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## 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 H<sup>3</sup>-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 folic 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 folic 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. Folic acid supplementation restores 5MTHF levels and serotonergic 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.<sup>1,2</sup> 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 2.<sup>3</sup> 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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**Table 1** Genetic data and clinical response after folinic acid treatment

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

\* 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.

islands within promotor 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* gene mutations in males.<sup>3-7</sup>

Previous studies in patients with Rett syndrome document diminished turnover of biogenic monoamines within the dopaminergic and serotonergic axis.<sup>8</sup> 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.<sup>9</sup> Disturbed folate metabolism or folate depletion in humans and animals are other known causes of reduced biogenic monoamine turnover.<sup>10-12</sup>

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. Fur-

ther 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.<sup>13</sup> 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 toward 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.

Patients 3 and 4 manifested a milder phenotype with no early signs of regression but moderately delayed psychomotor development, acquisition of a few words, and the ability to walk independently. However, both children developed regression after age 18 months with autistic features, hand stereotypies, and loss of speech. Patient 3 had petit mal status and clonic seizures with a good response to combined treatment with valproate and ethosuximide. Patient 4 did not have clinically manifest seizures but had typical electroencephalographic discharges within the vertex and paravertex region with intermittent generalized discharges. Genetic analysis of the *MECP2* gene showed a C to T transition at nucleotide 916 within the transcription repression domain in Patient 3 (R306C) and a C to T transition at nucleotide 316 in exon 2 within the methyl-binding domain in Patient 4 (R106W).<sup>3,4</sup>

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

standardized protocol between 8:30 and 10:00 AM for analysis of biogenic monoamine intermediate and end-metabolites—i.e., homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA), L-dopa, 5-hydroxytryptophan, 3-O-methyl-dopa, 3,4-dihydroxyphenylacetic acid (DOPAC), and 3-methoxy-4-hydroxy phenylglycol (MHPG). The content of the pterins bipterin and neopterin was measured as described previously.<sup>14-16</sup> The measured bipterin concentrations did not differentiate between the specific bipterin metabolites but represented the sum of tetrahydrobiopterin, quinonoid-dihydrobiopterin, and 7,8-dihydrobiopterin. The CSF concentration of 5-methyltetrahydrofolate (5MTHF) was measured in all patients. The intermediary metabolites of the methylation pathway homocysteine, methionine, S-adenosylmethionine (SAM), and S-adenosylhomocysteine (SAH) were determined before folic acid treatment.<sup>17</sup> The results were compared with previously described values in age-matched controls.<sup>16,18,19</sup> In addition, the substrates glycinamide ribotide and aminoimidazole carboxamide ribotide (AICAR) were measured in CSF. These substrates are expected to accumulate due to reduced activity of the N<sup>10</sup>-formyltetrahydrofolate dependent enzymes of purine de novo synthesis; i.e., glycinamide ribotide transformylase and aminoimidazole carboxamide ribotide transformylase. For each patient, folic acid, vitamin B12, homocysteine, and amino acids were determined in serum. Folic acid was also measured in erythrocytes.

**Investigation of folate binding protein (FBP).** The mammalian membrane-bound FBP is attached to the membrane by a glycosyl-phosphatidylinositol (GPI) anchor. The membrane-bound FBP and their derived soluble forms are highly preserved proteins with high-affinity folate binding and homology of their amino acid 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.<sup>20-23</sup> 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.<sup>20,24,25</sup> Binding of the radioligand H<sup>3</sup>-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.<sup>24</sup> The concentrations of FBP in two pooled samples of CSF collected from 30 to 40 healthy individuals and deprived of endogenous folates by acidic dialysis are 0.3 and 0.45 nmol folate bound/L<sup>24,26</sup>; binding affinity is 3.10<sup>10</sup> M<sup>-1</sup>.<sup>24</sup> The gel filtration profile of radioligand-bound FBP in Triton X-100 treated CSF samples exhibits a pattern<sup>24</sup> similar to that in other body fluids—e.g., milk, semen,<sup>27</sup> and saliva<sup>28</sup>—with a major peak (Mr~25 kDa) and a minor peak (Mr~100 kDa), the latter probably representing FBP containing a hydrophobic glycosylphosphatidylinositol anchor inserting into Triton X-micelles.

