Chapter One

Construction of a Fluorescent α5 Subunit

The significance of the α5 containing nAChR receptor (α5* receptor) has been a challenging question for researchers since its characterization by Role et al. in 1996 [19]. Elucidation of the unique contribution of the α5 subunit is complicated by several factors including the lack of α5 specific pharmacological agents. As an accessory subunit, α5 does not participate in ligand binding, making it difficult to selectively activate or block α5* receptors. This challenge, combined with the similarity in EC_{50} and dose response to agonist of α5* receptors to its parent receptors α4β2 or α3β4, and the lack of a reliably α5-specific antibody, increases the difficulty in isolating a pure population of α5* receptors for detailed analysis [7, 13, 20]. Lester et al. has demonstrated that individual nAChR subunits can be labeled with fluorescent proteins for detection of individual subunit expression using live cell, fluorescence microscopy [21]. Using a combination of fluorescence confocal microscopy, total internal reflection fluorescence (TIRF) microscopy and advanced fluorescence techniques, such as Försters resonance energy transfer (FRET), it is possible to detect changes in membrane expression, trafficking, and receptor complex formation of fluorescent nAChRs [6, 22-23]. We hypothesized that selective labeling of an α5 subunit would be possible for detection and observation of α5* receptor populations.

The mouse *Chrna*5 gene with partial 5' and 3' untranslated regions (UTR), as well as the mouse *Chrna*5 genes with D/N mutation and partial 5' and 3' UTRs were generously provided by Dr. Jerry Stitzel at the University of Colorado Boulder. These genes were subcloned into pcDNA3.1(+) mammalian expression vectors. *Chrna5* is heavily enriched

for guanine and cytosine nucleotides in the 5' region, making polymerase chain reaction (PCR) extension of the entire gene difficult. Many efforts were made to optimize the PCR protocol for efficient and consistent extension of the entire gene. Those optimized methods are reported in appendix i. Several pcDNA3.1(+) constructs containing variations of the *Chrna*5 gene with and without UTRs were constructed and are listed in table 1.

The nAChR M3-M4 loop is a preferred location for insertion of fluorescent protein (FP) tags [21-22]. It has been inferred from studies of other nAChR subunits that M3-M4 loop localized FP labels do not interfere with formation of the receptor complex, intracellular trafficking, or function of nAChRs [21]. Sequence alignment of mouse nAChR M3-M4 loops revealed that α5 contains the shortest loop (50 amino acids), and concerns were raised that the insertion of a ~ 200 amino acid fluorescent protein such as GFP would produce steric interference, preventing efficient incorporation of the labeled subunit into a receptor complex or the proper trafficking of an α5*-FP receptor to the plasma membrane. Consequently, several variants of α5-FP fusion products were constructed (see table 1).

Building on previous work by Lester et al., monomeric enhanced green fluorescent protein (mEGFP) was selected as the FP for insertion into the α5 gene. mEGFP exhibits enhanced fluorescence over wild-type GFP (wtGFP) and also contains an alanine-to-lysine mutation at position 206 that prevents multimerization of GFP molecules [24-25]. These modifications make mEGFP more compatible with biological imaging experiments and FRET analysis. Two intrasubunit locations for mEGFP were selected within the α5 M3-M4 loop.

Table 1

5' UTR	Sequence	Linker (A-G-A)	FP Insertion Site	Linker (G-A-G)	3' UTR	3'MUT	C – term FP
yes	Mouse α5				yes		
	Mouse α5						
	Mouse α5 D398N						
yes	Mouse α5		mEGFP 385		yes		
yes	Mouse α5	yes	mEGFP 385	yes	yes		
yes	Mouse α5		mGFP 378		yes		
	Mouse α5	yes	mEGFP 378	yes			
	Mouse α5	yes	mCherry 385	yes			
	Mouse α5	yes	mCherry 378	yes	yes		
	Mouse α5 D398N	yes	mEGFP 378	yes			
	Mouse α5	yes	mEGFP 378	yes		yes	
	Mouse α5 D398N	yes	mEGFP 378	yes		yes	
	Mouse α5						mEGFP
	Mouse α5 D398N						mEGFP

