RESEARCH ARTICLE

A high methionine, low folate and vitamin B_6/B_{12} containing diet can be associated with memory loss by epigenetic silencing of netrin-1

Anuradha Kalani[1, *, #], Pankaj Chaturvedi[1, *, #], Komal Kalani[2, 3], Pradip K. Kamat[1], Poonam Chaturvedi[4], Neetu Tyagi[1]

1 Department of Physiology, School of Medicine, University of Louisville, Louisville, KY, USA
2 Medicinal Chemistry Department, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India
3 Pharmacology Department and Toxicology, Higuchi Biosciences Center, University of Kansas, Lawrence, KS, USA
4 Department of Neurology, Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, India

Funding: *Part of this study was supported by NIH grant HL-107640 (to NT).*

Abstract

Memory-epigenetics which is the loss of memory due to epigenetic modifications can be due to the silencing of genes involved in cognitive functions and this is the basis of the current study. We hypothesize that a diet containing high methionine and low vitamins can lead to memory impairment by increasing global DNA methylation and therefore, silencing the netrin-1 gene, which encodes the glycoprotein involved in neurogenesis, axonal guidance and maintenance of the synaptic plasticity. Wild type (C57BL/6J) mice were fed with a diet containing excess methionine (1.2%), low-folate (0.08 mg/kg), vitamin B_6 (0.01 mg/kg), and B_{12} (10.4 mg/kg) for 6 weeks. Mice were examined weekly for the long-term memory function, using a passive avoidance test, which determined loss of fear-motivated long-term memory starting from the fourth week of diet. Similarly, an increase in brain %5-methyl cytosine was observed starting from the 4th week of diet in mice. Mice fed with a high methionine, low folate and vitamins containing diet showed a decrease in netrin-1 protein expression and an increase in netrin-1 gene promotor methylation, as determined by methylation-sensitive restriction enzyme-polymerase chain reaction analysis. The increase in methylation of netrin-1 gene was validated by high-resolution melting and sequencing analysis. Furthermore, the association of netrin-1 with memory was established by administering netrin that considerably restored long-term fear motivated memory. Taken together, these results suggest that a diet rich in methionine and lacking in folate and vitamin B_6/B_{12} can induce defects in learning and memory. Furthermore, the data indicates that decrease in netrin-1 expression due to hyper-methylation of its gene can be associated with memory loss. The animal procedures were approved by the Institutional Animal Care and Use Committee, University of Louisville, USA (No. A3586-01) on February 2, 2018.

Key Words: *Alzheimer's disease; epigenetics; memory; methionine; 5-methylcytosine; methylation; netrin-1*

Chinese Library Classification No. *R459.3; R363*

Correspondence to:
Anuradha Kalani, MS, PhD, anukalani@gmail.com; a0kala02@exchange.louisville.edu; Pankaj Chaturvedi, PhD, pcjuly4@gmail.com.

#Both authors contributed equally to this work.

orcid:
0000-0003-0856-7637 (Anuradha Kalani)
0000-0001-6752-2688 (Pankaj Chaturvedi)

doi: *10.4103/1673-5374.251333*

Received: *November 30, 2018*
Accepted: *January 7, 2019*

Introduction

Eggs, dairy products and red meat are the main sources of protein consumed in regular diet and their excessive intake can increase daily protein requirement in US individuals. Methionine, an essential amino acid, is the main constituent of red meat and has been studied as a risk factor for cerebrovascular pathologies including cognitive impairments and dementia (Dayal et al., 2005; Zhuo et al., 2010). Excessive dietary methionine consumption is reported to cause its accumulation which eventually metabolizes to an amino acid homocysteine by transferring a methyl (-CH3) group to S-adenosylhomocysteine from S-adenosylmethionine (Kalani et al., 2013). Our previous report suggested that high homocysteine levels can induce Alzheimer's disease-like pathology, blood brain disruption, and synaptic disorder (Kamat et al., 2016). Whereby methyl groups are important to execute several important physiological reactions of the body, higher levels can impair essential functional events by hyper-methylation of regulatory genes (Qureshi and Mehler, 2010; Kalani et al., 2013, 2014a). The process can be termed as "epigenetic silencing", which can affect the translation of important transcripts by hyper-methylation of genes. Methylation usually occurs at the 5th carbon of the cytosine residue at cytosine-guanine dinucleotide (CpG) site to form 5-methyl cytosine (5-mC) (Bender and Weber, 2013). The involvement of increased 5-mC with Alzheimer's disease has been reported earlier (Coppieters et al., 2014). A study conducted by Miller and Sweatt determined high transcript levels of methylating enzymes in rats after their training on a fear- conditioning test (Miller and Sweatt, 2007). Another study conducted on double knockout mice for methylating enzymes [DNA methyltransferase (DNMT)-3a and DNMT-1] showed compromised long-term potentiation and hippocampus-based spatial memory (Feng et al., 2010). In addition, increased DNA methylation of GABAergic interneurons, that downregulated reelin expression, was studied in schizophrenics (Lockett et al., 2010). These studies clearly indicate potential influence of DNA methylation that can be involved in regulation of memory and other brain disorders (Waterland, 2006). However, less information is available for the influence of dietary methionine on methylation and memory loss.

Memory is described as the capacity to contain the learned information and hence, can be divided into two parts: short-term memory, the retention of information for short time; and long-term memory, the retention of memory for long-period of time that depends on the neural pathways (Eriksson et al., 2015). Though there have been several models presented that described the role of different brain parts in memory, the role and interaction of hippocampus and frontal cortical regions

