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Ubiquilin-1 Regulates Nicotine-induced Up-regulation of Neuronal Nicotinic Acetylcholine Receptors*

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Mary Beth Ficklin[‡], Shengli Zhao[§], and Guoping Feng[‡§1]

From the Departments of [§]Neurobiology and [‡]Pathology, Duke University Medical Center, Durham, North Carolina 27710

Chronic exposure to nicotine, as in tobacco smoking, up-regulates nicotinic acetylcholine receptor surface expression in neurons. This up-regulation has been proposed to play a role in nicotine addiction and withdrawal. The regulatory mechanisms behind nicotine-induced up-regulation of surface nicotinic acetylcholine receptors remain to be determined. It has recently been suggested that nicotine stimulation acts through increased assembly and maturation of receptor subunits into functional pentameric receptors. Studies of muscle nicotinic acetylcholine receptors suggest that the availability of unassembled subunits in the endoplasmic reticulum can be regulated by the ubiquitin-proteosome pathway, resulting in altered surface expression. Here, we describe a role for ubiquilin-1, a ubiquitin-like protein with the capacity to interact with both the proteosome and ubiquitin ligases, in regulating nicotine-induced up-regulation of neuronal nicotinic acetylcholine receptors. Ubiquilin-1 interacts with unassembled α3 and α4 subunits when coexpressed in heterologous cells and interacts with endogenous nicotinic acetylcholine receptors in neurons. Coexpression of ubiquilin-1 and neuronal nicotinic acetylcholine receptors in heterologous cells dramatically reduces the expression of the receptors on the cell surface. In cultured superior cervical ganglion neurons, expression of ubiquilin-1 abolishes nicotine-induced up-regulation of nicotinic acetylcholine receptors but has no effect on the basal level of surface receptors. Coimmunostaining shows that the interaction of ubiquilin-1 with the α3 subunit draws the receptor subunit and proteosome into a complex. These data suggest that ubiquilin-1 limits the availability of unassembled nicotinic acetylcholine receptor subunits in neurons by drawing them to the proteosome, thus regulating nicotine-induced up-regulation.

The abundance and activity of ligand-gated ion channels at neuronal membranes are dynamically regulated by extracellular stimuli, and this regulation plays a key role in modulating neuronal excitability and synaptic plasticity (1, 2). For neuronal nicotinic acetylcholine receptors (nAChRs),[2] prolonged exposure to nicotine results in the up-regulation of surface receptors (3–6) as seen in the brains of human smokers (7, 8) and in chronically nicotine-treated animal models (9–12). This up-regulation is thought to play an important role in nicotine dependence, and the symptoms of nicotine withdrawal may be linked to both the recovery of desensitized receptors and the increased number of surface receptors (13, 14).

Neuronal nAChRs consist of subunits encoded by twelve different genes (α2–10 and β2–4), and their assembly in proper combinations into functional pentameric channels is necessary prior to trafficking to the surface membrane (15). In the central nervous system, α4 and β2 subunit-containing nAChRs account for the majority of high affinity nicotine-binding sites (9, 16, 17). In the rodent autonomic nervous system, the α3, α5, α7, β2, and β4 subunits are expressed (18–22). Immunostaining shows that α3, α5, β2, and β4 subunits are localized at mouse superior cervical ganglion (SCG) synapses (23–25). Thus, nAChRs expressed in rodent SCG contain the α3 subunit along with β2, β4, or both and, in some cases, the α5 subunit (22). Consistent with these data, mice lacking the α3 subunit or lacking both the β2 and β4 subunits show marked autonomic dysfunction and die shortly after birth (26, 27). Although several proteins have been shown to be either biochemically associated with neuronal nAChRs (28, 29) or functionally important for the surface expression of nAChRs (30–32), the mechanisms regulating the assembly/trafficking of neuronal nAChRs are still poorly understood.

Previous studies have shown that mRNA levels of nAChR subunits do not change after nicotine treatment (10, 33) and that nicotine-induced up-regulation occurs in the presence of protein synthesis inhibitors (4, 5, 34), suggesting that post-translational mechanisms are involved. Studies using heterologous cells, in which nicotine-induced up-regulation of nAChRs occurs in a fashion similar to that in neurons, have suggested several potential mechanisms underlying this up-regulation. Nicotine has been shown to affect a range of cellular mechanisms such as decreased turnover rates of nAChRs (4), increased assembly (4, 5), and changes in ligand binding affinity (34, 35). Recent studies suggest that the regulation of subunit assembly and maturation play an important role in nicotine-induced up-regulation. Studies using fluorescence resonance energy transfer to measure the assembly of nAChRs showed that nicotine stimulation results in increased assembly of α4β2 nAChRs in somatic compartments (36). In addition, nicotine treatment has been shown to promote the assembly of pentameric channels and maturation through the secretory pathway (37). Consistent with these results, a recent study also showed that interfering with receptor internalization, post-endocytic trafficking, and lysosomal degradation does not affect nicotine-induced up-regulation of surface receptors but that exocytic trafficking is required (6). Studies of muscle nAChRs further suggest that the ubiquitin-proteosome pathway plays a role in regulating surface expression of nAChRs by modulating the availability of an intracellular pool of assembly-competent nAChR subunits for assembly and subsequent trafficking (38). These studies suggest a model for nicotine-induced up-regulation of nAChRs based on the regulation of receptor subunit availability for assembly/maturation and exocytic trafficking.

Currently, regulatory molecules influencing the assembly and subse-

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[1] To whom correspondence should be addressed: Dept. of Neurobiology, Box 3209, Duke University Medical Center, Research Drive, Durham, NC 27710. Tel.: 919-668-1657; Fax: 919-668-1891; E-mail: feng@neuro.duke.edu.

[2] The abbreviations used are: nAChR, nicotinic acetylcholine receptor; SCG, superior cervical ganglion; HA, hemagglutinin; PBS, phosphate-buffered saline; UBA, ubiquitin-associated; UBQ, ubiquitin-like.

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quent trafficking of neuronal nAChRs to the surface membrane remain to be identified. Here we report the identification of ubiquilin-1 as a regulator of nAChR trafficking/assembly. Ubiquilin-1 (also known as Plic-1 (protein linking integrin-associated protein with cytoskeleton 1)) is a ubiquitin-like protein with the capacity to interact with both the proteosome and ubiquitin ligases (39, 40). We found that ubiquilin-1 directly interacts with unassembled α3 and α4 subunits. Immunostaining shows that ubiquilin-1 draws the α3 nAChR subunit to distinct puncta colocalizing with proteosomal subunits. Expression of ubiquilin-1 in an $\alpha3\beta2$ receptor-expressing stable cell line leads to down-regulation of surface receptors. In cultured SCG neurons, ubiquilin-1 prevents the up-regulation of nAChRs induced by nicotine. These data suggest a role for ubiquilin-1 in regulating the assembly/trafficking of neuronal nAChRs.

