Autoantibody-induced internalization of nicotinic acetylcholine receptor $\alpha 3$ subunit exogenously expressed in human embryonic kidney cells

Autoantibody-mediated internalization of nicotinic acetylcholine receptor α3

subunit exogenously expressed in human embryonic kidney cells

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Abstract

Autoantibody against nicotinic acetylcholine receptor (nAChR) $\alpha3$ subunit has been implicated in paraneoplastic neurological syndrome.   It has been proposed that this autoantibody impairs synaptic transmission, probably by inducing the internalization of nAChR.   To confirm this hypothesis, we incubated human embryonic kidney cells exogenously expressing $\alpha3$ subunit with seropositive serum, and visualized the intracellular localization of IgG and $\alpha3$ subunits by double-labeled immunofluorescence staining.   As a result, co-localization of internalized IgG and $\alpha3$ subunits was observed as overlapping punctate fluorescent staining.   Our results suggest that autoantibodies against nAChR $\alpha3$ subunit play a pathogenic role in synaptic transmission by inducing nAChR internalization.

Keywords: nicotinic acetylcholine receptor $\alpha3$ subunit, autoantibody, receptor internalization

1. Introduction

Autoantibody against nicotinic acetylcholine receptor (nAChR) α3 subunit has been implicated in paraneoplastic neurological syndrome (Vernino et al., 1998; McKeon et al., 2009). This autoantibody is also known as 'anti-ganglionic nAChR autoantibody' because it was first detected as an autoantibody specific for neuronal nAChR expressed in autonomic ganglia (Vernino et al., 2000). Some clinical and serological studies have revealed the positive correlation between serum levels of this autoantibody and the severity of autonomic symptoms including orthostatic hypotension, gastrointestinal hypomotility and sudomotor dysfunction (Klein et al., 2003; Winston and Vernino, 2010). In addition, recent studies have reported the presence of diverse neurological symptoms unrelated to autonomic nervous systems, such as peripheral neuropathy and psychiatric symptoms, in seropositive patients (McKeon et al., 2009; Gibbons et al., 2012).

Neuronal nAChRs are ligand-gated cation channels that mediate fast synaptic transmission. They are assembled as a homomeric or heteromeric pentamer from α (α2-α10) and β (β2-β4) subunits (Albuquerque et al., 2009). The α3 subunits are

widely distributed in both central and peripheral nervous systems (Flores et al., 1996; Genzen et al., 2001; Skok, 2002; Khan et al., 2003), where they are expressed in various combinations with α5, β2 and β4 subunits (Albuquerque et al., 2009).   The wide distribution of α3 subunits may explain the diversity of neurological symptoms observed in seropositive patients.   On the other hand, autoimmunity to α4 and α7 subunits has been thought to be involved in some neurological disorders, such as encephalopathy and cognitive impairment (Baker et al., 2009; Lykhmus et al., 2011).

There is increasing evidence that autoantibody-mediated dysfunction of nAChR is associated with the neurological symptoms observed in seropositive patients.   Some electrophysiological studies both *in vivo* and *in vitro* have revealed the pathogenic roles of anti-nAChR α3 subunit autoantibody.   Rabbits immunized with recombinant α3 subunits (Lennon et al., 2003) and mice given anti-nAChR α3 subunit autoantibodies by passive transfer (Vernino et al., 2004; Wang et al., 2010) developed autonomic dysfunction and showed the impairment of cholinergic neurotransmission.   It was suggested that cross-linking and internalization of cell-surface nAChR mainly contribute to the impairment of nAChR current (Wang et al., 2007).   However, there is

no evidence for autoantibody-mediated internalization of α3 subunits so far.

Therefore, we examined the internalization of IgG and the α3 subunit using a

seropositive patient's serum by double-labeled immunofluorescence staining.   To

examine the specific contribution of the autoantibody to the α3 subunits, we carried out

experiments on human embryonic kidney (HEK) 293 cells stably co-expressing α3 and

β4 subunits.   The seropositive patient's serum used in this study was confirmed to

include IgG specifically recognizing the α3 subunit.   This study reinforces the idea

that autoantibody against neuronal nAChR contributes to pathogenic processes by

affecting cell-surface nAChR α3 subunits.

