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Identification of α7 nicotinic acetylcholine receptor on hippocampal astrocytes cultured *in vitro* and its role on inflammatory mediator secretion★

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Abstract

The present study found expressions of α7 nicotinic acetylcholine receptor on hippocampal slices and hippocampal astrocytes using double immunofluorescence stainings. Expression of glial fibrillary acidic protein in the cultured hippocampal slices and hippocampal astrocytes significantly increased, and levels of macrophage inflammatory protein 1α, RANTES, interleukin-1β, interleukin-6, and tumor necrosis factor-α increased in the supernatant of cultured astrocytes following exposure to 200 nM amyloid β protein 1–42. Preconditioning of 10 μM nicotine, a nicotinic acetylcholine receptor agonist, could attenuate the influence of amyloid β protein 1–42 in inflammatory mediator secretion of cultured astrocytes. Experimental findings indicated that α7 nicotinic acetylcholine receptor was expressed on the surface of hippocampal astrocytes, and activated α7 nicotinic acetylcholine receptor was shown to inhibit inflammation induced by amyloid β protein 1–42.

Key Words

α7 nicotinic acetylcholine receptor; astrocytes; inflammation; cytokines; chemotactic factor; amyloid β protein; hippocampus; neural regeneration

Research Highlights

(1) The present study detected the expression of α7 nicotinic acetylcholine receptor on the surface of hippocampal astrocytes.
(2) Activated α7 nicotinic acetylcholine receptor was shown to inhibit inflammation induced by amyloid β protein 1–42.

Abbreviations

AD, Alzheimer's disease; $A\beta_{1-42}$, amyloid β protein 1–42; GFAP, glial fibrillary acidic protein

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INTRODUCTION

Previous studies have shown that cholinergic neurotransmitters released from the peripheral vagus nerve activate the α7 nicotinic acetylcholine receptor in macrophages, as well as inhibit synthesis and secretion of inflammatory mediators of macrophages, thereby resulting in decreased peripheral inflammation[1-2]. Alzheimer's disease (AD) is associated with cerebral chronic inflammation caused by genetic and environmental factors[3]. Astrocytes and microglia, which exhibit similar characteristics to peripheral macrophages, are predominant in chronic inflammation of the central nervous system. It is difficult to ascertain the role of α7 nicotinic acetylcholine receptor on astrocytes *in vivo*, because of the variety of cells. Therefore, *in vitro* studies are extremely helpful. Although cell culture is considered to be the best model for analyzing

cellular functions, the relationships between cells are different in cultured cells compared with those in the organisms. Therefore, the use of brain slice organotypic cultures has become a powerful tool for the analysis of neural mechanisms, allowing for close-to-normal relationships between neurons, glia, and other membrane structures[4]. However, the localization of α7 nicotinic acetylcholine receptor on astrocytes in organotypically cultured hippocampal slices remains poorly understood. Previous results have shown that cholinergic drugs delay AD development[5]. However, the role of astrocytes in this process remains unclear.

The present study analyzed the localization of α7 nicotinic acetylcholine receptor in rat hippocampal astrocytes to determine the role of α7 nicotinic acetylcholine receptor in inflammatory factor secretion induced by amyloid β protein 1–42 ($A\beta_{1-42}$).

RESULTS

Expression of α7 nicotinic acetylcholine receptor on astrocytes in cultured hippocampal slices

Expression of α7 nicotinic acetylcholine receptor on astrocytes in cultured hippocampal slices was observed using double immunofluorescence. As shown in Figure 1A, a large number of α7 nicotinic acetylcholine receptor-expressing cells were present in cultured hippocampal slices, as evident by fluorescence staining. Antibodies specific to glial fibrillary acidic protein (GFAP) were used to detect astrocytes, and Figure 1B shows astrocyte distribution. Both GFAP and α7 nicotinic acetylcholine receptor were expressed in the cells (Figure 1C).

