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Developmental Disability Animal Model Based on Neonatal Lipopolysaccharide with Altered 5-HT Function

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Developmental disability shows life-long behavioral abnormality with no significant physical malformation. This study was undertaken to develop an animal model for developmental disability by using two-factor approach. Lipopolysaccharide (LPS), a bacterial toxin, and NAN-190, a 5-HT_{1A} receptor antagonist, were administered to Sprague-Dawley rats on postnatal day (PND) 5 to induce inflammation and an altered 5-HT system, respectively. Long-term alteration of behavior occurred in the drug-treated groups. The LPS-treated group showed impaired motor coordination in the Rota-rod test. The LPS- treated or both LPS and NAN-190-treated groups showed impaired fore-paw muscle power in the wire maneuver test. These groups also showed decreased white matter volume and increased serotonergic fibers. The LPS and NAN-190-treated group also exhibited neurologic deficit in the placing reaction test and impaired equilibrium function in the tilt table test. The results showed that a variety of altered behaviors can be generated by two factor model, and suggested that combination of important etiologic factors and possible underlying defects is a promising strategy of establishing an animal model for developmental disabilities.

Key Words: Developmental disability, Lipopolysaccharide, 5-HT_{1A} receptor, Animal model

INTRODUCTION

Development of brain during developing period can be influenced by many factors, such as genetic characteristics, infection, exposure to drugs, environmental pollutants, trauma, physical stress, or unknown factors. Fetal death can occur when the brain is seriously damaged. Congenital malformation, growth retardation or abnormal functional development can also occur, depending on the severity of brain damage.

Developmental disability shows life-long alteration of behavior with no obvious physical malformation. Cerebral palsy, mental retardation, attention-deficit hyperactivity disorder, autism, and learning disability are included in this category of disorder. Many neurological and psychiatric symptoms accompany the developmental disability, and various symptoms are elicited from various damaged areas of brain, although the same etiological factor is related to the disorder (Grether et al, 1992; Rumeau-Rouguette et al, 1992; Roland & Hill, 1997). It is practically impossible to prevent the cause of developmental disability even if the cause is known. On the other hand, whatever the cause may be, altered behavior is elicited from altered function of brain. Therefore, understanding of pathophysiology is important to ameliorate symptoms which have occurred already. However, there is presently no intervention method targeting brain and even an accepted animal model for developmental disability. The animal model has been developed by applying environmental risk factors. Nevertheless, the failure of development of an animal model for developmental disability suggests that there may be another factor(s) in addition to the environmental risk factors. In the present study, we developed an animal model for developmental disability, based on the deranged 5-hydroxytryptamine (5-HT) system in addition to infection during development, an important environmental factor. Induction of inflammation was to generate periventricular leukomalacia, a typical pathological finding of cerebral palsy (Lin, 2003; Himmelmann et al, 2005), and the derangement of 5-HT system was to suppress active plasticity of developing brain.

In the present study, we applied two factors to develop an animal model for developmental disability. Lipopolysaccharide (LPS), a Gram-negative bacterial toxin, was administered to the lateral ventricle for generating inflammatory response in brain, and NAN-190, a 5-HT_{1A} receptor antagonist was also administered simultaneously because we found protection effect of 5-HT_{1A} receptor agonist on brain function (Lee et al, 2002).

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ABBREVIATIONS: 5-hydroxytryptamine, 5-HT; postnatal day, PND; lipopolysaccharide, LPS.

METHODS

Animals

To minimize and standardize unwanted environmental stimulation from in utero life, Sprague-Dawley rats were bred and the offsprings 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 in a SPF barrier area, and temperature (22±1°C) and humidity (55%) were constantly controlled with a 12 : 12 hour light:dark cycle (lights on 07:00 hour). Food (PMI Feeds, Inc, IN, USA) and tap water (membrane filtered purified water) were available ad libitum. Nulliparous female and proven breeder Sprague-Dawley male rats were used for breeding. Twelve hours after confirming delivery, pups were divided by gender and weighed. We used litters which included 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 was consistent with the NIH guideline Guide for the Care and Use of Laboratory Animals, 1996 revised (NIH, 1996).