sustaining the memory over the periods of time have been confirmed by various studies (Maren et al., 2013; Preston and Eichenbaum, 2013; Gilmartin et al., 2014). Identifying the molecular mechanisms responsible for long-term memory formation can help in strategizing future potential therapeutics. Netrins, named after Sanskrit word 'netr' meaning the 'one who 'guide', are highly evolutionarily conserved families (Cirulli and Yebra, 2007). As the name suggests, the family is involved in guiding axons and cells during development and differentiation (Cook et al., 1998). Out of different family members, netrin-1, netrin-2, netrin-3 and β netrin, netrin-1 exhibit various important functions for neurogenesis (O'Leary et al., 2015), axonal guidance (Cook et al., 1998; Mehlen and Rama, 2007), and synaptic plasticity (Bayat et al., 2012; Horn et al., 2013). The anti-inflammatory and angiogenetic roles of netrin-1 have also been confirmed (Ly et al., 2005; Li et al., 2011; Tian et al., 2011). Of most importance is the regulatory role of netrin-1 in maintaining spatial memory and synaptic plasticity (Bayat et al., 2012). However, the involvement of netrin-1 in diet-associated memory loss has not been studied. We hypothesize that a diet rich in methionine and low in vitamins B_6/B_{12} leads to memory impairment by increasing global DNA methylation and therefore, silencing the netrin-1 gene. To test this hypothesis, the effect of high methionine, low vitamin supplementation on memory dysfunction was analyzed in wild type mice. Alteration in brain methylation was assessed by determining total brain 5-mC level in mice fed with diet. The change in netrin-1 protein and gene, affected with epigenetic modifications, was determined. The therapeutic effect of netrin-1 was also evaluated for memory restoration in diet-fed mice.

Materials and Methods

Animals and experimental design

The animal procedures were carefully approved by the Institutional Animal Care and Use Committee, University of Louisville (No. A3586-01) on February 2, 2018. Male wild type (C57Bl6/J) mice were fed orally with a methionine-rich (1.2%) diet (TD.97345, Harlan Laboratories, Madison, WI, USA). The diet also contained low amounts of folate (0.08 mg/kg), vitamin B_6 (0.01 mg/kg), and B_{12} (10.4 mg/kg). Six individual groups of mice (6–8/group) received a high methionine and low vitamin diet (HMLVD) for 1, 2, 3, 4, 5, and 6 weeks, respectively. HMLVD was provided in a manner that all groups complete their diet plan by 8–10 weeks of age. Control mouse group was fed normal chow diet for 8–10 weeks of age. **Figure 1** shows the diet and experimental plans of the study.

Passive avoidance test

The test was performed as per our earlier report (Kamat et al., 2016). Briefly, different HMLVD-fed mice groups were studied for the loss of fear-motivated long-term memory using a passive avoidance test. Passive avoidance instrument (Columbus Instruments, Columbus, OH, USA) consisted of two compartments, one dark (poorly illuminated) and one white (white illuminated). The mice were subjected to the passive avoidance test by placing the animals in a compartment with light at an intensity of 8 [scale from 0 to 10 (brightest)] in a computerized shuttle box with a software program PACS 30 monitoring (Columbus Instruments). The light compartment was isolated from the dark compartment by an automated guillotine door. The guillotine door was opened and closed automatically after entry of the mouse into the dark compartment. The mice received a low-intensity foot shock (0.5 mA; 10 seconds) in the dark compartment. Infrared sensors monitored the transfer of the animals from one compartment to another, which was recorded as transfer latency time (TLT) in seconds. The 1st trial was termed as acquisition trial and the 2nd and 3rd trials (retention trials) were given after 24 and 48 hours of the 1st trial. The criteria for learning were taken as an increase in the TLT on retention (2nd or subsequent) trials as compared to acquisition (1st) trial.

Brain tissue collection

Mice were anesthetized with the overdose of pentobarbital. The frontal cortex and hippocampus regions of the brain tissues were dissected and cleaned in PBS and stored at –80°C until further used.

Quantification of 5-mC

Genomic DNA was isolated (Sigma, St. Louis, MO, USA) and total 5-mC levels was determined using the DNA-ELISA (enzyme-linked immunosorbent assay) kits (Epigentek, Farmingdale, NY, USA), as per the manufacturer's instructions.

Western blotting

Brain tissues were homogenized in 1× radioimmunoprecipitation assay buffer. Equal amounts of protein were separated onto a 10% polyacrylamide gel and run at a constant voltage. Separated proteins of the gels were transferred to polyvinylidene difluoride membranes using an electrotransfer apparatus (Bio-Rad, Hercules, California, USA). After blocking with 5% non-fat dry milk (1 hour), the membranes were probed overnight with primary antibodies [rabbit anti-Netrin-1, dilution 1:250 (Santa Cruz, Dallas, TX, USA); mouse anti-GAPDH, dilution 1:1000 (Millipore, Burlington, MA, USA)] at 4°C and then with species-specific secondary antibodies (dilution 1:5000) for 90 minutes at room temperature, which was conjugated to horse radish peroxidase (Santa Cruz). The membranes were developed with ECL Western blotting detection system (GE Healthcare, Piscataway, NJ, USA) and the images were recorded in the chemi-program of a gel documentation system (Bio-Rad). Each band density was normalized with a respective GAPDH density using Image Lab densitometry software (Bio-Rad).

Immunohistochemistry

The mice were euthanized with overdose of anesthesia. Mouse brain was exsanguinated by transcardially infusing 50 mM PBS (pH 7.4) and then with 4% paraformaldehyde. The cranium was opened and the brain was gently removed for fresh tissue processing. Brain was further fixed in 4% paraformaldehyde overnight and then processed in 30% sucrose for 3 days. After mounting in a protective matrix (Polyscience, Inc., Warrington, PA, USA), the brain was cryosectioned using a Leica CM 1850 Cryocut (Buffalo Grove, IL, USA). Twenty 20-μm thick slices were made and placed on charged microscope slides (VWR, West Chester, PA, USA) and stored at –20°C until used. For immunohistochemistry, slides were warmed at 37°C for 20 minutes and mounting matrix was carefully removed. After fixing sections with ice-cold 100% methanol

for 10 minutes, slides were washed thrice in Tris-buffered saline (TBS) and blocked for nonspecific epitope binding in 0.1% TritonX-100 TBS, 0.5% bovine serum albumin, and 10% normal donkey serum for 1 hour at room temperature. The sections were incubated with a primary antibody (rabbit anti-netrin-1, dilution 1:100, Santa Cruz) overnight at 4°C. After washing thrice in TBS, the sections were incubated with goat anti-rabbit conjugated to alexa flour 494 (dilution 1:500) for 60 minutes at room temperature. The slides were further stained with 4′,6-diamidino-2-phenylindole for 10 minutes (dilution 1:10,000) and mounted with anti-fade mounting media. The images were acquired using a laser scanning confocal microscope (60× objectives, FluoView 1000, Olympus, Center Valley, PA, USA). Fluorescence intensity was measured with image analysis software (Image-Pro Plus, Media Cybernetics, Rockville, MD, USA) and expressed as fluorescence intensity unit (FIU).