EXPERIMENTAL PROCEDURES

SCG cDNA Library—mRNA from mouse SCGs were isolated as previously described (41), and yeast two-hybrid cDNA libraries were constructed using the HybriZAP 2.1 vector system (Stratagene, La Jolla, CA). Briefly, double-stranded cDNAs were synthesized with either oligo(dT) primers or random primers. Oligo(dT) and random primed cDNAs were cloned directionally into the EcoRI/XhoI sites of the HybriZAP 2.1 vector. Phagemid libraries with a primary titer of 6×10^6 (oligo(dT)-primed) and 4.8×10^6 (random primed) were obtained. The average insert sizes were 1.9 and 1.4 kb for oligo(dT) and random primed libraries, respectively. The libraries were amplified once and kept at 4 °C.

Yeast Two-hybrid Screening—The large cytoplasmic loop of the α3 nAChR subunit was cloned into the pGBKT7 plasmid (Clontech) and transformed into the Saccharomyces cerevisiae AH109 reporter strain using a modified lithium-acetate protocol (42). The bait-bearing strain was subsequently cotransformed with the random primed SCG cDNA library. Selection for His3 reporter gene activation was performed on selection agar plates without histidine, leucine, and tryptophan, and colonies appearing after 4–5 days at 30 °C were restreaked on plates that also lacked adenine. Library plasmids were recovered from Ade2 reporter gene-positive clones following the Matchmaker 3 protocol (Clontech), and their inserts were sequenced.

Cell Culture and Transfection—Human embryonic kidney (HEK293T) cells were maintained at 37 °C in 5% CO_2 and passaged in Dulbecco's modified Eagle's medium/Ham's F-12 medium (Invitrogen) with 10% fetal bovine serum supplemented with penicillin/streptomycin. COS7 cells were maintained at 37 °C in 5% CO_2 and passaged in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum supplemented with penicillin/streptomycin. For transfection, the cells were plated in 6-well plates without antibiotics and grown until 50–80% confluent. The cells were transfected using FuGENE 6 reagent (Roche Applied Science). 48 h after transfection, the cells were fixed or harvested for further immunohistochemical or biochemical analysis.

Lentiviral Constructs—HA-tagged ubiquilin-1 was cloned into the FUGW vector (43), and this construct was transfected into HEK293T cells along with the Δ8.9 HIV-1 packaging vector and VSVG using Lipofectamine 2000 (Invitrogen). 48 h after transfection, media from the transfected plates were collected, and cell debris was spun down. The culture medium containing viruses was kept at −80 °C until it was thawed for infection.

Antibodies—The rabbit polyclonal antibodies to the α3, α7, and β2 subunits were generated and affinity-purified in the laboratory using fusion proteins from a unique region of the large cytoplasmic loop of each subunit (24). The rat monoclonal antibodies Mab299, Mab270, and Mab210 were obtained from the Developmental Studies Hybridoma Bank (44). The polyclonal antibody to human ubiquilin-2 was a generous gift from Dr. Peter Howley (Harvard Medical School) (39). The mouse monoclonal antibody to the 20 S proteosome subunit α7 (HC8) was obtained from Biomol International (Plymouth Meetings, PA).

Coimmunoprecipitations—For coimmunoprecipitations from heterologous cells, HA-tagged ubiquilin-1 was expressed in HEK293T cells along with the respective nAChR subunits. Transfected cells were harvested in ice-cold lysis buffer containing PBS, Complete protease inhibitors (Roche Applied Science), and 1% Triton. The lysate was incubated at 4 °C with rocking for 30 min and then centrifuged at $14{,}000 \times g$ for 10 min. The supernatant was transferred to a new tube, and immunoprecipitation was performed using subunit-specific antibodies. Western blotting was performed using the HA.11 monoclonal antibody (Covance). For coimmunoprecipitation experiments in cultured SCG neurons, infection with a lentivirus expressing HA-tagged ubiquilin-1 was performed after 2 days in culture. Lentiviral expression was allowed to proceed for 5 days, and neurons were then harvested in ice-cold PBS/Triton lysis buffer. Immunoprecipitation was performed using the anti-α3 rabbit polyclonal antibody, and subsequent Western blotting was performed using HA.11. For coimmunoprecipitation from brain tissue, 1.2 mg of mouse brain was collected in ice-cold PBS with Complete protease inhibitors and homogenized in a Dounce homogenizer (50 strokes). The homogenate was spun at $1000 \times g$ for 10 min at 4 °C. Supernatant was transferred to fresh tubes, incubated with 1% Triton for 1 h at 4 °C, and centrifuged at $14{,}000 \times g$ for 15 min. Aliquots representing 0.2 mg of homogenized tissue were used for each condition. Immunoprecipitation was performed using an antibody against human ubiquilin-2 (39) and subsequent Western blotting was performed with Mab299 recognizing the α4 nAChR subunit. Control immunoprecipitation was performed using rat IgG.

Immunohistochemistry—COS7 cells were transfected with the α3 nAChR subunit, HA-tagged ubiquilin-1, and/or green fluorescent protein. 48 h after transfection, the cells were washed in PBS, fixed for 5 min in 4% paraformaldehyde/PBS, and washed three times in PBS. Coverslips were then blocked with 2% bovine serum albumin, 5% normal goat serum, and 0.2% Triton in PBS for 1 h and incubated with primary antibodies in blocking solution minus Triton overnight. The coverslips were then washed four times for 5 min in PBS and incubated with the appropriate secondary antibodies for 2 h, washed again, and mounted in 90% glycerol with 0.1% p-phenylenediamine for imaging. For experiments in the HEK293 stable line, the cells were transfected with HA-tagged ubiquilin-1 or HA-tagged ubiquilin-1 missing the ubiquitin-associated (UBA) domain. The coverslips were washed and fixed as described above, but blocking occurred in the absence of permeabilization with 0.2% Triton. Surface staining of surface α3 nAChR subunits was performed using Mab210. The cells were then washed four times for 5 min with PBS prior to permeabilization for staining with an anti-HA polyclonal antibody (BD Biosciences) and subsequent secondary antibody immunostaining. For surface staining of cultured SCG neurons, the coverslips were incubated with Mab270 in culture media for 30 min at 37 °C. The cells were then washed three times for 5 min in PBS at 37 °C prior to being fixed for further secondary antibody immunostaining as described above. Immunostaining quantification was performed using data analysis software (NIH Image J) to determine fluorescence intensity/pixel. Student's t test was used to determine statistical significance.

