

Effect of tetrahydrobiopterin on nitric oxide synthase-containing cells in the rat hippocampus

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Abstract

We have observed that tetrahydrobiopterin (BH₄), a cofactor of nitric oxide synthase (NOS), acts as a self-protection factor against nitric oxide (NO) toxicity in PC12 cells. To further investigate the self-protection action of BH₄ in vivo, the effect of deletion of endogenous BH₄ on NO-producing cells was examined in the rat hippocampus. Following the peripheral infusion of 50 mM 2,4-diamino-6-hydroxypyrimidine (DAHP), an inhibitor of GTP cyclohydrolase I, using a miniosmotic pump for 14 days, BH₄ content in the hippocampus decreased as compared with the control group administered with vehicle solution, which had no effect on brain BH₄ content. When the rats were administered with 50 mM DAHP and 10 mM BH₄, the DAHP-induced decrease in BH₄ content was prevented. The extracellular concentration of NO metabolites remained unchanged following DAHP administration, suggesting that DAHP-induced decrease in BH₄ content had no effect on NO production. The number of NOS-positive cells decreased following DAHP administration in the hippocampal regions, while the number of NOS-negative cells remained unchanged. The DAHP-induced decrease in the NOS-positive cell number was prevented by the administration of 10 mM BH₄ and DAHP. These results suggest that endogenous BH₄ may affect NOS-positive cell number in the rat hippocampus.

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1. Introduction

Nitric oxide (NO) has diverse biological effects. NO is synthesized by NO synthase (NOS). There are several isoforms of NOS; two constitutive types (nNOS or NOS-1, and eNOS or NOS-3) and one inducible type (iNOS or NOS-2) (Nathan and Xie, 1994). Although these isoforms differ in some properties such as Ca²⁺ dependency, all these isoforms of NOS catalyze the conversion of arginine to citrulline and NO under aerobic conditions and require tetrahydrobiopterin (BH₄) as a cofactor (Mayer and Werner, 1995). In various tissues, NO acts as a signal messenger or a cytotoxic agent (McCall and Vallance, 1992; Schmidt and Walter, 1994). As a messenger, NO induces vasodilatation, synaptic plasticity, or neuronal, endocrine and exocrine secretion (Snyder and Brecht, 1991; Lowenstein and Snyder, 1992; Reid et al., 1996).

As a cytotoxic agent, NO is involved in immune responses mediated by macrophages and neutrophils (Corraçilia et al., 1993; Lewis et al., 1995; Evans et al., 1996; Brüne et al., 1997). Although activated macrophages and neutrophils secrete NO to exert their antimicrobial or antitumor function, these NO-producing cells themselves are resistant to NO (Stamler, 1994; Blanco et al., 1995; Brüne et al., 1997). Several lines of evidence indicate that apoptotic neuronal death is a critical pathological process in neurodegenerative disorders (Su et al., 1994; Lassmann et al., 1995; Portera-Cailliau et al., 1995; Schreiber and Baudry, 1995; Thompson, 1995). NO is produced in glia cells as well as neurons (Murphy et al., 1993) and NO is considered to play a key role in apoptosis in neurodegeneration (Dawson et al., 1991, 1994; Chao et al., 1992; McMillian et al., 1995; Jacobson, 1996; Troy et al., 1996). In the brain with neurodegenerative disorders, NO-producing neurons survive during the degeneration process (Ferrante et al., 1985; Kowall and Beal, 1988; Uemura et al., 1990; Lowenstein and Snyder, 1992). Thus, it is spec-

