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# Degeneration of the nigrostriatal pathway and induction of motor deficit by tetrahydrobiopterin: an in vivo model relevant to Parkinson's disease

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## Abstract

We determined whether the preferential toxicity of tetrahydrobiopterin (BH4) on dopamine-producing cells, which we have previously observed in vitro, might also occur in vivo and generate characteristics associated with Parkinson's disease. Intrastratial BH4 injection caused a loss of tyrosine hydroxylase immunoreactivity and decreased dopamine content. The dopaminergic cell bodies topologically corresponding to the lesioned terminals were selectively degenerated. This was accompanied by a dose-dependent and asymmetric movement deficit in the contralateral forepaw. Direct injection of BH4 into the substantia nigra caused a loss of tyrosine hydroxylase immunoreactivity, but injection into the dorsal raphe was without effect on the GTP cyclohydrolase-immunoreactive serotonergic neurons, demonstrating selectivity for the dopaminergic system. BH4 exhibited a range of potency comparable to that of 6-hydroxydopamine. Thus, this animal model generated by the administration of BH4, the molecule endogenously present in the monoaminergic neurons, exhibited morphological, biochemical, and behavioral characteristics associated with Parkinson's disease and may be useful for studies in dopaminergic degeneration.

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Parkinson's disease is one of the most common neurological disorders in the elderly, characterized by selective degeneration of dopaminergic neurons in the substantia nigra (SN)<sup>1</sup> pars compacta and their terminals in the striatum. The disease is accompanied by movement deficits as well as biochemical changes including loss of dopamine and its biosynthetic enzyme tyrosine hydroxylase (TH). While the physiological and neurochemical consequences of nigrostriatal degeneration have been extensively documented, the underlying cause of dopaminergic cell death or the mechanism by which these cells degenerate remains obscure. Decreased glutathione level (Sian et al., 1994) and increased iron concentration (Sofic et al., 1988) and lipid peroxidation (Dexter et al., 1989) have been noted in the SN

of Parkinson's patients and during aging, suggesting a role of oxidative stress in the degeneration.

We have previously observed that tetrahydrobiopterin (BH4), an obligatory cofactor for dopamine synthesis, exerts toxicity on dopamine-producing cells in vitro (Choi et al., 2000). This toxicity was shown to be selective, as the dopamine-producing cells such as CATH.a, SK-N-BE(2)C, and PC12 cells were vulnerable to BH4 whereas nondopaminergic cells were not. The BH4-induced toxicity could be prevented by antioxidant enzymes as well as sulfhydryl antioxidants, suggesting the involvement of oxidative stress. In vivo, BH4 is exclusively produced in monoaminergic neurons in the brain including the nigral dopaminergic neurons (Hwang et al., 1998; Nagatsu et al., 1995). We have also observed that BH4 synthesis can be readily upregulated by cellular changes such as calcium influx (Hwang et al., 1999), suggesting that the availability of BH4 in vivo might fluctuate in response to such changes. Based on these data, we hypothesized that BH4 might be involved in the selective degeneration of the dopaminergic pathway in vivo and produce characteristics associated with Parkinson's disease.

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<sup>1</sup> Abbreviations used: TH, tyrosine hydroxylase; BH4, tetrahydrobiopterin; GTPCH, GTP cyclohydrolase I; 6-OHDA, 6-hydroxydopamine; SN, substantia nigra; 5-HT, serotonin.

Considerable information regarding nigrostriatal vulnerability has come from studies using direct injection of the neurotoxins dopamine (Filloux and Townsend, 1993; Hastings et al., 1996) and 6-hydroxydopamine (6-OHDA) (Chang et al., 1999; Ichitani et al., 1994; Jin and Iacovitti, 1995) into the brain as well as transection of the nigrostriatal pathway (Joh and Weiser, 1993). Local injection of the toxins in the striatum has recently been shown to be a good model of early and moderate stages of Parkinson's disease, as this produces a dose-dependent loss of dopaminergic fibers in the striatum followed by degeneration of the nigral dopaminergic cell bodies (Ichitani et al., 1994; Przedborski et al., 1995; Sauer and Oertel, 1994) and corresponding behavioral abnormalities (reviewed by Mokry, 1995).

In the present study we determined whether the preferential toxicity of BH4 on dopamine-producing cells that we have observed *in vitro* might also occur *in vivo* and whether this might be accompanied by morphological, biochemical, and behavioral characteristics associated with Parkinson's disease. We report that intrastriatal injection of BH4 results in preferential and dose-dependent degeneration of the dopaminergic terminals and a decrease in dopamine content in the striatum and retrograde degeneration of the parent cell bodies in the SN and that this correlates with the behavioral deficit associated with nigrostriatal damage. BH4-induced degeneration *in vivo* also showed selectivity for the dopaminergic system.

