

Dwarfism and Low Insulin-like Growth Factor-1 Due to Dopamine Depletion in *Pts*^{-/-} Mice Rescued by Feeding Neurotransmitter Precursors and H₄-biopterin*

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The tetrahydrobiopterin (BH₄) cofactor is essential for the biosynthesis of catecholamines and serotonin and for nitric-oxide synthase (NOS). Alterations in BH₄ metabolism are observed in various neurological and psychiatric diseases, and mutations in one of the human metabolic genes causes hyperphenylalaninemia and/or monoamine neurotransmitter deficiency. We report on a knockout mouse for the *Pts* gene, which codes for a BH₄-biosynthetic enzyme. Homozygous *Pts*^{-/-} mice developed with normal morphology but died after birth. Upon daily oral administration of BH₄ and neurotransmitter precursors the *Pts*^{-/-} mice eventually survived. However, at sexual maturity (6 weeks) the mice had only one-third of the normal body weight and were sexually immature. Biochemical analysis revealed no hyperphenylalaninemia, normal brain NOS activity, and almost normal serotonin levels, but brain dopamine was 3% of normal. Low dopamine leads to impaired food consumption as reflected by the severe growth deficiency and a 7-fold reduced serum insulin-like growth factor-1 (IGF-1). This is the first link shown between 6-pyruvoyltetrahydropterin synthase- or BH₄-biosynthetic activity and IGF-1.

Tetrahydrobiopterin (BH₄)¹ plays a central essential role in metabolism, involving monoamine neurotransmitter biosynthesis, hepatic phenylalanine degradation, and nitric oxide (NO) production. The absolute requirement of BH₄ for such enzymatic functions is reflected by severe disturbances or even lethality in the case of cofactor limitation due to mutations in BH₄-metabolic genes. Patients with cofactor deficiency may

exhibit severe dopamine and serotonin neurotransmitter deficiency, hyperphenylalaninemia, and reduced NO production (1, 2).

The BH₄ cofactor is synthesized from guanosine triphosphate (GTP) by a set of reactions involving three enzymes. GTP cyclohydrolase I (GTPCH), the first enzyme in BH₄ biosynthesis, catalyzes the formation of dihydroneopterin triphosphate from GTP (3). GTPCH activity is regulated by its substrate GTP, BH₄, and phenylalanine. A physiological mechanism for post-translational control of GTPCH activity involves feedback inhibition by BH₄. Notably, feedback inhibition results from BH₄-induced complex formation of GTPCH with a regulatory protein known as GTPCH feedback regulatory protein (GFRP) (4–6). In the second step, the 6-pyruvoyltetrahydropterin synthase (PTPS) catalyzes the conversion of dihydroneopterin triphosphate to 6-pyruvoyltetrahydropterin. PTPS must be phosphorylated to be fully active (7, 8). Sepiapterin reductase (SR) is required for the final step reductions of the diketo intermediate, 6-pyruvoyl-tetrahydropterin to BH₄.

BH₄ is required as cofactor for phenylalanine hydroxylase (PAH), tyrosine hydroxylase, and tryptophan hydroxylase. The latter two are key enzymes in the biosynthesis of the neurotransmitters dopamine and serotonin (9). The complete hydroxylating system of aromatic amino acids consists of the two additional BH₄-regenerating enzymes: pterin 4 α -carbinolamine dehydratase and dihydropteridine reductase (DHPR) (10). BH₄ is also required for the nitric oxide synthase enzymes (11).

A deficiency of phenylalanine catabolism, leading to hyperphenylalaninemia (HPA), comprises a heterogeneous group of disorders caused by a partial or complete deficiency of the hepatic apoenzyme PAH, or by one of the enzymes involved in cofactor biosynthesis (GTPCH or PTPS) (12, 13), or regeneration (DHPR and pterin 4 α -carbinolamine dehydratase) (14–16). Whereas severe HPA, leading to classic phenylketonuria, can only be treated with a low phenylalanine diet, patients with BH₄-responsive PAH deficiency can be treated with BH₄ alone (17). Two disorders of BH₄ metabolism may present without HPA. These are dopa-responsive dystonia (Segawa disease) (18) and sepiapterin reductase deficiency (19, 20). Although dopa-responsive dystonia is caused by a mutation in the GTPCH gene and is inherited in an autosomal dominant manner, SR deficiency is an autosomal recessive trait. Both diseases manifest severe biogenic amine deficiencies.

To diagnose BH₄ deficiencies and follow-up of the resulting pathologies, only limited possibilities are available, including measurements of metabolites in body fluids, and follow-up of the disease development almost exclusively under treated conditions. Diagnosis starts in most cases with screening of HPA

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¹ The abbreviations used are: BH₄, tetrahydrobiopterin; NOS, nitric-oxide synthase; GTPCH, GTP cyclohydrolase I; GFRP, GTPCH feedback regulatory protein; PTPS, 6-pyruvoyltetrahydropterin synthase; SR, sepiapterin reductase; PAH, phenylalanine hydroxylase; DHPR, dihydropteridine reductase; HPA, hyperphenylalaninemia; HVA, homovanillic acid; 5-HIAA, 5-hydroxyindoleacetic acid; CSF, cerebrospinal fluid; IGF-1, insulin-like growth factor-1; ES, embryonic stem; HPLC, high-performance liquid chromatography; RIA, radioimmunoassay; GH, growth hormone; T₄, thyroxine; DOPAC, dihydroxyphenylacetic acid; TSH, thyroxin-stimulating hormone; dopa, L-dihydroxyphenylalanine.

with the Guthrie card and determination of plasma phenylalanine levels as an indirect measurement of hepatic phenylalanine hydroxylase (PAH) activity. Analysis of phenylalanine and tyrosine in serum or plasma before and after a BH₄ challenge are often applied as an additional diagnostic tool for differentiation between classic phenylketonuria and bipterin variants. Furthermore, urinary pterin analysis and enzymatic measurements in erythrocytes or skin fibroblasts are carried out to gather information on bipterin-metabolizing enzymes. These data are then combined with a neurotransmitter status. For neurotransmitters, the dopamine and serotonin neurotransmitter degradation products homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA), respectively, are measured in cerebrospinal fluid (CSF), thus following the activities of tyrosine and tryptophan hydroxylases. Furthermore, NO metabolites at least for brain nitric-oxide synthase (NOS) isoenzyme activity are determined in CSF.

Symptoms of BH₄ deficiency include a vast range of abnormalities of the central nervous system, including microcephaly, seizures, hypertonia, hypersalivation, temperature instability, feeding difficulties, and mental retardation. The goals of treatment are to control HPA by dietary restriction of phenylalanine (in PAH deficiency) or BH₄ administration (in GTPCH and PTPS deficiency) and to restore neurotransmitter homeostasis by oral administration of the dopamine and serotonin precursors L-dopa and 5-hydroxytryptophan, respectively, in BH₄ deficiencies. Late detection and introduction of treatment leads to irreversible brain damage. For patients with BH₄ deficiency, HPA is controllable with oral doses of 2–10 mg of synthetic BH₄/kg/day. However, such relatively low doses of BH₄ do not allow the cofactor to penetrate the blood-brain barrier efficiently (21, 22). To some extent, this problem can be overcome by administering higher doses of BH₄, up to 20 mg/kg/day, together with corresponding neurotransmitter precursors (23). The combined therapy is mandatory to avoid neurological damage; however, this treatment is not sufficient in every case (24).

