



Characterizing functional $\alpha 6\beta 2$ nicotinic acetylcholine receptors *in vitro*: Mutant $\beta 2$ subunits improve membrane expression, and fluorescent proteins reveal responsive cells

Cheng Xiao^{a,1}, Rahul Srinivasan^{a,1}, Ryan M. Drenan^a, Elisha D.W. Mackey^a, J. Michael McIntosh^b, Henry A. Lester^{a,*}

^a California Institute of Technology, 156-29 Caltech, Pasadena, CA 91125, USA

^b University of Utah, Salt Lake City, UT 84112, USA

ARTICLE INFO

Article history:

Received 8 April 2011

Accepted 9 May 2011

Available online 17 May 2011

Keywords:

Nicotinic acetylcholine receptor

$\alpha 6$

$\beta 2$

Fluorescent protein

Neuro2a cell

Endoplasmic reticulum exit sites

ABSTRACT

$\alpha 6^*$ nicotinic acetylcholine receptors (nAChRs) are highly expressed in mesostriatal and nigrostriatal dopaminergic systems, and participate in motor control, reward, and learning and memory. *In vitro* functional expression of $\alpha 6^*$ nAChRs is essential for full pharmacological characterization of these receptors and for drug screening, but has been challenging. We expressed eGFP-tagged- $\alpha 6$ and $\beta 2$ nAChR subunits in Neuro-2a cells, leading to functional channels. Inward currents were elicited with 300 μ M ACh in 26% (5/19) of cells with evenly expressed $\alpha 6$ -eGFP in cytoplasm and periphery. We dramatically increased chances of detecting functional $\alpha 6$ -eGFP $\beta 2$ nAChRs by (i) introducing two endoplasmic reticulum (ER) export-enhancing mutations into $\beta 2$ subunits, and (ii) choosing cells with abundant Sec24D-mCherry-labeled ER exit sites. Both manipulations also modestly increased $\alpha 6$ -eGFP $\beta 2$ nAChR current amplitude. $\alpha 6$ -eGFP $\beta 2$ nAChRs were also activated by nicotine and by TC-2403. The $\alpha 6$ -eGFP $\beta 2$ currents were desensitized by 1 μ M nicotine, blocked by α -conotoxin MII, partially inhibited by dihydro- β -erythroidine, and potentiated by extracellular Ca^{2+} . Single-channel recordings showed that $\alpha 6$ -eGFP $\beta 2$ nAChRs had similar single-channel conductance to, but longer open time than, $\alpha 4$ -eGFP $\beta 2$ nAChRs. These methods provide avenues for developing cell lines expressing subtypes of $\alpha 6^*$ nAChRs for both pharmacological study and drug screening.

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1. Introduction

Nicotinic acetylcholine receptors containing the $\alpha 6$ -subunits ($\alpha 6^*$ nAChRs) are expressed in only a few mammalian brain regions [1–8], with high expression in mesolimbic and nigrostriatal dopaminergic systems. Current knowledge regarding $\alpha 6^*$ nAChR function and drug screening is based primarily on mouse models, including lines lacking $\alpha 6$, $\alpha 4$, $\beta 2$, or $\beta 3$ subunits, as well as lines expressing hypersensitive $\alpha 6$ (L9'S) subunits [2,3,5,7–9]. α -Conotoxin MII is an $\alpha 6^*$ nAChR antagonist [5,6]. Functional $\alpha 6^*$ nAChR responses can be isolated by measuring α -conotoxin MII-sensitive components of nicotine- or ACh-induced responses [2,3,5,9]. $\alpha 6$ L9'S transgenic mice enable selective activation of $\alpha 6^*$ nAChRs with low concentrations of nicotine or other nicotinic ligands which activate $\alpha 6^*$ nAChRs [2,3].

These systems and protocols revealed that $\alpha 6^*$ nAChRs include a group of heteropentameric subtypes, including $\alpha 6\alpha 4\beta 2\beta 3$, $\alpha 6\alpha 4\beta 2$, $\alpha 6\beta 2\beta 3$, and $\alpha 6\beta 2$ nAChRs [2,5,7]. The $\beta 2$ subunit is present in all characterized $\alpha 6^*$ nAChR combinations in dopaminergic neurons [8]. $\alpha 6\alpha 4\beta 3\beta 2$ nAChRs are the most sensitive subtype [7,8], and deleting either $\alpha 4$ or $\beta 3$ subunits decreases receptor sensitivity [7]. $\alpha 6^*$ nAChRs participate in both motor control and reward. For instance, (1) knocking out $\alpha 4$ subunits, which decreases the sensitivity of $\alpha 6^*$ nAChRs, eliminates behavioral hyperactivity observed in $\alpha 6$ L9'S transgenic mice [2]; (2) $\alpha 6^*$ nAChRs are lost with degeneration of nigrostriatal dopaminergic system in Parkinson's disease [5,6,10]; (3) knocking out $\alpha 6$ subunits or blocking $\alpha 6^*$ nAChRs prevents nicotine self-administration [11–13]. Therefore, $\alpha 6^*$ nAChRs could be promising targets for both Parkinson's disease treatment and smoking cessation.

Progress in understanding $\alpha 6^*$ nAChR pharmacology is hindered by the fact that native expression systems currently available cannot adequately discriminate $\alpha 6^*$ nAChR subtypes. It is therefore important and necessary to establish robust and reproducible *in vitro* heterologous methods to express specific

* Corresponding author. Tel.: +1 626 395 4946; fax: +1 626 564 8709.
E-mail address: lester@caltech.edu (H.A. Lester).

¹ These authors contributed equally to this study.

$\alpha 6^*$ nAChR combinations for pharmacological characterization and high-throughput drug screening.

Successful expression of functional $\alpha 6^*$ nAChRs in heterologous systems has so far been challenging [14–19]. In *in vitro* expression systems, $\alpha 6$ subunits form mature, functional receptors more efficiently with $\beta 4$ subunits [15] than with $\beta 2$ and $\beta 3$ subunits [15–17,19]. Although $\alpha 6$ and $\beta 2$ subunits form agonist binding sites in human embryonic kidney cell lines, only 15% of cells show detectable ACh currents [20]. Kuryatov et al. constructed chimeras replacing the extracellular domain of $\alpha 3$ or $\alpha 4$ subunit with that of $\alpha 6$ subunit, and also dimeric and trimeric concatamers. These constructs do form functional $\alpha 6\beta 2\beta 3$ nAChRs [17,18]; however, they could alter either the machinery of channel gating or high order structures of the receptors.

In this study, we fused enhanced green fluorescent protein (eGFP) within the intracellular M3–M4 loop of $\alpha 6$ subunits, and detected functional $\alpha 6\beta 2$ nAChRs in 26% of Neuro-2a cells. Introducing a recently reported $\beta 2$ subunit with enhanced endoplasmic reticulum (ER) export ($\beta 2$ -DM, also called $\beta 2_{\text{enhanced-ER-export}}$) [21] dramatically increased the number of transfected cells expressing functional $\alpha 6\beta 2$ nAChRs. Further, transfecting an mCherry-tagged ER exit site (ERES) marker (Sec24D-mCherry) facilitated visualizing and choosing cells expressing functional receptors. Studying this heterologous expression system, we characterized pharmacological and electrophysiological properties of $\alpha 6\beta 2$ nAChRs with whole-cell and outside-out patch clamp recordings.