## Materials and methods

### Chemicals

BH4 was obtained from RBI (Natick, MA). Ketamine, xylazine, ascorbate, sodium octylsulfate, triethylamine, and bovine serum albumin were purchased from Sigma Chemical (St. Louis, MO). Tissue-Tek OCT compound was obtained from Sakura Finetek (Torrance, CA). Rabbit polyclonal antibodies to TH were obtained from Protos (New York, NY) and polyclonal antiserum against GTP cyclohydrolase I (GTPCH) was a gift from Dr. Ikuko Nagatsu of Fujita Health University. Rabbit polyclonal antibody to neuron-specific enolase (NSE) was purchased from BioGenex (San Ramon, CA). The Vectastain ABC kit and biotinylated secondary antibodies were from Vector Laboratories (Burlingame, CA) and Permunt was from Fisher Scientific (Pittsburgh, PA). All other chemicals were reagent grades from Sigma or Merck (Rahway, NJ).

### Animals

All procedures were performed in compliance with the guidelines set forth by the *Laboratory Animal Manual* of the Asan Institute for Life Sciences which adopt the National Institute of Health *Guide to the Care and Use of Animals*.

Female Sprague–Dawley rats were bred and maintained at the animal facility of the Asan Institute for Life Sciences. They were housed in groups of three per cage in a temperature- and humidity-controlled room with a 12-h light–dark cycle. Food and water were available *ad libitum*.

### Stereotaxic injection of BH4

Animals weighing 200–250 g were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (4 mg/kg ip). An injection cannula (26.5 gauge) was placed stereotaxically into the right striatum (AP, +0.7 mm from bregma; ML, 2.8 mm; DV, 4.6 mm below dura), the SN (AP, –4.8 mm from bregma; ML, 1.6 mm; DV, 8.2 mm), or the dorsal raphe (AP, –8.0 mm from bregma; ML, 0 mm; DV, 6.0 mm) according to the atlas (Paxinos and Watson, 1986). Injections of BH4 were made in 5  $\mu$ l (striatum and dorsal raphe) or 2.5  $\mu$ l (SN) containing 0.9% NaCl and 0.1% ascorbic acid, the vehicle shown to cause no significant changes in monoamine levels and TH immunoreactivity (Ichitani et al., 1994). BH4 was readily dissolved at all concentrations used in the experiments. The solutions were freshly prepared just prior to use and were kept on ice before (in a lightproof container) and during (in cannula) the injection. The final pH of the BH4 solution was somewhat acidic (pH 5.0) because BH4 was supplied as a hydrochloride salt, but the vehicle solution containing the same concentration of HCl did not cause a significant change in TH immunoreactivity. The infusion was done at a rate of 0.5  $\mu$ l/min using a microinfusion pump (Model No. 22, Harvard Apparatus). The cannula was left in place for 5 min before it was slowly withdrawn in order to avoid reflux along the injection track. The wound was closed with suture and the animals were allowed to recover before they were returned to their cage. The entire procedure was well-tolerated by the animals.

### Immunocytochemistry

Animals were deeply anesthetized (80 mg/kg ketamine and 20 mg/kg xylazine, ip) and transcardially perfused with normal saline containing heparin (100 USP units/ml) followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Immunocytochemistry was performed as described previously (Hwang et al., 1998), using antisera against TH (1:1,000), NSE (1:100), or GTPCH (1:1,000), Vectastain ABC kit, and biotinylated secondary antibodies.

### Forepaw adjusting steps

Motor deficit after the BH4 lesion was assessed by the animal's ability to adjust forepaw steps as described previously (Chang et al., 1999). Briefly, rats were held by one hand of the experimenter at the torso so that their hindlimbs were lifted. One forepaw was held with the other hand and the other forepaw was allowed to step along on the surface

of a treadmill (M.S.D., Seoul, Korea) moving at a rate of 7.5 cm/s. The number of forepaw steps during one round (90 cm) was counted. Each stepping test consisted of five trials for each forepaw, alternating between forepaws, and the average of the five trials for each forepaw was used for analysis. The test was performed by an experimenter blind to the treatment.

#### *Quantitative morphological analysis*

The area of striatal lesion after the BH4 injection was assessed after selecting up to five sections, each separated by 120  $\mu\text{m}$  near the injection site. The area of diminished TH immunoreactivity was quantified by computer-assisted image analysis. For this, the striatal sections were subjected to Bio-Rad Quantity One (version 4.2.1), which outlined and computed the area of low-density region representing the lesion. The section with the largest area of loss was used for comparisons across animals. TH-immunopositive cells in the SN were counted by the method of Burke et al. (1992). Briefly, every third SN sections from bregma  $-4.3$  to  $-6.3$ , based on the atlas (Paxinos and Watson, 1986), were scanned under light microscopy, and TH-immunopositive SN pars compacta cells were counted manually. The number of neurons was expressed as the average of the counts obtained from the representative sections. In planes between  $-4.8$  and  $-5.2$ , the boundary between the ventral tegmental area and SN pars compacta was defined as a line extending dorsally from the most medial boundary of the cerebral peduncle. For those posterior to  $-5.2$ , the immunopositive cells lateral to white matter bundles, the medial lemniscus and the tractus opticus basalis were counted.

