Deletion of one allele of *Mthfd1* (*methylenetetrahydrofolate dehydrogenase 1*) impairs learning in mice

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ABSTRACT

The *MTHFD1* gene encodes for methylenetetrahydrofolate dehydrogenase 1, an enzyme that has an important role in folate-mediated one-carbon metabolism. In people, a single nucleotide polymorphism of this gene (1958G > A; rs2236225) is associated with increased risk for bipolar disorder and schizophrenia, neural tube and other birth defects. Mice homozygous for a loss of *Mthfd1* via a gene-trap mutation are not viable, and heterozygotes, though they appear healthy, have metabolic imbalances in the folate- and choline-mediated 1-carbon metabolic pathways. In this study, we evaluated cognitive function in *Mthfd1*$^{gt/+}$ male and female mice using a behavioral battery composed of eight different tests. We found that these mice display impaired cue-conditioned learning, while other behaviors remain intact.

The gene *Mthfd1* encodes for methylenetetrahydrofolate dehydrogenase 1, a cytoplasmic enzyme that catalyzes the interconversion of tetrahydrofolate (THF) to 10-formyl-THF as well as to 5,10-methenyl-THF and 5,10-methylene-THF [1–3]. Thus, it regulates the routing of THF towards *de novo* purine synthesis or towards remethylation of homocysteine to form methionine [4]. In people, there are a number of known single nucleotide polymorphisms (SNPs) of *MTHFD1* (rs2236225, rs1956545 and rs56811449) that have been associated with increased risk of neural tube defects and other health risks [5]. *MTHFD1* rs2236225 (1958G > A) is common (AA occurring in ~20% Caucasians [6]) and the carriers of this SNP are at risk for folate-related pathologies; in women, rs2236225 is associated with an increased risk of pregnancy complications [7] and birth defects (including neural tube closure defects [8], cleft palate [9] and congenital heart disease [10]). Furthermore, carriers of rs2236225 are ~15x more likely to develop choline deficiency when fed a low choline diet than are noncarriers [11]. Men carrying this SNP are at increased risk for neurodevelopmental disorders, such as bipolar disorder and schizophrenia [12].

Mice homozygous for a loss of *Mthfd1* via a gene-trap mutation are not viable, while heterozygotes appeared healthy [13]. However, heterozygotes display metabolic imbalances in the folate-, as well as choline-mediated 1-carbon metabolic pathways [14]. These pathways are important for neurodevelopment and imbalances in the pathway affect cognitive functions [15]. In one study, adult male mice heterozygotes for the *Mthfd1* gene-trap mutation were reported to exhibit attentional deficits in the five choice serial task [16].

Here, we sought to explore whether other behavioral abnormalities occur in male and female mice with a loss of one allele of *Mthfd1* via a gene-trap mutation. We used a behavioral battery composed of eight behavioral tests, including assessment of locomotion, anxiety, as well as learning and memory.

The mice, backcrossed to C57BL/6J for more than 10 generations, were generated as previously described [13] and were kindly received as a gift from Dr. Patrick Stover. Studies were performed at the David H. Murdock Research Institute Center for Laboratory Animal Science facilities in accordance with the standards of the U.S. National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at this facility. The *Mthfd1* gene-trap mutation mice were maintained on a modified AIN 93G diet (in which casein was substituted for isolated soy protein, Dyets (catalog #103186), Bethlehem, PA; see Table S1 for composition). DNA was isolated from ear punches using GeneJET Genomic DNA purification kit (Thermo Scientific™). Genotype was determined using PCR; a duplex PCR reaction was used to detect the wild-type *Mthfd1* and *Mthfd1*^{gt} alleles. The primers and PCR conditions are listed in Table S2 and S3 respectively.