These locations were chosen due to their distance from the position of the identified SNP (amino acid 397 in the mouse protein, 398 in the human) and from identified trafficking motifs (see figure 1.1) [23, 26]. A flexible linker of alanine and glycine (A-G-A or G-A-G) was included with the inserted mEGFP sequence. Other α5-mEGFP fusion constructs with the mEGFP sequence fused to the c-terminal region of the α5 sequence

were also constructed using overlap PCR. Table 2 comprehensively displays all of the α5-mEGFP variants that have been used experimentally throughout the duration of this project.

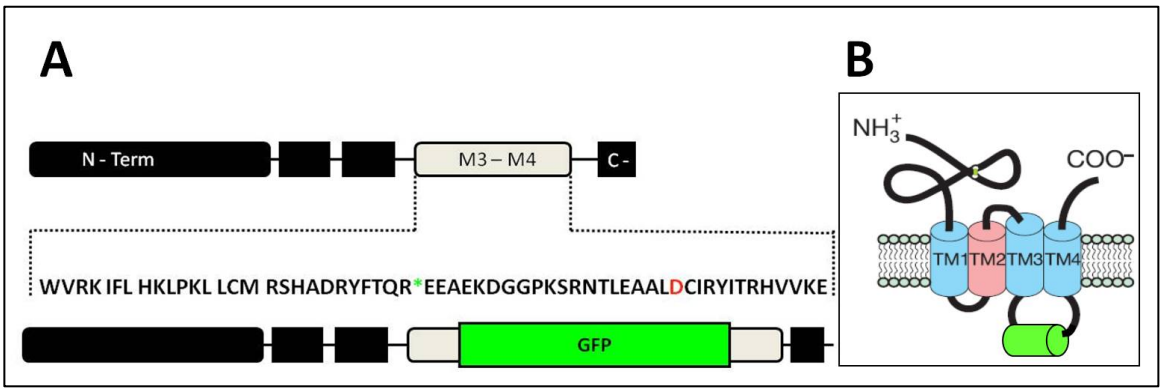


Figure 1.1
Schematic of the nAChR α5 subunit.
1.1A shows the amino acid sequence of the α5 M3-M4 loop. Asterisk indicates position 358, one of the two intracellular loop locations for mEGFP insertion.
1.1B Shows an alternative view of the α5 subunit, showing helical transmembrane domains and mEGFP barrel in the M3-M4 loop region (not to scale).

Table 2

5'UTR	Mouse α5 Template	mEGFP Location	3'UTR
✓	wt	385	✓
	wt	378	✓
	wt	378	
	V280S	378	
	D398N	378	✓
	D398N	378	
	V280S D398N	378	

Full length expression of the α5-mEGFP fusion constructs was verified using Western blot. α5-mEGFP constructs were expressed in either HEK293 or HEK293-T cells and immunoprecipitated using a mouse anti-GFP antibody. Extracts were then separated by gel electrophoresis and blotted with a rabbit anti-GFP primary and goat anti-GFP-HRP secondary antibodies for visualization. Resultant bands were then compared to an expressed α4-mEGFP control (see figure 1.2).

