

Disruption of *Shmt1* Impairs Hippocampal Neurogenesis and Mnemonic Function in Mice^{1–3}

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Abstract

Impaired folate-mediated one-carbon metabolism (OCM) has emerged as a risk factor for several diseases associated with age-related cognitive decline, but the underlying mechanisms remain unknown and thus hinder the identification of subpopulations most vulnerable to OCM disruption. Here we investigated the role of serine hydroxymethyltransferase 1 (SHMT1), a folate-dependent enzyme regulating de novo thymidylate biosynthesis, in influencing neuronal and cognitive function in the adult mouse. We observed *Shmt1* expression in the hippocampus, including the granule cell layer of the dentate gyrus (DG), and examined hippocampal neurogenesis and hippocampal-dependent fear conditioning in mice deficient for *Shmt1*. We used a 3 × 3 design in which adult male *Shmt1*^{+/+}, *Shmt1*^{+/-}, and *Shmt1*^{-/-} mice were fed folic acid control (2 mg/kg), folic acid-deficient (0 mg/kg), or folic acid-supplemented (8 mg/kg) diets from weaning through the duration of the study. Proliferation within the DG was elevated by 70% in *Shmt1*^{+/-} mice, yet the number of newborn mature neurons was reduced by 98% compared with that in *Shmt1*^{+/+} mice. Concomitant with these alterations, *Shmt1*^{+/-} mice showed a 45% reduction in mnemonic recall during trace fear conditioning. Dietary folate manipulations alone did not influence neural outcomes. Together, these data identify SHMT1 as one of the first enzymes within the OCM pathway to regulate neuronal and cognitive profiles and implicate impaired thymidylate biosynthesis in the etiology of folate-related neuropathogenesis. *J. Nutr.* 143: 1028–1035, 2013.

Introduction

Low folate status is associated with several adverse neurocognitive outcomes, but the underlying mechanisms remain poorly defined (1). In particular, memory function has emerged as acutely sensitive to perturbations in folate homeostasis (2,3). Mnemonic performance relies on the integrity of the hippocampus, and it has been suggested that decreased hippocampal neurogenesis as a result of aging may contribute to hippocampal dysfunction and memory impairment (4,5). Impaired hippocampal proliferation affects performance on certain memory tasks in rodents (6,7), and altered hippocampal proliferation has also been observed in the context of several neurological disorders (8–10). Interestingly, adult mice fed an FD diet exhibit

impaired hippocampal proliferation (11,12). The effect of folate deficiency on hippocampal proliferation may, therefore, underlie the association between disrupted one-carbon metabolism (OCM)⁹ and memory impairment; however, studies clearly identifying the responsible pathways and mechanism(s) are lacking.

Folates function as cofactors for the remethylation of homocysteine to methionine and for the de novo synthesis of purines and thymidylate (dTMP) (13) (**Supplemental Fig. 1**). Methionine can be adenosylated to form S-adenosylmethionine (AdoMet), the primary methyl donor for methylation of DNA and RNA and for neurotransmitter synthesis. Impaired dTMP biosynthesis causes uracil misincorporation into the genome, which contributes to hippocampal neurodegeneration (12). Elevated homocysteine induces oxidative stress and neurodegeneration (14) and impairs hippocampal proliferation (15). Thus, impaired folate metabolism may promote neuropathogenesis by affecting homocysteine levels, cellular methylation, and/or genomic stability. Unfortunately, dietary manipulation of folate status affects the entire OCM network and is thus

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⁹ Abbreviations used: AdoMet, S-adenosylmethionine; BrdU, 5-bromodeoxyuridine; DG, dentate gyrus; dTMP, thymidylate; FC, folate control; FD, folate deficient; FS, folate supplemented; NeuN, neuronal nuclei; OCM, one-carbon metabolism; RT, room temperature; SGZ, subgranular zone; SHMT1, serine hydroxymethyltransferase 1; 5-methylTHF, 5-methyltetrahydrofolate.

insufficient to discriminate among these potential causal pathways.

Serine hydroxymethyltransferase 1 (SHMT1) is a folate-dependent enzyme that regulates homocysteine remethylation and de novo dTMP biosynthesis (16,17) (Supplemental Fig. 1). In the cytoplasm, SHMT1 sequesters 5-methyltetrahydrofolate (5-methylTHF), a cofactor necessary for homocysteine remethylation and subsequent AdoMet production (Supplemental Fig. 1). During the S phase, SHMT1 translocates into the nucleus to provide thymidine triphosphate for DNA replication (18–20). Interestingly, *Shmt1* heterozygosity, but not homozygosity, elevates genomic uracil misincorporation and cellular methylation potential (16); however, only impaired dTMP biosynthesis mediates colon cancer risk and neural tube closure defects in *Shmt1*^{+/-} mice (21,22). Studying the consequences of *Shmt1* disruption may, therefore, clarify the underlying mechanisms connecting impaired folate metabolism and neurocognitive disorders.

In this study, we used *Shmt1*-deficient mice to determine the mechanism(s) by which impaired folate metabolism influences hippocampal function at both the cellular and behavioral levels. We hypothesized that disruption of dTMP biosynthesis in *Shmt1*^{+/-} mice would affect hippocampal neurogenesis and related behavioral outcomes, but we examined both *Shmt1*^{+/-} and *Shmt1*^{-/-} mice as each has been associated with a distinct metabolic and disease phenotype. Together, these experiments aimed to identify a causal relationship between disruption of specific pathways in folate-mediated OCM and folate-associated neuropathogenesis.

