An alpha7 nicotinic acetylcholine receptor gain-of-function mutant that retains pharmacological fidelity

Andon N. Placzek, Francesca Grassi, Edwin M. Meyer, and Roger L. Papke

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Department of Pharmacology and Therapeutics; A. N. P., E. M. M., R. L. P.
Box 100267 JMHSC
University of Florida
Gainesville, FL 32610-0267
(352) 392-4712
FAX (352) 392-9696

Istituto Pasteur-Fond. Cenci Bolognetti and Dip. di Fisiologia Umana e Farmacologia; F. G.
Universita' di Roma "La Sapienza"
p.le Aldo Moro 5, 00185 Roma, Italy
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Corresponding Author:

Roger L. Papke

Department of Pharmacology and Therapeutics
P. O. Box 100267
University of Florida
Gainesville, FL 32610-0267
(352) 392-4712
FAX (352) 392-9696
rlpapke@ufl.edu

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Abbreviations: nAChR, nicotinic acetylcholine receptor; TM2, second transmembrane domain; SEM, standard error of the mean; CRC, concentration-response curve; KRH, HEPES buffered Krebs Ringer
Abstract

The α7-type nicotinic acetylcholine receptor (nAChR) has been recognized as a potential therapeutic target for the treatment of a variety of pathologic conditions, including schizophrenia, Alzheimer’s disease, and peripheral inflammation. A unique feature of α7 nAChRs that tends to complicate functional assays intended to identify selective drugs for these receptors is the strong concentration-dependent desensitization of their agonist-evoked responses. At low agonist concentrations, voltage-clamp responses are small but tend to closely follow the solution exchange profile, while higher agonist concentrations produce responses that peak and then decay very rapidly, usually before the full drug concentration has been achieved. Here we report that an α7 T245S mutant, which has a point mutation at the sixth position in the α7 second transmembrane domain (T6’S), demonstrates a significant gain of function, sustaining current when exposed to relatively high agonist concentrations when expressed in Xenopus oocytes, and larger peak currents when expressed in mammalian GH4C1 cells. At the single-channel level, the T6’S mutant has a unitary conductance of 61.7 ± 5.8 pS, similar to that reported for wild-type α7, but a vastly longer average open duration. Also, channel burst activity indicates a greater than 40% probability of channel re-opening in the sustained presence of 30 μM acetylcholine, consistent with a greater overall open probability relative to wild-type α7. Unlike the α7 L248T gain-of-function mutant, the T6’S mutant exhibits a pharmacological profile that is remarkably similar to the wild-type α7 receptor, implicating it as a potentially useful tool for identifying therapeutic agents.
Introduction

Nicotinic acetylcholine receptors (nAChR) composed of the α7 subunit are pentameric, ligand-gated ion channels that are widely expressed in mammalian tissues, including both central and peripheral neurons (Dani, 2001; Skok, 2002), as well as non-neuronal cells (Shytle et al., 2004; Wang et al., 2003). Although the normal physiological functions of α7-type nAChRs are poorly understood, studies have shown behavioral effects of α7-selective agonists suggesting a role in learning and memory function (Rezvani and Levin, 2001). Other reports have implicated α7 nAChRs as potential therapeutic targets in schizophrenia (Freedman et al., 2000) and neurodegenerative conditions such as Alzheimer's disease (O'Neill et al., 2002). Recent studies have also shown that α7 receptors play an important role in peripheral inflammatory processes (Wang et al., 2003), suggesting that selective targeting of α7 nAChRs may be beneficial in preventing the deleterious effects of septicemia.

The α7-type nAChRs are unique among nicotinic receptors in that they display a high degree of concentration-dependence to the kinetics of agonist-evoked responses. That is, with the application of increasing concentrations of agonist, the macroscopic responses of α7 nAChRs become more and more transient to the point where the peak of the response occurs well before the maximum concentration is achieved (Papke and Thinschmidt, 1998). This is true even for very rapid drug application systems, indicating that the macroscopic kinetics of wild-type α7 receptors are faster than the rate of currently available agonist application devices (Papke et al., 2000a; Uteshev et al., 2002). The fact that α7 nAChRs have been reported to have a very high permeability to calcium (Seguela et al., 1993) suggests that this agonist concentration-dependent limitation of the channel's activity may help prevent excitotoxic injury in cells with high levels of α7 receptors expressed (Li et al., 1999).

Previous work in our laboratories has shown that the 6' position of the pore-forming, second transmembrane (TM2) domain (according to the numbering scheme proposed by Miller (Miller, 1989)) is an important regulator of several features that distinguish major subfamilies of nAChR (Placzek et al., 2004; Webster et al., 1999). This site corresponds to amino acid 245 in
the rat \( \alpha 7 \) nAChR sequence. In an earlier study of \( \alpha 7 \), the wild-type threonine residue was substituted with a phenylalanine residue at position 245 of the rat \( \alpha 7 \), resulting in a T6'F mutant (Placzek et al., 2004). Receptors containing this mutation generated only small currents which however lacked the agonist concentration-dependent kinetic effects characteristic of wild-type \( \alpha 7 \). Additionally, the T6'F mutant displayed some pharmacological properties that are like the muscle-type nAChR (Placzek et al., 2004). In an effort to test if an analogous effect could be produced with a similar single amino acid substitution in the \( \alpha 7 \) TM2 domain, an \( \alpha 7 \) T6'S mutant was constructed. In this case the \( \alpha 7 \) wild-type threonine at position 245 of the rat \( \alpha 7 \) was exchanged for the serine residue present at the homologous position of the neuronal \( \beta 2/\beta 4 \) subunit (see Figure 1), yielding a T6'S mutant \( \alpha 7 \).

As in the case of the T6'F mutant, much of the rationale for the hypothesized effects of the T6'S mutant are derived from previously published observations with heteromeric nAChRs. For example, sensitivity to the ganglionic blocker mecamylamine was increased in muscle-type receptors with chimeric \( \beta 1(\beta 4) \) subunits, and this effect could be attributed to the sequence differences at the TM2 6' and 10' positions (Webster et al., 1999).

