

# The Duplicated $\alpha 7$ Subunits Assemble and Form Functional Nicotinic Receptors with the Full-length $\alpha 7$ \*

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**Background:** Schizophrenia is linked to the  $\alpha 7$  nicotinic acetylcholine receptor and to duplicated genes encoding truncated dup $\alpha 7$  and dup $\Delta\alpha 7$  subunits.

**Results:** Fluorescently labeled duplicated subunits display FRET with  $\alpha 7$ . Electrophysiology shows that duplicated subunits and  $\alpha 7$  form heteropentamers with altered responses to choline and varenicline.

**Conclusion:** Duplicated subunits co-assemble with  $\alpha 7$ , forming functional receptors.

**Significance:** Such heteropentameric receptors could play a role in schizophrenia.

The  $\alpha 7$  nicotinic acetylcholine receptor gene (*CHRNA7*) is linked to schizophrenia. A partial duplication of *CHRNA7* (*CHRFAM7A*) is found in humans on 15q13–14. Exon 6 of *CHRFAM7A* harbors a 2-bp deletion polymorphism, *CHRFAM7A- $\Delta 2bp$* , which is also associated with schizophrenia. To understand the effects of the duplicated subunits on  $\alpha 7$  receptors, we fused  $\alpha 7$ , dup $\alpha 7$ , and dup $\Delta\alpha 7$  subunits with various fluorescent proteins. The duplicated subunits co-localized with full-length  $\alpha 7$  subunits in mouse neuroblastoma cells (Neuro2a) as well as rat hippocampal neurons. We investigated the interaction between the duplicated subunits and full-length  $\alpha 7$  by measuring Förster resonance energy transfer using donor recovery after photobleaching and fluorescence lifetime imaging microscopy. The results revealed that the duplicated proteins co-assemble with  $\alpha 7$ . In electrophysiological studies, Leu at the 9'-position in the M2 membrane-spanning segment was replaced with Cys in dup $\alpha 7$  or dup $\Delta\alpha 7$ , and constructs were co-transfected with full-length  $\alpha 7$  in Neuro2a cells. Exposure to ethylammonium methanethiosulfonate inhibited acetylcholine-induced currents, showing that the assembled functional nicotinic acetylcholine receptors (nAChRs) included the duplicated subunit. Incorporation of dup $\alpha 7$  and dup $\Delta\alpha 7$  subunits modestly changes the sensitivity of receptors to choline and varenicline. Thus, the duplicated proteins are assembled and transported to the cell membrane together with full-length  $\alpha 7$  subunits and alter the function of the nAChRs. The characterization of dup $\alpha 7$  and dup $\Delta\alpha 7$  as well as their influence on  $\alpha 7$  nAChRs may help explain the pathophysiology of schizophrenia and may suggest therapeutic strategies.

Schizophrenia is a complex neuropsychiatric disorder. Its heterogeneity, incomplete penetrance, and environmental factors provide challenges for identifying genetic defects. On-line Mendelian Inheritance in Man now lists more than 200 candi-

date genes for schizophrenia susceptibility. Many genes have been associated with schizophrenia, yet there is no definitive genetic model. The etiology may involve a set of genes predisposing to the illness (1, 2).

One of the replicated candidate genes for schizophrenia is the  $\alpha 7$  nicotinic acetylcholine receptor (nAChR)<sup>2</sup> subunit gene, *CHRNA7*. Both pharmacological and genetic studies support a potential link between the  $\alpha 7$  nAChR and the pathophysiology of schizophrenia. The expression of the  $\alpha 7$  receptor, as measured by  $\alpha$ -bungarotoxin binding, is decreased in postmortem hippocampus, cortex, and thalamus of schizophrenic subjects (3–6). Moreover, *CHRNA7* localized at 15q14 is linked to the P50 auditory sensory processing deficit in schizophrenia by genetic and neurobiological studies (7, 8). Polymorphisms in the 5' upstream regulatory region and intron 2 of *CHRNA7* are associated with both schizophrenia and the sensory processing deficit (9, 10).

The human *CHRNA7* gene is partially duplicated. Exons 5–10 of *CHRNA7* and additional DNA (~200 kbp) were duplicated upstream, interrupting a second partial duplication of four exons from the gene *ULK4* on chromosome 3 (11, 12). This duplication appears to be a relatively recent event unique to humans (13). The partially duplicated chimeric gene, *CHRFAM7A*, is located at 15q14 1.6 Mb centromeric to the full-length gene, and it is almost always in the opposite orientation to *CHRNA7*. In this duplication, exons 5–10, intervening introns, and the 3'-untranslated region of *CHRNA7* are conserved. The gene product, dup $\alpha 7$ , lacks the signal peptide and part of the binding site but contains all of the  $\alpha 7$  membrane-spanning regions. Mutation screening in human DNA and mRNA identified a 2-bp deletion polymorphism in exon 6 of *CHRFAM7A* (14). The partial duplication with the 2-bp deletion, *CHRFAM7A $\Delta 2bp$* , is almost always in the same orienta-

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<sup>2</sup> The abbreviations used are: nAChR, nicotinic acetylcholine receptor; dup $\alpha 7$ , partial duplication of human  $\alpha 7$ ; dup $\Delta\alpha 7$ , partial duplication of human  $\alpha 7$  with 2-bp deletion in exon 6; DRAP, donor recovery after photobleaching; FLIM, fluorescence lifetime imaging microscopy; SCAM, substituted cysteine accessibility method; MTSEA, ethylammonium methanethiosulfonate; ACh, acetylcholine; MTS, methanethiosulfonate; FP, fluorescent probe; GAT1, GABA transporter subtype 1.

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tion as *CHRNA7* (15). The 2-bp deletion in exon 6 causes a frameshift in translation, resulting in a smaller gene product, dup $\Delta\alpha 7$ , which lacks nearly the entire ligand-binding site compared with dup $\alpha 7$ . *CHRFAM7A $\Delta$ 2bp* is significantly associated with schizophrenia (16, 17), suggesting that the duplicated gene might contribute to cognitive impairment.

The gene products of *CHRFAM7A*, both mRNA and protein, are found in brain and also in the periphery (11). When expressed alone in cell lines or *Xenopus* oocytes heterologously, the protein product of *CHRFAM7A*, dup $\alpha 7$ , is detected at low levels, but no functional receptor is observed (18). When either dup $\alpha 7$  or dup $\Delta\alpha 7$ , the protein product of *CHRFAM7A $\Delta$ 2bp*, is co-expressed with  $\alpha 7$  in *Xenopus* oocytes, it reduces the amplitude of the acetylcholine (ACh)-evoked currents, and two previous studies show that the reduction increases with the dose of dup $\alpha 7$  (19, 20). The pharmacology of the expressed receptors shows differences from that of the  $\alpha 7$  heteromer (20). Immunological studies in GH4C1 cells show that dup $\alpha 7$  reaches the plasma membrane (20). The authors of these previous studies suggest that *CHRFAM7A* acts as a dominant negative modulator of *CHRNA7* function in humans and thus may be critical for receptor regulation (19, 20).

This study further characterizes the role of *CHRFAM7A* by investigating how dup $\alpha 7$  and dup $\Delta\alpha 7$  exert their effects on the full-length  $\alpha 7$  nAChR. We have studied the interaction between fluorescently labeled duplicated and full-length subunits in mouse neuroblastoma cells (Neuro2a) using imaging techniques. We concentrated on the possible stoichiometry of the assembled complexes. We have also performed electrophysiology recordings to examine the receptor properties. We confirm that the duplicated subunits are assembled and transported to the cell membrane together with full-length  $\alpha 7$  and thus alter the function of the nAChRs. Our results contribute to the understanding of the pathophysiology of schizophrenia and suggest therapeutic strategies.

### EXPERIMENTAL PROCEDURES

**Reagents**—Unless otherwise indicated, all chemicals for culture were purchased from Invitrogen, and all chemicals for electrophysiology were purchased from Sigma.

**Plasmid Constructs**—Human  $\alpha 7$ , dup $\alpha 7$ , and dup $\Delta\alpha 7$  cDNA clones in pcDNA 3.1 were described previously (19). The fluorescent protein (FP) cassette, containing an Ala-Gly-Ala linker flanking the FP open reading frame on both sides, was fused into the M3-M4 loop of the subunits (21). The fusion site was chosen as described previously (22). Mutations were introduced using the QuikChange kit from Stratagene (La Jolla, CA) according to the manufacturer's instructions.

**Cell Culture and Transfections**—Mouse Neuro2a cells obtained from American Type Culture Collection (ATCC) were maintained in 45% Dulbecco's modified Eagle's medium (DMEM), 45% Opti-MEM, 10% fetal bovine serum (FBS) and supplemented with penicillin and streptomycin. Neuro2a cells were transfected with BioT (Bioland Scientific LLC, Paramount, CA) according to the manufacturer's instructions. Plasmid concentrations used for transfection were as follows: for imaging, 1  $\mu$ g of each fluorescently labeled nAChR subunit; for competition experiments, 500 ng of each fluorescently labeled subunit + 1

$\mu$ g of untagged subunit; for recording, 1  $\mu$ g of each fluorescently labeled nAChR subunit + 200 ng of RIC-3. Rat hippocampal neurons were dissociated from day 18 rat embryos and plated on 35-mm glass-bottomed imaging dishes (MatTek, Ashland, MA) as described previously (23). Neurons were transfected after 7 days in culture using Lipofectamine 2000 in conjunction with Nupherin (Biomol Research Laboratories, Plymouth Meeting, PA) according to the manufacturer's instructions (24). 1  $\mu$ g of each fluorescently labeled subunit was used.

**Confocal Imaging**—Neuro2a cells were plated at 90,000 cells per dish and imaged live 48 h after transfection. Before imaging, cell culture medium was replaced with phenol red-free CO<sub>2</sub>-independent Leibovitz L-15 medium. Cells were imaged with a Nikon (Nikon Instruments, Melville, NY) C1 laser-scanning confocal microscope system equipped with a 63  $\times$  1.4 numerical aperture VC Plan Apo oil objective. Pinhole diameter was 30–60  $\mu$ m, and cells were imaged at 12-bit intensity resolution over 512  $\times$  512 pixels at a pixel dwell time of 6.12  $\mu$ s. Cellular mGFP and mCherry fluorescent signals were acquired by simultaneous excitation with 488-nm (for mGFP) and 561-nm (for mCherry) lasers. Emission spectra were acquired in 5-nm bins between 500 and 660 nm, and the signal of each expressed fluorophore was linearly unmixed from the raw spectral image using reference spectra from control cells expressing only mGFP or only mCherry fusion constructs. For each pixel of a spectral image, intensity of mGFP and mCherry was determined from fluorescence intensity values at the peak emission wavelength derived from the reference spectra.

