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Improvement by repeated administration of 6*R*-tetrahydrobiopterin of 5,7-dihydroxytryptamine-induced abnormal behaviors in immature rats

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Abstract

To clarify the therapeutic effects of 6*R*-L-erythro-5,6,7,8-tetrahydrobiopterin (6R-BH_4) on the abnormal behaviors induced by neonatal 5,7-dihydroxytryptamine (5,7-DHT, 100 μg; i.c.v.) treatment in immature rats, 6R-BH_4 (10–40 mg/kg) was administered intraperitoneally from 22nd to 28th days or only once on the 28th day. The locomotion activities decreased dramatically in 5,7-DHT-treated rats ($p < 0.01$; as compared to controls) on the 28th day. The reduced locomotion was recovered dose-dependently by repeated administration of 6R-BH_4, whereas it was not altered after a single injection of 6R-BH_4. In addition, repeated administration of 6R-BH_4 significantly facilitated 5-HT turnover ratio (5-HIAA/5-HT) in the striatum, cerebral cortex, and cerebellum. These findings suggest that the behavioral restoration by 6R-BH_4 might be due to the enhancement of 5-HT turnover by accumulated but not a single dose of 6R-BH_4.

Keywords: 6*R*-L-Erythro-5,6,7,8-tetrahydrobiopterin (6R-BH_4); Serotonin (5-HT); 5-HT turnover ratio; Locomotion activity; Open-field test; Rat

6*R*-L-Erythro-5,6,7,8-tetrahydrobiopterin (6R-BH_4) is a natural cofactor for three aromatic amino acid hydroxylases, phenylalanine hydroxylase (EC 1.14.16.1), tyrosine hydroxylase (EC 1.14.16.2), and tryptophan hydroxylase (EC 1.14.16.4) [1]. Tyrosine and tryptophan hydroxylases are the rate-limiting enzymes of catecholamine (dopamine and noradrenaline) and serotonin (5-HT) biosynthesis in the brain, respectively [2]. 6R-BH_4 is also a cofactor for nitric oxide synthase (NOS) [3]. Our group demonstrated that 6R-BH_4 showed the facilitation of dopamine (DA) [4] and 5-HT [5] release as well as promotion of their biosyntheses in the rat brain, when perfused through the dialysis membrane.

In clinical cases, Kaufman et al. [6] first reported that the deficiency of 6R-BH_4 is associated with atypical hyperphenylketonuria (malignant hyperphenylalaninemia) and administration of 6R-BH_4 to the patients was found to improve their clinical symptoms. Additionally, administration of 6R-BH_4 has been reported to show the therapeutic effects on the patients with a variety of neuropsychiatric disorders, such as Parkinson's disease [7], depression [8], familial dystonia [9], and infantile autism [10]. Although these diseases were more or less related to the abnormalities of monoaminergic neurons in the central nervous system, the detailed mechanisms on the therapeutic effect of 6R-BH_4 were not clear yet. Over the past decade, we have evaluated the effects of the administration of 6R-BH_4, mostly on the DAergic function in intact rats and monkeys including in vivo studies such as positron emission tomography (PET) [11,12], and we [13] also suggested that the repeated administration but not a single administration of 6R-BH_4 affects the expression level of 5-HT_{1A} and 5-HT_2 receptors, although the behavioral change was not

