Cerebrospinal Fluid Concentrations of Folate, Biogenic Amines and Pterins in Rett Syndrome: Treatment with Folinic Acid

Abstract

Background: Previous studies in Rett syndrome (RS) patients suggested various abnormalities in biogenic amines, pterins, and folate values in cerebrospinal fluid (CSF). Our aim was to analyse these metabolites in CSF of 16 RS patients (age range: 2 – 23 years). Biogenic amines, pterins, and 5-methyltetrahydrofolate were measured by HPLC with electrochemical and fluorescence detection. Results: CSF values of 5-methyltetrahydrofolate were decreased in 8 out of 16 RS patients (average: 53.6 nmol/L; range: 19 – 92) when compared with our reference values (average: 74.6 nmol/L; range: 45 – 127). These eight patients had epilepsy, while 4 out of 16 RS patients who did not have epilepsy showed normal CSF 5-methyltetrahydrofolate concentrations. Values of biogenic amines or pterins were decreased in four of the patients with low values of 5-methyltetrahydrofolate. No correlation was observed between CSF values of 5-methyltetrahydrofolate and pterins, biogenic amines, or age. Supplementation with folic acid was applied in six out of the eight patients with CSF 5-methyltetrahydrofolate deficiency. An improvement was noticed in all cases. Conclusions: An important percentage of RS patients showed 5-methyltetrahydrofolate concentrations under the reference values. Therefore, analysis of CSF 5-methyltetrahydrofolate seems advisable in RS, especially in patients with epilepsy and those resistant to antiepileptic drugs.

Key words
Rett Syndrome · 5-methyltetrahydrofolate · biogenic amines · folic acid

Abbreviations

BH₄ tetrahydrobiopterin
BP biopterin
CSF cerebrospinal fluid
5-HIAA 5-hydroxyindoleacetic acid
HVA homovanillic acid
MECp2 methyl-CpG binding protein 2
5-MTHF 5-methyltetrahydrofolate
NP neopterin
RS Rett syndrome
tHcy total homocysteine

Introduction

Rett syndrome (RS) is a progressive neurodevelopmental disorder occurring almost exclusively in females with onset in early childhood. Mutations in methyl-CpG binding protein 2 (MECp2) have been associated with some cases of RS, although at present this syndrome is a clinically but not a genetically defined condition. Diagnostic criteria have been established [6].

Pathogenic mechanisms are still unknown in RS. Previous studies showed a diminished turnover of dopamine and serotonin of unknown origin [15]. Recently, decreased cerebrospinal fluid (CSF) concentrations of 5-methyltetrahydrofolate (5-MTHF) were demonstrated in some cases of RS together with normal plasma folate, homocysteine, and methionine concentrations. This folate deficiency might be associated with a decreased pterin biosynthesis. Tetrahydrobiopterin (BH₄) is the main cofactor

Affiliation
1 Department of Clinical Chemistry, Hospital Sant Joan de Déu, Barcelona, Spain
2 Department of Neurology, Hospital Sant Joan de Déu, Barcelona, Spain

Correspondence
Rafael Artuch · Clinical Chemistry Department · Hospital Sant Joan de Déu · Passeig Sant Joan de Déu, 2 · 08095 Esplugues de Llobregat (Barcelona) · Spain · E-mail: rartuch@hsjdbcn.org

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Bibliography
We studied 16 female RS patients (age range: 2 – 23 years; average age: 11 years). All patients were diagnosed according to diagnostic criteria established in Baden-Baden [6]. In 8 out of 16 patients, mutations in the MECP2 gene were demonstrated (Table 1), while in the other eight patients no mutations were detected. Results of the biochemical studies in CSF (5-MTHF, biogenic amines, pterins, and amino acids) were compared with our reference ranges obtained in controls of similar ages (n = 38; age range: 2 – 18 years) examined in our laboratory under suspicion of viral or bacterial meningitis, encephalitis, or other neurological conditions of non-metabolic origin. Exclusion criteria for reference values were disturbance of folate, biogenic amines, or pterin metabolism, inborn errors of intermediary and energy metabolism, movement disorders and neuroimaging abnormalities, and diagnosis of viral or bacterial meningitis or other infectious diseases. Owing to the relationship between CSF biogenic amine and pterin concentrations and the age of controls, five reference intervals were established according to age (Table 1) [9].

