

Subcellular Trafficking, Pentameric Assembly, and Subunit Stoichiometry of Neuronal Nicotinic Acetylcholine Receptors Containing Fluorescently Labeled $\alpha 6$ and $\beta 3$ Subunits[§]

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ABSTRACT

Neuronal nicotinic acetylcholine (ACh) receptors are ligand-gated, cation-selective ion channels. Nicotinic receptors containing $\alpha 4$, $\alpha 6$, $\beta 2$, and $\beta 3$ subunits are expressed in midbrain dopaminergic neurons, and they are implicated in the response to smoked nicotine. Here, we have studied the cell biological and biophysical properties of receptors containing $\alpha 6$ and $\beta 3$ subunits by using fluorescent proteins fused within the M3-M4 intracellular loop. Receptors containing fluorescently tagged $\beta 3$ subunits were fully functional compared with receptors with untagged $\beta 3$ subunits. We find that $\beta 3$ - and $\alpha 6$ -containing receptors are highly expressed in neurons and that they colocalize with coexpressed, fluorescent $\alpha 4$ and $\beta 2$ subunits in neuronal soma and dendrites. Förster resonance energy transfer (FRET) reveals efficient, specific assembly of $\beta 3$ and $\alpha 6$ into nicotinic receptor pentamers of various subunit compositions.

Using FRET, we demonstrate directly that only a single $\beta 3$ subunit is incorporated into nicotinic acetylcholine receptors (nAChRs) containing this subunit, whereas multiple subunit stoichiometries exist for $\alpha 4$ - and $\alpha 6$ -containing receptors. Finally, we demonstrate that nicotinic ACh receptors are localized in distinct microdomains at or near the plasma membrane using total internal reflection fluorescence (TIRF) microscopy. We suggest that neurons contain large, intracellular pools of assembled, functional nicotinic receptors, which may provide them with the ability to rapidly up-regulate nicotinic responses to endogenous ligands such as ACh, or to exogenous agents such as nicotine. Furthermore, this report is the first to directly measure nAChR subunit stoichiometry using FRET and plasma membrane localization of $\alpha 6$ - and $\beta 3$ -containing receptors using TIRF.

$\alpha 6$ nicotinic ACh receptor subunits are expressed in several catecholaminergic nuclei in the central nervous system, including retinal ganglion cells (Gotti et al., 2005b), locus coeruleus (Léna et al., 1999), and dopaminergic neurons located in the substantia nigra and ventral tegmental area

(Whiteaker et al., 2000; Zoli et al., 2002; Champtiaux et al., 2003). Ligand-binding studies using the $\alpha 6$ -specific probe α -conotoxin MII suggest that many $\alpha 6^*$ (* indicates that other subunits may be present in the receptor) receptors are located on presynaptic terminals in the superior colliculus and striatum (Whiteaker et al., 2000). Indeed, this binding activity disappears in the brains of $\alpha 6$ knockout mice (Champtiaux et al., 2002). This strikingly specific expression pattern could indicate a unique function for $\alpha 6^*$ receptors, and $\alpha 6^*$ receptors are candidate drug targets for diseases or disorders such as Parkinson's disease or nicotine addiction (Quik and McIntosh, 2006).

Functional, voltage-clamped $\alpha 6$ -dependent responses are elusive in heterologous expression systems such as *Xenopus laevis* oocytes (Kuryatov et al., 2000; Broadbent et al., 2006), but native $\alpha 6^*$ receptors are readily studied using synaptosome preparations from brain tissue (Whiteaker et al., 2000;

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ABBREVIATIONS: HEK, human embryonic kidney; nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; FRET, Förster resonance energy transfer; TIRF, total internal reflection fluorescence; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; WT, wild-type; XFP, yellow or cyan fluorescent protein; NA, numerical aperture; *E*, FRET efficiency; GluCl, glutamate-gated chloride; HS, high sensitivity; LS, low sensitivity.

Grady et al., 2002; Champiaux et al., 2003; Gotti et al., 2005a). Indeed, α -conotoxin MII-sensitive receptors are pharmacologically and stoichiometrically distinct from α -conotoxin MII-resistant receptors in mediating [3 H]dopamine release from striatal synaptosomes (Grady et al., 2002; Salminen et al., 2007). Recent studies using $\alpha 4$ and $\beta 3$ knockout mice demonstrate the existence of functional $\alpha 6\beta 2$, $\alpha 6\beta 2\beta 3$, $\alpha 6\alpha 4\beta 2$, and $\alpha 6\alpha 4\beta 2\beta 3$ receptors (Salminen et al., 2007). It is noteworthy that native $\alpha 6\alpha 4\beta 2\beta 3$ receptors have the highest affinity ($EC_{50} = 0.23 \pm 0.08 \mu\text{M}$) for nicotine of any nicotinic receptor reported to date. Because nicotine is likely to be present at concentrations $\leq 0.5 \mu\text{M}$ in the cerebrospinal fluid of smokers (Rowell, 2002), only those receptors with the highest affinity for nicotine, including some $\alpha 4^*$ and $\alpha 6^*$ receptors, are likely to be important in nicotine addiction. Although previous studies offer major conceptual advances in our understanding of $\alpha 6^*$ receptors in the brain, there is a lack of information regarding the subcellular localization and biophysical properties of $\alpha 6$ subunits.

$\beta 3$ subunits are expressed in most of the same locations as $\alpha 6$, including midbrain dopaminergic neurons projecting to the striatum (Zoli et al., 2002). $\beta 3$ knockout mice demonstrate that $\beta 3$ subunits are important for the biogenesis of $\alpha 6^*$ receptors in the brain (Cui et al., 2003; Gotti et al., 2005a). This is corroborated by studies in *X. laevis* oocytes and tissue culture cells (Kuryatov et al., 2000). $\beta 3$ also increases $\alpha 6$ -specific binding activity in HEK293 cells (Tumkosit et al., 2006). Uncertainty exists, however, because others have reported that $\beta 3$ incorporation into nAChRs acts as a dominant negative (Boorman et al., 2003; Broadbent et al., 2006), suppressing ACh-evoked responses by an incompletely understood gating mechanism. This effect occurred apparently without significantly altering the surface expression of nAChRs. What is clear is that $\beta 3$ acts more like a muscle β subunit than a "typical" neuronal β subunit; it does not participate in forming the α :non- α interface that comprises the neuronal ligand binding site, and other β subunits, either $\beta 2$ or $\beta 4$, must be present to form functional nicotinic receptors (Broadbent et al., 2006). This presents a problem both for basic and therapeutic-oriented research on $\beta 3^*$ receptors, because there are no pharmacological ligands that can visually or functionally isolate $\beta 3$ -specific actions in cell culture systems or intact brain tissue. Given the precise localization and unique functional properties of $\beta 3^*$ receptors, potential for therapeutic intervention that would be afforded by $\beta 3$ -specific probes, and involvement in nicotine addiction (Bierut et al., 2007), it is important to develop and exploit tools to study $\beta 3$.

We have sought to compare characteristics of $\alpha 6$ and $\beta 3$ with the better understood $\alpha 4$ and $\beta 2$ subunits. We previously generated fluorescently labeled $\alpha 4$ and $\beta 2$ subunits, and we used these subunits to study assembly, trafficking, and nicotine-dependent up-regulation of $\alpha 4\beta 2$ receptors (Nashmi et al., 2003). We have now fluorescently labeled $\alpha 6$ and $\beta 3$ subunits by inserting a yellow fluorescent protein (YFP) or cyan fluorescent protein (CFP) in the M3-M4 intracellular loop. With this approach, one can optically monitor functional nicotinic ACh receptors containing these subunits in live cells and in real time. We measured 1) functional responses using two-electrode voltage-clamp and patch-clamp electrophysiology, 2) subcellular distribution and colocalization in neurons using confocal microscopy and spectral imaging, 3)

receptor assembly and subunit stoichiometry using Förster resonance energy transfer (FRET), and 4) plasma membrane localization and distribution patterns using total internal reflection fluorescence (TIRF) microscopy.

Materials and Methods

Reagents. Unless otherwise noted, all chemicals were from Sigma-Aldrich (St. Louis, MO). DNA oligonucleotides for PCR and site-directed mutagenesis were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Restriction enzymes for molecular biology were purchased from Roche Diagnostics (Indianapolis, IN) or New England Biolabs (Ipswich, MA). Glass-bottomed dishes (35 mm) coated with L-polylysine were purchased from MatTek (Ashland, MA).

Cell Culture and Transfection. N2a cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (high glucose with 4 mM L-glutamine; Invitrogen, Carlsbad, CA)/Opti-MEM (Invitrogen) mixed at a ratio of 1:1 and supplemented with 10% fetal bovine serum (Invitrogen), penicillin (Mediatech, Herndon, VA), and streptomycin (Invitrogen). N2a cells were transfected in DMEM without serum or antibiotics. Transfection was carried out using Lipofectamine/PLUS (Invitrogen) according to the manufacturer's instructions and with the following modifications. For a 35-mm dish, 1 to 2 μg of total plasmid DNA was mixed with 100 μl of DMEM and 6 μl of PLUS reagent. DMEM/DNA was combined with a mixture of 100 μl of DMEM and 4 μl of Lipofectamine reagent. Rat hippocampal neurons were dissociated and plated on glass-bottomed imaging dishes as described previously (Slimko et al., 2002). For primary neuron transfection, Lipofectamine 2000 (Invitrogen) was used in conjunction with Nupherin (BIOMOL Research Laboratories, Plymouth Meeting, PA) as described below. In brief, in total 1 μg of DNA was incubated with 20 μg of Nupherin in 400 μl of Neurobasal medium without phenol red (Invitrogen), whereas 10 μl of Lipofectamine 2000 was mixed in 400 μl of Neurobasal medium (Invitrogen). After 15 min, the two solutions were combined and incubated for 45 min. Neuronal cultures in 35-mm glass-bottomed culture dishes were incubated in the resulting 800- μl mixture for 120 min, followed by removal of transfection media and refeeding of the original, pretransfection culture media.

Plasmids and Molecular Biology. Mouse $\alpha 4$ and $\beta 2$ nAChR cDNAs in pCI-neo, both untagged and modified with YFP or CFP fluorescent tags, have been described previously (Nashmi et al., 2003). Mouse $\alpha 3$ and $\beta 3$ nAChR cDNAs in pCDNA3.1 were a generous gift of Jerry Stitzel (Institute for Behavioral Genetics, University of Colorado, Boulder, CO). A full-length mouse $\alpha 6$ I.M.A.G.E. cDNA (ID no. 4501558) was obtained from Open Biosystems (Huntsville, AL). A modified $\alpha 6$ cDNA was constructed that 1) lacked the 5' and 3' untranslated regions and 2) contained a Kozak sequence (GCC ACC) before the ATG start codon to facilitate efficient translation initiation. Rat $\beta 4$ cloned into pAMV was provided by Cesar Labarca (California Institute of Technology, Pasadena, CA). pEYFP-N1 and pECFP-N1 (Clontech, Mountain View, CA) were used to construct fluorescent nAChR cDNAs. mGAT1 labeled with CFP was provided by Fraser Moss (California Institute of Technology). YFP-Syntaxin was provided by Wolffhard Almers (Vollum Institute, Oregon Health and Science University, Portland, OR). CFP-tau was provided by George Bloom (University of Virginia, Charlottesville, VA). A Quik-Change (Stratagene, La Jolla, CA) kit was used to construct $\beta 3$ (WT or XFP-modified) cDNAs containing a V13'S point mutation.

To design fluorescently labeled $\alpha 6$ and $\beta 3$ subunits, we chose to insert the XFP moiety in the M3-M4 loop each subunit. We have previously found that this region is appropriate for insertion in nAChR $\alpha 4$ and $\beta 2$ subunits (Nashmi et al., 2003), the nAChR γ subunit (data not shown), and GluCl α and β subunits (Slimko et al., 2002). Similar to our previous studies, we inserted the XFP moiety in the M3-M4 loop at positions that avoided the conserved amphipathic

α -helix and putative cell sorting motifs and phosphorylation sites (Fig. 1, A and B). To construct nAChRs with XFP inserted into the M3-M4 loop, a two-step PCR protocol was used. First, YFP or CFP was amplified with PCR using oligonucleotides (sequences available upon request) designed to engineer 5' and 3' overhangs of 15 base pairs that were identical to the site where XFP was to be inserted, in frame, into the nAChR M3-M4 loop. A Gly-Ala-Gly flexible linker was engineered between the nAChR sequence and the sequence for YFP/CFP at both the 5' and 3' ends. In the second PCR step, 100 ng of the first PCR reaction was used as a primer pair in a modified QuikChange reaction using *Pfu* Ultra II (Stratagene, Cedar Creek, TX) polymerase and the appropriate nAChR cDNA as a template. All DNA constructs were confirmed with sequencing and, in some cases, restriction mapping.

cRNA for injection and expression in *X. laevis* oocytes was prepared using a T7 or SP6 in vitro transcription kit (mMessage mMachine; Ambion, Foster City, CA) according to the manufacturer's instructions. RNA yield was quantified with absorbance at 260 nm. RNA quality was assessed by observing absorbance profiles across a range of wavelengths between 220 and 320 nm. Spectrophotometric analysis was performed using a ND-1000 spectrophotometer (NanoDrop, Wilmington, DE).

