slopes, EC50 values, and R2 values, as well as their respective standard errors, were provided in the curve fit analysis. Grubb’s outlier tests were used to for all data sets (alpha=0.05). For statistical tests, p<0.05 is used as the level to determine significance (*). We report all means and standard errors of the mean as $\bar{x} \pm $SEM. GraphPad InStat v. 3 was used to calculate all ANOVAs. ANOVAs were calculated as “ordinary” and assumed to be Gaussian distributions. Tukey post-hoc tests were performed only if the p<0.05. Figure 2.A and 2.B are example traces that were collected using Clampex 9.2 and analyzed with Clampfit 9.2 (Axon Instruments). Screen shots were taken and then cropped in Paint (Windows 7). Figure 2.C and 2.D were generated using Adobe Illustrator CC (2017). Also, since each oocyte varies in the number of nAChR proteins it expresses it is necessary to normalize the data for comparison.

Materials

Plasmids: hα3 (Origene# SC126406), hβ2 (Origene# SC309051), pCMV6-XL4 plasmid: OriGene Technologies Inc., 9620 Medical Center Drive, Suite 200, Rockville, MD, USA, 20850. One Shot® E. coli chemically competent cells (Invitrogen), mMessage mMACHINE® T7 Ultra Kit: Thermo-Fischer Scientific 168 Third Avenue, Waltham, MA, USA, 02451. HiSPEED® plasmid purification kit: Qiagen, 19300 Germantown Road, Germantown, MD, USA, 20874.
SacI restriction enzyme: New England Biolabs, 240 Country Road, Ipswich, MD, USA, 01938-2723.

Tris-EDTA Buffer: BioExpress Corporation, 420 N Kays Dr, Kaysville, UT, USA 84037. Defolliculated *Xenopus laevis* oocytes. Animal husbandry and surgery using anesthesia and analgesia were performed by Ecocyte BioScience. 111 Ramble Ln #109, Austin, TX, USA, 78745.

Oocyte injection, Nanoject II microinjector: Drummond Scientific Company, 500 East Park Way, Broomall, PA, USA 19008.

Injection (1.12 mm OD x 0.51 mm ID) and recording (1.5 mm OD x 1.17 mm ID) capillary tubes: Harvard Apparatus, 84 October Hill Road, Holliston, MA, USA, 01746.

Needles were pulled using Model P-97 puller: Sutter Instrument Company, 1 Digital Drive, Novato, CA, USA, 94949.

Data acquisition software for electrophysiological recordings (Clampex 9.2) and recording analysis (Clampfit 9.2). GeneClamp 500B amplifier. Digidata 1322A digitizer. Axon Instruments, Molecular Devices, 1311 Orleans Drive, Sunnyvale, CA, USA, 94089.

Reagents for OR-2 Ca²⁺ solution: NaCl, KCl, Na₂HPO₄, HEPES, CaCl₂, MgCl₂, theophylline, penicillin, streptomycin; Acetylcholine chloride, Nicotine tartrate: Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO, USA, 63103.

Pinch valve perfusion system (product #s 13-pp-54, and 09-08). Automate Scientific Inc., 3271 Adeline Street, Unit B, Berkeley, CA, USA, 94703.

Microsoft Excel: Microsoft Building 92, 15010 NE 36th St, WA 98052-6399 USA.

Results

The qPCR data previously collected indicated that the α3 and β2 nAChR subunits are the highest co-expressed subunits in the rat CA1 hippocampal interneurons (specifically, the *stratum oriens* and *stratum radiatum*) (Jackson, 2017). Further analysis revealed that the mRNA is expressed in a 1:1 (α3:β2) ratio. However, no individual interneuron expressed a 1:1 ratio. Instead, we found that about half had greater α3 expression, while the other half had greater β2 expression. The two populations showed either a 3:1 ratio or a 1:3 of α3:β2 mRNA on average (Figure 1).

Injection of hα3 and hβ2 mRNA into *Xenopus laevis* oocytes at a 1:5 and 5:1 revealed two functional receptor subtypes. Both subtypes responded to ACh application; however,
extended ACh applications at their respective EC₈₅ revealed distinct kinetic differences (Figure 2).

The IV plot shows a similar reversal potential between the subtypes suggesting that there is no difference in ion selectivity. Also, the reversal potential suggests that, like other nAChR subtypes, the channels are permeable to both Na⁺ and K⁺ (Figure 3). The IV plot also shows that both subtypes are strong inwardly rectifying channels.

The dose-response curves (Figure 4) reveal a statistical difference in the ACh and nicotine affinities when comparing subtypes (ANOVA p<0.0001, p<0.0001 respectively, Table 1). The 1:5 injected ratio has greater affinity for ACh and nicotine than the 5:1 injected group. We also show that both subtypes have a lesser affinity for nicotine (Figure 4.B). The 1:5 injected ratio, like ACh, has a greater affinity for the ligand (Table 1). However, Figure 4.B reveals a bi-phasic curve for the 5:1 injected oocytes. Since, the dose-response curves for nicotine are more separated than the dose-response curves for ACh it is easier to fit the biphasic model.

In addition, we used t-tests to compare the peak amplitude for each injection ratio. When comparing the EC₈₅ and the EC₅₀ values on the ACh dose-response curve, the peak amplitudes for the 5:1 are statistically different at each (p<0.0001, p<0.001) with the 5:1 injections resulting in larger currents. Likewise, we compared the EMAX of nicotine (333 M for 1:5, 1 M for 5:1) and found a significant difference in the relative peak sizes as well. Like ACh, the 5:1 injection ratio resulted in larger peak amplitudes.

Our results of the kinetic properties show that when applying ACh for only 3 seconds, the two subtypes are more similar than different. However, there are two parameters that are statistically significant: the half-widths and rise-times (Figure 5). Yet, the results differ
depending on concentration. Figure 6 reveals the most distinguishable characteristic: with extended 60 second ACh applications, the two subtypes show marked differences in desensitization. The 1:5 injected oocytes are significantly more desensitized at their steady state than the 5:1 injected oocytes. After 30 seconds only 12.6±1.4% remained for the 1:5 oocytes while 81.6±2.1% (̄x±SEM) of the original peak amplitude remained for the 5:1 oocytes (Figure 6).

Discussion

The data suggests that there are at least two likely stoichiometries for the α3β2 nAChR. The nicotine dose-response curves appear to be a mixture of α3β2 subtypes. The 1:5 nicotine dose-response curve does not show a biphasic curve because it only represents the lower nicotine concentrations where certain subtypes may not be activated. The biphasic may not represent all of the possible stoichiometries, but the two most likely based on α4β2 and α2β2 stoichiometries (Covernton, 2000, Houlihan, 2001, Bussion, 2001, Exley, 2006). The ACh dose-response curve for the 1:5 injected oocytes, like the nicotine curve, appears best fit with a monophasic sigmoidal dose-response curve. However, even though the 5:1 injected oocytes also fit best with a monophasic sigmoidal dose-response curve, there are a few points on the curve that would suggest multiple contributing subtypes. However, the ACh dose-response curve are two close to distinguish considering. The 5:1 injection would increase the likelihood of α3(3)β2(2) subtypes expression over the α3(2)β2(3) subtype expression. In addition, our results indicate that the α3(2)β2(3) is either less efficient in formation resulting in overall smaller peak amplitudes, or has lower efficacy. To determine if efficiency or efficacy or both are contributing to peak amplitude size, single channel recordings would be necessary.
One explanation for the increase in peak amplitude with the 5:1 injected oocytes is that there is another possible binding site at the α3/α3 junction. Like the α4β2 nAChR, when more α4 is present it has been suggested than an additional binding site is found at the additional α4/α4 junction (Moroni, 2006). This may also be true of the α3β2 nAChR. However, this is simply one explanation. The α3(3)β2(2) may simply be a more favored protein expression. Since, the nH are similar, the different stoichiometries may have the same likelihood for channels opening. A larger nH could indicate greater cooperativity of the binding sites. Yet, by simply having more binding sites, this may increase the likelihood of binding without a change in the cooperativity of binding.

Regarding nicotine, our EC₅₀ values are larger than those previously reported (Wang, 1998), but previous studies do not distinguish between α3β2 subtypes. However, Wang et al. used nAChRs transfected in HEK cells whereas we used *Xenopus laevis* oocytes. We hope to follow up our research using HEK cells as well to confirm our findings regarding both injection ratios.

When considering how other nAChR subtypes assemble and the qPCR data from Jackson (2017), the data suggests that there are two likely stoichiometries: the α3(2)β2(3) (from a 1:5 mRNA ratio) and the α3(3)β2(2) (from a 5:1 mRNA ratio) (Papke, 1989, Zwart, 1998, Nelson, 2003, Khiroug, 2004). The injection ratios will likely not produce a homogenous population of one stoichiometry, but our results indicate that there are enough of each stoichiometry to distinguish their kinetic properties even though the nicotinic curve is biphasic (Figure 4.B). It has been shown previously that sometimes a low, or broad, nH (hill slope) may indicate multiple contributing stoichiometries, but considering the error bars and the fit of our curves, we are
likely getting very little contribution from the less likely stoichiometries of \((\alpha 3)_1(\beta 2)_4\) and \((\alpha 3)_4(\beta 2)_1\).

The most noteworthy difference between the two injected subgroups is desensitization with prolonged ACh application. The 1:5 injected oocytes are much more easily desensitized than the 5:1 injected oocytes. However, contrary to the \(\alpha 4\beta 2\) nAChR published results, the desensitization of ACh on the \(\alpha 4\beta 2\) nAChR also increases with more \(\beta 2\) subunit expression (Zwart 1998, Nelson, 2003, Carbone, 2009). Like the \(\alpha 4\beta 2\) and \(\alpha 2\beta 2\) ACh dose response curve increasing the number of \(\beta 2\) subunits in the subtype also increases the ACh affinity and shifts the dose-response curve left (Papke, 1989, Zwart, 1998, Nelson, 2003, Khirog, 2004).

The \(\beta 2\) nAChR subunit has been shown to be widely expressed throughout much of the rat brain (Hill, 1993). However, expression of the \(\alpha 3\) subunit is much more restricted, being previously identified in the sympathetic ganglion, medial habenula, and the autonomic ganglion (Vernallis, 1993, Ullian, 1997, Lindstrom, 1996, Yeh, 2001). They have also been identified outside of the nervous system in the adrenal gland (Campos-Caro, 1997), thymus (Mihovilovic, 1993), respiratory epithelial cells (Zia, 1997), and keratinocytes (Grando, 1995, 1996). With the identification of the \(\alpha 3\) subunit being highly expressed in hippocampal interneurons (Jackson 2017), the characterization of the \(\alpha 3\beta 2\) nAChRs is beneficial. We can now add it to the list of nAChR subtypes that should be screened for cognitive drug development.