In characterizing the effects of this TM2 T6'S mutation, we describe a mutant receptor that retains much of the pharmacology of wild-type \( \alpha 7 \), but with larger macroscopic responses and a dramatic lessening of agonist concentration-dependent limitation on response duration. The effects of this mutation are examined at the single-channel level and indicate that channel open time and burst properties are affected, rather than receptor expression, or single-channel conductance. Furthermore, the T6'S \( \alpha 7 \) mutant displays a pharmacological profile that is very similar to wild-type \( \alpha 7 \). Such a gain-of-function mutant that retains much of the pharmacology of the wild-type receptor may have significant utility for drug development.
Materials and Methods

cDNA Clones

These experiments used rat neuronal nAChR cDNA clones, which were obtained from Dr. Jim Boulter (UCLA). The sequences of the TM2 domains of the relevant subunits are shown in Figure 1. The 20 residues in the putative second transmembrane sequence are identified as 1' through 20' (Miller, 1989).

Site-directed Mutagenesis

Site-directed mutagenesis was performed using QuickChange (TM) kits (Strategene, LaJolla, CA). In brief, two complimentary oligonucleotides were synthesized which contained the desired mutation flanked by 10-15 bases of unmodified nucleotide sequence. Using a thermal cycler, *Pfu* DNA polymerase extended the sequence around the whole vector, generating a plasmid with staggered nicks. Each cycle built only off the parent strands, and therefore there was no amplification of misincorporations. After 12-16 cycles, the product was treated with *Dpn* I, which digested the methylated parent DNA into numerous small pieces. The product was then transformed into *E. coli* cells, which repaired the nicks.

Preparation of RNA

After linearization and purification of cloned cDNAs, RNA transcripts were prepared *in vitro* using the appropriate mMessage mMachine kit from Ambion Inc. (Austin, TX).

Expression in Xenopus Oocytes

Mature (>9 cm) female *Xenopus laevis* African frogs (Nasco, Ft. Atkinson, WI) were used as a source of oocytes. Prior to surgery, frogs were anesthetized by placing the animal in a 1.5 g/L solution of MS222 (3-aminobenzoic acid ethyl ester). Oocytes were removed from an incision made in the abdomen.

In order to remove the follicular cell layer, harvested oocytes were treated with collagenase from Worthington Biochemical Corporation (Freehold, NJ) for 2 hours at room temperature in calcium-free Barth’s solution (88 mM NaCl, 1 mM KCl, 15 mM HEPES pH 7.6, 0.81 mM MgSO4, 2.38 mM NaHCO3, 0.1 mg/ml gentamicin sulfate). Subsequently, stage 5
oocytes were isolated and injected with 5-10 ng each of the appropriate subunit cRNAs following harvest. Recordings were made 3 to 14 days after injection depending on the cRNAs being tested. Since all data were normalized using each cell as its own control, absolute differences in response magnitude did not affect comparisons between receptor subtypes.

**Voltage-clamp Recording of Oocyte Responses**

Data were obtained by means of two-electrode voltage-clamp recording. Recordings were made at room temperature (21-24 deg. C) in Frog Ringer’s solution (115 mM NaCl, 10 mM HEPES, 2.5 mM KCl, and 1.8 mM CaCl₂, pH 7.3) with 1 µM atropine to inhibit muscarinic acetylcholine receptor responses. This extracellular solution was used for all experiments unless otherwise noted. Voltage electrodes were filled with 3M KCl, and current electrodes were filled with 250 mM CsCl, 250 mM CsF, and 100 mM EGTA (pH 7.3).

Bath solution and drug applications were applied through a linear perfusion system to oocytes placed in a Lucite chamber with a total volume of 0.5 ml. Drug delivery involved pre-loading a 1.8 ml length of tubing at the terminus of the perfusion system, while a Mariotte flask filled with Ringer’s solution was used to maintain constant perfusion. Applications of drug solutions were then synchronized with acquisition. Current responses were recorded using a PC interfaced to either a Warner OC-725C (Warner Instruments, Hamden, CT) or a GeneClamp 500 amplifier via a Digidata 1200 digitizer (Molecular Devices, Union City, CA). In addition, some oocyte recordings were made using a beta version of the OpusXpress 6000A (Molecular Devices, Union City, CA). OpusXpress is an integrated system that provides automated impalement and voltage clamp of up to eight oocytes in parallel. Cells were automatically perfused with bath solution, and agonist solutions were delivered from a 96-well compound plate. In experiments using the OpusXpress system, the voltage and current electrodes were filled with 3 M KCl. In all experiments, bath flow rates were set at 2 ml/minute.

Current responses to drug application were studied under two-electrode voltage clamp at a holding potential of -50 mV unless otherwise noted (-60 mV for the OpusXpress system). Holding currents immediately prior to agonist application were subtracted from measurements of
the peak to agonist. All drug applications were separated by wash periods of 5 minutes unless otherwise noted. At the start of recording, all oocytes received two initial control applications of ACh. Subsequent drug applications were normalized to the second acetylcholine application in order to control for the level of channel expression in each oocyte. Means and standard errors (SEM) were calculated from the normalized responses of at least four oocytes for each experimental concentration.

For concentration-response relations, data were plotted using Kaleidagraph 3.0.2 (Abelbeck Software; Reading, PA), and curves were generated using the Hill equation (1).

\[
\text{Response} = \frac{I_{\text{Max}}[\text{agonist}]^n}{[\text{agonist}]^n + (EC_{50})^n}
\]

where \(I_{\text{max}}\) denotes the maximal response for a particular agonist/subunit combination, and \(n\) represents the Hill coefficient. \(I_{\text{max}}, n,\) and the \(EC_{50}\) were all unconstrained for the fitting procedures.

Calculations of peak amplitudes and net charge were made using pClamp either during acquisition or during subsequent Clampfit analysis. Note that measurement of net charge has been shown to be a more accurate indicator of fast responses of wild-type \(\alpha_7\) than measurement of peak response (Papke and Papke, 2002). Baseline was defined for Clampfit statistics based on 20 s before drug application, and the analysis region for peak and net charge analysis went from 5 s before the initiation of drug application and extended at least 135 s following. Area analysis data is provided for all receptor subtypes examined in this paper for comparison to wild-type \(\alpha_7\).

**Transfection and Patch-clamp Recording from GH4C1 Cells**

GH4C1 cells were cultured in F10 medium (Gibco, Carlsbad, CA) at 37 °C, 5% CO₂. Cells were transiently transfected using Fugene (Roche, Indianapolis, IN), according to the manufacturer instructions. One microgram of wild-type or T6'S mutant \(\alpha_7\) cDNA in the pClneo vector (Promega Corp., Madison WI) was added to each 35-mm Petri dish, together with 0.5 or 1 µg of the cDNA encoding the red fluorescent DsRed protein (BD Biosciences Clontech, Palo Alto, CA).
Alto, CA). Cells were used 48-72 hours after transfection. Typical transfection efficiency was 10-25% using this method.