Confocal Microscopy. N2a cells were plated on 35-mm glass-bottomed dishes, transfected with nAChR cDNAs, and they were imaged live 24–48 h after transfection. *X. laevis* oocytes were imaged 3 days after RNA injection. Oocytes were placed in an imaging chamber and allowed to settle for 20 min before imaging. To eliminate autofluorescence, growth medium was replaced with an extracellular solution containing the following components: 150 mM NaCl, 4 mM KCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 10 mM HEPES, and 10 mM D-glucose, pH 7.4. Cells were imaged with a Nikon (Nikon Instruments, Melville, NY) C1 laser-scanning confocal microscope system equipped with spectral imaging capabilities and a Prior (Rockland, ME) remote-focus device. For oocytes, a Nikon Plan Apo 20 \times 0.75 numerical aperture (NA) air objective was used, whereas a Nikon Plan Apo 60 \times 1.40 NA oil objective was used for mammalian tissue culture cells. Pinhole diameter was 30–60 μm , and cells were imaged at 12-bit intensity resolution over 512 \times 512 pixels at a pixel dwell

time of 4 to 6 μs . CFP was excited using a 439.5-nm modulated diode laser, and YFP was excited with an argon laser at 514.5-nm. In most cases, imaging was carried out using the Nikon C1si DEES grating and spectral detector with 32 parallel photomultiplier tubes. This allowed us to collect spectral images (λ stacks). In such images, each pixel of the X-Y image contains a list of emission intensity values across a range of wavelengths. We collected light between 450 and 600 nm at a bandwidth of 5 nm. The 515-nm channel was intentionally blocked while we used the 514.5-nm laser for YFP bleaching. Because the emission profile of YFP and CFP significantly overlap, we used the Nikon EZC1 linear unmixing algorithm to reconstruct YFP and CFP images. Experimental spectral images with both YFP- and CFP-labeled nAChR subunits were unmixed using reference spectra from images with only YFP- or CFP-labeled nAChR subunits. For each pixel of a spectral image, intensity of YFP and CFP was determined from fluorescence intensity values at the peak emission wavelength derived from the reference spectra.

Spectral FRET Analysis. To examine FRET between various nAChR subunits, the acceptor photobleaching method (Nashmi et al., 2003) was used with a modified fluorescence recovery after photobleaching macro built into the Nikon EZC1 imaging software. In this method, FRET was detected by recording CFP dequenching during incremental photodestruction of YFP. A spectral image was acquired once before YFP bleaching and at six time points every 10 s during YFP bleaching at 514.5 nm. Laser power during bleaching varied from cell to cell, but was between 25 and 50%. One bleach scan per cycle was used. This bleaching protocol was optimized to achieve 70 to 80% photodestruction of YFP while still enabling us to record incremental increases in CFP emission at each time point. In the confocal microscope, nAChRs labeled with XFP usually exhibit a uniform, intracellular distribution, regardless of the subunit being examined. This is consistent with our previous observations (Nashmi et al., 2003). To measure FRET, spectral images were unmixed to their CFP and YFP components as described above. We found little or no difference in FRET for various cellular structures or organelles in N2a cells, and we measured CFP and YFP mean intensity throughout the entire cell by selecting the cell perimeter as the boundary of a region of interest in Nikon's EZC1 software. CFP and

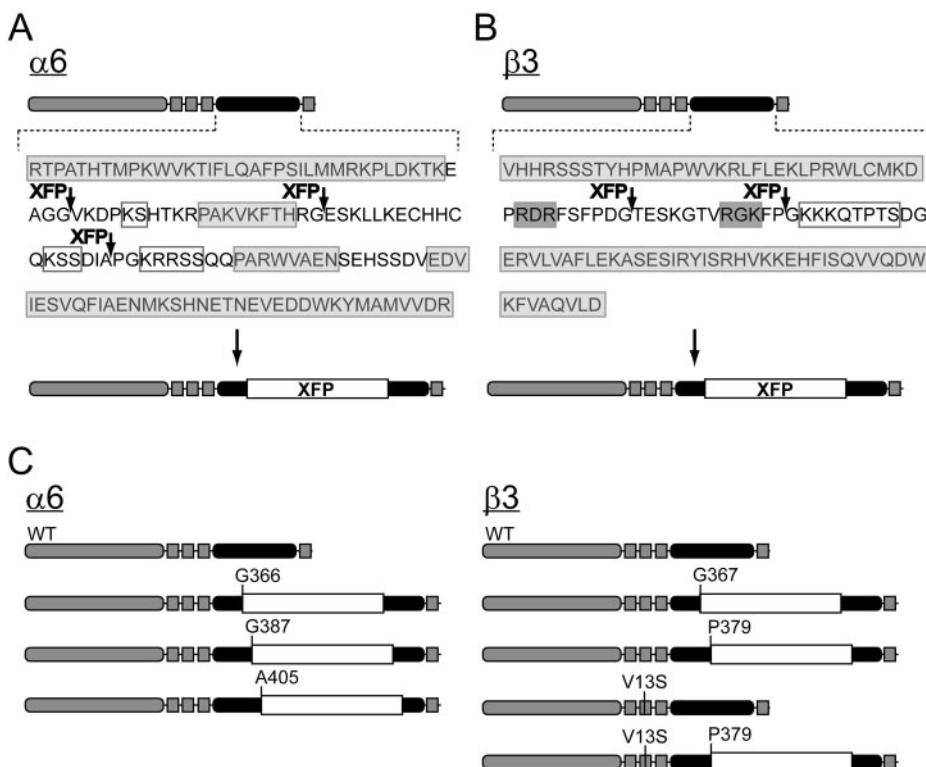


Fig. 1. $\alpha 6$ and $\beta 3$ nicotinic ACh receptor constructs used in this study. A, XFP insertion points in the $\alpha 6$ M3-M4 intracellular loop. The M3-M4 loop primary sequence of the mouse $\alpha 6$ nAChR subunit was analyzed for sequences predicted to be involved in forming α -helices (light gray boxes), phosphorylation sites (white boxes), or intracellular trafficking motifs (dark gray boxes); these were specifically avoided. Arrows adjacent to XFP indicate insertion points. The inserted XFP (YFP or CFP) protein was modified 1) to have a flexible Gly-Ala-Gly linker flanking the XFP coding sequence and 2) to lack its STOP codon. B, XFP insertion points in $\beta 3$ M3-M4 intracellular loop. Mouse $\beta 3$ -XFP fusion proteins were designed similarly to $\alpha 6$, as indicated in A. C, $\alpha 6$ and $\beta 3$ nAChR constructs used in this study. In addition to WT, three $\alpha 6$ -XFP and two $\beta 3$ -XFP fusions were constructed. A V13S mutation on the WT and XFP background was introduced into $\beta 3$ for characterization in *X. laevis* oocytes.

YFP components were saved in Excel format, and fluorescence intensities were normalized to the prebleach time point (100%). FRET efficiency (E) was calculated as $E = 1 - (I_{DA}/I_D)$, where I_{DA} represents the normalized fluorescence intensity of CFP (100%) in the presence of both donor (CFP) and acceptor (YFP), and I_D represents the normalized fluorescence intensity of CFP in the presence of donor only (complete photodestruction of YFP). The I_D value was extrapolated from a scatter plot of the fractional increase of CFP versus the fractional decrease of YFP. The E values were averaged from several cells per condition (see Table 1 for n values). Data are reported as mean \pm S.E.M.

TIRF Microscopy. N2a cells cultured in glass-bottomed, polyethylenimine-coated imaging dishes were transfected with cDNA mixtures as described above. Cells, superfused with the same imaging solution used for confocal microscopy, were imaged 18 to 24 h after transfection to minimize overexpression artifacts. TIRF images were obtained with an inverted microscope (Olympus IX71; Olympus America, Inc., Center Valley, PA) equipped with a 488-nm air-cooled argon laser (P/N IMA101040ALS; Melles Griot, Carlsbad, CA). Laser output was controlled with a UNIBLITZ shutter system and drive unit (P/N VMM-D1; Vincent Associates, Rochester, NY) equipped with a Mitutoyo (Mitutoyo America, City of Industry, CA) micrometer to control TIRF evanescent field illumination. TIRF imaging was carried out with an Olympus PlanApo 100 \times 1.45 NA oil objective, and images were captured with a 16-bit resolution Photometrics Cascade charge-coupled device camera (Photometrics, Tucson, AZ) controlled by SlideBook 4.0 imaging software (Intelligent Imaging Innovations, Santa Monica, CA).

Two-Electrode Voltage-Clamp Electrophysiology. Stage V to VI *X. laevis* oocytes were isolated as described previously (Quick and Lester, 1994). Stock RNAs were diluted into diethyl pyrocarbonate-treated water and injected 1 day after isolation. RNA was injected in a final volume of 50 nl per oocyte using a digital microdispenser (Drummond Scientific, Broomall, PA). After injection, oocytes were incubated in ND-96 solution (96 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES/NaOH, pH 7.6) supplemented with 50 μ g/ml gentamicin and 2.5 mM sodium pyruvate. After 1 to 4 days for nAChR expression, oocytes were used for recording or confocal microscopy.

TABLE 1

FRET efficiency calculations for nicotinic ACh receptors with various subunit compositions

Unless noted otherwise, all experiments with $\alpha 6$ and $\beta 3$ subunits were performed with $\alpha 6^{A405}$ and $\beta 3^{P379}$. Data are reported as mean FRET $E \pm$ S.E.M. n refers to the number of independently analyzed cells.

Subunit Composition	FRET E	n
	%	
$\alpha 4C/\beta 2Y$	29 \pm 2	10
$\alpha 4/\beta 2C/\beta 3Y$	22 \pm 2	9
$\alpha 4C/\beta 2/\beta 3Y$	20 \pm 2	8
$\beta 3Y/GAT1C$	-3 \pm 3	9
$\alpha 4/\beta 2C/\beta 3Y^{G367}$	22 \pm 1	7
$\alpha 6Y/\beta 2C$	36 \pm 2	12
$\alpha 6Y/GAT1C$	1 \pm 4	9
$\alpha 6Y^{G387}/\beta 2C$	38 \pm 2	7
$\alpha 6Y^{G366}/\beta 2C$	29 \pm 2	11
$\alpha 6Y/\beta 2C/\beta 3$	32 \pm 2	13
$\alpha 6Y/\beta 2/\beta 3C$	25 \pm 3	8
$\alpha 6/\beta 2Y/\beta 3C$	36 \pm 2	14
$\alpha 4Y/\alpha 4C/\beta 2$	22 \pm 2	10
$\alpha 4/\beta 2Y/\beta 2C$	16 \pm 2	9
$\alpha 6Y/\alpha 6C/\beta 2$	28 \pm 2	22
$\alpha 4/\beta 2/\beta 3Y/\beta 3C$	3 \pm 1	10
$\alpha 4Y/\alpha 4C/\beta 2/\beta 3$	14 \pm 1	10
$\alpha 6Y/\alpha 6C/\beta 2/\beta 3$	21 \pm 1	10
$\alpha 6Y/\alpha 4C/\beta 2$	22 \pm 1	21
$\alpha 6Y/\alpha 4C/\beta 2/\beta 3$	21 \pm 2	11
$\beta 3Y/GluCl \beta C$	6 \pm 4	5
$\alpha 6Y/GluCl \beta C$	14 \pm 2	5

Y refers to YFP, and C refers to CFP.

Agonist-activated nicotinic receptor responses were measured by two-electrode voltage-clamp recording using a GeneClamp 500 (Molecular Devices, Sunnyvale, CA) voltage clamp. Electrodes were constructed from Kwik-Fil borosilicate glass capillary tubes (1B150F-4; WPI, Sarasota, FL) using a programmable microelectrode puller (P-87; Sutter Instrument Company, Novato, CA). The electrodes had tip resistances of 0.8 to 2.0 M Ω after filling with 3 M KCl. During recording, oocytes were superfused with Ca²⁺-free ND-96 via bath-application and laminar-flow microperfusion using a computer-controlled application and washout system (SF-77B; Warner Instruments, Hamden, CT) (Drenan et al., 2005). The holding potential was -50 mV, and ACh was diluted in Ca²⁺-free ND-96 and applied to the oocyte for 2 to 10 s followed by rapid washout. Data were sampled at 200 Hz and low-pass filtered at 10 Hz using the GeneClamp 500 internal low-pass filter. Membrane currents from voltage-clamped oocytes were digitized (Digidata 1200 acquisition system; Molecular Devices) and stored on a PC running pCLAMP 9.2 software (Molecular Devices). Concentration-response curves were constructed by recording nicotinic responses to a range of agonist concentrations (six to nine doses) and for a minimum of six oocytes. EC₅₀ and Hill coefficient values were obtained by fitting the concentration-response data to the Hill equation. All data are reported as mean \pm S.E.M.

Whole-Cell Patch-Clamp Electrophysiology. N2a cells expressing YFP-labeled nicotinic receptors were visualized with an inverted microscope (Olympus IMT-2, DPlan 10 \times 0.25 NA and MPlan 60 \times 0.70 NA) under fluorescence illumination (mercury lamp). Patch electrodes (3–6 M Ω) were filled with pipette solutions containing 88 mM KH₂PO₄, 4.5 mM MgCl₂, 0.9 mM EGTA, 9 mM HEPES, 0.4 mM CaCl₂, 14 mM creatine phosphate (Tris salt), 4 mM Mg-ATP, and 0.3 mM GTP (Tris salt), pH 7.4 with KOH. The extracellular solution was 150 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, and 10 mM D-glucose, pH 7.4. Standard whole-cell recordings were made using an Axopatch 1-D amplifier (Molecular Devices), low-pass filtered at 2 to 5 kHz, and digitized online at 20 kHz (pClamp 9.2; Molecular Devices). Series resistance was compensated 80%, and the membrane potential was held at -70 mV. Recorded potentials were corrected for junction potential.

ACh was delivered using a two-barrel glass θ -shaped tube (outer diameter \sim 200 μ m; pulled from 1.5-mm-diameter θ -shaped borosilicate tubing) connected to a piezoelectric translator (LSS-3100, Burleigh Instruments, Fishers, NY) as described previously (Nashmi et al., 2003). ACh was applied for 500 ms (triggered by pCLAMP 9.2), and solution exchange rates measured from open tip junction potential changes during application with 10% extracellular solution were typically \sim 300 μ s (10–90% peak time). Data are reported as mean \pm S.E.M. for the peak current response to 1 μ M ACh, and statistical significance was determined using a Wilcoxon signed rank test.

Results

Design and Construction of $\alpha 6$ and $\beta 3$ XFP Fusions.

