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Nicotine decreases the activity of glutamate transporter type 3

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HIGHLIGHTS

- Nicotine (0.001–1 μM) resulted in a time- and dose-dependent decrease in EAAT3 activity in a *Xenopus* oocyte expression system.
- Nicotine decreases EAAT3 activity, but seems to be dependent on PKC and PI3K.
- Our results may provide an additional mechanism for nicotine-induced seizure.

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ABSTRACT

Nicotine, the main ingredient of tobacco, elicits seizures in animal models and cigarette smoking is regarded as a behavioral risk factor associated with epilepsy or seizures. In the hippocampus, the origin of nicotine-induced seizures, most glutamate uptake could be performed primarily by excitatory amino acid transporter type 3 (EAAT3). An association between temporal lobe epilepsy and EAAT3 downregulation has been reported. Therefore, we hypothesized that nicotine may elicit seizures through the attenuation of EAAT3 activity. We investigated chronic nicotine exposure (72 h) cause reduction of the activity of EAAT3 in a *Xenopus* oocyte expression system using a two-electrode voltage clamp. The roles of protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K) were also determined. Nicotine (0.001–1 μM) resulted in a time- and dose-dependent decrease in EAAT3 activity with maximal inhibition at nicotine concentrations of 0.03 μM or higher and at an exposure time of 72 h. V_{max} on the glutamate response was significantly reduced in the nicotine group (0.03 μM for 72 h), but the K_m value of EAAT3 for glutamate was not altered. When nicotine-exposed oocytes (0.03 μM for 72 h) were pretreated with phorbol-12-myristate-13-acetate (PMA, a PKC activator), the nicotine-induced reduction in EAAT3 activity was abolished. PKC inhibitors (staurosporine, chelerythrine, and calphostin C) significantly reduced basal EAAT3 activity, but there were no significant differences among the PKC inhibitors, nicotine, and PKC inhibitors + nicotine groups. Similar response patterns were observed among PI3K inhibitors (wortmannin and LY294002), nicotine, and PI3K inhibitors + nicotine. In conclusion, this study suggests that nicotine decreases EAAT3 activity, and that this inhibition seems to be dependent on PKC and PI3K. Our results may provide an additional mechanism for nicotine-induced seizure.

1. Introduction

Tobacco is a popular product world-wide despite the fact that nicotine causes toxicity and dependency. Pharmacologically, nicotine (methylpyridylpyrrolidine) is a water-soluble liquid alkaloid (Lavoie and Harris, 1991) which causes diverse central nervous system (CNS) effects, ranging from reduction of anxiety to seizure and coma (Picciotto et al., 2002). Smoking is regarded as a behavioral risk factor associated with epilepsy or seizure in epidemiological studies. People with epilepsy were found to smoke cigarettes more often than those without epilepsy (38.8% vs. 24.9%) (Kobau et al., 2004). In addition, participants reporting current cigarette smoking have an increased risk of seizure in comparison with those who never smoke (relative risk 2.60, 95% confidence interval 1.53–4.42) (Dworetzky et al., 2010). Moreover, accidental nicotine ingestion has been shown to cause seizures in children and adults (Lavoie and Harris, 1991; Smolinske et al., 1988).

Glutamate transporters, also known as excitatory amino acid transporters (EAATs), are a family of high-affinity,

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sodium-dependent transporters that span the plasma membrane of glia and neurons and contribute to the clearance of glutamate from the extracellular space in order to maintain synaptic glutamate concentrations and prevent excitotoxicity (Danbolt, 2001). Substantial alterations in glutamate transport may be implicated in a wide spectrum of neurologic disorders, such as amyotrophic lateral sclerosis, epilepsy, Huntington's disease, Alzheimer's disease, ischemic stroke injury, white matter injury, and schizophrenia (Beart and O'Shea, 2007; Maragakis and Rothstein, 2001; Sheldon and Robinson, 2007).

