

Tetrahydrobiopterin deficiency and dopamine loss in a genetic mouse model of Lesch–Nyhan disease

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Summary: Hypoxanthine–guanine phosphoribosyltransferase (HPRT) is an enzyme that catalyses the conversion of hypoxanthine and guanine into their respective nucleotides. Inherited deficiency of the enzyme is associated with a loss of striatal dopamine in both mouse and man. Although HPRT is not directly involved in the metabolism of dopamine, it contributes to the supply of GTP, which is used in the first and rate-limiting step in the synthesis of tetrahydrobiopterin (BH₄). Since BH₄ is required as a cofactor for tyrosine hydroxylase in the synthesis of dopamine, any limitation in the supply of GTP could interfere with the synthesis of dopamine. The current studies were designed to address the hypothesis that the reduced striatal dopamine in mice with HPRT deficiency results from reduced availability of BH₄. The mutant mice had small reductions in striatal BH₄, with normal BH₄ levels in other brain regions. Liver BH₄ was normal in HPRT-deficient mutant mice, and a phenylalanine challenge test failed to reveal any evidence for impaired hepatic phenylalanine hydroxylase, another BH₄-dependent enzyme. Although striatal BH₄ content is not normal, supplementation with BH₄ or L-dopa failed to correct the striatal dopamine deficiency of the mutant mice, suggesting that BH₄ limitation is not responsible for the dopamine loss.

Lesch–Nyhan disease is an inborn error of metabolism in which deficiency of the enzyme hypoxanthine–guanine phosphoribosyltransferase (HPRT) results in defective purine salvage (Jinnah and Friedmann 2000). Although the primary biochemical defect involves purine recycling, multiple studies have demonstrated abnormalities of striatal dopamine systems as well (Visser et al 2000). For example, neurochemical studies of autopsy material have revealed a 70–90% loss of striatal dopamine content (Lloyd et al 1981; Saito et al 1999). Neuroimaging studies of

the striatum have also revealed 60–70% reductions in fluoro-dopa uptake and the binding of WIN-35,428 to dopamine fibres (Ernst et al 1996; Wong et al 1996). Despite these findings, neuroanatomical studies of the brain at autopsy have revealed no evidence for dystrophy or degeneration of midbrain dopaminergic neurons (Crussi et al 1969; Saito et al 1999; Seegmiller 1968; Watts et al 1982).

Two strains of HPRT-deficient (HPRT⁻) mice have been produced as animal models for Lesch–Nyhan disease (Hooper et al 1987; Kuehn et al 1987). Like their human counterparts, these mutant mice display a significant loss of striatal dopamine, with no apparent anatomical abnormalities of the midbrain dopaminergic neurons or their fibres in the striatum (Finger et al 1988; Jinnah et al 1992, 1994, 1999). The loss of dopamine is selective in the HPRT⁻ mice, as these animals have normal levels of noradrenaline (norepinephrine), serotonin, and GABA. HPRT⁻ subclones of the PC12 rat pheochromocytoma cell line have also been produced as *in vitro* models for Lesch–Nyhan disease (Bitler and Howard, 1986; Yeh et al 1998). Despite a relatively normal morphological appearance *in vitro*, these HPRT⁻ PC12 subclones have a significantly lower dopamine content than normal PC12 cells. The consistent loss of dopamine in the HPRT-deficient human and mouse brain and cultured rat PC12 cells in the absence of any apparent morphological abnormality suggests an important metabolic connection between HPRT deficiency and dopaminergic systems.

Why HPRT deficiency is associated with impairment of dopamine metabolism remains enigmatic, since there is no direct relationship between purine salvage and the dopamine pathways. However, several investigators have noted that purine recycling indirectly contributes to the supply of GTP, which is utilized in the first and rate-limiting step in the synthesis of tetrahydrobiopterin (BH₄) as shown in Figure 1 (Giacomello and Salerno 1978; Goldstein, 1989; Nyhan 2000; Watts 1985). Since this cofactor is required by tyrosine hydroxylase for the synthesis of dopamine, any limitation in the supply of GTP could result in impaired dopamine synthesis. In addition, recent evidence has suggested that reductions in BH₄ can be associated with a loss of tyrosine hydroxylase protein (Furukawa et al 1999; Hyland and Munk-Martin 2001; Sumi-Ichinose et al 2001). Although there is no measurable loss of GTP in the HPRT⁻ mouse brain (Jinnah et al 1993), low GTP levels are associated with HPRT deficiency under certain conditions *in vitro* (Fairbanks et al 2002; Micheli et al 1999).