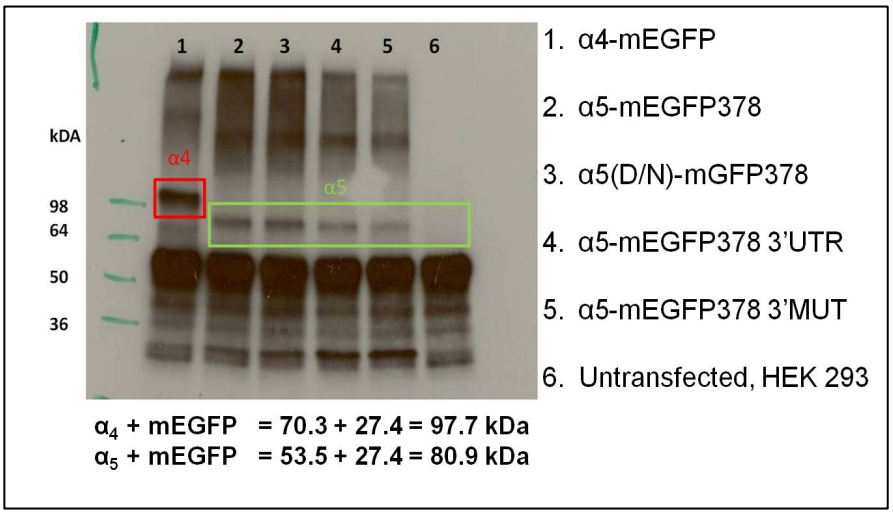


Figure 1.2
Western blot demonstrates full length (80.9 kDa) expression of α5-mEGFP constructs in HEK293 cells.
Lane 1 contains α4-mEGFP as a positive control.
Lanes 2-5 contain variants of α5-mEGFP
Lane 6 contains lysate from untransfected cells.

Next, α5-mEGFP constructs were assayed for fluorescence (see figure 1.3). Many of the fusion proteins were expressed in both N2a and HEK293 cell lines. N2a or HEK293 cells were transiently transfected with α5-mEGFP, α4, and β2 subunits and assayed for fluorescence using confocal microscopy. Fluorescence levels for all of the α5-mEGFP constructs were consistently low when compared to α4-mEGFP and β3-mYFP controls. This lack of expression was a consistent challenge moving forward with investigations using these constructs. However, α5-mEGFP387, a DNA construct lacking the 5' and 3' UTR regions, with A-G-A and G-A-G flanking linker sequences, and with the mEGFP inserted at position 378 was ultimately chosen as the optimum construct for use in future experiments.

