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Chronic Exposure of Nicotine Modulates the Expressions of the Cerebellar Glial Glutamate Transporters in Rats

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Rats were given nicotine (25 ppm) in their drinking water at the start of their mating period in order to study the expressions of glutamate transporter subtypes in cerebellar astrocytes following the chronic exposure of nicotine after mating. After the offspring were delivered, each group was divided into two subgroups. One group, the control group, was given distilled water only and the other group, the experimental group, was given distilled water containing nicotine. The cerebellar astrocytes were prepared from 7 day-old pups at each group. Ten days after the cells were cultured, the expression of the glutamate transporter subtypes (GLAST and GLT-1) was determined using immunochemistry and immunoblotting. During the continuous treatments, the developmental expression patterns of the GLAST and GLT-1 in the cerebellum were also determined from 2, 4 and 8 week-old rats. The expression levels of GLAST in cultured astrocytes of both the pre- or post-natally exposed groups were higher than those of the control group. However, these expression levels of the continuously exposed group were lower than those of the control group. Compared to those of the control group, the GLT-1 expression levels of all the nicotine-treated groups were higher, particularly in the continuously treated group.. According to the results from the immochemistry procedure, the cerebellar GLAST and GLT-1 expression levels of all nicotine-treated groups were lower than those of the control group at each age. However, the immunoblotting procedure showed that the cerebellar GLT-1 expression levels of all the nicotine-treated groups were higher than those of the control group, except for the rats that were continuously exposed for 8 weeks using immunoblotting. These results suggest that the expression of the glial GLAST and GLT-1 are altered differently depending on the initial exposure time and the partcicular period of nicotine exposure. In addition, nicotine exposure during gestation has persistent effects on glial cells.

Key words: Nicotine, Cerebellum, GLAST, GLT-1

INTRODUCTION

Nicotine is a well-known psychostimulant drug with both reinforcing and dependence-producing actions in animals as well as in humans. Nicotine enhances the ion flux and the release of neurotransmitters, and elicits a variety of physiological and behavioral effects, such as locomotor activity, cerebrovasodilatation, convulsions and antinociception (Martin, 1986). Nicotine also plays an important role in the development and flexibility of the synapses (Aramakis and Metherate, 1998). Although nicotine has many effects on the CNS functions, its actions are primarily focused on the catecholamine and acetylcholine producing neurons (Fung *et al.*, 1996; Li *et al.*, 1995; Zhang *et al.*, 1994). It has been reported that nicotine elicits the release of glutamate from a synaptosomal preparation (Perez De La Mora *et al.*, 1991) and that the nicotine-induced release of dopamine may be the result of a nicotine-induced release of glutamate (Garcia-Munoz *et al.*, 1996). Furthermore, nicotine can affect the cognitive abilities and protect the organism from the toxicity of glutamate-induced cellular damage (Akaike *et al.*, 1994; Borlongan *et al.*, 1995; Gattu *et al.*, 1997; Birtwistle and Hall, 1997). There is evidence suggesting that prenatal and early postnatal nicotine exposure affects the nervous system development and behaviors, such as locomotor activity and cognitive performance (Newman *et al.*, 1999). Prenatal nicotine exposure modifies behavior throughout the early stages of development. Such effects include impaired

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attention, impulsivity and deficits in cognitive control (Ajarem and Ahmad, 1998; Roth *et al.*, 2000). In addition, prenatal and neonatal nicotine exposure induces permanent changes in the brain nicotine receptors and causes hyperactivity during adolescence as well (Nordberg *et al.*, 1991; Thomas *et al.*, 2000; Tizabi *et al.*, 2000). This suggests that prenatal and postnatal nicotine exposure may alter the development of glutamatergic nervous activity.

Glutamate is a major rapid excitatory neurotransmitter in the mammalian brain. It has various important roles in neurotoxicity and neuronal plasticity (Collingridge and Lester, 1989; Meldrum and Garthwaite, 1990). Moreover, the released glutamate level in the synapse is maintained via glutamate uptake by the glutamate transporters of the astrocytes (Fairman *et al.*, 1995; Mennerick and Zorumski, 1994). In the brain, the majority of glutamate uptake is produced by astroglias, rather than neurons (Nicholis and Attwell, 1990; Rothstein *et al.*, 1996). It is known that excessive glutamate in the synapses can induce neuronal cell death. In addition, a mutual influence has been reported on the development, structure and function between neurons and glial cells during neuroembryogenesis (LoPachin and Aschner, 1993). Therefore, the changes in the glutamate uptake properties of glial cells may affect the glutamatergic nervous activities.

