Pharmacology, Biochemistry and Behavior 94 (2010) 454–463

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Systemic methotrexate induces spatial memory deficits and depletes cerebrospinal fluid folate in rats

Yan Li [a], Veena Vijayanathan [a], Maria E. Gulinello [b], Peter D. Cole [a,*]

[a] Department of Pediatrics, Albert Einstein College of Medicine, 1300 Morris Park Ave, Forchheimer 702, Bronx, NY 10461, United States
[b] Behavioral Core Facility, Dept of Neuroscience, Kennedy 925, 1410 Pelham PKWY S, Albert Einstein College of Medicine, Bronx, NY 10461, United States

ARTICLE INFO

Article history:
Received 3 September 2009
Received in revised form 1 October 2009
Accepted 22 October 2009
Available online 1 November 2009

Keywords:
Methotrexate
Spatial memory
Homocysteine
S-adenosylmethionine
S-adenosylhomocysteine
Cerebrospinal fluid

ABSTRACT

Purpose: Although most children with acute lymphoblastic leukemia (ALL) are cured, a subset manifests persistent, focal cognitive deficits. Methotrexate (MTX), a key component of leukemia treatment, is suspected to contribute to treatment-induced cognitive dysfunction. We sought to establish a rodent model in order to further investigate the underlying pathophysiology.
Procedures: Intraperitoneal MTX was given to Long–Evans rats on two schedules: acute (250 mg/kg once during adulthood), or chronic (1 mg/kg twice weekly ×4 doses, beginning at postnatal day 15, then weekly ×6). Control rats were given saline injections on the same schedules. All male rats subsequently underwent behavioral testing designed to assess cognitive domains frequently impaired among children treated for ALL. Cerebrospinal fluid and serum folate concentrations were measured by HPLC.
Findings: Both acute and chronic MTX administration produced spatial memory deficits, without significantly altering visual memory, general exploration, activity or motor coordination. MTX administration was also associated with a marked reduction in serum and CSF folate and a decrease in the ratio of CSF S-adenosylmethionine to S-adenosylhomocysteine.
Conclusions: Similar to children treated for ALL, rats given systemic MTX develop focal cognitive deficits along with expected alterations in folate physiology.

1. Introduction

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer, representing 23% of cancer diagnoses among patients younger than 15 years old (Smith et al., 1999). Advances in chemotherapy, including intensification of multi-agent treatment protocols, have greatly improved outcomes for pediatric ALL patients, with cure rates approaching 90% (Pui and Evans, 2006). However, intensification of therapy has been followed by a parallel increase in toxicity. Deficits in neurological and cognitive function are a particularly distressing complication of therapy for survivors, as these can persist in the years following treatment and affect school or occupational competence (Buizer et al., 2009; Lofstad et al., 2009; Peterson et al., 2008). As many as 40% of childhood leukemia survivors, for example, will have measurable defects in cognitive function and/or will need special education (Waber et al., 2007). Elucidating the underlying mechanisms of chemotherapy-induced adverse central nervous system (CNS) effects in patients is difficult, due to differences in administration route, dose, combination of reagents, and delivery schedule of chemotherapy. In addition, radiation usage and disease state further complicate this effort.

The chemotherapy drug methotrexate (MTX), a core component of therapeutic regimens for ALL, is associated with acute and chronic neurotoxicity (Vezmar et al., 2003). MTX exerts anti-neoplastic effects by competitively inhibiting folate-dependent biochemical processes, thus inhibiting DNA synthesis. The neurotoxic side effects of MTX may be explained both by these effects on normal folate physiology as well as the indirect consequences of depleting tissue folate stores (Kamen et al., 1984). The vitamin folic acid is essential in a variety physiology processes, as shown in Fig. 1. Folate is required, for example, to sustain concentrations of S-adenosylmethionine (SAM), the primary methyl donor for over 100 methylation reactions, including those necessary for biosynthesis of neurotransmitters, maintaining myelin basic protein, and gene regulation via DNA methylation. S-adenosylmethionine (SAH), an end-product and potent inhibitor of these methylation reactions, accumulates when low folate stores prohibit replenishment of SAM. Thus, the SAM/SAH ratio has been used to represent methylation potential. Both CNS folate deficiency and MTX administration have been associated with a decrease in the CSF SAM/SAH ratio and demyelination (Kishi et al., 2000; Surtees et al., 1991, 1998a). In addition, MTX administration increases cerebrospinal fluid (CSF) concentrations of homocysteine (Cole et al., 2009; Quinn et al., 1997), a vascular toxic amino acid that has been associated with acute MTX neurotoxicity (Drachtman et al., 2002).

* Corresponding author.
E-mail address: peter.cole@einstein.yu.edu (P.D. Cole).

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doi:10.1016/j.pbb.2009.10.008

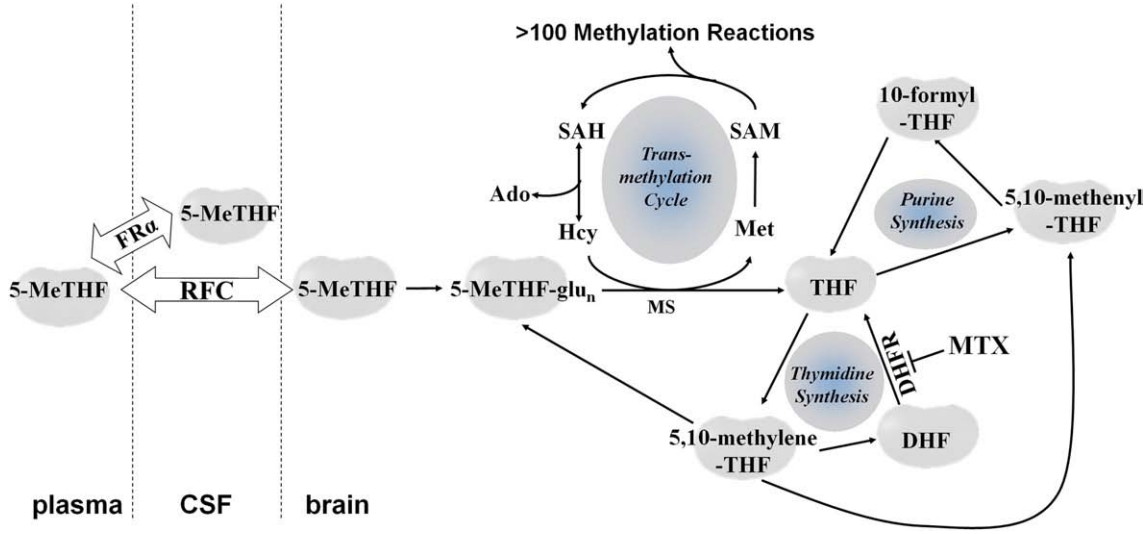

Fig. 1. Schematic of major folate-mediated biological pathways. Folates provide one carbon unit for methylation reactions and are therefore critical to a spectrum of biological processes, including purine and thymidine synthesis. MTX, a synthetic antifolate, primarily inhibits dihydrofolate reductase, thus inhibits thymidine synthesis, and exerts anti-neoplastic effects. Abbreviations for substrates are: 5-MeTHF: 5-methyltetrahydrofolate; 5-MeTHF-glu$_n$: 5-methyltetrahydrofolate polyglutamate; THF: tetrahydrofolate; DHF: dihydrofolate; Met: methionine; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; Ado: adenosine; Hcy: homocysteine. Other abbreviations: FRα: folate receptor-α; PCFT: proton coupled folate transporter; RFC: reduced folate carrier; MS: methionine synthase; DHFR: dihydrofolate reductase; MTX: methotrexate.

