原著論文

Alpha7 nicotinic ACh receptor mediated neuroprotective action by nicotine and GTS-21 ~An approach by the hippocampal organotypic slice cultures~

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Abstract

Nicotine, main constituent of tabaco, is known as a nicotinic acetylcholine receptor (nAChR) agonist and increases cognitive performance. 3-(2,4-dimethoxybenzylidene)-anabaseine (GTS-21) is derived from the marine worm toxin, anabaseine, and is alpha7-selective nAChR (α7-nAChR) agonist. Both nicotine and GTS-21 were expected as therapeutic agents of Alzheimer's disease. Several studies showed that nicotine and GTS-21 protected neuron by activating nAChR, especially α7-nAChR. It has been reported that α7-nAChR has been shown to be an essential regulator of inflammation. The purpose of this study is to examine the neuroprotective and anti-inflammatory effects of nicotine and GTS-21 using organotypic hippocampal slice cultures. Kainic acid (KA, 5-50 μM) induced concentration- and time-dependent neuronal cell death in the hippocampal organotypic slice cultures. The pretreatment with nicotine and GTS-21 tended to decrease in KA toxicity. In a CA3 area-specific analysis, pretreatment with nicotine resulted in significant inhibition of KA-induced neurotoxicity. The results suggest that nicotine may protect KA-induced neuronal cell death via α7-nAChR. We also examined anti-inflammatory effects of nicotine and GTS-21. Hippocampal slices were pretreated with nicotine or GTS-21, and then treated with lipopolysaccharide (LPS). LPS treatment induced concentration-dependent increases in TNFα and IL-1β gene expressions. LPS-induced TNFα gene expression, but not IL-1β was suppressed by GTS-21 pretreatment. These results suggest that α7-nAChR might be involved in the microglia activation towards a neuroprotective role by suppressing inflammatory cytokine.

Key Words : Alpha7 nicotinic ACh receptor, microglia, TNFα, Kainic acid, organotypic hippocampal slice cultures

Introduction

Organotypic hippocampal slice cultures have recently employed in the study of toxicological or disease oriented neurodegeneration. The hippocampus plays a key role in learning and memory, and thus it pays great attention in the study of neurodegenerative disorders. Since the primary culture of neuron destroys the environment for nerve and glial cells, it is difficult to reproduce their functions based on the intrinsic morphology of nerve, nerve activity or release of neurotransmitters. There is only electrophysiological method using slice section for evaluation of nerve activity. While, the hippocampal slice cultures make it possible to be cultivated under maintenance of neuron's morphology and function of neuron, and ganglionic layer in the hippocampus [1]. Furthermore, the organotypic hippocampal slice cultures are able to control their surroundings artificially, therefore, environments are easily modified by complying with the experimental purpose. Therefore, the organotypic hippocampal slice cultures are expected to be useful for *in vitro* study on effect of pharmacological or toxic agents on nerves system.

Nicotine, a main constituent of tabaco, is known to show a variety of pharmacological activities through nicotinic acetylcholine receptor (nAChR) in central and peripheral nervous system. A negative correlation is suggested between cigarette smoking and the incidence of Parkinson's disease or Alzheimer's disease [2], and nicotine is suggested to be a therapeutic agent for neurodegenerative diseases. Neurotoxicity or behavior disorders induced by glutamate receptor agonists including kainic acid (KA), glutamate and N-methyl-D-aspartate (NMDA) etc. have been reported to be inhibited by nicotine [3,4,5,6]. Therefore, neuroprotective effect of nicotine has been noted, and nAChR is thought to be engaged in this effect. nAChR consists of various subunits, and it has a wide distribution in the brain and peripheral nerves. Both α4β2 and α7 subunits are widely distributed especially in the brain [7], most notably α7-nAChR is calcium-permeant channel [8,9], and it is known to be distributed in high density in the area relevant to learning and memory including hippocampus [10,11,12].

3-(2, 4-dimethoxybenzylidene) anabasein (GTS-21), a synthetic nicotinic agonist was developed related to a compound, anabasein which produced by certain marine worms [13,14]. While benzyliden anabasein compounds have shown to be useful for the elucidation of nAChR drug-receptor structure-function relationships [15]. It has reported that α7 receptor activation is sufficient to improve both avoidance and spatial memory task [11]. Furthermore, when the roles of intracellular calcium ions and downstream of calcium channels in neuroprotection are assessed, α7 receptor activation is essential for cytoprotection against trophic factor deprivation, and protracted elevation in intracellular calcium ion is essential for such protection [16]. GTS-21 is expected to be a promising therapeutic agent for neurodegenerative diseases including Alzheimer's disease [17,18] and Schizophrenia [19,20,21]. In this study, we examined the effect of nicotine and GTS-21 on some neurotoxic chemicals, aiming at elucidating neuroprotective action by α7-nAChR. KA-induced neurotoxicity in the hippocampal slice cultures is reported to produce selectively neuronal cell death in CA3 area [22]. Based on these findings, we examined whether nicotine and GTS-21 is able to inhibit this specific neurotoxicity induced by KA in CA3 area.

