Catecholamines and Serotonin Are Differently Regulated by Tetrahydrobiopterin

A STUDY FROM 6-PYRUVOYLTETRAHYDROPTERIN SYNTHASE KNOCKOUT MICE*

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(6R)-L-erythro-5,6,7,8-Tetrahydrobiopterin (BH4) is an essential cofactor for tyrosine hydroxylase (TH), tryptophan hydroxylase, phenylalanine hydroxylase, and nitric-oxide synthase. These enzymes synthesize neurotransmitters, e.g. catecholamines, serotonin, and nitric oxide (NO). We established mice unable to synthesize BH4 by disruption of the 6-pyruvoyltetrahydropterin synthase gene, the encoded protein of which catalyzes the second step of BH4 biosynthesis. Homozygous mice were born at the almost expected Mendelian ratio, but died within 48 h after birth. In the brain of homozygous mutant neonates, levels of biopterin, catecholamines, and serotonin were extremely low. The number of TH molecules was highly dependent on the intracellular concentration of BH4 at nerve terminals. Alteration of the TH protein level by modulation of the BH4 content is a novel regulatory mechanism. Our data showing that catecholaminergic, serotonergic, and NO systems were differently affected by BH4 starvation suggest the possible involvement of BH4 synthesis in the etiology of monoamine-based neurological and neuropsychiatric disorders.

Catecholamines, e.g. dopamine (DA), norepinephrine (NE), and epinephrine, and serotonin (5-hydroxytryptamine; 5HT) are well known to be involved in many aspects of brain function, including mood, addiction, reward, and sleep. Alteration in the metabolism of these monoamines leads to abnormalities in behavior and neuropsychiatric states. For example, increased dopaminergic neurotransmission in the mesolimbic system induces hallucinations, illusions, and delusions, which are the major symptoms of schizophrenia (1). Depletion of monoamines may be the cause of depression, because drugs that can increase the level of monoamines and blockers of norepinephrine or serotonin uptake show therapeutic effects in patients with depression (2). Abnormal monoamine metabolism is also suspected to be involved in Tourette’s syndrome, obsessive-compulsive disorder, Rett syndrome, and infantile autism (3, 4).

Bioynthesis of catecholamines and serotonin is mainly regulated by the activity of the hydroxylation reaction catalyzed by tyrosine hydroxylase (TH) in catecholamine synthesis (5) and by tryptophan hydroxylase (TPH) in 5HT synthesis (6). The TH activity is tightly regulated by several factors (for review, see Ref. 7 and references therein). Short term regulation is achieved by phosphorylation of the enzyme and by feedback inhibition with the end products. Long term regulation is mainly governed at the transcriptional level.

(6R)-L-erythro-5,6,7,8-Tetrahydrobiopterin (BH4) is an essential cofactor for both TH and TPH. Although many cofactors such as folic acid and cobalamin (vitamin B12) are required to be dietary supplemented, mammals have a de novo biosynthetic pathway for BH4. In this pathway, guanosine triphosphate (GTP) is converted to 7,8-dihydrobiopterin triphosphate by GTP cyclohydrolase 1 (GCH), and 7,8-dihydrobiopterin triphosphate is then converted to 6-pyruvoyltetrahydropterin by pyruvoyltetrahydropterin synthase (PTPS). This tetrahydropterin is subsequently converted to BH4 by sepiapterin reductase (Fig. 1A). The intracellular concentration of BH4 is mainly regulated by the activity of GCH. Factors that can stimulate GCH activity, for example, phytohemagglutinin (8, 9), interferon-γ (10), and tumor necrosis factor-α (11), can increase the level of BH4.

BH4 also acts as the cofactor for phenylalanine hydroxylase (12) and nitric-oxide synthase (NOS) (13, 14). Phenylalanine hydroxylase in the liver is essential for metabolism of the essential amino acid phenylalanine, and converts it to tyrosine. Nitric oxide (NO) has a variety of physiological roles. NO synthesized by neural NOS (nNOS) in neuron functions as a neurotransmitter, whereas that generated by NOS in endothelial cells serves as a vasodilator. Additionally, NO generated by inducible NOS (iNOS) in macrophages acts as a chemical mediator, and iNOS activity in these cells is strongly stimulated by lipopolysaccharide and cytokines.

There are two types of inherited disorders that are caused by a genetic defect in BH4-synthesizing enzymes. One is atypical hyperphenylalaninemia (malignant type of phenylketonuria),
which is inherited as an autosomal recessive trait. Mutations in the BH4-synthesizing or BH4-recycling enzymes such as GCH, PTPS, or dihydropteridine reductase, cause this disease. In the patients, neurological symptoms, e.g. convulsion, drowsiness, and disturbance of muscle tone, are caused by decreased levels of neurotransmitters; e.g. catecholamines, 5HT, and NO, in addition to the excess amount of phenylalanine. The other is dopa-responsive dystonia (DRD; also called Segawa’s disease). We found in 1994 that autosomal dominant mutations in the GCH gene cause this disease (15). This disease is characterized by childhood-onset dystonia with remarkable responsiveness to low doses of L-3,4-dihydroxyphenylalanine (L-DOPA) (16, 17). The DRD patients have both mutated and wild-type alleles of the GCH gene, and the resultant partial depletion of BH4 causes a shortage in DA in the nigrostriatal dopaminergic neurons in the brain of DRD patients (15). In these patients, there are no symptoms other than dystonia, and dysfunction of serotonergic neurons is not obvious, even though BH4 is a cofactor for both TH and TPH.