#### *Determination of tissue dopamine level*

Animals were decapitated and the whole brain was immediately frozen. A 3-mm slice between bregma  $-1$  mm and  $+2$  mm was cut out in a brain matrix. The striatal region was excised by using a micropunch with an inner diameter of 2 mm and weighed. For analysis, the striatal tissue samples were homogenized in 0.1 N perchloric acid and the acid soluble fraction was obtained. Dopamine was separated by high-performance liquid chromatography using a C18 column (Novapak Corp., Eatontown, NJ) and the mobile phase consisting of 0.1 M sodium phosphate (pH 3.35), 8% methanol, 1 mM sodium octylsulfate, 0.1 mM EDTA, and 0.0003% triethylamine and detected electrochemically by a Waters 464 detector (Hwang et al., 1994, 1997). Amounts of dopamine were calculated using the Waters Millennium<sup>32</sup> integrator system and a standard curve.

#### *Data analyses*

Comparisons were made using ANOVA and Newman–Keuls multiple comparisons test.  $P < 0.05$  was considered statistically significant for all analyses.

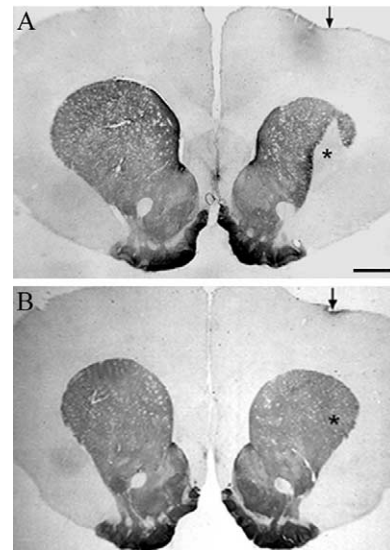


Fig. 1. Intrastratial BH4 injection causes degeneration of striatal dopaminergic terminals. Typical photomicrographs of TH-immunostained striatum 4 weeks after injection of (A) 200  $\mu\text{g}$  BH4 and (B) vehicle. Arrows indicate where injection needle entered the brain and asterisks indicate the injection site (scale bar = 1.1 mm).

## Results

### *Intrastratial BH4 injection causes degeneration of striatal dopaminergic terminals*

In order to determine whether BH4 might cause damages to the dopaminergic system, we first injected 200  $\mu\text{g}$  (0.6  $\mu\text{mol}$ ; 120 mM) BH4 stereotactically into the right striatum ( $n = 10$ ). The animals were sacrificed 4 weeks after the injection and the striatal sections were subjected to immunocytochemistry against TH, the marker enzyme responsible for dopamine synthesis. The results showed that the area surrounding the injection site exhibited a loss of TH immunoreactivity, demonstrating damages to the striatal dopaminergic terminals (Fig. 1A). Consistently reproducible lesions were produced in all animals that received BH4. On the other hand, the animals injected with the vehicle alone ( $n = 10$ ) maintained the dense TH immunoreactivity and only a very thin scar was visible along the needle track (Fig. 1B). Analysis of serial striatal sections revealed that the loss of TH immunoreactivity extended in all directions radially from the injection site.

### *Intrastratial BH4 injection leads to degeneration of nigral dopaminergic cell bodies*

Whether retrograde degeneration of the nigrostriatal pathway has occurred following the striatal damage by BH4 was assessed. For this, serial sections of the SN were taken and subjected to TH immunostaining and changes in the parent cell bodies were evaluated. As shown in Fig. 2, TH immunoreactivity in the sections corresponding to bregma