The purpose of the present studies was to determine whether the striatal dopamine deficiency of the HPRT⁻ mutant mice might result from reduced availability of BH₄. In comparison to controls, the HPRT⁻ mice had significantly lower BH₄ in the striatum. However, these mice had normal BH₄ in other brain regions and in the liver. In addition, the HPRT⁻ mutants did not exhibit a significant impairment in the hepatic conversion of phenylalanine to tyrosine by phenylalanine hydroxylase, a reaction also dependent upon BH₄. BH₄ supplements did not restore striatal dopamine to normal in the HPRT⁻ mutants, and administration of L-dopa provided no evidence that the striatal dopamine deficiency of the HPRT⁻ mutants could be normalized by bypassing the presumed defect in dopamine synthesis. These results

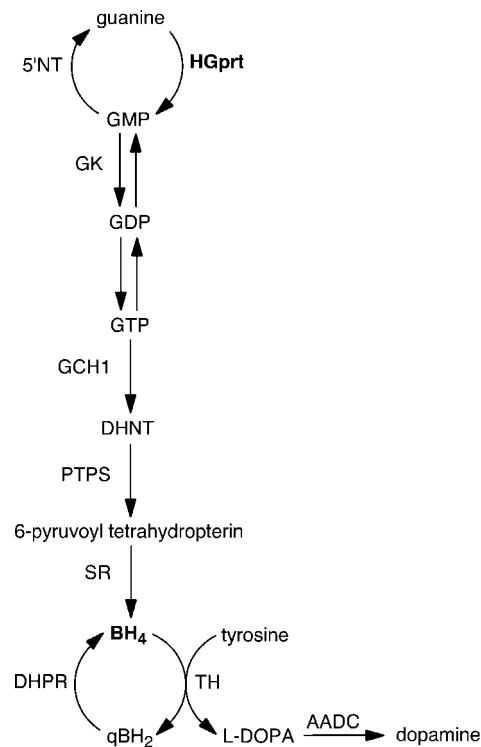


Figure 1 Proposed relationship between HPRT and BH₄ metabolism. HPRT indirectly contributes to the supply of GTP, which is a substrate in the first and rate-limiting step in BH₄ synthesis. 5'NT, 5'-nucleotidase; AADC, aromatic-amino-acid decarboxylase; BH₄, tetrahydrobiopterin; DHPR, dihydropteridine reductase; dihydroneopterin triphosphate; GCH1, GTP cyclohydrolase; GDP, guanosine diphosphate; GK, guanylate kinase; GMP, guanosine monophosphate; HGprt, guanosine triphosphate; HPRT, hypoxanthine-guanine phosphoribosyltransferase; PTPS, 6-pyruvoyltetrahydropterin synthase; qBH₂, quinoid dihydrobiopterin; SR, sepiapterin reductase; TH, tyrosine hydroxylase

reveal striatal BH₄ to be low in the HPRT⁻ mice, but they do not support the hypothesis that low BH₄ levels are responsible for impaired dopamine synthesis.

MATERIALS AND METHODS

Animals: HPRT⁻ (C57BL/6J^{HPRT.BM3}) mutants originally obtained from The Jackson Laboratories (Bar Harbor, ME, USA) were bred and raised in the Johns Hopkins University vivarium along with normal C57BL/6J mice as controls. Animals were housed 4–8 per cage, on a 14:10 hour light:dark cycle with free access to food and water at all times. All animal procedures were conducted with adult males 3–6 months of age in accordance with guidelines established by the Johns Hopkins

University Animal Care and Use Committee and described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

BH₄ measurement: Brains and livers were rapidly removed from 7 normal and 7 HPRT⁻ mice and placed on an ice-cooled platform. The brain was dissected as previously described to obtain striatum, cortex, hippocampus, brainstem and cerebellum (Jinnah et al 1994). Dissected samples were stored at -70°C until BH₄ was measured by HPLC with electrochemical detection as previously described (Hyland et al 1996).

Monoamine measurement: Monoamines were measured by HPLC with electrochemical detection as previously described (Jinnah et al 1999). Tissue extracts were eluted from a C18 reversed-phase MD-150 column (ESA Inc., Chelmsford, MA, USA) at a flow rate of 0.6 ml/min with a mobile phase consisting of 1.7 mmol/L 1-octanesulfonic acid sodium, 25 mmol/L EDTA, 0.01% tetraethylammonium and 8% acetonitrile in 75 mmol/L sodium phosphate buffer pH 2.9. Electrochemical detectors were set at 150, 250, 350 and 500 mV.