In this present study, for the first time, we established BH4-deficient mice by disrupting the PtpS gene to investigate the effects of BH4 depletion on the animals and the involvement of BH4 in regulating neural systems. Our results indicated that BH4 distinctively regulates the synthesis of catecholamines altering the amount of TH protein and that of 5HT, and suggested that BH4 metabolism may be deeply involved in the pathophysiology of Parkinson’s disease, stress responses, and neuropsychiatric disorders.

**EXPERIMENTAL PROCEDURES**

**Generation of a Targeted Mutation in 6-Pyruvoyltetrahydropterin Synthase Knockout Mice**—A 17.2-kb mouse PtpS genomic fragment was isolated from a 129/SVJ genomic library by using mouse PtpS cDNA as a probe. The DNA fragment was revealed to contain all exons of the mouse PtpS gene and 4.6-kb 5-flanking and 4.6-kb 3-flanking sequences. To construct the targeting vector containing a mouse PtpS 9.5-kb genomic fragment, we replaced 0.6-kb KpnI-BamHI fragment containing exon 1, in which the initiation codon is located, with a neo mutant resistance gene under the promoter of phosphoglycerate kinase (Pgk). Mc1-dipthitheria toxin A fragment and pBlueScript (+) sequence were ligated to the 5′ end of the vector. The vector was linearized and electroporated into 129/SVJ embryonic stem (ES) cells. ES cells were cultured in medium containing G418 for selection. Clones containing the targeted homologous recombinant ES cells were isolated by Southern hybridization (data not shown), amplified, and injected into C57BL/6 blastocysts, which were then transplanted into pseudopregnant female mice. Germline transmission occurred from one chimera, and we established C57BL/6 background line by repetitive crossing to compare their phenotype with that of 129/SVJ-C57BL/6 hybrid mice. Animals were handled according to the Guidelines for the Care and Use of Laboratory Animals in Fujita Health University. Animals were handled according to the Guidelines for the Care and Use of Laboratory Animals in Fujita Health University.

**Identification of Genotype**—DNA was extracted from the cells or mice tail, digested with SacI, and analyzed by Southern hybridization using a 0.45-kb EcoRI-EcoRI fragment, as a 3′ outside probe, as indicated in Fig. 1B.

**Northern Hybridization**—Total RNA was extracted from tissues with Trizol reagent (Life Technologies, Inc.). Eight μg (for PtpS) or 20 μg (for Th) of total RNA was electroblotted and blotted onto a nylon membrane. Specific RNA was detected by using mouse PtpS cDNA or mouse Th cDNA (18) as a probe. The same membranes were dehydrized and reprobed with mouse glyceraldehyde 3-phosphate dehydrogenase (G3pdh) cDNA as a control.

**Biochemical Analysis**—Mouse brains were homogenized in 10 volumes of buffer containing 25 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 10% glycerol. Protease inhibitors, i.e., 1 μg/ml pancreatic A, 2 μg/ml leupeptin, and 0.5 μg phenylmethylsulfonyl fluoride, were added before the TH assay. Protein concentration was determined by the method of Bradford (19).

For the assay of biopterin and neopterin, protein was removed by adding perchloric acid, and pteridines were then oxidized with 1% H2O2 and 2% KI in 1 N HCl for 60 min in the dark at room temperature, and analyzed by high performance liquid chromatography (HPLC) with fluorescence detection (20). Catecholamine and 5HT levels were analyzed by HPLC chemical and HPLC fluorescence detection, respectively.

**TH activity** was determined based on the formation of L-DOPA from L-tyrosine by HPLC electrochemical detection. The reaction mixture (2 ml) contained 0.01 mM sodium phosphate buffer (pH 6.0), 0.1 mM L-DOPA, 30 mCi/ml [3H]tyrosine, 0.2 mM ascorbic acid, 0.4 mM ferrous ammonium sulfate, and 0.4 mM L-tryptophan. 6-Methyltetrahydropterin (500 μM) was added to start the reaction, which was carried out for 10 min at 37 °C. The reaction was stopped by the addition of perchloric acid, and L-DOPA was extracted by an alumina column (21). TPH activity was determined by an HPLC fluorometric system monitoring the enzymatically formed 5-hydroxytryptaphan. The reaction mixture contained 0.03 mM sodium phosphate buffer (pH 7.2), 0.1 mM pargyline (a monoamine oxidase inhibitor), 0.1 mM ascorbic acid (pH 6.0), 100 μM L-DOPA, and 0.4 mM pyridoxal phosphate. The reaction was incubated for 20 min at 37 °C (23).

For measurement of NOS, the homogenate was centrifuged at 13,000 × g for 5 min, and the supernatant was used as an enzyme source. The reaction mixture contained 25 mM Tris-HCl (pH 7.4), 3 μM BH4, 1 μM flavin adenine dinucleotide, 1 μM flavin adenine mononucleotide, 1 mM NADPH, 1 mM L-arginine, and 0.6 mM CaCl2, and was incubated at 25 °C for 60 min. The reaction was stopped by the addition of EDTA, and enzymatically synthesized 14C-labeled citrulline was quantified by a scintillation counter (24) (NOS Detect assay kit, Stratagene).

