
Biochemical hallmarks of tyrosine hydroxylase deficiency

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We report the biochemical hallmarks of tyrosine hydroxylase deficiency with emphasis on reliable diagnostic strategies of four new cases of an inborn error of tyrosine hydroxylase (TH). Three of our patients from different parts of the Netherlands were found homozygous for a mutation in exon 6 (G698A) of the TH gene, and one patient was found compound heterozygous for the same mutation and an additional mutation in exon 3. The first clinical symptoms of hypokinesia, rigidity of arms and legs and axial hypotonia, developed between 3 and 7 months of age. Cerebrospinal fluid investigations revealed a characteristic metabolite constellation in every case: low homovanillic acid (HVA) and 3-methoxy-4-hydroxyphenylethyleneglycol concentrations in the presence of normal reference range 5-hydroxyindolacetic acid concentrations. Strict adherence to a standardized lumbar puncture protocol and adequate age-related reference values are essential for diagnosis of this “new” treatable neurometabolic disorder. Urinary measurements of HVA, vanillylmandelic acid, and catecholamines can lead to false-negative conclusions. All patients showed a remarkable clinical improvement on a low dose of l-dihydroxyphenylalanine/(-)-2-(3,4-dihydroxybenzyl)-2-hydrazinopropionic acid. During treatment, cerebrospinal fluid HVA, and 3-methoxy-4-hydroxy-phenylethyleneglycol increased substantially.

The conversion of l-tyrosine to l-dihydroxyphenylalanine (l-dopa), catalyzed by the enzyme tyrosine hydroxylase (TH; EC 1.14.16.2), is the rate-limiting step in the biosynthesis of the catecholamines dopamine, norepinephrine, and epinephrine. The stereospecific enzyme, an iron-containing mixed function oxidase, requires molecular oxygen and a tetrahydropteridine cofactor and is present in specific brain areas, in all sympathetically innervated tissues, and in the adrenal medulla (1). The major catabolic product of dopamine is homovanillic acid (HVA; Fig. 1). The major catabolic product in the central nervous system (CNS) from norepinephrine is 3-methoxy-4-hydroxy-phenylethyleneglycol (MHPG) (2), whereas vanillylmandelic acid (VMA) is the major norepinephrine catabolite outside the CNS.

TH deficiency can cause the autosomal recessive form of dopa responsive dystonia (DRD); in its clinical form, it is also known as Segawa’s disease. The autosomal dominant form of DRD results from a mutation in the GTP I cyclohydratase gene (3). Clayton et al. (4) were the first to suggest TH deficiency for the recessive form of DRD. The TH gene on chromosome 11p15.5 was sequenced by Lüdecke et al. (5). A point mutation (Q381K) in exon 11 (5, 6) and a point mutation (L205P) in exon 5 (7) were found as disease-causing mutations in the two patients with TH deficiency described thus far.

In this study, we report the diagnostic methodology and the biochemical hallmarks of TH deficiency in the CNS. Four unrelated families with the characteristic clinical signs and symptoms of recessive Segawa’s disease are described. The patients share a point mutation (R233H) in exon 6 in the TH gene recently identified by van den Heuvel et al. (8). Three of our patients are homozygous for the R233H mutation, and one is compound heterozygous for this mutation and for another mutation in exon 3 of this gene. Specific biochemical analyses are compatible with a markedly reduced biosynthesis of dopamine in the CNS because of a deficient function of the TH enzyme system and may enable the diagnosis of additional patients worldwide.

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5 Nonstandard abbreviations: l-dopa, l-dihydroxyphenylalanine; TH, tyrosine hydroxylase; HVA, homovanillic acid; CNS, central nervous system; VMA, vanillylmandelic acid; CSF, cerebrospinal fluid; MHPG, 3-methoxy-4-hydroxyphenylethyleneglycol; 5-HIAA, 5-hydroxyindolacetic acid; DRD, dopa-responsive dystonia; 3-OMD, 3-o-methyl-dopa; and carbidopa. (S)-2-(3,4-dihydroxybenzyl)-2-hydrazinopropionic acid.

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Several steps are involved. Phenylacetic acid; 3MT, 3-methoxytyramine; D, aldehyde; alld., aldehyde dehydrogenase; alcd., alcohol dehydrogenase; dotted arrow, several steps are involved.

