A Novel Effect of PDLIM5 In α7 Nicotinic Acetylcholine Receptors Up-Regulation And Surface Expression

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Abstract

α7 neuronal nicotinic acetylcholine receptors (α7nAChRs) are expressed widely in the brain, where they contribute to a variety of behaviors including arousal and cognition, participate in a number of neurodegenerative disorders including Alzheimer's and Parkinson's disease, and is responsible for nicotine addiction. Although recent studies indicate that the PDZ-containing proteins comprising PSD-95 family co-localize with nicotinic acetylcholine receptors and mediate downstream signaling in the neurons, the mechanisms by which α7nAChRs are regulated are still less well understood. Here we show that the regulation of the α7nAChRs is controlled by PDLIM5 in the endogenous PDZ domain proteins family. We find that chronic exposure to 1 μM nicotine up-regulated both α7, β2-contained nAChRs and PDLIM5 in primary cultured hippocampal neurons, and the up-regulation of α7nAChRs and PDLIM5 is increased more on the cell membrane than the cytoplasm. Interestingly, the α7nAChRs and β2nAChRs display distinct patterns of expression, with α7 co-localized more with PDLIM5. Meanwhile, PDLIM5 interacts with native brain α7 but not β2 nAChRs in neurons. Moreover, knocking down of PDLIM5 in heterologous cells abolishes nicotine-induced up-regulation of α7nAChRs. In cultured hippocampal neurons, shRNA against PDLIM5 decreased both surface clustering of α7nAChRs and α7nAChRs mediated currents. Proteomics analysis shows PDLIM5 interacts with α7nAChRs through the PDZ domain and the interaction between PDLIM5 and α7nAChRs can be promoted by nicotine. Collectively, our data suggest a novel cellular role of PDLIM5 in regulating α7nAChRs, which may be relevant to plastic changes in the nervous system.

Introduction

Nicotinic acetylcholine receptors (nAChRs) are widespread throughout the central nervous system. Endogenous signaling through nAChRs contributes to numerous higher order functions, including memory and cognition, participates in a number of neurodegenerative disorders, and is responsible for nicotine addiction[1–4]. The hippocampus is attractive for nicotinic cholinergic analysis because it is an ordered structure in the brain with known functions and expresses relatively high levels of the two main nicotinic receptors, the homopentameric α7-containing receptors (α7nAChRs) and the heteropentameric β2-containing nicotinic acetylcholine receptors (β2nAChRs)[5, 6]. Chronic exposure to nicotine, as in tobacco smoking, upregulates nicotinic acetylcholine receptor surface expression in neurons. This up-regulation has been proposed to play a role in nicotine addiction and withdrawal, and also has pivotal implications in psychiatric pathologies such as depression and schizophrenia[7, 8]. Up-regulation of neuronal nAChRs induced by chronic nicotine exposure has been demonstrated in vitro and animal models[9–11], and also has been shown in the post mortem brain of smokers[12]. Several models have been proposed to explain the different effects of nicotine on nAChRs subtypes[13, 14], but the mechanism and cellular machinery required for nAChRs up-regulation are still not completely understood.

The assembly and trafficking of nicotinic acetylcholine receptor subtypes and their expression on the cell surface are identified as the key steps in nicotine-induced up-regulation[15–17]. Recent studies indicate that the PDZ-containing proteins comprising postsynaptic density-95 (PSD-95) family serve as complex

molecular scaffolds for nAChRs at nicotinic synapses and contribute importantly to nicotinic signaling[18]. In mice, lacking PSD-93 leads synaptic clusters of nAChRs disperse much more rapidly[19]. In hippocampal neurons, α7nAChRs is co-localized with PSD-95, which is associated with α7nAChRs and constrains their mobility[20]. Study on the α7nAChRs clustering on the surface of the interneurons also shows that PICK1 (protein interacting with C kinase-1), a PDZ-containing protein interacts with α7nAChRs by its cytoplasmic loop[21].

PDLIM5, formerly known as Enigma Homolog (ENH), is a PDZ-LIM domain family protein, showing enriched expression in various brain regions. It contains one PDZ domain and three LIM domains[22, 23]. PDLIM5 has been implicated in susceptibility to bipolar disorder, major depression, and schizophrenia[24–26]. The precise mechanisms by which PDLIM5 regulates the functions of the nervous system remain to be determined. It has been shown that PDLIM5 regulates neuronal calcium signaling and co-localizes with neurotransmitter-protruding vesicles[27]. PDLIM5 can bind to spine-associated Rap GTPase-activating protein (SPAR) and cause dendritic spine shrinkage[28]. It has also been found that PDLIM5-PKCε interaction in the growth cone is essential for regulation of the neuronal growth cone morphology[29]. Moreover, PDLIM5 can interact with delta-catenin to form complex, which enhances dendrite branching at the expense of elongation during neuron development[30].

Here, we demonstrated a novel function of PDLIM5 in nicotine-induced upregulation of α7nAChRs. We found that chronic exposure to nicotine up-regulated α7nAChRs and PDLIM5, and the up-regulation was increased more on the cell membrane than the cytoplasm. Meanwhile, PDLIM5 interacted with α7 but not β2 nAChRs in primary cultured neurons. Knocking down of PDLIM5 abolished nicotine-induced up-regulation of the expression of α7nAChRs in SH-SY5Y cells. In primary cultured hippocampal neurons, using shRNA against PDLIM5 decreased both surface clustering of α7nAChRs and α7nAChRs mediated currents. Proteomics analysis showed that PDLIM5 interacted with α7nAChRs through the PDZ domain, and the interaction of PDLIM5 and α7nAChRs could be promoted by nicotine. These findings define a novel effect of PDLIM5 in α7 nicotinic acetylcholine receptors up-regulation and surface expression in neurons, and might be shed light on the PDLIM5 modulating cholinergic signaling in process thought to underlie synaptic plasticity, cognition, and nicotine addiction.

Materials And Methods

Construction of plasmids

The full-length PDLIM5 (NM053326.1) and α7nAChRs (AY574256.1) were amplified from rat brain (250g) tissue by RT-PCR. GST-tagged and polyhistidine (6×His)-tagged recombinant expression vector were generated by inserting the gene fragment into the pGEX vector (Amersham Pharmacia Biotech) and the pET-30 vector (Qiaqen). The full-length and subclone of PDLIM5 were generated as follow: PDZ domain (amino acid residues: 1–84), D1 (amino acid residues: 1-413), and LIM1-3 (amino acid residues: 414–591). Points mutations of PDLM5 within the highly conserved four residue GLGF at PDZ domain was made as MUTANT1 (GLGF → GAGA), MUTANT2 (GLGF → GAAA) and MUTANT3 (GLGF → AAAA). The

gene fragments are also inserted into pCMV-Tag2B (Stratagene) and pcDNA3.1(+) (Thermo Fisher) with flag-tag and HA-tag. The specific small hairpin RNA (shRNA) of PDLIM5 has been validated and used in our previous articles[29]. All constructs were verified by sequencing.

Drugs and antibodies

Nicotine (AJ0052, Express Technology), MLA (505208, Sigma), DHβE (2349, Tocris), Acetylcholine (A6625, Sigma) and PNU120596 (2498, Tocris) were dissolved in DMSO or water. Polyclonal PDLIM5 antibody (388800) was obtained from Thermo Fisher. Monoclonal anti-α7nAChRs antibody (sc-58607) and polyclonal anti-β2nAChRs antibody (sc-58596) were from Santa Cruz. Alexa 555-conjugated α-bungarotoxin (α-Bgtx) (B35451) was from Thermo Fisher. Monoclonal anti-GAPDH (HC301-01) and anti-β-tubulin (HC101-01) were from TRANS. Monoclonal Anti-Na^+/K^+ ATPase antibody (ab58475) was from Abcam. Monoclonal antibody Anti-DYKDDDDK (HT201-01), anti-HA (HT301-01), Anti-His (HT501-01), and anti-GST (HT601-01) were from TRANS. Secondary antibodies with peroxidase-conjugated were obtained from Jackson ImmunoResearch Laboratories. Secondary antibodies with Alexa Fluor 488 and 546 were obtained from Thermo Fisher.