For quantification of amino acids, the homogenate was deproteinized with sulfosalicylate, and analyzed by an L-8500 amino acid analyzer (Hitachi).

**Immunohistochemistry**—Immunohistochemistry was performed by the avidin-biotin peroxidase complex method (25). Ether-anesthetized newborn mice were fixed with 4% paraformaldehyde. The brains were dissected and sliced at 40 μm, and their adrenal glands and kidneys, at 25 μm, with a cryostat after having been rinsed with sucrose. Sections were pretreated with 0.5% hydrogen peroxide (H2O2), blocked with 5% normal swine serum or 5% normal goat serum, and incubated with rabbit anti-TH serum (26), rabbit anti-AADC serum (26), rabbit anti-tyrosine hydroxylase (27) and mouse anti-TNCH monocular antibody (Ab-1, Oncogene, Cambridge) diluted to 1:100,000, 1:2000, 1:1000, and 1 ng/ml respectively, followed by biotinylated goat anti-rabbit IgG or biotinylated anti-mouse IgG and avidin-biotin peroxidase complex (Vectorstain ABC kit, Funakoshi). Then the sections were visualized by reaction with 3,3′-diaminobenzidine tetrahydrochloride and 0.003% H2O2. At least two mice were analyzed.

**Western Blotting**—Antibodies were made against purified bovine TH (28), purified bovine AADC (23), and synthetic peptide for rat GCH (27). Monoclonal antibody against TPH was purchased from Oncogene. Brain homogenates containing 30 μg of protein were separated by 10% SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose membranes, and reacted with the respective first antibodies, and then with goat anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase (1:3000) for 1 h. Immune reactive bands were detected by use of an ECL Plus system (Amersham Pharmacia Biotech).

**Electrocardiogram of Mice**—Electrocardiograms of newborn mice were recorded on the day of delivery. Electrodes were attached to the right fore and left hind limbs, and surface electrocardiogram was monitored with an eight-channel oscilloscope using a high gain preamplifier (Nihon Koden, Tokyo, Japan). n = 15 (+/+), 37 (−/+), and 15 (−/−).

**Administration of BH4**—Ascorbic acid was dissolved to 0.25% in 0.9% NaCl 6R-BH4, which had generously been donated by Sunytone Inc. (Osaka, Japan), was dissolved in 0.25% ascorbic acid. The solution was administered to mice intraperitoneally. For examination of the acute effect of BH4, mice were sacrificed 1 h after the injection, and for that of the chronic effect, animals were injected with the drug once a day for 7 days. Then, on the following day, they were sacrificed for biochemical and Western blot analyses. For immunohistochemical analysis, mice were injected with the drug once a day for 7 days, and 3 h after the last injection their brains were fixed with 4% paraformaldehyde.
RESULTS

Ptps Knockout Mice Were Born but Died within 48 h of Birth—The Ptps gene was inactivated by homologous recombination in ES cells by use of a targeting vector, in which a 0.6-kb KpnI-BamHI Ptps genomic fragment containing exon 1 and a part of the 5′ promoter region of the Ptps gene was replaced with a neomycin resistance gene (shaded box). MC1-diphtheria toxin A fragment (hatched box) and pBlueScript were ligated to the 5′ end. The mutant allele is depicted on the bottom. Location of 3′ outside probe is indicated above the wild-type allele, and DNA fragments detected by Southern hybridization in wild-type allele and mutant allele are indicated above each allele. B, BamHI; K, KpnI; S, SacI.

C, Southern hybridization analysis of genotypes. Genomic DNA extracted from mouse tail was digested with SacI and hybridized with a 3′ probe. The 18-kb band corresponds to the wild-type allele, and the 9-kb one corresponds to the mutant allele. Genotypes of mice are indicated on the top of the panels (homozygotes, −/−; heterozygotes, +/−; wild type, +/+). D, Northern hybridization analysis of Ptps mRNA expression in the liver and the brain. Tissues and genotypes are indicated on the top of the panel. Total RNA was extracted from the liver and the brain of each mouse, electrophoresed, and blotted. The membrane was hybridized with mouse Ptps cDNA probe (PTPS; upper panel) and then re-hybridized with mouse G3pdh probe (G3PDH; lower panel).

identified three homologously recombinant clones out of 157 129/SVJ ES cells by Southern hybridization. We injected those into C57BL/6 blastocysts and obtained five chimeric mice. The genotype of the mice was also determined by Southern hybridization (Fig. 1C). Germline transmission occurred in one chimera; thus, we established C57BL/6 background mice by repetitive crossing and compared their phenotype with that of 129/
SVJ-C57BL/6 hybrid mice. Homozygous mice of 129/SVJ-C57BL/6 hybrids were born roughly according to the Mendelian ratio (wild type: 26.7%, heterozygote: 51.2%, homozygote: 22.2%; n = 424) without gross anomaly in major organs (data not shown). This means that mutant mice could survive in utero during embryogenesis; however, all homozygotes died within 48 h after birth. On the other hand, genotypes of newborn mice of C57BL/6 background in the fourth to fifth generation were identified as follows: wild type, 26.6%; heterozygote, 53.2%; homozygote, 20.3% (n = 79), and all homozygotes also died within 48 h. Thus all experiments were done with 129/SVJ-C57BL/6 hybrids.

