Methyl-donor deficiency in developmental period affects memory and epigenetic status in the mouse hippocampus

（発達期メチルドナー欠乏による記憶と海馬内エピジェネティクス機構への影響）

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Abstract

DNA methylation is one of the essential factors in the control of gene expression. Alteration of the DNA methylation pattern has been linked to various neurological, behavioral, and neurocognitive dysfunctions. Recent studies have pointed out the importance of epigenetics in brain development and functions. Nutrients related to one-carbon metabolism are known to play important roles in the maintenance of genomic DNA methylation. Previous studies have shown that the long-term administration of a diet lacking essential one-carbon nutrients such as methionine, choline and folic acid (methyl donors) caused global DNA hyper-methylation in the brain. Therefore, the long-term feeding of a methyl-donor-deficient diet may cause abnormal brain development.

To confirm this hypothesis, 3-week-old mice were maintained on a folate-, methionine- and choline-deficient (FMCD) or control (CON) diet for 3 and 6 weeks. We found that the methyl-donor deficiency impaired both novel object recognition and fear extinction after 3 weeks of treatment. The FMCD group showed spontaneous recovery of fear that differed from that in CON. In addition, we found decreased Gria1 gene expression and hyper-methylation of the Gria1 promoter region in the FMCD hippocampus. Our data suggest that a chronic dietary lack of methyl donors in the developmental period affects learning, memory and gene expressions in the hippocampus.
**Introduction**

DNA methylation is one of the important mechanisms altering gene expression without altering the DNA sequence. DNA methylation involves the addition of a methyl group to the DNA base cytosine within “CpG” sites containing cytosine residues succeeded by a guanine base. Evidence accumulated in recent years demonstrates the importance of healthy maintenance of the cellular epigenomic landscape for normal central nervous system development and function (Feng *et al.*, 2007). The deregulation of epigenetic mechanisms has been linked to the development of various neurological, behavioral, and neurocognitive dysfunctions (Tsankova *et al.*, 2007). Abnormal methylation has been reported in the brain of several neuropsychiatric disorders such as Alzheimer’s disease, schizophrenia, posttraumatic stress disorder (PTSD) and major depression (Fuchikami *et al.*, 2011, Levenson & Sweatt, 2005, Szyf, 2013). Nutrients related to one-carbon metabolism, such as methionine, choline and folic acids, work as methyl donors for maintaining genomic DNA methylation (Friso & Choi, 2002, James *et al.*, 2003). Previous studies have shown that the long-term administration of a diet lacking essential one-carbon nutrients after weaning caused global DNA hyper-methylation in the rat brain (Pogribny *et al.*, 2008). Accumulating evidence suggests that an adverse environment in early life such as neglect or child abuse could affect the developmental brain resulting in the alteration of hippocampal DNA methylation status with deteriorated response to stress; this has been found in both rodents (Weaver *et al.*, 2004) and humans (Mcgowan *et al.*, 2009). Developmental disorders such as autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD) might also be linked to epigenetic alterations (Ronald *et al.*, 2010, Yasui *et al.*, 2011). Thus, focusing on epigenetic alterations in the
developing brain would reveal some features in the pathogenesis of mental disorders (Szyf, 2014).

Recent studies have pointed out the importance of epigenetics in brain functions including learning and memory (Feng et al., 2007, Miller & Sweatt, 2007). Miller and Sweatt (2007) used Pavlovian fear conditioning and discovered that this behavioral paradigm leads to an upregulation of the mRNA of de novo DNA methyltransferases (DNMT), namely Dnmt3a and Dnmt3b, in the rat hippocampus. The infusion of a DNMT inhibitor into the hippocampus following fear conditioning caused impairment of fear memory in animals (Miller et al., 2008). Long-term potentiation (LTP) is the main underlying mechanism of memory formation, and two types of ionotropic glutamate receptors, alpha-amino-3-hydroxy-5-methyl-4 isoxazolepropionic acid (AMPA) receptor and N-methyl-D-aspartate (NMDA) receptor, have been reported as regulating LTP. Calcium influx from NMDA receptors is necessary for the activation of calmodulin-dependent protein kinase II (CaMKII). The consequence of CaMKII activation is the rapid recruitment of AMPA receptors to the synapses (Nicoll & Roche, 2013).

Taken together, we hypothesized that long-term methyl-donor deficiency in developmental mice would induce cognitive and behavioral alteration especially in learning and memory with rearrangement in hippocampal DNA methylation. In the present study, we fed a folate-/methionine-/choline-deficient (FMCD) diet to mice during the 3-week developmental period from three to six weeks of age to examine the effects on behavioral memory and the related gene expression of glutamate receptors.

