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Development of Animal Model for Cerebral Palsy Based on Impairment of Neonatal Gamma-aminobutyric Acid (GABA)

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ABSTRACT

In spastic diplegic cerebral palsy (CP) patients, we found increased ^{18}F-fluoroflumazenil binding in the bilateral precentral gyri, regardless of the presence of periventricular leukomalacia (PVL) in a positron emission tomography (PET) study. In this study, we aimed to develop an animal model that would corroborate the findings above by impairing GABA function of neonatal rats during development. Sprague-Dawley rat pups were daily injected with GABAergic drugs, and exposed to anoxic environment for 25 minutes using 100% N_2 gas on postnatal day (PND) 2, 3, and 4. GABAergic drugs we used were muscimol (0.1 mg/kg, s.c.), and biccuculine (1 mg/kg, s.c.), a $GABA_A$ receptor agonist and an antagonist respectively, and diazepam (1 mg/kg, s.c.), a benzodiazepine receptor agonist. Developmental and neurological tests, conducted on PND 16, 23, and 30, did not yield any significant differences. However, an impaired motor coordination was observed in the Anoxia-Muscimol group which showed decreased [^{3}H]flumazenil binding and an increased dopamine turnover in the cerebral cortex. Considering data of cerebral palsy patients, the present results suggest that the pathophysiological process of cerebral palsy occur in the early prenatal period, not in the perinatal period.

Key words: cerebral palsy, gamma-aminobutyric acid, animal model, developmental disability

INTRODUCTION

Cerebral palsy is an umbrella term covering a group of nonprogressive impairment of movement and posture that appears early in life and is due to an abnormality of the developing brain. The prevalence rate of cerebral palsy is 1.5 to 2.5 per 1,000 live births. There are many risk factors occurred in prenatal, perinatal or postnatal period. Among them, hypoxia-ischemia and infection were

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believed in the most important risk factors (Srivastava, 1992; Odding et al., 2006). The prevalence rate of cerebral palsy has not been decreased despite of much effort done for decreasing or preventing risk factors (Winter et al., 2002; Nelson, 2003).

Several types of cerebral palsy are accepted, and spastic type is the most common. The main etiology of the spastic type cerebral palsy has been believed in relation with periventricular leukomalacia (PVL) because PVL can be occurred by hypoxic-ischemic insult during 26 to 34 weeks of gestation (Lin, 2003; Himmelmann et al., 2005). For the development of an animal model for cerebral palsy many environmental manipulations during brain development were attempted based on known etiologic factors and pathophysiological mechanisms. However, currently no accepted animal model for cerebral palsy is available. In fact, children with PVL do not always show symptoms of spastic type cerebral palsy. Moreover, PVL is not always observed in patients with spastic type cerebral palsy. PVL can be observed in approximately 70～75% of spastic diplegia type cerebral palsy born before 32 weeks of gestation (Lin, 2003; Kwong et al., 2004).

Recently, we investigated the role of gamma-aminobutyric acid (GABA) in abnormal motor functions in cerebral palsy patients (Lee et al., 2007). In this study, central benzodiazepine receptor imaging was done in 20 patients with spastic diplegia type cerebral palsy. A positron emission tomography study was done using a recently developed positron emission tomography tracer, ^{18}F-fluoroflumazenil that binds to the central benzodiazepine receptor in the GABAA receptor-chloride ion channel complex (Olsen et al., 1990; Niimura et al., 1999; Chang et al., 2005; Yamauchi et al., 2005). We found increased ^{18}F-fluoroflumazenil binding in the bilateral precentral gyri in spastic diplegic cerebral palsy patients, regardless of the presence of PVL. Moreover, the same patients exhibited decreased ^{18}F-fluoroflumazenil binding in the brain stem. Based on these findings, we hypothesized that impaired GABA function, rather than PVL could be the etiological factor in cerebral palsy.

