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# Functional expression of nicotinic acetylcholine receptors containing rat α7 subunits in human SH-SY5Y neuroblastoma cells

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**Abstract** Neuronal nicotinic acetylcholine receptors (nAChR) are made from different combinations of subunits encoded by a diverse family of genes. However, the recently cloned α7 gene codes for subunits that can form homooligomeric nAChR complexes when expressed in *Xenopus* oocytes. Electrophysiological studies reveal that these α7-nAChR function as α-bungarotoxin (Bgt)-sensitive, quickly activating/inactivating ion channels with a unique pharmacological profile and an unusually high permeability to calcium ions. Although similar observations have been made in studies of Bgt-sensitive, functional nAChR subtypes that are naturally expressed in neuronal cells, all attempts until now to reconstitute functional α7-nAChR in cell lines have failed. Here we report the successful use of SH-SY5Y human neuroblastoma cells, which naturally express low levels of endogenous α7 transcripts, to stably overexpress heterologous rat nAChR α7 transgenes. These transgenes are expressed as the appropriately-sized α7 messages and protein, and stably transfected SH-SY5Y cells have over 30-times higher levels of specific Bgt binding sites than do wild-type cells. Whole cell current recordings confirm that transfected cells express functional nAChR that are sensitive to blockade by Bgt and display the typical physiological and pharmacological profiles of α7-nAChR. We conclude that stable, functional expression of α7 transgenes in a mammalian cell line has been achieved for the first time.

*Key words:* Acetylcholine; Nicotine; Nicotinic receptor; α-Bungarotoxin; SH-SY5Y cell line

## 1. Introduction

Nicotinic acetylcholine receptors (nAChR) exist as diverse members of the ligand-gated ion channel (LGIC) superfamily of neurotransmitter receptors [1–3]. Part of the diversity in nAChR is attributable to the existence of at least fifteen different nAChR subunit genes. Proteins encoded by these genes combine in different ways to generate several unique nAChR subtypes. However, roles of nAChR subunits, particularly in the formation and function of neuronal nAChR, are incompletely understood.

Neuronal/nicotinic alpha-bungarotoxin binding sites (nBgtS) represent a unique class of nAChR that was first discovered in autonomic and CNS neurons or nervous tissue based on their ability to bind radiolabeled, curaremimetic neurotoxins such as α-bungarotoxin (Bgt) with high affinity and specificity [2,4–9]. Found where nAChR expression was expected, nBgtS also display a clearly nicotinic ligand binding profile, possess many of the physical and chemical properties of other nAChR, and have drug reactivities consistent with roles as LGIC. However, whereas studies of autonomic or CNS neurons that express nBgtS detected functional nAChR responses involved in the mediation of excitatory neurotransmission, those responses were not demonstrated to be sensitive to blockade by curaremimetic neurotoxins (although there were some exceptions; *op. cit.*). Thus, functional relevance of nBgtS and their identity as LGIC was questioned.

Critical breakthroughs in our understanding of nBgtS were the cloning of what is now known as the chick nAChR α7 subunit gene and technical improvements allowing the detection of transient and novel functional responses of nAChR made up of α7 subunits [10–11]. The protein products of chick, rat or human α7 genes can form homooligomeric complexes in *Xenopus* oocytes that can bind curaremimetic neurotoxins and can mediate very short-lived, nicotine-gated, toxin-sensitive, ion channel responses [10,12–14]. These homooligomeric, transgenic α7 subunit-containing nAChR (α7-nAChR) have unique properties including high $Ca^{2+}$ permeability (*op. cit.*). Based on the breakthrough recognition that toxin-sensitive α7-nAChR responses have lifetimes of tens of milliseconds and include contributions from inward $Ca^{2+}$ currents, searches for functions of natural nBgtS were renewed. These studies uncovered short-lived, nicotine-gated, toxin-sensitive, inward currents and/or elevations of intracellular $Ca^{2+}$ in chick autonomic neurons [15–16] and in rat CNS neurons [17–20] that also have been shown to express natural α7 genes and entities corresponding to native nBgtS [2,5,21]. Thus, correlations have been drawn between the expression of nBgtS, α7 gene products, and toxin-sensitive, functional nAChR responses.

Described here are studies that further examine these relationships using a human ganglionic neuronal cell line as a model and as a host for expression of α7 subunit transgenes. Preliminary accounts of this work have appeared [22–23].