Five different types of EAATs have been cloned. EAAT1 and EAAT2 are localized mainly in glial cells, EAAT3 and EAAT4 in neurons, and EAAT5 in the retina. In the hippocampus, abundantly expressed EAAT3 (Danbolt, 2001) may play a major role as glutamate transporter because many synapses are not encompassed by astrocytes (Bergles et al., 1999). Temporal lobe seizures are related to hippocampal sclerosis, a characteristic feature of hippocampal pathology (Bouilleret et al., 1999; Wieser, 2004). In addition, dysfunction of EAAT3 has been reported to be related to temporal lobe epilepsy (Crino et al., 2002; Mathern et al., 1999), and in vivo electrophysiological studies have revealed that nicotine-induced seizures originate in the hippocampus (Cohen et al., 1981; Floris et al., 1964). Therefore, nicotine may elicit seizures through attenuation of EAAT3 activity.

In this study, we examined the effects of nicotine on EAAT3 activity expressed in *Xenopus* oocytes and investigated the roles of protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K), two intracellular signaling molecules, in the effect of nicotine on EAAT3.

2. Experimental procedures

The study protocol was approved by the Institutional Animal Care and Use Committee at Seoul National University College of Medicine (protocol number: 12-0175). Mature female *Xenopus laevis* frogs were purchased from Xenopus I (Dexter, MI). Molecular biology reagents were obtained from Ambion (Austin, TX). Nicotine and other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.1. Oocyte preparation

Xenopus oocytes were harvested and microinjected as previously described (Do et al., 2002a). We anesthetized frogs in 500 ml of 0.2% 3-aminobenzoic acid ethyl ester (Sigma, St. Louis, MO, USA) in water. Frogs underwent an operation on ice after checking for unresponsiveness to toe pinching. Following a 5 mm incision in the lower lateral abdominal region, an ovarian lobule, containing about 150–200 oocytes, was removed. Oocytes were instantly immersed in calcium-free OR-2 solution (NaCl 82.5 mM, KCl 2 mM, $MgCl_2$ 1 mM, HEPES 5 mM, and 0.1% collagenase type Ia; pH = 7.5) to remove the vitelline membrane. Oocytes were defolliculated by gentle shaking for nearly 2 h, and then incubated for one day in modified Barth's solution (NaCl 88 mM, KCl 1 mM, $NaHCO_2$ 2.4 mM, $CaCl_2$ 0.41 mM, $MgSO_4$ 0.82 mM, $Ca(NO_3)_2$ 0.3 mM, gentamicin 0.1 mM, and HEPES 15 mM; pH = 7.6) at 18 °C. Fully grown stage V or VI *Xenopus* oocytes were selected for the following experiments (Hollmann et al., 2000).

2.2. Expression of EAAT3

The complementary DNA (cDNA) of rat EAAT3 was provided by Dr. M.A. Hediger (Brigham and Women's Hospital, Harvard Institute of Medicine, Boston, MA, USA). The cDNA was subcloned into a commercial vector (Bluescript-SKm), and plasmid DNA was linearized using a restriction enzyme (Not I). We synthesized the messenger RNA (mRNA) in vitro using a transcription kit (Ambion, Austin, TX, USA). EAAT3 mRNA was quantified spectrophotometrically and diluted in sterile water. Thirty nanoliters of this mRNA (1 ng/nl) was injected into the cytoplasm of oocytes with an automated microinjector (Nanoject; Drummond Scientific Co., Broomall, PA, USA). The prepared oocytes were subsequently incubated at 18 °C for 3–4 days to express EAAT3 before electrophysiologic recording.