Gene trapped *Mthfd1*$^{gt/+}$ mice were bred and, *Mthfd1*$^{gt/+}$ and *Mthfd1*$^{+/+}$ (wild type; WT) male and female littermates from multiple litters (N = 12 per gender and per group) were tested starting at 12-weeks in the behavioral battery for locomotor activity (open field), sensorimotor function (accelerating rotarod), anxiety (open field and

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light-dark box), novel object and social investigation, spatial memory (Morris Water maze) and, in fear contextual and cue conditioning experiments (see supplementary materials for more detailed methods). The mice were tested in the light phase of the day and were given at least 48 h between the tests. Male and female mice were tested on separate days.

After we finished testing, hepatic tissue was collected in a subset of mice, to assess choline metabolite concentrations using liquid chromatography-stable isotope dilution-multiple reaction monitoring mass spectrometry (LC–SID–MRM/MS) as previously described with some modifications [17]. We observed decreased hepatic betaine concentrations in *Mthfd1*$^{gt/+}$ mice (421 ± 91 nmol/g versus 693 ± 138 nmol/g in WT; $t_{(2,10)} = -4.03$, $p = 0.002$), which is likely a result of increased utilization of betaine for methionine production, compensating for lack of 5-methyl tetrahydrofolate-mediated production of methionine. There were no changes in other metabolites (Table S4). We also measured MTHFD1 protein expression in the cerebellum and rest of the brain using Western Blot as previously described [18]. We observed a reduction (compared to WT) in MTHFD1 protein expression in the brain of the *Mthfd1*$^{gt/+}$ mice but not in cerebellum (Fig. S1).

From our behavioral evaluation, we found that heterozygote *Mthfd1*$^{gt/+}$ mice display impaired cue-dependent learning in fear conditioning test (Fig. 1). Contextual and cued fear conditioning was performed using a conditioned fear paradigm over 3-days using a Near-Infrared image tracking system (MED Associates, Burlington, VT) [19]. On the first day, after an initial 2-min exploration time (background activity), mice were exposed to a 30-s tone (85 dB, 2800 Hz), followed by a 2-s scrambled foot shock (0.4 mA) (CS1) under white light conditions. Mice received 2 additional tone-shock pairings (CS2 and CS3), with 80-s between the stimuli pairings, totaling a 7.5-min session. The response to the shock was measured with automatic assessment of the levels of freezing (immobility, except for breathing) using the Video Freeze (MED Associates Inc.) software. All mice learned the association between the tone and the shock, and the response to the tone increased with each pairing. There were no learning differences between the WT and *Mthfd1*$^{gt/+}$ mice (one way analysis of variance (ANOVA) with repeated measures $F_{(1,45)} = 1.17$, $p = 0.29$, Fig. 1A). On the following day, mice were evaluated for their context-dependent learning. Mice were placed back into the original test chamber, this time with no tone or foot shock, and response was determined across a 5-min session. All mice showed similar first minute response as well as throughout the 5-min ($t_{(2,45)} = 0.10$, $p = 0.92$ and ANOVA with repeated measures $F_{(1,45)} = 0.55$ $p = 0.46$, Fig. 1B). On the third day of testing, associative learning to the tone cue was evaluated. The conditioning chambers were modified by turning off the white light and keeping only Near-Infrared light, by modifying the chamber using a black Plexiglas insert in an A-shape to change the wall and another insert to change the floor surface, and, by adding a novel odor (vanilla flavoring). Mice were placed in the modified chamber and allowed to explore for a final 5-min session. After 2-min, the acoustic stimulus was presented continuously for a 3-min period. *Mthfd1*$^{gt/+}$ mice showed a decrease in response when the tone was presented in cue-dependent learning in the first exposure to the tone ($t_{(2,43)} = -2.27$, $p = 0.028$, Fig. 1C). *Mthfd1*$^{gt/+}$ mice maintained the same level of response to the tone during the 3-min tone (ANOVA with repeated measures $F_{(2,46)} = 2.43$ $p = 0.099$), while the WT mice showed a decrease in response to the tone (ANOVA with repeated measures $F_{(2,44)} = 16.52$, $p < 0.001$), though when the two groups were analyzed with ANOVA with repeated measures there was no significant difference ($F_{(1,45)} = 3.28$, $p < 0.08$). When the genders were analyzed separately, there was no difference between female mice, but we observed a trend towards decreased response ($p = 0.06$) in male mice (data not shown). This trend is in concordance with previously published reports describing behavioral impairments in male *Mthfd1*$^{gt/+}$ mice [16]. The *Mthfd1*$^{gt/+}$ mice did not show other behavioral abnormalities in any of the remaining behavioral tests, summarized in Table 1. The data is presented with both genders included. In a separate analysis, we evaluated whether there were gender-dependent effects in *Mthfd1*$^{gt/+}$ mice, and our analysis showed no such effect.