Rodent models are valuable for evaluating MTX effects without complication of a co-existing disease state. As in humans, administration of MTX causes learning and memory deficits in rodents (Madhyastha et al., 2002; Seigers et al., 2008, 2009). These cognitive deficits are associated with pathological changes in hippocampus and corpus callosum (Seigers et al., 2008, 2009). MTX-induced biochemical changes in rats are similar to those seen in human patients and include lowered liver folate levels, elevated homocysteine and alterations in amino acid profile (Phillips et al., 1986; Varela-Moreiras et al., 1995). However, widely varying dose, different routes of administration, and a lack of systematic examination of chronic *vs* acute treatment schedules make it difficult to summarize findings from animal studies. Furthermore, many behavioral assays used in rodents are difficult to compare with assays used to assess cognitive function in children.

We thus examined the effects of MTX administrated in schedules comparable to what is used in human subjects with ALL. This study had several aims: First, to characterize the nature of MTX-induced behavioral deficits in rats, using assays probing domains of function analogous to those found to be affected by therapy for childhood ALL; second, to compare the effects of chronic *vs* acute treatment schedules; and, last, to describe the effects of acute and chronic MTX administration on folate homeostasis within the CNS. Here, we report that both acute and chronic MTX caused specific cognitive deficits in short-term recognition/spatial memory without altering motor coordination or activity levels. These cognitive deficits were associated with decreased folate and altered methylation potential in the CSF.

2. Methods

2.1. Materials and subjects

For acute toxicity studies, outbred male adult (10 weeks old) Long Evans rats were purchased from Charles River Laboratories (Wilmington MA). For chronic toxicity experiments, Long–Evans rat pups were bred in-house. Rats were housed in groups of 2–3 with *ad lib* food (laboratory rodent diet 5001, LabDiet) and water in conventional rooms with 12–12 light/dark cycle. All studies were conducted following 'Guide For The Care And Use Of Laboratory Animals' (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council), and were approved by Animal Institute Committee of the Albert Einstein College of Medicine.

MTX (USP grade) and other chemicals were purchased from Sigma (Saint Louis, MO) unless otherwise stated. Final concentrations were confirmed spectrophotometrically, using the NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE). HPLC grade methanol was purchased from Fisher Scientific (Pittsburgh, PA).

2.2. MTX injection

Acute: In acute toxicity experiments, 12 week old adult male rats were intraperitoneally (ip) injected with either 250 mg/kg MTX, or equal volume (~5 ml) of saline (USP grade, Nurse Assist Corp, Fort Worth, TX). *Chronic:* In chronic toxicity experiments, rats of both sexes were injected IP with lower dose MTX (1 mg/kg). A total of 10 injections were carried out: twice a week for 2 weeks beginning when rats were 2 weeks old, followed by 6 weekly injections.

2.3. Behavioral tests

All behavioral tests were carried out in the Behavioral Core Facility at the Albert Einstein College of Medicine. In acute toxicity studies, behavioral testing was conducted between day 3 and day 7 after injection. In the chronic toxicity studies, behavioral tests were conducted when the rats reached 12 weeks of age (the same age as rats in acute toxicity studies). To avoid the potential effects of hormonal fluctuation on cognitive behavior among females with asynchronous estrous cycles, only behavioral data from male rats are shown. The order of tests was counterbalanced among cohorts, to prevent potential systematic order effects.

2.3.1. Open field

The open field has been extensively validated, ethologically and pharmacologically in both mice and rats (Pritchett and Mulder, 2003; Wilson et al., 1976). The testing arena consists of a 65 cm by 65 cm gray box with sides that were 30 cm high. High contrast visual cues in different shapes were placed on sidewalls. Animal activity was recorded for a total of 6 min and analyzed using Viewer tracking software (Biobsever, Bonn, Germany). Total activity was assessed as overall distance traveled (total track length). Thigmotaxis was assessed as distance traveled in the 23×23 cm center zone (center

track length), the duration of time spent in the center zone and the number of entries into the center zone.

2.3.2. Object placement and object recognition tests

Visual and spatial memory was tested in novel object recognition task and object placement task respectively, following established protocols (Ennaceur and Delacour, 1988; Ennaceur and Meliani, 1992). These tasks utilize the innate tendency of rats to preferentially explore novel objects to assess working and recognition memory, and they are similar to tests conducted in humans (Kessels et al., 2002; Salame et al., 2006). All objects used have been extensively validated to ensure that no intrinsic preference or aversion exists and that animals explore all objects for similar durations. Exploration of the objects was defined as any physical contact with an object (whisking, sniffing, rearing on or touching the object). Viewer software was used to obtain general activity levels (track length) in all sessions.

In object placement tests, spatial memory was assessed in a similar way. In trial 1, rats were allowed to explore two identical objects for 5 min in the arena. After spending a retention interval of 5 or 10 min in their home cages, rats were returned to the testing arena for 3 min with one object moved to a different location (trial 2). Care was taken to ensure the placement change alters both intrinsic relationship between objects and the position relative to visual cues. The exploration durations (in seconds) of the displaced (novel) and unmoved (familiar) objects were recorded.

In the object recognition test, rats were placed in the testing arena described earlier and allowed to freely explore two identical, nontoxic objects (e.g., plastic, glass, or ceramic items). Durations of exploration of each object (in seconds) were recorded for 3 min (trial 1). After spending a retention interval of 2 h in their home cages, rats were returned to the same arena for 3 min (trial 2), now containing one object from trial 1 (familiar object) and one novel object. The exploration durations of novel object and familiar object (in seconds) were recorded.

For both the object placement and object recognition tests, the results are reported as exploratory preference scores (100 × exploration duration new/exploration duration old + new). An exploratory preference score of 50 thus indicates that the rat spent equal time exploring the novel and familiar objects. Results were also reported as success rates — the proportion of animals in each group performing higher than chance (i.e. preferring the novel object). For this purpose, preference scores higher than 53 were defined as 'passing', based on the following rationale: during the initial and extensive validation of these tasks in Einstein Behavioral Core Facility, it was determined that animals with preference scores higher than 53 consistently demonstrate novel object preferences when retested (unpublished data); few animals had scores between 53 and 55; and the use of the stricter criterion (55) made little difference to the analysis (see supplementary data for success rate analysis).

2.4. Tissue sample collection

CSF was collected by transcutaneous cisternal puncture while rats were under anesthesia, using a modification of a previously published method (Consiglio and Lucion, 2000). Briefly, rats were positioned in the lateral decubitus position, while anesthetized by 5% isoflurane/95% oxygen. A 25-gauge butterfly needle was inserted into cisterna magna, and up to 250 µl CSF was collected by gravity. Samples with gross contamination by blood were discarded (approximately 25%). CSF samples were spun to remove any cellular elements and supernatants were stored at −80 °C until further analysis.