Drug treatment and grouping

On postnatal day (PND) 5, NAN-190 HBr (Sigma-Aldrich Chemical Co., Yongin-city, Korea), dissolved in polyethyleneglycol 400 (vehicle), was administered intraperitoneally (i.p.) at a dose of 10 mg/kg/2 ml. Thirty min after NAN-190, LPS (Sigma-Aldrich Chemical Co., Yongin-city, Korea) dissolved in physiological saline (1% LPS, 10 mg/ml), was administered intracerebroventricularly (i.c.v.) at a dose of 20㎍/2㎕ (Lu et al, 1995). The location of i.c.v. was 1.0 mm right lateral and 1.0 mm posterior to the bregma, and the tip of the needle of the Hamilton syringe was situated 2 mm below the scalp. The rat was moved to the cage with heating blanket, and it was then moved to the cage of mother after recovering body temperature.

We had 4 experimental groups: 1) Vehicle+ Saline (Control): physiological saline 2㎕ i.c.v. 30 min after vehicle 2 ml/kg i.p., 2) Vehicle+ LPS: LPS 2㎕ i.c.v. 30 min after vehicle 2 ml/kg i.p., 3) NAN+ Saline: physiological saline 2 ㎕ i.c.v. 30 min after NAN-190 10 mg/kg/2 ml i.p., and 4) NAN+ LPS: LPS 2㎕ i.c.v. 30 min after NAN-190 10 mg/kg/2 ml i.p.. All experimental animals were males, and each group contained at least 12 animals, and more than 6 animals were assigned to each group for the later individual experiment.

Behavioral measurements

Righting reflex, Placing reaction, Toe spreading test: Neuronal reflexes for developmental indices were performed PND 8 and 21. They were measured by slightly modified methods of Huston and Bures (1976). For the righting reflex, a rat was held in the lumbosacral region. When the body was tilted to the left and right, the head moved in the opposite direction in order to maintain the original position in the normal reflex. For the placing reaction, a rat was restrained at the edge of the table and one hindleg was displaced, so that it hung over the edge. The leg was withdrawn immediately to the table surface in the normal reflex. For the toe spreading test, a rat was placed on a plexiglass sheet suspended over a mirror, which made it possible to observe the position of the toes of the rat. When the plexiglass platform was suddenly tilted or moved up and down, the toes were extended and spread in the normal reflex.

Rotarod test: Rotarod test was performed on PND 21. 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). A session consisted of 3 trials with an interval of 5 min.

Wire maneuver test: Wire maneuver test was performed on PND 28. For the wire maneuver test, a rat was hung by both forelimbs on the wire 50 cm high from the floor. The diameter of the wire was 1 mm. The body of a rat was maintained parallel to the floor by hanging the tail by experimenter's hand, and the tail was then released. Its falling down latency was measured as an index of forelimb strength.

Tilt-table test: Tilt-table test was performed on PND 98. For the tilt-table test, a rat was placed on a platform positioned parallel to the floor. The platform (73.5 cm×47 cm) was made of rubber floor on the plexiglass not to slip. The platform was suddenly tilted by 30°, letting the head located downwards. It turned to face up the slop in the normal reflex. The latency to turn the body parallel to the floor was measured as an index of equilibrium ability.

Morris water maze test: For measuring spatial learning ability, Morris water maze test was performed. This test started on PND 196. A rat was allowed to find hidden platform in the circular water tank (diameter: 2 m), which was filled with black ink-mixed opaque water. A rat was required to find an invisible escape platform just beneath the surface of the water by using surrounding cues. A trial consisted of 60 sec, and an experimenter guided the rat to the platform if it could not find the platform. One daily session consisted of 4 trials, and the rat was released in 4 different quadrants in each trial. On the 7^{th} day of training, the probe test was done, in which the platform was removed and the time spent in platform-quadrant was measured as an index of spatial memory. From the 8^{th} day, four day session was added to the original training for the reversal training, in which the platform was moved to the opposite quadrant to the original location. Formation of new strategy could be measured in the reversal training. After finishing the training, a cue test was done to confirm that the rat's visual ability was normal. For the cue test, a rat was allowed to find the platform which had a visible flag on it.

