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Neuroprotective Effect of 8-OH-DPAT on Long-term Sequelae from Prenatal Ischemia in Rats

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The role of 5-hydroxytryptamine (5-HT)$_{1A}$ receptor activity in prenatal ischemia was studied, by injecting 8-hydroxy-dipropylaminotetraline (8-OH-DPAT; 50 μg/kg, s.c.), a 5-HT$_{1A}$ agonist on gestation day 17, and 30 min later inducing transient ischemia by ligating the uterine vessels for 30 min. On postnatal day 95, rats that had experienced prenatal ischemia showed impaired motor coordination and reduced concentration of 5-HT in the cerebellum compared with Sham-operated controls. In addition, they showed increased 5-HT$_{1A}$ receptor densities in the cerebral cortex. Pretreatment with 8-OH-DPAT ameliorated the behavioral and neurochemical sequelae measured in the present study. The results suggest that 5-HT$_{1A}$ receptors protect the brain from ischemic insult and/or facilitate recovery after prenatally experienced ischemia.

Key Words: 5-HT$_{1A}$ receptor, Neuroprotection, Prenatal ischemia, 8-OH-DPAT, Motor coordination, Rat

INTRODUCTION

Brain ischemia is one of the major causes of brain dysfunction, and perinatal hypoxic-ischemic brain damage is of special concern, because it can result in long-lasting functional disabilities such as, cerebral palsy, mental retardation, epilepsy, etc. The extent of hypoxic-ischemic damage during the perinatal period is far less than that in adulthood (Duffy et al, 1975; Boksa et al, 1995). The immature brain has higher levels of endogenous antioxidant concentrations, and lower energy requirements, and shows slower increases of intracellular Ca^{2+} concentration as a response to hypoxia than adults (Schurr & Rigor, 1987; Bickler et al, 1993). In addition to these mechanisms of resistance to hypoxic-ischemic injury, other enormous compensatory mechanisms of the immature brain can lessen functional sequelae. Therefore, we reasoned that it might be possible to reduce hypoxic-ischemic damage in the immature brain, rather than in the adult brain, by facilitating natural resistance to damage and/or triggering active compensatory mechanisms. For this reason, we focused on prenatal ischemia, and used an established method to induce prenatal ischemia by ligating the uterine vessels (Wiggleworth, 1964).

5-Hydroxytryptamine (5-HT) has been suggested to play a role in the hypoxic-ischemic brain damage. The concentration of 5-HT was found to decrease and 5-HT turnover rate increased, after a hypoxic-ischemic episode (Ishimaru et al, 1993). Moreover, the net amount of 5-HT available from the platelets invading the ischemic area increased, and these elevated 5-HT levels are known to aggravate the pathophysiological consequences via the 5-HT$_2$ receptors (Wiernsperger, 1990). However, considerable amounts of data indicate that 5-HT$_{1A}$ receptor agonists protect the brain against hypoxic-ischemic insult (Prehn et al, 1993). In addition, it has been suggested that increased 5-HT$_{1A}$ receptor activity induces hyperpolarization (Colino & Halliwell, 1987), decreases glutamate release (Mauler et al, 2001) and reduces apoptosis (Ahlemeyer et al, 2000) after ischemic-hypoxic insult.

On the other hand, 5-HT has long been regarded as a neurotrophic factor (Lauder & Krebs, 1978). Altered 5-HT function may alter neuronal wiring (El-Mallakh et al, 2000), by a mechanism that appears to involve a glial tropic factor S-100β via glial 5-HT$_{1A}$ receptor activation (Ramos et al, 2000).

The present study was conducted to elucidate the effect of a 5-HT$_{1A}$ receptor agonist, 8-hydroxy-2-(di-n-propylamino)-tetraline (8-OH-DPAT), on ischemic damage, especially during the prenatal period when the plasticity of the brain is high. We measured the behavioral and neurochemical consequences of prenatal ischemia in adulthood to evaluate effects of the 8-OH-DPAT on the post-ischemic adaptive plasticity as well as the ischemic insult itself.

