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# A Doxycycline Inducible HEK-293 Model for the Characterization and Screening of ∂3β2 Nicotinic Acetylcholine Receptors

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A thesis submitted to the faculty of
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Master of Science

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ABSTRACT

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Nicotinic acetylcholine receptors (nAChR) are found widely throughout the body. Like all members of the cys-loop family of receptors, nAChRs are composed of five protein subunits, each with a large extra-cellular domain and four transmembrane domains. Together these subunits form a binding domain, transmembrane pore, and selectivity filter. Neuronal nicotinic acetylcholine receptors, formed exclusively from α2-10 and β2-4 subunits, can form in many arrangements and stoichiometries. Each arrangement can have varying binding affinities and channel kinetics, resulting in great modulatory control. α3 and β2 subunit mRNA is found in CA1 interneurons in the stratum radiatum and stratum oriens of the rat hippocampus, and in surprising expression frequency and ratios. Further study of α3 and β2 subunit mRNA injected into *Xenopus laevis* oocytes yields interesting results about the potential for two α3β2 subtypes. These results were in intriguing, and prompted further study to better characterize and screen the α3β2 nAChR. In order to do so, a model was needed where the α3β2 nAChR could be studied in a more physiologically relevant mammalian environment, with consistent control over α3 and β2 subunit expression ratios, and sufficient protein expression and functionality. To this end, we created a doxycycline inducible HEK-293 cell line, stably transfected with the genetic sequences for the α3 and β2 subunits and NACHO, a transmembrane protein of the neuronal endoplasmic reticulum, which has been shown to mediate the assembly of α3β2 and other nAChRs. This new model is able to induce expression various ratios between α3 and β2 subunits in a consistent, manner, proving to be valuable tool in the characterization and screening of the α3β2 nAChR.

Keywords: neuronal nicotinic acetylcholine receptors, cell culture, HEK-293, electrophysiology, tetracycline inducible promoter

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF PROTOCOLS

## INTRODUCTION

Acetylcholine receptors (AChR), (named for their endogenous ligand acetylcholine), are transmembrane receptors found widely throughout the body in locations such as the neuromuscular junction, autonomic ganglia, and pre- and post-synaptic neurons in the brain (Albuquerque, Pereira, Alkondon, & Rogers, 2009). AChRs are divided into two categories, the metabotropic muscarinic AChRs, and the ionotropic nicotinic AChRs (nAChR) (Albuquerque et al., 2009), the latter of which will be the focus of this study. Similar to the other members of the cys-loop family of receptors, nAChRs are composed of five protein subunits which form a binding domain, transmembrane pore, and selectivity filter (Albuquerque et al., 2009). Combinations of different subunits yield receptors with varying kinetics (Albuquerque et al., 2009). Neuronal nAChRs, found in the central nervous system, are composed of α2-10, and β2-4 AChR subunits (Hurst, Rollema, & Bertrand, 2013). Though there are other nAChR subunits found outside the CNS (Hurst et al., 2013), many different functional receptors can be made from these neuronal subunits (Gotti et al., 2009). As neuronal nAChRs are associated with many physiological functions and pharmacological effects (Albuquerque et al., 2009), it is worthwhile to characterize all prevalent types of nAChRs to better understand their role in physiological functions and as potential therapeutic sites.

Hippocampal interneurons in CA1 stratum radiatum and stratum oriens contain many nAChR subtypes (Sudweeks & Yakel, 2000). These interneurons modulate the synchronous firing of pyramidal cells, the main neuron type important for learning and memory (Figure 1). Electrophysiology recordings from these interneurons show that the kinetics for nAChR current traces in response to ACh vary widely - some interneurons sharp, quickly desensitizing currents,

while others display rounded, slowly desensitizing currents, further demonstrating the diversity of nAChR found in the CA1 hippocampal interneurons (Alkondon, Pereira, & Barbosa, 1997).

Previous RT-qPCR work on aspirated CA1 interneurons from the stratum radiatum and stratum oriens in Wistar rats showed that α3 and β2 subunit mRNA was the most frequently co-expressed mRNAs in the tested interneurons, with 52% of interneurons expressing both α3 and β2 (Table 1A and 1B) (Unpublished data, Sudweeks lab).

When looking at the population of interneurons expressing both α3 and β2 mRNA, there is an overall average 1:1 ratio of α3:β2, however, no individual interneuron had a 1:1 α3:β2 ratio. Rather, there are two populations of interneurons with α3:β2 ratios of roughly 1:3 and 3:1, suggesting that there are two α3β2 subtypes. Knowing that the α4β2 nAChR makes two subtypes - $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$ (Nelson, Kuryatov, Choi, Zhou, & Lindstrom, 2003) - it is possible that the α3β2 receptor is also forming in these same stoichiometries - $(\alpha3)_3(\beta2)_2$ and $(\alpha3)_2(\beta2)_3$ (Figure 2, Figure 3).

Whole cell electrophysiology recordings show kinetic differences between *Xenopus laevis* ooctyes injected with either 1:5 or 5:1 ratios of α3:β2 mRNA, supporting the idea that the α3β2 receptors forms two subtypes. In response to ACh, the 1:5 dose-response curve was shifted to the left compared to 5:1 injection, demonstrating a higher affinity for ACh (Figure 4). Given the apparent differences in the two potential subtypes, the α3β2 nAChR could potentially provide two additional pharmaceutical targets for cognitive disorders.