Immunohistochemistry for myelin basic protein (MBP) and 5-HT

A set of rats were sacrificed on PND 130 for immunohistochemical study. Rats were overdosed with pentobarbital and transcardially perfused first with heparinized isotonic saline containing 0.5% $NaNO_2$, followed by ice-cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer. The brains were dissected out immediately, blocked, post-fixed for 2 hours, and transferred to 30% sucrose for cryoprotection. Forty micron coronal sections were cut on a freezing, sliding microtome (HM440E, Microm Co., Wall-

dorf, Germany). Free-floating tissue sections were treated for 30 min with 0.2% Triton X-100 (1% BSA: 0.2% Triton X-100: 0.1 M PBS). Then, the tissues were incubated for 16 hours with monoclonal anti-MBP antibody (Oncogene, Cambridge, USA) or polyclonal anti-5-HT antibody (Eugene Tech, USA). After washing twice in PBS-BSA (0.1 M PBS: 0.5% BSA), incubated for 1 hour with biotinylated anti-rabbit IgG (1 : 200 dilution, Vector Laboratories, CA, USA), and then bound secondary antibodies were amplified with the ABC kit (Vectastatin Elite Kit, Vector Laboratories, CA, USA). Antibody complexes were visualized with 0.05% diaminobenzidine for 5 min. Sections were mounted in anatomical order onto gelatin-coated slides from 0.05 M PB, air dried, dehydrated through a graded ethanol to xylene, and coverslipped. The slides were observed by using light microscope and MCID imaging system.

Data analysis

Data are expressed as means±S.E., and statistical analyses were completed with an aid of StatView program (version 5.01, The SAS Instiute, CA, USA). All data were analyzed by one-way analysis of variance (ANOVA), and preplanned comparisons were performed by Fisher's protected least significance difference (PLSD) test.

RESULTS

Effects of neonatal LPS and/or NAN-190 on body weight

The difference of body weight was first observed on PND 49, and the pattern was maintained thereafter. The Vehicle + LPS group showed lower body weight than that of the Vehicle+ Saline group and the Vehicle+ NAN group (Fig. 1). On the other hand, an increase of body weight during 24 hours after treatment with drugs on PND 5 was noted in the Vehicle+ Saline group and the NAN+ Saline group, and a decrease was noted in the Vehicle+ LPS group and the NAN+ LPS group (Fig. 2). However, body weights of all 4 groups of animals increased during the next 24 hours, suggesting normal feeding and development.

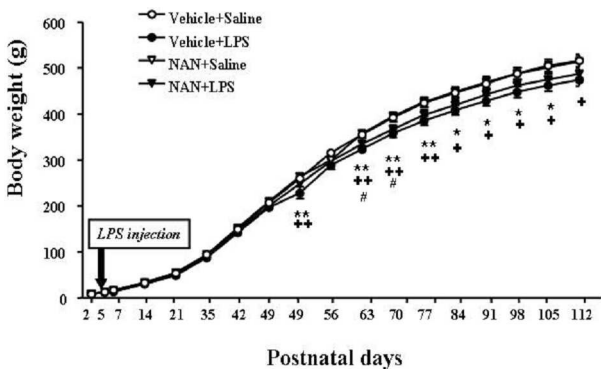

Fig. 1. Body weights after injection with lipopolysaccharide and/or NAN-190 on PND 5. In the Vehicle+ LPS group, $^{*}p<0.05$, $^{**}p<0.01$ compared with the Vehicle+ Saline group. $^{+}p<0.05$, $^{++}p<0.01$, compared with the NAN+ Saline group, #$p<0.05$, compared with the NAN+ LPS group.