In this study, we aimed to develop an animal model that would corroborate the findings above by impairing GABA function of neonatal rats during development. GABAergic function was modulated by several GABAergic drugs such as muscimol, a $GABA_A$ receptor agonist, bicuculline, a $GABA_A$ receptor antagonist, and diazepam, a benzodiazepine receptor agonist.

MATERIALS AND METHODS

Animals and experimental design

To minimize and to standardize unwanted environmental stimulation from *in utero* life, Sprague-Dawley rats were bred and the offspring were reared in a controlled manner. The animals were supplied from the Division of Laboratory Animal Medicine, Yonsei University College of Medicine. Animals were cared for in a SPF barrier area and the temperature ($22\pm1^{\circ}C$) and humidity (55%) were constantly controlled with a 12:12 h light:dark cycle (lights on 07:00 h). Food (PMI Feeds, Inc, IN, USA) and tap water (membrane filtered purified water) were available ad lib. Nulliparous female and proven breeder male Sprague-Dawley rats were used for breeding. Twelve hours after confirming delivery, pups were divided by sex and weighed. We used litters which had five males and five females or more, and pups were culled five males and five females in a litter. All animal experiments were approved by the Committee for Care and Use of Laboratory Animals at Yonsei University. The Guidelines and Regulations for the Use and Care of Animals in Yonsei University were consistent with the NIH guideline Guide for the Care and Use of Laboratory Animals, 1996 revised.

For the present study, we used pups from 15 litters. On postnatal day (PND, birthday=PND 0) 2, 3, and 4, pups were exposed to a temperature maintained acryl chamber (30×30×30 cm) filled and flowed (5 L/min) with 100% N_2 gas for 25 min in each day. One hour before exposure to anoxic environment, Saline, muscimol 0.1 mg/kg (Tocris Bioscience, Ellisville Mo, USA), diazepam 1 mg/kg (Daewon Pharmaceutical co., Seoul, Korea) or bicuculline 1 mg/kg (Tocris Bioscience, Ellisville Mo, USA), were injected subcutaneously in a volume of 30～35 μl. We have 5 groups: 1) Control (CON): Rats were not exposed to anoxic environment and drug. 2) the Anoxia-Saline (A-SAL) group: Rats were exposed to anoxic environment after injection with saline. 3) the Anoxia-Muscimol (A-MUS) group:

Rats were exposed to anoxic environment after injection with muscimol. 4) the Anoxia-Diazepam (A-DIA) group: Rats were exposed to anoxic environment after injection with diazepam. 5) the Anoxia-Bicuculline (A-BIC) group: Rats were exposed to anoxic environment after injection with bicuculline. Each group was assigned each pup in a litter in each sex. Thus there were no littermates in each group. This assignment method allowed us to minimize genetic variations between litters. These animals were raised normally, and behavioral tests were done on PND 16, 23 and 30. On PND 31, rats were sacrificed and the concentrations of neurotransmitters were measured in the frontal cortex by high performance liquid chromatography-electrochemical detection system.

Behavioral measurements

Developmental indices were measured by neuronal reflexes (forelimb flexion, torso twisting, righting reflex, placing reaction and toe spreading reflex), the wire maneuver test and the rotarod test. Neuronal reflexes were measured by slightly modified methods of Huston and Bures (1976). For the wire maneuver test, a rat was hung by both forelimbs on the wire 50 cm high from the floor. Its falling down latency was measured as an index of forelimb strength. For the rotarod test, a rat was placed on the rotating rod (7.5 cm diameter) and its falling down latency was measured as an index of motor coordination. The rotating speed was gradually increased from 0 (at 0 sec) to 40 rpm (at 300 sec). All behavioral measurements were tested in a day. Then, each rat exposed to the same behavioral measurement 3 times on PND 16, 23, and 30.