Although the expression of α7-nAChR is thought to be predominant in nerve cells, it has recently become evident in various non-nerve cells including immune cells [23]. α7-nAChR is identified in monocyte and α7-nAChR-mediated nicotine inhibits the release of tumor necrosis factor alpha (TNFα) from macrophages [24]. Microglia works as immune cells in the central nervous system (CNS), and it shows opsonic action by activation, and it thus contributes to neuronal degeneration [25, 26]. Activated microglia in these processes produces and releases inflammatory cytokines such as interleukin-1β (IL-1β) and TNFα [27, 28], and results in production of ROS including nitric oxide (NO). These actions produced by activated microglia, are thought to be a cause of neurodegenerative disorders through damage to normal cells. Therefore, it is interested to clarify whether α7-nAChR in microglia affects the production of inflammatory cytokines. In the present study, we examined the effects of nicotine and GTS-21 on lipopolysaccharide (LPS)-stimulated IL-1β and TNFα gene expressions in order to elucidate whether α7-nAChR is involved in functional regulation of microglia.

Methods

1. Materials and animals

GTS-21 was generously provided by Dr. William Kem of the University of Florida. The figure 1 shows chemical structures of nicotine and GTS-21 used in this study. LPS (from Escherichia coli serotype 055:B5) were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were analytical or of the highest grade commercially available. All animal experiments were conducted under the Showa University Animal Experiment and Welfare Regulations. Three days old Wistar rats were purchased with mother from Japan Laboratory Animal, Inc (Tokyo, Japan).

2. Preparation of organotypic hippocampal slice cultures

Organotypic hippocampal slice culture was prepared as described previously [29]. Seven days old rats were killed by decapitation and their brains were removed. The hippocampal slices were prepared by Mcllwain tissue chopper into 400 μm thick. Three of slices were placed on culture plate insert (Millicell-CM, 0.4 μm, Billerica, MA) and transferred to six well culture plate with 1 ml of MEM supplemented with 25% horse serum, 6.5 mg glucose and 0.5 mM glutamine. Culture was maintained at 32°C and the medium was changed every 2-3 days.

3. Drug treatment and assessment of neuronal cell death

Effects of nicotine and GTS-21 were tested in mature cultures, which had been maintained for 15-21 days. Slice cultures exposed to nicotine and GTS-21 for 12 hr followed by kainic acid treatment for 24 hr. And then neuronal cell death was assessed by propidium iodide (PI) uptake. The medium was changed to Locke solution [154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 5 mM Hepes, pH 7.4] and then added PI solution to the medium (final conc. 50 μg/ml). At 30 min after the addition of PI solution, neuronal cell death was assessed by microscopic observation (BZ-8000, KEYENCE, Osaka, Japan). The intensity of fluorescence was quantitatively analyzed using VH-H1A5 analyzer (KEYENCE). The mean ± S.E.M. of the fluorescence intensity in each group was calculated and the values are expressed as relative percentages of those in the only KA-treated group.

4. LPS treatment and inflammatory cytokine gene expressions

Slice cultures were exposed to nicotine and GTS-21 for 12 hr followed by LPS treatment for 3 hr. Three hippocampal slices were placed on one tube and then total RNA were extracted using the QIAGEN RNeasy Lipid Tissue Mini kit (QIAGEN, Hilden, Germany). Real time RT-PCR was carried out by QuantiTect SYBR Green RT-PCR (QIAGEN). Primers for IL-1β, TNFα and GAPDH were prepared by QuantiTect Primer Assays (QIAGEN). Amplification conditions were of 40 cycles of 94°C for 15 s, 55°C for 30 s and 72°C for 30 s. mRNA expression was normalized using GAPDH as an endogenous control.