Blood was collected from euthanized rats via cardiac puncture. Serum was separated from red blood cells by centrifugation. Blood samples were stored at −80 °C until further analysis.

After acute MTX injection, 250 mg/kg, CSF samples were collected at multiple time points, as stated in the figures. Blood was collected from these rats 7–10 days after MTX injection. Rats that underwent behavioral testing did not have CSF collection between MTX injection and behavioral testing. In chronically treated rats, CSF and blood were collected at 12 weeks of age, approximately three weeks after the final 1 mg/kg MTX injection.

2.5. High performance liquid chromatography

Total Folate, Homocysteine, S-Adenosyl Methionine (SAM) and S-Adenosyl Homocysteine (SAH) were measured using a Waters 2695 separation module equipped with a Waters 2475 fluorescence detector (Waters, Milford, MA). A reversed phase Waters Bondapak C18 analytical column (3.9 × 150 mm) was used for all HPLC analysis.

2.5.1. Folates

Rat CSF (50 µl) was mixed with 50 µl of water and 80 µl of extraction buffer (0.1 M phosphate buffer pH2.1, containing 1% β-Mercaptoethanol) and 20 µl of 50% Trichloroacetic acid. The mixture was centrifuged at 13,000 *g* for 10 min and supernatant was filtered through 0.2 µm syringe filters. The predominant folate in CSF, methyl tetrahydrofolate, was quantified by injecting 50 µl of the solution onto the analytical column using a mobile phase of 0.1 M phosphate buffer, pH3.5 containing 20% methanol at a flow rate of 0.5 ml/min. Methyl tetrahydrofolate was detected using the fluorescence detector set at 295 and 355 nm for excitation and emission respectively.

Calibration curves were constructed using standard solutions of 5-methyltetrahydrofolate, processed in the same methods as rat csf and plasma samples. A standard curve ranging from 10 to 500 nM was derived by linear regression analysis of the area under the curve using the Empower software supplied by the manufacturer. 5-methyltetrahydrofolate was identified both by retention time and also by repeating the HPLC separation after spiking the samples with known concentrations of 5-methyltetrahydrofolate. The standard curves were linear over the concentration range studied with a correlation coefficient of 0.996.

Precision and recovery of the extraction method was verified by spiking known concentrations of the 5-methyltetrahydrofolate into rat CSF and comparing the peak area of 5-methyltetrahydrofolate in CSF samples to that of standard solutions. The mean recovery of 97% indicates that no degradation of folate occurred during sample processing.

2.5.2. Homocysteine

Homocysteine levels were measured using the method of Pfeiffer et al with slight modifications (Pfeiffer et al., 1999). Briefly, 50 µl of CSF was mixed with an equal volume of water and 10 µl of 60 mg/ml tris-(2-carboxy ethyl phosphine), and the reaction mixture was incubated for 10 min at 37 °C to release the protein bound thiols. Samples were deproteinized using 50% trichloroacetic acid containing 2 mM ethylene diamine tetraacetic acid (EDTA) and centrifuged for 5 min at 13,000 *g*. Supernatant (100 µl) was mixed with 100 µl of 1 M borate buffer containing 4 mM EDTA, and 10 µl of 10 mg/ml ammonium-7-fluoro-benzo-2-oxa,1,3 diazole-4 sulphonate. Final pH of the reaction mixture was adjusted to 9.5 by the addition of 20 µl of 2.5 N sodium hydroxide. Samples were incubated at 60 °C for 60 min and the derivatized samples were filtered through 0.2 µM syringe filters before injecting on to the HPLC column.

The detector was set at an excitation wavelength of 385 nm and emission was monitored at 515 nm. Homocysteine was eluted isocratically using 0.1 M potassium phosphate buffer pH3.1 containing 20% methanol at a flow rate of 0.5 ml/min. Homocysteine was quantified by external calibration method.

2.5.3. S-Adenosyl methionine and S-Adenosyl homocysteine

S-adenosyl methionine (SAM) and S-Adenosyl homocysteine (SAH) in CSF samples were analyzed by HPLC after converting them to their 1,N^6 etheno derivative (Vezmar et al., 2009). Briefly, 75 µl of

CSF samples were mixed with 25 µl of water and pH was adjusted to 4 using 25 µl of 0.5 M sodium acetate pH4.5. Chloroacetaldehyde (20 µl) was added and samples were incubated at 60 °C in a heat block for 2 h. Derivatized samples were centrifuged for 10 min at 15,000 *g* and the supernatant was filtered through 0.2 µm syringe filters prior to injection into the HPLC system.

The mobile phase consisted of a gradient system with 40 mM potassium phosphate, pH 4.5 containing 8 mM Heptane sulphonic acid (Phase A) and Methanol as Phase B. After sample injection, 80% mobile phase A was pumped at a flow rate of 0.5 ml/min for 15 min, followed by 50% phase A at a flow rate of 1 ml/min. The column was returned to the starting condition by equilibrating with 80% Phase A for 10 min before the next sample injection. Sample (100 µl) was injected into the HPLC system and fluorescence intensities were measured with the detector set at an excitation wavelength of 280 nm and emission at 410 nm. Etheno derivatives were quantified by external calibration method. Standard solutions in water were used for calibration.

2.6. Statistics

There were no differences between saline-injected controls in the chronic *vs* acute groups in any of the behavioral assays and saline controls are thus combined in the statistical analyses and graphs.

In the novel object assays, preference scores were analyzed using one-way ANOVA followed by *post-hoc t*-test. Proportions of pass or fail were analyzed with the Fisher Exact test using GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego California USA.

Biochemical data was analyzed using one-way ANOVA followed by *post-hoc t-test*.

3. Results

There were no significant differences in biochemical analysis results between male and female rats in the chronic studies, thus both sexes are combined in the analyses and figures. Saline controls from acute and chronic studies were also tested at the same final age and were not different in behavioral or biochemical measure, and these are also combined and referred to as control, unless otherwise stated. In figures, one-time 250 mg/kg MTX-injected rats are designated 'MTX acute', repetitive 1 mg/kg MTX-injected rats are designated 'MTX chronic'.

