Reduced folate transport to the CNS in female Rett patients

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Abstract—Background: Previous CSF studies in Rett syndrome suggest reduced turnover of the biogenic monoamines serotonin and dopamine. Because diminished turnover may result from CNS folate depletion, the authors studied transport of folate across the blood–brain barrier. Methods: In four patients with Rett syndrome, the authors measured CSF values of 5-methyltetrahydrofolate (5MTHF), biogenic monoamine end-metabolites, and pterins together with serum and red blood cell folate. In CSF, the overall folate binding capacity by the two soluble folate-binding proteins FBP1 and FBP2 (sFBP) was measured using a radioligand binding method for H³-labeled folate. A specific immunoreactive test (ELISA) detected sFBP1, which normally contributes to 30 to 35% of the total folate binding capacity. Genetic analysis included DNA sequencing of the MECP2, FBP1, and FBP2 genes. Empirical treatment with oral folinic acid was evaluated. Results: Two patients without and two with mutations of the MECP2 gene had normal values for red blood cell folate, serum folate, homocysteine, and methionine. In CSF, all patients had low values for 5MTHF, neopterin, and the serotonin end-metabolite 5-hydroxyindoleacetic acid (5-HIAA). Genetic analysis of FBP1 and FBP2 genes had normal results. Compared to controls, patients with Rett syndrome had normal immunoreactive sFBP1 in CSF, whereas the total folate binding capacity was disproportionately lowered. Empirical treatment with oral folinic acid normalized 5-MHTF and 5-HIAA levels in CSF, and led to partial clinical improvement. Conclusion: Irrespective of the MECP2 genotype, 5MTHF transfer to the CNS is reduced in Rett syndrome. Folinic acid supplementation restores 5MTHF levels and serotoninergic turnover. The lowered folate binding capacity of FBP is not explained by a defect of the FBP1 or FBP2 gene, but most likely occurs as a secondary phenomenon in Rett syndrome.

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Rett syndrome is a progressive neurologic developmental disorder occurring in girls with an estimated incidence of 1:10,000 to 15,000. Clinical features in patients with Rett syndrome include normal development until the age of 6 to 18 months followed by microcephaly, gradual loss of speech, autistic signs, ataxia, loss of purposeful hand function, intermittent hyperventilation, and stereotypic hand-washing movements. After initial regression a stable phase can continue into adulthood but seizures, spasticity of the lower limbs, and scoliosis will complicate the course at some time. However, the clinical phenotype and course can be variable. Amir et al. reported on a genetic marker in 5 of 21 patients with sporadic Rett syndrome due to spontaneously occurring heterozygous mutations within the Xq28-linked MECP2 gene, which encodes the methyl-CpG-binding protein. The MECP2 protein is important for the transcription repression of methylated DNA. It serves as an epigenetic regulatory protein linking methyl-CpG

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islands within promoter sequences of genes with the transcription repression machinery composed of Sin3A and histone deacetylase. Differentiated cells expressing the mutated MECP2 allele undergo overexpression of some normally silenced genes with detrimental consequences upon nervous system maturation, whereas the presence of one normal MECP2 allele due to the random X-inactivation pattern in females enables survival of differentiated cells. In men the mutated allele is expressed in all cells, explaining the lethal outcome of MECP2 mutations in males.

Previous studies in patients with Rett syndrome document diminished turnover of biogenic monoamines within the dopaminergic and serotoninergic axis. The origin of reduced biogenic monoamine turnover remains obscure. In this context, several inborn errors of metabolism are known leading to reduced synthesis of dopamine and serotonin, including a group of inborn errors of synthesis of tetrahydrobiopterin, the common co-factor for tyrosine- and tryptophan-hydroxylases, which represent the rate-limiting enzymes in the synthesis of dopamine and serotonin. Disturbed folate metabolism or folate depletion in humans and animals are other known causes of reduced biogenic monoamine turnover.

In this article, we describe new findings among four female patients with Rett syndrome with low normal or reduced biogenic monoamine turnover, hypothesized to be due to brain folate depletion as a result of reduced folate transport to the CNS. Further analysis of the soluble FBP fraction in CSF was performed.