2.3. Electrophysiological recording

We measured electrophysiological changes at room temperature (21–23 °C). Microelectrodes were prepared with a micropipette puller and 10 μl glass capillary tubes (Drummond Scientific Co.). The diameter of the microelectrode tips was adjusted to approximately 10 μm by breaking the tip and the resistance of the microelectrode was estimated at 1–5 MΩ when filled with 3 M KCl. Oocytes were perfused with Tyrode's solution (NaCl 150 mM, KCl 5 mM, $CaCl_2$ 2 mM, $MgSO_4$ 1 mM, dextrose 10 mM, and HEPES 10 mM; pH = 7.5) at a flow rate of 3 ml/min before measuring the currents. A single oocyte was voltage-clamped using a two-electrode oocyte voltage clamp amplifier (OC725-C: Warner Co., New Haven, CT, USA), with a holding potential of −70 mV and the evoked currents were analyzed with the Ooclamp software program. Data from oocytes that did not show a stable holding current of less than 0.6 μA were discarded. L-Glutamate was diluted in Tyrode's solution and perfused over an oocyte for 20 s at a rate of 3 ml/min. Inward currents yielded by superfusion of L-glutamate were recorded at 125 Hz for 1 min (baseline: 5 s, L-glutamate application: 20 s, washing with Tyrode's solution: 35 s). We measured the response induced by L-glutamate, which was calculated by integration of the inward currents and described as microCoulombs (μC). The response should reflect the sum of the total amount of transported glutamate. At least three different frogs were used for analysis in all of the experiments.

2.4. Chemicals

Nicotine ((−)-nicotine hydrogen tartrate), phorbol-12-myristate-13-acetate (PMA), staurosporine, chelerythrine, calphostin C, LY294002, and wortmannin were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA).

2.5. Experimental chemical treatment

To evaluate the dose–response effect of nicotine on EAAT3 activity, oocytes were incubated in nicotine at serial concentrations (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, and 1 μM) by dilution in modified Barth's solution for 72 h. In the control group, oocytes were incubated in modified Barth's solution alone. In our previous study, the median effective concentration of glutamate that induced EAAT3 activity was 27.2 μM (Do et al., 2002b), so we used 30 μM glutamate as an agonist in this study. To study the effects of nicotine on the V_{max} and K_m of EAAT3 with L-glutamate, we used serial concentrations of L-glutamate (3, 10, 30, 100, and 300 μM).

To investigate PKC involvement in the effects of nicotine on EAAT3 activity, PKC activator (100 nM PMA) or PKC inhibitor (100 μM chelerythrine, 2 μM staurosporine, or 9 μM calphostin C) was applied to the oocytes for 10 min or 1 h, respectively, before recording the currents. Oocytes were exposed to the PI3K inhibitors (10 μM wortmannin and 50 μM LY294002) for 1 h to investigate the effect of PI3K inhibition on EAAT3 activity. To evaluate time course of the effects of nicotine exposure on the activity of EAAT3, oocytes were incubated in nicotine for 24, 48, and 72 h. The reversibility of the nicotine effect on EAAT3 activity was investigated by washing

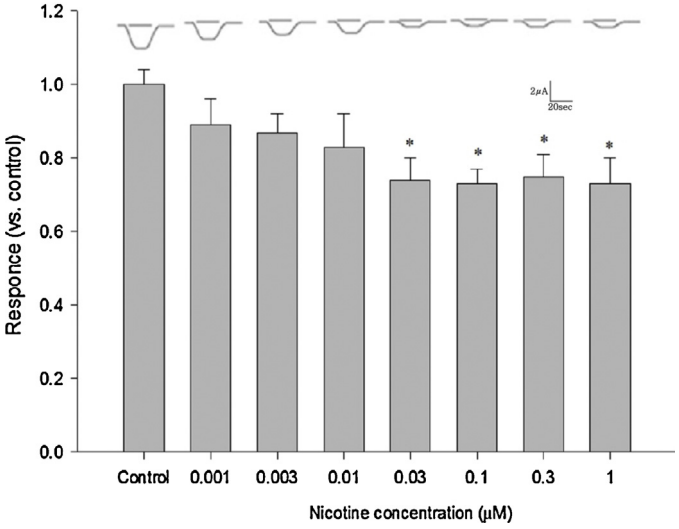

Fig. 1. Concentration–response of nicotine to the activity of EAAT3. Oocytes were exposed to serial concentrations of nicotine (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, and 1 μM) for 72 h before the response to 30 μM of L-glutamate was measured. The means are expressed as a bar plot with the standard error of the mean (S.E.M.) shown as an error bar (n = 15-27). *P < 0.05 compared to control.

