Identification and Characterization of a G Protein-binding Cluster in $\alpha_7$ Nicotinic Acetylcholine Receptors*

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Background: $\alpha_7$ nicotinic acetylcholine receptors are ligand-gated ion channels that bind intracellular signaling proteins.

Results: A mutation of the M3-M4 loop of the $\alpha_7$ nicotinic acetylcholine receptor abolishes G$_\alpha_q$ activation of intracellular calcium stores.

Conclusion: $\alpha_7$ nicotinic acetylcholine receptors functionally couple G proteins.

Significance: Nicotinic acetylcholine receptors engage metabotropic-signaling responses.

$\alpha_7$ nicotinic acetylcholine receptors (nAChRs) play an important role in synaptic transmission and inflammation. In response to ligands, this receptor channel opens to conduct cations into the cell but desensitizes rapidly. In recent studies we show that $\alpha_7$ nAChRs bind signaling proteins such as heterotrimeric GTP-binding proteins (G proteins). Here, we demonstrate that direct coupling of $\alpha_7$ nAChRs to G proteins enables a downstream calcium signaling response that can persist beyond the expected time course of channel activation. This process depends on a G protein-binding cluster (GPBC) in the M3-M4 loop of the receptor. A mutation of the GPBC in the $\alpha_7$ nAChR ($\alpha_7_{345-348A}$) abolishes interaction with G$_\alpha_q$ as well as G$_\beta\gamma$ while having no effect on receptor synthesis, surface trafficking, or $\alpha$-bungarotoxin binding. Expression of $\alpha_7_{345-348A}$ however, did significantly attenuate the $\alpha_7$ nAChR-induced G$_\alpha_q$ calcium signaling response as evidenced by a decrease in PLC-$\beta$ activation and IP$_3$-mediated calcium store release in the presence of the $\alpha_7$ selective agonist choline. Taken together, the data provides new evidence for the existence of a GPBC in nAChRs serving to promote intracellular signaling.

Nicotinic acetylcholine receptors (nAChRs)$^2$ comprise an important class of the Cys-loop ligand-gated ion channel superfamily, which mediate communication between neurons by conversion of chemical neurotransmitter signals into a transmembrane flux of ions (1). In most cell types, co-expression of ionotropic nAChRs as well as metabotropic muscarinic receptors ensures a fast and slow acetylcholine signaling response, respectively. At least nine different nAChR subunits are expressed in the mammalian brain. In the hippocampus and cortex homomeric $\alpha_7$ and heteromeric $\alpha_4\beta_2$ nAChRs have been shown to contribute to neurotransmitter release and dendritic plasticity (2). Upon ligand activation, $\alpha_7$ nAChRs conduct cations into the cell (1-4). However, because the receptor channel desensitizes within milliseconds (5) it is possible that ligand binding can also set into motion longer lived downstream signaling events.

Nicotinic receptors couple to a myriad of signaling, scaffolding, and trafficking proteins in neural and immune cells (4, 6). All nAChRs maintain an M3-M4 loop, which varies in length and sequence identity between different subunits (7-9). The M3-M4 loop contributes to the trafficking and clustering of the nAChR via an association with the cellular cytoskeleton (10). nAChRs are also found to associate with several types of G proteins, which can contribute to cross-talk between nAChRs and G protein-coupled receptors (4, 8, 11, 12).

Studies on the mechanisms of G protein binding to the glycine receptor 1 (GlyR1) reveal the existence of a G protein-binding cluster (GPBC) within the M3-M4 loop of Cys-loop receptors (8). A mutation at the GPBC in the human GlyR1 is sufficient to abolish G protein binding in HEK 293 cells (13). A protein sequence alignment of the M3-M4 loop reveals a conservation of the GPBC in the $\alpha_7$ nAChR. A mutation of these residues in $\alpha_7$ nAChRs attenuates interaction with G proteins and reduces the capacity of the receptor to activate phospholipase C (PLC) and inositol triphosphate (IP$_3$) calcium release in response to choline. These findings show a role for G protein coupling in $\alpha_7$ nAChR signaling.

Experimental Procedures

Animals—Male and female C57BL/6 adult mice (Jackson) were housed on a 12-h light/dark cycle and fed ad libitum. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

Cell Culture and Transfection—Pheochromocytoma line 12 (PC12) and Neuro-2A (N2a) cells were plated on dishes (Thermo Fisher, Rockville, MD) or 96-well glass bottom culture...
plates (Life Technologies) coated with either collagen (50 μg/ml) or poly-γ-lysine (100 μg/ml) as described (4, 15). Cells were differentiated by the addition of 50 ng/ml 2.5s mouse nerve growth factor (Millipore, Billerica, MA) for PC12 cells, and with low (1%) serum media for N2a cells (12). Transfection was performed using Lipofectamine 2000 (Life Technologies). Plasmids were purified by maxi prep (Xymo Research, Irvine, CA). Plasmids used were: GCaMP5G (Addgene, Cambridge, MA); PH-mCherry (Addgene); human α7 in pcDNA3.1 (14), Ric-3 Tetracycline (Abgent, San Diego, CA) at a 1:3 ratio of Dounce homogenizer on ice and then centrifuged at 1000 g for 1 min at 95 °C; 50 s at 95 °C; 50 s at 62 °C; 7 min at 68 °C for lygenesis kit from Stratagene (CA). Briefly, 10 ng of the DNA experiment using the QuikChange II XL site-directed mutagenesis kit from Stratagene (CA). Briefly, 10 ng of the DNA template were used in the PCR condition at the following settings: 1 min at 95 °C; 1 min at 95 °C; 1 min at 62 °C; 7 min at 68 °C for 18 cycles; and 68 °C for 7 min. The product was sequenced using the ABI 310 genetic analyzer (Applied Biosystems).

Preparation of the Soluble Membrane Fraction—Solubilized proteins were obtained from cultured cells using a non-denaturing Lysis Buffer (1% Triton X-100, 137 mM NaCl, 2 mM EDTA, and 20 mM Tris-HCl, pH 8) supplemented with protease and phosphatase inhibitors (Roche, Penzberg, Germany) as described (4, 18). Soluble membrane fractions were harvested from the prefrontal cortex, hippocampus, and striatum of adult C57BL6 mice (4). Mice were anesthetized using 5% isoflurane and all tissue was dissected in an ice-cold dissection buffer (Hanks’ balanced salt solution supplemented with 10 mM HEPES) and then placed into 10 volumes of a cold homogenization buffer (0.32 M sucrose, 10 mM HEPES, 2 mM EDTA, pH 7.4) supplemented with protease and phosphatase inhibitor mixtures (Roche). Tissue was homogenized with a glass Dounce homogenizer on ice and then centrifuged at 1000 × g at 4 °C to collect the supernatant, which was subsequently centrifuged for 60 min at 200,000 × g. Protein concentrations were determined via a Bradford assay (Thermo Scientific).