## 2. Materials and methods

### 2.1. Cell culture

Wild-type (WT) SH-SY5Y cells [24] were maintained in serum (Hyclone)-supplemented medium (Gibco BRL) as previously described [25].

### 2.2. Construction of α7/pCEP4 vector and stable transfection of SH-SY5Y cells

An *Eco*RI fragment of approximately 1.9 kb containing full length cDNA sequences of the rat nAChR α7 subunit was excised from its original clone [13] (generous gift of Dr. Jim Patrick, Baylor College of Medicine) and subcloned as a blunt-ended fragment into the HindIII site of the pCEP4 vector (Invitrogen). SH-SY5Y cells were transfected either with the insertless pCEP4 vector (as a control) or with the α7/pCEP4 vector via a standard electroporation technique (Bio-Rad).

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Briefly, a 200-V field was applied to a mixture of approximately $10^7$ cells and 20 μg of DNA. Selection of stably transfected cells was carried out by culturing as described above but in medium supplemented with 400 μg/ml of hygromycin B.

*2.3. Northern blot analysis*

Northern analysis of poly(A)$^+$RNA extracted by the Fast Track (Invitrogen) technique was performed as described [25–26] following resolution of RNA on a 1% agarose gel using a $^{32}$P-labeled, full-length, rat nAChR α7 subunit cDNA probe [13].

*2.4. Membrane fraction preparation and I-Bgt binding assays*

Cellular membrane fractions were obtained and $^{125}$I-labeled Bgt (I-Bgt; Amersham) binding assays using membrane fractions or intact cells in situ were performed as described [26] using native Bgt (purified according to [27] from crude venom obtained from Miami Serpentarium) to standardize specific activity of I-Bgt and to define non-specific binding.

*2.5. Affinity purification of Bgt-binding components*

Briefly (detailed protocol of Drisdel, Puchacz and Lukas to be published elsewhere), α7-transfected SH-SY5Y cells were lysed in Ringer's buffer [27] supplemented with protease inhibitors [28] and 2% Triton X-100. Toxin binding proteins were purified using najatoxin (purified according to [27] from crude venom obtained from Miami Serpentarium)-Acti-Gel (Sterogene) affinity chromatography [29], eluted using Acti-Sep (Sterogene) solution, and trace-radiolabeled with $^{125}$I (Amersham) using iodo-beads (Pierce). Samples were desalted and resolved via electrophoresis on 10% sodium dodecyl sulfate/polyacrylamide gels (SDS-PAGE). Dried gels were exposed at −84°C to Kodak XAR film juxtaposed with an intensifying screen for autoradiography to identify components of toxin-binding material.

*2.6. Electrophysiology*

Whole cell recordings were done using standard techniques initially described by [30]. Borosilicate electrodes fashioned on a BB-CH-PC pipette puller (Mecanex) and filled with intracellular salt solution (containing 120 mM KF, 20 mM KCl, 5 mM NaCl, 2 mM $MgCl_2$, 10 mM HEPES and 0.5 mM EGTA; pH was adjusted to 7.4 with KOH) were used for recording (Axopatch 200A amplifier, Axon Instruments). Data was filtered at 2 kHz, acquired at 5 kHz, and analyzed using a personal computer equipped with an analog to digital converter (ATMIO-16D, National Instrument) and the DATAC package [31]. Drugs were delivered in the millisecond range by means of a fast, multibarrel, puffer technique (Hu, Maury, Buisson and Bertrand, manuscript in preparation). Recordings were usually done one-to-four days after plating of cells onto coverslips attached to the bottoms of modified 35 mm Petri dishes to also allow high power microscopic examination of cultures. Cells were rinsed three times in fresh extracellular salt medium (120 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM D-glucose, 10 mM HEPES; pH was adjusted to 7.4 with NaOH) prior to recording and were continuously superfused with this medium between drug applications. Atropine (1 μM) was added to bath solutions to prevent activation of endogenous muscarinic receptors [32–33]. Bgt used in electrophysiological studies was purchased from Molecular Probes.

*2.7. Other materials*

Unless otherwise noted above, other reagents were obtained from commercial sources such as New England BioLabs, Sigma, Fluka, or RBI.