Expression of α7 nicotinic acetylcholine receptor in cultured hippocampal astrocytes

Expression of α7 nicotinic acetylcholine receptor on astrocytes, which were purified and cultured *in vitro*, was detected by double immunofluorescence. Green fluorescence represented α7 nicotinic acetylcholine receptor (Figure 2A), and astrocytes exhibited red fluorescence (Figure 2B). Superimposed images revealed a co-localization of astrocytes and α7 nicotinic acetylcholine receptor (Figure 2C).

$A\beta_{1-42}$ increased GFAP expression in astrocytes

Cultured hippocampal slices and astrocytes were treated with $A\beta_{1-42}$ (200 nM) for 48 hours, and then the expression of GFAP was detected. Results showed increased GFAP expression in cultured organic astrocytes and cultured single astrocytes (Figure 3). Astrocytes reacted to $A\beta_{1-42}$, by increasing GFAP expression, which is the first stage of an inflammatory reaction in astrocytes[6].

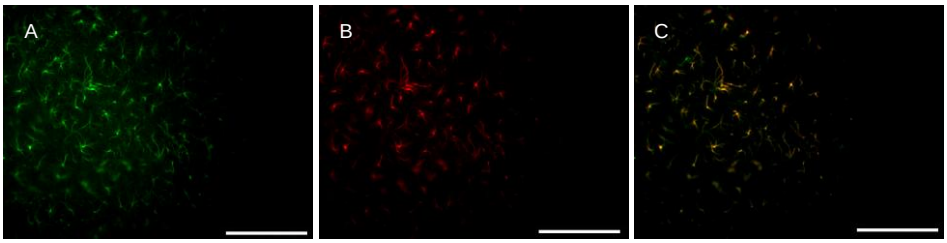

Figure 1 Expression of α7 nicotinic acetylcholine receptor on astrocytes in cultured hippocampal slices as detected by double immunofluorescence (fluorescence microscope, scale bars: 100 μm).

Expression of α7 nicotinic acetylcholine receptor in cultured hippocampal slices is shown in green (A; FITC-labeled), and expression of glial fibrillary acidic protein is shown in red (B; Texas Red-labeled). A merged image is shown in C, where yellow indicates co-localization of both proteins.

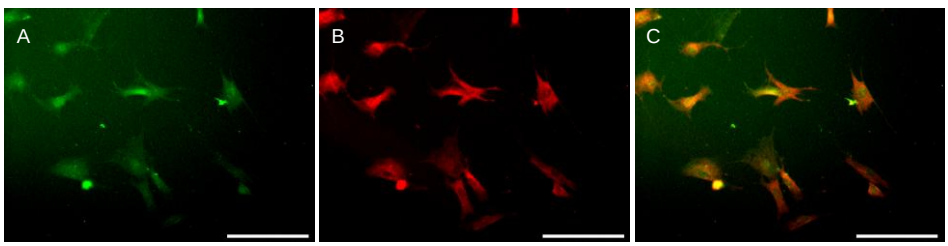

Figure 2 Double immunofluorescence of cultured hippocampal astrocytes using anti-glial fibrillary acidic protein and anti-α7 nicotinic acetylcholine receptor (fluorescence microscopy, scale bars: 100 μm).

The localization of α7 nicotinic acetylcholine receptor is shown in green (A; FITC-labeled), and localization of glial fibrillary acidic protein is shown in red (B; Texas Red-labeled). A merged image is shown in C, where yellow indicates co-localization of proteins.

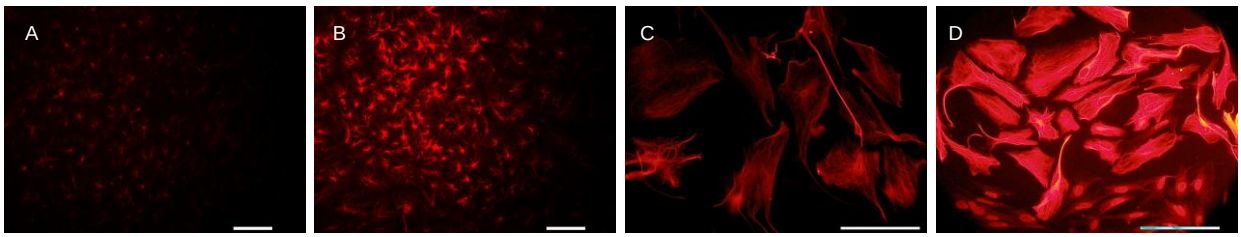

Figure 3 Expression of glial fibrillary acidic protein induced by amyloid β protein 1–42 (immunofluorescence staining, fluorescence microscopy, scale bars: 100 μm).