METHODS

Animals and model for prenatal ischemia

Sprague-Dawley rats bred and reared in a controlled

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid; 8-OH-DPAT, 8-hydroxy-dipropylaminotetraline; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; ECD, electrochemical detector; HPLC, high performance liquid chromaography; HVA, homovanillic acid.

manner. The animals were supplied by the Division of Laboratory Animal Medicine, Yonsei University College of Medicine, and cared for in a specific-pathogen-free barrier area at a temperature of 22±1°C and a humidity of 55% under a 12 : 12 h light : dark cycle (lights on 07 : 00 h). Food (Purina Rodent Chow, Purina Co., Seoul, Korea) and tap water (membrane filtered purified water) were available *ad libitum*. Nulliparous females and proven breeder male rats were used for the breeding. All animal experiments were approved by the Committee for the Care and Use of Laboratory Animals at Yonsei University (Project Licence Number #089). Animals were cared for according to The Guide for Animal Experiments, 2000, edited by the Korean Academy of Medical Sciences, which is consistent with the NIH Guideline Guide for the Care and Use of Laboratory Animals, 1996 revised.

On the morning of gestation day 17, the pregnant rats were anesthetized with equithesin i.p. (pentobarbital sodium 1 mg; chloral hydrate 42.5 mg; magnesium sulfate 21.3 mg; ethyl alcohol 0.1 ml in 1 ml of equithesin). A midline laparotomy was performed and the uterus was exposed. The uterine vessels were ligated with an atraumatic vascular clip (Micro-serrefines, Fine Science Tools Inc., B. C., Canada) at the body of the uterus and the uterus returned to its natural position. After being ligated for 30 min, the clip was released and the incision line sutured. The total operation time was about 45 min. R(+)-8-OH-DPAT (RBI/Sigma, MA, USA), 50 μg/kg was injected subcutaneously 30 min before the ligation.

Pups were delivered normally on the 21st day of pregnancy. To prevent nurturing biases of the operation-experienced mother, pups were fostered to normal mothers that delivered at same time as the operation-experienced mothers. Pups were culled to 5 males and 5 females in each sex in a litter on the fostering time, and they were raised normally in a controlled environment.

The experimental animals consisted of: pups delivered from dams that experienced the operational procedure without ischemia (the Sham group), pups were received ischemia for 30 min (the Ischemia group), pups that received 8-OH-DPAT and a sham operation (the 8-OH-DPAT group), and pups that received 8-OH-DPAT and ischemia for 30 min (the 8-OH-DPAT-Ischemia group). Only male pups were used in the present study.

Measures of developmental indices

To determine the effects of transient ischemia upon development, the pup's body weight and the outcome of pregnancy were determined on the fostering day. In addition, the eye-opening time on postnatal day 14 was recorded as an index of brain development. On postnatal day 22, rats were subjected to the wire maneuver test in which a rat was allowed to hang by a wire a height of 57 cm from the floor. Latency to falling down was measured as an index of power of forelegs.

Behavioral measurements

On postnatal day 65, exploratory behavior was assessed using an activity monitor system (ENV-515, MED Associates, VT, USA). Briefly, a rat was placed in a novel chamber (30.5 cm height, 43.2 cm wide, 43.2 cm length), and its ambulatory and stereotypic activities were monitored for 3 min intervals over a period of 30 min. The chamber was equipped with 16 infrared photocells 2.54 cm apart. The number of consecutive 3 beam interruptions was treated as ambulatory activity, and repetitive interruptions of one beam as stereotypic activity. Chambers were cleaned with alcohol (70%) after use to prevent any influence due to a previous test.

On postnatal day 95, a Rota-rod test was performed. A rat was placed on the rotating rod (15 cm diameter) and its falling down latency was measured as an index of motor coordination. The rotational speed used was 1.5 rpm for the first 30 s, and this was then increased to 4.5 rpm.

The autoshaped learning test was commenced on postnatal day 100. For this behavioral measurement, rats were gradually deprived of food and maintained at 85% of their free-feeding body weight. Animals were tested in standard operant conditioning chambers (ENV-007, MED Associates, VT, USA), enclosed in sound-attenuating cubicles with built-in CCTV systems. Behavioral boxes were 30.5 cm (height), 29 cm (wide), and 24 cm (length), with grid floors made of stainless steel bars (4.8 mm diameter, spaced 1.6 cm apart). Each box was equipped with a retractable lever (ENV-112B, MED Associates, VT, USA), a pellet dispenser (ENV-203, MED Associates, VT, USA) for the delivery of 45 mg food pellets (Formula: F Sucrose Pellets, P. J. Noyes Company, NH, USA) and a speaker for the introduction of white masking noise. The boxes were controlled and data collected by the MED-PC software for IBM compatible computers. During each autoshaping trial the lever was extended into the chamber for 15 s at a random intervals ranging from 22 to 68 s, with an average interval of 45 s. The lever was retracted when the animal made a lever touch response or after 15 s. One food pellet was delivered 4 s after the lever had retracted whether or not the rat made a lever touch response. A daily session consisted of 12 trials and 12 sessions were performed over a period of 12 consecutive days. The extended lever touch was defined as the correct lever touch. In this learning task, animals learned to associate food delivery with lever touching on condition that the lever was intermittently extended into the chamber and retracted, in conjunction with delivery of food reinforcement.