Based on previous work (Slimko et al., 2002; Nashmi et al., 2003), we chose to insert XFP fusions in the M3-M4 loop of mouse $\alpha 6$ and $\beta 3$ nAChR subunits. Like all members of the Cys-loop family, $\alpha 6$ and $\beta 3$ have predicted α -helices at the N- and C-terminal ends of their M3-M4 loop (Fig. 1, A and B) that may be important in ion permeation (Miyazawa et al., 1999). In addition to avoiding these regions, we also avoided potential phosphorylation sites and trafficking motifs (Fig. 1, A and B). Our XFP fusion cassette also consisted of a Gly-Ala-Gly flexible linker flanking the XFP open reading frame on both the N- and C-terminal side. We built three independent XFP fusions for $\alpha 6$ and two XFP fusions for $\beta 3$ (Fig. 1C). These were designated according to the residue immediately N-terminal to the beginning of the Gly-Ala-Gly linker (e.g.,

$\alpha 6$ -YFP^{G366} denotes that the GAG-YFP-GAG cassette was inserted between G366 and V367). Unless otherwise noted, all experiments were conducted with $\alpha 6$ -XFP^{A405} and $\beta 3$ -XFP^{P379}.

Functional Expression of $\alpha 6$ and $\beta 3$ Subunits. Despite exhaustive attempts to functionally reconstitute $\alpha 6^*$ nAChRs in *X. laevis* oocytes and mammalian tissue culture cells, we recorded no robust, reproducible responses from cells expressing $\alpha 6$, either with untagged subunits or the fluorescent subunits (Supplemental Data; Table 1). $\beta 3$ -YFP, however, was well expressed on the plasma membrane of *X. laevis* oocytes when coexpressed with $\alpha 3$ and $\beta 4$ subunits to support functional expression (Fig. 2A). As a control for oocyte autofluorescence, we imaged oocytes expressing untagged $\beta 3$ subunits (Fig. 2A). No fluorescence was detected in this case, indicating that our $\beta 3$ -YFP signal was specific.

$\beta 3$ subunits do not drastically alter the EC₅₀ for ACh or nicotine when incorporated into nAChRs (Boorman et al., 2003), but they do profoundly alter single-channel kinetics (Boorman et al., 2003). Channel burst duration was significantly shortened for nAChRs containing $\beta 3$ versus those without it (Boorman et al., 2003), suggesting that $\beta 3$ reduces the probability of channel opening, P_{open} . Consistent with this, macroscopic voltage-clamped responses from oocytes and mammalian cells expressing $\beta 3^*$ receptors were significantly smaller than for non- $\beta 3^*$ receptors (Broadbent et al.,

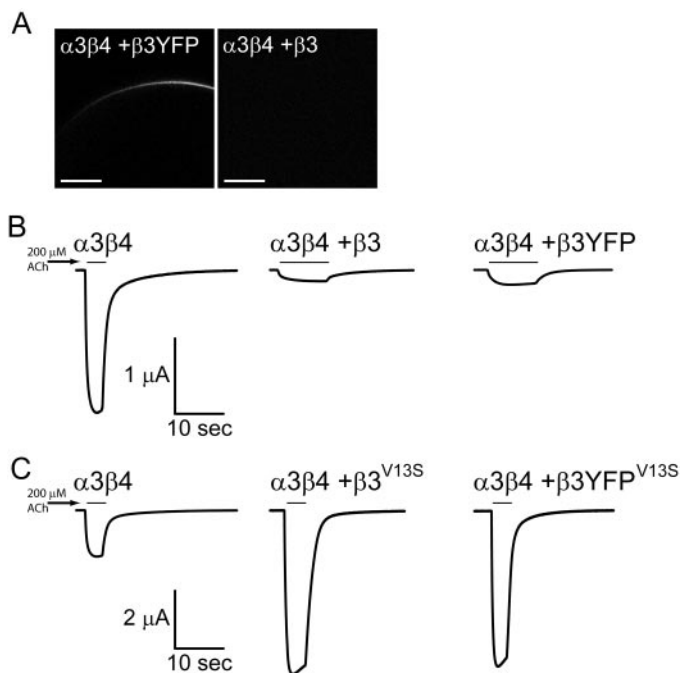


Fig. 2. Fluorescently labeled $\beta 3$ subunits are functional and expressed on the cell surface in *X. laevis* oocytes. A, *X. laevis* oocytes were injected with cRNA encoding WT (control) or YFP-labeled $\beta 3$ (15 ng) along with $\alpha 3$ (2 ng) and $\beta 4$ (3 ng). The oocyte surface was imaged with direct fluorescence confocal microscopy. True YFP signal was acquired by linear unmixing of the background fluorescence spectra (untagged $\beta 3$ with $\alpha 3\beta 4$) and a YFP reference spectrum. Scale bar, 54 μm . B, fluorescently labeled $\beta 3$ subunits are indistinguishable from WT subunits in their ability to attenuate nicotinic receptor responses. A representative voltage-clamped response from *X. laevis* oocytes expressing $\alpha 3\beta 4$, $\alpha 3\beta 4\beta 3$, or $\alpha 3\beta 4\beta 3$ -YFP is shown. Agonist (ACh; 200 μM) was applied and removed as indicated by the bar. C, reversal of $\beta 3$ -mediated suppression of nicotinic responses is identical in untagged and YFP-labeled hypersensitive $\beta 3$. Representative voltage-clamped responses from oocytes expressing $\alpha 3\beta 4$, $\alpha 3\beta 4\beta 3^{\text{V13S}}$, or $\alpha 3\beta 4\beta 3$ -YFP^{V13S} are shown. Agonist application is identical to B.

2006). To assess the functionality of our $\beta 3$ -YFP construct, we compared the ability of untagged and YFP-labeled $\beta 3$ subunits to attenuate nicotinic responses. $\beta 3$ must be coexpressed with other α and β subunits, so we chose to use $\alpha 3\beta 4$ receptors for this purpose. We did so because $\beta 3$ has been well characterized with this receptor combination (Boorman et al., 2003; Broadbent et al., 2006). When WT, untagged $\beta 3$ was coexpressed with $\alpha 3\beta 4$ receptors, we found a significant attenuation of the peak response to 200 μM ACh (Fig. 2B), consistent with previous findings (Broadbent et al., 2006). When $\beta 3$ -YFP was tested in this assay, it was also able to attenuate the maximal response in a manner identical to untagged $\beta 3$ (Fig. 2B). It is possible that, although $\beta 3$ -WT attenuates responses via a gating mechanism on the plasma membrane, YFP-labeled $\beta 3$ might do so via a different mechanism such as sequestering $\alpha 3$ or $\beta 4$ subunits inside the cell. To further test the functionality of YFP-labeled $\beta 3$, we took advantage of the fact that a gain-of-function TM2 mutation in $\beta 3$ is able to reverse the attenuation of peak responses seen for $\beta 3$ -WT (Broadbent et al., 2006). We reasoned that if the YFP label in the M3-M4 loop is not disturbing the function of $\beta 3$, we should detect the same gain-of-function response for unlabeled and YFP-labeled $\beta 3$ when they are engineered to express a mutation of this sort. When a Val13' to Ser mutation (V13S) was introduced into unlabeled $\beta 3$, we observed not only a reversal of this attenuation behavior, but a significant increase in the peak response to 200 μM ACh with $\alpha 3\beta 4$ receptors (Fig. 2C). When $\beta 3$ -YFP^{V13S} was tested in this assay, we observed an identical behavior. Taken together, these data suggest that $\beta 3$ -YFP is fully functional and incorporates into nAChRs in *X. laevis* oocytes.

To further characterize our $\beta 3$ -YFP construct, we constructed concentration-response curves for $\alpha 3\beta 4$ receptors containing either $\beta 3$ WT or $\beta 3$ -YFP. Consistent with previous reports (Boorman et al., 2000), we measured an EC₅₀ for ACh of 230 \pm 22 μM for $\alpha 3\beta 4\beta 3$ receptors, which is slightly higher than for $\alpha 3\beta 4$ (165 \pm 9 μM) (Fig. 3A). When $\beta 3$ -YFP was substituted for WT $\beta 3$, the EC₅₀ was shifted slightly, but acceptably, to 109 \pm 8 μM (Fig. 3A). We also noticed that the addition of $\beta 3$ to $\alpha 3\beta 4$ receptors increased the Hill coefficient from 1.5 \pm 0.1 to 2.0 \pm 0.3, and this effect was retained when $\beta 3$ -YFP was coexpressed with $\alpha 3\beta 4$ receptors. Likewise, we also constructed concentration-response relationship curves for oocytes expressing $\beta 3^{\text{V13S}}$ and $\beta 3$ -YFP^{V13S}. Compared with the EC₅₀ for $\alpha 3\beta 4\beta 3$ (230 \pm 22 μM), we measured an EC₅₀ for $\alpha 3\beta 4\beta 3^{\text{V13S}}$ of 28 \pm 3 μM (Fig. 3B). This is consistent with others who have reported an approximate 6-fold reduction in EC₅₀ for the inclusion of $\beta 3$ with a similar hypersensitive mutation, Val9'Ser (Boorman et al., 2000). We reasoned that if $\beta 3$ -YFP retained the WT function of $\beta 3$, then there should be a similar gain-of-function phenotype when it is coexpressed with $\alpha 3\beta 4$. We measured an EC₅₀ for $\alpha 3\beta 4\beta 3$ -YFP^{V13S} of 34 \pm 3 μM (Fig. 3B), confirming that this construct behaves identically to $\beta 3$ -WT. Collectively, our work in *X. laevis* oocytes with YFP-labeled $\beta 3$ subunits suggests that insertion of YFP into the M3-M4 loop does not significantly alter the assembly, subcellular trafficking, or function of this subunit.

Subcellular Localization and Trafficking of $\alpha 6$ and $\beta 3$ Subunits. To probe the subcellular localization and trafficking of $\alpha 6^*$ and $\beta 3^*$ receptors, we chose a mouse neuroblastoma cell line, N2a, to transiently express our fluorescent

nicotinic receptor subunits. We prefer these cells over, for example, HEK293 cells, because they 1) are of mouse origin, the same species as our fluorescent constructs; 2) are of neuronal origin, suggesting that they will be a permissive environment for correct expression, subcellular localization, and assembly of our ectopic nAChR subunits; and 3) express only moderate quantities of transfected membrane protein. To study the subcellular localization of $\beta 3^*$ receptors, we coexpressed $\beta 3$ -YFP with the previously described fluorescently labeled $\alpha 4$ and $\beta 2$ subunits (Nashmi et al., 2003). $\beta 3$ is able to assemble and function when coexpressed with $\alpha 4\beta 2$ receptors (Broadbent et al., 2006). When coexpressed with fluorescent $\alpha 4$ or $\beta 2$ receptors, $\beta 3$ -YFP was localized primarily in the endoplasmic reticulum of live N2a cells (Fig. 4A, i and B, i). We used CFP-labeled $\alpha 4$ (Fig. 4B, ii) or $\beta 2$ (Fig. 4, A and C, ii) subunits along with a confocal microscope with spectral imaging capabilities to unambiguously assign YFP and CFP signals to each pixel for the spectral images of the cells. In these experiments, YFP was assigned green, CFP was assigned red, and yellow indicated pixels where $\beta 3$ -YFP was colocalized with either $\beta 2$ -CFP (Fig. 4A, iii) or $\alpha 4$ -CFP (Fig. 4B, iii). We noted that $\beta 3$ -YFP was completely colocalized with either $\alpha 4$ or $\beta 2$ in this experiment, suggesting that these subunits are assembled in the same pentameric receptors. To further define the extent of this colocalization, we plotted the $\beta 3$ -YFP and $\alpha 4$ -CFP or $\beta 2$ -CFP pixel intensity across a two-dimensional region of interest transecting the cell (Fig. 4, A and B, iv). We noted that the YFP and CFP intensity profiles strongly resembled each other, suggesting that these subunits were indeed colocalized and coassembled in intracellular compartments of the cell. With respect to $\alpha 4\beta 2^*$ receptors, this localization pattern is not an artifact of

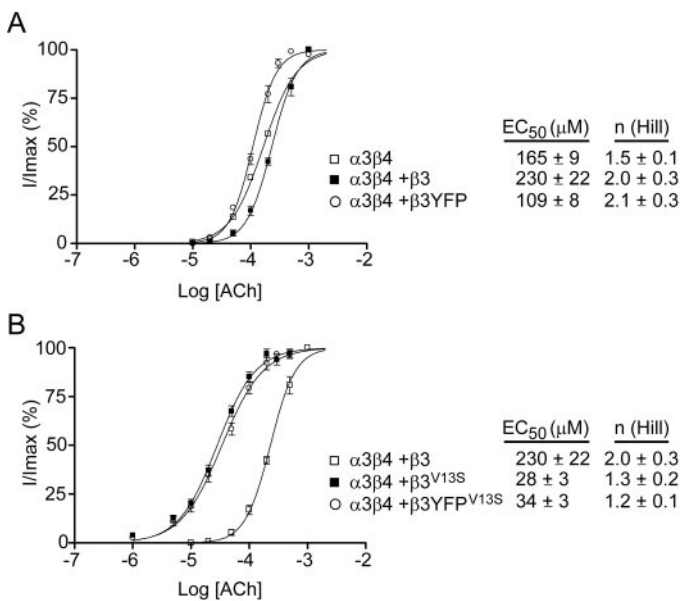


Fig. 3. $\beta 3$ nAChR subunit function is not affected by YFP insertion in M3-M4 loop. **A**, concentration-response relations for WT and fluorescently labeled $\beta 3$ -containing receptors are similar. *X. laevis* oocytes expressing the indicated receptor subunits were voltage-clamped during agonist application and washout. Peak responses to the indicated ACh concentration (molar) were normalized and the data were fitted to the Hill equation. **B**, concentration-response relation for $\beta 3$ subunits with a hypersensitive mutation is not affected by the presence of YFP in the M3-M4 loop. *X. laevis* oocytes were assayed and data were analyzed as described in **A**. $\alpha 3\beta 4$ data from **A** are shown for comparison. Error bars are \pm S.E.M., and $n = 6$ for each condition.

overexpression, because this is the same pattern we observed previously (Nashmi et al., 2003). This is also the expression pattern of endogenous, YFP-labeled $\alpha 4^*$ receptors in $\alpha 4$ -YFP knockin mice (Nashmi et al., 2007). This indicates that 1) a large pool of intracellular receptors exists in neurons, and 2) YFP tag does not interfere with the delivery of receptors to the plasma membrane. Thus, the localization pattern we observe here for $\beta 3$ subunits is the expected result if it is assembling with $\alpha 4\beta 2$ receptors.