**Whole-Cell and Fluorescence Measurements in GH4C1 Cells**

ACh-evoked currents and fluorescence measurements were simultaneously performed on cells loaded with Fura-2 (Molecular Probes, Eugene, OR) via the patch pipette. Whole-cell currents were recorded using an Axopatch 200B amplifier (Molecular Devices, Union City, CA) at room temperature. Cells were bathed in a solution containing 140 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM glucose, 10 mM HEPES/NaOH (pH 7.3). Patch electrodes (tip resistance, 3-5 MΩ) were filled with 140 mM N-methylglucamine, 10 mM HEPES/HCl (pH 7.3), plus 250 µM Fura-2. Cells were continuously superfused by a gravity-driven fast-exchanging perfusion system (RSC 200, BioLogic, France), with independent tubes for each solution placed approximately 100 µm from the cells. Currents were recorded at 2 kHz using pClamp 8 and a membrane holding potential of -70 mV unless otherwise indicated. Analysis was performed using pClamp 8.

Fluorescence determinations were made using a Zeiss Axioskop 2 FS microscope (Germany) equipped with a monochromator (Optoscan, Cairn, UK) and a high-sensitivity digital camera (SensiCam, PCO, Germany). The Axon Instrument Workbench 2 was used for data sampling and analysis. Fluorescence values were averaged over domains approximating the cell shape, assuming a homogeneous receptor density. Cells were loaded with fura-2 in the whole-cell configuration, until the basal fluorescence (F₀) reached a stable value (usually 3-5 min). The so-called "F/Q ratio" was determined as the ratio of the fluorescence change (ΔF/F₀) at the excitation wavelength of 380 nm over the total charge (Q) entering the cell. The F/Q ratio was calculated as the slope of the line fitting F vs Q points, taking into account only the data within 600 ms of transmitter application, when Ca²⁺ extrusion can be assumed to be negligible (Bollmann et al., 1998) and the relationship is indeed linear. Fractional Ca²⁺ permeability (Pᶠ) was obtained by normalizing the F/Q ratio obtained in standard medium (containing 2 mM Ca²⁺) to the F/Q ratio measured in calibration experiments, when Ca²⁺ is the only permeant cation. To
this purpose, cells were bathed in a solution containing 130 mM N-methylgucamine, 10 mM CaCl2, 10 mM HEPES/HCl, pH 7.3. Cells displaying high basal F340/F380 ratio values (>1 in our conditions) or low basal F380 values (< 150 a.u. in our conditions) were not considered for analysis. Experimental procedures and data analyses were as previously described (Ragozzino et al., 1998). Chemicals were purchased from Sigma (USA).

Single-Channel Patch Clamp Recordings from Transfected GH4C1 Cells

Single-channel currents were recorded in the cell-attached patch configuration using an Axopatch 200A amplifier (Molecular Devices, Union City, CA) at room temperature. Cells were bathed in a solution containing 140 mM NaCl, 2.8 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM glucose, 10 mM HEPES/NaOH (pH 7.3). Patch electrodes (tip resistances, 5-7 MΩ after fire polishing were coated with Sylgard (Dow Corning, Midland, MI) and filled with the same extracellular solution plus 1 µM atropine and 30 µM ACh. Currents were filtered at 10 kHz and digitized at 50 kHz using pClamp 8 (Molecular Devices, Union City, CA). Analysis was conducted using pClamp 9.

Radioligand Binding Studies

GH4C1 cells were harvested from 60 mm culture dishes using a sterile cell scraper and assayed for nicotine-displaceable, high-affinity [3H]methyllycaconitine binding using a modification of the procedure of Davies et al. (Davies et al., 1999). Cells were suspended in 20 volumes of ice cold HEPES buffered Krebs Ringer (KRH; 118 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM MgCl2, 2.5 mM CaCl2, and 20 mM HEPES; pH 7.5). After two 1-ml washes with KRH at 20,000g, the membranes were incubated in 0.5 ml KRH with 1, 3, 10, or 20 nM [3H]methyllycaconitine (Tocris, Ellisville, MO) for 60 min at 4°C with or without 5 mM nicotine. Tissues were washed three times with 5 ml cold KRH by filtration through Whatman GF/C filters that had been preincubated for 2 hours in blotto (KRH with 0.5% dry milk and 0.002% sodium azide). They were then assayed for radioactivity using liquid scintillation counting. Inhibition curves generated under two-electrode voltage-clamp with oocytes.
expressing either wild-type α7 or the TM2 T6'S mutant showed a less than 3-fold difference in methyllycaconitine potency between the two (data not shown).

Intact Oocyte Binding

[^3]HMLA binding in intact oocytes was performed similarly to the method described previously (Placzek et al., 2004). In brief, whole Xenopus oocytes that were either uninjected, or had been injected with mRNAs encoding either wild-type α7 or the T6'S mutant were placed in a single well of a 96-well plate containing either 20 nM[^3]Hmethyllycaconitine alone or 20 nM[^3]Hmethyllycaconitine with 5 mM nicotine. After four 4s washes in 2.5 ml KRH, total radioactivity was measured using an automated liquid scintillation counter as counts per minute (CPM). Nicotine displaceable binding was calculated for at least 4 cells in each condition.

Results

The TM2 T6'S mutation effects on the macroscopic ACh-evoked Currents

Compared to the wild-type α7 (Figure 2A (Placzek et al., 2004)), the α7 T6'S mutant showed a significant change in response-kinetics to ACh, with currents somewhat slower to rise and of longer duration at high agonist concentrations. Figure 2 also shows the decreased effect of increasing concentrations of acetylcholine on the macroscopic decay rate of the T6'S mutant responses (Figure 2B) compared to wild-type α7 (Figure 2A) with its characteristically strong concentration-dependent kinetics. An analysis of the rise times (Figure 2C) of these responses showed that the T6'S mutant actually exhibits a slowing of the response with increasing agonist concentration before the eventual increase in rise time dominates at the highest concentrations. This is particularly true for acetylcholine concentrations around 30 µM. The decay times of the T6'S mutant responses, much like those of the T6'F mutant (Placzek et al., 2004), showed little effect of high agonist concentrations, whereas the decay times of wild-type responses became progressively shorter at higher agonist concentrations (Figure 2D).