Fig. 1. Metabolism of serotonin and the catecholamines, showing the role of TH and the metabolites measured.

Neo, neopterin; GTP, guanosine triphosphate; NH₄TP, dihydroleoterin triphosphate; B₈H₄, tetrahydrobiopterin; qB₈H₄, quinonoid dihydrobiopterin; TR, tryptophan hydroxylase; COMT, catechol-ortho-methyltransferase; AADC, aromatic L-amino acid decarboxylase; MAO, monoamine oxidase; DOPAC, 3,4-dihydroxyphenylacetic acid; 3MT, 3-methoxytyramine; NM, normetanephrine; M, metanephrine; ALD, intermediate aldehyde (3-methoxy-4-hydroxyphenylhydroxylacetaldehyde); alld., alcohol dehydrogenase; dotted arrow, several steps are involved.

**Materials and Methods**

Standard substances, all of high analytical grade, and Sephadex G-10 were obtained from Sigma Chemical Co.; HPLC-grade methanol was obtained from Fisons; VMA urine standard, Iso-VMA internal standard, Lyphochek (quantitative urine control), and extraction columns for VMA (cat. no. 195-5005) and catecholamines (no. 189-2202) were obtained from Bio-Rad. All other chemicals were of analytical grade or higher.

**CSF and Urine**

By using a standardized protocol, lumbar CSF was collected between 0830 and 1200 and stored at −70 °C until analyzed. At the age of 0–1 year, the first 2 mL were used for HVA, 5-HIAA, and MHPG determination. At the age of 2–12 years, the first 5 mL of CSF were used for routine investigations; subsequently, the 3 mL were used for HVA, 5-HIAA, and MHPG determination. At the age >12 years, the first 8 mL were for routine CSF investigations, and the subsequent 3 mL were used for measurement of neurotransmitter metabolites. By using the same standardized protocol, reference values were established on CSF left over from routine investigations. Retrospectively, these patients were classified as suitable reference subjects because of lack of evidence for endocrine or metabolic abnormalities. Patients with epilepsy or extrapyramidal signs and symptoms were excluded. Appropriate studies ruled out immunological or chronic infectious diseases, deficiencies, and disorders caused by toxic agents. Samples containing red blood cells were eliminated. The study was approved by the ethical committee of the University of Marburg. CSF samples for the investigations of the gradient in the CSF column were obtained with informed consent of the patients involved.

Twenty-four-hour urine was collected into 5 mL of 3 mol/L hydrochloric acid and stored at −20 °C. Reference intervals for urine were obtained from children without metabolic disease, neuroblastoma, or phenochromocytoma.

**PROCEDURES**

(a) Measurement of HVA and 5-HIAA in CSF and urine. We adapted the method of Westerink and Mulder (9) for determination of HVA and 5-HIAA in CSF and urine with the following modifications: to 1 mL of CSF samples, 20 μL of 12.6 mol/L formic acid was added, and the pH ± 2.5 was controlled. The urine samples were centrifuged (10 min, 3000 rpm), and to 50 μL of supernatant, 5 mL Millipore water and 20 μL of 12.6 mol/L formic acid were added, and the pH ± 2.5 was controlled. Five hundred microliters of the CSF sample or urine sample were applied to the Sephadex G-10 column. After washing the Sephadex G-10 column successively with 3.5 mL of 25 mmol/L formic acid and 1.5 mL of 0.2 mol/L phosphate solution, the metabolites were eluted with 2 mL of 34 mmol/L ammonia in a tube containing 50 μL of 12.6 mol/L formic acid and 50 μL of 2.3 mmol/L ascorbic acid. HPLC was performed using a mobile phase of 0.2 mol/L phosphate solution and 0.1 mol/L citric acid (30:70, by volume), pH 3.5, and 350 mL of methanol; a Spectra-Physics SP 8800 gradient pump; a Spectra-Physics SP 8880 autosampler and a SP 8760 autosampler cooler (15 °C); and a 15-cm × 4.6-mm (i.d.) Nucleosil 5-μm RP-18 column. The flow rate was 1.0 mL/min. One hundred microliters of the eluate were injected into the system, and detection was by a Spark Holland amperometric detector with the analytical electrode set at +0.64 V, range × 1, offset × 0.1, and 50 nA full scale. Detector output was integrated by using the PC 1000 software system, Ver. 3.01 (Thermo Separations).