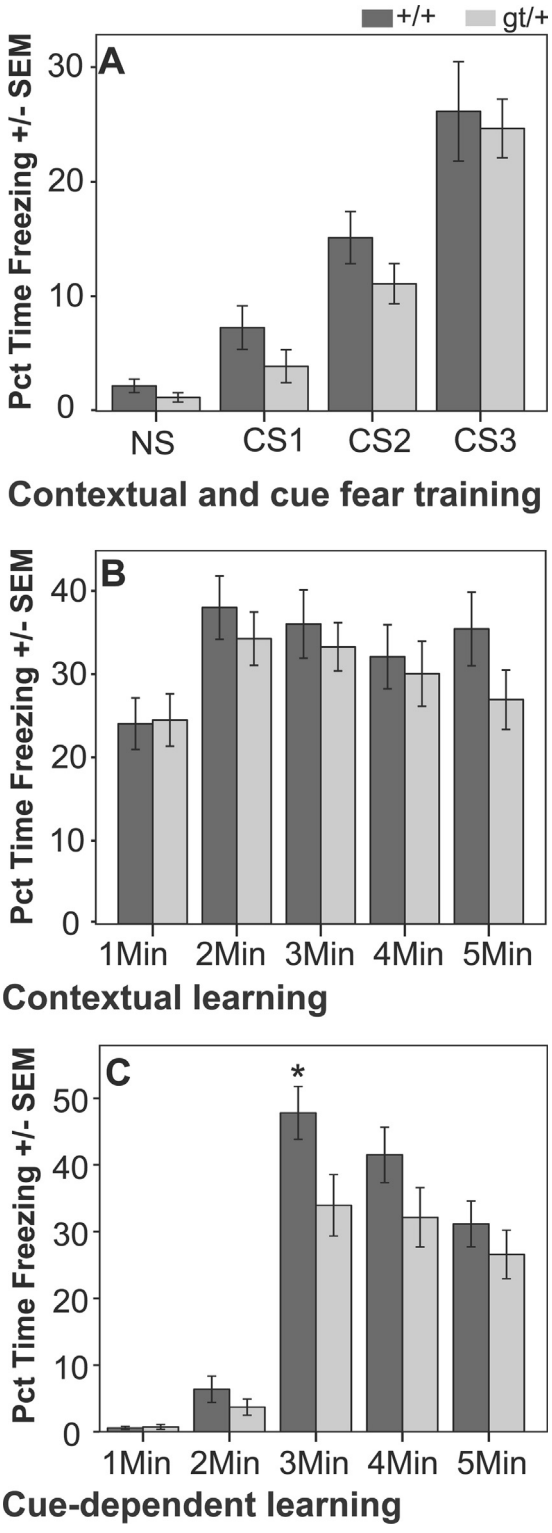

Fig. 1. Impaired cue-conditioned learning in *Mthfd1*$^{gt/+}$ mice. A) Mice were trained on Day-1 and the response (freezing behavior) increased with each stimulus. B) On Day-2, 24 h later, contextual learning was evaluated for 5-min. C) Cue-dependent learning was evaluated on Day-3 for 5-min, using the same 85 dB tone as on Day-1. *Mthfd1*$^{gt/+}$ mice showed a decrease in first response when the tone was presented. Mice were on a C57BL/6J background. NS – No stimulus, background and CS – conditioned stimulus. Data are shown as means ± SEM. *$p < 0.05$.