**Materials and methods**

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**Animals and diets**

3-week-old male and 8-week-old female C57BL/6J mice were obtained from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). Female mice were used as stimulus mice after ovariectomy (OVX). The animals were housed 3-5 per cage in plastic cages and placed on a 12-h light/dark cycle (light on at 7 a.m.) with *ad libitum* food and water. The mice were allocated randomly into two groups, one control and one experimental. The mice from the experimental group were maintained on a low methionine (0.18%) diet, completely lacking in choline and folic acid (Oriental Yeast Co., Ltd., Itabashi, Tokyo, Japan). The mice from the control group received a diet supplemented with 0.5% methionine, 0.3% choline, and 2 mg/kg folic acid. Diets were stored at 4 °C for future use, with once a week replacement. Body weights and food consumption were recorded weekly. All behavioral experiments were started at 6 weeks old. Open field test, Novel object recognition test, Social recognition test and Social interaction test were conducted in consecutive schedule. In other experiments, mice were used only once for each experiment. All the behavioral testing was conducted between 09:00 and 15:00 hours. The research and animal care were carried out according to the Guide for Animal Experimentation of the Chiba University Graduate School.

**Surgery**

Stimulus mice underwent bilateral OVX under pentobarbital anesthesia. The lower back skin was shaved and cleaned, and a 1.5 cm incision was made in the skin to expose the back muscles. A small incision was made in the muscles overlying the ovaries on each side, and then the ovary and the tip of the uterus were...
drawn out of the hole. The ovary was removed by cutting just above a ligation on the uterus, and then the uterus was replaced in the abdominal cavity. Mice were sutured these muscle and skin incisions, and were allowed 3 days of single-housed recovery; OVX stimulus mice were group-housed at least 7 days before the experiment.

The measurement of methionine and homocysteine levels in plasma

Methionine and homocysteine measurement protocol were modified from previous methods (Melnyk et al., 1999). The mice were anesthetized with CO₂, and bloods were collected into EDTA containing tubes and immediately centrifuged at 4100 rpm for 15 min at 4°C. Aliquots of the plasma layer were transferred into cryostat tubes and stored at −80°C until analysis. Plasma methionine and homocysteine are detected following high-performance liquid chromatograph (HPLC) separation with a model 5200A Coulrochem II EC detector (ESA, Inc., Bedford, MA, USA) equipped with a dual analytical cell (model 5010) and a guard cell (model 5020). The detector conditions were as follows: guard cell (5020) potential, E, 780 mV; analytical cell (5011) potentials, E1, 350 mV and E2, 730 mV; and sensitivity, 10 µA. Separation was performed using a 5-µm SunFire C18 (4.6 × 150 mm; Waters) column at 35 °C under Isocratic elution with a mobile phase, which consisted of 50 mM sodium phosphate monobasic, monohydrate, 1.0 mM ion-pairing reagent OSA, 2% acetonitrile (v/v), adjusted to pH 2.7 with phosphoric acid. The flow rate and injection volume were 1.0 ml/min and 20 µl, respectively.

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Behavioral experiment

Open field test

The open field test was performed according to our previous study (Matsuda et al., 2012). The open field apparatus was a square field (50 × 50 × 30 cm) made of white acrylic material. Each mouse was placed in the corner of the apparatus at the beginning of the test and allowed to move freely for 10 min. The total distance and total center time were recorded. The center area was assigned 16 × 16 cm. The total distance was evaluated as an index of locomotor activity, and the total center time was evaluated as an index of anxiety. The data analysis was performed using Image J OF4 (O'Hara & Co., Ltd., Nakano, Tokyo, Japan), modified software based on the public domain Image J program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij).

Contextual fear conditioning, extinction and spontaneous recovery test

Experiments were carried out in 22.8×19.7×13 cm experimental footshock chamber with transparent walls and metal-rod floor. The experimental footshock chamber was cleaned with 70% ethanol before each testing. Mice were placed into a conditioning chamber for 20 min (habituation) according to our previous studies (Ishii et al., 2010, Matsuda et al., 2013). The next day, after a 180 sec acclimation period (Pre), the mice received three footshocks (2 sec, 0.75 mA, inter-trial interval: 120 sec) in the testing chambers and were then returned to their home cages 60 sec after the last foot shock. We used the data from the average of % freezing during every 1 min. Fear extinction was started 24 hr after fear conditioning. Mice were placed for 20

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min without footshock in the same experimental chamber where the footshock was delivered. Fear extinction was performed on each of five consecutive days (FE1-FE5). We used the data from the average of % freezing during each day. To investigate spontaneous recovery, 28 days after fear conditioning, mice were placed for 5 min without footshock in the same experimental chamber (Recall: RE). We used the data from the average of % freezing during last 2 min of FE5 and first 2 min of RE according to our previous study (Matsuda et al., 2014). Freezing behavior was measured using a digital video camera connected to a computer with Actimetrics FreezeFrame software (Actimetrics Software, Wilmette, IL, USA). Freezing (no visible movement except for respiration) was scored and converted into a percentage [(freezing observations/total observations)×100].