(b) Measurement of MHPG in CSF. We used the same method as described above for HVA and 5-HIAA with the following modifications. After the washing step of the Sephadex G-10 column, MHPG was eluted with 2.0 mL of 25 mmol/L formic acid and 0.5 mL of 0.2 mol/L phosphate solution. Chromatography was performed with a mobile phase of 6.25 mmol/L phosphate buffer, pH 4.0, containing, per liter, 6 mmol of citric acid, 6 mmol of sodium chloride, 7 mmol of sodium perchlorate, 1 mmol of octyl sodium sulfate, 0.2 mmol of sodium EDTA, and 5 mL of methanol; a Spectra-Physics SP8810 isocratic pump; a Rheodyne 7125 injector; and a 25-cm × 4.6-mm (i.d.)
Nucleosil 5-μm C18 column. The flow rate was 0.6 mL/min. Fifty microliters of the eluate were injected into the system, and detection was by an electrochemical detector Decade (Antec) with the analytical cell set at +0.70 V, range × 2, and damping 5. Detector output was integrated using the PC 1000 software system, Ver. 3.01 (Thermo Separations).

(c) Measurement of l-dopa and 3-OMD in CSF. l-dopa and 3-OMD were measured by HPLC on a 25-cm × 4.6-mm (i.d.) Prodigy 5-m 5 ODS-2 column in combination with fluorescence detection (excitation, 278 nm; emission, 325 nm) as described by Hyland (10).

(d) Measurement of VMA in urine. VMA in urine was determined by HPLC on a Bio-Rad RP-ODS 5 column in combination with electrochemical detection after sample preparation using a commercially available kit (Bio-Rad, no. 1955001).

(e) Measurement of dopamine, norepinephrine, and epinephrine in urine. After extraction of the catecholamines with cation exchange columns (Bio-Rad, no. 189-2202), we included an additional cleaning step on a Sephadex G-10 column. Forty microliters of 86.1 mol/L perchloric acid were added to 2 mL of the boric acid eluate, and after mixing 1 mL of this solution, it was applied to a Sephadex G-10 column. The column was washed with 2.5 mL of 25 mmol/L formic acid, and the catecholamines were eluted with 2.5 mL of 25 mmol/L formic acid. The catecholamine concentrations were determined with HPLC on a 15-cm × 4.6-mm (i.d.) Supelcosil 5-μm C18 column with electrochemical detection. The system was calibrated by injecting 50 μL of eluate after cation exchange and by cleaning on Sephadex G-10 with a solution containing, per liter, 200 nmol of dopamine, norepinephrine, and epinephrine and 200 nmol of dihydroxybenzylamine (internal standard) in 4 mmol/L formic acid.

Our patients came from four unrelated families from different parts of the Netherlands. The parents were all healthy. All patients presented with signs and symptoms characteristic of the recessive form of DRD. A detailed clinical description will be published elsewhere. In short, after normal pregnancy and delivery, progressive severe motor retardation with predominant extrapyramidal symptoms first became obvious at ages between 3 and 7 months, whereas psychosocial development appeared relatively healthy. The children appeared hypokinetic with mask face, rigidity of arms and legs, and axial hypotonia. No diurnal fluctuation in the symptoms was observed. Routine clinical chemistry, electroencephalogram, and magnetic resonance imaging and computed tomography neuroimaging were within normal reference intervals in all cases. After establishing the diagnosis of TH deficiency and starting therapy with l-dopa together with (S)-2-(3,4-dihydroxybenzyl)-2-hydrazinopropionic acid (carbidopa) there was a clear improvement of symptoms. In all cases, a G698A transition was found by direct sequencing of exon 6 of the TH gene. This transition produces an amino acid change from arginine to histidine (R233H) (8). Patient I was heterozygous for the G698A mutation and also for a one-base deletion in exon 3 of the TH gene. The other three were homozygous for the G698A mutation. CSF and urine of the children were investigated before any medication was initiated and also during therapy with l-dopa.