Effects of neonatal LPS and/or NAN-190 on behavioral development

In the placing reaction test, the NAN+ LPS group, compared with the Vehicle+ Saline group, showed impaired reflex on PND 21, although this difference was not observed on PND 8. All pups showed intact reflex for the placing reaction. No abnormality was noted in the righting reflex (Fig. 3). Impaired motor coordination was noted in the Vehicle+ LPS group on PND 21 by the rotarod test (Fig. 4). Impaired forelimb muscle strength was also noted in the NAN+ LPS and the Vehicle+ LPS groups on PND 28 by the wire maneuver test (Fig. 5). The NAN+ LPS group also showed impaired equilibrium ability on PND 98 by the tilt-table test (Fig. 6). On PND196, Morris water maze test was started. After 6 days' training for spatial memory, the probe test on the 7th day and the reversal training for 4 days were performed. No significant differences between groups were found in this memory test (Fig. 7).

Effects of neonatal LPS and/or NAN-190 on the immunoreactivity of myelin basic protein

Animals were sacrificed on PND 130 for immunohistochemical studies. The lateral ventricles were widened in the LPS-treated groups, suggesting tissue damage by LPS. The defect of white matter was also observed in the groups treated with LPS neonatally (Fig. 8). When the width of white matter in the periventricular region was measured, the width of the Vehicle+ LPS and the NAN+ LPS groups were decreased more than that of other groups (Fig. 8).

Effects of neonatal LPS and/or NAN-190 on the immunoreactivity of 5-HT

The 5-HT immunoreactivity in the thalamus region was measured by the density of the serotonergic fibers after dark field examination. The relative optical density was measured by the MCID system. The immunoreactivity of the serotonergic fibers was higher in the Vehicle+ LPS and NAN+ LPS groups than that of the Vehicle+ Saline and the NAN+ Saline groups (Fig. 9). Similar pattern was observed also in the cerebral cortex (Fig. 10).

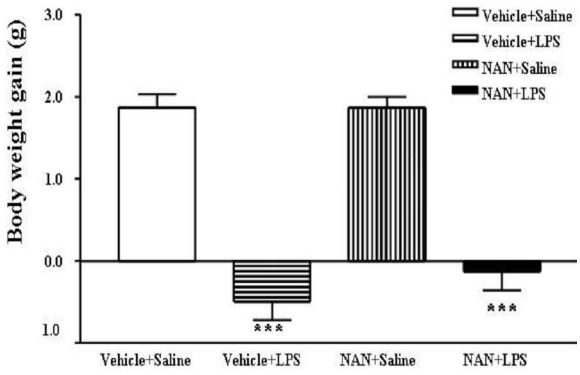

Fig. 2. Body weight gain 24 hours after injection with lipopolysaccharide and/or NAN-190 on PND 5. $^{***}p<0.001$, compared with the Vehicle+ Saline group.

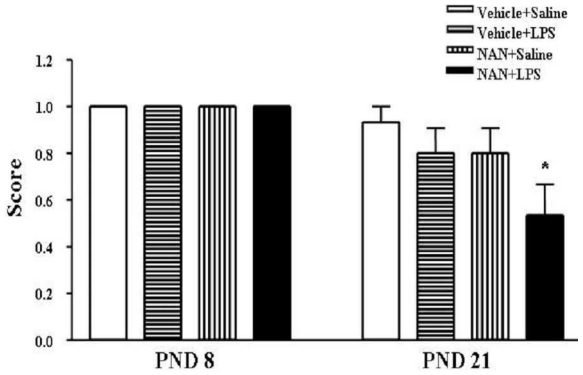

Fig. 3. Placing reaction measured on PND 8 and 21 after injection with lipopolysaccharide and/or NAN-190 on PND 5. *p < 0.05, compared with the Vehicle+ Saline group.