We expressed $\alpha 6$ -YFP along with $\beta 2$ -CFP in N2a cells, and we analyzed its localization pattern as described above for $\beta 3$. We also found that $\alpha 6$ was localized in intracellular compartments in the cell (Fig. 4C, i), and that it was completely colocalized with $\beta 2$ subunits (Fig. 4C, ii–iv). Although

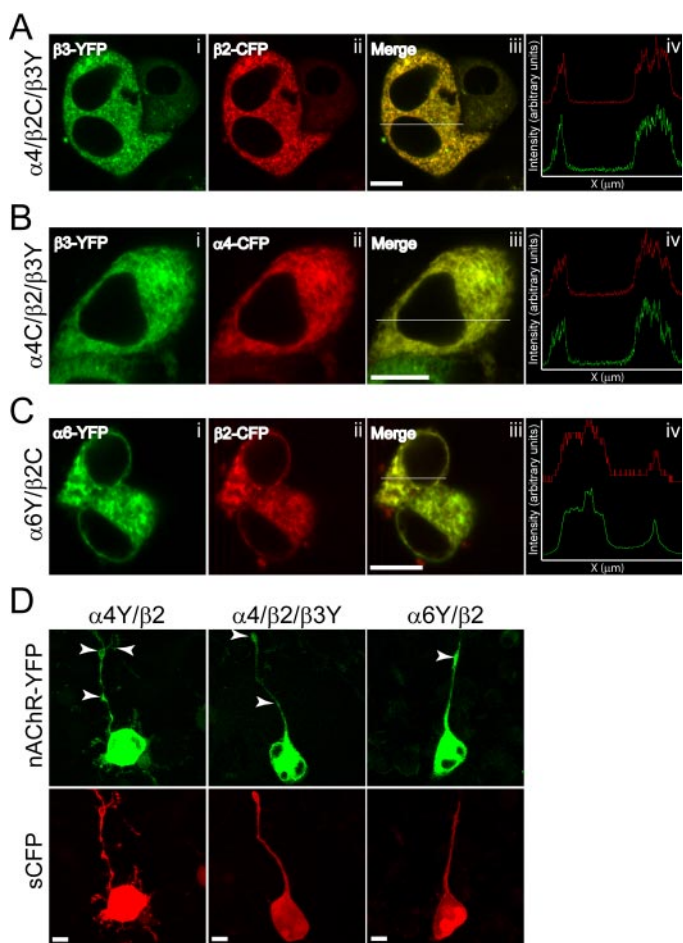


Fig. 4. $\beta 3$ -YFP and $\alpha 6$ -YFP expression in neuronal cells. **A**, $\beta 3$ and $\beta 2$ nAChR subunits are localized similarly in N2a cells. N2a cells expressing $\alpha 4\beta 2$ -CFP/ $\beta 3$ -YFP receptors were imaged live with spectral confocal microscopy. Spectral images were acquired and specific $\beta 3$ -YFP and $\beta 2$ -CFP signals were extracted with linear unmixing. Green ($\beta 3$ -YFP; i) and red ($\beta 2$ -CFP; ii) pseudocolor was assigned, and yellow (Merge; iii) indicates colocalized proteins. Pixel intensities for the YFP and CFP channel were plotted (iv) along a line (iii) transecting the imaged cell. **B**, $\beta 3$ and $\alpha 4$ nAChR subunits are localized similarly in N2a cells. N2a cells expressing $\alpha 4$ -CFP/ $\beta 2\beta 3$ -YFP receptors were imaged live as described in **A**. **C**, $\alpha 6$ and $\beta 2$ nAChR subunits are localized similarly in N2a cells. N2a cells expressing $\alpha 6$ -YFP/ $\beta 2$ -CFP were imaged live as described in **A** and **B**. **D**, $\alpha 4$ -YFP, $\beta 3$ -YFP, and $\alpha 6$ -YFP are localized intracellularly and in processes in differentiated neurons. N2a cells were differentiated for 2 days (see *Materials and Methods* for details) to induce neurite outgrowth, followed by transfection with the indicated nAChR cDNA combinations plus soluble CFP to mark cellular morphology. One day after transfection, cells were imaged live as described in **A** to **C**. Scale bar, 10 μm .

this is the first fluorescence imaging reported for $\alpha 6^*$ receptors, there is other evidence to corroborate our findings. Studies with [^3H]epibatidine demonstrate that a significant portion of $\alpha 6\beta 2$ and $\alpha 6\beta 2\beta 3$ receptors are intracellular (~ 50 and $\sim 20\%$, respectively), although some are delivered to the surface (Kuryatov et al., 2000; Tumkosit et al., 2006).

To further investigate the subcellular localization and trafficking of $\alpha 6$ and $\beta 3$ subunits, we imaged live, differentiated N2a cells and primary neurons. N2a cells can be induced to differentiate and undergo neurite outgrowth if serum is withdrawn and an activator of protein kinase A, dibutyryl-cAMP, is added (Fowler et al., 2001). In our previous work, $\alpha 4\beta 2$ receptors were localized to dendrites, but not axons, when expressed in primary midbrain neurons (Nashmi et al., 2003). We were interested in whether our fluorescent nicotinic receptor subunits were localized to N2a cell processes in a manner analogous to dendrites in primary neurons. Furthermore, we wanted to address the question of whether $\beta 3$ is localized with other subunits at distal sites such as dendrites. This is an unsolved question, as there is no high-affinity probe (pharmacological or immunological) that can reliably and unambiguously isolate $\beta 3^*$ receptors. N2a cells were plated on glass-bottomed dishes, and they were then differentiated for 2 days (see *Materials and Methods*) followed by transfection with various combinations of YFP-labeled and unlabeled nAChR subunits. Cells were also cotransfected with an expression plasmid for soluble CFP to mark total cell morphology. We found that $\alpha 4\beta 2$ receptors were indeed localized to neuronal processes in differentiated N2a cells (Fig. 4D, arrow), along with abundant expression in the cell soma. When $\beta 3$ -YFP was coexpressed with $\alpha 4\beta 2$, we observed a very similar pattern. We found that $\beta 3$ was present even at the most distant elements of neuronal processes (Fig. 4D, arrow). Because this pattern is identical to that of $\alpha 4\beta 2$ in differentiated N2a cells, we conclude that $\beta 3$ is likely assembling with $\alpha 4\beta 2$ receptors and that the YFP label in the M3-M4 loop is not disrupting the normal cellular trafficking of $\alpha 4\beta 2\beta 3$ pentamers. To further characterize the localization of $\beta 3^*$ receptors, we coexpressed $\beta 3$ -YFP with $\alpha 4\beta 2$ receptors in primary rat hippocampal neurons (Nashmi et al., 2003). To minimize overexpression artifacts, cells were imaged live only 18 to 24 h after transfection. We found that $\beta 3^*$ receptors were localized very similarly to $\alpha 4\beta 2$ receptors in our previous studies with primary neurons; we noted uniform localization in the soma, suggestive of endoplasmic reticulum, and dendritic localization (Fig. 5A, arrow) and an absence of localization in axons. A high-magnification micrograph demonstrates the dendritic localization of these putative $\alpha 4\beta 2\beta 3$ receptors (Fig. 5A, right). In cells coexpressing $\alpha 4\beta 2/\beta 3\text{Y}$ with soluble CFP [to mark total cell morphology, similar to Nashmi et al. (2003)], $\beta 3$ subunits did not traffic to a subregion of the cell interior likely to be axons (data not shown). To more directly determine whether $\beta 3^*$ receptors could be localized to axons in these neurons, we coexpressed $\alpha 4\beta 2\beta 3\text{Y}$ receptors with a CFP-labeled axonal marker, tau. The tau-CFP decorated axons in hippocampal neurons, with proximal (relative to the cell body) portions of the axon being labeled more strongly than distal portions (Fig. 5B). In all cells examined, we noted the presence of YFP-labeled $\beta 3$ subunits in these proximal axons but not distal axons (Fig. 5B, arrow). These data in differentiated N2a cells and primary neurons suggest that $\beta 3$ assembles efficiently with

$\alpha 4\beta 2$ receptors, and it is thus cotrafficked and targeted to distal sites in neurons.

Because $\alpha 6$ -YFP* receptors do not function in our hands, we wanted to determine whether this is due to a subtle trafficking defect that could prevent the correct delivery of $\alpha 6$ -YFP to the plasma membrane. Although we could readily detect $\alpha 6$ fluorescence in the cell body of undifferentiated N2a cells, we wanted to further probe the cellular trafficking of $\alpha 6^*$ receptors by expressing them in differentiated N2a cells that contain processes. To evaluate the subcellular localization of $\alpha 6^*$ receptors, we expressed $\alpha 6$ -YFP with $\beta 2$ in differentiated N2a cells. To our surprise, we found that $\alpha 6\beta 2$ receptors were trafficked to neuronal processes in a manner analogous to $\alpha 4\beta 2$ and $\alpha 4\beta 2\beta 3$ receptors (Fig. 4D, arrow). To further address this question, we expressed $\alpha 6$ -YFP $\beta 2$ receptors in rat hippocampal neurons as described for $\alpha 4\beta 2\beta 3$ -YFP. We observed a localization pattern for $\alpha 6$ -YFP that was very similar to $\alpha 4\beta 2\beta 3$ -YFP. These receptors were well expressed in the cell soma, but they were readily detectable in dendrites as well (Fig. 5A, arrow). In experiments with coexpressed soluble CFP and $\alpha 6$ -YFP $\beta 2$ receptors, $\alpha 6$ subunits were not detected in putative axons (data not shown). In tau-CFP/ $\alpha 6$ -YFP $\beta 2$ coexpression experiments, $\alpha 6$ subunits (similar to $\alpha 4\beta 2$ but not $\alpha 4\beta 2\beta 3$ receptors) were not detected in tau-labeled axons (Fig. 5B, arrow). These data indicate that, although $\alpha 6^*$ receptors produce little or no agonist-induced conductance in mammalian tissue culture cells, they are expressed well and trafficked similarly compared with $\alpha 4\beta 2$ and $\beta 3^*$ receptors.

FRET Revealed Assembly of $\alpha 6$ and $\beta 3$ Subunits into nAChR Pentamers. The fact that $\alpha 4\beta 2/\beta 3$ and $\alpha 6/\beta 2$ subunits are colocalized in the cell body and cotargeted to processes and dendrites in neurons suggests that they are assembled into pentameric receptors. The question of receptor assembly is often answered by simply measuring agonist-induced conductance increases in cells expressing a subunit combination of interest, or by applying a selective agonist or inhibitor to a pure receptor population of known pharmacological properties. This approach is not applicable, however, for $\alpha 6^*$ and $\beta 3^*$ receptors. $\alpha 6^*$ receptors do not function well in heterologous expression systems, so it is not straightforward to determine the extent to which free $\alpha 6$ subunits

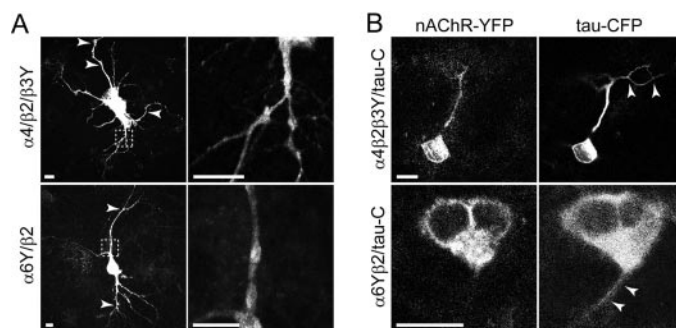


Fig. 5. Localization of $\beta 3^*$ and $\alpha 6^*$ receptors in primary neurons. **A**, $\beta 3$ -YFP and $\alpha 6$ -YFP are localized in the cell soma and in dendrites in primary hippocampal neurons. E18 rat hippocampal neurons were plated and cultured for 14 days followed by transfection with the indicated nAChR cDNAs. One day after transfection, cells were imaged live. Right, higher magnification image of the boxed area in the left panel. **B**, $\beta 3^*$ and $\alpha 6^*$ receptors are absent from axons. Neurons were transfected with the indicated nAChR cDNAs along with CFP-tau, followed by live confocal imaging as described in **A**. Scale bar, 10 μm .

assemble into pentameric receptors. Similarly for β_3 , although it is functional in oocytes (Fig. 2), there are no pharmacological probes that can be applied to β_3^* receptors to study their assembly or subunit composition. Others have indirectly measured receptor assembly of nicotinic subunits by using biochemical techniques such as immunoprecipitation (Zoli et al., 2002; Champetiaux et al., 2003) and centrifugation (Kuryatov et al., 2000) or by forcing subunits to assemble by using molecular concatamers (Tapia et al., 2007). To directly determine whether two nicotinic receptor subunits interact and, possibly, assemble to form pentameric receptors, we have used FRET coupled with our CFP- and YFP-tagged receptors. In the context of our nicotinic receptor subunits labeled with YFP or CFP in the M3-M4 loop, only subunits that interact will undergo FRET, because FRET occurs only when donors and acceptors are within 100 Å. Furthermore, we previously demonstrated that the efficiency of FRET directly correlates with the number of functional, plasma membrane-localized pentameric receptors (Nashmi et al., 2003). To measure FRET between subunits, we used the acceptor photobleaching method (Nashmi et al., 2003). In this method, we measure CFP dequenching during incremental photodestruction of YFP. CFP was excited at 439 nm, whereas YFP was bleached at 514 nm (Fig. 6A). Because the emission spectra for CFP and YFP overlap significantly, we imaged using a confocal microscope with spectral imaging capabilities along with a linear unmixing algorithm (described under *Materials and Methods*).

