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Neuroscience Letters 359 (2004) 69–72

Neuroscience
Letters

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Loss of striatal dopaminergic fibers after intraventricular injection of tetrahydrobiopterin in rat brain

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Received 15 September 2003; received in revised form 3 February 2004; accepted 8 February 2004

Abstract

We have reported previously that tetrahydrobiopterin (BH4), an obligatory cofactor for dopamine synthesis, exerts preferential toxicity on dopamine producing cells. We report in the present study that BH4 injection into the lateral ventricle leads to degeneration of the dopaminergic terminals in the striatum, evidenced by a loss of tyrosine hydroxylase (TH) immunopositive fibers, a decreased amount of TH protein, and decreased dopamine content. Thus, the results of our study further provide evidence that BH4, the molecule endogenously present in the dopaminergic neurons, may participate in the nigrostriatal degeneration as in Parkinson's disease.

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Keywords: Tetrahydrobiopterin; Dopamine; Tyrosine hydroxylase; Parkinson's disease

Parkinson's disease (PD) is a movement disorder characterized by a selective degeneration of dopamine (DA) neurons in the substantia nigra (SN) pars compacta and their terminals in the striatum. The disease is accompanied by biochemical changes including loss of DA and its biosynthetic enzyme tyrosine hydroxylase (TH). Despite extensive studies, the underlying cause of the DA cell death has not yet been fully understood.

Tetrahydrobiopterin (BH4), an obligatory cofactor for DA synthesis [13], is exclusively produced in monoaminergic neurons in the brain including the SN DA neurons [12,17]. It has been observed previously that exposure to BH4 leads to death of cultured DA producing cells [1,5], suggesting its possible role in the dopaminergic degeneration. In vivo, inhibitors of GTP cyclohydrolase, the enzyme responsible for BH4 synthesis, have been shown to be protective [4,9]. The toxic effect of BH4 seems to occur extracellularly once released, because we [5] and others [10,18] have observed that increased intracellular BH4 after administration of its precursor sepiapterin is nontoxic or even protective. BH4 is readily released from cells of its synthesis [5] and accumulates in the extracellular

space and cerebrospinal fluid (CSF). It has been observed that extracellular BH4 is increased after calcium influx in vitro [11] and that calcium influx into the SN dopaminergic cells occurs during activation of postsynaptic NMDA receptors [15] and during stress [16] in vivo.

Consistent with the in vitro observations, we have previously observed that direct injection of BH4 into the striatum leads to degeneration of striatal dopaminergic terminals as well as their cell bodies in the SN and behavioral deficit associated with PD [14]. While such intrastriatal administration allows generation of an asymmetric lesion that can be subsequently assessed for asymmetric motor deficit, direct injection can cause physical damage to the tissue. On the other hand, intraventricular injection would (1) allow exposure to BH4 in a less invasive condition, (2) ensure that BH4 acts from outside the cell, and (3) mimic pathological conditions in which an abnormally high concentration of BH4 is present in the CSF. In the present study we therefore determined whether BH4 injected into the lateral ventricle would cause morphological and biochemical changes relevant to selective dopaminergic damage in the striatum.

All procedures were performed in compliance with the guidelines set forth by the *Laboratory Animal Manual* of the Asan Institute for Life Sciences that adopt the National

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Institutes of Health *Guide to the Care and Use of Animals*. Stereotaxic injection of BH4 (RBI, Natick, MA) followed the general protocol described elsewhere [14] except that injection was made into the right lateral ventricle (AP, +0.7 mm from bregma; ML, 0.8 mm; DV, 4.0 mm below dura) in a total volume of 10 μ l. Immunocytochemistry was performed as described previously [12] using rabbit polyclonal antisera against TH (1:1000; Protos, New York) or neuron specific enolase (NSE; 1:100; BioGenex, San Ramon, CA), a Vectastain ABC kit and biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA). Adjacent brain sections were stained with hematoxylin.

For determination of the tissue DA level, animals were decapitated and the whole brain was immediately frozen. The right striatum was excised and its DA content was determined as described previously [14]. For Western blot analysis, striatal tissue was homogenized in 5 mM potassium phosphate buffer (pH 6.5) containing 0.2% Triton X-100 and 20 μ g protein was subjected to Western blot analysis [6] against TH (1:1000). Comparisons were made using a non-parametric Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant for all analyses.