Pharmacology—Drugs were added to the media 30 min prior to analysis: xestospongin C (1 μM) (Tocris, Bristol, United Kingdom); [7,9,10-3H]Trp substance P (SP; 1 μM; Tocris) (18); per-tussis toxin (100 ng/ml) (19)(Calbiochem, San Diego, CA); and nifedipine, 10 μM (20) (Sigma). 10 mM choline was used as a selective α7 agonist (18, 21). Experiments were performed in triplicate with data representing average values. Immunochemistry and Fluorescence Analysis—Cells were fixed in 1× PEM (80 mM PIPES, 5 mM EGTA, and 1 mM MgCl2, pH 6.8) containing 0.3% glutaraldehyde and permeabilized using 0.05% Triton X-100 (22). 2 mg/ml of sodium borohydride was used for glutaraldehyde quenching prior to immunoblocking with 10% goat serum (Life Technologies) and labeling with rhodamine phalloidin (Cytoskeleton, Denver, CO). α7 nAChRs were visualized using Alexa Fluor 488- or 647-conjugated α-bungarotoxin (Bgtx) (50 nm), or by visualizing the pEYFP tag. Imaging was performed using a Zeiss 710 LSM inverted microscope and images were captured using Zeiss software. Heat maps were created using Photoshop CS5 (Adobe Systems, San Jose, CA) and fluorescence intensities were measured using ImageJ (NIH).

Immunoprecipitation—Bungarotoxin Complex (BgtxC) Isolation, and Proteomics—Immunoprecipitation (IP) of protein complexes was performed as described (23). Protein G Dynabeads (Life Technologies) were preconjugated with 5 μg of the following antibodies (Abs): α7 nAChR (C-20) polyclonal (Santa Cruz); anti-Gab (New East Bioscience); and anti-Gβ (T-20) (Santa Cruz). 500 μg of cell lysate or 250 μg of brain tissue was used in each IP experiment. Proteins were eluted in LDS buffer (Life Technologies), separated onto BisTris gradient gels, and transferred onto nitrocellulose membranes (Invitrogen). HRP secondary antibodies were purchased from Jackson ImmunoResearch. Bands were visualized using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific) via the Gel Doc Imaging system (Bio-Rad). SeeBlue Ladder (Life Technologies) were used as a protein standard. Presented values are based on averages from three independent experiments.

BgtxC isolation was performed using a cyanogen bromide-activated α-bungarotoxin-Sepharose matrix as described (24). 1500 μg of solubilized membrane proteins was mixed with 100 μl of a Sepharose bead matrix overnight at 4 °C. Beads were centrifuged at 2,000 × g and unbound proteins were removed by washing in ice-cold TBS. BgtxC was eluted by a 1M carboxymethylcholine chloride solution followed by precipitation with acetone. Proteins were separated by a SDS-PAGE gel and select bands were excised from the Coomassie-stained gel for mass spectrometry (MS) protein analysis using liquid chromatography-electrospray ionization (LC-ESI) (4). Tandem MS collected by Xcalibur (version 2.0.2) was searched against the NCBI protein database using SEQUEST (Bioworks software from ThermoFishier, version 3.3.1). SEQUEST search results were filtered as: minimum X correlation of 1.9, 2.2, and 3.5 for 1+, 2+, and 3+ ions, respectively, and ΔCn > 0.1. Protein Score (PS) exclusion was set at X correlation < 0.1. Calcium Imaging—Cells were transfected with GCaMP5G (25) for detection of rapid calcium dynamics using a Zeiss Axio Observer Z.1 with attached mRM camera using an acquisition rate of 1 frame per 70 ms for 75 s at 2×2 binning. Phototoxicity and bleaching were minimized using low-wavelength and neutral density light filters (26). Drugs were applied to the recording chamber via a gravity fed perfusion system at a flow rate of...
1 ml/s after 70 frames of baseline recordings. For replacement experiments 1.26 mM barium was added to a calcium-free Hanks’ balanced salt solution (Life Technologies). Regions of interest (ROIs) were normalized as ΔF/FA0 and analyzed using ImageJ (NIH). Area under the curve (AUC) was determined using the formula 1/2(X1 + Xn)(time step difference between frames). ROIs were averaged over each condition as shown (18). Eight cells were imaged per experimental condition and all experiments were performed in triplicate to obtain group averages (n = 8).

PLC-β Translocation—PIP2 breakdown is detected quantitatively by imaging the translocation of a pleckstrin homology (PH) domain of PLC (δ) tagged with mCherry (18, 27). A PH-mCherry sensor was co-transfected with GCaMP5G into PC12 cells. PH-mCherry fluorescence (excitation λ = 555 nm) was visualized at an acquisition rate of 1 frame per 10 s for 1 min with 2 × 2 binning. Drugs were applied 10 s after a 1-frame (10 s) baseline recording. PH-mCherry translocation was determined using a 2-point ROI analysis (ROI 1, fluorescence at the plasma membrane (Fm); ROI 2, cytosol fluorescence (Fc)). Translocation of PH-mCherry was determined using the equation (Fm − Fc)/(Fm + Fc) (18, 27). Fluorescent values were normalized for the area (μm2) measured with Image J (NIH). Experiments were repeated in triplicate to obtain group averages (n = 8).

Statistical Analysis—Data were compared by one-way ANOVA and Tukey’s HSD post hoc or Student’s t test (p < 0.05). Data are presented as mean ± S.E. of at least three independent experiments.

Results

Proteomic Evidence on the Existence of α7 nAChR/G Protein Complexes in the Brain—Studies have revealed associations between nAChRs and G proteins in neural and non-neural cells (4, 9, 18, 23, 26, 28). We surveyed α7 nAChR associations with G proteins in the prefrontal cortex, hippocampus, and striatum of the mouse brain. α7 nAChR-specific antibodies were used to co-IP α7 along with G proteins from neuronal membranes fractions. A reverse co-IP experiment was performed using anti-G protein antibodies to detect α7 nAChRs in the same membrane fraction. Co-IP experiments were also performed from α7 knock-out mice (α7+/−) as a negative control (29). As shown in Fig. 1A, α7 nAChRs were found in association with Gαq, Gαq, and Gαi proteins within the hippocampus and prefrontal cortex. Striatal fractions suggest α7 interaction with Gαq and Gαi subunits (Fig. 1A). Comparable levels of Gαq, Gαq, and Gαi protein expression was seen on a Western blot in the three-brain structures (data not shown). An association between α7 nAChRs and Gβ was observed in the prefrontal cortex and hippocampus, whereas little to no detection of Gαq was seen in the adult brain (Fig. 1A). Complementary experiments in which the anti-α7 antibody C-20 was used to co-IP Gαq and Gβ from the hippocampus confirmed association between the α7 nAChR and these G proteins (Fig. 1A).