2. Materials and methods

2.1. Serum samples

Serum was collected from a patient with paraneoplastic neurological disorder who was seropositive for anti-ganglionic nAChR autoantibody. The titer of the autoantibody was estimated to be 2.20 nmol/L (normal<0.05 nmol/L) by the radioimmunoprecipitation assay described in a previous report (Vernino et al., 2008). Three samples of normal sera were obtained from healthy volunteers who received an annual health checkup at Keiju Medical Center (Nanao, Japan). Informed consent for this study was obtained from all subjects. This study was performed under the approval of the Medical Ethics Committee of Kanazawa University.

2.2. Construction of expression plasmids

cDNA fragments encoding human nAChR α3, α4, α5, α7, β2 and β4 subunits were obtained by reverse transcription-polymerase chain reaction (RT-PCR) as follows. Human brain total RNA (Clontech, Mountain View, CA, USA) was reverse-transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science,

Mannheim, Germany) according to the manufacturer's protocol.   The resulting cDNA

was then subjected to nested PCR using two sets of primers specific for each subunit

(Table 1).   Each reaction mixture (50 μl) contained cDNA (corresponding to 10 ng of

total RNA), KOD FX buffer (Toyobo, Osaka, Japan), 200 μM dNTPs, 300 nM each

primer and KOD FX DNA polymerase (Toyobo).   Amplification was carried out in a

thermal cycler (Applied Biosystems, Foster City, CA, USA) with the following

protocol: initial denaturation at 96°C for 1 min, followed by 30 cycles of 10 sec at 98°C,

30 sec at 65°C and 2 min at 68°C.   The resulting PCR products were purified using

MinElute PCR Purification Kit (QIAGEN, Hilden, Germany).   After addition of

deoxyadenosine to the 3' ends using Ex Taq polymerase (Takara Shuzo, Otsu, Japan),

the cDNA fragments encoding nAChR α3, α4, α5, α7, β2 and β4 subunits were cloned

into a pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) using a TOPO TA

Cloning Kit (Invitrogen) according to the manufacturer's protocol, to yield

pTOPO-CHRNA3, pTOPO-CHRNA4, pTOPO-CHRNA5, pTOPO-CHRNA7,

pTOPO-CHRNB2 and pTOPO-CHRNB4, respectively.   The cDNA inserts were

verified by nucleotide sequencing.   The 1.5-kb *Eco*RI fragments from

pTOPO-CHRNA3 and pTOPO-CHRNB4 were cloned into the *Eco*RI site of pcDNA3.1

(+) (Invitrogen); the resulting plasmids were designated pcDNA-CHRNA3 and

pcDNA-CHRNB4, respectively.   In addition, the 1.5-, 1.9-, 1.5-, 1.5- and 1.5-kb

*Eco*RI fragments from pTOPO-CHRNA3, pTOPO-CHRNA4, pTOPO-CHRNA5,

pTOPO-CHRNB2 and pTOPO-CHRNB4, respectively, and the 1.5-kb *Xho*I/*Bam*HI

fragments from pTOPO-CHRNA7 were ligated to the *Xba*I-cleaved pEF-BOS (a

generous gift from Professor Nagata, Kyoto University, Japan) (Mizushima and Nagata,

1990), after treatment with Klenow fragment of DNA polymerase I; likewise, the

resulting plasmids were designated pEF-CHRNA3, pEF-CHRNA4, pEF-CHRNA5,

pEF-CHRNA7, pEF-CHRNB2 and pEF-CHRNB4, respectively.

2.3. Cell culture and transfection

HEK293 cells were maintained in a humidified incubator with 5% CO_2 at 37°C in

Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries, Osaka,

Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-BRL,

Grand Island, NY, USA), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Wako

Pure Chemical Industries).

For transient transfection, COS-7 cells were seeded at a density of 1.0×10^4 cells/cm^2 on poly-L-ornithine (PLO; Sigma-Aldrich, St. Louis, MO, USA)-coated glass coverslips or 100-mm culture dishes. Twenty-four hours after seeding, cells were transiently transfected with pEF-CHRNA3, pEF-CHRNA4, pEF-CHRNA5, pEF-CHRNA7, pEF-CHRNB2, pEF-CHRNB4 and empty vector (mock) using FuGENE6 Transfection Reagent (Roche Applied Science) according to the manufacturer's protocol. Forty-eight hours after transfection, cells were subjected to autoantibody detection by immunofluorescence staining and immunoblot analysis.