HEK293 Stable Cell Line—We have shown previously that the Mab210 antibody, which recognizes an extracellular epitope of the

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human α3 nAChR subunit (44), does not recognize the α3 nAChR subunit from rodents because of differences in two amino acids in the major immunogenic region (23). To be able to stain surface α3 nAChRs using the Mab210 antibody, we changed these two amino acid residues to match those in the human α3 subunit. cDNAs encoding for the rat α3 (modified) and β2 nAChR subunits were cloned into the pBUD vector, which allows for coexpression of both subunits using a dual promoter system (Invitrogen). To obtain stably transfected lines, the construct was transfected into HEK293 cells using Lipofectamine 2000. After 2 days, the cells were switched to selection medium containing 250 μg/ml of Zeocin. The surviving clones were allowed to grow for 10–14 days prior to picking and expanding. Stable cell lines were subsequently maintained in Zeocin-containing media.

SCG Neuron Culture—SCGs were dissected from deeply anesthetized P2–P4 mouse pups. Ganglia were digested with 1 mg/ml collagenase (Worthington) in L15 medium for 30 min at 37 °C. Ganglia were spun down and collagenase removed and replaced with 1 ml of 0.25% Trypsin-EDTA (Invitrogen) for digestion for 30 min at 37 °C. Trypsin digestion was stopped using 2 ml of culture medium (Dulbecco's modified Eagle's medium/Ham's F-12 medium, 10% fetal bovine serum, 50 ng/ml nerve growth factor (Harlan) supplemented with penicillin/streptomycin). Digested SCGs were triturated up and down ~50 times using a polished pasteur pipette to dissociate the neurons for plating. The desired dilution of neurons was transferred to 60-mm dishes or 6-well plates containing culture medium. The next day fluorodeoxyuridine and uridine (Sigma) were added to concentrations of 20 μM each to the culture medium to limit the growth of dividing cells such as fibroblasts.

Biotinylation Experiments—After 2 days in culture, SCG neurons in 60-mm plates were infected with lentiviral constructs expressing HA-tagged ubiquilin-1. Lentiviral expression was allowed to proceed for 4 days, and neurons were then stimulated for 16 h with 50 μM nicotine diluted in culture media. Neurons were washed three times with progressively colder PBS++ (containing calcium and magnesium) and placed on ice. Each 60-mm plate of neurons was incubated with 14 mg of NHS-SS-biotin (Pierce), a membrane-impermeant biotinylation reagent, dissolved in 4 ml of PBS++ for 40 min on ice. The biotinylation reaction was then quenched with 10 mM glycine in PBS++ followed by two more 5-min washes with ice-cold 10 mM glycine. The neurons were harvested in ice-cold lysis buffer containing PBS, Complete protease inhibitors, 1% Triton, and 0.5% deoxycholate. Biotinylated proteins were pulled down using neutravidin beads (Pierce). Subsequent Western blotting for the α3 subunit and transferrin receptor was performed on biotinylated and total lysate samples. Quantification of protein bands was performed using data analysis software (NIH Image J), and levels of the α3 nAChR subunits were normalized to transferrin receptor levels. Student's *t* test was used to determine statistical significance.

RESULTS

Identification of Ubiquilin-1 as an α3 nAChR Subunit-interacting Protein—To better understand the molecular mechanisms regulating the assembly, trafficking, and targeting of neuronal nAChRs to the surface membrane, we chose the accessible mouse SCG as our model system. All SCG neurons receive cholinergic inputs and express neuronal nAChRs, thus providing a pure population of neurons for biochemical and molecular studies. Studies in chick ciliary ganglia indicate that the large cytoplasmic loop between transmembrane domains III and IV of the α3 subunit is both necessary and sufficient to target neuronal nAChRs to the postsynaptic membrane (45). To identify proteins that potentially regulate the trafficking and targeting of neuronal nAChRs,

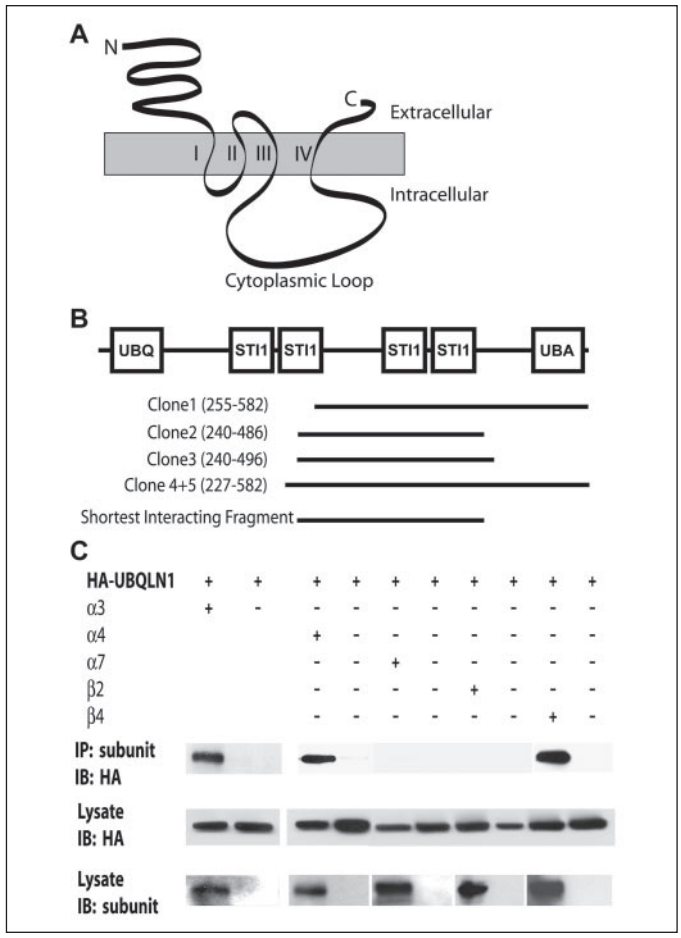

FIGURE 1. **Identification of ubiquilin-1 as a nAChR subunit-interacting protein.** *A*, the large cytoplasmic loop of the α3 nAChR subunit was used as bait for yeast-two hybrid screening of an SCG library. *B*, domain structure of ubiquilin-1 and locations of yeast two-hybrid clones relative to the full-length ubiquilin-1 sequence. Ubiquilin-1 has an amino-terminal UBQ domain and a carboxyl-terminal UBA domain, along with a central region rich in α-helices and four STI-1-like domains. *C*, coimmunoprecipitations of ubiquilin-1 with neuronal nAChR subunits from transfected 293T cells. HA-tagged ubiquilin-1 (*HA-UBQLN1*) was coexpressed with or without various single neuronal nAChR subunits. Immunoprecipitation was performed with subunit specific antibodies, and immunoprecipitated proteins were subjected to Western blotting for tagged ubiquilin-1 using an HA-specific antibody. Ubiquilin-1 was found to interact with the α3, α4, and β4 subunits but not with the α7 and β2 subunits. *IB*, immunoblot; *IP*, immunoprecipitation.