Materials and Methods

Experimental mice and diets. The generation and characterization of *Shmt1*^{-/-} mice have been previously described (16). *Shmt1*^{+/-} mice were maintained as a congenic heterozygous breeding colony on a C57BL/6 background (Jackson Laboratories). Mice received ad libitum access to food and water and were housed under a 12-h light/dark cycle under specific pathogen-free conditions. All experimental procedures were approved by the Cornell Institutional Animal Care and Use Committee according to the guidelines of the Animal Welfare Act and all applicable state and federal laws. At weaning, male *Shmt1*^{+/+}, *Shmt1*^{+/-}, and *Shmt1*^{-/-} mice were randomly assigned to an AIN93G folate-control (FC; 2 mg/kg folic acid; Dyets), folate-deficient (FD; 0 mg/kg folic acid), or folate-supplemented (FS; 8 mg/kg folic acid) diet. Assignment to each genotype and diet condition was balanced across litters. All mice remained on their respective diet until they were killed. Mice were housed up to 4 to a cage and grouped according to diet with the exception of mice used for behavioral analysis, which were housed individually at least 1 wk prior to training. Unless otherwise noted, experiments were conducted on male mice between 14–16 mo of age. All experiments were conducted by an investigator blinded to genotype and diet group.

Experimental design. Mice were assigned to 1 of 3 experimental groups, and group assignment was balanced across litters. Experimental group A underwent behavioral testing as described below. Three weeks after behavioral analysis, these same mice were killed by cervical dislocation, and their brains, livers, plasma, and RBCs were flash-frozen for biochemical analysis, including measurement of metabolite concentrations and genomic uracil content. Hippocampal proliferation was assessed in mice assigned to group B 24 h after pulse treatment with the thymidine analog 5'-bromodeoxyuridine (BrdU; Sigma). A 50-mg/kg dose of BrdU was injected intraperitoneally once daily for 4 consecutive days. Survival and neuronal differentiation of proliferating cells were assessed in mice assigned to group C 3 wk after this same BrdU pulse.

In situ hybridization. Localization of *Shmt1* mRNA by in situ hybridization was performed by using digoxigenin-labeled RNA probes

as previously described (23). Probes were synthesized to the 5' end of *Shmt1* with the following primers: 1) forward, 5'-TGGCGGGACC-GAGTTCATC-3'; and 2) reverse, 5'-CCAGGTTCCGGGAGTAG-CAG-3'. Expression was analyzed in adult mice that were 6–8 wk of age.

BrdU immunofluorescent staining. Twenty-four hours (group B) or 3 wk (group C) after the last BrdU injection, mice were transcardially perfused with PBS followed by 4% paraformaldehyde. Thirty-micrometer sections were treated for 20 min at 95°C in 1× Antigen Retrieval Solution (Dako), and DNA was denatured with 2 N HCl for 1 h at 37°C. Sections were incubated with rat anti-BrdU (1:50; Abcam) overnight at 4°C and with Alexa Fluor 488 conjugated goat anti-rat secondary antibody (1:400; Molecular Probes) for 1 h at room temperature (RT).

BrdU/NeuN immunofluorescence. Free-floating sections were processed by using the same procedure as for BrdU-alone immunostaining but without antigen retrieval. Sections were incubated with rat anti-BrdU (1:600; Abcam) and mouse anti-NeuN (1:1000; Chemicon) for 1 h at RT, followed by an incubation of Alexa Fluor 488 conjugated goat anti-rat (1:400; Molecular Probes) and Cy5 conjugated goat anti-mouse (1:300; Chemicon) for 1 h at RT.

Imaging and cell counts. Every 10th (BrdU alone, group B) or 20th (BrdU/NeuN, group C) section throughout the rostral-caudal extent of the hippocampus was visualized on a confocal microscope (Leica TCS SP2) at a magnification of 20×. A z-series was taken throughout the depth of each section and reconstructed in Volocity 4.4 (PerkinElmer). BrdU-labeled cells were counted in the subgranular zone (SGZ) of the DG, defined as a 2-cell width spanning the intersection between the granule cell layer and the hilus. BrdU/NeuN-labeled cells were counted within the entirety of the granule cell layer. Cell counting of BrdU+ cells was performed automatically in Volocity 4.4. Colabeling of BrdU+ cells with NeuN was determined manually.

Volume measurements. The volume of the DG was measured in every section adjacent to those used in immunofluorescent staining. The area of the DG was measured in Nissl-stained sections by manual tracing using Metamorph Image Analysis (Molecular Devices). Volume was calculated by multiplying the area of each section with its depth (30 μm) and summing these values across the rostral-caudal extent of the hippocampus.

Auditory trace fear conditioning. Auditory trace fear conditioning was used to assess associative memory deficits related to hippocampal proliferation (6). Whereas trace fear conditioning measures subtle defects associated with altered hippocampal proliferation, contextual conditioning serves as a measure of general hippocampal integrity (24,25). The protocol closely followed Smith et al. (26) and is summarized in Figure 3. Briefly, mice were first placed individually into the training chamber (context A, vanilla scent; Coulbourn) and acclimated for 720 s. Percentage freezing, defined as complete immobility for at least 1 s other than that required for respiration, was recorded by Actimetrics FreezeFrame software (Coulbourn) throughout the entirety of all procedures. Twenty-four hours after acclimation, mice were returned to context A and presented with six 100-s trials consisting of a 20-s sound (80 db, 2 kHz) and a 2-s shock (0.7 mAmp), separated by an interval of 15 s (the “trace”) between the offset of the tone and the onset of the shock. The first tone was presented after a 3-min acclimation period, and subsequent trials were separated by a 63-s intertrial interval. The following day, mice were returned to context A for 300 s to assess contextual conditioning. Freezing behavior in response to the context was measured across the entirety of the 300-s session. Approximately 2 to 4 h later, mice were placed into a novel context with a different set of visual and olfactory cues (context B, bedding scent; Coulbourn) and presented with 4 trials that were identical to those from training, except that the foot-shock was not delivered. Baseline freezing was measured during the first 30 s after placement into context B, prior to the presentation of the first tone. Trace conditioning was measured as freezing during the 35-s interval including the presentation of the tone

and the trace period. All testing occurred during the light phase of the light/dark cycle.