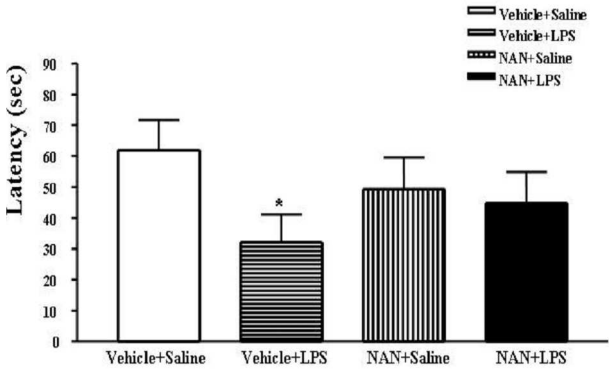

Fig. 4. Rota-rod test on PND 21 after injection with lipopolysaccharide and/or NAN-190 on PND 5. *p < 0.05, compared with the Vehicle+ Saline group.

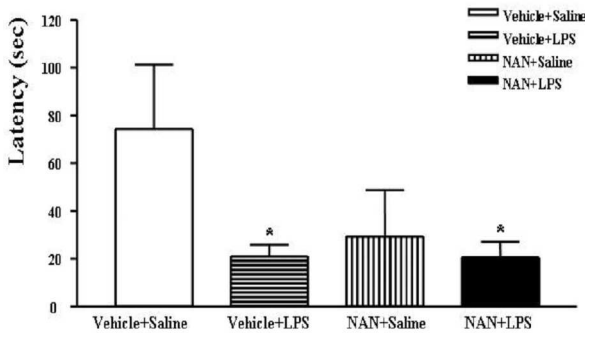

Fig. 5. Wire maneuver test on PND 28 after injection with lipopolysaccharide and/or NAN-190 on PND 5. *p < 0.05, compared with the Vehicle+Saline group.

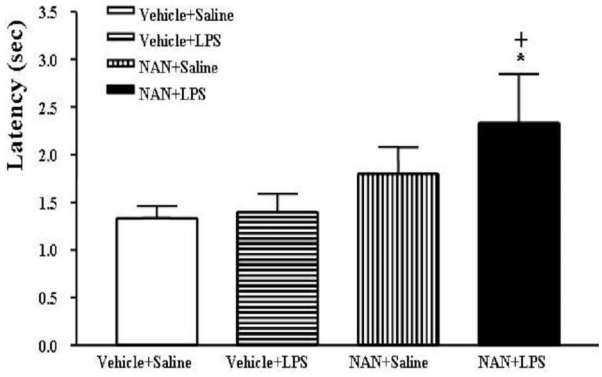

Fig. 6. Tilt-table test on PND 98 after injection with lipopolysaccharide and/or NAN-190 on PND 5. *p < 0.05, compared with the Vehicle+Saline group, + p < 0.05, compared with the Vehicle+LPS group.

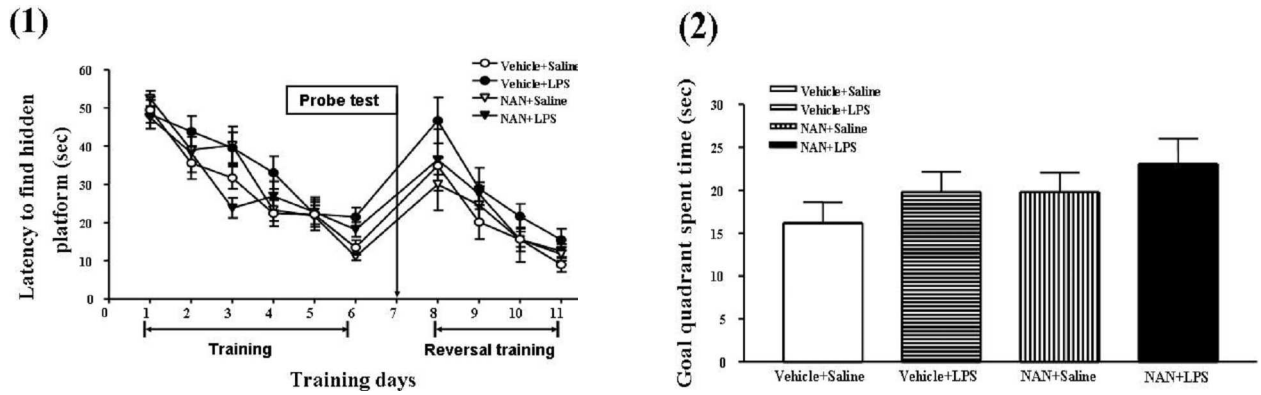

Fig. 7. Morris water maze test (1) started on PND 196 and the probe test (2) after injection with lipopolysaccharide and/or NAN-190 on PND 5.