Fluorescent α_4 and β_2 subunits are functional and undergo robust FRET in mammalian cells (Nashmi et al., 2003), so we used these subunits in our acceptor photobleaching assay with XFP-tagged β_3 and α_6 . We expressed β_3 -YFP with untagged α_4 and β_2 -CFP in N2a cells, followed by live cell FRET imaging. We recorded the whole-cell fluorescence intensity for β_3 -YFP and β_2 -CFP before and after photobleaching of YFP with the 514-nm laser, and we expressed with pseudocolor intensity scaling (Fig. 6B). In this experiment, β_2 -CFP was clearly dequenched after β_3 -YFP photodestruction (Fig. 6B), indicating that the two subunits had been undergoing FRET. In a similar experiment, we recorded multiple spectral images at several time points during YFP photodestruction. This revealed a corresponding increase in CFP intensity (Fig. 6C). A reciprocal experiment was also done, where β_3 -YFP was coexpressed with α_4 -CFP and untagged β_2 . We recorded a similar dequenching for α_4 -CFP after YFP photobleaching (Fig. 6, D and E), indicating FRET between these subunits as well. Both for β_2/β_3 and α_4/β_3 FRET, we found no difference between FRET inside the cell versus FRET at the cell periphery at or near the plasma membrane. These results directly demonstrate that β_3 is able to assemble with $\alpha_4\beta_2$ receptors in neuronal cells. This assembly likely occurs in the endoplasmic reticulum, which is consistent with previous findings (Nashmi et al., 2003).

There are many different putative α_6^* receptor subtypes in brain, including $\alpha_6\beta_2$, $\alpha_6\beta_2\beta_3$, $\alpha_6\alpha_4\beta_2$, and $\alpha_6\alpha_4\beta_2\beta_3$ (Salminen et al., 2007). To begin to study α_6^* receptor assembly, we measured FRET between α_6 -YFP and β_2 -CFP. In response to YFP bleaching, we recorded a robust dequenching of β_2 -CFP throughout the cell, indicating FRET between these subunits (Fig. 6, F and G). The pattern of localization and FRET pattern was identical to $\alpha_4\beta_2\beta_3$ receptors.

To further quantify FRET between α_4/β_2 subunits and

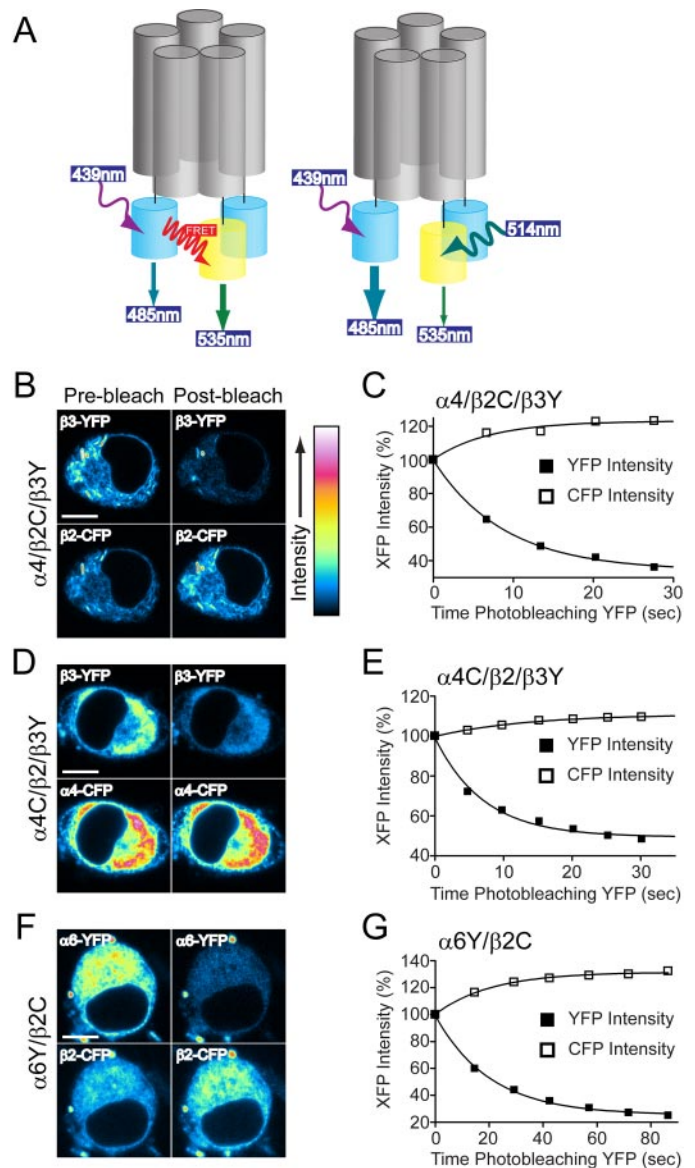


Fig. 6. FRET reveals assembly of β_3 and α_6 nAChR subunits with α_4 and β_2 . A, Nicotinic receptor FRET schematic diagram. Gray cylinders indicate nAChR subunits, whereas cyan or yellow cylinders attached to nAChR subunits indicate CFP or YFP, respectively. FRET between nAChR subunits used the acceptor photobleaching method. When subunits are assembled, 439-nm excitation of CFP (donor) results in some emission of CFP at 485 nm, and some nonradiative transfer of energy (FRET) to YFP (acceptor) and resulting in emission at 535 nm. Acceptor photobleaching reveals FRET by measuring incremental dequenching of CFP during photodestruction of YFP with high-power 514-nm excitation. B, β_3 assembles with β_2 in the presence of α_4 . N2a cells expressing $\alpha_4\beta_2$ -CFP/ β_3 -YFP receptors were imaged live for FRET. The 439-nm laser line was coupled to a confocal microscope equipped with spectral imaging capabilities; this instrument generated spectral images before and after photodestruction of YFP using the 514-nm laser. Specific CFP and YFP signals were generated with linear unmixing as described under *Materials and Methods*. YFP and CFP intensity throughout the cell before and after YFP photodestruction is shown using intensity scaling. C, plot of β_3 -YFP and β_2 -CFP intensity during incremental photodestruction of β_3 -YFP. Normalized data from a representative cell were fitted to an exponential decay. D, β_3 assembles with α_4 in the presence of β_2 . N2a cells expressing α_4 -CFP/ $\beta_2\beta_3$ -YFP were imaged live for FRET as described in B. E, plot of β_3 -YFP and α_4 -CFP intensity during incremental photodestruction of β_3 -YFP, similar to C. F, α_6 assembles with β_2 . N2a cells expressing α_6 -YFP/ β_2 -CFP were imaged live for FRET as described in B. G, plot of α_6 -YFP and β_2 -CFP intensity during incremental photodestruction of α_6 -YFP, similar to C. Scale bar, 10 μ m.

$\beta 3$ or $\alpha 6$, we measured FRET E values for various receptor subtypes. $\alpha 4$ -CFP $\beta 2$ -YFP, $\alpha 4\beta 2$ -CFP $\beta 3$ -YFP, and $\alpha 4$ -CFP $\beta 2\beta 3$ -YFP receptors were expressed in N2a cells followed by acceptor photobleaching FRET (Fig. 7A). We acquired spectral images with 439-nm laser excitation before and during incremental photobleaching of YFP-labeled subunits, followed by extraction of true CFP and YFP image data using linear unmixing (see *Materials and Methods*). A scatterplot of CFP intensity in response to YFP photobleaching reveals FRET between the subunits in question (Fig. 7B) when the slope of the linear regression line is >0 . This slope was used to calculate FRET efficiency values, which were expressed as bar graphs (Fig. 7C) or listed (Table 1). As shown qualitatively in Fig. 6, significant FRET occurred in all nAChR pentamer conditions. We noted a higher FRET E for $\alpha 4$ / $\beta 2$ Y than for $\beta 3$ Y with either $\beta 2$ C or $\alpha 4$ C (Y, YFP; C, CFP). To assess the specificity of this measurement, we also measured FRET between $\beta 3$ and a non-nAChR, CFP-labeled protein, mGAT1. GAT1 is also a multipass transmembrane protein with a CFP-tag at its C terminus, which faces the cytoplasm. This protein is mainly localized to the endoplasmic reticulum (data not shown). These two points are important, because it was critical for a specificity probe to have 1) the same membrane topology as our labeled nicotinic recep-

tors, with respect to the attached fluorophore; and 2) the same subcellular localization such that they are capable of interacting with each other. In N2a cells expressing $\beta 3$ -YFP and mGAT1-CFP, we could not detect any FRET between these proteins (Table 1; Fig. 7C). In an even more rigorous test, we assessed FRET between $\beta 3$ -YFP and another Cys-loop receptor labeled in the M3-M4 loop, the CFP-labeled GluCl β subunit (Slimko et al., 2002; Nashmi et al., 2003). FRET between $\beta 3$ and the GluCl β subunit was significantly smaller (FRET $E = 6 \pm 4\%$) than for $\alpha 4$ or $\beta 2$ nAChR subunits (Table 1; Fig. 7C). Thus, our FRET results between $\beta 3$ and other labeled nAChR subunits cannot be explained by random collision or interaction with unassembled subunits. Finally, we were interested in whether subtle changes in the location of the fluorophore within the $\beta 3$ M3-M4 loop could influence its ability to undergo FRET with another subunit. FRET E decreases strongly with the distance between fluorophores. We reasoned that changes in the insertion point of YFP in $\beta 3$, while holding the position of CFP in $\beta 2$ constant, might alter FRET between these two subunits. To address this, we compared the FRET E between $\beta 2$ -CFP and two different $\beta 3$ -YFP constructs, $\beta 3$ -YFP^{P379} and $\beta 3$ -YFP^{G367}, which have different insertion points for YFP within the M3-M4 loop. To our surprise, there was no change in the FRET E for these two subunits (Table 1; Fig. 7D).

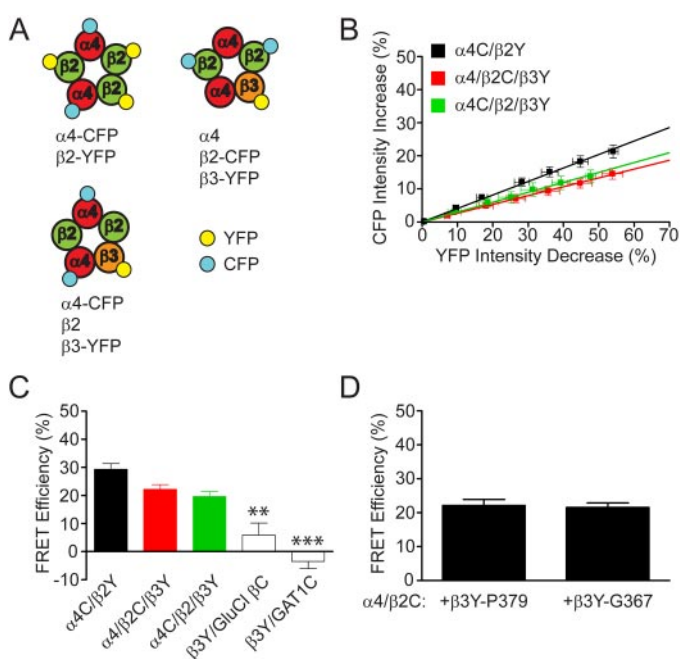


Fig. 7. $\beta 3$ specifically assembles with $\alpha 4\beta 2$ nAChRs. A, fluorescently labeled nicotinic receptor pentamers assayed for FRET in this experiment. N2a cells expressing the indicated receptor pentamers were assayed live for FRET using the acceptor photobleaching method. B, linear plots of donor (CFP) dequenching versus acceptor (YFP) photodestruction for nAChRs with the indicated fluorescent subunits. FRET efficiency was calculated by extrapolating linear regression plots to 100% YFP photodestruction as described under *Materials and Methods*. C, specific FRET signal detected between $\beta 3$ and $\alpha 4$ or $\beta 2$. The FRET efficiency for the given donor-acceptor pair was calculated from the linear plot shown in B as described under *Materials and Methods*. FRET between $\beta 3$ -YFP and mGAT1-CFP or GluCl β -CFP was measured as a specificity control. D, FRET efficiency for $\beta 3$ -YFP does not depend on the insertion site in the M3-M4 loop. Two $\beta 3$ -YFP constructs, $\beta 3$ -YFP^{P379} and $\beta 3$ -YFP^{G367}, were compared for their ability to assemble with $\alpha 4\beta 2$ -CFP as measured by FRET. FRET efficiency was calculated as described in C. Error bars are \pm S.E.M., and $n = 5$ to 15 cells for each condition. ***, $p < 0.001$; **, $p < 0.01$.

We quantitatively measured FRET between $\alpha 6$ and $\alpha 4/\beta 2$ subunits as well. We expressed either $\alpha 6\gamma\beta 2$ C or $\alpha 6\gamma\alpha 4\beta 2$ in N2a cells to measure FRET (Fig. 8A). The latter receptor was studied because recent work indicates that nAChR receptors containing both $\alpha 6$ and $\alpha 4$ (1) exist and are functional in mouse brain tissue (Salminen et al., 2007), and 2) are both necessary to form the nAChR subtype with the highest affinity for nicotine yet reported in a functional assay (Salminen et al., 2007). Acceptor photobleaching FRET experiments reveal robust CFP dequenching in response to YFP photobleach for both of these receptor subtypes, indicating FRET (Fig. 8B). Similar to $\beta 3$ -YFP* receptors, we measured FRET E values for these two subtypes, and we found a FRET E of $36.0 \pm 2.4\%$ for $\alpha 6\gamma\beta 2$ C and $21.9 \pm 1.1\%$ for $\alpha 6\gamma\alpha 4\beta 2$ (Table 1; Fig. 8C). We also assessed the specificity of our FRET measurements for $\alpha 6$ by measuring FRET between $\alpha 6$ -YFP and mGAT1-CFP as described above for Fig. 7. Similar to $\beta 3$ and mGAT1, we could record no significant FRET between $\alpha 6$ and mGAT1 (Table 1; Fig. 8C). FRET experiments between $\alpha 6$ and the GluCl β subunit, the most rigorous test conducted, yielded a small FRET signal (FRET $E = 14 \pm 2\%$) (Table 1; Fig. 8C). Because these subunits presumably do not form functional channels, there may be a small distortion of our $\alpha 6$ FRET signals that is due to partially assembled receptors. Because this signal is significantly smaller than for all other $\alpha 6$ combinations, FRET between subunits in pentameric receptors remains the most plausible explanation for the energy transfer we observe for $\alpha 6$. Finally, we studied FRET between $\beta 2$ -CFP and three $\alpha 6$ -YFP constructs ($\alpha 6$ -YFP^{A405}, $\alpha 6$ -YFP^{G387}, and $\alpha 6$ -YFP^{G366}) that differed only in their insertion point for YFP within the M3-M4 loop. Again, we were surprised to find no significant difference in FRET E between these three $\alpha 6$ constructs (Table 1; Fig. 8D).