Determination of metabolites. Blood was collected by cardiac puncture into heparin-coated tubes (BD Vacutainer) and centrifuged at $2000 \times g$ for 5 min to isolate plasma. Tissues were immediately flash-frozen in liquid nitrogen and stored at -80°C . Plasma metabolite concentrations were determined by stable isotope dilution capillary GS-MS as described elsewhere (27,28). Folate concentrations in RBCs and cortex samples were measured by a *Lactobacillus casei* assay as previously described (29).

Statistical analysis. Analyses of cell counts, genomic uracil content, and metabolite measurements were conducted in JMP, version 8.02 (SAS Institute). Cell counts and metabolite measurements were analyzed by 2-way ANOVA with Tukey's post hoc analysis. *Shmt1* genotype and diet were included as independent variables. BrdU cell counts and serine concentrations were log-transformed in order to normalize the data. For BrdU/NeuN quantification, 3 mice (2 *Shmt1*^{+/-} mice and 1 *Shmt1*^{-/-} mouse) were excluded from the analysis due to the complete absence of BrdU+ cells. Analyses of behavior data were performed in SAS (version 9.0) by using a mixed-model procedure (PROC Mixed) followed by post hoc analysis with Student's *t* test and Bonferroni's correction. Values in the text indicate means \pm SEMs. Differences were considered significant at $P \leq 0.05$.

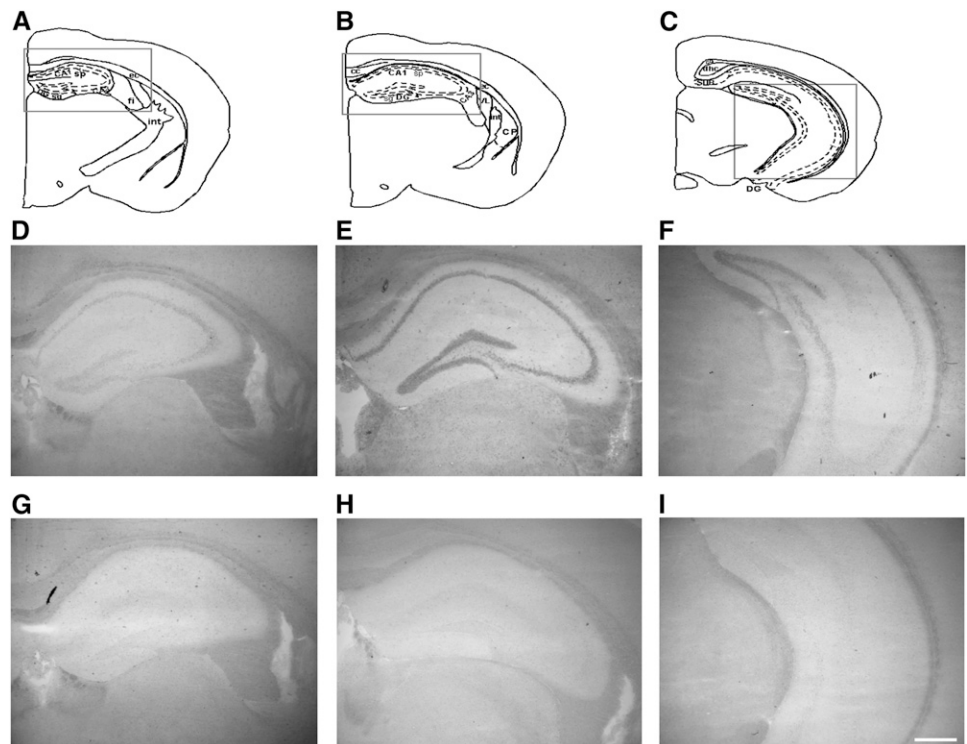
Results

***Shmt1* is specifically expressed in regions of constitutive neurogenesis.** Examination of *Shmt1* expression revealed restricted expression within the adult mouse brain. In situ hybridization localized *Shmt1* mRNA to the hippocampus, including the DG and cornu ammonis regions (Fig. 1D–F), as well as the subventricular zone, rostral migratory stream, and the cerebellum (data not shown). Thus, *Shmt1* mRNA is expressed in regions of cellular proliferation, as well as in regions implicated in memory function.

***Shmt1* disruption enhances proliferation in the SGZ of the DG.** Analysis of hippocampal cell proliferation, as determined by the total number of BrdU+ cells in the SGZ of the DG, revealed that *Shmt1*^{+/-} mice exhibited 70% more BrdU+ cells compared with the *Shmt1*^{+/+} and *Shmt1*^{-/-} mice ($P = 0.012$; Fig. 2A, B). Two-way ANOVA also revealed a significant gene \times diet interaction ($P = 0.0011$); however, post hoc analysis revealed that none of the groups were significantly different from *Shmt1*^{+/+} mice fed an FC diet ($P > 0.10$), and the limited sample size of a few groups obstructed clear interpretation of the interaction effect (see Materials and Methods). Dietary folate intake alone did not independently affect hippocampal proliferation ($P > 0.10$). Together, these data indicate that partial loss of *Shmt1* expression results in greater proliferation in the SGZ independent of dietary folic acid intake.