we therefore performed a yeast two-hybrid screen using the large cytoplasmic loop of the α3 subunit as the bait (Fig. 1A). To facilitate the identification of proteins interacting with neuronal nAChRs, we constructed yeast two-hybrid cDNA libraries from mouse SCG. These SCG libraries have a significant advantage over brain libraries for the isolation of proteins interacting with the α3 nAChR subunit because the α3 subunit is not widely expressed in the central nervous system (46–49).

Screening of the mouse SCG library with the large cytoplasmic loop of the α3 nAChR subunit resulted in the isolation of several overlapping clones partially encoding for a protein showing high sequence homology with the human ubiquitin-like protein ubiquilin-1. The clones from the yeast two-hybrid screen were further used to isolate full-length clones by screening a mouse SCG cDNA library. Complete sequencing of these clones revealed that they encode the mouse ortholog of the human ubiquilin-1 gene product, and the full sequence completely matches the mouse ubiquilin-1 gene recently reported in the GenBank™ data base (accession number BC028857). The ubiquilin family of proteins (ubiquilin-1, -2, and -3) has been shown to play a role in the trafficking and degradation of a variety of proteins (39, 50, 51). Struc-

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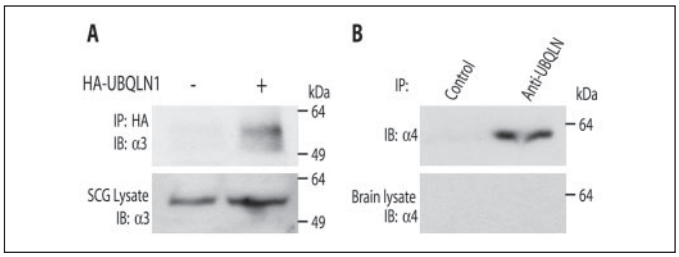

FIGURE 2. **Ubiquilin-1 interacts with endogenous nAChRs.** *A*, cell lysates from cultured SCG neurons in the presence or absence of viral expression of HA-tagged ubiquilin-1 were subjected to immunoprecipitation using an HA-specific antibody and blotted for the α3 nAChR subunit. The α3 subunit was specifically coimmunoprecipitated in the presence of ubiquilin-1 (*HA-UBQLN1*). *B*, proteins from brain lysates were immunoprecipitated using a human ubiquilin-2 antibody that also recognizes mouse ubiquilin-1 and blotted for the α4 nAChR subunit. The α4 subunit was specifically coimmunoprecipitated by the ubiquilin-2 antibody but not by rabbit IgG used as control. The α4 subunit is undetectable in brain lysate without prior concentration through immunoprecipitation. *IB*, immunoblot; *IP*, immunoprecipitation.

turally, ubiquilins contain a ubiquitin-like (UBQ) domain at the amino terminus and a ubiquitin-associated (UBA) domain at the carboxyl terminus (Fig. 1*B*); both domains have been shown to bind the proteosome complex (39, 40). Protein domain analysis using the SMART (Simple Modular Architecture Research Tool) program also predicts that the central region of ubiquilin-1 is rich in α-helices and contains four STI-1-like motifs (Fig. 1*B*). The STI-1 motif was initially found in the stress-inducible phosphoprotein-1 and binds to heat shock chaperones (52, 53). Analysis of our overlapping yeast two-hybrid clones showed the shortest α3 subunit-interacting region to be the central α-helical region of ubiquilin-1 containing two of the STI-1-like motifs (Fig. 1*B*). This suggests that these domains may be involved in the protein-protein interaction with the α3 nAChR subunit, thus leaving the UBA and UBQ domains available for functional roles in regulating α3 nAChR subunit assembly and trafficking.

Ubiquilin-1 Interacts with Endogenous Neuronal nAChR Subunits—To confirm the interaction of ubiquilin-1 with the α3 nAChR subunit in mammalian cells, we coexpressed HA-tagged ubiquilin-1 and the full-length α3 subunit in 293T cells. Following immunoprecipitation with an α3 subunit-specific antibody, HA-tagged ubiquilin-1 was detected by Western blotting using an anti-HA antibody (Fig. 1C, *first* and *second lanes*). To determine whether ubiquilin-1 interacts with other neuronal nAChR subunits, we coexpressed HA-tagged ubiquilin-1 with a panel of nAChR subunits found in the central and peripheral nervous systems. Coimmunoprecipitations using nAChR subunit-specific antibodies and subsequent Western blotting for HA-tagged ubiquilin-1 showed that in addition to the α3 subunit, ubiquilin-1 also interacts with the α4 and β4 subunits, but not with the α7 and β2 subunits (Fig. 1*C*).

To determine whether ubiquilin-1 interacts with endogenous α3 subunit-containing nAChRs, we performed coimmunoprecipitation experiments using cultured SCG neurons. Cultured SCG neurons were infected with a lentivirus expressing HA-tagged ubiquilin-1, and coimmunoprecipitation was performed using an antibody against the HA epitope. Subsequent Western blot analysis for the endogenous α3 nAChR subunit showed that the α3 nAChR subunit was coimmunoprecipitated only in the presence of HA-tagged ubiquilin-1 (Fig. 2*A*).

We next tested whether ubiquilin-1 interacts with endogenous α4 subunit-containing nAChRs in the brain. We used a polyclonal antibody generated against human ubiquilin-2 (39), which we showed to recognize mouse ubiquilin-1 in transfected cell lysates by Western blot (data not shown). Coimmunoprecipitation experiments were performed with mouse brain lysate using this anti-ubiquilin-2 antibody followed by Western blotting for the α4 subunit. As shown in Fig. 2*B*, the α4 subunit was specifically coimmunoprecipitated by the anti-ubiquilin-2 antibody. These data strongly suggest that ubiquilin-1 interacts with endogenous α3 and α4 subunits, which are the major α subunits in the peripheral and central nervous system, respectively.