Several results described above gave us confidence that our YFP-labeled $\beta 3$ and $\alpha 6$ constructs were performing as expected. After confirming that these subunits assemble and

traffic normally when expressed independently of each other, we used these constructs together to study $\alpha 6\beta 2\beta 3$ nAChRs. This receptor represents a modest population of the total striatal nAChR pool, and it contributes to nicotine-stimulated dopamine release (Salminen et al., 2007). $\alpha 6\beta 2\beta 3$ receptors, where one subunit is untagged and the remaining subunits are either YFP- or CFP-tagged ($\alpha 6Y\beta 2C\beta 3$, $\alpha 6Y\beta 2\beta 3C$, and $\alpha 6\beta 2Y\beta 3C$), were expressed in N2a cells (Fig. 9A). We measured robust donor dequenching for all receptor subtypes (Fig. 9B), which was confirmed with FRET E measurements (Table 1; Fig. 9C). Thus, aside from $\alpha 6$ functional measurements, we conclude that XFP-labeled $\alpha 6$ and $\beta 3$ subunits exhibit normal subcellular trafficking and assembly compared with our well characterized fluorescent $\alpha 4$ and $\beta 2$ subunits.

$\alpha 6$ and $\beta 3$ Subunit Stoichiometry Probed with FRET.

Having established that fluorescently labeled $\alpha 6$ and $\beta 3$ are functional ($\beta 3$ only), have a reasonable subcellular localization pattern, and assemble into nicotinic receptor pentamers with other subunits, we used these tools to probe an important question facing the nicotinic receptor field: subunit stoichiometry. A variety of creative approaches have been taken to understand subunit stoichiometry, including immunoprecipitation (Zoli et al., 2002; Champtiaux et al., 2003), density gradient centrifugation (Kuryatov et al., 2000), molecular concatamers/linked subunits (Tapia et al., 2007), reporter mutations (Boorman et al., 2000), and mouse genetic approaches (Gotti et al., 2005a; Salminen et al., 2007). We now

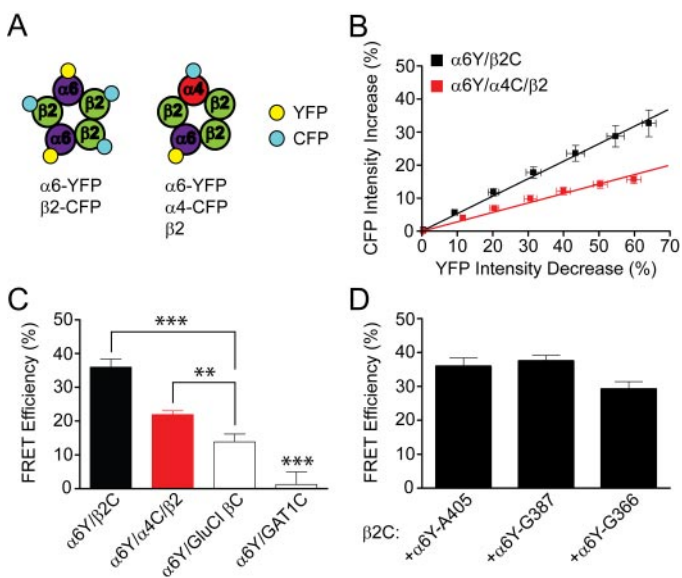


Fig. 8. $\alpha 6$ specifically assembles with $\alpha 4$ and $\beta 2$ nAChR subunits. A, fluorescently labeled nicotinic receptor pentamers assayed for FRET in this experiment. N2a cells expressing the indicated receptor pentamers were assayed live for FRET using the acceptor photobleaching method. B, linear plots of donor (CFP) dequenching versus acceptor (YFP) photobleaching for nAChRs with the indicated fluorescent subunits. FRET efficiency was calculated by extrapolating linear regression plots to 100% YFP photobleaching as described under *Materials and Methods*. C, specific FRET signal detected between $\alpha 6$ and $\alpha 4$ or $\beta 2$. The FRET efficiency for the given donor-acceptor pair was calculated from the linear plot shown in B as described under *Materials and Methods*. FRET between $\alpha 6$ -YFP and mGAT1-CFP or GluCl β -CFP was measured as a specificity control. D, FRET efficiency for $\alpha 6$ -YFP does not depend on the insertion site in the M3-M4 loop. Three $\alpha 6$ -YFP constructs, $\alpha 6$ -YFP^{A405}, $\alpha 6$ -YFP^{G387}, and $\alpha 6$ -YFP^{G366}, were compared for their ability to assemble with $\beta 2$ -CFP as measured by FRET. FRET efficiency was calculated as described in C. Error bars are \pm S.E.M., and $n = 5$ to 15 cells for each condition. ***, $p < 0.001$; **, $p < 0.01$.

use FRET to address the problem of subunit stoichiometry because FRET occurs only when subunits are directly interacting, and often assembled, with one another.

We have previously shown that FRET efficiency correlates directly with functional receptor pentamers (Nashmi et al., 2003). To examine the number of $\alpha 6$ and $\beta 3$ subunits in a nicotinic receptor pentamer, we first used FRET to examine the stoichiometry of a well studied receptor, namely, $\alpha 4\beta 2$ receptors. It is widely accepted that $\alpha 4$ and $\beta 2$ subunits assemble to form both high-sensitivity (HS) and low-sensitivity (LS) receptors. Cells often produce a mixture of these two receptors (Buisson and Bertrand, 2001; Nashmi et al., 2003), although they can be induced to express a pure population of one or the other (Nelson et al., 2003; Briggs et al., 2006). The subunit stoichiometry of HS receptors is postulated to be $(\alpha 4)_2(\beta 2)_3$, whereas the LS receptors is thought to be $(\alpha 4)_3(\beta 2)_2$ (Nelson et al., 2003). Regardless of the fraction of HS and LS receptors, we took advantage of the fact that all $\alpha 4\beta 2$ receptors presumably contain two or more $\alpha 4$ and two or more $\beta 2$ subunits. We reasoned that when cells express $\alpha 4$ -YFP and $\alpha 4$ -CFP along with $\beta 2$ (Fig. 10A), a fraction of the receptors will contain both YFP- and CFP-labeled $\alpha 4$ subunits, and they will therefore be detectable by FRET. Confirming this hypothesis, we did detect modest dequenching of $\alpha 4$ -CFP upon incremental $\alpha 4$ -YFP photobleaching (Fig. 10B). FRET E for $\alpha 4Y\alpha 4C\beta 2$ receptors was $22.2 \pm 2.3\%$ (Table 1; Fig. 10C). We also conducted a similar experiment with $\beta 2$, and we found a modest FRET signal (FRET $E = 16.3 \pm 1.7\%$) between $\beta 2$ -YFP and $\beta 2$ -CFP within the same pentamer (Table 1; Fig. 10, B and C). We next used this assay to determine whether $\alpha 6^*$ and $\beta 3^*$ receptors have one or more than one $\alpha 6$ or $\beta 3$ subunit per pentamer. N2a cells expressing either $\alpha 6Y\alpha 6C\beta 2$ or $\alpha 4\beta 2\beta 3Y\beta 3C$ receptors were

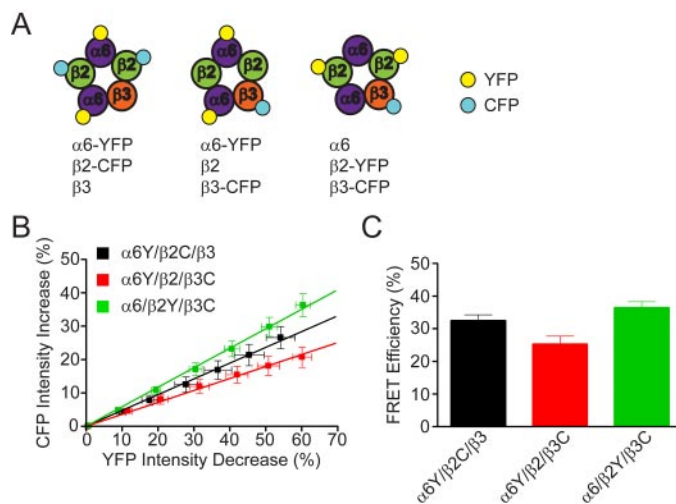


Fig. 9. FRET reveals assembly of $\alpha 6\beta 2\beta 3$ nAChRs. A, fluorescently labeled nicotinic receptor pentamers assayed for FRET in this experiment. N2a cells expressing the indicated receptor pentamers were assayed live for FRET using the acceptor photobleaching method. B, linear plots of donor (CFP) dequenching versus acceptor (YFP) photobleaching for nAChRs with the indicated fluorescent subunits. FRET efficiency was calculated by extrapolating linear regression plots to 100% YFP photobleaching as described under *Materials and Methods*. C, specific FRET signal detected between $\alpha 6$ -YFP and $\beta 2$ -CFP with $\beta 3$ present, $\alpha 6$ -YFP and $\beta 3$ -CFP with $\beta 2$ present, and $\beta 2$ -YFP and $\beta 3$ -CFP with $\alpha 6$ present. The FRET efficiency for the given donor-acceptor pair was calculated from the linear plot shown in B as described under *Materials and Methods*. Error bars are \pm S.E.M., and $n = 10$ to 15 cells for each condition.

analyzed for FRET (Fig. 10A). We measured a strong FRET signal between $\alpha 6$ -YFP and $\alpha 6$ -CFP in donor dequenching (Fig. 10B), corresponding to a robust FRET E of $27.8 \pm 1.7\%$ (Table 1; Fig. 10C). Thus, these data are the first to directly demonstrate that $\alpha 6^*$ receptors are capable of containing at least two $\alpha 6$ subunits, similar to other α subunits such as $\alpha 3$ and $\alpha 4$. In contrast to $\alpha 6$, $\beta 3$ is thought to be an “ancillary subunit”, only able to incorporate into nAChRs with other α and β subunits (Groot-Kormelink et al., 1998). We could detect little or no FRET between $\beta 3$ -YFP and $\beta 3$ -CFP (FRET $E = 2.6 \pm 1.3\%$) (Table 1; Fig. 10, B and C). This was a specific result, because $\beta 3$ -YFP and $\beta 3$ -CFP were able to FRET with other subunits (Figs. 7 and 9), thus ruling out the notion that one of these subunits is not able to undergo FRET. These data are the first to directly demonstrate that receptors containing $\beta 3$ subunits are only able to incorporate a single copy of this subunit. We interpret this to mean that $\beta 3$ incorporates into the “accessory” position in a nAChR pentamer (Tumkosit et al., 2006), and it likely does not contribute to either of the two α :non- α interfaces that form the ligand-binding sites.

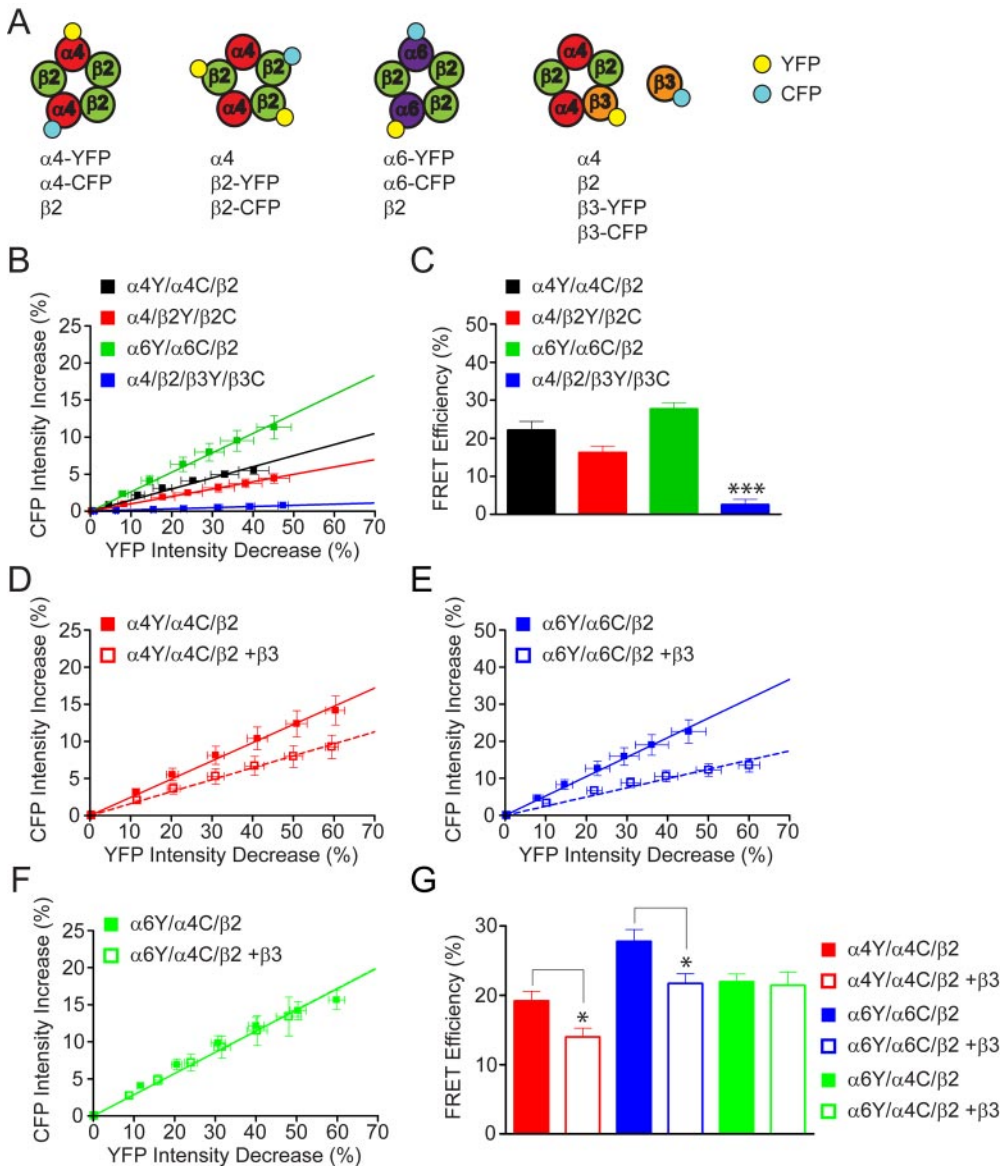


Fig. 10. $\beta 3$ and $\alpha 6$ subunit stoichiometry studied by FRET. A, fluorescently labeled nicotinic receptor pentamers assayed for FRET in this experiment. N2a cells expressing the indicated receptor pentamers were assayed live for FRET using the acceptor photobleaching method. B, linear plots of donor (CFP) dequenching versus acceptor (YFP) photodestruction for nAChRs with the indicated fluorescent subunits. FRET efficiency was calculated by extrapolating linear regression plots to 100% YFP photodestruction as described under *Materials and Methods*. C, $\alpha 6$ -containing receptors have multiple $\alpha 6$ subunits whereas $\beta 3$ -containing receptors have only one $\beta 3$ subunit. The FRET efficiency for the given donor-acceptor pair was calculated from the linear plot shown in B as described under *Materials and Methods*. D to F, linear plots of donor (CFP) dequenching versus acceptor (YFP) photodestruction for nAChRs with the indicated fluorescent subunits. FRET efficiency was calculated by extrapolating linear regression plots to 100% YFP photodestruction as described under *Materials and Methods*. G, $\beta 3$ coexpression with $\alpha 4\beta 2$ or $\alpha 6\beta 2$ receptors reduces FRET between YFP- and CFP-labeled α subunits. FRET E value for a given subunit combination was calculated from the linear plot in D, E, or F. Error bars are \pm S.E.M., and $n = 10$ to 15 cells for each condition. ***, $p < 0.001$; *, $p < 0.05$.

results in a decrease in $\alpha 4$ to $\alpha 4$ FRET for pentamers with a mixed subunit stoichiometry.

