Nicotinic acetylcholine receptor α7 subunit maybe a risk factor of seizure susceptibility

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Abstract

Previous studies have confirmed that α7 nicotinic acetylcholine receptors (nAChRs) play a pivotal role in cognition, memory, and neuropsychiatric diseases, but their effect on seizure susceptibility is not fully understood. Here, we show that decreased activity of α7 nAChRs increases the excitability of the CA1 pyramidal neurons and shortens the onset time of epilepsy in pilocarpine-induced mouse model. Compared with control group, however, there was no apparent difference upon increased activity of α7 nAChRs. Moreover, expression of α7 nAChRs was downregulated in human epileptogenic tissue. Taken together, our findings suggest that α7 subunit of nAChRs maybe a risk factor of seizure susceptibility.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are a family of ligand-gated, pentameric ion channels. Understanding the precise role of nAChRs has remained a challenge because these can modulate cholinergic activities in the postsynaptic and presynaptic neurons. In humans, there are 16 different subunits (α1-7, α9-10, β1-4, ε, γ, δ) of nAChRs that are found in both the peripheral and the central nervous system. Of these, 12 are associated with a wide spectrum of physiological and pharmacological functions [1]. The main function of nAChRs is to regulate neuronal plasticity and neuroprotection [7,8]. The most abundant nAChRs units in the central nervous system are α4β2 and α7 subunits. Mutations in the membrane region of the neuronal α4 subunit receptor in the neocortex and thalamus can cause autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) [9], which is a type of epilepsy in the frontal lobe with attacks typically arising during non-rapid eye movement (NREM) sleep [10]. The α7 subunit is expressed widely in the brain with highest levels in the hippocampus and cortex [11], and it has been identified to play a role in both cognitive impairment and neuropsychiatric phenotypes [12–15].

Our previous studies have indicated that activation of α7 nAChRs could decrease the seizure susceptibility in Chat-Mecp2\textsuperscript{−/−} mice [16], but the role of α7 nAChRs in seizure susceptibility in C57BL/6 wildtype mice and humans has remained uncertain. Therefore, in the present study, we explored the role of α7 nAChRs in seizure susceptibility in the wildtype mice and its expression in human epileptogenic tissue. We reveal that decreased α7 nAChRs activity in CA1 pyramidal neurons in hippocampus increases seizure susceptibility in the wildtype mice, while increased activity of α7 nAChRs has no significant effect on seizure susceptibility. Additionally, we also determined that the expression of α7 nAChRs was downregulated in human tissue that had suffered epilepsy after traumatic brain injury or intracerebral hemorrhage. Our findings suggest that α7 nAChRs may be a regulator of seizure susceptibility.

2. Materials and methods

All human experiments were reviewed and approved by the Medical Ethical Committee of Zhejiang Provincial People’s Hospital, People’s Hospital of Hangzhou Medical College (NO. 2018KT098) based on The Code of Ethics of the World Medical Association and the written informed consent was obtained from the participants. All animal-related experiments were examined and approved by the Animal Advisory Committee of the Department of Neurology, Zhejiang Provincial People’s Hospital, People’s Hospital of Hangzhou Medical College, Hangzhou, Zhejiang, China.

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Committee of Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College based on the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.1. Animals and reagents
Ten-weeks old, male wildtype C57BL/6 mice (25–30 g weight) were used for acute pilocarpine induced epilepsy model. ChAT-ChR2–EYFP mice were used for immunohistochemistry. Before behavior test, the mice were kept under a 12 h light/dark cycle and controlled environmental conditions. Mice with abnormal body weight and appearance were excluded from the behavior test. Primary antibodies used were rabbit polyclonal anti-α7 nAChRs (Santa Cruz Biotechnology, USA, Cat#: sc-5544), rabbit polyclonal anti-GAPDH (Cell Signaling Technology, USA, Cat#: 5014S), mouse polyclonal anti-NeuN (Millipore, USA, clone A60). Other chemicals purchased were methyllycaconitine citrate (MLA) from Abcam (UK), pilocarpine and PNU282987 from Sigma-Aldrich (USA).