To analyze in more detail the consequences of BH₄ deficiency and its treatment, and to study pathologies in the organism, the use of animal models is required. Here we report on the generation of an animal model for PTPS deficiency by knocking out the *Pts* gene in the mouse. This led to perinatal lethality of otherwise normal born animals. Treatment studies with daily oral administration of different concentrations of BH₄, L-dopa, and 5-hydroxytryptophan for BH₄ led to the observation that mice can be rescued but exhibit severe growth deficiency leading to dwarfism due to low serum insulin-like growth factor-1 (IGF-1).

EXPERIMENTAL PROCEDURES

Pts Gene Targeting

A genomic clone containing the *Pts* gene encoding the mouse PTPS was isolated from a 129/Sv-λ phage library and characterized previously (25). To construct a targeting vector, a *KpnI-NcoI* fragment, generated by PCR and spanning exon 1, and the first nine codons from exon 2 were used for the short arm of homology (Fig. 1A). Exon 2 of this fragment was ligated in-frame with an *NcoI-BamHI* fragment containing the prokaryotic *lacZ* gene, followed by a phosphoglycerate kinase promoter (*Pgk-neo*) cassette. A *Pgk-tk* cassette was added 5' to this short arm of homology. The long arm of homology was a 5.4-kb *HindIII* fragment containing exons 5 and 6 of the *Pts* gene. The final targeting vector, plasmid pMSY23, was linearized, electroporated into 129/Sv embryonic stem (ES) cells, and selected for G418 and FIAU (1(1,2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil) resistance as described previously (26). For PCR screening of ES clones, a nested PCR with two rounds of 40 cycles under standard amplification conditions with an annealing temperature of 55 °C was applied. For the first PCR, the 5' primer MSY69 annealed outside (upstream) of the short arm of homology, and the 3' primer PLACZ6 matched to the *lacZ* gene. For the second round of amplification, the 5' primer MSY70 was upstream of exon 1, and the 3' primer PLACZ7 again in the *lacZ* gene (MSY69:

TABLE I
Treatment protocol for oral application of BH₄ and neurotransmitter precursors

Compound ^a	Treatment		
	Low ^b	Medium	High
	μg/g body weight/day		
BH ₄	10	60	122
L-Dopa	5	10.6	13.5
5-Hydroxytryptophan	3	7.4	9.5
Carbidopa ^c	2.5	2.5	2.5
Ascorbic acid ^d	10	50	100
<i>N</i> -Acetyl-L-cysteine ^d	5	25	50

^a The daily doses for the medium and high treatments were given in two applications, one in the morning, and one in the late afternoon.

^b The low treatment corresponds to the recommended standard concentrations for treatment of human patients (23).

^c Decarboxylase inhibitor.

^d Antioxidant.

5'-TATATGCCATCTCTGACTGACAACA-3'; MSY70: 5'-CTGTCTCTGTTTGGAGGAAGTCTCT-3'; PLACZ6: 5'-CAGTTTGAGGGGACGACACAGTAT-3'; PLACZ7: 5'-TGCTGTTTCTGGTCTTCAACCACCG-3'). Upon additional confirmation of correct double cross-over by Southern blot analyses with an outside probe A (see Fig. 1C) or an internal probe B (not shown), positive *Pts*^{+/-} ES cell sub-clones were used for injection into C57BL/6 blastocysts, generation of chimeras (derivatives of ES cell-subclone 7-E3), and breeding of homozygous *Pts*^{-/-} 129/Sv-C-57BL/6 hybrid mice. Screening of mouse tissue material (tail biopsies) was performed by a complex PCR with 40 cycles, 3 primers, and an annealing temperature of 53 °C. Primers were MSY107 (a), 5'-TGAC-TATGGGCAGAGTTGTT-3'; MSY108 (b), 5'-GATTGTTGCATTTCCCA-AAC-3'; and PLACZ8 (c), 5'-GGCTCAGTTGAGGTGCT-3' (see also Fig. 1B). β-Galactosidase activity was determined with extracts from ES cells according to a published protocol (27).

Replacement Therapy

For an overview of concentrations of drugs used for treatments, see Table I. For the "low" treatment, tablets from Schircks Laboratories (Jona, Switzerland) containing 50 mg of BH₄, 50 mg of ascorbic acid, and 25 mg of *N*-acetyl-L-cysteine were dissolved in 10 ml of H₂O. For L-dopa, tablets containing 100 mg of levodopa and 25 mg of carbidopa (Sinemet MSD, Glattbrugg, Switzerland), and for 5-hydroxytryptophan, tablets containing 100 mg of oxitriptanum (Sigma-tau) were each dissolved in 20 ml of H₂O. For the medium treatment, the following stock solution was prepared: 65 mg of BH₄-2HCl, 50 mg of ascorbic acid, 10 mg of L-dopa, 6 mg of 5-hydroxytryptophan, 2.5 mg of carbidopa and 25 mg of *N*-acetyl-L-cysteine dissolved in 1 ml of H₂O. For the high treatment, the following stock solution was prepared: 130 mg of BH₄-2HCl, 100 mg of ascorbic acid, 10 mg of L-dopa, 6 mg of 5-hydroxytryptophan, 2.5 mg of carbidopa, and 50 mg of *N*-acetyl-L-cysteine dissolved in 1 ml of H₂O. To determine the actual concentrations of BH₄, L-dopa and 5-hydroxytryptophan, the dissolved compounds were analyzed by standard HPLC (see below). Aliquots were kept frozen, thawed before used, and buffered with sodium citrate to pH 5. The solutions were diluted with water and 10–20 μl were orally administered using yellow tips and a Gilson pipette. For the medium and high treatment protocols, the daily aliquots were divided into two daily doses.

Preparation of Mouse Tissues

After sacrificing the animals, brains and livers were withdrawn immediately for preparing tissue homogenates. Homogenizing buffer containing 50 mM Tris-HCl, pH 7.5, 0.1 M KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 1 μM pepstatin was added to whole brain (4 μl/mg tissue), or liver (5 μl/mg tissue). Tissues were blended at 4 °C with an electric homogenizer (Kinematic GmbH, Littau-Luzern, Switzerland) and centrifuged at 15,000 × *g* for 20 min at 4 °C. Supernatants were kept frozen at -80 °C.

Neopterin, Biopterin, and Neurotransmitter Measurements

A volume of 50 μl of liver or 50 μl of brain tissue homogenates were adjusted to 100 μl and oxidized with 10 μl of oxidizing solution (5 g/liter iodine and 10 g/liter potassium iodide in 1 M HCl). After oxidation in the dark for 60 min, the reaction was stopped by adding 10 μl of freshly prepared ascorbic acid (20 g/liter). A total of 14 μl of 1 M NaOH was added to adjust the mixture to pH 8.5, followed by incubating with 20 μl of an alkaline phosphatase solution at 37 °C for 1 h (300 units/ml calf

intestine alkaline phosphatase from Roche Applied Science in 0.1 M Tris-HCl, pH 8.0, 1 mM MgCl₂, 0.1 mM ZnCl₂). The mixture was adjusted to pH 2 by adding 5 μ l of 2 M HCl and deproteinized through an Ultrafree-MC filter (Millipore). Neopterin and biopterin are measured from the filtrate by HPLC (28). The concentrations are expressed as picomoles per mg of protein. Monoamine neurotransmitters were determined according to a published method (46).