**Förster Resonance Energy Transfer (FRET) by Donor Recovery after Acceptor Photobleaching (DRAP)**—To examine FRET between nAChR subunits, the acceptor photobleaching method (21) was used with a modified DRAP macro built into the EZC1 imaging software (Nikon). The fluorescence intensity of mGFP and mCherry was recorded simultaneously using a 488- and 561-nm laser, respectively, during a six-step incremental photodestruction of mCherry with a 561-nm laser and normalized to the prebleach time point.  $I_D$  value was extrapolated from a scatterplot of the fractional increase of mGFP versus the fractional decrease of mCherry. FRET efficiency was calculated as  $E = 1 - (I_{DA}/I_D)$ , where  $I_{DA}$  represents the normalized fluorescence intensity of mGFP (100%) in the presence of both donor (mGFP) and acceptor (mCherry), and  $I_D$  represents the normalized fluorescence intensity of mGFP in the presence of donor only (complete photobleaching of mCherry). Data were averaged from 20 to 30 cells per condition and reported as mean  $\pm$  S.E.

FRET measurements may include contributions from pairs of fluorophores that are nearby but not in a macromolecular complex. In the biophysical literature, this has also been termed stochastic FRET, bystander FRET, background FRET, and proximity FRET. King *et al.* (25) calculated and measured that proximity FRET became negligible for tetramers at membrane densities less than  $\sim 10^{-3}$  receptors/nm<sup>2</sup> or 10<sup>3</sup> receptors/ $\mu$ m<sup>2</sup>, and similar estimates would apply to pentamers. The closely packed acetylcholine receptors at the nerve-muscle synapse have densities of 10<sup>4</sup>/ $\mu$ m<sup>2</sup>. For this study, densities on the plasma membrane were probably  $<10^2/\mu$ m<sup>2</sup>, judging by the

small size of the currents we observed, by the fact that receptors were not evident in membrane profiles, and by the fact that we have rarely observed signals from single  $\alpha 7$  nAChRs in membrane patches studied by zero-mode waveguides (26); measurements from Simonson *et al.* (27) yielded a similar conclusion. A more substantial, but less measurable, source of stochastic FRET would arise from fluorophores in the endoplasmic reticulum membrane. Total internal reflection fluorescent measurements of endoplasmic reticulum-resident heteromeric nAChRs (28) have led us to the assumption that these densities are  $<10^3/\mu\text{m}^2$ ; and the much lower signals observed in this study led us to an estimate of  $<10^2/\mu\text{m}^2$  in the endoplasmic reticulum. Thus, we conclude that proximity FRET has played little or no role in our measurements. An abundance of caution leads us to assume that in DRAP mode, FRET efficiencies of  $<10\%$  may include a contribution from proximity FRET.

**FRET by Fluorescence Lifetime Imaging Microscopy (FLIM)**—We have monitored the fluorescence lifetime  $\tau$  by time-correlated single-photon counting. YFP (donor)- and mCherry (acceptor)-tagged nAChRs were co-expressed in Neuro2a cells. We excited YFP with a picosecond pulsed laser (514 nm) and monitored the fluorescence emission using a time-correlated single-photon counting module (PicoQuant, West Springfield, MA). Data were collected at  $530 \pm 20$  nm through a bandpass filter. Laser power was adjusted to give average photon-counting rates of the order  $10^4$  to  $10^5$  photons/s to avoid pulse pile up. The decay of the YFP fluorescent intensity was fitted into either a single exponential (when YFP is present alone) or a double exponential (when YFP and mCherry are present) with SymPhoTime software (PicoQuant) using Equation 1,

$$I(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} \quad (\text{Eq. 1})$$

where  $\tau_1$  is the fluorescence lifetime of a YFP molecule when it is present alone, and  $\tau_2$  is the fluorescence lifetime of a YFP molecule when it interacts with its acceptor mCherry. We calculated the “binding fraction” and FRET efficiency using Equations 2 and 3,

$$P_{\text{FRET}} = \frac{A_2}{A_1 + A_2} \quad (\text{Eq. 2})$$

$$E_{\text{FRET}} = 1 - \frac{\tau_2}{\tau_1} \quad (\text{Eq. 3})$$

where  $P_{\text{FRET}}$  is the binding fraction, the fraction of YFP molecules that interact with mCherry, and  $E_{\text{FRET}}$  is the FRET efficiency.

**Whole-cell Patch Clamp Recordings**—Neuro2a cells were plated on 12-mm glass coverslips within 35-mm plastic bottom cell culture dishes at the density of 50,000 cells per dish. 48–72 h after transfection, glass coverslips were transferred to the recording chamber on the microscope stage. Expressing cells were identified and visualized as follows. Green (mGFP) and red (mCherry) fluorescence was visualized with an upright microscope (BX50WI, Olympus) and UV illumination with appropriate excitation filters. Cells were voltage-clamped at a holding potential of  $-65$  mV. Agonists were applied using a focal drug application system to minimize desensitization (29).

For concentration-response studies, ACh was applied from the lowest to highest concentration at 3-min intervals to get the dose-response curve. Whole-cell patch clamp recordings were performed with a MultiClamp 700B amplifier, a 1322 analog-to-digital converter, and pCLAMP 9.2 software (all from Axon Instruments, Molecular Devices). Data were sampled at 10 kHz and filtered at 2 kHz.

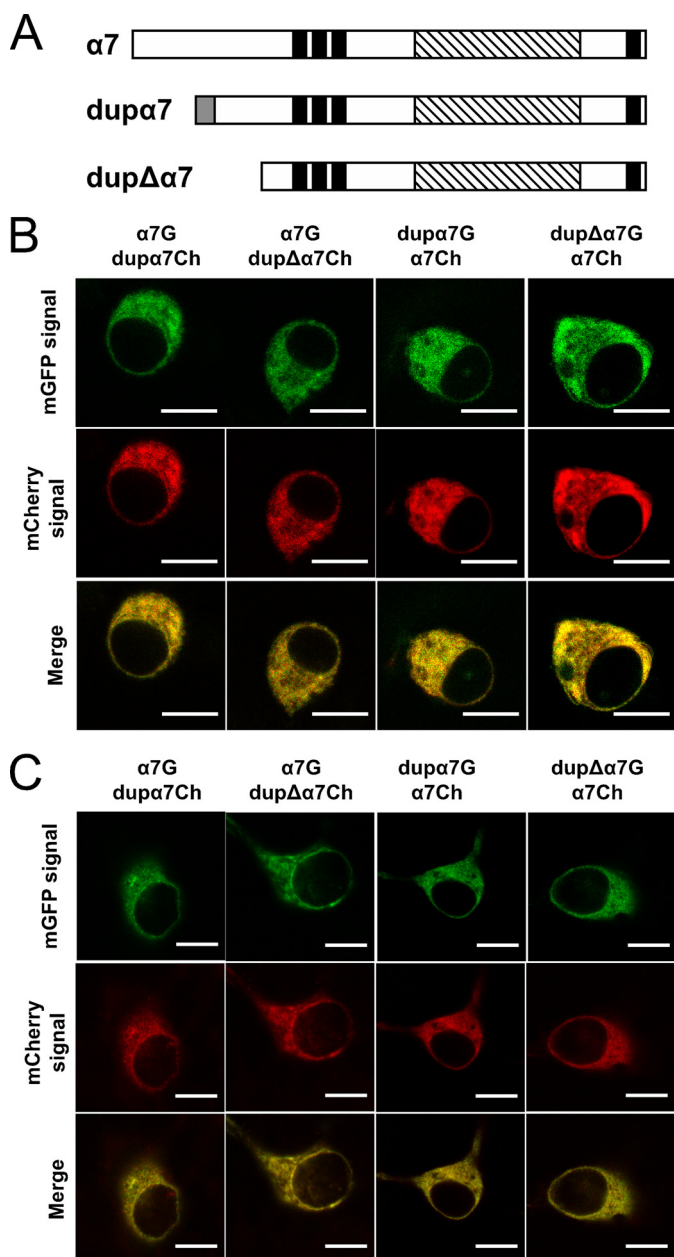
The intrapipette solution contained (in mM) the following: 135 potassium gluconate, 5 KCl, 5 EGTA, 0.5  $\text{CaCl}_2$ , 10 HEPES, 2 Mg-ATP, and 0.1 GTP; the pH was adjusted to 7.2 with Tris base and the osmolarity to 300 mOsm with sucrose. The extracellular solution contained (in mM) the following: 140 NaCl, 5 KCl, 2  $\text{CaCl}_2$ , 1  $\text{MgCl}_2$ , 10 HEPES, and 10 glucose; pH was adjusted to 7.3 with Tris base. The Nernst potential for  $\text{Cl}^-$  in the intrapipette solution is  $-82.9$  mV. The bath was continuously perfused with extracellular solution at room temperature. The patch electrodes had resistances of 5–8 megohms. The junction potential between the patch pipette and the bath solutions was nulled just before forming a seal. Series resistance was monitored and compensated by 70–80% throughout the recordings. The data were ignored if the series resistance (15–30 megohms) changed by  $>20\%$  during the recording session.

For the substituted cysteine accessibility method (SCAM), each cell received one initial baseline application of ACh (300  $\mu\text{M}$ ), one control ACh application, an application of ethylammonium methanethiosulfonate (MTSEA, 2 mM, Toronto Research Chemicals Inc., North York, ON, Canada), and then a follow-up experimental application of ACh. The peak amplitude of control responses and experimental responses were calculated relative to baseline ACh responses to normalize the data.