α-Bgtx is a selective antagonist of the α7 nAChR. We determined subtypes of G proteins that associated with a BgtxC matrix from the mouse brain using tissue from α7−/− as a negative control. This purification method enabled us to examine G proteins that interact with nAChRs (24). Western blotting confirmed expression of α7 subunits within the BgtxC fraction (Fig. 1B). As shown in Fig. 1C, SDS-PAGE fractionation of the BgtxC indicates greater specificity and stronger yield when the nicotinic agonist, carbachol, is used to elute from the Bgtx matrix. Gel lanes of the BgtxC were divided into 3 molecular mass fractions: F1, 190–90 kDa; F2, 89–45 kDa; and F3, 44–15 kDa. D, G proteins identified within F2 and F3 fractions. Protein score (Score); molecular weight (MW); and GenBank™ accession number (GI). Total: 10% of the total membrane fraction loaded as a positive control.

Identification of a GPBC within the M3-M4 Loop of nAChRs—We examined the existence of a G protein-binding motif within nAChRs. Mutagenesis experiments in the structurally related GlyR1 have demonstrated that amino acids RFRR at position 360–363 within the M3–M4 loop enable G protein coupling (13). This binding sequence is similar to a Gβγ binding motif (RFRR) within the inward rectifying potassium channel (GIRK1) (30). Sequence alignment of mammalian nAChRs revealed a conservation of residues at the GPBC site in the M3-M4 loop (Fig. 2A). Based on amino acid similarity we found that α7 nAChRs possess 88% homology with the GlyR1 at the GPBC (Fig. 2A). Other nAChRs such as the α4, which have been shown to functionally couple to G proteins in immune cells (28), also demonstrate sequence similarity at the GPBC (63%). Because sequence similarity at the putative GPBC varies
between nAChR subunits, it is plausible that nAChRs could differentially couple G proteins.

Generation of a G Protein Binding Dominant Negative α7 nAChR—To test if the GPBC in α7 nAChRs directs G protein binding, we mutated amino acids at position 345 to 348 in the human α7 subunit. All four amino acids were changed to alanine thereby generating a α7<sub>345–348A</sub> nAChR proposed to lack a G protein coupling capacity. A similar mutation at the GPBC (RFRR, residues 360–364) in the GlyR1 was found to abolish association with G proteins in HEK293 cells (13). Constructs encoding α7 nAChRs with an amino terminus YFP tag (YFP-α7 and YFP-α7<sub>345–348A</sub>) were transfected into PC12 cells, which endogenously express the α7 receptor (4), YFP labeling of the nAChR at the amino terminus has been shown to not interfere with the trafficking or function of the nAChR (31). As shown in Fig. 2B, YFP-α7<sup>+</sup> and YFP-α<sub>7</sub><sup>345–348A</sup> cells presented similar receptor expression patterns in the soma, neurite, and at the growth cone (GC) suggesting that the mutation in α<sub>7</sub><sup>345–348A</sup> does not impair receptor synthesis or trafficking (Fig. 2B).

Direct visualization of assembled α7 nAChRs using fBgtx in non-permeablized cells was used to determine the spatial distribution of cell surface receptors in α7, α7<sup>+</sup>, and α7<sub>345–348A</sub>
expressing PC12 cells (Fig. 2C). Recently we have shown a role for α7 nAChRs in the development of hippocampal neurons and PC12 cells via receptor localization at the GC (26). Strong fBgtx labeling was seen at the GC in α7, α7+, and α7345–348A nAChR expressing cells (Fig. 2C). Distribution analysis of the fBgtx signal indicates that overexpression of either receptor variant does not alter fBgtx labeling at the cell surface (Fig. 2C). The topographic distribution of the fBgtx signal was comparable between α7+, α7345–348A, and α7 controls (Fig. 2C). Statistical analysis indicates no significant difference in fBgtx distribution between the experimental groups.

We examined the expression of the α7345–348A nAChR in an N2a cell line that does not express endogenous α7 nAChRs. N2a cells were co-transfected with Ric-3, which has been shown to promote the trafficking of α7 nAChRs to the cell surface (32–34). Visualization of fBgtx labeling in non-permeabilized N2a cells transfected with α7 or α7345–348A suggest that a mutation of the GPBC does not interfere with α7 nAChR cell surface expression in a heterologous system (Fig. 2D). Statistical analysis of the fBgtx signal indicates no significant difference in the level of α7 nAChR expression in the soma or GC of N2a cells transfected with either the wild-type or mutant construct (Fig. 2D). The findings confirm that the mutation in α7345–348A does not interfere with the trafficking or expression of the nAChR.

A Loss in G Protein Coupling in α7345–348A Expressing Cells—

We determined the expression of α7 nAChRs in transfected cells. Western blot analysis confirms that transfection of α7 nAChRs augments total α7 subunit expression in PC12 cells, which express endogenous α7 nAChRs. Transfection with α7 (α7+) increased the immunoreactive α7 signal by over 60% from endogenous mock-transfected control cells, whereas transfection with the mutant α7345–348A increased the total α7 signal by 48% over the endogenous α7 from control cells (Fig. 3A). Transfection of PC12 cells with α7 siRNA significantly reduces α7 nAChR protein expression and confirms the specificity of mAb306 on the Western blot (Fig. 3A) (16, 26). Studies in N2a cells indicate that transfection with α7345–348A yields a similar α7 nAChR expression as the wild-type (α7) and supports the finding that mutation at the GPBC does not impact the synthesis of the nAChR in cells.