To determine whether $\alpha 6^*$ receptors have a fixed or a mixed subunit stoichiometry, we coexpressed $\beta 3$ in excess with $\alpha 6$ -YFP, $\alpha 6$ -CFP, and $\beta 2$. If $\alpha 6^*$ receptors only incorporate two $\alpha 6$ subunits, we expect to observe little or no change in FRET between $\alpha 6$ -YFP and $\alpha 6$ -CFP because $\beta 3$ will only displace one unlabeled $\beta 2$ subunit. However, if $\alpha 6^*$ receptors exist as a mixture of $(\alpha 6)_2(\beta 2)_3$ and $(\alpha 6)_3(\beta 2)_2$ subtypes similar to $\alpha 4^*$ receptors, we expect to observe a similar decline in FRET when $\beta 3$ is present to induce only the $(\alpha 6)_2(\beta 2)_2\beta 3$ stoichiometry. The latter was the case. We noted a significant decline in the slope of the donor dequenching profile for $\alpha 6Y\alpha 6C^*$ receptors when $\beta 3$ was present (Table 1; Fig. 10E) and a decline in the FRET E for $\alpha 6Y\alpha 6C\beta 2\beta 3$ ($21.7 \pm 1.4\%$) versus $\alpha 6Y\alpha 6C\beta 2$ ($27.8 \pm 1.7\%$) (Table 1; Fig. 10G). Thus, fluorescent $\alpha 6^*$ receptors behave identically to $\alpha 4^*$ receptors, and these results suggest that $\alpha 6^*$ receptors are capable of forming either of two subunit stoichiometries: $(\alpha 6)_2(\beta 2)_3$ and $(\alpha 6)_3(\beta 2)_2$.

Several groups have reported the existence of $\alpha 4\alpha 6^*$ receptors in brain tissue (Zoli et al., 2002; Salminen et al., 2007), and $\alpha 4\alpha 6\beta 2\beta 3^*$ receptors (presumably $\alpha 4_1\alpha 6_1\beta 2_2\beta 3_1$) have high affinity for nicotine (Salminen et al., 2007). To learn about the subunit stoichiometry of $\alpha 4\alpha 6^*$ receptors, we expressed $\alpha 6$ -YFP and $\alpha 4$ -CFP along with $\beta 2$ subunits in N2a cells. We noted a modest FRET signal, indicating that these subunits are present in some of the same nicotinic receptor pentamers (Fig. 8, B and C; Fig. 10F). In contrast to our results with $\alpha 6\beta 2\beta 3$ and $\alpha 4\beta 2\beta 3$ receptors, there was no difference between cells transfected with $\alpha 6Y/\alpha 4C/\beta 2$ and $\alpha 6Y/\alpha 4C/\beta 2/\beta 3$ subunits (Fig. 10, F and G). This shows that addition of excess $\beta 3$ subunits did not reduce FRET between $\alpha 6Y$ and $\alpha 4C$. Thus, this experiment is not informative regarding $\alpha 4\alpha 6\beta 2\beta 3$ receptors in N2a cells. For instance, the $\alpha:\beta$ subunit stoichiometry as measured by FRET may not change in the presence of $\beta 3$.

TIRF Revealed $\alpha 4^*$, $\alpha 6^*$, and $\beta 3^*$ Receptor Plasma Membrane Localization. Having confirmed that fluorescent $\alpha 6$ and $\beta 3$ subunits assemble to form nicotinic receptor pentamers, we probed the plasma membrane localization of nAChRs containing these subunits using TIRF microscopy. For nicotinic receptor subunits fused to fluorescent proteins, TIRF illumination selectively excites only receptors at or very close to the plasma membrane. We imaged live N2a cells expressing $\alpha 4\beta 2\beta 3$ -YFP, $\alpha 6$ -YFP $\beta 2$, and $\alpha 4$ -YFP $\beta 2$. In epifluorescence mode (Fig. 11, epi), these receptors exhibited an intracellular, endoplasmic reticulum-like localization identical to our confocal imaging data in Fig. 4. In TIRF mode, however, we noted robust plasma membrane fluorescence for all receptor combinations. We were surprised to find $\alpha 4Y\beta 2$ receptors to be localized to distinct, filamentous structures protruding from the cell body (Fig. 11A, arrow). This specific filamentous pattern was seen for >90% of the plasma membrane fluorescence. These structures were reminiscent of filopodia, which are actin-dependent plasma membrane protrusions. To test whether these structures contain actin, a hallmark of filopodia, we imaged cells stained with rhodamine-phalloidin, a marker of polymerized actin. We noted distinct, actin-containing protrusions (Fig. 11B, arrow). These structures were actin-dependent, because they were destroyed by treatment with latrunculin B, an actin-disrupting

agent (Fig. 11B, right). These data indicate that, in N2a cells, $\alpha 4Y\beta 2$ nicotinic receptors are localized to membrane protrusions that strongly resemble filopodia.

Similar to $\alpha 4Y\beta 2$, we imaged live N2a cells, in TIRF mode, expressing either $\alpha 4\beta 2\beta 3Y$ or $\alpha 6Y\beta 2$. To our surprise, we noted a very different localization pattern compared with $\alpha 4Y\beta 2$. $\beta 3^*$ and $\alpha 6^*$ receptors were well expressed on the plasma membrane, but there was no evidence of membrane

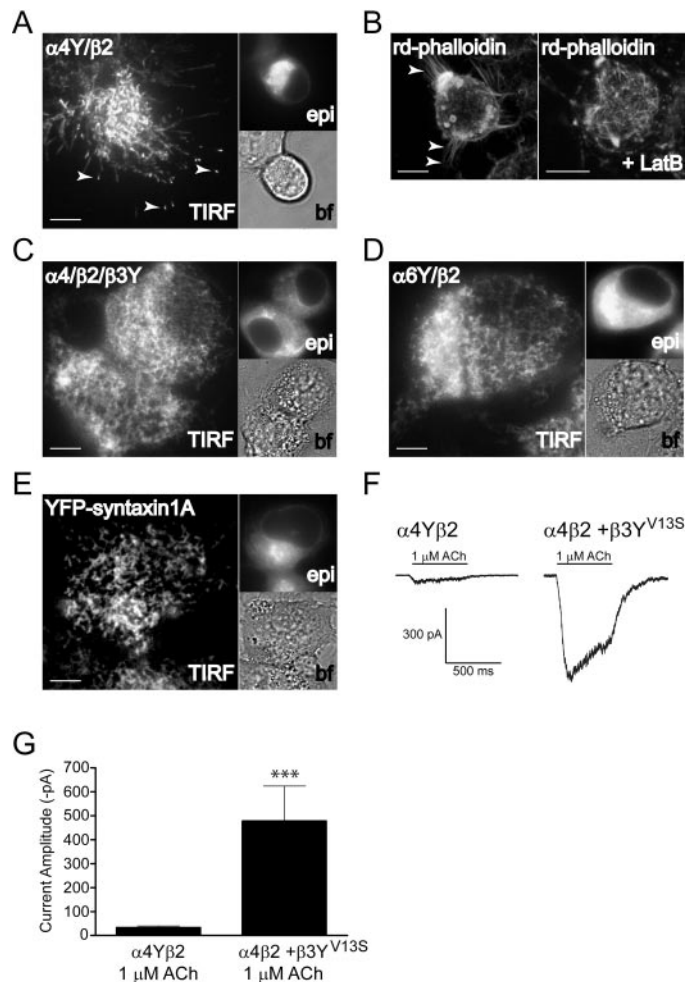


Fig. 11. Distinct plasma membrane localization for $\alpha 4\beta 2$ versus $\beta 3^*$ and $\alpha 6^*$ receptors. A, plasma membrane localization of $\alpha 4\beta 2$ receptors. N2a cells plated on polyethylenimine, expressing $\alpha 4$ -YFP $\beta 2$ receptors were imaged live under TIRF illumination. Arrows indicate $\alpha 4\beta 2$ receptors in distal parts of membrane protrusions. Epifluorescence (non-TIRF) images and bright-field images are shown for reference. Scale bars, 10 μm . B, filopodia in N2a cells. N2a cells were plated as described in A and stained with rhodamine-phalloidin to mark actin filaments (left, arrows) in the cytoplasm and in filopodia-like protrusions. Cells were imaged with confocal microscopy. Cells were treated with latrunculin B to disrupt actin filaments (right). C and D, lattice-like and punctate localization of $\alpha 4\beta 2\beta 3$ -YFP and $\alpha 6$ -YFP $\beta 2$ receptors on the plasma membrane. N2a cells expressing the indicated nAChR subunits were imaged in TIRF mode as described in A. Epifluorescence and bright-field images are shown for reference. E, syntaxin1A plasma membrane localization is similar to $\beta 3$ and $\alpha 6$ nicotinic receptors. N2a cells expressing YFP-syntaxin1A were imaged as described in A, C, and D. F, $\beta 3$ -YFP subunits on the plasma membrane are functional. N2a cells expressing either $\alpha 4$ -YFP $\beta 2$ or $\alpha 4\beta 2\beta 3$ -YFP V13S were studied using whole-cell patch-clamp electrophysiology. Voltage-clamped cells were stimulated with 1 μM ACh for 500 ms. A representative response from the indicated nAChR subtype is shown. Scale bar, 300 pA and 500 ms. G, quantification of electrophysiology data in F. Peak current responses from $\alpha 4$ -YFP $\beta 2$ and $\alpha 4\beta 2\beta 3$ -YFP V13S (1 μM stimulation) were averaged for four cells. ***, $p < 0.001$.

protrusion or filopodia localization for these receptors. Rather, these proteins exhibited a punctate, lattice-like localization pattern on the plasma membrane (Fig. 11, C and D). This pattern was consistently seen in other cells types such as HEK293 (data not shown), and it suggests that $\beta 3^*$ or $\alpha 6^*$ receptors cluster in microdomains distinct from $\alpha 4\beta 2$ receptors. Alternatively, some of these puncta could be clusters of assembled receptors adjacent to the plasma membrane within the 100-nm evanescent wave. We also recorded movies to monitor plasma membrane nAChRs, and we noted that although they exhibited localized, stochastic movements, most of these receptor clusters did not travel or translocate to any significant degree (data not shown). This localization pattern resembles that of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor protein syntaxin1, which was localized to distinct granules or microdomains in the plasma membrane when observed in TIRF (Ohara-Imaizumi et al., 2004). Because soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor proteins are important regulators of ion channel subcellular trafficking and function in neuronal soma and synaptic terminals (Bezprozvanny et al., 1995), we compared the plasma membrane localization pattern of YFP-syntaxin1A with $\beta 3$ -YFP* and $\alpha 6$ -YFP* receptors in N2a cells. We observed a plasma membrane distribution pattern for syntaxin1A that was very similar to $\beta 3$ and $\alpha 6$ subunits; syntaxin1A was also localized to distinct clusters adjacent to the plasma membrane or microdomains on the plasma membrane (Fig. 11E).

It is possible that the different plasma membrane localization pattern observed for $\alpha 4\beta 2$ versus $\alpha 4\beta 2\beta 3\gamma$ reflects the localization pattern of functional versus nonfunctional nicotinic receptors, respectively. To address this, we used whole-cell patch-clamp electrophysiology to record voltage-clamped responses from functional, fluorescent nAChRs expressed in N2a cells. Because WT (Broadbent et al., 2006) or YFP-tagged $\beta 3$ subunits (Fig. 2B) significantly attenuated nAChR responses, we used $\beta 3$ -YFP^{V13S} subunits to reverse this attenuation. We reasoned that, if coexpressed and coassembled with $\alpha 4\beta 2$ receptors, $\beta 3$ -YFP^{V13S} subunits should 1) induce the high-sensitivity ($\alpha 4$)₂($\beta 2$)₂($\beta 3$)₁ subunit stoichiometry similar to previous work (Broadbent et al., 2006); and 2) lower the EC₅₀ for activation of this high-sensitivity form by approximately 1 order of magnitude (Fig. 3B). When voltage-clamped N2a cells expressing $\alpha 4\beta 2$ receptors were stimulated with 1 μ M ACh, a dose that induces minimal (20–30 pA) responses in our previous work with HEK293 cells (Nashmi et al., 2003), we observed an identical phenotype (Fig. 11F). Responses to 300 μ M ACh were robust (200–400 pA), indicating significant plasma membrane expression of these receptors (data not shown). However, cells expressing $\alpha 4\beta 2\beta 3\gamma$ ^{V13S} receptors exhibited robust responses to 1 μ M ACh (Fig. 11F), which were significantly larger than the response size for $\alpha 4\beta 2$ receptors (Fig. 11G). This is the expected result if $\beta 3$ -YFP^{V13S} subunits are incorporated into functional nAChRs in N2a cells, and it is consistent with our *X. laevis* oocyte experiments (Figs. 2 and 3), and with the work of others (Broadbent et al., 2006). These data confirm that both $\alpha 4\beta 2$ and $\alpha 4\beta 2\beta 3\gamma$ receptors are functional in N2a cells and that the observed plasma membrane localization pattern for functional $\alpha 4\beta 2$ and $\alpha 4\beta 2\beta 3\gamma$ receptors is significantly different.