Ubiquilin-1 Redistributes the α3 nAChR Subunit to the Proteosome—To further examine the interaction of ubiquilin-1 with the α3 nAChR subunit and its functional consequences, we performed immunostaining experiments in heterologous cells. HA-tagged ubiquilin-1 and the α3 nAChR subunit were transfected into COS-7 cells, which allow for good visualization of the cytoplasmic space, its subcellular compartments, and internal protein distribution. When expressed alone, ubiquilin-1 forms distinct intracellular puncta (Fig. 3, A and *D*). In the absence of ubiquilin-1, the α3 subunit is distributed throughout the cytoplasm in a lacy appearance reminiscent of the endoplasmic reticulum (Fig. 3*B*). This is consistent with previous studies showing that single subunits of nAChRs accumulate intracellularly when expressed in heterologous cells (15, 54, 55). When coexpressed in COS7 cells, we found that the α3 nAChR subunit colocalizes with HA-tagged ubiquilin-1 at distinct intracellular puncta as determined by immunostaining for the receptor subunit and HA epitope (Fig. 3*C*). Thus, the interaction of ubiquilin-1 with α3 subunits leads to the redistribution of the α3 subunits. This redistribution of α3 nAChR subunits to the intracellular puncta by ubiquilin-1 is a specific effect as determined by the continued diffuse distribution of green fluorescent protein in the presence of ubiquilin-1 (Fig. 3*A*).

To determine the identity of the intracellular ubiquilin-1 puncta, coimmunostaining of HA-tagged ubiquilin-1 with a variety of intracellular organelle and compartment markers was performed, including markers for the early endosome, endosome, lysosome, Golgi, aggresome, and proteosome. We found that ubiquilin-1 specifically colocalized with the 20 S proteosomal subunit in puncta (Fig. 3*D*). In the absence of ubiquilin-1, both the α3 nAChR subunit and 20 S proteosomal subunit were diffusely distributed (Fig. 3*E*). However, in the presence of ubiquilin-1, the α3 subunit and 20 S proteosomal subunit were drawn together to form distinct puncta (Fig. 3*F*). These data suggest that ubiquilin-1 draws the α3 nAChR subunit and proteosome into a complex.

Both the UBA and UBQ domains in ubiquilin-1 have been previously shown to interact with the proteosome (39, 40). To determine whether the redistribution of the α3 nAChR subunit to the proteosome depends on either domain, we created HA-tagged ubiquilin-1 deletion constructs missing either the UBA or UBQ domain. These constructs were then transfected into COS-7 cells, and their localization with the 20 S proteosomal subunit was assessed by immunostaining. UBQ deletion constructs were found to aggregate in transfected cells and result in toxicity (data not shown), thus preventing further studies. The UBA deletion construct was observed to be diffusely distributed in transfected COS-7 cells and to not colocalize with the 20 S proteosomal subunit (Fig. 3*G*), indicating that the UBA domain is necessary for the colocalization of ubiquilin-1 with the proteosome.

Ubiquilin-1 Decreases the Surface Expression of nAChRs in an α3β2 Receptor-expressing Stable Cell Line—To investigate the functional consequences of the interaction between ubiquilin-1 and the α3 nAChR subunit, we examined the effect of ubiquilin-1 on the surface expression of nAChRs. To circumvent the problems inherent in studying surface expression of nAChRs in transiently transfected heterologous cells, such as variability in expression levels from cell to cell and variability in transfection efficiency, we created a HEK293 stable cell line expressing the α3 and β2 subunits of nAChRs. The use of this stable cell line allowed us to investigate effects on the surface expression of nAChR

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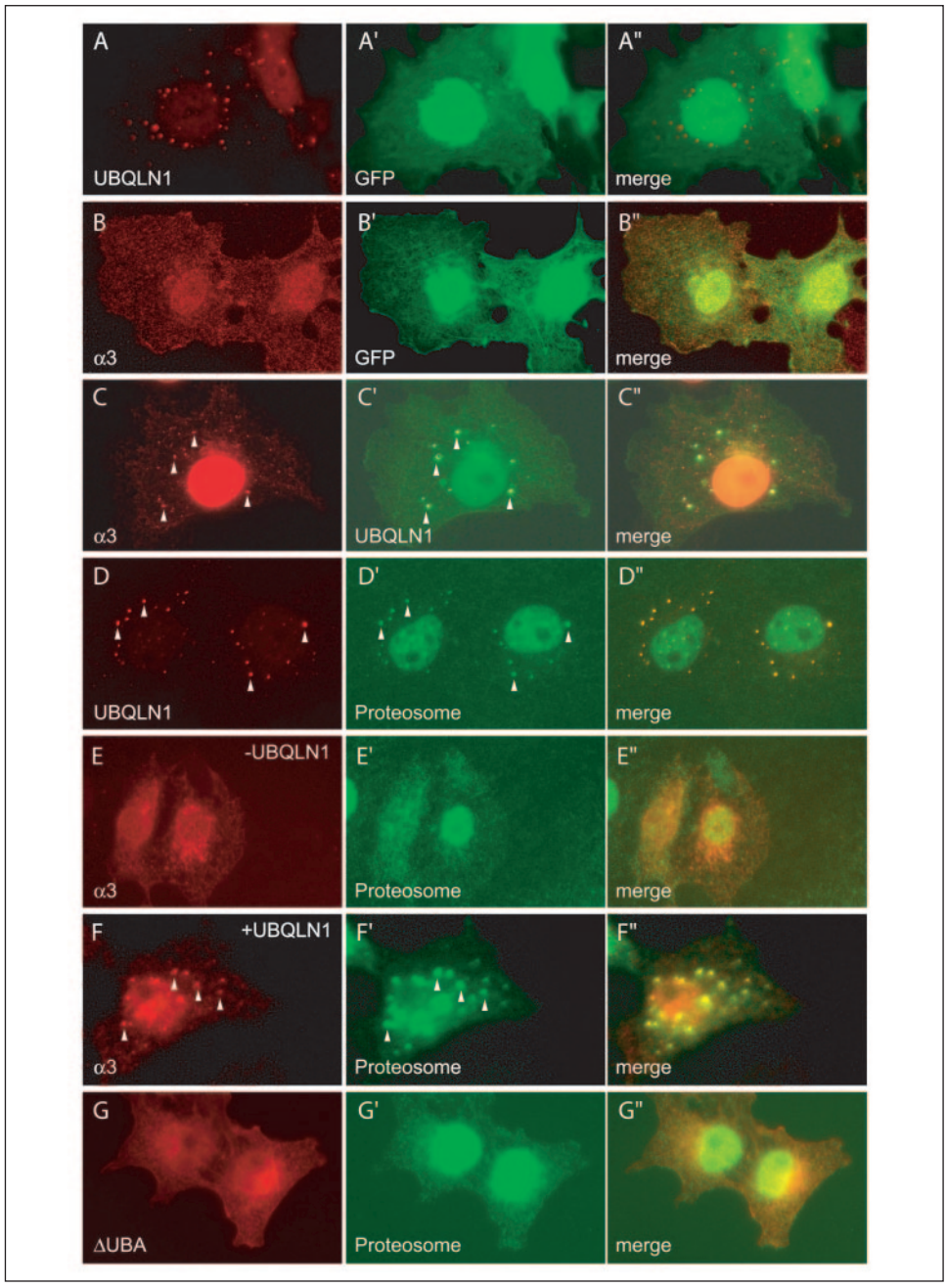