2. Methods and materials

2.1. Cell culture and nicotinic receptor transfection

$\alpha 6$ -eGFP was subcloned from an $\alpha 6$ -eYFP construct [14]. We transfected mouse $\alpha 6$ -eGFP $\beta 2$ -wt (wild type), $\alpha 6$ -eGFP $\beta 2$ -DM, or $\alpha 4$ -eGFP $\beta 2$ -wt nAChRs into Neuro-2a cells (mouse neuroblastoma 2a, CCL-131). For transfections, sterilized 12 mm ϕ glass coverslips (Deckgläser, Czech Republic, Prague) were placed in 35-mm culture dishes. Fifty thousand Neuro-2a cells in culture medium, composed of 45% DMEM, 45% Opti-MEM, 10% fetal bovine serum, were plated onto coverslips and cultured in an incubator (37 °C, 95% air, 5% CO₂). Twenty-four hours after plating, a mixture of 4 μ l Expressfect Transfection Reagent (E2600, Denville Scientific Inc.) and appropriate plasmids were added to 0.2 ml DMEM, and equilibrated for 20 min at room temperature. Cells were washed with DMEM twice to remove culture medium, and incubated with 0.2 ml transfection mixture in 1 ml culture medium for 4 h (37 °C, 95% air, 5% CO₂). Cells were then washed twice with culture medium and incubated in a final volume of 3 ml culture medium. Forty-eight hours later, the cells were ready for electrophysiological recording or imaging. Plasmid concentrations for transfection were as follows: 500 ng $\alpha 6$ -eGFP and 500 ng $\beta 2$ -wt/ $\beta 2$ -DM subunits, with or without 250 ng Sec24D-mCherry. We previously reported the expression of other nAChRs and transporters with this expression system, with regard to dependence on DNA levels, trafficking, and surface density [21–23]. The sources of plasmids have been previously described [21].

2.2. Total internal reflection fluorescence microscopy (TIRFM)

Neuro-2a cells were cultured in glass-bottom poly-D-lysine-coated imaging dishes (MatTek Corporation, Ashland, MA). Forty-eight hours after nAChR transfection, the culture dishes were transferred to a stage-mounted dish incubator (37 °C) (Warner Instruments, Hamden, CT) on an inverted microscope (IX71; Olympus). The microscope was equipped with an Olympus Plan Apo 100 \times 1.45 numerical aperture oil objective and a Mitutoyo

micrometer to control the position of the fiber optic and TIRF evanescent field illumination. TIRFM visualizes fluorescently labeled molecules within ~ 200 nm above the glass dish. Such molecules include those expressed in the cell membrane and in near-membrane intracellular structures [24–26]. eGFP fluorophores were excited with a 488-nm air-cooled argon laser, and an Optosplit II image splitter (Cairn Research, Faversham, UK) was used to simultaneously detect fluorescence emission from eGFP and pCS2-mCherry, a plasma membrane (PM) marker.

Images were captured with an iXON DU-897, back-illuminated EM-CCD camera. Sample exposure rate, percent laser transmission, and gain parameters were initially adjusted, then maintained constant across all samples for each imaging session. 488-nm laser lines were linearly s-polarized as revealed using an achromatic 400–800-nm half-wave plate (AQWP05M-600; Thorlabs, Newton, NJ).

The methodology for quantification of TIRFM images is described in detail elsewhere [21]. In brief, $\alpha 6$ -eGFP served as a subcellular marker for nAChR localization. To obtain ER regions of interest (ROIs), average PM fluorescence intensity and background signal was subtracted from the entire TIRF image. PM fluorescence was extracted as follows: Raw TIRF images were converted to background-subtracted images and the ER fluorescence was thresholded and selected. ER fluorescence was then subtracted from the original image to generate images with PM fluorescence signals. These procedures yielded a dataset of several hundred thousand pixel intensities over 15–50 cells in each experimental group. Integrated densities are simply the sum of pixel values for either PM or ER or ER + PM (using whole TIRF footprint images). Mean pixel-based PM integrated densities were derived: dividing the total integrated density of all pixels of imaged cells from each experimental group by the number of imaged cells, and were used as pixel-based measure of the mean population of PM localized receptors. Ratios of integrated densities for whole TIRF footprint (ER + PM) to ER were used to determine the post-Golgi fraction of receptors. For PM integrated density measurements, rather than plotting SEMs (which would be indistinguishably small on the plots), we have used “error bars” to depict 99% confidence intervals based on a two-tailed *t*-test.

2.3. Spectral confocal microscopy

A spectrally resolved laser-scanning confocal microscope (Eclipse C1si, Nikon) was equipped with a 63 \times 1.4 numerical aperture VC Plan Apo oil objective. Before imaging, cell culture medium was replaced with phenol red-free CO₂-independent Leibovitz L-15 medium. All images were taken from live cells 48 h after transfection at 37 °C. Cellular eGFP and mCherry fluorescence signals were acquired after sequential excitation with 488-nm (for eGFP) and 561-nm (for mCherry) lasers. Full-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 eGFP or only mCherry fusion constructs.

2.4. Patch clamp recordings

Recorded cells were visualized with an upright microscope (BX50WI; Olympus) in either bright field or fluorescence (eGFP and mCherry) mode. Electrophysiological signals were recorded with a MultiClamp 700B amplifier (Molecular Devices, Union City, CA), Digidata 1322 analog-to-digital converter (Axon Instruments), and pClamp 9.2 software (Axon Instruments). Patch pipettes were filled with solution containing (in mM): 135 K gluconate, 5 KCl, 5 EGTA, 0.5 CaCl₂, 10 HEPES, 2 Mg-ATP, and 0.1 GTP (pH was adjusted to 7.2 with Tris-base, and osmolarity was adjusted to 280–

300 mOsm with sucrose). The resistance of patch pipettes was 4–6 M Ω for whole-cell recordings, and 12–15 M Ω for outside-out patch single-channel recordings. Junction potential was nulled just before forming a gigaseal. Series resistance was monitored without compensation throughout the experiment (Multiclamp 700B). The data were discarded if the series resistance (10–25 M Ω) changed by more than 20% during recordings. All recordings were done at room temperature.

Data were sampled at 10 kHz and filtered at 2 kHz for whole-cell recordings, and sampled at 20 kHz and filtered at 4 kHz for single-channel recordings. Nicotinic agonists were dissolved in extracellular solution containing (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose (320 mOsm, pH set to 7.3 with Tris-base), and were puffed (0.1 or 3 s, 20 psi) onto voltage-clamped Neuro-2a cells (holding potential (V_H), –65 mV), or outside-out patches (V_H , –65 or –60 mV). To avoid receptor desensitization by repetitive ACh application, we applied ACh at ~3 min intervals, and continually perfused the recording chamber with extracellular solution [27,28].

2.5. Chemicals and applications

Mouse neuroblastoma 2a (Neuro-2a; CCL-131) cells were obtained from ATCC (Manassas, VA). pcDNA3.1(+) expression vectors and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). Expressfect was purchased from Denville Scientific (South Plainfield, NJ). Acetylcholine chloride (ACh), dihydro- β -erythroidine hydrobromide (DH β E), and (–)-nicotine hydrogen tartrate salt (nicotine) were from Sigma–Aldrich (St. Louis, MO). TC-2403 was provided by Targacept Inc. (Winston-Salem, NC).

2.6. Data analysis

Clampfit 9.2 was used to analyze both whole-cell and single-channel currents. For whole-cell currents, we low-pass filtered traces at 1 kHz, measured peak amplitude, and estimated decay time constant by fitting 10–90% of decay with one or two exponential terms. A chi-square test was used to compare incidences of α 6 β 2 nAChR current among cells with different transfections (α 6-eGFP β 2-wt, α 6-eGFP β 2-DM, and α 6-eGFP β 2-

DM plus Sec24D-mCherry). The difference of decay time constant between groups of cells was assayed by one-way ANOVA. When drug effects were tested, the amplitude of ACh-induced currents during the application of antagonists or 1 μ M nicotine was normalized to its mean value observed during control period (average of 3 responses). Drug effects were expressed as % inhibition (mean \pm SEM), while the recovery after drug washout was expressed as % of control values. The statistical significance of drug effects was assessed by a paired two-tailed *t*-test.

In outside-out patch recordings, we selected single-channel events that occurred within 5 s after ACh puffs (Fig. 6A₁ and B₁). This time window matches the decay of whole-cell currents (Figs. 1 and 4). We low-pass filtered signals at 4 kHz by Gaussian or Butterworth-8-pole filters, and used the event detection feature (single-channel search) in Clampfit to detect channel openings that were longer than 0.1 ms (Fig. 6A₂ and B₂). We pooled all events from cells transfected with either α 6-eGFP β 2-DM or α 4-eGFP β 2 nAChRs, and plotted histograms for both amplitude (0.1 pA per bin) and dwell time (0.2 ms per bin) of one-level openings (Fig. 6). The mean of each parameter was estimated after curve fitting. The amplitude distribution was fitted by a Gaussian relation, and the distribution of open times was fitted to a single exponential relation. We performed a two-tailed *t*-test to compare single-channel conductance, and used the Kolmogorov–Smirnov test to compare open time between α 6-eGFP β 2-DM and α 4-eGFP β 2-wt nAChRs.