(A, B): Cultured hippocampal slice; (C, D): cultured astrocytes. The cultured organic astrocytes and cultured single astrocytes are activated by amyloid β protein 1–42, and glial fibrillary acidic protein expression is increased. A, C: Control group; B, D: amyloid β protein 1–42-treated group.

Effects of $A\beta_{1-42}$ on secretion of inflammatory mediators in astrocytes

Following exposure to 200 nM $A\beta_{1-42}$, chemokine levels in the supernatants of cultured astrocytes were measured. $A\beta_{1-42}$ increased macrophage inflammatory protein 1α and RANTES release from astrocytes in a time-dependent manner (Figure 4).

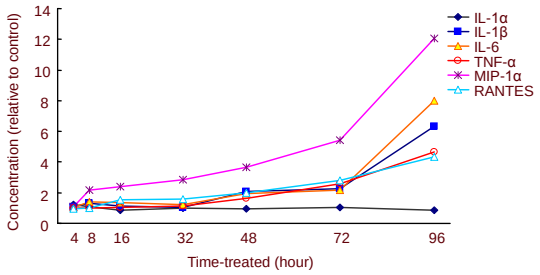

Figure 4 Effect of amyloid β protein 1–42 on secretion of astrocyte cytokines.

The cultured astrocytes were treated with amyloid β protein 1–42 for 4–96 hours, and then concentrations of macrophage inflammatory protein 1α (MIP-1α), RANTES, interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) were measured in the supernatants.

Data are expressed as means, from three independent experiments.

Concentrations of macrophage inflammatory protein 1α increased at 8 hours after treatment with $A\beta_{1-42}$ and reached 12-fold expression levels at 96 hours ($F = 85.510$, $P < 0.01$). Increased RANTES expression was less than macrophage inflammatory protein 1α; expression increased by 16 hours after treatment, and reached a 4.6-fold increase by 96 hours ($F = 40.424$, $P < 0.01$). To determine whether interactions between $A\beta_{1-42}$ and astrocytes upregulated pro-inflammatory cytokines, supernatants were collected from cultured astrocytes treated with $A\beta_{1-42}$. Concentrations of interleukin-1α, interleukin-1β, interleukin-6, and tumor necrosis factor-α were measured. As shown in Figure 4, $A\beta_{1-42}$ stimulated interleukin-1β, interleukin-6, and tumor necrosis factor-α release, but not interleukin-1α. The increases in cytokines profiles varied according to time. After 96 hours, changes in interleukin-6 and interleukin-1β levels were most significant, with approximately 8-fold and 6.3-fold increased expression, respectively ($F = 196.317$, $P < 0.01$; $F = 368.181$, $P < 0.01$). In addition, tumor necrosis factor-α levels were most significant at 96 hours, with approximately 4.8-fold increased expression ($F = 43.955$, $P < 0.01$).

Effects of activated α7 nicotinic acetylcholine receptor on secretion of inflammatory mediators of astrocytes mediated by $A\beta_{1-42}$

To explore the role of astrocyte α7 nicotinic acetylcholine receptor in secretion of inflammatory mediators mediated by $A\beta_{1-42}$, cultured astrocytes were pre-activated with nicotine (10 μM), a nicotinic acetylcholine receptor agonist, for 60 minutes, and then the concentration of inflammatory mediators was measured at 96 hours after $A\beta_{1-42}$ exposure. Results showed that α7 nicotinic acetylcholine receptor was pre-activated by nicotine, which decreased secretion of inflammatory mediators (except interleukin-1α) mediated by $A\beta_{1-42}$ ($P < 0.01$; Figure 5).