A comparison of[^3]HMLA binding between intact oocytes expressing either wild-type α7 or the T6'S mutant showed that the average difference in nicotine-displaceable binding was not
statistically significant (Table 1). Despite this similarity in receptor binding, the ACh-evoked currents in cells from the same injection set differed substantially in the amount of net charge. Currents from oocytes expressing either wild-type α7 or the T6’S mutant, all of which received mRNA injections on the same date, approximately 72 hours prior to recording indicated that the total area under the curve for the averaged wild-type α7 current was 11.5% of that of the T6’S mutant, a difference that cannot be attributed to differences in receptor expression (Table 1).

**Ca2+ permeability of α7T6’S mutant receptors**

The GH4C1 cell line has previously been shown to functionally express transfected wild-type α7 receptors at levels higher than other commonly used mammalian cell expression systems (Sweileh et al., 2000). The wild-type receptors heterologously expressed in this cell line have also been shown to have a high degree of pharmacological similarity to native α7 receptors expressed in rat brain (Quik et al., 1996), indicating GH4C1 cells as useful for studying α7-type nAChRs in small mammalian cells. GH4C1 cells successfully co-transfected with wild-type or the T6’S mutant α7 nAChR and DsRed protein showed a bright red fluorescence when excited at 550 nm. ACh-evoked currents in both wild-type (not shown) and T6’S α7-expressing cells (Figure 3A) showed a marked rectification of the current evoked at positive membrane potentials. However, acetylcholine elicited whole-cell currents (I_ACh) with much larger peak amplitude in cells expressing T6’S α7 nAChR than wild-type α7 nAChR (p < 0.01, Table 2). Addition of 5-hydroxyindole (5HI) increased the peak amplitude of I_ACh ACh-evoked currents in wild-type and T6’S α7-expressing cells without affecting current rise and decay times (Figure 3B). However, this potentiating effect of 5-hydroxyindole was significantly less (p <0.05) for the T6’S mutant receptor than for wild-type α7 nAChR (Table 2). By contrast, ACh-evoked currents recorded from oocytes expressing T6’S mutant α7 receptors showed only a relatively slight potentiation by 5HI. The small but significant (p < 0.05) 26% potentiation observed was dramatically lower than the average potentiation wild-type α7 receptors expressed in Xenopus oocytes (904 %). This difference in 5-hydroxyindole effect was very significant (p < 0.0001)
and greater than the difference in 5-hydroxyindole effects obtained with GH4C1 cells transfected with either wild-type or T6'S α7.

The Ca\(^{2+}\) fractional permeability (P\(_{\text{fCa}}\)) was investigated in the presence of 5HI, since in the absence of 5-hydroxyindole ACh-induced Ca\(^{2+}\) transients in wild-type cells were too small to be detected. In other studies, it was demonstrated that 5-hydroxyindole does not affect P\(_{\text{fCa}}\) (Fucile et al., 2003). Simultaneous recording of I\(_{\text{ACh}}\) and the ACh-induced change of fura-2 fluorescence (Figure 3C, D) indicated that P\(_{\text{fCa}}\) was 7% for the T6'S mutant α7 nAChR (Fig. 3E). For comparison, we repeated the same measurements on wild-type α7 nAChR and found a P\(_{\text{fCa}}\) value of 8% (Table 2).

**Single-channel conductance of T6'S mutant α7 receptors**

Single-channel patch-clamp experiments were conducted in order to identify the specific channel properties affected by the T6'S mutation. One aspect of the receptor that may have been altered with significant effect on the amount of charge carried by the mutant receptor is the unitary conductance. Therefore in order to obtain high resolution single-channel currents, cell-attached patches were obtained from cells transiently transfected with the T6'S mutant. Figure 4 shows a representative recording from a cell exposed to 30 µM acetylcholine in the patch pipette. Control untransfected GH4C1 cells (n=6) showed no channel openings in the presence of ACh. A current-voltage relationship was established for the T6'S mutant in order to quantify the single-channel conductance.

Figure 5 shows a representative current-voltage plot. Linear regression analysis gave an average slope conductance of 61.7 ± 5.8 pS. This is less than the wild-type α7 single-channel conductance of 91.5 ± 8.5 pS previously reported (Mike et al., 2000), however, this difference might at least in part be due to differences in experimental solutions, since there was no extracellular magnesium and lower calcium in the previously published experiments (Mike et al., 2000). In any case, it seems unlikely that the T6'S mutant has a greater single channel conductance than wild-type α7. This suggests that the difference in response magnitude between mutant and wild-type macroscopic currents is not attributable to a change in unitary conductance.
It should be noted that while our analysis was conducted on the predominant 61.7 pS conductance, openings of lower conductance channels were sometimes observed. These smaller conductance channels were not seen in control patches, and represented no more than 16% of the total number of openings in the presence of 30 µM ACh. Furthermore, these smaller conductances were not present in all cells that responded to 30 µM ACh, and only appeared in less than 20% of the total number of recordings.

**Single channel open times of T6'S mutant α7 receptors**

Analysis of channel dwell time distributions (Figure 6) showed that the T6'S mutant channel open times were best fit by two exponentials with the average shorter open time being 580 ± 110 µs and the longer open time being 4.3 ± 0.9 ms. Since T6'S mutant channel open times were fit by two exponential components, a weighted average was used to give an estimate of the total average open time for a macroscopic current (2.5 ms). Comparing this to the previously published apparent mean channel open time of roughly 100 µs (Mike et al., 2000), gave an approximate 25 fold increase in the channel open time.

**Burst activity of T6'S mutant α7 receptors**

Figure 7A shows raw single channel events where bursts of openings were observed under steady-state conditions. Figure 7B shows a representative closed time distribution for the T6'S mutant from patches exposed to 30 µM ACh. The requirement for multiple exponentials to fit the distribution is consistent with channel bursting. In contrast, the wild-type α7 nAChR has been shown to have little or no burst activity under steady-state conditions (Mike et al., 2000), or in other words, at the detection limits of those studies, events appeared to consist of single openings (bursts of one). In such a case the apparent average channel open time approximates the average burst duration. However, for the currents through the T6'S mutant receptors, we could identify intraburst closures (i.e., brief gaps between sequential openings of the same single channel).