Enzymatic Assays

A volume of 100- μ l tissue homogenates was desalted on a spin column (MicroSpinTM G-25 columns, Amersham Biosciences), and 100 μ g of protein from the liver filtrate or 200 μ g of protein from the brain filtrate was used for GTPCH and PTPS assays, respectively.

GTPCH Assay—A final volume of filtrate was adjusted to 50 μ l and added to 148 μ l of homogenizing buffer and 2 μ l of 100 mM GTP (Roche Applied Science). This mixture was divided into two 100- μ l portions. One portion was immediately oxidized as blank with cell extract, and the second portion was incubated for 60 min at 37 °C. The reaction was stopped by cooling the sample on ice and adding 10 μ l of oxidizing solution (5 g/liter iodine and 10 g/liter potassium iodide in 1 M HCl). After oxidation in the dark for 60 min, the reaction was stopped by adding 10 μ l of 20 g/liter ascorbic acid (freshly prepared). The mixture was adjusted to pH 8.5 by adding 14 μ l of 1 M NaOH, and the sample was incubated with 20 μ l of alkaline phosphatase solution at 37 °C for 1 h (300 units/ml calf intestine alkaline phosphatase (Roche Applied Science); see above). The mixture was adjusted to pH 2 by adding 5 μ l of 2 M HCl and deproteinized through an Ultrafree-MC filter (Millipore). Neopterin was measured from the filtrate by HPLC. One unit of GTPCH produces 1 μ mol of neopterin per minute at 37 °C.

PTPS Assay—A final volume of filtrate was adjusted to 50 μ l and added to 60 μ l of reaction mixture (100 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1 mM NADPH, 1 mM NADH, 3 milliunits of SR, 140 milliunit of DHPR from Roche Applied Science, and 60 μ mol/liter dihydroneopterin triphosphate) in a final volume of 110 μ l. This mixture was divided into two portions, one 50- μ l aliquot was incubated for 2 h at 37 °C, and another 50 μ l was used as a blank. A blank without cell extract was incubated at the same time; it contained 50 μ l of reaction buffer and 60 μ l of reaction mixture. The reaction was stopped by adding 15 μ l of 300 g/liter trichloroacetic acid for protein precipitation, and cooling on ice for at least 10 min, followed by oxidation with 10 μ l of oxidizing solution (10 g/liter iodine and 10 g/liter potassium iodide in H₂O) for 60 min in the dark. Excess iodine is destroyed by adding 15 μ l of ascorbic acid (10 g/liter ascorbic acid in H₂O). For the blanks, the same procedure was used. After 2 min of centrifugation at 15,000 \times g, the supernatant was deproteinized through an Ultrafree-MC filter (Millipore) and analyzed by HPLC. One unit of PTPS produces 1 μ mol of biopterin per minute at 37 °C.

NO Assay—The NO, which is the product of NOS, is extremely reactive and undergoes a series of reaction. Nitrite (NO₂⁻) and nitrate (NO₃⁻) are the final products. The sum of these two products (nitrite plus nitrate) was measured using a commercial Colorimetric assay kit (Cayman Chemical, Ann Arbor, MI). During this assay, nitrate was converted to nitrite utilizing nitrate reductase and measured by using the Griess reagent. Absorbance was read at 570/620 nm in a MicroELISA autoreader MR 530 (Dynatech, Chantilly, VA).

Phenylalanine Hydroxylase Assay—This assay was adapted from Ledley *et al.* (29). Liver homogenate containing 50–100 μ g of total protein was used for PAH assay. For the blank, the appropriate amount of liver homogenate was adjusted to a final volume of 104 μ l with water and incubated for 5 min in a 96 °C heating block. For the sample, the appropriate amount of liver homogenate was adjusted to a final volume of 77.5 μ l with water. A volume of 22 μ l of master mixture was added to each sample. The master mixture contained 0.6 mM phenylalanine, 3.6 units of catalase (Sigma), 0.15 M KCl in a 0.2 M potassium phosphate buffer, pH 6.8. After preincubation at room temperature, the reaction was started by adding 2 μ l of 0.1 M dithiothreitol and 2 μ l of 4.5 mM 6-methyltetrahydropterin (Schircks Laboratories) to the samples and incubated for 60 min at 25 °C. The reaction was stopped by incubation for 5 min in a 96 °C heating block and centrifuged for 5 min at 13,000 rpm. The supernatant was filtrated in an Ultrafree-MC filter device and centrifuged again at 5000 \times g for 15 min. Phenylalanine and tyrosine were quantified with a standard amino acid analyzer (Biochrom 20 Plus, Amersham Biosciences).

Phenylalanine Concentration in the Blood

The blood from the mice was collected on filter paper cards (Guthrie card). Phenylalanine (and tyrosine) concentrations were measured using electrospray ionization tandem mass spectrometry.

Protein Measurement

Protein concentrations in homogenized tissues were determined by the spectrophotometric method described by Bradford, using γ -globulin as a calibrator (30). The activities of the various enzymes are expressed as units per milligram of protein.

Immunoassays

Blood was collected from 35- and 44-day-old mice at the time animals were sacrificed.

Insulin-like Growth Factor-1—Serum IGF-1 was separated from clotted blood by centrifugation. It was measured after extraction with acid-ethanol (40 μ l of serum and 160 μ l of acid-ethanol). The mixture was incubated for 30 min at room temperature and centrifuged, and 100 μ l of supernatant was diluted 1:6 before analysis. Serum IGF-1 was determined by radioimmunoassay (RIA) using a rat IGF-1 RIA kit (DSL-2900, Böhmann Laboratories AG, Switzerland).

Growth Hormone—Serum GH was measured by RIA using a specific rabbit anti-rat antiserum and rat GH as standard. The rat GH RIA kit (AH R012) was obtained from Böhmann Laboratories AG.

Blood Thyroxin—T₄ was measured by fluoroimmunoassay using the mouse anti-thyroxine IgG as first antibody and the anti-mouse IgG as second antibody. The blood was dried on filter paper cards (Guthrie card). The total amount of T₄ was determined in the test. The AutoDELFLIA™ neonatal thyroxine (T₄) kit was obtained from PerkinElmer Life Sciences, Wallac-ADL AG (Switzerland).

RESULTS

Targeted Deletion of the Mouse *Pts* Leads to Perinatal Death—A targeting construct was generated based on the previously isolated and characterized mouse gene structure *Pts*, encoding the 6-pyruvoyltetrahydropterin synthase (25). As shown in Fig. 1A, the pMSY32-targeting vector contained an in-frame *lacZ* gene fusion at exon 2 of the *Pts* gene, expressing a putative PTPS- β -galactosidase fusion with 35 N-terminal amino acids from PTPS. Downstream of the *lacZ* gene, a *Pgk-neo* cassette was inserted in the opposite direction. Upon correct homologous recombination in ES cells, a putative mutant allele was generated with the *lacZ* and *Pgk-neo* inserted, and a deletion of exons 3 and 4, plus most of exon 2. The targeting frequency for correct double cross-over in the 129/Sv ES cells, as verified by PCR, was approximately 5% (not shown). These ES cells had a β -galactosidase activity of 0.05–0.12 OD/mg (wild-type activity < 0.001 OD/mg) and a PTPS activity indistinguishable from wild-type (5.4–6.5 microunits/mg).