## 3. Results and discussion

SH-SY5Y cells stably transfected with the rat nAChR α7 subunit-based α7/pCEP4 construct appropriately express the transgene as mRNA. Northern analysis (Fig. 1) shows that endogenous α7 transcripts are evident (although at low levels; see legend) in control SH-SY5Y human neuroblastoma cells transfected with only the insertless vector (or in WT cells; not shown here). However, much higher levels of mRNA hybridizing with α7 cDNA probes are expressed in SH-SY5Y cells

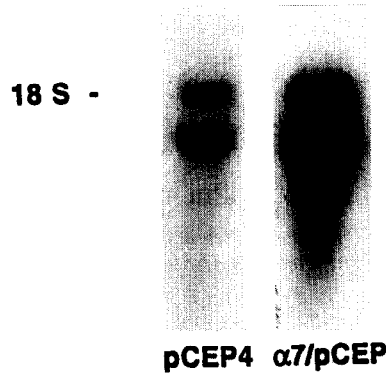


Fig. 1. Northern blot for RNA preparations from control SH-SY5Y cells transfected with the pCEP4 vector alone (pCEP4; 10 μg poly(A)$^+$RNA) or from SH-SY5Y cells transfected with the α7/pCEP4 vector (α7/pCEP4; 8.5 μg poly(A)$^+$RNA) probed with full-length α7 nAChR cDNA. Autoradiogram was exposed for 4 days at −84°C with an intensifying screen to permit visualization of native α7 mRNA from control cells. Densitometric analyses of this and other Northern blots indicates that α7-transfected cells have at least 6-fold more α7 message than do control cells. Approximate position of 18S ribosomal RNA as a reference is indicated.

transfected with full-length rat α7 transgenes (Fig. 1). Moreover, these transgene products are processed to the same two, predominant sizes as is seen for native human α7 messages in control SH-SY5Y cells, suggesting that transcription and processing to these forms of mRNA are the same from endogenous or transgenic templates. Continuing studies will determine whether long-term expression of α7 transgenes has any effect on expression of native α7 or other nAChR genes in SH-SY5Y cells.

SH-SY5Y cells stably transfected with α7 cDNA also express the appropriate α7 protein product. Toxin binding proteins affinity purified from non-ionic detergent-solubilized membrane extracts of α7-transfected SH-SY5Y cells, when resolved on SDS-PAGE and visualized by trace radioiodination and autoradiography, include a predominant polypeptide of about 67 kDa (Fig. 2). This polypeptide corresponds to the highest molecular mass species from a possibly multi-component, native nBgtS identified in WT SH-SY5Y cells [34]. The ~67 kDa polypeptide also comigrates with the peptide from native nBgtS that is reactive on Western immunoblots with an antisera raised against a unique peptide corresponding to sequences in the putative cytoplasmic domain of the α7 protein [34]. Yet to be

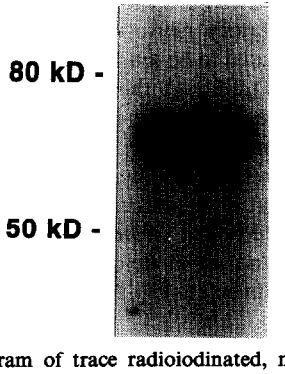


Fig. 2. Autoradiogram of trace radioiodinated, najatoxin-Acti-Gel-purified components from SH-SY5Y cells transfected with rat α7 transgenes. Positions of protein standards of know size are indicated.

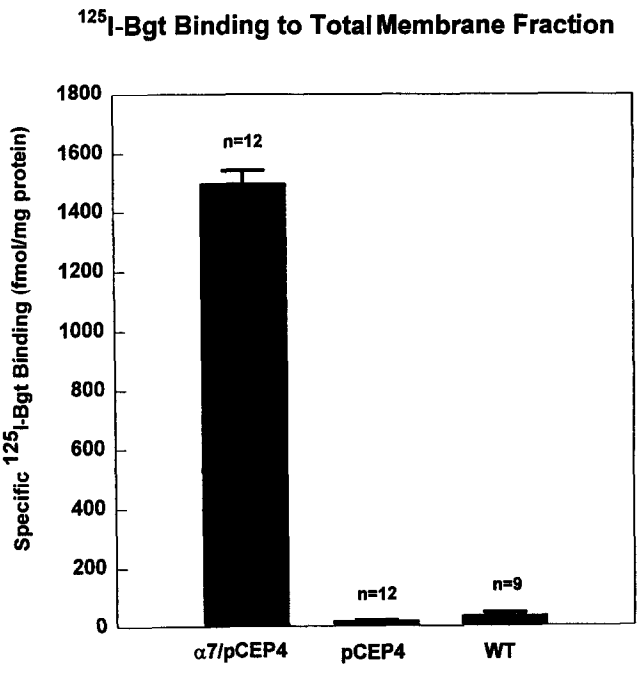


Fig. 3. Comparison of specific I-Bgt binding levels in membrane preparations from wild-type (WT), pCEP4-transfected (pCEP4), or α7/pCEP4-transfected (α7/pCEP4) SH-SY5Y cells. Values represent means plus/minus standard deviation for the indicated number of replicate studies ($n$ = 9–12) each done using samples in triplicate.