Recently, we have shown that coupling to Gαq enables α7-mediated calcium release from the endoplasmic reticulum (ER) leading to changes in neurite growth (18). We tested the ability of transfected α7345–348A nAChRs to function as dominant negative regulators of G protein binding in PC12 cells, which endogenously express α7 nAChRs. Using an anti-Gαq antibody we observed interactions between α7 nAChRs and G proteins in an IP experiment from transfected PC12 cells. As shown in Fig. 3A, coupling between Gαq and α7 nAChRs was virtually abolished by expression of α7345–348A. A noticeable loss in Gαq (−62.18%) and Gβγ (−20.03%) expression within the α7 nAChR complex IP was seen in cells transfected with α7345–348A (Fig. 3A). An increase in G protein association within the α7 complex was observed in α7+ cells (Gαq + 16.71%; Gβγ + 19.90%) (Fig. 3A). Similar findings in transfected N2a cells indicate a loss in G protein association within the α7 complex when α7345–348A nAChRs are expressed (Fig. 3B). In particular, when the α7345–348A nAChR was expressed by itself in N2a cells, little to no Gαq/Gβγ was detected within the α7 complex IP (Fig. 3B). Compared with N2a cells transfected with the wild-type α7 nAChR, α7345–348A expressing N2a cells show little to no nAChR/G protein association (Fig. 3B). The data complements earlier findings on the ability of α7345–348A to function as a dominant negative blocker of G protein coupling in PC12 cells, and suggests that the GPBC directs nAChR association with Gαq and Gβγ.

An Attenuation of the Calcium Transient Response to Choline

in α7345–348A Cells—Upon binding ligands, α7 nAChRs conduct both calcium and sodium into the cell but rapidly desensitize resulting in a short-lived ionotropic response (21, 35). Recent studies indicate that activation of α7 nAChRs can promote calcium release from the ER (18). In hippocampal slices this α7 nAChR-mediated calcium response persists for several minutes thereby modulating synaptic plasticity (6). To determine the role of Gαq activation in α7 nAChR calcium signaling, we examined responses to choline, a selective α7 nAChR agonist using GCaMP5G imaging in PC12 cells (18, 25). Choline-mediated calcium transients were seen in the neurite and GC (Fig. 4, A–C) consistent with fBgtx labeling at these sites (Fig. 2) (18). Calcium transients in the neurite peaked at 620% ΔF/F0 (±88.7%). In α7+ cells, choline application resulted in a calcium signal that peaked at 1050% ΔF/F0 (±176.4%) in the neurite. Calcium transients in the GC were found to last for ~1.6 s in both α7 and α7+ cells peaking at 1396% (±154.4%) and 1316% (±146.9%) ΔF/F0 relatively (Fig. 4, A and B).

PC12 cells transfected with α7345–348A showed a reduction in choline-mediated calcium responses. A one-way ANOVA revealed a significant difference between α7, α7+, and α7345–348A transfected cells (F(2,24) = 6.013, p = 0.008). Most notably, in the GC, the calcium peak values were significantly lower in α7345–348A transfected cells compared with α7 cells after Tukey’s HSD post hoc comparisons (peak: 741 ± 159.8% ΔF/F0, p = 0.006). This represents a 46.92% reduction from the α7 baseline calcium response (Fig. 4, A and B). This reduction approached significance in the neurite of α7345–348A cells (peak: 561 ± 124.9% ΔF/F0) (Fig. 4, A and B). The total cal-
G Protein Binding in nAChRs

A one-way ANOVA of the AUC values in the GC demonstrated a statistically significant reduction (333.4% ΔF/F₀ ± 35.7%) when compared with N2a cells that express the α7nACHR (peak: 581% ΔF/F₀ ± 122.7%; p = 0.04) (Fig. 4, D and E). AUC measures for choline responses in N2a cells showed a 60% reduction in calcium transients in α7.345-348A nACHR expressing cells relative to α7nACHR (α7: 83.15% ΔF/F₀² × s ± 25.5%; α7.345-348A: 37.54% ΔF/F₀² × s ± 7.86%) (Fig. 4E). These findings in N2a cells are consistent with data from PC12 cells and suggest that α7.345-348A nACHR expression impairs nACHR calcium signaling.

Choline-induced Calcium Transients Depend on Gαq and Calcium Channel Activity—We tested the role of a proposed Gαq inhibitor SP in α7 calcium signaling in PC12 cells (18). As shown in Fig. 5, A and B, choline peak responses were significantly diminished (−53.87%) in cells pretreated with SP compared with vehicle controls (p = 0.005). SP pretreatment did not significantly alter calcium peaks in α7.345-348A nACHR expressing cells, showing a small (−31.24%) reduction in calcium responses relative to the α7.345-348A baseline measure (p > 0.05). Similar results were seen when examining the AUC values for α7 (291.00% ΔF/F₀² × s ± 113.78%) or α7.345-348A (253.75% ΔF/F₀² × s ± 138.63%, p = 0.003) nACHR expressing cells pretreated with SP (Fig. 5C). Because α7.345-348A-transfected cells did not show any responsiveness to SP, these findings suggest this receptor mutant is not functionally coupled to Gαq.

To test if choline-induced calcium transients depended on calcium flow through the nACHR, imaging was performed in a calcium-free solution supplemented with 1.26 mM barium. As shown in Fig. 5, A–C, barium replacement had little to no effect on the peak and total calcium transient observed in α7 (peak: 1474.83% ΔF/F₀ ± 162.00%; AUC: 693.5% ΔF/F₀² × s ± 154.15%) and α7.345-348A expressing cells (peak: 794% ΔF/F₀ ± 81.36%; AUC: 543.5% ΔF/F₀² × s ± 89.59%). Because barium is impermeable through voltage-gated calcium channels (36) and studies indicate a role for α7 nACHRs in the modulation of calcium-induced calcium release via local VGCCs (37, 38), we tested the effect of the L-type channel blocker nifedipine on choline-induced calcium signals. As shown in Fig. 5, A–C, nifedipine was found to decrease the peak calcium response to choline in PC12 cells (peak: 795.00% ΔF/F₀ ± 107.1%) by 56.94% (p = 0.003), whereas prolonging the duration of the choline-induced calcium transient (AUC: 7.86% ΔF/F₀² × s ± 64.02%) in the same cell. In α7.345-348A nACHR expressing cells, nifedipine had no effect on the peak or the duration of the calcium transient (peak: 957.00% ΔF/F₀ ± 252.2%; AUC: 333.33% ΔF/F₀² × s ± 91.53%) relative to choline treatment alone (Fig. 5, A–C). The findings suggest that choline-induced calcium responses in PC12 cells involve the activity of VGCC (37, 38).

A one-way ANOVA revealed a significant difference in both peak calcium responses (F(3,31) = 8.048, p = 0.001) and total AUC calcium transients (F(3,31) = 8.191, p < 0.005) of SP, barium, and nifedipine on choline-induced calcium transients in α7 nACHR expressing PC12 cells. This effect was not observed in PC12 cells transfected with the α7.345-348A mutant.