Results

The reference values

Table 1 shows our age-related reference values of the CSF metabolites HVA, 5-HIAA, HVA/5-HIAA ratio, and MHPG. All CSF samples included in this reference range study were obtained with the standardized protocol described in Materials and Methods. For the first 2 years of life, there were only relatively few data points available that could be included in the statistical calculation. There

| Table 1. Age-related reference values for CSF concentrations of HVA, 5-HIAA, HVA/5-HIAA ratio, and MHPG (all concentrations in nmol/L). |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | P<0.10          | 0.10 to 0.17    | 0.17 to 0.27    | 0.27 to 0.37    | 0.37 to 0.47    | 0.47 to 0.57    | 0.57 to 0.67    | 0.67 to 0.77    | 0.77 to 0.87    |
| n (HVA, 5-HIAA) | P 75.0 1142     | 895 664        | 789 769        | 716 668        | 668 540        | 540 372        |
| HVA             | P 2.5 543       | 478 488        | 429 384        | 346 339        | 339 211        | 211 87         |
|                 | P 50 780        | 566 557        | 605 510        | 505 449        | 449 397        | 397 213        |
|                 | P 97.5 1142     | 895 664        | 789 769        | 716 668        | 668 540        | 540 372        |
| 5-HIAA          | P 2.5 383       | 231 190        | 156 110        | 100 109        | 109 95         | 95 58          |
|                 | P 50 531        | 322 229        | 207 183        | 166 152        | 152 148        | 148 105        |
|                 | P 97.5 1028     | 618 301        | 275 265        | 245 214        | 214 173        | 173 190        |
| ratio HVA/5-HIAA| P 2.5 0.8       | 1.3 2.1        | 1.6 1.8        | 2.3 1.9        | 1.9 1.2        |
|                 | P 50 1.4        | 1.7 2.5        | 2.8 2.9        | 3.1 3.1        | 3.1 2.0        |
|                 | P 97.5 1.9      | 3.1 2.9        | 3.3 4.4        | 4.0 3.8        | 3.8 3.1        |
| n (MHPG)        | P 2.5 7        | 11 8           | 14 12          | 18 10          | 10 7           | 7 74           |
| MHPG            | P 50 116        | 67 49          | 54 50          | 49 54          | 54 53          | 53 44          |
|                 | P 97.5 277      | 116 71         | 71 64          | 75 68          | 68 82          | 82 64          |

*P, percentile.
was a marked effect of age on CSF metabolite concentrations. The median concentration of free MHPG decreases rapidly in the first year of life. In later life, the reference range for MHPG does not change substantially any more. The metabolites HVA and 5-HIAA decrease rapidly in early neonatal life and decrease steadily until they reach adult concentrations at the age of 15 years. The HVA/5-HIAA ratio increases from birth up to the age of 12 years. Lower values for this ratio are found in adults.

THE ROSTRO CAUDAL GRADIENT
To investigate a concentration gradient in CSF for the metabolites HVA, 5-HIAA, and MHPG in CSF from different levels of the spinocisternal system, consecutive CSF fractions from adults without deficiency in the neurotransmitter metabolism were taken and analyzed. There was a steep gradient for HVA and 5-HIAA in spinal CSF of two adult patients, with an increase of 60% in HVA concentrations and of 95% in 5-HIAA concentrations in the first two CSF fractions. Fig. 2 gives a representative example. From the first fraction to the last CSF fraction (35 mL), there was an increase of 124% for HVA and 173% for 5-HIAA. The HVA/5-HIAA ratio varied between 1.03 and 1.48 in the various fractions. The metabolite MHPG was unaffected by the volume fraction (Fig. 2).

THE STABILITY OF THE METABOLITES IN CSF
To investigate the stability of the monoamine metabolites in CSF, we took fresh CSF samples from two adult patients without neurometabolic disease. One portion of the CSF was immediately stored at −70 °C. Separate volumes of the sample of the first patient were left at room temperature (25 °C) for 1.5, 4, 20, 27, and 51 h and of the second patient for 1.5, 3, 4, 6, 23, and 27 h and subsequently stored at −70 °C.