## RESULTS

**Co-localization of Fluorescently Labeled *dup* $\alpha 7$ /*dup* $\Delta\alpha 7$  Subunits and the Full-length  $\alpha 7$** —Based on previous work (21, 22), we fused fluorescent protein to the M3-M4 loop of full-length human  $\alpha 7$  nAChRs as well as the *dup* $\alpha 7$  and *dup* $\Delta\alpha 7$  subunits (Fig. 1A). Fluorescently labeled subunits are co-expressed in Neuro2a cells and imaged alive using a confocal microscope (Fig. 1B). We noted that the heterologous expression of duplicated subunits was low. The chimeric gene, *CHRFAM7A*, is transcribed efficiently, but translation is poor (19). The intensity of the FP attached to these subunits was  $\sim 5\%$  of the intensity of the FP attached to the full-length subunits. When co-expressed with fluorescent  $\alpha 7$ , the duplicated subunits were localized primarily in the endoplasmic reticulum, similar to the localization of the full-length receptors. We did not systematically study localization at the plasma membrane. The merged images of the GFP and mCherry signals indicate that both *dup* $\alpha 7$  and *dup* $\Delta\alpha 7$  subunits co-localize with the full-length  $\alpha 7$  receptor very well. Pearson correlation coefficients were as follows:  $\alpha 7$ GFP with *dup* $\alpha 7$ mCherry, 0.96 ( $n = 26$ );  $\alpha 7$ GFP with *dup* $\Delta\alpha 7$ mCherry, 0.95 ( $n = 25$ ); *dup* $\alpha 7$ GFP with  $\alpha 7$ mCherry, 0.96 ( $n = 27$ ); and *dup* $\Delta\alpha 7$ GFP with  $\alpha 7$ mCherry, 0.97 ( $n = 26$ ). We then co-expressed the duplicated and the full-length subunits in primary rat hippocampal neurons. Because of the low intensity of the labeled duplicated

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**FIGURE 1. Co-localization of fluorescently labeled duplicated subunits with full-length  $\alpha 7$  in representative confocal cross-sections.** *A*, schematics of fluorescently labeled constructs.  $\alpha 7$  is full-length human  $\alpha 7$  nAChR;  $dup\alpha 7$  is partial duplication of human  $\alpha 7$ ;  $dup\Delta\alpha 7$  is partial duplication of human  $\alpha 7$  with a 2-bp deletion. *Black boxes* represent transmembrane domains; *gray box* represents duplication of sequences from gene *ULK4* on chromosome 3, and *striped boxes* represent fluorescent proteins fused into the M3-M4 loop of nAChRs. *B*, duplicated subunits and full-length  $\alpha 7$  receptors are localized similarly in Neuro2a cells. Neuro2a cells were transfected with the indicated nAChR cDNAs and were imaged live with spectral confocal microscopy 48 h after transfection. Spectral images were acquired, and specific mGFP and mCherry signals were extracted with linear unmixing. Fluorophore abbreviations are as follows: G, mGFP; Ch, mCherry. *Green* (mGFP signal) and *red* (mCherry signal) pseudocolor was assigned, and *yellow* (Merge) indicates co-localized proteins. *C*, duplicated subunits and full-length  $\alpha 7$  receptors are localized similarly in the cell soma in primary 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 using confocal microscopy. Normalization was done to emphasize co-localization and differed for the full-length versus duplicated constructs. Without normalization, the merged signal is mostly green (when  $\alpha 7$ mGFP is expressed) or red (when  $\alpha 7$ mCherry is expressed) and provides little information about co-localization. All images in *C* are less bright than *B* because the overall protein expression level is lower in primary neurons (*C*) than in Neuro2a cells (*B*). Scale bar, 10  $\mu\text{m}$ .

subunits, they were not detected in the distal sites such as axons and dendrites. In the cell soma, where we were able to detect the duplicated subunits, both  $dup\alpha 7$  and  $dup\Delta\alpha 7$  were co-localized with the full-length  $\alpha 7$  (Fig. 1C). Pearson correlation coefficients were as follows:  $\alpha 7$ GFP with  $dup\alpha 7$ mCherry, 0.85 ( $n = 11$ );  $\alpha 7$ GFP with  $dup\Delta\alpha 7$ mCherry, 0.86 ( $n = 11$ );  $dup\alpha 7$ GFP with  $\alpha 7$ mCherry, 0.93 ( $n = 10$ ); and  $dup\Delta\alpha 7$ GFP with  $\alpha 7$ mCherry, 0.91 ( $n = 12$ ).

**Interaction between  $dup\alpha 7$ / $dup\Delta\alpha 7$  Subunits and the Full-length  $\alpha 7$ , DRAP Assays**—The fact that the duplicated and the full-length subunits are co-localized both in Neuro2a cells and in the cell body of neurons suggests that they may be assembled into oligomers. The receptor assembly of nicotinic subunits is often measured by immunoprecipitation (30). This approach has limited applicability, however, for  $dup\alpha 7$  and  $dup\Delta\alpha 7$ , due both to the extremely low protein levels and the lack of immune reagents selective for the duplicated subunits.

To directly determine whether the duplicated subunits interact with the full-length  $\alpha 7$ , we measured FRET between fluorescently tagged receptor subunits. FRET occurs only when donors and acceptors are within  $\sim 100 \text{ \AA}$ . When the fluorescent proteins are fused into the M3-M4 loop of nicotinic receptor subunits, subunits within pentamers undergo FRET. We have previously developed experimental approaches, well supported by theory, to analyze the assembly and subunit stoichiometry of such subunits within Cys loop receptors (23, 28, 31–33). For the present experiments, important guidelines arise from the strong distance dependence of FRET. Thus, for DRAP experiments adjacent subunits have considerably higher FRET values than nonadjacent subunits, and a donor adjacent to two acceptors displays higher FRET than one adjacent to zero or one acceptor.

We first employed DRAP to measure FRET between subunits (34). An mGFP/mCherry FRET pair was used for good separation of the excitation and emission spectra. We incrementally bleached mCherry at 561 nm while monitoring fluorescence intensity of both mGFP and mCherry, at 488 and 561 nm, respectively. Changes in fluorescence intensity versus time were plotted for each cell, and FRET efficiency was calculated as described under “Experimental Procedures.” DRAP data revealed several types of co-assembly as follows:  $\alpha 7$  with  $\alpha 7$ ;  $\alpha 7$  with  $dup\alpha 7$ ;  $\alpha 7$  with  $dup\Delta\alpha 7$ ;  $dup\alpha 7$  with  $dup\alpha 7$ ; and  $dup\Delta\alpha 7$  with  $dup\Delta\alpha 7$  (Fig. 2A). The measured FRET efficiencies were different between reciprocal pairs. FRET efficiency was greater when  $dup\alpha 7$ , or  $dup\Delta\alpha 7$ , was the donor. This is likely due to the low expression of the duplicated gene; a  $dup\alpha 7$  subunit is more likely to lie adjacent to an  $\alpha 7$  subunit in the heteromeric receptor.

To confirm the interaction between the duplicated subunits and the full-length subunit, we measured FRET between  $\alpha 7$ -mGFP and  $\alpha 7$ -mCherry when untagged competing subunits were co-expressed. Calculated FRET efficiency showed that co-expression of untagged subunits, either duplicated or full-length, significantly decreased the FRET efficiency of the  $\alpha 7$ -mGFP/ $\alpha 7$ -mCherry pair (Fig. 2B), suggesting the specific interaction between  $dup\alpha 7$ / $dup\Delta\alpha 7$  subunits and the full-length  $\alpha 7$ .

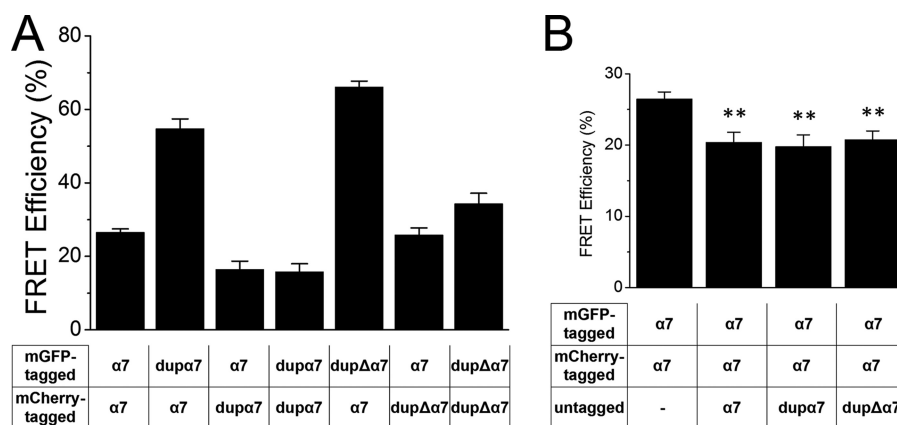


FIGURE 2. DRAP revealed assembly of duplicated subunits with full-length  $\alpha 7$ . A, FRET efficiency was measured by DRAP in Neuro2a cells. Several types of co-assembly were detected as follows:  $\alpha 7$  with  $\alpha 7$ ;  $\alpha 7$  with  $\text{dup}\alpha 7$ ;  $\alpha 7$  with  $\text{dup}\Delta\alpha 7$ ;  $\text{dup}\alpha 7$  with  $\text{dup}\alpha 7$ ; and  $\text{dup}\Delta\alpha 7$  with  $\text{dup}\Delta\alpha 7$ . B, co-expression of untagged competing subunits decreased the FRET efficiency of the  $\alpha 7$ -mGFP/ $\alpha 7$ -mCherry pair. Error bars are  $\pm$  S.E., and  $n = 20$ – $30$  cells for each condition. \*\*,  $p < 0.01$  compared with first condition.

*Potential Stoichiometry of  $\alpha 7 / \text{dup}\alpha 7$  and  $\alpha 7 / \text{dup}\Delta\alpha 7$  Heteromers, FLIM Measurements*—DRAP is not optimal for determining subunit stoichiometry in the present experiments because there are many more full-length  $\alpha 7$  subunits than duplicated subunits when they are co-expressed (32). We also employed FLIM to measure FRET. The fluorescence decay profile of the donor, YFP molecules, was acquired from the entire cell with the use of a time- and space-correlated single-photon counting detector and was fitted to a double exponential time course (Fig. 3A). Neuro2a cells transfected with various nAChR cDNAs were monitored alive. The binding fraction, the fraction of donor that interacts with the acceptor, and FRET efficiency were calculated as described under “Experimental Procedures.” In the context of analyses presented elsewhere, we believe that the binding fraction is best interpreted as the fraction of donors adjacent to one or more acceptors (23, 28, 31, 32). These two metrics are relatively insensitive to fluorophore concentration and light-path length, conditions that are poorly controlled inside a cell (35, 36). FLIM data confirmed several types of co-assembly as follows:  $\alpha 7$  with  $\alpha 7$ ;  $\alpha 7$  with  $\text{dup}\alpha 7$ ;  $\alpha 7$  with  $\text{dup}\Delta\alpha 7$ ;  $\text{dup}\alpha 7$  with  $\text{dup}\alpha 7$ ; and  $\text{dup}\Delta\alpha 7$  with  $\text{dup}\Delta\alpha 7$  (Fig. 3B).