[^{3}H]flumazenil binding assay

Frontal cortices without frontal poles of six male and six female rats in each group were collected on PND 31. Frontal poles were used for measuring concentrations of biogenic amines. Tissues from 2 animals were gathered for the assay because the amount of brain tissue from single animal was not sufficient to proceed. Tissue was thawed and homogenized by a teflon-on-glass tissue homogenizer in 10 volumes ice-cold 0.32 M sucrose solution and centrifuged at 900 g for 10 min at 4°C to pellet nuclei and cell debris. The supernatant fraction was centrifuged at 11,500 g for 30 min. Membrane pellets were resuspended in assay buffer (50 mM Tris-HCl buffer, pH 7.4 with 120 mM NaCl and 5 mM KCl) with protease inhibitor mixture and used for ligand binding. Resuspended membranes were incubated (in a volume of 0.5 ml) for 30 min at 37°C in the presence of [^{3}H]flumazenil (87 Ci/mmol, NEN, Boston, USA). Membranes (150 μg of protein per filter) were collected by rapid filtration on GF/B filters glass fiber filters (Whatman). After three washing steps with 5 ml of ice-cold wash buffer (50 mM Tris-HCl buffer, pH 7.4), the filter-retained radioactivity was counted in a TR-2300 liquid scintillation counter (Packard). Nonspecific binding was determined in the presence of 10 μM diazepam.

Concentrations of biogenic amines in the cerebral cortex

On PND 31, animals were sacrificed by decapitation and the frontal pole was dissected out, frozen on dry ice, and stored at −70°C until assay. Concentrations of biogenic amines were analyzed by the high performance liquid chromatography (HPLC) fitted with an electrochemical detector (ECD) system. Tissue concentrations of norepinephrine, epinephrine, and 5-hydroxytryptamine (5-HT) and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), dopamine and its metabolite, 3, 4-dihydroxyphenylacetic acid (DOPAC) were determined. Briefly, tissues were homogenized on ice in a solution consisting of 0.1 M perchloric acid (containing 0.25% disodium EDTA). Extracts were then centrifuged at 12,500 g for 20 min. Supernatants were filtered (Super-200 membrane filter, 0.2 mm, Gelman Sciences Inc., CA, USA), and 20 μl samples injected into the HPLC system. The HPLC-ECD system consisted of a Waters 600 syringe pump, a 7125 Rheodyne injector (Waters model 700, Waters Instruments, MA, USA), a column C18 ODS 5 μm; 250 mm×4.5 mm diameter (Bio Analytical System, IN, USA) and an electrochemical detector (Coulochem II #5200A; ESA Inc., MA, USA), operated at a at Guard cell voltage of 320 mV, Electrode cell voltage of 240 mV, and a sensitivity of 200 nA. The mobile phase consisted of 8% acetonitrile within 92% 0.15 M monochloroacetic acid buffer (containing: 0.55 mM sodium octyl sulfonate and 2 mM disodium EDTA),

pH 3.35. The flow rate was 1.0 ml/min, the area of the peak (μV×sec) was measured as data and the concentrations were calculated by the external standard method.

RESULTS

Effects of altered neonatal GABA function on behavioral development

Behavioral measurements were done 3 times on PND 16, 23 and 30 to measure developmental changes if any. Neonatal exposure to anoxic environment and GABAergic drugs did not elicit abnormal reflexes (forelimb flexion, torso twisting, righting reflex, placing reaction and toe spreading reflex) in all 3 serial measurements. No significantly altered forelimb strength was observed in the wire maneuver test.