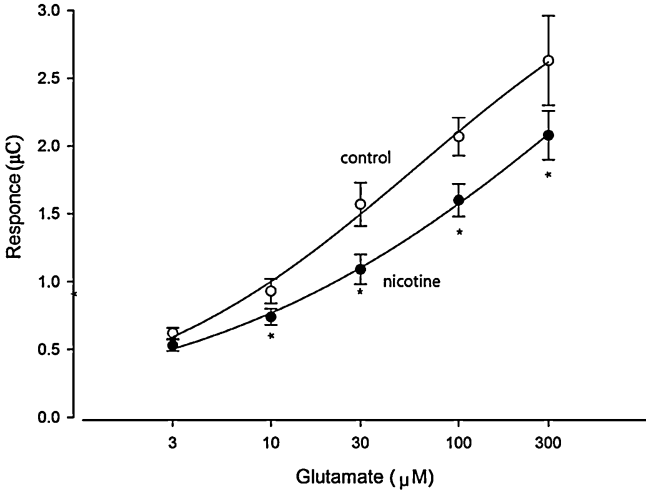

Fig. 2. Dose–response effects of EAAT3 to L-glutamate in the presence or absence of nicotine. In the nicotine group, oocytes were exposed to 0.03 μM nicotine for 72 h. Nicotine significantly decreased the responses induced by 10, 30, 100, and 300 μM L-glutamate. Data are shown as the mean ± S.E.M. (n = 16–20). *P < 0.05 versus the corresponding controls.

oocytes in modified Barth's solution for 12 or 24 h after incubation in nicotine for 72 h.

2.6. Statistical analysis

Data are expressed as mean ± S.E.M. Responses were sometimes normalized to the same-day controls for each oocyte batch because of batch-to-batch variations of EAAT3 expression in oocytes. Statistical analysis was performed using the one-way analysis of variance (ANOVA) with Student–Newman–Keuls as post hoc comparison or unpaired Student's t-test, when appropriate after Kolmogorov–Smirnov normality test. Data were analyzed using SigmaStat version 4.0 (San Jose, CA) and Prism version 5.0 (Graphpad, San Diego, CA). P values < 0.05 were considered significant.

3. Results

Inward currents were produced by oocytes that were provided with L-glutamate only when they had been injected with EAAT3 mRNA, compared to no response to L-glutamate from oocytes that had not been injected with EAAT3 mRNA independent of incubation in 0.03 and 0.3 μM nicotine for 72 h (data not shown). When oocytes were exposed to sequentially higher concentrations of nicotine, the responses decreased in a concentration-dependent manner, with a statistically significant decrease at 0.03 μM (P = 0.004), 0.1 μM (P = 0.024), 0.3 μM (P = 0.033), and 1 μM (P = 0.02) nicotine compared to the control group (Fig. 1). Nicotine resulted in a concentration-dependent reduction in EAAT3 activity at low concentrations, although the IC_{50} value of nicotine could not be calculated from the dose–response data because they did not fit the Hill equation. Since the inhibition reached a maximum at concentrations of 0.03 μM or higher, we used 0.03 μM for further experiments. The EAAT3 response to L-glutamate in the presence of 0.03 μM nicotine was reduced by approximately 25% (Fig. 1).

When oocytes were exposed to serial concentrations of L-glutamate at 3–300 μM with or without nicotine, the responses decreased significantly in the nicotine group (Fig. 2). Further analysis of the data showed that V_{max} on the glutamate response was significantly reduced in the nicotine group (control: 2.7 ± 0.1 μC and nicotine: 2.1 ± 0.1 μC, P < 0.001), but the K_m value of EAAT3 for glutamate was not altered (control: 18.6 ± 2.9 μM and nicotine: 20.4 ± 2.9 μM, P = 0.662; Fig. 2).

EAAT3 activity was significantly increased in oocytes pretreated with PMA (control: 1.00 ± 0.07 and PMA: 1.28 ± 0.11, P = 0.01; Fig. 3). When nicotine-treated oocytes (0.03 μM for 72 h) were exposed to PMA, the nicotine-induced inhibition of EAAT3 activity was abolished (nicotine: 0.73 ± 0.05 and PMA + nicotine: 0.99 ± 0.06, P = 0.02; Fig. 3).