The closed time distributions of four patches which had greater than 300 events (318-6854) events were best fit by 4 exponential components, the average time constants for which were
0.21 ± 0.03 ms, 3.6 ± 0.4 ms, 141 ± 40, and 2650 ± 1500 ms. Under our experimental conditions, the overall $P_{\text{open}}$ was low, with channels open no more than 1% of the time. The $P_{\text{open}}$ values ranged from 0.9% to .001% time in the open state, presumably reflecting variations in the total number of channels in the patch. The two patches which had the highest frequency of openings also had a low number of simultaneous openings, accounting for 2.9% and 1.7% of the total number of events when the event frequencies were 3.8 and 1.8 events per second, respectively. As shown in Figure 7C the two brief time constants did not correlate to the frequency of events in the record and therefore most likely represented closed times associated with the reopenings of the same channel. However, the longer closed times were of greater duration when overall frequency of events was low, so that these intervals likely reflected the total number of channels in the patch.

Accepting just the briefest closed times as intraburst closures and using the method of Colquhoun and Sakmann (Colquhoun and Sakmann, 1985) a critical time value of 759 µs was determined as a threshold for defining intraburst closures. The average burst duration distributions (Figure 8A) for the T6'S mutant defined with these criteria were best fit by two exponential components (560 ± 100 µs and 5.5 ± 1.4 ms). Histograms of the number of bursts with more than one opening were plotted and fitted by a Poisson distribution (Figure 8B), providing a prediction of the probability of channel reopening. In this case, the T6'S mutant had a 42.1 ± 6% probability of opening more than once.

Hypothetically, the brief intraburst closed times ($\tau_1 = 0.21 ± 0.03$ ms) represent time spent back in the same resting, ligand-bound, closed state that normally precedes opening (Colquhoun and Sakmann, 1985). As noted above, the closed times fit with the time constant of 3.6 ± 0.4 ms ($\tau_2$, Figure 7C) may also represent gaps between reopenings of the same channel. The frequency of these events was 60 ± 13% that of the briefer intraburst closures. These closures may represent times when channels close, dissociate and rebind agonist, and then reopen. Alternatively, they may represent time in a short-lived desensitized or predesensitized state (Sine and Steinbach, 1987).
Pharmacology of T6’S mutant α7 receptors

The concentration-response relationship for acetylcholine applied to either the T6’S mutant or wild-type α7 are shown in Figure 9, showing a slight decrease in the apparent acetylcholine potency for the T6’S mutant compared to wild-type (see Table 3). Previous reports have shown that wild-type α7 nAChRs have significantly different peak amplitude and net charge concentration-response curves (Papke and Papke, 2002; Placzek et al., 2004; Uteshev et al., 2002). This is indicative of the strong agonist concentration-dependence for the time course of their macroscopic responses. The T6’S mutant showed less of a difference between the two methods (Figure 9B), again indicating that response kinetics are less sensitive to agonist concentration.

Agonist selectivity profiles

Concentration-response relationships for a series of nicotinic receptor agonists indicated that the T6’S mutant has a pharmacology that is similar to wild-type α7 with regard to both potency and efficacy of the agonists examined. Figure 10 compares the effects of several α7-selective agonists on oocytes expressing either the T6’S mutant or wild-type rat α7. Although some differences in potency and efficacy were evident, with the exception of the partial agonist tropisetron, which was nearly identical between the two receptor types (Figure 10D), all agonists for wild-type α7 receptors tested also activated the mutant receptor. Furthermore, the potency differences observed were similar to that seen with acetylcholine (Figure 9), suggesting a general trend in modest potency reduction for agonists. The similarity in agonist response profiles also extended to agonists that are not selective for α7-type nAChRs. The β4 subunit-selective agonist cytisine (Papke and Heinemann, 1994) and the α4β2-selective agonist metanicotine (Papke et al., 2000b) displayed agonist activity at the T6’S mutant (Table 3). Again, there was a slight reduction in potency and efficacy with the mutant, particularly in the case of cytisine, but these shifts were consistent with the general trend observed for most of the agonists examined (Table 3).
Antagonist selectivity profiles

Another feature of the T6'S mutant that is similar to wild-type α7 is its sensitivity to nAChR antagonists. A somewhat peculiar feature of the α7 L9'T (L248T) mutant is that several drugs that function as antagonists of wild-type α7 have been shown to activate this mutant (Bertrand et al., 1992b; Demuro et al., 2001; Palma, 1996). By contrast, each of these drugs inhibited the T6'S mutant (Table 4). Furthermore, for the concentrations tested, no agonist activity of these same drugs was observed (not shown).

Discussion

Kinetic effects of the T6'S mutation

The mutant α7 receptor described here displayed a profound change in response kinetics, with a significant increase in responsiveness to the application of relatively high concentrations of agonist. Based on the results of receptor binding experiments, the overall increase in response magnitude observed in the T6'S mutant is apparently not due to an increase in receptor expression. This, however, is not particularly surprising, since an increase in receptor number alone would not be a likely cause of altered response kinetics. Likewise, although mutations in the pore-forming TM2 domain frequently affect single-channel conductance (Imoto et al., 1988), such a change alone would not affect the kinetics of macroscopic currents, and our data suggest that the conductance of the T6'S receptors is not substantially different from that of the wild-type.

An examination of the mean channel open time indicates that the T6'S mutant has far longer open durations than observed for wild-type α7 (Mike et al., 2000). This increased stability of the open state made it possible to record responses from cells expressing the T6'S mutant under cell-attached patch conditions. No similar data have ever been reported from wild-type α7 receptors under steady-state conditions of exposure to low concentrations of agonist, presumably due to the intrinsically low open probability (P_open) and brief lifetime of α7-mediated single channel currents.
Burst analysis indicates that there is a significant likelihood of rapid re-opening for the T6’S mutant channel, a property not reported for wild-type α7 channels. This increase in channel re-opening most likely reflects a general increase in overall P_open for the mutant receptors. However, since no estimates were made of the number of T6’S mutant channels present in each patch, absolute P_open values could not be determined. Aside from the gaps associated with the rapid re-openings detected in our burst analysis, our data are not sufficient to determine whether other closed times also represent gaps between the re-openings of single channels or closed times between the independent openings of multiple channels within the patches.