**Patients.** Rett syndrome was diagnosed in four girls according to the criteria adopted by an international group. Consanguinity and other diseases did not occur in any of the families. Patients 1 and 2 manifested a severe phenotype of early onset with regression starting between age 6 and 12 months leading to a bedridden state (table 1). Patient 1 had sporadic clonic seizures, for which no anticonvulsant therapy was necessary. Her EEG revealed localized spike discharges within the vertex region with extension towards the temporo-occipital regions. In Patient 2, generalized myoclonic seizures with right hemispheric and secondary generalized EEG discharges began between age 2 and 3 years and responded to ethosuximide monotherapy. For both patients, high-resolution chromosome studies and MECP2 genetic analysis were normal. Neuroimaging revealed moderate frontotemporal brain atrophy. Metabolic investigations excluded disorders of amino- and organic acid metabolism; mitochondrial, lysosomal, and peroxisomal disease; and disorders of purine- and pyrimidine metabolism.

**Methods.** CSF analysis of biogenic amine metabolites, pterins, folate, and folate metabolites. After informed parental consent, lumbar punctures were performed according to a stan-

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### Table 1 Genetic data and clinical response after folinic acid treatment

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mut</th>
<th>RO, y</th>
<th>Motor</th>
<th>Seizures</th>
<th>EEG</th>
<th>Medication</th>
<th>Start (dur.), Cogn</th>
<th>Motor</th>
<th>Seizures</th>
<th>EEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>1</td>
<td>Bedridden</td>
<td>Sporadic clonic seizures</td>
<td>Vertex spikes, temp-occ spikes</td>
<td>None</td>
<td>5.9 (3.1)</td>
<td>Eye contact, recognition, looks TV</td>
<td>Pull to sit, rabbit hop, mobility ↑</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>0.5</td>
<td>Bedridden, floppy infant, hand washing</td>
<td>Gen. myoclonic seizures</td>
<td>Right hemispheric and sec. gen. discharges</td>
<td>Ethosuximide</td>
<td>4.3 (2)</td>
<td>Eye contact, recognition, looks TV</td>
<td>Stable sit, bear weight, tone ↑, stopped hand washing</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>R306C (TRD)</td>
<td>1.5</td>
<td>Gait ataxia, stable sitting, hand washing</td>
<td>Petig mal and clonic seizures</td>
<td>Multifocal and sec. gen discharges</td>
<td>Valproate, ethosuximide</td>
<td>4.5 (1.6)</td>
<td>Improved contact and recognition, looks TV</td>
<td>Less ataxic, able to run, grasps and eats with hands</td>
<td>Sporadic</td>
</tr>
<tr>
<td>4</td>
<td>R106W (MBD)</td>
<td>1.5</td>
<td>Gait ataxia, stable sitting, hand washing, rabbit hop</td>
<td>None</td>
<td>Vertex and gen. discharges</td>
<td>None</td>
<td>3.8 (2.5)</td>
<td>Improved contact</td>
<td>Tries to grasp, no motor improvement</td>
<td>Focal</td>
</tr>
</tbody>
</table>

* Poor compliance. Mut = MECP2 mutation; RO = regression onset; Start (dur.) = start and duration of folinic acid treatment; Cogn = cognitive functions; NI = normal; path = pathological; Temp-occ = temporo-occipital region; gen = generalized; sec = secondary; TRD = transcription repression domain; MBD = methyl-binding domain of the MECP2 gene.
We established a reference range for the concentration of immunoreactive FBP in CSF samples from 20 apparently healthy individuals by use of the present ELISA method (median value 0.24; range 0.14 to 0.38 nmol/L). The gel filtration profile of immunoreactive FBP in human CSF coincided with that of radioligand-bound FBP (functional FBP), both possessing a major peak at 100 kDa and a minor peak at 50 kDa. The results indicated above. Furthermore, the gel filtration profile did not contain any peaks of immunoreactivity not associated with functional radioligand-bound FBP.

For these reasons the calculated ratio between the concentration of immunoreactive FBP and radioligand-bound FBP in CSF from healthy individuals varies between 0.3 and 0.35 and must always be below 1.0. If the ratio of immunoreactive FBP to radioligand-bound FBP is near 1.0, this indicates the presence of a fraction of immunoreactive FBP, which does not possess any folate binding capacity; in other words, nonfunctional FBP.