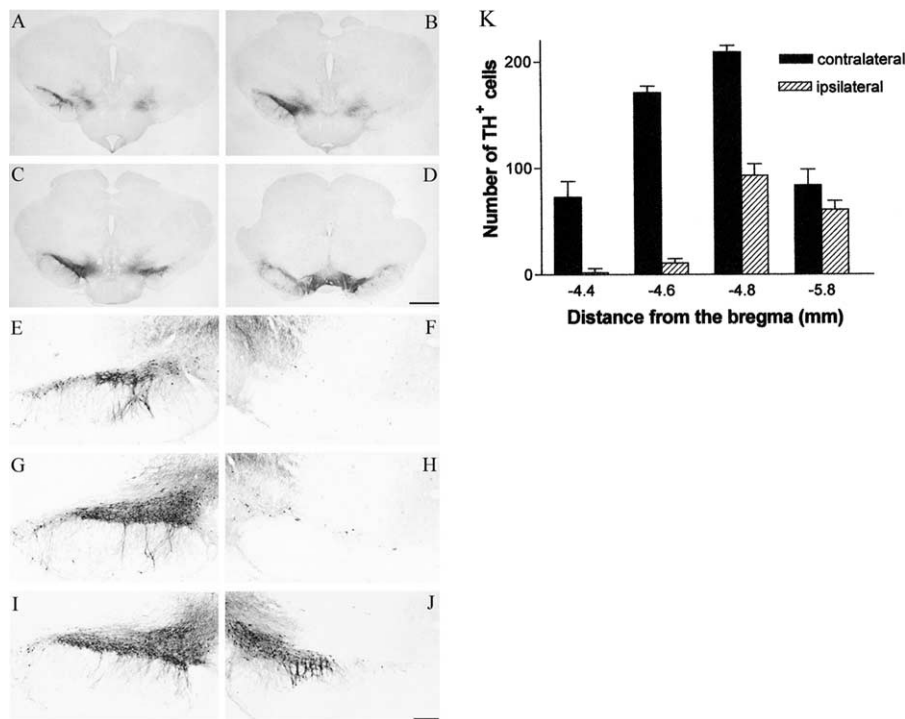


Fig. 2. Intrastratial BH4 injection causes degeneration of the nigral dopaminergic cell bodies. Typical photomicrographs of the SN sections immunostained for TH 4 weeks after injection of 200  $\mu$ g BH4. A–D, in low magnification; E–J, in high magnification. A, E, and F, –4.4 mm from the bregma; B, G, and H, –4.6 mm; C, I, and J, –4.8 mm, and D, –5.8 mm; E, G, and I, contralateral side; and F, H, and J, ipsilateral side (scale bar = 2 mm for A–D and 320  $\mu$ m for E–J); K, Mean numbers of TH-positive cells on sections of the SN pars compacta of rats 4 weeks after intrastratial injection with 200  $\mu$ g BH4. The number of neurons was expressed as the average of the counts obtained from the representative sections for each animal. Data are means  $\pm$  SEM ( $n = 10$ ) and  $P < 0.005$  vs respective contralateral sides for all sections except for the –5.8 mm.

–4.4 to –4.8 (lower magnification, Fig. 2A–C; higher magnification, Fig. 2E–J) was abolished only on the side ipsilateral to the injection. The dopamine cells of the ventral tegmental area were unaffected, demonstrating that this absence of nigral neurons was specifically caused by retrograde degeneration subsequent to the striatal damage. The cell loss was not as evident in the more caudal sections, and the section at –5.8 showed no sign of such degeneration (Fig. 2D). Thus, the striatal damage at around +0.7 caused nigral cell loss between –4.4 and –4.8. We also observed that a lesion in the more posterior part of the striatum led to degeneration of dopamine cells in the more posterior SN (not shown).

For more quantitative analysis, the TH-immunopositive cells of the SN were counted. As shown in Fig. 2K, almost complete abolishment of the TH-immunopositive cells was observed in the anterior part of the SN and only 1–6% of total cells remained between the planes –4.4 and –4.6. More posteriorly at plane –4.8, 44% of cells were intact. No significant changes were noted in planes posterior to –5.8.

#### BH4-induced nigrostriatal lesion causes motor deficit

Nigrostriatal lesions by other neurotoxins have been shown to accompany motor deficits (Chang et al., 1999;

Przedborski et al., 1995; Schwarting and Huston, 1996) and we (Chang et al., 1999) and others (Olsson et al., 1995; Winkler et al., 1996) have previously demonstrated that deficits in forepaw adjusting steps provide a simple and consistent behavior phenomenologically similar to akinesia in early to moderate stages of Parkinson's disease. Using this model, whether the BH4-induced lesion might also cause this deficit was tested. Because the 6-OHDA-induced deficit occurred within 2 weeks and was not recovered for up to 4 weeks (Chang et al., 1999), we tested the animals 25 days postinjection. As shown in Table 1, the ipsilateral forepaw of the BH4-injected animals showed adjusting steps that were not significantly different from the vehicle-injected animals. On the other hand, the affected forepaw,

Table 1  
Effect of intrastratial BH4 injection on forepaw adjusting steps

	Contralateral forepaw	Ipsilateral forepaw
Vehicle-injected ( $n = 10$ )	11.70 $\pm$ 0.42	12.30 $\pm$ 0.42
BH4-injected ( $n = 10$ )	5.60 $\pm$ 0.81*	12.20 $\pm$ 0.37

Note. Animals were injected with 200  $\mu$ g BH4 into the striatum and tested for their ability to adjust forepaw steps 25 days later. Data are mean numbers of forepaw stepping  $\pm$  SEM.

\*  $P < 0.005$  vs contralateral forepaw stepping of the vehicle-injected control animals.

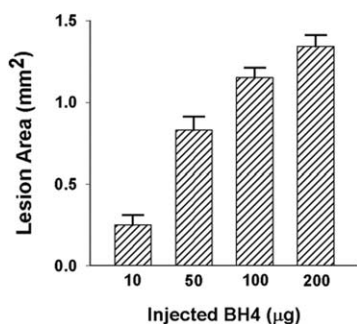


Fig. 3. Quantitative analysis of striatal areas of diminished TH immunoreactivity after intrastriatal injection of various amounts of BH4. The lesion areas were quantified using a computer-assisted image analysis system and the sections with the largest area of loss were used for comparisons across animals. Data are means  $\pm$  SEM ( $n = 9$  in each group).

i.e., the side contralateral to the lesioned striatum, showed a dramatic reduction in stepping ability (48% of the control). Thus, the BH4-induced damage to the nigrostriatal pathway accompanied a motor deficit related to dopamine depletion.