In the interpretation of the kinetic effects of the T6’S mutation, there are several possible explanations for the increased macroscopic responses and prolonged responses at high agonist concentrations. We have proposed (Papke et al., 2000a) that wild-type α7 receptors are unlikely to open at high levels of agonist occupancy, and have a higher P_open when the multiple agonist binding sites are only partially occupied. One possibility is that one or more states that are closed in the wild-type receptor have been converted to an open state(s) in the mutant. However our data clearly indicate that the open state itself is more stable (i.e., long-lived) in the mutant receptor than in the wild-type. This observation alone accounts for a large increase in P_open under steady-state conditions.

Wild-type α7 shows little or no apparent burst activity (Mike et al., 2000) and therefore has a probability of re-opening that approaches zero. Under our experimental conditions, with 30 µM acetylcholine in the patch pipette, for the T6’S receptors the transition rates between the bound, non-desensitized states and the open states were sufficiently high relative to the dissociation and desensitization rate constants to make multiple opening bursts more likely. This indicates that the T6’S mutation alters the equilibrium between the open and activatable resting states of the receptor and the desensitized states at the level of agonist occupancy corresponding to our single-channel conditions. The fact that macroscopic currents were prolonged across the range of agonist concentrations suggests that a similar reduction or slowing of equilibrium desensitization occurs at all levels of agonist occupancy.
Although it may seem as though the T6'S mutation has the effect of reducing agonist potency, this effect should not be interpreted outside of the context of the kinetic effects. This is because the apparent reduction in potency may in fact be due to a broadening of the concentration-response functions for agonists. That is, at the higher agonist concentrations that impose limitations on the responses of the wild-type receptor, the T6'S mutant showed significant increases in response. This essentially stretches out the agonist concentration-response curves (CRCs) for the mutant, producing an apparent reduction in potency.

Pharmacological effects of the T6'S mutation

The rapid desensitization of the wild-type α7 nAChR creates significant difficulty when attempting to study this receptor subtype, particularly in mammalian expression systems. The use of the *Xenopus* oocyte expression system circumvents this problem to some degree by providing a large cell with sufficient tolerance for application systems that are relatively slow and less likely to produce leakage. In addition, the presence of calcium-dependent chloride currents in the oocyte produces a secondary amplification of α7-mediated currents at holding potentials that are more negative than the chloride reversal potential (Miledi and Parker, 1984). Automated oocyte recording systems have been developed but, in general, have been considered medium rather than high throughput screening systems. Truly high throughput drug screening is best accomplished with automated systems that generate fluorescence signals and are capable of delivering hundreds of compounds in a single day to cells in multiwell plates. Due to their small transient responses, wild-type α7 receptors are generally not suitable to use in these systems, and so consequently, some efforts in the area of drug discovery have attempted to make use of gain-of-function mutant α7 receptors.

The α7 gain-of-function mutant that has been most well studied corresponds to an a change in the TM2 sequence only 3 amino acids away from the T6'S site (L247T in chick α7 (Bertrand et al., 1992b) and L248T in human α7 (Palma et al., 1999)). Receptors with this L9'T mutation produce large non-desensitizing currents but show very unusual pharmacology compared to the wild-type α7 nAChR. For example, there are several drugs that have been
reported to be antagonists of the wild-type receptor that are agonists of the L9'T mutant including: bicuculline, strychnine, dihydro-beta-erythroidine, 5-hydroxytryptamine, hexamethonium, and tubocurarine (Bertrand et al., 1992b; Demuro et al., 2001; Palma et al., 1999; Palma, 1996). Another gain-of-function mutation (V13’T or V274T) also has pharmacological properties that are quite distinct from the wild type (Briggs et al., 1999). Although cells expressing these gain-of-function mutants can generate large signals in automated fluorescence detection systems, their failure to recapitulate the pharmacological properties of wild-type receptors, even to the point of distinguishing antagonists from agonists, limits their utility for drug screening.

One pharmacological aspect of the T6'S mutant that does differ significantly from wild-type α7 is the reduced potentiation by 5HI. This difference was more pronounced in the oocyte studies than in the transfected cell experiments. This may be related to the fact that the brief ACh-evoked currents recorded in oocytes expressing wild-type α7 are augmented by transient calcium-dependent chloride currents. While the T6'S receptor-mediated component of the current in oocytes may be potentiated by 5-hydroxyindole to the same degree as the currents in GH4C1 cells, there may not be a linear increase in the chloride-mediated component during the prolonged responses of the mutant channels. Since the mechanism of 5-hydroxyindole potentiation of wild-type α7 remains unknown, the reason why the T6's receptors are less sensitive to 5-hydroxyindole than wild-type α7 receptors is unclear. It is possible that the effect of 5-hydroxyindole on the wild-type receptor is functionally similar to the effect of the T6'S mutation, in essence suggesting that the T6'S mutant is in a perpetually potentiated state. However, this explanation is not adequate to explain the observed differences. Potentiation by 5-hydroxyindole is unusual by itself, in that there is little or no effect on the macroscopic kinetics of potentiated responses compared to unpotentiated responses (Zwart et al., 2002). The fact that the T6'S mutation appears to have significant effects on the kinetics of response to agonist suggests that the mechanisms of response amplification for 5-hydroxyindole potentiation are different.
The data presented here show that the $\alpha_7$ T6'S mutation produces a functionally enhanced receptor that retains important characteristics of the wild-type $\alpha_7$ receptor, including high calcium permeability and inward rectification. The relative lack of significant changes in pharmacology indicate that this mutant receptor can prove useful in the identification of compounds that will affect $\alpha_7$. In support of this, the T6'S mutant expressed in *Xenopus* oocytes has recently been used to screen a panel of over sixty compounds, and results were directly compared to those obtained with wild-type rat $\alpha_7$ receptors expressed in *Xenopus* oocytes (unpublished data). The compounds tested included agonists, partial agonists, and antagonists. Activity for the mutant correlated well with that for wild-type $\alpha_7$. Additionally, some $\alpha_7$ agonists such as GTS-21 produce long-lived desensitization of wild-type $\alpha_7$ receptors, while others such as 4OH-GTS-21 and AR-R17779 do not (Meyer et al., 1998; Papke et al., 2004). In the side-by-side comparison of 60 compounds it was noted that the T6'S mutant was also useful to distinguish compounds that produced prolonged desensitization from those that did not (unpublished data). These observations confirm the great potential utility of the T6'S mutant $\alpha_7$ for drug development.
Acknowledgements

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References


Mike A, Castro NG and Albuquerque EX (2000) Choline and acetylcholine have similar kinetic properties of activation and desensitization on the alpha7 nicotinic receptors in rat hippocampal neurons. *Brain Res.* **882**:155-68.