For stable transfection, HEK293 cells were seeded at a density of 1.0×10^4 cells/cm^2 on 100-mm culture dishes. Forty-eight hours after seeding, cells were co-transfected with pcDNA-CHRNA3 and pcDNA-CHRNB4, or transfected with an empty vector (mock) using FuGENE6 Transfection Reagent. Forty-eight hours after transfection, culture medium was replaced with DMEM containing 10% FBS and 800 μg/ml G418 (Roche Applied Science). Cell colonies resistant to G418 were isolated and maintained in DMEM containing 10% FBS and 100 μg/ml G418. Co-expression

of α3 and β4 subunits was confirmed by both immunofluorescence staining and immunoblot analysis.

2.4. Immunofluorescence staining

COS-7 and HEK293 cells were grown on PLO-coated glass coverslips, fixed in ice-cold 4% paraformaldehyde (PFA) for 20 min, washed in PBS, three changes, each 10 min, blocked in PBS containing 1% BSA and 0.3% Triton X-100 for 30 min and sequentially incubated with (1) primary antibodies against each nAChR subunit (Table 2) in the blocking solution for 3 hr at room temperature (RT); (2) washing solution (PBS containing 0.3% Triton X-100), three changes, each 10 min; (3) secondary antibodies (Table 2) in the blocking solution for 1 hr at RT; and (4) washing solution, three changes, each 10 min.   For staining of cell-surface nAChR subunits, Triton X-100 was omitted from all solutions.   In the case of autoantibody detection, COS-7 cells were first incubated with the patient's or normal serum (1:500−10,000) overnight at 4°C, and then with secondary antibody (Table 2) for 1 hr at RT.   The coverslips were mounted on glass slides with Dapi-Fluoromount-G (SouthernBiotech,

Birmingham, AL, USA).   Images were captured by a BX51 fluorescence microscope

(Olympus, Tokyo, Japan) using DP controller (Olympus) and processed using DP

Manager (Olympus).   Fluorescence Mirror Units were U-MNIBA3 (excitation,

470−495 nm; emission, 510−550 nm), U-MWIG3 (excitation, 530−550 nm; emission,

575 nm) and U-MWU2 (excitation, 330−385 nm; emission, 420 nm).

Immunofluorescent intensities were quantitated densitometrically using ImageJ

software (version 1.43; National Institute of Health, Bethesda, MD, USA).   The ratio

of red (Alexa Fluor 594) to green (DyLight 488) fluorescent signals was calculated after

subtracting green fluorescent intensities obtained from non-transfected COS-7 cells.

One hundred and eighty cells were analyzed in twenty-eight random fields obtained

from two independent experiments.

2.5. Immunoblot analysis

COS-7 and HEK293 cells on culture plates were harvested by scraping in PBS

containing 0.1% (w/v) EDTA and pelleted by centrifugation at $1,500 \times g$ for 5 min at

4°C.   The pellets were homogenized in ice-cold homogenization solution containing

0.32 M sucrose, 2 mM EDTA, 2 mM EGTA, 20 mM HEPES and protease inhibitor

cocktail (BioVision Research Products, Milpitas, CA, USA) at pH 7.2 using a Dounce

homogenizer.   Protein concentration of homogenates was determined using DC protein

assay (BIO-RAD, Hercules, CA, USA).   The homogenates were solubilized in

Laemmli sample buffer, subjected to 10% SDS-polyacrylamide gel electrophoresis

(SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Millipore,

Billerica, MA, USA).   Then, the blots were blocked in TBS containing 3% BSA and

0.04% NP-40 for 1 hr and sequentially incubated in (1) primary antibody (Table 2) or

serum (1:100) in antibody-incubation solution (TBS containing 1% BSA and 0.04%

NP-40) overnight at 4°C; (2) washing solution (TBS containing 0.04% NP-40), three

changes, each 15 min; (3) secondary antibodies (Table 2) in antibody-incubation

solution for 1 hr at RT; and (4) washing solution, three changes, each 15 min.   The

blots were developed using AP color development reagent (BIO-RAD).