Table 1
Behavioral test battery results summary.

Schedule	Test	Behavior analysis	WT	n	*Mthfd1*$^{gt/+}$	n
1	*Open field test*	Total distance (cm)	3197 ± 194	24	3181 ± 134	24
		Total time in central area (s)	11.7 ± 1.2		11.0 ± 0.8	
		Latency to reach the central arena (s)	8.06 ± 2.25		10.30 ± 2.17	
2	*Object investigation test*	Total time (s)	7.36 ± 1.50	24	10.39 ± 2.09	24
3	*Social interaction test*	Social behavior time (s) (no aggressive behavior was observed)	35.8 ± 2.6	16	39.3 ± 4.1	16
4	*Accelerated rotarod test*	Time trial 1 (s)	199 ± 18	24	174 ± 23	24
		Time trial 2 (s)	234 ± 15	24	235 ± 18	24
5	*Light dark box*	Latency to go in the light zone (s)	48.3 ± 10.7	23	45.6 ± 11.6	21
		Percent time in light zone (s)	17.4 ± 1.9	23	20.6 ± 2.0	21
		Total distance moved in light zone (cm)	351 ± 38	23	415 ± 41	21
6	*Morris Water Maze*	Probe trial− % time spent in the target quadrant	33.4 ± 2.3	23	37.0 ± 1.9	23
7	*Fear conditioning test*	Response to stimuli presented as % freezing time.	See Fig. 1	23	See Fig. 1	24
8	*Homecage analysis*	Activity D1 (X + Y + Z beam break counts)	84,183 ± 17,980	4	103,105 ± 7,498	4
		Activity D2 (X + Y + Z beam break counts)	98,276 ± 7,951		91,034 ± 17,599	
		Activity D3 (X + Y + Z beam break counts)	68,246 ± 2,648		57,808 ± 7,314	

Loss of one allele of *Mthfd1* via a gene-trap mutation in mice did not have a significant effect on spontaneous activity, balance and motor coordination on an accelerating rotarod, anxiety-like behavior, response to novelty and social interaction. Data for fear conditioning learning are presented separately in Fig. 1. Data are presented as mean ± SEM. Units are indicated in parens in 'Behavior analysis' column; n- indicates number of mice studied per group.

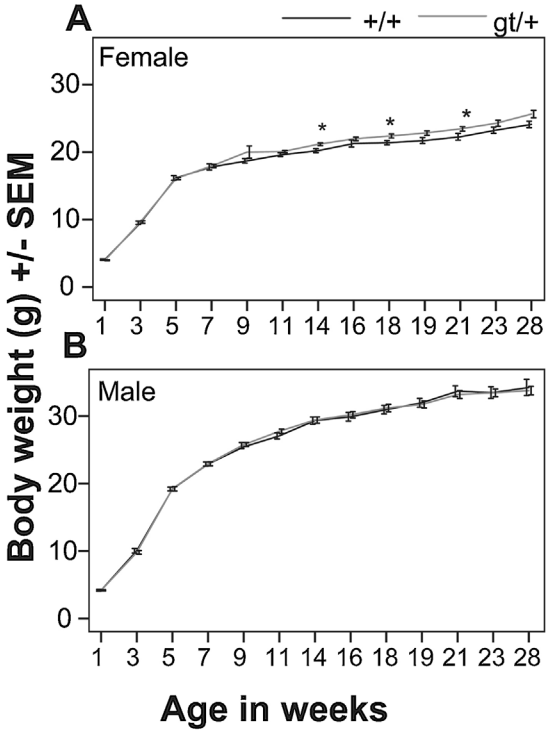

Fig. 2. Body weight curves. A) *Mthfd1*$^{gt/+}$ female mice had higher body weight compared to their WT littermates at 14, 18 and 21 weeks. B) Male *Mthfd1*$^{gt/+}$ were not different from WT mice at any age. Data are shown as means ± SEM. *p < 0.05 is considered significant.