Current nAChR therapies target either the \(\alpha 7\) or the \(\alpha 4\beta 2\) subtypes (Sarter, 2009). Also since the \(\alpha 3\beta 2\) appears to be more restricted to hippocampal interneurons, drugs targeting the \(\alpha 3\beta 2\) may be more selective than current therapies. Likewise, drugs targeting the \(\alpha 3\beta 2\) subtype may alter the hippocampal firing to a different degree, or in a different manner current therapies. There is even the possibility of combining \(\alpha 3\beta 2\) targeted therapies with current therapies in cases
where such would be beneficial. Many cognitive diseases like AD and ASD currently have few effective treatment options. Therefore, characterization of a new protein target in the hippocampus may widen the possible therapeutics and give further insight into disease development.

In conclusion, multiple \( \alpha_3\beta_2 \) nAChR subtypes have been identified. Although similar in many respects they do have distinguishable properties. Both subtypes are valid novel targets for cognitive therapies and further research may yield great implications.

Supporting Information

Controls were done to ensure that the h\( \alpha_3 \) nor the h\( \beta_2 \) mRNA are not able to form a functional nAChR by themselves. Following the methods previously outlined, we injected solely h\( \alpha_3 \) mRNA or h\( \beta_2 \) mRNA, waited 7 days and then performed electrophysiological recordings with ACh. The recordings were under the same conditions and restraints as all other recordings. We found no evidence that the h\( \alpha_3 \) or the h\( \beta_2 \) forms a functional homomeric receptor.
Table 2.1: Summary of ACh and Nic Dose-Response Curves.

<table>
<thead>
<tr>
<th></th>
<th>1:5 ACh</th>
<th>5:1 ACh</th>
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<tbody>
<tr>
<td><strong>EC-50 (µM)</strong></td>
<td>12±1.7</td>
<td>264±1.6</td>
</tr>
<tr>
<td>Hill Slope</td>
<td>0.48±0.13</td>
<td>0.55±0.15</td>
</tr>
<tr>
<td>R²</td>
<td>0.74</td>
<td>0.77</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p&lt;0.0001</td>
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<table>
<thead>
<tr>
<th></th>
<th>1:5 Nic</th>
<th>5:1 Nic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EC-50 (mM)</strong></td>
<td>25±1.5</td>
<td>21±1.6</td>
</tr>
<tr>
<td>Hill Slope</td>
<td>1.0±0.46</td>
<td>1.4±0.90</td>
</tr>
<tr>
<td>EC-50₂ (mM)</td>
<td>N/A</td>
<td>415±7E18</td>
</tr>
<tr>
<td>Hill Slope₂</td>
<td>N/A</td>
<td>15±1680</td>
</tr>
<tr>
<td>R²</td>
<td>0.67</td>
<td>0.57</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p&lt;0.0001</td>
<td></td>
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</table>
Figure 2.1: Ratio of α3 and β2 Expression in Hippocampal Interneurons. The most commonly co-expressed subunits, the α3 and β2, were analyzed further to identify two populations. (A) 56% of the interneurons tested in Jackson (2017) expressed in a 1:3 α3:β2 ratio. (b) 44% of the interneurons tested in Jackson (2017) expressed in a 3:1 α3:β2 ratio. Of the 93 cells analyzed, 34 co-expressed α3 and β2. Therefore, n=19 for 1.A and n=15 for 1.B (total n=34).
Figure 2.2: Sample Traces and Likely Stoichiometries. Injection of hα3 and hβ2 mRNA into *Xenopus laevis* oocytes at a (A) 1:5 and (B) 5:1 ratio formed functional and distinguishable receptors. The respective oocytes were perfused with 333 µM and 10 mM ACh for 60 seconds to characterize desensitization. The likely stoichiometries are shown respectively.
Figure 2.3: IV Plot. 1:5 injected oocytes fit a linear trendline of $y = 0.0356x + 1.033$ ($R^2 = 0.9892$) ($n=5$) and fit a linear trendline for the 5:1 injected oocytes $y = 0.0537x + 1.7478$ ($R^2 = 0.9362$) ($n=8$) when fitting points between -90 mV and -30 mV. The reversal potential for both is approximately -30 mV.
Figure 2.4: Dose-Response Curves. A. ACh Dose-Response. 1:5) EC50=12.2±1.7 μM, \(n_H=0.49±0.13\) \((R^2=0.74)\) (n=14 oocytes, replicates of 4, 1 individual data point identified as an outlier and removed). 1:5) EC50=263.8±1.6 μM, \(n_H=0.55±0.15\) \((R^2=0.77)\) (n=12, replicates of 4, 2 individual data points outliers). The 1:5 injected ratio has a minimum response at \(~100\) nM and an \(E_{\text{max}}\) at \(~1000\) μM ACh. The 5:1 injected ratio has a minimum response at \(~10\) μM and an \(E_{\text{max}}\) at \(~33\) mM ACh (ANOVA, \(F_{[15, 258]}=54.644\)).

A. Nicotine Dose-Response. 1:5) EC50=25.3±1.5 mM, \(n_H=1.04±0.46\) \((R^2=0.6705)\) (n=6 oocytes, replicates of 3, but not at all concentrations were used for each oocyte, 1 individual data point was identified as an outlier and removed.) (ANOVA, \(p<0.0001, F_{[12, 238]}=30.396\)) 5:1) EC50,1=21.2±1.6 mM, \(n_H,1=1.41±0.91\), EC50,2=414±7E18 mM, \(n_H,2=14.85±1680\) \((R^2=0.57)\) (n=11 oocytes, replicates of 3, but not at all concentrations were used for each oocyte, 3 individual data points were identified as outliers and removed.)
Figure 2.5: Comparison of Rise Time and Half-Width of ACh Dose-Response Curve.
Comparisons were made at the EC$_{50}$ (10 µM, 333 µM) and the EC$_{85}$ (333 µM, 10 mM) for the
1:5 and 5:1 mRNA injected ratios respectively. A. Comparison of 10% to 90% rise time. 1:5) 10
µM ($\bar{x}$=mean±SEM) $\bar{x}$=1166±237 ms (n=6, replicates of 3), 333 µM $\bar{x}$=169±9 ms (n =21,
replicates of 4, 3 replicate outliers removed). 5:1) 333 µM $\bar{x}$=821±123 ms (n=13, replicates of 4),
10 mM $\bar{x}$=1424±171 ms (n=25, replicates of 4, 3 replicate outliers removed). B. Comparison of
half-width. 1:5) 10 µM $\bar{x}$=3084±130 ms (n=5, replicates of 3, 3 replicate outliers removed), 333
µM $\bar{x}$=3522±654 ms (n=11, replicates of 4). 5:1) 333 µM $\bar{x}$=3988±163 ms (n=13, replicates of 4,
2 replicate outliers removed), 10 mM $\bar{x}$=3003±297 ms (n=21, replicates of 4).
Figure 2.6: Desensitization. Comparisons were made after 30 seconds of continuous ACh application at the EC$_{85}$. The 1:5 injected oocytes (n=7, replicates of 3, 1 replicate outlier removed) were much more desensitized with very little of the original peak remaining while the 5:1 injected oocytes (n=3, replicates of 3, 1 replicate outlier removed) was only desensitized minimally with much of the original peak remaining at 30 s. A t-test was used to test for significance.
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(α4)2(β2)3 and (α4)3(β2)2 nicotinic acetylcholine receptors: subunit arrangement

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CHAPTER 3: The Highly Selective Positive Allosteric Modulator
3-furan-2-yl-N-p-tolyl-acrylamide Potentiates α7 and α7β2 Nicotinic Receptors With Different Efficacy


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Key words: 3-furan-2-yl-N-p-tolyl-acrylamide, PAM-2, positive allosteric modulator, nicotinic acetylcholine receptor (AChR), acetylcholine (ACh), alpha 7, beta 2, homomeric, heteromeric, dose-response, molecular modeling, GABA

No conflicts of interest to be reported.

TABLES, FIGURES AND LEGENDS: 7 Figures, 4 Tables
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Abstract

The activity of 3-furan-2-yl-N-p-tolyl-acrylamide (PAM-2) was compared among human (h) $\alpha_7$ and $\alpha_7\beta_2$ nicotinic acetylcholine receptors (AChRs), heteromeric $\alpha_1\beta_2\gamma_2$ and homomeric $\rho_1$ GABA$_A$ receptor subtypes (GABA$_A$Rs), and homomeric $\alpha_1$ glycine receptors (GlyRs) by electrophysiological methods. The patch-clamp results indicated that although PAM-2 did not affect GlyRs and slightly decreased the activity of $\rho_1$ GABA$_A$Rs (i.e., 300 µM PAM-2 decreased the activity by 30 ± 1%), it moderately enhanced the activity of $\alpha_1\beta_2\gamma_2$ GABA$_A$Rs ($EC_{50} = 56 \pm 7 \mu M$; $E_{\text{max}} = 131 \pm 4\%$) compared to control (100%). These results confirmed the high selectivity of PAM-2 for $\alpha_7$ AChRs. The voltage-clamp results indicated that PAM-2 potentiates $\alpha_7$ AChRs mainly by increasing current intensity ($EC_{50} = 141 \pm 1.74 \mu M$; $E_{\text{max}} = 230\%$) with higher efficacy than that observed in $\alpha_7\beta_2$ AChRs ($EC_{50} = 186 \pm 1.35 \mu M$; $E_{\text{max}} = 190\%$). The molecular docking and dynamics results show that PAM-2 interacts differently between the $\alpha_7$ and $\alpha_7\beta_2$ models. More specifically, PAM-2 interacts with the archetypic intrasubunit site as well as with the ECD-TMD junction in the $\alpha_7$ model, whereas it interacts with different ECD-TMD sites in the $h(\alpha_7)_{2}(\beta_2)_{3}$ and $h(\alpha_7)_{3}(\beta_2)_{2}$ stoichiometries. The combination of functional and structural results suggest that the potentiating effect elicited by PAM-2 is determined by its interaction with the intrasubunit site, whereas its activity is mediated by its interaction with junctional sites.
Introduction

Positive allosteric modulators (PAMs) with high selectivity for α7 nicotinic acetylcholine receptors (AChRs) have generated a lot of expectation since these compounds might be used for the treatments of different neurological disorders producing fewer side effects than agonists (reviewed in Arias, 2010; Chatzidaki and Millar, 2015). Among them, PAM-2 (3-furan-2-yl-N-p-tolyl-acrylamide) enhances agonist-induced α7 AChR activity in a temperature-sensitive manner and reactivates desensitized α7 AChRs supporting a type II PAM classification, with certain resemblance to type I PAMs when studied at the single-channel level (Arias et al., 2011; Targowska-Duda et al., 2014; Andersen et al., 2016). In conclusion, PAM-2 has pharmacological properties that are in between of type I and type II PAMs, which makes it a distinct α7 PAM.

