Neurochemistry International 125 (2019) 16–24

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Administration of tetrahydrobiopterin restored the decline of dopamine in the striatum induced by an acute action of MPTP

Hiroki Kurosaki[a,1], Kentaro Yamaguchi[a,1], Kohei Man-yoshi[a], Shin-ichi Muramatsu[b,c], Satoshi Hara[a], Hiroshi Ichinose[a,*]

[a] School of Life Science and Technology, Tokyo Institute of Technology, Yokohama, Japan
[b] Division of Neurology, Jichi Medical University, Shimotsuke, Tochigi, Japan
[c] Center for Gene & Cell Therapy, The Institute of Medical Science, University of Tokyo, Tokyo, Japan

ARTICLE INFO

Keywords:
Tetrahydrobiopterin
Dopamine
Oxidative stress
Parkinson's disease
Tyrosine hydroxylase

ABSTRACT

Parkinson's disease (PD) is the second common neurodegenerative disorder. Deficit of the nigro-striatal dopaminergic neurons causes the motor symptoms of PD. While the oxidative stress is thought to be deeply involved in the etiology of PD, molecular targets for the oxidative insults has not been fully elucidated. 6*R*-5,6,7,8-Tetrahydrobiopterin (BH4) is a cofactor for tyrosine hydroxylase (TH), the rate-limiting enzyme for production of dopamine, and easily oxidized to its dihydro-form. In this study, we examined the alteration in the metabolism of BH4 caused by a parkinsonian neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP reduced the dopamine content and the *in vivo* activity of TH in the striatum prior to degeneration of the dopaminergic neurons. We found that administration of BH4 could restore the dopamine content and *in vivo* TH activity in the striatum of MPTP-treated mice. Unexpectedly, when BH4 was administered with MPTP, BH4 contents in the brain were far higher than those injected without MPTP even at 23 h after the last injection. Because MPTP has been shown to increase ROS production in the dopaminergic neurons, we assumed that the increased ROS oxidizes BH4 into its dihydro-form, excreted from the dopaminergic neurons, taken-up by the neighboring cells, reduced back to BH4, and then accumulated in the brain. We also investigated the action of MPTP in mice lacking *quinonoid*-dihydropteridine reductase (Qdpr), an enzyme catalyzing regeneration of BH4 from *quinonoid* dihydrobiopterin. The dopamine depletion induced by MPTP was severer in *Qdpr*-deficient mice than in wild-type mice. The present data suggest that perturbation of the BH4 metabolism would be the cause of early and persistent dopamine depletion in the striatum.

1. Introduction

Parkinson's disease (PD) is a common progressive neurodegenerative disorder that is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta leading to severe loss of dopamine (DA) in the striatum (Bernheimer et al., 1973). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin that induces symptoms similar to those of PD in humans (Langston et al., 1983). Since mice treated with MPTP also showed a decrease of DA in the striatum, MPTP-treated mice are widely used as a model of PD (Heikkila et al., 1984; Kurosaki et al., 2004; Pileblad et al., 1985).

6*R*-5,6,7,8-Tetrahydrobiopterin (BH4) is a cofactor for tyrosine hydroxylase (TH; EC 1.14.16.2), the enzyme that catalyzes the rate-limiting step in the biosynthesis of DA, as well as a cofactor for phenylalanine and tryptophan hydroxylases, nitric oxide synthases, and glyceryl-ether monooxygenase (Supplementary Fig. 1) (Nichol et al., 1985; Matsuura et al., 1985; Thöny et al., 2000). BH4 is synthesized from GTP through three enzymatic steps catalyzed by GTP cyclohydrolase I (GCH; EC 3.5.4.16), pyruvoyltetrahydropterin synthase (PTS; EC 4.2.3.12), and sepiapterin reductase (SPR; EC 1.1.1.153). In addition to *de novo* synthesis, BH4 levels are maintained via recycling pathways mediated by *quinonoid* dihydropteridine reductase (QDPR; EC 1.5.1.34)

Abbreviations: AADC, aromatic L-amino acid decarboxylase; BH4, tetrahydrobiopterin; BH2, dihydrobiopterin; BP, biopterin; DA, dopamine; DHFR, dihydrofolate reductase; L-DOPA, 3,4-dihydrocxyphenylalanine; DOPAC, 3,4-dihydroxyphenylacetic acid; GCH, GTP cyclohydrolase 1; HVA, homovanillic acid; DAT, dopamine transporter; MPTP, 1-metyl-4-phenyl-1,2,3,6-tetrahydropyridine; NOS, nitric oxide synthase; PD, Parkinson's disease; *q*BH2, *quinonoid* dihydrobiopterin; QDPR, *quinonoid* dihydropteridine reductase; TH, tyrosine hydroxylase

* Corresponding author. School of Life Science and Technology, Tokyo Institute of Technology, Yokohama 226-8501, Kanagawa, Japan.
E-mail address: hichinos@bio.titech.ac.jp (H. Ichinose).

[1] Both authors contributed equally to this work.

https://doi.org/10.1016/j.neuint.2019.02.005
Received 16 October 2018; Received in revised form 19 January 2019; Accepted 6 February 2019
Available online 07 February 2019
0197-0186/

and dihydrofolate reductase (DHFR; EC 1.5.1.3). In the recycling pathways, *quinonoid* dihydrobiopterin (*q*BH2) and dihydrobiopterin (BH2) are reduced back into BH4 by QDPR and DHFR, respectively, while *q*BH2 is easily converted to BH2 through tautomerization.

Genetic defect of BH4-synthesizing enzymes causes malignant-type hyperphenylalaninemia and dystonia/parkinsonism depending on the defective enzymes and the mode of inheritance (Werner et al., 2011). Genetic mutations in an allele of the GCH gene or mutations in both alleles of SPR gene cause dopa-responsive dystonia (DRD) due to DA deficiency in the brain (Ichinose et al., 1994; Bonafe et al., 2001). Mouse models which are defective in BH4-synthesizing enzymes showed a decrease in the amount of TH in the brain, and exhibited dystonia- and parkinsonism-like phenotypes (Sumi-Ichinose et al., 2001; Sato et al., 2008; Takazawa et al., 2008). Dystonia is a hyperkinetic disorder, while brady-kinesia is a typical feature of PD. It has not been clarified why the same deficiency of DA in the brain causes dystonia in some cases and parkinsonism in other cases.

Mencacci et al. (2014) reported presence of PD patients in family members of four independent DRD pedigrees in which pathogenic GCH mutations were identified, and found that the frequency of GCH variants were significantly higher in PD patients than in controls (Mencacci et al., 2014). Genome-wide association study identified the GCH gene as having significant association with PD (Mencacci et al., 2014; Nalls et al., 2014). These studies strongly suggested that BH4 metabolism should be involved in the PD etiology. There is, however, little study on the relation of the BH4 metabolism with PD pathogenesis.

In 1985, Hirata and Nagatsu found that MPTP strongly inhibits *in vivo* TH activity, evaluated by accumulation of L-DOPA under the inhibition of aromatic L-amino acid decarboxylase (AADC) using tissue slices of the rat striatum (Hirata & Nagatsu 1985, 1986). They reported that MPTP itself did not directly inhibit the TH activity *in vitro*, and that the inhibition of the *in vivo* TH activity by MPTP was detected also in the mouse brain as early as 1 h after administration (Hirata and Nagatsu, 1986). The molecular mechanism, however, was remained to be solved. In this study, we explored the action of MPTP on the BH4 metabolism especially relating to the *in vivo* TH activity. Our present data suggest that perturbation of the BH4 metabolism should be the cause for the inhibition of the *in vivo* TH activity, and for early and persistent depletion of striatal DA by MPTP.