Preincubation of oocytes with 2 μM staurosporine significantly reduced EAAT3 activity (control: 1.00 ± 0.05 and staurosporine: 0.76 ± 0.06, P = 0.004). Oocytes exposed to staurosporine, nicotine, or staurosporine plus nicotine showed a significant decrease in EAAT3 activity as compared with untreated controls. However, the EAAT3 activity was not significantly different among oocytes treated with staurosporine, nicotine, or staurosporine plus nicotine (Fig. 4), suggesting the absence of an additive or synergistic interaction between the effects of staurosporine and nicotine on EAAT3 activity. Similar patterns of responses were recorded for other PKC

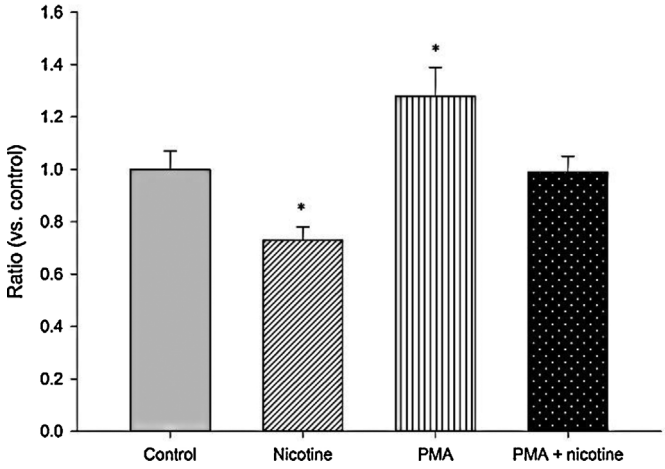

Fig. 3. Effects of protein kinase C (PKC) activation on EAAT3 activity in the presence or absence of 30 μM nicotine for 72 h. When nicotine-exposed ooctyes (0.03 μM for 72 h) were pretreated with phorbol-12-myristate-13-acetate (PMA, PKC activator: 100 nM for 10 min), the nicotine-induced reduction in EAAT3 activity was abolished. Data are described as mean ± S.E.M. (n = 20–23). *P < 0.05 compared to control.

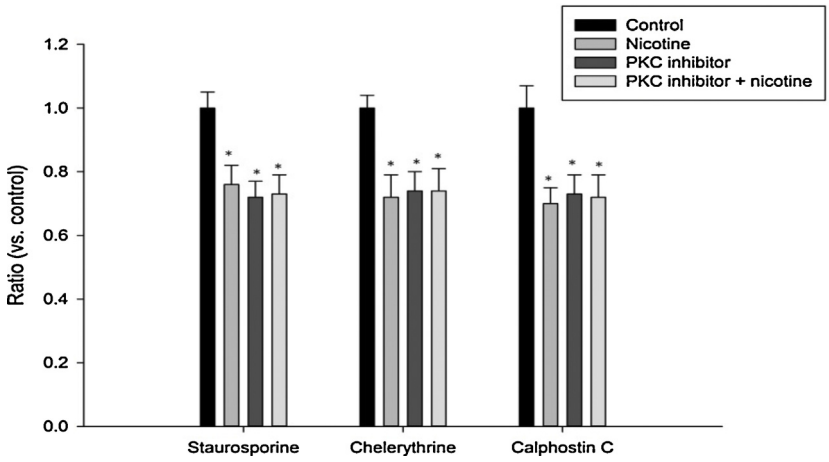

Fig. 4. Effects of protein kinase C (PKC) inhibition on EAAT3 activity in the presence or absence of 0.03 μM nicotine for 72 h. Oocytes exposed to PKC inhibitor, nicotine, or PKC inhibitor plus nicotine showed a significant decrease in EAAT3 activity compared to control, but the EAAT3 activity in oocytes treated by PKC inhibitor, nicotine, or PKC inhibitor plus nicotine were not different significantly. Data are expressed as mean ± S.E.M. (n = 17–23). *P < 0.05 compared to control.

inhibitors (chelerythrine [100 μM for 1 h] and calphostin C [9 μM for 1 h]) (Fig. 4).