Effects of altered neonatal GABA function on motor coordination

Motor coordination was measured by the rotarod

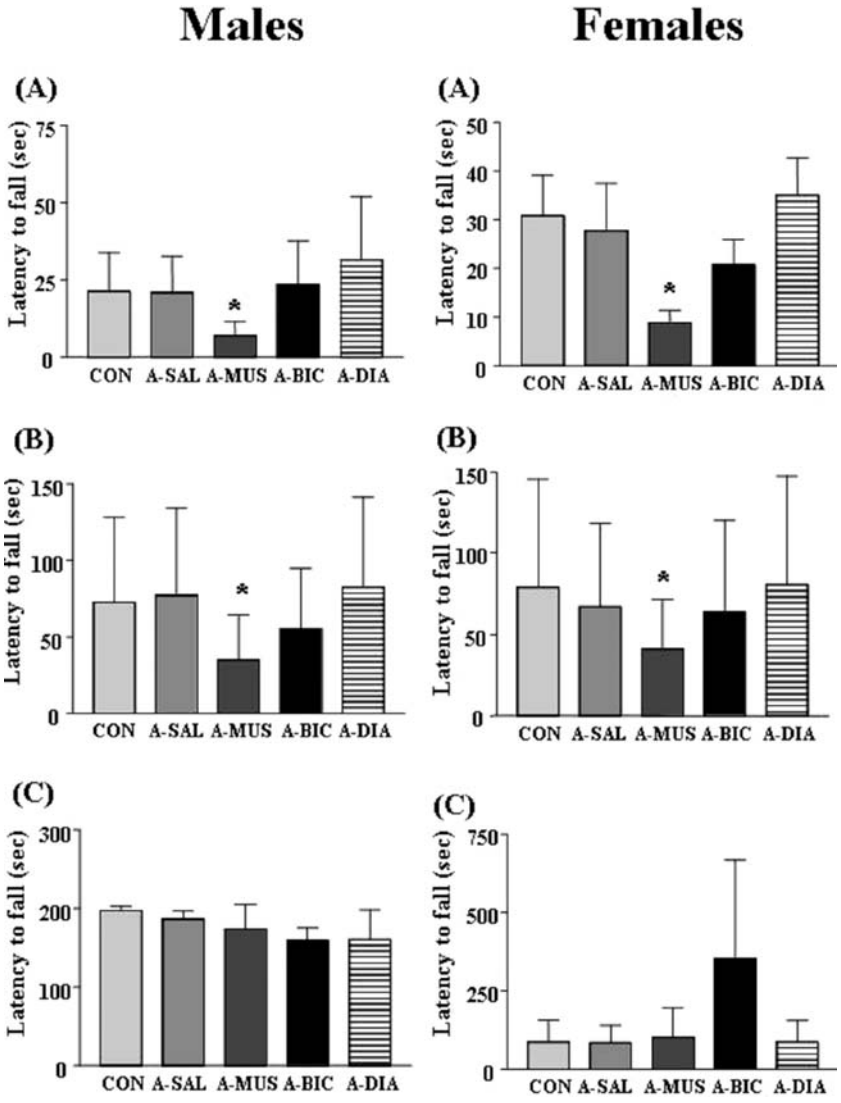

Fig. 1. Effects of altered neonatal GABA function on motor coordination on PND 16, 23 and 30 in the rotarod test. The left panel is data of males (n=6 per group) and the right panel is data of females (n=6 per group). Rats were exposed to the anoxic environment after injection with saline (A-SAL), muscimol (A-MUS), biccuculline (A-BIC) or diazepam (A-DIA) on PND 2, 3 and 4. (A) Rotarod test on PND 16, (B) Rotarod test on PND 23, and (C) Rotarod test on PND 30. *$p < 0.05$ vs. A-SAL (Fisher PLSD test).

test (Fig. 1). As the age increased, the latency to fall increased, suggesting the ability of motor coordination developed and improved till adolescent. The latencies to fall of the CON group were 33±9 sec on PND 16, 128±17 sec on PND 23 and 197±6 sec on PND 30 in males (n=6), and 31±8 sec on PND 16, 146±13 sec on PND 23 and 157±16 sec on PND 30 in females (n=6). GABAergic drug-treated groups were compared with the A-SAL group because there was no difference between the CON and the A-SAL groups. Among GABAergic drug-treated groups, only the A-MUS group showed short latency to fall on PND 16 and 23 in both sexes. The latencies to fall of males in the A-MUS group were 35.1% and 48.3% of those of the A-SAL group on PND 16 and 23, respectively. Similarly, the latencies to fall of females in the A-MUS group were 35.1% and 48.3% of those of the A-SAL group on PND 16 and 23, respectively.