Concentrations of biogenic amines in the cerebellum

Neurochemical studies were conducted on animals, which had not been used for behavioral study above. On postnatal day 100, animals were sacrificed by decapitation and the cerebellum was dissected, 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 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), dopamine (DA) and its metabolite, 3, 4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) 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; 250×4.5 mm diameter (Bio Analytical System, IN, USA) and an electrochemical de-

tector (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 octylsulfonate 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 of 5-HT were calculated by the external standard method.

5-HT$_{1A}$, 5-HT$_{2A/2C}$ receptor binding assays

Receptor binding assays for the 5-HT$_{1A}$ or the 5-HT$_{2A/2C}$ receptors were performed with slight modification. Briefly, membrane fractions were obtained from the cerebral cortices. The membrane fraction was incubated at 37°C for 15 min in a final volume of 1.0 ml Tris-HCl, pH 7.6, containing [^{3}H]8-OH-DPAT (0.25~12 nM) for the 5-HT$_{1A}$ receptor and [^{3}H]ketanserin (0.0625~5 nM) for the 5-HT$_{2A/2C}$ receptor. The reaction was terminated by rapidly filtering the mixture through Whatman GF/B glass-fiber filters and washed three times with 3.5 ml ice-cold Tris-HCl buffer using a cell harvester (Brandel cell harvester; Biomedical Research & Developmental Laboratories, MD, USA). Filters were then transferred to polyethylene vials. A volume of 6 ml Ready Safe (Beckman, CA, USA) scintillation cocktail was added to each vial, which was stored overnight at room temperature, and then counted using a Liquid Scintillation Counter (Beckman, CA, USA). Nonspecific binding for the 5-HT$_{1A}$ or the 5-HT$_{2A/2C}$ receptor was defined as the hot ligand bound in the presence of 100 μM 5-HT or 10 μM methysergide, respectively. Data were analyzed using the Ligand program.

RESULTS

Outcome of pregnancies and developmental indices

Average litter size was 10.5 heads, sex ratio was 1 : 0.93 (M : F), and birth weight of all animals 9.53±0.12 g. The ischemic stress and/or treatment with 8-OH-DPAT on gestation day 17 had no influence on the litter size, sex ratio, birth weight, eye-opening time, and performance in the wire maneuver test.

Adult behavior after prenatal ischemia

Pups were raised normally and their behavioral performances were determined in adulthood. No significant differences were found in the exploratory behavior tested on postnatal day 65. However, in the Rota-rod test on postnatal day 95, the Ischemia group showed a decreased latency to falling (21.8±2.8 s), indicating a decreased motor coordination (Fig. 1). This prenatal ischemia-induced impairment of motor coordination was prevented by the 8-OH-DPAT treatment before the ischemic insult on gestational day 17. The latency to falling of the 8-OH-DPAT-Ischemia group was 32.3±4.9 s, which was similar to that of the Sham group (35.9±4.5 s). Motor coordination of the 8-OH-DPAT-Ischemia group was actually much higher than that of the Ischemia group, because the speed of the rotating rod increased 3 times after 30 s stay on the rod.

To assess possible impairment of learning and memory after prenatal ischemia, the autoshaped learning test was performed on postnatal day 100. Numbers of the extended lever touches reached a plateau level after the 9th session in all groups, and no intergroup significant differences were observed for in this task (Fig. 2).