Upon PCR and Southern blot analyses (Fig. 1, B and C), a few *Pts*^{+/-} ES cell clones were used for blastocyst injection and subsequent generation of PTPS-null mice. Homozygous mice developed with normal morphology *in utero* and were born at the expected Mendelian ratio (25% wild-type, 48% heterozygotes, 27% *Pts*-null mice; *n* = 159). However, most of the *Pts*^{-/-} mice died within the first hours after birth; at maximum we found 4 mice out of 26 surviving for 7 days. A more detailed analysis of brain development and fine structure of *Pts*^{-/-} mice is now in preparation.² Southern blot analyses with genomic mouse tail DNA and a 5'-external probe (Fig. 1C), or an internal *neo*-probe (*Probe B* in Fig. 1A; results not shown) revealed correct homologous recombination at the single mouse *Pts* gene locus. As compiled in Table II, newborn knockout mice at day 1 had no PTPS (<0.05 microunit/mg) and normal GTPCH activity (0.1–0.3 microunit/mg). Heterozygous animals showed intermediate PTPS activity (1.5 microunit/mg) compared with normal activity in wild-type mice (8.0 microunits/mg). Liver neopterin was almost 200-fold higher than normal (59.0 pmol/mg in *Pts*^{-/-}), and biopterin was only 4% of wild-type (0.9 pmol/mg). Furthermore, the *Pts*^{-/-} animals had hyperphenylalaninemia with blood values of 1352 μ mol/liter phenylalanine (normal control levels were between 34 and 85 μ mol/liter), and

² L. Elzaouk, W. Leimbacher, M. Turri, B. Ledermann, K. Bürki, N. Blau, and B. Thöny, manuscript in preparation.

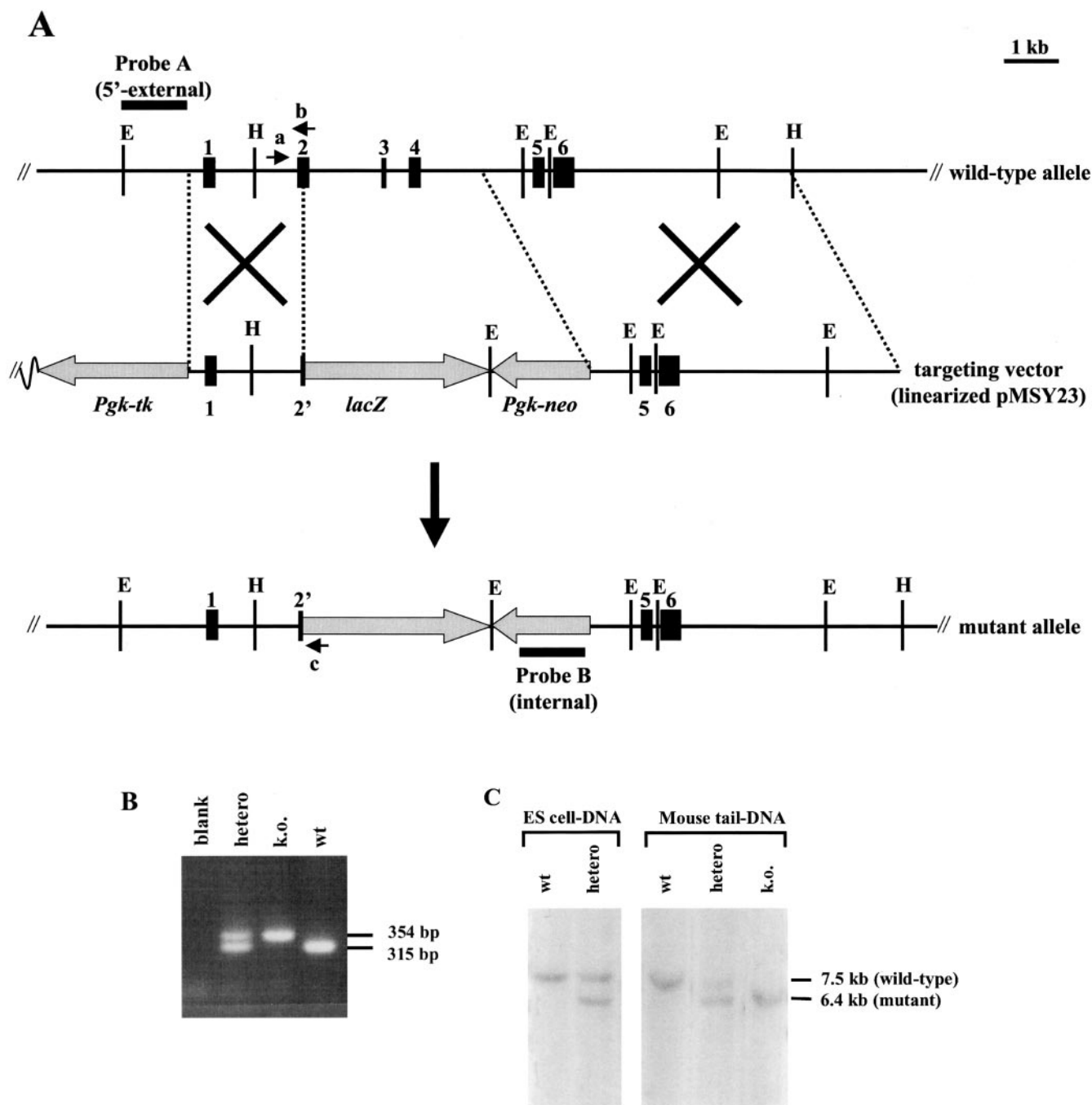


FIG. 1. Targeted disruption of the murine *Pts* gene. *A*, genomic structure of the *Pts* wild-type allele containing the six exons, the targeting vector (pMSY23), and the expected targeted allele with the in-frame *lacZ* and the *Pkg-neo* insertion, replacing exons 3 and 4 plus most of exon 2. Position of the primers *a-c* for PCR analysis and probes for Southern analysis are indicated. *E*, *EcoRI*; *H*, *HindIII*. *B*, genetic analysis of F1-hybrid mice by multiplex PCR with genomic DNA as template and the three primers *a-c*: while primer *a* is upstream of exon 2 and thus present in wild-type and mutant alleles, primer *b* is specific for exon 2, and primer *c* is specific for *lacZ*. The multiplex PCR with primer pair *a/b* results in a DNA fragment of 315 bp and with primers *a/c* in 354 bp. *C*, Southern blot analyses of genomic DNA with either wild-type ES cell clone 129/Sv (*wt*) and the targeted ES cell clone (7-E3; *hetero*), or mouse tail DNA from wild-type, heterozygous or *Pts*-knockout mice. Genomic DNA was digested with *EcoRI* and hybridized with the 5'-external probe *A*. The endogenous wild-type fragment is 7.5 kb, whereas the targeted fragment is 6.4 kb in size.

no detectable or very low brain dopamine and serotonin levels. Expression of β -galactosidase was observed in heterozygous and homozygous *Pts* mutants (not shown). A more detailed study on developmental expression of the PTPS-LacZ fusion is now in preparation.²