***Shmt1* disruption decreases the number of newly born, mature neurons in the DG.** To determine whether the increased proliferation in the DG of *Shmt1*^{+/-} mice resulted in greater net neurogenesis, the survival and differentiation of BrdU-incorporating cells was analyzed 3 wk after a BrdU pulse. In this paradigm, BrdU+ cell counts reflected both initial proliferation and subsequent survival of newly generated cells. Further phenotypic analysis of BrdU-labeled cells was performed by costaining with the mature neuronal marker NeuN. Analysis throughout the granule cell layer of the DG revealed that the total number of BrdU-labeled cells remaining 3 wk after BrdU pulse did not differ across *Shmt1* genotypes or diet conditions ($P > 0.10$; Fig. 2D). However, analysis of the number of BrdU/NeuN-labeled cells showed a trend toward a significant effect of *Shmt1* genotype ($P = 0.05$; Fig. 2D). Post hoc analysis revealed that *Shmt1*^{+/-} mice exhibited significantly fewer BrdU/NeuN-labeled cells compared with *Shmt1*^{+/+} mice (Fig. 2D). Diet and gene \times diet interactions did not significantly affect the total number of BrdU/NeuN-labeled cells. These results indicate that

FIGURE 1 *Shmt1* is expressed in the CA regions and dentate gyrus of the hippocampus. (A–C) Schematics showing coronal sections at 3 rostro-caudal levels corresponding to -1.60 , -2.10 , and -5.70 mm relative to Bregma. (D–F) Photomicrographs indicating in situ hybridization signal with antisense *Shmt1* probes in coronal brain sections. (G–I) Corresponding photomicrographs depicting in situ hybridization signal with a sense (control) probe. CA, cornu ammonis; cc, corpus callosum; CP, caudate putamen; DG, dentate gyrus; dhc, dorsal hippocampal commissure; ec, entorhinal cortex; fi, fimbria; int, internal transverse artery; *Shmt1*, serine hydroxymethyltransferase 1; sg, stratum granulosum; sp, stratum pyramidale; SUB, subiculum; VL, lateral ventricle.



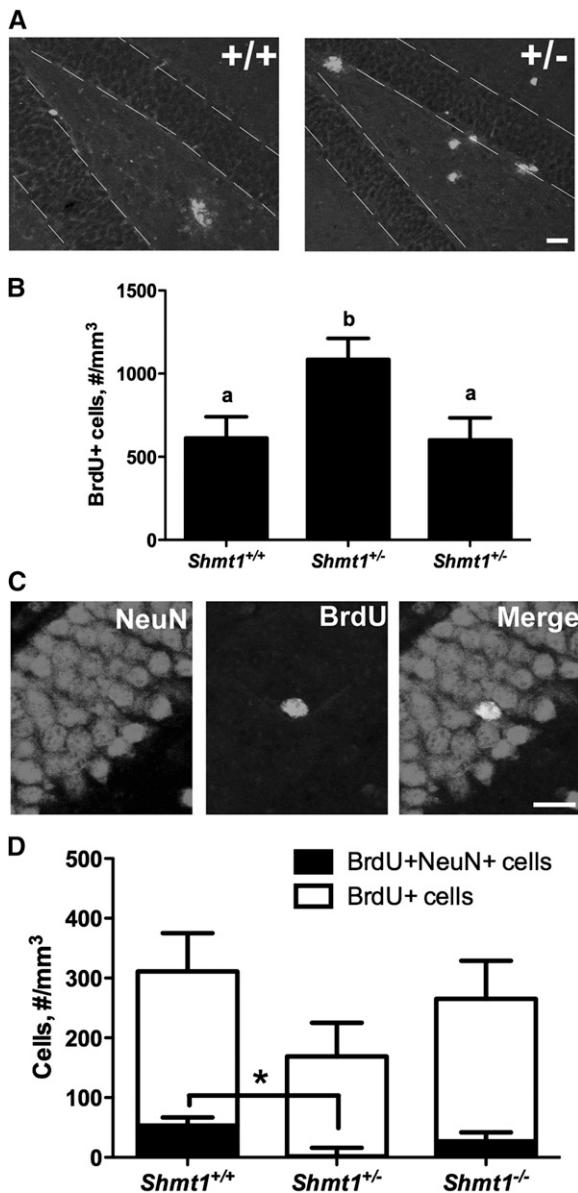


FIGURE 2 *Shmt1* heterozygosity, independent of diet, results in greater hippocampal cellular proliferation but reduced numbers of mature neurons in the DG. (A) Representative images of BrdU+ cells (green) in the DG of *Shmt1*^{+/+} and *Shmt1*^{+/-} mice 24 h after BrdU pulse. (B) Quantification of BrdU-labeled cells from panel A. Values are means \pm SEMs. Groups designated with different letters are significantly different from one another: *Shmt1*^{+/-} mice are significantly different than *Shmt1*^{+/+} and *Shmt1*^{-/-} mice ($P = 0.012$, $n = 9$ – 10 per genotype). (C) A representative image of a BrdU/NeuN-labeled cell in the DG 3 wk after BrdU pulse. (D) Quantification of BrdU+ cells and BrdU/NeuN-labeled cells 3 wk after BrdU pulse. Values are means \pm SEMs. No significant differences in the number of BrdU+ cells were observed among groups ($P > 0.1$, $n = 9$ – 12 per genotype). Analysis of BrdU/NeuN-labeled cells, however, showed a trend toward a significant effect of genotype, and post hoc analysis revealed that *Shmt1*^{+/-} mice were significantly different from *Shmt1*^{+/+} mice ($*P = 0.05$, $n = 8$ – 10 per genotype). BrdU, 5-bromodeoxyuridine; DG, dentate gyrus; NeuN, Neuronal Nuclei; *Shmt1*, serine hydroxymethyltransferase 1.