2.2. Immunohistochemistry
Mice were anesthetized and the brain vessel was perfused with 4% paraformaldehyde, which was dissolved in phosphate-buffered saline (PBS). Whole brains were isolated and fixed in paraformaldehyde at 4 °C overnight, and further dehydrated in 30% sucrose dissolved in PBS. According to anatomical landmarks, 35-μm-thick brain sections were cut from the regions of interest on a freezing microtome. The brain sections were treated with 10% (vol/vol) normal donkey serum in PBS containing 0.3% Triton X-100 for immunolabeling. Then, the brain sections were incubated with anti-α7 nAChRs (1:100) antibody and 4°C overnight. The immunoreactivity was detected with the secondary antibodies, Alexa Fluor 594 donkey anti-mouse, Alexa Fluor 633 donkey anti-goat, and Alexa Fluor 488 donkey anti-rabbit IgG (1:400). The fluorescent images were visualized and captured by confocal microscopy (Olympus FV1000 Laser Scanning Confocal Microscope, USA).

2.3. Human tissue preparation and western blot analysis
One gram of epileptic tissue was dissected from fresh frozen brain sections maintained at −80 °C. The tissues were homogenized in lysis buffer (Beyotime Biotechnology, China) with 1 mM protease inhibitor PMSF (Beyotime Biotechnology, China). The homogenates were collected after centrifugation at 10,000 g at 15,000 rpm. After the protein concentration was measured in Bradford’s reagent (Beyotime Biotechnology, China), samples in loading buffer containing equivalent amount of protein were boiled for 5 min (Beyotime Biotechnology, China). The samples were run on SDS-PAGE and the separated proteins were transferred to an Immobilon PVDF membrane (Millipore, USA) at 300 mA. The membranes were incubated with a 1:2000 nAChR primary antibody (1:100) in 5% skimmed milk, after blocking with TBST solution (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20), for 1 h at room temperature. After washing with TBST, the blots were incubated with secondary antibodies (1:7500) for 1 h at room temperature. The signals were detected with enhanced chemiluminescence and were developed on an X-ray film. The immunobots were digitized on a flatbed scanner and were quantified using the US National Institute of Health ImageJ program for densitometric quantification.

2.4. Surgeries and EEG measurement
The mice were anesthetized by isoflurane and were secured in a stereotactic head frame. A midline incision was made and 8% H2O2 was used to expose bregma to posterior. The electrode was placed over the cranium with three screws (3 positions: the first screw (left): A-P: + 1.5 mm, lateral: + 1.5 mm; the second and the third screws: A-P: −3 mm, lateral: ± 3 mm). The cannula (intraventricular drug administration) was placed in the CA1 region of the hippocampus (position (right): A-P: -1.94 mm, lateral: ± 1.1 mm depth: -2.0 mm).

The EEGs were recorded in freely moving mice after the surgery for one week. The abnormal wave was defined as the amplitude more than 400 mV.

2.5. Pilocarpine-induced seizures and monitoring
The pilocarpine-induced seizure stages were defined as follows: 1. The onset time, measured as the time from pilocarpine injection to the first epileptic wave. 2. Latency of generalized tonic-clonic (GTC), assessed from the time of pilocarpine injection to the first GTC seizure epileptic waves. 3. Latency of death, calculated from the time of injection pilocarpine until the mice dead.

2.6. Electrophysiology
The neurons were identified by the brain sections with an upright microscope equipped with ×100 water immersion lens (Nikon, ECLIPSE FN1, Japan) and the membrane activity was recorded using whole-cell patch-clamp (MultiClamp 700B Amplifier, Digidata 1440 A analog-to-digital converter, and pClamp 10.2 software, Axon Instruments, Molecular Devices, USA). Pyramidal neurons were recorded from the CA1 region in the hippocampus. Glass pipettes (3.0, 5.0 MΩ) used for whole-cell recording were filled with internal recording solution containing 10 mM K-glucuronate, 40 mM KCl, 10 mM HEPES, 2 mM Mg-ATP, 1 mM NaGTP, and 0.2 mM EGTA; pH was adjusted to 7.25 ± 0.05 with KOH.

Statistics
Unless otherwise stated, the data are expressed as mean ± s.e.m.; error bars denote s.e.m. (n = number of samples). A two-tailed Student’s t-test was used for comparison of means from the same group. Diff; differences between more than two group were tested with two-way analysis of variance (ANOVA). Diff; differences were considered significant if P < 0.05.