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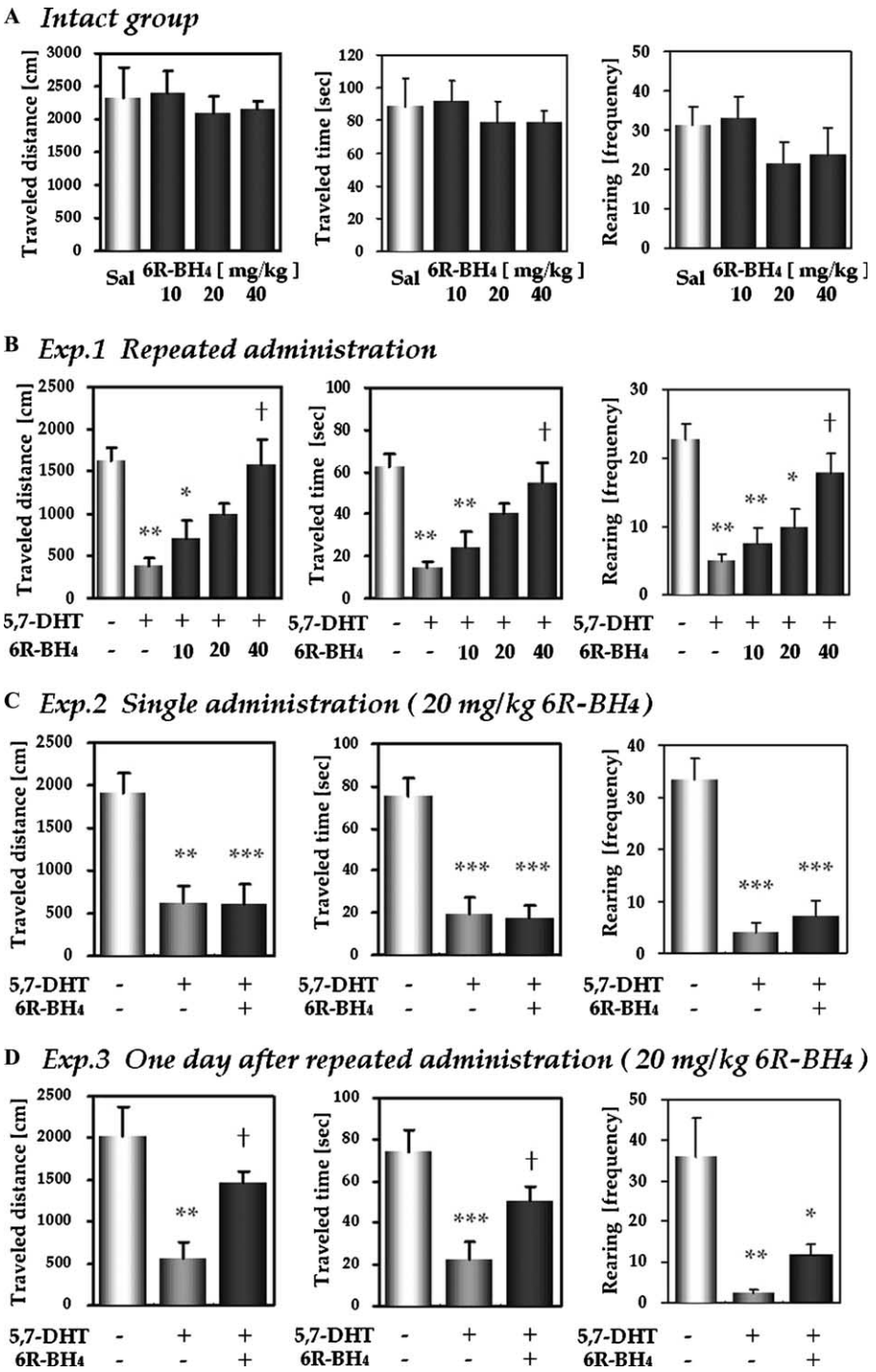

Fig. 1. Effects of the repeated intraperitoneal administration of 6R-BH_4 on the traveled distance in immature rats with neonatal 5,7-DHT treatment. The effects of 1-week repeated administration of 6R-BH_4 on the locomotion activities were investigated in 4-week-old intact (Intact group; A) and 5,7-DHT-treated rats. Detailed protocols for 5,7-DHT treatment and for 6R-BH_4 administration with controls in Experimental groups 1 (Exp. 1; B), 2 (Exp. 2; C), and 3 (Exp. 3; D) are described under "Materials and methods." Data are presented as means ± SEM. Statistical analyses were performed by unpaired Student's t test in intact group and by Scheffe's F test in Exp. 1–3. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, as compared with vehicle/saline group; $^{\dagger}p < 0.05$, as compared with 5,7-DHT/saline group.

studied at that time. In this study, we attempted to clarify the therapeutic effect of 6R-BH_4 on the abnormal behaviors induced by neonatal 5-HTergic depletion model.