Gene analysis of the FBP1 and FBP2 genes. The FBP1 gene maps to chromosome 11q13.3-q13.5 and is composed of seven exons spanning 6.7 kb, whereas the FBP2 gene maps to the same chromosome locus not more than 23 kb removed from the FBP1 gene. The FBP2 gene comprises five exons. After isolation of genomic DNA from whole blood, DNA sequence analysis was performed on PCR products of about 250 bp derived from specific primers based on the genomic sequence of the intron-exon boundaries for the FBP1 and FBP2 genes, as reported previously. FBP expression analysis was performed by quantitative reverse transcriptase PCR on peripheral blood lymphocyte RNA. Primers specific for the FBP1 isoform 1 and for sequences, which explains their similarity with respect to ligand binding characteristics and physicochemical properties. The soluble FBP of human and bovine milk, the membrane-bound and soluble form of epithelial FBP1, nonepithelial FBP2, and the soluble FBP-gamma show very similar characteristics. The membrane-bound FBP1 and FBP2 possess a signal peptide and GPI-anchor, whereas gamma-FBP in plasma does not.

Previous studies show that the two membrane-anchored high-affinity folate binding proteins FBP1 and FBP2 are mainly expressed at choroid plexus epithelium and mediate at this site the transfer of 5MTHF across the blood–brain barrier into the CNS. The choroid plexus FBP, encoded by the FBP1 and FBP2 genes, are composed of a signal peptide, attached to the membrane by a GPI-anchor, and a protein with one folate binding site. Part of the FBP-protein, called the soluble FBP fraction, will be released into CSF without losing its folate binding properties.

Binding of the radioligand H3-folate to FBP is used as the method of choice to determine the overall concentration of functional FBP in terms of exogenous folate binding capacity in CSF. The concentrations of FBP in two pooled samples of CSF collected from 30 to 40 healthy individuals and deprived of endogenous folates by matched controls. In addition, the substrates glycine, arginine, methionine, S-adenosylmethionine (SAM), and S-adenosylhomocysteine (SAH) were determined before folic acid treatment. The results were compared with previously described values in age-matched controls.

The results for biogenic monoamine metabolites, pterins, and the intermediary metabolites of the methylation pathway homocysteine, methionine, S-adenosylmethionine (SAM), and S-adenosylhomocysteine (SAH) were determined before folic acid treatment.

Blood cell folate values (data not shown). For all patients, lowered 5MTHF values in the presence of normal plasma folate were observed. For Patient 1 the CSF analysis before treatment demonstrated much higher initial dose of 1.8 mg/kg/day, the effect of which was not measured for an internal standard, the SOD1 RNA (primers 5′-ATGCAGCTGGCGCAAGAC-3′ and 5′-GAGAACTCGTCC-3′) were recorded at 95 °C, 1 minute at 60 °C, and 1 minute at 72 °C for 35 cycles. PCR products were run on automated gels (ABI 310, Applied Biosystems, Weiterstadt) and evaluated using ABI GeneScan software.

Treatment protocol. After the observation of low 5MTHF levels in CSF in Patients 1 through 4, empirical treatment for Patients 3 and 4 consisted of starting oral supplementation with folic acid at a dose of 0.8 to 1.3 mg/kg/day (leucovorin) combined with 1 mg vitamin B12 three times weekly. Because Patient 2 had to leave for Turkey during a period of 1 year, she was started on a much higher initial dose of 1.8 mg/kg/day, the effect of which was controlled by CSF analysis after 4 weeks. Follow-up studies during treatment of Patients 1, 3, and 4 included repeated clinical assessments, EEG records, and control lumbar punctures for measurement of biogenic monoamines, folate, and pterins after 3 to 6 months following treatment. If CSF 5MTHF did not normalize, the substitution of folic acid was increased. All studies and investigations were performed after informed parental consent was obtained.

Results. Biogenic monoamine metabolites, pterins, folate, and intermediary metabolites. Table 2 shows the CSF results for biogenic monoamine metabolites, pterins, and 5MTHF before and after folic acid substitution. In all patients the CSF analysis before treatment demonstrated lowered 5MTHF values in the presence of normal plasma levels of folic acid and vitamin B12 as well as normal red blood cell folate values (data not shown). For all patients, blood values for hemoglobin, erythrocyte indices, homocysteine, and amino acids were normal, thereby excluding macrocytic anemia or inborn errors of folate metabolism. At the time of low 5MTHF values, both the serotonin end metabolite 5HIAA and the neopterin values in Patients 1, 2, and 4 were well below the normal range of age-matched controls, whereas 5HIAA and neopterin values were still within the lower limit of the normal range in Patient 3. In Patient 2, a repeated CSF analysis 1 year later showed a...
further decrease of 5MTHF together with further decreases of 5HIAA, neopterin, and biopterin.