We measured animal body weights as an indicator of general development. Because of the expected difference in weight gain between male and female mice, already visible at week four, we analyzed the two genders separately (Fig. 2). Body weight was measured regularly starting at postnatal day seven (P7). Pups were marked with tattoo ink (green paste, Ketchum Manufacturing Inc, Canada) in their paws at P7 and identified later for the genotype. *Mthfd1*$^{gt/+}$ female mice had higher body weight compared to their WT littermates at 14, 18 and 21 weeks of age ($t_{(2,44)} = -2.33$, $p = 0.024$; $t_{(2,30)} = -2.17$, $p = 0.038$; $t_{(2,30)} = -2.06$, $p = 0.048$ respectively Fig. 2A). *Mthfd1*$^{gt/+}$ female mice were not different in activity in open field and homecage 3-day analysis (Table 1). *Mthfd1*$^{gt/+}$ did not affect weight gain in male mice (Fig. 2B), even during the first 3 weeks, contrary to what has been previously reported [13]. This could be due to a difference in the rodent diet used. While in our study, animals were raised and maintained on modified AIN93G diet, the other reported study [13] was conducted using mice bred and maintained on a standard rodent chow diet until postnatal day 21. Furthermore, mouse genetic background could play a role in animal weight gain. While our mice had been backcrossed to C57BL/6J strain for more than 10 generations, the mice in the other study were on a mixed background.

The present study provides an extensive behavioral profile of *Mthfd1*$^{gt/+}$ mice, which includes both males and females. Compared to WT mice, *Mthfd1*$^{gt/+}$ mice show reduced response in the cue fear conditioning, this effect was larger in male mice. This level of response was maintained through the cue interval, whereas the WT mice showed a decrease in response with time. These results suggest impaired learning in *Mthfd1*$^{gt/+}$ mice and are consistent with behavioral findings from previous studies [16]. Interestingly, no other behavioral abnormalities were observed in male and female *Mthfd1*$^{gt/+}$ mice, suggesting intact locomotor and anxiety-like behavior. In females, we observed an effect of the mutation on body weight that was not seen in male mice.

MTHFD1 regulates the routing of THF towards *de novo* purine synthesis or towards remethylation of homocysteine to form methionine [4], and it is likely that the behavioral effects of a mutation in this gene are due to a perturbation in these pathways. We did not measure metabolites in these pathways in the brain, but in liver we observed that betaine concentrations were decreased. This is consistent with previous observation that *Mthfd1*$^{gt/+}$ mice have reduced expression of the choline dehydrogenase (*Chdh*) gene compared to WT mice [16]; CHDH converts choline to betaine [20]. Betaine is important because of its role as a methyl donor and as an osmoregulator [21].

Mthfd1$^{gt/+}$ mice also have reduced expression of the nicotinic acetylcholine receptor, subunit α7 (*nAChR7*) gene, relative to WT mice [16]. Reduced *nAChR7* gene expression could contribute to the impaired learning observed in these mice as this gene and the cholinergic system have been implicated in learning and memory [22].

The *Mthfd1*$^{gt/+}$ mice evaluated here represent a model where all three functions of the enzyme are affected. The next step would be to dissect the enzyme's separate functions and it would be interesting to

see whether we can observe similar, or additional behavioral effects, with specific functions of the enzyme inactivated. An example would be the 10-formyltetrahydrofolate synthetase-deficient mouse which impairs purine synthesis in a manner similar to the MTHFD1 rs2236225 (1958G > A) polymorphism; this mouse has deficits in development [23]. Future studies are necessary to assess how *Mthfd1* affects brain development and what can be done to attenuate or mitigate these effects.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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