Materials and methods

Animals. Pregnant Wistar rats were obtained at 15–18 days of gestation from Ishikawa Animal Laboratory (Saitama, Japan). Each

litter size was adjusted 8–10 pups to each mother rat before weaning (22 days after birth). Following weaning, the male pups were used in this study and separated to be four in the cage until behavioral tests. They were housed with free access to food and water under a 12-h light-dark cycle (lights on at 6:00 a.m.) at 22 ± 1 °C.

Chemicals. 5,7-Dihydroxytryptamine creatinine sulfate (5,7-DHT) was purchased from I.C.N. Biomedicals (OH, USA); desipramine hydrochloride from Sigma Chemical (MO, USA); and 6R-L-erythro-5,6,7,8-tetrahydrobiopterin hydrochloride (6R-BH_4) from Schircks Lab (Jona, Switzerland).

Neonatal lesions. 5-HT lesions were made with 5,7-DHT, injected into each lateral ventricle on the 3rd and 6th day after birth. On the 3rd day, 5,7-DHT (50 μg as a free base in 5 μl) dissolved in 0.9% saline containing 0.1% ascorbic acid or vehicle was given into the right ventricle (3.0 mm through the skull, 1.0 mm lateral to sagittal suture, at the level of bregma) [14] using 27-G needle Hamilton syringe. The injection was repeated in the left ventricle on the 6th day (total dose 100 μg) [15]. All groups were pretreated with desipramine hydrochloride (25 mg/kg, s.c.) at 30 min before the intraventricular injections of 5,7-DHT, in order to protect catecholamine neurons [16].

Administration of 6R-BH_4. As was done in the previous study by Muguruma et al. [13], 6R-BH_4 dissolved in 0.9% saline was administered intraperitoneally at 10, 20, or 40 mg/kg dose (once a day for 1 week, 22nd–28th day after birth) in 5,7-DHT-lesioned or intact rats. In acute single dose experiment, 6R-BH_4 (20 mg/kg) was administered intraperitoneally on 28th day in the rats treated with 5,7-DHT. As controls for repeated and single administration, 0.9% saline alone was injected according to the same protocols.

Behavioral studies. The locomotor activities were assessed in an open-field apparatus (diameter, 75 cm; height, 40 cm) with the trucking system (BAS-2 system, Muromachi, Tokyo, Japan), which was equipped with the CCD camera above the apparatus and the personal computer in the adjacent room. During 20-min session the traveled distance (cm) and time (s) were recorded automatically, and the numbers of rearing (frequency) were counted manually via the observation monitor. Rats were placed on an open-field apparatus at 1 h after the administration of saline or 6R-BH_4. Behavioral observations in all experiments were performed at around 10:00 a.m.

Analyses of 6R-BH_4 and monoamines. The effects of various compounds were confirmed by analyzing 6R-BH_4 and monoamine levels in the rat brain. Under deep anesthesia with diethyl ether, the brain was removed and dissected into 9 regions, i.e., hippocampus, striatum, frontal cortex, medial cortex, posterior cortex, pons/medulla, thalamus/midbrain, hypothalamus, and cerebellum at 1 h after administration of saline or 6R-BH_4. The specimens were weighed and homogenized with 5 volumes of 0.1 M perchloric acid containing 3 mM L-cysteine and 6 mM ascorbic acid. The homogenates were centrifuged at 10,000 rpm for 20 min at 4 °C and the resulting supernatant was filtered (0.45 μm pore size). The samples were stored at −80 °C until analysis.