3. Results
3.1. α7 nAChR expression in the CA1 pyramidal neurons in hippocampus
The mice expressing channelrhodopsin-2 (ChR2) protein under the control of the choline acetyltransferase (ChAT, a marker for cholinergic neurons) promoter (ChAT–ChR2–EYFP) were used in this research. It has been reported that the hippocampus is the main target for the projection of basal forebrain (BF) cholinergic neurons [18]. To confirm this, we stained hippocampal CA1 stratum pyramidal neurons with NeuN in ChAT–ChR2–EYFP mice (Fig. 1a left). We also detected positive cholinergic neurons in the area (Fig. 1a middle). The merged image shows co-expression in the hippocampal CA1 stratum pyramidal neurons (Fig. 1a right). The results showed that the expression of cholinergic neurons in the hippocampal CA1 stratum pyramidal neurons. Next, we stained α7 nAChRs in the CA1 stratum pyramidal neurons (Fig. 1b left) and found α7 nAChR co-expression with pyramidal neurons in the CA1 (Fig. 1b right). Together, these results indicate that α7 nAChRs are concentrated in the CA1 pyramidal neurons.

3.2. Decreased activity of α7 nAChRs increases the seizure susceptibility in C57BL/6 wildtype mice
To investigate the effect of α7 nAChRs on the seizure susceptibility in C57BL/6 wildtype mice, we injected α7 nAChRs agonist (PNU282987, a selective α7 nAChR agonist) and antagonist
(methyllycaconitine citrate (MLA), a specific α7 nAChR blocker) into the CA1 pyramidal neurons in the hippocampus by microtubules administration. The seizure susceptibility was measured by injecting the cholinergic agonist pilocarpine (250 mg/kg i.p.), which acts upon the muscarinic receptors. To observe the electrographic changes caused by pilocarpine-induced seizures between the vehicle and α7 nAChRs agonist and antagonist, electroencephalogram (EEGs) was recorded using bilateral epidural screw electrodes (see Materials and Methods), and the electrographic seizures were quantified by the latency onset time, the latency of GTC, and the latency of death. Administration of vehicle and PNU282987 in the CA1 of the hippocampus in wildtype mice 15 min before the injection of pilocarpine showed no significant difference in the onset time of epilepsy and the latency of GTC (Fig. 2a-c). The possible reason might be related to the α7 nAChRs saturation. Moreover, we observed that the latency of death was slightly prolonged after administration of PNU282987, but there was no significant difference compared to the vehicle-treated control group (Fig. 2d). However, after bath application of MLA, a specific α7 nAChR blocker, the number of APs was found to be significantly increased compared to the control group (Fig. 2c, d). Together these results reveal that decrease in the activity of α7 nAChRs could increase the excitability of the CA1 pyramidal neurons.

3.3. Decreased activity of α7 nAChRs increases the excitability of the CA1 pyramidal neurons in the hippocampus in C57BL/6 wildtype mice

To further demonstrate the effect of α7 nAChRs, we recorded the excitability of CA1 pyramidal neurons by checking the number of action potentials (APs) elicited by current injections of various amplitudes (1 s, 0 to +250 pA) in the cortical slices. All pyramidal neurons showed high-frequency discharges with increasing current. We assessed the effect of PNU282987, a selective α7 nAChR agonist, on hippocampal slices from C57BL/6 mice. In contrast to the control group, there was no significant change in the excitability of pyramidal neurons (Fig. 3a, b). However, after bath application of MLA, a specific α7 nAChR blocker, the number of APs was found to be significantly increased compared to the control group (Fig. 3c, d). Together these results reveal that decrease in the activity of α7 nAChRs could increase the excitability of the CA1 pyramidal neurons in the hippocampus.

3.4. Decreased α7 nAChRs expression in human epileptogenic tissue

To further establish the link between the activity of α7 nAChRs and the seizure susceptibility, we assessed the expression of α7 nAChRs in cell membranes by immunoblotting the epileptogenic foci tissue from the subjects with secondary and intractable epilepsy, and the individuals with traumatic brain injury and intracerebral hemorrhage (Fig. 4a). The results showed that the level of α7 nAChRs in epilepsy samples was only about 50% of that in the control group (Fig. 4b).

