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PRIMARY CULTURES OF RAT CORTICAL MICROGLIA TREATED WITH NICOTINE INCREASES IN THE EXPRESSION OF EXCITATORY AMINO ACID TRANSPORTER 1 (GLAST) VIA THE ACTIVATION OF THE α7 NICOTINIC ACETYLCHOLINE RECEPTOR

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Abstract—Although the clearance of glutamate from the synapse under physiological conditions is performed by astrocytic glutamate transporters, their expression might be diminished under pathological conditions. Microglia glutamate transporters, however, might serve as a back-up system when astrocytic glutamate uptake is impaired, and could have a prominent neuroprotective function under pathological conditions. In the current study, the effect of nicotine, well known as a neuroprotective molecule, on the function of glutamate transporters in cultured rat cortical microglia was examined. Reverse transcription polymerase chain reaction and pharmacological approaches demonstrated that, glutamate/aspartate transporter (GLAST), not glutamate transporter 1 (GLT-1), is the major functional glutamate transporter in cultured cortical microglia. Furthermore, the α7 subunit was demonstrated to be the key subunit comprising nicotinic acetylcholine (nACh) receptors in these cells. Treatment of cortical microglia with nicotine led to a significant increase of GLAST mRNA expression and ^{14}C-glutamate uptake in a concentration- and time-dependent manner, which were markedly inhibited by pretreatment with methyllycaconitine, a selective α7 nACh receptor antagonist. The nicotine-induced expression of GLAST mRNA and protein is mediated through an inositol trisphosphate (IP_3) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) depend intracellular pathway, since pretreatment with either xestospongin C, an IP_3 receptor antagonist, or KN-93, a CaMKII inhibitor, blocked GLAST expression. Together, these findings indicate that activation of nACh receptors, specifically those expressing the α7 subunit, on cortical microglia could be a key mechanism of the neuroprotective effect of nACh receptor ligands such as nicotine.

Key words: nicotinic acetylcholine receptor, microglia, glutamate transporter, inositol triphosphate, Ca^{2+}/calmodulin-dependent protein kinase II.

INTRODUCTION

Microglia are known to act as the main immunologic effecter cells in the central nervous system (CNS) (Kettenmann and Verkhratsky, 2008). In general, it is well recognized that activated microglia, in response to illness or injury, produce various cytotoxic molecules, such as proinflammatory cytokines and chemokines, which are associated with processes that could lead to the initiation and development of neurodegenerative diseases (Gao et al., 2002; Sargsyan et al., 2005). On the other hand, emerging evidence also suggests that activated microglia under pathological conditions could act as effectors of processes that promote tissue recovery. Recent studies have demonstrated that activated microglia produce neuroprotective molecules, such as brain-derived neurotrophic factor, glial cell-derived neurotrophic factor, transforming growth factor (TGF)-β and tumor necrosis factor (TNF)-α (Suzuki et al., 2004; Lai and Todd, 2008; Polazzi and Monti, 2010). It has been demonstrated that transient middle cerebral artery occlusion induces a significant increase in the size of brain tissue infarction and the number of apoptotic cells in the region of injury in transgenic mice in which microglia were selectively ablated compared to that of wild-type mice (Lalancette-Hébert et al., 2007). Moreover, in in vitro analysis of cultured cells, treatment of cerebellar granule neurons with microglial-conditioned medium led to decreased neuronal death induced by 6-hydroxydopamine-TGF-β2 produced in the conditioned medium could be involved in the neuroprotective effect of microglia (Polazzi et al., 2009). Together, these observations demonstrate that microglia have a significant neuroprotective function under pathological conditions.

Since excessive glutamate neurotransmission could be involved in the initiation of various neurodegenerative diseases, the regulation of synaptic glutamate

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Abbreviations: BSS, balanced salt solution; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; DHK, dihydrokainic acid; DMEM, Dulbecco's modified Eagle's medium; EAATs, excitatory amino acid transporters; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GLAST, glutamate/aspartate transporter; GLT-1, glutamate transporter 1; Iba1, ionized Ca^{2+}-binding adaptor 1; IP_3, inositol trisphosphate; MLA, methyllycaconitine; nACh, nicotinic acetylcholine; SDS, sodium dodecyl sulfate; t-PDC, L-*trans*-pyrrolidine-2,4-dicarboxylic acid; TGF, transforming growth factor; TNF, tumor necrosis factor; xesC, xestospongin C.

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concentration through excitatory amino acid transporters (EAATs) located in glial cells, mainly EAAT1 (glutamate/aspartate transporter; GLAST) and EAAT2 (glutamate transporter 1; GLT-1), is important for maintaining low concentrations of excitatory amino acids (Robinson and Dowd, 1997). In general, under physiological conditions, the clearance of glutamate in the synapse is performed by astrocytic glutamate transporters, the expression of which might be significantly diminished under pathological conditions (Fine et al., 1996; Xin et al., 2009). Microglia glutamate transporters, however, might serve as a back-up system when astrocytic glutamate uptake is impaired (López-Redondo et al., 2000; Xin et al., 2009). Thus, microglia glutamate transporters could have greater prominence under pathological compared to normal conditions. The expression and function of glutamate transporters in microglia, and their involvement in pathological conditions have been previously documented (Morioka et al., 2008). In addition, microglia themselves are neuroprotective in that they remove excess extracellular synaptic glutamate through glutamate transporters (Shaked et al., 2005; Beschorner et al., 2007a). However, a specific mechanism that regulates glutamate transporter function in microglia has yet to be elaborated.