Because of the unique distribution of α3 and β2 subunit mRNA found in CA1 stratum radiatum and stratum oriens interneurons, and the varying responses to ACh found in *Xenopus laevis* oocytes, the α3β2 nAChR is an intriguing point for research. To further characterize and screen the α3β2 nAChR for pharmaceutical purposes, we needed a more physiologically relevant

and consistent model than the *Xenopus laevis* oocytes. As part of this model, we would need the ability to reliably be able to alter the expression ratios between the α3 and β2 subunits, and would need robust protein expression for future studies. Unfortunately, previous studies have had difficulty getting robust protein expression of α3β2 nAChRs in mammalian cell lines. Recently, however, a transmembrane protein of the neuronal endoplasmic reticulum, called NACHO, has been found to help mediate the assembly of α3β2 and other nAChRs, and increase surface protein expression (Matta et al., 2017). To this end, an α3, β2, and NACHO stably transfected, inducible mammalian cell culture model was created to screen and characterize the α3β2 nAChR in a way that is physiologically relevant and consistent. The construction and testing of this model is described in this report.

## MATERIALS AND METHODS

### *Plasmid Cloning*

Three plasmids were constructed for transfection into HEK-293 Flp In T-Rex cells: FUNW-PGK-CHRNA3-HA, pCDNA5-FRT-TO-CHRNB2-FLAG, and Lenti-NACHO-Puro.

#### FUNW-PGK-CHRNA3-HA:

The PGK and CHRNA3 component sequences of FUNW-PGK-CHRNA3-HA were PCR amplified from pBS/Pac1 PGK and pCMV6-XL5 (Origene), respectively (Protocol 1). PCR primers for the CHRNA3 sequence were designed such that following PCR amplification, an HA epitope tag is attached to the C-terminus of the protein. All PCR products were designed to have overlapping end sequences for Gibson Assembly. PCR products were run on an agarose gel to confirm band size, and gel extracted using the Monarch DNA Gel Extraction Kit and protocol (New England BioLabs). The backbone plasmid, FUNW, was linearized using the restriction

enzyme, Pac1, and gel extracted. The linearized FUNW, and PGK and CHRNA3 PCR products were combined in a Gibson Assembly Reaction (Protocol 2). This plasmid confers neomycin resistance. (Figure 5)

pCDNA5-FRT-TO-CHRNB2-FLAG:

CHRNB2 was PCR amplified from the plasmid pCMV6-XL5 (Origene) (Protocol 1). Primers were designed to encode the epitope tag, FLAG at the C terminus. The backbone plasmid, pCDNA5-FRT-TO was linearized with restriction enzymes KPN1 and NOT1, and gel extracted. CHRNB2 was also restricted with KPN1 and NOT1 and gel extracted in preparation for directional cloning, and then ligated with the linearized pCDNA5-FRT-TO using T4 Ligase (Protocol 3). The backbone, pCDNA5-FRT-TO, is an inducible expression vector to be used with the Flp-In T-REx system in our HEK-293 cells. This system allows for our gene of interest, the β2 subunit to be inserted at one specific genetic location, the FRT site, and to have inducible expression in response to doxycycline due to the TET-On system, just upstream from the β2 sequence. When tetracycline, or one of its derivatives bind the tet-promoter, downstream expression will occur. The more doxycycline present, the more β2 expression. This plasmid confers hygromycin resistance. (Figure 6).

Lenti-NACHO-Puro:

The NACHO sequence was PCR amplified from a geneblock ordered from Integrated DNA Technologies, and the puromycin sequence was PCR amplified from Lenti-OsTIR-Puro (Protocol 1). Primers designed for Gibson Assembly. The backbone plasmid, Lenti-Blast, was linearized and restricted with ECOR1 and BamH1 to remove the blasticidin sequence. All

component pieces and linearized backbone were gel extracted and then combined via Gibson Assembly (Protocol 2). This plasmid confers puromycin resistance. (Figure 7)

All PCR reactions utilized α-Phusion polymerase and all primers were ordered from Invitrogen (Carlsbad, CA). Primers designed with SnapGene software. After Gibson Assembly or T4 Ligation, plasmids were tested for correct assembly by restriction and Sanger Sequencing.

*Transformation, Colony Selection, and Plasmid Extraction*

Following successful assembly and ligation, plasmids were transformed into bacterial cells for colony selection using Top10 cells (Protocol 4). After plating transformed cells onto ampicillin agar plates and incubating overnight, colonies were selected, and grown in ampicillin containing LB broth for 14-18 hours, at 37C and 250 rpm in the incushaker. Resultant bacteria was then collected, and mini-preped, utilizing the Monarch Plasmid Mini Prep Kit (New England BioLabs). Plasmid concentrations were measured using a NanoDrop Spectrophotometer.

*Cell culture*

HEK-293 Flp-In T-Rex cells were obtained from Dr. Alder (Univesity of Pittsburgh), and cultured in Dulbecco's Modified Eagle Medium (DMEM) (+4.5g/L D-Glucose, + L-Glutamine, -Sodium Pyruvate) (10% FBS DMEM), purchased from Simga-Aldrich, with 10% Fetal Bovine Serum, purchased from Gibco Life Sciences. Cells were split every 48-72 hours to promote continual growth and decrease contamination and incubated at 37C and 5% CO2.