Novel object recognition test

The novel object recognition (NOR) test comprised the following three sessions. First, in a habituation, mice were pre-exposed to the open field arena for 10 min twice in the absence of objects (Open field test was regarded as first habituation). Second, a training session was performed 24 hr after the habituation. Two objects (A and A’) were placed approximately 10 cm from the walls of the open field arena. Mice were individually placed in the middle front of the open field arena and the time spent in exploring the two objects was recorded. Exploration behavior of the mice was defined as directing the nose toward the object at a distance of less than 2 cm. Third, a retention session was performed 24 hr after the training session. One of the familiar objects (A or A’) used in the training session was replaced by a novel object B, and these
objects were placed as in the previous session. Mice were then individually placed in the box and allowed to explore freely for 10 min, with recording of the time spent in exploring each of the two objects. Climbing or sitting on objects was not scored as object exploration. The open field arena and all objects were thoroughly wiped down with 70% ethanol before and after all behavioral procedures. The discrimination index was expressed as a ratio of the time spent exploring the novel object (TB) over that spent on the two objects (TA+TB): discrimination index (%) = TB/(TA+TB) × 100. The data analysis was performed using TOP scan (CleverSys Inc. Reston, VA, USA).

**Social recognition test**

Social recognition test was conducted 24hr after novel object recognition test. We modified the social recognition protocol used by Kogan and colleagues (Kogan et al., 2000). Mice were habituated to the cage with two empty plexiglas perforated cylinder for 15 min. Following the habituation, each mouse was exposed to a stimulus mouse (OVX) held inside a cylinder four times (T1–T4). Intertest intervals were 30 min. To investigate long-term social memory, 24 hours after T4, on a fifth exposure (T5) a novel stimulus mouse was introduced. Exploration behavior of the mice was defined as directing the nose toward the cylinder at a distance of less than 2 cm. All tests lasted 5 min and were videotaped for subsequent analysis with specific software. Plexiglas cylinders were cleaned with 70% ethyl alcohol after each part. The data analysis was performed using TOP scan (CleverSys Inc. Reston, VA, USA).
Social interaction test

Social interaction test was conducted 48hr after social recognition test. The social interaction test was carried out in the same box in which open field test was performed. Each mouse was randomly assigned to a male C57BL/6J mouse of similar age and body weight, as an unfamiliar partner. The two mice were placed in the test box for 10 min and the time spent by the two in active social interaction with each other (active/passive contact, approaching, leaving, following, sniffing and grooming) was measured and recorded. The data analysis was performed using TOP scan (CleverSys Inc. Reston, VA, USA).

Tissue dissection and nucleic acid extraction

Mice were killed by cervical dislocation at 6 weeks old. The brains were quickly removed and immediately rinsed in ice-cold phosphate-buffered saline (PBS). Slices 1 mm thick were prepared using a mouse coronal brain matrix (RBM-2000C; ASI Instruments, MI, USA), and the tissue was dissected out in ice-cold PBS under a stereoscopic microscope. Dorsal hippocampus was dissected out by a sharp scalpel. These tissues were placed in dry-ice-chilled microcentrifuge tubes, immediately frozen in liquid nitrogen and stored in a deep freezer (−80 °C). The DNA and total RNA was extracted from frozen hippocampus using the AllPrep DNA/RNA Mini Kit (Qiagen, Toronto, Canada) according to the manufacturer’s instructions.

Quantitative real-time RT-PCR

The cDNAs were synthesized with the High Capacity cDNA Reverse Transcription Kit (Applied
Biosystems, Foster City, CA, USA) using 0.4 µg of total RNA, which had been primed with Random primer, as the template. The assays were chosen to span exons so as to avoid amplification of trace genomic DNA.

The following assays were designed using the Roche Universal ProbeLibrary Assay Design Center (www.roche-applied-science.com): \( G\text{ria1} \) (probe #62 and primers 5’ -agggatgcagatccagag-3’ and 5’ -tgcacattctgtcacaacc -3’ ), \( G\text{ria2} \) (probe #32 and primers 5’ -cagttcgeagtcaccaatg -3’ and 5’ -acccaaatcgeataagc-3’ ), \( G\text{ria3} \) (probe #109 and primers 5’ -actgettagcaatctgtgc -3’ and 5’ -tcattcctgtactgca -3’ ), \( G\text{rin1} \) (probe #63 and primers 5’ -tgcatcccaaatgacagga -3’ and 5’ -gggttcatccgaatgacagga -3’ ), \( G\text{rin2a} \) (probe #48 and primers 5’ -atccggaaggggacatc -3’ and 5’ -ttcaagacaggtgcatagca -3’ ), \( G\text{rin2b} \) (probe #29 and primers 5’ -tcatggtacgcatc -3’ and 5’ -atccggaaggggacatc -3’ ), and \( G\text{apdh} \) (probe #9 and primers 5’ -agttgcagatcagagga -3’ and 5’ -tttcatgccgcatc -3’ ). The probes were taken from the Universal ProbeLibrary Set, Mouse (Roche Diagnostics, Tokyo, Japan).

For each reaction, a FastStart Universal Probe Master (ROX) (Roche Diagnostics) was used with 0.2 µM of each primer, 0.1 µM probe, and 50 ng cDNA in a total volume of 15 µl. Then, the cDNA levels were quantified in duplicate using an 7300/7500 Real-Time PCR system (Applied Biosystems) under the manufacturer's recommended conditions: 2 min at 50 °C, followed by 10 min at 95 °C, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. To assess amplification efficiency, standard dilution curves were generated for all genes, and quantitative normalization of cDNA in each sample was performed using the expression of the \( G\text{apdh} \) gene as an internal control. Real-time PCR assays were tested in duplicate for each
sample, and a mean value was used for calculation of expression levels.