Samples from patients and controls were obtained in accordance with the Helsinki Declaration of 1964, as revised in 2000. Informed consent was obtained from parents, and the ethics committee of the Hospital Sant Joan de Déu approved the study.

**Clinical examination**

Antiepileptic drug monitoring was performed in all epileptic patients. In baseline conditions and one year after initiating oral folinic acid treatment, neurological examination was performed, evaluating gait, social contact, motor functions, presence of stereotypic movements, and epilepsy control. EEG studies were performed in all patients, and in the six patients under folinic therapy, control of EEG was performed again one year after supplementation.

**Biochemical analysis**

**Blood**

Haemoglobin concentration and erythrocyte indices were analysed by standard automated procedures (PENTRA 120, ABX haematologic analyser, France). Plasma folate and vitamin B12 concentrations were analysed by automated chemiluminescent immunoassays (ADVIA Centaur, Bayer, Tarrytown, NY). Plasma total homocysteine (tHcy) was analysed by HPLC with fluorescence detection of SBDF derivatives, and plasma amino acids were analysed by ion exchange chromatography with ninhydrin detection using a Biochrom 30 analyser (Pharmacia Biotech, UK), in accord with previously reported procedures [7, 14].

**CSF**

Samples were collected between 8:00 – 10:00 a.m. following a previously reported protocol [9]. Samples were immediately frozen at –70°C until the moment of the analysis. Haematic samples were centrifuged immediately and the clear CSF supernatant was stored at –70°C.

For 5-MTHF analysis, CSF samples were diluted 1:2 in 5 mg/mL of ascorbic acid diluted in phosphate buffer 5 mmol/L (pH = 2.3), centrifuged at 1500 × g (10 min), and filtered through 0.45 μm nylon filters (Millipore). The 5-MTHF calibrator was obtained from Sigma (M-0132) and it was diluted to attain a final concentration of 22 nmol/L. Twenty μL of sample or calibrator were injected into the HPLC. 5-methyltetrahydrofolate was analysed by HPLC with fluorescence detection (Waters, MA, USA) following a previously reported procedure [2] with some modifications. Briefly, the mobile phase consisted of phosphate buffer (5 mmol/L, pH 2.3) mixed with acetonitrile (93/7 v/v) for five minutes followed by a linear gradient (curvature = 6) to achieve a proportion of 83/17 (v/v). Fluorescent conditions were excitation 295 nm and emission 355 nm. 5-MTHF was separated in a Nucleosil C-18 column (150 mm × 0.4 mm, 5 μm particle size; Teknokroma, Barcelona, Spain). Chromatographic data were processed with the Breeze 3.3 GP software (Waters). Flow rate was 0.8 mL/min.

Biogenic amines in CSF (3-orthomethyl Doddopa, 3-methoxy-4-hydroxyphenylglycol, 5-hydroxytryptophan, 5-HIAA and HVA) were measured by ion pair HPLC with electrochemical detection following a previously reported procedure [9]. Pterins (neopterin [NP] and biopterin [BP]) were analysed by reverse phase HPLC with fluorescence detection (Waters, MA, USA) following a previously reported method [3]. Amino acids were analysed by ion exchange chromatography with ninhydrin detection using a Biochrom 30 analyser (Pharmacia Biotech, UK).