EAAT3 activity was significantly decreased by pretreatment of oocytes with PI3K inhibitor wortmannin (10 μM for 1 h) (control: 1.0 ± 0.04 and wortmannin: 0.74 ± 0.06, P = 0.013; Fig. 5), however, there were no differences in response among the wortmannin, nicotine, and wortmannin plus nicotine groups (Fig. 5). Likewise, another PI3K inhibitor, LY294002 (50 μM for 1 h) showed a similar pattern of responses (Fig. 5).

A time course of the effect of nicotine exposure on EAAT3 activity showed a time-dependent reduction in EAAT3 activity with a significant difference only between the control and 72 h treated groups (P = 0.031; Fig. 6).

Oocytes exposed to nicotine were removed and then incubated in modified Barth's solution. In this washout study, EAAT3 activity still showed a significant inhibition compared to that of the control at 12 h (P = 0.006). However, EAAT3 activity recovered toward the control level at 24 h, which indicated that the effects of nicotine exposure on EAAT3 activity were reversible (P = 0.231; Fig. 7).

4. Discussion

The results of the present study demonstrate that nicotine decreases EAAT3 activity in a time- and dose-dependent manner. In addition, the effects of nicotine on EAAT3 activity seem to be mediated by PKC and PI3K.

The brain/blood ratio for nicotine concentration was 3–4 when nicotine concentrations plateaued in both blood and brain, indicating preferential distribution of nicotine toward brain tissue (Benowitz, 1990; Ghosheh et al., 2001; Rowell and Li, 1997). Considering that plasma nicotine concentrations in smokers range from 0.625 nM to 0.31 μM (median value: 0.07 μM) (Schneider et al., 2001; Taylor et al., 1986), the expected concentration in the brain would be 1.875 nM–1.24 μM (approximate median value: 0.245 μM). To approximate the putative brain concentration of nicotine while smoking, we thus studied nicotine concentrations from 1 nM to 1 μM for evaluating the dose–response influence of nicotine on EAAT3 activity. In addition, we exposed oocytes to nicotine for 72 h to simulate the chronic effects of nicotine.

A 0.03 μM concentration of maximal effect found in this study is much lower than the median value of the nicotine concentration in the brain (0.245 μM). A prospective study of smoking as a risk factor for seizure or epilepsy found no correlation between the risk of seizure and the number of cigarettes smoked daily, but seizure development increased incrementally with pack-years of smoking (relative risk 1.03, 95% confidence interval 1.02–1.05, per

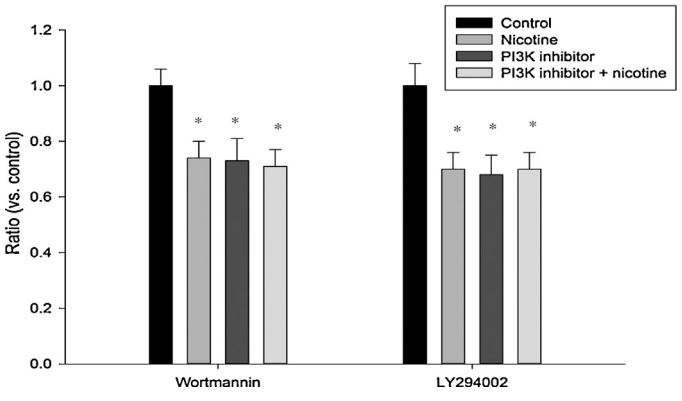

Fig. 5. Effects of phosphatidylinositol 3-kinase (PI3K) inhibition on EAAT3 activity in the presence or absence of 0.03 μM nicotine for 72 h. When oocytes were preincubated with a PI3K inhibitor before measurement, EAAT3 activity was significantly reduced. The response was the same in oocytes exposed to PI3K inhibitor, nicotine, or PI3K inhibitor plus nicotine. Data are expressed as mean ± S.E.M. (n = 17–22). *P < 0.05 compared to control.