**Bisulfite sequencing**

The 5-6 DNA samples of CON and FMCD were pooled each group, and 500 ng DNA was subjected to sodium bisulfite treatment using EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA, USA). The C-to-T conversion was conducted for 10 min at 98 °C and then for 2.5 hr at 64 °C in a TaKaRa PCR Thermal Cycler Dice (TAKARA BIO Inc., Otsu, Shiga, Japan). The converted DNA was cleaned using Zymo-Spin IC columns, and then subjected to PCR amplification. Primers for each promoter were designed using MethPrimer (http://urogene.org/methprimer/index1.html) and synthesized by Eurofins Genomics Inc. (Tokyo, Japan). The amplification procedure was performed in a total volume of 20 µl employing AmpliTaq Gold 360 Master Mix (Applied Biosystems) using primers that cover the Gria1 promoter. For the -1461 to -812 analysis, these primers sequences are the following: sense, 5′- gtgaattttaggatgtatggtttattttg-3′; antisense, 5′-cttatctaatcatcttaataatac-3′. For the first PCR, the conditions were: 95°C for 5 min, 30 cycles at 95°C for 30 sec followed by 52°C for 30 sec, and 72°C for 1 min. The product from the first PCR reaction was re-amplified by PCR using the following same primers under the same conditions. For the -262 to -48 analysis, these primers sequences are the following: sense, 5′- gaggttgagaggtggtgtctg-3′; antisense, 5′-taatctctctctctctctta-3′. For the PCR, the conditions were: 95°C for 5 min, 30 cycles at 95°C for 30 sec followed by 60°C for 30 sec, and 72°C for 1 min.

The isolated PCR products were cloned into the pCR®2.1-TOPO® vector using TOPO TA Cloning...
The vectors were transfected into One Shot® TOP10 Chemically Competent cells (Invitrogen). The cultivated colonies were PCR-amplified (25 cycles of 94 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 1 min) using the M13 primers. The PCR products were treated the Exosap, and sequenced on an ABI PRISM 3100 system (Applied Biosystems) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

### Statistical analysis

Student’s *t*-test was applied for plasma methionine and homocysteine measurement, openfield test, object recognition test and gene expression. Two-way repeated measures of analysis of variance (ANOVA) was applied for comparing body weight, fear conditioning and fear extinction, between the groups. Two-way ANOVA was used for spontaneous recovery. Post hoc *Bonferroni* tests for multiple comparisons were applied for further analyses. For the bisulfite sequencing analysis, we used the quantification tool for methylation analysis (QUMA) ([http://quma.cdb.riken.jp/](http://quma.cdb.riken.jp/)) (Kumaki *et al.*, 2008). Low quality sequences were excluded depending on identify score (<95% or >10 mismatches) and the conversion efficacy (conversion efficacy <95% or >6 unconverted CpH). For all the analyses, the level of statistical significance was set at *p* < 0.05. All statistical analyses were performed using SPSS 22.0 J (SPSS Japan, Inc., Tokyo, Japan). The data are presented as the mean ± SEM.

### Results
**Loss of body weight in FMCD mice**

For body weight, two-way ANOVA showed a significant main effect of group ($F(1,22) = 4.49, p<0.05$) and age ($F(2,41) = 460.10, p<0.01$) with no significant interaction of group × age ($F(2,41) = 3.11$).

Although there was a significant difference, the mean weight loss in the FMCD mice group was no larger than 1.75g at 5 weeks age (Fig. 1). We previously reported that an FMCD diet completely deficient in methionine induced extreme weight loss (Ishii *et al.*, 2014). Thus, in the current study, we attempted to suppress the weight loss as small as possible to prevent its potential effect on behaviors in 6-week-old FMCD mice.

**Methionine and homocysteine levels were altered in the FMCD mice plasma**

Plasma homocysteine accumulation has been observed with one-carbon metabolism cycle impairment such as methyl-donor deficiency (Obeid, 2013). In the current study, as a result of methyl-donor deficiency, HPLC showed that the plasma methionine and homocysteine levels were significantly altered in the FMCD mice compared to the CON mice. The Student's $t$-test showed that total plasma methionine levels of FMCD were significantly lower than these of CON ($t(5) = 2.96, p<0.05$) (Fig. 2a). The Student's $t$-test showed that total plasma homocysteine levels of FMCD were significantly higher than these of CON ($t(8) = -12.39, p<0.001$) (Fig.2b). The reduction of plasma methionine and accumulation of plasma homocysteine by methyl-donor deficiency that we found were consistent with findings in previous reports (Herrmann & Obeid, 2011, Pogribny *et al.*, 2008).
Normal exploratory behavior and anxiety-like behavior of FMCD mice

For total distance traveled, the Student's *t*-test showed no significant difference between groups (*t*(18) = -0.55) (Fig. 3a). For the time spent in the center area, the Student's *t*-test showed no significant difference between groups (*t*(18) = -0.79)(Fig. 3b). These results suggest that locomotion and anxiety-like behavior were not changed by the FMCD diet.