More than three types of glutamate transporters have been reported to exist in the rat brain, such as GLAST, GLT-1 and rEAAC1 (Seal and Amara, 1999). Two of these glutamate transporters (GLAST and GLT-1) exist in glial cells (Kondo *et al.*, 1995). In a recent study, changes in the glutamate uptake properties of the cerebellar glial cells have been reported in both the *in vitro* subacute nicotine-exposed primary cultured cells (Lim *et al.*, 2000) and in the cells prepared from the perinatal nicotine exposed pups (Lim and Kim, 2001). Therefore, the evidence suggests that nicotine may affect the development of the glutamate uptake transporters in glial cells. However, little is known regarding the developmental changes in the glutamate uptake transporters in glial cells from different dose schedules throughout the perinatal periods. Furthermore, in order to regulate the use of nicotine, it is necessary to elucidate the effects of prenatal and postnatal nicotine exposure on the development of the glutamate uptake transporters in glial cells.

This study has thus examined the development of the GLAST and GLT-1 in the cerebellar glial cells and in the cerebellum to determine whether or not they are affected after prenatal or postnatal nicotine exposure.

MATERIALS AND METHODS

Animals and materials

Sixweek-old Spraque-Dawley male and female rats used in this study were purchased from the Taehan Experimental Animal Co. (Taejon, Korea). The animals were housed at room temperature (22-24°C) in a 12 h light/dark cycle. Guinea pig anti-GLAST and anti-GLT-1 antibodies were purchased from Chemicon Int. (Temecula, Ca., U.S.A.). Rabbit anti-GLT-1 was obtained from Oncogene Research Products (Boston, Ma., U.S.A.). The ECL Plus Western Detection System was purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). The fetal bovine and bovine calf serum were purchased from JRH Biosciences (Lenexa, Ks., U.S.A.). All other chemicals were obtained from the Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

Treatment protocol

Eightweek -old male and female rats were treated with either distilled water or 25 ppm nicotine in their drinking water from the beginning of their mating period with access to food *ad libitum*. The offspring rats were subdivided into two groups. The drinking water for half of the distilled water-feeding rats was replaced with a solution containing nicotine, while the drinking water for half of the nicotine-feeding rats was replaced with distilled water. Each group contained more than eight animals. Based on preliminary experiments, the nicotine concentration was chosen so that neither the appearance of the mother nor that of her offspring was affected. Seven days after delivery, the pups in each group were sacrificed and their cerebella were cultured to obtain their granule and glial cells. Two, four and eight weeks after the birth, the male rats in each group were sacrificed and their cerebella were removed.

Determination of the levels of nicotine in the pup's brain and the cerebellum of young rats

The nicotine levels were determined using a slight modification of the method reported by Rop *et al.* (1993). The pup's brain except for the cerebellum and the dissected cerebellum of the young rats were homogenized separately in 50 mM Tris-HCl buffer (pH 7.4). The homogenate was transferred into a screw-capped Teflon tube, mixed with 5 mM pargyline, 5 M KOH, methylene chloride and an internal standard?. The mixture was then shaken in an ice bath for 30 min. This homogenate was subsequently centrifuged at 2,000×g for 20 min at 4°C. After centrifuging, the organic phase was transferred into a new Teflon tube containing 0.5 M HCl and centrifuged at 2,000 ×g for 20 min at 4°C. The resulting acidic aqueous layer was mixed with 5 M KOH and methylene chloride. It was then agitated vigorously for 30 min, and centrifuged at 2,000×g for 20 min at 4°C. The separated organic layer was mixed with 10 mM HCl in methanol and evaporated under nitrogen gas. After one repetition of this procedure, the extract was diluted to the required concentration and analyzed by High Performance Liquid Chromatography

(HPLC) using a UV detector at 260 nm.