**FIGURE 4.** α7.345-348A expression attenuates choline-mediated calcium transients. A, GCaMP5G measurements of calcium following application of the α7-selective agonist choline (10 mM). Calcium transients were detected in the neurite and the GC. B, average peak calcium responses in the neurite and growth cone. C, average AUC of the calcium transient in the GC. D, calcium transients at the growth cone of N2a cells transfected with α7 or α7.345-348A nACHRs and treated with choline (10 mM). E, % change in peak ΔF/F₀ and AUC between α7.345-348A and α7 nACHR expressing N2a cells. Arrow, time of drug application. The results represent the mean ± S.E. (error bars) of independent experiments compared by one-way ANOVA and Tukey's HSD post test. n = 8; *, p < 0.05; **, p < 0.005.
**G Protein Binding in nAChRs**

**A**

α7 and α7<sub>345–348A</sub> nAChRs Do Not Activate PLC—Activation of G<sub>αq</sub> sets into motion PLC-associated signaling leading to the formation of IP<sub>3</sub> and the mobilization of calcium release from the ER (23). This second messenger pathway can be examined via the genetically encoded PLC(δ) sensor pleckstrin homology mCherry probe (PH-mCherry) (27). A translocation of PH-mCherry from the cell surface to the cytosol has been found to quantitatively relate to PIP<sub>2</sub> breakdown and IP<sub>3</sub> production (18, 27). Treatment of PC12 cells with 10 mM choline was associated with a translocation of PH-mCherry from the cell surface as determined by the presence of the fluorescence signal within 1 μm of the edge of the cell into the cytosol of the GC (Fig. 6, A and B). Pre-treatment of cells with SP abolished this translocation (Fig. 6B). In PC12 cells expressing α7<sub>345–348A</sub> nAChRs choline had a weak effect on PH-mCherry translocation relative to empty plasmid-transfected controls. Expression α7<sub>345–348A</sub> nAChRs was surprisingly associated with strong levels of PH-mCherry at the cell surface in the absence of drug treatment (Fig. 6B).

Sequential imaging of PH-mCherry and GaMgP5G confirms that choline promotes a rise in intracellular calcium and PH-mCherry translocation in the same cellular compartment (Fig. 6, B and C). Cytoplasmic translocation of PH-mCherry occurred on a slower time scale (40 s after choline application) than peak calcium responses (∼1 s after choline application). These kinetics are consistent with the translocation of the PH domain sensor in the cell (20, 29).

α7 receptor activation and the generation of IP<sub>3</sub> via PLC promotes calcium release via IP<sub>3</sub>Rs in neurons (18, 26). We confirmed the involvement of IP<sub>3</sub>Rs in choline-induced calcium transients at the GC of PC12 cells. Cells were preincubated with the IP<sub>3</sub>R blocker xestospongin C (1 μM) prior to imaging. As shown in Fig. 7, A–C, pretreatment with xestospongin C reduced the α7 nAChR calcium response peak by 46.82% in α7 cells (peak: 1308.43% ΔF/F<sub>0</sub> ± 238.13%; + xestospongin C = 695.80% ΔF/F<sub>0</sub> ± 101.46%). A one-way ANOVA confirmed that this effect of xestospongin C is significant (F(3,31) = 5.128, p = 0.006) (AUC: 690.50% ΔF/F<sub>0</sub> ± 66.98%; + xestospongin C = 343.54% ΔF/F<sub>0</sub> ± 112.86%; p = 0.007). Xestospongin C treatment did not impact the calcium peak (628.87% ΔF/F<sub>0</sub> ± 69.43%) or total calcium transient (AUC: 443.86% ΔF/F<sub>0</sub> ± 54.72%; + xestospongin C = 340.39% ΔF/F<sub>0</sub> ± 96.99%) in α7<sub>345–348A</sub> expressing cells (α7 to α7 + xestospongin C, p = 0.017; α7 to α7<sub>345–348A</sub> + xestospongin C, p = 0.017) (Fig. 7, B and C). The data underscores the inability of α7<sub>345–348A</sub> nAChRs to activate intracellular calcium via IP<sub>3</sub>Rs (18).

**Ligand Stimulation of the α7 Receptor Reduces Its Interaction with G Proteins**—We examined the dynamics of α7 nAChR/G protein coupling in response to ligand stimulation. PC12 cells were treated with the selective α7 nAChR agonist choline (10 mM) for 2 min prior to analysis. An IP assay was utilized to examine changes in nAChR/G protein coupling in drug-treated versus vehicle-treated control cells. As shown in Fig. 8, A and B, IP of the α7 using the C-20 antibody suggests that choline application attenuates G protein binding with the nAChR. Choline treatment resulted in a 56% reduction in G<sub>αq</sub> and 47% reduction in Gβ association within the α7 nAChR complex (Fig. 8B). In cells transfected with the α7<sub>345–348A</sub> nAChR a surprising 38.81% increase in Gβ detection was seen in the α7 nAChR IP experiment following choline treatment (Fig. 8, A and B). The data suggest a role for ligands in the modulation of α7 nAChR interaction with G proteins.

**Discussion**

A Motif for G Protein Binding in Nicotinic Receptors—Cys-loop receptors such as the GlyR1 have been shown to bind G proteins via specific amino acids within the M3-M4 intracellular loop (13). In this article, we show a similar GPBC in α7 nAChRs and propose the existence of a GPBC in other
nAChR subunits such as α4 and α1 based on sequence homology at this site. This is consistent with recent studies that indicate that nicotine stimulation of α4 nAChRs activates Goq signaling in CD4+ T-cells leading to cytokine release in mice (28). A mutation of the GPBC within α7 nAChRs was found sufficient to eliminate association with Goq as well as Gβ subunits in both PC12 and N2a cells. In preliminary studies, we found that individual mutations at the GPBC did not significantly impact G protein binding, confirming that all four residues contribute to a G protein binding core within the α7 nAChR. In light of the similarity of this site with the G protein binding residues (RFFP) within the GIRK1 channel, it is plausible that a minimal consensus sequence consisting of RXYR

3 J. R. King and N. Kabbani, unpublished data.
**G Protein Binding in nAChRs**

![Diagram](https://via.placeholder.com/150)

**FIGURE 8. Ligand activation of the α7 nAChR reduces interaction with G proteins.** PC12 cells were treated with 10 μM of the α7 agonist choline (+) or vehicle (Hanks’ balanced salt solution) (−) for 2 min. A, 500 μg of a membrane fraction were used in an IP with the anti-α7 C-20 antibody. Blots were probed with anti-Gαq or anti-Gβγ antibodies. B, band density analysis of the IP showing the percent change in the levels of detected G proteins in response to choline (n = 3).