**Bioterin, Catecholamines, and Serotonin Were Severely Reduced in the Brain of Newborn Ptps Knockout Mice**—Northern hybridization using total RNA extracted from the brain and the liver of newborn mice showed that homozygotes were null mutants, for Ptps mRNA was not detected (Fig. 1D). Biochemical analysis revealed a dramatic decrease in the level of bioterin (6.3% of wild type), the oxidized form of BH4, and the accumulation of neopterin (17.6 times higher than wild type), a metabolite of 7,8-dihydroypterin triphosphate (Fig. 2A). Since BH4 is an essential cofactor for TH and TPH, we also quantified catecholamines and 5HT in the brain of newborn mice. As we expected, the levels of DA, NE, and 5HT were all decreased in homozygotes (14.6, 6.3, and 7.8% of wild type, respectively). There was no significant difference between heterozygotes and wild-type mice (Fig. 2B). These results show that in vivo synthesis of catecholamines and 5HT was diminished by the lack of BH4. The phenylalanine content in both brain and liver of homozygotes was 2 times higher than that of the wild type (Fig. 2C).

**TH Activity Was Severely Impaired by Depletion of BH4**—We assayed the activity of TH, AADC, TPH, GCH, and NOS in the brain of newborn mice. AADC decarboxylates L-DOPA and 5-HTP to form DA and 5HT, respectively, and co-exists with TH and TPH in catecholaminergic and serotonergic neurons. Although both TH and TPH are BH4-dependent enzymes, only TH activity was severely impaired in the homozygotic mutant mice (7.0% of wild type; Table I). AADC, GCH, and TPH activities were not altered at all. Activity of NOS was significantly decreased (72% of wild type), but far less than that of TH (Table I).

**TH Immunoreactivity Was Reduced in the Nerve Terminals but Not in the Cell Bodies**—Next, we examined the localization of TH protein by using an immunohistochemical technique. Dopaminergic and noradrenergic neural cell bodies are located in the substantia nigra, ventral tegmental area, and locus ceruleus. Those neural cell bodies in homozygotic mutant mice as well as in wild-type ones were stained with anti-TH antibody equally (Fig. 3, A and B; locus ceruleus, data not shown), and were also stained with anti-AADC antibody (data not shown). Although there was no significant difference in TH staining on cell bodies, we found a striking difference in the terminal region. In the brain of wild-type mice, nerve fibers were strongly stained with anti-TH antibody, and many positive varicosities were easily seen at higher magnification in the substantia nigra project (Fig. 3, C and E). In the same region of the homozygotic mutant mice, nerve fibers were not stained; instead, a few unusual TH-immunopositive cells were found in the striatum (Fig. 3, D and F). In order to examine the presence of dopaminergic fibers, we stained the same sections with anti-

![Table I](image)

**Table I**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>TH (pmol/min/mg protein)</th>
<th>AADC (pmol/min/mg protein)</th>
<th>TPH (pmol/min/mg protein)</th>
<th>GCH (pmol/min/mg protein)</th>
<th>NOS (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>++</td>
<td>5</td>
<td>4.12 ± 0.47</td>
<td>685 ± 30</td>
<td>19.7 ± 1.7</td>
<td>2.87 ± 0.14</td>
<td>211,000 ± 12,000</td>
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<tr>
<td>+/−</td>
<td>6</td>
<td>4.07 ± 0.11</td>
<td>671 ± 21</td>
<td>16.7 ± 1.4</td>
<td>3.10 ± 0.22</td>
<td>236,000 ± 21,000</td>
</tr>
<tr>
<td>−/−</td>
<td>4</td>
<td>0.29 ± 0.08*</td>
<td>627 ± 21</td>
<td>21.0 ± 2.0</td>
<td>2.86 ± 0.20</td>
<td>152,000 ± 15,000*</td>
</tr>
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*Significant difference from the corresponding values of wild-type, p < 0.01 (Student’s t test).

*Significant difference from the corresponding values of wild-type, p < 0.05 (Student’s t test).
AADC antibody. This time nerve fibers in the striatum were positively stained in homozygotes similarly as in the wild type (Fig. 3, G and H). These results indicate that the amount of TH protein was markedly decreased only in nerve terminals, but not in cell bodies. These findings were identical to those obtained from the other homozygous mouse.

A reduction in TH protein in the brain of homozygous newborn mice was also shown by Western blotting (Fig. 9, lanes 1 and 2), although we could not see a big difference in the mRNA level of Th by Northern blot analysis (Fig. 4). This Western blot analysis clearly confirmed that the reduction in TH activity was caused by decrease in the amount of the TH protein itself.

We also stained brain sections of newborn mice with anti-GCH and with anti-TPH antibody. Neural cell bodies in the substantia nigra, ventral tegmental area, and locus ceruleus of homozygotes were immunopositively stained with anti-GCH antibody as in the wild type (data not shown). Serotonergic cell bodies in the dorsal raphe nucleus of the homozygous mice were also well stained with anti-TPH antibody in the Ptps-null mice as well as in the wild type (data not shown).