despite the high levels of proliferation initially observed in the *Shmt1*^{+/-} mice, the majority of these newborn cells did not persist after their generation. Indeed, of the newborn cells that did remain in the DG of *Shmt1*^{+/-} mice, significantly fewer mature neurons were observed than in wild-type mice. Despite

this reduction in newborn neurons, no differences in total volume of the DG were observed among groups ($P > 0.10$; data not shown).

***Shmt1* disruption impairs trace fear conditioning.** We next investigated whether altered proliferation and neurogenesis observed in *Shmt1*^{+/-} mice correlated with behavioral outcomes in a fear-conditioning paradigm (Fig. 3A). Two-way ANOVA revealed that *Shmt1*^{+/-} mice froze significantly less compared with *Shmt1*^{+/+} mice during presentation of the tone alone when freezing was averaged across all trials (freezing was analyzed during the tone and trace; $P = 0.02$; Fig. 3B). No significant effect of diet or gene \times diet interaction was observed ($P > 0.10$). Acquisition freezing, baseline freezing, or freezing behavior during the context test did not differ among groups ($P > 0.10$; Fig. 3B and data not shown). These results demonstrate that *Shmt1*^{+/-} mice exhibit a specific deficit in hippocampal function consistent with the alterations observed in hippocampal neurogenesis.

***Shmt1* disruption and folate intake influence folate status in brain and RBC tissue.** We examined the effect of *Shmt1* deficiency and dietary folate manipulation on blood and brain folate concentrations. Dietary folic acid deficiency significantly reduced RBC folate concentrations by 40% ($P < 0.0001$; Table 1),

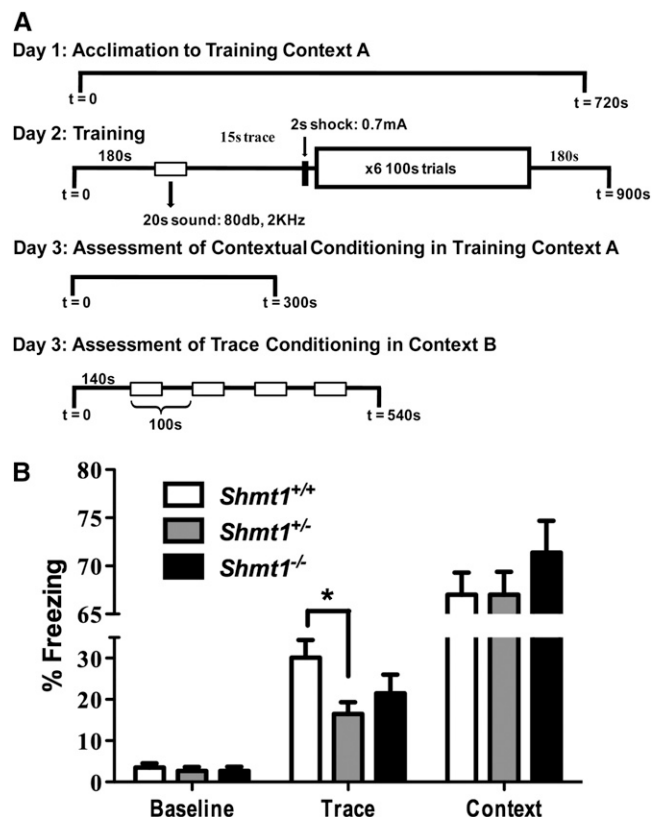


FIGURE 3 Mice heterozygous for *Shmt1* exhibit deficits in auditory trace fear conditioning independent of diet. (A) Schematic representation of the behavior protocol. (B) *Shmt1*^{+/-} mice showed a significant reduction in percentage freezing during the trace test compared with *Shmt1*^{+/+} mice ($*P = 0.020$, $n = 7$ – 10 per condition). No differences in freezing were observed at baseline (measured during the first 30 s in context A on training day 2, prior to presentation of the first tone) or across all 5 min of testing during the context test ($P > 0.10$). Values are means \pm SEMs. *Shmt1*, serine hydroxymethyltransferase 1.

but dietary folate supplementation did not affect RBC folate concentrations. In the brain, 2-way ANOVA revealed a significant gene \times diet interaction on folate concentrations ($P = 0.037$; Table 1). Student's t test post hoc analysis indicated significantly reduced brain folate concentrations in both $Shmt1^{+/+}$ and $Shmt1^{+/-}$ mice in response to the FD diet, but the FS diet did not significantly elevate folate concentrations relative to the FC diet (Table 1). In contrast, brain folate concentrations in $Shmt1^{-/-}$ mice did not differ between the FC and FD diets ($P > 0.10$; Table 1); only the FS diet significantly elevated folate concentrations above those observed with the FD diet in these mice (Table 1). Overall, these results suggest that the FD diet reduced brain folate concentrations, whereas the FS diet did not significantly elevate brain folate concentrations. These results are consistent with both a significant main effect of diet and the absence of an effect of the FS diet on blood folate concentrations across all 3 genotypes. Additionally, $Shmt1^{-/-}$ mice were differentially sensitive to dietary manipulation of folate concentrations.