Effects of altered neonatal GABA function on [^{3}H]flumazenil binding

GABAergic drug-treated groups were compared with the A-SAL group because the radioactivities of the CON and A-SAL groups were not different. The A-MUS group showed lower binding of [^{3}H]fluma-

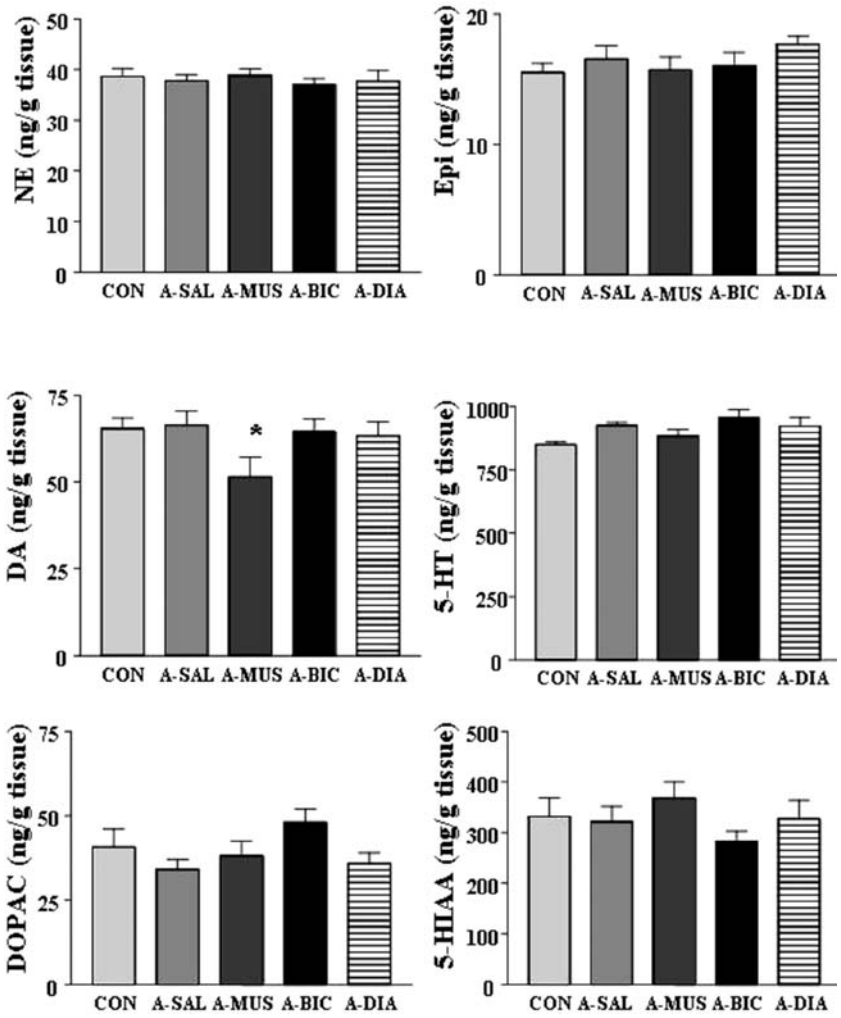

Fig. 2. Effects of altered neonatal GABA function on concentrations of biogenic amines in the frontal cortex. Rats were exposed to the anoxic environment after injection with saline (A-SAL), muscimol (A-MUS), biccuculline (A-BIC) or diazepam (A-DIA) on PND 2, 3 and 4, and the cortical tissues were obtained on PND 31 (n=12 per group in both sexes). Each group has 12 rats in both sexes. NE: norepinephrine, Epi: epinephrine, DA: dopamine, 5-HT: 5-hydroxytryptamine, 5-HIAA: 5-hydroxyindoleacetic acid, DOPAC: 3, 4-dihydroxyphenylacetic acid. *$p<0.05$ vs. A-SAL (Fisher PLSD test).