Reduced fear extinction and emerged spontaneous recovery in FMCD mice

A schedule of contextual fear conditioning paradigm is shown in Fig. 4a. As shown in the Fig.4b, fear conditioning process did not show difference between the FMCD and the CON groups. Two-way ANOVA with repeated measures showed a significant main effect of trials (*F*(2, 51) = 72.51, *p* < 0.001) whereas no significant main effect of group (*F*(1, 23) = 0.52) or interaction of group × trials (*F*(2, 51) = 0.62).

However, in the fear extinction, the two-way ANOVA with repeated measures showed a significant main effect of group (*F*(1, 115) = 9.34, *p* < 0.01) and day (*F*(4, 115) = 28.31, *p* < 0.001), but no significant interaction of group × day (*F*(4, 115) = 0.64). Post-hoc Bonferroni comparison indicated that the %freezing at FE1 and FE3 were significantly higher in the FMCD group than the CON group (*p* < 0.05) (Fig. 4c). To examine spontaneous recovery, a recall assessment was conducted 28 days after the fear conditioning. The two-way ANOVA showed a significant main effect of group (*F*(1, 46) = 7.14, *p* = 0.01) and trial (*F*(1, 46) = 8.70, *p* < 0.01) and significant interaction of group × trial (*F*(1, 46) = 4.49, *p* < 0.05). Post-hoc Bonferroni comparison indicated that the %freezing at Recall (RE) was significantly higher than that at extinction day 5.
(FE5; last 5 min) in FMCD ($p<0.01$) but not in CON. In addition, %freezing of FMCD during the recall was significantly higher than that of CON ($p<0.01$) (Fig. 4d).

These results suggest that overall delay of fear extinction and spontaneous recovery appeared in developmental mice fed a FMCD diet in a contextual fear paradigm.

**Reduced novel object (NOR) recognition memory in FMCD mice**

During the training session, Student’s $t$-test showed that the exploration times of A and A’ were not significantly different in the CON and FMCD groups ($t(18) = 1.00$, $t(18) = 0.60$, respectively) (Fig. 5a). However, total exploration time was significantly longer in FMCD compared to CON ($t(18) = 2.15$ $p<0.05$) (Fig. 5b). Twenty-four hours after the training, we performed a retention session. The exploration time of a new object (B) was significantly greater than that of a familiar object (A) in CON ($t(11) = -2.96$ $p<0.05$) but not in the FMCD group ($t(18) = -1.34$) (Fig. 5c). As a result, the FMCD group showed a significantly lower discrimination index (%) ($t(18) = -3.69$ $p<0.01$) (Fig. 5d). Such patterns of exploratory behavior suggested impairments in the encoding of recognition memory in the FMCD group. Furthermore, these results indicated that memory impairment of FMCD was not caused by reduced exploration during the training day.

**Social memory and interaction were not changed in FMCD mice**

Next, we focused social behavior of FMCD mice. In the social recognition test, for the total investigation time during the training, two-way ANOVA with repeated measures showed a significant main
effect of trial \((F(3, 48) = 7.12, p< 0.01)\) but did not showed significant main effect of group \((F(1, 16) = 1.82)\),
or the interaction of group \(\times\) trials \((F(3, 48) = 2.68)\) (Fig. 6a). Twenty-four hours after the training, we performed a test session. The recognition index did not showed significant difference between FMCD and CON \((t(16) = -2.08, p= 0.054)\) (Fig. 6b).

In the social interaction test, each social behavior were not changed between the two groups; contact \((t(18) = 0.92)\), active contact \((t(18) = -0.003)\), passive contact \((t(18) = 1.31)\), approach \((t(18) = -0.83)\), leaving \((t(18) = 0.52)\), following \((t(18) = -0.31)\), sniffing \((t(18) = -0.58)\) and grooming \((t(18) = 1.05)\) (Fig. 7).

**Gria1 expression of FMCD mice was reduced in the hippocampus**

We speculate that methyl-donor deficiency affects hippocampus-dependent learning by the above-mentioned behavioral results. It was previously reported that NOR and contextual fear extinction is associated with the hippocampus (De Lima et al., 2006, Maren et al., 2013). Thus, we focused on expression of the glutamate receptor genes in the hippocampus because they play an important role in long-term memory and synaptic plasticity (Nicoll & Roche, 2013).

The Student's \(t\)-tests showed significantly lower hippocampal Gria1 gene expression in FMCD than CON \((t(9) = 2.59 p< 0.05)\) (Fig. 8a). Student's \(t\)-test did not show significant differences in group for other glutamate receptor gene (Gria2, Gria3, Grin1, Grin2a and Grin2b) expressions, \((t(9) = 0.61, t(9) = -0.60, t(9) = 0.51, t(9) = -0.33\) and \(t(9) = -0.72, \) respectively) (Fig. 8a, b).