Cerebellar glial cell cultures

The cells were prepared using a slight modification of the method reported by McCaslin and Morgan (1987). The seven day-old pups were sacrificed and the cerebella were removed. The cells obtained from four to five cerebella were then mechanically dissociated in Dulbecco's Modified Eagle's Medium (DMEM, pH 7.4). The growth medium containing the DMEM supplemented with 40 mM $NaHCO_3$, 0.15 mM $CaCl_2$, 66 μM, $MgSO_4$, 0.44 mM KCl, 6% fetal bovine serum and 6 % bovine calf serum was replaced. The cells were incubated at 37°C with 10% CO_2. The culture medium was changed after 2 days of stabilization. The growth medium containing 0.15 mM dibutyryl cAMP was used to prepare the cerebellar glial cells. The cells were then returned to the CO_2 incubator. All cells were used 10 days after plating.

Immunocytochemistry of cultured glial cells and cerebellum

The cultured glial cells were washed with phosphate-buffered saline (pH 7.5) and then fixed with cold methanol. The cerebellum were sliced to 20 μm using a microtome and fixed with para-formaldehyde. Each slide was consecutively pretreated with 50 mM Tris-buffered saline (TBS) containing 0.3% H_2O_2 for 30 min, 3.3% goat serum for 1 h and 50 mM TBS containing 0.5% Triton X-100 (TBST) for 1 h. The slides were incubated with the primary antibodies (guinea pig anti-GLAST and guinea pig anti-GLT-1 1:2000) in TBST at 4°C overnight. The excess primary antibodies were removed with three washes and the slides were incubated with peroxidase-conjugated goat anti-guinea pig antibodies (1:400) for 2 h. The excess secondary antibodies were removed with three washes and the slides were then incubated with guinea pig peroxidase-anti-peroxidase (1:600) in TBST for 2 h at room temperature. After three washes, the staining was developed with diaminobenzidine and H_2O_2 for 10 min. After an additional three washes, the slides were dried overnight. The stained slides were defatted with ethanol and xylene, and observed with optical microscopy.

Western blotting for GLT-1

The cultivated cells were homogenized with a 25 mM Tris-HCl (pH 8.0) buffer containing 1 mM EDTA, 150 mM NaCl 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (RIPA buffer) and the protease inhibitors (2 μM leupeptin, 2 μM pepstatin A, 1 μg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 12,800×g for 60 min at 4°C. The supernatant was then subjected to electrophoresis. The dissected cerebellum was homogenized with a 25 mM Tris-HCl (pH 7.4) buffer containing 2 mM EDTA and various protease inhibitors. The homogenate was centrifuged at 70,000×g for 60 min at 4°C and the pellet was then resuspended with the RIPA buffer containing the protease inhibitors. After centrifuging, a similar quantity of protein in each sample (20 μg for the cultured cells and 2.5-10 μg for the tissue) was electrophoresed on 8% polyacrylamide gels. The proteins were transferred electrophoretically to a polyvinylidene fluoride membrane. After the membranes were blocked in TBS containing 1% non-fat dry milk, the blots were washed in TBS containing 0.1% Tween 20 (TTBS) and incubated with rabbit anti-GLT-1 (1:2,000 for the cultured cells and 1:2,500 for the tissues) for 1 h at room temperature. After washing in TTBS, the blots were incubated with HRP-conjugated donkey anti-rabbit (1:5,000) for 1 h. After an additional washing with TTBS, the protein bands were visualized using the ECL Plus Western Blotting Kit. The band intensities were quantified by scanning, and the data is expressed as a ratio to each control group.

Determination of protein concentration

The protein concentrations were determined using the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Statistics

The values are expressed as a mean±S.E.M. The statistical significance was determined by an Analysis of Variance and Dunnett's Test. A *p*-value (under) <0.05 was considered significant.

RESULTS

The nicotine levels in the pup's brain and the cerebellum of young rats

Table I shows that nicotine was detected in all the nicotine-treated groups. The nicotine levels were similar in the brains of both the post- and continuously nicotine-treated pups. A low nicotine concentration was still detected in the brains of the pre-natal nicotine-treated pups. The nicotine levels were also similar in both the cerebellum of the post- and continuously nicotine-treated groups. In addition, the nicotine level in the cerebellum from the prenatal nicotine-treated rats was still detected and was almost the same as that in the nicotine-exposed pups.