2. Materials and methods

2.1. Animals and treatment

Wild-type male C57BL/6J mice 8–10 weeks old were used and maintained at 21–23 °C in a 12-h light-dark cycle with free access to water and food. *Qdpr*-KO mice were produced by Lexicon Pharmaceuticals, Inc. (Woodlands, Texas, USA) with 129SvEv-derived ES cells. A C57BL/6J background line was established by repetitive crossing. All animal experiments were carried out in accordance with the Tokyo Institute of Technology guidelines for the care and use of laboratory animals and approved by the Animal Care Committee of Tokyo Institute of Technology [Approved No. D2016003]. MPTP (20 mg/kg; Sigma-Aldrich, USA) was injected into the abdominal cavities of the mice once or four times at 2 h intervals. Control groups received saline instead of MPTP. For BH4 administration studies, BH4/2HCl (provided by Suntory Inc. Osaka, Japan) was dissolved in PBS containing 1 mM ascorbic acid and 0.1 M Na_2HPO_4 to adjust pH around 7, stored in a deep-freezer, and used within 1 week to prevent the undesirable oxidation and degradation. As a control of BH4 administration, PBS containing 1 mM ascorbic acid and 0.1 M Na_2HPO_4 was administered to the mice. BH4 was intraperitoneally administered at a dose of 50 mg/kg 1 h after the every MPTP injections as depicted in Figs. 4A and 5A. The dose of BH4 was determined according to our previous literature (Homma et al., 2013). In all experiments, mice were arbitrarily divided into each treatment group.

2.2. Sample preparation for biochemical analysis

After decapitation, the striatum and dorsal midbrain were immediately dissected from coronal slices of the brain, frozen in liquid nitrogen and kept at −80 °C until analysis. The striatal tissues were homogenized with 350 µl of the buffer containing 15 mM Na_2HPO_4, 5 mM NaH_2PO_4, 0.1 mM EDTA, 100 mM NaCl and 1 mM ascorbic acid, followed by sonication. The obtained lysates were analysed as follows.

2.3. Quantification of pteridines by HPLC with fluorescence detection

One hundred 10 µL of each lysate were deproteinized by the addition of 12 µl of 2 M perchloric acid (PCA) containing 1 mM EDTA and 1 mM ascorbic acid, followed by centrifugation. The supernatants were filtered through a 0.2 µm filter and diluted with 0.1 M HCl to prevent oxidation of BH4. BH4, BH2 and biopterin were quantified separately using HPLC by a post-column oxidation method as previously described (Tani and Ohno, 1993).

2.4. Quantification of DA by HPLC with electrochemical detection

One hundred microliters of each lysate were deproteinized by the addition of 11 µl of 2 M PCA containing 1 mM EDTA, followed by centrifugation. The supernatants were filtered through a 0.2 µm filter, and isoproterenol was added as an internal standard. Catecholamines including DA and its metabolites were analysed using HPLC with a reversed-phase column (SC5-ODS, Eicom, Kyoto, Japan) and electrochemical detection. The data were normalized based on the protein concentrations.

2.5. Detection of protein expression by western blotting

Aliquots of the brain homogenates combined with 0.1 M dithiothreitol and 2% SDS were centrifuged at 20,400 × *g* for 20 min. The obtained supernatants were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. The membranes were analysed by immunoblotting with a primary antibody against TH (1:5000, Millipore) and DAT (1:1000, Millipore), and the horseradish peroxidase-conjugated secondary antibody against rabbit IgG (1:5000, GE Healthcare). Images were assessed using a chemiluminescent substrate (Millipore).

2.6. Assay for in vivo TH activity

To evaluate *in vivo* TH activity, NSD-1015 (Sigma-Aldrich), an inhibitor of aromatic L-amino acid decarboxylase (AADC), was injected to the mice 30 min prior to sacrifice, and L-DOPA accumulation was measured (Carlsson and Lindqvist, 1973). Striatal tissue extracts were prepared as described above, and 140 µl of lysate was deproteinized by centrifugation with 15 µl of 2 M PCA containing 1 mM EDTA. The supernatants were neutralized by the addition of 400 mM K_2CO_3, and the L-DOPA formed was purified with Al_2O_3. The amount of L-DOPA was analysed using HPLC with a reversed-phase column (Nucleosil7C18, GL Science) and electrochemical detection. The mobile phase was composed of 0.1 M sodium phosphate (pH 3.5), 8 µM EDTA and 1% (v/v) methanol.

2.7. Assay for in vitro TH activity

The TH reaction was carried out at 37 °C for 10 min in a reaction mixture (200 µl) containing 0.2 M sodium acetate (pH 6.0), 40 µg catalase, 0.1 M 2-mercaptoethanol, 0.05 mM L-tyrosine, 1 mM BH4, and brain tissue homogenates. The reaction was quenched by the addition of PCA. The amounts of synthesized L-DOPA were measured by using HPLC as described above and normalized by protein concentrations (Ogawa and Ichinose, 2006).

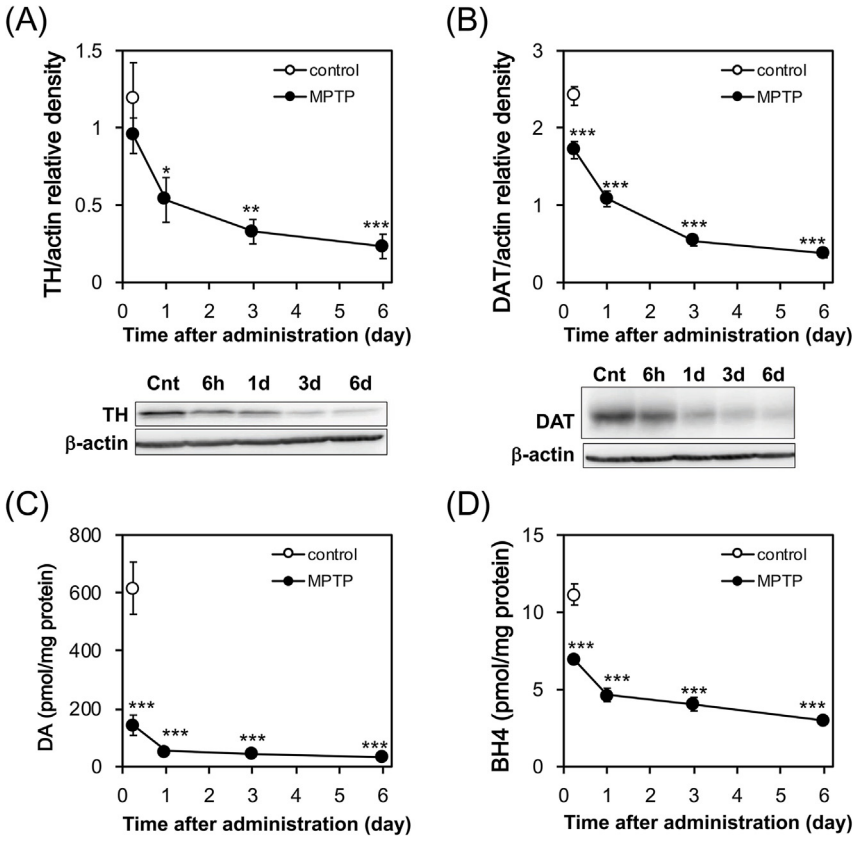

Fig. 1. Alteration of striatal TH, DAT, DA and BH4 content in mice treated with MPTP. Mice were dissected and the striatum was collected at 4 different time points (6 h, 1 day, 3 days, and 6 days after the final 4 injections of 20 mg/kg MPTP). Western blotting analysis provide TH (A) and DAT (B) signal density, quantified with ImageJ. (C) DA levels were measured by HPLC with electrochemical detection, (D) BH4 levels were evaluated by HPLC with fluorimetric detection by post-column sodium nitrite oxidation. Each value represents the mean ± S.E.M. $n = 4$ mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; one-way ANOVA followed by Dunnett's test. Significance test was performed versus PBS-injected mice.