Phenylalanine challenge: The phenylalanine challenge test for evaluating BH₄ utilization was performed as previously described for the BH₄-deficient *hph-1* mutant mouse (McDonald and Bode 1988). Mice were given intraperitoneal injections of 1 g/kg phenylalanine and whole blood was collected at specific time points (0, 1, 2 and 4 h). A total of 5 normal and 5 HPRT⁻ mice were studied at each time point, except for the 1 h time point where 10 normal and 10 HPRT⁻ mice were studied. Whole blood was centrifuged at 500g for 5 min, and phenylalanine and tyrosine were measured in the serum as previously described (Hyland et al 1985).

BH₄ supplements: BH₄ supplements were provided to the HPRT⁻ mice to determine whether striatal dopamine synthesis or content could be restored to normal by correcting the presumed BH₄ deficiency. A total of 8 normal and 8 HPRT⁻ mice were given subcutaneous injections of 50 mg/kg BH₄ freshly dissolved in 1% ascorbic acid as a preservative every 4 h for six doses, a procedure previously reported to persistently increase basal brain BH₄ levels above control values for 24 h (Brand et al 1996). An additional 8 normal and 8 HPRT⁻ mice were treated in parallel with 1% ascorbic acid as controls. Half of the mice received intraperitoneal injections of 100 mg/kg NSD-1015 after the last BH₄ injection and 30 min before tissue collection to block the activity of aromatic-amino-acid decarboxylase for the measurement of tyrosine hydroxylase activity *in vivo* as previously described (Carlsson et al 1972; Jinnah et al 1994). At the conclusion of the drug treatments, animals were deeply anaesthetized with methoxyflurane and briefly perfused through the heart with 20 ml of ice-cold saline to eliminate BH₄ from the brain vasculature. Brains were rapidly removed and the striatum was dissected on a cooled platform. Tissues were stored frozen at -70°C prior to measurement of tissue BH₄ and monoamines as described above.

L-Dopa supplementation: L-Dopa supplements were provided to the HPRT⁻ mice to determine whether the striatal dopamine content could be restored by bypassing the presumed defect in tyrosine hydroxylase activity. The procedure followed that

previously shown to restore striatal dopamine content to normal in another mouse mutant with severe dopamine depletion due to complete absence of tyrosine hydroxylase (Chartoff et al 2001). In brief, 7 normal and 7 HPRT⁻ mice were given a single intraperitoneal injection of 50 mg/kg L-dopa plus 25 mg/kg carbidopa. A total of 6 normal and 6 mutant mice treated with carbidopa alone served as controls. Brains were collected 3 h after injection, and the monoamine content of the striatum was determined as described above.

Data analysis: BH₄ levels among different brain regions were compared by two-way ANOVA with genotype and region as the main factors, and post-hoc Tukey *t*-tests at $p < 0.05$ as the level of significance. Phenylalanine and tyrosine levels were compared by MANOVA, with genotype and time as the main factors. Monoamines were compared by MANOVA, with genotype and treatment condition as the main factors. A Bonferroni correction for four different measures was applied to arrive at $p < 0.0125$ as the level for significance to assure an overall $p < 0.05$, with $0.0125 < p < 0.05$ considered a borderline significant result.

RESULTS

Tissue BH₄: Tissue BH₄ concentrations were first measured to assess for overt BH₄ deficiency. The BH₄ content varied significantly among different tissues and brain regions of normal C57BL/6J mice (Table 1). The highest levels were measured in the liver. In the brain, the highest levels were in the striatum, with lower levels in other brain regions. Compared to normal mice, the HPRT⁻ mutants had significantly lower BH₄ levels in the striatum (-22.9%). Lower BH₄ levels in the cortex (-20.4%) and liver (-4.7%) of the HPRT⁻ mutants both fell short of statistical significance. The mutants had normal BH₄ levels in the hippocampus, brainstem and cerebellum. These results confirm the possibility that HPRT deficiency might be associated with reduced availability of BH₄.