3.1. Behavior tests

3.1.1. MTX induces spatial memory deficits in the object placement test

In both the acute and chronically treated rats, MTX induced deficits in spatial memory when the retention interval between novel object exposure (trial 1) and testing (trial 2) was 10 min (Fig. 2A). One-way

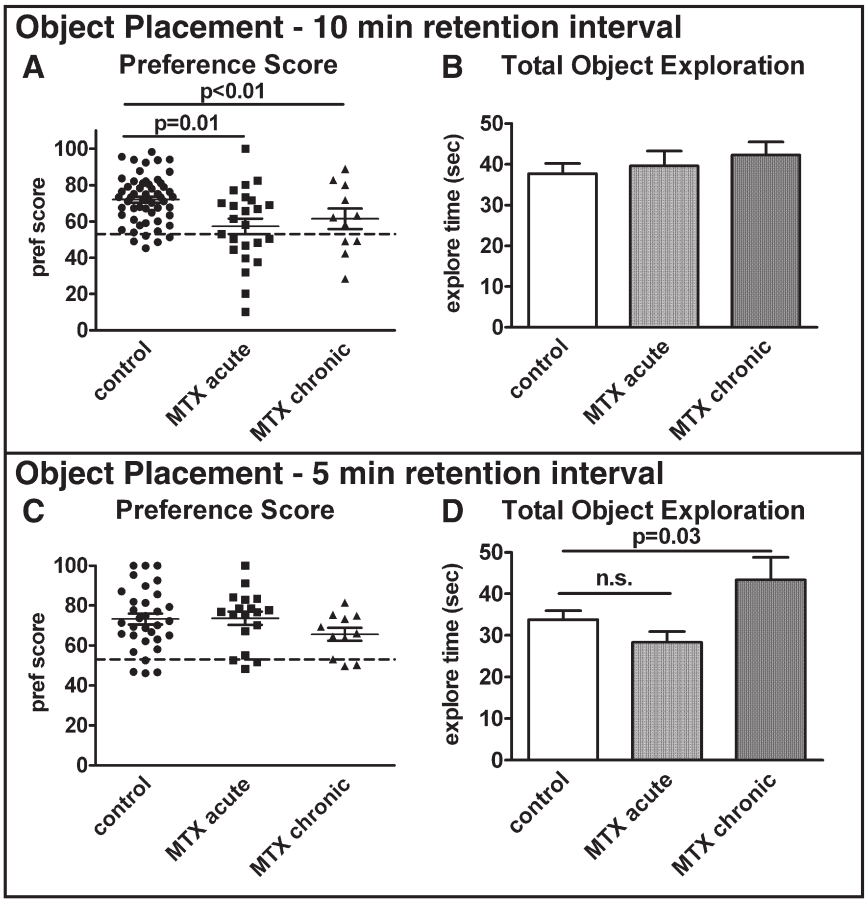

Fig. 2. MTX induces deficits in spatial memory, measured with the object placement test. A. Preference scores of individual rats with group means and standard errors, in object placement tests with 10 min retention interval. Dotted line represents a preference score of 53. Group means are: control ($n=55$) 72.0 ± 1.8, MTX acute ($n=24$) 57.4 ± 4.2, MTX chronic ($n=11$) 61.5 ± 5.6. Difference among groups is significant ($p<0.01$, one-way ANOVA). *Post-hoc t*-test, both MTX acute ($p=0.01$) and MTX chronic ($p<0.01$) are significantly different from control. B. Exploring time in trial 1 in the object placement test with 10 min retention interval. Mean exploration times are: control 37.71 ± 2.50 s, MTX acute 39.61 ± 3.68 s, and MTX chronic 42.33 ± 3.17 s. Difference among groups is not significant. C. Preference scores of individual rats with group means and standard errors, in object placement tests with 5 min retention interval. Dotted line represents the preference score of 53. Group means are: control ($n=32$) 73.2 ± 2.7, MTX acute ($n=18$) 73.6 ± 3.3, MTX chronic ($n=11$) 65.6 ± 3.2. The difference is not significant ($p=0.3$, one-way ANOVA). D. Exploring time in trial 1 in the object placement test with 5 min retention interval: control 33.77 ± 2.18 s, MTX acute 28.29 ± 2.64 s, MTX chronic 43.38 ± 5.43 s. One-way ANOVA showed there is a difference among groups ($F_{(2,58)}=4.463$, $p=0.02$).

ANOVA analysis of preference scores revealed a main effect of treatment ($F_{(2,87)}=7.645$, $p<0.01$). *Post-hoc t*-test revealed significant difference between control and acute MTX groups ($p=0.01$), as well as between control and chronic MTX groups ($p<0.01$). There was no significant difference between MTX-injected rats and control rats in the total duration of novel object exploration in trial 1 (Fig. 2B).

Using a pass criterion of preference score greater than 53 (Fig. S1A), the proportion of rats with a novel object preference was lower in both acutely and chronically MTX-treated groups than in controls. While the majority of saline-injected controls (91%) displayed a novel object preference after a 10 min retention interval, 41% of acute MTX animals and 36% chronic MTX animals failed to display a preference for the displaced object (chi-square test $p<0.01$). Similar results are seen using a preference score of 55 as the pass criterion (Fig S1D).

Interestingly, there were no significant differences in the extent of cognitive deficits between chronically and acutely treated MTX rats, assessed either by preference score or by proportion of failures.

All cohorts displayed intact spatial memory in the object placement test when tested with shorter retention intervals (Fig. 2C). There was no significant difference in mean preference scores between control acute MTX and chronic MTX groups ($F_{(2,58)}=1.308$, $p=0.28$). Similarly, there was no difference in the proportion of animals displaying a preference for the displace object the test, using the same pass criterion of preference score higher than 53 ($p=0.87$, chi-square analysis, Fig. S1B) or higher than 55 (Fig S1E). There were no differences in total novel object exploration in trial 1 between control and MTX groups in this object placement test (Fig 2D).

3.1.2. MTX does not induce deficits in object recognition memory or open field activity

In contrast to deficits in spatial memory induced by MTX, object recognition memory, assessed by the object recognition test, was intact after acute or chronic MTX exposure, with retention intervals as long as 2 h (Fig. 3). There were no significant differences between saline-injected subject and either acute or chronic administration of MTX in preferences scores ($F_{(2,74)}=0.4875$, $p=0.62$, Fig. 3A). Chi-square analysis of success rates also did not show difference between treatment groups (Fig S1C for pass score of 53, chi-square $p=0.47$, or Fig S1F for pass score of 55). Total object exploration times were not different among groups either ($F_{(2,74)}=0.4021$, $p=0.67$, Fig. 3B).

Furthermore, MTX-treated animals did not show alterations in general activity, assessed by total track length in the open field test (Fig. 4A, $F_{(2,46)}=2.317$, $p=0.11$). There were no significant differences in thigmotaxis as assessed by center track length (control 132.6 ± 20.4 cm, MTX acute 202.8 ± 33.4 cm, and MTX chronic 100.2 ± 15.7 cm, $p=0.07$ between control and MTX acute, $p=0.22$ between control and MTX chronic, *post-hoc t*-test). However, acutely treated MTX rats spent significantly more time in the center zone than either saline-injected controls or chronically treated MTX rats (Fig. 4B: $F_{(2,47)}=4.697$, $p<0.01$; $p=0.02$, *post-hoc t*-test).

We also monitored the effects of chronic MTX exposure on weight gain. Many rat pups had diarrhea at the beginning of receiving MTX, leading to delays in weight gain. However, the rate of weight gain was similar in all groups thereafter. By 12 weeks of age, MTX-treated rats weighed no more that 10% less than controls of same sex (male control 518 ± 9 g, male MTX 481 ± 7 g, female control 323 ± 8 g, female MTX 292 ± 8 g).

3.1.3. MTX decreases serum and CSF folate concentrations

Single high-dose systemic MTX administration induced a significant decrease in CSF folate concentration, to less than 10% of baseline within 24 h of injection (Fig. 5A). By 7 days after injection, CSF folate was still less than 50% of control ($F_{(3,39)}=69.45$, $p<0.01$). *Post-hoc t*-test showed significant decreases in folate at all time points (24 h, 48 h, 7 day) compared to baseline ($p<0.01$).