Values of *p* < 0.05 were considered significant.

3. Results

3.1. ACh-induced currents in Neuro-2a cells transfected with α 6-eGFP β 2-wt and α 4-eGFP β 2-wt nAChRs

In order to visualize α 6 β 2 nAChRs, we transfected Neuro-2a cells with α 6-eGFP and β 2-wt nAChR subunits. Forty-eight hours after transfection, most eGFP-positive cells showed cytoplasmic localization of eGFP aggregates. In a small fraction of cells, eGFP was evenly expressed in cytoplasm and periphery (Fig. 3A). Since peripheral receptors may include those inserted in PM, which could respond to agonist, we selected cells that evenly expressed

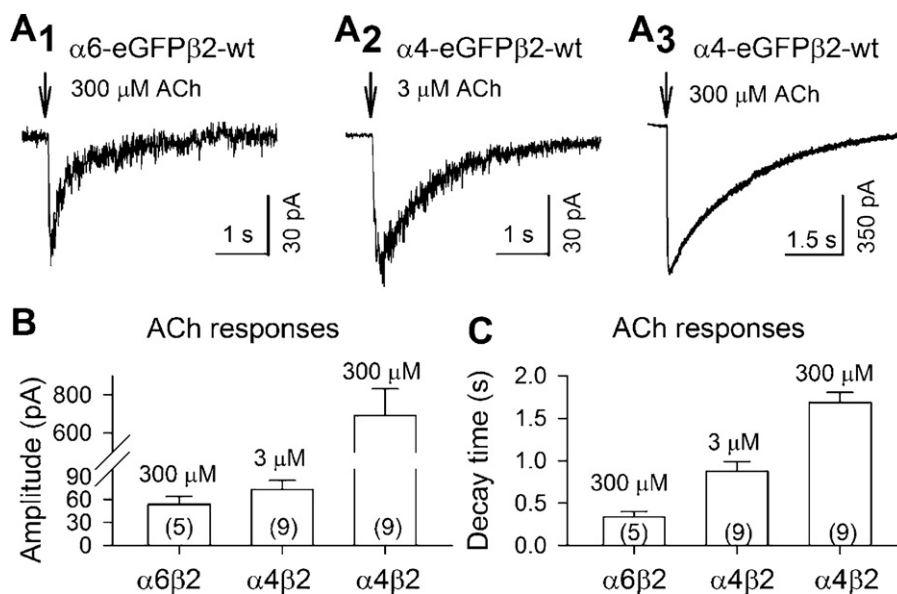


Fig. 1. α 6 β 2 and α 4 β 2 nAChR currents in transfected Neuro-2a cells. (A_{1–3}) Representative traces showing 300 μ M ACh-induced current in an α 6-eGFP β 2-wt cell (A₁), and 3 μ M (A₂) and 300 μ M (A₃) ACh-induced currents in an α 4-eGFP β 2-wt cell. Arrows indicate 100 ms ACh puffs. Summaries of peak amplitude and decay time constants, respectively, are shown in (B) and (C). Transfected nAChR subunits are indicated on the x-axis. α 6 β 2: α 6-eGFP β 2-wt. α 4 β 2: α 4-eGFP β 2-wt. ACh concentrations are indicated above each vertical bar (mean \pm SEM). Cell numbers are shown in parentheses.

$\alpha 6$ -eGFP in cytoplasm and periphery (Fig. 3A) for patch clamp recording. We recorded inward currents in 26% (5/19) of cells in response to puffed ACh (300 μ M, 0.1 s, 20 psi) (Fig. 1A₁). Using the same methods, we transfected $\alpha 4$ -eGFP plus $\beta 2$ -wt subunits into Neuro-2a cells [21]. Forty-eight hours after transfection, we detected ACh-induced inward currents from all recorded $\alpha 4$ -eGFP-positive cells (Fig. 1A_{2–3}).

300 μ M ACh-induced inward currents in $\alpha 6$ -eGFP $\beta 2$ -wt cells (54 ± 10 pA, $n = 5$, Fig. 1A₁ and B) were much smaller than those in $\alpha 4$ -eGFP $\beta 2$ -wt cells (690 ± 140 pA, $n = 9$, Fig. 1A₃ and B). Although 3 μ M ACh induced no current in $\alpha 6$ -eGFP $\beta 2$ -wt cells that responded to 300 μ M ACh ($n = 2$, data not shown), it evoked inward currents (73 ± 12 pA, $n = 9$, Fig. 1A₂ and B) in cells transfected with $\alpha 4$ -eGFP $\beta 2$ -wt nAChRs. The averaged decay time constant of 300 μ M ACh-induced $\alpha 6$ -eGFP $\beta 2$ -wt currents was 333 ± 67 ms ($n = 5$), while that of 3 and 300 μ M ACh-induced $\alpha 4$ -eGFP $\beta 2$ -wt currents was respectively 932 ± 69 ms ($n = 9$) and 1686 ± 120 ms ($n = 9$) (Fig. 1C).

All known nAChRs are at least 50% activated by 300 μ M ACh used in the experiment of Fig. 1 [29,30]. $\alpha 6\beta 2$ nAChRs are not exceptional in this regard [20]. Therefore, it is unlikely that the small size of the observed ACh-induced $\alpha 6$ -eGFP $\beta 2$ -wt currents arose from the activation of a small fraction of functional receptors, although we have not gathered systematic dose–response data. It seemed more appropriate to pursue the hypothesis that a large majority of $\alpha 6$ -eGFP $\beta 2$ -wt nAChRs do not insert into the PM. This supposition motivated the experiments described below.

3.2. $\beta 2$ subunits with enhanced ER export mutations increased membrane insertion of $\alpha 6$ -eGFP $\beta 2$ nAChRs

The mouse $\beta 2$ subunit has an ER retention/retrieval motif (365RRQR368), but lacks an ER export motif (347LFL349, instead of 347LFM349) [21]. We previously measured the effects of disrupting the ER retention motif (365AAQA368) and reconstituting ER export motif (347LFM349) in the $\beta 2$ subunit; the doubly mutant

$\beta 2$ subunit is called $\beta 2$ -DM or $\beta 2_{\text{enhanced-ER-export}}$. Incorporation of $\beta 2$ -DM increases the number of ERES [21] and subsequently facilitates PM insertion of $\alpha 4$ -eGFP $\beta 2$ nAChRs. We were encouraged to conduct analogous experiments with $\alpha 6$ subunits, because the $\beta 4$ subunit possesses an ER export motif and lacks an ER retention/retrieval motif [21]; and both $\alpha 4\beta 4$ and $\alpha 6\beta 4$ nAChRs show much more functional expression than the corresponding $\alpha 4\beta 2$ and $\alpha 6\beta 2$ heteropentamers [15,21]. Therefore, we hypothesized that these mutants could enhance membrane insertion of $\alpha 6$ -eGFP $\beta 2$ nAChRs.

TIRF microscopy enables visualization of fluorophores within ~ 200 nm of PM [24–26]. Our studies showed that TIRFM detects fluorescent protein-tagged nAChRs localized in the PM and peripheral ER [14,21]. In TIRF footprints, ER is characterized by reticulate labeling, while PM shows uniform fluorescence. Subcellular differences in fluorescence labeling became apparent in PM and ER subtracted TIRF images (Fig. 2A). We consistently observed that ER was 2–3 fold brighter than PM. We exploited subcellular differences in morphology and fluorescence intensity to separately demarcate and quantify PM and ER fluorescence (Fig. 2A, see Section 2).