Cell culture and transfection

Hippocampi or cortex were dissected from the brain tissue of postnatal days 0 to 1 (P0-P1) Sprague-Dawley (SD) rat. To dissociate hippocampal neurons, papain (Worthington) was used, and the neurons were plated at a density of 1×10^5cell/cm^2 for immunostaining and 2×10^5cell/cm^2 for electrophysiology onto 12 mm diameter poly-D-lysine (sigma) coated glass coverslips. The cells were grown in Neurobasal™-A Medium (Gibco) supplemented with B-27™ supplement (Gibco) and 1×GlutaMAX™ Supplement (Gibco) at 37°C in a 5% CO_2 humidified incubator (HERA CELL150). Half of the culture medium was replaced every 3 days. The culture neurons were maintained for 1, 3 or 7 days with either nicotine, MLA, DHβE or DMSO (as control). PDLIM5 shRNA or scramble transfections with calcium phosphate was carried out on DIV 6–7 cultured hippocampal neurons and assays were performed on DIV 9–10.

SH-SY5Y human neuroblastoma cells and HEK293T cells were maintained in DMEM (Gibco), 10% fetal bovine serum (Gibco) and 1×Penicillin-Streptomycin (Gibco) at 37℃ in a 5% CO_2 humidified incubator. Lipofectamine 3000 (Thermo Fisher) was used for cell transfection. To generate stable cell lines with PDLIM5 knockdown, SH-SY5Y cells were transfected with PDLIM5 shRNA or a scramble plasmid by Lipofectamine 3000, and selected in Puromycin (5ug/mL, Cayman). Then, cells were maintained with either nicotine (1 μM) or DMSO (as control) being present for about 3 days.

Western blotting

Proteins were separated on SDS-PAGE, transferred to PVDF membranes (Millipore), and then blocked with 5% skimmed milk in TBST buffer (in mM, 20 Tris/HCl, pH 7.5, 150 sodium chloride, 0.1% Tween 20) for 1 h followed by incubation with various primary antibodies at 4°C overnight. Quantitation was obtained

from densitometric measurements of immunoreactive bands and analyzed with the Image J 1.53e and GraphPad Prism 7.00.

Expression of recombinant proteins in vitro

GST fusion proteins or 6×His-tagged proteins were expressed in the BL21 (DE3) pLys S strain of Escherichia coli (Tiangen). Production of GST-fused proteins was induced by the addition of 0.1 mM isopropyl-1-thio-hd-galactopyranoside (IPTG) (Sigma) at 16℃ for 16 hours in LB broth (Invitrogen), and cells were spun down and resuspended in a buffer containing (in mM) 350 NaCl, 30 Tris, 0.2 EDTA, 1 DTT and 10% glycerol, at pH 8.0, along with 1×protease Inhibitor Cocktail (APE×BIO). As for 6×His-tagged proteins, cells were induced by the addition of 0.2 mM IPTG at 16°C for 16 hours in LB broth encompassing 500 mM sorbitol and 10 mM choline, then spun down and resuspended in a pH 8.0 buffer with (mM) 350 NaCl, 30 Tris, 500 Sucrose, 10 choline, 20 imidazole, 1 × β-Mercaptoethanol (Gibco), 10% glycerol, and 1×cocktail of protease inhibitors specially for His-tagged proteins. The cell suspension was treated with 1×lysozyme on ice for about 30 min. After some time of sonication, the cell debris was removed by centrifugation at 10,000 g for 20 min. For the purification, the supernatant was incubated with corresponding agarose beads. The GST-fused proteins using Glutathione-Sepharose beads (GE) or the 6×His-tagged protein using Ni-NTA beads (GE) with gentle rocking at 4℃. The duration of incubation changed for 6 hours to overnight. After incubation, the samples were solved by SDS-PAGE and examined by immunoblotting.

Pulldown and co-immunoprecipitation (CO-IP)

Mole ratio of GST fusion protein and the 6×His-tagged proteins is 1:1. The mixture was incubated vortically at 4℃ for at least 4 hours. The ice-cold binding buffer encompassed (in mM): 150 NaCl and 20 Tris, pH 7.4, along with 0.2% Triton X-100, 5% glycerol and 1×Protease Inhibitor Cocktail. The binding samples were solved by SDS-PAGE and analyzed by immunoblotting. For CO-IP, cultured cells were rinsed with ice cold 1×PBS solution and cracked with IP buffer containing the following (in mM): 20 Tris/HCl, pH 7.6, 100 NaCl, 20 KCl, 1.5 MgCl2, 0.5% NP-40, 0.25% deoxycholate and protease inhibitors. 2μg of specific antibodies or IgG was mixed with 1000μg total protein of lysates and 50μl protein G plus/protein A agarose (Merck Millipore), then incubated with at 4°C overnight. After incubation, the samples were solved by SDS-PAGE and examined by immunoblotting.

Immunostaining assay

Cultured cells grown on coverslips were washed with ice-cold 1×PBS and fixed 4% paraformaldehyde (PFA) in PBS solution, along with 4% sucrose for 15 min at RT. Then, the cells were permeabilized with 0.25% Triton X-100 in 1×PBS and blocked with 5% normal donkey serum in PBS for 1 hour. Neurons were labeled the nAChRs at the surface without Triton X-100. Cells were labeled with primary antibodies in 3% donkey serum in 1×PBS solutions, incubated overnight at 4°C. Then incubated with secondary antibodies at an optimal dilution for 1 h at room temperature. After rinsed, coverslips were mounted with Fluoromount-G (Electron Microscopy Sciences). All images were captured with a 63×objective on a

confocal microscope LSM800 (Carl Zeiss AG) and analyzed with the Image J 1.53e and GraphPad Prism 7.00.

Electrophysiology

Recordings of Ach (acetylcholine)-evoked currents were made 48–72 h after transfection as described before[31]. Coverslips containing primary cultured hippocampal neurons were transferred into a recording chamber containing ACSF containing the following (in mM): 150 NaCl, 5 KCl, 10 HEPES, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 0.0005 tetrodotoxin. For drug application by bath perfusion, the external solution was applied adjacent to the patched cell at a constant flow rate of 4 ml/min at room temperature (22–25°C) using a ValveLink8.2 Pinch Valve Perfusion System (Automate). Cells were visualized using a 40×water-immersion lens (Olympus). For ACh-evoked currents data were acquired using a Multiclamp 700B amplifier (Molecular Devices) with cells clamped at -60 mV. Patch pipettes (resistance 5–10 M) were filled with internal recording solution containing the following (in mM): 130 K-gluconate, 10 KCl, 0.2 EGTA, 4 Mg-ATP, 0.5 Na-ATP, 10 Na-Phosphocreatine, and 10 HEPES, pH 7.3 (300 mOsm). Agonist concentrations were as follows: 1mM acetylcholine and 10 μM PNU120596. For all data analysis, currents were digitized at 10 kHz and filtered at 2 kHz. ACh-evoked currents were analyzed using pClamp 10.4 (Molecular Devices).

Isothermal Titration Calorimetry (ITC) experiments

ITC experiments were performed on NANO-ITC instrument (TA). Experiment buffer containing the following (in mM): 50 Tris, pH 7.5, 100 NaCl. The PDLIM5-GST (100 μM) was titrated into the α7nAChRs (20 μM) using a 500 μL syringe. Each titration consisted of a preliminary 1.01 μL injection followed by 24 subsequent 2.02 μL additions. The heat for each injection was subtracted from the heat of dilution of the injectant, which was measured by injecting the PDLIM5-GST and α7nAChRs-His solution into the experimental buffer. With or without nicotine treatment as the experimental solution. The thermodynamic parameters were normalized to the blank of GST and α7nAChRs-His for each experiment. Each corrected heat was divided by the molar concentration of PDLIM5-GST injected and was analyzed on the basis of a “one set of sites” model with Launch NanoAnalyze software 3.8.0 supplied by the manufacturer. The experiment temperature was 4°C.

Statistics analysis

All data are representative of at least three independent experiments. Data are presented as mean ± SEM. Unless otherwise indicated, statistical significance was assessed with Student's t-test for unpaired values and with one/two-way analysis of variance (ANOVA) for multiple values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Pearson correlation coefficient was used to indicate the relationship of proteins in double-immunostaining assay.