**Function of the Peripheral Nervous System Was Impaired in Ptps Knockout Mice**—NE is a neurotransmitter of the peripheral sympathetic neurons, and NE and epinephrine are synthesized in adrenomedullary cells as hormones. Since cardiovascular function, respiratory rate, metabolism, and body temperature are regulated by both sympathetic and parasympathetic nervous systems, physical catastrophe could be caused by failure of the peripheral nervous systems as well as by dysfunction of the central nervous system in Ptps knockout mice. So we recorded the surface electrocardiogram of newborn homozygotic mutant mice and compared their heart rates with those of their littermates. Except for one ventricular rhythm, all homozygous mutants showed sinus rhythms that tended toward bradycardia (64% of the wild type, Fig. 5, A and B). Next, with anti-TH antibody we immunostained the adrenal gland and the kidney where many terminals of sympathetic nerves exist. Adrenomedullary cells in the homozygotic mutants were immunopositive as well as those in the wild type, but sympathetic nerve terminals in the kidney of homozygotes showed less positive staining for TH compared with those of the wild-type animals (Fig. 6). These results indicate that TH protein was decreased selectively in nerve terminals and that

![Fig. 3. Immunostaining of TH and AADC in the brain of wild-type and homozygous mutant mice.](image)

AADC antibody. This time nerve fibers in the striatum were positively stained in homozygotes similarly as in the wild type (Fig. 3, G and H). These results indicate that the amount of TH protein was markedly decreased only in nerve terminals, but not in cell bodies. These findings were identical to those obtained from the other homozygous mouse.
dysfunction of peripheral nervous system could thus be involved in the cause of death for homozygous mutant mice.

Effect of Acute BH4 Administration—To explore the acute effect of administration of BH4 on catecholamine and 5HT levels in the brain, we injected newborn mice with BH4 at a dose of 50 mg/kg body weight by the intraperitoneal route on day 0 (postnatal day 0), and sacrificed them 60 min later. Monoamine contents and the monoamine-synthesizing enzyme activities in their brain were analyzed and compared with those of vehicle-injected mice (0.25% ascorbic acid used to protect BH4 from oxidization). The biopterin content in the brains of both BH4-injected wild type and homozygous mutants was 6–7 times higher than that of the ascorbic acid-injected wild-type mice (Fig. 7A). This finding indicates that BH4 passed through the immature blood-brain barrier well in neonates. Despite the great elevation in the amount of BH4, the DA content in the brain was not altered at all in the wild-type mice, whereas the 5HT content was slightly increased (1.4 times). In the Ptps knockout mice, the 5HT level in the brain of BH4-injected animals was markedly elevated to more than 10 times the level for the ascorbic acid-injected ones, reflecting a recovering up to 73% of the level for the wild type injected with ascorbic acid (Fig. 7C). In contrast, the increase in DA levels in the Ptps-null mice was only 1.5-fold the original level (Fig. 7B). TH activity was increased 3-fold in the homozygous mutants by 60 min after the administration of this dose of BH4, whereas the TPH activity was not altered (Fig. 7, D and E).

Effect of Repeated BH4 Administration—We tried to rescue the Ptps knockout mice by multiple intraperitoneal injections of BH4 (Table II). We administered BH4 at 5 or 50 mg/kg once a day intraperitoneally seven times, and then sacrificed the mice on the next day (postnatal day 7) after the last injection. The knockout mice could survive up to postnatal day 7, although growth retardation was obvious. The body weight of the homozygous mutants given the low and high doses was only 29 and 64%, respectively, of that of their heterozygous littermates. Accumulation of phenylalanine in the brain of homozygotes rescued with 5 mg/kg BH4 was observed, being 22.3 times higher than that for the heterozygote given the same dose of BH4 (Table II).
Then we analyzed the content of pteridines and monoamines, as well as TH, GCH, and TPH activities, in the brain. The biopterin content in the brain of homozygous mice was not so improved by the 5 mg/kg BH4 injection, and the TH activity remained low. In contrast, the biopterin content in the brain of homozygotes rescued with 50 mg/kg BH4 was 44% of that of the heterozygotes used as a control. By this subchronic administration of BH4, TH activity was recovered to a greater extent than the DA level in the brain. In the homozygotes we observed 50% of the TH activity found for the 50 mg/kg BH4-injected heterozygous mice, although the DA level remained very low (16% of the level for heterozygous mice). The 5HT content in the brain of the homozygous mutants was not remarkably improved 1 day after the last injection. The activity of TPH and GCH tended to increase in the homozygous mice rescued with 5 mg/kg BH4, but the TH activity was still low (Table II).

To examine whether TH protein was recovered in the striatum, we stained with anti-TH antibody brain sections of mice rescued by 7 days’ administration of 50 mg/kg BH4. The mouse brains were fixed 3 h after the last injection. Strikingly, the caudate-putamen of the rescued homozygous mice was positively stained with anti-TH antibody as was that of the wild-type one. The kidney of the wild-type mouse (C), whereas those of the homozygous mutant were weakly stained (D). Scale bar in B, 100 μm; in D, 50 μm.

**Fig. 6. Immunostaining of TH in the adrenal gland and kidney.** A and B, the adrenal gland of a wild type (+/+) and a homozygous mutant (−/−) mouse, respectively. The adrenal medulla in the homozygous mutant mouse was positively stained with anti-TH antibody as well as the wild-type one. C and D, the kidney of wild type and homozygous mutant, respectively, at higher magnification. Sym pathetic nerve terminals were well stained with anti-TH antibody in the kidney of the wild-type mouse (C), whereas those of the homozygous mutant were weakly stained (D).