The CSF HVA, 5-HIAA, and MHPG concentrations of the first patient were stable at room temperature (25 °C) for at least 27 h (HVA: mean 303 nmol/L, SD 10; 5-HIAA: mean 129 nmol/L, SD 6; MHPG: mean 47 nmol/L, SD 2; HVA/5-HIAA ratio: mean 2.4, SD 0.1) after CSF collection without any addition of antioxidants or buffers in the analyzed samples. After 51 h, the HVA concentration increased >2 SDs. In the second CSF sample, the metabolites were also found to be stable during 27 h at room temperature.

PRETREATMENT METABOLITE CONCENTRATIONS IN BODY FLUIDS
Clinical chemical routine investigations were within normal reference intervals in all patients, as were metabolic investigations including organic acids in urine and amino acids in urine, blood, and CSF. Phenylalanine was within normal reference intervals in all body fluids investigated. Tyrosine as substrate of tyrosine hydroxylase was in the normal reference interval in urine, plasma, and CSF [for CSF, patient I: 14 μmol/L (reference, 6–19 μmol/L); patient II, 13 μmol/L (reference, 6–19 μmol/L); patient III, 10 μmol/L (reference, 5–13 μmol/L); and patient IV, 12 μmol/L (reference, 5–13 μmol/L) per method according to Gerrits et al. (11). The pterin concentrations in CSF and urine (biopterin, neopterin, and their ratio) were all completely within the normal reference interval. Determinations of the dihydropteridine reductase activity in blood showed results within the normal reference intervals in all four patients (data not given). These findings exclude a defect of tetrahydrobiopterin biosynthesis or recycling.

Fig. 3 shows a characteristic chromatogram of the CSF metabolites HVA and 5-HIAA of patient III and of a control subject. In four patients with TH deficiency, analyses of the CSF revealed low concentrations of the dopamine metabolite HVA and the norepinephrine metabolite MHPG, whereas the serotonin metabolite 5-HIAA was always in the normal reference interval (Table 2). The HVA/5-HIAA ratio in CSF was abnormally low in all patients. These results were confirmed in repeat CSF samples from all four patients (data not shown).

The CSF concentration of HVA ranged from 8% of the
age-related lower reference range limit (percentile, 2.5) in patient IV to 30% in patient I. The CSF concentration of MHPG ranged from 6% to 37% of the lower limit of the age-related reference range (percentile, 2.5). Concentrations of CSF l-dopa were not detectable in all patients (reference, 0–10 nmol/L). Similarly, 3-OMD, a metabolite deriving from l-dopa (Fig. 1), was not detectable in all patients (reference, 0–50 nmol/L), and the concentration of vanillactic acid in urine was not increased, thus excluding aromatic l-amino acid decarboxylase deficiency.

Pretreatment urinary HVA (Table 3) was found decreased in three patients and within normal reference intervals in patient IV. The VMA concentration was decreased in all four cases. 5-HIAA concentrations in all samples were within the normal reference interval. Remarkable were the values within the normal reference intervals of the catecholamines norepinephrine, dopamine, and epinephrine in most of the urine samples, except the urine of patient III, which had decreased values of norepinephrine and dopamine. In all four cases, the epinephrine/norepinephrine ratio was increased (1.0 to 5.4; reference, <1). Unexpectedly, epinephrine was repeatedly found increased in the urine of patient I (about 255% above the upper limit of the age-related reference range). The concentrations of normetanephrine and metanephrine in two different urine samples of patient I were also measured: the normetanephrine concentrations were within the normal reference intervals, and the metanephrine concentrations were increased (about 38% to 257% above the upper limit of the reference range in the two samples).

**Table 2. CSF metabolite concentrations in four patients with TH deficiency before treatment (all concentrations in nmol/L).**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>HVA (P&lt;sub&gt;2.5&lt;/sub&gt;:384)</th>
<th>5-HIAA (P&lt;sub&gt;2.5&lt;/sub&gt;:110)</th>
<th>HVA/5-HIAA ratio (P&lt;sub&gt;2.5&lt;/sub&gt;:1.8)</th>
<th>MHPG (P&lt;sub&gt;2.5&lt;/sub&gt;:35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I, male</td>
<td>2 y 1 mo</td>
<td>117</td>
<td>151</td>
<td>0.77</td>
<td>13</td>
</tr>
<tr>
<td>II, female</td>
<td>2 y 5 mo</td>
<td>111</td>
<td>233</td>
<td>0.48</td>
<td>6</td>
</tr>
<tr>
<td>III, male</td>
<td>3 y 3 mo</td>
<td>76</td>
<td>268</td>
<td>0.28</td>
<td>12</td>
</tr>
<tr>
<td>IV, female</td>
<td>3 y 1 mo</td>
<td>31</td>
<td>234</td>
<td>0.13</td>
<td>2</td>
</tr>
</tbody>
</table>

*P, percentile; reference ranges of the metabolites are given in the top bar.