Previous functional studies demonstrated the PAM-2 has positive allosteric modulatory activity on α7 AChRs, whereas it inhibits human (h)α3β4, hα4β2, hα4β4 AChRs, slightly inhibits N-methyl-D-aspartate (NMDA)-sensitive glutamate receptors (GluRs), slightly potentiates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-sensitive GluRs, and affects neither kainate-sensitive GluRs, serotonin type 3 receptors, Na_1.2 and K_3.1 voltage-gated ion channels, acetylcholinesterase, nor β-amyloid content, indicating that this ligand presents high receptor selectivity (Arias et al., 2011; 2015a; 2016; Andersen et al., 2016). Nevertheless, the activity of PAM-2 between homomeric α7 and heteromeric α7β2 AChRs has not been studied yet. Interestingly, functional α7β2 AChRs have been characterized in different neuronal types, including hippocampal GABAergic interneurons (Khiroug et al., 2002, Liu et al., 2012; Wu et al., 2016), and forebrain cholinergic neurons (Liu et al., 2009). Since inhibition of hippocampal α7β2 AChRs by β-amyloid might be significant for the observed memory and learning disabilities found in Alzheimer’s disease patients (Liu et al., 2012), the modulatory activity
elicited by PAM-2 might alleviate these cognitive problems. To start delineating this supposition, we initiated this study by comparing the activity of PAM-2 between hα7 and hα7β2 AChRs expressed on *Xenopus laevis* oocytes by whole-cell voltage-clamp recordings.

Glycine receptors (GlyRs) and γ-aminobutyric acid type A (GABA\textsubscript{A}Rs), two major inhibitory receptor members from the Cys-loop ligand-gated ion channel superfamily where AChRs also belong (reviewed in Arias, 2011), are important for learning and memory processes (reviewed in Xu and Gong, 2010). GABA\textsubscript{A}Rs are potentiayed by structurally different compounds, including neurosteroids, benzodiazepines, and barbiturates, and the major adult isoform, α1β2γ2 GABA\textsubscript{A}R, is expressed in brain areas related to cognitive function such as cerebral cortex and hippocampus (reviewed in Sigel and Steinmann, 2012). In addition, GlyRs are potential targets for analgesic and anti-inflammatory drugs, and α1 containing GlyRs are selectively potentiated by ginkgolic acids which are the active components for the antidepressant activity found in *Ginkgo biloba* lipophilic extracts (Maleeva et al., 2015, and references therein). This evidence supports the notion that these receptor subtypes could be modulated by other PAMs. To determine whether the observed procognitive (Potasiewicz et al., 2015; 2017), promnesic (Targowska-Duda et al., 2016), antidepressant-like (Targowska-Duda et al., 2014; Arias et al., 2015), anti-nociceptive and anti-inflammatory (Bagdas et al., 2015) effects elicited by PAM-2 are related to these receptor subtypes, the activity of this ligand was determined on heteromeric hα1β2γ2 and homomeric hρ1 GABA\textsubscript{A}R subtypes as well as on homomeric hα1 GlyRs by whole-cell patch-clamp techniques.

Considering that the PAM-2 structure has an amide link susceptible to the metabolic activity of amidases from the liver and/or brain (Pop, 1997), the activity of the potential metabolites p-toluidine and 3-(2-furyl)acrylic acid were also tested on the hα7 AChR. Since N,N′-
dicyclohexylurea was found as a byproduct in the synthesis of PAM-2, the PAM activity of this compound was also tested on the hα7 AChR.

Previous molecular docking and molecular dynamics experiments using the Torpedo AChR as the template for the construction of the hα7 AChR, demonstrated that the potential active sites for PAM-2 are localized in the transmembrane domain (TMD) (Arias et al., 2016). More specifically, PAM-2 interacted with the intrasubunit site already characterized for PNU-120596, the archetype of type II PAMs, and also with an intersubunit site located between two α7-TMDs that had not been previously characterized and might be important to define the pharmacological differences observed between PNU-120596 and PAM-2 and other type I PAMs (Andersen et al., 2016). By comparing the structural components of the docking sites between the homomeric hα7 and heteromeric hα7β2 AChRs, we demonstrated that PAM-2 binds to the archetypical intrasubunit site in the hα7 AChR as well as to an intersubunit site in the h(α7)2(β2)3 model. Interestingly, this structural difference correlates with the functional results indicating that PAM-2 potentiates hα7 AChRs mainly by increasing current intensity with higher efficacy than that at hα7β2 AChRs.

Materials and Methods

Materials

Glycine (Gly), γ-aminobutyric acid (GABA), penicillin (5000 U/mL)-streptomycin (5 mg/mL), acetylcholine chloride (ACh), fetal bovine serum (FBS), poly-L-lysine hydrobromide, and Dulbecco’s Modified Eagle Medium (DMEM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). PAM-2 was synthesized as described previously (Arias et al., 2011). Xenopus laevis oocytes were purchased from Ecocyte Bioscience (Austin, TX, USA). Plasmids containing
the hα7 and hβ2 subunit genes were purchased from Origene Technologies (Rockville, MD, USA). One Shot® E. coli chemically competent cells were obtained from Invitrogen (Carlsbad, CA, USA). HiSpeed® plasmid purification kit was obtained from QIAGEN Inc. (Valencia, CA, USA). TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) was purchased from Bioexpress (Layton, UT, USA). PolyJet™ DNA In Vitro transfection reagent was obtained from SignaGen Laboratories (Rockville, MD, USA). Trans-IT 293 transfection reagent was purchased from Mirus, Bio LLC (Madison, WI, USA). Salts, solvents, and reagents were purchased from commercial suppliers and used as received.

Voltage-Clamp Recording on Xenopus laevis Oocytes Expressing hα7 or hα7β2 AChRs

The hα7 (Origene# SC124074-20) and hβ2 (Origene# SC309051) mRNAs were prepared using the respective subunit gene in the pCMV6-XL4 plasmid. The plasmids were transformed into One Shot® E. coli chemically competent cells, and then isolated and purified using the HiSpeed® plasmid purification kit. Plasmids containing the hα7 and hβ2 genes were linearized with XBaI and AvrII, respectively (New England BioLabs Inc., Ipswich, MA, USA). The mRNA was then transcribed, capped on the 5’ end, and a PolyA tail was added using the mMessage mMachine® T7 Ultra Kit (Ambion, Carlsbad, CA, USA). mRNAs were isolated by LiCl purification, resuspended in Tris-EDTA Buffer, aliquoted, and stored at -20 °C until used.

Human α7 (0.2 or 1.0 µg/µL) and β2 (1.0 µg/µL) AChR subunit mRNAs were injected into X. laevis oocytes using a Nanoject II microinjector (Drummond Scientific Company, Broomall, PA, USA). The injection and recording needles of borosilicate glass were pulled by using Model P-97 puller (Sutter Instrument Company, Novato, CA, USA). For homomeric hα7 AChRs, oocytes were injected with 50.6 nL of 0.2 µg/µL hα7 mRNAs. For heteromeric hα7β2 AChRs, the hα7 and hβ2 mRNAs were injected at either the [1:1] or [1:5] hα7:hβ2 ratio (1.0
µg/µL: 1.0 µg/µL, 0.2 µg/µL: 1.0 µg/µL). The rationale being that a [1:5] (i.e., 0.2:1 µg/µL) ratio would be more likely to produce a higher yield of heteromeric hα7β2 AChRs and relatively less homomeric hα7 AChRs. However, the resulting recordings using either ratio were not statistically different from each other (One-way ANOVA of rise times, decay times, and peak currents, p>0.05), indicating the presence of hα7β2 AChRs in oocytes injected with either hα7:hβ2 ratio.

Following injection, the oocytes were incubated for 7-9 days on a rocker between 17-19 °C and stored in OR-2-Ca²⁺ (Ringer’s solution, extracellular solution) (pH 7.5) with penicillin-streptomycin (10,000 units/mL; 10 mg/mL).

For the two-electrode whole-cell voltage clamp recordings, ligands were perfused on oocytes clamped at -60 mV. All ligands were dissolved in OR-2-Ca²⁺, whereas PAM-2 was dissolved in 1% DMSO. Control experiments showed no significant difference in responses with solutions containing 1% DMSO. Solutions were perfused using an 8-valve, computer controlled, pressurized perfusion system (Automate Scientific, Berkeley, CA, USA). Each oocyte was impaled with microelectrodes filled with 3 M KCl and a resistance between 0.1-2 Ω.

Clampex 9.2 was used to run the electrophysiology protocols through a GeneClamp 500B amplifier and Digidata 1322A digitizer. Data was acquired at 5 kHz and filtered at 2 kHz. The data was analyzed using Clampfit 9.2 (Molecular Devices, Sunnyvale, CA, USA).

Patch-Clamp Recording of HEK293 Cells Expressing GABA₄Rs or GlyRs

The HEK293 cell line stably expressing recombinant hα1β2γ2 GABA₄R subunits or transiently transfected with hα1 GlyR or hρ1 GABA₄R subunits were used to investigate the effect of PAM-2 on these receptors. The PolyJet™ DNA In Vitro and Trans-IT 293 transfection reagents were used for transient transfection of the respective hα1 and hρ1 subunits. Briefly, 0.5 µg of hα1
subunit cDNA was added to cells growing exponentially on poly-L-lysine coated coverslips placed in a 35-mm culture dish. In the case of hρ1, 2 µg of hρ1 subunit cDNA and 2 µg of enhanced green fluorescent protein cDNA was added to cells growing on glass coverslips. Cells that showed expression of the green fluorescent protein were subjected to patch-clamp electrophysiology, as described previously (Snell and Gonzales, 2015). Transfected cells were used for electrophysiological analysis 24-48 h after the transfection.

Whole-cell patch clamp recordings were performed at room temperature (RT) (22-25 °C) at a holding potential of -60 (hα1β2γ2 and hα1) or -70 mV (hρ1). Patch pipettes of borosilicate glass (M1B150F, World Precision Instruments, Inc., Sarasota, FL, USA) were pulled using a P-87 Flaming/Brown or a Sutter P-97 horizontal puller (Sutter Instrument Co., Novato, CA) to a tip resistance of 6-9 MΩ. The pipette solution for the hα1β2γ2 GABAARs and hα1 GlyRs contained (in mM): 140 CsCl, 10 EGTA, 10 HEPES, 4 Mg-ATP; pH 7.2. A coverslip containing transfected cells was placed in a small chamber (~1.5 mL) on the stage of an inverted light microscope (Olympus IMT-2) and superfused continuously (5-8 mL/min) with the following external solution containing (in mM): 125 NaCl, 5.5 KCl, 0.8 MgCl2, 3.0 CaCl2, 10 HEPES, 10 glucose, pH 7.3. The external solution for the hρ1 GABAAR contained (in mM): 137 NaCl, 5.4 KCl, 1.8 CaCl2, 1 MgCl2, and 5 HEPES; pH 7.4, whereas the internal recording solution contained: 120 CsCl, 20 tetraethylammonium chloride, 1 CaCl2, 2 MgCl2, 11 EGTA, and 10 HEPES; pH 7.2 (Snell and Gonzales, 2015). In this case, a Perfusion Fast-Step 77B (Warner Instrument LLC., Hamden, CT, USA) equipped with an array of square capillary tubes was used.

GABA or Gly was prepared in extracellular solution and applied (10-s) to cells containing the hα1β2γ2 GABAARs or hα1 GlyRs via gravity flow using a Y-shaped tube positioned near the target cell. With this system, the 10-90% rise time of the junction potential at
the open tip was 60-120 ms (Huang and Dillon, 1999). In the case of the hρ1 GABAAR, the GABA control and PAM-2 containing test solutions were applied for 5 s. A washout period of 90 s followed each recording to ensure a return to baseline current readings was achieved before proceeding to the next recording. Receptor currents were obtained using a patch clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA, USA) equipped with a CV201A headstage, whereas a pClamp10.0 (Molecular Devices, Sunnyvale, CA, USA) on an inverted fluorescence microscope equipped with a FITC fluorescence cube was also used to record hρ1 GABAAR currents.