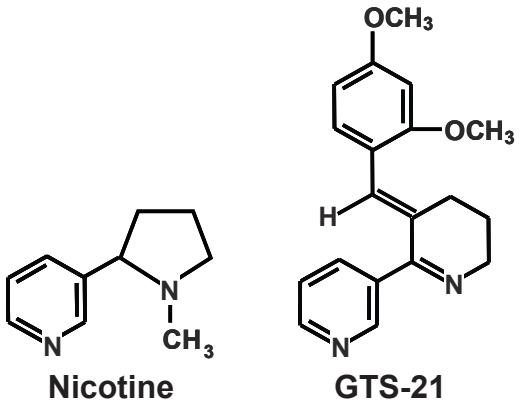

Fig. 1. Structures of nicotine and GTS-21.

5. Statistical analysis

Statistical analysis was performed with Dunnett's test.

Results

1. KA-induced neurotoxicity

Neurotoxicity induced by KA which was an agonist of glutamate receptors was assessed in areas of CA1, CA3 and dendate gyrus (DG) in the hippocampal slices (Fig. 2A top panel). Selective cell death in pyramidal cell layer of CA3 area was observed by the treatment with 5 and 10 μM KA. When slices were treated with more higher concentration of KA, cell death was observed in not only CA3 area, but also the pyramidal cell layer of CA1 and granular cell layer of DG area (Fig. 2A). KA-induced cell death in hippocampus was induced in a concentration-dependent manner.

Figure 2B shows the time course of 20 μM KA-induced neurotoxicity. Cell death was not observed in the hippocampal

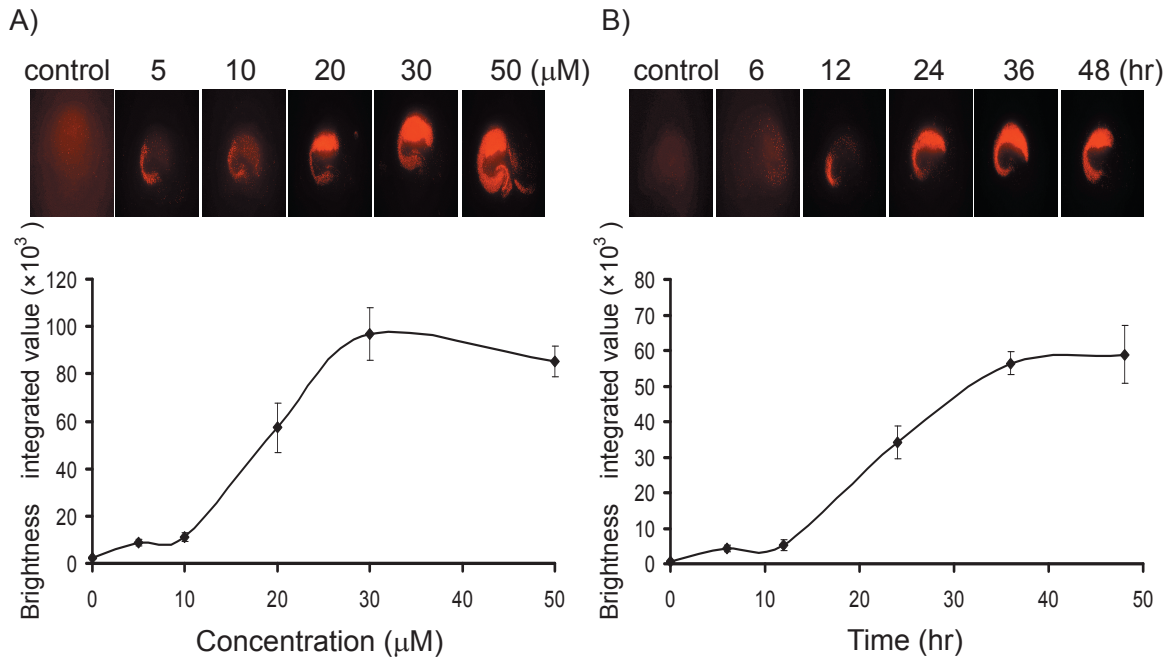

Fig. 2. Toxicity of KA in organotypic hippocampal slice cultures. Cell death was assessed at 48 hr after kainic acid treatment. Toxicity of KA was assessed on concentration- (a) and time-dependent analysis (b). Representative data from three different experiments are shown. Values are expressed as mean ± S.E.M.