5-HT content in the cerebellum and 5-HT receptor densities in the cerebral cortex after prenatal ischemia

We were able to reliably measure the concentrations of 5-HT in cerebellar homogenates. Prenatal treatment with 8-OH-DPAT did not change the concentration of 5-HT or 5-HIAA, or the ratio of 5-HIAA/5-HT, at postnatal day 100 days. On the other hand, prenatal experience of ischemia resulted in a slight but significant decrease (11.2%) in the concentration of 5-HT, and the ratio of 5-HIAA/5-HT was 35.9% higher of that of the Sham group, although we were

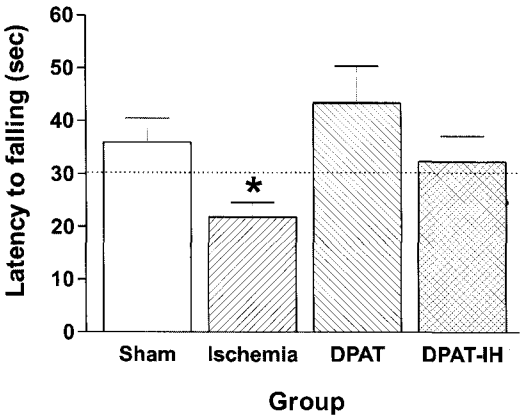

Fig. 1. Effect of 8-OH-DPAT on prenatal ischemia in the Rota-rod test performed on PND 95. The rotation rate of the bar was 1.5 rpm for initial 30 seconds, and then increased to 4.5 rpm (the dotted line). *$p<0.05$ vs. the Sham, and the 8-DPAT-ischemia groups by Scheffe's F test. Sham: the Sham group; Ischemia: the Ischemia group; DPAT: the 8-OH-DPAT group; DPAT-IH: the 8-OH-DPAT-ischemia group.

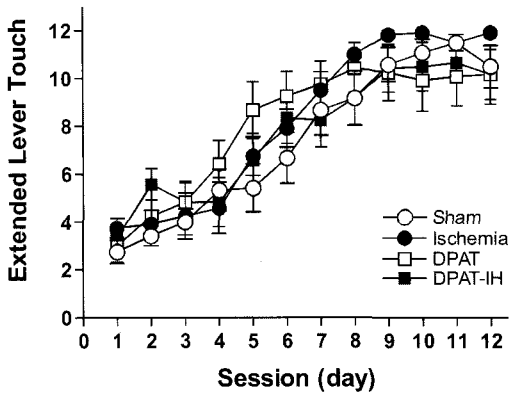

Fig. 2. Effect of 8-OH-DPAT on prenatal ischemia in the autoshaped learning test performed on PND 110. Rats were maintained 85% of free feeding body weight. On the test day, a rat was put into the standard operant chamber. The lever was presented 12 times/daily session and retracted when the rat made a lever touch response or after 15 sec. Abbreviations are the same as Fig. 1.

Table 1. Effect of 8-OH-DPAT on prenatal ischemia in the concentrations of 5-HT and 5-HIAA and the ratio of 5-HIAA/5-HT in the cerebellum of 100 day-old rats

Group	5-HT (ng/g tissue)	5-HIAA (ng/g tissue)	5-HIAA/5-HT (ratio)
Sham (8)	229.5±7.1	58.2±4.6	0.256±0.022
DPAT (8)	239.1±6.9	70.5±2.1	0.298±0.016
IH (8)	203.8±8.3[ab]	69.6±5.3	0.348±0.034[a]
DPAT-IH (6)	225.6±11.1	71.1±5.8	0.323±0.031

Values are mean±S.E.M. [a]$p<0.05$ vs. the Sham group by Scheffe's F test. [b]$p<0.05$ vs. the 8-OH-DPAT group by Scheffe's F test. The numbers in the parentheses are the number of animals. Each group consisted of 9 rats. Abbreviations are the same as Fig. 1.

Table 2. Effect of 8-OH-DPAT on prenatal ischemia in 5-HT_{1A} receptor binding assay in the cerebral cortex of 100 day-old rats

Group	B_{MAX} (pmole/g tissue)	pK_d (−log M)
Sham (8)	3.20±0.25	8.54±0.14
DPAT (8)	3.14±0.34	8.47±0.15
IH (8)	4.26±0.48[a]	8.56±0.11
DPAT-IH (6)	2.67±0.24	8.69±0.04

Values are mean±S.E.M. [a]$p<0.05$ vs. the Sham, the 8-OH-DPAT, and the 8-OH-DPAT-ischemia groups by Scheffe's F test. The numbers in the parentheses are the number of animals. Abbreviations are the same as Fig. 1.

unable to find significant differences in the concentrations of 5-HIAA. These neurochemical sequelae attributed to prenatal ischemia did not occur in the 8-OH-DPAT-Ischemia group (Table 1). A 5-HT_{1A} receptor binding assay was performed in the cerebral cortex of the same brain. Prenatal treatment with 8-OH-DPAT, at the dose of the 50 μg/kg s.c., did not change the B_{MAX} and pK_d values, measured at age of 100 days. However, we found increased values of B_{MAX} in the Ischemia group, which were 33.1% higher than that of the Sham group. Once again, increased B_{MAX} was not found in the 8-OH-DPAT-Ischemia group (Table 2).