The N-terminal amino acid sequence of human milk FBP shows for more than 120 residues an identical sequence to FBP1.<sup>29</sup> To determine the concentration of immunoreactive FBP in CSF, we use a previously described ELISA, utilizing rabbit antibodies against human milk FBP.<sup>25,30</sup> Previous studies established that the used antibodies only cross-react with FBP1 in choroid plexus and renal tubules, known to have the highest expression of FBP1. There was no cross-reactivity with gamma-FBP in serum, nor with FBP2 known to dominate in prostrate, liver, testicular, and ovarian tissues. In accordance herewith antibodies against human milk FBP only immunoreacted with FBP1—e.g., in the choroid plexus.<sup>20</sup> However, even in choroid plexus cross-reactivity varied between 50% in homogenate and less than 20% in some subfractions, indicating that at least half of the membrane-bound FBP is composed of FBP2. Likewise, using these anti-human milk FBP1 antibodies, a CSF pool containing a mixture of sFBP1 and sFBP2 possessed a cross-reactivity of 30 to 35% in ELISA, whereas the remaining 65 to 70% of the radioligand-binding concentration represented the nonimmunoreactive FBP2.

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).<sup>25,30</sup> 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 25 kDa and a minor peak at 100 kDa,<sup>25</sup> similar to the results indicated above. Furthermore, the gel filtration profile did not contain any peaks of immunoreactivity not associated with functional radioligand-bound FBP.<sup>25</sup>

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 lower than 1.0. If the ratio exceeds 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,<sup>31</sup> 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.<sup>31</sup> 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.<sup>32</sup>

**FBP expression analysis** was performed by quantitative reverse transcriptase PCR on peripheral blood lymphocyte RNA. Primers specific for the RNA isoform 1 and 2 were designed (5'-Fam-ATCAGAGCTGGCGCAAAGAG-3' and 5'-GAACAG-TGGGTGTGGGGAAG-3' for isoform 1; 5'-Fam-CCTTTGTGAAG-GCCTCTGGA-3' and 5'-AGCATCAGGGCCAGACTGAG-3' for isoform 2) and amplification products were compared to those obtained for an internal standard, the SOD1 RNA (primers 5'-Fam-TGGGCCAAAGGATGAAGAGA-3' and 5'-CCACAA-GCCAAACGACTTCC-3'). PCR cycling conditions were 1 minute 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 1, 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 assessment, 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

**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

Patient	Age, y	Therapy	5-HIAA, nmol/L	HVA	Ratio HVA/5-HIAA	5MTHF, nmol/L	Neo	Bio
1	5.8	None	36	327	9.1	30.9	5.6	29.7
	6	Folinic acid	52	249	4.8	39.4	7.1	27.1
	6.2	Folinic acid	92	428	4.6	43.7	5.3	16.1
	6.6	Folinic acid	150	316	2.1	57.5	14.5	24.4
2	3.1	None	153	481	3.1	32	7.5	17
	4.3	None	42	341	8.1	12	5.5	10.1
	4.4	Folinic acid	98	405	4.1	40	7.8	28
	5.6	Folinic acid	164	440	2.7	50	5.1	17.7
3	4.1	None	129	410	3.2	0	16.2	25.1
	5	Folinic acid	198	449	2.3	65	11.8	18.8
4	3.8	None	105	462	4.4	14	7.7	23.7
	4.2	Folinic acid	142	411	2.9	13.9	8	21.8
	5.6	Folinic acid	134	381	2.8	58.8	10.3	27.9
Controls	2–4		202 (105–299)	603 (211–871)*	1.5–3.5†	63–111†	9–30†	10–30†
Controls	5–10		133 (88–178)	523 (144–801)*	1.5–3.5†	41–117†	9–20†	10–30†

\*Median (range).

† Range.

Neo = neopterin; Bio = biopterin.

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 glycylamide ribotide and AICAR in CSF was found in Patients 2 and 3 (courtesy of Prof. Dr. G. van den Berghe).