#### BH4-induced toxicity is dose-dependent

In order to assess the potency of BH4, we tested whether lower amounts of BH4 also caused the striatal damage and behavioral deficit. For this, animals were injected with various amounts of BH4 down to 30 nmol (10  $\mu$ g; 6 mM) and were sacrificed after 4 weeks. TH immunocytochemistry of their striatal sections revealed lesions at the injected site for all BH4 doses tested. The lesion caused by 10  $\mu$ g BH4 was smaller, but generally similar in morphology as those caused by the higher amounts (not shown). Quantitative analysis of the largest area of each lesion showed  $0.25 \pm 0.06$ ,  $0.83 \pm 0.08$ ,  $1.15 \pm 0.06$ , and  $1.34 \pm 0.07$  mm<sup>2</sup> for 10  $\mu$ g (30 nmol; 6 mM), 50  $\mu$ g (150 nmol; 30 mM), 100  $\mu$ g (300 nmol; 60 mM), and 200  $\mu$ g (600 nmol; 120 mM) BH4, respectively (Fig. 3). Thus, BH4 as low as 10  $\mu$ g effectively caused degeneration of the dopamine terminals in the striatum and the size of lesion increased in a dose-dependent manner. In addition, the number of adjusting steps of the contralateral forepaw was reduced with increasing BH4 dose (Fig. 4). Doses of 50, 100, and 200  $\mu$ g significantly reduced the stepping abilities to 76, 52, and 48% of the vehicle-injected controls, respectively. On the other hand, 10  $\mu$ g BH4 caused no significant stepping disability. Thus, although lower amounts were not tested, BH4 as low as 30 nmol (10  $\mu$ g) was sufficiently toxic to the dopaminergic striatal terminals and nigral cell bodies.

#### BH4 causes dopamine loss in the striatum

Dopaminergic degeneration should accompany a decrease in dopamine content in addition to the reduced immunoreactivity against the marker enzyme TH. In order to obtain biochemical evidence, dopamine contents in the stri-

atum were measured. Animals were injected with 100  $\mu$ g BH4, the dose sufficient to cause the striatal degeneration and motor deficit (Figs. 3 and 4). Four weeks later, the striatal tissues were excised by micropunch with an internal diameter of 2 mm, which was large enough to encompass the lesion area. The dopamine content of the vehicle-injected striatal tissue was  $18.5 \pm 1.6$   $\mu$ g dopamine/g tissue, whereas that of the BH4-injected tissue was  $10.1 \pm 1.6$   $\mu$ g dopamine/g tissue ( $n = 6$  each,  $P < 0.0001$  vs the vehicle-injected control). Thus, BH4 caused a decrease in dopamine content in the injected area to  $46.75 \pm 7.24\%$  of the vehicle-treated control. Because the TH-immunonegative area ( $1.15 \pm 0.06$  mm<sup>2</sup>, Fig. 3) would be roughly 40% of the total micropunched area (approximately 3.14 mm<sup>2</sup>), the data indicated that the TH-immunonegative area would most likely be devoid of dopamine. Thus, BH4 caused not only the loss of TH immunoreactivity but also a decrease in dopamine content in the striatum.

#### BH4-induced striatal damage occurs as early as 24 h

In order to determine the time required for the BH4-induced damage, we tested TH immunoreactivity of striatal and nigral sections at 1, 4, 7, or 28 days after injection of 100  $\mu$ g BH4. The results revealed that sufficient damage to the striatal dopaminergic terminals occurred even after 1 day (Table 2). The 1-, 4-, and 7-day treatments caused striatal damages to similar degrees, accounting for approximately 75% of the size of the lesion obtained in 4 weeks. Thus, the primary lesion occurred shortly after the BH4 injection and further degeneration might subsequently occur as a secondary reaction. Analysis of their parent nigral sections, on the other hand, showed no statistically significant decrease in the TH-positive nigral cells up to 7 days

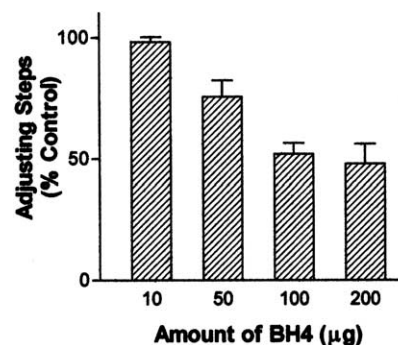


Fig. 4. Ability to adjust forepaw step is diminished with increasing amounts of injected BH4. Animals were injected with the various amounts of BH4 into the right striatum and the number of forepaw adjusting steps was determined. Each stepping test consisted of five trials for each forepaw, alternating between forepaws, and the average of the five trials for each forepaw was used for analysis. Data are means  $\pm$  SEM of the left forepaw steps in percentages of those of the vehicle-injected control animals ( $n = 9$  in each group).  $P < 0.05$  for all values except 10  $\mu$ g ( $P > 0.01$ ). The right forepaw steps did not differ between the BH4-treated and control groups ( $P > 0.05$ ).