Promoter methylation analysis

The sequence of the netrin-1 region was taken from NCBI database: *Mus musculus* netrin 1 (Ntn1) NM_008744 and primer sequences were designed through Methprimer website (Li and Dahiya, 2002) (http://www.urogene.org/methprimer/). The genomic DNA was isolated from the diet-fed mice brains using a DNA isolation kit (Sigma) and subjected to sodium bisulfite treatment using the EZ-DNA methylation kit (Zymo Research Corporation, Irvine, CA, USA) as per supplier's protocol. Bisulfite-converted DNA was amplified using PCR. The amplified product was run on 1.2% agarose gel and band was excised from the agarose gel. After isolating DNA with the gel extraction kit (Qiagen, Germantown, MD, USA), it was further processed for Sanger DNA sequencing at the core facility, University of Louisville.

Methylation sensitive restriction enzyme analysis

Genomic DNA was isolated from mice brain and subjected to methylation sensitive restriction enzyme (MSRE) analysis. The process was followed as described elsewhere (Melnikov et al., 2005). Genomic DNA (1 μg) was digested with HaeII and then PCR amplified with MSRE primers designed from the promoter region of the netrin-1 gene. The amplified product was run on horizontal agarose gel (prepared in 1× tris-acetate-EDTA buffer). The presence of strong band at ~485 bp on agarose gel confirmed methylation while absence indicated un-methylation of netrin-1 gene.

Methylation sensitive-high resolution melting analysis

Genomic DNA was isolated from diet-fed mice. Sodium bisulfite conversion of genomic DNA was performed with EZ-DNA methylation kit (Zymo Research Corporation). The methylated primers were designed from methprimer online website (Li and Dahiya, 2002) (http://www.urogene.org/methprimer/). HRM analysis of the sodium bisulfite treated DNA was performed using a Light cycler 480 system (Roche Diagnostics Corporation, Indianapolis, IN, USA) as per manufacturer's instructions. Reaction mixtures were amplified using high resolution melting dye as described elsewhere (Krypuy, 2008) with some modification. The shift of melting curves with methylated primer represents higher methylation of the netrin-1 gene.

Intracerebral administration of netrin-1

Mice (aged 8 weeks, body weight 25–30 g) fed on HMLVD for 6 weeks were randomly divided into three groups (4–5 mice per group) and treated with netrin-1 (Minneapolis, MN, USA). We used 100 ng of netrin-1 dissolved in artificial cerebro-spinal fluid (119 mM NaCl, 26.2 mM $NaHCO_3$, 2.5 mM KCl, 1 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 10 mM glucose). The above dose of netrin-1 was chosen according to an earlier report (Bayat et al., 2012), and pilot experiments performed in mice (data not shown). The administration procedure of netrin-1 was followed as per our earlier reports (Kamat et al., 2013; Kalani et al., 2014b). Briefly, mice were anesthetized intraperitoneally with tribromoethanol (2.5 g, 2,2,2 tribromoethanol; 5 mL 2-methyl-2-butanol (tertiary amyl alcohol), 200 mL distilled water - neutral pH) at 200 μg/g. A 27-gauge hypodermic needle, attached to a Hamilton syringe, was inserted (2.5 mm depth) perpendicularly through the skull into the brain. Netrin-1 (100 ng/2 μL) was administered very slowly through intracerebral route. The site of injection was 2 mm from either side of the midline on a line drawn through the anterior base of the ears. The syringe was left in the place for further 80–90 seconds for proper diffusion of netrin-1.

Passive avoidance test on netrin-1 administered mice

Mice (aged 8 weeks, Body weight 25–30g) fed on HMLVD for 6 weeks were randomly divided into four groups (4–5 mice/group). One group of HMLVD-fed mice was treated with artificial cerebrospinal fluid. All other three groups of HMLVD-fed mice were treated with netrin-1 as described earlier. The first group of HMLVD-fed mice was performed with acquisition trial on the 1st day and retention trial on the 2nd day post netrin administration. Similarly, the second and third groups of HMLVD-fed mice were subjected to acquisition trials on the 3rd and 7th days post-netrin administration. The corresponding log-term retention memory or retention trials for the second and third groups of mice were performed on the 4th and 8th days, respectively.

Statistical analysis

All data are expressed as the mean ± SEM and analyzed with GraphPad version 5.0 (GraphPad Software, San Diego, CA, USA. A value of *P* less than 0.05 was considered statistically significant.

Results

HMLVD induces memory impairment

The control and individual mice groups fed on HMLVD for 1, 2, 3, 4, 5, and 6 weeks were first tested for the loss in fear motivated long-term memory through passive avoidance test. We did not find significant difference in transfer latency time in retention trial compared with the acquisition trial in mice groups fed on HMLVD for 4, 5, and 6 weeks. However, The mice fed with HMLVD for 1, 2 and 3 weeks and control mice fed with normal chow showed a significant increase in the transfer latency time (**Figure 2**).

HMLVD increases methylated cytosine levels in the brain

Total methylated cytosine levels were evaluated in control and six individual HMLVD-fed mice to address epigenetic remodeling. Total 5-mC levels were evaluated in DNA samples

derived from the brains in different mice groups using ELISA with specific subset of methylation controls. DNA-based ELISA assay confirmed a significant increase of total 5-mC levels in HMLVD-fed mouse groups for 4, 5, and 6 weeks compared with control ($P < 0.05$). Mice fed with HMLVD for 1, 2, and 3 weeks did not show significant alterations in 5-mC levels compared with control (**Figure 3**).