Table 1 summarizes the baseline data and clinical response after folinic acid substitution. Before treatment all patients manifested autism and complete loss of hand function with stereotypic hand washing; Patients 1, 2, and 3 had clinical seizures. Evaluation of the clinical effect after folinic acid and vitamin B12 supplementation showed improvement of social contact with recognition of persons and interest to surroundings among all patients. In contrast to the lack of interest before treatment, all patients now enjoyed watching television for about 30 minutes.

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

Patient	Age, y	Methionine, $\mu$ mol/L	Homocysteine, $\mu$ mol/L	SAM, nmol/L	SAH, nmol/L
1	5.8	—	<0.5	185	7.72
2	3.1	1.2	<0.5	135	15
3	4.2	—	<0.5	130.9	41
4	3.8	2.5	<0.5	129.6	7.9
Control values		1.6–6.4	<1	172–450	46–52

**Table 4** Concentrations of immunoreactive (ELISA) and radioligand-bound folate binding protein (FBP) among four patients with Rett syndrome

Patient	Age, y	ELISA, nmol/L	Radioligand binding, nmol/L	Ratio
1	5.8	0.24	0.05	4.8
2	3.1	0.01	No binding	>1
	4.3	0.20	No binding	>1
3	4	0.14	0.07	2
	4.2	0.26	0.12	2.16
4	3.8	0.44	0.14	3.14
		0.14–0.38*	0.3 and 0.45†	0.3–1‡

\*Reference values (n = 20).

† Values determined in two pooled samples of CSF.

‡ The ratio between the concentrations of immunoreactive and radioligand-bound FBP in CSF from healthy individuals varies between 0.3 and 0.35 and must always be lower than 1.0.

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 folic acid, Patient 4 showed no substantial clinical improvement and even started to have additional focal clonic seizures. For this reason the anticonvulsant drug sulthiame had to be started. After folic 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 folic acid, because she feared that folic 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—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 H<sup>3</sup>-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 promoter 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).<sup>33–35</sup> In contrast to RFC1, which is a ubiquitous membrane protein operating at high folate concentrations within the micromolar range,<sup>33</sup> the membrane-attached FBP1 and FBP2 proteins show a tissue specific distribution,<sup>36,37</sup> possess high affinity for folate in the nanomolar range, and therefore bind physiologic levels of folate.<sup>36,38,39</sup> *FBP* expression is inversely regulated by the extracellular concentration of folates.<sup>36,37</sup> The mechanism of FBP-mediated transport from extracellular compartments to the cell interior occurs via typical endocytosis or through the use of caveolae.<sup>37,40,41</sup> An intact RFC1 is needed for intestinal folate resorption<sup>23</sup> as well as for the uptake of folates by neuronal axons and dendrites,