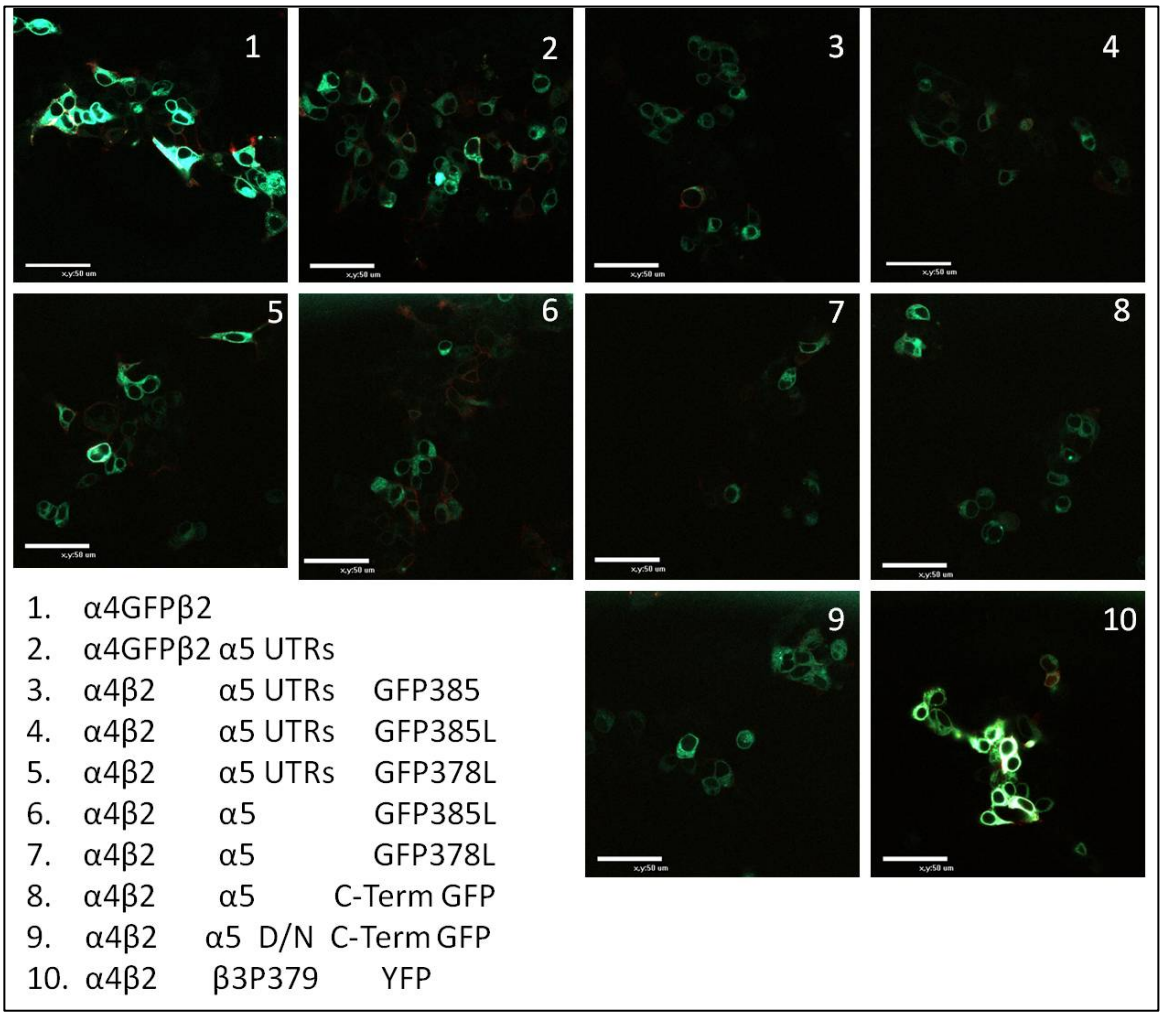


Figure 1.3A
Images of N2a cells expressing fluorescent nAChR constructs and a red fluorescent plasma membrane marker PM-mCherry. Images were taken 24 h post-transfection with a scanning confocal microscope after sample excitation with 488 nm laser. Images 1, 2, and 10 display images of HEK293 cell expressing α4-mEGFP or β3-mYFP as positive controls for relative expression. Images 3 – 9 show the low relative fluorescence of the α5-mEGFP constructs.