Table 1 Tissue BH₄ content (nmol/g tissue)

	HPRT ⁺	HPRT ⁻	% change	<i>p</i> -Value ^a
Liver	5.92 ± 0.09	5.65 ± 0.11	-4.7	0.06
Brain				
Striatum	1.29 ± 0.05	1.00 ± 0.03	-22.9	<0.001
Cortex	0.39 ± 0.01	0.33 ± 0.01	-20.4	0.08
Hippocampus	0.37 ± 0.01	0.38 ± 0.01	+ 8.1	0.84
Brainstem	0.71 ± 0.02	0.74 ± 0.03	+ 4.2	0.30
Cerebellum	0.09 ± 0.02	0.10 ± 0.02	+ 11%	0.52

^a BH₄ values (nmol/g tissue) for liver were compared via Student's *t*-test, and for brain were compared via two-way ANOVA, with HPRT status and brain region as the main factors. This analysis revealed significant main effects for both HPRT status ($F = 13.5$, $p < 0.001$) and brain region ($F = 328.4$, $p < 0.001$). The interaction between HPRT and brain region was also significant ($F = 15.1$, $p < 0.001$). The table provides *p*-values for post-hoc Tukey *t*-tests

Phenylalanine challenge: Because steady-state tissue levels of BH₄ might underestimate an even greater defect of BH₄ synthesis in the HPRT⁻ mice, the phenylalanine loading test was used as a probe of hepatic BH₄ metabolism. This test is based on the rapid consumption of BH₄ by hepatic phenylalanine hydroxylase during the conversion of phenylalanine to tyrosine. Normal mice treated with 1 g/kg phenylalanine typically display a small transient increase in serum phenylalanine, but BH₄-deficient mice display a marked increase in serum phenylalanine that persists for >5 h (McDonald and Bode 1988).

The HPRT⁻ mice had normal serum phenylalanine and tyrosine concentrations at baseline (Figure 2). Serum phenylalanine increased 1 h after administration of 1 g/kg phenylalanine and returned towards normal within 4 h in both normal and HPRT⁻ mice. Serum tyrosine also increased 1 h after phenylalanine administration and returned to normal by 4 h in both normal and mutant mice. Neither phenylalanine nor tyrosine differed significantly between the normal and HPRT⁻ mice at any time. These results imply no limitation of hepatic BH₄ availability in the HPRT⁻ mice.

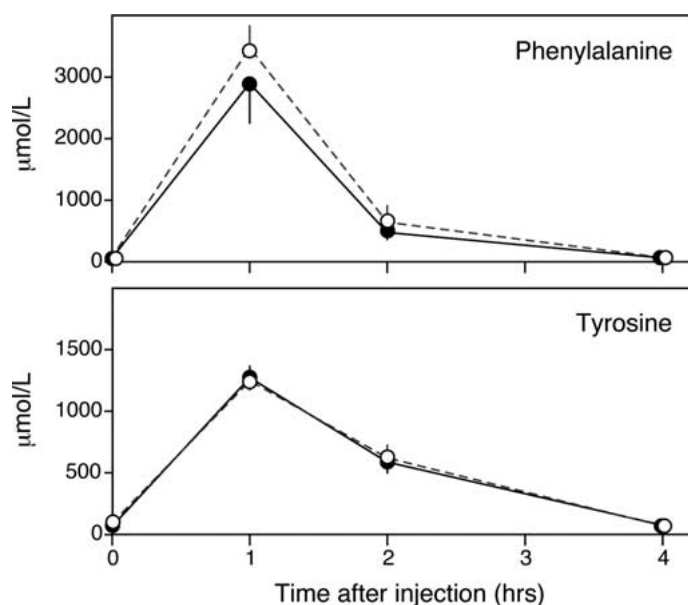


Figure 2 Phenylalanine challenge. Serum phenylalanine and tyrosine concentrations are shown as average values \pm SEM for 5–10 normal mice (closed circles) and 5–10 HPRT⁻ mice (open circles). Data for each amino acid were analysed separately by MANOVA with genotype and time after treatment as the main factors. For phenylalanine, there was a significant main effect for time ($F=140.3$, $p < 0.001$) but no significant effect for genotype ($F=0.4$, $p=0.55$) or interaction between time and genotype ($F=0.3$, $p=0.85$). For tyrosine, there was a significant main effect for time ($F=32.7$, $p < 0.001$) but no significant effect for genotype ($F=0.02$, $p=0.89$) or interaction between time and genotype ($F=0.08$, $p=0.99$)

BH₄ supplementation: A similar challenge to probe the physiological significance of the low striatal BH₄ is not available, so a different strategy was required. The first approach was to determine whether striatal dopamine synthesis or content could be normalized in the HPRT⁻ mutant mice by supplementing them with BH₄ for 24 h. Concentrations of L-dopa, the immediate product of the rate-limiting step in dopamine synthesis (Figure 1), are normally very low because of rapid metabolism to dopamine by excess aromatic-amino-acid decarboxylase. To measure L-dopa production more directly, some animals in this experiment were also treated with NSD-1015 to block aromatic-amino-acid decarboxylase 30 min before tissue collection. NSD-1015 does not typically have a significant effect on total dopamine stores in 30 min (Carlsson et al 1972; Jinnah et al 1994), so the total dopamine content could be measured simultaneously to determine whether it had been restored to normal during the preceding 24 h of BH₄ supplementation.