Table 2  
Size of the striatal lesion in relation to postinjection period

	Lesion size (mm <sup>2</sup> )	n
1 day	0.85 ± 0.05	5
4 days	0.88 ± 0.03	8
7 days	0.86 ± 0.14	6
28 days	1.15 ± 0.06	18

(not shown). Thus, BH4-induced striatal damage could occur as early as 24 h postinjection, but the subsequent retrograde degeneration of the nigrostriatal pathway seemed to take longer.

#### BH4-induced vulnerability exhibits selectivity

We have previously observed in vitro that BH4 causes death of the dopamine-producing cells but not the cells that produce serotonin (5-HT), another monoaminergic neurotransmitter, although they also synthesize BH4 (Choi et al., 2000). We tested whether such selectivity might exist in vivo. For this, BH4 was stereotactically injected into the SN or the dorsal raphe regions and the animals were sacrificed 24 h later. As shown in Fig. 5B and D, the number of

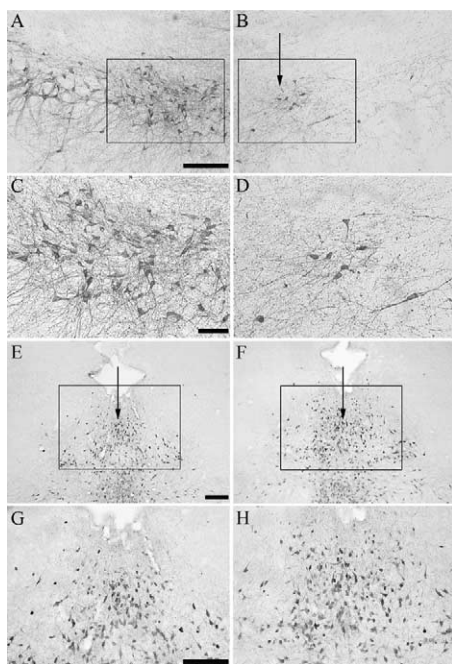


Fig. 5. Selectivity of BH4-induced toxicity on the nigral system. (A–D) Typical photomicrographs of nigral section of rats intranigally injected with 10 µg BH4, sacrificed after 24 h, and immunostained against TH (B, D) and compared with contralateral side injected with vehicle (A, C). Scale bar = 200 µm (A, B) and 50 µm (C, D); (E–H) Typical photomicrographs of dorsal raphe section of rats sacrificed 24 h after injection of (F) 10 µg BH4 or (E) vehicle into the dorsal raphe and immunostained for the 5-HT-containing cell bodies with GTPCH antibody (scale bar = 200 µm). The arrows indicate the injection sites and directions.

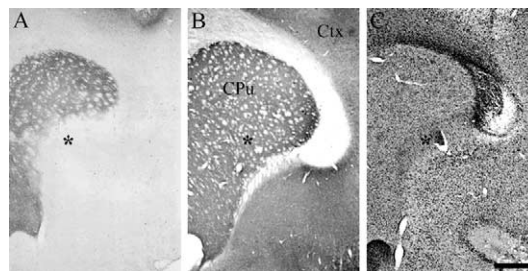


Fig. 6. The striatal degeneration after BH4 injection is selective for the dopaminergic terminals. Typical photomicrographs of striatum of rats sacrificed 4 weeks after injection of 100 µg BH4 and immunostained for (A) TH and (B) NSE and (C) stained with hematoxylin. Asterisks indicate same position in the adjacent sections. (scale bar = 600 µm); CPu, caudate putamen; Ctx, cortex.

TH-positive dopamine neurons in the SN pars compacta dramatically decreased after BH4 injection (Fig. 5A and C;  $17.0 \pm 5.7\%$  of the contralateral side (Fig. 5B and D);  $P < 0.0001$ ). The damage appeared to occur by apoptosis, as determined by in situ TUNEL staining (not shown). For observation of the 5-HT neurons in the raphe region, we used antibody against GTPCH, which is a well-known marker enzyme for BH4 synthesis and is abundantly and specifically present in the 5-HT dorsal raphe neurons in this region of the brain (Nagatsu et al., 1995). The results showed that the GTPCH-immunoreactive serotonergic cells in the BH4-injected dorsal raphe region were intact (Fig. 5H), indistinguishable from the vehicle-injected control (Fig. 5G) ( $P = 0.07$ ). The same results were observed by immunocytochemistry against tryptophan hydroxylase, another marker enzyme of the 5-HT system (not shown).

For further verification of the selectivity, we assessed the relative effects of the intrastriatal BH4 injection on the dopaminergic terminals and nondopaminergic cell bodies in the striatum. For this, we analyzed the striatal sections of the animals treated with various concentrations of BH4, and adjacent striatal sections were subjected to immunocytochemistry against TH and NSE, as well as to hematoxylin staining. Typical photographs of striatal sections 4 weeks after injection of 100 µg BH4 are shown in Fig. 6. TH immunocytochemistry again demonstrated the loss of dopaminergic terminals at the site of injection (Fig. 6A). On the other hand, NSE, present in all neuronal cells, revealed no loss of immunoreactivity (Fig. 6B), demonstrated that the nondopaminergic neurons of the striatum were undamaged. Similar results were obtained with another neuron-specific marker, MAP-2, and as early as 24 h after the injection with as low as 10 µg BH4 (not shown). Hematoxylin staining of the adjacent section revealed the presence of dense nuclei, showing that both neurons and glial cells were viable (Fig. 6C). Taken together, the intrastriatally injected BH4 led to selective damage of the dopaminergic terminals, leaving the cells in the region undamaged. Thus, the cell bodies of nondopaminergic neurons in the striatum appeared to be largely resistant to BH4.