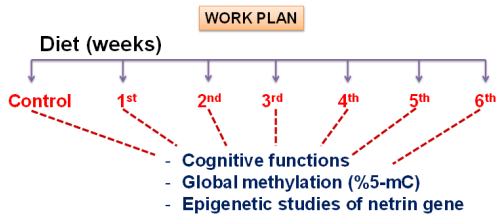

Figure 1 Schematic representation of experimental work plan in mice fed with a high Methionine, low folate and vitamin B_6/B_{12} diet.
5-mC: 5-Methylcytsine.

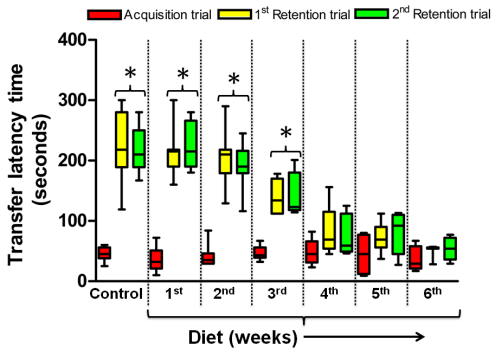

Figure 2 Memory performance of mice fed with a high methionine, low folate and vitamin B_6/B_{12} diet.
Box and whisker plot showing passive avoidance test results in high methionine, low vitamin B_6/B_{12} diet-fed mice. Diet was administered for 1 to 6 weeks in six different groups of mice. Control mice were fed with a normal chow diet. Retention memory test, entry of mouse to dark compartment from white compartment, was performed after 24 and 48 hours of acquisition test. During acquisition test, mice experienced a mild current when entered to dark compartment from the light compartment. Retention test was performed after 24 and 48 hours of acquisition trial and the time duration for every trial was set for 300 seconds (5 minutes). Mice with higher freezing behavior showed more learned experience through acquisition trials. Data is expressed as transfer latency time. Data are expressed as the mean ± SEM. $*P < 0.05$ (two-way analysis of variance followed by Tukey's *post hoc* test).

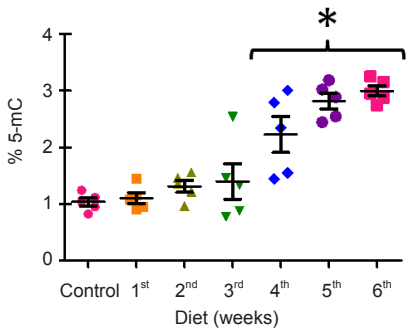

Figure 3 5-methylcytsine (5-mC) levels in high methionine, low folate and vitamin B_6/B_{12} diet-fed mice.
Scatter plot represents total brain %5-mC levels in high methionine, low vitamin B_6/B_{12} diet-fed mice. Diet was administered for 1 to 6 weeks in six different mice groups. By a DNA based enzyme-linked immunosorbent assay, %5-mC levels were estimated in the presence of specific controls for methylation. Data are expressed as the mean ± SEM ($n = 5$/group). $*P < 0.05$ (one-way analysis of variance followed by Tukey's *post hoc* test)

HMLVD-fed mice show decrease in netrin-1 expression level

A decrease of netrin-1 protein expression was determined in individual mice groups fed for 4, 5, and 6 weeks of HMLV diet by western blot analysis (**Figure 4A** and **B**). However, no change in netrin-1 protein expression was observed in individual mice groups fed on HMLV diet for 1, 2, and 3 weeks. The changes in netrin-1 protein expression in different mice groups were also confirmed by immunohistochemistry studies. Immunohistochemistry analysis revealed decrease of netrin-1 expression started after 3 weeks of HMLV diet and that was consistent till 6 weeks of diet (**Figure 4C** and **D**).

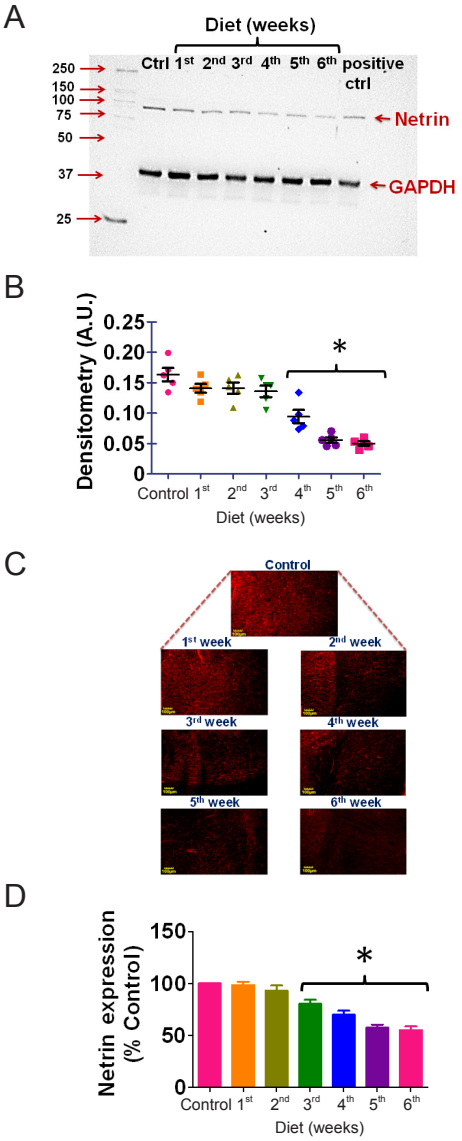

Figure 4 Netrin expression in mice fed with a high methionine, low folate and vitamin B_6/B_{12} diet.
(A) Western blots represent netrin-1 protein expression in different groups of mice fed with a high methionine, low vitamin B_6/B_{12} diet for 1 to 6 weeks. Normal chow diet fed mice were used as control. (B) Scatter plot represents densitometry analysis for netrin-1 protein expression in different groups of mice ($n = 5$/group). (C) Immunohistochemistry images for netrin-1 expression in different groups of mice brains (red represents netrin-positive cells), scale bars: 100 µm. (D) Bar graph represents quantitation of netrin-1, as determined through immunohistochemistry analysis, in diet-fed mice brains ($n = 3$/group). Data are expressed as the mean ± SEM. $*P < 0.05$ (one-way analysis of variance followed by Tukey's *post hoc* test).