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The promoter region of \textit{Gria1} was hyper-methylated in the FMCD mice’s hippocampus

A previous study reported that \textit{Gria1} promoter activity was stronger in neurons, with neuronal specificity appearing to reside mainly within the neuronal expression-enhancing regions, -1395 to -743 and -253 to -48 in rats (Borges & Dingledine, 2001). To study the alteration of \textit{Gria1} promoter methylation, we focused on the CpG-rich region of the \textit{Gria1} promoter -1461 to -812 and -262 to -48 (these sequences in mice include the equivalent promoter region in rats); these areas were sequenced after bisulfite modification of genomic DNA (Fig. 9). As shown in Fig. 5a, the FMCD diet resulted in a significant increase in methylated CpG sites in the -1461 to -812 region by Fisher's exact test ($p < 0.05$). In particular, CpG site 9 was hyper-methylated in FMCD compared to CON ($p < 0.01$, Fisher's exact test) (Fig. 10a,b). However, the methylation level of the -262 to -48 region showed no difference between the groups (data not shown).

Discussion

We demonstrated that FMCD diet feeding in developmental mice induced hippocampal-related cognitive dysfunctions. The FMCD group mice showed impairment in novel object recognition and exacerbated spontaneous recovery of contextual fear memory. In the hippocampus of the FMCD-group mice, significantly decreased expression of the AMPA receptor subunit gene \textit{Gria1} was observed, with increased CpG methylation in the promoter region, compared to CON-group mice.

Impaired memory consolidation by the methyl-donor deficiency

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In the current study, FMCD group mice showed impairment in fear extinction and reduced discrimination index of NOR (Fig. 6, 7). As shown in Fig. 6, FMCD mice showed significantly higher freezing than control mice at FE1 and FE3, which suggests that the fear extinction process was delayed in the FMCD mice compared to the control group. Moreover, spontaneous recovery of fear was observed in the FMCD mice, suggesting that FMCD impaired consolidation of fear extinction memory. It has been revealed that sufficient consolidation of fear extinction is capable of preventing spontaneous recovery. For example, one study using an avoidance task showed that a 5-day extinction paradigm prevented the spontaneous recovery of fear in male rats (Matsuda et al., 2014, Rossato et al., 2010). Recovery of extinguished fear was reduced with the administration of D-cycloserine, a partial NMDA receptor agonist, which is considered to play an enhancing role on the consolidation of fear extinction memory (Myers & Carlezon, 2012, Vervliet, 2008). It is believed that fear extinction does not erase the original fear memory but forms a new memory of safety that inhibits the original association (Bouton et al., 2006). Thus, our data indicate that new memory consolidation might be inhibited by methyl-donor deficiency, resulting in the spontaneous recovery of fear.

In this study, methyl-donor deficient affected only memory tasks not activity and social tasks. Several previous studies suggested that association of methyl-donor with memory. Folate deficiency impaired active avoidance learning in rats (Bachevalier & Botez, 1978) and methotrexate-induced folate deficiency impaired passive avoidance learning in chicks (Crowe & Ross, 1997). Administration of choline in the dorsal hippocampus reverses scopolamine-induced memory impairment (Blake et al., 2012). Supplemental choline during the periweaning period protects against trace conditioning impairments by post-training ethanol

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exposure in rats (Hunt, 2012). These data lend support our current study. Methylene tetrahydrofolate reductase (MTHFR), which required for homocysteine remethylation to methionine, knockout study showed not only short and long-term memory impairments but also decreased hippocampal volumes and increased apoptosis (Jadavji et al., 2012). Taken together, characteristic of one-carbon metabolism failure was assumed to hippocampal hypofunction. Thus, methyl-donor deficiency might affect hippocampal dependent memory compared to activity, anxiety and social related behavior.

**Reduced expression of Gria1 gene**

In the process of memory consolidation including contextual fear extinction, neuroplasticity based on the enhancement of glutamatergic neurotransmission recruiting AMPA and NMDA receptors is required (Cleva et al., 2010). Thus, we investigated AMPA and NMDA receptor expressions as related to LTP, and as a result, we found that Gria1 gene expression was significantly reduced in the FMCD group (Fig. 4a, b). An increase in synaptic strength during LTP is achieved by exocytosis and synaptic insertion of GluR1-containing AMPA receptors (Shi et al., 2001). Dynamic movement of GluR1 containing AMPA receptors from the intracellular pool to the synapse was observed to play a role on the synaptic plasticity (Makino & Malinow, 2009). PEPA, a pharmaceutical potentiator of AMPA receptors, facilitated fear extinction (Zushida et al., 2007), and that enhancement of recognition memory was accompanied by GluR1 phosphorylation in the hippocampus (Uslaner et al., 2009). Our study suggest that methyl-donor deficiency in the adolescent brain decreases the expression of Gria1 gene and that sufficient enhancement of glutamatergic process was impaired
in the consolidation process of the fear extinction in FMCD mice group.

As to fear acquisition, we did not observe any significant change between FMCD and CON groups (Fig. 3b). This conflicts with results of previous studies that fear conditioning recruited newly synthesized GluR1 to spines in adult hippocampal CA1 neurons (Matsuo et al., 2008) and that fear conditioning enhances spontaneous AMPA receptor-mediated synaptic transmission in the hippocampus (Zhou et al., 2009). However, these conflicts may be due to a ceiling effect because we used a strong shock (0.75mA) as the unconditioned stimuli. Fear conditioning memory was considered to be a stronger memory than extinction because fear responses could recover after extinction such as spontaneous recovery and renewal (Bouton et al., 2006, Vervliet et al., 2013).