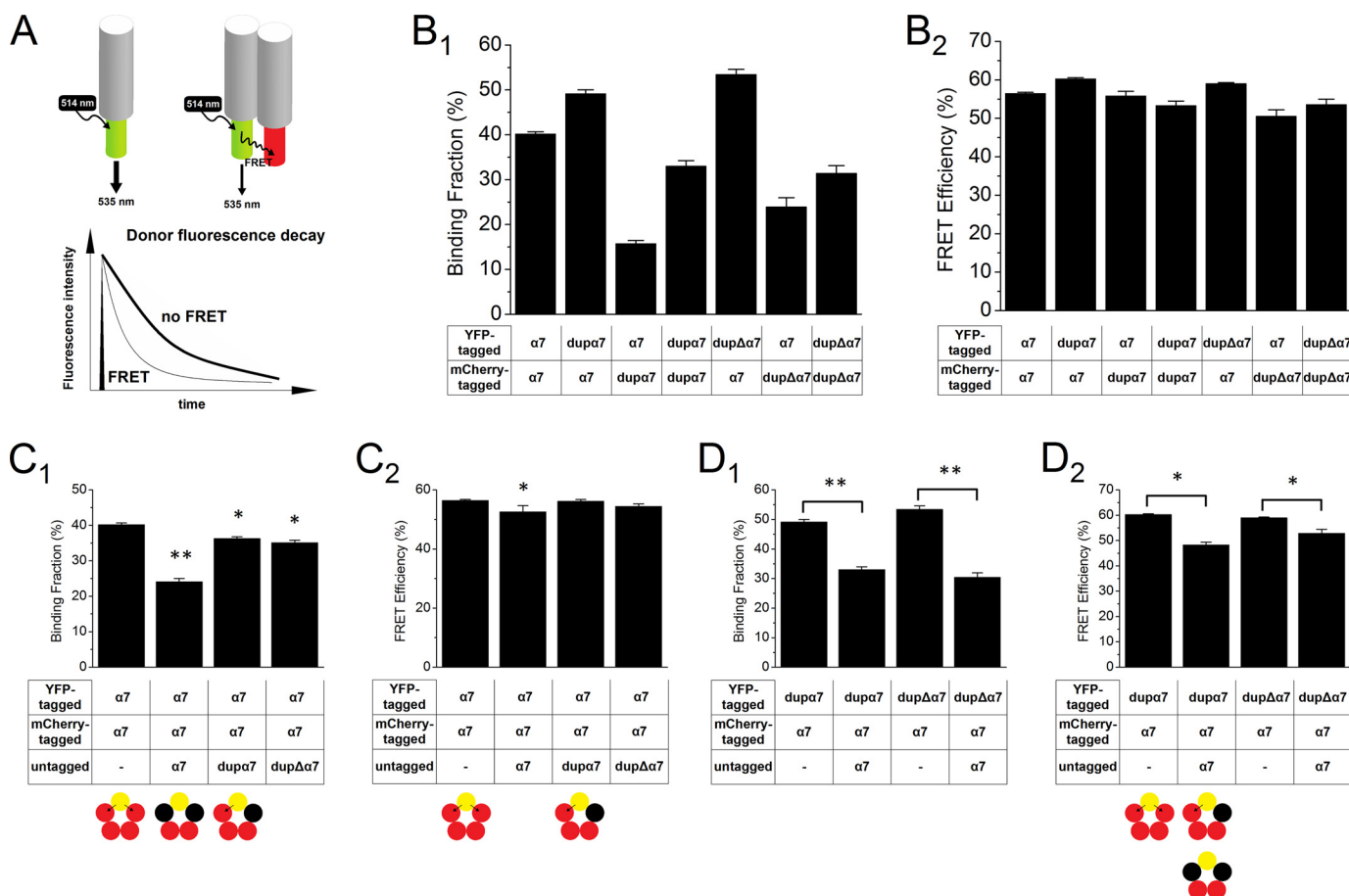
To investigate the stoichiometry of  $\alpha 7 / \text{dup}\alpha 7$  and  $\alpha 7 / \text{dup}\Delta\alpha 7$  heteromers, we measured FRET between various pairs when untagged competing subunits were co-expressed. Both binding fraction and FRET efficiency of the  $\alpha 7$ -YFP/ $\alpha 7$ -mCherry pair were decreased, as expected, by untagged full-length  $\alpha 7$  (Fig. 3,  $C_1$  and  $C_2$ ). Interestingly, co-expression of untagged duplicated  $\alpha 7$  decreased binding fraction but not FRET efficiency of the  $\alpha 7$ -YFP/ $\alpha 7$ -mCherry pair, suggesting a limited incorporation of the duplicated subunits. In an  $(\alpha 7\text{-YFP})_1(\alpha 7\text{-mCherry})_4$  configuration, as the number of untagged full-length subunits increases, the donor is less likely to be adjacent to an acceptor, which results in a decrease in the binding fraction. In contrast, if only one acceptor can be replaced by the duplicated subunit, the donor is still adjacent to an acceptor; thus one expects only a modest change in the binding fraction, and this is observed experimentally (Fig. 3C<sub>1</sub>). Furthermore, when untagged full-length  $\alpha 7$  was co-expressed for competition, both binding fraction and FRET efficiency of  $\text{dup}\alpha 7$ -YFP/ $\alpha 7$ -mCherry and

$\text{dup}\Delta\alpha 7$ -YFP/ $\alpha 7$ -mCherry pairs were decreased significantly. The difference between the reciprocal conditions,  $\text{dup}\alpha 7$  competing for  $\alpha 7$ -YFP/ $\alpha 7$ -mCherry pair and  $\alpha 7$  competing for  $\text{dup}\alpha 7$ -YFP/ $\alpha 7$ -mCherry pair, also supports the limited incorporation of duplicated subunits; more than one acceptor can be replaced by untagged subunits when  $\alpha 7$  is competing, decreasing FRET substantially.

*FRET Shows Assembly of  $\text{dup}\alpha 7$  and  $\text{dup}\Delta\alpha 7$  Subunits with Other Nicotinic Acetylcholine Subunits*—We examined whether the duplicated subunits interact with other nicotinic receptor subunits. We co-expressed  $\alpha 4$ -mCherry or  $\alpha 3$ -mCherry subunits with YFP-tagged  $\alpha 7$  subunits, either duplicated or full-length, in Neuro2a cells and measured FRET using FLIM (Fig. 4). Extensive quantitative analysis is not possible because of two complicating factors (32). 1) The  $\alpha 4$ ,  $\alpha 3$ , and  $\alpha 7$  subunits differ substantially in the size of their M3-M4 regions. 2) The fluorescent proteins may be inserted at differing orientations within these nonhomologous loops. Nonetheless, the results showed that both duplicated and full-length  $\alpha 7$  interacted with  $\alpha 4$  subunits (Fig. 4,  $A_1$  and  $A_2$ ). The FRET efficiency of a reciprocal pair,  $\alpha 4$ -YFP/ $\alpha 7$ -mCherry, was comparable with that of the  $\alpha 7$ -YFP/ $\alpha 4$ -mCherry pair, but the binding fraction is lower, probably due to larger numbers of  $\alpha 4$ -YFP subunits in the cells. The FRET efficiency of  $\text{dup}\alpha 7$ -YFP/ $\alpha 4$ -mCherry was higher than that of the  $\alpha 7$ -YFP/ $\alpha 4$ -mCherry pair, suggesting that  $\alpha 7$  subunits, both duplicated and full-length, assembled with  $\alpha 4$  into trimers or higher order multimers. If  $\alpha 7$ -YFP and  $\text{dup}\alpha 7$ -YFP only form dimers with  $\alpha 4$ -mCherry, the FRET efficiency will be the same for  $\alpha 7$ -YFP/ $\alpha 4$ -mCherry and  $\text{dup}\alpha 7$ -YFP/ $\alpha 4$ -mCherry.

To confirm the specificity of the interaction detected by FLIM, we co-expressed untagged competing  $\alpha 4$  subunits with the  $\alpha 7$ -YFP/ $\alpha 7$ -mCherry pair; both binding fraction and FRET efficiency were decreased (Fig. 4,  $C_1$  and  $C_2$ ). In contrast, co-expression of untagged GABA transporter GAT1 (31) had no effect on the  $\alpha 7$ -YFP/ $\alpha 7$ -mCherry pair. When untagged  $\alpha 7$  was used for competition, binding fraction but not FRET efficiency of the  $\alpha 7$ -YFP/ $\alpha 4$ -mCherry pair was decreased, whereas both binding fraction and FRET efficiency of  $\text{dup}\alpha 7$ -YFP/ $\alpha 4$ -mCherry and  $\text{dup}\Delta\alpha 7$ -YFP/ $\alpha 4$ -mCherry pairs were affected (Fig. 4,  $D_1$  and  $D_2$ ). Furthermore, both full-length and dupli-

## $\alpha 7$ / $dup\alpha 7$ and $\alpha 7$ / $dup\Delta\alpha 7$ Heteropentameric nAChRs



**FIGURE 3. FLIM confirmed assembly of duplicated subunits with full-length  $\alpha 7$ .** *A*, schematic of FRET occurring when a donor, the YFP (yellow-green cylinder) molecule attached to a nAChR subunit (gray cylinder), and an acceptor, the mCherry (red cylinder) molecule attached to a nAChR subunit, are in close proximity, usually  $< 10$  nm. The nonradiative transfer of excited state energy of the donor to the acceptor shortens the donor fluorescence lifetime and results in a faster decay in the fluorescence intensity of the donors. *B<sub>1</sub>* and *B<sub>2</sub>*, Neuro2a cells were transfected with the indicated nAChR subunits and were imaged alive using FLIM. Binding fraction (*B<sub>1</sub>*) and FRET efficiency (*B<sub>2</sub>*) were calculated as described under "Experimental Procedures." Several types of co-assembly were detected as follows:  $\alpha 7$  with  $\alpha 7$ ,  $\alpha 7$  with  $dup\alpha 7$ ,  $\alpha 7$  with  $dup\Delta\alpha 7$ ,  $dup\alpha 7$  with  $dup\alpha 7$ , and  $dup\Delta\alpha 7$  with  $dup\Delta\alpha 7$ . *C<sub>1</sub>* and *C<sub>2</sub>*, additional expression of untagged competing subunits (black circles) decreased the binding fraction of the  $\alpha 7$ -YFP/ $\alpha 7$ -mCherry pair. \*\*,  $p < 0.01$ ; \*,  $p < 0.05$  versus first condition. *D<sub>1</sub>* and *D<sub>2</sub>*, additional expression of untagged full-length  $\alpha 7$  subunit decreased both binding fraction and FRET efficiency of the  $dup\alpha 7$ -YFP/ $\alpha 7$ -mCherry and  $dup\Delta\alpha 7$ -YFP/ $\alpha 7$ -mCherry pairs. Error bars are  $\pm$  S.E., and  $n = 20$ – $30$  cells for each condition. \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ .

cated  $\alpha 7$  display robust FRET with  $\alpha 3$  nAChR subunits (Fig. 4, *E<sub>1</sub>* and *E<sub>2</sub>*).