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ulated that NO-producing cells have self-protective factors against NO under physiological and pathological conditions. Substrates the expression of which is triggered by NO or essential factors for NO production are possible candidates for self-protective agents against NO toxicity. The transcription of some genes encoding antioxidant agents such as superoxide dismutase, glucose-6-phosphatase and glutathione S-transferase is induced by NO (Stamler, 1994). BH₄, a cofactor of NOS, was reported to degrade NO in a cell-free system (Mayer et al., 1995). Since BH₄ is consistently present in NO-producing cells, this report raised the possibility that BH₄ acts as a self-protective factor against NO-induced cytotoxicity in NO-producing cells. We have observed that the inhibition of BH₄ synthesis by 2,4-diamino-6-hydroxypyrimidine (DAHP), an inhibitor of GTP cyclohydrolase I, decreases cellular BH₄ content and the number of viable PC12 cells. The inhibitory effects of DAHP are prevented by exogenous BH₄ (Koshimura et al., 1998). These results support the possibility that BH₄ acts as a self-protection factor against NO toxicity. To investigate whether BH₄ acts as a protective agent against NO toxicity in vivo, the effect of depletion of BH₄ on NO-producing cells was examined in the rat hippocampus, which is vulnerable to diverse stress associated with neurodegeneration (Ferchmin et al., 2003; Katsuse et al., 2003; Rami, 2003; Shinoda et al., 2003).

The present study demonstrates that endogenous BH₄ affects the number of NO-producing cells in the rat hippocampus.

2. Experimental procedures

2.1. Drugs

BH₄ is a generous gift from Suntory Biomedical Research Center, Osaka, Japan. 2,4-Diamino-6-hydroxypyrimidine (DAHP) was purchased from Nacalai Tesque Co. (Kyoto, Japan). A rabbit anti-nNOS polyclonal antibody was purchased from Chemicon International (Temecula, CA, USA). All other chemicals are of the purest grade available from regular commercial sources.

2.2. Rats

Male Wistar rats (250–300 g) were anesthetized with pentobarbital (50 mg/kg body weight, intraperitoneal injection), and miniosmotic pumps (Model 2002, Alza, Palo Alto, CA, USA) filled with drugs were implanted subcutaneously. DAHP was dissolved in 100 mM NaOH and BH₄ in 1% ascorbate. The drugs were infused by the miniosmotic pumps (200 μ l) at a flow rate of 0.5 μ l/h for 14 days at the maximum. As a control group, the vehicle of each drug was filled in each miniosmotic pump, which was then implanted subcutaneously. The effect of vehicle was examined by comparing with the rats implanted with the miniosmotic pump filled with saline. At the end of the drug-

administration period, the rats were decapitated. The brains were removed to cut into two hemispheres. Hippocampal tissues in one hemisphere were used for biopterin assay, and those in the other hemisphere were used for NOS immunostaining.

2.3. Biopterin assay

Brain biopterin content was measured as reported previously (Koshimura et al. 1998). In brief, brain tissues were placed in 1 ml of 0.2 M perchloric acid (PCA) with 10 mM dithiothreitol and sonicated. After centrifugation at 18,000 \times g for 5 min, 0.9 ml of the supernatant was mixed with 0.15 ml of iodine solution (0.2 M PCA containing 2.5% I₂ and 10% KI) and incubated at 30 °C for 60 min under reduced light conditions. Then, 5% ascorbic acid was added to the mixture, which was then centrifuged at 18,000 \times g for 5 min. The resulting supernatant was applied on a DOWEX column (H⁺ form) equilibrated with 20 mM HEPES (pH 5.5). Following washing with 6 ml of Milli Q water, biopterin was eluted with 3 ml of 1 M NH₄OH. The eluate was neutralized with 0.24 ml of concentrated PCA and applied on the high performance liquid chromatography (HPLC) system, of which mobile phase was 0.1 M Na acetate (pH 4.5). Biopterin was detected using with a fluorescence detector (excitation at 350 nm and emission at 455 nm). Since most of biopterin exists as BH₄ in the brain (Fukushima and Nixon, 1980), biopterin content was used as a measure of BH₄ content in the present study.

2.4. Extracellular NO metabolites (NO_x) assay

After the administration of drugs for 14 days, the rats were anesthetized with diethylether, and microdialysis probes (A-I-4-02, EICOM, Kyoto, Japan) were stereotactically implanted in the hippocampus. The probes were perfused with Ringer solution at a flow rate of 6.3 μ l/min (Koshimura et al., 1990). After a complete recovery from the anesthesia, dialysis fluid was collected for 120 min at 20-min intervals. NO_x in the dialysis fluid was measured with a NO_x-analyzing HPLC system (EICOM, Kyoto, Japan) as previously reported (Koshimura et al., 1998). The mean content of NO_x in the fractions collected represented the extracellular NO_x concentration in the hippocampus of an examined rat.