## Discussion

We demonstrate in the present study that intrastriatal injection of BH4, an obligatory cofactor of dopamine synthesis, reproducibly causes (1) dose-dependent degeneration of the dopaminergic terminals and a decrease in dopamine content; (2) degeneration of the parent nigral cell bodies topologically corresponding to the lesioned terminals in the striatum; and (3) asymmetric and quantifiable motor deficit associated with dopamine depletion of the nigrostriatal pathway. We also show that this toxicity exhibits selectivity for the dopaminergic system.

Studies have shown that degeneration of striatal terminals followed by retrograde degeneration of the pathway is a good model for Parkinson's disease (Ichitani et al., 1994; Przedborski et al., 1995; Sauer and Oertel, 1994). We observed that intrastriatal administration of BH4 led to retrograde abolishment of the TH-positive neurons in the SN pars compacta. This occurred within 4 weeks, but not within 1 week, showing a time window similar to that reported for retrograde degeneration subsequent to 6-OHDA administration (Chang et al., 1999; Ichitani et al., 1994). We have also observed that there is a topological relationship between the site of injection in the striatum and the region of TH immunonegativity in the SN. That is, a lesion in the more posterior part of the striatum led to degeneration of dopamine cells in the more posterior SN (not shown). This topological relationship between the striatal terminals and their parent nigral cell bodies corresponded well with the previously published tracing studies on the nigrostriatal pathway (Beckstead et al., 1979; Fallon and Moore, 1978; Gerfen, 1992; Medina and Reiner, 1995). Interestingly, a relatively small lesion in the striatum seems to lead to a massive loss of TH cells in the topologically relevant nigral sections. Although the reason for this is unknown, one might attempt to speculate that the nigral cells may project to a diffuse area in the coronal plane of the striatum and that damage to some of the terminals may lead to retrograde degeneration of the parent cell body.

The clinical manifestations of Parkinson's disease include gait abnormalities, bradykinesia, rest tremor, rigidity, and akinesia. We demonstrate that the BH4-induced degeneration of the nigrostriatal pathway leads to the behavioral deficit associated with dopamine depletion. A correlation between size of the lesion and the behavioral deficit was observed: the striatal lesion area of 0.83 mm<sup>2</sup> or greater was sufficient to cause the behavioral deficit associated with dopamine depletion whereas the lesion of about 0.19 mm<sup>2</sup>, created by 10 μg BH4, was not. Thus, unlike the drug-induced rotation tests that require severe dopamine depletion (Chang et al., 1999), the forepaw stepping analysis seems to provide fine gradation of deficit associated with nigrostriatal damage.

In Parkinson's disease, the dopaminergic neurons undergo selective degeneration while the serotonergic neurons in the dorsal raphe are mostly unaffected (Chinaglia et al.,

1993). We show in the present study that direct exposure to BH4 leads to abolishment of the dopaminergic neurons in the SN and ventral tegmental area whereas the serotonergic cells in the dorsal raphe were spared. This was consistent with our *in vitro* finding that the serotonin-producing (nondopaminergic) cell line RBL-2H3 was resistant to BH4 (Choi et al., 2000). The selectivity of the BH4 toxicity is also evident after intrastriatal injection, in that the TH-immunoreactive dopaminergic terminals are vulnerable to BH4 whereas the NSE-positive nondopaminergic neurons seem to be intact. Thus, the BH4-induced degeneration appears to exhibit selectivity for the dopaminergic system.

The toxicity of BH4 seems to be in the range comparable to that of the known dopaminergic toxins. An amount of BH4 as low as 30 nmol (10 μg) was sufficiently toxic to the striatal dopaminergic terminals. In comparison, Filloux and Townsend (1993) reported that injection of 500 nmol, but not 250 nmol, dopamine caused a lesion in the striatum. Hastings et al. (1996) later observed dopamine terminal lesions at 100 nmol dopamine, which was still higher than what was observed for BH4 in the present study. On the other hand, 6-OHDA was reported to cause 92 and 10–20% reduction in dopamine uptake after intrastriatal injection of 85 and 10.5 nmol, respectively (Przedborski et al., 1995). We have observed that approximately 10% of the striatum has lost TH immunoreactivity after injection of 30 nmol BH4. Thus, the fact that a compound with toxicity comparable to that of the well-known synthetic neurotoxin and selectivity for the dopaminergic system exists *in vivo* is of interest and may suggest the possibility that BH4 might have some physiological relevance to the selective dopaminergic degeneration in Parkinson's disease. While the animal models of Parkinson's disease utilizing the synthetic toxins 6-OHDA (Lotharius et al., 1999) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Chun et al., 2001) have been very useful in attempts to elucidate the mechanism of the nigrostriatal vulnerability (Bloem et al., 1990; Kaakkola and Teravainen, 1990; Tolwani et al., 1999), our animal system using a molecule that is endogenously present in and causes selective damage to the dopaminergic system may prove to be a good model for such studies.