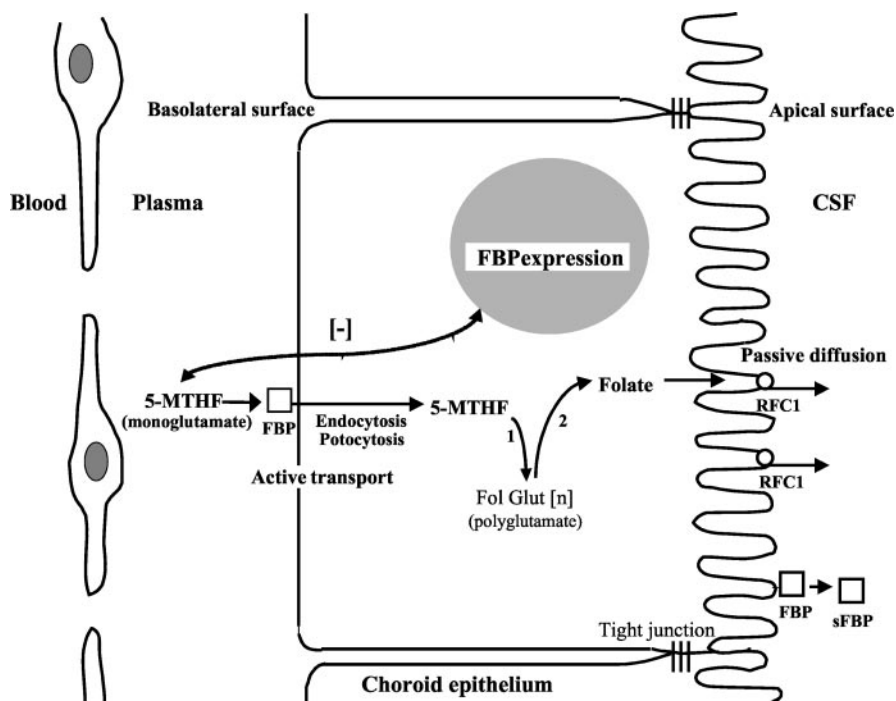


Figure 1. Mechanisms of active transport for folates across the blood–brain barrier are mainly localized within the choroid epithelium of the choroid plexus. The choroid plexus capillaries have open fenestrations between their lining endothelial cells (left side of the figure). The monoglutamate 5MTHF is bound to the folate binding proteins (FBP) located at the basolateral surface of choroid epithelium and transported actively against a concentration gradient within the cell interior. At the apical surface passive diffusion or facilitated diffusion through the reduced folate carrier 1 (RFC1) delivers folates to the CSF compartment where diffusion to interstitial spaces and subsequent uptake by neurons and neuroglia takes place. Intracellular storage of 5MTHF inside cells occurs by polyglutamation to folyl-polyglutamates (Fol Glut [n]) by the enzyme folylpolyglutamate synthase

(1), while pteroyl-polyglutamate conjugase (2) is responsible for the conversion toward monoglutamates. The extracellular folate concentration correlates inversely with the expression of FBP proteins at the membrane surface (indicated by the – sign between brackets). Part of the FBP proteins can be isolated from CSF (secreted or soluble FBP, indicated as sFBP) and used for analysis.

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,<sup>20,22</sup> CSF folate levels are 1.5 to 2 times higher than blood folate levels.<sup>42–44</sup>

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.<sup>45</sup> 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.<sup>25,46</sup> 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-