2.8. Statistics

All data were expressed as the mean ± S.E.M. Pairwise significant differences were determined by Student's *t*-test. Multiple comparisons were performed using one-way ANOVA or two-way ANOVA followed by a post hoc Tukey's HSD test or Dunnett's test when there were more than three groups.

3. Results

3.1. Time-dependent alterations in the amounts of DA, BH4, TH and DAT after repeated MPTP administration

First, we examined the time-dependent alterations in the amounts of DA, BH4, TH and DAT in the striatum after 4 times injections of 20 mg/kg MPTP with 2 h-intervals. Control mice were injected with PBS instead of MPTP and sacrificed at 6 h after the last injection. The TH and DAT proteins were quantified by intensity of the immunoreactive bands of western blotting. As shown in Fig. 1, the amounts of TH and DAT proteins were gradually decreased after MPTP administration, showing the gradual degeneration of the dopaminergic nerve terminals in the striatum (Fig. 1A and B). The reductions of the DA levels were far greater than those of TH and DAT, and the DA levels were decreased to approximately 30% of the control as early as 6 h after the last MPTP injection (Fig. 1C), when the amounts of TH proteins were not decreased (Fig. 1A). As shown in Fig. 1D, the BH4 levels were significantly decreased to about 60% of the control at 6 h after the last MPTP injection, and gradually decreased at 1, 3, and 6 days after MPTP. Because the decreases in the protein amount of TH and DAT would reflect the degree of neurodegeneration in the dopaminergic neurons, the acute decreases of DA and BH4 soon after the MPTP treatment suggest that MPTP acutely affects the metabolism of DA and BH4.

3.2. In vivo TH activity after repeated MPTP administration

The significant loss of DA content prior to the reduction in TH protein by MPTP treatment implied the existence of an unrevealed mechanism of control over the DA level. To examine the mechanism, we focused on the inhibition of *in vivo* TH activity by MPTP reported by Hirata and Nagatsu (Hirata and Nagatsu, 1985). *In vivo* TH activity was evaluated by accumulation of L-DOPA in the presence of NSD-1015, an inhibitor of AADC, the enzyme that catalyzes the conversion of L-DOPA to DA. The *in vivo* TH activities in striatum and substantia nigra of MPTP-treated mice were markedly reduced compared with control mice (4.5% and 22%, respectively) (Fig. 2Band C). Then, we performed *in vitro* TH assay using enough amounts of BH4 and tyrosine, a substrate for TH, to examine whether the TH protein in the brain homogenates retained the catalytic activity after the repetitive MPTP treatment. There were no significant differences on the TH activity by MPTP treatment (Fig. 2D and E). Therefore, the observed decreases in the *in vivo* TH activities were thought to be caused by any changes in cellular environments induced by MPTP.

3.3. Time-dependent alteration of in vivo TH activity after single MPTP administration

Next, we examined time-dependent alterations in the *in vivo* TH activity, TH protein, and the amounts of BH4 and BH2 after single MPTP administration. The *in vivo* TH activity started to decrease at 1 h after the administration, reached 24% of the control value at 3 h, and then recovered to the original level at 6 h (Fig. 3A). The TH protein levels evaluated by a western blotting technique, showed no significant alteration during 6 h after MPTP treatment, indicating that a single MPTP administration did not cause degeneration of the dopaminergic neurons (Fig. 3B).

The transient decrease in the *in vivo* TH activity suggests that it should be caused by alteration in the cellular environment. Because

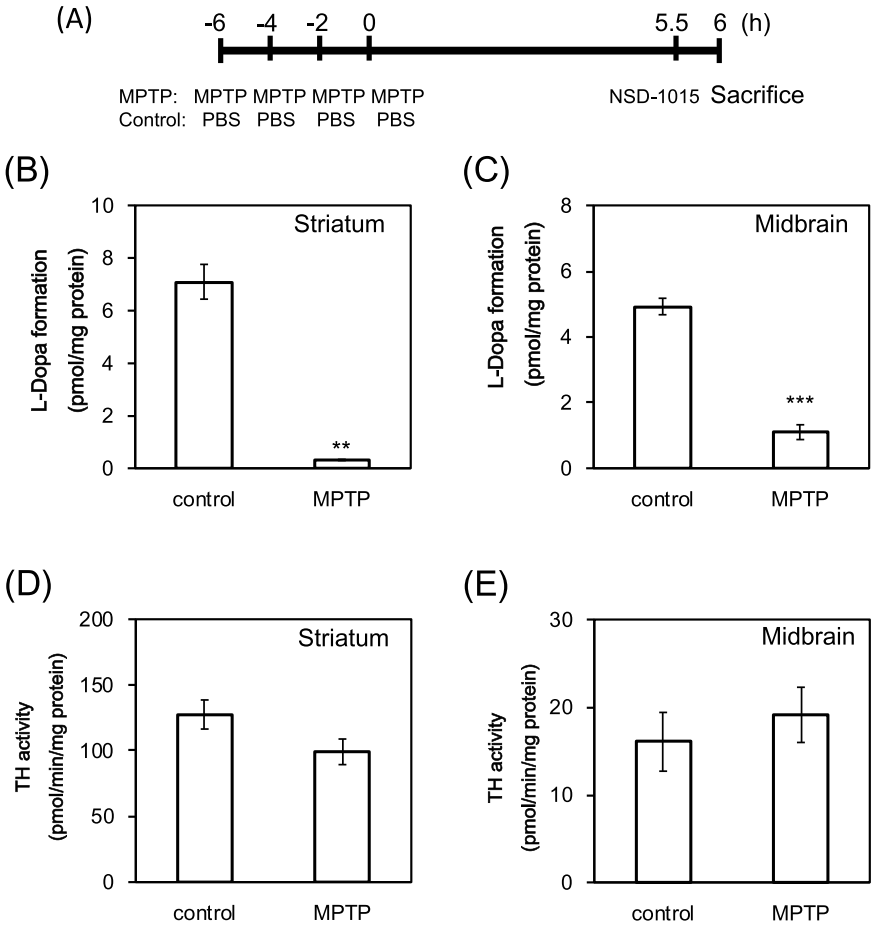

Fig. 2. *In vivo* TH activity and *in vitro* TH activity at 6 h after 4-times MPTP injection. At a delay of 5.5 h after 4 injections of 20 mg/kg MPTP, NSD-1015, an AADC inhibitor, was injected (A). L-DOPA formation in 30 min in the striatum (B) and midbrain (C) was measured. To evaluate *in vitro* TH activity, tissue homogenates of the striatum (D) and midbrain (E) were incubated with sufficient amounts of tyrosine and BH4 under an optimal condition, and L-DOPA was measured by HPLC with electrochemical detection. Each value represents the mean ± S.E.M. $n = 4$ mice per group. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Student's *t*-test.)

MPTP has been known to induce ROS and oxidative stress in the dopaminergic neurons, we hypothesized that the increased ROS may oxidize BH4, an essential cofactor for TH, into BH2 through *q*BH2. Then, we measured the amounts of BH4 and BH2 in the tissue homogenates treated with MPTP. As shown in Fig. 3C and D, the levels of BH2 exhibited an increasing trend compared with the control (vehicle treatment), whereas the BH4 contents were unchanged.