Table 3 summarizes the CSF values for methionine, homocysteine, SAM, and SAH. Before treatment the CSF analysis among all patients showed no marked increase of homocysteine. Methionine was low in one but normal in the other patient in which this could be measured. Subnormal values were found for SAM in three and for SAH in each of the four patients.

No accumulation of the intermediary purine metabolites glycaminamide ribotide and AICAR in CSF was found in Patients 2 and 3 (courtesy of Prof. Dr. G. van den Berghe).

Because Patient 1 showed neither a clinical nor a substantial CSF change on a daily folinic acid dose of 1.3 mg/kg, the dose was increased to 2.2 mg/kg/day. After treatment during 3 years, the previously bedridden Patient 1 was able to pull herself to a sitting position and managed to move herself forward in a kneeling/sitting position like a rabbit. Disappearance of clonic seizures and the previously described spike discharges in her EEG was noted.

After introduction of an initial higher dose of 1.8 mg/kg/day folinic acid in Patient 2, because she had to leave for Turkey, the CSF 5MTHF values raised after 4 weeks. On follow-up after 1 year, her seizures had stopped completely with normalization of the EEG. After treatment over a period of 2 years, she became more responsive and regained the ability to sit independently. In general, her muscle tone increased and she became able to bear her weight. Her hand-washing movements stopped but purposeful hand function was not regained.

In Patient 3 with petit mal status and clonic seizures, folinic acid substitution for 6 months (1 mg/kg/day) resulted in normalization of the CSF concentrations for

Table 2 Results of CSF measurements of biogenic amine metabolites (5-HIAA, HVA), 5-methyltetrahydrofolate (5MTHF), and pterins (neopterin, biopterin) before and after folinic acid replacement

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, y</th>
<th>Therapy</th>
<th>5-HIAA, nmol/L</th>
<th>HVA</th>
<th>Ratio HVA/5-HIAA</th>
<th>5MTHF, nmol/L</th>
<th>Neo</th>
<th>Bio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.8</td>
<td>None</td>
<td>36</td>
<td>327</td>
<td>9.1</td>
<td>30.9</td>
<td>5.6</td>
<td>29.7</td>
</tr>
<tr>
<td>2</td>
<td>3.1</td>
<td>None</td>
<td>153</td>
<td>481</td>
<td>3.1</td>
<td>32</td>
<td>7.5</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>4.3</td>
<td>None</td>
<td>42</td>
<td>341</td>
<td>8.1</td>
<td>12</td>
<td>5.5</td>
<td>10.1</td>
</tr>
<tr>
<td>4</td>
<td>4.4</td>
<td>Folinic acid</td>
<td>98</td>
<td>405</td>
<td>4.1</td>
<td>40</td>
<td>7.8</td>
<td>28</td>
</tr>
<tr>
<td>5</td>
<td>5.6</td>
<td>Folinic acid</td>
<td>164</td>
<td>440</td>
<td>2.7</td>
<td>50</td>
<td>5.1</td>
<td>17.7</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>None</td>
<td>129</td>
<td>410</td>
<td>3.2</td>
<td>0</td>
<td>16.2</td>
<td>25.1</td>
</tr>
<tr>
<td>7</td>
<td>3.8</td>
<td>None</td>
<td>105</td>
<td>462</td>
<td>4.4</td>
<td>14</td>
<td>7.7</td>
<td>23.7</td>
</tr>
<tr>
<td>8</td>
<td>4.2</td>
<td>Folinic acid</td>
<td>142</td>
<td>411</td>
<td>2.9</td>
<td>13.9</td>
<td>8</td>
<td>21.8</td>
</tr>
<tr>
<td>9</td>
<td>5.6</td>
<td>Folinic acid</td>
<td>134</td>
<td>381</td>
<td>2.8</td>
<td>58.8</td>
<td>10.3</td>
<td>27.9</td>
</tr>
<tr>
<td>Controls</td>
<td>2–4</td>
<td></td>
<td>202 (105–299)</td>
<td>603 (211–871)*</td>
<td>1.5–3.5†</td>
<td>63–111†</td>
<td>9–30†</td>
<td>10–30†</td>
</tr>
<tr>
<td>Controls</td>
<td>5–10</td>
<td></td>
<td>133 (88–178)</td>
<td>523 (144–801)*</td>
<td>1.5–3.5†</td>
<td>41–117†</td>
<td>9–20†</td>
<td>10–30†</td>
</tr>
</tbody>
</table>

*Median (range).
† Range.