*Transfection and Selection*

Two different transfection protocols were required of this study, depending on the plasmid design. After successful pCDNA5-FRT-TO-CHRNB2-FLAG stable transfection and

selection, cells were grown out and FUNW-PGK-CHRNA3-HA and Lenti-NACHO-Puro were subsequently stably transfected and selected for, such that by the end of Lenti-NACHO-Puro selection, all three plasmids were stably transfected into the HEK-293 Flp-In T-REx cells.

pCDNA5-FRT-TO-CHRNB2-FLAG:

Because the β2 subunit is to be controlled by the tetracycline inducible promoter, it was vital that the β2 sequence be inserted at only one, specific site. To achieve this, the pCDNA5-FRT-TO-CHRNB2-FLAG plasmid was co-transfected along with pOGG44, a plasmid encoding Flp Recombinase, an enzyme that causes the recombination of the Flp-In site in the HEK-293 Flp-In T-Rex cells and our gene of interest, the β2 subunit. HEK-293 Flp-In T-REx cells were transfected with pCDNA5-FRT-TO-CHRNB2-FLAG and pOGG44 via TransIT-293 Transfection Reagent and protocol (Mirus Bio). Cells stably transfected with pCDNA5-FRT-TO-CHRNB2-FLAG were then selected for by adding hygromycin to the growth media (150 ug hygromycin/ 1 mL 10% FBS DMEM) after 48 hours, and colonies selected 10-14 days following hygromycin addition.

FUNW-PGK-CHRNA3-HA and Lenti-NACHO-Puro:

FUNW-PGK-CHRNA3-HA and Lenti-NACHO-Puro are both lentivirus plasmids, resulting in stable transfections once they are virally packaged and transfected onto target cells. FUNW-PGK-CHRNA3-HA and Lenti-NACHO-Puro were transfected into HEK-293 FT cells via TransIt-293 Transfection Reagent and protocol and lentivirus packaging protocol (Protocol 5), along with co-transfected plasmids Δ8.9 and VSV.g. Following viral packaging and subsequent and viral transfection of HEK-293 Flp-In T-REx cells, FUNW-PGK-CHRNA3-HA

and Lenti-NACHO-Puro were selected for by adding neomycin (800 ug/1 mL FBS DMEM) and puromycin (2 ug / 1 mL 10% FBS DMEM) 24 hours post viral transfection. Colonies were picked 10-14 days following selection agent addition.

*Doxycyline Dosing*

Doxycycline, ordered from Sigma-Aldrich, was diluted in 10% FBS DMEM to make concentrations of 0, 3.3, and 10.0 uM. Normal 10% FBS DMEM was removed, replaced with varying concentrations of doxycycline containing 10% FBS DMEM, and incubated for 48 - 96 hours.

*RNA Isolation and Reverse Transcription*

Following doxycycline dosing, RNA was extracted and isolated using TRIzol RNA Isolation Reagent and protocol (Invitrogen). Reverse transcription reactions were carried out to create cDNA from the template RNA strands using SuperScript III First-Strand Synthesis Supermix and protocol (Invitrogen).

*Quantitative Polymerase Chain Reaction (qPCR)*

Triplicate qPCR reactions were carried out using the reverse transcribed cDNA as template, the previously designed PCR primers and iTaq Universal SYBR Green Supermix and associated protocol (BioRad) (Protocol 6). Ct values were measured and relative yield was calculated using the ΔCt method.

RESULTS

Following the transfection of HEK-293 Flp-In T-REx cells with our three genes of interest, the α3 nAChR subunit, the β2 nAChR subunit, and NACHO, treating with doxycycline

at various time points and concentrations to induce β2 expression, and isolating RNA, qPCR reactions were carried out to 1.) Positively determine that the transfections were successful, 2.) Determine if the inducible tetracycline promoter was functional, and 3.) Determine the relative amounts of α3 nAChR subunit mRNA expression compared to β2 nAChR mRNA expression in response to doxycycline.

All three genes of interest were positively identified in the qPCR findings. At 48 hours of incubation without doxycycline, the average Ct for α3, β2, and NACHO was 27.8, 33.3, and 25.7, respectively. Calculated using the $2^{\Delta Ct}$ method (Livak & Schmittgen, 2001), α3 expression is 45.05 times greater than β2 expression at 0 uM doxycycline (Table 2). Because β2 expression is controlled by the presence of doxycycline, it makes sense that β2 expression crosses threshold so many cycles later.

As the doxycycline concentration increased, the relative expression ratio between α3 and β2 decreased. After 48 hours incubation in 3.3 and 10 uM doxycycline media, α3 was expressed 12.8 and 6.9 times greater than β2, respectively, which was expected with the increasing doxycycline concentration (Table 2). These findings not only help quantify the relative amounts of α3 and β2 expression, but also show that the tetracycline inducible promoter is functional (Figure 6).