Results

Chronic nicotine exposure induces up-regulation of α7nAChRs, β2nAChRs, and PDLIM5

To clarify the role of PDLIM5 in nAChRs' upregulation with chronic nicotine treatment, 1μM nicotine, a concentration in the range of peak serum levels found in smokers[32], was chosen in our study. Primary cultured hippocampal neurons were treated with control medium (Con) or with medium containing 1μM nicotine (Nic) for 1, 3, and 7 days respectively, and then quantitated with immunoblotting at DIV10. Nicotine treatment did not affect the amount of total α7nAChRs, β2nAChRs, and PDLIM5 proteins for one day, but increased the amount of these proteins for 3 and 7 days gradually, with 7-day nicotine treatment the most obvious (Fig. 1a). The relative protein levels showed that 7-day exposure to 1μM nicotine significantly increased the expression of total α7nAChRs for about 2.7-fold (Con vs Nic7d, 1.01 ± 0.11 vs 2.79 ± 0.23, $p < 0.001$), β2nAChRs for about 2.9-fold (Con vs Nic7d, 1.08 ± 0.18 vs 2.93 ± 0.30, $p < 0.001$), and PDLIM5 for about 2.5-fold (Con vs Nic7d, 0.89 ± 0.19 vs 2.26 ± 0.31, $p < 0.001$) (Fig. 1b). Immunofluorescence imaging performed at DIV10 also showed that 7-day nicotine exposure increased the puncta number of α7nAChRs, β2nAChRs and fluorescence intensity of PDLIM5. The puncta number of α7nAChRs and β2nAChRs increased about 1.7-fold (Fig. 1c, e). The fluorescence intensity of PDLIM5 was about 1.3-fold increase (Fig. 1d-e). These results indicated that chronic nicotine exposure could induce the up-regulation of α7nAChRs, β2nAChRs, and PDLIM5 in primary cultured hippocampal neurons.

To illustrate whether nicotine-induced up-regulation of PDLIM5 is dependent on AChRs, α7nAChRs and β2nAChRs were inhibited by methyllycaconitine (MLA, 200 nM) and dihydro-β-erythroidine (DhβE, 10 μM) respectively. The antagonists were added with nicotine at DIV3 and treated for 7-day in primary hippocampal neurons. As shown in Fig. 1f-g, nicotine-induced up-regulation of PDLIM5 protein levels could be blocked by MLA (Con vs Nic + MLA, 1.09 ± 0.28 vs 1.48 ± 0.35, $p > 0.05$), but not by DhβE (Con vs Nic + DhβE, 1.09 ± 0.28 vs 2.01 ± 0.17, $p < 0.05$). These results showed that the nicotine-induced up-regulation of PDLIM5 has a similar pattern with α7nAChRs, which implied a close relationship between these two proteins.

PDLIM5 associates with α7nAChRs, but not β2nAChRs in hippocampal neurons

It has been reported that α7nAChRs are presented at both presynaptic and postsynaptic sites[33], similar cellular localization was also found in PDLIM5[34, 35]. To reveal the relationship between these two proteins, we performed immunostaining to detect the subcellular colocalization of PDLIM5 and nAChRs in primary hippocampal neurons (DIV10). As the results shown in Fig. 2a, PDLIM5 colocalized more with α7nAChRs than β2nAChRs. Next, lysates from primary cultured hippocampal neurons were assessed by co-immunoprecipitation with nAChRs or PDLIM5 antibody respectively. The results showed that PDLIM5 interacted with α7nAChRs, but not β2nAChRs (Fig. 2b, left panel). Similar results have been observed in the lysates prepared from the rat hippocampi (Fig. 2b, right panel).

Since α7nAChRs and PDLIM5 are both located on the plasma membrane and in the cytoplasm of a neuron, then we examined the distribution of these two proteins in membrane and cytoplasm fractions in DIV10 cultured neurons. Immunoblot assay showed that nicotine exposure for 7 days caused α7nAChRs at the cell membrane for about 2.4-fold increase (Con vs Nic7d, 1.00 ± 0.13 vs 2.39 ± 0.11, $p < 0.001$) and

in the cytoplasm for about 1.5-fold increase (Con vs Nic7d, 1.02 ± 0.05 vs 1.55 ± 0.02, $p < 0.05$) (Fig. 2c-d). Similarly, the upregulation of PDLIM5 caused by nicotine treatment appeared to be more on the membrane than the cytoplasm (Membrane: Con vs Nic7d, 0.99 ± 0.08 vs 1.99 ± 0.26, $p < 0.001$; Cytoplasm: Con vs Nic7d, 1.04 ± 0.23vs 1.45 ± 0.11, $p < 0.05$) (Fig. 2c-d). Our data suggest that nicotine-induced up-regulation of α7nAChRs and PDLIM5 is mainly located on the membrane.

PDLIM5 is necessary for the up-regulation and surface expression of α7nAChRs

To investigate the role of PDLIM5 in nicotine-induced α7nAChRs up-regulation, SH-SY5Y cells were transfected with a scramble-GFP plasmid, or a GFP-tagged PDLIM5 shRNA plasmid and then treated with 1 μM of nicotine for 3 days. The knockdown effect was validated through western blot analyses, as indicated in Fig. 3a-b (scramble vs PDLIM5 shRNA, 0.95 ± 0.08 vs 0.13 ± 0.01, $p < 0.001$). Compared to the β2nAChRs, chronic nicotine induced up-regulation of α7nAChRs was significantly reduced in PDLIM5 knockdown cells (Fig. 3b, α7nAChRs: Con vs Nic3d, 1.08 ± 0.11 vs 1.09 ± 0.09, $p > 0.05$; β2nAChRs: Con vs Nic3d, 0.92 ± 0.13 vs 2.88 ± 0.32, $p < 0.01$). To confirm the abolished effect of nicotine induced α7nAChRs up-regulation in PDLIM5 knockdown cells, we next tested the distribution of α7nAChRs in SH-SY5Y cells. As the data showed in Fig. 3c and 3d, up-regulation of α7nAChRs was impaired both in cell membrane and cytoplasm in nicotine treated PDLIM5 knockdown cells (Membrane: Con vs Nic3d, 1.00 ± 0.02 vs 0.94 ± 0.15; Cytoplasm: Con vs Nic3d, 1.03 ± 0.04 vs 1.01 ± 0.19, $p > 0.05$).

Since nicotine-induced up-regulation of α7nAChRs and PDLIM5 was mainly at the membrane, we hypothesized that PDLIM5 might induce surface α7nAChRs changes in hippocampal neurons. To test this hypothesis, knockdown effect against PDLIM5 in primary cultured hippocampal neurons was first evaluated by immunofluorescence. Compared to scramble, relative PDLIM5 protein intensity was significantly reduced in neurons expressing PDLIM5 shRNA (Fig. 4a,c, from 0.95 ± 0.06 to 0.32 ± 0.07, $p < 0.001$). Then, surface α-Bgtx labeling of α7nAChRs in neurons infected with PDLIM5 shRNA was observed (Fig. 4b-c). Knockdown of PDLIM5 significantly reduced the surface α-Bgtx labeling of α7nAChRs puncta number for about 35% and area for about 41% (puncta number, from 3.40 ± 0.27 to 2.20 ± 0.20, $p < 0.01$; area, from 0.49 ± 0.03 to 0.29 ± 0.04,$p < 0.01$) (Fig. 4c). As the expression of α7nAChRs is associated with its functional regulation, we next recorded the whole-cell nicotinic currents medicated by α7nAChRs. As expected, once knockdown the PDLIM5 in primary neurons, the α7nAChRs currents was reduced for about 50% compared to the scramble cells (Fig. 4d). Collectively, these results suggest that PDLIM5 plays an important functional role in α7nAChRs.

PDLIM5 interacts with α7nAChRs through the PDZ domain

As protein-interaction domains, PDZ domains are usually found in multi-domain scaffolding proteins[36]. To test whether PDZ domain in PDLIM5 plays such a role with α7nAChRs interaction, we generated the full-length PDLIM5, the corresponding truncations PDZ domain, LIM1-3 and D1 fragment (Fig. 5a), and verified by GST pulldown assays. PDLIM5-GST, PDZ-GST and D1-GST could be detected in α7nAChRs-His complexes, but not in LIM1-3-GST (Fig. 5b). It appeared that PDLIM5 and α7nAChRs interacted with each other in a fashion independent of the LIM domain. To test the specificity of the PDZ domain for

α7nAChRs binding, the mutants target at the GLGF sequence was generated as MUTANT1 (GLGF → GAGA), MUTANT2 (GLGF → GAAA), and MUTANT3 (GLGF → AAAA) (Fig. 5a), and verified by GST pulldown assays (Fig. 5c). With the increase of mutant amino acids at the GLGF, PDLIM5 mutants found in the α7nAChRs-His complex were gradually decreased. This was also confirmed by the CO-IP assay, in which Flag-tagged α7nAChRs co-transfected with HA-tagged PDLIM5 or MUTANT3 in cultured HEK293T cells was used. HA-PDLIM5 was coimmunoprecipitated with Flag-α7nAChRs, but MUTANT1, MUTANT2, MUTANT3 had a much weaker interaction, with MUTANT3 had the weakest interaction (Fig. 5d). Besides, immunofluorescent staining also showed that the fluoresce intensity of surface α7nAChRs was decreased in the HEK293T cells expressing MUTANT3 (Fig. 5e). Therefore, our results strongly support that the interaction between PDLIM5 and α7nAChRs was mainly mediated by the PDZ domain of PDLIM5.