determined in future work is whether exogenous α7 subunits are expressed in transfected SH-SY5Y cells as homooligomers, as chimeric homooligomers containing exogenous rat and native human α7 subunits, and/or as chimeric heterooligomers containing transgenic rat α7 subunits plus other, presently unidentified subunits.

Our previous studies demonstrated expression of native nBgtS in SH-SY5Y cells [25]. However, α7-transfected SH-SY5Y cells have over 30-fold higher levels of specific, I-Bgt binding sites than do control WT cells or control cells transfected with the insertless pCEP4 vector alone (Fig. 3). This higher level of expression of I-Bgt binding sites in transfected cells is observed in assays using total membrane preparations or intact cells in situ, suggesting that exogenous α7 subunits can form complexes that are inserted into the plasma membrane.

α7-transfected cells stably express functional, Bgt-sensitive nAChR that quickly activate and inactivate on exposure to nicotinic agonists. Passive or voltage-dependent electrical responses of WT or transfected SH-SY5Y cells are not significantly different (data not shown). Whole cell currents recorded in a large sample of either WT or α7-transfected cells ($n$ = 120) show peak responses ranging from 50 to 3,000 pA when cells are challenged with short pulses of agonist (50 ms of 100 μM nicotine; sample traces shown in Fig. 4). Data obtained using WT cells are comparable to those previously reported for SH-SY5Y cells in response to longer agonist applications [35]. However, analysis of peak current amplitudes and times-to-peak reveals important differences between functional nAChR responses in WT and transfected cells. Transfected cells mainly