The measurement of 6R-BH_4 was performed according to the modified method of Tani and Ohno [17] using high-performance liquid chromatography (HPLC) system, consisting of DG-300 degasser, EP-300 pump and ATC-300 column heater (Eicom, Kyoto, Japan) with fluorimetric detection (L-7485; Hitachi, Tokyo, Japan), and reversed-phase column (ϕ4.6 mm × 250 mm; 5C18-MS-II, Nacalai Tesque, Kyoto, Japan). The mobile phase was 0.1 M sodium phosphate buffer containing 4.5 mM sodium 1-octanesulfonate (SOS), 0.1 mM ethylenediaminetetraacetic acid disodium salt (EDTA-2Na), 0.1 mM ascorbic acid, and 5% (v/v) methanol (pH 2.9). The flow rate was 1.0 ml/min. After the separation by the HPLC column, 6R-BH_4 was introduced to nitrite oxidation by $NaNO_2$ in S-3850 reaction heater (Soma, Tokyo, Japan) at 80 °C and detected fluorometrically by excitation at 350 nm and emission at 440 nm.

For monoamines and their metabolites, the aliquots of the samples were analyzed by the HPLC system with electrochemical detection (ECD-300; Eicom, Kyoto, Japan) and reversed-phase column (ϕ3 mm × 100 mm, SC-3ODS; Eicom, Kyoto, Japan). The mobile phase adjusted to pH 3.5 was 80% of the mixed solution of 0.1 M citrate and 0.1 M sodium acetate (composition ratio = 10:9), 20% (v/v) methanol including 0.97 mM (210 mg/L) SOS and 0.013 mM (5 mg/L) EDTA-2Na. The flow rate was 0.33 ml/min. The electrochemical condition executed to a glassy carbon working electrode was set at 750 mV vs. an Ag/AgCl reference electrode. The index of turnover was expressed by the ratio of 5-HIAA to 5-HT in various brain regions [14].

Statistical analysis. All data are expressed by means ± SEM. The evaluation of the effects of the administration of 6R-BH_4 or saline on the behavior in intact rats and the concentration change of 6R-BH_4 between 6R-BH_4 or saline injection in 5,7-DHT-treated rats was conducted using unpaired Student's t test. Furthermore, a comparison of behavioral effects and monoamine/metabolite contents by the administration of different doses of 6R-BH_4 or saline in 5,7-DHT-treated rats was subjected to a one-way analysis of variance (one-way ANOVA) followed by the post-variance test of Scheffe's F test. A probability level of $p < 0.05$ was considered statistically significant.

Table 1
Accumulation of 6R-BH_4 (ng/g wet tissue) in the different brain regions of 5,7-DHT-treated rats with repeated administration of saline or 6R-BH_4

6R-BH_4 Brain regions	5,7-DHT		Fold
	Saline	6R-BH_4	
Hippocampus	73 ± 3	232 ± 37**	3.2
Striatum	316 ± 10	441 ± 23***	1.4
Frontal cortex	98 ± 3	322 ± 26***	3.3
Medial cortex	122 ± 7	320 ± 19***	2.6
Posterior cortex	71 ± 2	287 ± 28***	4.0
Thalamus/midbrain	250 ± 10	453 ± 23***	1.8
Hypothalamus	435 ± 20	788 ± 67***	1.8
Pons/medulla	204 ± 27	476 ± 106***	2.3
Cerebellum	103 ± 2	412 ± 40***	4.0

6R-BH_4 contents were analyzed as described under "Materials and methods." Data are presented as means ± SEM ($n = 7$).
**$p < 0.01$, and ***$p < 0.001$, as compared with saline injection.

Results

Repeated administration of 6R-BH$_4$ in intact and 5,7-DHT-lesioned rats

In intact rats, the traveled distance and time and count of rearing were not significantly altered by the repeated administration of 6R-BH$_4$ (Fig. 1). 5,7-DHT-treated rats showed less traveled distance and time and rearing behavior than control group ($p < 0.01$). The repeated administration of 6R-BH$_4$ dose-dependently recovered the decrease of locomotion induced by 5,7-DHT. In the group with the 6R-BH$_4$ dose of 40 mg/kg, their locomotion activities were almost at the same level as those of the control group in an open-field apparatus (Fig. 1). However, a single injection of 6R-BH$_4$ in 5,7-DHT-treated rats did not cause any significant improvement as compared with saline group (Fig. 1).