To confirm our hypotheses, we used TIRFM to compare PM localization of $\alpha 6$ -eGFP $\beta 2$ -wt and $\alpha 6$ -eGFP $\beta 2$ -DM nAChRs. The average pixel-based measure of PM integrated density for $\alpha 6$ -eGFP $\beta 2$ -DM receptors was ~ 2 fold greater than that of $\alpha 6$ -eGFP $\beta 2$ -wt nAChRs (Fig. 2B). We utilized ratios of whole cell footprint to ER integrated density to quantify ER-associated changes in receptor localization. The ratio for $\alpha 6$ -eGFP $\beta 2$ -DM nAChRs (~ 1.2) was only slightly higher than that for $\alpha 6$ -eGFP $\beta 2$ -wt nAChRs in Neuro-2a cells (Fig. 2C), while it is substantially lower than the corresponding parameter for $\alpha 4\beta 2$ nAChRs (3–4) and $\alpha 4\beta 2$ -DM nAChRs (7–9) [21].

To enhance our chances of detecting functional $\alpha 6$ -eGFP $\beta 2$ nAChRs, we performed electrophysiological recordings in Neuro-2a cells transiently expressing $\alpha 6$ -eGFP $\beta 2$ -DM nAChRs. As

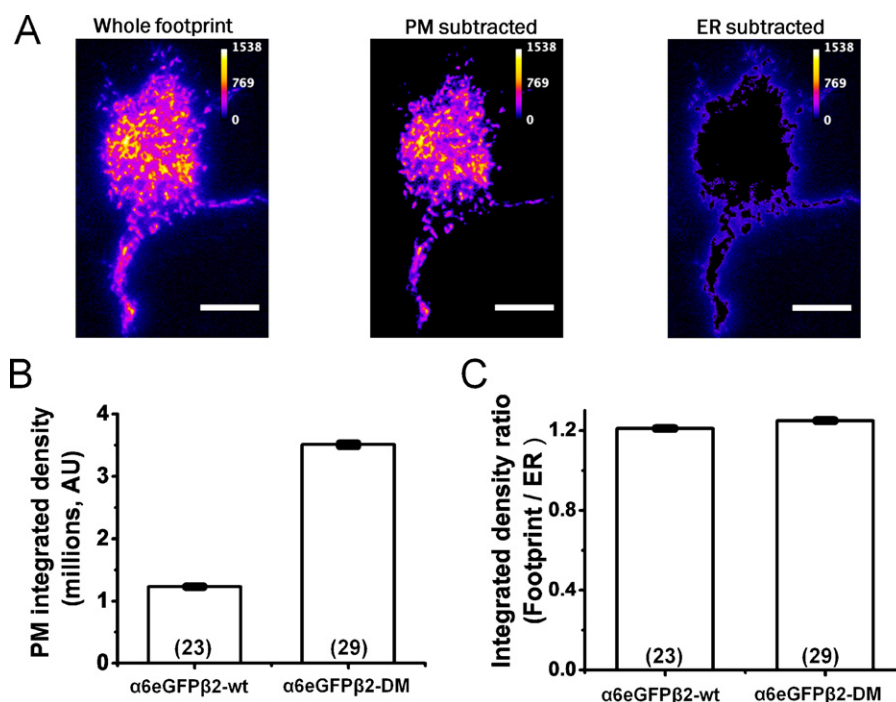


Fig. 2. The $\beta 2$ -DM subunit (also called $\beta 2_{\text{enhanced-ER-export}}$) increased fluorescent $\alpha 6\beta 2$ nAChR density in the PM. (A) Representative TIRFM footprint of a Neuro-2a cell expressing $\alpha 6$ -eGFP $\beta 2$ -wt. The TIRF footprint was processed post-acquisition to obtain plasma membrane (PM) subtracted and ER subtracted footprint as indicated. Images are pseudo-colored using the fire-color look up table (LUT), as shown in each panel. Scale bars, 10 μ m. (B) Quantification of averaged PM integrated density for TIRF images. Transfected subunits are shown on x-axis. Error bars are 99% confidence interval. (C) Quantification of footprint/ER ratios from TIRF images. Transfected subunits are indicated on x-axis. Error bars are $\pm 99\%$ confidence limits. Numbers of cells are indicated in parentheses for each graph.

expected, 300 μ M ACh evoked inward currents in a significantly larger proportion of $\alpha 6$ -eGFP $\beta 2$ -DM cells (28 out of 51) than in $\alpha 6$ -eGFP $\beta 2$ -wt cells (5 out of 19) ($p = 0.03$) (Fig. 3C).

These results only partially confirm the hypothesis that the $\beta 2$ -DM subunits could enhance ER export and, eventually, PM insertion of $\alpha 6$ -eGFP $\beta 2$ nAChRs. Most $\alpha 6\beta 2$ -DM nAChRs remained trapped in the ER, although the ER exit rate was apparently great enough to produce an increased fraction of cells with functional PM nAChRs.

3.3. Visualization of a fluorescently tagged ERES marker

We continued to pursue the hypothesis that ER trapping dominates the poor surface expression of $\alpha 6\beta 2$ nAChRs. We transfected Neuro-2a cells with $\alpha 6$ -eGFP $\beta 2$ -DM, and Sec24D-mCherry, a fluorescently tagged ERES marker, as well. When we chose cells with moderate to high density of $\alpha 6$ -eGFP (Fig. 3B₁), and abundant, large and bright ERES (Fig. 3B₂), we detected inward currents (300 μ M ACh) in 86% (24/28) of cells (Fig. 3C). Therefore, choosing cells with more ERES dramatically increased our chances of detecting functional $\alpha 6$ -eGFP $\beta 2$ -DM nAChRs. The rather small number of cells displaying $\alpha 6$ -eGFP $\beta 2$ -wt nAChR currents vitiated systematic study of these responses. However Fig. 3D conveys the impression that, compared with $\alpha 6$ -eGFP $\beta 2$ -wt nAChR currents, the distribution of $\alpha 6$ -eGFP $\beta 2$ -DM nAChR currents in cells co-transfected with or without Sec24D-mCherry was similar at the two lower quartiles (<50%), but larger at the highest quartile (>75%). The average responses for the three groups were similar within 25% ($\alpha 6$ -eGFP $\beta 2$ -DM: 70 ± 11 pA, $n = 28$; $\alpha 6$ -eGFP $\beta 2$ -DM plus Sec24D-mCherry: 66 ± 10 pA, $n = 24$; $\alpha 6$ -eGFP $\beta 2$ -wt: 54 ± 10 pA, $n = 5$). The increased incidence of $\alpha 6$ -eGFP $\beta 2$ nAChR currents suggested that introducing $\beta 2$ -DM increased membrane insertion of $\alpha 6$ -eGFP $\beta 2$ nAChRs, while the ERES marker could facilitate choosing cells expressing functional receptors. We do not

know whether the effects of the fluorescent Sec24D arise solely from our ability to visualize cells with increased ERES, or also from the increased COPII levels in those cells.

Lack of difference in response waveforms among $\alpha 6$ -eGFP $\beta 2$ -wt, $\alpha 6$ -eGFP $\beta 2$ -DM, and $\alpha 6$ -eGFP $\beta 2$ -DM plus Sec24D-mCherry cells (Fig. 3E) suggested that neither $\beta 2$ -DM nor Sec24D-mCherry altered the intrinsic functional properties of $\alpha 6\beta 2$ nAChRs. The observations gave us the confidence to conduct electrophysiological and pharmacological studies of the expressed $\alpha 6\beta 2$ nAChRs.

3.4. Pharmacological properties of heterologously expressed $\alpha 6\beta 2$ nAChRs in Neuro-2a cells

We observed that both nicotine and TC-2403 activated inward currents in cells expressing functional $\alpha 6$ -eGFP $\beta 2$ -DM nAChRs (Fig. 4A and B). This is consistent with a previous study showing that nicotine and TC-2403 stimulate both dopamine release and locomotion in $\alpha 6$ L9/S transgenic mice [3].