At 96 hours incubation with 0, 3.3, and 10 uM doxycycline, the expression ratio between α3 and β2 widened greatly. α3 expression was 49.5, 103.3, and 128.8 times higher compared to β2 expression at 96 hours incubation with 0, 3.3, and 10 uM doxycycline (Table 3). Although these are early findings and more work needs to be done to confirm, it appears that the tetracycline promoter does not increase β2 expression over longer periods of time.

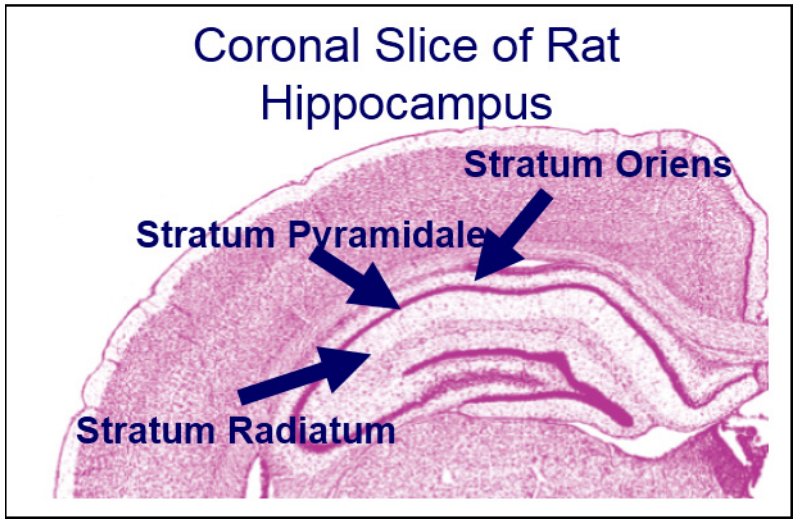