Footnotes:

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Please direct reprint requests to:

Roger L. Papke  
Department of Pharmacology and Therapeutics  
P. O. Box 100267  
University of Florida  
Gainesville, FL 32610-0267  
(352) 392-4712  
FAX (352) 392-9696  
rlpapke@ufl.edu

1Department of Pharmacology and Therapeutics  
Box 100267 JHMHSC  
University of Florida  
Gainesville, FL 32610-0267  
(352) 392-4712  
FAX (352) 392-9696

2Istituto Pasteur-Fond. Cenci Bolognetti and Dip. di Fisiologia Umana e Farmacologia  
Universita' di Roma "La Sapienza"  
p.le Aldo Moro 5, 00185 Roma, Italy
Figure Legends

Figure 1. Amino acid sequences for wild-type and the T6'S mutant α7 nAChR TM2 domains based on the numbering of specific residues of the second membrane-spanning region (Miller, 1989). The corresponding sequences for neuronal beta subunits are also given as a reference. The 6' position is indicated by the box.

Figure 2. The time course and concentration-dependence of α7 nAChR macroscopic kinetics are altered by the T6'S mutation. Two-electrode voltage clamp responses from oocytes expressing A) wild-type α7, or B) the T6'S mutant. The traces show the relative effect of increasing concentrations of ACh. The T6'S mutant responses are slower and the effect of higher concentrations of agonist on the macroscopic kinetics reaches a maximum, whereas for wild-type α7, no maximum is achieved. C) The T6'S mutant shows a slowing of the rise time near 30 µM before the higher agonist concentrations dominate. D) The decay times also show that the T6'S mutant is insensitive to higher agonist concentrations while the wild-type decay times become increasingly rapid. The wild-type data (panels A and C) was previously published (Placzek et al., 2004).
Figure 3. Whole-cell current and Ca\(^{2+}\) determinations in GH4C1 cells expressing T6'S \(\alpha7\) nAChR.  

A) \(I_{\text{ACH}}\) recorded in a single cell, in the presence of 5HI, at different test potentials.  Inset: currents recorded at -70 mV (#) or +23 mV (*).  

B) Typical ACh-evoked current responses recorded in the same cell with or without 5HI, as indicated.  Test potential -70 mV, [ACh] = 200 µM.  

C) Simultaneous recordings of whole-cell current and Ca\(^{2+}\) responses elicited by acetylcholine (100 µM, -70 mV) in standard medium.  Top: \(I_{\text{ACH}}\); Bottom: fluorescence intensity, expressed as \(\Delta F/F\) (squares) and current integral (line).  

D) As in C, but in a different cell, bathed in calibration medium (10 mM Ca\(^{2+}\) as the only permeant cation).  

E) Relationships between the fluorescence variation and the charge \(Q\), fitted by linear regressions, from the same cells as in C (slope = 0.5 nC\(^{-1}\)) and D (slope = 7.04 nC\(^{-1}\)).  The Pf for the T6'S \(\alpha7\) nAChR was 7.1%.

Figure 4. Single-channel currents recorded from a GH4C1 cell expressing the T6'S mutant.

Transiently transfected GH4C1 cells were studied using the cell-attached patch configuration.  The representative trace shown above is from a cell with 30 µM acetylcholine in the patch pipette, at a holding potential +50 mV depolarized from resting potential.  No currents were observed in untransfected control cells (n=6, data not shown).

Figure 5. The T6'S mutant \(\alpha7\) single-channel conductance.  Single-channel current-voltage plots were generated from cell-attached patch recordings from transfected GH4C1 cells and indicated a slope conductance of 62 ± 6 pS.  The data shown here are representative of those used to produce an average conductance value (n=5).
Figure 6. T6'S single-channel open times are fit by two exponentials and indicate relatively prolonged average open times. A representative open time distribution for a 30 min. cell-attached patch recording from transfected GH4C1 cells in the sustained presence of 30 µM ACh.

Figure 7. T6'S mutant single-channel burst activity in transfected GH4C1 cells. A) Raw data traces recorded in the presence of 30 µM acetylcholine showing channel bursts. B) Closed time distribution for the T6'S mutant showing a fit by multiple exponentials. The existence of multiple closed times is an indicator of channel bursting. To identify bursts, the critical time threshold \( t_{\text{crit}} \) for identifying closures within bursts was determined using the method described by Colquhoun and Sakmann (Colquhoun and Sakmann, 1985). C) Four patches which had between 300 and 7000 events had event frequencies which varied from 0.5 to 3.8 events per second. The closed time distributions from these patches were best fit with four exponential components. The time constants fit to the two briefest closed times (\( \tau_1 \) and \( \tau_2 \)) did not vary systematically with event frequency, while the two slower time constants (\( \tau_3 \) and \( \tau_4 \)) were longest when event frequency was low.

Figure 8. T6'S mutant burst durations and number of intraburst openings recorded from transfected GH4C1 cells. A) The average burst duration distribution was best fit by two exponential components. B) The number of bursts with intraburst openings greater than unity, indicating a significant probability of the mutant channel re-opening.
Figure 9. Peak and area CRCs for wild-type and T6’S mutant nAChRs. Each data point represents the mean normalized response (± SEM) obtained from at least four oocytes.

Figure 10. Selective agonists of the wild-type α7 nAChR. All of the agonists selective for α7 nAChR tested, including A) choline, B) GTS-21, C) AR-R17779, and D) tropisetron, retained their agonist activity for the T6’S mutant. However, the concentration-response functions for the indicated drug for either wild-type α7 or the T6’S mutant showed potency and efficacy differences, but these were relatively moderate and consistent with the potency shifts seen with non-α7 selective and nonselective agonists, including ACh. The wild-type rat α7 CRCs for AR-R17779 and tropisetron are taken from Papke et al., 2004 and Papke et al., 2005, respectively.
Table 1. Intact oocyte [³H] methyllycaconitine binding.