**Mutation analysis of MECP2**

Genomic DNA was extracted from the leukocytes of RS patients. DNA amplification of the MECP2 coding exons was performed by PCR and the products were sequenced in an ABI-Prism 3100 (Applied Biosystems), as previously reported [1].
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<th>HVA nmol/L</th>
<th>HVA/5-HIAA</th>
<th>NP nmol/L</th>
<th>BP nmol/L</th>
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<sup>*</sup> Values below control range, <sup>(1)</sup> Resistant to antiepileptic drugs (RAEDs), <sup>(2)</sup> Cbz = carbamazepine, <sup>(3)</sup> Etho = ethosuximide, <sup>(4)</sup> Pri = primidone, <sup>(5)</sup> Clom = clonazepam, <sup>(6)</sup> Topi = topiramate
Whitney U test: P = 0.003) (Fig. 1).

**Table 1**
The Spearman test was applied to search for correlation among the different variables of the study, and the Mann-Whitney U test was used to investigate differences between patients and controls. Statistical calculations were performed with the SPSS 12.0 program.

**Results**
Clinical, biochemical, genetic, and treatment data are reported in Table 1. Blood analysis of haemoglobin, erythrocyte count, plasma folate, vitamin B12, plasma tHcy, and plasma amino acids were normal. CSF phenylalanine and other amino acids were also within the normal range.

CSF values of 5-MTHF were significantly lower in RS patients (average: 53.6 nmol/L; range: 19 – 92) when compared with the control group (average: 74.6 nmol/L; range: 45 – 127) (Mann-Whitney U test: P = 0.003) (Fig. 1). Eight out of 16 RS patients showed 5-MTHF concentrations below the lower limit of our reference intervals. These eight patients had epilepsy (cases 9 to 16: Table 1), while 4 out of 16 RS patients who did not have epilepsy showed normal CSF 5-MTHF concentrations (average: 80.7; range: 56 – 92). Regarding values of biogenic amines and pterins, four out of the eight patients with low 5-MTHF presented decreased values of these metabolites. 5-HIAA concentrations were below the lower limit of our reference interval in two out of the eight patients (cases 14 and 16, Table 1), while HVA concentrations and the other biogenic amines analysed in CSF were all normal. Neopterin concentrations in CSF were below the normal range in two out of the eight RS patients with low 5-MTHF (cases 13 and 15, Table 1), although they did not show low 5-HIAA concentrations.

No correlation was observed between CSF values of 5-MTHF and NP, BP, 5-HIAA, HVA, or age, either in controls or in RS patients.

No association was observed between the presence or absence of mutations in the MECP2 gene and CSF 5-MTHF deficiency (Table 1). Two patients with the 763 C>T mutation showed decreased CSF 5-MTHF and 5-HIAA values, while two patients with the 880 C>T mutation showed normal results.

Supplementation with folic acid (in combination with antiepileptic drug regimen) was applied in six out of the eight patients with CSF 5-MTHF deficiency (Table 1). An improvement in social contact and behaviour problems, and a decrease in stereotypic hand-washing movements, was noticed by parents in all cases. Concerning the epilepsy, folic acid supplementation was associated with control of epilepsy in two patients with resistant seizures (cases 10 and 11, Table 1) and a reduction in the number of seizures in one patient (case 12, Table 1). In case 10, EEG showed posterior-right bilateral paroxysmal discharges which became normal after one year of folic acid therapy. Cases 11 and 12 showed the same EEG record before and after therapy. The other three patients (with controlled epilepsy prior to the start of the therapy) remained stable after folic acid treatment (Table 1). EEG showed no changes in two out of these three cases, and an improvement was obtained in case 9: the parietal bilateral paroxysmal discharges disappeared after folic acid therapy.

**Discussion**
Cerebral folate deficiency with normal folate metabolism in the peripheral system has been reported in several conditions [11]. This status could be caused either by disturbed folate transport or by increased folate turnover in the central nervous system [11]. Recently, the presence of autoantibodies against the folate binding proteins in cerebral folate deficiency has been demonstrated [12]. A cerebral 5-MTHF deficiency in some RS patients has also recently been described, and in these cases deficient transport by the folate binding proteins seems to be the main cause, although there is no clear explanation for these findings [10].