Accumulating evidence has demonstrated that the neuroprotective effect of nicotine in the CNS is mediated through nicotinic acetylcholine (nACh) receptors. For example, it has been shown that persistent stimulation of nACh receptors prevents glutamate- and amyloid β protein-induced neurotoxicity (Kihara et al., 2001). Furthermore, activation of nACh receptors with nicotine prevents cytotoxicity of dopaminergic neurons in acute and chronic Parkinson's disease models in rats (Takeuchi et al., 2009; Suzuki et al., 2013). In addition, the stimulation of the α7 nACh receptor prevents the production of reactive oxygen species in microglia that were stimulated with fibrillar β amyloid peptide (Moon et al., 2008). Although nicotine and nACh receptors might have essential roles in ameliorating neurodegenerative disorders, the regulatory mechanism underlying the neuroprotective action, especially the function of microglial glutamate transporters, of nicotine-nACh receptors is not fully understood.

The current study aimed to further support a neuroprotective role of microglia-expressed glutamate transporters by characterizing the effect of nACh receptor stimulation on glutamate transporters expressed in rat cortical microglia. In addition, the intracellular process induced by nACh receptor activation that led to changes in GLAST expression was elucidated.

EXPERIMENTAL PROCEDURES

Materials

Nicotine was obtained from Nacalai Tesque (Kyoto, Japan). Methyllycaconitine citrate (MLA), U0126, wortmannin and xestospongin C (xesC) were purchased from Tocris Cookson (Bristol, UK). PP2 was obtained from Calbiochem (La Jolla, CA). AG-490, Gö6983, H89 and KN-93 were purchased from Cayman Chemical (Ann Arbor, MI). L-*trans*-pyrrolidine-2,4-dicarboxylic acid (t-PDC) was from Sigma Chemical Co. (St. Louis, MO). Dihydrokainic acid (DHK) and UCPH-101 were obtained from Abcam Biochemicals (Cambridge, UK).

Cell culture

The preparation of cultured rat microglia has been described previously (Morioka et al., 2009, 2013). All procedures used in the experiments were approved by the Animal Care and Use Committee of Hiroshima University Graduate School of Biomedical & Health Sciences, Hiroshima, Japan. In brief, the isolated cerebral cortices were minced, and then incubated with trypsin and DNase I. Dissociated cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and penicillin/streptomycin (100 units/ml and 100 μg/ml, respectively). Thereafter, cell suspensions were plated in 75cm^2 tissue culture flasks (7.5–10 $\times$ 10^6 cells/flask) precoated with poly-L-lysine (10 μg/ml). Cells were maintained in an incubator in 10% CO_2 at 37 °C. After 8–13 days, microglia were prepared as floating cell suspensions by gentle shaking of the culture flask. Aliquots were transferred to 35-mm (5×10^5 cells), 60-mm (2×10^6 cells) diameter dishes or 24-well plate (2×10^5 cells/well). After 1 h, unattached cells were removed by washing with serum-free DMEM. Prepared microglia showed a purity >98% as determined by ionized Ca^{2+}-binding adaptor 1 (Iba1) immunoreactivity.

RT-PCR analysis

Total RNA in cultured cortical microglia was prepared by a previously described method (Chomczynski and Sacchi, 1987) and used to synthesize cDNA with MuLV reverse transcriptase (Applied Biosystems, Foster City, CA) and a random hexamer primer. PCRs were performed with the specific primers indicated in Table 1 and AmpliTag Gold™ (Applied Biosystems) at 95 °C for 10 min followed by 35 cycles of 95 °C for 30 s, the annealing temperature indicated in Table 1 for 30 s, and 72 °C for 2 min with a final extension at 72 °C for 5 min. The resulting PCR products were analyzed on a 1.5% agarose gel and had the size expected from the known cDNA sequence.

Real-time PCR analysis

cDNA synthesized using 1 μg of total RNA in each sample was subjected to real-time PCR assays with specific primers and EXPRESS SYBR® GreenER™ qPCR SuperMix (Invitrogen). The sequences of primers are as follows: GLAST, 5′-CCATCCAGGCCAACGAAA-3′ (forward) and 5′-GCCGAAGCACATGGAGAA-3′ (reverse), GLT-1, 5′-CCACCCTGATGTGGTCAT-3′ (forward) and 5′-CAGCACAGCGGCAATGAT-3′ (reverse), glyceraldehydes-3-phosphate dehydrogenase (GAPDH), 5′-AGCCCAGAACATCATCCCTG-3′ (forward) and 5′-CACCACCTTCTTGATGTCATC-3′ (reverse). Real-time PCR assays were conducted using a DNA engine Opticon