DISCUSSION

The present study showed that various long-term behavioral alterations can be made by neonatal manipulation of two factors rather than one factor, and these altered behaviors can be one of the symptoms of developmental disability.

In this study, we tried to establish an animal model for developmental disability on the basis of brain infection

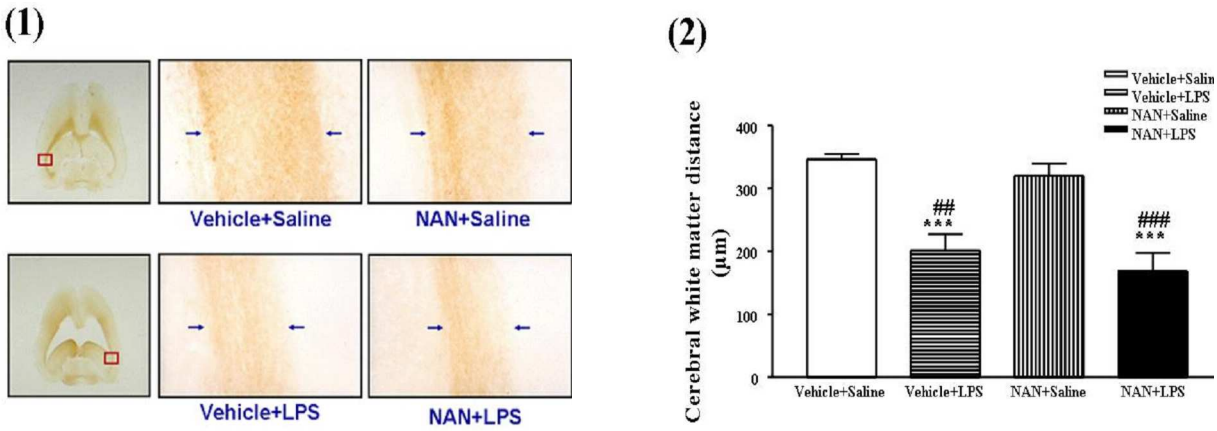

Fig. 8. (1) Myelin basic protein (MBP) immunohistochemistry on PND 130 after injection with lipopolysaccharide and/or NAN-190 on PND 5. Left 2 panels: whole brain. Right 4 panels: periventricular region (×400). (2) Distance of white matter in the periventricular region on PND 130 after injection with lipopolysaccharide and/or NAN-190 on PND 5. ***$p < 0.001$ vs. the Vehicle+ Saline group, ##$p < 0.01$, ###$p < 0.001$ vs. the NAN+ Saline group. n=3.