***dup $\alpha 7$  and dup $\Delta\alpha 7$  Subunits Form Functional Receptors with  $\alpha 7$  Subunits***—The SCAM is appropriate to identify the residues lining the lumen of ion channels (37, 38). The cysteine sulfhydryl forms a disulfide bond with sulfhydryl-specific reagents, methanethiosulfonate (MTS) derivatives, including MTS-ethylammonium (MTSEA), ethyltrimethylammonium, and ethylsulfonate. A cysteine mutation is generated to replace a particular residue; if this residue lies in the lumen of the ion channel and is accessible from the extracellular solution, MTS derivatives alkylate the cysteine and alter ion channel function.

Leucine at the 247-position of  $\alpha 7$  nicotinic receptors resides  $\sim 9$  residues downstream from the cytoplasmic beginning of the M2 pore-lining region (termed the 9'-position in most studies, because the M2 domain is rather well conserved among Cys loop receptors). The M2 domain of  $dup\alpha 7$  and  $dup\Delta\alpha 7$  has the same sequence as  $\alpha 7$  in this domain. We replaced the Leu-9' residue with cysteine (L9'C mutation) in  $dup\alpha 7$  and  $dup\Delta\alpha 7$ . We transfected these subunits individually with  $\alpha 7$  subunits and measured receptor function with the whole-cell patch

clamp technique. If  $dup\alpha 7$  and  $dup\Delta\alpha 7$  subunits assemble with  $\alpha 7$  subunits to form functional receptors, we expect the introduced cysteines in  $dup\alpha 7$  and  $dup\Delta\alpha 7$  to be accessible for MTSEA and MTSEA to change receptor function. As the  $\alpha 7$  subunit has a cysteine at the 116-position, to eliminate the possibility that MTS derivatives bind to the endogenous cysteine and distort the results, we replaced this cysteine with serine in all of the constructs. The aligning mutation in  $dup\alpha 7$  and  $dup\Delta\alpha 7$  has different numbers but are termed C116S for consistency. Previous reports show that the C116S does not alter  $\alpha 7$  receptor function (39).

Using the whole-cell patch clamp technique and a focal drug application system (29, 40), we examined  $300 \mu\text{M}$  ACh-evoked inward current ( $I_{\text{ACh}}$ ) at 3-min intervals (Fig. 5*A<sub>1</sub>*). In cells satisfying the criterion of stable baseline responses ( $< 15\%$  variation in 10 min), we subsequently perfused the cell with standard extracellular solution for 3 min to wash out ACh, applied 2 mM MTSEA for 2 min, washed out MTSEA for 5 min, and puffed  $300 \mu\text{M}$  ACh to evoke  $I_{\text{ACh}}$  (Fig. 5*A<sub>2</sub>*). We compared  $I_{\text{ACh}}$  before and after MTSEA application to test whether MTSEA changed receptor function. Changes in agonist-induced currents indi-

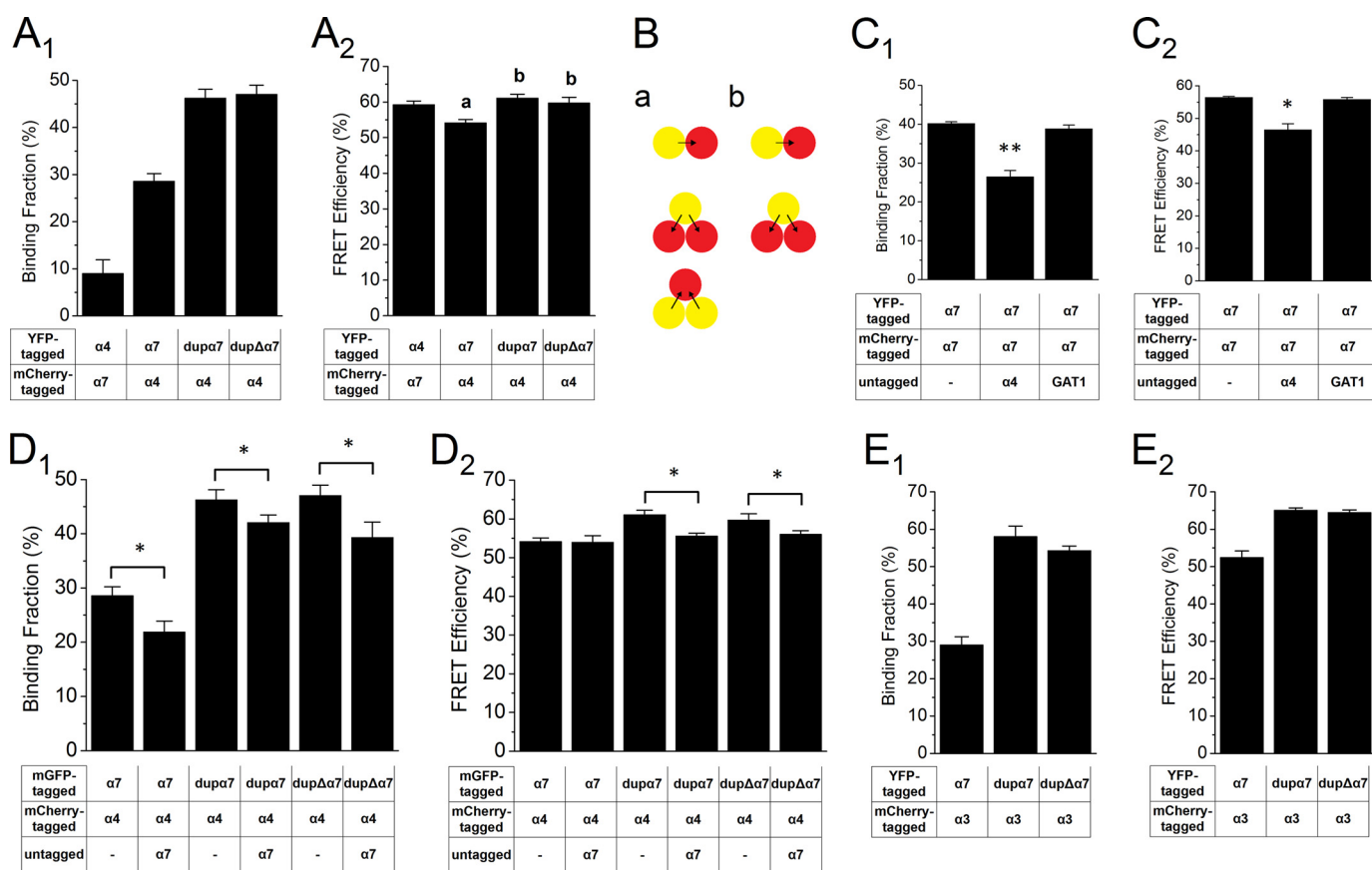


FIGURE 4. FLIM revealed potential interaction of duplicated subunits with other nAChR subtypes. *A*<sub>1</sub> and *A*<sub>2</sub>, Neuro2a cells were transfected with indicated nAChR subunits and were imaged alive using FLIM. Binding fraction (*A*<sub>1</sub>) and FRET efficiency (*A*<sub>2</sub>) were calculated.  $\alpha 7$ ,  $\text{dup} \alpha 7$ , and  $\text{dup} \Delta \alpha 7$  interact with the human  $\alpha 4$  subunit. *B*, schematic of FRET between donors and acceptors in a dimer or trimer. The yellow circle represents the donor (YFP molecule attached to a nAChR subunit), and the red circle represents the acceptor (YFP molecule attached to a nAChR subunit). An arrow represents energy transfer between the donor and the acceptor. *C*<sub>1</sub> and *C*<sub>2</sub>, co-expression of untagged  $\alpha 4$ , but not GAT1, decreased the FRET between the  $\alpha 7$ -YFP/ $\alpha 7$ -mCherry pair. *D*<sub>1</sub> and *D*<sub>2</sub>, co-expression of untagged full-length  $\alpha 7$  subunit decreased the FRET between the  $\alpha 7$ -YFP/ $\alpha 4$ -mCherry,  $\text{dup} \alpha 7$ -YFP/ $\alpha 4$ -mCherry, and  $\text{dup} \Delta \alpha 7$ -YFP/ $\alpha 4$ -mCherry pairs. *E*<sub>1</sub> and *E*<sub>2</sub>,  $\alpha 7$ ,  $\text{dup} \alpha 7$ , and  $\text{dup} \Delta \alpha 7$  interact with the human  $\alpha 3$  subunit. Error bars are  $\pm$  S.E., and  $n = 20$ –30 cells for each condition. \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ .

cate that the receptors include subunits with the cysteine mutation.