Nicotine promotes the interaction between PDLIM5 and α7nAChRs

We have shown that nicotine can induce the up-regulation of PDLIM5, it remains unclear how nicotine affects this up-regulation. To determine whether nicotine promotes stronger interaction between α7nAChRs and PDLIM5, binding thermodynamics of the interaction incubated with nicotine was analyzed using isothermal titration calorimetry (ITC). PDLIM5-GST and α7nAChRs-His was used for the ITC experiments. All interactions between PDLIM5-GST and α7nAChRs-His showed exothermic reactions. Binding affinity is typically measured and reported by the equilibrium dissociation constant (Kd), which is used to evaluate and rank order strengths of bimolecular interactions. In comparison with control, the binding affinity of the nicotine group was about 1 order of magnitude higher (Fig. 6 a, b). The Kd (μM) for the control group and nicotine group was 0.54 ± 0.19 vs 1.07 ± 0.52, respectively. This was also confirmed by the CO-IP assay, in which Flag-tagged α7nAChRs co-transfected with HA-tagged PDLIM5 in cultured HEK293T cells was used. The CO-IP assays also showed that nicotine significantly increased the protein level of PDLIM5 in α7nAChRs complexes and vice versa. (Fig. 6c). Collectively, these results demonstrated that nicotine promoted the affinity of a7nAChRs and PDLIM5.

Discussion

PDLIM5 is a cytoskeleton-related protein that acts as a binding protein[22]. In neurons, PDLIM5 has been found that present at both presynaptic and postsynaptic sites[34, 35]. The wide distribution of PDLIM5 provides the possibility of a host of functions as exemplified by regulating cell signaling transduction and involving in neuronal development, mature and differentiation. Here, we described a novel role of PDLIM5, an Enigma family protein, in regulating the surface expression of α7nAChRs (Fig. 7).

While nicotine-induced upregulation of nAChRs is well-established, its role in nicotine addiction and the mechanisms of upregulation are not fully understood. Most studies of the up-regulation of nAChRs are based on the α4β2 subtype, however, very little is known about protein interactions of α7nAChRs. Receptor localization and trafficking in neurons are fundamental mechanisms involved in synaptic plasticity, the pathophysiology of diseases and drug activities[37]. It has been suggested that a key step

in the regulation of surface expression of nAChRs, in particular nicotine-induced up-regulation, is the assembly and trafficking of receptor subunits[17]. We found that chronic exposure to 1 μM nicotine caused up-regulation of both α7nAChRs, β2nAChRs and PDLIM5 in hippocampal neurons. It is tempting to speculate that chronic exposure to nicotine may change the level and/or activity of endogenous PDLIM5, thus permitting more surface α7nAChRs expression.

α7nAChRs were observed in the CA1-CA3 pyramidal cell layer of the hippocampus, and also in cells of the granular layer of the dentate gyrus, where cholinergic fibers converge[38]. Increasing evidence indicates that α7nAChRs are concentrated both pre- and post-synaptic at a variety of glutamatergic and GABAergic synapses[33, 39–41]. α-Bgtx for α7nAChRs immunostaining was used to detect subcellular location of α7nAChRs with PDLIM5 as the nonspecific immunolabeling of α7nAChRs[42, 43]. It has shown that α7nAChRs and β2nAChRs displayed distinct patterns of expression[44]. Here, we found that compared to β2nAChRs, PDLIM5 colocalized more with endogenous α7nAChRs in somatodendritic compartments of primary cultured hippocampal neurons. Furthermore, interaction between PDLIM5 and α7nAChRs, but not with β2nAChRs was observed in the primary cultured hippocampal neurons and hippocampal tissue. These results suggest that PDLIM5 can act as a chaperone at subunit level by interacting or regulating interactions with α7nAChRs. Indeed, it was shown that reducing PDLIM5 in SH-SY5Y cells by shRNA could block nicotine-induced α7nAChRs but not β2nAChRs. The result is not surprising, the effect of chronic nicotine exposure among various nAChRs subtypes differs[15, 17].

It is known that nAChRs can be up-regulated by nicotine, leading to export of receptors from the endoplasmic reticulum and increased levels at the plasma membrane[17, 45–47]. Our finding suggests that chronic exposure to nicotine up-regulated α7nAChRs and PDLIM5, and the up-regulation was increased more on the cell membrane than the cytoplasm. To address the role of PDLIM5 in clustering of α7nAChRs on the surface, PDLIM5 was knocked down in neurons to determine whether PDLIM5 affected native receptor clusters and functions. We found that nicotine-induced up-regulation of α7nAChRs was impaired by knockdown of PDLM5 both in cell membrane and cytoplasm, meanwhile, knockdown of PDLIM5 could reduce α7 nAChRs clustering on the surface as well as α7nAChRs mediated currents in primary cultured hippocampal neurons. These evidence suggest that PDLIM5 knockdown might reduce delivery of newly synthesized α7nAChRs to the plasma membrane or promote receptor internalization, and further functionally affect the α7nAChRs currents. This mechanism may depend on PDLIM5 expressed in neurons that binds to the α7nAChRs and affect its targeting and/or transport processes. In such a manner, α7nAChRs-PDLIM5 complexes may have a defined molecular composition in neurons.

The PDZ domain is a protein interaction motif consisting of 80–100 amino acid residues with a highly conserved four residue GLGF sequence[48]. The PDZ domain are crucial in the formation and stability of protein complexes, establishing as important bridge between extracellular stimuli and intracellular responses[49]. It has been shown that the PDZ domain of PDLIM5 interacts with α-actinin in cardiomyocytes[30]. PDLIM5 also acts as a direct binding partner for Anion exchanger 1 (AE1) via its PDZ domain for membrane targeting[50]. Determining how and which PDZ protein family members specify

nAChRs expression, localization, and activity will be an important part of understanding the nature of nicotinic signaling.

The cytoplasmic aggregates of α7nAChRs may reflect large intracellular receptor pools, which have been suggested to provide a mechanism for the rapid ACh-induced upregulation of the nAChRs[51–53]. Though nAChRs do not have intracellular N- or C-terminals, they do participate in PDZ-scaffolds in a variety of systems mediate downstream signaling in the neurons, especially α7nAChRs[20, 21, 54, 55]. It has also been shown that specific disruption of PDZ-containing scaffolds increases α7nAChRs mobility on chick neurons[56], while PSD-95 constrains α7nAChRs mobility at the neuron surface[20]. PDLIM5 consists of a PDZ domain at the N-terminus and three LIM domains at the C-terminus[23]. PDLIM5 binds to the cytoskeleton and membrane proteins through its PDZ domain and interacts with various signaling molecules, including protein kinases and transcription factors, through its LIM domain. Here, we found that the PDZ domain of PDLIM5 showed certain interaction with α7nAChRs, while the LIM domain did not. Further experiment indicated that the PDZ domain mediated the interaction between PDLIM5 and α7nAChRs, and the interaction could be affected when the conserved sites of PDZ domain in PDLIM5 were replaced. Mutations in the main conserved site of PDZ domain GLGF motif attenuate the interaction of PDLIM5 to α7nAChRs. Furthermore, ITC experiment indicated that the PDLIM5 and α7nAChRs binding affinity was increased by nicotine. Combined with our data, it can be concluded that α7nAChRs-PDLIM5 complexes may have a defined molecular composition in neurons, determining the intracellular targeting and clustering, and this process can be modulated by nicotine. PDZ domains can bind to internal sequences in proteins, it seems that the large cytoplasmic loop of the α7nAChRs subunit is required for the association[20], which needs further investigation.

To summarize, our findings implicate PDLIM5 in a novel pathway defining α7nAChRs up-regulation and surface expression in hippocampal neurons. α7 subunits have a high permeability to Ca^{2+}. A high level of free Ca^{2+} activates protein kinase, which then upregulates gene expression and protein production, leading to alterations in the structures and functions of neurons[57]. Proteins that regulate α7nAChRs may thus be important for normal neurophysiology and could in theory contribute to derangement of brain function under certain conditions, including neuronal survival, nicotine addiction, synaptic plasticity in learning, and neurological diseases.