**DISCUSSION**

For the first time, we established mice in which de novo biosynthesis of BH4 was impaired by disruption of the Ptps gene, whose product catalyzes the conversion of 7,8-dihydronopterin triphosphate to 6-pyruvoyl-5,6,7,8-tetrahydropterin. Homozygous mutants were born nearly according to the Mendelian law, but all of them died within 48 h. In the brain of newborn Ptps-null mutants, only a trace amount of biopterin was present in the brain, neopterin derived from 7,8-dihydronopterin triphosphate accumulated, and catecholamine and 5HT levels were markedly reduced. It was surprising that TH activity was selectively diminished compared with that of TPH and NOS, for BH4 is an essential cofactor for those enzymes as for TH. The reduction of TH activity was accompanied by a decreased amount of the enzyme only at the nerve terminals. Whereas a single administration of BH4 to the null mice led to a great increase in the 5HT content, 1 h after the administration, the DA content remained low. These results suggest differential action of BH4 between the regulation of catecholamines and that of 5HT in the brain.

We observed accumulation of phenylalanine in the brain and liver of homozygous mutant mice. Most cases of hyperphenylalaninemia are caused by a genetic defect in phenylalanine hydroxylase. However, in ~10% of the cases it is caused by genetic defects in biopterin metabolism. More than 400 patients were reported to have this “atypical” form of hyperphenylalaninemia, and ~40% of them had the genetic defect in the PTPS gene (28). The present Ptps knockout mouse is an animal model for this type of atypical hyperphenylalaninemia.
result thus indicates that the Ptps knockout mouse is not an embryonically lethal. Previous studies on Th knockout mice (26, 29) and dopamine β-hydroxylase (Dbh) knockout mice (30) showed that catecholamines are essential for embryonic development, because these knockout mice died in utero at a late stage of embryonic development or shortly after birth. Dbh knockout mice die at embryonic day 11 or 12 if the mother was also Dbh-null. Our findings raise the possibility that a minor side pathway for BH4 biosynthesis may exist, because it was reported that BH4 hardly passed through placenta (31).

We succeeded in rescuing homozygous mutants from death for up to 7 days by intraperitoneal administration of BH4. BH4, being a hydrophilic molecule, is believed to hardly permeate into the blood-brain barrier. However, in the neonatal stage, peripherally administered BH4 could pass through the immature blood-brain barrier well and was incorporated into cells to increase neurotransmitter levels (Fig. 7). Their increase by BH4 administration of BH4 was more remarkable in the Ptps knockout mice than in the wild-type mice. It is possible that a BH4 transporter or carrier protein to maintain the BH4 concentration constant in the cell exists.

Mice lacking nNos (32), eNos (33), and iNos (34) were established by gene targeting. Hypertrophy of the pyloric sphincter and the circulate muscle layer along with the lack of NADPH-diaphorase-containing neurons occurred in the nNos knockout mice (32). eNos mutant mice were hypertensive, and acetylcholine-induced relaxation was absent in their vascular system (33). Hypotension caused by endotoxin in wild-type mice was not observed in mice deficient in iNos, and antibacterial and anti-tumor activities in the latter were poor (34). Since we did not subfractionate the brain homogenate, the NOS activity that we assayed was that of mixed types of NOS. However, the major subtype of NOS in the brain is nNOS in neurons, and iNOS activity remained low before induction with immunological stimulation including cytokines. Thus, the NOS activity that we assayed would mainly reflect that of nNOS. In the present study, the NOS activity was slightly reduced by the lack of BH4 in vitro, but no phenotype relating to NO systems was evident in vivo. We could not determine whether in vivo NO production was impaired or not, because the levels of NO metabolites such as nitrite and nitrate were too low to be quantified under the unstimulated condition. However, as the \(K_m\) value (0.02–0.03 \(\mu\)M) of NOS against BH4 is very low (35), the binding between BH4 and NOS protein may be so strong that a trace amount of BH4 may be sufficient for constitutive NOS activity. It is likely that depleted BH4 may affect the immune response or host defense mechanisms when iNOS induction is required. We are currently planning an experiment to examine this possibility.

**Cause of Death in PTPS Knockout Mice—**Th knockout mice, which were embryonic lethal but could be rescued by administration of L-DOPA or L-three-dihydroxyphenylserine, died apparently of cardiovascular failure as shown by congestion in the liver and vessels and by an abnormal electrocardiogram (26, 29). Although the cause of neonatal death in our Ptps knockout mice remains unclear, the severe decrease in catecholamine levels, bradycardia, and the marked reduction in TH immunoreactivity in sympathetic nerve terminals suggest that failure in the peripheral nervous system in addition to abnormality in the brain could be a causative factor leading to death. BH4 could be maternally supplied in utero; however, it would be gradually depleted after birth. This assumption is further supported by the prolonged survival period with administration of BH4. The mortality of Ptps knockout mice resembled that of Phox2a (36) or Nurr1-deficient mice (37). In the former, the locus ceruleus and subsets of sympathetic, parasympathetic, and sensory ganglia were absent; and in the latter, TH-positive cells in the ventral midbrain were not generated. DA-deficient mice were reported to be hypoactive and showed a feeding disturbance after weaning (38). However, as thinning out of littersmates did not seem to be very helpful for survival of homozygous mutants of Ptps knockout mice (data not shown), simple competition with their littersmates was not a critical factor. The phenylalanine content in the brain of homozygous mutant neonates was only 2 times higher than that of the wild type. The contribution of hyperphenylalaninemia to the early death seems to be low, as it is known that the symptom of human phenylketonuria is not remarkable at the neonatal stage, but becomes worse by intake of phenylalanine in the milk. Further, the level of 5HT also decreased in Ptps knockout mice, and NO system could be impaired by BH4 starvation. Although symptoms based on dysfunction of 5HT and NO systems were not observed in human PTPS deficiency, these factors also may have contributed to the death of knockout mice.