Chronic low-dose MTX administration also significantly decreased CSF folate (Fig. 5B), measured three weeks following the final injection ($p<0.01$).

Serum folate concentrations are shown in Fig. 5C. Seven days after acute MTX injection, serum folate was significantly lower than after saline control ($p<0.01$). There was no significant decrease in serum folate three weeks after the final chronic MTX injection compared with control.

3.1.4. Chronic MTX increases serum homocysteine but not CSF homocysteine

No significant change in CSF homocysteine was observed seven days after acute MTX injection or three weeks after the last chronic injection (Fig. 6A; $F_{(2,29)}=0.2613$, $p=0.77$). In all groups, serum homocysteine was 50–75 fold higher than in CSF. Chronic low-dose MTX increased serum homocysteine concentration by 30% (Fig. 6B; $p<0.01$) compared to controls, when measured three weeks after the last injection. In contrast, serum homocysteine one week after acute MTX was not significantly different than controls ($p=0.61$).

3.1.5. Acute MTX injection induces a transient change in CNS methylation potential

Methylation potential can be altered by MTX, as it can decrease levels of SAM, a primary methyl donor, and increase the levels of SAH, a potent inhibitor of SAM-mediated methylation reactions. CSF SAM concentration was not significantly altered by high-dose one-time MTX administration (Fig. 7A, $F_{(3,42)}=0.6032$, $p=0.61$). CSF SAH concentration increased 24 h after high-dose MTX (Fig. 7C), and returned to baseline level by 4 days after injection. One-way ANOVA

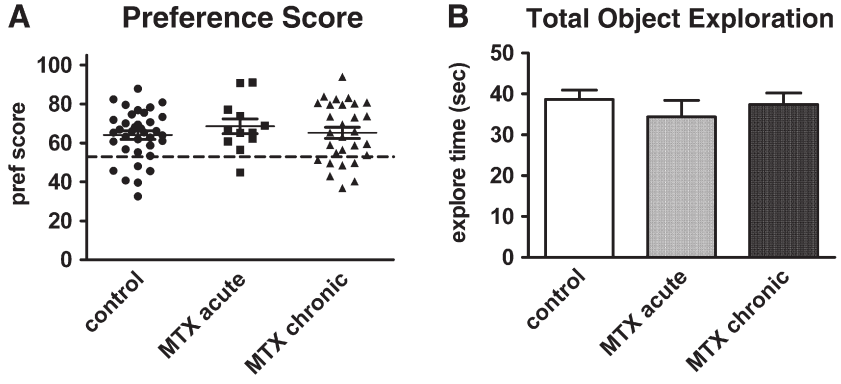

Fig. 3. MTX does not induce deficits in object recognition. A. Preference scores of individual rats with group means and standard error in object recognition test, with 2 h retention interval. Dotted line represents preference score of 53. Group means are: control ($n=36$) 64.1 ± 2.1, MTX acute ($n=12$) 68.6 ± 3.8, MTX chronic ($n=31$) 65.2 ± 2.8. Difference is not significant ($p=0.62$, one-way ANOVA). B. Exploring time in trial 1 of object recognition test. Group means are (in seconds): control 38.76 ± 2.29, MTX acute 34.41 ± 3.99, MTX chronic 37.41 ± 2.79. Difference among groups is not significant ($p=0.67$, one-way ANOVA).

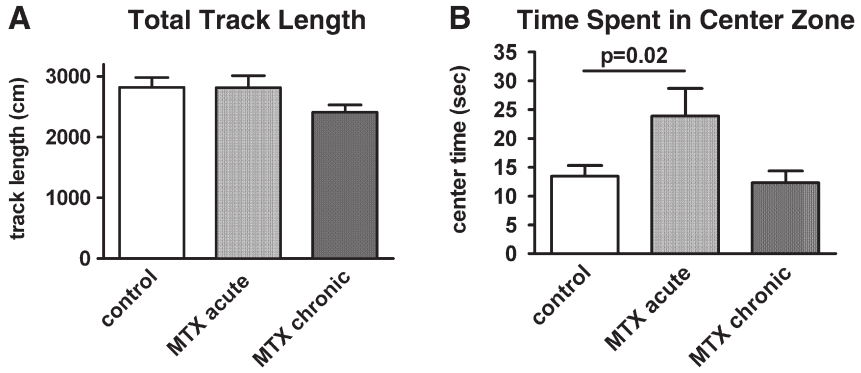

Fig. 4. MTX-injected rats behaved the same as control rats in the open field test. A. Total track lengths in open field tests. Control animals ($n=19$) traveled an average of 2820 ± 164 cm in 6 min, acute MTX rats ($n=12$) traveled 2814 ± 199 cm, and chronic MTX rats ($n=18$) traveled 2411 ± 118 cm. There is no significant difference among groups ($F_{(2,46)}=2.317$, $p=0.11$, one-way ANOVA). B. Length of time rat spent in the center zone in open field test. Group means are: control ($n=20$) 13.45 ± 1.85 s, acute MTX ($n=12$) 23.92 ± 4.77 s, and chronic MTX ($n=18$) 12.33 ± 2.04 s. There is a significant difference between groups ($p<0.01$, one-way ANOVA) due to acute MTX group of rats spent more time in the center zone ($p=0.02$, *post-hoc t*-test).

analysis showed significant treatment effect ($F_{(3,42)}=0.6032$, $p=0.04$), due to the increase at 24 h ($p<0.01$, *post-hoc t*-test). The increase in CSF SAH, with unchanged SAM, suggests a transient decrease in methylation potential, as indicated by the SAM/SAH ratio following acute MTX injection (Fig. 7E; $p<0.01$, *post-hoc t*-test following significant one-way ANOVA analysis).

Interestingly, a decrease in both CSF SAH and SAM was observed three weeks after the last chronic MTX injection (Fig. 7B, D). However, there was no significant difference in CSF SAM:SAH, relative to controls (Fig. 7F).

4. Discussion

A substantial subset of patients demonstrates clinically significant deficits in neurocognitive function after completion of treatment for ALL, even in the current era of treatment protocols that limit the use of cranial radiation for CNS prophylaxis (Buizer et al., 2009; Peterson et al., 2008). Among chemotherapeutic agents used for childhood ALL, many might cause neurotoxicity. In this study, we focus on MTX, because more is known about the multifactorial pathophysiology of MTX-induced cognitive dysfunction (Cole and Kamen, 2006). Here we demonstrated systemic MTX administration causes short-term spatial memory deficits in rats treated both acutely and chronically with MTX, and that the cognitive deficit is concomitant with altered folate homeostasis and CNS methylation potential.