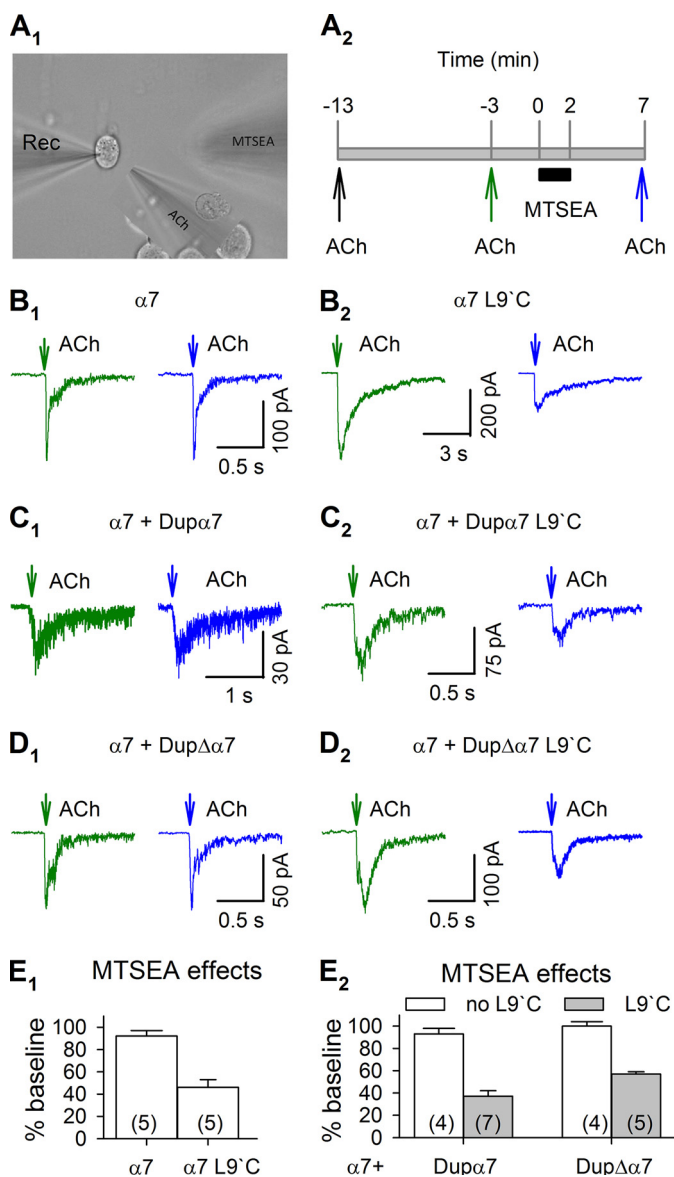
We transfected Neuro2a cells with  $\alpha 7$  DNA constructs containing the C116S mutation. As a control, we transfected  $\alpha 7$ -mCherry,  $\alpha 7$ -mCherry +  $\text{dup} \alpha 7$ -GFP, or  $\alpha 7$ -mCherry +  $\text{dup} \Delta \alpha 7$ -GFP into Neuro2a cells and tested whether MTSEA changed receptor function. As expected, MTSEA did not change  $I_{\text{ACh}}$  in the transfected cells (Fig. 5, *B*<sub>1</sub>, *C*<sub>1</sub>, and *D*<sub>1</sub>), confirming that introducing the C116S mutation was sufficient to prevent MTSEA from affecting the receptor's function. We next transfected  $\alpha 7$ -mCherry with both C116S and L9'C mutations into Neuro2a cells. In this situation, all of the subunits in the functional receptors are accessible to MTSEA. Indeed, we observed that MTSEA inhibited  $I_{\text{ACh}}$  by  $54 \pm 7\%$  ( $n = 5$ ,  $p = 0.002$ ) (Fig. 5, *B*<sub>2</sub> and *E*<sub>1</sub>). These results indicated that the assay was valid to reveal whether cysteine-containing subunits exist in functional receptors.

We next tested whether MTSEA changes receptor function in Neuro2a cells transfected with  $\alpha 7$ -mCherry containing the C116S mutation and either the  $\text{dup} \alpha 7$ -GFP or  $\text{dup} \Delta \alpha 7$ -GFP construct containing both C116S and L9'C mutations. We recorded  $I_{\text{ACh}}$  from cells showing both GFP and mCherry fluorescence and found that MTSEA, respectively, inhibited  $I_{\text{ACh}}$  by

$63 \pm 5\%$  ( $n = 7$ ,  $p < 0.0001$ ) and by  $43 \pm 2\%$  ( $n = 6$ ,  $p < 0.0001$ ) in cells expressing  $\text{dup} \alpha 7$ -GFP +  $\alpha 7$ -mCherry and  $\text{dup} \Delta \alpha 7$ -GFP +  $\alpha 7$ -mCherry (Fig. 5, *C*<sub>2</sub>, *D*<sub>2</sub>, and *E*<sub>2</sub>). Note that the MTSEA effects on these cells were similar to those on cells expressing  $\alpha 7$ -L9'C subunits only. These results suggested that both  $\text{dup} \alpha 7$  and  $\text{dup} \Delta \alpha 7$  subunits were able to form functional receptors with  $\alpha 7$  subunits, and in this expression system, most functional receptors had  $\text{dup} \alpha 7$  or  $\text{dup} \Delta \alpha 7$  subunits.

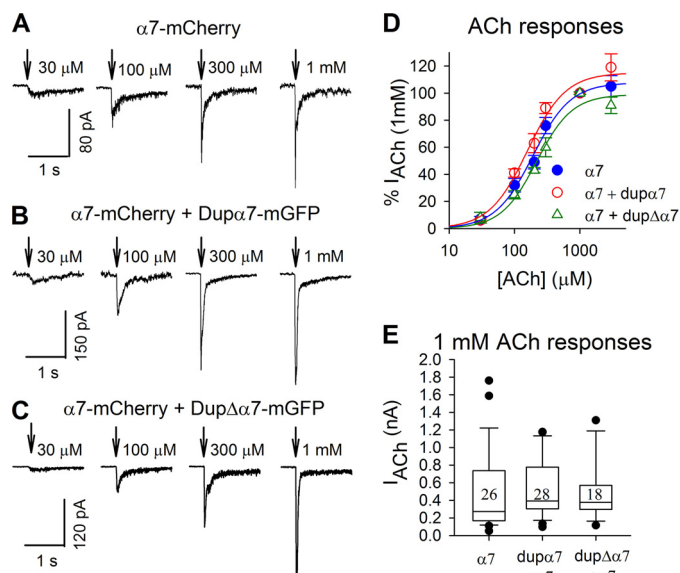
SCAM analysis suggests that in Neuro2a cells  $\text{dup} \alpha 7$  or  $\text{dup} \Delta \alpha 7$  subunits commonly assemble with  $\alpha 7$  subunits forming functional receptors. To understand whether the presence of  $\text{dup} \alpha 7$  or  $\text{dup} \Delta \alpha 7$  subunits changes function of  $\alpha 7$  receptors, we tested the dose-response relationship of  $I_{\text{ACh}}$  in Neuro2a cells transfected with  $\alpha 7$  only,  $\alpha 7$  +  $\text{dup} \alpha 7$ , or  $\alpha 7$  +  $\text{dup} \Delta \alpha 7$  subunits (Fig. 6, *A*–*C*).  $I_{\text{ACh}}$  in all transfected cells reached maximum levels when ACh exceeded 1 mM (Fig. 6*D*). The shifts of dose-response curves were not statistically significant (Fig. 6*D*). The Hill efficient was similar in  $\alpha 7$  only ( $1.51 \pm 0.25$ ),  $\alpha 7$  +  $\text{dup} \alpha 7$  ( $1.48 \pm 0.34$ ), or  $\text{dup} \Delta \alpha 7$  +  $\alpha 7$  ( $1.60 \pm 0.44$ ), and the  $EC_{50}$  value was similar in  $\alpha 7$  only ( $192 \pm 21 \mu\text{M}$ ),  $\alpha 7$  +  $\text{dup} \alpha 7$  ( $158 \pm 24 \mu\text{M}$ ), and  $\alpha 7$  +  $\text{dup} \Delta \alpha 7$  cells ( $218 \pm 31 \mu\text{M}$ ), respectively. These data suggest that the presence of  $\text{dup} \alpha 7$  and  $\text{dup} \Delta \alpha 7$  does not alter the ACh sensitivity of  $\alpha 7$  receptors.

## $\alpha 7$ /*dup* $\alpha 7$ and $\alpha 7$ /*dup* $\Delta\alpha 7$ Heteropentameric nAChRs

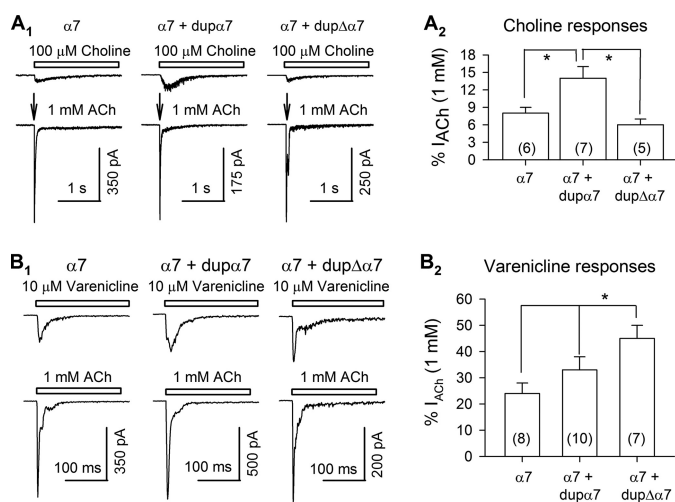


**FIGURE 5. SCAM evidence for the co-assembly of  $\alpha 7$  and *dup* $\alpha 7$  or *dup* $\Delta\alpha 7$  subunits in functional nAChRs.** *A<sub>1</sub>*, picture showing the whole-cell patch clamp recording from a Neuro2a cell, and the application of ACh and MTSEA. *A<sub>2</sub>*, timeline of ACh and MTSEA application. MTSEA did not change  $\alpha 7$  receptor function in cells expressing  $\alpha 7$  subunit alone (*B<sub>1</sub>*),  $\alpha 7 + \text{dup}\alpha 7$  subunits (*C<sub>1</sub>*), and  $\alpha 7 + \text{dup}\Delta\alpha 7$  subunits (*D<sub>1</sub>*). When the L9'C mutation was introduced into  $\alpha 7$  subunits, MTSEA inhibited ACh-induced currents (*B<sub>2</sub>*). When the L9'C mutation was introduced into *dup* $\alpha 7$  and *dup* $\Delta\alpha 7$ , MTSEA attenuated ACh-induced currents in cells expressing  $\alpha 7 + \text{dup}\alpha 7$  subunits (*C<sub>2</sub>*) and  $\alpha 7 + \text{dup}\Delta\alpha 7$  subunits (*D<sub>2</sub>*). *E<sub>1</sub>* and *E<sub>2</sub>*, summary of MTSEA effects on each group of cells. All subunits also included the C116S or aligning mutations. Numbers in parentheses indicate the number of cells examined.

**Channel Properties of *dup* $\alpha 7$ / $\alpha 7$  and *dup* $\Delta\alpha 7$ / $\alpha 7$  Heteromers**—We next tested whether *dup* $\alpha 7$  and *dup* $\Delta\alpha 7$  subunits affect the responses of  $\alpha 7$  receptors to choline, another endogenous agonist, and to varenicline, a full agonist. We activated  $\alpha 7$  receptors by puffing (20 p.s.i.) 100  $\mu\text{M}$  choline or 10  $\mu\text{M}$  varenicline (both are less than  $\text{EC}_{50}$ ) (41, 42) (Fig. 7, *A<sub>1</sub>* and *B<sub>1</sub>*, upper panels). On the same cell, we applied 1 mM ACh to evoke maximal activation of  $\alpha 7$  receptors (Fig. 7, *A<sub>1</sub>* and *B<sub>1</sub>*, lower panels). Then we normalized choline- and varenicline-induced currents to 1 mM ACh-evoked currents (Fig. 7, *A<sub>2</sub>* and *B<sub>2</sub>*). This process reduced the variability introduced by differences in plasma



**FIGURE 6. *dup* $\alpha 7$  and *dup* $\Delta\alpha 7$  subunits did not change  $\alpha 7$  receptor responses.** Responses to 30–300  $\mu\text{M}$  ACh were recorded from cells expressing  $\alpha 7$  subunits (*A*),  $\alpha 7 + \text{dup}\alpha 7$  subunits (*B*), and  $\alpha 7 + \text{dup}\Delta\alpha 7$  subunits (*C*). The peaks of ACh currents were normalized to those of the 1 mM ACh-induced ones in individual cells, and the normalized currents were used to depict dose-response curves (*D*). Each point is averaged from 5–9 cells and is shown as mean  $\pm$  S.E. The 1 mM ACh-induced currents are shown in box plots (*E*). Numbers in the box indicate the numbers of cells.