Treatment of *Pts*^{-/-} Mice Resulted in Rescue from Lethality but Severe Dwarfism—In the next step, we sought to rescue the knockout mice by applying a replacement therapy protocol based on the recommended standard concentrations for treat-

ment of human PTPS patients (1). This included daily oral administration of BH₄ to control blood phenylalanine and the neurotransmitter precursors L-dopa and 5-hydroxytryptophan. Unexpectedly, we learned that, with this standard treatment protocol, *Pts*^{-/-} mice survived not longer than for about 3 weeks. We thus extended our treatment studies with three types of application levels (Table I; see also "Experimental Procedures"): standard or low treatment, a medium treatment with 2- to 5-fold higher concentrations of BH₄ and neurotrans-

TABLE II
 Mouse liver values for GTPCH and PTPS enzymes and their metabolites neopterin and biopterin

Age (days after birth)	Genotype	Treatment ^a	GTPCH ^b	PTPS ^b	Neopterin ^b	Biopterin ^b	Phenylalanine ^c
			<i>microunits/mg</i>		<i>pmol/mg</i>		<i>μmol/liter</i>
1	k.o. ^d	None	0.21 (0.1–0.3)	<0.05	59.0 (37.1–77.7)	0.9 (0.7–1.1)	1352 (1290–1415)
	Heterozygote		0.21 (0.1–0.3)	1.5 (0.9–4.3)	1.2 (0.9–1.6)	12.5 (12.1–13.2)	n.m. ^e
	Wild-type		0.27 (0.2–0.3)	8.0 (3.6–14.8)	0.3 (0.3–0.3)	21.0 (20.6–21.6)	n.m.
23	k.o.	Low	1.3 (0.9–1.7)	<0.05	144 (73.8–213.3)	0.6 (0.6–0.6)	993 (02–1060)
	Heterozygote		2.2 (1.3–3.2)	8.7 (4.5–12.0)	0.1 (0.1–0.3)	26.1 (12.3–35.9)	92 (52–140)
	Wild-type		2.3 (1.4–4.9)	18.4 (15.3–23.3)	ND ^f	22.9 (9.3–28.9)	94 (58–112)
31	k.o.	Medium	0.8 (0.7–0.8)	<0.1	75.6 (39.8–140.8)	64.3 (10.2–152.5)	437 (152–717)
	Heterozygote		1.6 (1.5–1.6)	8.6 (7.7–9.6)	0.5 (0.1–1.0)	20.0 (17.1–22.3)	47 (39–57)
	Wild-type		1.2 (1.1–1.6)	21.4 (18.2–24.8)	0.5 (0.2–0.9)	18.6 (14.7–20.8)	43 (34–56)
44	k.o.	High	0.6 (0.5–0.9)	<0.1	19.8 (16.4–26.7)	24.5 (0.9–54.3)	56 (42–85)
	Heterozygote		1.8 (1.6–1.9)	6.5 (5.9–7.0)	1.4 (1.3–1.5)	23.5 (22.1–24.2)	50 (37–73)
	Wild-type		1.5 (0.8–1.9)	17.5 (13.0–20.3)	0.7 (0.1–1.4)	23.3 (17.6–29.7)	55 (40–73)

^a See Table I and “Experimental Procedures” for treatment conditions.

^b Indicated are mean values and ranges (in parentheses); for each age group 3–7 animals were tested, except only 2 k.o.-animals for none and low treatments.

^c Phenylalanine was measured by Tandem-MS in blood spots (Guthrie cards). For the non-treated mice, blood phenylalanine was determined at day 7.

^d k.o., knockout.

^e n.m., not measured.

^f ND, not detectable.

mitter precursors, and a high treatment with roughly 3- to 10-fold higher concentrations. Each treatment group contained 7–14 control animals, *i.e.* wild-type or heterozygotes, and 3–6 *Pts*^{-/-} mice. The body weight of each animal was monitored daily and is depicted for each treatment group in Fig. 2.

As mentioned before, the *Pts*^{-/-} animals with the low treatment did not survive for more than 3 weeks. A similar situation was encountered with the medium treatment, where survival of the *Pts*^{-/-} animals was prolonged but they eventually died between day 31 and 40 after birth (at a certain point knockout animals had to be sacrificed due to progressively poor conditions and in agreement with the Rules and Guidelines for the Care and Use of Laboratory Animals of the State of Zurich). In contrast, all *Pts*^{-/-} animals with the high treatment survived and were in relatively good health conditions. The experiment was stopped after 6 weeks of treatment, where all animals were sacrificed for biochemical analysis (see below). As shown in Fig. 2 (A–C), newborn mice regardless of the treatment mode gained weight without significant differences between genotypes for about 2 weeks and underwent pronounced growth retardation during week 3 (days 15–21). Only the *Pts*^{-/-} mice with low treatment seemed to be slightly different from their normal littermates, as they had reduced growth rate almost from birth (see low at day 3 in Fig. 3). This developmental difference in the low treatment group was even more pronounced later, as illustrated also in Fig. 2D, which shows a 7-day-old *Pts*^{-/-} animal in comparison with an age-matched heterozygous littermate. After the period of growth stagnation, the *Pts*^{-/-} animals stopped gaining weight independently of the treatment level, whereas all wild-type and heterozygous mice grew normally. The diminutive body size of *Pts*^{-/-} mice was best visible for those that survived due to high treatment, where the body weight was 34% of control at the age of 7 weeks (7.8 ± 1.5 g for *Pts*^{-/-} mice compared with 23.1 ± 2.4 g for combined controls; Fig. 3 medium and high at days 23, 31, and 44). Further qualitative characteristics of these otherwise healthy dwarf mice included reduced activity, hypersalivation, difficulty in swallowing, dystonia, tremor, and no signs of sexual maturation. Additional but less pronounced or steady characteristics were fair hair due to light pigmentation, hair loss, and hypothermia. In summary, treatment of *Pts*^{-/-} mice with BH₄ and

neurotransmitter precursors resulted in rescue from lethality but severe dwarfism.

Biochemical Analysis of Sacrificed Mice following Different Treatment Protocols—For enzymatic and metabolite analyses in liver, blood, and brain, all groups of treated mice were sacrificed at day 23 for low, day 31 for medium, and day 44 for high treatments. Liver analysis was also carried out with untreated mice at day 1 after birth (see above), whereas the limited brain material from newborns allowed us to measure only nitrite plus nitrate (compare with Tables II and III).

As expected, PTPS activity in liver and brain was completely abolished in the *Pts*-knockout mice and reduced to roughly 45% in heterozygous animals compared with wild-type. Furthermore, in liver and brain of phenotypically normal mice, *i.e.* wild-type and heterozygotes, PTPS activity increased with age. Independent of the treatment level, blood phenylalanine, and liver BH₄ and GTPCH activity decreased in 23, 31, and 44-day-old knockout mice compared with controls. Low GTPCH activity in *Pts*^{-/-} mice was surprising, because it was expected that hyperphenylalaninemia and low BH₄ levels, as observed in these mice, result in an increase of GTPCH activity via the stimulatory action of GFRP (6) (see also “Discussion”).