(where X is any amino acid and Y is a basic amino acid) facilitates G protein binding in various proteins (30).

Coupling to Gαq appears essential for α7 nAChR-mediated calcium release from the ER; however, α7 nAChRs can also couple to other G proteins (24). This may be determined by factors such as the localization of the nAChR within subcellular domains such as lipid rafts which may enable proximity to specific G proteins (39), or the interaction between the nAChR and adaptor molecules, such as G protein-regulated inducer of neurite outgrowth 1, which may facilitate coupling to specific G proteins (4, 26). Receptor associations with Gβγ can also influence coupling to Gαo (40). This is in agreement with our proteomic findings that indicate that α7 nAChRs bind both Gαq as well as Gβγ proteins and that this association is reduced in the presence of a ligand. The existence of a nAChR/G protein trimer complex is also consistent with FRET and chemical cross-linking evidence on functional associations between trimeric G proteins and their targets (41).

The M3-M4 loop is presumed to constitute an important intracellular protein-protein interaction interface within nAChRs controlling functional features of the receptor such as its targeting to axons or dendrites (31, 42, 43). Our findings expand this theory and indicate that binding of G proteins enables a downstream signaling response by α7 nAChRs. Based on this study we can infer a dominant negative function for α7345–348A nAChR subunit expression in cells. Whether 1 or more α7345–348A subunits are incorporated into the homomeric receptor is sufficient to entirely disrupt G protein signaling is an important focus of future studies. Based on our findings in heterologous N2a cells, expression of the α7345–348A subunit alone enables the formation of fBgtx binding nAChRs at the cell surface. Compared with cells that expressed the wild-type α7, α7345–348A expressing N2a cells were found to respond to choline with a noticeably reduced calcium transient. A similar reduction (>50%) in calcium responsiveness to choline is also found in PC12 cells that were transfected with α7345–348A subunits and suggests that expression of this dominant negative disrupts the G protein coupling of endogenous α7 nAChRs. Similarly, co-IP experiments demonstrate that expression of α7345–348A in PC12 and N2a cells is associated with a prominent reduction in G protein interaction with α7 nAChRs.

A G protein signaling response may account for some of the non-conducting channel functions of new compounds termed “silent agonists” such as NS6740, which may be able to engage intracellular calcium stores (44). Although it will be of interest to test the effects of such compounds on the G protein signaling response of the α7 nAChR, proteomic findings presented in this study suggest that G protein association is detectable in the Bgtx bound state. It will be important to determine whether agonist currents through the α7345–348A nAChR are altered relative to the wild-type. Based on previous findings on the impact of mutations in the M3-M4 loop, it is expected that currents are unaffected in the α7345–348A nAChR (45).

**G Protein Coupling Activates a Local Rise in Intracellular Calcium**—Ligand activation of the nAChR leads to a depolarization of the neuronal membrane and changes in neuronal firing (38). Activation of nAChRs can also mediate longer-lived cellular signals including the modulation of survival and plasticity genes (15). Positive allosteric modulation of the α7 nAChR promotes cytotoxicity in SH-SY5Y cells via the release of calcium from the ER (46). In adult hippocampal neurons, α7 nAChR activation of ER calcium release promotes calcium transients that modulate glutamate release at synapses (6). Our earlier studies indicate a role of α7/G protein signaling in regulating the release of calcium from the ER during axon development via their interactions at the GC (18). Functional coupling to G proteins, such as Gαq, provide an important mechanistic explanation on how α7 nAChRs regulate intracellular calcium release from the ER and may play a role in receptor distribution.

Based on the current state of evidence, we propose a model by which direct G protein interactions enable metabolotropic signaling via the α7 nAChR receptor (Fig. 9). In this model, nAChR signaling via the G protein pathway may involve cross-talk with nearby G protein-coupled receptors, which can mediate GTP exchange on the Gαq subunit. An analysis of the peak response and duration of the PLC sensor (PH-mCherry) clearly shows that choline activation of α7 nAChRs enhances the activity of PLC in the same cellular compartment as measured calcium responses. Here and previously, we have shown that Bgtx, a GPBC mutation, as well as the Gαq blocking peptide SP, attenuates this process in cells (18). Moreover, in cells expressing the α7345–348A mutant, a loss in PLC activation is consistent with the role of Gαq coupling in nAChR-associated PLC signaling and IP3-mediated calcium store release. However, it is not clear why expression of the α7345–348A mutant increases the PH-mCherry signal at the cell surface.

Pharmacological inhibition of IP3Rs by xestospongin C appears sufficient to attenuate the α7 calcium signal at the GC. Varied responses to xestospongin C exist throughout the cell and suggest a role for compartment specific mechanisms. At pharmacological levels consistent with IP3R inhibition (47), xestospongin C attenuates the choline-induced calcium
response to the same extent as α7345–348A nAChR expression. The evidence suggests that α7 nAChRs in concert with G proteins promote a local elevation in intracellular calcium that can persist for seconds beyond the expected time frame of α7 channel activation. α7–Mediated calcium transients are strongest near the ER consistent with the role of IP3R activation downstream of nAChR signaling (35, 38). Interestingly, this time period of intracellular calcium signaling may persist during α7 nAChR desensitization (35). Replacement of extracellular calcium with barium, which does not pass through the α7 nAChR, did not alter the calcium transient response to choline. Because barium is permeable via VGCCs, however, our findings underscore a role of calcium channels in the G protein-associated nAChR signaling response (37, 38). This is evidenced by the finding that choline-mediated calcium transients are noticeably attenuated in the presence of the L-type channel blocker nifedipine in α7 but not α7345–348 nAChR expressing cells. Because VGCC are regulated by Gβγ, the findings suggest a mechanism of VGCC regulation by nAChRs via G proteins (48). This process may contribute to a local rise in intracellular calcium via choline-induced calcium release (46).

Author Contributions—N. K. and J. R. K. conceived and coordinated the study and wrote the paper. J. R. K. designed, performed, and analyzed the experiments. J. C. N., S. P. B., and M. K. L. provided technical assistance and contributed to the preparation of the figures. All authors reviewed the results and approved the final version of the manuscript.