Neo = neopterin; Bio = biopterin.

Table 3 CSF analysis before folinic acid substitution demonstrating the levels for methionine, homocysteine, S-adenosylmethionine (SAM), and S-adenosyl-homocysteine (SAH)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, y</th>
<th>Methionine, µmol/L</th>
<th>Homocysteine, µmol/L</th>
<th>SAM, nmol/L</th>
<th>SAH, nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.8</td>
<td>—</td>
<td>&lt;0.5</td>
<td>185</td>
<td>7.72</td>
</tr>
<tr>
<td>2</td>
<td>3.1</td>
<td>1.2</td>
<td>&lt;0.5</td>
<td>135</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>4.2</td>
<td>—</td>
<td>&lt;0.5</td>
<td>130.9</td>
<td>41</td>
</tr>
<tr>
<td>4</td>
<td>3.8</td>
<td>2.5</td>
<td>&lt;0.5</td>
<td>129.6</td>
<td>7.9</td>
</tr>
<tr>
<td>Control values</td>
<td>1.6–6.4</td>
<td>&lt;1</td>
<td>172–450</td>
<td>46–52</td>
<td></td>
</tr>
</tbody>
</table>

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5MTHF, an increase of 5HIAA, and gross reduction of clinical seizures with a dramatic recovery of her EEG record. The previously marked gait ataxia became less and she became able to run. Her hands regained the ability to grasp objects and she managed to eat with her hands.

Despite substitution with folinic acid, Patient 4 showed no substantial clinical improvement and even started to have additional focal clonic seizures. After folinic acid substitution for 5 months (0.8 mg/kg/day), 5MTHF in spinal fluid remained low, but after supplementation with higher dosages (1.6 mg/kg/day) for another 14 months, the 5MTHF, 5HIAA, neopterin concentration, and HVA/5HIAA ratio returned to normal. The mother admitted poor compliance to folinic acid, because she feared that folinic acid induced more seizures. She recently noticed improved contact and periods where her daughter made attempts to grasp for objects.

Folate binding protein studies. In Patient 1, the concentration of immunoreactive FBP was 0.24 nmol/L—i.e., within the reference range (0.14 to 0.38; n = 20)—but the concentration of radioligand-bound FBP was very low at 0.05 nmol/L. In Patient 2, the two CSF samples with lowered 5-methyltetrahydrofolate were analyzed for FBP at the age of 3.1 and 4.3 years. The first CSF analysis at the age of 3.1 years demonstrated an extremely low concentration of immunoreactive FBP in ELISA, 0.01 nmol/L (reference range 0.14 to 0.38; n = 20), whereas binding studies with H3-folate showed no detectable folate binding activity. The second sample analyzed at age 4.3 years showed a further decline of 5-methyltetrahydrofolate with a concentration of immunoreactive FBP of 0.20 nmol/L—i.e., below the values of 0.3 and 0.45 nmol/L observed in two pooled samples of CSF (table 4).

In Patient 2, the two CSF samples with lowered 5-methyltetrahydrofolate were analyzed for FBP at the age of 3.1 and 4.3 years. The first CSF analysis at the age of 3.1 years demonstrated an extremely low concentration of immunoreactive FBP in ELISA, 0.01 nmol/L (reference range 0.14 to 0.38; n = 20), whereas binding studies with H3-folate showed no detectable folate binding activity. The second sample analyzed at age 4.3 years showed a further decline of 5-methyltetrahydrofolate with a concentration of immunoreactive FBP of 0.20 nmol/L—i.e., within the reference range—however, without any detectable folate binding activity.