**FIGURE 7. Choline- and varenicline-evoked  $\alpha 7$  currents.** Choline (100  $\mu\text{M}$ ) (*A<sub>1</sub>*) and varenicline (10  $\mu\text{M}$ ) (*B<sub>1</sub>*) induced inward currents in Neuro2a cells expressing  $\alpha 7$  subunits,  $\alpha 7 + \text{dup}\alpha 7$  subunits, and  $\alpha 7 + \text{dup}\Delta\alpha 7$  subunits. In the same cells, 1 mM ACh-induced currents were also recorded (lower panels in *A<sub>1</sub>* and *B<sub>1</sub>*). The responses of choline and varenicline were normalized to those of 1 mM ACh, and the normalized responses were illustrated in *A<sub>2</sub>* and *B<sub>2</sub>*, respectively. Numbers in parentheses indicate the number of cells examined. \*,  $p < 0.05$ .

membrane receptor numbers among individual cells, and by the process of deliberately selecting fluorescent cells. Choline induced a larger relative current in cells expressing *dup* $\alpha 7$  +  $\alpha 7$  subunits ( $14 \pm 2\%$ ,  $n = 8$ ) than those expressing either  $\alpha 7$  subunits alone ( $8 \pm 1\%$ ,  $n = 6$ ) or *dup* $\Delta\alpha 7$  +  $\alpha 7$  subunits ( $6 \pm 1\%$ ,  $n = 7$ ) ( $p = 0.01$ , one-way analysis of variance) (Fig. 7*A<sub>2</sub>*). Varenicline-induced currents were significantly larger in cells expressing *dup* $\Delta\alpha 7$  +  $\alpha 7$  subunits ( $45 \pm 5\%$ ,  $n = 7$ ) than in those expressing  $\alpha 7$  subunits alone ( $24 \pm 4\%$ ,  $n = 8$ ) or those expressing *dup* $\alpha 7$  +  $\alpha 7$  subunits ( $33 \pm 5\%$ ,  $n = 10$ ) ( $p = 0.02$ ,



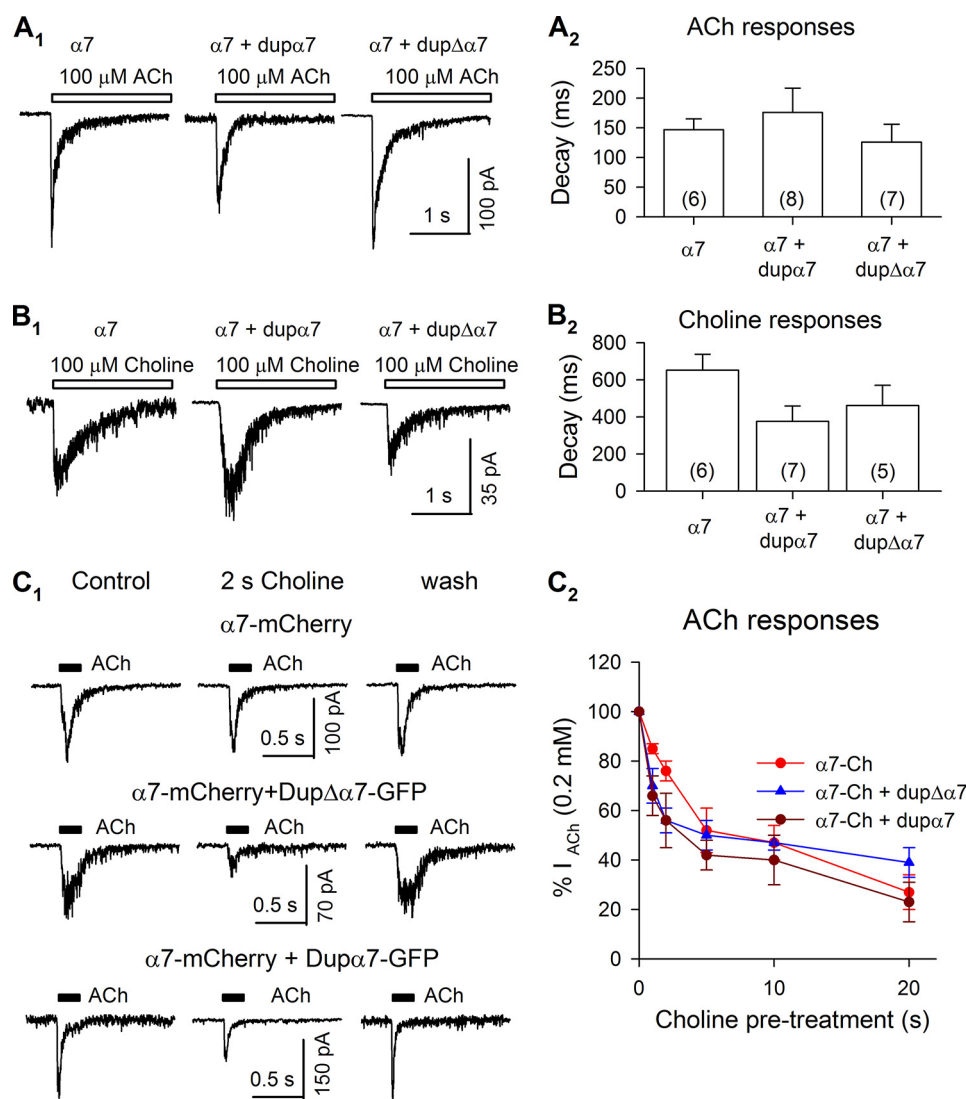


FIGURE 8. **Desensitization of  $\alpha 7$  receptors.** 100  $\mu\text{M}$  ACh ( $A_1$ ) or 100  $\mu\text{M}$  choline ( $B_1$ ) was applied for 2 s to Neuro2a cells expressing  $\alpha 7$  subunits,  $\alpha 7 + \text{dup} \alpha 7$  subunits, and  $\alpha 7 + \text{dup} \Delta \alpha 7$  subunits. The decay time constants of these responses were measured and summarized in  $A_2$  and  $B_2$ .  $C_1$ , prolonged preincubation with 20  $\mu\text{M}$  choline reversibly attenuated  $\alpha 7$  receptor currents induced by 200  $\mu\text{M}$  ACh.  $C_2$ , time course of desensitization by 20  $\mu\text{M}$  choline. Each point in  $C_2$  was averaged from 5 to 9 cells expressing  $\alpha 7$  subunits only (red circle),  $\alpha 7 + \text{dup} \Delta \alpha 7$  subunits (blue triangle), or  $\alpha 7 + \text{dup} \alpha 7$  subunits (brown circle).

one-way analysis of variance) (Fig. 7B<sub>2</sub>). These data suggest that the presence of dup $\alpha 7$  subunits may increase the sensitivity of  $\alpha 7$  receptors to choline, but co-assembly of dup $\Delta \alpha 7$  with  $\alpha 7$  subunits produced no significant change in sensitivity compared with homopentameric  $\alpha 7$  receptors. However, the presence of dup $\Delta \alpha 7$  subunits may enhance the sensitivity of  $\alpha 7$  receptors to varenicline.

Because 1 mM ACh evoked near-saturating currents, activating the great majority of  $\alpha 7$  receptors (Fig. 6D), the size of 1 mM ACh-induced currents may correlate well with the number of functional receptors. We compared 1 mM ACh-induced currents in cells expressing  $\alpha 7$  subunits alone,  $\alpha 7 + \text{dup} \alpha 7$  subunits, and  $\alpha 7 + \text{dup} \Delta \alpha 7$  subunits 72 h after transfection. As illustrated in Fig. 6E, the peak amplitude of  $I_{\text{ACh}}$  did not differ among groups of cells. These data suggest that dup $\alpha 7$  and dup $\Delta \alpha 7$  did not alter the number of  $\alpha 7$  receptors. We acknowledge that ascertainment bias could have been introduced by the process of identifying and studying the fluorescent cells.

We applied 100  $\mu\text{M}$  ACh and 100  $\mu\text{M}$  choline for relatively prolonged pulses lasting 2 s, whereas we measured voltage-clamp currents (Fig. 8,  $A_1$  and  $B_1$ ). Such experiments represent the classical tests for desensitization kinetics of nAChRs. As shown in Fig. 8,  $A_2$  and  $B_2$ , we found that the presence of dup $\alpha 7$  and dup $\Delta \alpha 7$  did not alter the desensitization kinetics of  $\alpha 7$  receptors in the presence of ACh and choline. In all cases, desensitization was nearly complete during the pulse.

Endogenous choline levels are around 35  $\mu\text{M}$  in the neonatal nervous system, and become  $\sim 3$  times lower in the adult (43). It remains unknown whether the loss of function in a schizophrenia patient carrying dup $\Delta \alpha 7$  receptors comes from less activation by choline, accelerated desensitization, or more complete desensitization of  $\alpha 7$  receptors in the presence of choline at circulating concentrations. To address these issues, we tested receptor activation by 20  $\mu\text{M}$  choline, and we compared 200  $\mu\text{M}$  ACh-induced currents before and after preincubation with 20  $\mu\text{M}$  choline for 1–60 s (Fig. 8,  $C_1$  and  $C_2$ ). 20  $\mu\text{M}$  choline induced

## $\alpha 7$ /*dup* $\alpha 7$ and $\alpha 7$ /*dup* $\Delta\alpha 7$ Heteropentameric nAChRs

no detectable currents in all cells we recorded (data not shown). Preincubation with 20  $\mu\text{M}$  choline up to 5 s reversibly attenuated ACh currents by  $\sim 50\%$  in cells expressing  $\alpha 7$  subunits alone, *dup* $\alpha 7$  +  $\alpha 7$  subunits, and *dup* $\Delta\alpha 7$  +  $\alpha 7$  subunits (Fig. 8,  $C_1$  and  $C_2$ ). The briefest preincubations with choline, 1 or 2 s, reversibly attenuated ACh-induced currents in cells expressing  $\alpha 7$  subunits alone by  $14 \pm 3\%$  ( $n = 6$ ) and  $24 \pm 3\%$  ( $n = 5$ ), respectively. Interestingly, the attenuations were significantly greater in cells expressing  $\alpha 7$  + *dup* $\alpha 7$  subunits (1-s choline by  $34 \pm 8\%$ ,  $n = 7$ ; 2-s choline by  $44 \pm 11\%$ ,  $n = 5$ ) and  $\alpha 7$  + *dup* $\Delta\alpha 7$  subunits (1-s choline by  $30 \pm 7\%$ ,  $n = 5$ ; 2-s choline by  $44 \pm 5\%$ ,  $n = 5$ ) (Fig. 8 $C_2$ ). These results support the hypothesis that the presence of *dup* $\alpha 7$  and *dup* $\Delta\alpha 7$  enhances some aspects of choline-induced desensitization at  $\alpha 7$  receptors.