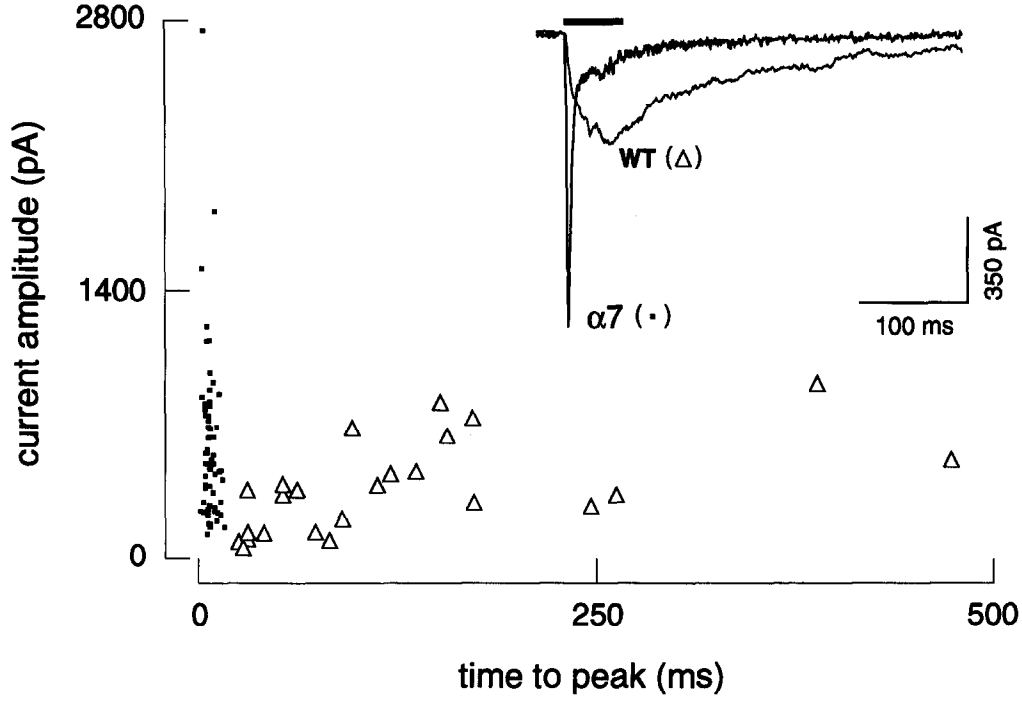


Fig. 4. α7-transfected cells display a fast inactivating, nicotine-evoked current. Each cell was held at −100 mV, and amplitude and time-to-peak of current evoked by a 50 ms pulse of saturating nicotine (100 μM) was measured. Values of current amplitudes determined in 58 α7-transfected cells (dots) and 26 WT cells (triangles) are plotted as a function of their time-to-peak. Typical recordings obtained in response to a pulse of saturating nicotine (300 μM) for WT cells and for α7-transfected (α7) cells are shown in the inset.

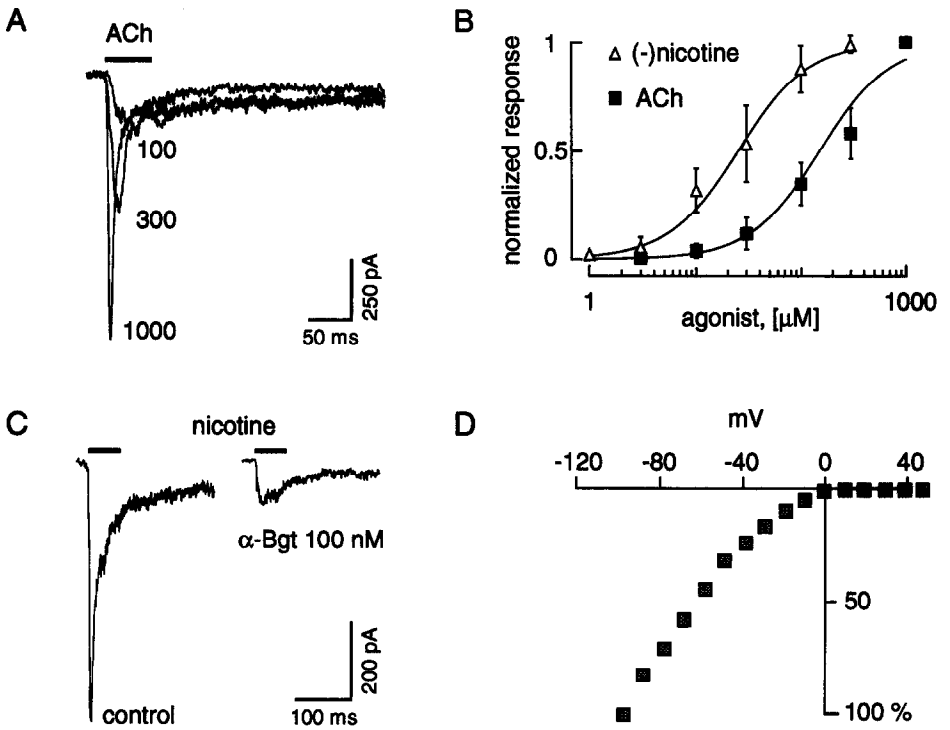


Fig. 5. Physiological characteristics of the fast inactivating, Bgt-sensitive responses of α7-transfected cells. (A) Whole cell recordings of currents evoked by ACh at three concentrations are superimposed. Pulse duration (50 ms) is illustrated by the bar. Applications were done once every 3 s beginning with the lowest concentration. (B) Dose–response curves for ACh (filled squares, 12 cells) and nicotine (open triangles, 11 cells). Currents evoked by short pulses of agonists given from lowest to higher concentrations were measured in cells held at −100 mV (see panel A). Currents were normalized to their maximum value and plotted as a function of the logarithm of the agonist concentration. Continuous lines were obtained using the empirical Hill equation. The best fit for ACh was obtained with an $EC_{50}$ of 150 μM and a Hill coefficient of 1.3, whereas for nicotine the values were, respectively, 25 μM and 1.3. (C) Nicotine-evoked current (100 μM, 50 ms) is blocked by α-Bgt. Nicotine-evoked current recorded as described above prior to (control) and after (α-Bgt; 100 nM) 1 min perfusion with α-Bgt as indicated. (D) Nicotine-evoked currents are strongly voltage dependent. Currents evoked by short pulses (300 μM, 50 ms) were recorded (in 3 cells) at several different holding potentials. Current–voltage relationships were then obtained by plotting the peak of the evoked current as a function of the holding potential. Currents were plotted relative to their maximal values determined at −100 mV. Error bars, within the symbol size, are omitted.