To minimize the number of animals to sacrifice, we first assessed the amount of BH4 to be injected. We have observed that BH4 is in a similar range of toxicity as the published amount of 6-hydroxydopamine (6-OHDA) used for intrastriatal injection [14]. A literature search revealed that the amounts of 6-OHDA used for intraventricular injection varied between 50 μ g (0.2 μ mol) and 1000 μ g (4 μ mol) with 250 μ g (1 μ mol) used most widely [3,8,19,20]. We therefore decided to use 1 μ mol (10 μ l of 100 mM solution) of BH4 for our study. BH4 was stereotaxically injected into the right lateral ventricle and the animals were sacrificed 7 days after the injection.

For morphological analysis, striatal sections were subjected to immunocytochemistry against TH. The results showed that TH-positive dopaminergic fibers were lost in the striatal area adjacent to the ventricle (Fig. 1B). Higher magnification showed gradation in the TH immunoreactivity: the area closest to the lateral ventricle was most affected, presumably due to exposure to a higher concentration of BH4 (Fig. 1D). Consistently reproducible lesions were produced in all animals that received BH4 ($n = 7$). The left (contralateral to the injection) striatum revealed a small degree of loss in TH immunoreactivity (data not shown). Control animals injected with vehicle ($n = 7$) maintained the dense TH immunoreactivity (Fig. 1A,C). Compared to the dramatic decrease in TH immunoreactivity by BH4, NSE, which is present in all neuronal cells, revealed no loss of immunoreactivity (Fig. 1E,F), demonstrating that the non-dopaminergic neurons of the striatum were undamaged. Hematoxylin staining of the adjacent section revealed the presence of dense nuclei, indicating that both neurons and glial cells were viable (Fig. 1G,H) and

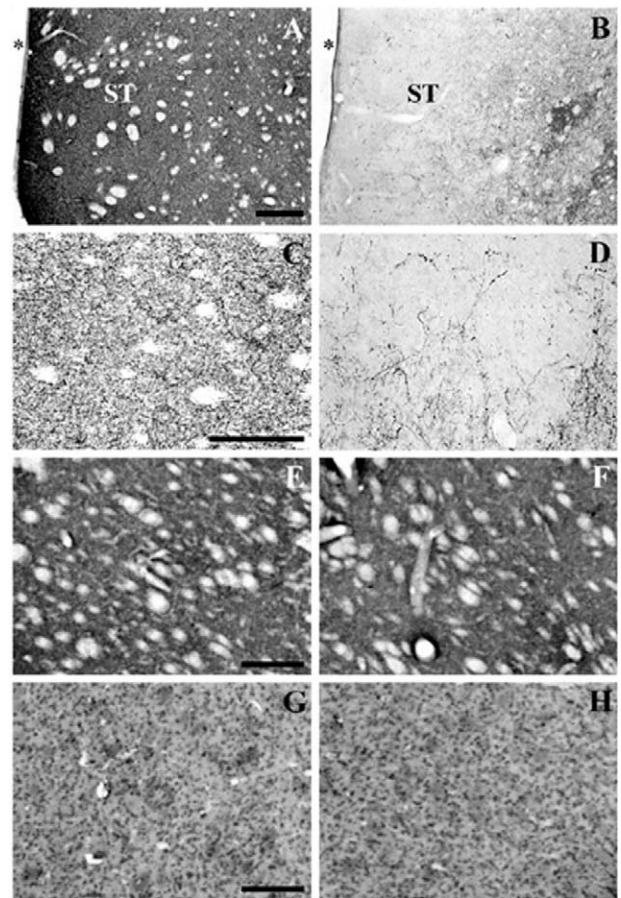


Fig. 1. Intraventricular BH4 injection causes selective degeneration of striatal dopaminergic terminals. Typical photomicrographs of TH-immunostained striatum 7 days after injection of 10 μ l of 100 mM BH4 (B,D) and vehicle (A,C) into the lateral ventricle. Adjacent sections were also immunostained against NSE (F, BH4-injected; E, vehicle-injected) or stained with hematoxylin (H, BH4-injected; G, vehicle-injected). *Lateral ventricle; scale bar, 1 mm.

GFAP immunoreactivity was not significantly changed (data not shown). Thus, the cell bodies of non-dopaminergic neurons in the striatum appeared to be largely resistant to BH4. Taken together, intraventricularly injected BH4 led to selective damage of the dopaminergic fibers in the adjacent striatal region. We have previously observed that the lesion size increases dose-dependently but stays the same during the first 7 days after intrastriatal injection of BH4 [14].