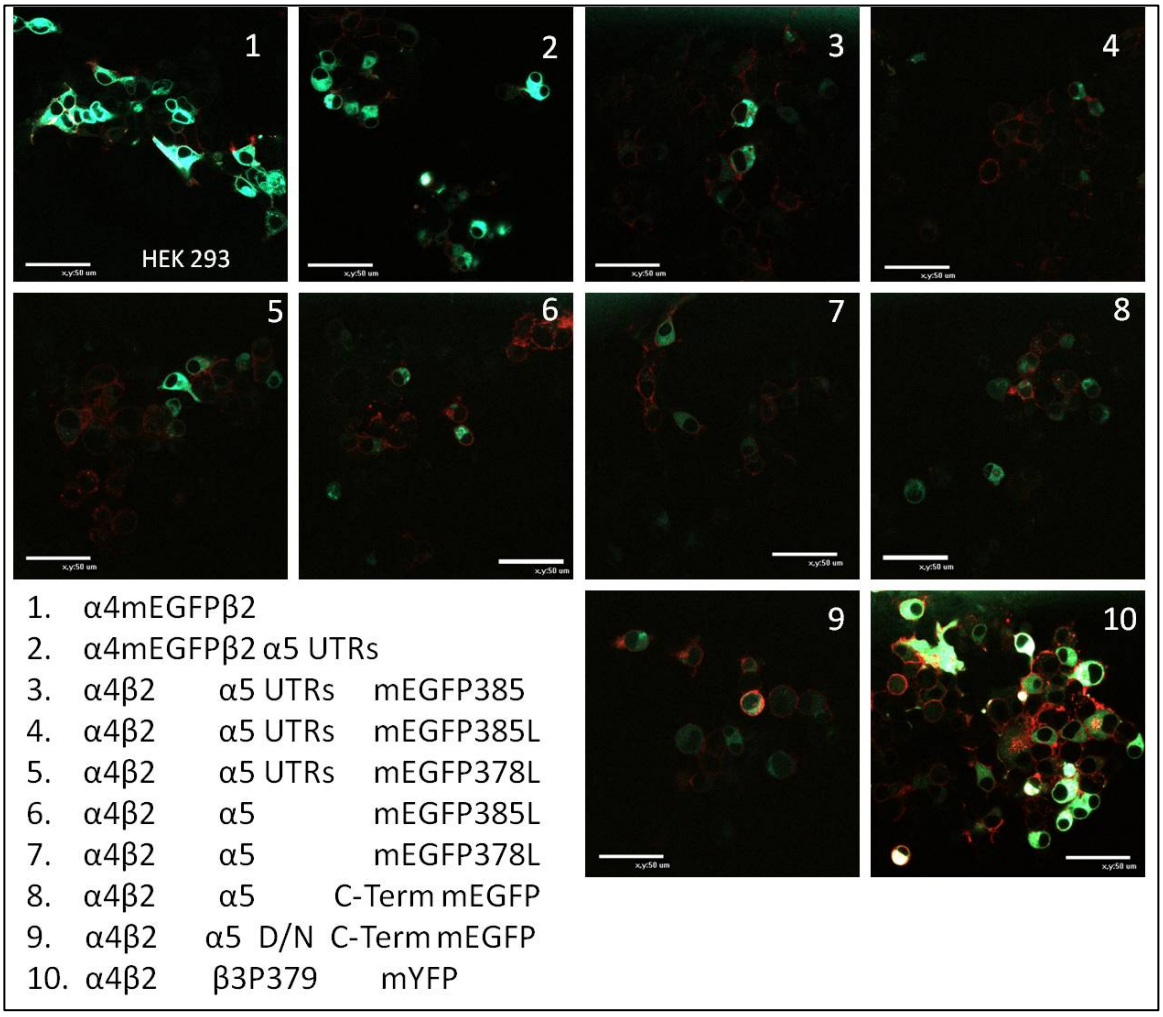


Figure 1.3B

Images of HEK293 cells expressing fluorescent nAChR constructs and a red fluorescent plasma membrane marker PM-mCherry. Images were taken 24 h post-transfection with a scanning confocal microscope after sample excitation with 488 nm laser. Images 1, 2, and 10 display images cells expressing α4-mEGFP or β3-mYFP as positive controls for relative expression. Images 3 – 9 show the low relative fluorescence of the α5-mEGFP constructs.

Intracellular assembly of α5-mEGFP378 (α5-mEGFP) with α4 and β2 subunits to form an α4β2α5-mEGFP receptor was evaluated using normalized Försters resonance energy transfer (NFRET). NFRET uses the distance dependence of energy transfer between a donor and acceptor fluorophore to measure interactions between proteins. Attention is paid to the theory of FRET and NFRET in chapter 4. FP labeled α4 and/or β2 subunits were co-expressed in N2a cells, and NFRET measurements were performed. In this case the α5-mEGFP acted as the FRET donor, and a red fluorescent protein, mCherry, labeling the α4 subunit, functioned as the FRET acceptor (see chapter 4) [6, 23, 26]. Low incidence of expression of the α5-mEGFP (15%) made finding cells expressing both α5-mEGFP and α4-mCherry at levels sufficient for FRET signal detection challenging, but we are able to demonstrate intracellular association of α5-mEGFP and α4-mCherry when co-expressed with β2 in transiently transfected N2a cells (figure 1.4).