HMLVD-fed mice show silencing of netrin-1 gene through high-methylation

To determine whether decrease of netrin-1 expression in HMLVD-fed mice is due to increased methylation of netin-1 gene promotor, we preformed MSRE, methylation sensitive-high resolution melting (MS-HRM) and Sanger sequencing analysis. Several CpG islands were confirmed at the promotor region of netrin-1 gene through methprimer site (http://www.urogene.org/methprimer/). Few sites for restriction enzyme HaeII were confirmed in netrin-1 gene (**Figure 5A**). MSRE analysis showed a PCR product of 485 bp starting in different mice groups fed on HMLVD for 3, 4, 5, and 6 weeks. However, no PCR product was observed in control and mice groups fed on HMLVD for 1 or 2 weeks (**Figure 5B**). The amplification product after *Hae*II restriction digestion suggested netrin-1 gene methylation in mice fed with HMLVD. Next, MS-HRM analysis performed on 4 and 6 weeks of HMLVD administered mice groups showed considerable shift in melting temperature (Tm) with methylated primer as compared to unmethylated primer (**Figure 5C** and **D**). Additionally, Sanger sequencing data also confirmed CpG island methylation in the netrin-1 gene in 6 weeks HMLVD-fed mouse brain (**Figure 5E** and **F**). Cumulatively, these results are suggestive of netrin-1 gene methylation in mice fed with HMLVD.

Netrin administration alleviated memory impairment in HMLVD-fed mice

To determine the association of netrin-1 with memory, we administered netrin-1 intracerebrally in 6 weeks of HMLVD-fed mice. Passive avoidance test determined that TLT was not significantly changed in retention trials as compared to acquisition trial in vehicle (artificial cerebrospinal fluid) administered HMLVD-fed mice group. Similarly, no change in TLT during retention trials was observed on the 2nd day of netrin-administration in 6 weeks of HMLVD-fed mice (1st group). Second, HMLVD-fed mice group was tested for TLT change on the 4th day of netrin administration that showed significant changes in retention trials. Similarly netrin administered third HMLVD group was tested on the 8th day of netrin administration which also showed high TLT in retention trial as compared to acquisition trial (**Figure 6A** and **B**).

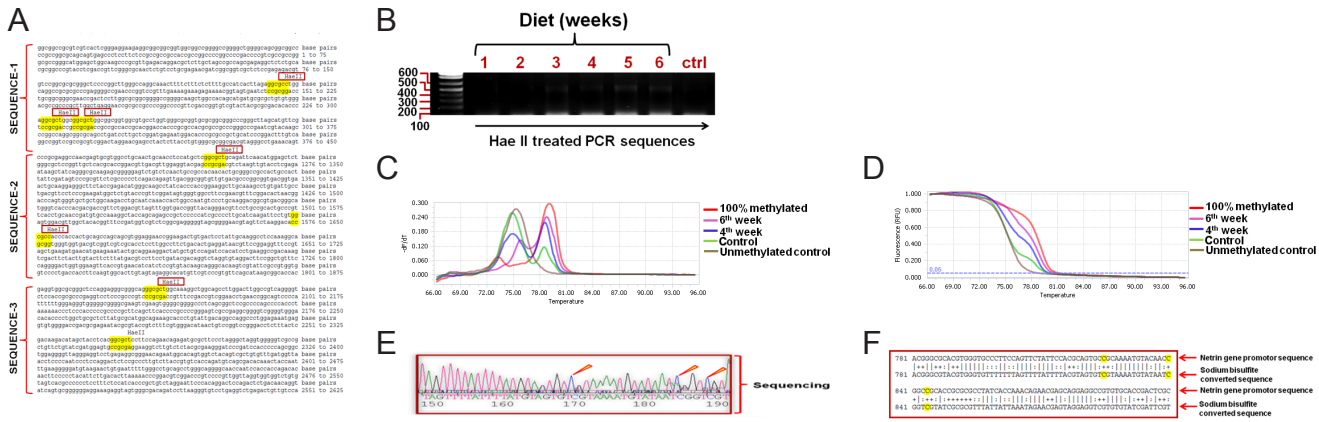

Figure 5 Methylation of netrin gene in a high methionine, low folate and vitamin B_6/B_{12} diet-fed mice.
(A) The presence of *Hae*II sites, represented with yellow color, at various sites in netrin-1 gene. (B) Agarose gel image represents methylation specific restriction enzyme analysis in control and high methionine, low vitamin B_6/B_{12} diet fed mice for 1 to 6 weeks. The genomic DNA from different mice brains were isolated, digested with *Hae*II restriction enzyme and amplified using netrin-1 based primers. The presence of band at 485 bp showed methylation of the netrin-1 gene. Molecular weight ladder (100 bp) represents at leftmost side. Diet-fed mice samples (1 to 6 weeks) are shown in continuity and the right most lane shows control mouse sample. (C, D) MS-HRM analysis (C, difference curve; D, melt curve). Sodium bisulfite treated DNA was used for MS-HRM analysis and the shift in Tm (melting temperature) shows methylation of the promotor region of netrin gene. Brain samples of mice fed on HMLVD for 4 and 6 weeks showed more methylation as compared to control. (E) Confirmation of the methylation at CpG island by sequencing: Sodium-bisulfite treated DNA was PCR amplified and the amplified product was sequenced to confirm the methylation at the CpG islands. Arrowheads represent cytosine (C) residue which is not converted to thiamine (T) after bisulfite sequencing. (F) The sequence of netrin-1 gene and bisulfite converted DNA is shown. Yellow color shows the methylated sites. MS-HRM: Methylation specific-high resolution melting; HMLVD: high methionine low vitamin diet.