2.6. Assay for antibody-mediated internalization of nAChR α3 subunit

HEK293 cells stably co-expressing $\alpha 3$ and $\beta 4$ subunits (HEK293-$\alpha 3\beta 4$) and

mock-transfected cells were seeded at a density of 5.0 x 10^4 cells/cm^2 on PLO-coated

glass coverslips in a 24-well culture plate.   On the second day, the cells were washed

in PBS and incubated with heat-inactivated serum (5%) or rat anti-nAChR $\alpha 3$

monoclonal antibody (1 μg/ml; Abcam, Cambridge, UK) in the culture medium for 30

min at 4°C or 37°C.   CELL LAB Rat IgG Isotype Control (Beckman Coulter, Brea,

CA, USA) was used as a negative control for the rat anti-nAChR $\alpha 3$ monoclonal

antibody.   The cells were then subjected to immunofluorescence staining using

secondary antibody for human and rat IgG (Table 2).   For double-labeled

immunofluorescence staining, the cells were stained with antibody against nAChR $\alpha 3$

subunit and Alexa Fluor 594-conjugated secondary antibody (Table 2).   To evaluate

the autoantibody-mediated internalization of nAChR $\alpha 3$ subunits, the number of

overlapping punctate dots per cell was counted under a fluorescence microscope.   We

counted 200 cells in eight random fields obtained from two independent experiments.

2.7. Statistical analysis

Data are shown as mean $\pm$ S.D.　Results were compared using two-tailed *t*-test.

The analyses were performed with the statistical analysis system StatMateIII (ATMS

Co., Ltd., Tokyo, Japan).

3. Results

3.1. Detection of autoantibodies against neuronal nAChR subunit

Using sera from a patient with paraneoplastic neurological syndrome or normal

volunteers and commercial antibodies, we performed double-labeled

immunofluorescence staining on COS-7 cells transiently expressing $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$

and $\beta 4$ subunits (Fig. 1).　Overlapping staining of human IgG and $\alpha 3$ subunit was

observed in the patient's serum, but not in normal serum (Fig. 1A).　Fig. 1B shows the

relationship between serial serum dilution (1:500−10,000) and relative fluorescent

intensities of human IgG.　Reduction of relative immunofluorescent intensities was

observed in the serial dilution of the patient's serum, but not in that of normal serum.

Staining of the patient's IgG was not detected in the cells expressing $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$ and

$\beta 4$ subunits (Fig. 1C).　These results clearly suggested the presence of autoantibodies

against α3 subunit in the patient's serum, but not in the normal sera.

We also performed immunoblot analysis to examine the affinity of the patient's IgG for the α3 subunit (Fig. 2).   We confirmed the expression of α3 subunit in COS-7 cells using commercial antibody.   Two main protein bands with predicted molecular masses of 50 kD and 55 kD were detected in the COS-7 cells transfected with pEF-CHRNA3, but not in the mock-transfected cells (Fig. 2A).   In the patient's serum, a similar blot pattern of recognition was found with anti-human IgG3 antibody, but not with anti-human IgG1, 2 and 4 antibodies (Fig. 2B).

3.2. Stable co-expression of nAChR α3 and β4 subunits in HEK293 cells

To examine the autoantibody-mediated internalization of α3 subunit, we established HEK293 cells stably co-expressing α3 and β4 subunits (HEK293-α3β4). We confirmed the co-expression of both subunits by immunoblot analysis and immunofluorescence staining (Fig. 3).   Fig. 3A shows the result of immunoblot analysis.   The predicted bands of α3 (50 kD) and β4 (55 kD) were detected in HEK293-α3β4 cells, but not in mock-transfected cells.   The band at 42 kD is most

likely a proteolytic form of β4 subunit.   The bands at 38 kD and 70 kD might be

non-specific reactions of anti-nAChR β4 antibody because these bands were also seen

in mock-transfected cells.   To detect the co-expression of α3 and β4 subunits on the

cell surface, we performed immunofluorescence staining under non-permeable

conditions using specific antibodies recognizing the extracellular epitope of α3 or β4

subunit.   Immunoreactivities of α3 and β4 subunits were clearly detected in

HEK293-α3β4 cells, but not in mock-transfected cells (Fig. 3B).