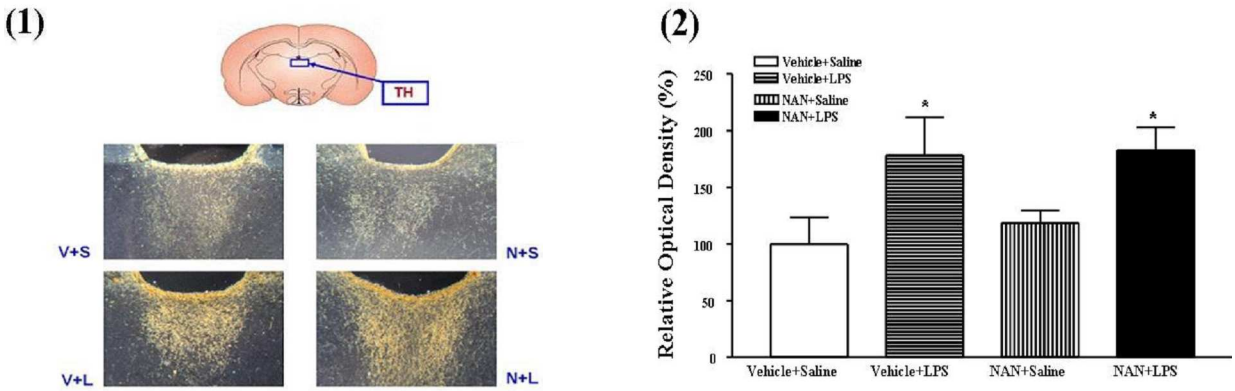

Fig. 9. 5-HT immunoreactive fiber (1) and measured density (2) of 5-HT immunoreactive fiber in the thalamus on PND 130 after injection with lipopolysaccharide and/or NAN-190 on PND 5. *$p < 0.05$ compared with the Vehicle+ Saline group.

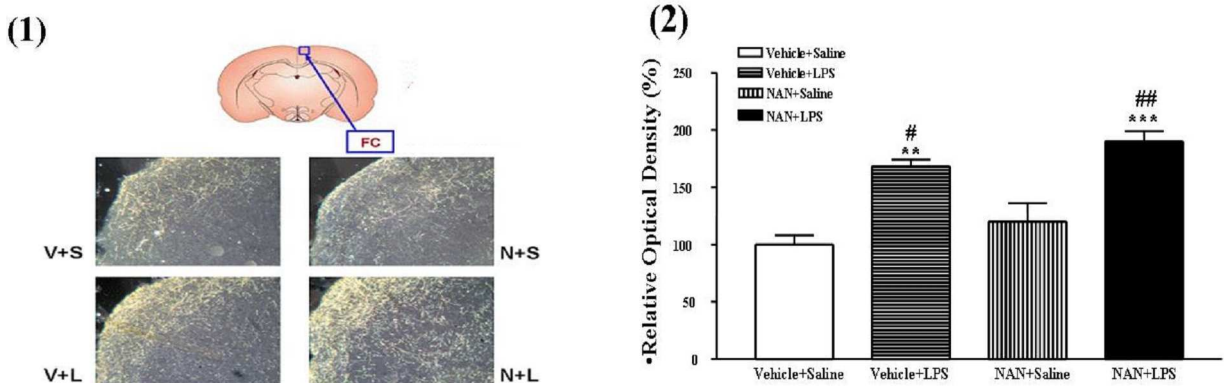

Fig. 10. 5-HT immunoreactive fiber (1) and measured density (2) of 5-HT immunoreactive fiber in the frontal cortex on PND 130 after injection with lipopolysaccharide and/or NAN-190 on PND 5. **$p < 0.01$, ***$p < 0.001$ compared with the Vehicle+ Saline group. #$p < 0.05$, ##$p < 0.01$ compared with the NAN+ Saline group.

together with altered 5-HT system. Periventricular leukomalacia (PVL) was elicited by LPS-induced inflammatory reaction, and altered 5-HT system was elicited by injecting 5-HT_{1A} receptor antagonist, NAN-190. PVL is a phenomenon of disappearance of periventricular white matter (Yi et al, 2000; Olaf et al, 2001). PVL is a representative pathologic finding of developmental disability, especially in the diplegic type of cerebral palsy (Elie & Marret, 2001). In this type of cerebral palsy, 90% of patients show PVL (Krageloh-Mann et al, 1995), and children with PVL suffer from 60~100% cerebral palsy (Leviton & Panetth, 1990). PVL can be elicited experimentally by hypoxia-ischemia (Rice et al, 1981; Hisakazu et al, 1999), excitotoxicity (Marret & Muckendi, 1995), thyroid deficiency (Anderson et al, 1987; Gressens et al, 1998), and inflammatory reaction (Gilles et al, 1977; Yoon et al, 1997; Zhengwei et al, 2000). Many of these methods, however, are not specific for PVL because hypoxia-ischemia can damage gray matter as well as white matter, and hormone deficiency can affect various function of the body. Various cytokines play a role in the inflammatory reaction induced by LPS. Tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), interleukin-6 (IL-6) and nitric oxide (NO) are generated, and these cytokines destroy immature or developing oligodendrocytes, resulting in damages in the white matter (Jhodie, 2002; Pang et al, 2003).