The striatal concentration of BH4 *in vivo* has been estimated to be 100 μM (Levine et al., 1981), but the concentration of extracellular BH4, which is thought to exert the toxic effect, is not known. The lowest concentration of BH4 we tested was 6 mM, which is probably much higher than what the cells and terminals would be exposed to in the striatum *in vivo*. However, because lower concentrations were not tested, it is possible that the minimal amount required for such lesion is lower. That this apparent toxicity of BH4 is not a nonspecific high-dose effect is demonstrated by several lines of evidence. First, the BH4 toxicity showed selectivity and the dorsal raphe neurons were not damaged by the same treatment that led to dopaminergic degeneration (the present results). *In vitro*, a series of dopamine-producing cells were shown to be sensitive to BH4 treatment

whereas those cells that do not produce dopamine were not (Choi et al., 2000). In addition, we have observed that the level of BH4 may easily increase under aberrant conditions. In fact, calcium influx into the nigral dopaminergic cells has been observed during activation of postsynaptic NMDA receptors (Loopuijt and Schmidt, 1998) and during stress (Mamczarz et al., 1999), and we have recently observed dramatic increases in the level of BH4 in the nigrostriatal system of stressed animals (Kim et al., in preparation).

The BH4 toxicity in dopaminergic cells is associated with generation of reactive oxygen species (Choi et al., 2000). In vivo, the nigral dopaminergic neurons are under a substantial level of oxidative stress owing to oxidation of dopamine (Hirsch et al. 1988), producing the highly reactive quinones and hydrogen peroxide during its auto- and enzymatic oxidations (Stokes et al., 1999). On the other hand, BH4 can also be oxidized to generate hydrogen peroxide (Davis et al., 1988; Davis and Kaufman, 1993; Fisher and Kaufman, 1973), which can be easily reduced in the presence of ferrous iron to yield the highly reactive hydroxyl radical. As a cofactor for TH, a rise in BH4 may lead to increased dopamine synthesis and synergistically augment the level of reactive oxygen species. In fact, exogenously applied BH4 is reported to increase TH activity in the rat striatum (Miwa et al., 1985) and to stimulate dopamine release both in vivo (Koshimura et al., 1990) and in vitro (Koshimura et al., 1999). Furthermore, BH4 is an essential cofactor for the synthesis of nitric oxide, which can form the potentially toxic 6-nitroderivatives with catecholamines (d'Ischia and Costantini, 1995) and/or can be oxidized to the highly toxic peroxynitrite. Anatomically, neurons expressing the neuronal nitric oxide synthase are abundant in the striatum and form a close contact with the BH4-containing dopaminergic terminals (Hwang et al., 1998; Fujiyama and Masuko, 1996). Microglia expressing inducible nitric oxide synthase may also contribute to the degeneration. In fact, we have observed that OX-42-expressing microglial cells have completely infiltrated the TH-negative lesion in the striatum 4 weeks postinjection, giving a discrete and demarcated appearance to the lesion, whereas the striatum 24 h postinjection showed a rather diffuse appearance (not shown). It is possible, then, that the injected BH4 may generate oxidative stress by itself and/or by enhancing dopamine and nitric oxide syntheses.

The results of the present study provide implication that the amount of BH4 might be finely regulated in vivo in order to maintain functions of the dopaminergic system without the development of toxicity. Deficiency in BH4 leads to DOPA-responsive dystonia (Ichinose et al., 1999) and hyperphenylalaninemia (Liu et al., 1998), demonstrating that the availability of BH4 is crucial in proper physiological functions. However, excess amounts of BH4 may be harmful to the dopamine cells as demonstrated in the present study. In order to prevent such toxicity, then, careful monitoring of its in vivo level

would be essential in administration of BH4 and application of gene therapy toward correction of such deficiencies (Canevari et al., 1999; Laufs et al., 2000). For instance, BH4 is clinically administered at a dose of 20–40 mg/kg body wt/day to hyperphenylalaninemia patients, and simple estimation of the concentration of BH4 in the body fluid after such administration would be in the range of 0.7 to 1.9 mM. While this is lower than what we have tested in the present study, in light of the fact that this is about 10-fold higher than the normal range in the striatum and our in vitro finding shows that 100  $\mu$ M extracellular BH4 is toxic, caution in administration of a high-dose BH4 should be warranted.

Thus far, no neuroprotective therapy that significantly stops or delays the progression of the nigrostriatal degeneration is available. Development of such treatment will become possible only with better understanding of the mechanism underlying the degeneration and availability of good experimental models. As demonstrated in the present paper, the BH4-induced lesion resembles the nigrostriatal degeneration in Parkinson's disease in several aspects. Further studies on the physiological and pathological role of BH4 on dopaminergic degeneration may provide some knowledge with which a possible neuroprotective therapy may be designed.

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