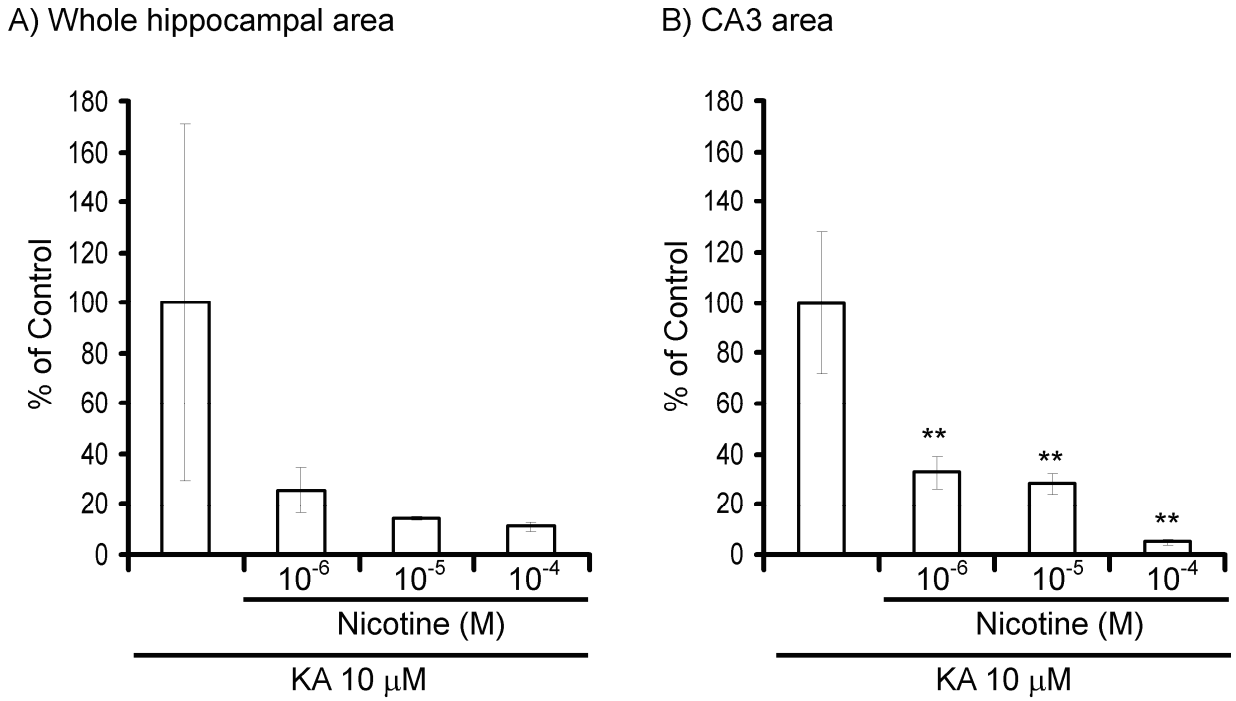

Fig. 3. Effect of nicotine on KA-induced neurotoxicity. Organotypic hippocampal slice culture were pretreated with nicotine for 12 hr and followed by KA treatment for 24 hr. And then neuronal cell death was assessed by PI uptake in whole hippocampal area (a) and CA3 area (b). Representative data from three different experiments are shown. The mean ± S.E.M. of the fluorescence intensity in each group was calculated and the values are expressed as relative percentages of those in the only KA-treated group. **$p<0.01$ compared with the KA-treated group.