Compared to normal mice, the overall analysis revealed the HPRT⁻ mice to have significantly lower striatal dopamine (-40.3%) and 3,4-dihydroxyphenylacetic acid (DOPAC) (-31.0%), borderline lower homovanillic acid (HVA) (-19.6%), and normal L-dopa (Table 2). Consistent with the results of Table 1, the HPRT⁻ mutants also had significantly lower striatal BH₄ (-13.3%) with normal cerebellar BH₄ (Table 3). The treatments with 50 mg/kg of BH₄ for 24 h increased BH₄ by an overall

Table 2 Striatal monoamines (ng/mg protein) after BH₄ and/or NSD-1015 supplementation

	<i>L-Dopa</i>	<i>Dopamine</i>	<i>DOPAC</i>	<i>HVA</i>
No NSD-1015				
No BH ₄				
HPRT ⁺ (n = 4)	ND	198.6 ± 14.1	12.1 ± 0.9	16.3 ± 1.9
HPRT ⁻ (n = 4)	ND	95.7 ± 16.3	7.7 ± 0.6	11.6 ± 0.7
Plus BH ₄				
HPRT ⁺ (n = 4)	ND	191.7 ± 9.5	14.1 ± 1.9	20.5 ± 1.6
HPRT ⁻ (n = 4)	ND	116.5 ± 5.2	9.9 ± 1.6	16.1 ± 1.5
Plus NSD-1015				
No BH ₄				
HPRT ⁺ (n = 4)	11.4 ± 0.8	190.1 ± 13.9	3.7 ± 0.9	14.2 ± 2.5
HPRT ⁻ (n = 4)	10.8 ± 1.8	116.3 ± 7.2	3.1 ± 0.5	13.1 ± 2.2
Plus BH ₄				
HPRT ⁺ (n = 4)	11.1 ± 1.3	181.6 ± 11.6	4.8 ± 1.2	14.3 ± 3.0
HPRT ⁻ (n = 4)	10.9 ± 1.6	103.0 ± 14.8	3.2 ± 0.7	11.8 ± 2.6

Monoamines (ng/mg protein) are shown as average values ± SEM. ND = not detectable. The data for each analyte were examined separately by MANOVA with HPRT status, NSD-1015 treatment and BH₄ treatment as the main factors. A Bonferroni correction for four measures was used to stipulate $p < 0.0125$ for a statistically significant result, and $p < 0.05$ for a borderline result. The genotype effect was significant for dopamine ($F = 123.4$, $p < 0.001$) and DOPAC ($F = 15.0$, $p < 0.001$), borderline for HVA ($F = 6.3$, $p = 0.02$), and not significant for L-dopa ($F = 0.1$, $p = 0.8$). The effect of NSD-1015 treatment was significant for L-dopa ($F = 315.0$, $p < 0.001$) and DOPAC ($F = 108.9$, $p < 0.001$), borderline for HVA ($F = 4.8$, $p = 0.04$), and not significant for dopamine ($F = 0.2$, $p = 0.7$). The effect of BH₄ treatment was not significant for any metabolite: dopamine ($F = 0.1$, $p = 0.8$), L-dopa ($F = 0.01$, $p = 0.9$), DOPAC ($F = 3.9$, $p = 0.6$), HVA ($F = 2.1$, $p = 0.2$). Interactions between genotype and NSD-1015 treatment or genotype and BH₄ treatment were not significant for any metabolite.

Table 3 Brain BH₄ (nmol/g tissue) after BH₄ and/or NSD-1015 supplementation

	<i>Striatum</i>	<i>Cerebellum</i>
No NSD-1015		
No BH ₄		
HPRT ⁺ (<i>n</i> = 4)	1.46 ± 0.02	0.58 ± 0.03
HPRT ⁻ (<i>n</i> = 4)	1.27 ± 0.04	0.59 ± 0.04
Plus BH ₄		
HPRT ⁺ (<i>n</i> = 4)	1.88 ± 0.06	1.14 ± 0.08
HPRT ⁻ (<i>n</i> = 4)	1.76 ± 0.15	1.27 ± 0.17
Plus NSD-1015		
No BH ₄		
HPRT ⁺ (<i>n</i> = 4)	1.41 ± 0.04	0.60 ± 0.05
HPRT ⁻ (<i>n</i> = 4)	1.33 ± 0.04	0.57 ± 0.02
Plus BH ₄		
HPRT ⁺ (<i>n</i> = 3)	2.26 ± 0.15	1.43 ± 0.38
HPRT ⁻ (<i>n</i> = 4)	1.73 ± 0.08	1.22 ± 0.09