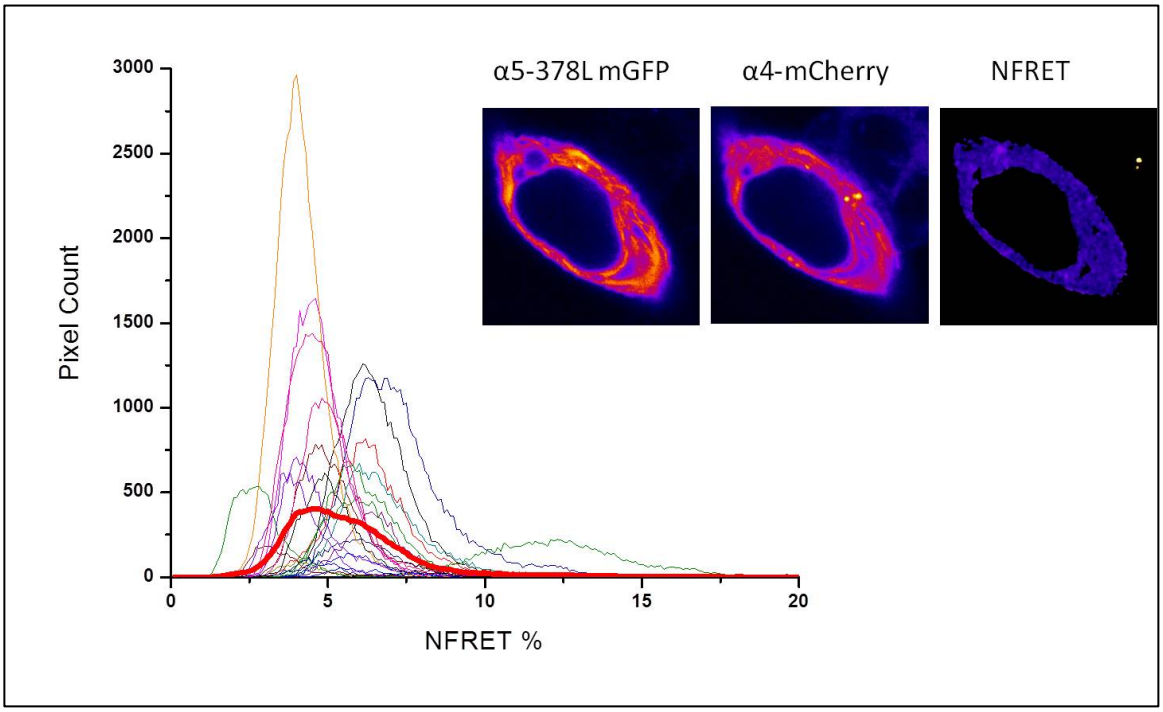
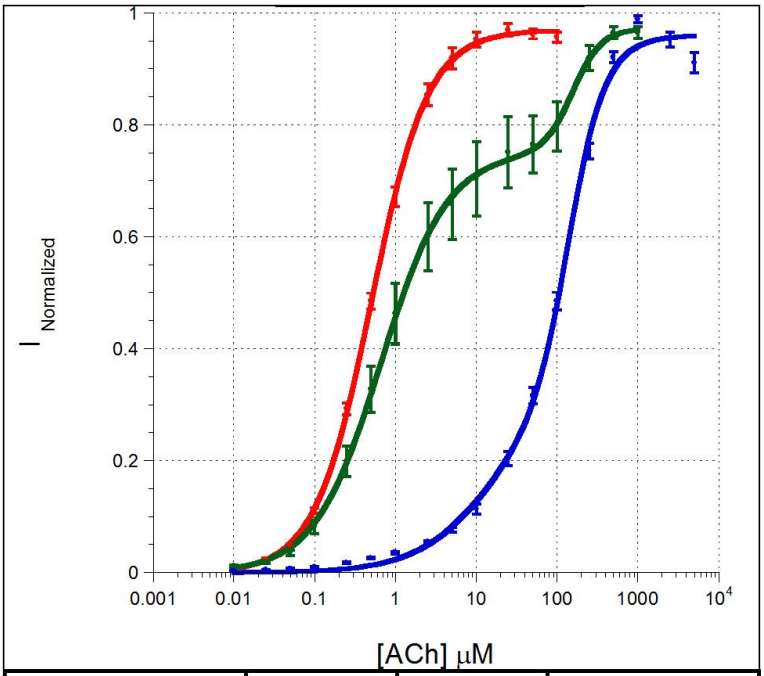


Figure 1.4
23 individual cell histograms of NFRET positive pixels in images of HEK293 cells expressing α5-mEGFP378, α4-mCherry, and β2 subunits. Bolded histogram describes the average of all 23 cells.
Boxes on the right display sample images of an HEK293 cell expressing α5-mEGFP378, α4-mCherry, and the associate NFRET values illustrated as a heat map of expression intensity.