The hα1β2γ2 GABAAR and hα1 GlyR currents were low-pass filtered at 5 kHz, monitored on an oscilloscope and a chart recorder (Gould TA240), and stored on a computer (pClamp 6.05, Axon Instruments) for subsequent analysis. Receptors were typically activated with agonist concentrations corresponding roughly to the EC30 (10 µM; hα1β2γ2) (Huang et al., 2001) or EC50 (9.4 µM; hρ1) value for GABA and the EC30 value for Gly (15 µM; hα1) (Chen et al., 2004), respectively. These concentrations were chosen because produce minimal receptor desensitization, which may confound interpretation of results. To monitor the possibility that access resistance changed over time or during different experimental conditions, the current response to a -5 mV voltage pulse was measured at the initiation of each recording and stored on our digital oscilloscope. This stored trace was continually referenced throughout the recording. If a change in access resistance was observed throughout the recording period, the patch was aborted and the data were not included in the analysis.

The concentration-response relationship for PAM-2 on hα1β2γ2 GABAARs was analyzed using the following logistic equation (Potasiewicz et al., 2015): \[ I_{PAM-2}/I = 1/[1 + (\text{apparent EC50}/[PAM-2])^{nH}] \], where I is the current amplitude determined in the absence of PAM-2 (control
assigned as 100%), $I_{PAM-2}$ is the potentiation induced by different concentrations of PAM-2 [PAM-2], apparent $EC_{50}$ is the PAM-2 concentration to produce half-maximal potentiation, and $n_H$ is the Hill coefficient.

**Statistical Analysis**

The obtained data, expressed as the mean ± SEM, were analyzed using the Origin 6.0 Microcal Software. Statistical analyses were performed using one-way analysis of variance (ANOVA) and t-test (paired or unpaired). Differences will be accepted as significant with $p \leq 0.05$.

**Homology Modeling, Molecular Docking, and Molecular Dynamics**

The amino acid sequences and numbering of the $h\alpha 7$, $h\alpha 4$, and $h\beta 2$ subunits were obtained from the UniProt implemented in ExPASy Molecular Biology Server (http://www.us.expasy.org) (Gasteiger et al., 2003). The $h\alpha 7$ sequence was aligned with the $h\alpha 4$ and $h\beta 2$ sequences using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Structural models of the $h\alpha 7$ as well as of the $h(\alpha 7)_2(\beta 2)_3$ and $h(\alpha 7)_3(\beta 2)_2$ stoichiometries were built applying homology modeling methods using the crystal structure of the $h\alpha 4\beta 2$ nAChR obtained at 3.94 Å atomic resolution [PDB ID: 5KXI; www.rcsb.org/pdb/home/home.do (Morales-Perez et al., 2016)] as template. Modeller 9.14 was used to obtain 100 homology models for each template, and subsequently, their Discrete Optimized Protein Energy profiles (Eswar et al., 2006) were assessed. The best model of each template was assessed by its quality by Ramachandran plots, generated by using the Schrödinger suite software (Schrödinger Release 2015-3: Maestro, version 10.3). The $\beta 2$ subunit in both $h(\alpha 7)_2(\beta 2)_3$ and $h(\alpha 7)_3(\beta 2)_2$ stoichiometries was taken from the $h\alpha 4\beta 2$ nAChR crystal structure, and each model optimized using Desmond (Schrödinger suite software).
For the molecular docking procedure, PAM-2 was first built using the semiempirical AM1 method included in Spartan 10 V.1.1.0 (Wavefunction, Inc. Irvine, CA, USA) as previously described (Arias et al., 2016). AutoDock Vina (Trot and Olson, 2010) was used for docking simulations of the flexible ligand into the extracellular (ECD) or transmembrane (TMD) (including ECD-TMD junction) of the whole, rigid, nAChR model under study. The energetically lower poses were selected from each cluster of superposed poses as described previously (Arias et al., 2013; 2016).

To investigate the stability of the best scored complexes, 15-ns molecular dynamics (MD) simulations were subsequently performed for PAM-2 docked to each binding site in the respective AChR model, using Desmond v.3.0.3.1 (Bowers et al., 2006) and OPLS-2005 force field. Each receptor-PAM-2 complex was inserted into 1-palmitoyl-2-oleoyl phosphatidylcholine membranes, solvated with water, and the protein charges neutralized with 0.15 M NaCl as described elsewhere (Arias et al., 2015; 2016). Each complex was first minimized and then subjected to 1-ns MD in NVT (constant number of particles, volume, and temperature) ensemble, followed by 15-ns MD in NPT (constant number of particles, pressure, and temperature) ensemble with fixing constrains on protein backbone as previously described (Arias et al., 2015; 2016). Finally, each complex was simulated in NPT ensemble for 15-ns without any fixing constraints. The total potential energy of each docked model was calculated using the OPLS-2005 force field according to Bowers et al. (2006).

Results

*Different PAM-2 Activity Between ha7 and ha7β2 AChRs*

Injection of ha7 mRNAs with or without hβ2 mRNAs into *Xenopus laevis* oocytes resulted in functional ha7 and ha7β2 AChRs. For heteromeric ha7β2 AChRs, the ha7 and hβ2
mRNAs were injected at either the [1:1] or [1:5] hα7:hβ2 ratio. The rationale being that a [1:5] (i.e., 0.2:1 µg/µL) ratio would be more likely to produce a higher yield of heteromeric hα7β2 AChRs and relatively fewer homomeric hα7 AChRs. However, the resulting recordings using different ratios were not statistically different from each other (One-way ANOVA of rise times, decay times, and peak currents to PAM-2, p>0.05), indicating the presence of hα7β2 AChRs in oocytes injected with either hα7:hβ2 ratio.

Regarding the first series of experiments, the electrophysiological recordings illustrate that the pre-application of PAM-2 caused kinetic changes on both ACh-activated hα7 (Figure 1.A) and hα7β2 (Figure 1.B) AChRs. PAM-2 increased the peak amplitude on both receptor subtypes. To differentiate the effect of PAM-2 between the hα7 and hα7β2 AChRs, two protocols were developed. The concentration-response curves for PAM-2 (Figure 1.C) in the presence of a fixed concentration of ACh was determined on each AChR subtype. Additionally, the concentration-response curves for ACh were determined in the absence and presence of a fixed concentration of PAM-2 (Figures 3.A-B). PAM-2 increased the peak amplitude of both hα7 and hα7β2 (Table 1). At the maximal PAM-2 effect, there is not a significant difference in the fold increase of the peak amplitudes between hα7 and hα7β2. However, because of the differences in their PAM-2 EC50 values we observe that there is a statistical difference in relative peak amplitude at 10 µM PAM-2 (p<0.01, t-test).

The concentration-response curves for PAM-2, with a fixed ACh concentration, showed significant increases at all PAM-2 concentrations in rise time, half-width, and decay tau when compared to controls that were not exposed to PAM-2 (Figs 2.A-C). These effects were similar at all PAM-2 concentrations (i.e. ANOVA testing showed no significant difference between PAM-2 concentrations). Therefore, we pooled the data from multiple PAM-2 concentrations for
the following t-tests (significance level of 0.05, two-tailed). The rise time values for PAM-2 α7 and PAM-2 α7β2, although both significantly different from their respective baselines, were not significantly different from each other (t-test against baselines p<0.01, p<0.01, respectively, Figure 2.B). Likewise, half-width values from PAM-2 α7 and PAM-2 α7β2 were significantly different from their respective baselines (α7 p<0.001, α7β2 p<0.00000001), but not from each other.

The decay phase of the ACh induced currents was fit using a single exponential equation, yielding tau values. Unlike the rise time and half-widths, the PAM-2 effect on the tau values is statistically different for α7 than for α7β2 (p=0.00816, Figure 2.C). The α7 does not show a significant increase in the tau decay, but the trend shows a possible increase. Whereas, the α7β2 subtypes show a significant increase in the tau decay following PAM-2 exposure when compared to their respective baselines (p=0.104, p<0.001). Additionally, the α7β2 has a significantly longer tau value following PAM-2 application compared to the α7. This allows the two subtypes to be distinguished using PAM-2.

The ACh concentration-response curves for α7 and α7β2 are not statistically different from each other. Additionally, in the presence of PAM-2, the concentration-response curves remain similar to each other (Figures 3.A-B; Table 2). PAM-2 significantly increased the rise time values at the lower ACh concentrations for both α7 and α7β2 (Figures 3.C-D). Additionally, 20 µM PAM-2 significantly increased the total area at ACh concentrations between 100 µM and 10 mM for both subtypes. In fact, at the maximum ACh concentration (10 mM) PAM-2 (20 µM) differentiates between α7 and α7β2 AChRs because of the larger increase in total area of the α7 subtype (ANOVA, p<0.001, Figure 3.F). The α7 26–fold as compared to
the hα7β2 which AChRs by 26- and 6-fold, respectively (Figure 3.F, Table 1). This is a 4.5-fold
difference between the total current in the hα7 and hα7β2 treated with 20 µM PAM-2.

Although there is an increase in rise time for both subtypes when compared to the
controls, there is only a slight difference in the effect of PAM-2 either subtype (Figures 3.C-E).
The trend shows that hα7β2 AChRs may have longer rise times after PAM-2 applications. This
could indicate changes in the channel opening, ACh affinity, or desensitization properties. See
Table 2 for the summary of the fits and kinetic changes of Figures 3A-F.

Figure 4 shows the time dependence of the PAM-2’s effect on the peak amplitude and
current area for both hα7 and hα7β2 AChRs. Both parameters were significantly different than
that for the control after only 10 seconds of 20 µM PAM-2 application on both subtypes.
Interestingly, the effects became more pronounced over time with continued application of
PAM-2. We also observed that the PAM-2 effect was reversible over time. To more easily
differentiate the AChR subtypes and to ensure that the maximum effect on peak amplitude was
reached, the application of PAM-2 was continued for 4.5 min. The maximal effect of PAM-2 on
peak amplitude was reached at 1.5 min, whereas its effect on the current area continued to
increase during the entire application. Therefore, we selected 3 min as our point of comparison
for the experiments used in Figures 1-3.