Declarations

Funding

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Conflict of interest

Authors disclose no conflicts of interests.

Availability of data and material

The data that support the findings of this study are available within the article.

Contributions

Yuan Chen and Jingjing Duan designed the study. Zilin Li performed the experiments, made the analysis and wrote the manuscript. Jingjing Duan processed in electrophysiology. Chenyu Gou and Wenhui Wang processed in cell cultured. Yuan Li and Yu Cui processed in construction of plasmids. All authors gave their consent for publication of this manuscript.

Ethics approval

All primary neuron culture experiments were approved by the Animal Care Committee of School of Medicine of Sun Yat-sen University.

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Figures

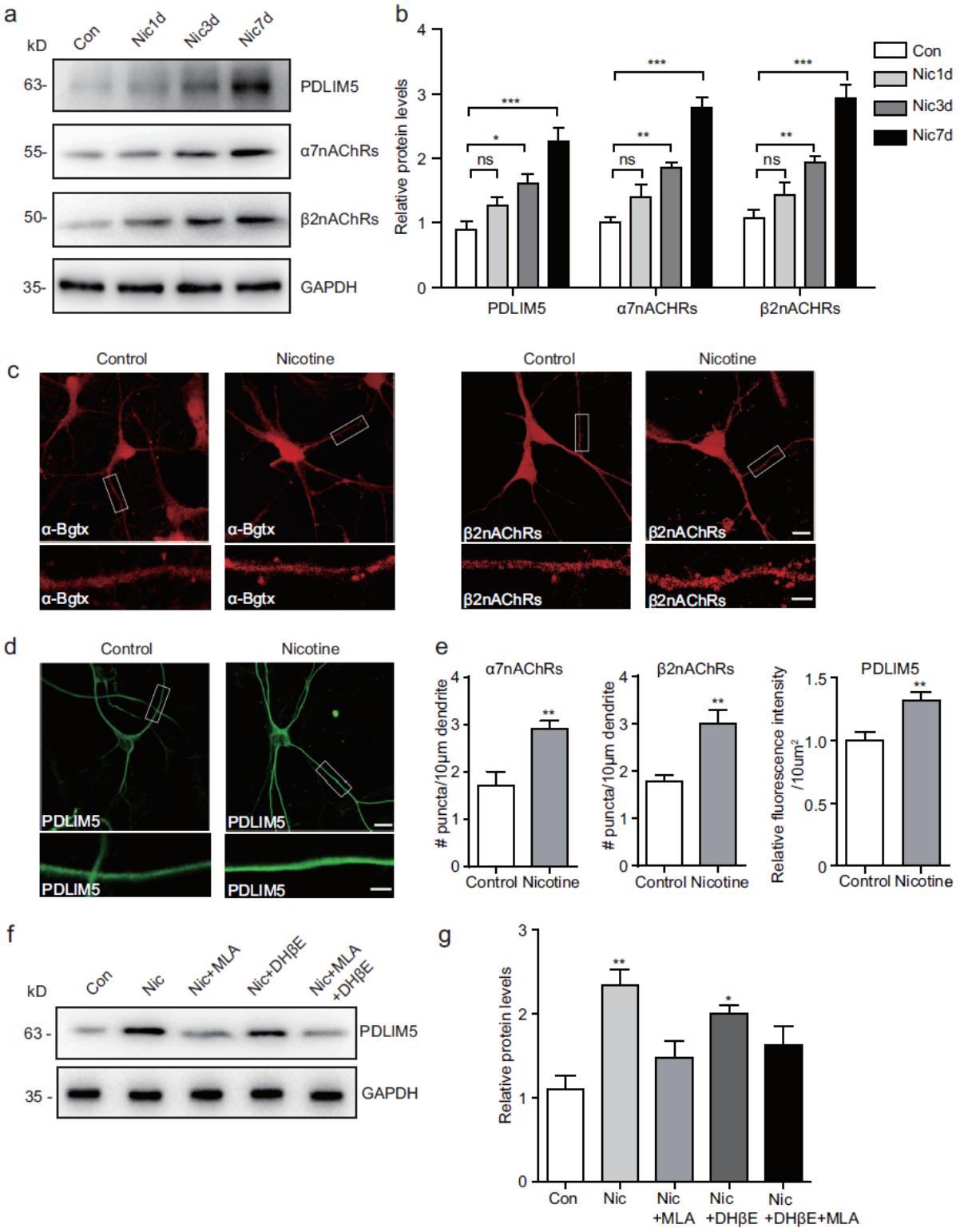


Figure 1

Chronic nicotine induces up-regulation of α7nAChRs, β2nAChRs and PDLIM5. a Primary cultured hippocampal neurons were treated with 1 μM nicotine for 1, 3, or 7 days, respectively. Western blot examined the expression of total α7nAChRs, β2nAChRs and PDLIM5 in DIV10 hippocampal neurons lysates. b Quantitative analysis of total α7nAChRs, β2nAChRs and PDLIM5 (n=3, two-way ANOVA, *p<0.05, **p<0.01,***p<0.001). c Primary cultured hippocampal neurons at DIV3 were treated with 1 μM

nicotine for 7 days. Immunofluorescence examined the expression of α7nAChRs and β2nAChRs (red) (Scale bar, 10 μm (up), 1 μm (down) for each set). d Primary cultured hippocampal neurons at DIV3 were treated with 1 μM nicotine for 7 days. Immunofluorescence examined the expression of PDLIM5 (green) (Scale bar, 10 μm (up), 1 μm (down) for each set). e Quantification of puncta number of α7nAChRs , β2nAChRs, and the intensity of PDLIM5 (Control, n=10; Nicotine, n=10, t-test, **p<0.01). f Representative western blot showed the effects of α7nAChRs or β2nAChRs antagonists MLA or DhβE on the expression of PDLIM5 in neurons. The antagonists were added to the media of primary cultured hippocampal neurons at DIV3 for 7 days in the presence of nicotine. g Quantitative analysis of total PDLIM5 (n=3, one-way ANOVA, *p<0.05, **p<0.01).