BH₄ contents (nmol/g tissue) are shown as average values ± SEM. The data were analysed by MANOVA with HPRT status, NSD-1015 treatment and BH₄ treatment as the main factors. The genotype effect was significant for the striatum ($F=22.1, p < 0.001$) but not for the cerebellum ($F=0.1, p=0.8$). The effect of BH₄ treatment was significant for both striatum ($F=126.3, p < 0.001$) and cerebellum ($F=82.0, p < 0.001$). No interactive effects were significant except for a three-way interaction among all three variables in the striatum ($F=7.2, p < 0.02$)

average of 39.8% in the striatum and 115.9% in the cerebellum (Table 3). The BH₄ supplements had no significant effect on dopamine or its metabolites, in keeping with previously published studies (Brand et al 1996). Because of differences in baseline dopamine, the possibility that BH₄ supplements were selectively increasing dopamine in the HPRT⁻ mutants was investigated by examining a statistical interaction between genotype and treatment. This interaction was not significant for dopamine, providing no evidence that BH₄ was restoring dopamine stores in the mutants (Table 2).

Treatments with NSD-1015 increased L-dopa in both normal and HPRT⁻ mice, with no evidence for significantly slower accumulation of L-dopa in the HPRT⁻ mice (Table 2). NSD-1015 also caused a decrease in DOPAC and a borderline decrease of HVA, with no influence on total dopamine levels. Taken together, these results do not provide evidence for significantly reduced tyrosine hydroxylase activity in the HPRT⁻ mice *in vivo*, whether or not they were treated with BH₄.

L-Dopa supplementation: Because exogenous BH₄ supplements might not penetrate the brain in sufficient quantities for a sufficient duration to influence striatal monoamines (Brand et al 1996; Levine et al 1987), a second strategy was used to probe the physiological significance of the low striatal BH₄. The reasoning for this experiment was that if low striatal BH₄ of the HPRT⁻ mutants was responsible for low striatal dopamine levels as a result of impaired tyrosine hydroxylase activity, then supplementation with L-dopa should restore dopamine levels by bypassing the presumed defect in dopamine synthesis. This approach has been shown to be effective for restoring even more severe dopamine deficiency of the tyrosine hydroxylase-deficient mutant mouse (Chartoff et al 2001).

Table 4 Striatal monoamines (ng/mg protein) after L-dopa challenge

	<i>L-Dopa</i>	<i>Dopamine</i>	<i>DOPAC</i>	<i>HVA</i>
Carbidopa controls				
HPRT ⁺ (<i>n</i> = 6)	1.72 ± 0.09	196.7 ± 10.5	10.4 ± 1.0	21.6 ± 2.3
HPRT ⁻ (<i>n</i> = 6)	1.29 ± 0.07	104.4 ± 5.6	8.7 ± 0.8	16.4 ± 1.2
L-Dopa + carbidopa				
HPRT ⁺ (<i>n</i> = 7)	2.61 ± 0.33	212.0 ± 10.6	26.2 ± 6.0	84.5 ± 9.9
HPRT ⁻ (<i>n</i> = 7)	2.01 ± 0.14	129.5 ± 1.41	20.8 ± 2.2	63.7 ± 4.4

Monoamines (ng/mg protein) are shown as average values ± SEM. The data for each metabolite were analysed separately by MANOVA, with HPRT status and treatment group as the main factors, and a Bonferroni correction for four measures stipulating $p < 0.0125$ for statistical significance and $p < 0.05$ for a borderline result. The genotype effect was significant for dopamine ($F = 119.0$, $p < 0.001$), borderline for L-dopa ($F = 6.9$, $p = 0.02$) and HVA ($F = 5.4$, $p = 0.03$), and nonsignificant for DOPAC ($F = 1.2$, $p = 0.3$). The treatment effect was significant for L-dopa ($F = 16.4$, $p < 0.001$), DOPAC ($F = 22.9$, $p < 0.001$), and HVA ($F = 114.6$, $p < 0.001$). The treatment effect was borderline for dopamine ($F = 4.8$, $p = 0.04$). Interactions between genotype and treatment were not significant for any metabolite (L-dopa, $p = 0.66$; dopamine, $p = 0.30$; DOPAC, $p = 0.62$; HVA, $p = 0.21$)