3.4. Administration of BH4 restored the decreased in vivo TH activity by MPTP

Then, we examined the effect of BH4 supplementation on the *in vivo* TH activity inhibited by MPTP. When BH4 was injected into the abdominal cavity, all mice showed significant elevation of BH4 levels in the brain (Fig. 4C and E). Associated with BH4 accumulation, the reduced *in vivo* TH activity at 3 h after MPTP administration was significantly recovered both in the striatum and ventral midbrain, whereas administration of BH4 alone did not increase the L-DOPA formation (Fig. 4B and D). These data showed that the administration of BH4 increased the BH4 levels in the brain and restored the *in vivo* TH activity suppressed by MPTP, suggesting that MPTP impaired the availability of BH4 in the dopaminergic neurons and that the impaired availability of BH4 should be the cause of the reduced *in vivo* TH activity under MPTP.

3.5. Administration of BH4 reversed the depletion of DA by MPTP in the striatum

Because BH4 administration restored the MPTP-induced reduction of the *in vivo* TH activity by a single MPTP treatment, we assumed that DA depletion in the early stage of MPTP toxicity might also be due to BH4 deficiency in the dopaminergic neurons. To confirm this assumption, we examined whether the DA content in the striatum could be maintained by BH4 administration after repeated MPTP treatment causing neurodegeneration. Mice were given 4 injections of 20 mg/kg MPTP at 2 h intervals, followed by 20 mg/kg BH4 administrations 1 h after each MPTP injection (0 h MPTP, 1 h BH4, 2 h MPTP, 3 h BH4, and so on). Twenty-four hours after the final MPTP injection, the striatal tissues were collected and analysed (Fig. 5A). Control groups were given the vehicle (PBS containing ascorbic acid without BH4). Substantially high levels of striatal BH4 were observed in mice treated with both MPTP and BH4 (MPTP-BH4), despite no significant difference between the PBS-PBS group and the PBS-BH4 group, suggesting that MPTP affected BH4 metabolism in the brain (Fig. 5B). In the MPTP-PBS group, BH4 levels tended to be lower compared with the PBS-PBS group (Fig. 5B), probably due to partial degeneration of the dopaminergic neurons as shown in Fig. 1A. We next quantified the DA, DOPAC, and HVA contents in the four groups to evaluate whether the BH4 treatment could rescue the MPTP-induced DA depletion. We found that the drastic reduction of striatal DA content by MPTP-PBS (17% of control) was mitigated to 51% of the control group in the MPTP-BH4 mice (2.9-fold

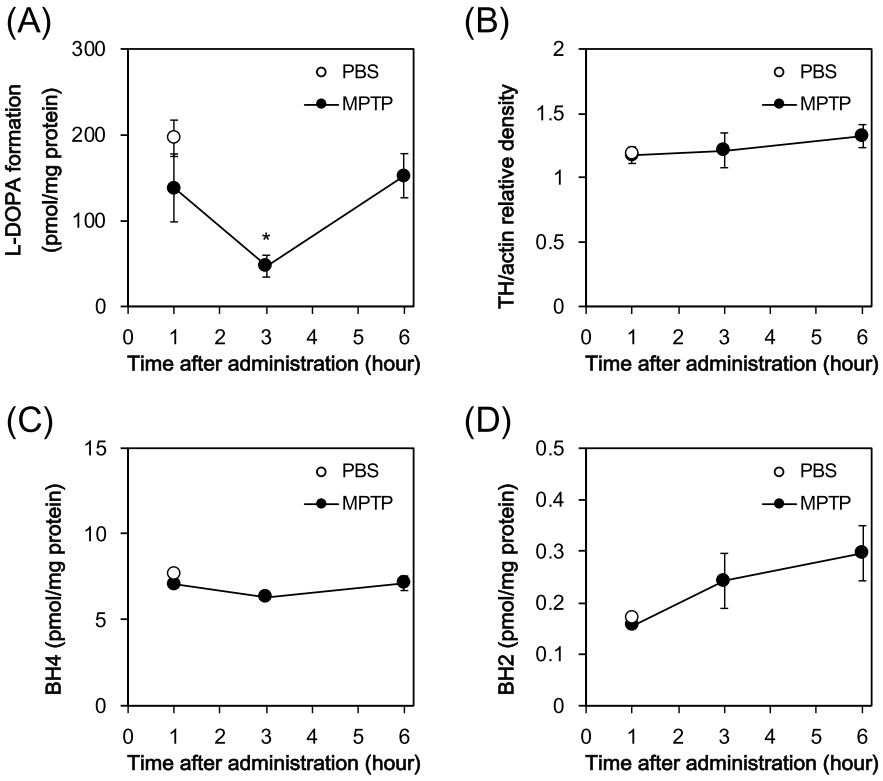

Fig. 3. Time-dependent alterations in *in vivo* TH activity, TH protein, BH4 and BH2 levels after a single MPTP treatment. Mice were dissected and the striatum was analysed at 4 different time points (1 h, 3 h, and 6 h after single injections of 20 mg/kg MPTP). (A) L-DOPA formation in 30 min, recognized as an indicator of TH activity *in vivo*, was measured by HPLC with electrochemical detection. (B) TH signal was quantified by western blotting. Levels of BH4 (C) and BH2, an oxidized form of BH4 (C), were measured by HPLC with fluorescent detection. Each value represents the mean ± S.E.M. $n = 4$ mice per group. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; one-way ANOVA followed by Dunnett's test.)

increase compared with MPTP-PBS) (Fig. 5C).

We summarized the contents of DA and its metabolites, HVA and DOPAC, in the striatum after MPTP and BH4 administration in Table 1. The HVA and DOPAC concentrations were not altered by the BH4 treatment (Table 1). Since the recovery of DA content by BH4 administration might protect degeneration of dopaminergic neurons from MPTP, we examined the TH protein level in the striatum, which is regarded as an index of neurodegeneration. However, despite the increase of BH4 and DA in the striatum, the TH protein levels of the MPTP-BH4 group remained unchanged compared with those of MPTP-PBS, suggesting that the BH4 supplementation does not prevent DA neurons from degenerating (Fig. 5D).

3.6. *Qdpr-KO mice were more susceptible to MPTP toxicity*

The above experiments suggest that MPTP oxidizes BH4 into BH2 by ROS. When BH4 is oxidized to BH2 through a chemical reaction with ROS, it is plausible that *q*BH2 is first formed then converted to BH2 through tautomerization. Because QDPR reduces *q*BH2 to BH4, MPTP toxicity may be enhanced in the *Qdpr*-defective mice. To address this assumption, we examined the effect of MPTP in the *Qdpr*-KO mice, in which BH4 is not regenerated via a QDPR-mediated pathway. We quantified DA content and BH4 levels 24 h after 4-times injections of 20 mg/kg MPTP administration. *Qdpr*-KO mice showed relatively lower levels of BH4, concurrent with higher contents of oxidized forms (BH2 + BP), in the striatum (Fig. 6A and B). In control mice with MPTP treatment, the level of DA in the striatum was 24% of that in PBS treated mice, whereas only 4% of DA remained in *Qdpr*-KO mice (Fig. 6C). These data suggest that BH4 recycling pathway plays an important role in the presence of BH4 against MPTP-induced striatal DA depletion.

4. Discussion

In this study, we showed that MPTP transiently inhibited *in vivo* TH activity in the mice brain (Fig. 3), and that administration of BH4 restored the decreased *in vivo* TH activity (Fig. 4) and DA levels (Fig. 5) in the striatum after MPTP treatment. These data suggest that MPTP oxidizes BH4 through production of ROS, and that the depletion of the intraneuronal BH4 concentration available for the TH reaction should be the cause of the inhibition of *in vivo* TH activity and of the reduction of DA in the early stage of the MPTP toxicity. In support of this idea, DA reduction induced by MPTP was exacerbated in *Qdpr*-KO mouse, which lacks a BH4 regenerating pathway (Fig. 6). Furthermore, we found that the larger amounts of BH4 was retained in the brain when BH4 was administered with MPTP (Fig. 5B). Taken together, our findings suggest that MPTP perturbs the BH4 metabolism resulting in a decrease of *in vivo* TH activity and in long-lasting DA depletion.