Next we tested whether functional $\alpha 6$ -eGFP $\beta 2$ -wt/ $\alpha 6$ -eGFP $\beta 2$ -DM nAChRs could be blocked by either $\alpha 6^*$ or $\beta 2^*$ nAChR antagonists. In this set of experiments, antagonists were added in the perfusate at the intended concentration, until stable effects were obtained. Indeed, the $\alpha 6\beta 2$ currents were blocked by 80 nM α -conotoxin MII, an antagonist of $\alpha 3^*$ and $\alpha 6^*$ nAChRs (Fig. 4C₁₋₂). The currents recovered to $70 \pm 8\%$ ($n = 5$, $p = 0.01$) of initial values after 20 min washout. DH β E (300 nM), a selective antagonist for $\beta 2^*$ nAChRs, blocked 300 μ M ACh-induced $\alpha 4$ -eGFP $\beta 2$ nAChR currents (by $97 \pm 1\%$, $n = 3$, $p = 0.00001$, data not shown), and the currents recovered to about 50% of baseline values after 15 min washout (data not shown). However, $\alpha 6$ -eGFP $\beta 2$ -DM nAChRs showed lower sensitivity to DH β E, along with faster and greater recovery (Fig. 4D₁₋₂). In particular, 300 nM and 2 μ M DH β E inhibited $\alpha 6$ -eGFP $\beta 2$ -DM nAChR currents by $39 \pm 3\%$ ($n = 4$, $p = 0.0005$) and $59 \pm 9\%$ ($n = 4$, $p = 0.003$), respectively, while the currents recovered

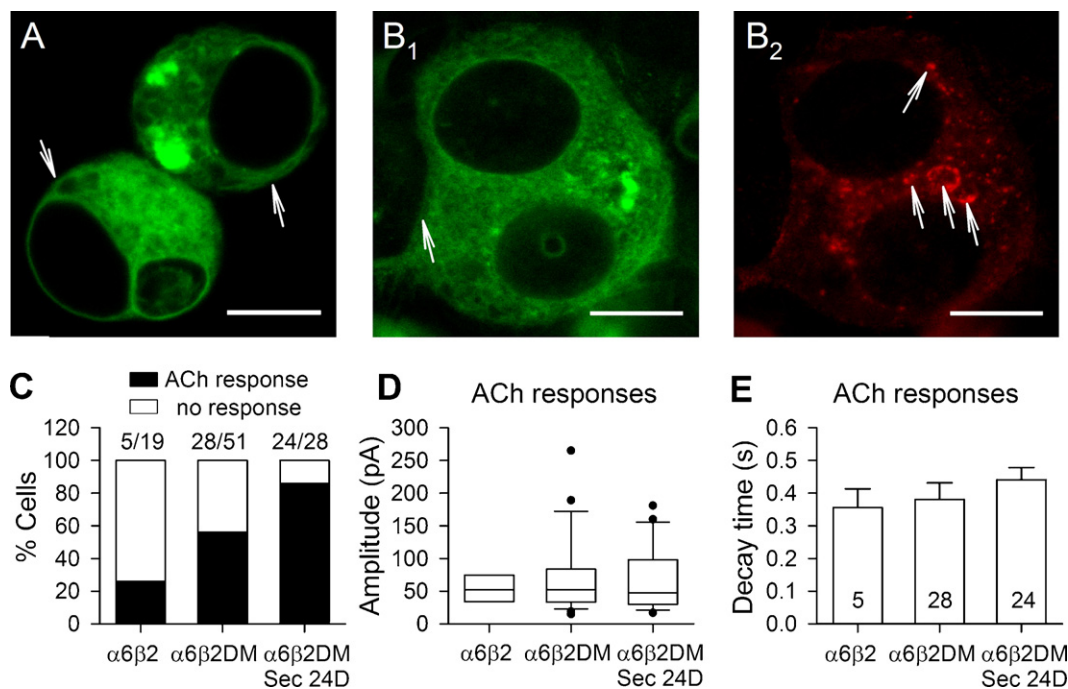


Fig. 3. Improved strategies to detect functional $\alpha 6\beta 2$ nAChRs *in vitro*. (A) Representative images of recorded Neuro-2a cells transfected with $\alpha 6$ -eGFP $\beta 2$ -wt. Arrow: plasma membrane. Scale bar: 10 μ m. (B₁₋₂) Representative images of a Neuro-2a cell transfected with $\alpha 6$ -eGFP $\beta 2$ -DM plus Sec24D-mCherry, showing $\alpha 6$ -eGFP (B₁) and Sec24D-mCherry (B₂). An arrow in (B₁) indicates plasma membrane, which is not well defined. Arrows in (B₂) indicate ER exit sites. Scale bar: 10 μ m. (C) Incidences of ACh-induced currents in cells transfected with $\alpha 6$ -eGFP $\beta 2$ -wt, $\alpha 6$ -eGFP $\beta 2$ -DM and $\alpha 6$ -eGFP $\beta 2$ -DM plus Sec24D-mCherry. Black square: % of cells responding to ACh. White square: % of cells not responding to ACh. Cell numbers to calculate these percentages are indicated above stacked bars. (D) Box plots of current amplitude in cells transfected with $\alpha 6$ -eGFP $\beta 2$ -wt ($n = 5$), $\alpha 6$ -eGFP $\beta 2$ -DM ($n = 28$), or $\alpha 6$ -eGFP $\beta 2$ -DM plus Sec24D-mCherry ($n = 24$). (E) Summary of decay time constant of 300 μ M ACh-induced currents in cells transfected with $\alpha 6$ -eGFP $\beta 2$ -wt, $\alpha 6$ -eGFP $\beta 2$ -DM, and $\alpha 6$ -eGFP $\beta 2$ -DM plus Sec24D-mCherry (mean \pm SEM). Cell numbers are indicated in each vertical bar.