**Table 3. Urine metabolite concentrations in four patients with TH deficiency before treatment: HVA, 5-HIAA, and VMA concentrations in μmol/mmol creatinine and norepinephrine (NE), epinephrine (E), and dopamine (DA) concentrations in nmol/mmol creatinine.**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>HVA</th>
<th>Reference HVA</th>
<th>5-HIAA</th>
<th>Reference 5-HIAA</th>
<th>VMA</th>
<th>Reference VMA</th>
<th>NE</th>
<th>Reference NE</th>
<th>E</th>
<th>Reference E</th>
<th>E/NE ratio (reference, &lt;1.0)</th>
<th>DA</th>
<th>Reference DA</th>
</tr>
</thead>
<tbody>
<tr>
<td>I, male</td>
<td>2 y 1 mo</td>
<td>2.5</td>
<td>5–15</td>
<td>4.1</td>
<td>3–12</td>
<td>1.1</td>
<td>2–15</td>
<td>9.5</td>
<td>8–70</td>
<td>50.9</td>
<td>1–20</td>
<td>5.4</td>
<td>70</td>
<td>50–700</td>
</tr>
<tr>
<td>II, female</td>
<td>2 y 7 mo</td>
<td>4.1</td>
<td>5–15</td>
<td>7.6</td>
<td>3–12</td>
<td>1.2</td>
<td>2–15</td>
<td>13.5</td>
<td>10–100</td>
<td>21.2</td>
<td>1.5–30</td>
<td>1.6</td>
<td>195</td>
<td>70–975</td>
</tr>
<tr>
<td>III, male</td>
<td>3 y 3 mo</td>
<td>1.4</td>
<td>2–10</td>
<td>3.4</td>
<td>1–10</td>
<td>1.0</td>
<td>2–10</td>
<td>3.8</td>
<td>7–85</td>
<td>4.5</td>
<td>1.5–30</td>
<td>1.2</td>
<td>14</td>
<td>70–825</td>
</tr>
<tr>
<td>IV, female</td>
<td>3 y 1 mo</td>
<td>5.3</td>
<td>2–10</td>
<td>10.0</td>
<td>1–10</td>
<td>1.2</td>
<td>2–10</td>
<td>10.6</td>
<td>7–85</td>
<td>10.6</td>
<td>1.5–30</td>
<td>1.0</td>
<td>293</td>
<td>70–825</td>
</tr>
</tbody>
</table>
CSF neurotransmitter metabolites could be observed (Table 4). However, their concentrations did not normalize (compared with the lower reference range limit of percentile 2.5: HVA, 48–58%; MHPG, 51–94%; and HVA/5-HIAA, 44–84%). The CSF concentrations of l-dopa and 3-OMD were 1 to 55 times higher than the upper limit of the reference range in different samples (Table 4).

Using the treatment with the low dose of l-dopa, we also analyzed the catecholamines and metabolites in the urine of the patients and found a similar picture in all samples. As expected, we found high concentrations of dopamine (6 to 25 times higher than the upper limit) and HVA (within normal reference intervals to 4 times higher than the upper limit) and values within normal reference intervals for 5-HIAA, VMA, norepinephrine, and epinephrine. Without exception, the ratio of epinephrine/norepinephrine reached values within the normal reference intervals. In patient I, the increased concentration of epinephrine that was observed in two samples before treatment also fully reached values within normal reference intervals.