2.5. NOS immunostaining

The brains were immersed in the Bouin solution at 4 °C overnight. The fixed brains were washed with 10 mM phosphate buffer (pH 7.2) for 2–5 days, dehydrated in a graded series of ethanol (70, 90, 99 and 100%) and chloroform, and then embedded in paraffin. The paraffin-embedded tissues were cut into 5- μ m-thick serial sections and placed on 0.05% poly-L-lysine-coated glass slides. The paraffin-embedded serial sections were deparaffinized in 100% xy-

lene, then rehydrated in the graded series of ethanol with a step-wise decrease in concentration (99, 95, 90, 80 and 70%) and water. After autoclaving the sections in 10 mM citrate buffer (pH 6.0) for 15 min, the sections were incubated in 0.3% H₂O₂ in 100% methanol for 10 min at room temperature to block endogenous peroxidase activity. The sections were washed with phosphate-buffered saline (PBS) containing 0.05% Tween-20, and nonspecific binding was blocked by 30-min incubation in 1% normal goat serum in PBS at room temperature. Then, the sections were incubated with the rabbit anti-nNOS polyclonal antibody (Chemicon International, Temecula, CA, USA), which was diluted with PBS containing 0.05% Tween-20 and 1% goat serum at 1:500 at 4 °C overnight. For the negative staining of nNOS, the sections were incubated with a normal rabbit IgG fraction (Vector Laboratories, Burlingame, CA, USA) at 4 °C overnight. After washing with PBS containing 0.05% Tween-20, the sections were incubated with biotinylated goat anti-rabbit immunoglobulins (Dako, Glostrup, Denmark) diluted with PBS containing 0.05% Tween-20 and 1% goat serum at 1:600 for 30 min at room temperature. For visualization, the sections were incubated with streptavidin/biotinylated horseradish peroxidase (StreptAB-Complex/HRP, Dako, Glostrup, Denmark) for 40 min at room temperature and developed with diaminobenzidine. All the specimens compared were stained in the same experiment.

2.6. Cell counting

In each rat, five slices were taken at 100 μm interval. The total numbers of NOS-positive cells and NOS-negative cells were counted in the pyramidal cell layers of CA1 and CA3 and the granule cell layer of the dentate gyrus in each slice. Cell counting of specimens was carried out by two

independent researchers who are blind to the experimental conditions.

2.7. Protein content

Protein content was measured by Bio-Rad protein assay using bovine serum albumin as the standard (Bradford, 1976).

2.8. Statistical analysis

All results were expressed as means ± S.E.M. The significance of difference was tested by ANOVA and Fisher's test. A probability level of $P < 0.05$ was considered statistically significant.

3. Results

Drugs were administered using miniosmotic pumps for 7 or 14 days, and BH₄ content in the hippocampus was measured. The seven-day administration of DAHP had no effect on the hippocampal BH₄ content at any given doses (Fig. 1A). Following the administration of DAHP for 14 days, BH₄ content decreased as compared with that of the control group treated with vehicle (100 mM NaOH). The effect of DAHP reached the maximum at 50 mM (Fig. 1B). Implantation of a miniosmotic pump containing saline did not affect BH₄ content in the hippocampus. Vehicle (100 mM NaOH) had no effect on BH₄ content as compared with saline solution (saline-treated group ($n = 20$) 2.82 ± 0.55 pmol/mg protein versus 100 mM NaOH group ($n = 20$) 3.00 ± 0.61 pmol/mg protein).