The rat model of MTX-induced neurotoxicity described in this report is representative of chemotherapy-induced cognitive dysfunction among survivors of childhood ALL therapy (Buizer et al., 2005, 2009; Espy et al., 2001; Lofstad et al., 2009; Mennes et al., 2005; Moleski, 2000; Peterson et al., 2008; Waber et al., 2007) in several important aspects. First, the doses of MTX used in our acute studies are analogous to the high-dose MTX employed in the consolidation phases of ALL therapy, while the low-dose chronic MTX treatment is similar to the prolonged weekly MTX given in the maintenance phase of ALL therapy. Second, cognitive deficits were not universal among the rats after MTX, although they occurred at a rate that was statistically greater than among the control animals. Like children treated for ALL, some MTX exposed rats retained intact cognitive function, even though all rats within each of the two treatment cohorts received identical treatment. Third, the observed deficits were focal, involving specific domains of cognitive function, while sparing others. Both acute and chronic administration of MTX induced cognitive deficits in spatial short-term memory without alterations in motor coordination, locomotor activity, visual memory or novel object exploration. Fourth, as has been described among children with ALL (Cole et al., 2009; Drachtman et al., 2002; Vezmar et al., 2009), both the acute and chronic administration of MTX were associated

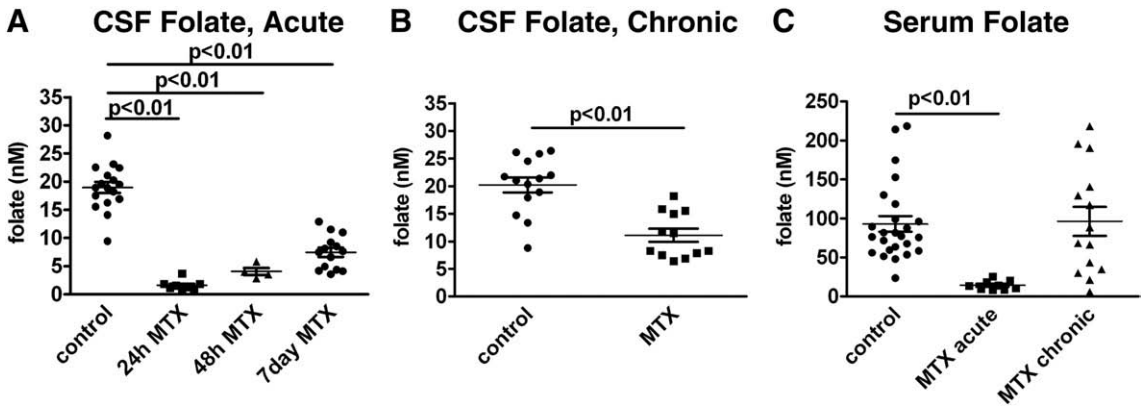

Fig. 5. IP MTX injection decreases CSF and serum folate concentrations. A. Acute 250 mg/kg MTX injection decreases CSF folate concentration. Cerebrospinal fluid folate concentrations (in nM) of these groups are: 18.97 ± 1.01 (control, $n=17$), 1.60 ± 0.33 (24 h after MTX injection, $n=8$), 4.06 ± 0.63 (48 h after MTX, $n=4$), 7.44 ± 0.80 (7 days after MTX, $n=14$). Differences between groups are significant ($F_{(3,39)}=69.45$, $p<0.01$, one-way ANOVA), and *post-hoc t*-tests showed significant differences between control and 24 h, control and 48 h, as well as control and 7 days after MTX injection ($p<0.01$ for all comparisons). B. Weekly MTX injection, 1 mg/kg decreases CSF folate concentration. Samples were collected 3 weeks after last injection. Folate concentrations (nM) are: control ($n=14$) 20.22 ± 1.39, MTX ($n=12$) 11.10 ± 1.20. Difference is significant ($p<0.01$, *t*-test). C. Serum folate levels of different groups are: 93.0 ± 10.0nM (control, $n=25$), 14.2 ± 2.0nM (MTX acute, $n=9$), and 96.4 ± 18.7nM (MTX chronic, $n=14$). The difference between treatments is significant ($F_{(2,45)}=8.452$, $p<0.01$ one-way ANOVA), due to the significant reduction in folate concentration in MTX acute rats ($p<0.01$, *post-hoc t*-test).

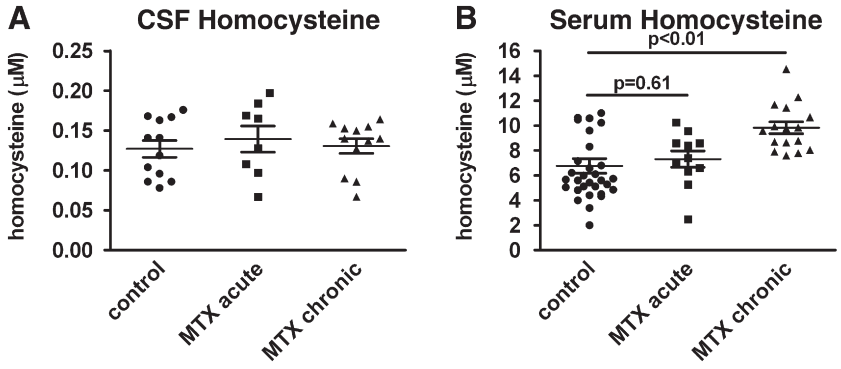

Fig. 6. MTX effects on homocysteine levels in serum and CSF. A. CSF was collected one week after acute MTX injection and three weeks after the last chronic injection. CSF homocysteine concentrations (in μM) were measured by HPLC: control ($n=12$) 0.13 ± 0.01, MTX acute ($n=8$) 0.14 ± 0.02, MTX chronic ($n=12$) 0.13 ± 0.03. One-way ANOVA showed no difference among groups ($F_{(2,29)}=0.2613$, $p=0.77$). B. Serum was collected one week after acute MTX injection and three weeks after the last chronic injection. Serum homocysteine concentrations from different groups are (in μM): control ($n=31$) 6.77 ± 0.59, MTX acute ($n=11$) 7.32 ± 0.66, and MTX chronic ($n=16$) 9.83 ± 0.48. Difference among groups is significant ($F_{(2,55)}=6.542$, $p<0.01$ one-way ANOVA). *Post-hoc t*-test showed that serum homocysteine was higher after chronic MTX exposure than in controls. ($p<0.01$).

with measurable changes in folate-related parameters in blood and cerebrospinal fluid. Fifth, the cognitive deficits induced by chronic MTX were detectable in adult rats, at least three weeks after cessation of treatment, similar to human studies, where cognitive deficits were evident up to 8 years (Carey et al., 2007) after MTX treatment. Because of these parallels, the animal model described in this report is particularly appropriate to the further study of the pathophysiology of treatment-induced cognitive dysfunction as well as of interventions to prevent this toxicity.

4.1. Cognitive deficits

We chose to use the object recognition and object placement tests as our primary assessment of cognitive function for several reasons. First, these tests are analogous to those used in assessing cognitive function in human subjects (Kessels et al., 2002; Salame et al., 2006). Second, they do not require food or water deprivation, or the application of stressors (such as shock). Third, these tests do not require high levels of motor coordination or muscle stamina.