The expressions of GLAST and GLT-1 in cultured cerebellar glial cells

Fig. 1 shows the immunochemical photograph of GLAST and GLT-1 in the cerebellar glial cells prepared from each group. The GLAST expression levels in the cerebellar glial cells prepared from the prenatally nicotine-exposed pups was higher, but those from either the postnatally or

continuously nicotine-exposed pups were lower than those of the control pups. The GLT-1 expression levels in the cerebellar glial cells prepared from all the nicotine-exposed pups were higher and more widely distributed than those in the glial cells prepared from the control pups. Fig. 2 shows the immunoblot analysis of the GLT-1 in the cerebellar glial cells prepared from each group. Compared to the glial cells prepared from the control pups, the GLT-1 expression levels were small, but significantly higher in the glial cells prepared from the continuously nicotine-treated pups.

Table I. The nicotine levels in the pup's brain and the cerebellum of young rats after prenatal, postnatal or continuous nicotine exposure

Treatments	Pup's brain	Cerebellum
Control	–	–
Post-treatment	0.186±0.005	0.422± 0.003
Pre-treatment	0.132±0.004	0.153±0.004
Continuous	0.199±0.003	0.450±0.004

At the beginning of their mating period, the rats were treated with nicotine (25 ppm) in their drinking water. After delivery of the offspring pups, each group was divided into two groups, the nicotine group or the distilled water group. Seven days after delivery, the female pups were sacrificed and their cerebellum were obtained to analyze the effects ofnicotine exposure. The same procedure was carried out for the male pups at 8 weeks after birth. The unit used is μg/g weight. The values are a mean±S.E.M. for 5 to 8 different determinations. Nicotine was not detected in either the pup's brain or the cerebellum of young rats in the control.

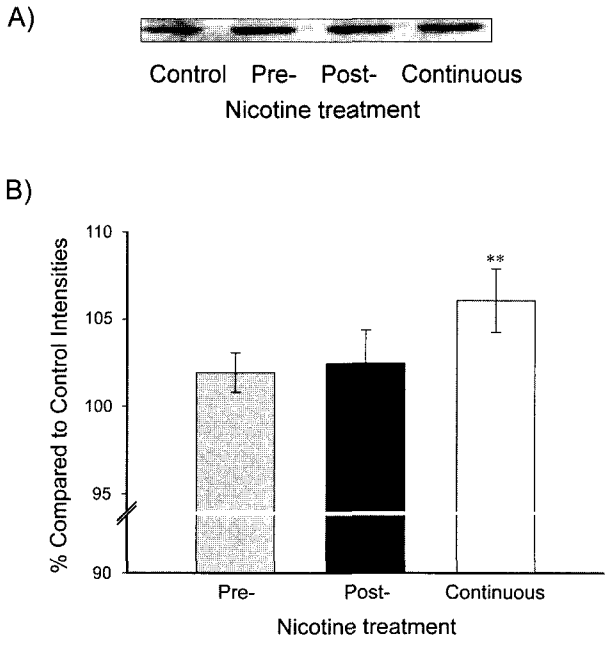

Fig. 2. Typical immunoblotting (A) and changes in the intensities of the immunoblots of cerebellar glial cells prepared from the various nicotine exposures (B). The cerebellar glial cells were prepared from 7 day-old rat pups. On the 10th day of culturing, the cerebellar astrocytes were scrapped out and subjected to SDS-PAGE electrophoresis. The membranes were treated with GLT-1 antibodies and the GLT-1 immunoreactivities were visualized using the ECL Plus Detection System. The intensities were calculated and compared to the control intensity in each strip. The values are a mean±S.E.M. for 7 different determinations. The p-value (**P 0.01) is significant.