Discussion

Subcellular Localization of Nicotinic ACh Receptors. $\alpha 4\beta 2$ receptors were localized intracellularly in our previous studies in HEK293 cells and primary midbrain neurons (Nashmi et al., 2003), although enough receptors are expressed on the cell surface to record responses using electrophysiology. Furthermore, knockin mice with YFP-labeled $\alpha 4$ subunits show uniform intracellular and plasma membrane localization of $\alpha 4^*$ receptors (Nashmi et al., 2007). Other investigators have found a similar localization pattern for $\alpha 4\beta 2$ (Xu et al., 2006), $\alpha 3\beta 4$ (Grailhe et al., 2004), $\alpha 7$ (Xu et al., 2006), and 5-hydroxytryptamine_{3A} (Grailhe et al., 2004) receptors. In light of these studies, it is not surprising that we found fully assembled $\beta 3^*$ and $\alpha 6^*$ receptors in intracellular stores in N2a cells. This suggests that neurons produce more assembled nAChRs than they can use at any particular time and that they may require the ability to rapidly change either their total number or specific stoichiometry of receptor subtypes on the plasma membrane in response to different extracellular signals. The specific phenomenon of up- or down-regulation of nicotinic receptors occurs during chronic nicotine exposure (Marks et al., 1983; Nashmi et al., 2007) and in other neurological disorders including autism and Alzheimer's disease (for review, see Graham et al., 2002).

$\alpha 4\beta 2$ receptors were localized to actin-dependent membrane protrusions akin to filopodia, whereas $\beta 3^*$ and $\alpha 6^*$ receptors were not. Filopodia are critical sensory components of growth cones, influencing growth cone orientation and turning toward extracellular cues. Nicotinic receptors are required for growth cone orientation in some neuronal types (Zheng et al., 1994). Interestingly, $\alpha 4$ and $\beta 2$ subunits are highly expressed during embryogenesis (Azam et al., 2007), suggesting a role for these subunits in neuronal development and in adult function. $\alpha 6$ and $\beta 3$ subunits, in contrast, are not expressed at appreciable levels until after birth (Azam et al., 2007). We speculate that the differences we observe for $\alpha 4\beta 2$ versus $\beta 3$ or $\alpha 6$ receptors on the plasma membrane could reflect their involvement (or lack thereof) in neuronal development.

$\alpha 6$ Functional Expression. Although it remains unclear why it is very difficult to record functional responses from $\alpha 6^*$ receptors, this study advances our knowledge of this problem. In 20 different attempts, using several expression/assay systems, we could record no $\alpha 6$ functional responses (Supplemental Data; Table 1). This could be a result of many different problems, such as $\alpha 6$ mRNA stability, $\alpha 6$ protein production, proper assembly of $\alpha 6^*$ receptors, intracellular trafficking, or plasma membrane delivery. We (herein) and others have demonstrated that cells do not have an apparent problem synthesizing $\alpha 6$ subunits (Kuryatov et al., 2000; Tumkosit et al., 2006), and either partial or full assembly of $\alpha 6^*$ receptors occurs in mammalian tissue culture cells and *X. laevis* oocytes (Kuryatov et al., 2000; Tumkosit et al., 2006). We found robust ectopic expression of $\alpha 6$ -YFP subunits in tissue culture cells and in primary neurons. Furthermore, based on our FRET measurements, $\alpha 6$ subunits are fully capable of assembling with $\alpha 4$, $\beta 2$, and $\beta 3$ subunits in a manner indistinguishable from that of $\alpha 4$ and $\beta 2$. These are the presumptive subunits necessary for expression of $\alpha 6^*$ receptors in vivo (Salminen et al., 2007). Assembled $\alpha 6^*$

receptors are also localized identically to $\alpha 4^*$ receptors in our experiments. Finally, and most surprisingly, we ruled out the possibility that $\alpha 6^*$ receptors are not delivered to the plasma membrane. Plasma membrane localization for $\alpha 6^*$ receptors was identical to that of assembled, functional $\beta 3^*$ receptors. From these data, we conclude that, although fully assembled and partially localized on the plasma membrane, $\alpha 6^*$ receptors do not yield responses in standard functional assays. This information should facilitate the design of new experimental approaches to develop robust, reproducible reconstitution of $\alpha 6$ function in vitro.

Because we found no difference in FRET between $\alpha 4\alpha 6\beta 2$ receptors $\pm \beta 3$, the experiments probing the stoichiometry of $\alpha 4\alpha 6\beta 2\beta 3$ receptors are uninformative. It is possible that there are no assembled $\alpha 4\alpha 6\beta 2\beta 3$ receptors in N2a cells. We suggest that catecholaminergic or retinal ganglion cells, which are those cells in vivo that produce high levels of functional $\alpha 6^*$ receptors (Léna et al., 1999; Whiteaker et al., 2000; Champiaux et al., 2002, 2003; Zoli et al., 2002; Gotti et al., 2005b), express a unique protein or factor that is essential for proper function of these receptors. It is also possible that these cells are specially suited to traffic $\alpha 6^*$ and/or $\beta 3^*$ receptors to distal axons/presynaptic terminals. Our results probing $\alpha 6$ and $\beta 3$ axonal targeting (Fig. 5B) may be negative as a result of differences in the cell trafficking machinery in hippocampal ($\alpha 6$ -negative) versus catecholaminergic ($\alpha 6$ -positive) neurons, and they highlight the need for a more detailed study of $\alpha 6/\beta 3$ axonal targeting in $\alpha 6$ -positive neurons. Perhaps there is an $\alpha 6$ -associated protein similar to other nicotinic receptor-associated proteins, such as lynx1 (Miwa et al., 1999), which remains to be characterized.

$\alpha 6^*$ and $\beta 3^*$ Receptor Assembly and Subunit Stoichiometry. We previously demonstrated that FRET between XFP-labeled nicotinic receptor subunits not only reveals proximity between subunits but that increased FRET efficiency correlates with increased assembled, functional receptors (Nashmi et al., 2003). Because we cannot measure functional responses from $\alpha 6^*$ receptors, we must draw conclusions about their behavior inferentially by comparing it to $\alpha 4\beta 2$ receptors, which are functionally expressed. Using these criteria, we conclude that $\alpha 6$ subunits assemble with $\alpha 4$, $\beta 2$, and $\beta 3$ subunits. Furthermore, we conducted several specificity controls (FRET with GAT1C and GluCl β) that revealed that FRET between $\alpha 6$ or $\beta 3$ and other nAChR subunits is robust and likely explained by pentameric assembly. These experiments also reveal that a subpopulation of $\alpha 6$ subunits may be contained in partially assembled receptors. It may be this feature that precludes routine measurement of functional responses, further supporting the notion of a special factor in vivo that promotes $\alpha 6^*$ nAChR assembly and function.

We noted a slightly higher FRET efficiency for $\alpha 6^*$ versus $\alpha 4^*$ receptors in all assays reported herein. Because FRET E depends on distance, we speculate that this is largely due to the smaller M3-M4 intracellular loop of $\alpha 6$ (~136 residues) versus $\alpha 4$ (~270 residues). When the same XFP-labeled $\beta 2$ construct is expressed with $\alpha 4$ versus $\alpha 6$, the relative distance between fluorophores, and therefore the efficiency of FRET, will be different. Although the lack of structural information about these M3-M4 loops precludes us from making any quantitative predictions or correlations with our observed FRET E values, we assert that, at a qualitative

level, the differences in FRET E for $\alpha 4$ versus $\alpha 6$ are likely explained by the differences in M3-M4 loop length.

In this study, we use FRET to describe $\alpha 6$ and $\beta 3$ subunit stoichiometry in assembled pentamers containing these subunits. Based on our data, only one $\beta 3$ subunit is able to incorporate into a nicotinic receptor pentamer with other α and β subunits. It is not clear whether $\beta 3$ does so because it lacks residues required for formation of an $\alpha:\beta$ ligand-binding interface, or whether it is due to specific residues in the transmembrane segments or intracellular loops. What is clear is that $\beta 3$ is able to displace one subunit in a pentamer, which affords the ability to alter the subunit stoichiometry of receptors containing this subunit. Although this has been assumed based on indirect experiments (Broadbent et al., 2006), the present study is the first to directly elucidate the stoichiometry of $\beta 3^*$ receptors.

Based on our data, $\alpha 6^*$ receptors, like $\alpha 4^*$, are stoichiometrically heterogeneous. Previous reports using immunoprecipitation or genetic techniques have identified the specific subunits coassembled with $\alpha 6$, but not their stoichiometry (Zoli et al., 2002; Salminen et al., 2007). For example, Salminen et al. (2007) demonstrated the existence of native $\alpha 6\beta 2$, $\alpha 6\beta 2\beta 3$, and $\alpha 4\alpha 6\beta 2\beta 3$ subtypes, among others. But for $\alpha 6\beta 2$ receptors, how many $\alpha 6$ and $\beta 2$ subunits are present in a given pentamer? Our FRET results suggest that a mixture of stoichiometries exist for $\alpha 6^*$ receptors. We interpret the $\beta 3$ -induced decline in FRET between $\alpha 6Y$ and $\alpha 6C$ in $\alpha 6\beta 2$ receptors to mean, at least in part, that $\beta 3$ is displacing a third $\alpha 6$ subunit and stabilizing a stoichiometry of $(\alpha 6)_2(\beta 2)_2(\beta 3)_1$. The alternative, that $\alpha 6^*$ receptors adopt a strict $(\alpha 6)_2(\beta X)_3$ stoichiometry, is less likely. Such a scenario would require that $\beta 3$ is able to significantly reduce FRET between $\alpha 6Y$ and $\alpha 6C$ without changing $\alpha 6$ stoichiometry. It is more reasonable to assume that $\alpha 6$ is behaving similar to $\alpha 4$, whose stoichiometry is varied and can be altered by $\beta 3$ coexpression (Broadbent et al., 2006). $\alpha 6$ and $\beta 3$ subunits are present in $\alpha 4\alpha 6\beta 2\beta 3$ receptors in striatum and nucleus accumbens, which have the highest affinity for nicotine of any nicotinic subtype yet reported (Salminen et al., 2007). Their localization on dopaminergic nerve terminals coupled with this high affinity for nicotine ensures that they are among the first nicotinic subtypes activated during a smoking-induced bolus of nicotine. These and other high-affinity receptors are important targets for smoking cessation and Parkinson's disease medications, so understanding their subunit stoichiometry is important for the rational design of small molecule modulators of nAChR function.

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Supplemental Data

Table 1. nAChRs containing $\alpha 6$ subunits assayed for functional activity. No significant nAChR function was detected in cells expressing the following subunit combinations. The identity of the injected/transfected subunits, the expression/assay system, and reference (if applicable) using the specific $\alpha 6$ expression plasmid is listed.

Unless indicated otherwise, mouse subunits were used. For non-mouse subunits, the following notation indicates the species: ^H – human, ^R – rat, ^C – chick. In some cases, “sensitizing”, hypersensitive mutations ($\alpha 6^{\text{L9'S}}$ or $\beta 3^{\text{V13'S}}$) in $\alpha 6$ or $\beta 3$ were used.

Abbreviations: TEVC – two electrode voltage clamp; HEK – human embryonic kidney 293 cells; WCP – whole cell patch clamp; N2a – mouse N2a neuroblastoma cells; Fura – Fura-2 Ca^{++} flux assay.

<u>Subunits expressed</u>	<u>Expression/Assay system</u>	<u>Reference</u>
$\alpha 6\beta 2$	<i>Xenopus</i> oocyte - TEVC	this study
$\alpha 6\beta 2\beta 3$	<i>Xenopus</i> oocyte – TEVC	this study
$\alpha 6^{\text{L9'S}}\beta 2$	<i>Xenopus</i> oocyte – TEVC	this study
$\alpha 6^{\text{L9'S}}\beta 2\beta 3$	<i>Xenopus</i> oocyte – TEVC	this study
$\alpha 4\alpha 6^{\text{L9'S}}\beta 2$	<i>Xenopus</i> oocyte – TEVC	this study
$\alpha 6\beta 4$	<i>Xenopus</i> oocyte – TEVC	this study
$\alpha 6\beta 4\beta 3$	<i>Xenopus</i> oocyte – TEVC	this study
$\alpha 6^{\text{H}}\beta 2$	<i>Xenopus</i> oocyte – TEVC	(1)
$\alpha 6^{\text{R}}\beta 2$	<i>Xenopus</i> oocyte – TEVC	N/A
$\alpha 6^{\text{C}}\beta 2$	<i>Xenopus</i> oocyte – TEVC	(1)

$\alpha 3\alpha 6^{L9'S}\beta 4$	<i>Xenopus</i> oocyte – TEVC	this study
$\alpha 6\beta 3^{V13'S}\beta 4$	<i>Xenopus</i> oocyte – TEVC	this study
$\alpha 6$ -YFP $\beta 2$	<i>Xenopus</i> oocyte – TEVC	this study
$\alpha 6$ -YFP $\beta 4$	<i>Xenopus</i> oocyte – TEVC	this study
$\alpha 6\beta 2$ +Ric-3	<i>Xenopus</i> oocyte – TEVC	this study
$\alpha 6$ -YFP $\beta 2$	N2a – Fura, WCP	this study
$\alpha 6$ -YFP $\beta 2\beta 3$	N2a – Fura, WCP	this study
$\alpha 6$ -YFP $\beta 4$	N2a – WCP	this study
$\alpha 6\beta 2$	HEK – Fura, WCP	this study
$\alpha 6$ -YFP $\beta 2$	HEK – Fura, WCP	this study

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