### DISCUSSION

Exons 1–6 of *CHRNA7* correspond to the receptor's extracellular N-terminal region, which contains the ligand-binding domain. Exons 7 and 8 correspond to the first three transmembrane regions, M1, M2 and M3, and M2 constitutes the ion channel of the receptor (44). Exons 9 and 10 encode its intracellular cytoplasmic loop, the fourth transmembrane region, M4, and the extracellular C terminus. *CHRFAM7A*, containing only exons 5–10 of *CHRNA7*, lacks the signal peptide as well as part of the extracellular N-terminal region, including one of the three N-glycosylation sites. This lack of a ligand-binding domain renders it unsurprising that *dup* $\alpha 7$ , when expressed alone in *Xenopus* oocytes or Neuro2a cells, fails to produce ACh-induced current. In addition, previous studies have demonstrated that the N-terminal extracellular domain of nAChR plays a leading role in mediating assembly and determines the specificity of the intersubunit recognition (45–47). This study confirms that in a heterologous expression system, a truncated nAChR subunit lacking both signal peptide and part of the N-terminal extracellular domain is 1) processed in a similar way as the full-length subunit; 2) assembled with the full-length subunit; 3) trafficked to the cell membrane; and 4) incorporated into a functional channel.

Although the N-terminal extracellular domain is important for the initial association between subunits, a downstream domain, the cytoplasmic loop between M1 and M2, participates in the subsequent interaction and stabilization of the oligomeric complex (48). Further downstream, the M3–M4 intracellular loop also affects nAChR assembly (49, 50). Assembly of *dup* $\alpha 7$  and *dup* $\Delta\alpha 7$  with full-length  $\alpha 7$  suggests that other regions of sequence may mediate the interaction between subunits and thus compensate for the lack of the N terminus.

The duplicated subunits also interact with other nAChR subtypes, such as  $\alpha 4$  and  $\alpha 3$ , suggesting that they may have broad effects on nAChR function, depending on the cell types and brain regions in which they are expressed. Because the N-terminal extracellular domain controls specificity of subunit interaction, future studies should examine whether duplicated subunits assemble with other Cys loop receptors, such as serotonin 5-HT<sub>3</sub>, that do not normally interact with full-length  $\alpha 7$ . We do not know whether the interactions of the duplicated  $\alpha 7$  subunit, found here in the admittedly forced heterologous system, also occur in human neurons.

Immunostaining of *dup* $\alpha 7$  overexpressed in SH-EP cells confirmed that translated peptide contains the sequence for the  $\alpha 7$  subunit (19). The translation of *CHRFAM7A* $\Delta 2\text{bp}$ , however, is based only on prediction. When the 2-bp deletion in exon 6 is present, translation is likely to start at one of the two initiating methionines in exon 6. The peptide is out of frame for either 6 or 13 amino acids, depending on which ATG is used, until the 2-bp deletion is reached. At that point, the amino acid sequence returns to the reading frame of the  $\alpha 7$  subunit. The resulting protein, *dup* $\Delta\alpha 7$ , is smaller than *dup* $\alpha 7$  and lacks a larger portion of the agonist-binding domain. We fused a fluorescent protein into the M3–M4 loop of *dup* $\Delta\alpha 7$  and expressed the labeled protein in Neuro2a cells. Fluorescent signals were observed, and they overlap with the signals from fluorescently labeled full-length  $\alpha 7$ . Our confocal imaging data confirmed that *dup* $\Delta\alpha 7$  is indeed translated, and the protein is in-frame with  $\alpha 7$ .

We have studied the effect of the duplicated  $\alpha 7$  genes on  $\alpha 7$  nAChR function. In theory, they could regulate receptor expression and function in other ways, such as at the RNA level. Furthermore, several ATGs in *CHRFAM7A* could result in truncated transcripts that do not contain *CHRNA7* coding sequence. Because of the frameshift in *CHRFAM7A* $\Delta 2\text{bp}$ , use of the *dup* $\alpha 7$  subunit initiating methionine would encode a unique 40-amino acid peptide, terminated by a stop codon. With no homology to  $\alpha 7$ , it is unlikely that such a truncated peptide would assemble with  $\alpha 7$  subunits.

The duplicated subunits may have a role in inflammatory responses. Recent studies described  $\alpha 7$  nAChR as a link in the cholinergic anti-inflammatory pathway and an anti-inflammatory target (51–53). *dup* $\alpha 7$  is highly expressed in macrophages, and it is down-regulated at the mRNA level by IL-1, LPS, and nicotine (20, 54). Duplicated subunits could therefore modulate  $\alpha 7$  receptor-mediated cholinergic anti-inflammatory responses. We noted that co-expression of  $\alpha 7$  increased the proportion of *dup* $\alpha 7$ -expressing cells by 2-fold (data not shown); we do not know whether this occurred because  $\alpha 7$  enhances *dup* $\alpha 7$  mRNA expression or acts as a protein chaperone for *dup* $\alpha 7$  survival. Thus, investigating *dup* $\alpha 7$ / $\alpha 7$  pathways may have medical applications in the treatment of inflammatory disorders.

We found that co-expression of either duplicated subunit with  $\alpha 7$  subunits in mammalian cells had no marked effects on ACh-induced currents. This finding contrasts with previous studies in oocytes, showing a dominant negative effect of *dup* $\alpha 7$  on the full-length receptor (19, 20). Many previous studies show that ion channels and receptors assemble differently in these two expression systems, probably in several ways. First, *dup* $\alpha 7$  and *dup* $\Delta\alpha 7$  genes and proteins are expressed at extremely low levels in mammalian cells. Although we used equimolar amounts of  $\alpha 7$  and *dup* $\alpha 7$  DNA for transfection, the actual mRNA and protein ratio of *dup* $\alpha 7$ / $\alpha 7$  was probably 1:10 or lower. Because small amounts of *dup* $\alpha 7$  subunits were present, few  $\alpha 7$ /*dup* $\alpha 7$  heteromers included more than one *dup* $\alpha 7$  subunit. A previous study shows that nAChRs containing only a single non- $\alpha 7$  subunit, and therefore lacking two  $\alpha 7$ - $\alpha 7$  interfaces, do function rather well (55); but an additional non- $\alpha 7$  subunit does abolish function (55). This surfeit of *dup* $\alpha 7$  or

$\text{dup} \Delta \alpha 7$  subunits may be possible in oocytes, producing the apparent dominant negative effect, but apparently not in the present mammalian system. Second, our studies in Neuro2a cells also included RIC-3 (resistant to inhibitor of cholinesterase) expression. RIC-3 is an endoplasmic reticulum chaperone for nAChRs (56–58). The effects of the RIC-3 level on  $\alpha 7$  receptors display an inverted U-shape dose-response relation (57). RIC-3 also shows differential effects when co-expressed with various ligand-gated ion channels as follows: enhancing functional expression of multiple nAChR subtypes and inhibiting 5-HT<sub>3</sub> receptors and several nAChR subtypes, including  $\alpha 3 \beta 4$ , but had no effect on either GABA or glutamate receptors (59–61). We co-expressed RIC-3 in Neuro2a cells for all our electrophysiological experiments to enable functional expression of  $\alpha 7$  on the cell membrane. However, we do not know what influence RIC-3 may have on duplicated subunits and how RIC-3 may regulate the effect of duplicated subunits on full-length  $\alpha 7$  receptors.

Although we did not observe a significant difference in the ACh response of  $\alpha 7$  receptors when  $\text{dup} \alpha 7$  or  $\text{dup} \Delta \alpha 7$  was co-expressed,  $\text{dup} \Delta \alpha 7 / \alpha 7$  did display a lower choline sensitivity than  $\text{dup} \alpha 7 / \alpha 7$  heteromers. In addition,  $\text{dup} \alpha 7 / \alpha 7$  or  $\text{dup} \Delta \alpha 7 / \alpha 7$  receptors desensitize more quickly during brief (1 to 2 s) exposure to physiological concentrations of choline. Genetic and neurobiological studies suggest that choline availability may contribute to the development of schizophrenia-associated sensory gating deficits (62), and perinatal dietary choline supplementation has been tested for lowering schizophrenia risk, with positive results (63). The copy of the duplicated gene with the 2-bp deletion, *CHRFAM7A $\Delta$ 2bp*, is found much more frequently in schizophrenic patients than in control subjects with no mental illness (16) and is associated with abnormal sensory processing (64). Our electrophysiological data, suggesting that  $\text{dup} \Delta \alpha 7$  lacks the augmentation of choline sensitivity produced by  $\text{dup} \alpha 7$ , may be relevant to a decreased efficacy of choline during development in subjects that carry this 2-bp deletion.

Varenicline produces improved cognitive performance in schizophrenic patients (65–67). The underlying mechanisms remain unclear because varenicline activates at least three types of nAChRs. It is a partial agonist for  $\alpha 4 \beta 2$  ( $EC_{50} = 2.3 \mu\text{M}$ , with maximal efficacy of 13% relative to ACh) and  $\alpha 3 \beta 4$  ( $EC_{50} = 55 \mu\text{M}$  with maximal efficacy of 75% relative to ACh) nAChRs but a full agonist for  $\alpha 7$  nAChRs ( $EC_{50} = 18 \mu\text{M}$ ) (42). We observed a moderately enhanced sensitivity of  $\alpha 7 / \text{dup} \Delta \alpha 7$  nAChRs relative to  $\alpha 7$  and  $\alpha 7 / \text{dup} \alpha 7$  receptors, supporting the present concepts that  $\alpha 7$  nAChRs are promising targets for development of drugs to treat cognitive impairment in schizophrenia.

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