Shmt1 and dietary folate deficiency influence metabolic markers of homocysteine remethylation. Both dietary folate status and $Shmt1$ disruption significantly influenced several biomarkers of homocysteine metabolism. The FD diet resulted in a doubling of homocysteine concentrations compared with the FC and FS diets ($P < 0.0001$; Table 2), whereas homocysteine concentrations were significantly reduced by 25% in $Shmt1^{-/-}$ mice compared with the $Shmt1^{+/+}$ mice ($P = 0.032$; Table 2). ANOVA also revealed significant effects of both diet ($P = 0.005$; Table 2) and $Shmt1$ genotype ($P = 0.034$; Table 2) on plasma concentrations of cystathionine, a product of homocysteine degradation via the folate-independent transsulfuration pathway, catalyzed by the enzyme cystathionine β -synthase. Cystathionine concentrations were $\sim 20\%$ higher in mice fed the FD diet compared with those fed the FC and FS diets (Table 2), consistent with the observed increase in homocysteine concentrations associated with the FD diet. In contrast, cystathionine concentrations were reduced by 15% in $Shmt1^{+/-}$ mice, suggesting that $Shmt1^{+/-}$ mice may metabolize less homocysteine through the transsulfuration pathway (Table 2). Serine concentrations were also significantly affected by diet and $Shmt1$ genotype. Post hoc analysis of a significant gene \times diet interaction ($P = 0.034$; Table 2) revealed that serine concentrations were significantly elevated in $Shmt1^{+/-}$ males fed the FS diet compared with those fed the FC and FD diets (Table 2).

Discussion

Epidemiological studies have identified impaired folate metabolism resulting from either dietary deficiency and/or genetic

variation as a possible risk factor for age-related cognitive decline and neurodegenerative diseases [for review see (1)]. However, study of the mechanisms by which impaired folate metabolism affects neurocognitive function has been obstructed by the lack of available genetic mouse models that target individual folate-dependent pathways without causing severe disruption to the broader OCM network. In this study, we report that deficiency of the folate-metabolizing enzyme SHMT1, which regulates folate-dependent de novo dTMP biosynthesis, affects hippocampal function at both the cellular and behavioral levels in adult mice. $Shmt1$ showed enriched expression within the hippocampus (Fig. 1), and adult $Shmt1^{+/-}$ mice exhibited elevated hippocampal cellular proliferation (Fig. 2A, B). However, the majority of proliferating cells did not persist after their generation. Although we did not explore the alternative fates of these newborn cells, we demonstrated that of the BrdU+ cells remaining after 3 wk, significantly fewer expressed the mature neuronal marker NeuN (Fig. 3C, D). Concomitant with these alterations in neurogenesis, $Shmt1^{+/-}$ mice performed significantly worse on a trace fear-conditioning task, a test of associative memory that has been demonstrated to reflect changes in hippocampal proliferation (6,7). We have previously reported that $Shmt1^{+/-}$ mice are impaired in their ability to synthesize dTMP de novo and, therefore, accumulate uracil within nuclear DNA (16). Together, these data implicate SHMT1 in regulating hippocampal function and suggest that impaired dTMP biosynthesis may contribute to neurocognitive dysfunction.

In the present study, we observed neural and cognitive deficits in mice that were heterozygous for $Shmt1$ disruption, but mice that were homozygous null for $Shmt1$ were not affected. Our laboratory has recently shown that $Shmt1$ heterozygosity correlates with both elevated genomic uracil misincorporation and disease susceptibility (21,22). $Shmt1^{+/-}$ mice maintained on an APC^{min} background were susceptible to colon cancer that was associated with greater nuclear uracil accumulation (21). Importantly, neither elevated genomic uracil misincorporation nor cancer susceptibility was observed in $Shmt1$ null mice. This absence of disease vulnerability in $Shmt1^{-/-}$ mice was associated with an upregulation of dTMP synthase levels, as well as enzymes within the nucleotide salvage pathway, which ameliorated the disruption of dTMP biosynthesis caused by the complete lack of SHMT1 protein (21). $Shmt1^{+/-}$ mice, on the other hand, did not exhibit these compensatory mechanisms. The presence of both neurobehavioral and cellular phenotypes associated with $Shmt1$ heterozygosity in the present study therefore suggests that impaired dTMP biosynthesis in newborn hippocampal neurons underlies the neurocognitive pathology observed in response to $Shmt1$ deficiency. Although we did not detect differences in the genomic uracil content of whole

TABLE 1 Total RBC and brain folate concentrations in $Shmt1^{+/+}$, $Shmt1^{+/-}$, and $Shmt1^{-/-}$ mice fed FD, FC, or FS diets¹

Genotype	RBCs					Brain				
	Diet			P value		Diet			P value	
	FD	FC	FS	Diet \times genotype effect	Diet effect	FD	FC	FS	Diet \times genotype effect	Diet effect
	<i>fmol/μg protein</i>					<i>fmol/μg protein</i>				
$Shmt1^{+/+}$	11.4 \pm 5.0	36.9 \pm 5.0	53.4 \pm 7.0	NS	N/A	1.56 \pm 0.2 ^b	3.05 \pm 0.2 ^a	2.73 \pm 0.2 ^a	0.037	N/A
$Shmt1^{+/-}$	11.0 \pm 5.0	33.4 \pm 5.0	42.5 \pm 5.0	NS	N/A	1.00 \pm 0.2 ^b	2.66 \pm 0.2 ^a	3.46 \pm 0.2 ^a	0.037	N/A
$Shmt1^{-/-}$	20.0 \pm 5.0	42.9 \pm 5.0	41.4 \pm 5.0	NS	N/A	1.21 \pm 0.3 ^b	2.16 \pm 0.2 ^b	3.21 \pm 0.2 ^a	0.037	N/A
All	14.1 \pm 3.2 ^b	37.7 \pm 3.2 ^a	41.8 \pm 3.4 ^a	N/A	<0.0001	1.26 \pm 0.2 ^c	2.62 \pm 0.1 ^b	3.14 \pm 0.1 ^a	N/A	<0.0001