3.3. Autoantibody-mediated internalization of cell-surface nAChR α3 subunits

To assess the autoantibody-mediated internalization of cell-surface α3 subunits,

we incubated HEK293-α3β4 cells with the patient's heat-inactivated serum at 4°C or

37°C.   After 30-min incubation at 4°C, IgG was distributed uniformly over the cells

(Fig. 4A).   In contrast, after 30-min incubation at 37°C, IgG was detected as punctate

dots (Fig. 4B).   When the cells were incubated with rat anti-nAChR α3 monoclonal

antibodies (rat anti-α3) as a positive control, the formation of punctate dots of rat IgG

occurred in a temperature-dependent manner, as seen for the patient's IgG (Fig. 4D and

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E). Only a few punctate dots were seen under non-permeable staining conditions (data not shown), suggesting the internalization and intracellular localization of the patient's and rat IgG. Internalization of IgG was α3 subunit-specific because no punctate dots were seen in mock-transfected cells incubated with both the patient's serum and rat anti-α3 (Fig. 4C and F). Punctate dots were not seen in the HEK293-α3β4 cells incubated with normal serum (Fig. 5A).

Furthermore, we performed double-labeled immunofluorescence staining to examine the co-localization of internalized IgG and the α3 subunit (Fig. 5). The α3 subunit was stained using rabbit anti-nAChR α3 antibody that recognizes different epitopes of rat anti-α3. To test the time-dependent effect of IgG on α3 subunit internalization, we compared the formation of overlapping staining at 5 min and 30 min after incubation. As observed in Fig. 4B and E, internalized patient's and rat IgG were detected as punctate dots. The α3 subunits were also stained as a punctate pattern and overlapped with each IgG. The formation of overlapping punctate dots was observed in a time-dependent manner (Fig. 5A). Fig. 5B shows the quantitative results of overlapping punctate dot formation per cell at the indicated times after incubation.

HEK293-α3β4 cells incubated with the patient's serum or rat anti-α3 for 5 min formed

5.3 ± 2.4 or 5.6 ± 3.2 punctate dots (n = 50), whereas those incubated for 30 min formed

19.0 ± 5.4 or 17.8 ± 5.7 punctate dots (n = 50), respectively (p<0.001).   When the cells

were incubated with normal serum, no punctate dot formation of IgG was observed, and

α3 subunits were diffusely stained over the cell.   These results clearly suggested the

autoantibody-mediated internalization of α3 subunits.

4. Discussion

Autoantibody-mediated internalization of cell-surface antigen is now recognized

as a key mechanism for some immune-mediated disorders, such as myasthenia gravis

(MG) (Drachman et al., 1978), anti-N-methyl-D-aspartate receptor encephalitis (Hughes

et al., 2010) and neuromyelitis optica (Hinson et al., 2012).   In this study, we directly

showed the involvement of autoantibodies in the internalization of nAChR α3 subunit

in vitro.

In the first part of this study, we demonstrated the affinity of the patient's IgG3

for α3 subunit using immunofluorescence staining and immunoblot analysis.   The

commonly used assay for the detection of anti-nAChR $\alpha 3$ subunit autoantibody is radioimmunoprecipitation (RIP) using iodine 125 (^{125}I)-labeled epibatidine, a potent but non-selective agonist for neuronal nAChR, and solubilized neuronal nAChR from human neuroblastoma IMR-32 cells as antigenic sources (Vernino et al., 2000). IMR-32 cells are confirmed to express at least six neuronal nAChR subunits: $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$ and $\beta 4$ (Groot Kormelink and Luyten, 1997). Although autoantibodies detected in this RIP assay have been suggested to be highly specific for $\alpha 3$ subunit, cross-reaction between different nAChR subunits can occur (Balestra et al., 2000; Vernino et al., 2008; Baker et al., 2009). In contrast, the immuofluorescence staining and immunoblot analysis established in this study can determine both the subunit specificity and the IgG subclass of the autoantibodies. Differences in these factors of autoantibody could affect the diversity of clinical symptoms between seropositive patients. Therefore, our assay methods may contribute to our understanding of the pathogenic roles of the autoantibody against neuronal nAChRs.

In contrast to anti-nAChR $\alpha 3$ subunit autoantibody, pathogenic roles of anti-muscle nAChR autoantibodies in MG patients have been well characterized.