Compared to normal mice, the overall analysis revealed the HPRT⁻ mutants to have significantly lower dopamine (-46.9%), borderline lower L-dopa (-25.4%) and HVA (-23.9%), and normal DOPAC (Table 4). The slight changes in the profile of statistically significant differences among the metabolites between this experiment and the BH₄ supplementation experiment (Table 2) can be attributed to the relatively small magnitude of some of the differences, the differing numbers of animals in each experiment yielding varying statistical power, statistical effects that were sometimes close to a borderline result, and/or the varying influence of the supplements provided (BH₄ or L-dopa) on the dopamine metabolites. Additionally, the result for L-dopa must be interpreted with caution because it is close to the limit of detection (~1 ng/mg protein) without NSD-1015. However, the most relevant measure, dopamine, was consistently reduced in the HPRT⁻ mutants in both experiments (Tables 2 and 4).

Treatment with 50 mg/kg L-dopa plus 25 mg/kg carbidopa significantly increased striatal L-dopa and related metabolites in both normal and HPRT⁻ mutants. However, these treatments did not appear to restore striatal dopamine in the HPRT⁻ mutants to normal. Because of differences in baseline dopamine contents, the possibility that the treatments altered dopamine metabolism in the HPRT⁻ mutants more than in the normal mice was investigated by examining a statistical interaction between genotype and treatment. This interaction was not significant for any metabolite, providing no evidence that the L-dopa was being metabolized differently in the HPRT⁻ mice compared to normal.

DISCUSSION

Empirical evaluation of the BH₄ hypothesis: The results of these studies reveal the HPRT⁻ mutant mice to have significantly lower striatal BH₄ than normal littermates.

However, BH₄ was normal or borderline in four other brain regions and in the liver, which uses far more BH₄ than brain. In the HPRT⁻ mutants, phenylalanine loading provided results similar to those of normal mice, indicating no significant limitation of hepatic BH₄ availability. Similarly, supplementing the HPRT⁻ mutants with either BH₄ or L-dopa did not restore striatal dopamine content to normal, providing no evidence that the low striatal BH₄ was limiting dopamine synthesis by slowing the activity of tyrosine hydroxylase. Therefore, the small reductions of tissue BH₄ do not appear to be physiologically relevant for any tissue in the HPRT⁻ mice, and they do not support the hypothesis that the low striatal dopamine is due to the low BH₄.

Comparisons with other rodent models for BH₄ deficiency: Observations from other experiments also argue against the BH₄ deficiency hypothesis of dopamine loss in HPRT deficiency. Because of the proposed role of BH₄ in the HPRT⁻ mutants, it is valuable to compare them with other rodents with BH₄ deficiency. The *hph-1* mutant mice have a partial BH₄ deficiency as a result of a presumed defect in the gene encoding GTP cyclohydrolase, the rate-limiting step in the synthesis of BH₄ (McDonald et al 1988). As adults, the *hph-1* mutants exhibit >50% reduction of BH₄ in whole-brain homogenates (Hyland et al 1996). The reduced availability of BH₄ slows the activity of two BH₄-dependent enzymes, tyrosine hydroxylase and tryptophan hydroxylase, resulting in small reductions in brain dopamine, noradrenaline and serotonin (Hyland et al 1996). The *hph-1* mutants also have low hepatic BH₄, slowing BH₄-dependent phenylalanine hydroxylase and producing high baseline serum phenylalanine levels in pre-weanling animals. Administration of a phenylalanine load to the *hph-1* mutants also results in a marked elevation of serum phenylalanine that persists for >5 h (McDonald and Bode 1988). These abnormalities of brain monoamine and hepatic phenylalanine metabolism are not unique to the *hph-1* mouse. They can also be reproduced in normal rats by inducing partial BH₄ deficiency with the GTP-cyclohydrolase inhibitor 2,4-diaminohydroxypyrimidine (Suzuki et al 1988). Mice with more complete BH₄ deficiency as a result of targeted disruption of the gene encoding 6-pyruvoyltetrahydropterin synthase display a near-complete absence of all brain monoamines and do not survive more than 2 days after birth (Sumi-Ichinose et al 2001).