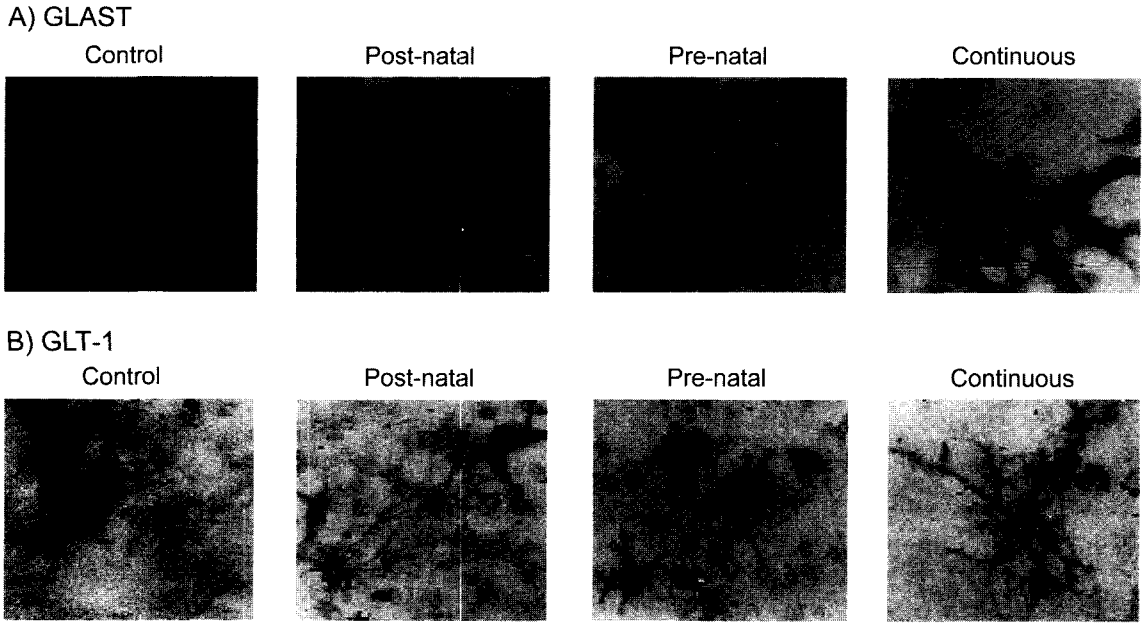

Fig. 1. Typical immunochemical micrographs of cerebellar glial cells prepared from various nicotine exposures. The cerebellar glial cells were prepared from 7 day-old rat pups. On the 10th day after the culture, the cerebellar astrocytes were treated with either GLAST antibodies (A, B, C, D) or GLT-1 antibody (E, F, G, H) and visualized using diaminobenzidine. The detailed procedure is described in the methods section.

The expressions of GLAST and GLT-1 in the cerebellum of young rats

Fig. 3 shows the immunochemical photograph of GLAST and GLT-1 in the cerebellum of 2, 4, and 8 week-old rats from each nicotine-treated group. In the 4 week-old rats, the glial glutamate transporters, GLAST and GLT-1, were strongly expressed. Moreover, the GLAST expression level in the cerebellum was higher than that of GLT-1 in 2 week-old rats. Compared to the control, the appearance of GLAST in the cerebellum from both the postnatally or continuously nicotine-exposed group was strongly expressed at 2 to 8 weeks after birth. However, that of the prenatally nicotine-exposed group was widely expressed at 2 and 8 weeks after birth.

Compared to the control, the appearance of GLT-1 in the cerebellum from the postnatally nicotine-exposed group was densely expressed 2 weeks after birth., In addition, GLT-1 in the cerebellum of the prenatally nicotine-exposed group was strongly expressed 2 weeks after birth. From 4 to 8 week-treatments, the appearances of GLT-1 levels in the cerebellum of both the pre- or post-natally nicotine-exposed rats were slightly lower than those of the control rats. However, the appearance of GLT-1 expression level in the cerebellum of the continuously nicotine-treated rats was even lower than that of the control. Fig. 4 shows the immunoblot analysis of GLT-1 in the cerebellum of the 2,