Brain serotonin was severely lowered only in 23-day-old knockout mice with the low treatment, but ~50% of normal in knockouts with medium or high treatment. Instead, brain dopamine was not detectable in knockouts with the low treatment and also very low, between 3–11% of control, in the medium and high treatment. The brain metabolites for dopamine and serotonin, HVA and 5-HIAA, were only slightly reduced in *Pts*^{-/-} mice even under the high treatment conditions, with 52% of normal for HVA and 60% of normal for 5-HIAA (day 44 of treatment). Brain dihydroxyphenylacetic acid (DOPAC), which is the first degradation product of dopamine following the action of dopamine hydroxylase, was indistinguishable among knockout and wild-type or heterozygous animals from the medium and high treatment groups (between 30.2 and 67.9 pmol/mg; not shown).

The NOS activity in brain, as determined by measuring the sum of nitrate plus nitrite, revealed no difference among knockout and control animals and was independent of treatment levels. NOS activity in the brain of untreated *Pts*^{-/-} or normal

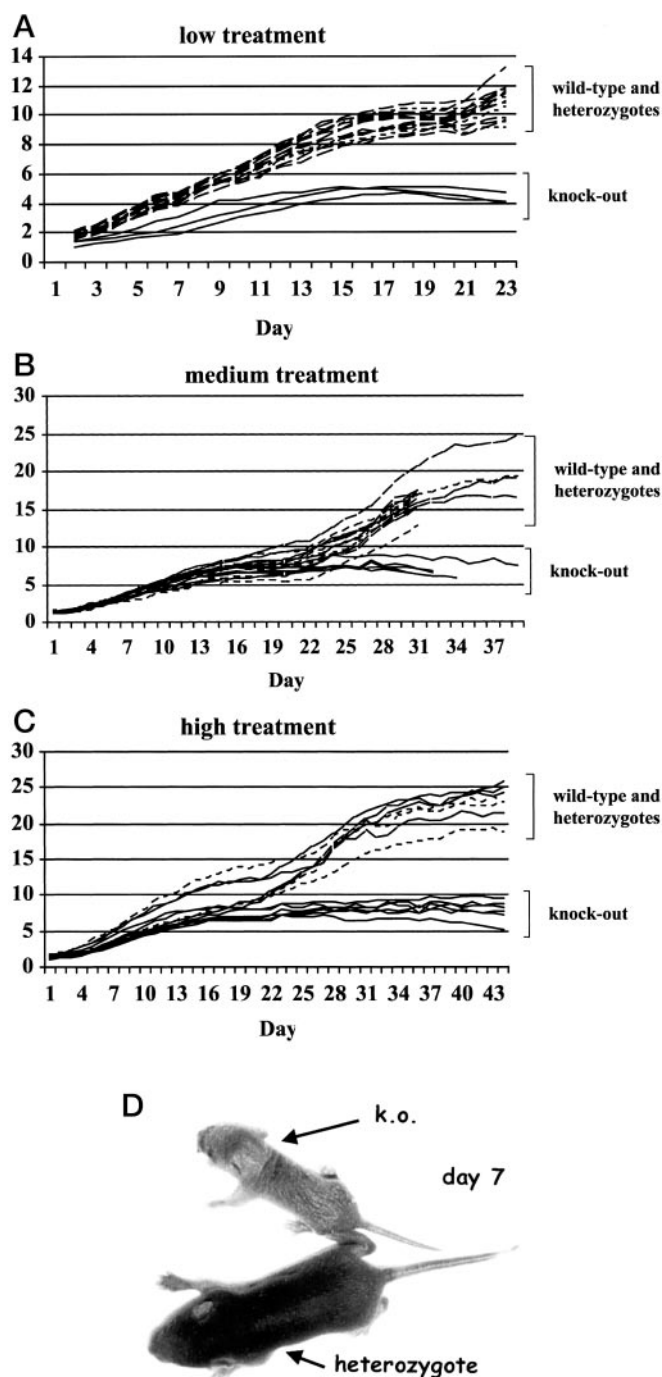


FIG. 2. Growth behavior following BH_4 , L-dopa, and 5-hydroxytryptophan treatment of *Pts*^{-/-} and control mice. Growth curves of colonies with low (A), medium (B), and high (C) treatments are shown. Full lines are for *Pts*^{-/-} animals (3 in A, 5 in B, and 6 in C), whereas the dashed lines are for the wild-type or heterozygous animals (14 in A, 13 in B, and 7 in C). D, Comparison of a 7-day-old *Pts*^{-/-} mouse with a heterozygous healthy littermate. Both animals were treated with the low dose. Details are described in the text.

mice at day 1 was severalfold higher compared with older animals but was also indistinguishable between the phenotypes (100.0–117.3 nmol/g of tissue at day 1).

The *Pts*^{-/-} knockout animals, independent of medium or high treatment conditions, exhibited extremely low dopamine and sub-optimal levels of BH_4 and serotonin. Furthermore, the only metabolic difference we observed among these two treatment conditions, which eventually lead to the death of animals with only the medium treatment, was the intermediate HPA.

*IGF-1 Is Severely Lowered in Rescued *Pts*^{-/-} Mice with Dwarfism*—Because the phenotypic characteristics of the dwarf mice might be a consequence of abnormal feeding behavior due to low dopamine and/or of hormonal deregulation, we wondered whether the pituitary growth hormone (GH), the thyroid hormone thyroxin (T_4), and the insulin-like growth factor-1 (IGF-1) in serum of knockout animals were reduced. As shown in Fig. 4, the serum IGF-1 levels in the knockout mice were reduced by a factor of 7 in comparison with age-matched controls (knockouts 79 ± 36 ng/ml; controls 541 ± 155 ng/ml). The expected sexual dimorphism between females (482 ± 170 ng/ml) and males (613 ± 113 ng/ml) is also clearly visible as published before (31). Furthermore, we also tested whether the pituitary-derived growth hormone (GH) and the thyroxin-stimulating hormone (TSH)-dependent thyroxin (T_4) were also reduced in these animals. However, we found no change in GH and T_4 (not shown), indicating that the pituitary gland is normally developed and thus not the primary reason for dwarfism in these treated *Pts*^{-/-} mice. Low IGF-1 might thus be the biochemical reason for the dwarfism, probably caused by the limiting brain dopamine, because abnormal feeding behavior was reported in dopamine-deficient mice (see “Discussion”) (32).

DISCUSSION

Here we report on treatment studies with a BH_4 -deficient mouse that was generated by targeted disruption of the *Pts* locus that encodes the second enzyme in the BH_4 -biosynthetic pathway. This model was used to study the role of the cofactor in metabolism and treatment. The importance of BH_4 for dopamine and serotonin production has been well established in patient studies, where treatment of cofactor deficiency by replacement with the precursors L-dopa and 5-hydroxytryptophan is required for neurotransmitter homeostasis and essential for survival (1). The observation that a complete knockout of BH_4 biosynthesis in the mouse leads to a phenotype with perinatal death fits the expectations regarding the absolute requirement of a cofactor with central metabolic importance. The lack of biosynthetic activity for catecholamines, which includes dopamine and norepinephrine, and for serotonin must be one of the primary reasons for the perinatal death, because these neurotransmitters are essential for postnatal survival (33). However, in contrast to human patients, we were surprised to find that the mice died almost immediately after birth with no visible abnormalities, an observation that was also made by Sumi-Ichinose and co-workers, and published during the course of our study (34).