The causal involvement of oxidative stress would be a leading hypothesis for the pathogenesis of PD. Because dopaminergic neurons have low levels of glutathione and high levels of pro-oxidant iron, whose conjugation with superoxide produces strong oxidative stress, the dopaminergic neurons seem to be vulnerable to oxidative stress (Smeyne M & Smeyne R, 2013). Many reports demonstrated increased oxidative stress and generation of ROS caused by the action of 1-methyl-4-phenyl-pyridinium ion (MPP^+), an active toxin derived from MPTP (Sriram et al., 1997; Drechsel and Patel, 2008). However, it has not been clarified the target molecule(s) for oxidative stress relating to the neurotoxicity. We propose that oxidation of BH4 could be one of the molecular targets relating to the neurotoxicity by MPTP and pathogenesis of PD.

Besides monoamine synthesis, BH4 is required for the synthesis of nitric oxide (NO), an important signaling molecule, by the action of nitric oxide synthase (NOS; EC 1.14.13.39). Previous reports demonstrated the involvement of neuronal NOS (nNOS) and inducible NOS (iNOS) for neurodegeneration of the dopaminergic neurons by MPTP. The inhibition of nNOS by 7-nitroindazole, knock-out of nNOS and iNOS ameliorated the MPTP toxicity (Schulz et al., 1995; Przedborski et al., 1996; Liberatore et al., 1999), suggesting the exacerbation of MPTP-induced neurodegeneration by NOS and NO. The generation of

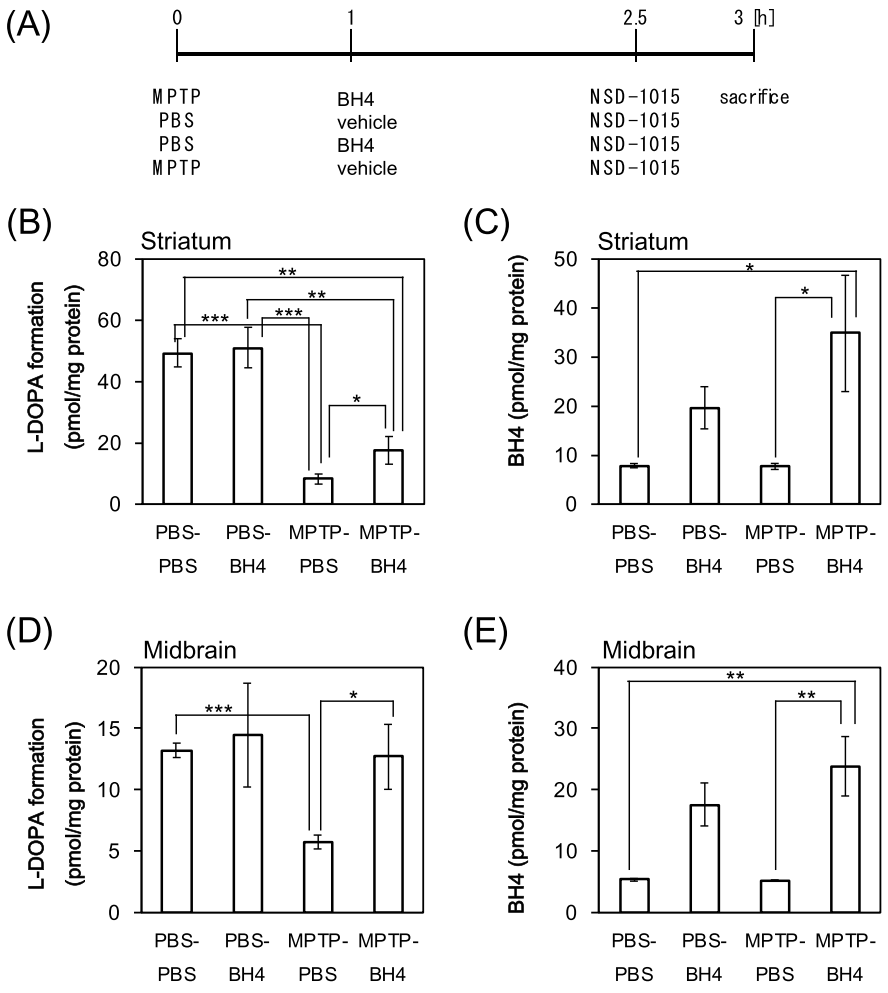

Fig. 4. Effect of BH4 to MPTP-induced inhibition of the *in vivo* TH activity. Mice were treated with 20 mg/kg MPTP followed by a 50 mg/kg BH4 injection 1 h after MPTP treatment, and NSD-1015 was injected 30 min before brain slices were collected 3 h after MPTP administration (A). The control groups received PBS instead of MPTP and BH4. L-DOPA formation in the striatum (B) and dorsal midbrain (D) was measured by HPLC with electrochemical detection. BH4 levels in the striatum (C) and dorsal midbrain (E) were evaluated by HPLC with fluorimetric detection. Each value represents the mean ± S.E.M. $n = 4$ mice per group. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; two-way ANOVA followed by Tukey's HSD test.)

ROS by NOS uncoupling reaction when BH4 was favor to be oxidized to BH2 (Landmesser et al., 2003; Vásquez-Vivar et al., 2002), and the increased expression of NOS and production of NO in the striatum by MPTP (Tsai et al., 2011) would accelerate the oxidative stresses and BH4 depletion falling in a vicious cycle of MPTP toxicity.

MPTP has been reported to induce the release and oxidation of DA (Chang and Ramirez, 1986; Obata et al., 2001; Lotharius and O'Malley, 2000). MPTP acutely depletes DA by releasing DA from its vesicles. In this study, BH4 administration did not alter intracellular or extracellular DA metabolites (DOPAC, HVA) after 24 h of MPTP treatment (Table 1). Judging from this, it is more likely that the administered BH4 was used for DA synthesis rather than changing the storage and turnover of DA. Furthermore, BH4 injection restored the *in vivo* TH activity as well as the striatal DA content from restriction by MPTP. Therefore, we suppose that the recovery of striatal DA by BH4 is attributable not to a decline in DA release but to an increase in *in vivo* TH activity.

Nigrostriatal DA neurons may be subjected first to an intracellular and then to an extracellular oxidative insult due to activation of inflammatory processes (Wu et al., 2003). Judging from that description, the effect of BH4 administration, as we revealed in Fig. 5, fulfil the role of relieving impairment of the DA synthesis but not cellular toxicity since TH protein level was not affected. There would be distinct mechanisms mediating the regulation of DA synthesis in the early stage and cell death in the late stage after MPTP treatment. Because there are reports for protective effects of anti-oxidants against MPTP toxicity (Park et al., 2004; Perry et al., 1985), preventing oxidation of BH4 might be involved in the cellular toxicity but not the decline in the amount of BH4. It will be necessary to elucidate the association between intracellular and extracellular oxidative stress in MPTP models and the etiology of PD.