In addition, we previously reported that 3-week feeding with a FMCD diet during adolescence affected fear acquisition and Grin2b expression at 6 weeks of age (Ishii et al., 2014) contrary to our current study, in which no alteration was observed. However, Ishii et al. could not exclude the effects of greatly decreased feed intake and weight loss (Ishii et al., 2014). Food restriction induces several alterations of behavioral and physiologic responses (Speakman & Mitchell, 2011). Previously, we used a diet completely lacking methionine, which induced very low body weights because methionine is associated with feed intake (Khalil et al., 1968). In the current study, we used a low-methionine compound to FMCD diet according to previous study (Pogribny et al., 2008) to prevent decrease of feed intake and weight loss as much as possible, and that might explain the inconsistent results.
Hypermethylation in the \textit{Gria1} promoter region

In the current study, the \textit{Gria1} promoter region showed hyper-methylation in the FMCD mice. A previous study reported that GluR1 promoter activity was stronger in neurons, with neuronal specificity appearing to reside mainly within the neuronal expression-enhancing regions (Borges & Dingledine, 2001). In this study, methylation of a \textit{Gria1} promoter region (-1461 to -812 in mice) was significantly increased. Specifically, the CpG site 9 showed the most prominent change (Fig. 5a, b). We tried speculative transcription factor binding in this region using TFSERCH (http://www.cbrc.jp/research/db/TFSEARCH.html), and found one candidate gene, upstream stimulatory factor 1 (USF1) (data not shown). Although there was an interaction between USF1 and \textit{Gria1} regulation, whether USF1 affects \textit{Gria1} expression via promoter methylation status remains unknown.

Although the methyl-donor deficiency in mice was confirmed by reduction of plasma methionine (Fig. 1a), the \textit{Gria1} promoter showed hyper-methylation in the FMCD. Our result was consistent with previous reports in which long-term feeding of a methyl-donor-deficient diet induced hyper-methylation and increase of methionine levels in the brain (Bagnyukova \textit{et al.}, 2008, Pogribny \textit{et al.}, 2008) whereas this diet induced hypo-methylation in the liver (Pogribny \textit{et al.}, 2008). Thus, a lack of methyl donors might induce different effects on different organs and/or tissues. The hyper-methylation induced in the brain by methyl-donor deficiency is opposite to the effects in other organs and the blood.

Methyl-donor feeding and brain development during adolescence

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Maternal one-carbon nutrient deficiency has been well researched and shown to affect various disorders and symptoms: e.g., fetal size, insulin resistance, diabetes risk, cardiovascular function, neural tube defect and cognitive functions (Jiang et al., 2014, Reynolds, 2014, Yajnik & Deshmukh, 2012). However, there are few studies that have focused on methyl-donor deficiency during the developmental period. Folate deficiency impaired active avoidance learning in post-weanling rats (Bachevalier & Botez, 1978). Likewise, methotrexate-induced folate deficiency impaired passive avoidance learning in chicks (Crowe & Ross, 1997). Adolescent brain maturation involves peaks in cholinergic, dopaminergic, and serotoninergic inputs to the frontal cortex (Dori et al., 1998, Gould et al., 1991, Kalsbeek et al., 1988). Hippocampus development is still in the developmental stage at postnatal day 21 (Albani et al., 2014). Alterations of the DNA methylation pattern in the developmental period may disrupt normal brain development.

It is noteworthy that abnormal DNA methylation patterns have been linked to several psychiatric disorders, such as Alzheimer’s disease, schizophrenia, PTSD and major depression in postmortem brain studies (Fuchikami et al., 2011, Levenson & Sweatt, 2005, Szyf, 2013). Moreover, folate and choline deficits and elevated plasma homocysteine also might be associated with the pathogenesis or the vulnerability of those psychiatric diseases, cognitive impairments or the risk of Alzheimer’s disease (Herrmann & Obeid, 2011, Moore et al., 2012). A higher GluR2-to-GluR1 subunit ratio seems to be related to individual stress vulnerability (Schmidt et al., 2010). In this study, methyl-donor deficiency did not prevent spontaneous recovery of fear, a phenomenon might be related to the onset of PTSD (state of failure to overcome fear). Methyl-donor deficiency during the developmental period might potentially induce vulnerability to several psychiatric conditions.
psychiatric disorders.

In conclusion, methyl-donor deficient during adolescence in mice induced hippocampal related some memory reductions with altered methylation status in the AMPA receptor gene. Our study suggests that altering the one-carbon metabolic pathway in the developmental brain could affect memory formation and gene expression.

Acknowledgements

This work was supported by grants from the Urakami Foundation and from the JSPS KAKENHI Grant Number 24791196.

Figure legends

Figure 1 Body weight of CON and FMCD mice. Body weights were measured every week. Data represent means ± SEMs (n = 12/group). * indicate $p < 0.05$, CON vs. FMCD.