DISCUSSION

In this study, we revealed that higher 5-HT_{1A} receptor activity at the time of a prenatal ischemic insult can protect the brain from behavioral and neurochemical sequelae.

We found impaired motor coordination and decreased 5-HT concentrations and increased 5-HT turnover rates in the cerebellum in 3 month-old rats, which had experienced transient ischemia *in utero* on gestational day 17. Interestingly, these behavioral and neurochemical impairments were not found in rats that had been treated with 8-OH-DPAT 30 min before the ischemic insult. Since impaired motor coordination was noted, we examined the cerebellum, which is known to play a key role in regulating body and limb movements. We focused on 5-HT because 5-HT is not only a neurotransmitter but also a potent neurotrophic factor, which acts during both the developmental and adult stages (Whitaker-Azmitia, 2001). Generally, 5-HT1A receptor agonists have been reported to be able to prevent acute ischemic damage (Mauler et al, 2001). However, 8-OH-DPAT has been previously reported to be fairly (Piera et al, 1995) or minimally (Bielenberg & Burkhardt, 1990) effective or wholly ineffective (Bode-Greuel et al, 1990). In the present study, therefore, it can be postulated that the preventive effect of 8-OH-DPAT was primarily due to its role in facilitation of post-ischemic adaptation rather than direct protection from ischemic insult.

Cerebellar 5-HT system has been suggested to modulate motor function via modulating excitatory amino acid neurotransmission in cerebellar Purkinje cells (Netzeband et al, 1993; Strazielle et al, 1996; Le Marec et al, 1998). We don't know, at this point, how low concentration of 5-HT was resulted from prenatal ischemia. One suggestion is that a massive increase of synaptic 5-HT during ischemic insult (Binienda et al, 1994) somehow leads to the loss of 5-HT terminals in the adult rat, as occurred in adult animals that experienced increased synaptic 5-HT by pharmacological manipulations during development (Akbary et al, 1994; Cabrera-Vera et al, 2000). Unfortunately, we were unable to perform receptor binding assay in the same cerebellar tissues because of a lack of tissue materials, and instead performed a 5-HT_{1A} receptor binding assay in the cerebral cortices of the same animals. If we assume the same regulatory changes in 5-HT_{1A} receptors occur in the cerebellum, our results suggest that decreased level of 5-HT terminals result in decreased levels of 5-HT, and that the brain compensates by increasing 5-HT turnover and by attenuating the negative feedback mechanism of 5-HT release. It is well known that the activation of presynaptic 5-HT_{1A} receptor leads to an inhibition of the release of 5-HT.

It is possible that the neuronal plasticity needed for adequate response to environmental stimuli may be inadequate in rats that have experienced prenatal ischemia, because we found altered 5-HT levels long after the experience of prenatal ischemia. In the present study, the ischemic insult was not severe enough to cause structural malformations, and the outcome of pregnancies was not different from the control. Therefore, it can be generalized that long-lasting inadequate brain plasticity can be caused by early life environmental stress even at levels below those required to produce structural abnormality.

We did not find any significant difference in the autoshaped learning test which is known to have both factors of the classical and the operant conditioning (Hughes & Sparber, 1978). Take into account the finding of impaired motor coordination, it may be that the present model of prenatal ischemia offers a good animal model for cerebral palsy that shows motor impairments mainly.

This study shows that the long-term behavioral and neurochemical sequelae resulting from prenatal ischemia can be prevented by increasing 5-HT_{1A} receptor activity at the time of ischemic insult. This suggests that the 5-HT_{1A} receptor protects the brain from ischemic insult and/or facilitates recovery after prenatally experienced ischemia. In addition to conventional treatments for brain ischemia, we propose to manipulate 5-HT system for brain ischemia, especially during developing period.

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