FIGURE 3. **Ubiquilin-1 draws the α3 neuronal nAChR subunit and proteosome into an intracellular complex.** *A*, ubiquilin-1 is localized to distinct intracellular puncta when transfected into COS7 cells and does not affect the distribution of transfected green fluorescent protein. *B* and *C*, the α3 subunit is diffusely distributed when cotransfected with green fluorescent protein. However, when cotransfected with ubiquilin-1, the α3 subunit is redistributed and colocalizes with ubiquilin-1 at the intracellular puncta. *D*, the ubiquilin-1 intracellular puncta colocalize with the proteosome as determined by immunostaining with an antibody against the 20 S proteosomal subunit. *E*, in the absence of ubiquilin-1, the α3 nAChR subunit and proteosome are diffusely distributed. *F*, in the presence of ubiquilin-1, however, the α3 subunit and proteosome are drawn to intracellular puncta and colocalize. *G*, ubiquilin-1 lacking the UBA domain is diffusely distributed and no longer colocalizes with the proteosomal 20 S subunit.

subunits through immunostaining using the Mab210 antibody, which recognizes an extracellular epitope on the α3 nAChR subunit (see "Experimental Procedures").

Surface staining of the α3β2 receptor-expressing stable cell line revealed robust and uniform expression of α3 and β2 subunits on the surface membrane (Fig. 4A and data not shown), indicative of proper assembly and trafficking of the expected heteromeric receptors. Staining for the α3 subunit after permeabilization also showed the existence of a large intracellular pool of the α3 subunit (Fig. 4*B*), consistent with previous observations in both heterologous cells and neurons (6, 36, 37, 56, 57). To investigate whether expression of ubiquilin-1 affects the presence of nAChRs at the surface membrane, we transfected HA-tagged ubiquilin-1 into the α3β2 receptor-expressing stable cell line and immunostained for surface α3 subunits using Mab210. Subsequent permeabilization and staining against the HA epitope was used to identify transfected cells. We found that the presence of ubiquilin-1 dramatically reduced the level of nAChRs at the membrane (Fig. 4*C*, compare transfected and untransfected cells). Together with the intracellular immunostaining results, these data suggest that the interaction of ubiquilin-1 with the α3 subunit sequesters it to the proteosome, thus leading to reduced expression of nAChRs at the cell surface. Consistent with this idea, deletion of the UBA domain from ubiquilin-1, which disrupts its interaction with the proteosome (Fig. 3*G*), abolished the effect of ubiquilin-1 on the surface expression of nAChRs (Fig. 4*D*). Interestingly, expression of ubiquilin-1 lacking the UBA domain resulted in a slight but statistically significant increase of surface nAChRs (Fig. 4*E*).

Ubiquilin-1 Abates Nicotine-induced Up-regulation of Surface nAChRs in SCG Neurons—Next, to test the effect of ubiquilin-1 on surface expression of nAChRs in neurons under basal and nicotine-stimulated conditions, we turned to the cultured mouse SCG neuron system. To date, most studies into the regulatory mechanisms of

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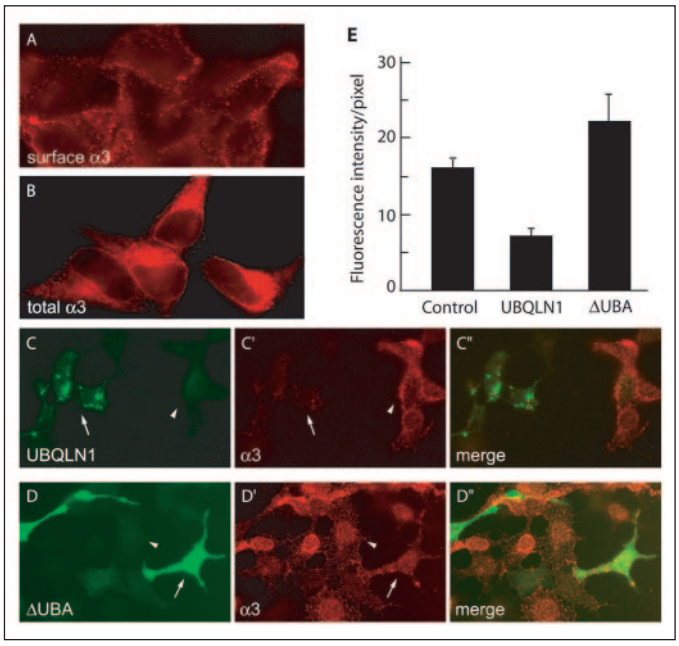

FIGURE 4. **Ubiquilin-1 decreases surface expression of nAChRs in an $\alpha3\beta2$ nAChR-expressing stable cell line.** *A*, surface staining using Mab210, which recognizes an extracellular epitope of the $\alpha3$ subunit, demonstrates proper assembly and trafficking of nAChRs in the stable cell line. *B*, staining of permeabilized cells shows the existence of a large cytoplasmic pool of $\alpha3$ subunits. *C*, the $\alpha3\beta2$ nAChR-expressing stable cell line was transfected with HA-tagged ubiquilin-1 (*UBQLN1*). Surface staining for $\alpha3$ subunits (*C'*) was performed 48 h post-transfection, and the cells were subsequently permeabilized for staining for HA-tagged ubiquilin-1 (*C*). Transfected cells are denoted with arrows, untransfected cells with arrowheads. Expression of ubiquilin-1 results in decreased surface expression of $\alpha3$-containing nAChRs. *D*, expression of ubiquilin-1 missing the UBA domain does not reduce the surface expression of $\alpha3$-containing nAChRs. *E*, quantification of the fluorescence intensity of surface $\alpha3$ nAChR subunit staining shows a significant reduction of surface $\alpha3$ nAChR subunits in the presence of ubiquilin-1 ($p < 0.001$) and a slight increase of surface $\alpha3$ nAChR subunits in the presence of ubiquilin-1 missing the UBA domain ($p < 0.05$).