Accumulating evidence suggests that the following three effects are major contributing

factors to MG pathogenesis (Keefe et al., 2009).   When bound to muscle nAChR,

autoantibodies induce (1) blocking of the cholinergic neurotransmission at

neuromuscular junction (Drachman et al., 1982), (2) cross-linking and internalization of

the muscle nAChR (Drachman et al., 1978) and (3) complement-dependent cell damage

(Engel et al., 1979).   On the other hand, some electrophysiological studies

demonstrated that the anti-nAChR $\alpha3$ subunit autoantibody impaired the synaptic

transmission (Lennon et al., 2003; Vernino et al., 2004), probably by mechanisms

involving the autoantibody-mediated blocking and/or internalization of functional

nAChR on the cell surface as reported in MG.   In this study, we performed the assay

for autoantibody-mediated internalization of nAChR using a seropositive patient's

serum.   For this assay, we established HEK293 cells stably co-expressing $\alpha3$ and $\beta4$

subunits (HEK293-$\alpha3\beta4$ cells).   In accordance with previous reports that $\alpha3$ subunits

could form functional receptors when expressed in combination with $\beta4$ subunits on the

HEK293 cell surface (Krashia et al., 2010), we successfully co-expressed the $\alpha3$ and $\beta4$

subunits on the surface of HEK293 cells.   When the HEK293-$\alpha3\beta4$ cells were

incubated with the patient's serum, antibody-mediated internalization of $\alpha3$ subunits

was observed within 30 min, as predicted by a previous study (Wang et al., 2007). We

also demonstrated the internalization of $\alpha3$ subunits using rat anti-nAChR $\alpha3$

monoclonal antibody as a positive control. This result suggests that antibodies against

the extracellular domain of the $\alpha3$ subunit can induce internalization of the cell-surface

$\alpha3$ subunit. Internalization of antibody was remarkably inhibited at low temperature,

indicating that autoantibodies could be internalized via endocytosis because endocytic

membrane trafficking is inhibited at low temperature (Arancibia-Carcamo et al., 2006).

On the other hand, it also remains to be seen whether complement-mediated

neuronal cell damage is involved in the progression of neuronal symptoms. Some

recent studies suggested that long-standing exposure to anti-nAChR $\alpha3$ subunit

autoantibodies may cause the loss of neuronal cells and irreversible neuronal cell

damage (Koike et al., 2010; Manganelli et al., 2011). In this study, we detected the

IgG3 subclass autoantibody, which activates complement, in the patient's serum.

Although we have not examined the complement-dependent cell damage, differences in

IgG subclasses of autoantibodies could underlie the variety of clinical symptoms in

seropositive patients.

In conclusion, we obtained evidence that autoantibodies against nAChR α3

subunit induced the internalization of cell-surface nAChR.   Our results support the

hypothesis that the internalization of cell-surface nAChR is involved in the

pathogenesis of the autoantibody against the nAChR α3 subunit.   However, this study

was restricted in that serum was only obtained from one seropositive patient.   Further

accumulation of patients and pathophysiological evaluation should be carried out in

future studies.

Acknowledgement

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Figure Legends

Fig. 1.   Detection of autoantibodies against neuronal nAChR subunits by

double-labeled immunofluorescence staining.   (A) COS-7 cells transiently expressing

α3 subunit were stained with the patient's serum (top) and normal serum (bottom**)**.

IgG, human IgG detected with DyLight 488-conjugated anti-human IgG antibody

(green); α3, commercially available anti-nAChR α3 antibody detected with Alexa Fluor

594-conjugated anti-rabbit IgG antibody (red); Merge, merged images (yellow).

Nuclei were stained with DAPI (blue).   Note that staining of IgG was detectable from

the patient's serum, but not from normal control.   (B) Relationship between serum

dilution and relative fluorescent intensity.   Abscissa, serum dilution; ordinate, relative

intensity.   The fluorescent intensity for human IgG (green) was normalized to that for

nAChR α3 (red)**.**   (C) COS-7 cells transiently expressing nAChR α4, α5, α7, β2 and

β4 subunits were stained with the patient's serum and commercially available

antibodies specific for the indicated subunits as described above for α3.   Merged

images are shown.   Note that no staining of human IgG (green) is detectable.   Scale

bars, 50 μm.