Effect of PAM-2 on GABA<sub>4</sub>R and GlyR Function

To examine whether PAM-2 affects hα1β2γ2 and hρ1 GABA<sub>4</sub>Rs or hα1 GlyR activity,
different PAM-2 concentrations (i.e., 10-300 µM) were co-applied with 10 µM (Figure 4.A) or
9.4 µM GABA (Figure 4.B) or 15 µM Gly (Figure 5). The results indicated that PAM-2
potentiated GABA-activated hα1β2γ2 GABA<sub>4</sub>R currents (Figure 4.A) as well as inhibited hρ1
GABA<sub>4</sub>R activity (Figure 4.B) in a concentration-dependent manner. The maximum effects were
observed with 300 µM PAM-2, where $h\alpha1\beta2\gamma2$ currents were increased to $131 \pm 4\%$ (p<0.05), whereas the $h\rho1$ currents were decreased to $70 \pm 1\%$ (p<0.001) compared to control (100%) (Table 1). The potentiating EC$_{50}$ value for PAM-2 on $h\alpha1\beta2\gamma2$ GABA$_A$Rs was $56 \pm 7$ µM (Table 1). The calculated $n_H$ value ($1.50 \pm 0.20$; Table 1) suggests a cooperative mechanism, which is in agreement with the fundamental mechanism of PAMs. In the $h\alpha1\beta2\gamma2$ subtype, the onset of the PAM-2 effect was rapid and completely reversible within 2-3 min. On the other hand, PAM-2 had no effect on GlyR response (p>0.05) (Figure 5; Table 1).

**Homology Modeling, Molecular Docking, and Molecular Dynamics of PAM-2 at the $h\alpha7$, $h(\alpha7)2(\beta2)3$, and $h(\alpha7)3(\beta2)2$ Models**

PAM-2 was docked into the ECD or TMD (including ECD-TMD junction) of the whole receptor model. Since PAM-2 activity does not involve the orthosteric sites (Arias et al., 2011), this interaction was not included in Figure 6. Although the ECD is not involved in the PAM-2 activity (Andersen et al., 2016), the observed allosteric site in the $h(\alpha7)2(\beta2)3$ model is shown in Figure 6A. The MD results indicated that the interactions summarized in Table 3 are stable during the 50-ns simulations. This is based on the consideration that a ligand in its respective pocket is stable when the RMSD variance value is ≤0.5 Å (Arias et al., 2016). The RMSD mean values for the 50-ns simulation and the RMSD variance values calculated during the last 10-ns are included in Table 4.

In the $h\alpha7$ model, PAM-2 interacted with both the archetypical intrasubunit and ECD-TMD junction 1 sites (Figure 6). The results using this new $h\alpha7$ model showed that PAM-2 interacts with the intrasubunit site as previously described in the $h\alpha7$ model built using the *Torpedo* AChR as template (Arias et al., 2016). More specifically, PAM-2 interacted with residues from M1 (i.e., Leu216, Cys219, Val220, and Ser223, and M4 (i.e., Thr461, Ile464, and Leu465). In
junction 1, PAM-2 interacted with residues from the Cys-loop (i.e., Trp134, Phe135, Pro136, and Phe137), pre-M1 segment (i.e., Thr208, Tyr210, and Gly212), M1 (Leu216), M2-M3 loop (i.e., Leu270 and Ile271), and M4 (i.e., Leu465, Ala468, and Pro469) (Figure 6A).

In the h(α7)2(β2)3 model, PAM-2 interacted with the ECD-TMD junction at three sites called junction 2, and 3 (orientation 1 and 2), which are different from that characterized on the hα7 nAChR (Figure 6A; Table 3). In junction 2, PAM-2 intercalated between both α7 and β2 subunits, interacting with the β1-β2 loop (i.e., α7-Lys46, β2-Glu47, and β2-Arg48), pre-M1 (i.e., β2-Phe211, β2-Tyr212, β2-Asn215, and β2-Leu216), and M2 (i.e., α7-Leu255, α7-Ala258, α7-Glu259, α7-Ala263, β2-Leu257, β2-Lys260, and β2-Ile261) (Figures 6.A-B; Table 3). An H-bond is formed between the PAM-2 nitrogen of the amide moiety and the carbonyl oxygen from the α7-Ala258 backbone. In junction 3 orientation 1, located within the β2 subunit, PAM-2 interacted with residues from the M2-M3 loop (i.e., Lys274 and Tyr275), M3 (i.e., Phe278, β2-Thr279, and Leu282), and M4 (i.e., Cys361, Phe363, Gly364, Gly367, and Met368) (Figures 6.A-C). An H-bond is formed between the carbonyl oxygen of PAM-2 and the α7-Lys274 nitrogen moiety. In junction 3 orientation 2, PAM-2 also interacted with β2 residues from the Cys-loop (i.e., His136, Phe137, Pro138, and Phe139, M1 (i.e., Ile217, Cys220, and Val221), M2-M3 loop (i.e., Leu271, Val272, Tyr275, and Leu276), and M4 (i.e., Met368, Phe369, and Pro372) (Figures 6.A-D; Table 3). Although two residues (i.e., β2-Tyr275 and β2-Met368) are common for two orientations of PAM-2 at ECD-TMD junction 3 site at h(α7)2(β2)3 model, there are three possible β2 subunits so these two bindings sites might not be overlapped.

In the h(α7)3(β2)2 model, PAM-2 interacted with ECD-TMD junction 1, 2, and 4, and luminal sites. The junction 1 and 2 sites are observed in the hα7 and h(α7)2(β2)3 model, respectively. In junction 4, PAM-2 docked between two α7 subunits, interacting with residues
from the β1-β2 loop (i.e., Lys46 and Gln48) (this site has the same residue, α7-Lys46, as observed in junction 2 from the h(α7)2(β2)3 model, so change the number but junction 2 is between a7 and b2, while this junction 4 is between two a7 subunits. These are homologous sites that need to be clearly stated, if you use different numbers, you are saying that are two totally different sites), Cys-loop (i.e., Tyr129), and M2 (i.e., Glu259, Pro262, Ala263, and Thr264) from one α7 subunit, and with residues from the ECD (i.e., Asp42, Val43), β1-β2 loop (i.e., Asp44 and Glu45), Cys-loop (i.e., Asn171, Gly172, Glu173, and Trp174), pre-M1 (i.e., Tyr211) from an adjacent α7 subunit. Finally, PAM-2 interacted with the ion channel lumen, more specifically with residues from positions 6’, 2’, and -2’, as well as with additional residues from positions 5’, 1’, -3’, and -4’. Since the binding to this luminal site presented higher energy compared to other non-luminal sites, and considering that PAM-2 does not block hα7 nAChRs at potentiating concentrations (Arias et al., 2011; 2016), this interaction was not further considered.

Discussion

In electrophysiological recordings, the hα7 and hα7β2 are almost indistinguishable. However, with enough replicates we can resolve that the rise time for the hα7 is slightly faster than the hα7β2. This is likely because there are 5 possible ACh binding sites on the hα7 (Corringer, 2000, Changeux, 1998). In addition, the hα7 AChR only requires that one binding site needs to be occupied for full receptor activation and subsequent channel opening (Andersen et al., 2013). Therefore, the likelihood of channel opening is higher for the hα7. However, saturating concentrations of agonists may induce rapid desensitization (Papke et al., 2000). Whereas, the hα7β2 likely only has 2 or 3 ACh binding sites, depending on the subunit stoichiometry, (likely hα7β2 or hα7β2) and multiple molecules of ACh are required for
receptor activation. Since a $\alpha_7:\beta_2$ 1:5 ratio was injected, both $h\alpha_7\beta_2$ and $h\alpha_7\beta_2^2$ might be expressed, producing events that are the result of both receptor stoichiometries. In sum, it is likely that ACh more easily activates and desensitizes the $h\alpha_7$ than the $h\alpha_7\beta_2$; yet, the differences are so minute that it can be difficult to distinguish \textit{in vivo}. Additionally, there are no current pharmacological ways to distinguish the two subtypes \textit{in vivo} during an electrophysiological recording. Therefore, we thought it imperative to see if PAM-2 has a distinguishable effect on the $h\alpha_7$ and $h\alpha_7\beta_2$.

We found that the area under the curve, or total current, distinguishes the $h\alpha_7$ and $h\alpha_7\beta_2$ in the presence of PAM-2. Since, there is a dramatic increase in area this may be evidenced of a change in desensitization. This change in total area may be more evident in the $h\alpha_7$ because it is natively in a more desensitized state. As a metaphor, the $h\alpha_7$ has a greater working distance, and that the change in desensitization is more pronounced since it starts at a higher desensitized state. The change in desensitization for each subtype is supported by the increase in rise time and the increase in peak amplitude for each.

PAM-2 may slow the process of receptors moving from their open to their desensitized states. The voltage clamp results also indicated that the activity of PAM-2 differs between $h\alpha_7$ and $h\alpha_7\beta_2$ AChRs. Namely, PAM-2 increased the tau for the single exponential fit of decay more for the $h\alpha_7\beta_2$ than for the $h\alpha_7$. Both subtypes show an increase in the tau as compared to the baselines, but the changes in the $h\alpha_7\beta_2$ were more significant. Considering, there is not a significant difference in the PAM-2 effect on $h\alpha_7$ and $h\alpha_7\beta_2$ half-width and rise times, but only on the decay phase, it may highlight distinct binding properties of PAM-2 on either subtype.

Interestingly we observed a shift in the ACh EC$_{50}$ value to the right in the $h\alpha_7\beta_2$ ACh concentration-response curve in the presence of PAM-2. A shift to the left is expected for
PAMs, however, in the presence of a PAM the higher the agonist concentration, the higher the ion channel blockade. For this reason, we used 50 µM ACh (represents ~25% of the total peak current for the hα7β2 and the hα7) for our PAM-2 concentration-response curve (Figure 1.C) In the ACh concentration-response curve, even though the ACh EC\text{50} in the presence of PAM-2 was shifted to the left, the fold response is still greater compared to the control (Figure 3.B).

Regarding the PAM-2 concentration-response curve (Figure 1.C) we see sharp increases in the nH, as well as maximum fold increases of 2.5 for the hα7 and 2.1 for the hα7β2. For both subtypes, there is a concentration at which you reach the maximum fold increase and increasing the PAM-2 concentration does not result in a more enhanced response. At 10 µM PAM-2, we see a distinguishing difference between the effect of PAM-2 on the hα7 and hα7β2. In sum, even though there are many indistinguishable properties between the hα7 and hα7β2, PAM-2 serves to distinguish the hα7 and hα7β2 from one another.

The patch clamp results indicated that PAM-2 induces only a modest potentiation of GABA-activated hα1β2γ2 GABA\textsubscript{A}R currents and slight inhibition of hρ1 GABA\textsubscript{A}R currents, but does not influence homomeric hα1 GlyR activity. Since the observed effects on GABA\textsubscript{A}Rs are produced at non-clinically relevant concentrations, it is unlikely that GABA\textsubscript{A}Rs and GlyRs, members of the same Cys-loop receptor superfamily as for α7 AChRs, might mediate the beneficial effects elicited by PAM-2, including procognitive (Potasiewicz et al., 2015; 2016), promnesic (Targowska-Duda et al., 2016), antidepressant-like (Targowska-Duda et al., 2014; Arias et al., 2015), anti-nociceptive and anti-inflammatory (Bagdas et al., 2015) activities.

Considering that the PAM-2 structure has an amide link susceptible to the metabolic activity of amidases from the liver and/or brain (Pop, 1997), the activity of the potential metabolites p-toluidine and 3-(2-furyl)acrylic acid were tested on the hα7 AChR. The functional results
indicated that neither $p$-toluidine nor 3-(2-furyl)acrylic acid have activity (Table 1), discarding the possibility of PAM-2 active metabolites produced by amidases from the liver and/or brain. However, we cannot rule out the existence of other metabolic pathways producing additional metabolites (e.g., methoxy metabolites) with potential activity. Since N,N’-dicyclohexylurea was found as a byproduct in the synthesis of PAM-2, the PAM activity of this compound was also tested on the hα7 AChR. The results showed no activity for N,N’-dicyclohexylurea, indicating that this precursor is not responsible for the observed PAM-2 activity.