Table 1. Effects of altered neonatal GABA function on [^{3}H]flumazenil binding in the cerebral cortical tissues

	CON	A-SAL	A-MUS	A-BIC	A-DIA
[^{3}H]Flumazenil binding (Percent of CON)	96.6±3.7	100.4±3.7	79.4±6.1*	93.6±5.7	86.5±15.3

Data are means±S.E. (n=6, both sexes). Rats were exposed to the anoxic environment after injection with saline (A-SAL), muscimol (A-MUS), biccuculline (A-BIC) or diazepam (A-DIA) on PND 2, 3 and 4, and the cortical tissues were obtained on PND 31. *$p<0.05$ vs. A-SAL (Fisher PLSD test).

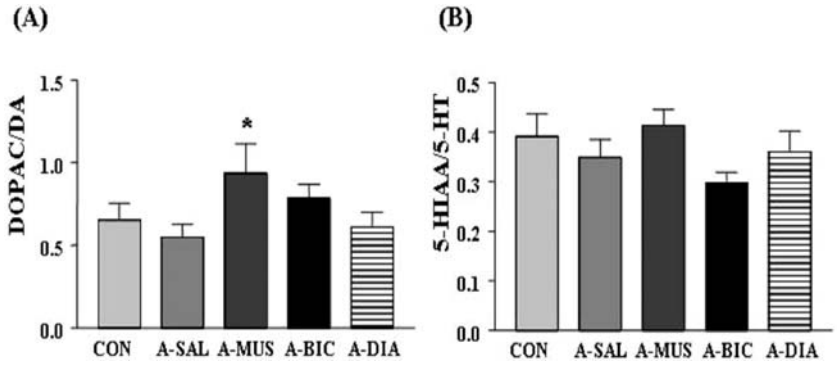

Fig. 3. Effects of altered neonatal GABA function on the ratio of DOPAC/DA and 5-HIAA/5-HT in the frontal cortex. Rats were exposed to the anoxic environment after injection with saline (A-SAL), muscimol (A-MUS), biccuculline (A-BIC) or diazepam (A-DIA) on PND 2, 3 and 4, and the cortical tissues were obtained on PND 31 (n=12 per group in both sexes). (A) the ratio of DOPAC/DA, (B) the ratio of 5-HIAA/5-HT. DA: dopamine, DOPAC: 3, 4-dihydroxyphenylacetic acid, 5-HT: 5-hydroxytryptamine, 5-HIAA: 5-hydroxy-indoleacetic acid. *$p<0.05$ vs. A-SAL (Fisher PLSD test).

zenil than the A-SAL group in the frontal cortex on PND 31. The [^{3}H]flumazenil binding of the A-MUS group was 79.4±6.1% (n=6, both sexes) of the A-SAL group (Table 1). Other groups did not show significant alteration of [^{3}H]flumazenil binding.

Effects of altered neonatal GABA function on concentrations of biogenic amines in the frontal cortex

GABAergic drug-treated groups were compared with the A-SAL group because concentrations of biogenic amines of the CON and A-SAL groups were not different. The concentration of dopamine was lower in the A-MUS group (51.5±5.8 ng/g, n=12, both sexes) than that of the A-SAKL group (66.4±3.1 ng/g, n=12, both sexes) in the frontal cortex on PND 31. Concentrations of epinephrine, norepinephrine, 5-HT, 5-HIAA and DOPAC were not different from those of the A-SAL group (Fig. 2).

Turnover rates of 5-HT and dopamine were calculated by the ratio of 5-HIAA/5-HT and DOPAC/dopamine, respectively. As expected, the ratio of DOPAC/dopamine in the A-MUS group (0.9±0.2, n=12, both sexes) was higher than that of the A-SAL group (0.7±0.1, n=12, both sexes) (Fig. 3), suggesting that neonatal muscimol resulted in increased turnover rate of dopamine in the frontal cortex of an adolescent rat.