display fast activating/inactivating currents (7 ms mean time-to-peak) whereas slower current responses are observed in WT cells (119 ms mean time-to-peak). Nicotine-activated responses in cells transfected only with the insertless pCEP4 vector ($n = 6$; data not shown) are indistinguishable from those for WT cells, indicating that fast inactivating currents are attributable only to expression of rat α7 cDNA and not to simple vector transfection. WT SH-SY5Y cells naturally express human nBgtS and α7 subunits as message and/or protein (Fig. 1) [14,25,34]. However, no fast transient was observed in any of the WT cells challenged with either acetylcholine (ACh) or nicotine. This suggests either that endogenous α7 subunits (and/or nBgtS) are expressed only by a small fraction of WT cells or that protein levels are so low that the resulting currents are masked by the response(s) of another class(es) of endogenous nAChR [25,35]. It was previously shown that homooligomeric nAChR composed of either chicken or rat α7 subunits and expressed in *Xenopus* oocytes are very permeable to calcium ions [8,13,36, 37] and indirectly mediate activation of a calcium-dependent chloride conductance. However, no chloride contribution was detected in the ACh-evoked responses recorded in α7-expressing cells. This result probably derives from the calcium buffering capacity of the EGTA/KF-containing intracellular medium and to good exchange between the recording pipette solution and the cytosol. Other studies indicate that the physiological and pharmacological profiles of the nAChR mediating transient currents in α7-transfected cells closely resemble those observed for native, Bgt-sensitive nAChR and/or for homooligomeric α7-nAChR expressed in *Xenopus* oocytes [13,17–19]. First, for nicotine and acetylcholine (ACh), respective $EC_{50}$ values are 25–27 and 129–150 μM for rat nBgtS or α7-nAChR (*op. cit.*) compared to 25 and 150 μM for peak currents in α7-transfected cells (Fig. 5B; sample traces shown in Fig. 5A) and in contrast to 20 and 63 μM for the sustained and longer time-to-peak currents in WT SH-SY5Y cells (data not shown). Second, one minute superfusion of α7-transfected cells with 100 nM Bgt strongly inhibits agonist-evoked currents ($n = 3$; Fig. 5C) whereas nicotinic currents of WT cells are unaffected under the same conditions ($n = 6$; data not shown). Similarly, preincubation of transfected cells with 60 nM Bgt reduces nicotine-evoked, fast-transient currents to undetectable levels. Moreover, methyllycaconitine (MLA), which is a highly-selective antagonist of Bgt-sensitive nicotinic responses [38], produces 92% inhibition (at 1 nM) of the current evoked by 1 mM ACh (50 ms) in transfected cells ($n = 3$). Two surprising observations warrant mention. One is that slower, nicotine- or ACh-evoked currents resembling those seen in WT cells were still observed in some, but not all, of the transfected cells investigated after

Bgt or MLA blockade. Another is that nicotine-evoked currents measured 120 ms after the onset of agonist application, which should be dominated by activation of Bgt-insensitive nAChR, were 166 ± 32 pA in α7-transfected cells ($n$ = 27) but were 358 ± 47 pA in WT cells ($n$ = 26). The idea that transgenic α7 subunit expression can influence expression of other nAChR subunits and functional nAChR subtypes will be explored in continuing studies with α7-transfected SH-SY5Y cells. Finally, the current–voltage relationship for nicotine-evoked responses in α7-transfected cells strongly rectifies (Fig. 5D) and compares well with that previously described either for homooligomeric α7-nAChR heterologously expressed in *Xenopus* oocytes [10,13–14] or for transient functional responses of native, Bgt-sensitive nAChR [18–19].

Collectively, these results show, for the first time and after failures to achieve such a result in work in a variety of other laboratories, (i) that a mammalian cell line can be stably transfected to overexpress α7 mRNA and protein from a transgenic template and (ii) that transgenic α7 subunits can assemble into a nAChR subtype(s) that is capable of binding curaremimetic neurotoxins and nicotinic ligands and functioning as a toxin-sensitive LGIC with unique physiological and pharmacological properties. The results also support a role for α7 subunits in the ganglionic nAChR subtype (nBgtS) identified by I-Bgt binding [25]. The development of this transfected cell line provides opportunities for a number of studies employing multidisciplinary techniques that could lend further insight into the composition, structure and function of the physiologically important nAChR family.

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