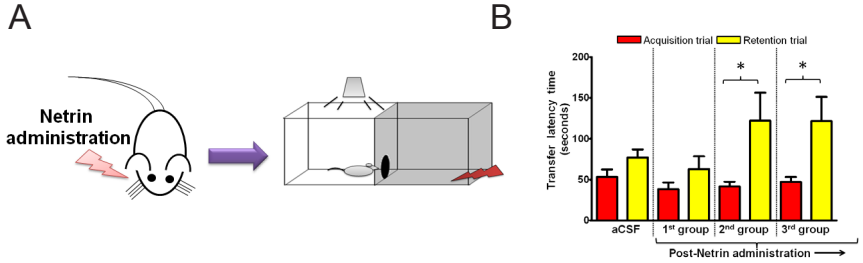

Figure 6 Netrin administration restores memory in high methionine, low folate and vitamin B_6/B_{12} diet-fed mice.
(A) Cartoon representing netrin-1 administration in mice and long-term fear motivated memory assessment by passive avoidance test. Netrin-1 was administered after 6 weeks of high methionine, low vitamin B_6/B_{12} diet (HMLVD) in three groups of mice. The 6 weeks HMLVD-fed mice group received vehicle (artificial cerebrospinal fluid; aCSF) and used as control. (B) The Box and whisker plot shows the transfer latency time recorded after 24 hours of acquisition test in vehicle- and three groups of netrin-administered HMLVD-fed mice. The retention tests of different netrin-1 administered HMLVD-fed mice groups were recorded on days 2, 4 and 8 (n = 4–5/group). Data are expressed as the mean ± SEM. $*P < 0.05$ (paired-samples t-test).

Discussion

In the present study, we determined the effect of HMLV diet on long-term memory impairment in mice. The impairment in memory was opposite to total brain 5-mC levels, which was found to be increased in HMLVD-administered mice. We next confirmed that HMLVD reduced netrin-1 protein expression by increasing promotor methylation of netrin-1 gene. Interestingly, netrin-1 administration restored long-term fear motivated memory function to a significant extent in HMLVD-fed mice.

Studies have shown that nutrient-rich diet positively supports cognitive processes (Bourre, 2006; Gomez-Pinilla, 2008; Smyth et al., 2015; Sorensen et al., 2015), whereas unhealthy food negatively affects brain health (Gibson, 2006). Hence, it is of importance to understand how food can impact on our ability to remember things and enhance our probability of developing dementia. A methionine rich diet, such as red meat, is routinely consumed and has been studied as a cardiac threat (Arshad et al., 2015; Chaturvedi et al., 2016). We have provided a diet high in methionine and low in vitamin B_6/B_{12} to our experimental wild-type mice and tested time course of memory loss by examining individual mice groups administered with 1 to 6 weeks of HMLVD. The study determined impairment in fear motivated long-term memory started in mice fed with 4 weeks of HMLVD. The study also determined alterations of total methylation in different HMLVD-fed mice groups by measuring an epigenetic mark, %5-mC. Our results suggested a loss of fear-motivated long-term memory and increase of %5-mC level starting after 4 weeks of HMLVD supplementation in mice. By applying Pearson's rank correlation test, we determined highly significant association between long-term memory loss and %5-mC (data not shown). In agreement with our findings, previous studies have shown the loss of memory in rodents when administered with the currently studied diet (Sudduth et al., 2013, 2014). Alteration in methylation process with excess methionine supplementation was also suggested in previous studies (Niculescu and Zeisel, 2002; Waterland, 2006). The reports indicated that dietary methionine is a precursor of S-adenosylmethionine which is a donor of methyl group in methionine metabolism. However, it is still no very clear how dietary methionine affects DNA methylation? Though, it is evident from the some of the previous studies that high dietary methionine induces DNA hyper-methylation (Waterland, 2006; Kalani et al., 2013, 2014a). In support, our report also suggests increase in total 5-mC level with high methionine diet feeding. Since methylation usually occurs at 5^{th} carbon of cytosine residues to form 5-mC (Khare et al., 2012; Chopra et al., 2014; Kalani et al., 2015), induced levels of %5-mC in HMLVD-fed mice suggest hyper-methylation of genes. Hyper-methylation of genes may convert transcriptionally active chromatin to inactive. Hence, chromatin silencing can affect transcription and then translation of regulatory genes.

Netrins have been reported to be crucial for neuronal and vascular developments (Freitas et al., 2008; Sun et al., 2011). Among different family members, netrin-1 has been extensively studied for its role in neurogenesis, axonal guidance and synaptic plasticity maintenance (Cirulli and Yebra, 2007; Bayat et al., 2012). For our study, the selection of netrin-1 was based on strong reports that suggest its protective role against ischemia-reperfusion injury of the kidney (Reeves et al., 2008; Wang et al., 2008), and myocardial infarction (Zhang and Cai, 2010). Of major interest, netrin-1 has been shown to improve spatial memory and synaptic plasticity following global ischemia in a rat model (Bayat et al., 2012). In the present study, we determined that in HMLVD-fed mice, high methylation can affect netrin-1 protein expression by hyper-methylation of netrin-1 gene promotor. Our results confirmed a reduction in netrin-1 protein expression starting after 3 to 4 weeks of HMLVD supplementation in mice. The reduction of netrin-1 was similar to decline in memory impairment as these two processes started at the same time in mice fed with HMLVD. To confirm netin-1 gene methylation, several experiments were conducted, for example; MSRE, MS-HRM and Sanger sequencing. First, through MSRE experiment, target restriction sites of *Hae*II enzyme in netrin gene were confirmed and primers were designed. The appearance of an amplified PCR product starting in 3 weeks of HMLVD fed mice suggested beginning of netrin-1 gene methylation. Since *Hae*II cleaved unmethylated-cytosine residues, no amplified product was observed in control and HMLVD-fed mice groups for 1 and 2 weeks. The products detected (3 weeks of diet supplementation onwards) were due to the methylated DNA, which was not digested with *Hae*II in netrin-1 gene. Similar assay has been used earlier for confirming pathological hallmarks in the brain (Guo et al., 2011; Masliah et al., 2013). Second, methylation in the netrin-1 gene was confirmed through MS-HRM experiment. In the experiment, the bisulfite treated DNA of HMLVD-fed mice showed amplification pattern which was found quite similar to methylation-specific control. MS-HRM was successfully used earlier for establishing the association of SH2B1 CpG-SNP with reduction in body weight during a dietary restriction program study conducted on obese subjects (Mansego et al., 2015). Similarly, the technique was used to determine the increased methylation of serotonin receptor 5HTR1A gene promoter in schizophrenia and bipolar disorder patients (Carrard et al., 2011). Third, through sequencing analysis of bisulfite treated DNA, methylation of netrin-1 gene was confirmed in HMLVD-fed mice. The primers of the study were specifically designed from the promotor region of netrin-1 gene. Hence, all these results suggest promotor methylation of netrin-1 gene following HMLVD supplementation in mice.