**TH-positive Cells in the Striatum—**Interestingly, we found TH-positive cells in the striatum of the BH4-deficient mice, which cells were not detectable in the wild-type mice. AADC-positive cells were also not visible, and GCH-immunopositive cell bodies were not detected in the striatum of wild-type and
Mice were injected with 0.25% ascorbic acid (AA) and 5 or 50 mg/kg BH4. Values are the means ± S.E. Body weight is indicated in grams. Phenylalanine content is expressed as nmol/mg protein. Neopterin, biotin, DA, and serotonin (5HT) are expressed as pmol/mg protein. TH, TPH, and GCH activities are indicated by pmol/min/mg protein. Values in parentheses are percentages to those of their heterozygous littersmates.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Body weight</th>
<th>Phenylalanine</th>
<th>Neopterin</th>
<th>Biotin</th>
<th>DA</th>
<th>TH</th>
<th>5HT</th>
<th>TPH</th>
<th>GCH</th>
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<td>AA</td>
<td>4</td>
<td>4.63 ± 0.09</td>
<td>1.6 ± 0.2</td>
<td>0.77 ± 0.04</td>
<td>9.25 ± 0.32</td>
<td>16.4 ± 1.3</td>
<td>14.9 ± 0.6</td>
<td>1.50 ± 0.11</td>
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<tr>
<td>(+/-)</td>
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<td>5.88 ± 0.19</td>
<td>1.3 ± 0.04</td>
<td>0.80 ± 0.05</td>
<td>9.86 ± 0.29</td>
<td>19.9 ± 1.3</td>
<td>10.6 ± 0.8</td>
<td>1.08 ± 0.30</td>
<td>22.7 ± 0.8</td>
<td>5.32 ± 0.35</td>
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<td>29 ± 10^5</td>
<td>17.8 ± 2.5^3</td>
<td>1.64 ± 0.38</td>
<td>0.85 ± 0.3^4</td>
<td>1.49 ± 0.2^5</td>
<td>ND</td>
<td>34.9 ± 0.8^6</td>
<td>6.60 ± 0.57</td>
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<tr>
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<tr>
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<td>0.93 ± 0.08</td>
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<td>24.0 ± 5.1</td>
<td>5.60 ± 0.09</td>
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</tbody>
</table>

Significant difference from the corresponding values for heterozygotes, p < 0.01 (Student’s t test).

Significant difference from the corresponding values for heterozygotes, p < 0.05 (Student’s t test).

Not detected.

Fig. 8. Immunohistochemical staining of TH in the striatum of mice rescued with BH4 administration. A, an ascorbic acid (vehicle)-administered heterozygous mouse (+/-); B, a 50 mg/kg BH4-administered heterozygous mouse (+/-); C, a 50 mg/kg BH4-administered homozygous mouse (-/-). These injections were done for 7 days. CP, caudate-putamen; Cx, cortex. Scale bar, 500 μm.

Fig. 9. Western analysis using the brain homogenate of mice rescued with BH4 administration. Thirty μg of protein was applied to each lane and stained with specific antibodies for TH, AADC, GCH, and TPH, respectively, as indicated at the left side of panels. Lane 1, wild type (+/+) newborn; lane 2, homozygous (-/-) newborn; lane 3, ascorbic acid (vehicle)-administered heterozygous mouse (+/-); lane 4, 50 mg/kg BH4-administered heterozygous mouse (+/-); lane 5, 50 mg/kg BH4-administered homozygous mouse (-/-). These injections were done for 7 days.

Homozgyous mice. It is possible that TH- and/or AADC-positive cells in the wild type were masked by the extensively stained nerve terminals. However, such intrinsic TH-positive cells were also observed in the striatum of rats with 6-hydroxydopamine lesions (39) and monkeys treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (a dopaminergic neurotoxin that induces parkinsonism) (40), and these cells were reported to be increased in number after the treatment. In the latter case, the cells were reported to be doubly positive when stained with anti-TH and anti-DA transporter antibodies, and they were further reactive with anti-glutamate decarboxylase (GAD_{67}) antibodies. TH-positive neurons in the striatum were also observed in DA transporter knockout mice, in which the DA content and TH protein were reduced by 95 and 90%, respectively (41). Physiological and pathological meanings of these cells are still unclear; however, they could represent an endogenous mechanism compensating for DA deficiency in the striatum and may be common in BH4-and/or DA-deficient states of the human brain.