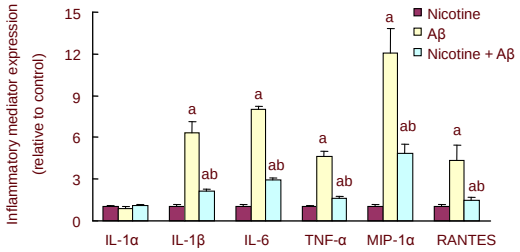

Figure 5 Effect of activated astrocytic α7 nicotinic acetylcholine receptor on secretion of inflammatory mediators mediated by amyloid β protein 1–42 (Aβ).

Cultured astrocytes were pre-treated with nicotine, a nicotinic acetylcholine receptor agonist, for 60 minutes prior to amyloid β protein 1–42 treatment. Action of amyloid β protein 1–42 on inflammatory mediators is decreased. Data are expressed as mean ± SD from five independent experiments.

[a]$P < 0.01$, *vs*. nicotine group; [b]$P < 0.01$, *vs*. amyloid β protein 1–42 group [one-way analysis of variance followed by least significant difference method for interleukin-1α (IL-1α), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α); Welch method for macrophage inflammatory protein 1α (MIP-1α), RANTES, and interleukin-1β (IL-1β)].

DISCUSSION

Cognitive functional impairment in AD patients has been shown to be related to cholinergic deficiency in the central nervous system. However, nicotinic acetylcholine receptor excitomotors improve cognition in animals and humans[7-8]. In the clinic, cholinergic agents are the most effective and most generally used, although little is known about the mechanisms of action. It is thought that transmitters released by the vagus nerve, which activates α7 nicotinic acetylcholine receptor on macrophages, can also inhibit peripheral inflammation[2]. In contrast to macrophages, astrocytes are abundant in the central nervous system and play a crucial role in central inflammatory reaction. Therefore, α7 nicotinic acetylcholine receptor expression on hippocampal astrocytes cultured *in vitro* was analyzed, as well as the role of α7 nicotinic acetylcholine receptor in inducing secretion of inflammatory mediator.

Double immunofluorescence was used to identify astrocytes and expression of α7 nicotinic acetylcholine receptor in cultured hippocampal slices. Results showed that cells other than astrocytes also expressed α7 nicotinic acetylcholine receptor, as previously reported[9].

In hippocampal cells, astrocytes are mixed with other cells in the hippocampal slice. Therefore, detailed functions of α7 nicotinic acetylcholine receptor on astrocytes remains poorly understood due to limitations with astrocyte purity. Therefore, it is important to study the mechanisms of astrocytes in astrocytic cultures. As described in a previous study[10], pooled hippocampal cells were cultured for 8–10 days and then transferred to a new culture flask to purify astrocytes from microglia and oligodendroglia. Purified astrocytes were further confirmed by immunofluorescence with GFAP-specific antibody.

Expression of α7 nicotinic acetylcholine receptor is influenced by many factors. In different encephalic regions, astrocytes can express different α7 nicotinic acetylcholine receptor expression patterns[11]. In addition, there is a significant difference in growth conditions between organotypic cultures and cultured single cells. Therefore, it is necessary to determine whether cultured single astrocytes express α7 nicotinic acetylcholine receptor. Double immunofluorescence staining was used in the present study, and results demonstrated that α7 nicotinic acetylcholine receptor was expressed on cultured single astrocytes. These results suggested that it is possible to analyze the function of α7 nicotinic acetylcholine receptor in astrocytes using cultured single astrocyte models.

In the brain, astrocytes sustain and nourish neurons, and they have been shown to play a role in intracephalic inflammatory reactions, with direct effects on the central nervous system[12]. Diffuse plaques, consisting of Aβ, results in the accumulation of astrocytes and microglia, as well as other pathological change. Previous studies have shown that Aβ directly injures neurons[13], as well as induces cytokine secretion resulting in neuronal damage *via* the activation of astrocytes and microglia. To determine the effects of Aβ on astrocytes, GFAP expression was measured in cultured hippocampal slices following exposure to $A\beta_{1-42}$. Results showed that $A\beta_{1-42}$ significantly increased GFAP expression. However, when combined with other cells, it becomes difficult to determine that the effect of $A\beta_{1-42}$ on astrocytes is direct or indirect. Therefore, $A\beta_{1-42}$ was added to cultured single astrocytes, demonstrating that $A\beta_{1-42}$ increased GFAP expression, enlarged astrocytic cell bodies, and created rough synapses. These results suggested that $A\beta_{1-42}$ directly activated astrocytes, resulting in increased GFAP expression.