Fig. 2.   Detection of anti-nAChR α3 autoantibodies by immunoblot analysis.   (A)

Detection with rabbit anti-nAChR α3 subunit (Rabbit anti-α3).   Homogenates (1 μg of

protein) prepared from COS-7 cells transiently transfected with pEF-CHRNA3 (α3) and

empty vector (Mock) subjected to immunoblot analysis as described in the Materials

and methods.   Note that two bands of 55 and 50 kD are specifically detected for

pEF-CHRNA3-transfected cells.   (B) Detection with the patient's serum.   Note that

two bands of 55 and 50 kD as in (A) are detectable with anti-human IgG3 antibody, but

not with anti-human IgG1, 2 and 4 antibodies.   The positions of molecular weight

markers (kD) are indicated on the left.

Fig. 3.   Establishment of human embryonic kidney (HEK) 293 cells stably

co-expressing nAChR α3 and β4 subunits.   (A) Immunoblot analysis of homogenates

(10 μg of protein) from HEK293 cells stably co-transfected with pcDNA-CHRNA3 and

pcDNA-CHRNB4 **(**α3β4**)** and those with empty vector (Mock).   The blots were

probed with rabbit anti-nAChR α3 antibody (Anti-α3) or rabbit anti-nAChR β4

antibody (Anti-β4).   The positions of molecular weight markers (kD) are indicated.

(B) Immunofluorescence staining of HEK293-α3β4 cells (α3β4) and mock-transfected

cells (Mock).   Cells were stained with rat anti-nAChR α3 (Anti-α3) and rabbit

anti-nAChR β4 (Anti-β4) under non-permeable staining conditions, and detected with

Alexa Fluor 488-conjugated anti-rat IgG antibody (green) and Alexa Fluor

594-conjugated anti-rabbit IgG antibody (red), respectively.   Nuclei were stained with

DAPI (blue).   Scale bar, 100 μm.

Fig. 4.   Detection of internalized human and rat IgG in HEK293 cells stably

co-expressing nAChR α3 and β4 subunits by immunofluorescence staining.   HEK293

cells stably expressing both α3 and β4 subunits (HEK293-α3β4) and mock-transfected

cells (Mock) were incubated with the patient's serum (A−C) or rat anti-nAChR α3

monoclonal antibody (Rat anti-α3; D−F) for 30 min at 4°C or 37°C.   Cells were then

fixed, permeabilized and stained with DyLight 488-conjugated anti-human IgG

antibody (A−C) or Alexa Fluor 488-conjugated anti-rat IgG antibody (D−F).   Note that

punctate staining of both patient's and rat IgG is detectable in HEK293-α3β4 cells at

37°C, but not at 4°C.   Scale bar, 20 μm.

Fig. 5.   Co-localization of internalized human and rat IgG with nAChR α3 subunit in

HEK293 cells stably co-expressing α3 and β4 subunits (HEK293-α3β4).   (A) Cells

were incubated with the patient's serum, normal control serum or rat anti-nAChR α3

monoclonal antibody (Rat anti-α3) for 5 min and 30 min at 37°C.   Then, the cells were

fixed, permeabilized and stained with rabbit anti-nAChR α3 antibody, followed by

detection with DyLight 488-conjugated anti-human IgG or Alexa Fluor 488-conjugated

anti-rat IgG (green), and Alexa Fluor 488-conjugated anti-rabbit IgG (red).   Nuclei

were stained with DAPI (blue).   Scale bar, 20 μm.   (B) The number of overlapping

punctate dots formed by patient's serum and rat anti-α3 was counted at the indicated

time points.   Data are expressed as mean ± S.D.; *, p<0.001, two-tailed *t*-test.   Note

that overlapping punctate dots were observed in the cells incubated with the patient's

serum and rat anti-α3, and increased in a time-dependent manner.   No overlapping

staining was observed in the cells incubated with normal serum.