Figure 2 Plasma methionine and homocysteine levels. (a) Plasma methionine levels of FMCD and CON mice. (b) Plasma homocysteine levels of FMCD and COM mice. Data represent means ± SEMs (n = 5/group). * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, CON vs. FMCD.
Figure 3 Methyl-donor deficient did not alter locomotion and anxiety behavior. (a) Total distance of open field test. (b) Time spent in center area of open field test. Data represent means ± SEMs (n = 10/ group).

Figure 4 Contextual fear extinction and spontaneous recovery affected by FMCD. (a) Schedule of contextual fear conditioning paradigm. (b) Percent freezing during the contextual fear conditioning. (c) Percent freezing during the contextual fear extinction. (d) Percent freezing in last 2 min in last day of extinction (FE5) and first 2 min in spontaneous recovery test (RE) in CON and FMCD. Data represent means ± SEMs (n = 12-13/ group). * and ** indicate p < 0.05 and p < 0.01, respectively, CON vs. FMCD. # indicate p < 0.05, FE5 vs. RE.

Figure 5 NOR memory was reduced in FMCD mice. (a) Time spent exploring objects (A and A’) at the training session. (b) Time spent exploring object (Familiar; A or A’ and Novel; B) at the retention session. (c) The ratio of exploring Familiar to Novel. The dotted line represents 50 % of recognition index. Data represent means ± SEMs (n = 10/ group). * and ** indicate p < 0.05 and p < 0.01, respectively, CON vs. FMCD.

Figure 6 Social recognition memory were not changed in FMCD mice. (a) Time spent exploring mice at the four training session. (b) Recognition index (The ratio of exploring Familiar mice to Novel mice). The dotted line represents 50 % of recognition index. Data represent means ± SEMs (n = 9/ group).

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Figure 7 Social interaction were not changed in FMCD mice. Duration of eight social behaviors to age and weight matched partner mice (contact, active contact, passive contact, approaching, leaving, following, sniffing and grooming). Data represent means ± SEMs (n = 10/ group).

Figure 8 Effects on AMPAR and NMDAR gene expressions in the hippocampus by FMCD exposure. (a) Gene expressions of Gria1, Gria2 and Gria3. Data were normalized to CON values. (b) Gene expressions of Grin1, Grin2a and Grin2b. Data were normalized to CON values. Data represent means ± SEMs (n = 5-6/ group). * indicate p < 0.05, CON vs. FMCD.

Figure 9 Gria1 sequence. The 5’ sequence of the mice and rats Gria1 gene, part of the promoter region, and important features are shown. CpG sites are shown in bold and numbered 1–10. The primers used for bisulfite sequence are underlined. Numbering is relative to the first ATG.

Figure 10 Hyper-methylation of Gria1 promoter in FMCD mice hippocampus. (a) Methylation pattern of Gria1 in hippocampus of CON and FMCD mice; CpG sites are numbered 1–10. Each row of circles represents an individual clone sequenced. Unmethylated (○) or methylated (•) CpGs are indicated. (b) Percentage of DNA methylation at Gria1 in hippocampus of CON and FMCD mice. * indicate p < 0.05, CON vs. FMCD.
References


Figure 1

![Graph showing body weight over time for CON and FMCD groups.](image1)

Figure 2

(a) Plasma Met (μM)

(b) Plasma Hcy (μM)

![Bar graphs comparing plasma levels for CON and FMCD groups.](image2)
Figure 3

(a) Total distance (cm) - CON vs. FMCD
(b) Total center time (sec) - CON vs. FMCD

Figure 4

(a) Day 1 Fear Cond. → Day 2-6 Fear Ext. → Day 29 Spontaneous Recovery
(b) % Freezing vs. Day
(c) % Freezing vs. Fear Ext. (FE1-5)
(d) % Freezing vs. Fear Ext. (FE5 RE CON vs. FMCD)
Figure 5

(a) Exploration time (sec) for group CON and FMCD, with comparisons A vs A' and A vs A'.

(b) Total exploration time (sec) for group CON and FMCD, with a significant difference denoted by an asterisk (*).

(c) Exploration time (sec) for groups CON and FMCD, with comparisons A vs B and A vs B'.

(d) Discrimination index (%) for group CON and FMCD, with a significant difference denoted by two asterisks (**) and a dotted line.

Figure 6

(a) Exploration time (sec) over time T1, T2, T3, T4 for groups CON and FMCD.

(b) Recognition index (%) for groups CON and FMCD, showing a significant difference denoted by a dotted line.
Figure 7

![Graph showing duration (sec) for different behaviors: Contact, Active Contact, Passive Contact, Approaching, Leaving, Following, Sniffing, Grooming. Comparison between CON and FMCD groups.]

Figure 8

(a) AMPA receptor

![Bar graph showing relative expression of Gria1, Gria2, Gria3 comparing CON and FMCD groups.]

(b) NMDA receptor

![Bar graph showing relative expression of Grin1, Grin2a, Grin2b comparing CON and FMCD groups.]

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Figure 9

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primer