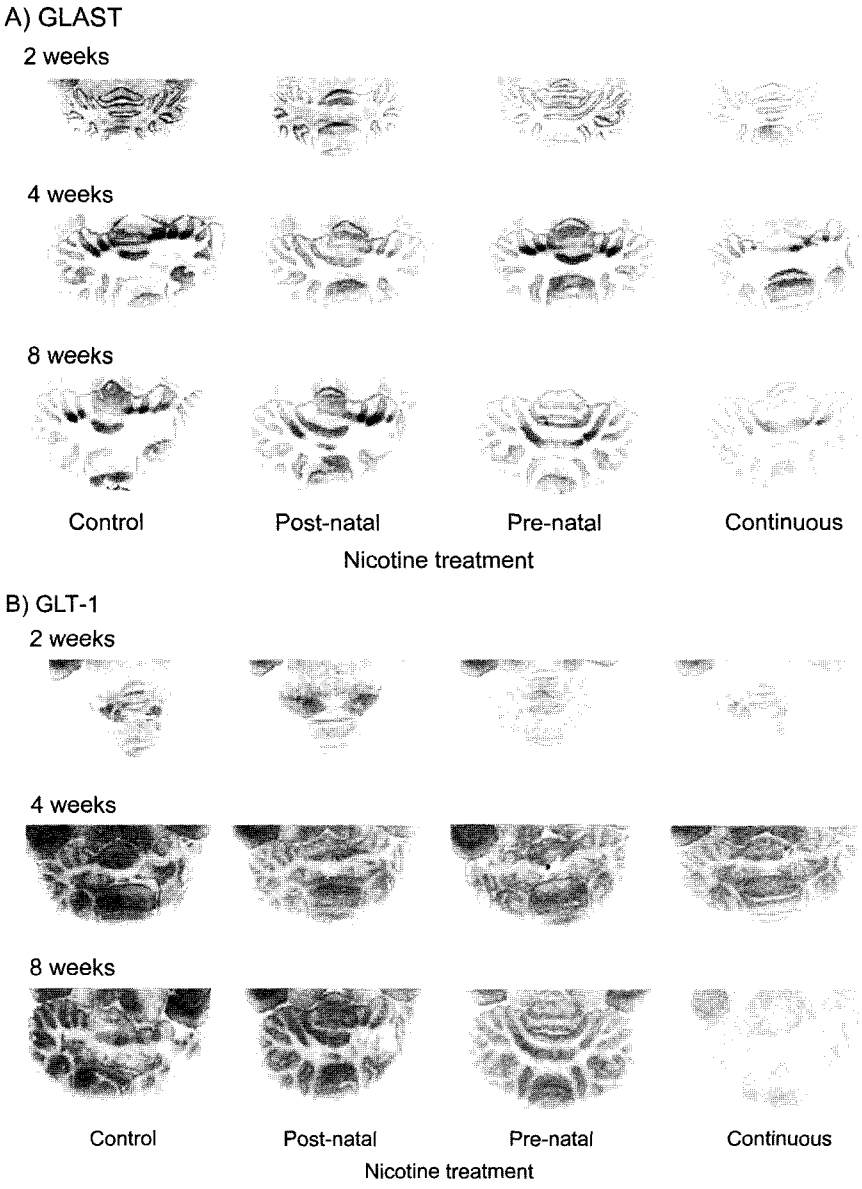

Fig. 3. Typical immunochemical photomicrographs of the cerebellum prepared from various nicotine exposures. The rats were treated as shown in Table 1. On the 2nd , 4th and 8th week day, they were subjected to the immunohistochemistry. The tissue slices were treated with either GLAST antibodies or GLT-1 antibodies and visualized using diaminobenzidine. The detailed procedure is described in the methods section.