Figure 1. Coronal slice of Rat Hippocampus

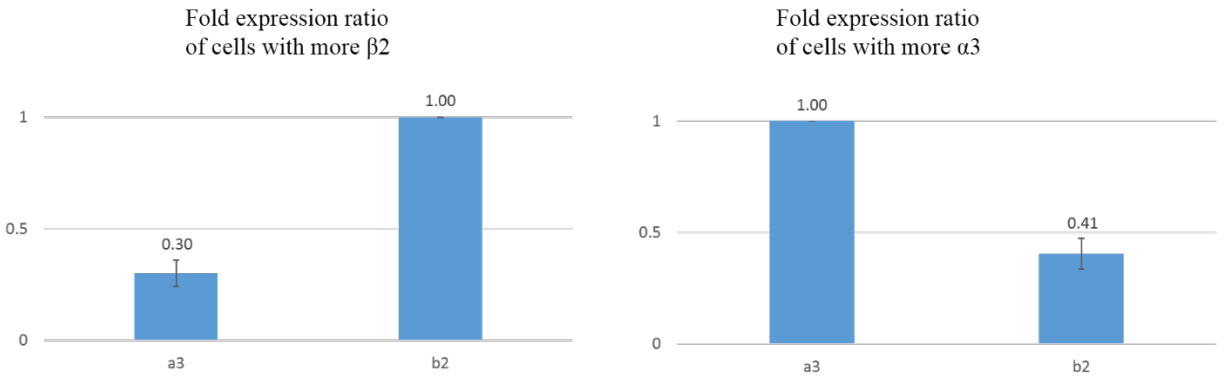


Figure 2. Fold expression ratio of cells with more α3 or β2

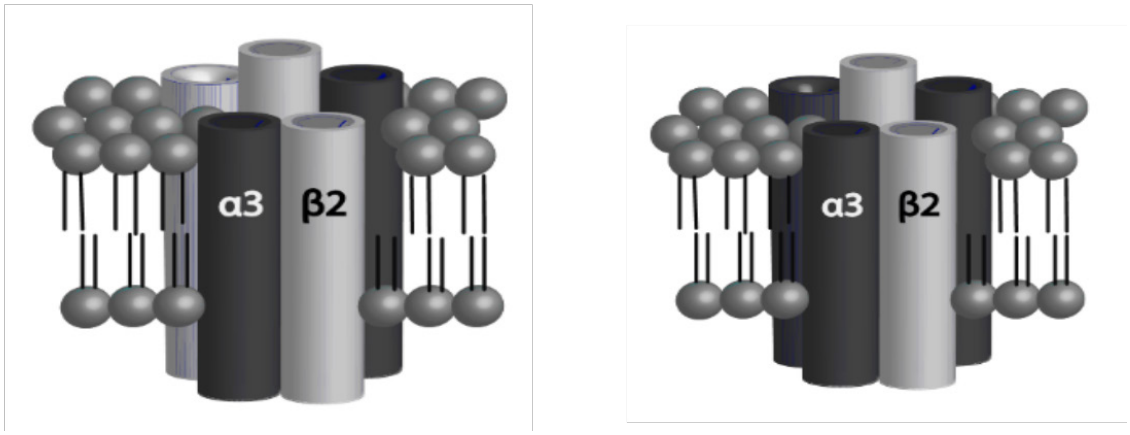


Figure 3. Proposed stoichiometries of potential α3β2

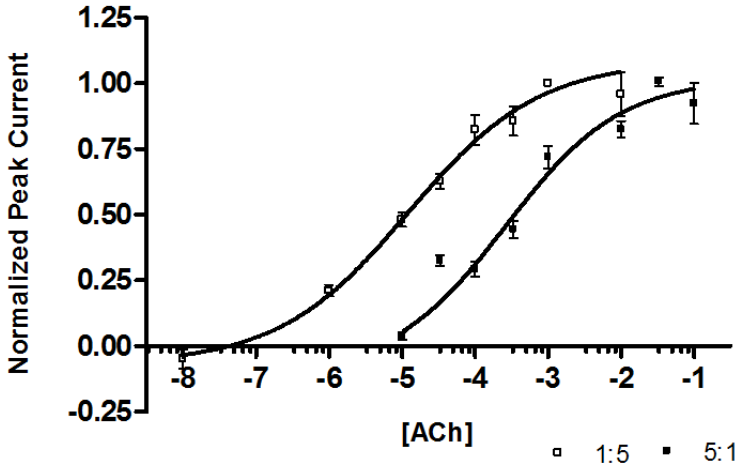


Figure 4. Dose response curve of Xenopus laevis oocytes injected with α3 and β2 mRNA in ratios of 1:5 and 5:1

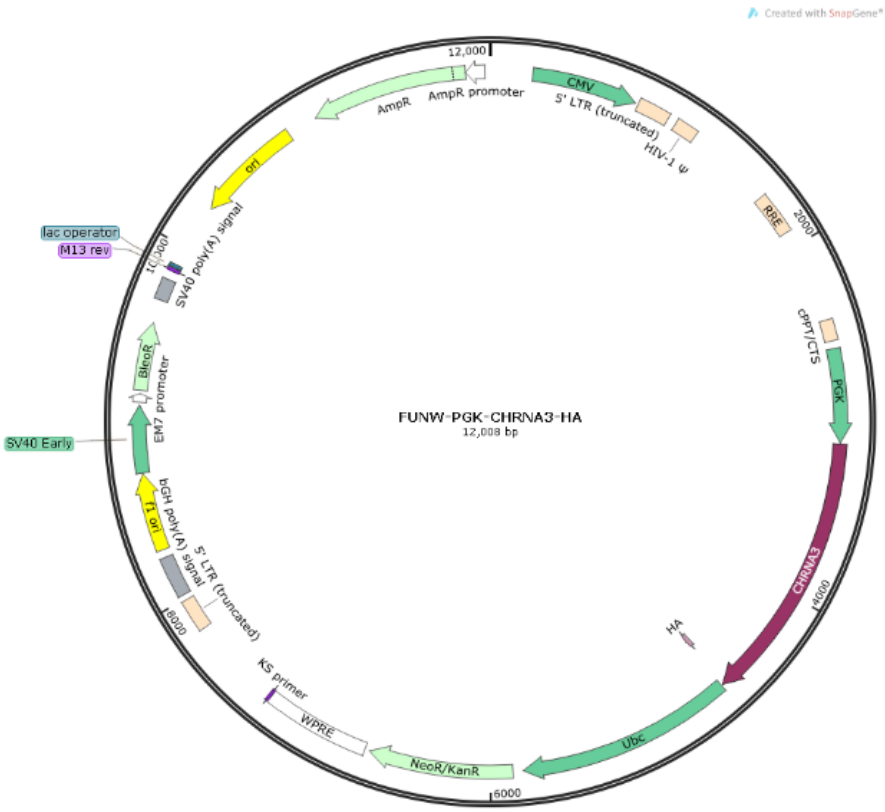


Figure 5. Plasmid map of FUNW-PGK-CHRNA3-HA. Made with SnapGene software.