Then, the effect of exogenous BH₄ on DAHP-induced decrease in the hippocampal BH₄ content was examined. BH₄ was dissolved at 10 mM in 1% ascorbate and administered us-

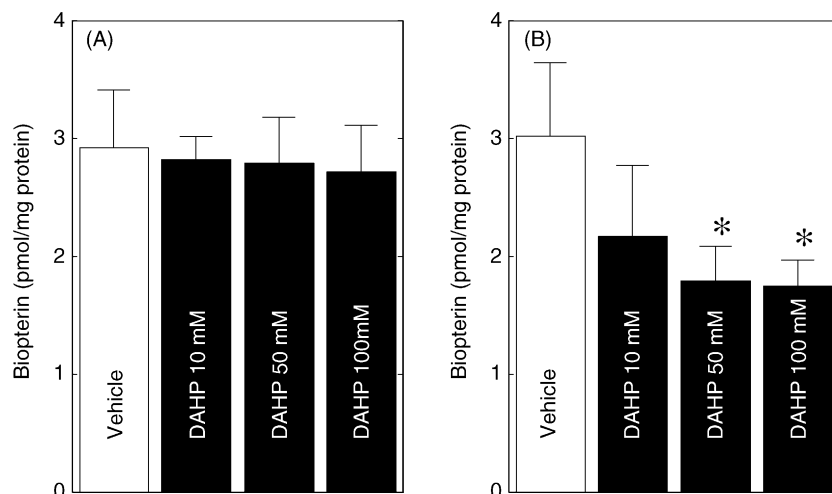


Fig. 1. Effect of DAHP on BH₄ content in the rat hippocampus. Various concentrations of DAHP solution were administered to the rats using miniosmotic pumps for 7 (A) or 14 (B) days. Vehicle solution (100 mM NaOH) was administered to the control rats. Each value was the mean ± S.E.M. of 10 experiments. (*) $P < 0.05$ vs. the control group.

ing a miniosmotic pump. In each rat, two miniosmotic pumps were implanted subcutaneously. In the control rats, the miniosmotic pumps were filled with vehicle for DAHP (100 mM NaOH) and BH₄ (1% ascorbate). In the DAHP-treated rats, the pumps were filled with 50 mM DAHP and 1% ascorbate. The pumps of the BH₄-treated rats contained 10 mM BH₄ and 50 mM DAHP. BH₄ content in the control rats was not significantly different from that in the saline-treated rats (saline-treated group ($n = 20$) 3.05 ± 0.86 pmol/mg protein versus control group ($n = 20$) 2.95 ± 0.88 pmol/mg protein). BH₄ content in the hippocampus of rats treated with 50 mM DAHP decreased to a similar extent as those shown in Fig. 1. When 10 mM BH₄ was administered together with 50 mM DAHP, BH₄ content in the hippocampus was restored (Fig. 2). The population of NOS-positive cells was examined in CA1, CA3 and the dentate gyrus of the hippocampus of the corresponding hemisphere of the rats in which BH₄ content in the hippocampus was measured. Representative photomicrographs are shown in Fig. 3. NOS-positive cells were stained in the pyramidal cell layers of CA1 (Fig. 3A) and CA3 (Fig. 3D) and in the granule cell layer of the dentate gyrus (Fig. 3G). Following the treatment with 50 mM DAHP for 14 days, the number of NOS-positive cells was decreased in CA1 (Fig. 3B), CA3 (Fig. 3E) and the dentate gyrus (Fig. 3H). The number of NOS-negative cells remained unchanged following the DAHP treatment in all the examined regions (vehicle-treated group ($n = 20$) CA1 526 ± 26 , CA3 290 ± 16 , dentate gyrus 1470 ± 45 versus DAHP group ($n = 20$) CA1 512 ± 30 , CA3 286 ± 24 , dentate gyrus 1481 ± 38).