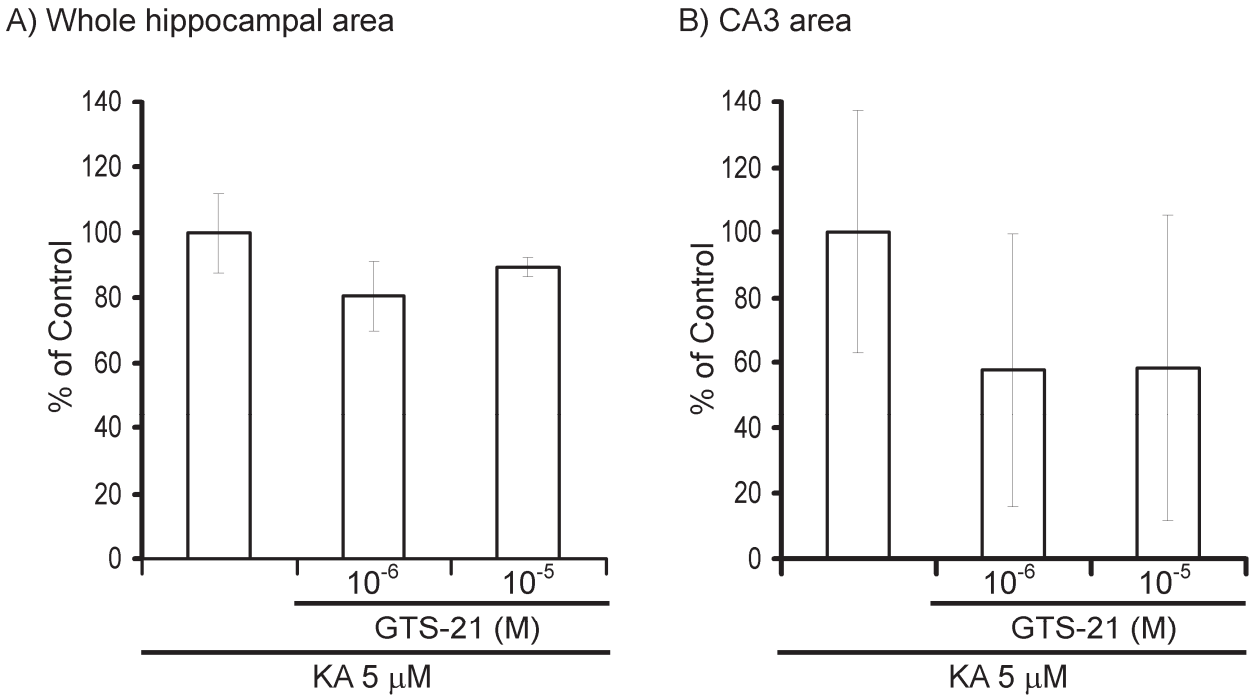

Fig. 4. Effect of GTS-21 on KA-induced neurotoxicity. Organotypic hippocampal slice cultures were pretreated with GTS-21 for 12 hr and followed by KA treatment for 24 hr. And then neuronal cell death was assessed by PI uptake in whole hippocampal area (a) and CA3 area (b). The mean ± S.E.M. of the fluorescence intensity in each group was calculated and then the values are expressed as relative percentages of those in the only KA-treated group.

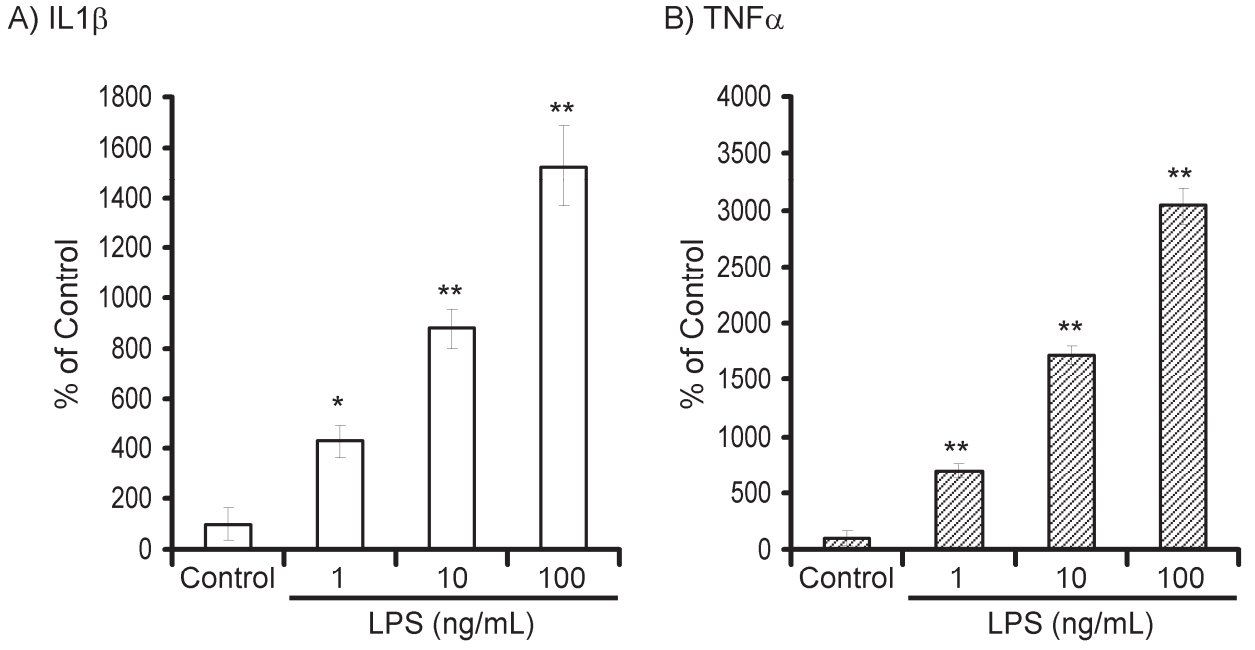

Fig. 5. LPS-induced inflammatory cytokine gene expression. Organotypic hippocampal slice culture were treated with LPS for 3 hr. Gene expressions of IL-1β (a) and TNFα (b) were analysed by real time RT-PCR. mRNA expression was normalized using GAPDH as an endogenous control. The mean ± S.E.M. of the mRNA expression in each group was calculated and then the values are expressed as relative percentages of those in the control group. *p<0.05, **p<0.01 compared with the control group.

slices at 0 and 6 hr after KA treatment. However, a selective cell death was seen in pyramidal cell layer of CA3 area at 12 hr, and it was detected not only in CA3 area, but also in pyramidal cell layer of CA1 area and granular cell layer of DG area at 24, 36 and 48 hr after KA treatment. Neurotoxicity produced by KA was increased in an exposure time-dependent manner.