Within the cell nAChRs, like other receptor complexes, are synthesized and assembled in the endoplasmic reticulum (ER). Once assembled, the complete pentameric complex is trafficked to the plasma membrane (PM) for insertion. It is understood that only complete nAChR pentamers reside in the PM. Functional response of α5-mEGFPα4β2 receptors to agonist would confirm proper synthesis, incorporation, and trafficking of α5-mEGFPα4β2 receptors. Electrophysiology on *Xenopus* oocytes expressing α5α4β2 receptors was performed in collaboration with Chris Marotta, and some results are reported in Marotta et al. [20]. Both α5α4β2 and $(\alpha4)_2(\beta2)_3$ have a similar dose response to acetylcholine (ACh), and no pharmacological agents for selective activation of α5 receptors have yet been reported.

Mutation of a conserved leucine (L) at the 9' position within a nAChR subunit confers "hypersensitivity" to agonist in receptors containing L9' mutant subunits [27-29]. Thus, 9' mutations can act as a reporter for subunit incorporation. Interestingly, the "accessory" subunits α5 and β3 do not have the conserved amino acid at their 9' location. Both the α5 and β3 subunit sequences have a valine (V) occupying the 9' position. Hypersensitive β3 subunits have been constructed by mutation of either the 9' or 13' positions [22]. To construct a potentially hypersensitive α5 subunit, the 9'V at position 280 was mutated to an S to create α5V9'S and α5V9'S-mEGFP constructs. These constructs were then subcloned into the oocyte expression vector pGEMhe for use in electrophysiology experiements. Injection of α59'S mRNA with α4 and β2 in a 10:10:1 ratio was sufficient to cause a leftward shift in dose response to ACh. We conclude that α59'Sα4β2 receptors are formed, and the increase in receptor sensitivity indicates that the 9'S mutation can act as a reporter for α5 incorporation (see figure 1.5). Injection of α59'S–mEGFP mRNA with α4 and β2 in a 10:10:1 ratio results in a biphasic dose response to ACh. This shift indicates that the fluorescently labeled subunit is incorporated into an α59'S-mEGFPα4β2 receptor, but subunit incorporation may not be as efficient.



Receptor	Expression System	mRNA Injection Ratio α5:α4:β2	EC_{50} (µM)
$(\alpha4)_3(\beta2)_2$	Oocyte	0:10:1	141.4 ± 26.9
α59'Sα4β2	Oocyte	10:10:1	0.503 ± 0.01
α59'S- mEGFP378 α4β2	Oocyte	10:10:1	0.660 ± 0.27 156.1 ±14.3

Figure 1.5
Acetylcholine dose response curves from $(\alpha4)_3(\beta2)_2$ –bottom curve α59's-mEGFPα4β2-middle curve α59'Sα4β2 –top curve. Receptors expressed in *Xenopus* oocytes and recorded with an OpusExpress.

Table below describes the mRNA injection ratio of each subunit and calculated EC_{50} value from the dose response curve.

With these data we are able to demonstrate construction of a functional fluorescent α5-mEGFP subunit. Complete protein expression was verified using IP and Western blot. α5-mEGFP was shown to be fluorescent via heterologous expression in transiently transfected N2a, HEK293, and HEK293-T cells using fluorescence confocal microscopy. Expression of fluorescent α5 is consistently low compared to other fluorescent α subunits, and low expression of many α5-mEGFP constructs has been observed in N2a and HEK293 clonal cell lines. Functional verification of α5-mEGFP was performed by oocyte electrophysiology, and the optimized α5-mEGFP construct, α5-mEGFP378, was selected for use in additional experiments.