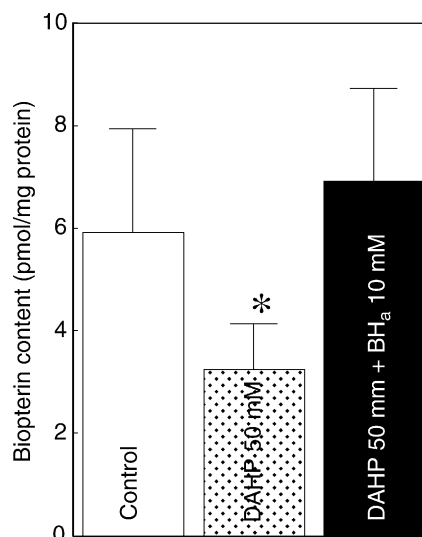


Fig. 2. Effect of BH₄ on DAHP-induced decrease in the hippocampal BH₄ content. BH₄ solution (10 mM) and 50 mM DAHP were coadministered for 14 days. DAHP (50 mM) and vehicle solution for BH₄ (1% ascorbate) were coadministered to the DAHP-treated rats, while, vehicle solutions for DAHP (100 mM NaOH) and BH₄ (1% ascorbate) were coadministered to the control rats. Each value was the mean \pm S.E.M. of 10 experiments. (*) $P < 0.05$ vs. the control group. (#) $P < 0.05$ vs. DAHP-treated group.

The number of NOS-positive cells was restored following the administration of 10 mM BH₄ and 50 mM DAHP in all areas (Fig. 3C, F and I). The population of NOS-positive cells was calculated in each examined area (Fig. 4). The effect

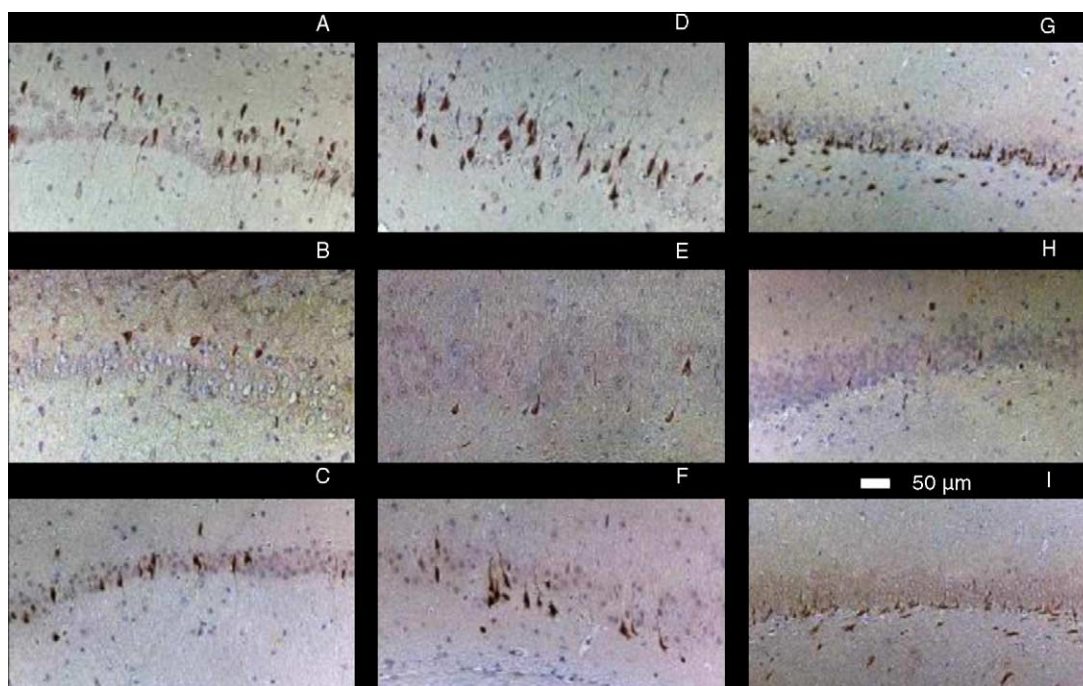


Fig. 3. Photomicrographs of NOS-positive cells in CA1, CA3 and dentate gyrus of rats. Rats were treated with drugs for 14 days and hippocampal tissue was stained with the anti-nNOS antibody. CA1 (A), CA3 (D) and dentate gyrus (G) of the control group treated with vehicle of DAHP (100 mM NaOH) and BH₄ (1% ascorbate). CA1 (B), CA3 (E) and dentate gyrus (H) of the group treated with 50 mM DAHP (100 mM NaOH) and vehicle of BH₄ (1% ascorbate). CA1 (C), CA3 (F) and dentate gyrus (I) of the group treated with 50 mM DAHP and 10 mM BH₄.