One day after repeated administration

Then the open-field test was performed on 1 day (24 h) after the cessation of the repeated administration of 6R-BH$_4$. 6R-BH$_4$ administration group still showed

Table 2
Recovery effects by repeated administration of 6R-BH$_4$ on 5-HT and 5-HIAA contents, and 5-HT turnover index in 5,7-DHT-treated rats

Brain regions Group (i.c.v./i.p.)	Conc. [ng/g wet tissue]		5-HT turnover ratio [5-HIAA/5-HT]
	5-HT	5-HIAA	
Hippocampus			
Vehicle/saline	286 ± 47 (100)	332 ± 11 (100)	1.30 ± 0.11 (100)
5,7-DHT/saline	28 ± 2 (9.7)***	37 ± 8 (11.3)***	1.33 ± 0.23 (102)
5,7-DHT/6R-BH$_4$	25 ± 2 (8.8)***	56 ± 8 (16.8)***	2.30 ± 0.44 (177)*
Striatum			
Vehicle/saline	229 ± 8 (100)	294 ± 13 (100)	1.29 ± 0.05 (100)
5,7-DHT/saline	21 ± 7 (9.2)***	24 ± 10 (8.3)***	0.98 ± 0.13 (75.9)
5,7-DHT/6R-BH$_4$	32 ± 9 (13.8)***	49 ± 14 (16.5)***	1.48 ± 0.15 (115)†
Frontal cortex			
Vehicle/saline	233 ± 8 (100)	216 ± 5 (100)	0.94 ± 0.03 (100)
5,7-DHT/saline	18 ± 2(7.7)***	12 ± 3(5.6)***	0.63 ± 0.06 (67.3)**
5,7-DHT/6R-BH$_4$	15 ± 1 (6.5)***	13 ± 2 (5.9)***	0.83 ± 0.09 (88.6)
Medial cortex			
Vehicle/saline	300 ± 10 (100)	250 ± 4 (100)	0.84 ± 0.03 (100)
5,7-DHT/saline	51 ± 18 (17.0)***	29 ± 11 (11.8)***	0.53 ± 0.03 (62.8)***
5,7-DHT/6R-BH$_4$	69 ± 16(23.0)***	47 ± 12 (18.9)***	0.66 ± 0.03 (78.2)**†
Posterior cortex			
Vehicle/saline	227 ± 6 (100)	201 ± 8 (100)	0.89 ± 0.04 (100)
5,7-DHT/saline	29 ± 7 (13.0)***	20 ± 5 (9.8)***	0.66 ± 0.03 (73.9)***
5,7-DHT/6R-BH$_4$	26 ± 5 (11.6)***	27 ± 5 (13.3)***	1.01 ± 0.09 (114)††
Thalamus/midbrain			
Vehicle/saline	632 ± 32 (100)	564 ± 13 (100)	0.91 ± 0.04 (100)
5,7-DHT/saline	374 ± 103 (59.2)*	253 ± 61 (9.8)***	0.71 ± 0.05 (77.7)*
5,7-DHT/6R-BH$_4$	638 ± 80 (101)	451 ± 62 (13.3)††	0.71 ± 0.06 (78.3)*
Hypothalamus			
Vehicle/saline	525 ± 38 (100)	604 ± 44 (100)	1.20 ± 0.12 (100)
5,7-DHT/saline	452 ± 98 (86.0)	319 ± 69 (52.7)	0.71 ± 0.06 (59.3)*
5,7-DHT/6R-BH$_4$	673 ± 112 (128)	631 ± 140 (104)	1.00 ± 0.16 (83.5)
Pons/medulla			
Vehicle/saline	699 ± 24 (100)	562 ± 24 (100)	0.48 ± 0.08 (100)
5,7-DHT/saline	728 ± 76 (104)	395 ± 69 (70.4)	0.34 ± 0.09 (65.9)
5,7-DHT/6R-BH$_4$	795 ± 55 (114)	565 ± 53 (101)	0.50 ± 0.09 (88.5)
Cerebellum			
Vehicle/saline	53 ± 3 (100)	84 ± 4 (100)	1.60 ± 0.08 (100)
5,7-DHT/saline	30 ± 6 (56.7)**	31 ± 7 (36.4)***	0.95 ± 0.09 (59.5)**
5,7-DHT/6R-BH$_4$	32 ± 6 (60.6)*	51 ± 9 (60.3)**	1.59 ± 0.18 (99.3)††

5-HT and 5-HIAA contents, and 5-HT turnover index were analyzed as described under "Materials and methods." Data are presented as means ± SEM ($n = 7$–10).