In the next sets of experiments, we confirmed the therapeutic aspects of netrin-1 in restoring long-term memory in HMLVD-fed mice. The results suggested that netrin-1 administration restored freezing behavior in diet-fed mice by around ~50%, which was significant when compared to vehicle-treated HMLVD fed mice. In agreement with this finding, Bayat et al. (2012) have also confirmed the therapeutic role of netrin-1 in recovering spatial memory and synaptic plasticity following global ischemia in rats. Previous supportive findings along with our study clearly indicate association of netrin-1 with memory function and provide an innovative therapeutic direction using netrin-1. Alongside, the results also clearly indicate the ill-effects of HMLV diet. The current diet was earlier used and termed as hyperhomocysteinemic diet. Studies using HMLVD showed that the diet can cause vascu-

lar dementia pathology and alterations in blood-brain barrier integrity (Kamath et al., 2006; Sudduth et al., 2013). Hyperhomocysteinemia condition arises with high plasma homocysteine levels and is a risk factor to develop brain pathology (Kalani et al., 2013, 2014b). HMLVD was also found to induce mild to moderate levels of plasma homocysteine in rodents (Kamath et al., 2006). It is not well established how the elevated plasma homocysteine levels affect brain integrity. However, accumulating evidence supports that the elevated levels of homocysteine is involved in brain pathology through altered methionine metabolism, simple diffusion, and crossing of blood-brain barrier *via* specific saturable receptor (Grieve et al., 1992; Griffiths et al., 1992). In our study, although we did not measure plasma homocysteine levels in HMLVD mice, the possibility for the involvements of increased homocysteine levels in memory impairment cannot be ignored. It would be exciting to assess homocysteine periodically to correlate disease pathology with homocysteine levels. Also, since we found total increase of 5-mC in HMLVD-fed mouse brain samples which can be related to more genomic methylation and suppression of regulatory genes, the possibility of under expression of other regulatory genes cannot be overlooked and may be further studied in future.

In summary, we have determined the effect of high methionine, low vitamin supplementation in long-term memory loss in mice. The week-wise study confirmed that loss of memory was associated with increased DNA methylation. High methionine, low vitamin fed mice also showed decrease in netrin-1 protein expression, which can be due to hyper-methylation of netrin-1 gene promotor. The role of netrin and memory function can be further explored in the future studies and these studies will be important in bringing a potential candidate(s) for future therapeutics.

Acknowledgments: *The authors acknowledge Prof. David Lominadze University of Louisville, for the valuable suggestions during the study.*
Author contributions: *Study design, experimental studies, data analysis, and manuscript preparation: AK and PC; experimental studies, data analysis, and manuscript editing: AK and PC; valuable help in writing and editing the manuscript: PC and KK; help in performing memory experiments and data analysis: PKK; discussing the data and obtaining supported financially: NT. All authors approved the final manuscript.*
Conflicts of interest: *The authors claim that there are not conflicts of interest.*
Financial support: *Part of this study was supported by NIH grant HL-107640 (to NT).*
Institutional review board statement: *The animal procedures were approved by the Institutional Animal Care and Use Committee, University of Louisville, USA (No. A3586-01) on February 2, 2018.*

Data sharing statement: *A part of the study was shown in Experimental Biology meeting 2015 and abstract was shared by a press release by American Physiological Society: http://www.the-aps.org/mm/hp/Audiences/Public-Press/2015-11.html*
Plagiarism check: *Checked twice by iThenticate.*
Peer review: *Externally peer reviewed.*

Open peer reviewer: *Matin Ramezani, Neuroscience Research Institute, Arak University, Iran.*

Additional file: *Open peer review report 1.*

References

Arshad MS, Zulfiqar A, Anjum FM, Saeed F, Khan MI, Sohaib M (2015) Red meat consumption: a threat towards cardiovascular diseases. Pak J Food Sci 25:86-91.
Bayat M, Baluchnejadmojarad T, Roghani M, Goshadrou F, Ronaghi A, Mehdizadeh M (2012) Netrin-1 improves spatial memory and synaptic plasticity impairment following global ischemia in the rat. Brain Res 1452:185-194.
Bender A, Weber M (2013) DNA methylation: an identity card for brain cells. Genome Biol 14:131.
Bourre JM (2006) Effects of nutrients (in food) on the structure and function of the nervous system: update on dietary requirements for brain. Part 2 : macronutrients. J Nutr Health Aging 10:386-399.
Carrard A, Salzmann A, Malafosse A, Karege F (2011) Increased DNA methylation status of the serotonin receptor 5HTR1A gene promoter in schizophrenia and bipolar disorder. J Affect Disord 132:450-453.
Chaturvedi P, Kamat PK, Kalani A, Familtseva A, Tyagi SC (2016) High methionine diet poses cardiac threat: a molecular insight. J Cell Physiol 231:1554-1561.
Chopra P, Papale LA, White AT, Hatch A, Brown RM, Garthwaite MA, Roseboom PH, Golos TG, Warren ST, Alisch RS (2014) Array-based assay detects genome-wide 5-mC and 5-hmC in the brains of humans, non-human primates, and mice. BMC Genomics 15:131.
Cirulli V, Yebra M (2007) Netrins: beyond the brain. Nat Rev Mol Cell Biol 8:296-306.
Cook G, Tannahill D, Keynes R (1998) Axon guidance to and from choice points. Curr Opin Neurobiol 8:64-72.
Coppieters N, Dieriks BV, Lill C, Faull RL, Curtis MA, Dragunow M (2014) Global changes in DNA methylation and hydroxymethylation in Alzheimer's disease human brain. Neurobiol Aging 35:1334-1344.
Dayal S, Devlin AM, McCaw RB, Liu ML, Arning E, Bottiglieri T, Shane B, Faraci FM, Lentz SR (2005) Cerebral vascular dysfunction in methionine synthase-deficient mice. Circulation 112:737-744.
Eriksson J, Vogel EK, Lansner A, Bergstrom F, Nyberg L (2015) Neurocognitive architecture of working memory. Neuron 88:33-46.
Feng J, Zhou Y, Campbell SL, Le T, Li E, Sweatt JD, Silva AJ, Fan G (2010) Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult forebrain neurons. Nat Neurosci 13:423-430.
Freitas C, Larrivee B, Eichmann A (2008) Netrins and UNC5 receptors in angiogenesis. Angiogenesis 11:23-29.
Gibson EL (2006) Emotional influences on food choice: sensory, physiological and psychological pathways. Physiol Behav 89:53-61.
Gilmartin MR, Balderston NL, Helmstetter FJ (2014) Prefrontal cortical regulation of fear learning. Trends Neurosci 37:455-464.
Gomez-Pinilla F (2008) Brain foods: the effects of nutrients on brain function. Nat Rev Neurosci 9:568-578.
Grieve A, Butcher SP, Griffiths R (1992) Synaptosomal plasma membrane transport of excitatory sulphur amino acid transmitter candidates: kinetic characterisation and analysis of carrier specificity. J Neurosci Res 32:60-68.
Griffiths R, Grieve A, Allen S, Olverman HJ (1992) Neuronal and glial plasma membrane carrier-mediated uptake of L-homocysteate is not selectively blocked by beta-p-chlorophenylglutamate. Neurosci Lett 147:175-178.
Guo JU, Ma DK, Mo H, Ball MP, Jang MH, Bonaguidi MA, Balazer JA, Eaves HL, Xie B, Ford E, Zhang K, Ming GL, Gao Y, Song H (2011) Neuronal activity modifies the DNA methylation landscape in the adult brain. Nat Neurosci 14:1345-1351.
Horn KE, Glasgow SD, Gobert D, Bull SJ, Luk T, Girgis J, Tremblay ME, McEachern D, Bouchard JF, Haber M, Hamel E, Krimpenfort P, Murai KK, Berns A, Doucet G, Chapman CA, Ruthazer ES, Kennedy TE (2013) DCC expression by neurons regulates synaptic plasticity in the adult brain. Cell Rep 3:173-185.
Kalani A, Kamat PK, Tyagi N (2015) Diabetic stroke severity: epigenetic remodeling and neuronal, glial, and vascular dysfunction. Diabetes 64:4260-4271.