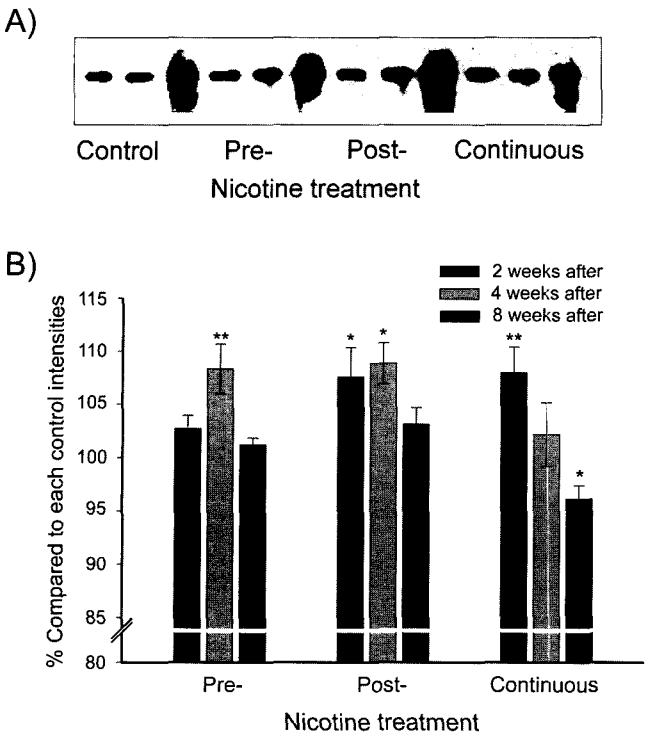

Fig. 4. Typical immunoblotting (A) and the changes in the intensities of the immunoblots of the cerebellum prepared from the various nicotine exposures (B). The rats were treated as shown in Table I. They were subjected to immunoblotting at three different stages: at 2, 4 and 8 weeks after birth. The cerebellum was homogenized and subjected to 8% SDS-PAGE electrophoresis. The membranes were treated with GLT-1 antibodies and the GLT-1 immunoreactivities were visualized using the ECL Plus Detection System. The intensities were calculated and compared with the intensity of the control group at each age. The values are a mean±S.E.M. for 7 different determinations. The *p*-values (*P<0.05, **P 0.01) are significant. The detailed procedure is described in the methods section.

4, and 8 week-old rats from each of the nicotine-treated groups.

Compared to the control, the immuno-intensities of GLT-1 in the cerebellum of both the prenatally-exposed and the continuously nicotine-treated groups were higher 2 weeks after birth. In addition, those of both the prenatally-exposed- and post-natally nicotine-treated groups were higher 4 weeks after birth than those of the control. The immuno-intensities of the continuously nicotine-treated group was decreased at 8 weeks after birth.

DISCUSSION

Glutamate uptake is the primary mechanism for inactivating synaptically-released glutamate (Nicholls and Attwell, 1990). It has been reported that astrocytes, rather than neurons, perform the majority of glutamate uptake in the brain (McLennan, 1976). This is important because neuronal injury can result from excessive stimulation of the NMDA (name?) receptor. It is known that more than three types of glutamate transporters exist in the rat brain. Astrocytes contain two of these types of glutamate transporters, GLAST and GLT-1, which are responsible for glutamate uptake in the synapses. They have a different affinity to the glutamate uptake inhibitors (Kondo *et al.*, 1995) and their expression patterns in the brain regions vary during development (Furuta *et al.*, 1997; Sutherland *et al.*, 1996). In addition, their metabolic turnover rates have been reported to be regulated differentially depending on the environmental cues and signaling pathways (Gegelashvili and Schousboe, 1997). It has been reported that two glial glutamate transporters (GLAST and GLT-1) undergo contradictory functional changes in response to either oxidation or reduction of the reactive sulfhydryl agents (Trotti *et al.*, 1997). These results suggest that the expression levels of the glial GLAST and GLT-1 are modulated in the cultured cerebellar glial cells prepared from nicotine-exposed pups during either gestation or lactation. The GLAST expression levels in the glial cells were either higher or lower depending on the nicotine exposure level with either the prenatal or postnatal periods, while those of GLT-1 were increased as a result of nicotine exposure, particularly in the continuously nicotine-treated group. These results suggest that the expression levels of the glial GLAST and GLT-1 are altered differently depending on the initial exposure time and the duration of nicotine exposure.

It has been shown that the inhibitory potency of PDC for EAAT2 (the human homologue of the astrocyte transporter, GLT-1) is ten times more sensitive than it is for EAAT1 (the human homologue of GLAST) (Arriza *et al.*, 1994). In a recent study, the glutamate uptakes from the cerebellar glial cells prepared from either the prenatally-exposed- or continuously nicotine-exposed rat pups were either higher or lower, and the response to PDC in the cerebellar glial cells prepared from the nicotine-exposed pups was higher than that for the control group. (Lim and Kim, 2001). Therefore, although the GLAST and GLT-1 expression ratios in the glial cells prepared from the various nicotine-treated pups are unknown, these results suggest that nicotine exposure during gestation has persistent effects on the development of the glial GLAST and GLT-1. Furthermore, the altered expressions of the glial GLAST and GLT-1 following the perinatal nicotine exposure suggest that the intrinsic GLAST and GLT-1 expression levels might have been modulated during nicotine exposure.