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References
1. Changeux, J.-P. (2010) Nicotine addiction and nicotinic receptors: lessons from genetically modified mice. Nat. Rev. Neurosci. 11, 389–401
2. Halíř, A. W., Gómez-Varela, D., John, D., and Berg, D. K. (2014) A novel mechanism for nicotinic potentiation of glutamatergic synapses. J. Neurosci. 34, 2051–2064
3. Campbell, N. R., Fernandes, C. C., Halíř, A. W., and Berg, D. K. (2010) Endogenous signaling through α7-containing nicotinic receptors promotes maturation and integration of adult-born neurons in the hippocampus. J. Neurosci. 30, 8734–8744
4. Nordman, J. C., and Kabbani, N. (2012) An interaction between α7 nicotinic receptors and a G-protein pathway complex regulates neurite growth in neural cells. J. Cell Sci. 125, 5502–5513
5. Liu, Q., and Berg, D. K. (1999) Actin filaments and the opposing actions of CaM kinase II and calcineurin in regulating α7-containing nicotinic receptors on chick ciliary ganglion neurons. J. Neurosci. 19, 10280–10288
6. Zhong, C., Talmage, D. A., and Role, W. L. (2013) Nicotine elicits prolonged calcium signaling along ventral hippocampal axons. PLoS ONE 8, e82719
7. Lin, L., Jeanclos, E. M., Treuil, M., Braunerewell, K.-H., Gundelfinger, E. D., and Andan, R. (2002) The calcium sensor protein visinin-like protein-1 modulates the surface expression and agonist sensitivity of the α4β2 nicotinic acetylcholine receptor. J. Biol. Chem. 277, 41872–41878
8. Kabbani, N., Nordman, J. C., Corgiat, B. A., Velthuijzen, D. P., Shehu, A., Seymour, V. A., and Adams, D. J. (2013) Are nicotinic acetylcholine receptors coupled to G proteins? Bioessays 35, 1025–1034
9. McClure-Begley, T. D., Stone, K. L., Marks, M. J., Grady, S. R., Colangelo, C. M., Lindstrom, J. M., and Picciotto, M. R. (2013) Exploring the nicotinic acetylcholine receptor-associated proteome with iTRAQ and transgenic mice. Genomics Proteomics Bioinformatics 11, 207–218
10. Gomez-Varela, D., and Berg, D. K. (2013) Lateral mobility of presynaptic α7-containing nicotinic receptors and its relevance for glutamate release. J. Neurosci. 33, 17062–17071
11. Quarta, D., Ciruela, F., Patkar, K., Borycz, J., Solinas, M., Lluis, C., Franco, R., Wise, R. A., Goldberg, S. R., Hope, B. T., Woods, A. S., and Ferré, S. (2007) Heteromeric nicotinic acetylcholine-dopamine autoreceptor complex modulates striatal dopamine release. Neuropsychopharmacology 32, 35–42
12. Jacovina, A. T., Zhong, F., Khazanova, E., Lev, E., Deora, A. B., and Hajjar, K. A. (2001) Neuritogenesis and the nerve growth factor-induced differentiation of PC-12 cells requires annexin II-mediated plasmin generation. J. Biol. Chem. 276, 49350–49358
13. Yevenes, E. G., Moraga-Cid, G., Guzmán, L., Haeger, S., Oliveira, L., Olate, J., Schmalzing, G., and Aguayo, L. G. (2006) Molecular determinants for G protein βγ modulation of ionotropic glycine receptors. J. Biol. Chem. 281, 39300–39307
14. Saragoza, P. A., Modir, J. G., Goel, N., French, K. L., Li, L., Nowak, M. W., and Stittzel, I. A. (2003) Identification of an alternatively processed nicotinic receptor α7 subunit RNA in mouse brain. Brain Res. Mol. Brain Res. 117, 15–26
15. Lozada, A. F., Wang, X., Gounko, N. V., Massey, K. A., Duan, J., Liu, Z., and Berg, D. K. (2012) Glutamatergic synapse formation is promoted by α7-containing nicotinic acetylcholine receptors. J. Neurosci. 32, 7651–7661
16. Qi, X.-L., Nordberg, A., Xiu, J., and Guan, Z.-Z. (2007) The consequences of reducing expression of the α7 nicotinic receptor by RNA interference and of stimulating its activity with an alpha7 agonist in SH-SY5Y cells indicate that this receptor plays a neuroprotective role in connection with the pathogenesis. Neurochem. Int. 51, 377–383
17. Feng, D. F., Johnson, M. S., and Doolittle, R. F. (1984) Aligning amino acid sequences: comparison of commonly used methods. J. Mol. Evol. 21, 112–125
18. Nordman, J. C., and Kabbani, N. (2014) Microtubule dynamics at the growth cone are mediated by α7 nicotinic receptor activation of Gαq and IP3 receptor pathway. FASEB J. 28, 2995–3006
19. Tso, P. H., Morris, C. I., Jung, L. Y., Ip, N. Y., and Wong, Y. H. (2009) Multiple G proteins participate in nerve growth factor-induced activation of c-Jun N-terminal kinases in PC12 cells. Neurochem. Res. 34, 1101–1112
G Protein Binding in nAChRs

20. Kumar, A. (2010) Carbachol-induced long-term synaptic depression is enhanced during senescence at hippocampal CA3–CA1 synapses. J. Neurophysiol. 104, 607–616

21. Khiroug, S. S., Harkness, P. C., Lamb, P. W., Sudweeks, S. N., Khiroug, L., Millar, N. S., and Yakel, J. L. (2002) Rat nicotinic ACh receptor α7 and β2 subunits co-assemble to form functional heteromeric nicotinic receptor channels. J. Physiol. 540, 425–434

22. He, Y., Francis, F., Myers, K. A., Yu, W., Black, M. M., and Baas, P. W. (2005) Role of cytoplasmic dynein in the axonal transport of microtubules and neurofilaments. J. Cell Biol. 168, 697–703

23. Kabbani, N., Woll, M. P., Levenson, R., Lindstrom, J. M., and Changeux, J.-P. (2007) Intracellular complexes of the β2 subunit of the nicotinic acetylcholine receptor in brain identified by proteomics. Proc. Natl. Acad. Sci. U.S.A. 104, 20570–20575

24. Xu, J., Zhu, Y., and Heinemann, S. F. (2006) Identification of sequence motifs that target neuronal nicotinic receptor subtypes in mammalian cells. Mol. Pharmacol. 68, 1431–1438