Folate active transport across the blood-brain barrier is carried out by folate-binding proteins. CSF 5-MTHF concentrations are 1.5 to 2 times higher than blood folate [10]. According to our results, 50% of RS patients had decreased 5-MTHF values in CSF. Our results suggest that CSF folate deficiency may be a common
Therefore, the contribution of decreased pterin and biogenic amine concentrations to the pathogenesis of RS as a consequence of the net reduction of the GTP pool secondary to folate deficiency seems plausible. However, this pathogenic mechanism might be more evident in some cases, and taking into account the involvement of folate in many reactions, other folate-dependent mechanisms are probably involved in the pathogenesis of RS.

Concerning MECP2 gene mutations and 5-MTHF status, the presence or absence of mutations was not related with CSF folate status. The two patients with the 763 C>T (R255X) mutation showed folate deficiency together with decreased 5-HIAA values. This is a severe nonsense mutation involving three MECP2 domains (translation repression, nuclear and binding region). However, another nonsense mutation with the same involvement in functional domains (R270X) showed normal folate values. Conversely, the missense R306C mutation, which only involves the translation repression domain of MECP2, was associated with low CSF 5-MTHF values. Therefore, there is no clear relationship between MECP2 mutations and folate status, although a large series with a full mutation analysis should be conducted in order to determine whether there is a clear relationship between the two variables, or not. On the other hand, severe epileptic encephalopathies and antiepileptic treatment have also been associated with decreased folate values [5]. Therefore, it would also be possible that antiepileptic treatment was associated with folate deficiency in our RS patients, and the study of CSF 5-MTHF in a larger series of patients with active epilepsy from different origins and with or without antiepileptic treatment seems advisable.

It has been suggested that folinic acid supplementation may be effective in RS patients [10]. A mild improvement in behaviour and cognitive functions, stereotypic hand-washing movements, and seizure control with supplementation was demonstrated [10]. We started folinic acid therapy in cases with low CSF 5-MTHF values, and our results agree with previously reported data. All treated patients showed an improvement in behaviour and visual and social contact, as was documented by parents and teachers. Stereotypic hand-washing movements were reduced in all of the cases after folinic acid supplementation. Concerning epilepsy, all patients who showed low 5-MTHF values had seizures (either controlled or resistant to antiepileptic drugs), while all patients without epilepsy showed normal CSF 5-MTHF values. These results suggest that RS patients without epilepsy would have normal CSF 5-MTHF values, while the presence of seizures was associated with low folate concentrations. However, a larger series of patients should be evaluated to establish whether RS patients without epilepsy always shows normal CSF folate concentrations. Furthermore, it is possible that antiepileptic treatment was associated with folate deficiency prior to the start of the therapy. The third patient improved, with a lowering of the number of crises, although complete control of epilepsy was not achieved. A further increment in daily folinic acid doses is probably advisable in this case. Patients with low CSF 5-MTHF values with controlled epilepsy prior to the start of folinic acid treatment remained stable. Therefore, RS patients with controlled or resistant epilepsy would be candidates for CSF 5-MTHF and biogenic amine quantification, since treatment seems important to improve social contact and epilepsy control, and can improve quality of life. As previously reported [10], there was also an improvement in EEG records in two cases, supporting the effectiveness of folinic acid therapy in RS.

Previously reported data showed a correction of 5-MTHF values over treatment. Unfortunately, lumbar puncture was not possible after folinic acid supplementation in our patients. Most of our patients under treatment presented severe scoliosis, and lumbar puncture was extremely difficult and not recommended for ethical and technical reasons. However, the improvement in clinical conditions supports the correction of central nervous system folate values.
In conclusion, an important percentage of RS patients presented 5-MTHF concentrations below the reference range values and in half of them, CSF biogenic amine or pterin values were decreased as well. All the patients with low CSF 5-MTHF values presented epilepsy, and an improvement in behaviour and social contact, as well as seizure control, was observed after folic acid treatment. Therefore, analysis of CSF 5-MTHF seems advisable in RS, especially in patients with epilepsy and those resistant to antiepileptic drugs.

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