2. Neuroprotective effects of nicotine and GTS-21 on KA-induced neurotoxicity

Nicotine induced no neurotoxicity up to 10^{-3} M (data not shown). Therefore, we chose nicotine concentration of 10^{-6}, 10^{-5} and 10^{-4} M. Nicotine tended to inhibit KA-induced neurotoxicity in a concentration-dependent manner in whole hippocampal area. When analysed in CA3 area, pretreatment with nicotine resulted in a significant inhibition of KA-induced neurotoxicity (Fig. 3).

GTS-21 itself showed no neurotoxicity under a long–term exposure to hippocampal slice cultures up to 10^{-4} M (data not shown). Pretreatment of slices with 10^{-6} and 10^{-5} M of GTS-21 for 12 hr resulted in a tendency to inhibit KA-induced neurotoxicity slightly. When examined CA3 area-specific analysis, pretreatment with GTS-21 showed the tendency of additional protective effect (Fig. 4).

3. Effects of nicotine and GTS-21 on LPS-induced cytokine inductions

Inflammatory cytokines mRNA expressions were assessed by treatment of hippocampal slices with 1, 10 and 100 ng/mL LPS. Marked mRNA expressions of IL-1β and TNFα were observed in a concentration-dependent manner (Fig. 5). Next, we examined the pretreatment effects of nicotine and GTS-21 on LPS-induced cytokine gene expressions. As shown in Fig. 6, both nicotine and GTS-21 failed to inhibit LPS-induced IL-1β gene expression (Fig. 6A). On the other hand, pretreatment with 10^{-5} M nicotine and 10^{-5} M GTS-21 significantly inhibited LPS-induced TNFα gene expression (Fig. 6B).

Discussion

GTS-21 is an α7-nAChR agonist synthesized on basis of chemical structure of anabasein [13]. Anabasein closely resembles chemical structure to anabasine, which is a major product in tabaco plant *Nicotiana glauca* cultivated in the Eastern Europe. Pharmacological difference between anabasein (or anabasine) and nicotine is that the former has high affinity to α7-nAChR than α4β2-nAChR. However, anabasein also stimulates almost all nAChR. Therefore, disubstituted benzylidene-anabasein derivatives were developed which character had not ganglionic and neuromuscular stimulating effects [14]. Under a variety of preclinical studies, 2, 4-dimethoxybenzaldehyde-added anabasein was selected for further development, and it is called GTS-21 in code name [17]. It has a high selectivity for α7-nAChR as compared to anabasein.

In this study, we examined neuroprotective effects of nicotine and GTS-21 on KA-induced neurotoxicity in the hippocampal slice cultures. Since KA receptors are distributed profoundly in CA3 area rather than CA1 and DG areas of juvenile rats [30,31], the nerves in CA3 area are selectively vulnerable to KA stimulation, and KA is thought to exert a toxic effect selectively on CA3 area [22]. α7-nAChR also expresses abundantly in CA3 area of hippocampus [32,33]. Therefore, we chose KA to examine neuroprotective effects of nicotine and GTS-21 in respect of their α7-nAChR activities.

When KA at concentration of 5 ~ 50 μM added to the hippocampal slice cultures, it produced cytotoxic effect on hippocampal CA3 area, and its concentrations more than 20 μM resulted in cell death not only CA3 area but also CA1 or DG in a concentration-dependent manner. At lower concentrations, KA-induced toxicity was observed selectively in CA3 area, probable due to be high distribution of KA receptors in this area. The findings are also compatible to time-dependent toxic effect of KA on CA3 area. Based on these findings, we examined possible neuroprotective effects of nicotine and GTS-21 on 5 and 10 μM KA at which concentrations it induces a selective cell death in CA3 area of hippocampus. Nicotine has been shown to activate α7-nAChR in the nerve ending of glutamatergic neurons in hippocampus and to stimulates a calcium influx into the nerve ending [33]. We found that the pretreatment with nicotine and GTS-21 tended to decrease KA toxicity, especially in CA3 area of hippocampus. These results are compatible to many reports indicating that nicotine is able to protect effect on KA-induced neurotoxicity [34,35], probably mediating through α7-nAChR [36]. Chronic