The striatal BH4 content did not show a salient change in response to MPTP despite a significant decrease in the *in vivo* TH activity (Figs. 3C and 4C), whereas we assume that oxidative stress induced by MPTP reduces the BH4 amounts available for the TH reaction in the dopaminergic neurons. The reason would be attributed to the intercellular transportation of BH4 and BH2. BH2 was reported to be excreted easily across the plasma membrane through transporters, ENT2 and OAT1 (Ohashi et al. 2011, 2017). Therefore, it is probable that BH2 generated by the action of MPTP is excreted from the dopaminergic neurons, taken up by surrounding neurons and glia, and is converted to BH4 via DHFR in non-dopaminergic neurons and glia, resulting in the stable BH4 levels in the striatum. In support of this idea, the BH2 content in the striatum showed an increasing trend after MPTP administration (Fig. 3D). Furthermore, the BH4 content in the striatum was greatly elevated after both MPTP and BH4 treatments as shown in Fig. 5B. Although the BH4 content was unchanged in the PBS-BH4-treated mice, the MPTP-BH4-treated mice showed 5-fold larger amounts of BH4 (Fig. 5B). The great increases in the BH4 content in the MPTP-BH4 group would suggest that MPTP certainly affects BH4 metabolism and that BH4 might be regenerated from BH2 in the proximal non-DA cells by the action of DHFR. Indeed, it was reported that MPP^+ decreased the BH4 content in primary-culture cells derived from cerebellar granule neurons (Shang et al., 2004). This report supports our hypothesis that oxidized BH4 by MPP^+ should be easily excreted out of the cells.

NO can readily react with ROS and form strong toxic oxidant,

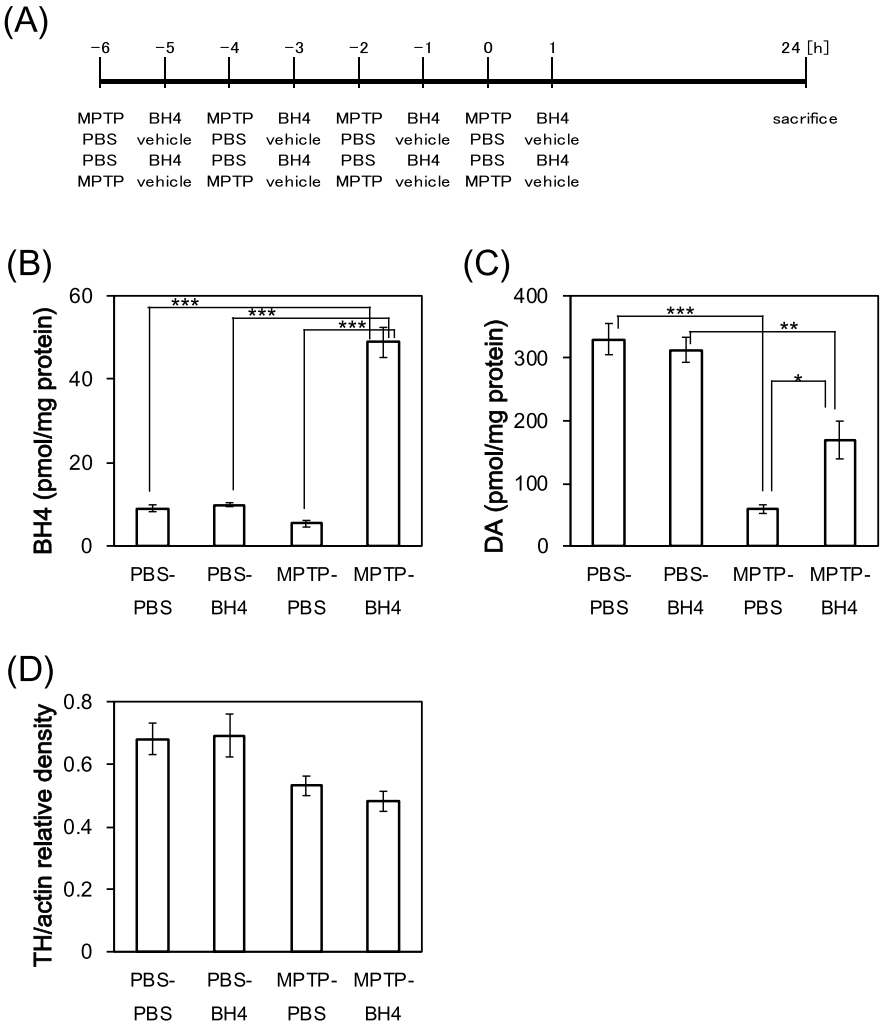

Fig. 5. Effect of BH4 on DA depletion by 4 injections of MPTP. Mice were given 4 injections of 20 mg/kg MPTP followed by 20 mg/kg BH4 injection 1 h after each MPTP administration (0 h MPTP, 1 h BH4, 2 h MPTP, 3 h BH4, and so on). The control groups received PBS instead of MPTP and BH4 (A). BH4 levels (B) were evaluated by HPLC with fluorimetric detection. Striatal DA content (C) was measured by HPLC with electrochemical detection. TH signal was quantified by western blotting (D). Each value represents the mean ± S.E.M. $n = 4$ mice per group. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; two-way ANOVA followed by Tukey's HSD test.)

peroxynitrite. It was reported that the peroxynitrite caused an inactivation of TH activity through the reversible oxidative modification of cysteine (Kuhn et al., 1999), and nitration of tyrosine residues which was improved by BH4 (Kuhn and Geddes, 2003). In addition to the oxidation and subsequent decrease of available BH4 for TH, these oxidative and nitrosative stresses might affect to DA deficit through TH inactivation.

Penetrance of BH4 into the brain is controversial. Hoshiga et al. (1993) reported that peripherally administered BH4 hardly entered the brain by examining the autoradiographic distribution of [^{14}C]-labeled BH4 administered at a low dose, 45 μg/kg. However, others reported that substantial amounts of BH4 passed through the blood-brain barrier (Kapatos and Kaufman, 1981; Miller et al., 1986). We also reported that peripherally administered BH4, intraperitoneally 50 mg/kg, was effective to increase the amounts of BH4 and dopamine in the brains of BH4-deficient mice (Homma et al., 2013). Our present data showed that the BH4 levels in the brain was increased by the intraperitoneal injections of 50 mg/kg BH4, and that it was effective to increase the DA levels in the brain after MPTP treatment (Figs. 4 and 5). Therefore, we think that the BH4 contents in the brain may be saturated under a normal condition and that it can be increased by large amounts of intraperitoneal administration of BH4 under pathological conditions.

There is evidence that MPTP changes the permeability of the blood brain barrier (Thiollier et al., 2016). We dissolved BH4 into PBS containing 1 mM ascorbic acid to prevent BH4 from oxidation, while we administered the vehicle containing ascorbic acid to the control mice.

Table 1
DA and its metabolites after injection of MPTP and BH4. Mice were treated with indicated reagents as shown in Fig. 4. Striatal HVA and DOPAC contents were measured by HPLC. Each value represents the mean ± S.E.M. $n = 4$ mice per group.

	PBS-PBS	PBS-BH4	MPTP-PBS	MPTP-BH4
DA (pmol/mg protein)	330.8 ± 25.0	313.7 ± 20.0	58.8 ± 6.9	169.4 ± 30.2
HVA (pmol/mg protein)	41.3 ± 2.7	39.0 ± 2.2	16.1 ± 2.3	20.0 ± 1.5
DOPAC (pmol/mg protein)	96.2 ± 11.8	79.5 ± 6.9	11.1 ± 3.3	12.4 ± 3.8