Statistical significance is analyzed by Scheffe's F test: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, as compared with vehicle/saline group; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, as compared with 5,7-DHT/saline group.

the increase in the traveled distance and time, and count of rearing than saline group in 5,7-DHT-treated rats. Therefore, the recovery from the 5-HT depletion states might be established by repeated administration of 6R-BH_4 and that state was not caused by the acute effect of the last administration (Fig. 1).

6R-BH_4 level

As shown in Table 1, 6R-BH_4 concentrations in the brain were elevated after the repeated administration of 6R-BH_4. The increase was significant in all brain regions as compared with saline group in 5,7-DHT-treated rats. However, there was an apparent regional difference in the increment: It was considerably higher in the posterior cortex and cerebellum (approximately 4.0-fold) by the intraperitoneal administration of 6R-BH_4.

Monoamine levels

As shown in Table 2, in 5,7-DHT-treated rats, the levels of 5-HT and 5-HIAA were reduced in all brain regions, especially highly reduced in the 5-Htergic terminal regions such as the hippocampus, striatum, and cortical regions. Sharp contrast was observed in neuronal somata regions (pons/medulla) and some relatively proximal projection regions such as the cerebellum, hypothalamus, and thalamus/midbrain. In addition, 5-HT turnover ratio was significantly decreased in the frontal, medial, and posterior cortices, thalamus/midbrain, hypothalamus, and cerebellum.

By the repeated administration of 6R-BH_4, 5-HT and 5-HIAA levels in 5,7-DHT-lesioned rats were recovered in some regions (Table 2). Especially, the 5-HIAA level was recovered in most of the regions and therefore the 5-HT turnover ratio was enhanced in the regions in which the accumulation fold of 6R-BH_4 was higher than other regions (Table 1, posterior cortex and cerebellum).

Other monoamines, NE, DA and its metabolites, were not significantly changed by 5,7-DHT-lesion nor by the repeated and single administration of 6R-BH_4 (data not shown).

Discussion

In the present study, we found that the locomotion activities at 4 weeks old markedly decreased by 5,7-DHT treatment in neonatal period (Fig. 1). In such model rats, the levels of 5-HT and 5-HIAA were dramatically decreased in the remote terminal regions such as hippocampus, striatum, and cerebral cortices, which was associated with the decrease of 5-HT turnover ratio in almost all regions (Table 1). Lucot and Seiden [18] first demonstrated that intracerebral injection of 5,7-DHT on the 3rd day after birth resulted in a decrease of locomotion activities 15 days later, and this behavioral change was thought to be due to the depletion of 5-HT contents mostly in the rostral regions of the brain by other reports [14,18,19]. The depletion also led to an alteration of the sensitivity of 5-HT receptors, in particular, 5-HT_1 receptor in the brain regions during development [20,21]. These results suggest that low activity of spontaneous behavior in 5,7-DHT-lesioned rats might be induced by altered sensitivity of 5-HT receptors through the depletion of 5-HT in the developing brain. The model animal for explaining certain features of infantile autism (hypoactivity and less exploring activity under the novel environment) was thus established.

In the previous report [13], we demonstrated the change of 5-HT_{1A} and 5-HT_2 receptors by repeated intraperitoneal administration of 6R-BH_4 for 2 weeks in intact young adult rats. In this case, too, a single injection of 6R-BH_4 could not alter the level of 5-HT receptors. Here in a 5,7-DHT-treated animal model, we could demonstrate both behavioral recovery and the recovery of 5-HT and 5-HIAA levels in some brain regions and 5-HT turnover ratio (5-HIAA/5-HT) in wider brain regions. The present data suggested the necessity of some effective period for such restoration, possibly through the change of receptor density and sensitivity. Such studies in the receptor level and other studies focusing on the change of gene expression are currently in progress in our laboratory.

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