We detected spatial working memory deficits in rats receiving either acute or chronic MTX, in a test with comparatively short retention interval (10 min, Fig. 2A). These deficits were specific to memory in the spatial domain, since MTX subjects were indistinguishable from control subjects in novel object recognition test (Fig. 3A), they also had comparable general activity levels (open field test, Fig. 4), and no gross deficits were evident in balance or coordination. Further, the observation that MTX groups had normal spatial memory in the object placement task with shorter retention intervals (5 min, Fig. 2C, D) indicates that the animals did not lack the normal endogenous preference for novel objects. Thus, we are truly detecting a specific MTX-induced deficit in short-term spatial memory with the object placement test.

4.2. Biochemical mechanisms for MTX causing cognitive deficits

As in humans, the pathophysiology of MTX-induced cognitive deficits in rats is likely to be a result of multiple mechanisms. In humans, MTX exposure results in depletion of folate concentrations within CSF (Cole et al., 2009), directly inhibits nucleotide synthesis, and increases concentrations of the vasculopathic and neurotoxic amino acid homocysteine (Cole et al., 2009; Quinn et al., 1997). Furthermore, MTX inhibits methylation processes by diminishing the ratio of the methyl donor SAM to the methylation inhibitor SAH. Interplay of these mechanisms might contribute to white matter damage and neuronal dysfunction.(Surtees et al., 1991, 1998b) In this study, we observed a spectrum of biochemical changes might contribute to cognitive deficits in rat.

4.2.1. Depletion of folate

Folate is critical to normal brain development and to optimal cognitive function of the mature brain. It plays critical roles in metabolism of the neurotoxic amino acid homocysteine, neurotransmitter synthesis, and methylation reactions necessary for the maintenance of the myelin sheath.

This report is the first to document the significant decrease in CSF and serum folate after MTX exposure in rats (Fig. 5), consistent with the reported decrease in CSF folate among ALL patients treated with MTX (Cole et al., 2009). A decrease in CSF folate was detected as early as 24 h after 250 mg/kg MTX injection, and lasted at least for one week. Even low-dose MTX injection caused a decrease in CSF folate, detectable three weeks after the final injection; serum folate was no different from control rats at this point. These results support our hypothesis that cognitive deficits after MTX exposure are more closely related to the decrease in bioactive folate available to the brain, than to changes in serum folate concentrations.

This decrease in CSF and serum folate following MTX exposure occurred in spite of the fact that these rats were consuming standard rat chow, folate intake of which was calculated to be approximately 300–400 μg/kg. This amount far exceeds recommended daily requirement in humans (400 μg total per day, approximately 6 μg/kg).

The rapid and persistent depletion of CSF folate by systemic MTX may be explained by the multitude of effects that MTX exerts on folate transport. MTX transiently increases urinary folate excretion (Deutsch and Kolhouse, 1989), decreases folate uptake across choroid plexus into CSF in spite of sufficient serum level (Chen and Wagner, 1975; Spector and Lorenzo, 1975), and directly inhibits uptake of folate by primary cultured rat astrocytes (Cai and Horne, 2003). Previous rat studies demonstrated that treatment with MTX decreases folate levels in solid tissues, such as brain (Alonso-Aperte and Varela-Moreiras, 1996) and liver (Varela-Moreiras et al., 1995). Similar results were observed in chronically treated rhesus monkeys, where the brain was the tissue with the greatest decrease (>90%) in folate content (Winick et al., 1987).

4.2.2. Homocysteine

Homocysteine and its metabolites are excitotoxic amino acids, agonists at the N-methyl-D-aspartate receptor, and are suspected to relate to the neurotoxic effects of MTX (Quinn and Kamen, 1996). A lack of folate may inhibit homocysteine conversion to methionine by removing the methyl group substrate (Fig. 1). Homocysteine has been reported to increase in CSF after MTX exposure in human (Cole et al.,