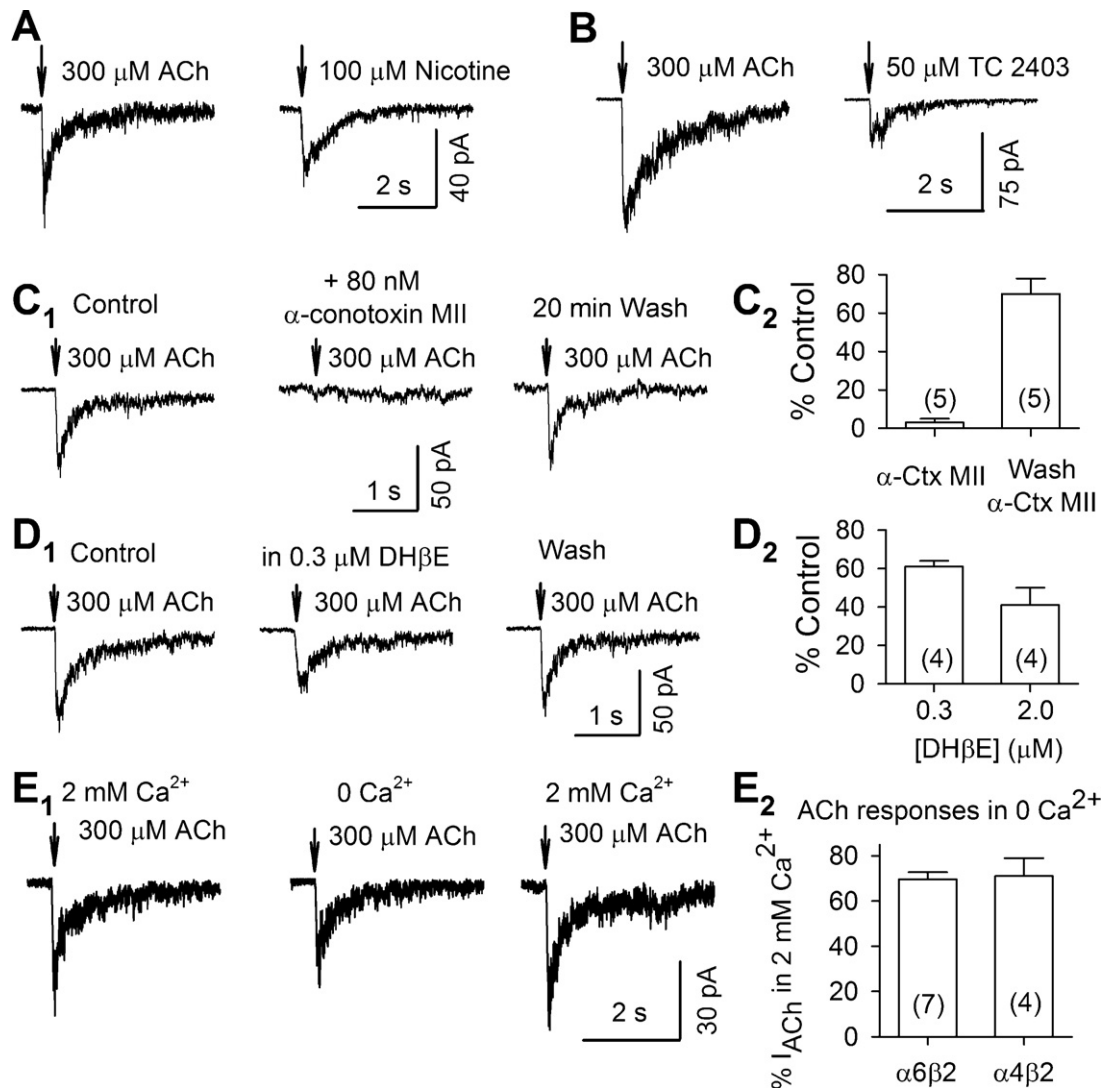


Fig. 4. Pharmacology of $\alpha 6 \beta 2$ nAChRs *in vitro*. $\alpha 6$ -eGFP $\beta 2$ -DM nAChRs were activated by either nicotine (A) or TC 2403 (B). (C₁–2) Typical traces (C₁) and summary (C₂) showing that $\alpha 6 \beta 2$ nAChR currents were blocked by 80 nM α -conotoxin MII, and recovered 20 min after washout. (D₁–2) Typical traces (D₁) and summary (D₂) showing that 0.3 and 2 μ M DH β E partially and reversibly inhibited $\alpha 6$ -eGFP $\beta 2$ -DM nAChR currents. (E₁–2) Both $\alpha 6$ -eGFP $\beta 2$ -DM and $\alpha 4$ -eGFP $\beta 2$ -wt nAChR currents significantly decreased after removal of extracellular Ca^{2+} (0 Ca^{2+}). Cell numbers are shown in parenthesis. Vertical bars show mean \pm SEM. Arrows indicate agonist applications (0.1 s, 20 psi).

to $90 \pm 5\%$ ($n = 5$) of initial currents following 10 min washout of 2 μ M DH β E. These data suggest that $\alpha 6 \beta 2$ nAChRs might have lower affinity to DH β E than $\alpha 4 \beta 2$ nAChRs.

We found that removing extracellular Ca^{2+} by replacing 2 mM Ca^{2+} with 2 mM Mg^{2+} (0 Ca^{2+}) significantly decreased $\alpha 4$ -eGFP $\beta 2$ -wt nAChR currents by $30 \pm 7\%$ ($n = 4$, $p = 0.008$, Fig. 4E₂). A similar phenomenon was observed in $\alpha 6$ -eGFP $\beta 2$ -DM nAChRs (Fig. 4E₁). In 0 Ca^{2+} medium, 300 μ M ACh-induced currents diminished by $30 \pm 3\%$ ($n = 4$, $p = 0.00004$, Fig. 4E₂). These data on Ca^{2+} modulation of $\alpha 6 \beta 2$ nAChRs resemble results with other nAChR subunit combinations [31,32].

3.5. Desensitization of $\alpha 6$ -eGFP $\beta 2$ -DM nAChRs

Desensitization is a common property of nAChRs. The ACh puffs (0.1 s) used to evoke $\alpha 6$ -eGFP $\beta 2$ -DM nAChR currents were one order of magnitude briefer than the decay time constant of the currents (Figs. 1 and 4). The current decay could be a consequence of either agonist diffusion, deactivation of receptors due to agonist dissociation, receptor desensitization, or a combination of these processes. To isolate the role of desensitization in response waveforms, we puffed

300 μ M ACh for 3 s (Fig. 5A₁). The $\alpha 6$ -eGFP $\beta 2$ -DM nAChR current decay was described successfully by a two-exponential function (see in Section 2). The faster ($\tau = 283 \pm 24$ ms, $n = 7$) and slower component ($\tau = 3528 \pm 349$ ms, $n = 7$) respectively account for $66 \pm 5\%$ ($n = 7$) and $23 \pm 4\%$ ($n = 7$) of peak amplitude.

Next we examined $\alpha 6$ -eGFP $\beta 2$ -DM nAChR desensitization by 1 μ M nicotine. We puffed 300 μ M ACh (0.1 s) onto Neuro-2a cells to induce $\alpha 6$ -eGFP $\beta 2$ -DM nAChR currents at 3 min intervals. After a stable baseline response was established (~ 10 min), we added 1 μ M nicotine into the perfusate, and found that ACh-induced currents diminished to nearly zero within a few minutes (Fig. 5B₁–₂). The currents recovered to $58 \pm 3\%$ of initial values ($n = 3$) after 6 min washout, and reached $88 \pm 2\%$ ($n = 3$) of initial values after 20 min washout.

3.6. Single-channel analysis of $\alpha 6 \beta 2$ and $\alpha 4 \beta 2$ nAChR currents in Neuro-2a cells

To characterize the function of $\alpha 6 \beta 2$ nAChRs at the single-channel level, and to compare with $\alpha 4 \beta 2$ nAChR function, we performed outside-out patch single-channel recordings from cells

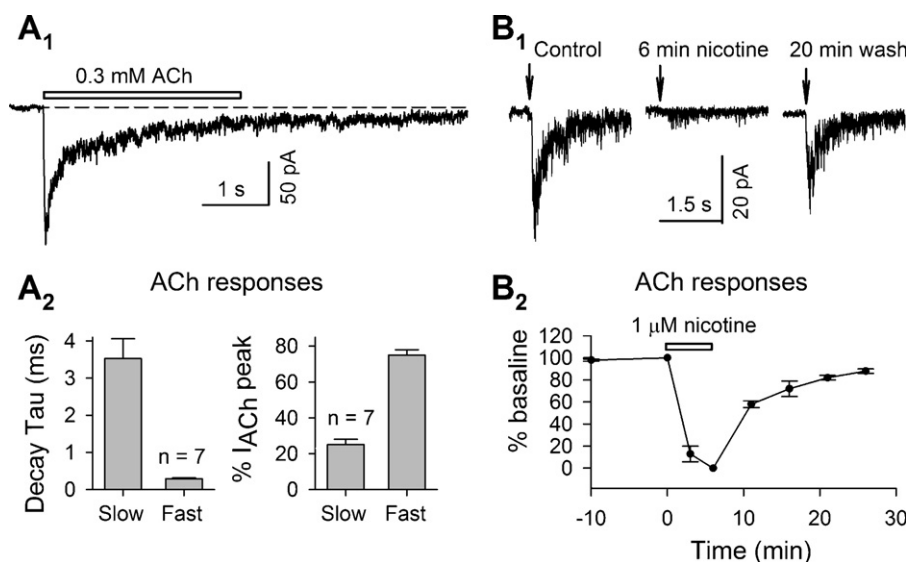


Fig. 5. Desensitization of $\alpha 6\beta 2$ nAChRs *in vitro*. (A₁) A typical trace of 3 s ACh (300 μ M) induced $\alpha 6$ -eGFP $\beta 2$ -DM nAChR current. (A₂) The current decay was fitted by two exponential components. The faster component contributed >70% to the peak amplitude. Cell numbers are indicated in each panel. (B_{1–2}) Typical traces (B₁) and summarized time course (B₂, $n = 3$) showing that $\alpha 6$ -eGFP $\beta 2$ -DM nAChRs desensitized within a few minutes in 1 μ M nicotine, and recovered gradually after nicotine was washed out. Arrows in B₁ indicate ACh applications (0.1 s, 20 psi).