We found only a few knockout mice surviving for up to 7 days after birth, probably due to the BH_4 present in mother's milk (35), while the amount of milk available is in turn dependent on the litter size and/or the mother's behavior. Data from ~250 PTPS patients, as compiled in the data base www.bh4.org, does not reveal perinatal lethality, and, although symptoms may be noted during the neonatal period, abnormalities develop typically during the first weeks of life (1). Furthermore, low birth weight and microcephaly, which are typical for PTPS deficiency in human newborns (36), were not observed in our mice. On the other hand, symptoms like hypersalivation and temperature instability are found in PTPS mice and human patients. Analysis of metabolites showed HPA and neopterin accumulation due to complete absence of PTPS activity, as expected (day 1, Table II). Brain metabolites at this age were only determined for NOS due to the limited material. However, monoamine neurotransmitters must be low as inferred from measurements of 23-day-old knockouts under low treatment that died despite initial treatment and had very low or not detectable brain serotonin and dopamine levels. Although BH_4 has a central

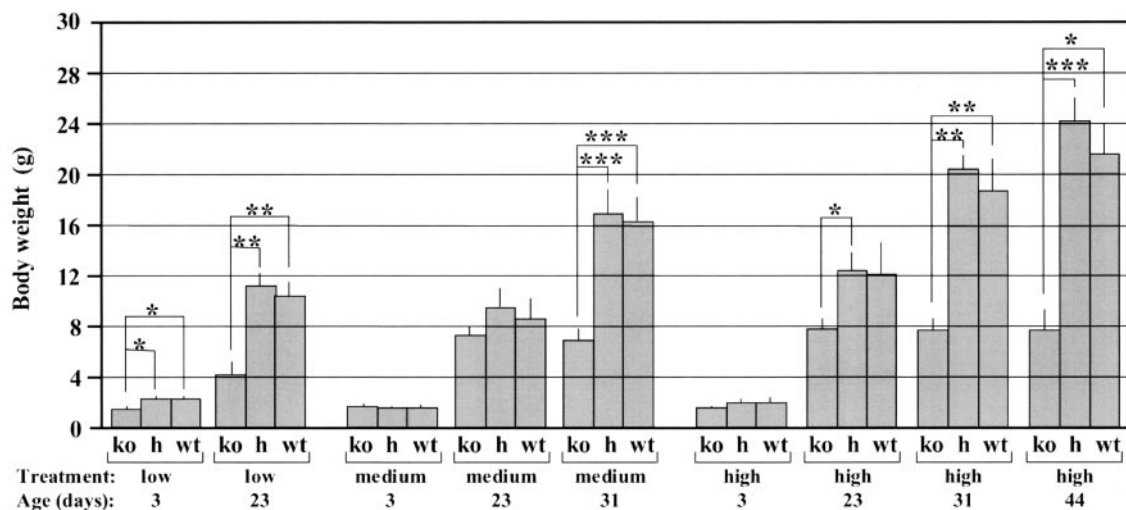


FIG. 3. Statistical analysis of data points from growth curves with low, medium, and high treatment. Shown are mean values and standard deviations for animals with low treatment at days 3 and 23, medium treatment at days 3, 23, and 31, and high treatment at days 3, 23, 31, and 44 (ko, knockout; h, heterozygous; wt, wild-type). Significant difference is indicated by asterisks: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ (Student's t test).

role for the function of the three NOS isoenzymes, *i.e.* vasorelaxation, immune response, and neurotransmission, no direct association with such pathologies in BH₄-deficient patients has been made. Only recently, however, we found that patients revealed reduced NO metabolites in cerebrospinal fluids independent of treatment. Implications from this study are that, under BH₄-deficient conditions NOS is uncoupled and produces by-products that are neurotoxic and thus responsible for neuronal cell pathology through peroxynitrite generation (2, 37, 38). Regarding the NOS activity in our mutant mouse presented here, we found no alterations in brain nitrate and nitrite levels, independently of treatment level or age (Table III). Also the mouse brain NOS activity was not reduced in untreated newborn knockouts, a phenomenon that may be explained by the fact that mother's milk contains high concentrations of BH₄ and that all NOS have a low K_D for BH₄ binding compared with the aromatic amino acid hydroxylases (K_D of 100–600 μM compared with K_D of 0.2 μM for nNOS) (39). Despite lethality during the first days of life, which is not typical for BH₄ deficiency in humans, we think that this *Pts* knockout is a suitable animal model for studying the pathophysiology and treatment of BH₄ deficiency.

During the initial treatment study where the recommended concentration of compounds and precursors for the treatment of human patients was administered orally (low treatment), we learned that this therapy did not rescue the mice. Accordingly, we found no normalization of the metabolites that are followed today to control treatment in human patients, *i.e.* plasma phenylalanine, brain bipterin, HVA, and 5-HIAA (Tables II and III). Furthermore, brain dopamine and serotonin were extremely low in the knockout mice with low treatment. These neurotransmitters are below detection levels in human CSF and thus can not be determined in patients. A simple explanation for the requirement of higher doses of precursors and compounds for treatment may be the fact that mice have a much higher metabolic rate than humans. For instance, the enzymatic efficiency of PTPS is roughly ten times higher for the mouse compared with the human enzyme (k_{cat}/K_m of recombinant PTPS from mouse is 2.5×10^4 and from human 2.8×10^3) (25). This hypothesis was corroborated by the fact that we could eventually rescue the animals by increasing the treatment doses.

The biochemical parameters under high treatment conditions revealed that plasma phenylalanine was normalized, and

biopterin was in the same range as in controls (Tables II and III). Serotonin and the neurotransmitter metabolites HVA and 5-HIAA were in the subnormal range (~50% of normal), whereas brain dopamine was unexpectedly low at 3% of normal (see below). Furthermore, plasma and brain neopterin remained elevated, and GTPCH was below normal activity, although hyperphenylalaninemia is expected to result in an increase of GTPCH activity. Moreover, under conditions of low BH₄ levels, as in *Pts*^{-/-} mice with low treatment, stimulation of GTPCH by the GTPCH-GFRP complex was expected to be even more pronounced, because the inhibitory action of BH₄ should also be diminished (4, 6). From the data presented here, we conclude that PTPS may have a direct or indirect effect on GTPCH expression or GTPCH-GFRP activity. Regarding hyperphenylalaninemia, increasing levels of oral BH₄ lead to a gradually decrease of blood phenylalanine. Whereas the medium treatment exhibited an intermediate phenylalanine level, the high treatment conditions were required to completely normalize blood phenylalanine levels. A further observation that cannot be explained sufficiently at this point is that knockout animals under medium treatment consistently did not survive for more than 4 weeks, whereas under high treatment none of the mutants died. The only metabolic difference we observed between these two treatment procedures was the slightly elevated plasma phenylalanine levels in the animals treated with the medium dose (see Tables II and III). It is unlikely that a mild HPA has such a dramatic effect on growth and development, and additional treatment studies must be conducted to learn more about these differences.