25. Nordman, J. C., Phillips, W. S., Kodama, N., Clark, S. G., Del Negro, C. A., Akerboom, J., Chen, T.-W., Wardill, T. J., Tian, L., Marvin, J. S., Mutlu, S., Paulo, J. A., Brucker, W. J., and Hawrot, E. (2009) Proteomic analysis of an α7 nicotinic acetylcholine receptor interactome. J. Proteome Res. 8, 1849–1858

26. Kabbani, N., Woll, M. P., Levenson, R., Lindstrom, J. M., and Changeux, J.-P. (2007) Intracellular complexes of the β2 subunit of the nicotinic acetylcholine receptor in brain identified by proteomics. Proc. Natl. Acad. Sci. U.S.A. 104, 20570–20575

27. Nordman, J. C., Phillips, W. S., Kodama, N., Clark, S. G., Del Negro, C. A., and Kabbani, N. (2014) Axon targeting of the α7 nicotinic receptor in developing hippocampal neurons by Gprin1 regulates growth. J. Neurochem. 129, 649–662

28. Chisari, M., Saini, D. K., Cho, J.-H., Kalyanaraman, V., and Gautam, N. (2009) G protein subunit dissociation and translocation regulate cellular response to receptor stimulation. PLoS ONE 4, e7797

29. Nordman, J. C., Muldoon, P., Clark, S., Damaj, M. I., and Kabbani, N. (2014) The α4 nicotinic receptor promotes CD4+ T-cell proliferation and a helper T-cell immune response. Mol. Pharmacol. 85, 50–61

30. Orr-Urtreger, A., Göldner, F. M., Saeki, M., Lorenzo, I., Goldberg, L., De Blasi, M., Duni, J. A., Patrick, J. W., and Beaudet, A. L. (1997) Mice deficient in the α7 neuronal nicotinic acetylcholine receptor lack α-bungarotoxin binding sites and hippocampal fast nicotinic currents. J. Neurosci. 17, 9165–9171

31. Mahajan, R., Ha, J., Zhang, M., Kawano, T., Kozasa, T., and Logothetis, D. E. (2013) A computational model predicts that Gβγ acts at a cleft between channel subunits to activate GIRK1 channels. Sci. Signal. 6, ra69

32. Xu, J., Zhu, Y., and Heinemann, S. F. (2006) Identification of sequence motifs that target neuronal nicotinic receptors to dendrites and axons. J. Neurosci. 26, 9780–9793

33. Landsell, S. J., Gee, V. J., Harkness, P. C., Doward, A. I., Baker, E. R., Gibb, A. J., and Millar, N. S. (2005) RIC-3 enhances functional expression of multiple nicotinic acetylcholine receptor subtypes in mammalian cells. Mol. Pharmacol. 68, 1431–1438

34. Dau, A., Komal, P., Truong, M., Morris, G., Evans, G., and Nashmi, R. (2013) RIC-3 differentially modulates αβ2 and α7 nicotinic receptor assembly, expression, and nicotine-induced receptor upregulation. BMC Neurosci. 14, 47

35. Castillo, M., Mulet, J., Gutiérrez, L. M., Ortiz, J. A., Castelán, F., Gerber, S., Sala, S., Sala, F., and Criado, M. (2006) Role of the RIC-3 protein in trafficking of serotonin and nicotinic acetylcholine receptors. J. Mol. Neurosci. 30, 153–156

36. Pape, R. L. (2014) Merging old and new perspectives on nicotinic acetylcholine receptors. Biochem. Pharmacol. 89, 1–11

37. Bourinet, E., Zamponi, G. W., Stea, A., Soong, T. W., Lewis, B. A., Jones, L. P., Yue, D. T., and Snutch, T. P. (1996) The α1E calcium channel exhibits its permeation properties similar to low-voltage-activated calcium channels. J. Neurosci. 16, 4983–4993

38. Rathouz, M. M., and Berg, D. K. (1994) Synaptic-type acetylcholine receptors raise intracellular calcium levels in neurons by two mechanisms. J. Neurosci. 14, 6935–6945

39. Shen, J. X., and Yakel, J. L. (2009) Nicotinic acetylcholine receptor-mediated calcium signaling in the nervous system. Acta Pharmacol. Sin. 30, 673–680

40. Luoma, J. L., Boulware, M. I., and Mermelstein, P. G. (2008) Caveolin proteins and estrogen signaling in the brain. Mol. Cell. Endocrinol. 290, 8–13

41. Bünemann, M., Frank, M., and Loehse, M. J. (2003) G protein activation in intact cells involves subunit rearrangement rather than dissociation. Proc. Natl. Acad. Sci. U.S.A. 100, 16077–16082

42. Robishaw, J. D., and Berlot, C. H. (2004) Translating G protein subunit diversity into functional specificity. Curr. Opin. Cell Biol. 16, 206–209

43. Corringer, P.-J., Poitevin, F., Prevost, M. S., Sauguet, L., Delarue, M., and Changeux, J.-P. (2012) Structure and pharmacology of pantemeric receptor channels: from bacteria to brain. Structure 20, 941–956

44. Le Novère, N., Corringer, P.-J., and Changeux, J.-P. (2002) The diversity of subunit composition in nAChRs: evolutionary origins, physiologic and pharmacologic consequences. J. Neurobiol. 53, 447–456

45. Papke, R. L., Chojnacka, K., and Horenstein, N. A. (2014) The minimal pharmacophore for silent agonism of the α7 nicotinic acetylcholine receptor. J. Pharmacol. Exp. Ther. 350, 665–680

46. Murray, T. A., Liu, Q., Whiteaker, P., Wu, J., and Lukas, R. J. (2009) Nicotinic acetylcholine receptor α7 subunits with a C2 cytoplasmic loop yield fluorescent protein insertion form functional receptors. Acta Pharmacol. Sin. 30, 828–841

47. Guerra-Álvarez, M., Moreno-Ortega, A. J., Navarro, E., Fernández-Morales, J. C., Egea, J., López, M. G., and Cano-Abad, M. F. (2015) Positive allosteric modulation of α7 nicotinic receptors promotes cell death by inducing Ca2+ release from the endoplasmic reticulum. J. Neurochem. 133, 309–319

48. Gafni, J., Munsch, J. A., Lam, T. H., Catlin, M. C., Costa, L. G., Molinski, T. F., and Pessah, I. N. (1997) Xestospongin: potent membrane permeable blockers of the inositol 1,4,5-trisphosphate receptor. Neuron 19, 723–733

49. Fischer, H., Liu, D.-M., Lee, A., Harries, J. C., and Adams, D. J. (2005) Selective modulation of neuronal nicotinic acetylcholine receptor channel subunits by Gαq-protein subunits. J. Neurosci. 25, 3571–3577