For more quantitative analysis, animals were treated as above and the right striatal tissue was subjected to Western blot analysis against TH ($n = 7$ each). A typical Western blot is shown in Fig. 2A. The intensity of the TH immunoreactive 60 kDa band was lower in the animals treated with BH4. The densitometry of the bands from all animals showed a 45% decrease on average compared to the vehicle-injected control (Fig. 2B; $P < 0.05$). As dopaminergic degeneration should accompany a decrease in DA content as well, we measured DA contents in the right striatum. As shown in Table 1, BH4 treatment caused a decrease in DA content to 53% of the vehicle-treated

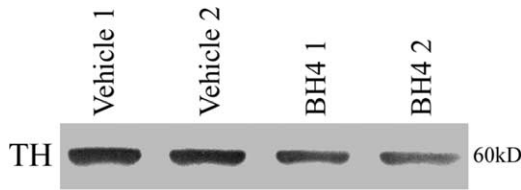


Fig. 2. Intraventricular BH4 injection causes a reduction in the amount of TH. The figure is a representative Western blot analysis against TH showing striatal samples from two vehicle-injected controls and two BH4-injected animals. Quantification of TH band from all experimental animals showed a 45% decrease compared to vehicle-injected control ($n = 7$; $P < 0.05$).

control. Although we have not counted the dopaminergic cell bodies in the SN in this study, our previous data showed dramatic decreases in their number following striatal fiber loss by BH4 [14]. We have also observed in mesencephalic neuronal culture that TH-positive neurons are selectively lost after exposure to BH4 and that the fiber loss is followed by a decreased size of soma, and ultimately cell death (data not shown).

We demonstrate in the present study that intraventricular injection of BH4, an obligatory cofactor of DA synthesis, causes a decrease in (1) the number of TH-positive fibers, (2) the amount of TH protein, and (3) DA content in the striatum. These findings further corroborate our previous reports that BH4 causes toxicity to dopaminergic cells in vitro [5–7] and after direct injection into the dopaminergic brain regions [14]. That BH4-induced damage is selective for the dopaminergic system is demonstrated by several lines of evidence: (1) BH4 toxicity is selective on dopaminergic cells among various dopaminergic and non-dopaminergic cell lines [5]; (2) BH4 toxicity requires the presence of intracellular DA and is attenuated in the presence of DA synthesis inhibitors in dopaminergic cells [7]; (3) in vivo, BH4 injected into SN results in TUNEL-staining positive neurons corresponding to TH-positive DA cells [6]; (4) the serotonergic neurons in dorsal raphe were unaffected after direct injection of BH4 into this region [14]; (5) the relatively intact staining pattern of NSE (the present results) [14] and MAP-2 (unpublished results) after BH4 administration in vivo indicates that the non-DA neurons in the striatum are unaffected by BH4; (6) GFAP immunoreactivity is not significantly changed during the first week

after the intraventricular (the present results) or intrastriatal injection (unpublished results).

Therefore, it is possible that aberrant conditions that elevate the tissue BH4 level may lead to selective damage to the dopaminergic system. The mechanism by which BH4 exerts selective toxicity on dopaminergic cells seems to be mediated by the generation of oxidative stress [5,7] and involves apoptosis [6], as reported for PD [2]. Taken together, the findings that BH4 (1) is endogenously present in the dopaminergic system, (2) has selective toxicity for dopaminergic cells both in vivo and in vitro, (3) generates oxidative stress, and (4) leads to apoptosis point to the possibility that BH4 has physiological relevance to PD.

Acknowledgements

We are grateful to Y.J. Lee, H.J. Choi and S.W. Kim for technical assistance and help in preparation of the manuscript. This work was supported by a Korea Research Foundation Grant (KRF-2002-005-E00003). S.T. Kim was supported in part by the BK21 Life Sciences Program.

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Table 1
Effect of intraventricular BH4 injection on striatal DA contents

| | Striatal DA content ($\mu\text{g DA/g tissue}$) | n | % control |
|------------------|---|-----|-----------|
| Vehicle-injected | 17.8 ± 1.9 | 7 | 100 |
| BH4-injected | 9.4 ± 1.3 | 7 | 53 |

Animals were intraventricularly injected with BH4 or vehicle and decapitated after 7 days. The whole brain was immediately frozen and the right striatum was excised. DA content was determined by HPLC and electrochemical detection. Data are mean numbers \pm SEM. $P < 0.05$ vs. vehicle-injected.

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