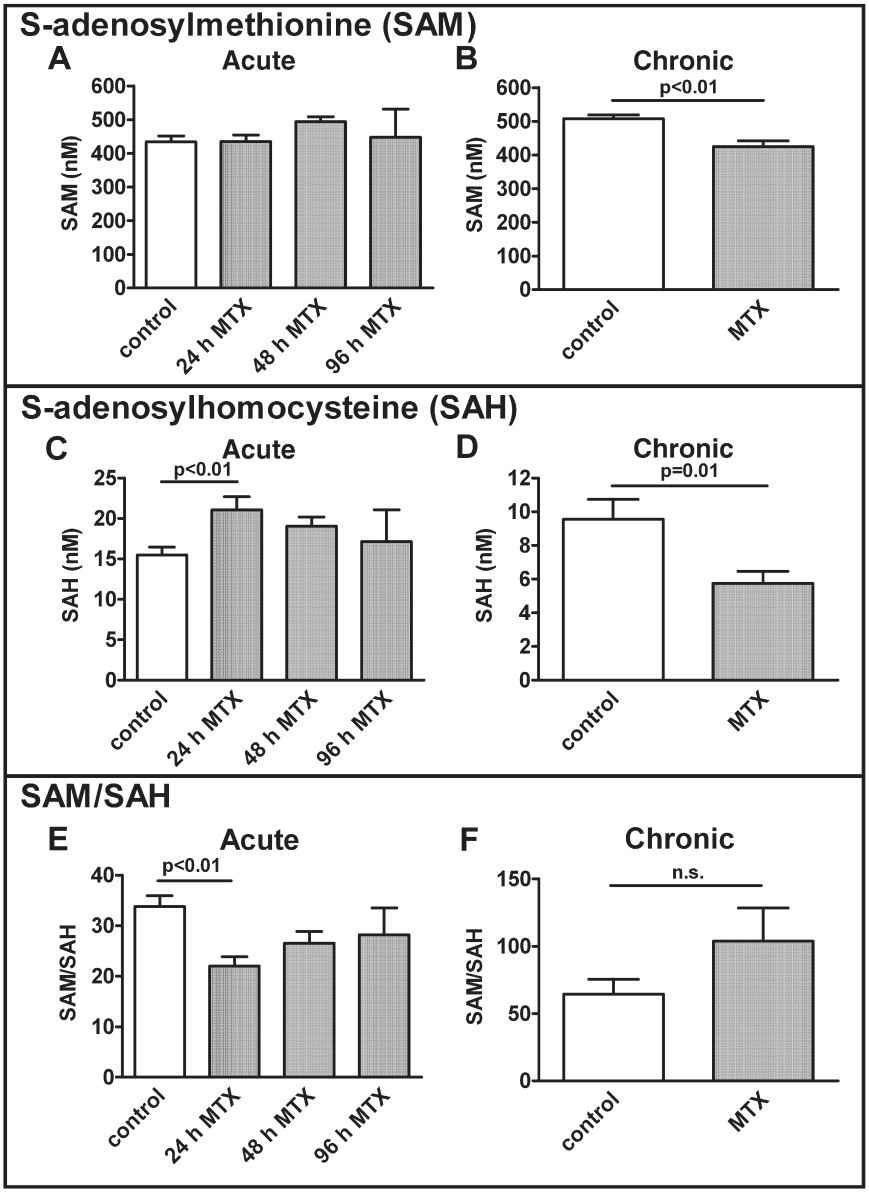

Fig. 7. Effects of MTX on S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), and SAM/SAH ratio. A. Acute 250 mg/kg MTX injection did not change SAM concentration in CSF. Mean concentrations (in nM) are: 433.8 ± 17.9 (control, $n = 24$), 435.1 ± 19.3 (24 h after MTX, $n = 12$), 494.0 ± 14.6 (48 h after MTX, $n = 5$), and 447.9 ± 83.0 (96 h after MTX, $n = 5$). Difference among groups is not significant ($F_{(3,42)} = 0.6032$, $p = 0.61$ one-way ANOVA). B. Repetitive low-dose MTX injection decreased CSF SAM concentration. Mean concentrations (in nM) are: 507.9 ± 10.5 (control, $n = 10$), and 424.5 ± 17.4 (MTX, $n = 15$). The difference is significant ($p < 0.01$, t-test). C. Acute 250 mg/kg MTX injection increased CSF SAH 24 h after injection. Mean concentrations (nM) of each group are: 15.47 ± 0.98 (control, $n = 24$), 21.04 ± 1.63 (24 h after MTX, $n = 12$), 19.02 ± 1.14 (48 h after MTX, $n = 5$), and 17.14 ± 3.93 (96 h after MTX, $n = 5$). Difference among groups is significant ($F_{(3,16)} = 8.759$, $p = 0.04$ one-way ANOVA), and it was due to the increase of SAH at 24 h after MTX injection ($p < 0.01$, *post-hoc* t-test). D. Repetitive low-dose MTX injection decreased CSF SAH concentration. Mean concentrations (in nM) are: 9.56 ± 1.19 (control, $n = 9$), and 5.75 ± 0.72 (MTX, $n = 11$). The difference is significant ($p = 0.01$, t-test). E. Acute 250 mg/kg MTX injection decreases the SAM/SAH ratio in CSF. SAM/SAH ratios: 33.78 ± 2.10 (control, $n = 15$), 22.01 ± 1.83 (24 h after MTX, $n = 12$), 26.52 ± 2.33 (48 h after MTX, $n = 5$), and 28.17 ± 5.36 (96 h after MTX, $n = 5$). Significant difference among groups ($F_{(3,33)} = 5.048$, $p < 0.01$, one-way ANOVA) was due to the decrease at 24 h after MTX ($p < 0.01$, *post-hoc* t-test). F. No significant change in CSF SAM/SAH ratio was observed after chronic MTX. SAM/SAH ratio was calculated based on measurements from panels B and D: 64.40 ± 10.99 (control, $n = 10$), 103.7 ± 24.60 (MTX, $n = 10$). The difference is not significant ($p = 0.16$, t-test).

2009; Quinn et al., 1997) and in animal studies (Varela-Moreiras et al., 1995).

We did not observe significant changes in CSF homocysteine concentrations one week following acute MTX or three weeks after chronic MTX exposure (Fig. 6A), despite significant decrease in CSF folate concentrations (Fig. 5A, 5B) at these time points. It is possible that increased CSF homocysteine occurs only at earlier time points and then resolves, due to the rapid clearance of MTX from CSF (Bode et al., 1980). We have previously noted that CSF homocysteine returns to baseline by one week after intrathecal MTX in children with ALL (Cole et al., 2009). The difference in MTX effects on serum and CSF homocysteine concentrations shown here (Fig. 7) is consistent with other evidence that there is no correlation between CSF and plasma/serum homocysteine level (Vrethem et al., 2003).

4.2.3. Brain methylation potential

In any given compartment, the ratio of SAM:SAH indicates the folate-dependent methylation potential. SAM is the methyl donor for steps in neurotransmitter synthesis and maintenance of the myelin sheath (Chiang et al., 1996), while the end-product SAH is the most potent inhibitor of all SAM-mediated reactions. A decrease in methylation potential, indicated by a decrease in the ratio of SAM:

SAH, is associated with demyelination, leukoencephalopathy, and clinical toxicity among humans treated with MTX (Hyland et al., 1988; Kishi et al., 2000; Surtees et al., 1991, 1998a). Consistent with the human data, we observed a significant, albeit transient, increase in CSF SAH following high-dose MTX exposure (Fig. 7C), with a consequent decrease in the SAM:SAH ratio (Fig. 7E).

Both SAM and SAH were decreased in CSF three weeks after the last chronic MTX injection, relative to control rats (Fig. 7B, 7D). In addition to enzymatic synthesis by the combination of adenosine with homocysteine, SAH is generated when SAM participates in a methylation reaction. It is therefore possible that the observed decrease in SAH was simply a result of decreased SAM.

It is also possible that significant changes in the SAM:SAH ratio were present earlier in the course of chronic MTX exposure, that resolved or reversed by three weeks after the final injection. Investigating these earlier changes, however, was limited by the difficulty in collecting CSF in younger rats without contamination by blood.

4.3. Other possible mechanisms by which MTX causes neurotoxicity

Even though our results emphasize that MTX depletes folate stores available to the CNS, other mechanisms are likely to be involved in the pathophysiology of MTX-induced neurotoxicity. For example, MTX induces signs of oxidative stress in the phospholipids of the CNS (Miketova et al., 2005), is directly toxic to neuronal tissue in vitro (Gilbert et al., 1989; Gregorios et al., 1989) and may inhibit hippocampal neurogenesis (Seigers et al., 2008, 2009). We have not yet examined the contribution of these processes to cognitive deficits in our rat model.

4.4. Limitations on the applicability of this rat model to studies of patients with ALL

Extrapolation of our results to patients is restricted by biological differences between rats and humans. For example, our laboratory rats have significantly higher mean serum folate concentrations (93.0 ± 10.0 nM) than children with ALL (20.2 ± 4 nM) (Cole et al., 2009), possibly due to high folate content in standard rat chow. In addition, CSF folate concentrations are lower in rats (19.0 ± 1.0 nM) than humans (62.7 ± 3.4 nM) (Cole et al., 2009). The marked difference in the ratio of CSF to serum folate between these species suggests that the regulation of folate transport from serum to the CNS also differs. Consequently, findings relative to prediction or prevention of neurotoxicity will have to be confirmed in translational and clinical trials.

Furthermore, in addition to systemic (oral or intravenous) MTX, all therapeutic protocols for patients with ALL include direct administration of MTX into the intrathecal space, to prevent CNS relapse. It is likely that prophylactic intrathecal MTX is a significant contributor to treatment-induced cognitive dysfunction, as the CSF concentration of MTX following IT administration (>100 μM) (Bleyer and Dedrick, 1977) is many orders of magnitude greater than CSF MTX concentrations seen after systemic standard-dose therapy (~20 nM) (Cole et al., 2006). We are, therefore, currently investigating the effects of intrathecal MTX on rat cognitive function and CNS folate physiology.

5. Conclusion

In this study, we demonstrate that either a single high-dose IP MTX injection or repetitive low-dose MTX injection causes measurable cognitive deficits in rats, associated with significant changes in CSF folate physiology. In addition to allowing further study of the pathophysiology of MTX-induced cognitive dysfunction, this model provides us a useful tool to test protective pharmacologic strategies.

Acknowledgements

We are thankful of the technical support of Zareen Mehmud. This work was supported in part by the Damon Runyon Cancer Research Foundation (CI-16-03).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pbb.2009.10.008.

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