Once activated, astrocytes evoked inflammatory reactions in the brain and secreted inflammatory mediators that induced neurotoxicity. The present study observed concentration changes of cytokines following treatment with $A\beta_{1-42}$. All tested cytokines, with exception to interleukin-1α, exhibited expression changes in a time-dependent manner. Astrocytes have been shown to express tumor necrosis factor-α in response to an inflammatory response[14]. However, in the present study, expression increased by only 4.6 folds, compared with 8-fold increases in interleukin-6.

Expression of chemotactic factors increased earlier and more dramatically than cytokines, and macrophage inflammatory protein 1α exhibited the most significant change. Chemotactic factors promote the migration and infiltration of other phagocytes, as well as enhance inflammatory reactions and prolong inflammatory processes, which can subsequently exacerbate the symptoms of AD[15].

The ion channel α7 nicotinic acetylcholine receptor exists in the peripheral and central nervous systems and exhibits a high affinity to nicotine[9]. To identify the influence of activated astrocytic α7 nicotinic acetylcholine receptor on secretion of inflammatory mediators mediated by $A\beta_{1-42,}$ the cultured astrocytes were pretreated with nicotine for 60 minutes prior to $A\beta_{1-42}$ exposure. Results suggested that once astrocytes expressed α7 nicotinic acetylcholine receptor, action of $A\beta_{1-42}$ in inflammatory mediators increased. Levels of cytokines and chemotactic factors significantly decreased compared with non-activated α7 nicotinic acetylcholine receptor. Therefore, results suggested that activated astrocytic α7 nicotinic acetylcholine receptor decreased secretion of inflammatory mediators and chemotactic factors, suppressed migration and infiltration of other phagocyte, and decreased formation of a self-toxic circuit loop. α7 nicotinic acetylcholine receptor activation did not completely return cytokine or chemotactic factors to normal levels, which suggested that activated α7 nicotinic acetylcholine receptor only de-

creased the inflammatory reaction of astrocytes. These mechanisms could help to explain why cholinergic treatment only improves AD symptoms and postpones the pathological processes of AD, but does not cure it.

MATERIALS AND METHODS

Design

A comparative, *in vitro* study.

Time and setting

The experiment was performed at the Department of Pharmacy, Zhujiang Hospital, Southern Medical University, China from August 2006 to May 2010.

Materials

Neonatal Sprague-Dawley rats (10 days old) and newborn rats (within 24 hours after birth) were obtained from the Experimental Animal Center of Southern Medical University, China (Certificate No. 2005A060). All experimental animal procedures complied with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[16].

Methods

Preparation of amyloid-beta peptide

$A\beta_{1-42}$ was prepared in fresh stock solution according to previously described methods, with slight modifications[17]. Briefly, $A\beta_{1-42}$ (Sigma, St. Louis, MO, USA) was dissolved in 35% acetonitrile and further diluted to 10 μM with incremental additions of PBS while vortexing between additions. The peptide solution was subsequently incubated at 37°C for 72 hours to promote fibrillization and aggregation, followed by storage at −20°C. The final working concentration used in the experiment was 200 nM $A\beta_{1-42}$ in culture medium[18].

Preparation of hippocampal slices

The hippocampus was isolated from 10-day-old neonatal rats and sectioned for culture. The rinsed hippocampus slices were placed onto a membrane in a solution-air culture. The culture medium comprised 50% Eagle's-modified medium (Gibco, Carlsbad, CA, USA), 25% Earle's balanced salt solution, and 25% horse serum containing 6.5 mg/L D-glucose. In the first 3 days, the slices were cultured at 37°C with 5% CO_2, and medium was replaced every day. Afterwards, the medium was replaced every 3 days, and culture temperatures were reduced to 33°C[19].