Discussion

ANALYTICAL ASPECTS WITH DIAGNOSTIC RELEVANCE

There are many pitfalls in accurate measurements and interpretation of neurotransmitter metabolites in CSF from sampling to analytical procedures. In agreement with previous reports (12–15), HVA and 5-HIAA concentrations were significantly correlated (r = −0.74 for HVA and −0.45 for 5-HIAA) with age. The metabolites HVA and 5-HIAA decrease with age over the first years of life and for MHPG over the first months of life. Therefore, adequate age-related reference values are of utmost importance. In accordance with previous studies (16, 17), a steep gradient from lumbar to ventricular CSF was observed for the CSF metabolites HVA and 5-HIAA. No gradient was found for the metabolite MHPG and for the HVA/5-HIAA ratio. For correct interpretation of neurotransmitter metabolite analysis, a standardized protocol including standardization of the time of lumbar puncture and of the CSF volume fraction used for analyses of neurotransmitter metabolite is essential. We have presented reference values for a specific volume fraction (see Materials and Methods). To our knowledge, this is the first study on neurotransmitter metabolites in CSF that uses defined CSF volume fractions and gives appropriate age-related reference values based on a standardized protocol for the lumbar puncture. Because of the interplay between neurotransmitters and their receptors, small deviations of metabolite concentrations or of ratios of metabolites can be of diagnostic importance. Over the last years, we were involved in the workup of several patients in whom a diagnosis could not be established initially because of sampling errors or interpretation difficulties because of inadequate reference ranges.

In CSF samples of two adult patients, we found the neurotransmitter metabolites HVA, 5-HIAA, and MHPG stable for at least 27 h at room temperature, in agreement with an earlier publication (18). In contrast, pterin concentrations in CSF are unstable at room temperature and are also light sensitive (13). Because pterin analysis is important in the differential diagnosis of genetic defects of biogenic monoamine metabolism, CSF samples for all of these investigations have to be kept in the dark and stored immediately at −70 °C.

When interpreting concentrations of conjugated catecholamines in urine, it is important to consider dietary influences. Particular caution is necessary with food known to contain biogenic amines, i.e., bananas (19, 20). An influence of diet on CSF catecholamine metabolites has not yet been fully investigated.

TH is mainly expressed in brain and adrenal medulla. Therefore, direct measurement of TH enzymatic activity in tissue samples is not a diagnostic option, and final proof can only be obtained by molecular genetic analysis. Until now, only two patients have been described in the literature with DNA-confirmed defects in TH (6, 7). Our experience suggests that TH deficiency is a rare but widely underdiagnosed inborn error of metabolism.

Table 4. Effect of l-dopa therapy of the TH-deficient patients on metabolite concentrations in CSF (all concentrations in nmol/L).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>l-dopa* doses, mg/kg/day</th>
<th>HVA (P 2.5:384)</th>
<th>5-HIAA (P 2.5:110)</th>
<th>HVA/5-HIAA ratio (P 2.5:1.8)</th>
<th>MHPG (P 2.5:35)</th>
<th>l-dopa (reference, 0–10)</th>
<th>3-OMD (reference, 0–50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I, male</td>
<td>3 y 7 mo</td>
<td>2.9</td>
<td>195</td>
<td>178</td>
<td>1.09</td>
<td>16</td>
<td>10</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>4 y 7 mo</td>
<td>5.0</td>
<td>223</td>
<td>148</td>
<td>1.51</td>
<td>33</td>
<td>22</td>
<td>741</td>
</tr>
<tr>
<td>II, female</td>
<td>3 y 2 mo</td>
<td>3.0</td>
<td>187</td>
<td>263</td>
<td>0.70</td>
<td>16</td>
<td>25</td>
<td>407</td>
</tr>
<tr>
<td></td>
<td>4 y 1 mo</td>
<td>4.4</td>
<td>193</td>
<td>206</td>
<td>0.94</td>
<td>17</td>
<td>18</td>
<td>506</td>
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<tr>
<td>III, male</td>
<td>3 y 11 mo</td>
<td>5.7</td>
<td>220</td>
<td>263</td>
<td>0.80</td>
<td>21</td>
<td>41</td>
<td>365</td>
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<tr>
<td></td>
<td>4 y 10 mo</td>
<td>6.8</td>
<td>185</td>
<td>199</td>
<td>0.93</td>
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<td>55</td>
<td>577</td>
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<tr>
<td>IV, female</td>
<td>3 y 7 mo</td>
<td>3.1</td>
<td>136</td>
<td>268</td>
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<tr>
<td></td>
<td>4 y 6 mo</td>
<td>4.2</td>
<td>147</td>
<td>191</td>
<td>0.77</td>
<td>18</td>
<td>15</td>
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* Carbidopa dose is 25% of l-dopa dose.