that displayed 300 μ M ACh-induced inward currents in whole-cell mode. As illustrated in Fig. 6A₁, 300 μ M ACh (0.1 s)-induced events show 1–2 opening levels in voltage-clamped patches (V_H , –60 mV) from the cells transfected with $\alpha 6$ -eGFP $\beta 2$ -DM nAChRs. However, 300 μ M ACh evoked 20–40 pA currents in voltage-clamped patches (V_H , –65 mV) from the cells transfected with $\alpha 4$ -eGFP $\beta 2$ nAChRs (data not shown). We decreased ACh concentration to 3 μ M to limit the number of $\alpha 4$ -eGFP $\beta 2$ nAChRs being activated. This strategy provided us with unambiguous single-channel events for further analysis. As illustrated in Fig. 6B₁, 3 μ M ACh evoked ≤ 3 levels of channel openings. In both $\alpha 6$ -eGFP $\beta 2$ -DM and $\alpha 4$ -eGFP $\beta 2$ patches, we observed that ACh produced channel opening over a time course of ~ 5 s (Fig. 6A₁ and B₁), similar to the waveform of whole-cell responses to ACh puffs (Figs. 1 and 4). Fig. 6A₂ and B₂ shows expanded traces of single level openings. We pooled single level opening events from 10 $\alpha 6$ -eGFP $\beta 2$ -DM patches and 7 $\alpha 4$ -eGFP $\beta 2$ -wt patches. The single-channel conductance of $\alpha 6$ -eGFP $\beta 2$ -DM nAChRs (24 pS) was similar to that of $\alpha 4$ -eGFP $\beta 2$ -wt nAChRs (26.2 pS) ($p = 0.35$, two tailed t -test) (Fig. 6A₃ and B₃). The single-channel open time of $\alpha 6$ -eGFP $\beta 2$ -DM nAChRs ($\tau = 1.4$ ms, $n = 279$) was significantly longer than that of $\alpha 4$ -eGFP $\beta 2$ -wt nAChRs ($\tau = 0.6$ ms, $n = 875$) ($p < 0.0001$, Kolmogorov–Smirnov test) (Fig. 6A₄ and B₄).

4. Discussion

4.1. Increasing the membrane expression of $\alpha 6\beta 2$ nAChRs by enhancing ER export

After transfecting Neuro-2a cells with $\alpha 6$ -eGFP $\beta 2$ nAChRs, we selected cells with uniform expression of $\alpha 6$ -eGFP in cytoplasm and periphery (Fig. 3A), presumably indicating surface expression. This provided a modest success rate (26%, Figs. 1A₁ and 3C), higher than previous studies [14,18,20], which showed zero to 15%. When we replaced $\beta 2$ -wt with $\beta 2$ -DM subunits, we observed (1) that PM insertion of $\alpha 6$ -eGFP was increased by 2 fold (Fig. 2B), (2) that functional $\alpha 6\beta 2$ nAChRs were detected in more than half of transfected cells (Fig. 3C), and (3) that ACh induced larger currents in some transfected cells (Fig. 3D).

To achieve even greater success rates, we also fluorescently tagged Sec24D molecules. Following assembly, nAChRs bind to Sec24D through LFM motifs in M3-M4 loop, resulting in the formation of specialized ERES [21,33,34]. ERES number increases in direct proportion to nAChR export rate [21]. Therefore, cells having more ERES could more efficiently export nAChRs to PM. Indeed, 86% of cells with abundant, bright, and large ERES (Fig. 3B₂), displayed functional $\alpha 6$ -eGFP $\beta 2$ -DM nAChRs (Fig. 3C). Although current amplitude in the selected cells was modestly (22%) larger than that in cells transfected with $\alpha 6$ -eGFP $\beta 2$ -wt nAChRs, it was similar to that in cells transfected with $\alpha 6$ -eGFP $\beta 2$ -DM nAChRs (Fig. 3D). Apparently ACh responses occur in $\alpha 6$ -eGFP $\beta 2$ -DM cells with abundant and large ERES; and the ERES marker enabled the visualization of $\alpha 6$ -eGFP $\beta 2$ -DM cells expressing functional receptors.

4.2. Pharmacology of $\alpha 6\beta 2$ nAChRs

In this study, $\alpha 6\beta 2$ nAChRs were activated by ACh, nicotine (Fig. 4A), and TC-2403 (Fig. 4B), consistent with a previous study using $\alpha 6L9/S$ transgenic mice [3], although another study found that TC-2403 has no detectable agonist activity at $\alpha 6\beta 2^*$ nAChRs [9]. The $\alpha 6$ -eGFP $\beta 2$ -DM nAChRs were blocked by α -conotoxin MII, recovering after 20 min washout (Fig. 4C_{1–2}). This recovery is not directly comparable with the slower recovery shown in previous studies using native systems, including brain slices and synaptosomes [3,8,28]. It is possible that $\alpha 6^*$ nAChR subtypes (e.g. those containing $\beta 3$ and/or $\alpha 4$ subunits) could have different dissociation rates for α -conotoxin MII. Interestingly, $\alpha 6\beta 2$ nAChRs were partially and reversibly blocked by DH β E (Fig. 4D_{1–2}), contrasting with the fact that $\alpha 4\beta 2$ nAChRs are blocked by DH β E at low hundreds nM.

We also observed that at levels achieved during smoking, nicotine desensitized $\alpha 6\beta 2$ nAChRs (Fig. 5B_{1–2}). The effect is roughly as robust as previous studies on $\alpha 4\beta 2$ nAChRs [35,36]. However, when nicotine was washed out, $\alpha 6\beta 2$ nAChR currents recovered more rapidly and thoroughly than $\alpha 4\beta 2$ nAChR currents [35]. These data suggested (1) that the desensitized state(s) of $\alpha 6\beta 2$ nAChRs might have lower affinity to nicotine than $\alpha 4\beta 2$