¹ Values are means \pm SEMs; $n = 4$ per group. $P \leq 0.05$ was considered significant; NS = $P > 0.10$. Means in a row with superscripts without a common letter differ, $P \leq 0.05$. No significant genotype effects were detected. FC, folate control; FD, folate deficient; FS, folate supplemented; N/A, not applicable; $Shmt1$, serine hydroxymethyltransferase 1.

TABLE 2 Metabolic profile of plasma isolated from *Shmt1*^{+/+}, *Shmt1*^{+/-}, and *Shmt1*^{-/-} mice fed FD, FC, or FS diets¹

Metabolite	FD			FC			FS			P value					
	<i>Shmt1</i> ^{+/+}	<i>Shmt1</i> ^{+/-}	All	<i>Shmt1</i> ^{+/+}	<i>Shmt1</i> ^{+/-}	All	<i>Shmt1</i> ^{+/+}	<i>Shmt1</i> ^{+/-}	All	Genotype	Diet × Genotype				
	Homocysteine, $\mu\text{mol/L}$	8.96 ± 0.72 ^a	7.70 ± 0.72 ^{ab}	6.13 ± 0.72 ^b	7.59 ± 0.41 ^c	3.77 ± 0.72 ^d	4.41 ± 0.63 ^{ab}	3.47 ± 0.72 ^b	3.88 ± 0.40 ^d	4.12 ± 0.72 ^e	3.61 ± 0.72 ^{ab}	2.88 ± 0.72 ^b	3.54 ± 0.41 ^d	0.0322 ^{ab}	<0.0001 ^{c,d}
Cystathionine, nmol/L	1040 ± 77 ^a	1030 ± 77 ^{ab}	1120 ± 73 ^b	1060 ± 44 ^c	976 ± 77 ^b	665 ± 67 ^{ab}	871 ± 77 ^b	837 ± 43 ^d	936 ± 77	782 ± 77 ^{ab}	831 ± 73 ^b	850 ± 44 ^d	0.0337 ^{ab}	0.0005 ^{c,d}	NS
Cysteine, $\mu\text{mol/L}$	208 ± 8	209 ± 8	210 ± 7	209 ± 4	211 ± 8	216 ± 7	194 ± 8	207 ± 4	199 ± 8	209 ± 8	203 ± 7	204 ± 4	NS	NS	NS
α -Aminobutyric acid, $\mu\text{mol/L}$	13.0 ± 1.6 ^a	13.1 ± 1.6 ^a	13.6 ± 0.9 ^a	13.3 ± 1.5	14.5 ± 1.6 ^a	14.0 ± 1.4 ^a	17.2 ± 1.6 ^a	15.2 ± 0.9	10.7 ± 1.6 ^a	11.2 ± 1.6 ^a	16.3 ± 1.5 ^a	12.7 ± 0.9	NS	0.0292	NS
Methionine, $\mu\text{mol/L}$	44.7 ± 10.9	38.0 ± 10.9	44.0 ± 10.4	43.7 ± 6.2	66.0 ± 10.9	43.7 ± 9.5	43.1 ± 10.9	59.3 ± 6.0	42.7 ± 10.9	44.7 ± 10.9	49.7 ± 10.4	49.0 ± 6.2	NS	NS	NS
Glycine, $\mu\text{mol/L}$	201 ± 16	183 ± 16	213 ± 16	203 ± 9	257 ± 16	210 ± 14	201 ± 16	231 ± 9	204 ± 16	222 ± 16	209 ± 16	231 ± 9	NS	NS	NS
Serine, $\mu\text{mol/L}$	149 ± 19 ^{ab}	130 ± 19 ^b	154 ± 18 ^{ab}	144 ± 11	196 ± 19 ^a	129 ± 17 ^b	136 ± 19 ^{ab}	151 ± 11 ^{ab}	137 ± 19 ^{ab}	145 ± 19 ^{ab}	152 ± 18	144 ± 11	N/A	N/A	0.0340
Dimethylglycine, $\mu\text{mol/L}$	6.40 ± 0.49	5.20 ± 0.49	5.21 ± 0.49	5.60 ± 0.28	6.04 ± 0.49	6.29 ± 0.43	5.42 ± 0.49	5.92 ± 0.28	5.10 ± 0.49	5.34 ± 0.49	5.14 ± 0.49	5.18 ± 0.31	NS	NS	NS
Methylglycine, $\mu\text{mol/L}$	1.0 ± 0.10 ^a	0.85 ± 0.10 ^a	1.0 ± 0.10 ^a	1.0 ± 0.06	1.1 ± 0.10 ^a	0.89 ± 0.10 ^a	0.78 ± 0.10 ^a	0.93 ± 0.06	0.86 ± 0.10 ^a	0.77 ± 0.10 ^a	0.85 ± 0.10 ^a	0.83 ± 0.06	NS	0.0640	NS

¹ Values are means ± SEMs; n = 10–14 per group. P ≤ 0.05 was considered significant; NS = P > 0.10. Means in a row with superscripts without a common letter differ, P ≤ 0.05. All mice within a genotype are marked to indicate genotype effects irrespective of diet designation. FC, folate control; FD, folate deficient; FS, folate supplemented; N/A, not applicable; *Shmt1*, serine hydroxymethyltransferase 1.

hippocampus preparations from *Shmt1*-deficient mice (data not shown), this result is likely due to the limited population of proliferating *Shmt1*-expressing cells within the hippocampus. Nevertheless, newly generated neurons have been shown to be more sensitive to telomere-associated DNA damage than either neuronal progenitors or mature neurons (30). Furthermore, uracil misincorporation in uracil-DNA-glycosylase-deficient mice impairs hippocampal neurogenesis and also diminishes survival of hippocampal neurons in vivo (12,14). Together, these results suggest that disrupted folate status may affect hippocampal function by altering dTMP biosynthesis and/or genomic uracil levels in proliferating cells within the DG.