Differential Regulation of Monoaminergic Neurons by BH4—The fact that catecholaminergic, serotonergic, and NO systems were differently affected by BH4 starvation was clearly shown by our results. In the catecholaminergic system, depletion of BH4 resulted in a reduced level of TH protein (accompanied by reduced TH activity) at nerve terminals. A reduction in TH immunoreactivity was observed in the striatum and sympathetic nerve terminals, whereas cell bodies of catecholaminergic neurons in the substantia nigra, ventral tegmental area, and locus ceruleus, as well as adrenomedullary cells, were well stained with the anti-TH antibody. In the serotonergic system, however, TPH activity was not affected by the severe reduction in the BH4 level. These differences were clearly reflected by responses to acute BH4 administration. In the Ptpes knockout mice, the 5HT content in the brain was dramatically increased 60 min after the administration, although the DA content was only slightly elevated. These differences were also observed to a lesser extent even in normal animals, which could synthesize BH4 (Fig. 7, B and C).

The BH4 concentration in the cell had been thought to be not high enough to saturate TH (42–44). However, our results showed that acute administration of BH4 to the wild-type mice was not effective to increase the DA content in the brain,
although the bipterin content in the brain was elevated up to 7-fold of that of the vehicle-injected mice (Fig. 7). Instead, BH4 depletion caused a reduction in the content of TH protein. These findings suggest that BH4 regulates the catecholamine synthesis through altering the amount of TH molecules, especially at nerve terminals.

One possible explanation for the reduction only at nerve terminals would be that TH forms a stable complex with BH4 and/or catecholamines, and that only the stable complex is carried to nerve terminals through axonal flow. We showed previously that human TH shows regulatory kinetic properties for BH4 (45). Flatmark et al. (46) also observed the negative cooperativity of BH4 binding to TH. On the other hand, TH has been reported to make a stable and inactive complex with catechol compounds (47). An alternative possibility is that TH is carried to the terminals with or without BH4. TH protein and mRNA levels are normal in neural cell bodies because that is where the protein is synthesized. A shortened half-life would only be revealed at nerve terminals where TH accumulates. We observed an additional immunoreactive band of small molecular size in the brain homogenate of Ptps-null mutants by Western blot analysis (data not shown), which could represent degraded TH protein. Although more study is required to clarify the molecular mechanism, these data strongly suggest that the turnover of TH protein is very rapid in the absence of the bipterin cofactor.

A similar reduction in the level of TH protein only in the striatum was shown also in patients with DRD (Segawa's disease). DRD is caused by disturbed BH4 synthesis (15). Autopsied brains of patients with the disease showed a marked reduction in the content of TH protein in the striatum (48, 49).

Our present results would explain the reason why only dysfunction of nigrostriatal dopaminergic neurons is apparent, and why serotoninergic systems can function properly in the DRD patients. Because of decreased synthesis of BH4, the amount of TH protein carried to the nerve terminal is reduced. At the nerve terminal (striatum), although a small amount of BH4 is present there, the ability to synthesize DA is not sufficient due to the decreased number of the TH molecules. In the case of serotoninergic neurons, the stability of the TPH protein is not affected by depletion of BH4. Thus, the number of TPH molecules at the nerve terminal is the same as the normal one, and a sufficient amount of 5HT can be synthesized, although the rate of de novo synthesis of BH4 is partially impaired. The presence of a recycling system powered by dihydropteridine reductase may help to facilitate the monoamine synthesis through re-redox of quinonoid-dihydrobipterin, which is formed from the reaction with aromatic amino acid hydroxylases, to BH4.

**Reduction in the Level of TH Protein in the Striatum Was Reversed by BH4 Administration**—Our results also showed that the reduction in the TH protein level was reversible by supplementation with BH4. Intraperitoneal administration of 50 mg/kg BH4 increased TH activity in the brain even after 1 h, and this increase was remarkable in mice continuously injected with 50 mg/kg BH4 for 7 days. This is a novel regulatory mechanism for TH protein by BH4.

In the brain of homozygous mutant mice, DA and 5HT levels were very low (16 and 5%, respectively, of those of ascorbic acid-treated heterozygous mice), although the BH4 level was elevated up to 44% of the control 24 h after the last of the repeated injections of 50 mg/kg BH4 (Table II). The low levels of 5HT and DA would be explained by the time after the last injection. We think that the local concentration of BH4 in monoaminergic neurons would not be as high as 44% of the control, because BH4 incorporated into monoaminergic neurons might be consumed during the 24 h before the analysis was made.

There have been several reports describing the dissociation between Th mRNA levels and expression of the enzyme activity (7). For example, a transgenic mouse bearing extra copies of the human TH gene expressed the transgene mRNA in dopaminergic neurons at levels 50 times over that of the endogenous gene. Despite this increase in the amount of mRNA, TH immunoreactivity and enzyme activity in the striatum were altered by only 2–3-fold (50). A part of the dissociation between Th mRNA and the protein could be explained by the stabilizing effect of the cofactor.

In summary, our results showed that the amount of TH protein at the nerve terminal is governed by the intracellular concentration of BH4, that transient elevation in the BH4 content increases the biosynthesis of 5HT but not that of catecholamines, and that a continuous elevated level of BH4 leads to an increase in the catecholamine levels. We think that this paper opens a new insight into regulation of monoamines by BH4.

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