** P, percentile; reference ranges of the metabolites are given in the top bar.
DIAGNOSTIC HALLMARKS OF TH DEFICIENCY
TH deficiency leads to depressed concentrations of L-dopa and consecutively to low concentrations of the catecholamines dopamine, norepinephrine, and epinephrine. The measurement of phenylalanine and tyrosine in body fluids does not provide any clues to the diagnosis of TH deficiency. Furthermore, urinary measurements of VMA, HVA, and catecholamines can give values within normal reference intervals and lead to erroneous interpretations. The ratio of urinary epinephrine/norepinephrine may prove to be a useful indicator. As yet we conclude that the biochemical diagnosis of TH deficiency can only be made reliably by measuring neurotransmitter metabolites in CSF. Reduced dopamine synthesis leads to decreased CSF concentrations of HVA and MHPG. Together with unaffected pterin and CSF tyrosine and 5-HIAA concentrations, these findings are the diagnostic hallmarks of isolated TH deficiency.

RELATION BETWEEN MUTATION AND CSF NEUROTRANSMITTER METABOLITES
For all four patients, the CSF HVA concentration ranged between 8% and 30% of the lower reference range limit, whereas MHPG ranged from 6% to 37%. The patient (I) with the highest CSF HVA concentration is heterozygous for the G698A mutation in exon 6 of the TH gene and for another mutation in exon 3 of the TH gene. The other three patients are homozygous for the same mutation. Different mutations in the gene may produce a variable residual enzyme activity and therefore various CSF HVA and MHPG concentrations between patients. The different mutations may give rise to a wider clinical and biochemical spectrum.

THERAPY
There was a marked improvement of all clinical signs and symptoms under treatment with low doses of L-dopa/carbidopa. HVA concentrations in CSF increased substantially but still were below the lower reference range limit. After increasing the dose (Table 4), an additional clinical improvement could be achieved corresponding to an additional increase in HVA concentrations in CSF but still below the lower limit of the reference range. In earlier publications (4, 7), higher doses of L-dopa (up to 10 mg L-dopa/kg/day) were given, and a healthy HVA concentration in CSF could be achieved. However, some of our patients responded with hyperkinesia to increasing doses. Also, it should be considered that adverse effects may occur in the long run because the L-dopa therapy has to be continued life-long. Therefore, the therapy strategy in each patient has to find a balance between the short-term beneficial aspects and the longer term side effects of the therapy.

URINE ANALYSIS
The biochemical picture in urine warrants additional comments. Low to healthy HVA and VMA concentrations, together with healthy 5-HIAA concentrations, occur in all samples. The catecholamines are in the normal reference interval in many urine samples of our patients. Because TH in the adrenals and in the CNS derives from the same gene, TH in the adrenals is expected to be equally deficient in the patients. Therefore, the often healthy urinary catecholamine concentrations and the lack of clinical evidence for adrenal malfunction are ill-understood. Dietary influences may play a role in the healthy urinary concentrations of the catecholamines (19, 20). We have no explanation for the increased concentration of epinephrine in patient I in different urine samples and the disturbed epinephrine/norepinephrine ratio in all four cases. The high epinephrine concentration in the urine of patient I was also found in another laboratory using an independent technique. Because we also found high concentrations of metanephrine in the urine of the patient, these findings are unlikely to be artifacts. There has been speculation in the literature about alternative pathways in catecholamine metabolism (1, 21). Attempts have been made to find alternative pathways in vivo and to determine whether they are functionally important. Biosynthetic pathways from L-tyrosine to norepinephrine and epinephrine are possible via p-tyramine, octopamine, and synephrine (1, 21). Such alternative pathways of epinephrine and norepinephrine synthesis may become important in TH-deficient patients.

We thank N. Blau for measurements of pterins as well as of the activity of dihydropteridine reductase, N. Abeling for measurement of metanephrines, and the statistical department of the University Nijmegen for deliberation.

References