In addition to regulating dTMP biosynthesis, SHMT1 also influences cellular methylation potential by binding 5-methylTHF in the cytoplasm and preventing its entrance into the homocysteine remethylation cycle (16,17) (Supplemental Fig. 1). Although hippocampal methylation potential was not directly measured in this study, *Shmt1*^{+/-} mice exhibited lower concentrations of cystathionine, a product of homocysteine catabolism through the folate-independent transsulfuration pathway, suggesting greater availability of folate-derived methyl groups for homocysteine remethylation and subsequent AdoMet production. AdoMet-dependent methylation is required for methylation of DNA, RNA, proteins, lipids, and neurotransmitters (31), and the effects of chromatin hypomethylation on hippocampal proliferation and neurocognitive function have been well documented (32). However, it is unclear whether a higher cellular methylation potential affects methyltransferase activity or whether chromatin hypermethylation affects neurocognitive function. Furthermore, no differences in methylation potential or gene expression have been observed in the colons of cancer-susceptible *Shmt1*-deficient mice (21). Together, these data suggest that the changes in methylation potential resulting from *Shmt1* disruption contribute minimally toward neurocognitive disease.

In contrast to previous reports (11,12,14,33), we did not observe an effect of dietary folic acid deficiency on hippocampal neurogenesis or on hippocampal-dependent memory function. The deficient diets used in our study differed from those in previous investigations in that they did not include the addition of the antibiotic succinyl sulfathiazole in the diet to eliminate folate-producing bacteria found in the murine digestive system. Although mice fed the FD diet exhibited significant reductions in RBCs and brain total folate concentrations, as well as elevated plasma homocysteine concentrations (~1.5-fold), reports of mice fed deficient diets with the addition of succinyl sulfathiazole indicate more extreme deficiency (e.g., ~9-fold elevated homocysteine concentrations) (11,33). Dietary folate supplementation also showed little effect on neurocognitive outcomes in our study, but analysis of brain folate concentrations revealed that the FS diet did not significantly raise brain folate concentrations above those observed in mice fed the FC diet within each genotype group (Table 1).

The absence of an effect of dietary folate deficiency on neurobehavioral outcomes in our study also is in contrast to aforementioned reports of folate deficiency affecting cognitive function in humans. One possible explanation for this discrepancy is that human dietary folate deficiency, particularly in aging humans, is often comorbid with deficiency of other micronutrients, including vitamin B-12, which influences folate metabolism. Additionally, genetic diversity in the human population, including common polymorphisms in folate-related genes, leaves certain subpopulations more vulnerable to the effect of folate deficiency (34). Our study with mice does not address such genetic variation. Nevertheless, our dietary manipulations,

which resulted in moderate folate deficiency, may better model human dietary folate fluctuations and highlights the necessity of studying the causal mechanisms underlying OCM disruption as folate supplementation alone may not ameliorate every condition.

Homocysteine is a biomarker of impaired folate metabolism, and increased concentrations of homocysteine have also been identified as an independent risk factor for age-related cognitive decline and neurodegenerative disease [for review see (35)]. Whether homocysteine is a direct causal factor in age-related neurological disorders remains to be determined. Both *in vitro* and *in vivo* studies have indicated that highly elevated homocysteine concentrations, caused either by dietary manipulation or direct treatment with homocysteine, can lead to impaired hippocampal proliferation, impaired cognitive function, and neurodegeneration (11,12,14,33). In our study, mice fed an FD diet exhibited elevated plasma homocysteine, but elevated homocysteine was not associated with any of our behavioral or molecular outcomes. Furthermore, homocysteine was not significantly elevated in affected *Shmt1*^{+/-} mice (Table 2). Another recent study that used equivalent dietary manipulations reported that folate deficiency did not affect hippocampal morphology or cell density, despite elevated homocysteine concentrations (36). Together, these results indicate that, in our model, homocysteine does not play a causal role in the neural and cognitive alterations associated with *Shmt1* heterozygosity and suggest that homocysteine may be a biomarker of impaired folate status as opposed to a causal factor in neurocognitive pathology.

Human epidemiological studies have identified memory function as particularly sensitive to folate status. Here, by using a novel mouse model to understand the role of folate metabolism in normal brain function, we have shown that memory performance is sensitive to perturbations in folate metabolism independent of dietary folate intake. We have demonstrated that disruption of *Shmt1* is associated with specific cellular changes within the hippocampus that underlie the observed cognitive impairments. Although further investigation is required to elucidate the mechanism(s) whereby *Shmt1* disruption affects hippocampal function, we present the first, to our knowledge, genetic evidence implicating altered *de novo* thymidylate biosynthesis in the etiology of neurocognitive dysfunction. Future work will be required to assess the impact of dTMP supplementation on the prevention and treatment of neurocognitive disease.

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