This new study using the crystal structure of the hα4β2 nAChR as the homology template confirms previous results obtained with the hα7 model built using the Torpedo AChR as the homology template (Arias et al., 2016). More precisely, these new results indicate that PAM-2 binds to the intrasubunit site characterized for PNU-120596, the archetypical type II PAM (Young et al., 2008; daCosta et al., 2011; Arias et al., 2016). Recent findings using the α7TSLMF mutant (i.e., Ser223Thr, Ala226Ser, Met254Leu, Ile281Met, and Val288Phe) also showed that PAM-2 and PNU-120596 failed to potentiate the quintuple mutant, supporting the notion that these two PAMs share the similar structural determinants within the intrasubunit site (Andersen et al., 2016). This new study also confirms previous results (Arias et al., 2016), indicating that PAM-2 interacts with the ECD-TMD junction at sites that are also observed at both hα7 and h(α7)3(β2)2 nAChRs (i.e., junction 1), and at both h(α7)2(β2)3 and h(α7)3(β2)2 stoichiometries (i.e., junction 2-5), respectively. Interestingly, junction 1 is formed by residues from α7 subunit, junction 3 from the β2 subunit, while junction 2 and 4 is formed by residues located at the interface between α7 and β2 subunits or between two adjacent α7 subunits, respectively.
The existence of the ECD-TMD junction sites, located apart from the archetypical intrasubunit site, might be important to understand the mechanisms underlying the dissimilar activity of PAM-2 between the $\alpha_7$ and $\alpha_7\beta_2$ nAChRs (this work) as well as the different features found between type I and type II PAMs (Andersen et al., 2016; Arias et al., 2016).
Table 3.1: Pharmacologic Activity of PAM-2 on hα7 and hα7β2 AChRs, hα1β2γ2 and hp1 GABA_ARs, and hα1 GlyRs.

<table>
<thead>
<tr>
<th>Receptor Type</th>
<th>Method</th>
<th>Ligand (concentration range)</th>
<th>Pharmacologic activity</th>
<th>$E_{\text{max}}$ (% control)</th>
<th>EC$_{50}$ (µM)</th>
<th>nH</th>
</tr>
</thead>
<tbody>
<tr>
<td>hα7 AChR</td>
<td>Two-electrode voltage-clamp on <em>Xenopus</em> oocytes injected with hα7 subunit mRNA</td>
<td>PAM-2 (1-80 µM)</td>
<td>Potentiation</td>
<td>246 ± 25</td>
<td>8.8 ± 1.15</td>
<td>12.23 ± 13.26</td>
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<tr>
<td></td>
<td></td>
<td>(&gt;100 µM)</td>
<td>Inhibition</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>hα7β2 AChR</td>
<td>Two-electrode voltage-clamp on <em>Xenopus</em> oocytes injected with hα7 and hβ2 subunit mRNAs (1:5 ratio)</td>
<td>PAM-2 (1-80 µM)</td>
<td>Potentiation</td>
<td>207 ± 26</td>
<td>19.5 ± 1.14</td>
<td>11.74 ± 28.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(&gt;100 µM)</td>
<td>Inhibition</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>hα1β2γ2 GABA_A</td>
<td>Whole-cell patch clamp on HEK293 cells permanently expressing hα1β2γ2 GABA_ARs</td>
<td>PAM-2 (10-300 µM)</td>
<td>Slight Potentiation</td>
<td>131 ± 4</td>
<td>56 ± 7</td>
<td>1.50±0.2</td>
</tr>
<tr>
<td>hp1 GABA_A</td>
<td>Whole-cell patch-clamp on HEK293 cells transiently expressing hp1 GABA_ARs</td>
<td>PAM-2 (10-300 µM)</td>
<td>Slight Inhibition</td>
<td>70 ± 1 $^a$</td>
<td>~540 $^b$</td>
<td>—</td>
</tr>
<tr>
<td>hα1 GlyR</td>
<td>Whole-cell patch clamp on HEK293 cells transiently expressing hα1 GlyRs</td>
<td>PAM-2 (30-300 µM)</td>
<td>No effect</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>hα7 AChR</td>
<td>Two-electrode voltage-clamp on <em>Xenopus</em> oocytes injected with hα7 subunit mRNA</td>
<td>N,N′-dicyclohexylur ea (30-300 µM)</td>
<td>No effect</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>hα7 AChR</td>
<td>Two-electrode voltage-clamp on <em>Xenopus</em> oocytes injected with hα7 subunit mRNA</td>
<td>p-Toluidine (10 µM)</td>
<td>No effect</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>hα7 AChR</td>
<td>Two-electrode voltage-clamp on <em>Xenopus</em> oocytes injected with hα7 subunit mRNA</td>
<td>3-(2-Furyl)acrylic acid (10 µM)</td>
<td>No effect</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

$a$ This value corresponds to PAM-2-induced inhibition relative to control (100%).

$b$ This IC$_{50}$ value is just an estimation using three different concentrations.
Table 3.2: Kinetics Parameters for ACh in the Absence (Control) and Presence of PAM-2 at the α7 and α7β2 AChRs.

<table>
<thead>
<tr>
<th>Kinetics parameter</th>
<th>hα7 Control</th>
<th>hα7 Control + PAM-2</th>
<th>hα7β2 Control</th>
<th>hα7β2 Control + PAM-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak amplitude</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACh EC50 (µM)</td>
<td>194±1.17</td>
<td>141±1.74</td>
<td>144±1.12</td>
<td>186±1.35</td>
</tr>
<tr>
<td>nH</td>
<td>2.33±0.58</td>
<td>1.06±0.51</td>
<td>2.37±0.50</td>
<td>0.99±0.25</td>
</tr>
<tr>
<td>Maximal increase (x-fold)</td>
<td>—</td>
<td>2.3</td>
<td>—</td>
<td>1.9</td>
</tr>
<tr>
<td>R²</td>
<td>0.734</td>
<td>0.424</td>
<td>0.646</td>
<td>0.552</td>
</tr>
<tr>
<td>ANOVA</td>
<td>—</td>
<td>p&lt;0.0001</td>
<td>—</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>Rise time</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximal increase (x-fold)</td>
<td>—</td>
<td>2.1</td>
<td>—</td>
<td>3.6</td>
</tr>
<tr>
<td>R²</td>
<td>0.760</td>
<td>0.132</td>
<td>0.445</td>
<td>0.520</td>
</tr>
<tr>
<td>ANOVA</td>
<td>—</td>
<td>p&lt;0.0001</td>
<td>—</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>Current area</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximal increase (x-fold)</td>
<td>—</td>
<td>26.3</td>
<td>—</td>
<td>5.9</td>
</tr>
<tr>
<td>R²</td>
<td>0.537</td>
<td>0.258</td>
<td>0.684</td>
<td>0.439</td>
</tr>
<tr>
<td>ANOVA</td>
<td>—</td>
<td>p&lt;0.0001</td>
<td>—</td>
<td>p&lt;0.0001</td>
</tr>
</tbody>
</table>

The kinetics parameters are obtained from Figures 2A-F, respectively.
Table 3.3: Molecular Interactions of PAM-2 with Allosteric Sites at the hα7 and hα7β2 Models.

<table>
<thead>
<tr>
<th>nAChR subtype</th>
<th>Binding site location</th>
<th>ECD</th>
<th>Cys loop</th>
<th>Pre-M1</th>
<th>M1 (position)</th>
<th>M2-M3 loop</th>
<th>M3</th>
<th>M4</th>
</tr>
</thead>
<tbody>
<tr>
<td>hα7</td>
<td>ECD-TMD junction 1</td>
<td></td>
<td>W134</td>
<td>T208</td>
<td>L216</td>
<td>L270 I271</td>
<td></td>
<td>L465 A468 P469</td>
</tr>
<tr>
<td></td>
<td>Intrasubunit</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>L216 C219 V220 S223</td>
<td>F275</td>
</tr>
<tr>
<td>h(α7)2(β2)3</td>
<td>ECD</td>
<td>α7-P17 α7-L18 α7-G83 α7-K87 α7-D89 α7-W86 α7-H105 α7-Y151 β2-P83 β2-H86 β2-F106 β2-N109</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ECD-TMD junction 2</td>
<td>β1-β2 loop: α7-K46 β2-E47 β2-R48</td>
<td>β2-F211 β2-Y212 β2-N215 β2-L216</td>
<td></td>
<td>α7-L255 α7-A258 α7-E259 α7-A263 β2-L257 β2-K260 β2-I261</td>
<td>β2-K274 β2-T279 β2-L282</td>
<td></td>
<td>β2-C361 β2-F363 β2-G364 β2-G367 β2-M368</td>
</tr>
<tr>
<td></td>
<td>ECD-TMD junction 3 Orientation 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ECD-TMD junction 3 Orientation 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tbody>
</table>
Table 3.3: Continued: Molecular Interactions of PAM-2 with Allosteric Sites at the hα7 and hα7β2 Models.

<table>
<thead>
<tr>
<th>h(α7)3(β2)2</th>
<th>ECD-TMD junction 1</th>
<th>ECD-TMD junction 2</th>
<th>ECD-TMD junction 4</th>
<th>Luminal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α7-W134 α7-F135 α7-P136 α7-F137</td>
<td>α7-T208 α7-G212</td>
<td>α7-L216 α7-V220</td>
<td>α7-L270 α7-I271 α7-Y274 α7-F275</td>
</tr>
<tr>
<td></td>
<td>β2-Y275 β2-L276</td>
<td>α7-L215</td>
<td>β2-L256 β2-V257 α7-E259 β2-S259 α7-1260 β2-K260 β2-P264</td>
<td>α7-T349 α7-I352 α7-L353</td>
</tr>
<tr>
<td></td>
<td>β1-β2 loop: α7-E45 α7-K46 β2-R48</td>
<td>α7-Y120 α7-Y211 α7-N214</td>
<td>α7-Y210</td>
<td>α7-L215</td>
</tr>
<tr>
<td></td>
<td>α7-D42 α7-V43 α7-D44 α7-E45 α7-K46 α7-Q48</td>
<td>α7-Y120 α7-G172 α7-E173 α7-W174</td>
<td>α7-Y211</td>
<td>α7-E259 (1') α7-P262 (1') α7-A263 (1') α7-T264 (1')</td>
</tr>
<tr>
<td></td>
<td>α7-G237 (3') β2-C237 (4') α7-E238 (2') β2-G238 (3') β2-E239 (2') β2-M241 (1') α7-S241 (2') β2-T242 (2') α7-T245 (6') β2-I245 (5')</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>h(α7)3(β2)2</th>
<th>ECD-TMD junction 2</th>
<th>ECD-TMD junction 4</th>
<th>Luminal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α7-W134 α7-F135 α7-P136 α7-F137</td>
<td>α7-T208 α7-G212</td>
<td>α7-L216 α7-V220</td>
</tr>
<tr>
<td></td>
<td>β2-Y275 β2-L276</td>
<td>α7-L215</td>
<td>β2-L256 β2-V257 α7-E259 β2-S259 α7-1260 β2-K260 β2-P264</td>
</tr>
<tr>
<td></td>
<td>β1-β2 loop: α7-E45 α7-K46 β2-R48</td>
<td>α7-Y120 α7-Y211 α7-N214</td>
<td>α7-Y210</td>
</tr>
<tr>
<td></td>
<td>α7-D42 α7-V43 α7-D44 α7-E45 α7-K46 α7-Q48</td>
<td>α7-Y120 α7-G172 α7-E173 α7-W174</td>
<td>α7-Y211</td>
</tr>
<tr>
<td></td>
<td>α7-G237 (3') β2-C237 (4') α7-E238 (2') β2-G238 (3') β2-E239 (2') β2-M241 (1') α7-S241 (2') β2-T242 (2') α7-T245 (6') β2-I245 (5')</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.4: RMSD Mean and RMSD Variance Values for PAM-2 Interacting with Different Nicotinic Receptor Subtypes, Including hα7, h(α7)(β2)3, and h(α7)(β2)2 nAChRs.