In Patient 3, two consecutive CSF samples showed normal concentrations of immunoreactive FBP of 0.14 at age 4 and 0.26 nmol/L at age 4.2 years. The concentration of radioligand-bound FBP was lowered to 0.07 at age 4, and 0.12 nmol/L at age 4.2 years.

In Patient 4, the CSF concentration of immunoreactive FBP at 0.44 nmol/L was slightly elevated, but the concentration of radioligand-bound FBP was low at 0.14 nmol/L.

Compared to healthy individuals, whose ratio between the concentration of immunoreactive and radioligand-bound FBP will vary between 0.3 and 1, the ratio for all patients with Rett syndrome exceeded a value well above 1.0. These findings in Rett syndrome suggested functional loss of FBP1 and FBP2 proteins (see table 4).

Genetic analysis. The promotor region, open reading frame, and intron-exon boundaries for both the FBP1 and FBP2 genes in Patients 1 and 2 showed no abnormalities as determined by genomic DNA sequence analysis. FBP expression studies in peripheral blood lymphocytes showed no differences in quantities of isoform 1 RNA and isoform 2 RNA in the patients compared to controls (data not shown).

Discussion. Irrespective of their MECP2 genotype the four female patients with Rett syndrome demonstrate lowered 5MTHF levels in CSF in the presence of normal serum folate, homocysteine, and methionine as well as folate content within red blood cells. Because de novo folate synthesis is not present in the CNS, it depends on adequate folate transport across the blood–brain barrier. Our findings suggest disturbed transport of folate across the blood–brain barrier due to nonfunctional FBP.

To understand the pathogenesis of disturbed folate transfer to the CNS, the two main folate transport mechanisms in humans have to be considered; i.e., the reduced folate carrier 1 (RFC1) and the folate binding proteins (FBP1 and FBP2). In contrast to RFC1, which is a ubiquitous membrane protein operating at high folate concentrations within the micromolar range, the membrane-attached FBP1 and FBP2 proteins show a tissue specific distribution. In the nanomolar range, the membrane-attached FBP1 and FBP2 proteins show a tissue specific distribution.

In Patient 3, two consecutive CSF samples showed normal concentrations of immunoreactive FBP of 0.14 at age 4 and 0.26 nmol/L at age 4.2 years. The concentration of radioligand-bound FBP was lowered to 0.07 at age 4, and 0.12 nmol/L at age 4.2 years.

In Patient 4, the CSF concentration of immunoreactive FBP at 0.44 nmol/L was slightly elevated, but the concentration of radioligand-bound FBP was low at 0.14 nmol/L.
whereas normal FBP1 and FBP2 protein function at the choroid plexus is a prerequisite for the active folate transport across the blood–brain barrier to the CSF compartment.

Figure 1 summarizes the mechanisms of active folate transport across the choroid plexus and intracellular folate accumulation. Due to the active vectorial folate transport across the blood–brain barrier,20,22 CSF folate levels are 1.5 to 2 times higher than blood folate levels.42-44 Based on this knowledge and the normal intestinal and red blood cell folate uptake in our patients, we assume a normal RFC1 function and therefore focused on the investigation of the folate binding proteins FBP1 and FBP2. In a first step we investigated the immunoreactive concentration of FBP1, which was normal. Analyzing the folate binding capacity of FBP, however, suggests the presence of nonfunctional FBP1 and FBP2. There is an obvious analogy between the present data for CSF and normal findings in human saliva where two species of immunoreactive FBP are found; i.e., a major fraction of nonfunctional FBP and a small fraction of functional (2%) FBP.45 Basically, the finding of a nonfunctional FBP may be due to several possibilities: 1) a cross-reacting protein with a common epitope but different from FBP, 2) occupation of folate binding sites by drugs or endogenous folate resistant to acidic hydrolysis, 3) genetic alterations of FBP1 and FBP2 affecting the binding site for folate, 4) a nonfunctional (unprocessed) precursor form of FBP or expression of a nonfunctional pseudogene, 5) errors in the process of post-translational folding of FBP and tertiary structure, necessary for folate binding, 6) post-translational modifications at the binding site of FBP, 7) disturbed mechanisms of folate accumulation and release within the choroid plexus, and 8) factors disturbing the process of endocytosis and the use of caveolae (i.e., potocytosis).