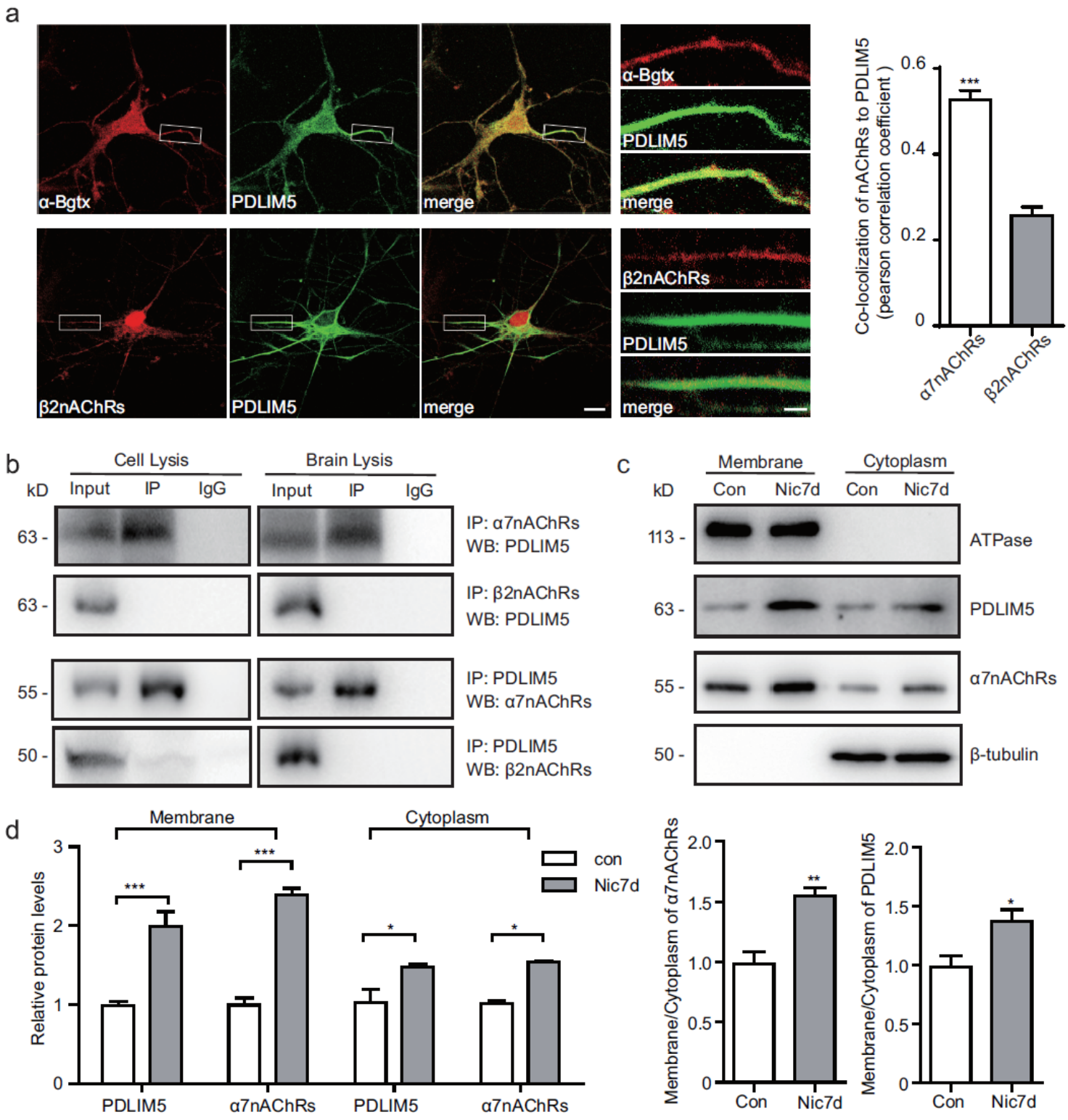


Figure 2

PDLIM5 associates with α7nAChRs, but not β2nAChRs. a Colocalization of α7nAChRs (red), β2nAChRs (red) with PDLIM5 (green) in primary cultured hippocampal neurons (n=9, t-test, ***p<0.001. Scale bar, 10 μm (left), 1 μm (right) for each set). b PDLIM5 interacts with α7nAChRs but not β2nAChRs in rat hippocampal neurons and hippocampi tissue lysates(n=3). c Allocate expression of α7nAChRs and PDLIM5 in nicotine treated primary cultured hippocampal neurons. Na+/K+ ATPase and β-tubulin were

detected as loading controls for membrane and cytoplasm proteins respectively. d Nicotine treatment induced upregulation of α7nAChRs and PDLIM5 in membrane and cytoplasm fractions (n=3, two-way ANOVA, *p<0.05, ***p<0.001; t test, *p<0.05, **p<0.01).

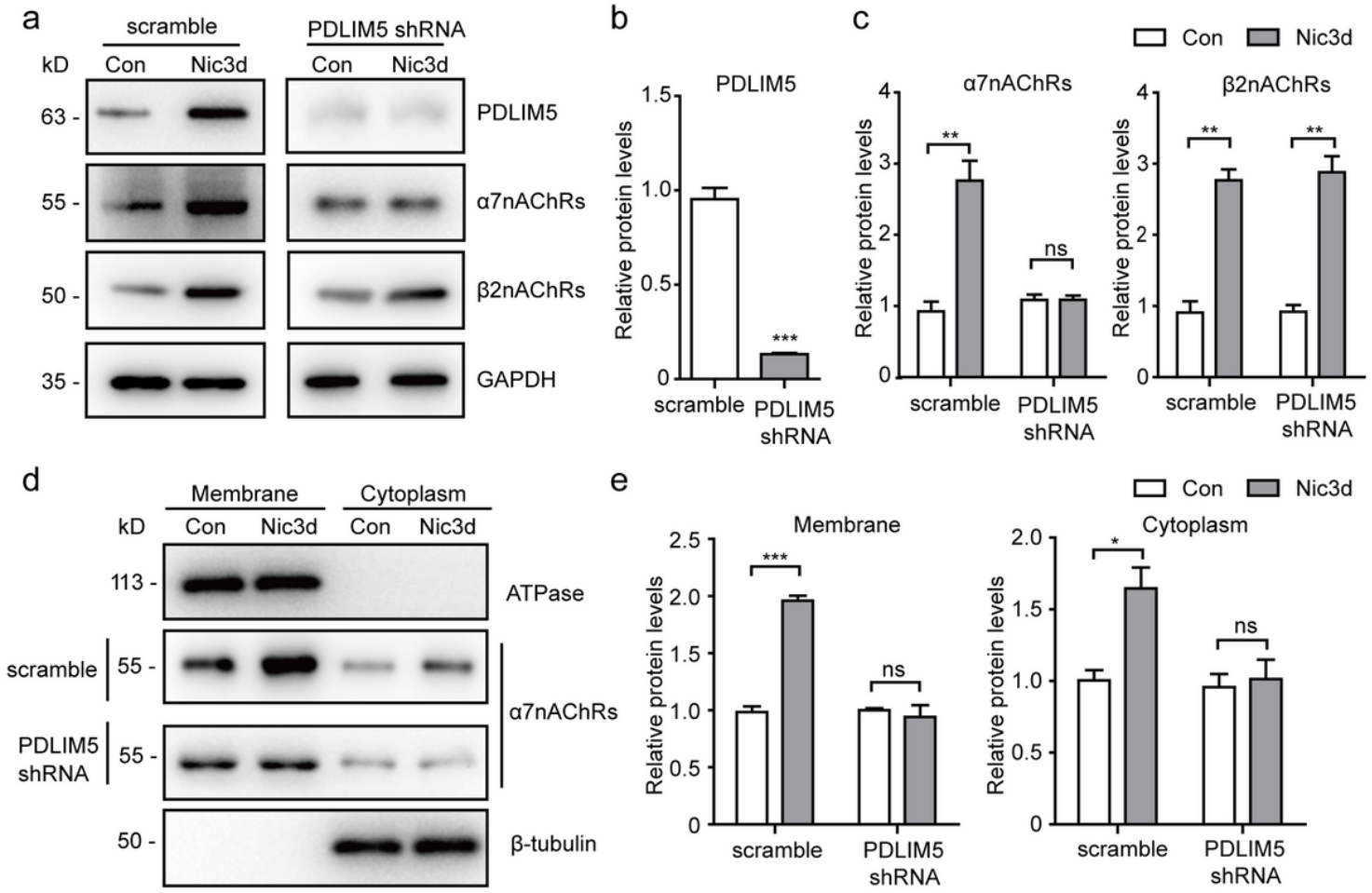


Figure 3

Knockdown of PDLIM5 inhibits nicotine-induced up-regulation and surface expression of α7nAChRs. a Representative western-blot showed the effect on α7nAChRs, β2nAChRs and PDLIM5 within 3-day nicotine on SH-SY5Y cells transfected with scramble or PDLIM5 shRNA. b Quantitative analysis of total α7nAChRs, β2nAChRs and PDLIM5 (n=3, t-test, ***p<0.001; two-way ANOVA, **p<0.01). c Allocate expression of α7nAChRs within 3-day nicotine on SH-SY5Y cells transfected with scramble or PDLIM5 llshRNA. Na+/K+ ATPase and β-tubulin were detected as loading controls for membrane and cytoplasm proteins respectively. d Quantitative analysis of α7nAChRs in membrane and cytoplasm fractions in nicotine treated cells (n=3, two-way ANOVA, *p<0.05, ***p<0.001).