<table>
<thead>
<tr>
<th>Binding site</th>
<th>nAChR subtype</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hα7</td>
<td>h(α7)(β2)3</td>
<td>h(α7)(β2)2</td>
</tr>
<tr>
<td>ECD</td>
<td>-</td>
<td>2.051 ± 0.014 (0.002)</td>
<td>-</td>
</tr>
<tr>
<td>ECD-TMD junction 1</td>
<td>0.6825 ± 0.008 (0.087)</td>
<td>-</td>
<td>1.599 ± 0.006 (0.017)</td>
</tr>
<tr>
<td>ECD-TMD junction 2</td>
<td>-</td>
<td>0.9509 ± 0.010 (0.026)</td>
<td>0.9043 ± 0.008 (0.059)</td>
</tr>
<tr>
<td>ECD-TMD junction 3</td>
<td>1.441 ± 0.008 (0.074)</td>
<td>1.892 ± 0.009 (0.059)</td>
<td>-</td>
</tr>
<tr>
<td>Orientation 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECD-TMD junction 3</td>
<td>-</td>
<td>1.014 ± 0.012 (0.004)</td>
<td>-</td>
</tr>
<tr>
<td>Orientation 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECD-TMD junction 4</td>
<td>-</td>
<td>-</td>
<td>1.785 ± 0.007 (0.002)</td>
</tr>
<tr>
<td>Luminal</td>
<td>-</td>
<td>-</td>
<td>1.446 ± 0.014 (0.007)</td>
</tr>
</tbody>
</table>

The RMSD mean values correspond to the 50-ns simulation, and the RMSD variance values were calculated during the last 10-ns.
Figure 3.1: Effect of PAM-2 on (A) Homomeric hα7 and (B) Heteromeric hα7β2 AChRs. Acetylcholine (50 µM) was applied for 3 seconds activating each AChR subtype. Although PAM-2 (50 µM) did not appear to affect AChR activity independently, a 3 minute pre-treatment of PAM-2 altered the kinetic properties of both AChRs. We observed an increase in the peak amplitude and total area. The hα7 and hα7β2 were both altered but, the kinetic changes of the hα7 were more pronounced. (C) Effect of PAM-2 on the ACh-induced peak amplitude at the hα7 (■) and hα7β2 (□) AChRs. Increasing concentrations of PAM-2 (1-100 µM) were applied to either the hα7 (n=11) AChR, while ACh was held constant at 50 µM. A 90 second PAM-2 wash was done between each ACh application. The potentiating EC$_{50}$, E$_{max}$, and n$_H$ values for PAM-2 at each AChR subtype are summarized in Table 1.
Figure 3.2: Change in Properties of Desensitization After a 3 minute PAM-2 Application.

(A) Pooled data of the half-width measurement (width of the peak in ms at 50% of the amplitude) after PAM-2 application when compared to the baseline measurements before the PAM-2 application are statistically different for each subtype ($\alpha_7$ Pre-PAM-2 v. $\alpha_7$ PAM-2, p-value<0.0001, $\alpha_7\beta_2$ Pre-PAM-2 v. $\alpha_7\beta_2$ PAM-2, p-value<0.00000001, t-tests). However, the PAM-2 effect on the half-width for $\alpha_7$ and $\alpha_7\beta_2$ are not statistically different, as well as their comparison on their pre-PAM-2 baselines.  

(B) Pooled data of the rise-time (time required to increase from 10% to 90% of the peak amplitude) after PAM-2 application when compared to the baseline measurements before the PAM-2 application are statistically different for each subtype ($\alpha_7$ Pre-PAM-2 v. $\alpha_7$ PAM-2, p-value=0.0042, $\alpha_7\beta_2$ Pre-PAM-2 v. $\alpha_7\beta_2$ PAM-2, p-value<0.0014, t-tests). However, the PAM-2 effect on the rise-times for $\alpha_7$ and $\alpha_7\beta_2$ are not statistically different, as well as their comparison on their pre-PAM-2 baselines. 

(C) Pooled data of the tau for single exponential fit of decay after PAM-2 application when compared to the baseline measurements before the PAM-2 application are statistically different for the $\alpha_7\beta_2$ AChR ($\alpha_7$ Pre-PAM-2 v. $\alpha_7$ PAM-2, p-value=0.104) ($\alpha_7\beta_2$ Pre-PAM-2 v. $\alpha_7\beta_2$ PAM-2, p-value<0.001, t-tests). In addition, the PAM-2 effect on the tau for single exponential fit of decay for $\alpha_7$ and $\alpha_7\beta_2$ were statistically different (p-value=0.00816), even though the comparisons of their pre-PAM-2 baselines were not. ANOVAs revealed no significant differences in the desensitization properties with various concentrations of PAM-2 (1 µM-100 µM), therefore, all data was pooled.
Figure 3.3: Effect of 20 µM PAM-2 on Various Kinetic Parameters Obtained from ACh-induced hα7 and hα7β2 AChR Responses.
Figure 3.3 Continued: The activity of ACh on the respective hα7 (■) (n = 7) and hα7β2 (▲) (n = 14) AChR was compared to that obtained in the presence of 20 µM PAM-2 applied for 3 minutes [hα7 (□), n = 4; hα7β2 (△), n = 9]. The comparison of the relative peak amplitude for the (A) hα7 (■) and (B) hα7β2 (▲) AChRs in the absence versus the presence of PAM-2 [(□), F[13, 118]=11.126, p<0.0001; (△), F[12, 234]=35.956, p<0.0001] indicated a statistically significant effect by PAM-2. The comparison of the time required for a pulse to rise from 10% to 90% of the peak amplitude (i.e., relative rise time) for the (C) hα7 (■) and (D) hα7β2 (▲) AChRs in the absence versus the presence of PAM-2 [(□), F[12, 111]=4.697, p=0.0011; (△), F[9, 227]=44.602, p<0.0001] indicated a statistically significant effect by PAM-2. The error bars are the S.E.M. values. The apparent absence of error bars on certain points is due to the scale required for comparison. (*p<0.05, **p<0.01, ***p<0.001). The quantitative parameters for ACh in the absence and presence of PAM-2 at each AChR subtype are summarized in Table 2. (E) Comparison of the difference between the largest PAM-2 effects and the largest control values on both hα7 (black bars) and hα7β2 (white bars) AChRs. Although the peak amplitude and current area are compared at 10 mM ACh, the rise time comparison is made at the minimum ACh concentration because the effect inversely related. (F) The results summarize that PAM-2 has a much greater potentiating effect on homomeric hα7 AChRs compared to that for heteromeric hα7β2 AChRs. The quantitative parameters for ACh in the absence and presence of PAM-2 at each AChR subtype are summarized in Table 2. (F) Fold increase in total current (area) increases with increasing ACh concentrations during 20 µM PAM-2 applications for the hα7 AChRs. However, even though the hα7β2 AChRs show a ~5 fold increase in total area during 20 µM PAM-2 applications, the increase is not observed with increasing ACh concentrations.
Figure 3.4: Time Dependence of the Change in Peak Amplitude and Current Area of the Respective ha7 (■,□) and ha7β2 (▲,△) AChR Perfused with 50 µM PAM-2. A significant effect on peak amplitude (▬) and area (…) was observed after 10 seconds of perfusion on both ha7 (peak, p<0.0001; area, p<0.0001; n = 6) and ha7β2 (peak, p<0.05; area, p<0.001; n = 3) AChRs. T-tests were performed at 10 seconds in comparison to the control.
Figure 3.5: Effect of PAM-2 on Heteromeric \( \alpha_1\beta_2\gamma_2 \) (A) and Homomeric \( \rho_1 \) (B) GABA\(_A\)Rs. (A) Top, representative traces showing GABA-activated \( \alpha_1\beta_2\gamma_2 \) GABA\(_A\)R currents, in the absence and presence of PAM-2, by using whole-cell patch-clamp. Different concentrations of PAM-2 (i.e., 10-300 \( \mu \)M) were co-applied with 10 \( \mu \)M GABA for 10 s. Bottom, the results indicated that PAM-2 potentiated \( \alpha_1\beta_2\gamma_2 \) currents in a concentration-dependent manner (n = 3-8). The potentiating EC\(_{50}\), n\(_H\), and E\(_{max}\) values were summarized in Table 1. (B) Top, representative traces showing GABA-activated \( \rho_1 \) GABA\(_A\)R currents, in the absence and presence of PAM-2, by using whole-cell patch-clamp. Different concentrations of PAM-2 (i.e., 10, 100 or 300 \( \mu \)M) were co-applied with 9.4 \( \mu \)M GABA for 5 s. Bottom, the results indicated that PAM-2 slightly inhibited \( \rho_1 \) currents in a concentration-dependent manner (n = 3-8; paired t-test; p<0.01 and p<0.001 for 100 and 300 \( \mu \)M, respectively). All current amplitudes are normalized to the response in the absence of PAM-2 (assigned as 100\%). Each data point represents Mean ± SEM. The estimated IC\(_{50}\) and E\(_{max}\) (%) values are summarized in Table 1.
Figure 3.6: Effect of PAM-2 on Recombinant hα1 GlyRs. Top, representative traces showing Gly-activated hGlyR currents, in the absence and presence of PAM-2, by using whole-cell patch-clamp. Different concentrations of PAM-2 (i.e., 30, 100 or 300 µM) were co-applied with 15 µM Gly for 10 s. Bottom, the results indicated that PAM-2 did not affect GlyR function (n = 6-7; paired t-test; p>0.05). All current amplitudes were normalized to the response in the absence of PAM-2 (assigned as 100%). Each data point represents Mean ± SEM.
Figure 3.7: Molecular Docking of PAM-2 to the hα7 and h(α7)2(β2)3 Models. (A) PAM-2 binding sites at the hα7 [i.e., the intrasubunit (light blue) and ECD-TMD junction 1 (purple) sites] and h(α7)2(β2)3 models [i.e., the ECD allosteric (orange), ECD-TMD junction 2 (magenta), ECD-TMD junction 3 (yellow), and ECD-TMD junction 4 (green) sites]. For clarity, one α7 subunit is hidden from the h(α7)2(β2)3 model, thus, the order of explicitly shown subunits is (from left): β2 (gray), α7 (blue), β2 (gray), and β2 (gray). (B) Molecular interactions of PAM-2 with the ECD-TMD junction 2 site (magenta) at the h(α7)2(β2)3. This site, located in the α7/β2 interface, is formed by residues from the β1-β2 loop (i.e., α7-Lys46, β2-Glu47, and β2-Arg48), pre-M1 (i.e., β2-Phe211, β2-Tyr212, β2-Asn215, and β2-Leu216), and M2 (i.e., α7-Leu255, α7-Ala258, α7-Glu259, α7-Ala263, β2-Leu257, β2-Lys260, and β2-Ile261). The black arrow shows the H-bond formed between the PAM-2 nitrogen and the carbonyl oxygen from the α7-Ala258 backbone. (C) Molecular interactions of PAM-2 with the ECD-TMD junction 3 site at orientation 1(yellow) at the h(α7)2(β2)3. This site, located within the β2 subunit, is formed by residues from the M2-M3 loop (i.e., Lys274 and Tyr275), M3 (i.e., Phe278, Thr279, and Leu282), and M4 (i.e., Cys361, Phe363, Gly364, Gly367, and Met368). An H-bond is formed between the carbonyl oxygen of PAM-2 and the α7-Lys274 nitrogen moiety. (D) Molecular interactions of PAM-2 with the ECD-TMD junction 3 site at orientation 2 (green) at the h(α7)2(β2)3. This site is formed only by β2 residues from the Cys-loop (i.e., His136, Phe137, and Pro138, and Phe139), M1 (i.e., Ile217, Cys220, and Val221), M2-M3 loop (i.e., Leu271, Val272, Tyr275, and Leu276), and M4 (i.e., Met368, Phe369, and Pro372). PAM-2 is rendered in ball (A) or stick (B-D) mode, whereas residues as stick mode (element color code). All non-polar hydrogen atoms are hidden.
References