The HPRT⁻ mutants differ from these other rodent models of BH₄ deficiency in several respects. First, the HPRT⁻ mutants have only small reductions of BH₄ limited to specific brain regions, most notably the striatum (Table 1). Second, the HPRT⁻ mutants exhibit selective reductions of dopamine; noradrenaline and serotonin are both normal (Jinnah et al 1994). Third, hepatic BH₄ is essentially normal, with no evidence for significant impairment of the metabolism of a phenylalanine load (Table 1 and Figure 2). Aside from the lowered striatal BH₄, the condition of the HPRT⁻ mutant mice therefore does not resemble that of other rodents with bona fide BH₄ deficiency (Table 5).

The low striatal BH₄ levels in the HPRT⁻ mutant mice merit an explanation. The normal steady-state GTP levels previously reported for the brains of these mutants (Jinnah et al 1993) argue against the proposal that BH₄ levels are low because of limited availability of GTP as a substrate for GTP-cyclohydrolase in the synthesis

Table 5 Comparison of HPRT⁻ and *hph-1* mutant mice

	HPRT ⁻	<i>hph-1</i>
Tissue BH ₄		
Liver	Normal	Reduced
Brain ^a	Reduced	Reduced
Brain ^a monoamines		
Dopamine	Reduced	Reduced
Noradrenaline	Normal	Reduced
Serotonin	Normal	Reduced
Serum phenylalanine		
Baseline	Normal	Increased
Challenge	Normal	Markedly increased

^a The current study includes data for regional brain BH₄ and monoamine content in the HPRT⁻ mouse, but only whole-brain BH₄ and monoamine contents have been reported for the *hph-1* mouse (Hyland et al 1996)

of BH₄. Therefore, alternative explanations must be considered. First, a loss of nigrostriatal dopaminergic neurons or fibres could account for reduced BH₄, since the majority of striatal BH₄ is contained within these fibres. As noted in the introduction, however, dopamine neurons and fibres appear normal in the HPRT⁻ mutant mice (Finger et al 1988; Jinnah et al 1994). Another potential mechanism for reducing BH₄ is provided by observations that certain purines, such as guanine or 8-hydroxyguanine, can inhibit GTP cyclohydrolase (Yoneyama et al 2001). Any buildup of these compounds might therefore reduce BH₄ levels, but at present there is no empirical evidence for the accumulation of either of these purines in the HPRT⁻ mice. A third possibility is that BH₄ is being consumed by some other process, such as scavenging of excess superoxide radicals (Nakamura et al 2001; Visser et al 2002). Finally, there may be mechanisms for coordinated regulation of BH₄ with the related hydroxylases. Low levels of tyrosine hydroxylase protein are associated with BH₄ deficiency in human GTP cyclohydrolase deficiency (Furukawa et al 1999), the *hph-1* mouse (Hyland and Munk-Martin, 2001) and the 6-pyruvoyltetrahydropterin synthase knockouts (Sumi-Ichinose et al 2001). Coordinated regulation might be achieved because enzyme-cofactor complexes stabilize each against degradation, or it might occur at the level of gene transcription. Perhaps the most likely explanation for the low striatal BH₄ levels is that this region is metabolically more compromised by HPRT deficiency than other brain regions or tissues. The low striatal BH₄ levels may merely reflect a by-product of an unhealthy brain.

Relevance for Lesch-Nyhan disease: Although it is difficult to extrapolate these results with confidence to HPRT deficiency in humans, BH₄ deficiency provides an unsatisfactory explanation for the loss of dopamine in the Lesch-Nyhan brain as well. First, the neurobehavioural phenotype of Lesch-Nyhan disease does not resemble that of other patients with inherited defects in BH₄ metabolism, such as deficiency of GTP cyclohydrolase or dihydropteridine reductase (Blau et al 2001). Second, individuals with Lesch-Nyhan disease do not display hyperphenylalaninaemia or sensitivity to diets containing phenylalanine. Third, tissue BH₄ levels have not been measured

in Lesch–Nyhan disease, but urinary BH₄ levels are normal or even increased (Hatanaka et al 1990; Manzke et al 1986; Sebesta et al 1991). Fourth, there is a selective loss of striatal dopamine in the Lesch–Nyhan brain; noradrenaline is normal and serotonin is actually increased (Lloyd et al 1981). Finally, treatments effective for the inborn errors of BH₄ metabolism (L-dopa and/or 5-hydroxytryptophan) are of little benefit in Lesch–Nyhan disease (Jankovic et al 1988; Manzke et al 1986). Other mechanisms must therefore be sought to explain the association between HPRT deficiency and striatal dopamine loss.

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