<table>
<thead>
<tr>
<th></th>
<th>CPM/Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rat α7 wild-type</strong></td>
<td></td>
</tr>
<tr>
<td>20 nM methyllycaconitine alone</td>
<td>143 ± 4 (n = 5) *</td>
</tr>
<tr>
<td>20 nM MLA+ 5 mM nicotine</td>
<td>100 ± 12 (n = 4)</td>
</tr>
<tr>
<td><strong>Rat α7 T6'S mutant</strong></td>
<td></td>
</tr>
<tr>
<td>20 nM methyllycaconitine alone</td>
<td>169 ± 10 (n = 5) *</td>
</tr>
<tr>
<td>20 nM MLA+ 5 mM nicotine</td>
<td>106 ± 6 (n = 5)</td>
</tr>
<tr>
<td><strong>Uninjected oocytes</strong></td>
<td></td>
</tr>
<tr>
<td>20 nM methyllycaconitine alone</td>
<td>98 ± 17 (n = 4)</td>
</tr>
</tbody>
</table>

Data represent the mean (± SEM) counts per minute per cell (CPM/Cell) for the indicated treatments.

* p ≤ 0.05 by Student's t compared to the same receptor subtype in the presence of 20 nM methyllycaconitine with 5 mM nicotine.
Table 2. Whole-cell voltage clamp and fractional calcium current recorded from transiently transfected GH4C1 cells.

<table>
<thead>
<tr>
<th></th>
<th>Wild-type α7 nAChR</th>
<th>T6'S α7 nAChR</th>
</tr>
</thead>
<tbody>
<tr>
<td>$I_{Ah}$ (nA)</td>
<td>$-0.06 \pm 0.02$ (13)**</td>
<td>$-1.03 \pm 0.36$ (9)**</td>
</tr>
<tr>
<td>$I_{Ah+hI} / I_{Ah}$ (%)</td>
<td>$540 \pm 75$ (7)*</td>
<td>$280 \pm 50$ % (5)*</td>
</tr>
<tr>
<td>$P_{Ca}$ (%)</td>
<td>$8.0 \pm 0.7$ (10)</td>
<td>$7.1 \pm 0.6$ (12)</td>
</tr>
</tbody>
</table>

Holding potential, -70 mV. [ACh]= 200 µM. Results as mean ± SEM (n. of cells).

* Significantly different (1-way ANOVA, P < 0.05).
** Significantly different (1-way ANOVA, P < 0.01)
Table 3. Agonist profile comparison for wild-type α7 and the T6'S mutant expressed in *Xenopus* oocytes.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Wild-type α7</th>
<th>T6'S</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh EC\textsubscript{50}</td>
<td>30 µM 100% agonist</td>
<td>100 µM 100% agonist</td>
</tr>
<tr>
<td>Choline</td>
<td>300 µM 100% agonist</td>
<td>2 mM 95% agonist</td>
</tr>
<tr>
<td>GTS-21</td>
<td>5 µM 32% agonist</td>
<td>3 µM 12% agonist</td>
</tr>
<tr>
<td>4OH-GTS-21</td>
<td>1.4 µM 46% agonist</td>
<td>3.3 µM 20% agonist</td>
</tr>
<tr>
<td>AR-R17779</td>
<td>10 µM † 78% agonist</td>
<td>30 µM 90% agonist</td>
</tr>
<tr>
<td>Tropisetron</td>
<td>0.3 µM †† 38% agonist</td>
<td>0.9 µM 30% agonist</td>
</tr>
<tr>
<td>Metanicotine</td>
<td>240 µM * 16% agonist</td>
<td>400 µM 18% agonist</td>
</tr>
<tr>
<td>Cytisine</td>
<td>13 µM 73% agonist</td>
<td>43 µM 80% agonist</td>
</tr>
</tbody>
</table>

EC\textsubscript{50} values and maximum efficacy relative to acetylcholine for each of the indicated agonists.

* Potency and efficacy values derived from peak CRC analysis (Papke et al., 2000b).

† Potency and efficacy values derived from net charge analysis (Papke et al., 2004).

†† Potency and efficacy values derived from net charge analysis (Papke et al., 2005).
Table 4. Antagonists of wild-type α7 and their effect on the T6'S mutant expressed in *Xenopus* oocytes.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Wild-type α7</th>
<th>T6'S *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bicuculline, 10 µM</td>
<td>~ 63% inhibition&lt;sup&gt;a&lt;/sup&gt;</td>
<td>88 ± 3% inhibition</td>
</tr>
<tr>
<td>5-HT, 20 µM</td>
<td>~ 25% inhibition&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100% inhibition</td>
</tr>
<tr>
<td>Tubocurarine, 1µM</td>
<td>~ 28% inhibition&lt;sup&gt;c&lt;/sup&gt;</td>
<td>97 ± 2% inhibition</td>
</tr>
<tr>
<td>Hexamethonium, 100 µM</td>
<td>100% inhibition&lt;sup&gt;d&lt;/sup&gt;</td>
<td>60 ± 12% inhibition</td>
</tr>
</tbody>
</table>

<sup>*</sup> Percent reduction in peak amplitude when co-applied with 30 µM acetylcholine relative to a 30 µM acetylcholine control application. Each mean and SEM represents data obtained from at least four oocytes.

<sup>a</sup> (Demuro et al., 2001)
<sup>b</sup> (Palma, 1996)
<sup>c</sup> (Briggs et al., 1999)
<sup>d</sup> (Bertrand et al., 1992a)
<table>
<thead>
<tr>
<th></th>
<th>intracellular</th>
<th>TM2 Domain</th>
<th>extracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type α7</td>
<td>ISLGIITVLLSLTFTVMLLLVAEIMPAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wild-type β2</td>
<td>MTLCISVLALTVFLLLLISKIVPPT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wild-type β4</td>
<td>MTLCISVLALTFLLLLLISKIVPPT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α7 T6'S mutant</td>
<td>ISLGIISVLSSLTFTVMLLLVAEIMPAT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1
Figure 2

(A) Wild-type α7

(B) TM2 T6'S mutant

(C) Rise Times (10 - 90% Peak)

(D) Decay Times (90 - 70% Peak)
Figure 3


Figure 5

T6'S Mutant Current-Voltage Relationship

Holding Potential (mV)

Current Amplitude (pA)
A

Average T6'S Burst Duration

Count (N) vs. Log Duration (ms)

B

T6'S Channel Probability of Re-Opening

Count (N) vs. Number of Events in Burst

Bursts with openings of n > 1
Figure 9

(A) Wild-type α7 ACh CRCs

(B) TM2 T6'S ACh CRCs

[Graphs showing normalized response vs. [ACh], μM for each condition]
Figure 10