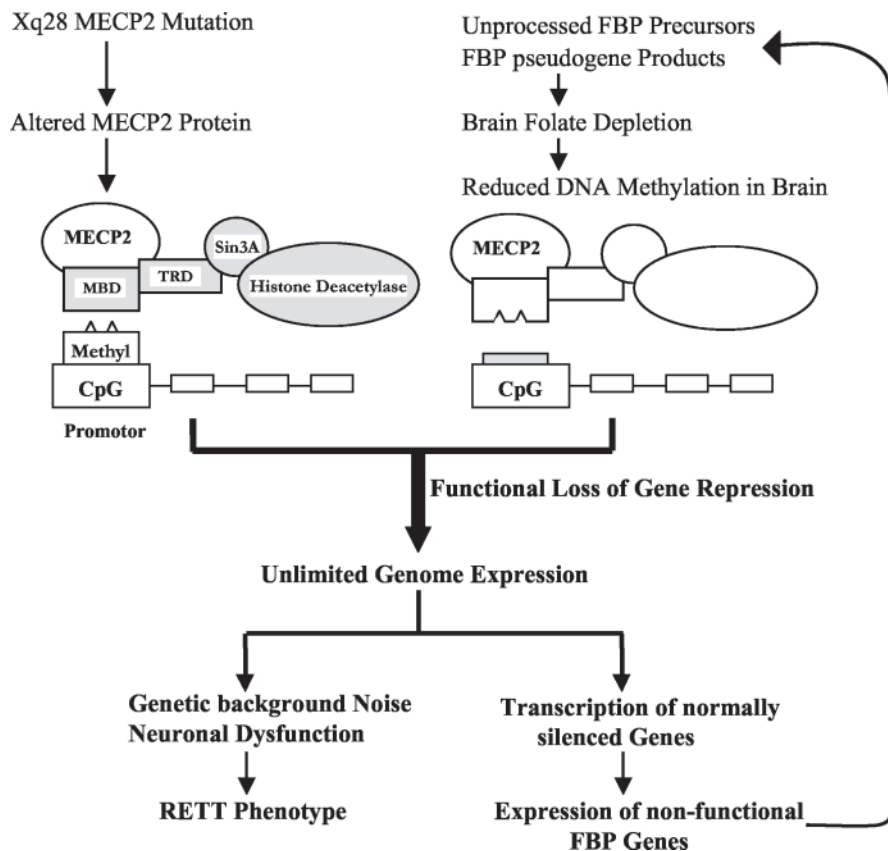


Figure 2. Hypothetical scheme shows the sequence of events caused by *MECP2* gene alterations (gray shading represents the loss of *MECP2* protein function), resulting in loss of transcription repression of the methylated genome leading to expression of normally silenced genes, nonfunctional genes, or pseudogenes. Expression of nonfunctional folate binding protein (FBP) genes or pseudogenes at the blood-brain barrier is expected to reduce the transport capacity for folates. The consequent brain folate depletion will further compromise the methyl-transfer processes in the brain including DNA methylation. The further loss of methylated CpG promotor sites within methylated DNA sequences of normally silenced genes will reduce the number of recognition sites to the interacting methyl-binding domain of the *MECP2* protein (encoded by the normal allele). This will further enhance genetic background noise and transcription of nonfunctional FBP and other nontissue specific genes. MBD = methyl-binding domain; TRD = transcription repression domain.

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 mod-

erately 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.<sup>47</sup> 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

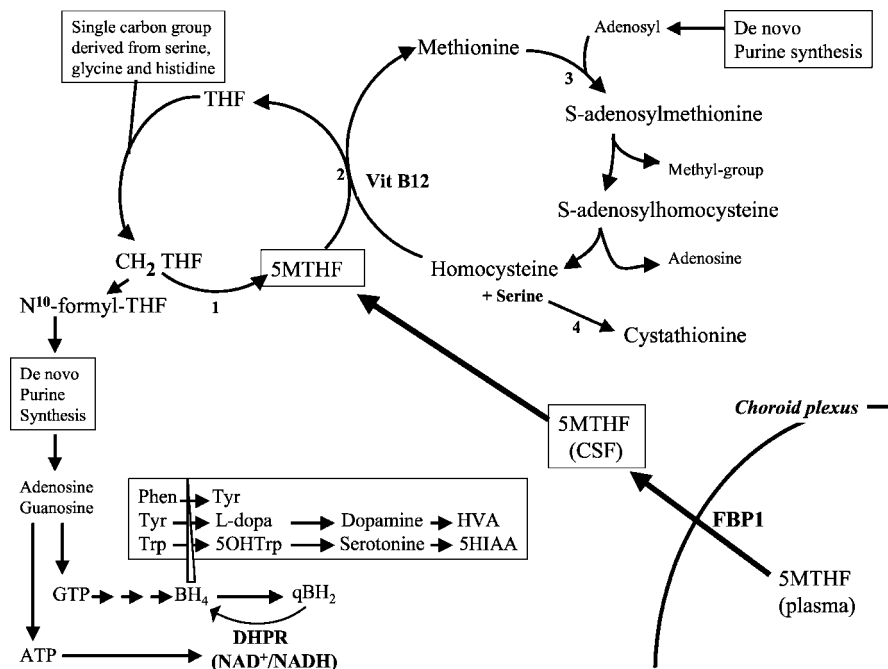


Figure 3. Metabolic fate and pathways of folate compounds. After transport of 5-methyltetrahydrofolate (5MTHF) across the choroid plexus the brain folate pool is involved in the methyl-transfer and donor pathways, de novo purine synthesis, and possible interactions with GTP synthesis, which serves as the substrate for BH4 production. Diminished BH4 cofactor synthesis for the aromatic amino acid hydroxylases will reduce dopamine and serotonin production. 1) Methylene tetrahydrofolate reductase; 2) Methionine synthase; 3) S-Adenosyl-methionine transferase; 4) Cystathionine synthase. DHPR = dihydropterin reductase.