Table 1

Primers used for amplification of cDNAs encoding human nAChR subunits

Subunit	Sequence (5′–3′)	
α3	F1: ggtctggggtctgcgctgga	F2: gagaggccgtctctgcgacc
	R1: gaagcagcctcctcctgccc	R2: ggcaggcacacagcttagtgc
α4	F1: cccacaggagaagacgaac	F2: catctagagcccgcgaggtg
	R1: ccaaggccgtcttacagcag	R2: gagtccagggagaagccagc
α5	F1: cacgactcacactcagtgctc	F2: aagagttcgcgttcccccgcg
	R1: ataggtgccatcagatatatgtgtg	R2: acttcagtcccttgggaggc
α7	F1: gcgacagccgagacgtggag	F2: gctgcagctccgggactcaa
	R1: gtggcgtgtaatgctgtcctgg	R2: ggccttgcccatctgtgagt
β2	F1: ggcttcagcaccacggacag	F2: ggtgtaggcgaggcagcgag
	R1: gcccctcttcctggtagctc	R2: tactgtgcagcagagggtggc
β4	F1: aggaccggcgctcactcgac	F2: tgtgaccccacagcggagct
	R1: gggcctcatcagccacaacc	R2: ctctcaccccacaacccagg

F1 and R1 refer to forward and reverse primers for the 1st PCR; F2 and R2 to those for the 2nd PCR.

Table 2. List of antibodies used in this study

Antibody	Species / Clonality	Source (Catalogue No.)	Dilution	Usage
Primary antibodies				
Anti-nAChR α3 subunit	Rabbit / Polyclonal	Santa Cruz (sc-5590)	1:1,000	IF, IB
Anti-nAChR α4 subunit	Goat / Polyclonal	Santa Cruz (sc-1772)	1:1,000	IF
Anti-nAChR α5 subunit	Rabbit / Polyclonal	Abcam (ab26099)	1:1,000	IF
Anti-nAChR α7 subunit	Goat / Polyclonal	Santa Cruz (sc-1447)	1:1,000	IF
Anti-nAChR β2 subunit	Rabbit / Polyclonal	Santa Cruz (sc-11372)	1:1,000	IF
Anti-nAChR β4 subunit	Rabbit / Polyclonal	Millipore (AB15327)	1:2,000	IF, IB
Anti-nAChR α1, α3, α5 subunit	Rat / Monoclonal	Abcam (ab24719)	1:1,000	IF
Secondary antibodies				
Alexa Fluor 594-conjugated anti-rabbit IgG	Donkey	Invitrogen (A21207)	1:2,000	IF
Alexa Fluor 594-conjugated anti-goat IgG	Donkey	Invitrogen (A11058)	1:2,000	IF
Alexa Fluor 594-conjugated anti-rat IgG	Donkey	Invitrogen (A21209)	1:2,000−5,000	IF
Alexa Fluor 488-conjugated anti-rabbit IgG	Donkey	Invitrogen (A21206)	1:2,000	IF
DyLight 488-conjugated anti-human IgG	Donkey	Jackson ImmunoRes.Lab. (709-486-149)	1:2,000	IF
Alkaline-phophatase-conjugated anti-rabbit IgG	Goat	BIO-RAD (170-6518)	1:2,000	IB
Alkaline-phophatase-conjugated anti-human IgG1	Mouse	Merck KGaA (401459)	1:5,000	IB
anti-human IgG2	Mouse	Merck KGaA (401462)	1:5,000	IB
anti-human IgG3	Mouse	Merck KGaA (401465)	1:5,000	IB
anti-human IgG4	Mouse	Merck KGaA (401468)	1:5,000	IB

IF, immunofluorescence staining; IB, immunoblot analysis

Fig. 1.

A

| | IgG | α3 | Merge |

Patient

Normal

B

- Patient
- Normal-1
- Normal-2
- Normal-3

Relative fluorescence intensity (Green / Red)

Dilution

C

α4   α5   α7   β2   β4

Fig. 2.

A
Rabbit anti-α3

(kD)

| | α3 | Mock |

220 —
140 —
80 —
60 —
50 —
40 —
30 —

B
Patient's serum

IgG1   IgG2   IgG3   IgG4

(kD)   α3   Mock   α3   Mock   α3   Mock   α3   Mock

220 —
140 —
80 —
60 —
50 —
40 —
30 —

Fig. 3.

A

B

Fig. 4.

HEK293-α3β4

Mock

4℃

37℃

37℃

Patient's serum

A

B

C

Rat anti-α3

D

E

F

Fig. 5.