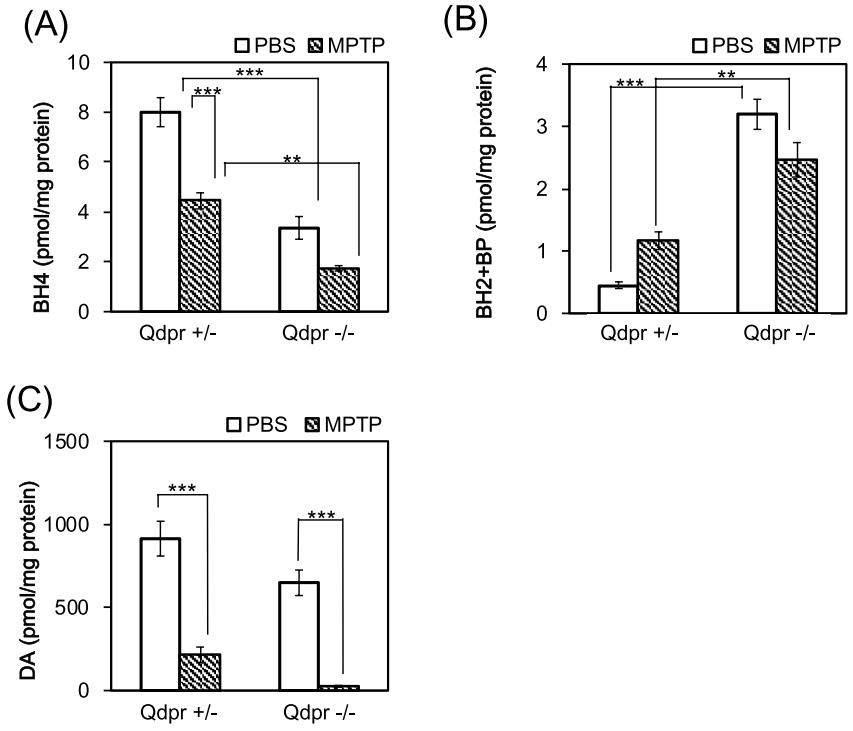

Fig. 6. Difference between *Qdpr*-KO mice and control mice in sensitivity to MPTP. Twenty-four hours after *Qdpr*-KO (*Qdpr*−/−) mice and control (*Qdpr*+/-) mice received 4 injections of 20 mg/kg MPTP, the striatum and dorsal midbrain were dissected and analysed. BH4 levels (A) and BH2+BP levels in the striatum (B) were evaluated by HPLC with fluorimetric detection. DA content in the striatum (C) was measured by HPLC with electrochemical detection. Each value represents the mean ± S.E.M. $n = 4$ mice per group. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; two-way ANOVA followed by Tukey's HSD test.)

Possible alteration in the permeability of the blood-brain barrier or a synergistic action of BH4 and ascorbic acid may contribute to the beneficial action of BH4 in the present experiments.

Our data suggest possible therapeutic efficacy of BH4 for the treatment of PD patients. However, BH4 had been administered in PD patients with only minimal effect: 10–30 mg i.v. in mild to moderate PD with an DA-potentiating effect of about 50%, while there was no anti-akinetic efficacy at all in severe PD (Birkmayer and Riederer, 1986; Nagatsu et al., 1982; LeWitt et al., 1982; Curtius et al., 1984). We suppose that administration of BH4 would be effective only in the early or prodromal stages of PD when the dopaminergic neurons are remaining, before the dopaminergic neurons have been lost. Further studies would be required to clarify this point.

In conclusion, we showed that the inhibition of *in vivo* TH activity and the DA depletion caused by the early action of MPTP was recovered by administration of BH4. Our data strongly suggest that MPTP causes oxidation of BH4 possibly through increased ROS in the cells, and that the perturbation of the BH4 metabolism should be involved in the toxic action of MPTP.

Declarations of interest

None.

Author contributions

HI conceived the project. HK, KY, and KM conducted experiments and analysed data. KY, SM, SH, and HI wrote and edited the manuscript.

Acknowledgments

We thank Naomi Takino and Mika Ito for their technical assistance. This work was supported by Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Adaptive circuit shift, 15H01422 and 17H05555), and by a research grant from the Ministry of Health, Labour and Welfare of Japan, and by the Uehara Memorial Foundation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.02.005.

References

Bernheimer, H., Birkmayer, W., Hornykiewicz, O., Jellinger, K., Seitelberger, F., 1973. Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. J. Neurol. Sci. 20, 415–455.

Birkmayer, W., Riederer, P., 1986. second ed. Die Parkinson-Krankheit 1986. 1980, Springer Publishers Vienna, New York, pp. 166–167 pp 148, respective the.

Bonafe, L., Thony, B., Penzien, J.M., Czarnecki, B., Blau, N., 2001. Mutations in the sepiapterin reductase gene cause a novel tetrahydrobiopterin-dependent monoamine-neurotransmitter deficiency without hyperphenylalaninemia. Am. J. Hum. Genet. 69, 269–277.

Carlsson, A., Lindqvist, M., 1973. In-vivo measurements of tryptophan and tyrosine hydroxylase activities in mouse brain. J. Neural. Transm. 34, 79–91.

Chang, G.D., Ramirez, V.D., 1986. The mechanism of action of MPTP and MPP+ on endogenous dopamine release from the rat corpus striatum superfused in vitro. Brain Res. 368, 134–140.

Curtius, H.C., Niederwieser, A., Levine, R., Muldner, H., 1984. Therapeutic efficacy of tetrahydrobiopterin in Parkinson's disease. Adv. Neurol. 40, 463–466.

Drechsel, D.A., Patel, M., 2008. Role of reactive oxygen species in the neurotoxicity of environmental agents implicated in Parkinson's disease. Free Radic. Biol. Med. 44, 1873–1886.

Heikkila, R.E., Hess, A., Duvoisin, R.C., 1984. Dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine in mice. Science 224, 1451–1453.

Hirata, Y., Nagatsu, T., 1985. Inhibition of tyrosine hydroxylation in tissue slices of the rat striatum by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Brain Res. 337, 193–196.

Hirata, Y., Nagatsu, T., 1986. Early and late effects of systemically administered 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on tyrosine hydroxylase activity in vitro and on tyrosine hydroxylation in tissue slices of mouse striatum. Neurosci. Lett. 68, 245–248.

Homma, D., Kato, S., Tokuoka, H., Ichinose, H., 2013. The role of tetrahydrobiopterin and catecholamines in the developmental regulation of the tyrosine hydroxylase level in the brain. J. Neurochem. 126, 70–81.

Hoshiga, M., Hatakeyama, K., Watanabe, M., Shimada, M., Kagamiyama, H., 1993. Autoradiographic distribution of [^{14}C]tetrahydrobiopterin and its developmental change in mice. J. Pharmacol. Exp. Therapeut. 267, 971–978.

Ichinose, H., Ohye, T., Takahashi, E., et al., 1994. Hereditary progressive dystonia with