The first possibility that CSF sampled from the patients with Rett syndrome contains a cross-reacting protein, different from FBP, is highly unlikely because the anti-FBP antibodies used possess a high degree of specificity.25,46 The possibility that the anticonvulsant drugs valproate and ethosuximide, used in Patients 2 and 3, occupy the folate binding sites at the FBP is highly unlikely, because identical results are found among Patients 1 and 4, who received no medication at the time of investigation. Genetic mutations or deletions affecting the FBP gene sequence encoding for the folate binding site have been ruled out by the normal DNA sequence and expression of DNA transcripts for FBP1 and FBP2 in peripheral lymphocytes.

Expression of nonfunctional (unprocessed) FBP precursors or illegitimate expression of a FBP pseudogene within the choroid plexus can be speculated on, assuming the MECP2 mutation in Rett syndrome may lead to loss of appropriate transcription repression of methylated genes or pseudogenes, encoding for nonfunctional FBP or unprocessed precursors of FBP (figure 2). However, outside the nervous system there is little evidence for this hypothesis because in peripheral blood lymphocytes the quantitative ex-
pression of mRNA isoforms for FBP1 and FBP2 is shown to be normal, as is the folate content within erythrocytes. Defective post-translational folding and disturbed build-up of the tertiary structure of FBP, necessary for folate binding, can also result from overexpression of many other proteins of normally silenced genes interfering with the post-translational process and allocation of FBP to the cell membrane. Post-translational modification affecting the binding site of FBP caused by an unknown factor remains another option for which further FBP protein analysis is needed. Disturbed folylpolyglutamate storage and release within the choroid plexus appears highly unlikely because this does not fit with our finding of nonfunctional FBP in CSF. Finally, disturbed folate transfer across the blood–brain barrier may originate from many disturbing factors due to the mutated MECP2 gene in patients with Rett syndrome that compromise the proper expression of the complex machinery necessary for the process of endocytosis and potocytosis responsible for folate transfer across the blood–CSF barrier. However, on the assumption of disturbed endocytosis and lost functionally active caveolae, the FBP analysis will be expected to be normal, which is not the case.

Disturbed folate transport to the CNS associated with a nonfunctional FBP does not appear to be specific for Rett syndrome. In a recent article, we detected a group of eight patients (four boys and four girls, aged between 3 and 8.5 years) without MECP2 gene abnormalities whose CSF also contained moderately or severely decreased 5MTHF levels. The reported neurologic phenotype showed some resemblance to Rett syndrome and included postnatal microcephaly, severe retardation, spastic diplegia, ataxia, dyskinesia, and occasional seizures. In the two eldest patients a central visual disorder developed. Radioligand binding for folate was found to be equally lowered in all patients (0.02 to 0.11 nmol/L). In the four patients with the lowest CSF folate concentration (ranging from 0 to 17 nmol/L), the immunoreactive FBP concentration (ELISA) appeared to be elevated and upregulated to a value between 0.48 to 0.56 nmol/L, whereas the group of four patients with moderately decreased CSF folate (29 to 34.7 nmol/L) demonstrated FBP concentrations within the normal range between 0.24 to 0.48 nmol/L. Comparison of these preliminary FBP data among the two groups with the lowest range of CSF 5MTHF values from 0 to 17 nmol/L suggests that the four children without Rett syndrome are capable of upregulating their FBP expression (FBP ELISA 0.48 to 0.56 nmol/L) to some extent, whereas patients with Rett syndrome fail to upregulate their FBP values in a similar way at the time of lowest 5MTHF values in CSF (see tables 2 and 4).

Although the cause of the low CSF folate concentration itself remains uncertain, it is an important finding, as brain folate subserves many physiologic processes like turnover of pterins and biogenic monoamines, methyl-transfer processes, and de novo purine synthesis. Several studies among humans and animals reported on the connection between low
brain folate status and reduced turnover of pterins and biogenic monoamines as well as disturbed methyl-transfer processes associated with leukoencephalopathy and subacute combined degeneration of the spinal cord.\textsuperscript{10-12,48-50} One hypothesis assumed that 5MTHF is required as substrate together with the aid of the enzyme methylenetetrahydrofolate reductase as an alternative salvage pathway to regenerate tetrahydrobiopterin from quinonoid-dihydrobiopterin, the latter reaction normally being catalyzed by the enzyme dihydropterin reductase.\textsuperscript{51,52} In one patient with methylenetetrahydrofolate reductase deficiency, the reduced folate concentrations were accompanied by reduced biogenic amine metabolites and total biopterins in CSF.\textsuperscript{48} Several articles have provided indirect evidence for the connection between folate, pterin, and monoamine turnover.\textsuperscript{48-52} Our results confirm low neopterin concentrations and a reduction of biogenic monoamine turnover that can be corrected by folinic acid treatment.\textsuperscript{10-12} Our study only measured total biopterin but does not differentiate between the specific biopterin metabolites.