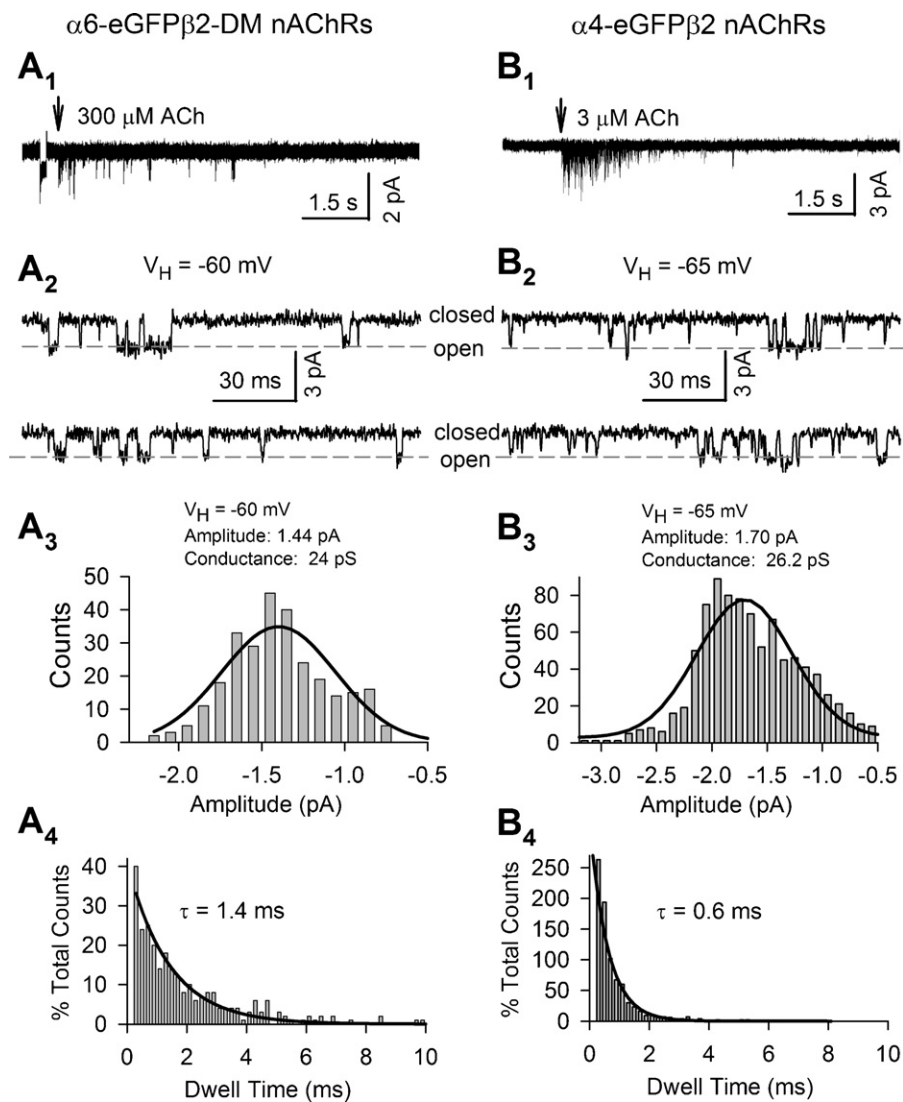


Fig. 6. Single-channel properties of $\alpha 6\beta 2$ and $\alpha 4\beta 2$ nAChRs. A typical trace (8 s) of ACh-induced response in an outside-out patch from a Neuro-2a cell transfected with either $\alpha 6$ -eGFP $\beta 2$ -DM (A₁) or $\alpha 4$ -eGFP $\beta 2$ (B₁) nAChRs. Arrows indicate ACh applications. An inward current before ACh application in A₁ was caused by a -5 mV voltage step. Typical traces of single level openings of $\alpha 6$ -eGFP $\beta 2$ -DM (induced by 300 μ M ACh) and $\alpha 4$ -eGFP $\beta 2$ nAChRs (induced by 3 μ M ACh) are respectively shown in (A₂) and (B₂). Holding potential (V_H), and closed and open states are indicated. Histograms of $\alpha 6$ -eGFP $\beta 2$ -DM and $\alpha 4$ -eGFP $\beta 2$ nAChR single-channel event amplitude are respectively shown in (A₃) and (B₃). Both were fit to a Gaussian equation (thick black curve). Histograms of $\alpha 6$ -eGFP $\beta 2$ -DM and $\alpha 4$ -eGFP $\beta 2$ nAChR single-channel open-time distributions are respectively shown in (A₄) and (B₄). Both were fitted to a single exponential component (thick black curve). Mean open time (τ) is indicated.

nAChRs; (2) that $\alpha 6\beta 2$ nAChRs would regain function more readily than $\alpha 4\beta 2$ nAChRs if desensitized.

Extracellular Ca^{2+} allosterically modulates nAChRs, and increases channel opening probability [31,32]. In the brain, high levels of neuronal activity deplete extracellular Ca^{2+} , and this could lower opening probability of nAChRs [37,38]. We observed that removal of extracellular Ca^{2+} decreased both $\alpha 4\beta 2$ and $\alpha 6\beta 2$ nAChR currents to roughly the same extent (Fig. 4E), suggesting that $\alpha 6\beta 2$ nAChRs could also be modulated by neuronal activity *in vivo*.

Our pharmacological data thus indicate that $\alpha 6\beta 2$ nAChRs could be distinguished from $\alpha 4\beta 2$ nAChRs by their resistance to DH β E and rapid recovery from nicotine desensitization. In some situations, desensitization could be physiologically relevant by changing the balance of excitation and inhibition in neural circuits [39,40].

4.3. Electrophysiological characterization of $\alpha 6\beta 2$ nAChRs

We observed that the decay of $\alpha 6$ -eGFP $\beta 2$ -DM nAChR currents, evoked by 3 s puff of 300 μ M ACh, was described by a two-

exponential function (Fig. 5A₁₋₂). This suggested either that multiple processes account for “desensitization” of individual $\alpha 6\beta 2$ nAChRs, or that more than one population of $\alpha 6\beta 2$ nAChRs exist, with different desensitization rates (Fig. 5A₂). By analogy with $\alpha 4\beta 2$ nAChRs (see next paragraph), it would not be surprising to find that these two components could reflect different receptor stoichiometries: $(\alpha 6)_3(\beta 2)_2$ and $(\alpha 6)_2(\beta 2)_3$. Further investigations would test this hypothesis by performing (1) Förster resonance energy transfer [21,22], (2) biased transfections of $\alpha 6$ and $\beta 2$ subunits with different ratios to assess changes in the predominance of faster and slower components [41,42], and (3) systematic dose–response studies [41–43]. Additionally, the physiological significance of these two components also warrants further investigations.

$\alpha 4\beta 2$ nAChRs assemble into two stoichiometries: $(\alpha 4)_3(\beta 2)_2$ and $(\alpha 4)_2(\beta 2)_3$ [21,22,41–44]. Low concentrations (<10 μ M) of ACh primarily activate high sensitivity $\alpha 4\beta 2$ nAChRs, while high concentrations (≥ 100 μ M) of ACh activate both. Previous studies revealed that high sensitivity $\alpha 4\beta 2$ nAChRs have smaller single-channel conductance (17–31 pS) than low sensitivity receptors

(44–46 pS) [32,43–48]. In this study, we activated $\alpha 4\beta 2$ nAChRs on voltage-clamped outside-out patches by 3 μM ACh (Fig. 6B₁). Given this concentration and the single-channel conductance of 26.2 pS (Fig. 6B₂), we suggest that most of the $\alpha 4\beta 2$ single-channel events were mediated by high sensitivity receptors.

A previous study reported that the EC₅₀ of ACh for $\alpha 6\beta 2$ nAChRs is 150 μM [20]. Therefore, 300 μM ACh can activate both high and low sensitivity $\alpha 6\beta 2$ nAChRs if any. We observed a single conductance component in 300 μM ACh-induced single $\alpha 6\beta 2$ nAChR currents (Fig. 6A_{2–3}). It could be (1) that, in our transfection condition, the majority of $\alpha 6\beta 2$ nAChRs assembled in one stoichiometry, and the number of recorded events were too low to show a minority component, or (2) that $\alpha 6\beta 2$ nAChRs in two stoichiometries could have the same single-channel conductance or even a single sensitivity component as previously reported [20]. The average conductance of $\alpha 6\beta 2$ nAChRs was 24 pS, which was similar to that of $\alpha 4\beta 2$ nAChRs (26.2 pS) (Fig. 6A₃ and B₃). The single-channel duration of $\alpha 6\beta 2$ nAChRs was significantly longer than that of $\alpha 4\beta 2$ nAChRs. We applied 3 μM ACh to activate $\alpha 4\beta 2$ nAChR single-channel events, but applied 300 μM ACh to activate $\alpha 6\beta 2$ nAChR single channel events, and still observed that $\alpha 4\beta 2$ nAChRs opened more frequently (875 events/25.3 s) than $\alpha 6\beta 2$ nAChRs (279 events/50 s). The low open probability of $\alpha 6\beta 2$ nAChRs could result from the high concentration of ACh, which could cause receptor desensitization.

4.4. Additional subunits and stoichiometries?

Note that native $\alpha 6^*$ nAChRs include several subunit combinations and stoichiometries. The $\alpha 6\beta 2$ nAChRs studied here may be the subtype with the least sensitivity to agonists, the lowest binding affinity to α -conotoxin MII and DH β E, and the least biological relevance. The $\alpha 6\alpha 4\beta 2^*$ nAChRs could be the major physiologically relevant subtypes for dopamine release [2]. Including $\alpha 4$ and/or $\beta 3$ subunits in $\alpha 6\beta 2^*$ nAChRs increases receptor sensitivity [7,8], and including $\beta 3$ subunits also increases expression levels [15–17,19].

Thus we can hope that the present techniques for expressing purely $\alpha 6\beta 2$ nAChRs ($\beta 2$ -DM subunits, fluorescently tagged $\alpha 6$ subunits, and ERES markers) may have overcome the greatest hurdle. However, we do not yet know whether one or more of the strategies introduced here could also increase chances of detecting functional $\alpha 4\alpha 6\beta 2$ and $\alpha 4\alpha 6\beta 2\beta 3$ nAChRs in Neuro-2a cells. If so, these systems could be used to investigate pharmacological and electrophysiological properties of $\alpha 6^*$ nAChRs subtypes, and may facilitate drug screening for both Parkinson's disease protection and smoking cessation.

Acknowledgements

This work was supported by grants from the US National Institutes of Health (DA17279, NS11756, AG033954, DA19375, DA12242, MH53631, and GM48677); from Targacept Inc.; from the California Tobacco-Related Disease Research Program (TRDRP); and from Louis and Janet Fletcher. R.S. was supported by a postdoctoral fellowship from TRDRP (18FT-0066), and R.D. by an NIH National Research Service Award (DA021492) and an NIH Pathway to Independence Award (DA030396).

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