marked diurnal fluctuation caused by mutations in the GTP cyclohydrolase I gene. Nat. Genet. 8, 236–242.
Kapatos, G., Kaufman, S., 1981. Peripherally administered reduced pterins do enter the brain. Science 212, 955–956.
Kuhn, D.M., Aretha, C.W., Geddes, T.J., 1999. Peroxynitrite inactivation of tyrosine hydroxylase: mediation by sulfhydryl oxidation, not tyrosine nitration. J. Neurosci. 19, 10289–10294.
Kuhn, D.M., Geddes, T.J., 2003. Tetrahydrobiopterin prevents nitration of tyrosine hydroxylase by peroxynitrite and nitrogen dioxide. Mol. Pharmacol. 64, 946–953.
Kurosaki, R., Muramatsu, Y., Kato, H., Araki, T., 2004. Biochemical, behavioral and immunohistochemical alterations in MPTP-treated mouse model of Parkinson's disease. Pharmacol., Biochem. Behav. 78, 143–153.
Landmesser, U., Dikalov, S., Price, S.R., McCann, L., Fukai, T., Holland, S.M., Mitch, W.E., Harrison, D.G., 2003. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. J. Clin. Invest. 111, 1201–1209.
Langston, J.W., Ballard, P., Tetrud, J.W., Irwin, I., 1983. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science 219, 979–980.
LeWitt, P.A., et al., 1982. In: Abstract of the 7th International Symposium on Parkinson's Disease, pp. 83.
Liberatore, G.T., Jackson-Lewis, V., Vukosavic, S., Mandir, A.S., Vila, M., McAuliffe, W.G., Dawson, V.L., Dawson, T.M., Przedborski, S., 1999. Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease. Nat. Med. 5, 1403–1409.
Lotharius, J., O'Malley, K.L., 2000. The parkinsonism-inducing drug 1-methyl-4-phenylpyridinium triggers intracellular dopamine oxidation. A novel mechanism of toxicity. J. Biol. Chem. 275, 38581–38588.
Matsuura, S., Sugimoto, T., Murata, S., Sugawara, Y., Iwasaki, H., 1985. Stereochemistry of biopterin cofactor and facile methods for the determination of the stereochemistry of a biologically active 5,6,7,8-tetrahydropterin. J. Biochem. 98, 1341–1348.
Mencacci, N.E., Isaias, I.U., Reich, M.M., et al., 2014. Parkinson's disease in GTP cyclohydrolase 1 mutation carriers. Brain : J. Neurol. 137, 2480–2492.
Miller, L., Insel, T., Scheinin, M., Aloi, J., Murphy, D.L., Linnoila, M., Lovenberg, W., 1986. Tetrahydrobiopterin administration to Rhesus Macaques. Its Appearance in CSF and effect on neurotransmitter synthesis. Neurochem. Res. 11, 291–298.
Nagatsu, T., et al., 1982. In: Abstract of the 7th International Symposium on PD, pp. 82.
Nalls, M.A., Pankratz, N., Lill, C.M., et al., 2014. Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease. Nat. Genet. 46, 989–993.
Nichol, C.A., Smith, G.K., Duch, D.S., 1985. Biosynthesis and metabolism of tetrahydrobiopterin and molybdopterin. Annu. Rev. Biochem. 54, 729–764.
Obata, T., Yamanaka, Y., Kinemuchi, H., Oreland, L., 2001. Release of dopamine by perfusion with 1-methyl-4-phenylpyridinium ion (MPP(+)) into the striatum is associated with hydroxyl free radical generation. Brain Res. 906, 170–175.
Ogawa, S., Ichinose, H., 2006. Effect of metals and phenylalanine on the activity of human tryptophan hydroxylase-2: comparison with that on tyrosine hydroxylase activity. Neurosci. Lett. 401, 261–265.
Ohashi, A., Mamada, K., Harada, T., Naito, M., Takahashi, T., Aizawa, S., Hasegawa, H., 2017. Organic anion transporters, OAT1 and OAT3, are crucial biopterin transporters involved in bodily distribution of tetrahydrobiopterin and exclusion of its excess. Mol. Cell. Biochem. 435, 97–108.
Ohashi, A., Sugawara, Y., Mamada, K., Harada, Y., Sumi, T., Anzai, N., Aizawa, S., Hasegawa, H., 2011. Membrane transport of sepiapterin and dihydrobiopterin by equilibrative nucleoside transporters: a plausible gateway for the salvage pathway of tetrahydrobiopterin biosynthesis. Mol. Genet. Metabol. 102, 18–28.
Park, S.W., Kim, S.H., Park, K.H., Kim, S.D., Kim, J.Y., Baek, S.Y., Chung, B.S., Kang, C.D., 2004. Preventive effect of antioxidants in MPTP-induced mouse model of Parkinson's disease. Neurosci. Lett. 363, 243–246.
Perry, T.L., Yong, V.W., Clavier, R.M., Jones, K., Wright, J.M., Foulks, J.G., Wall, R.A., 1985. Partial protection from the dopaminergic neurotoxin N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine by four different antioxidants in the mouse. Neurosci. Lett. 60, 109–114.
Pileblad, E., Fornstedt, B., Clark, D., Carlsson, A., 1985. Acute effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine on dopamine metabolism in mouse and rat striatum. J. Pharm. Pharmacol. 37, 707–711.
Przedborski, S., Jackson-Lewis, V., Yokoyama, R., Shibata, T., Dawson, V.L., Dawson, T.M., 1996. Role of neuronal nitric oxide in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurotoxicity. Proc. Natl. Acad. Sci. U. S. A. 93, 4565–4571.
Sato, K., Sumi-Ichinose, C., Kaji, R., Ikemoto, K., Nomura, T., Nagatsu, I., Ichinose, H., Ito, M., Sako, W., Nagahiro, S., Graybiel, A.M., Goto, S., 2008. Differential involvement of striosome and matrix dopamine systems in a transgenic model of dopa-responsive dystonia. Proc. Natl. Acad. Sci. U.S.A. 105, 12551–12556.
Schulz, J.B., Matthews, R.T., Muqit, M.M., Browne, S.E., Beal, M.F., 1995. Inhibition of neuronal nitric oxide synthase by 7-nitroindazole protects against MPTP-induced neurotoxicity in mice. J. Neurochem. 64, 936–939.
Shang, T., Kotamraju, S., Kalivendi, S.V., Hillard, C.J., Kalyanaraman, B., 2004. 1-Methyl-4-phenylpyridinium-induced apoptosis in cerebellar granule neurons is mediated by transferrin receptor iron-dependent depletion of tetrahydrobiopterin and neuronal nitric-oxide synthase-derived superoxide. J. Biol. Chem. 279, 19099–19112.
Smeyne, M., Smeyne, R., 2013. Glutathione metabolism and Parkinson's disease. Free Radical Biol. Med. 62, 13–25.
Sriram, K., Pai, K.S., Boyd, M.R., Ravindranath, V., 1997. Evidence for generation of oxidative stress in brain by MPTP: in vitro and in vivo studies in mice. Brain Res. 749, 44–52.
Sumi-Ichinose, C., Urano, F., Kuroda, R., Ohye, T., Kojima, M., Tazawa, M., Shiraishi, H., Hagino, Y., Nagatsu, T., Nomura, T., Ichinose, H., 2001. Catecholamines and serotonin are differently regulated by tetrahydrobiopterin. A study from 6-pyruvoyltetrahydropterin synthase knockout mice. J. Biol. Chem. 276, 41150–44160.
Takazawa, C., Fujimoto, K., Homma, D., Sumi-Ichinose, C., Nomura, T., Ichinose, H., Katoh, S., 2008. A brain-specific decrease of the tyrosine hydroxylase protein in sepiapterin reductase-null mice–as a mouse model for Parkinson's disease. Biochem. Biophys. Res. Commun. 367, 787–792.
Tani, Y., Ohno, T., 1993. Analysis of 6R- and 6S-tetrahydrobiopterin and other pterins by reversed-phase ion-pair liquid-chromatography with fluorimetric detection by post-column sodium nitrite oxidation. J. Chromatogr. 617, 249–255.
Thiollier, T., Wu, C., Contamin, H., Li, Q., Zhang, J., Bezard, E., 2016. Permeability of blood-brain barrier in macaque model of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson disease. Synapse 70, 231–239.
Thöny, B., Auerbach, G., Blau, N., 2000. Tetrahydrobiopterin biosynthesis, regeneration and functions. Biochem. J. 347 (Pt 1), 1–16.
Tsai, S.J., Chao, C.Y., Yin, M.C., 2011. Preventive and therapeutic effects of caffeic acid against inflammatory injury in striatum of MPTP-treated mice. Eur. J. Pharmacol. 670, 441–447.
Vásquez-Vivar, J., Martasek, P., Whitsett, J., Joseph, J., Kalyanaraman, B., 2002. The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study. Biochem. J. 362, 733–739.
Werner, E.R., Blau, N., Thony, B., 2011. Tetrahydrobiopterin: biochemistry and pathophysiology. Biochem. J. 438, 397–414.
Wu, D.C., Teismann, P., Tieu, K., Vila, M., Jackson-Lewis, V., Ischiropoulos, H., Przedborski, S., 2003. NADPH oxidase mediates oxidative stress in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. Proc. Natl. Acad. Sci. U. S. A. 100, 6145–6150.