4. Discussion

In this study, we have investigated the relationship between the α7 nAChRs activity and the seizure susceptibility. We present evidence that decreased activity of α7 nAChRs could increase the seizure susceptibility in C57BL/6 wildtype mice. First, we proved that cholinergic neurons exist and its subunit receptor-α7 nAChRs express in the CA1 pyramidal neurons. Second, we studied the seizure susceptibility after stimulation and inhibition of the α7 nAChRs. To address this, we used C57BL/6 wildtype mice with pilocarpine-induced seizures as the
experimental model. The results showed that the seizure susceptibility between the PNU282987-treated mice and control group has no significant difference, but MLA-treatment could significantly decrease the seizure susceptibility. Third, the number of APs significantly increased after MLA treatment, however, there was no significant difference after infusion of PNU282987 in CA1 pyramidal neurons. Finally, we showed that the expression of α7 nAChRs protein was reduced in the epileptogenic foci tissue from individuals with secondary and intractable epilepsy. In summary, our study establishes that decrease in the α7 nAChRs maybe a risk factor for seizure susceptibility.

It has been reported that α7 nAChRs are widely expressed, with highest expression in the hippocampus and thalamus, but with low levels in the post mortem human brain [19,20]. The α7 nAChRs have been found to be concentrated on almost all synapses, both presynaptically and postsynaptically, in the CA1 region of the hippocampus [21]. The initial evidence about α7 nAChRs activity in a synapse was found in the rat hippocampal CA1 pyramidal neurons by examining spontaneous activity [22–24]. It has been reported that loss of α7 nAChRs protein may play a pivotal role in cognitive function damage [25–28]. Previous studies have implicated neuroprotective effects of α7 nAChRs [29]. Our previous studies have shown that activation of α7 nAChRs decreased the seizure susceptibility in Chat-Mecp2−/− mice. So, we hypothesized that α7 nAChR may have a role to play in epilepsy. In the present study, immunofluorescence of hippocampal neurons showed that α7 nAChRs are expressed in pyramidal neurons in the hippocampus. We also revealed that the treatment of MLA to block α7 nAChRs on CA1 pyramidal neurons directly increased the excitability of these neurons. Previous studies have also shown that α7 nAChRs are expressed on GABAergic interneurons in the hippocampus [21] and parvalbumin (PV) interneurons, which project their axons into the perisomatic region of the target neurons, thus, regulating the output of pyramidal neurons [30]. Therefore, we presumed that the effect of α7 nAChRs on pyramidal neuron excitability could be caused by two possibilities: either as excitatory input from PV interneurons to pyramidal neurons, or as direct modulation of α7 nAChRs on the pyramidal neurons. However, further studies are required to substantiate these hypotheses.

5. Conclusion

Decreased excitability of α7 nAChRs in the hippocampus led to a high seizure sensitivity in mice. Moreover, the expression of α7 nAChRs was found to be reduced in human epileptogenic tissue. Our findings suggest that downregulation of α7 nAChRs contributes to human epilepsy. However, increasing the activity of α7 nAChRs on the CA1 pyramidal neurons in hippocampus has no significant effect on seizure susceptibility. Overall, the study suggests that α7 nAChRs may be a risk factor of seizure susceptibility.
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Fig. 3. Decreased activity of α7 nAChRs increases the excitability of the CA1 pyramidal neurons in the hippocampus of C57BL/6 wildtype mice. (a) Voltage responses of a representative to in pyramidal neuron to various injections (1 s) from top to bottom, 50, 100, 150, 200 pA. Left: Control group. Right: PNU282987 group. Membrane potential was kept at −70 mV by injecting a small DC current through the recording pipette. (b) Summary histogram showing the number of pyramidal neuron APs. Two-way ANOVA was used for analysis. n = 10. (c) Voltage responses of a representative pyramidal neuron to various injections (1 s) from top to bottom, 50, 100, 150, 200 pA. Left: Control group. Right: MLA group. Membrane potential was kept at −70 mV by injecting a small DC current through the recording pipette. (d) Summary histogram showing the number of pyramidal neuron APs. Two-way ANOVA was used for analysis. n = 11. *P < 0.05; **P < 0.01; ***P < 0.001. Error bars are means ± s.e.m.

Fig. 4. Decreased α7 nAChRs expression in human epileptogenic tissue. (a) Immunoblotting of α7 nAChRs in the membrane fraction of cortex from control individuals or individuals with epilepsy. Each lane was loaded with 40 μg of protein with GAPDH as the loading control. (b) Quantification of α7 nAChRs proteins in control and epilepsy groups. n = 3. Protein level of epilepsy tissue was normalized to the that of non-epilepsy tissue. +++P < 0.001; Error bars are means ± s.e.m.