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.<sup>10-12,48-50</sup> 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.<sup>51,52</sup> In one patient with methylenetetrahydrofolate reductase deficiency, the reduced folate concentrations were accompanied by reduced biogenic amine metabolites and total biopterins in CSF.<sup>48</sup> Several articles have provided indirect evidence for the connection between folate, pterin, and monoamine turnover.<sup>48-52</sup> Our results confirm low neopterin concentrations and a reduction of biogenic monoamine turnover that can be corrected by folic acid treatment.<sup>10-12</sup> 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<sup>10</sup>-formyltetrahydrofolate dependent steps of de novo purine synthesis with consequent limited production of ATP and GTP.<sup>53</sup> Because GTP is the substrate for GTP cyclohydrolase-I in the first step of tetrahydrobiopterin synthesis,<sup>54</sup> 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. Al-

though we find no accumulation of CSF purine metabolites and normal biopterin 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<sup>+</sup>/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.<sup>55</sup> 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.<sup>56</sup> 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.<sup>57</sup> 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 folic acid apparently enables sufficient binding and transfer of folate to the CNS. Possibly, institution of folic 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 folic acid in Rett syndrome may be warranted.

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### References

- Rett A. Über ein eigenartiges hirnatrophisches Syndrom bei Hyperammonaemie im Kindesalter. *Wien Med Wochenschr* 1966;116:723–738.
- Hagberg B, Aicardi J, Dias K, Ramos O. A progressive syndrome of autism, dementia, ataxia, and loss of purposeful hand use in girls: Rett's syndrome: report of 35 cases. *Ann Neurol* 1983;14:471–479.
- Amir RE, Van den Veyver IB, Wan M, et al. Rett syndrome is caused by mutations in X-linked *MECP2*, encoding methyl-CpG-binding protein. *Nat Genet* 1999;23:185–188.
- Wan M, Sung Jae Lee S, Zhang X, et al. Rett syndrome and beyond: recurrent spontaneous and familial *MECP2* mutations at CpG hotspots. *Am J Hum Genet* 1999;65:1520–1529.
- Jones PL, Veenstra GJ, Wade PA, et al. Methylated DNA and *MECP2* recruit histone deacetylase to repress transcription. *Nat Genet* 1998;19:187–191.
- Nan X, Ng HH, Johnson CA, et al. Transcriptional repression by the Methyl-CpG-binding protein *MECP2* involves a histone deacetylase complex. *Nature* 1998;393:386–389.
- Ng HH, Bird A. DNA methylation and chromatin modification. *Curr Opin Genet Dev* 1999;9:158–163.
- Zoghbi HY, Milstien S, Butler LJ, et al. Cerebrospinal fluid biogenic amines and bioppterin in Rett syndrome. *Ann Neurol* 1989;25:56–60.
- Blau N. The hyperphenylalaninemia. A differential diagnosis and international database of tetrahydrobiopterin deficiencies. Tectum Verlag Marburg, 1996.
- Surtees R, Heales S, Bowron A. Association of cerebrospinal fluid deficiency of 5-methyltetrahydrofolate, but not S-adenosylmethionine, with reduced concentrations of the acid metabolites of 5-hydroxytryptamine and dopamine. *Clin Sci (Colch)* 1994;86:697–702.
- Gospe SM, Gietzen DW, Summers PJ, et al. Behavioral and neurochemical changes in folate-deficient mice. *Physiol Behav* 1995;58:935–941.
- Bottiglieri T, Hyland K, Laundry M, et al. Folate deficiency, biopterin and monoamine metabolism in depression. *Psychol Med* 1992;22:871–876.
- Trevathan E, Moser HW. Diagnostic criteria for Rett syndrome. *Ann Neurol* 1988;23:425–428.
- Blau N, Thöny B, Renneberg A, et al. Variant of dihydropteridine reductase deficiency without hyperphenylalaninemia: effect of oral phenylalanine loading. *J Inher Metab Dis* 1999;22:216–220.
- Curtius HC, Blau N, Kuster T. Pterins. In: Hommes FC, ed. *Techniques in diagnostic human biochemical genetics*. New York: Wiley-Liss, 1991; 377–396.
- Blau N, Duran M, Blaskovics ME, Gibson KM. *Physician's guide to the laboratory diagnosis of metabolic diseases*. 2nd ed. Heidelberg: Springer-Verlag, 2002.