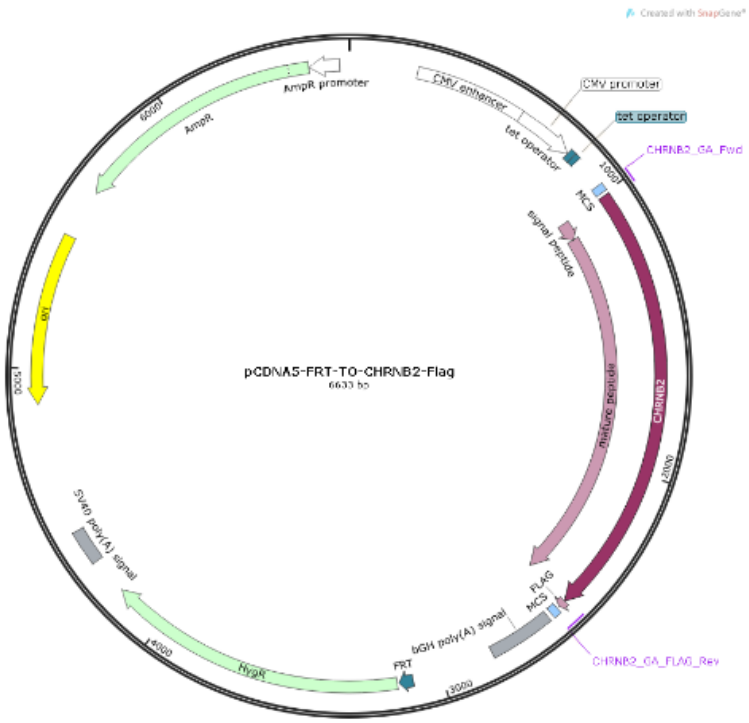


Figure 6. Plasmid map of pCDNA5-FRT-TO-CHRNB2-FLAG. Made with SnapGene

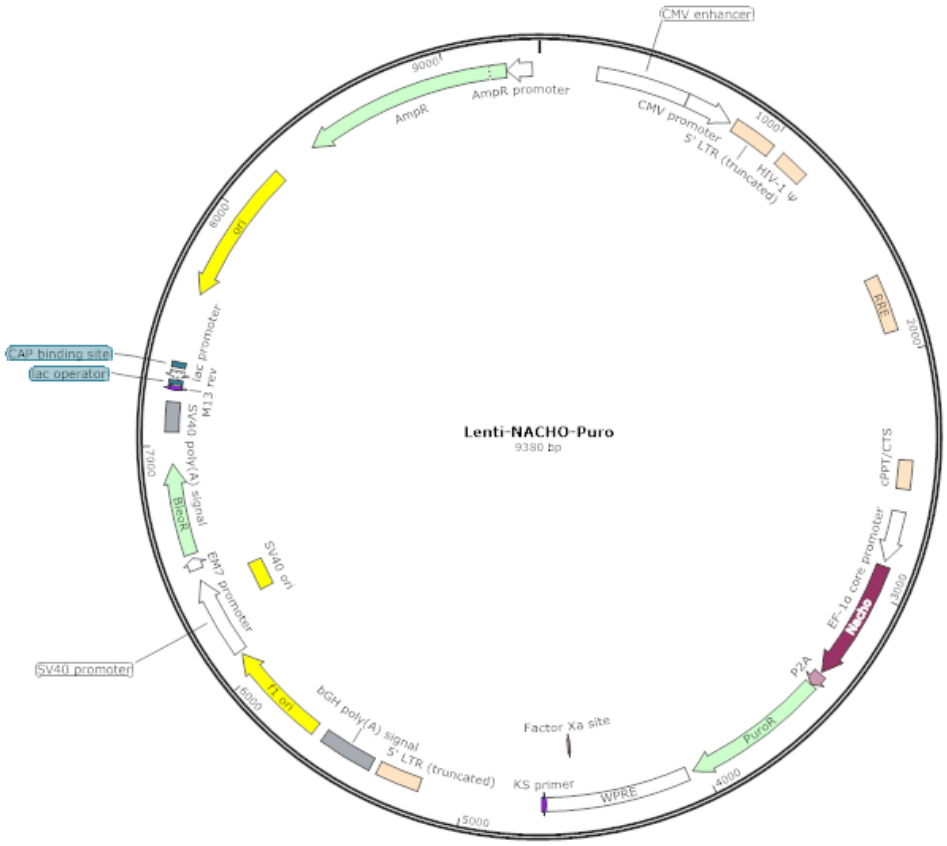


Figure 7. Plasmid map of Lenti-NACHO-Puro. Made with SnapGene software**.**

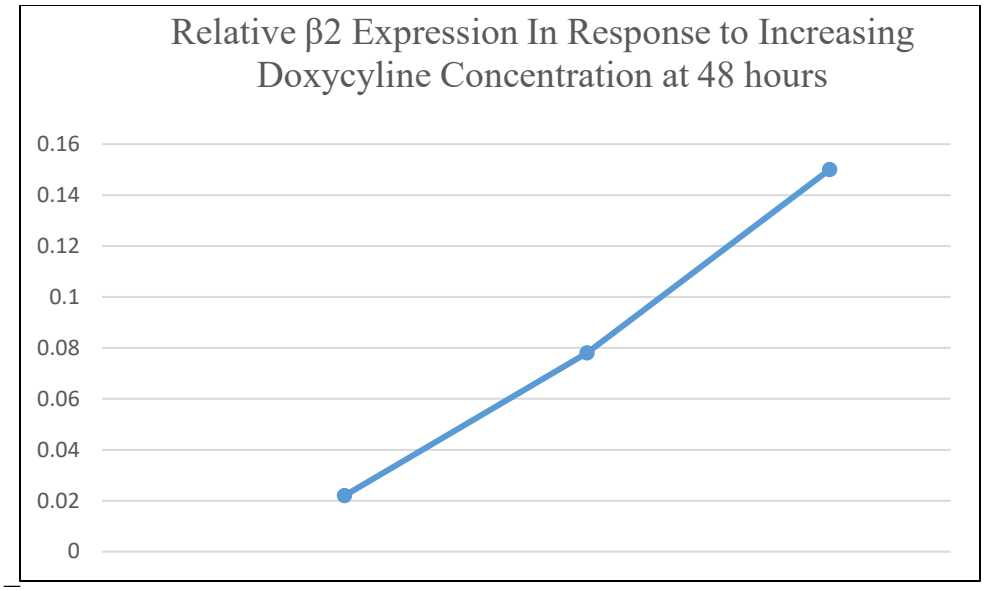


Figure 8. Relative β2 Expression In Response to Increasing Doxycycline Concentration at 48 hours. Inverse of relative α3 expression ratio.