The most remarkable observation made while treating the *Pts*^{-/-} mice was the consistently reduced growth starting almost from the first days of life, leading to dwarfism. Normal growth and development are largely programmed during the first weeks of postnatal life by the pituitary growth hormone (GH) and thyroxin-stimulating hormone (TSH) (40). Furthermore, somatic growth is mediated mainly by circulating IGF-I, an insulin-like hormone produced mainly in the liver but also in many other tissues. At least two examples of dwarf mice are well described: the so-called Ames and Snell dwarf mice with recessive mutations in the homeotic genes *Pit-1* or *Prop-1*, respectively, with developmental arrest in pituitary ontogeny (40). Phenotypic characteristics are decreased growth rate post-weaning and reduced body size of adults, having approximately one-third of the weight of their normal siblings, similar

TABLE III
 Mouse brain values for GTPCH and PTPS enzymes and their metabolites, plus neurotransmitter and nitrite plus nitrate values

Age ^a	Genotype	Treatment ^b	GTPCH ^c micromoles/mg	PTPS ^c	Neopterin ^c	Biopterin ^c	Dopamine ^c pmol/mg	HVA ^c	Serotonin ^c	5-HIAA ^c	NO ^{c,d} nmol/g tissue
23	k.o. ^e	Low	0.07 (0.06-0.08)	n.m. ^f	7.0 (6.1-7.9)	0.9 (0.8-1.0)	ND ^g	0.5 (ND-1.0)	0.3	7.5 (4.9-10.1)	21.0 (20.5-21.5)
	Heterozygote		16.3 (2.4-24.3)	<0.01	6.4 (3.3-10.1)	16.8 (14.5-21.1)	22.6 (19.9-26.9)	4.8 (3.3-6.4)	63.3 (56.2-75.1)	30.9 (27.1-36.8)	
	Wild-type		26.0 (1.9-46.8)	<0.05	4.6 (2.3-6.2)	n.m.	15.4	n.m.	57.2	29.7 (29.4-30.0)	
31	k.o.	Medium	0.09 (0.09-0.10)	<0.05	5.3 (4.6-6.9)	4.8 (2.2-7.9)	3.5 (0.9-5.1)	18.4 (2.3-28.7)	6.0 (1.8-8.8)	44.9 (19.7-66.5)	22.0 (20.0-24.8)
	Heterozygote		0.10 (0.08-0.12)	31.9 (25.7-42.0)	<0.01	6.9 (6.6-7.0)	34.1 (28.9-41.9)	9.7 (8.9-10.1)	58.2 (52.9-62.6)	37.9 (19.7-69.1)	
	Wild-type		0.12 (0.09-0.14)	72.5 (69.7-75.5)	0.13 (0-0.4)	6.9 (6.4-7.3)	28.2 (26.6-29.0)	9.4 (9.1-9.5)	63.6 (55.2-71.2)	21.3 (14.9-30.9)	
44	k.o.	High	0.08 (0.07-0.09)	<0.05	4.5 (4.0-5.5)	5.5 (2.1-11.4)	1.2 (0.4-2.1)	12.0 (0.7-22.8)	4.8 (1.0-7.0)	36.8 (9.9-50.5)	20.7 (17.4-28.0)
	Heterozygote		0.12 (0.09-0.13)	29.7 (26.9-33)	<0.05	12.8 (6.6-24.8)	37.2 (30.7-44.9)	12.0 (10.6-13.3)	60.0 (58.7-62.5)	21.2 (20.5-22.8)	
	Wild-type		0.12 (0.11-0.15)	72.0 (67-74.8)	<0.01	7.0 (6.6-7.8)	38.1 (32.1-43.7)	22.1 (20.1-24.8)	63.3 (55.5-74.5)	29.6 (26.1-34.2)	

^a Days after birth. Note that from the brain of 1-day-old mice, not enough material could be prepared for these analyses.

^b See Table I and "Experimental Procedures" for treatment conditions.

^c Indicated are mean values and ranges (in parentheses); for each age group 3-4 animals were tested, except for low treatment, where in some instances only one or two animals were available for analysis.

^d NO, nitric oxide; sum of nitrite (NO₂⁻) and nitrate (NO₃⁻).

^e k.o., knockout.

^f n.m., not measured.

^g ND, not detectable.

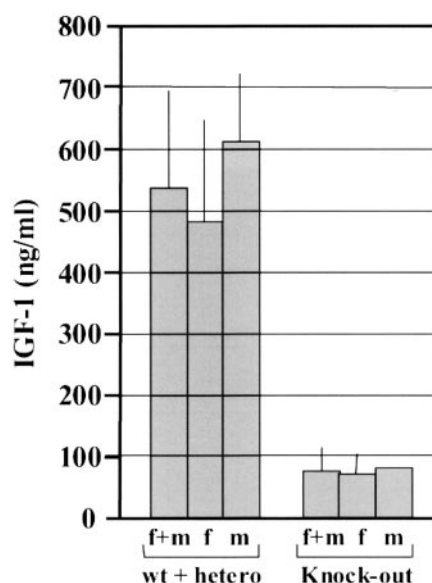


FIG. 4. Serum IGF-1 levels for 44-day-old mice under high treatment. Mean values and standard deviations in nanograms/ml are shown for normal animals (wild-type and heterozygote; $n = 9$) and knockouts ($n = 6$). The first bar is IGF-1 for both sexes ($f + m$), whereas f is for females and m for males only.

to what we found in our *Pts* knockout mice. Further characteristics reminiscent of our dwarf mice include delayed puberty, reduction of body temperature, tendency to experience hair loss, and reduction in plasma IGF-1. This prompted us to determine these hormones in our treated mice. In contrast to the Ames and Snell dwarf mice, we found normal levels for the pituitary GH- and TSH-dependent T₄, indicating that the pituitary gland developed normally and can thus be excluded as the primary reason for dwarfism of treated *Pts*^{-/-} mice. The IGF-1 level is influenced by GH but also by the nutritional status and food intake, which in turn is regulated by the dopamine and norepinephrine levels (41-43). For instance, it was reported that a knockout mouse that does not express tyrosine hydroxylase was unable to initiate feeding, an ability that can be restored by gene delivery of tyrosine hydroxylase into the striatum (32, 33, 44, 45). As also mentioned before, we found that the brain serotonin, and the neurotransmitter metabolites HVA, DOPAC, and 5-HIAA, although slightly below normal, were not much different from control levels. This is in sharp contrast with the actual dopamine levels of 3% of normal in the brain of mice under high treatment. Furthermore, the DOPAC/dopamine ratio (and the HVA/dopamine ratio), which is an index of dopamine turnover rate, was 1.8 in the wild-type and heterozygous mice group under medium and high treatment, but was 48 in both knockout mice groups, presumably reflecting the high turnover of the small dopamine pool (not shown). The extremely low brain dopamine together with the wealth of literature on the feeding behavior in dopamine-deficient mice mentioned above supports the assumption that abnormal (hypothalamic) neurotransmission is associated in our mice with disturbance of eating behavior. Although we did not measure daily food or water intake, we consistently observed difficulties in swallowing in our knockout mice groups and conclude that control of appetite is compromised in the treated *Pts*^{-/-} mice, and thus chronic undernutrition is responsible for low IGF-1 and dwarfism. Such feeding difficulties have been described for BH₄-deficient patients, but so far there was no indication of growth retardation or dwarfism. Nevertheless, we tested for IGF-1 in a first study with a very small group of BH₄-deficient patients and found specifically reduced plasma

IGF-1 levels in newborns with PTPS deficiency. Although these results are only preliminary, we believe it will be important to collect more data on IGF-1 levels and to follow growth and development in human patients with BH₄ deficiency.

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