- Löhrer FM, Angst CP, Brunner FP, et al. Evidence for disturbed S-adenosylmethionine: S-adenosylhomocysteine ratio in patients with end-stage renal failure: a cause for disturbed methylation reactions? *Nephrol Dial Transplant* 1998;13:656–661.
- Surtees R, Hyland K. A method for the measurement of S-adenosylmethionine in small volume samples of cerebrospinal fluid using high pressure liquid chromatography. *Anal Biochem* 1989;181: 331–335.
- Surtees R, Hyland K. Cerebrospinal fluid concentration of S-adenosylmethionine, methionine and 5-methyltetrahydrofolate in a reference population: cerebrospinal fluid S-adenosylmethionine falls with age in humans. *Biochem Med Metab Biol* 1990;44:192–199.
- Holm J, Hansen SI, Hoier-Madsen M, Bostad L. High-affinity folate binding in human choroid plexus. Characterization of radioligand binding, immunoreactivity, molecular heterogeneity and hydrophobic domain of the binding protein. *Biochem J* 1991;280:267–271.
- Weitman SD, Lark RH, Coney LR, et al. Distribution of the folate receptor GP38 in normal and malignant cell lines and tissues. *Cancer Res* 1992;52:3396–3401.
- Wu D, Partridge WM. Blood-brain barrier transport of reduced folic acid. *Pharm Res* 1999;16:415–419.
- Wang Y, Zhao R, Russell RG, Goldman ID. Localization of the murine reduced folate carrier as assessed by immunohistochemical analysis. *Biochim Biophys Acta* 2001;1513:49–54.
- Hansen SI, Holm J, Lyngbye J. A high-affinity folate binding protein in human cerebrospinal fluid. *Acta Neurol Scand* 1985;71:133–135.
- Hansen SI, Holm J, Hoier-Madsen M. Quantitation of the folate binding protein in human cerebrospinal fluid by an enzyme-linked immunosorbent assay (ELISA). *Pteridines* 1989;1:231–233.
- Holm J, Hansen SI, Hoier-Madsen M. Immunological heterogeneity of folate binding protein in human cerebrospinal fluid. *Med Sci Res* 1998; 26:71–72.
- Hansen SI, Holm J. Conversion of an apparent 100 kDa folate binding protein from human milk, choroid plexus and semen to a 25 kDa molecular species by phosphatidylinositol-specific phospholipase C. *Biosci Rep* 1992;12:87–93.
- Holm J, Hansen SI, Hoier-Madsen M, Nichols CW. Characterization of a folate receptor in parotid gland and a folate binding protein in saliva from humans. Epitope relatedness to human milk folate binding protein. *APMIS* 2000;108:517–524.
- Ross JF, Chaudhuri PK, Ratnam M. Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. Physiologic and clinical implications. *Cancer* 1994;73: 2432–2443.
- Hoier-Madsen M, Hansen SI, Holm J. Rabbit Antibodies against the low molecular weight folate binding protein from human milk. Use for immunological characterization of human folate binding proteins in an enzyme-linked immunosorbent assay (ELISA). *Biosci Rep* 1987;7:553–557.
- Elwood PC, Nachmanoff K, Saikawa Y, et al. The divergent 5' termini of the alpha human folate receptor (hFR) mRNAs originate from two tissue-specific promoters and alternative splicing: characterization of the alpha hFR gene structure. *Biochemistry* 1997;36:1467–1478.
- Heil SG, Van der Put NMJ, Trijbels FJM, et al. Molecular genetic analysis of human folate receptors in neural tube defects. *Eur J Hum Genet* 1999;7:393–396.
- Said HM, Nguyen TT, Dyer DL, et al. Intestinal folate transport: identification of a cDNA involved in folate transport and the functional expression and distribution of its mRNA. *Biochim Biophys Acta Biomembranes* 1996;1281:164–172.
- Shen F, Ross JF, Wang X, et al. Identification of a novel folate receptor, a truncated receptor, and receptor type beta in hematopoietic cells—cDNA cloning, expression, immunoreactivity and tissue specificity. *Biochemistry* 1994;33:1209–1215.
- Wang TTY, Chandler CJ, Halsted CH. Intracellular pteroylpolyglutamate hydrolase from human jejunal mucosa: isolation and characterization. *J Biol Chem* 1986;261:13551–13555.
- Kamen BA, Capdevila A. Receptor-mediated folate accumulation is regulated by the cellular folate content. *Proc Natl Acad Sci USA* 1986;83: 5983–5987.
- Kane MA, Elwood PC, Portillo RM, et al. Influence on immunoreactive folate-binding protein of extracellular folate concentration in cultured human cells. *J Clin Invest* 1988;81:1398–406.