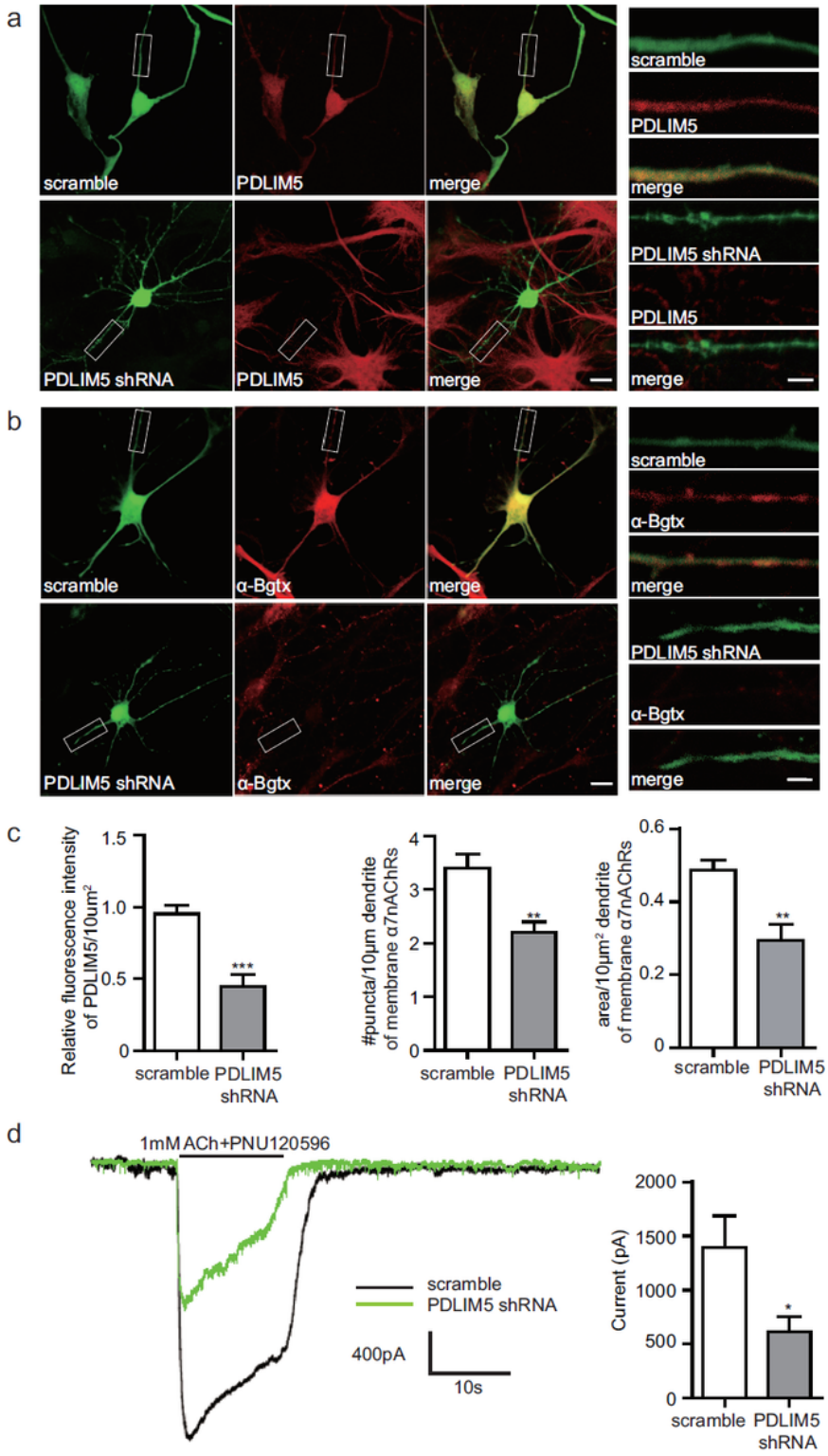


Figure 4

PDLIM5 mediates surface clustering of α7nAChRs and α7nAChRs currents in primary cultured hippocampal neuron. a Representative images showed the reduction of PDLIM5 in neurons expressing PDLIM5 shRNA compared to the scramble vector (Scale bar, 10 μm (left), 1 μm (right) for each set). b Representative images of α7nAChRs in neurons expressing GFP with PDLIM5 shRNA or scramble (Scale bar, 10 μm (left), 1 μm (right) for each set). c Quantitative analysis of PDLIM5 (scramble, PDLIM5 shRNA,

n=10; t-test, ***p<0.001) and α7nAChRs puncta and area (scramble, n=10; PDLIM5 shRNA, t-test, **p<0.01). d Representative α7nAChRs currents from neurons transfected with PDLIM5 shRNA (green trace) or scrambled control (black trace). Quantification of neuronal recordings in current density with or without knockdown of PDLIM5 (scramble, n = 11; PDLIM5 shRNA, n = 14, t-test, *p<0.05. Scale bar, 400 pA and 10s).

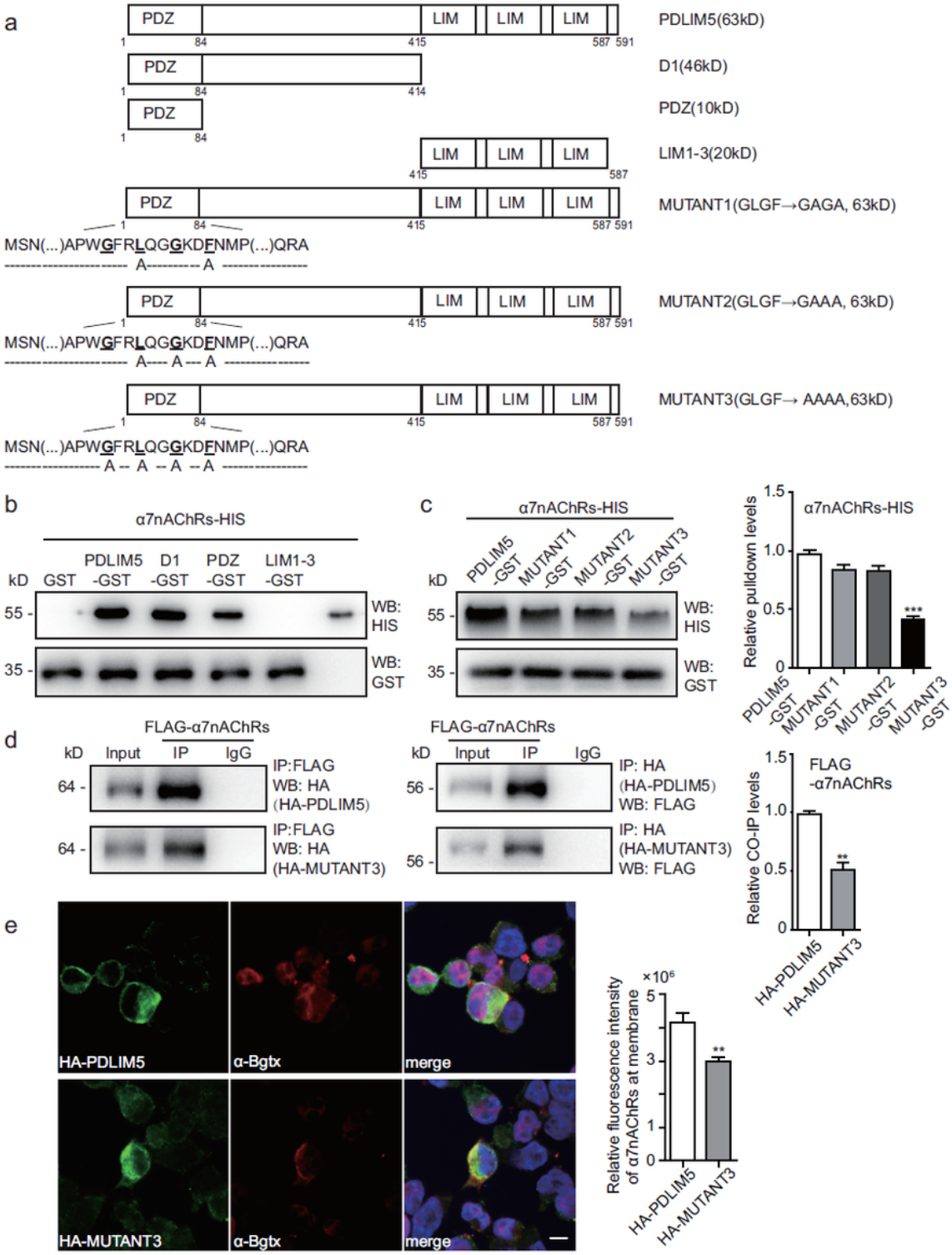
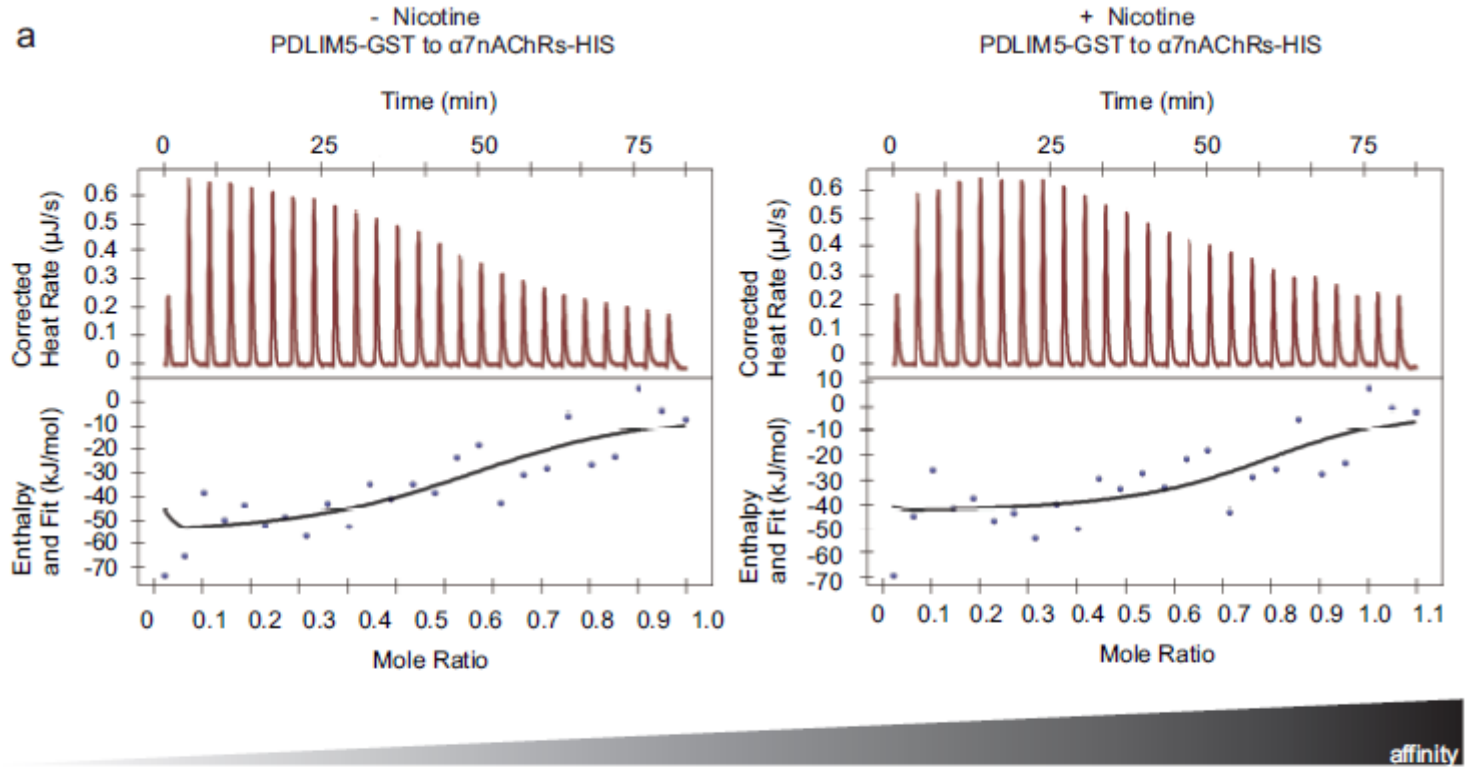