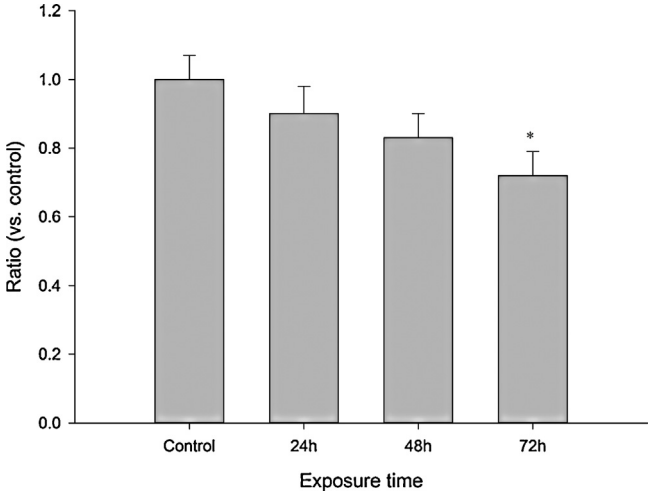

Fig. 6. Time course of the effects of nicotine exposure on the activity of EAAT3. Oocytes were exposed to 0 03 μM nicotine for 24, 48, and 72 h. Incubation in 0.03 μM nicotine for 72 h showed a significantly lower EAAT3 activity than controls. Data are shown as mean ± S.E.M. (n = 18–23). *P < 0.05 compared to control.

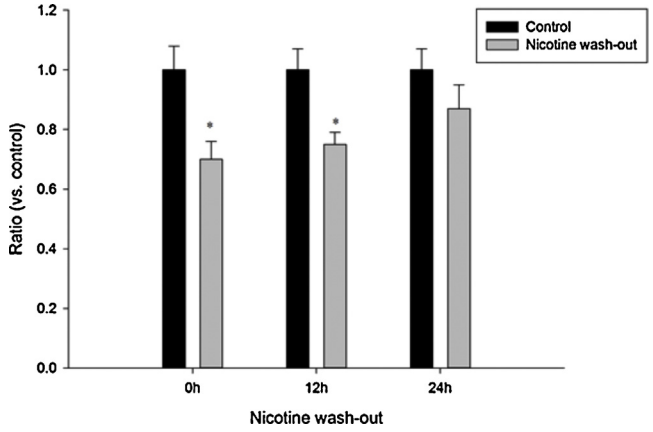

Fig. 7. Effects of nicotine washout on the activity of EAAT3. Oocytes were exposed to 0.03 μM nicotine for 72 h and washed out in modified Barth's solution for 12 or 24 h. EAAT3 activity returned to the control level at 24 h. Data are described as mean ± S.E.M. (n = 14–16). *P < 0.05 compared to control.

pack-year) (Dworetzky et al., 2010). Thus, the duration of exposure to nicotine could be more important for seizure development than the amount of smoking or blood concentration of nicotine. In our time course study, nicotine attenuated the activity of EAAT3 in a time-dependent manner. In this sense, continuous smoking since school age may be a risk for seizure development. According to a recent study, educational games may provide superior performance about student' knowledge about smoking to lectures (De Vitta et al., 2013).

Several underlying mechanisms of nicotine-induced seizure have been proposed, all of which involve initial activation of central nicotinic acetylcholine receptor (nAChR). First, glutamate release and increased glutamatergic synaptic transmission could elicit seizures in animal experimental models. Damaj et al. (1999) suggested the involvement of mainly α7 nAchR, L-type calcium channels, N-methyl-D-aspartate (NMDA) receptors, and nitric oxide (NO) formation in nicotine-induced seizures. In addition, nicotine is known to enhance the production of NO through glutamate release and activation of NMDA receptors in the rat hippocampus (Fedele et al., 1998). Second, reduced GABAergic input to CA1 pyramidal cells through the nAchR in the hippocampus may mediate nicotine-induced excitability or seizures (Chiodini et al., 1999; Dobelis et al., 2003). Third, oxidative stress resulting from the depletion of glutathione (GSH) may trigger nicotine-induced seizure (Yildiz et al., 1998, 1999). Oxidative free radicals may play a pivotal role in epileptogenesis (Aguiar et al., 2012). Oxidative stress has been shown to be associated with decreased bioavailable NO, and increased production of reactive oxygen species and reactive nitrogen species (Tabima et al., 2012).