5-HT is an important neurotransmitter in the brain, and it exists in the raphe nucleus with a high concentration. When 5-HT activity increases, food intake and aggressiveness decrease. On the other hand, obesity and aggressiveness can occur when 5-HT activity decreases. Following points may explain that 5-HT plays a key role in conduction of brain function. 1) 5-HT is a phylogenetically very old compound and found in insects or plants. This facts suggest that there is a possibility that 5-HT plays a key role in adapting to varing environment, which is a basic function of the life (Berendsen & Broekkamp, 1990). 2) 5-HT exists ubiquitously in the brain and shows seldom varicosities (Descarries et al, 1990). This distribution characteristic suggests that 5-HT plays a role in regulating, rather than specifically acting, brain function, and promotes a harmony of the whole brain function. 3) 5-HT shows lower activity than other neurotransmitters, such as norepinephrine or dopamine, in the normal physiological condition (Sanders-Busch, 1990). This characteristic enables it to act promptly under the situation of adapting to varing environment, although it may not have an important role in the resting condition. 4) Changed activities of serotonergic neurons result in metabolism and blood flow of the brain (Cohen et al, 1996). This is an evidence of direct regulation of brain function by 5-HT. 5) 5-HT plays a role as a neurotransmitter as well as a neurotrophic factor (Lauder, 1990, 1995). 5-HT is intimately related with S-100β which is released from astrocytes. S-100β is synthesized and released by the activation of 5-HT_{1A} receptors in the astrocyte, and S-100β plays a role of a neurotrophic factor for the serotonergic neuron (Lauder & Liu, 1994; Whitaker-Azmitia & Azmitia, 1994). S-100β is an important factor for synaptic formation. The synaptic formation is decreased, and learning ability is impaired after eliminating 5-HT on PND 2 (Mazer et al, 1997). All the above characteristics of 5-HT indicate that it plays an important role in the neuronal plasticity to adapt to changing environment.

The present study showed that two-factor manipulation was better than single factor in terms of generation of variable symptoms in an animal model for developmental disability. Decrease of body weight gain and impaired motor coordination were observed in the Vehivle+ LPS group. In both the Vehicle+ LPS and the NAN+ LPS groups, no weight gain was noted during 24 hours after injection, and impaired forelimb muscle strength was observed. These groups also showed decreased white matter distance and increased serotonergic fibers in the thalamus and cortical areas. Impaired placing reaction and equilibrium ability were observed only in the NAN-LPS group.

Developmental disabilities in human are usually diagnosed after 2 years of age as cerebral palsy, mental retardation or learning disability, etc. Late appearance of definite human symptoms of developmental disabilities was also shown in the animal in the present study, in which a decreased body weight gain occurred after 49 days of age, and an impaired placing reaction occurred on PND 21, but not on PND 8.

A decrease in the amount of white matter was observed in the Vehicle+ LPS group as well as in the NAN+ LPS group. This finding suggests that 5-HT is not related to the content of white matter, as expected. An increase of serotonergic fibers was observed in the Vehicle+ LPS group as well as NAN+ LPS group. It is consistent with earlier report which showed that 5-HT release in the hippocampus, thalamus, and hypothalamus was increased by LPS (Nolan et al, 2000). This reaction to the inflammation could be a compensatory mechanism of the brain, and the increased 5-HT release may play a role in neuronal plasticity of the damaged brain. In the present study, NAN-190 antagonized 5-HT_{1A} receptors activated by the function of increased serotonergic fibers, therefore, the NAN-190+ LPS group showed more impaired neuronal reflex in the placing reaction test and more impaired equilibrium ability in the tilt table test than the Vehicle+ LPS group.

In summary, we tried to establish an animal model for developmental disability by adding a derangement of 5-HT system to an important etiologic factor, inflammation. This two-factor animal model could generate several behavioral expressions similar to human developmental disabilities. Combination of important etiologic factors and possible underlying defects could provide a promising strategy of establishing an animal model for developmental disability, although two factor animal model of the present study is not perfect.

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