Culture of hippocampal astrocytes

Hippocampi were dissected from the cerebrum of newborn rats within 24 hours after birth, and the tissue was digested with trypsin and centrifuged. Subsequently, the supernatant was discarded, and dissociated cells were plated in culture flasks. After 8–10 days, the culture flasks were shaken at 37°C for 4 hours in a rotator, and then the adherent cells were collected for further culture. To assess astrocytic purity, GFAP expression was detected[20].

Identification of astrocytes

The hippocampal slices and cultured astrocytes were fixed in 4% paraformaldehyde (pH 7.2) for 20 minutes and washed with 0.01 M PBS. Following a blocking step in PBS containing 10% goat serum and 0.3% Triton X-100, the specimens were incubated with mouse anti-GFAP polyclonal antibody (1:200; Chemicon, Santa Cruz, CA, USA) at 4°C overnight. The sections were then washed three times with PBS for 10 minutes each and incubated with Texas Red-conjugated anti-mouse IgG secondary antibody (1:100; Chemicon) at room temperature for 1 hour. Then sections were washed three times with PBS, coverslipped, and examined under a fluorescence microscope (Olympus, Tokyo, Japan).

Double immunofluorescence of α7 nicotinic acetylcholine receptor in cultured hippocampal slices and cultured astrocytes

Double immunofluorescence was used to identify expression of astrocytic α7 nicotinic acetylcholine receptor in hippocampal organotypic-cultured hippocampal slices and cultured astrocytes. Sections and cultured astrocytes were fixed with 4% paraformaldehyde, and then incubated with 0.5% Triton X-100 for 30 minutes. Rinsed sections and cultured astrocytes were pre-blocked with goat serum prior to primary antibody incubation. Mouse anti-GFAP (1:200; Chemicon) and rabbit anti-α7 nicotinic acetylcholine receptor (1:200; Chemicon) polyclonal antibodies served as the primary antibodies at 4°C overnight, respectively. Normal rabbit serum was used as the negative control. Neuronal signals served as a putative positive control. After three washes with PBS for 5 minutes each, Texas Red-conjugated anti-mouse IgG (1:100; Chemicon) and FITC-labeled anti-rabbit IgG (1:100; Chemicon) were used as secondary antibodies, respectively. Then slides were washed three times with PBS and examined under a fluorescence microscope (BX51; Olympus).

Determination of inflammatory factor expression in supernatant of astrocytes using the liquid phase technique

Following treatment of cultured astrocytes with 200 nM $A\beta_{1-42}$ for 4, 8, 16, 32, 48, 72, and 96 hours, supernatants were collected to detect concentrations of cytokines and chemokines. To determine the role of α7 nicotinic acetylcholine receptor, the cells were pretreated with 10

μM nicotine (Sigma) for 1 hour prior to $A\beta_{1-42}$ administration. Control cultures were from the same culture set, but were treated with media only. Cytokines/ chemokines, including interleukin-1α, interleukin-1β, interleukin-6, tumor necrosis factor-α, macrophage inflammatory protein 1α, and RANTES in the culture supernatants of astrocytes were measured simultaneously using a LiquiChip work station, which employed bead-based xMAP (flexible multi-analyte profiling) technology, according to manufacture instructions[21].

Statistical analysis

All statistical analyses were performed using SPSS 11.0 software (SPSS, Chicago, IL, USA). Data were expressed as mean ± SD or mean. One-way analysis of variance was used to compare means between groups, and multiple comparisons for further steps. With homogeneity of variance, least significant difference was adopted for each two-group comparison. Otherwise, the Welch method was utilized. Differences were considered statistically significant if the *P* value < 0.05.

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Author contributions: The experiment was designed by Yong Wang, Kewan Wang, performed by Yong Wang, Yan Wang, Ning Zhu and Zhongyi Zhang. Yan Wang and Yong Wang were responsible for data integrity and manuscript writing.

Conflicts of interest: None declared.

Ethical approval: The pilot was approved by Medical Animal Ethical Committee of Guangdong Province in China.

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