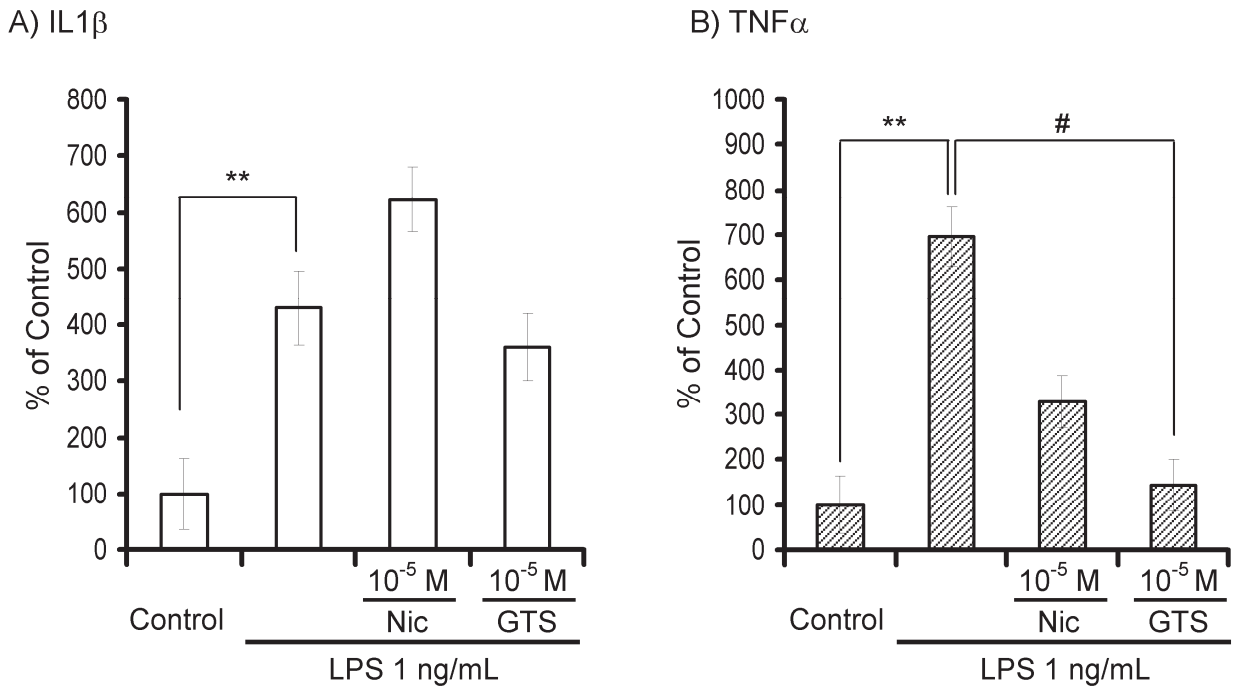

Fig. 6. Effects of nicotine and GTS-21 on LPS-induced cytokine inductions. Organotypic hippocampal slice cultures were pretreated with 10^{-5} M nicotine and GTS-21 for 12hr and followed by LPS treatment for 3 hr. Gene expressions of IL-1β (a) and TNFα (b) were analysed by real time RT-PCR The mean ± S.E.M. of the mRNA expression in each group was calculated and then the values are expressed as relative percentages of those in the control group. **p<0.01 compared with the control group. #p<0.05 compared with LPS-treated group.

exposure of cigarette smoking has also shown to inhibit KA-induced neurotoxicity, significantly [37]. The present results have also revealed that nicotine and GTS-21 suppresses KA-induced neurotoxicity in a limited area of hippocampal CA3 area. Therefore, it became apparent that neuroprotective effect of nicotine and GTS-21 on KA-induced toxicity is produced in an area-dependent manner. However, a tendency of decrease in KA-induced toxicity by GTS-21 was weak as compared to nicotine. The reason comes from the hypothesis that since nicotine binds to other nAChR which is found extensively in the CNS such as not only α7-nAChR but α4β2-nAChR, the neuroprotective effect on KA-induced toxicity may be produced through α4β2-nAChR alone or both α4β2-nAChR and α7-nAChR. Involvement of other nAChR remains to be clarified.