| Proportion of Cells containing each subunit | |
|---|---|
| Subunit | Proportion |
| α3 | 0.54 |
| β2 | 0.54 |
| α5 | 0.43 |
| α7 | 0.42 |
| β4 | 0.42 |
| β3 | 0.40 |
| α2 | 0.37 |
| α4 | 0.27 |

Table 1. qPCR nAChR Subunit Expression - Proportion of Cells Containing Each Subunit

| Subunit Co-expression of nAChR mRNA | |
|---|---|
| Percentage | Subunit Co-expression |
| 52% | α3 and β2 |
| 49% | α3 and α5 |
| 44% | α7 and β2 |
| 42% | α5 and β2 |
| 41% | α3 and β4 |
| 41% | β2 and β4 |
| 39% | α3 and β3 |
| 38% | β2 and β3 |

Table 2. qPCR nAChR Subunit Expression - Subunit Coexpression of nAChR Subunits

| Doxycycline Concentration | Average α3 Ct | Average β2 Ct | Times More α3 Than β2 |
|---|---|---|---|
| **0 uM** | **27.8** | **33.3** | **45.0** |
| **3.3 uM** | **26.5** | **30.2** | **12.8** |
| **10 uM** | **27.3** | **30.1** | **6.9** |

Table 3. Relative yield of α3 and β2 expression following 48 hours incubation with varying concentrations of doxycycline containing media.

| Doxycycline Concentration | Average α3 Ct | Average β2 Ct | Times More α3 Than β2 |
|---|---|---|---|
| **0 uM** | **26.1** | **31.7** | **49.5** |
| **3.3 uM** | **25.6** | **32.4** | **103.3** |
| **10 uM** | **26.6** | **33.6** | **128.9** |

Table 4. Relative yield of α3 and β2 expression following 96 hours incubation with varying concentrations of doxycycline containing media.

## DISCUSSION

nAChRs are found widely throughout the brain. Given the modulatory role of hippocampal interneurons and unique α3 and β2 nAChR subunit mRNA profile found in these interneurons, we wanted to create a model in which the α3β2 nAChR could be further studied in a more physiologically relevant environment, with consistent control over mRNA expression ratios. By creating the described doxycycline inducible, α3β2 and NACHO stably transfected HEK-293 cell line, we have created a model that can be used to study the α3β2 nAChR with control over the expression ratio between α3 and β2 subunits by increasing or decreasing the concentration of doxycycline in the media.

From the qPCR data, it is clear that the all three genes of interest were successfully transfected. NACHO's relative expression seems to be similar to that of α3's expression and as long as NACHO is expressed, for this study, that is ultimately sufficient. The addition of NACHO expression in the cells should result in robust protein expression. Robust α3 mRNA is consistently detected and β2 expression ratio relative to α3 changes with increasing doxycycline concentration, though this needs further elucidation.

We found that the tetracycline inducible promoter in our cells responded well to low uM concentrations of doxycycline, similar to the findings of Das, Zhou, Metz, Vink, and Berkhout (2016) at 48 hours. We considered the possibility that increased incubation time could increase relative β2 expression. Interestingly, incubations of 96 hours with any doxycycline dosing did not seem effective. Meehan, Puett, and Narayan (2004) found that the greatest downstream expression utilizing a Tet-On system in HEK-293 cells occurred between 18 and 24 hours following the addition of doxycycline. This seems to fit with our data, where the shorter time

point shows more effective expression. Future studies should focus on time points earlier than 48 hours to increase β2 expression compared to α3.

Though this study focuses on the presence and relative abundance of α3, β2, and NACHO mRNA, this model will be used to study the functionality of the α3β2 nAChR as a protein. To that end, the HA and FLAG tags attached to the c-terminus ends of α3 and β2, respectively, will prove useful in protein studies. Another (and perhaps better) confirmation of functional protein will be to perform whole cell electrophysiology recordings to look at channel kinetics and study drug-receptor interactions.

The purpose of this study was to create a new, consistent, mammalian model in which the α3β2 nAChR can be studied. In creating the described doxycycline inducible HEK-293 cell line, a model now exists that can consistently control relative expression between α3 and β2, enhanced by the presence of the nAChR mediator, NACHO. This model can now be used for further characterization and screening of the α3β2 nAChR.

PROTOCOLS

Protocol 1. αPhusion PCR

On ice, for 50 µL reaction, add:

10 µL HF Buffer (10 µM)

1.0 µL dNTPs (5 µM)

5.0 µL FWD primer (5 µM)

5.0 µL REV primer (5 µM)

1.0 µL DNA template

5.0 µL 10x PCRx Enhancer Solution

0.5 µL αPhusion

H2O for volume total to equal 50 µL

| **Temperature** | **Time (minutes)** |
|---|---|
| **98 °c** | **2:00** |
| **58 °c** | **1:00** |
| **72 °c** | **1:00** |
| **Repeat 35x** | |
| **22 °c** | **5:00** |
| **4 °c** | ∞ |

***Depending on the reaction, designed primer annealing temperatures, and length of reaction, these times and temperatures may vary some. Primers designed to have annealing temperatures around 60°c.**

Protocol 2. Gibson Assembly

Sample

5 uL Gibson Assembly Master Mix

5 uL of component pieces, in 3:2 ratio of whatever component piece is smaller in size