38. Anderson RG, Kamen BA, Rothberg KG, Lacey SW. Potocytosis: sequestration and transport of small molecules by caveolae. *Science* 1992; 255:410–411.
39. Fan J, Vitols KS, Huennekens FM. Multiple folate transport systems in L 1210 cells. *Adv Enzyme Regul* 1992;32:3–15.
40. Mineo C, Anderson RGW. Potocytosis. Robert Feulgen Lecture. *Histochem Cell Biol* 2001;116:109–118.
41. Rothberg KG, Ying Y, Kolhouse JF, et al. The glycopospholipid-linked folate receptor internalizes folate without entering the clathrin-coated pit endocytotic pathway. *J Cell Biol* 1990;110:637–649.
42. Spector R, Lorenzo A. Folate transport in the central nervous system. *Am J Physiol* 1975;229:777–782.
43. Spector R. Micronutrient homeostasis in mammalian brain and cerebrospinal fluid. *J Neurochem* 1989;53:1667–1674.
44. Weitman SD, Weinberg AG, Coney LR, et al. Cellular localization of the folate receptor: potential role in drug toxicity and folate homeostasis. *Cancer Res* 1992;52:6708–6711.
45. Verma RS, Anthony AC. Immunoreactive folate-binding proteins from human saliva. Isolation and comparison of two distinct species. *Biochem J* 1992;286:707–715.
46. Holm J, Hansen SI, Høier-Madsen M, et al. High-affinity folate receptor in human ovary, serous ovarian carcinoma, and ascites: radioligand binding mechanism, molecular size, ionic properties, hydrophobic domain, and immunoreactivity. *Arch Biochem Biophys* 1999;366:183–191.
47. Ramaekers VT, Häusler M, Opladen T, Heimann G, Blau N. Psychomotor retardation, spastic paraplegia, cerebellar ataxia and dyskinesia associated with low 5-methyltetrahydrofolate in cerebrospinal fluid: a novel neurometabolic condition responding to folinic acid substitution. *Neuropediatrics* 2002;33:301–308.
48. Clayton PT, Smith I, Harding B, Hyland K, Leonard JV, Leeming RJ. Subacute combined degeneration of the cord, dementia and parkinsonism due to an inborn error of folate metabolism. *J Neurol Neurosurg Psychiatry* 1986;49:920–927.
49. Smith I, Howells DW, Hyland K. Pteridines and monoamines: relevance to neurological damage. *Postgrad Med J* 1986;62:113–123.
50. Botez MI, Young SN, Bachevalier J, Gauthier S. Effect of folic acid and vitamin B12 deficiencies on 5-hydroxyindoleacetic acid in human cerebrospinal fluid. *Ann Neurol* 1982;12:479–484.
51. Kaufman S. Some metabolic relationships between bipterin and folate: implications for the “Methyl Trap Hypothesis.” *Neurochem Res* 1991; 16:1031–1036.
52. Matthews RG, Kaufman S. Characterization of the dihydropterin reductase activity of pig liver methylenetetrahydrofolate reductase. *J Biol Chem* 1980;255:6014–6017.
53. Wijngaarden JB, Kelley WN. Gout and hyperuricemia. New York: Grune and Stratton, 1976;1–512.
54. Blau N, Thöny B, Cotton RGH, Hyland K. Disorders of tetrahydrobipterin and related biogenic amines. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Vogelstein B, eds. *The metabolic and molecular bases on inherited disease*. 8th ed. New York: McGraw-Hill, 2000;1275–1776.
55. Blom HJ, Wevers RA, Verrips A, et al. Cerebrospinal fluid homocysteine and the cobalamin status of the brain. *J Inherit Metab Dis* 1993; 16:517–519.
56. Surtees R. Biochemical pathogenesis of subacute combined degeneration of the spinal cord and brain. *J Inherit Metab Dis* 1993;16:762–770.
57. Oldfors A, Hagberg B, Nordgren H, Sourander P, Witt-Engerstrom I. Rett syndrome: spinal cord neuropathology. *Pediatr Neurol* 1988;4:172–174.