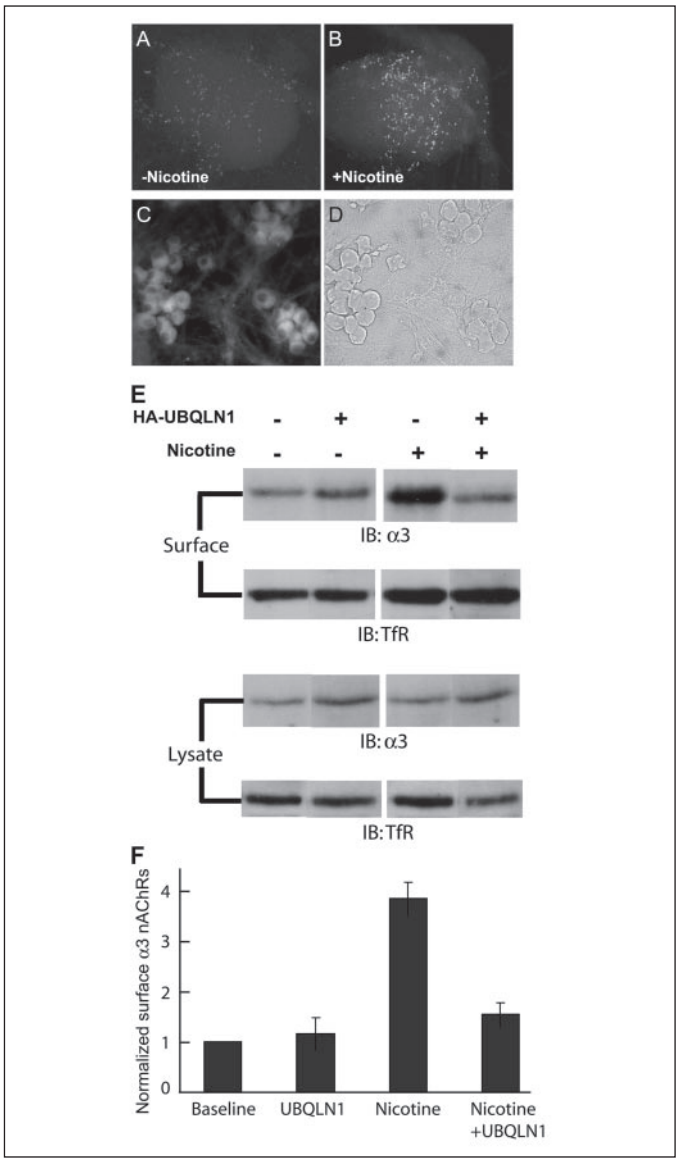

FIGURE 5. **Overexpression of ubiquilin-1 in SCG neurons abates nicotine-induced up-regulation of neuronal nAChRs.** *A* and *B*, nicotine treatment (100 μm) up-regulates surface expression of nAChRs in cultured SCG neurons. Live surface staining for the $\beta2$ subunit using Mab270 was performed on cultured SCG neurons under basal (*A*) or nicotine-treated conditions (*B*). *C* and *D*, infection of cultured SCG neurons with a lentivirus expressing HA-tagged ubiquilin-1 leads to the expression of HA-tagged ubiquilin-1 in the vast majority of neurons as determined by HA staining (*C*). The Image in *D* is a phase contrast view of the same field as in *C*. *E*, surface levels of nAChRs on SCG neurons were determined using biotinylation assays and subsequent Western blotting for the $\alpha3$ nAChR subunit. Overexpression of ubiquilin-1 had no significant effect on surface levels of the $\alpha3$ subunit containing nAChRs under basal conditions. However, expression of ubiquilin-1 greatly reduced nicotine-induced up-regulation of surface $\alpha3$-subunit containing nAChRs in cultured SCG neurons. Transferrin receptor (*TfR*) levels were used as internal controls for normalization. *F*, quantification of the biotinylation experiments ($n = 4$). Nicotine treatment resulted in a 3.5-fold increase of surface $\alpha3$ subunit containing nAChRs in cultured SCG neurons ($p < 0.001$), and this up-regulation was abolished in the presence of ubiquilin-1 ($p < 0.001$). *IB*, immunoblot.

nAChR trafficking have been performed in heterologous cells, and in particular, nicotine-induced up-regulation of $\alpha3$ subunit-containing nAChRs has only been studied in heterologous cells (5, 58, 59). We therefore first confirmed that nicotine stimulation was able to induce surface up-regulation of nAChRs in SCG neurons. Nicotine stimulation was performed by adding nicotine to the media of cultured SCG neurons for 16 h. Maximal up-regulation of nAChRs in heterologous cells has previously been shown to occur between concentrations of 10 and 100 μM nicotine (6). We found that both 10 and 100 μM nicotine were able to increase surface expression of nAChRs as determined by surface immunostaining for the $\beta2$ nAChR subunit using Mab270. Up-regulation by 100 μM nicotine was easily discernible by immunostaining (Fig. 5, *A* and *B*).

Having demonstrated nicotine-induced up-regulation of nAChRs in SCG neurons, we investigated the gain of function effect of ubiquilin-1 on both the basal levels and nicotine-stimulated levels of surface nAChRs in cultured SCG neurons. To reliably compare populations of SCG neurons with or without the overexpression of ubiquilin-1, we generated a lentivirus expressing HA-tagged ubiquilin-1. Infection of cultured neurons by the ubiquilin-1 lentivirus resulted in the expression of ubiquilin-1 in the vast majority of SCG neurons as determined by immunostaining for the HA-tagged ubiquilin-1 (Fig. 5, *C* and *D*).

To provide a quantitative measure of surface receptors and confirm the nicotine-induced up-regulation seen through immunostaining, we performed surface biotinylation assays using a membrane impermeable biotin. Cultured SCG neurons were infected with the HA-tagged ubiquilin-1 lentivirus after 2 days in culture. Five days after infection, the surface biotinylation assay was performed. Subsequent Western blotting of surface proteins for the $\alpha3$ nAChR subunit showed that viral expression of ubiquilin-1 did not significantly affect the basal level of $\alpha3$-containing nAChRs found at the membrane (Fig. 5, *E* and *F*). However, the robust increase in surface $\alpha3$-containing nAChRs resulting from nicotine stimulation in neurons was greatly abated by the overexpression of ubiquilin-1 (Fig. 5, *E* and *F*), suggesting that the interaction of ubiquilin-1 with nAChRs in neurons regulates the surface expression of nAChRs under stimulated conditions.