It has been reported that GLAST is the primary developing glutamate transporter in glial cells, and GLT-1 is developed later in the cerebellum (Furuta *et al.*, 1997). Although the neuronal inputs are believed to be regulating the expression of the glial transporters (Swanson *et al.*, 1997), the glutamate transporter expression is altered after

its exposure to various substances as well as pathogens (Gegelashvili and Schousboe, 1997). According to the present results, the GLAST expression levels are increased during nicotine exposure, while the GLT-1 expression levels first increase and then decrease during nicotine exposure. Although the exact mechanism of the interaction between nicotine and GLAST and GLT-1 expression require further investigation, these results suggest that the expression levels of the glial GLAST and GLT-1 are modulated in the cerebellum during nicotine exposure.

Nicotine is reported to induce the phosphorylation of the cAMP response element binding protein (Nakayama *et al.*, 2001) and mecamylamine-precipitated nicotine withdrawal is reported to increase adenylate cyclase activity in a certain brain region (Tzavara *et al.*, 2002). It has been reported that GLT-1 expression in the cultured glial cells is increased as a result of cAMP addition (Swanson *et al.*, 1997). In addition, phosphorylation stimulates GLT-1 activity (Casado *et al.*, 1993) but inhibits GLAST function (Conradt and Stoffel, 1997). The evidence suggests that nicotine may be involved in modulating the cAMP pathway. In accordance, these results suggest that nicotine might affect the expression of the glutamate transporters via the cAMP pathways. However, the changes in the expression levels of GLAST and GLT-1 in the *in vivo* results are somewhat different to those in the glial cell culture. It is known that nicotine enhances the ion flux and the release of neurotransmitters, and elicits a variety of physiological and behavioral effects (Martin, 1986). It has also been reported that various cellular components, such as the potassium channel and calcium influx, are affected by the exposure of nicotine (Tang *et al.*, 1999). Nicotine modulates the various neuronal activities as explained in the introduction. In addition, the neuronal activity influences the proliferation and differentiation of glial cells. This indicates that, unlike a glial cell culture, subacute nicotine exposure after the birth may also affect the activity and expression of the glial GLAST and GLT-1. Therefore, the difference between the two experimental results might be due to both the preparation and the duration of nicotine exposure: seven days exposure in the glial cell culture and eight weeks exposure in the *in vivo* study. Interestingly, the changes in the GLT-1 expression levels are dependent on the time of nicotine exposure. During nicotine exposure, the GLT-1 expression level increases after the initial nicotine exposure, but decreases after longer nicotine exposure. The bi-phasic changes in the GLT-1 expression levels found in these results suggest that nicotine may be involved in the development of GLT-1 through either many factors or through cellular adaptation. Furthermore, the changes in the GLAST and GLT-1 expression levels also occurred in the prenatal nicotine exposed group as observed in the glial cell culture. Therefore, these results suggest the possibility that nicotine affects the expression patterns of the glutamate transporter subtypes by modulating the intrinsic expression in addition to the cAMP pathway.

It has been reported that glial GLT-1 was down-regulated due to cerebral ischemia and is specifically decreased due to amyotrophic lateral scleosis (Bristol and Rothstein, 1996; Rao *et al.*, 2000). In addition, the glial GLAST and GLT-1 were down-regulated due to glutamatergic denervation and Wernickes Encephalopathy (Hazell *et al.*, 2001; Levy *et al.*, 1995). Therefore, although the GLAST and GLT-1 expression ratios in the cerebellum during the various nicotine treatments are not known, these results suggest that the development of GLAST and GLT-1 might be affected by perinatal nicotine exposure, even in the fetal periods. Furthermore, the decrease in the expression levels of the glial GLT-1 after subacute nicotine exposure may have detrimental effects on either the external excitatory stimulus or on the disease.

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