Neuronal nicotinic acetylcholine receptors are highly targeted protein targets used in treating many neurological conditions including cognition, depression, schizophrenia and neurodegeneration (Freedman, 2014, Hurst, 2013). This research is the first of its kind identifying and characterizing the subtypes of the α3β2 nAChR. We have hypothesized that considering the high levels of mRNA α3 and β2 subunit coexpression that the α3β2 nAChR may play a more significant role in hippocampus circuitry than previously believed. In addition, considering the ratio of the mRNA subgroups we sought to differentiate α3β2 nAChR subtypes using electrophysiology. Our results suggest there are at least two distinguishable subtypes of the α3β2 nAChR. This novel result provides new drug targets for neurological conditions.

In addition, we found it intriguing and valuable to characterize PAM-2, an α7* selective positive allosteric modulator, that may be promising drug in cognitive therapeutics (Uteshev, 2014). Positive allosteric modulators that target GABA receptors have been effective in the treating anxiety, depression, and other mood disorders (Nickols, 2014). The current research also suggests in addition to positive allosteric modulators being more effective in treating cognitive disorders, they may also lend fewer negative side effects. Our research using PAM-2 sought to different the PAM-2 effect on α7 nAChR homomers and α7β2 nAChR heteromers. We found that although PAM-2 does have significant effects on both subtypes the effect is distinguishable. Therefore, PAM-2 serves as one of the only methods of distinguishing α7 from α7β2 nAChR in vivo.

The results of each chapter are significant to the field of pharmacology and neuroscience. The enhanced understanding of nAChR subtypes and their positive allosteric modulators are a gateway to many additional studies.
References


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Brigham Young University, Provo, UT current
Neuroscience, PhD

Brigham Young University, Provo, UT Apr 2013
Neuroscience, B.S., GPA: 3.83 Minor: Chemistry

Publications

“Expression of nAChR mRNA in Rat Hippocampal Interneurons.” In Preparation
Doris C Jackson, Spencer Thompson, Richard M Burgon, and Sterling N Sudweeks.

“The Human alpha 3 beta 2 Neuronal Nicotinic Acetylcholine Receptor Forms Two Distinguishable Subtypes.”
Doris C Jackson, Marcel K Hall, Sterling N Sudweeks. In Preparation

“The highly selective positive allosteric modulator 3-furan-2-yl-N-p-tolyl-acrylamide potentiates α7 and α7β2 nicotinic receptors with different efficacy.”

Presentations

Dissertation Defense May 2017
“Characterization of Neuronal Nicotinic Acetylcholine Receptors and their Positive Allosteric Modulators.”

International Conference on Nicotinic Acetylcholine Receptors, Greece May 2017
“The α3β2 Neuronal Nicotinic Acetylcholine Receptor Forms Two Distinguishable Subtypes.” Young Scientist, Oral Presentation.
Mary Lou Fulton Mentored Research Conference, Provo, UT

BYU Graduate Student Society, 3 Minute Thesis (3MT), Provo, UT
“Saving the Synapse through Cognitive Therapies.” Oral Presentation. Feb 2017

Graduate-Faculty Discussion, Provo, UT

Society for Neuroscience, San Diego, CA

Alzheimer’s Association International Conference, Toronto, Canada

LDS Life Sciences Symposium, Lehi, UT

Mary Lou Fulton Mentored Research Conference, Provo, UT
“Novel Pharmacological Target: Characterization of a3b2 nAChRs expressed in Xenopus Laevis Oocytes.” See BYU Library Student Archives. 1st place Graduate Student-Neuroscience. Poster Presentation. Apr 2016

Mary Lou Fulton Mentored Research Conference, Provo, UT

Graduate-Faculty Discussion, Provo, UT

Graduate-Faculty Discussion, Provo, UT

Society for Neuroscience, Washington, DC
“Interactions of neuronal a7 nAChR with β-Amyloid and the kinase inhibitor Genistein in Xenopus Oocytes.” Poster Presentation. Nov 2014
Neuroscience Symposium, SFN Chapter, University of Utah Oct 2014
“Interactions of neuronal a7 nAChR with β-Amyloid and the kinase inhibitor Genistein in Xenopus Oocytes.” Poster Presentation.

Undergraduate Lecture, Neurobiology, Brigham Young University Apr 2013
Taught 90 students 1-hour lecture on neuronal control of movement.

Journal Club, Biophysics Great Lab, Provo, UT 2009-10, 2013-14
Presented various peer-reviewed articles that were of significance to our lab group. Sterling Sudweeks Lab Meeting.

Alzheimer’s Association International Conference, Honolulu, HI June 2010
“Characterizing the effects of b-amylloid on neuronal nicotinic acetylcholine receptor subtypes found in the rat hippocampus.” Poster Presentation.

Other Conferences Attended

Autism Translation Research Conference, BYU 2017

Human Anatomy and Physiology Society Conference 2017

Intermountain Chapter: Society of Neuroscience, Salt Lake City, UT 2013, 2014

Work Experience

Graduate Teacher’s Assistant, Physiology of Drug Mechanisms, PDBio 561 F 2016
Graded Student Presentations
Taught review sessions and held regular office hours

Graduate Instructor, Physiology and Developmental Biology, PDBio 362 S 2015
Taught 14 hours of lecture and assisted students outside of class
Helped to generate quizzes and maintain online course software

Teacher’s Assistant, Neuroanatomy, Neuro 360 F 2014-W 2016
Taught weekly review sessions
Assisted with in classroom activities and graded weekly quizzes

Beginning Tennis Instructor, BYU, Stac 181 F 2014, W 2015
Developed and executed a unit plan
Instructed groups and individuals on the rules and techniques
Teacher’s Assistant, Behavioral Neuroscience, Neuro 380  W 2014
Met with students individually to prepare for examinations
Taught tests review for the students

Teacher’s Assistant, Cellular Biology, PDBio 360  F 2013, S 2014
Prepared the classroom for each class period
Provided tutoring and review sessions on a weekly basis

Research Assistant, Dr. Sudweeks  2010-2017
Mentored and trained new lab members
Designed experiments that lead to publications

Teacher’s Assistant, Neurobiology, Neuro 205  Dec 2012-Apr 2013
Wrote quizzes, reviewed exams, and taught review sessions weekly.

Certified Nursing Assistant, Country View Manor, Provo, UT  Dec 2009-Jan 2011
Promoted to provide daily restorative care for ~20 residents
Evaluated, designed, and implemented care plans

Honors and Awards

Research Presentation Award, BYU GSS  F 2014, W 2017
byugss@byu.edu

Research Assistantship Award  F 2015, S 2016, W 2017
Paul Reynolds
paul_reynolds@byu.edu, (801)422-1933

Mary Lou Fulton Mentoring Research Conference, 1st Place Neuroscience  2016
Patricia Wilson
patricia_wilson@byu.edu, (801)422-1355

Half-Tuition Academic Scholarship, Brigham Young University  2009-2011, 2013
Christian Hansen
christian_hansen@byu.edu, (801)422-7075

Invitation for Membership

Undergraduate Research Travel Award  2010
Richard Bobo
richard_bobo@byu.edu, (801)422-7860
Summer Medical and Dental Education Program, Stipend Recipient  
University of Nebraska Medical Center, Omaha, Nebraska  
Jul 2009

Memberships

Human Anatomy and Physiology Society  
2017

Society for Neuroscience  

ISTAART (Alzheimer’s Association)  
2016, 2010

Service and Volunteer Experience

Brain Awareness Week  
2010, 2013  
Collaborated with local elementary and high schools  
Engaged students in activities about the brain  
Trenton Simmons, simmons.trenton@gmail.com

Full-time Missionary Service  
Mar 2011-Aug 2012  
Missionary: The Church of Jesus Christ of Latter-Day Saints  
Improved communities through weekly service  
Brent Olson, brent@theolson.net

Science Fair Judge, Provo, UT  
2010  
Judged K-12 district level science fair projects

4-H Youth Mentor  
2008-2010  
Designed weekly activities concerning social, family, and academics  
Autumn Linsley, autumn.linsley@usu.edu

Software

Microsoft Word  
Microsoft Excel  
Microsoft Powerpoint  
EndNote  
Clampex 9.0  
Clampfit  
Snap Gene  
Adobe Connect  
GraphPad Prism, InStat  
Brain Storm
References

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Mike Brown, Associate Professor, Department of Physiology and Developmental Biology, Neuroscience Center, BYU
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R Paul Evans, Assistant Professor, Microbiology & Molecular Biology, BYU
3139 LSB, Provo, UT 84602
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Johnathan Wisco, Associate Professor, Physiology and Developmental Biology (Human Anatomy), BYU
2028 LSB, Provo, UT 84602
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Glenna Padfield, Student Activities Program Coordinator
203C RB, Provo, UT 84602
801.422.1601
glenna_padfield@byu.edu

Arminda Suli, Assistant Professor, Department of Physiology and Developmental Biology, Neuroscience Center, BYU
3048 LSB, Provo, UT 84602
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asuli@byu.edu