Run at 50C for 1 hour

Control

5 uL H2O

5 uL of component pieces, in 3:2 ratio of whatever component piece is smaller in size

Run at 50C for 1 hour

Protocol 3. T4 Ligase

Sample

1 uL T4 Ligase buffer

1 uL T4 Ligase enzyme

3:1 molar equivalency of plasmid insert to linearized back bone

Add H2O until total volume equals 10 uL

Run for 1 hour at 22c

Control

1 uL T4 Ligase buffer

3:1 molar equivalency of plasmid insert to linearized back bone

Add H2O until total volume equals 10 uL

Run for 1 hour at 22c

Protocol 4. Top10 Bacterial Cell Transformation

On ice:

Thaw Top10 cells

Add 1-5 uL of sample to 50 uL of thawed Top10 cells

Add 1-5 uL of control to 50 uL of thawed Top10 cells

For both the sample and control:

Chill for 30 minutes on ice

Heat shock for 30 seconds in 42c $H_2O$

Immediately put samples back on ice

Add 250 uL Lysogeny Broth (LB)

Shake for 1 hour at 37c and 225 rpm

Plate sample and control onto ampicillin agar plates in the following amounts

100 uL of sample or control

10 uL of sample or control, and 90 uL LB

Invert plates and incubate overnight at 37C

Protocol 5. Lentiviral Packaging and Collecting

Have HEK-293 FT cells plated to reach 80% confluence at the time of transfection in 10 cm plate in complete media (10% FBS DMEM)

24 hours after plating, follow TransIt-293 Transfection Reagent protocol.

The TransIt-293 Transfection Reagent protocol recommends 2.5 ug of total plasmid DNA. Use the following ratios to calculate the needed amounts of each plasmid.

| **Plasmid** | **10 cm Plate** |
|---|---|
| **LV Vector (i.e. FUNW plasmid)** | **0.95 µg** |
| **Δ8.9** | **1.25 µg** |
| **VSV.G** | **0.3 µg** |

48 hours after transfection, collect media with pipette, and replace media on cells with complete media. Repeat collection at 72 hours post transfection.

Dispense collected media onto target cells (HEK-293 Flp-In T-Rex cells) at 50% confluency.

After second collection has been made, remove media on target cells, and dispense collected media on target cells.

48 hours following transduction, add selection reagent.

Protocol 6. Quantitative Polymerase Chain Reaction

Volumes and concentration amounts of all reagents were according to iTaq Universal SYBR Green Supermix protocol (BioRad).

The following is the time and temperature protocol used for the thermocycler.

| **Temperature (ºC)** | Time (Minutes) |
|---|---|
| 95.0 | 3:00 |
| 1x | |
| 95.0 | 0:15 |
| 55.0 | 0:20 |
| 72.0 | 0:30 |
| 40x | |

REFERENCES

Albuquerque EX; Pereira EFR; Alkondon M; Rogers SW. Mammalian Nicotinic Acetylcholine Receptors: From Structure to Function. Physiological Reviews. 2009;89(1):73-120.

Alkondon M; Pereira EFR; Barbosa CTF; Albuquerque EX. Neuronal nicotinic acetylcholine receptor activation modulates gamma-aminobutyric acid release from CA1 neurons of rat hippocampal slices. Journal of Pharmacology and Experimental Therapeutics. 1997;283(3):1396-411.

Das, A. T., Zhou, X., Metz, S. W., Vink, M. A., & Berkhout, B. (2016). Selecting the optimal Tet-On system for doxycycline-inducible gene expression in transiently transfected and stably transduced mammalian cells. *Biotechnology Journal, 11*(1), 71-79. doi:10.1002/biot.201500236

Gotti C; Clementi F; Fornari A; Gaimarri A; Guiducci S; Manfredi I, et al. Structural and functional diversity of native brain neuronal nicotinic receptors. Biochemical Pharmacology. 2009;78(7):703-11.

Hurst R; Rollema H; Bertrand D. Nicotinic acetylcholine receptors: From basic science to therapeutics. Pharmacology & Therapeutics. 2013;137(1):22-54.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods, 25*(4), 402-408.

Matta JA; Gu SY; Davini WB; Lord B; Siuda ER; Harrington AW, et al. NACHO Mediates Nicotinic Acetylcholine Receptor Function throughout the Brain. Cell Reports. 2017;19(4):688-96.

Meehan, T. P., Puett, D., & Narayan, P. (2004). Tightly regulated and inducible expression of a

yoked hormone-receptor complex in HEK 293 cells. *Journal of Molecular Endocrinology, 32*(1), 247-255. doi:10.1677/jme.0.0320247

Nelson, M. E., Kuryatov, A., Choi, C. H., Zhou, Y., & Lindstrom, J. (2003). Alternate stoichiometries of alpha 4 beta 2 nicotinic acetylcholine receptors. *Molecular Pharmacology, 63*(2), 332-341. doi:10.1124/mol.63.2.33

Sudweeks SN; Yakel JL. Functional and molecular characterization of neuronal nicotinic ACh receptors in rat CA1 hippocampal neurons. Journal of Physiology-London. 2000;527(3):515-28.

Zoli M; Pistillo F; Gotti C. Diversity of native nicotinic receptor subtypes in mammalian brain. Neuropharmacology. 2015;96:302-11