DISCUSSION

This study showed that neonatal increase in GABA activity with an exposure to the anoxic environment resulted in an impaired motor coordination later, one of the major symptoms of cerebral palsy. This finding was consistent with our human data in which ^{18}F-fluoroflumazenil binding increased in the bilateral precentral gyri of patients with cerebral palsy (Lee et al., 2007). Moreover, the present study suggested that an increased ^{18}F-fluoroflumazenil binding in patients might not result from a compensatory increase of the chloride ion channel-$GABA_A$ receptor complex induced by the insufficient GABA activity because the results of the present study showed that a behavioral impairment was caused by an increased, not by a decreased

GABA activity. GABA is the major inhibitory neurotransmitter in the human brain acting at the chloride ion channel-$GABA_A$ receptor complex. For the harmonious motor function, the GABAergic signaling plays important roles in linking of sensory and motor cortical points. It has been reported that one of the important pathophysiological mechanisms of the motor dysfunction is the dissociation between sensory and motor representations at cortical level (Thickbroom et al., 2001; Hoon et al., 2002). The role of GABA activity can be speculated in the studies that biccuculline produced expansion of receptive fields in the motor cortex (Schneider et al., 2002; Capaday and Rasmussen, 2003), and that muscimol increased error rates during motor sequence production (Lu and Ashe, 2005). The present study also showed an increased GABAergic inhibition by muscimol resulted in abnormal motor coordination.

GABA also plays an important role in the development of plasticity after brain damage. The use-dependent plasticity was facilitated by the decreased GABAergic inhibition in the expansion of trained representations (Zieman et al., 2001). On the contrary, lorazepam, a positive allosteric modulator of the chloride ion channel-$GABA_A$ receptor complex, reduced the use-dependent plasticity in the motor cortex after brain damage (Butefisch et al., 2000). Excessive GABA activity during developmental period also brought about adverse effects on motor function by inhibiting migration of embryonic or postnatal neurons (Behar et al., 1998; Boletus and Bordey, 2004). Moreover, GABA generates depolarization in the immature neuron due to high intracellular chloride concentration (Ben-Ari, 2002; Owen and Kriegstein, 2002). GABA-induced depolarization may trigger glutamate-induced excitotoxicity by unplugging magnesium in the NMDA receptor. In fact, it has been reported that GABA or muscimol produced a concentration-dependent exacerbation of neuronal cell death in in vitro hypoxic ischemic model with cortical cell culture (Muir et al., 1996), and that neonatal increase in GABA activity resulted in long-term deficit of hippocampal-dependent behavior (Nuñez et al., 2003), similar to our results. In the present study, neonatal muscimol could alter brain plasticity because muscimol-induced impairment of motor function showed on PND 23 although muscimol was administered on PND 2～4.

We have an unexpected finding in which the [^{3}H]flumazenil binding of the A-MUS group was decreased. The [^{3}H]flumazenil binding should increase if an increased GABA activity resulted in behavioral impairments. Together with the finding of disappearance of behavioral impairment on PND 30, our finding suggested that the GABA-related functional abnormality of the brain in patients with cerebral palsy might occur early in the development, not in the late development as in the present study.

We do not know yet the meaning of muscimol-induced increase in dopamine turnover rate in the cortex. Further study is needed to clarify this finding.

In summary, we tried to develop an animal model for cerebral palsy according to our previous human study in that an increased ^{18}F-fluoroflumazenil binding was found in the motor cortex. We used rats as the experimental animal, and they were exposed to the anoxic environment after injection with drugs modifying GABA activity on PNDs 2, 3, and 4. We found neonatal increase in GABA activity resulted in impaired motor coordination up to PND 23. However, a decreased [^{3}H]flumazenil binding was found in the cortex. Taken altogether, the animal model used in the present study is not sufficient to the human cerebral palsy although there are some behavioral similarities. However, the results of this study strongly suggest that an impaired GABA in the early developmental period is the key factor in the development of cerebral palsy.

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