Figure 5

PDLIM5 interacts with α7nAChRs through the PDZ domain. a Schematic diagrams of the full-length PDLIM5, its truncations and mutant fragments. b GST-fusion protein pulldown assays of PDLIM5-GST, D1-GST, PDZ-GST and LIM1-3-GST with α7nAChRs-His. c GST-fusion protein pulldown assays of PDLIM5-GST and three mutants with α7nAChRs-His (t-test, ***p<0.001). d CO-IP analysis of HEK293T cells co-transfected with Flag-tagged α7nAChRs and HA-tagged PDLIM5 or PDLIM5 MUTANT3 (t-test, **p<0.01,). e Representative images showed that mutation of PDLIM5 reduce the membrane expression of α7nAChRs in HEK293T cells (HA-PDLIM5, n=10; HA-MUTANT3, n=10, t-test, **p<0.01. Scale bar, 5 μm).



b

Protein	ITC	- Nicotine	+ Nicotine
PDLIM5-GST +α7nAChRs-HIS	n	0.71	0.81
	Kd(μM)	1.07 ± 0.52	0.54 ± 0.19
	△H	-55.30	-45.21
	△S	-85.21	-43.14

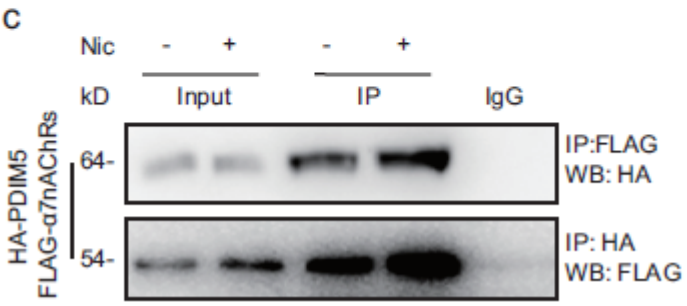


Figure 6

Nicotine promotes the interaction between α7nAChRs and PDLIM5. a Isothermal titration calorimetry (ITC) measurements for the interaction between PDLIM5-GST and α7nAChRs-His with or without 1 μM nicotine. ITC experiments were performed as triplicates. b The parameters of the ITC experiments. c CO-IP analysis of HEK293T cells co-transfected with FLAG-tagged α7nAChRs and HA-tagged PDLIM5, treated with or without nicotine (1 μM) for 3 days (n=3).

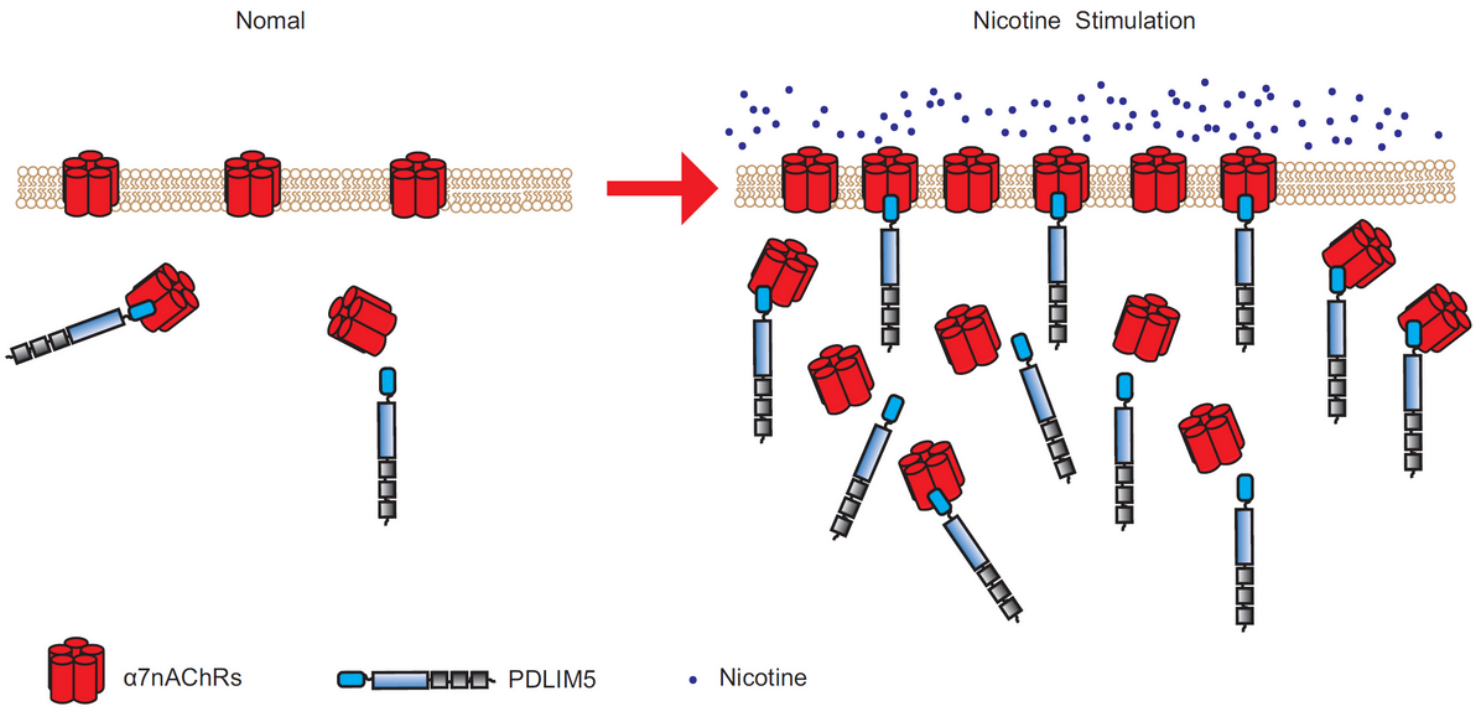


Figure 7

A Model of the nicotine-induced upregulation and surface expression of α7nAChRs at the membrane. In hippocampal neurons, PDLIM5 interacts with α7nAChRs to facilitate α7nAChRs surface and cytoplasm expression, and this process can be accelerated by nicotine.