Figure 3 summarizes the mechanisms through which folate influences brain metabolism. Considering these metabolic pathways, impaired transport with consequent folate depletion in the CNS will be expected to affect the two N\textsuperscript{10}-formyltetrahydrofolate dependent steps of de novo purine synthesis with consequent limited production of ATP and GTP.\textsuperscript{53} Because GTP is the substrate for GTP cyclohydrolase-I in the first step of tetrahydrobiopterin synthesis,\textsuperscript{54} the low availability of its substrate GTP leads to lower production of neopterin and tetrahydrobiopterin with reduced activity of the three tetrahydrobiopterin-dependent aromatic amino acid hydroxylases, of which tryptophan- and tyrosine-hydroxylases represent the first rate-limiting enzymes for serotonin and dopamine synthesis. Although we find no accumulation of CSF purine metabolites and normal bioperin values, correction of brain folate depletion in Patients 1 and 4 is accompanied by a return of the previously low neopterin and 5HIAA toward normal. In Patient 2, in whom folic acid treatment restores the previously low 5HIAA levels, neopterin remains at subnormal values. However, the overall period with folic acid substitution is perhaps too short, as confirmed by the mother who admits to having interrupted treatment for some undetermined time during her 1-year stay in Turkey. In summary, a partial compromise by a low folate pool upon net GTP production remains an important hypothesis.

Limited de novo purine synthesis may also diminish the ATP pool with the derived NAD$^+$/NADH reserve, which functions as cosubstrate for the regeneration of tetrahydrobiopterin from quinonoid dihydrobiopterin by the enzyme DHPR. This provides another possible explanation for the link between folate, pterin, and monoamine metabolism.

CNS folate depletion may also be expected to lower methionine synthase activity, leading to homocysteine accumulation with reduced concentrations of methionine, SAM, and SAH.\textsuperscript{55} In our patients, CSF homocysteine concentrations are normal. Possibly this may be due to a sufficient capacity of the alternative transsulphuration pathway or due to an exhaustion of the described metabolic pathways because of a lack of intermediary substrates. The lack of the intermediary substrates SAM and SAH may result from the low precursor methionine and from diminished adenosyl-substrates feeding into the cycle due to reduced de novo purine synthesis associated with folate depletion.

In support of our findings is that the neurologic features in Rett syndrome such as microcephaly, mental retardation, epilepsy, and ataxia are reminiscent of
part of the spectrum of clinical manifestations encountered in hereditary defects of folate metabolism, like hereditary folate malabsorption, methylenetetrahydrofolate reductase deficiency, and methionine synthase deficiency. In addition, acquired folate or cobalamin deficiency leads to macrocytic anemia associated with serious neurologic deficits such as subacute combined degeneration of the brain and spinal cord, the latter being present in Rett syndrome. One neuropathologic report on two young women with Rett syndrome who died at ages 20 and 30 years showed degenerative spinal cord changes affecting the gray and white matter of both the ascending and descending tracts with loss of spinal ganglion nerve cells and reduction of the number of motor neurons. This resemblance of features between Rett syndrome and diseases with acquired folate deficiency and inborn errors of folate metabolism strongly supports our hypothesis about the important role that folate deficiency may play and exert upon the developing nervous system in Rett syndrome. The most important finding, however, is the clinical response after folate treatment leading to clinical stabilization with better social contact, some motor improvement, and reduction of seizures. Although the concentration of functional FBP is extremely low, the high supply with folinic acid apparently enables sufficient binding and transfer of folate to the CNS. Possibly, institution of folinic acid supplementation at an earlier age before the onset of regression may be of more sustained benefit and must be considered, and longer clinical follow-up is warranted. Based on these preliminary and suggestive data, clinical trials of folinic acid in Rett syndrome may be warranted.

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