EAAT3 was reported to have functions which are relevant to the suggested mechanisms of nicotine-induced seizure: (1) EAAT3 could block excessive NMDA activity (Nieoullon et al., 2006); (2) EAAT3 plays a role in direct transport of glutamate which is the precursor for GABA synthesis in GABAergic neurons of the hippocampus (Sepkuty et al., 2002) and reduced EAAT3 activity may cause seizures as a result of diminished presynaptic GABA release (Maragakis and Rothstein, 2001); and (3) since the affinity of EAAT3 for cysteine is equivalent to that for glutamate, EAAT3 may mediate uptake of cysteine into neurons (Nieoullon et al., 2006; Shanker et al., 2001), which is related to the synthesis of glutathione, a major antioxidant agent (Dringen, 2000). Neuronal glutathione deficiency was reported in EAAT3-deficient mice (Aoyama et al., 2006).

Our results showed that nicotine attenuated the activity of EAAT3. Therefore, dysfunction of EAAT3 may be involved in the mechanism and pathway of nicotine-induced seizure.

Xenopus oocytes used in our study may have cholinergic receptors but these receptors are muscarinic (Kusano et al., 1982). Furthermore, oocytes that were not injected with EAAT3 mRNA and exposed to nicotine were unresponsive to L-glutamate in our study. Most of the CNS effects of nicotine are mediated by nAChR. However, nicotine could have effects on brain independent of nAChR (Ferrea and Winterer, 2009).

The activity of EAAT3 can be regulated by cell surface expression (redistribution between plasma membrane and cytosol, trafficking) and/or affinity for glutamate (Davis et al., 1998; Lee et al., 2005). Our kinetic study showed decreased V_{max} but no significant change in K_m of EAAT3 for glutamate. This suggested that nicotine redistributed EAAT3 from the plasma membrane to intracellular pools without changing the affinity of EAAT3 for glutamate (Davis et al., 1998). Interestingly, in our previous study, effects of caffeine on EAAT3 activity showed similar kinetic results (Shin et al., 2013).

We investigated the involvement of PKC and PI3K in nicotine effects on EAAT3 activity. PKC activation was shown to increase the expression of the transporter at the plasma membrane (Guillet et al., 2005) and to stimulate glutamate uptake in brain homogenates (Casado et al., 1993). In terms of glutamate transporter, enhanced PKC activity was shown to increase EAAT3 activity, whereas it was reported to reduce EAAT2 function (Gonzalez and Robinson, 2004).

PI3K has also been demonstrated to modulate EAAT3 activity independently by cell trafficking, like PKC (Davis et al., 1998; Sims et al., 2000). However, PKC could function as a downstream signal of PI3K (Ettinger et al., 1996; Frey et al., 2006; Toker and Cantley, 1997). This study using wortmannin and LY294002, the PI3K inhibitors, also suggested the involvement of PI3K in mediating the effect of nicotine on EAAT3 activity.

In experiments using PKC inhibitors (staurosporine, chelerythrine, and calphostin C) and PI3K inhibitors (wortmannin and LY294002), we could not find additive or synergistic interactions between nicotine and PKC inhibitors or PI3K inhibitors. In addition, nicotine may inhibit PKC activity in deactivation of the major neuronal nicotinic receptor (Eilers et al., 1997). Taken together, the above results suggest that nicotine attenuates EAAT3 activity through PKC and PI3K. To confirm this conclusion, further study will be needed testing directly if nicotine is inhibiting or altering in any way PKC and testing directly the effect of nicotine on PKC and PI3K enzymatic activities in oocytes, probably in vitro. Our study demonstrated that downregulation of EAAT3 activity by nicotine is time-dependent and reversible.

In conclusion, we found that nicotine exposure decreased EAAT3 activity presumably by mediation of PKC and PI3K. This study may suggest a novel mechanism for nicotine-induced seizure.

Conflict of interest statement

The authors report no conflicts of interests related to this experiment.

Acknowledgments

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