There are reports that nicotine and GTS-21 showed neuroprotective effect on various stimulations including glutamate, aspartate, Aβ peptide, and nerve growth factor receptor withdrawal [3,38,39,40]. These reports have in common in that in order to exert neuroprotective effect, the administration of nicotine agonist is required before neurological damage occurs by stimulation. Based on this fact, neuroprotective effect by nicotine agonist is induced by the mechanism that continued over influx of calcium due to calcium influx into cells is inhibited [17]. On the other hand, co-administration of nicotine agonist and neurotoxic substances may result in actually excessive influx of calcium into cells than usual. The mechanism of this nicotinic neuroprotective effect has not been yet completely clarified, therefore, we intend to examine the mechanism of action of nicotine and GTS-21 in this field. Furthermore, we estimated the effect of nicotine and GTS-21 by cell death in this study. However, it would be more important that we study not only the result of cell death but also the process to elucidate the mechanism of neuroprotection.

LPS is a polysaccharide which constitutes cell wall of gram-negative bacteria, it is an immune cell stimulating material known to be an endotoxin [41]. Furthermore, LPS combines with specifically Toll like receptor 4(TLR4) to activate microglia resulting in the release of inflammatory cytokines and NO. These reactions are suggested to exert on survival or functions of nerve cells [27, 28, 42]. In the present study, we clarified that both nicotine and GTS-21 were able to inhibit

LPS-stimulated TNFα gene expressions but not IL-1β. The results support the finding that nicotine regulates the release of TNFα in microglial primary cultured cells [43]. Thus, nicotine could regulate TNFα production at gene level through α7-nAChR in the hippocampal slice cultures. In contrast, lack of the inhibitory effects of nicotine and GTS-21 on LPS-stimulated IL-1β expression suggest that there exists different control mechanism in inflammatory cytokine production in microglia. In this respect, further detailed study is required. Also, whether the abilities of nicotine and GTS-21 to inhibit LPS-induced TNFα are really mediated through α7-nAChR remains to be determined. Activation of microglia including the release of inflammatory cytokines such as IL-1β and TNFα has also been observed during the development of neurodegenerative conditions such as Alzheimer's and Parkinson's diseases [44]. It will be very useful to develop a therapeutic drug, which has neuroprotective and anti-inflammatory effects via α7-nAChR for neurodegenerative diseases including Alzheimer's disease.

In conclusion, the present study has revealed α7-nAChR agonist, nicotine and GTS-21 are able to inhibit KA-induced neurotoxicity and LPS-induced TNFα gene expression.

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ニコチンおよびGTS-21によるa7ニコチン受容体を介した神経保護作用について

～海馬切片培養系を用いた検討～

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要　旨

ニコチンはたばこに含まれる主な化合物であり，ニコチン様アセチルコリン受容体（nAChR）の作用薬として知られ，認知能力を向上する等の報告がある．3-(2,4-ジメトキシベンジリジン)-アナバセイン(GTS-21）は海洋虫から得られたアナバセインの誘導体で，α7 選択的な nAChR (α7-nAChR）の作用薬である．ニコチンと GTS-21はアルツハイマー病の治療薬として期待されていた．いくつかの研究は，ニコチンと GTS-21はnAChR特にα7-nAChRに作用する事によりニューロンを保護することを示している．α7-nAChR は炎症の調節に重要な役割を担っているという報告がある．この研究の目的は海馬切片培養系を用いて，ニコチンおよびGTS-21の神経保護および抗炎症作用について検討することである．カイニン酸(KA，5-50 μM）は海馬切片培養において，用量依存的なそして時間依存的な神経細胞死を生じる．ニコチンおよびGTS-21の前処置はKA毒性を減弱する傾向が認められた。海馬 CA3領域特異的な解析においてはニコチンの前処置によりKA で生じる毒性を有意に抑制することがわかった．この結果は，ニコチンはα7-nAChR を介してKA で生じる神経細胞死を保護すること可能性を示唆している．我々はまた，ニコチンおよびGTS-21の抗炎症効果についても検討を行った．海馬切片をニコチンおよび GTS-21で前処置してから，リポポリサッカライド(LPS）で刺激を行った．LPS は TNFα および IL-1β の遺伝子発現を濃度依存的に増加させた．LPS で生じる TNFα遺伝子発現は GTS-21の前処置で抑制されたが，IL-1β の遺伝子発現は抑制されなかった．これらの結果はα7-nAChR はミクログリアの活性化において，炎症性サイトカインを抑制することにより神経保護的な役割を演じている事が示唆された．

Key Words：α7ニコチン様アセチルコリン受容体，ミクログリア, TNFα, カイニン酸，
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