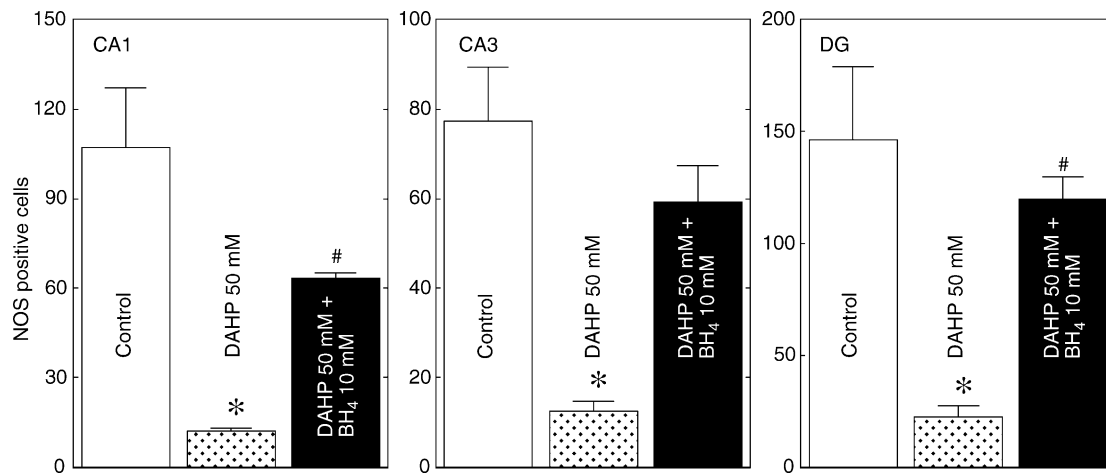


Fig. 4. The numbers of NOS-positive cells in CA1, CA3 and dentate gyrus (DG) of the rats. The total number of NOS-positive cells was calculated in the granular layer of CA1 and CA3 and molecular layer of dentate gyrus of 5 slices in each rat. Each value was the mean \pm S.E.M. of 10 experiments. (*) $P < 0.05$ vs. the control group. (#) $P < 0.05$ vs. DAHP-treated group.

of DAHP on NOS-positive cell number was similar among the examined areas. Exogenous BH₄ significantly restored the DAHP-induced decrease in BH₄ content, particularly in CA3 and the dentate gyrus.

Since BH₄ acts as a cofactor of NO synthase, the effect of DAHP-induced decrease in BH₄ content on NO production in the hippocampus was examined using brain microdialysis. Following the administration of 50 mM DAHP for 14 days, extracellular NO metabolites (NO₂⁻ plus NO₃⁻ as NO_x) were measured as an index of NO production. The NO_x concentrations did not vary among collected fractions in each rat. Extracellular NO_x concentration remained unchanged after DAHP treatment as compared with that after saline treatment (Fig. 5).

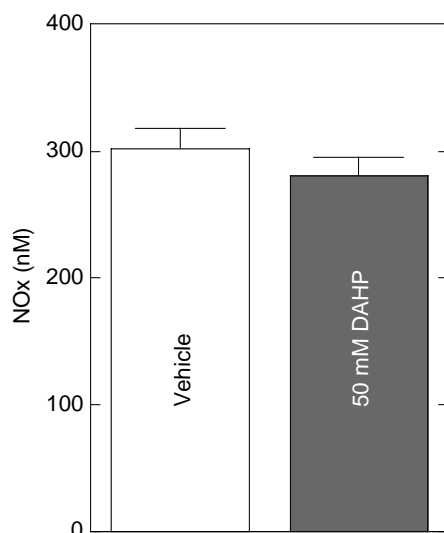


Fig. 5. Effect of DAHP on extracellular NO_x concentration in the rat hippocampus. Following the administration of 50 mM DAHP for 14 days, extracellular NO_x concentration was measured by brain microdialysis. Each value was the mean \pm S.E.M. of six experiments.