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DISCUSSION

Recent lines of evidence suggest 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 (6, 36, 37) and may involve the ubiquitin-proteosome pathway (38). In this study, we identified ubiquilin-1 as a nAChR subunit-interacting protein. In heterologous cells, coexpression of ubiquilin-1 with nAChR subunits sequestered α3 subunits to the proteosome and decreased surface expression of assembled receptors, suggesting that the interaction of ubiquilin-1 with nAChR subunits limits their availability for assembly/trafficking to the surface. Consistent with this idea, in cultured SCG neurons ubiquilin-1 abated nicotine-induced up-regulation of surface nAChRs. These data suggest a role for ubiquilin-1 in regulating the assembly/trafficking of surface nAChRs.

In the brain, the majority of high affinity nicotine-binding sites are $\alpha4\beta2$ nAChRs (9, 16, 17), and these receptors are highly up-regulated in response to nicotine treatment (9, 34, 60, 61). Our data show that ubiquilin-1 interacts with the α4 subunit both in heterologous cells and in the brain. Therefore, it is likely that ubiquilin-1 also plays a role in regulating nicotine-induced up-regulation of $\alpha4\beta2$ nAChRs in the brain. Interestingly, high affinity nicotine-binding sites ($\alpha4\beta2$ nAChRs) are found throughout the brain, yet nicotine-induced up-regulation differs regionally (10, 62). It will be interesting to examine the expression pattern of ubiquilin-1 in the brain because it may suggest a mechanism for the region-specific up-regulation of nAChRs by nicotine.

Previously, most studies on nicotine regulation of nAChRs were performed in heterologous expression systems (5, 6, 37, 58–61). In this study we have utilized cultured SCG neurons that express endogenous receptors as a model system. The major nAChR subunits expressed in SCG neurons are α3, α5, β2, and β4. $\alpha3\beta2$ nAChRs have been shown to be up-regulated by nicotine in heterologous cells (5, 58, 59). In this study, we show that similar up-regulation of nAChRs occurs in cultured SCG neurons, thus providing a neuronal culture system for studying the mechanisms of nicotine-induced up-regulation of nAChRs. The ubiquilin family of proteins (ubiquilin-1, -2, and -3) contain a UBQ domain and a UBA domain. Both domains have been shown to interact with the proteosome (39, 40) and may target interacting proteins to the proteosomal machinery (39). Although the *in vivo* function of ubiquilins has not been elucidated, expression of ubiquilins with target proteins in heterologous cells often results in the intracellular accumulation of these proteins (50, 51, 63–65), suggesting that ubiquilins either sequester these proteins for further processing or that degradation of these proteins requires other factors. In either case, target proteins are no longer available for their function. In our study, we found that coexpression of ubiquilin-1 and nAChR subunits resulted in the targeting of receptor subunits to the proteosome. We propose that ubiquilin-1 regulates the availability of intracellular nAChR subunits for assembly and exocytic trafficking through their redistribution to the proteosome. Consistent with this hypothesis, a large proportion of nAChR subunits, both in heterologous cells and neurons, are found intracellularly (6, 36, 37, 56, 57), and regulation of the availability of assembly-competent nAChR subunits in the endoplasmic reticulum by the ubiquitin-proteosome pathway has been shown to affect surface expression of nAChRs (38). In addition, recent studies strongly suggest that nicotine-induced up-regulation of surface receptors occurs through increased assembly/maturation and exocytic trafficking of these existing nAChR subunits (6, 36, 37). Our study suggests that ubiquilin-1 may provide a potential molecular link in this regulatory pathway. If indeed ubiquilin-1 functions as a "gatekeeper" in receptor assembly/trafficking under normal conditions, it is tempting to speculate that chronic exposure to nicotine may change the level and/or activity of endogenous ubiquilin-1, thus permitting more surface receptor expression. Currently, mechanisms regulating the expression and activity of ubiquilin family members are unknown. Further studies in this area may help us to better understand the mechanisms of nicotine-induced up-regulation.

Our data show that overexpression of ubiquilin-1 in SCG neurons does not affect basal levels of nAChR surface expression in SCG neurons but does abolish nicotine-induced up-regulation of surface nAChRs. One possible explanation is that the large intracellular pool of nAChR subunits mainly functions as a reserve supply for response to cellular stimuli. Binding of ubiquilin-1 to these not fully assembled subunits may serve as a regulatory mechanism to monitor the progression of these receptor subunits into further assembly/maturation and subsequent trafficking to the surface. In this case, overexpression of ubiquilin-1 would mainly interfere with the availability of these receptors for assembly/trafficking in response to stimulation.

In addition to the UBQ and UBA domains, ubiquilin-1 also contains four STI-1 motifs. The STI-1 motif was originally identified in stress-inducible phosphoprotein 1 (52). This protein plays a role in the cellular stress response by binding to Hsp90 and serving as a cochaperone in the regulation of protein trafficking and degradation (53, 66). Interestingly, the STI-1 motifs in ubiquilins have also been shown to bind Hsp90 (67). Thus, ubiquilins might be part of the stress-inducible chaperone complex that regulates protein trafficking and degradation in response to noxious stimuli. Both the expression of nAChRs in heterologous cells and chronic exposure of neurons to nicotine may be considered as stress to these cells. Therefore, limiting the surface expression of nAChRs by ubiquilin-1 under these conditions may be viewed as a stress response aimed at reducing potential toxicity. This may explain our observation that ubiquilin-1 only affects nicotine-induced up-regulation, but not basal levels, of surface nAChRs. In this regard, it is interesting to note that ubiquilin-1 has been found, together with other stress response proteins, within neuropathological lesions, such as neurofibrillary tangles and Lewy bodies, in the brains of Parkinson disease and Alzheimer disease patients (63, 64). More recently, genetic variations within the STI-1 motif region of the ubiquilin-1 gene have been associated with an increased risk of familial Alzheimer disease (68). Therefore, understanding the regulatory function of ubiquilin-1 in response to stressors may also shed light on some of the pathological mechanisms of these neurodegenerative diseases.

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Ubiquilin-1 Regulates Nicotine-induced Up-regulation of Neuronal Nicotinic Acetylcholine Receptors

Mary Beth Ficklin, Shengli Zhao and Guoping Feng

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