4. Discussion

The present study demonstrates that endogenous BH₄ has a critical role in NOS-positive cells in the rat hippocampus. It was reported that an intraperitoneal injection of DAHP suspension decreased brain BH₄ content (Suzuki et al., 1988). These data supported the report that the peripheral administration of DAHP is a valid method of delivering DAHP to the central nervous system. In the present study, the subcutaneous administration of DAHP using a miniosmotic pump effectively decreased hippocampal BH₄ content without any stressful stimulation. It is conceivable that BH₄ content in peripheral organs including those in nervous system is reduced by the subcutaneous administration of DAHP. However, the vital conditions of rats such as blood pressure, food intake, or weight gain remained unchanged following the treatment with DAHP in this study (data not shown). Thus, it is likely that the effect of DAHP on BH₄ content in the hippocampus is the primary effect of centrally delivered DAHP, not secondary to the systemic effect of peripherally delivered DAHP.

BH₄ administration using a miniosmotic pump prevented the decrease in hippocampal BH₄ content. BH₄ was administered peripherally because several lines of evidence showed that peripherally administered BH₄ reached the brain (Levine et al., 1983; Brand et al., 1996). These results taken together suggest that BH₄ administration using a miniosmotic pump increased brain BH₄ content.

NOS-positive cells were widely distributed in rat hippocampal CA1, CA3 and dentate gyrus. The continuous administration of 50 mM DAHP for 14 days markedly decreased the number of NOS-positive cells in the examined hippocampal areas. In contrast, DAHP infusion had little effect on the number of NOS-negative cells. It is possible that DAHP decreased the number of NOS molecules in the NOS-positive cells rather than NOS-positive cells themselves. Further studies are necessary to clarify this possibility. Exogenous BH₄ increased the number of NOS-positive cells in

the DAHP-treated rats. The number of NOS-negative cells was apparently unchanged by BH₄. Thus, it is assumed that BH₄ prevented the DAHP-induced decrease in the number of NOS-positive cells in the hippocampus. It remains to be investigated whether exogenous BH₄ induces a selective generation of NOS-positive cells in the hippocampus.

Previously, we reported that BH₄ stimulates monoamine synthesis and release in the hippocampus (Ohue et al., 1992). Since BH₄ is a cofactor of aromatic L-amino acid hydroxylases (Kaufman, 1959; Nagatsu et al., 1964; Lovenberg et al., 1967), the treatment with DAHP may reduce monoamine content and release in the hippocampus. Further studies are necessary to clarify the possibility that monoamines are involved in the expression of effect of BH₄ on NOS-positive cells.

While a DAHP-induced decrease in BH₄ content had little effect on NO production, it markedly decreased the number of NOS-positive cells in the hippocampus. This discrepancy in vulnerability to the decrease in BH₄ content could be due to as follows. First, since NO is diffusible gas, the concentration of NO at the production site in a cell is assumed to be much higher than the extracellular concentration. Thus, it is speculated that a large amount of BH₄ is necessary to degrade intracellular NO. Second, although the recycling system and salvage system for BH₄ are reported (Abou-Donia et al., 1986; Kwon et al., 1989; Mayer and Werner, 1995), it is possible that these complementary systems for BH₄ are not appropriately functioning when BH₄ content decreased following DAHP treatment.

Although the hippocampal BH₄ content and the number of NOS-positive cells significantly decreased following DAHP treatment, DAHP treatment had little effect on extracellular NO_x concentration, an index of NO production, in the hippocampus. Although *in vivo* NOS activity in each cell in the hippocampus after treatment with DAHP was not examined, it is possible that NO is compensatorily produced in the remaining NOS-positive cells by some mechanisms such as the elevation of intracellular Ca²⁺ concentration or induction of NOS molecule, and that NOS is saturated with BH₄ in the rat hippocampus. Further studies are necessary to clarify the regulation of NO production in the hippocampal NOS-positive cells of DAHP-treated rats.

These results taken together suggest that endogenous BH₄ plays a critical role in NOS-positive cells in the rat hippocampus.

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