Kalani A, Kamat PK, Tyagi SC, Tyagi N (2013) Synergy of homocysteine, microRNA, and epigenetics: a novel therapeutic approach for stroke. Mol Neurobiol 48:157-168.

Kalani A, Kamat PK, Givvimani S, Brown K, Metreveli N, Tyagi SC, Tyagi N (2014a) Nutri-epigenetics ameliorates blood-brain barrier damage and neurodegeneration in hyperhomocysteinemia: role of folic acid. J Mol Neurosci 52:202-215.

Kalani A, Kamat PK, Familtseva A, Chaturvedi P, Muradashvili N, Narayanan N, Tyagi SC, Tyagi N (2014b) Role of microRNA29b in blood-brain barrier dysfunction during hyperhomocysteinemia: an epigenetic mechanism. J Cereb Blood Flow Metab 34:1212-1222.

Kamat PK, Kyles P, Kalani A, Tyagi N (2016) Hydrogen sulfide ameliorates homocysteine-induced Alzheimer's disease-like pathology, blood-brain barrier disruption, and synaptic disorder. Mol Neurobiol 53:2451-2467.

Kamat PK, Kalani A, Givvimani S, Sathnur PB, Tyagi SC, Tyagi N (2013) Hydrogen sulfide attenuates neurodegeneration and neurovascular dysfunction induced by intracerebral-administered homocysteine in mice. Neuroscience 252:302-319.

Kamath AF, Chauhan AK, Kisucka J, Dole VS, Loscalzo J, Handy DE, Wagner DD (2006) Elevated levels of homocysteine compromise blood-brain barrier integrity in mice. Blood 107:591-593.

Khare T, Pai S, Koncevicius K, Pal M, Kriukiene E, Liutkeviciute Z, Irimia M, Jia P, Ptak C, Xia M, Tice R, Tochigi M, Moréra S, Nazarians A, Belsham D, Wong AH, Blencowe BJ, Wang SC, Kapranov P, Kustra R, et al. (2012) 5-hmC in the brain is abundant in synaptic genes and shows differences at the exon-intron boundary. Nat Struct Mol Biol 19:1037-1043.

Krypuy M, Wojdacz TK, Dobrovic A (2008) Rapid High-Throughput Methylation Analysis Using the LightCycler® 480 System. Biochemica 1:11-13.

Li LC, Dahiya R (2002) MethPrimer: designing primers for methylation PCRs. Bioinformatics 18:1427-1431.

Li Q, Yao D, Ma J, Zhu J, Xu X, Ren Y, Ding X, Mao X (2011) Transplantation of MSCs in combination with netrin-1 improves neoangiogenesis in a rat model of hind limb ischemia. J Surg Res 166:162-169.

Lockett GA, Wilkes F, Maleszka R (2010) Brain plasticity, memory and neurological disorders: an epigenetic perspective. Neuroreport 21:909-913.

Ly NP, Komatsuzaki K, Fraser IP, Tseng AA, Prodhan P, Moore KJ, Kinane TB (2005) Netrin-1 inhibits leukocyte migration in vitro and in vivo. Proc Natl Acad Sci U S A 102:14729-14734.

Mansego ML, Milagro FI, Zulet MA, Martinez JA (2015) SH2B1 CpG-SNP is associated with body weight reduction in obese subjects following a dietary restriction program. Ann Nutr Metab 66:1-9.

Maren S, Phan KL, Liberzon I (2013) The contextual brain: implications for fear conditioning, extinction and psychopathology. Nat Rev Neurosci 14:417-428.

Masliah E, Dumaop W, Galasko D, Desplats P (2013) Distinctive patterns of DNA methylation associated with Parkinson disease: identification of concordant epigenetic changes in brain and peripheral blood leukocytes. Epigenetics 8:1030-1038.

Mehlen P, Rama N (2007) Netrin-1 and axonal guidance: signaling and asymmetrical translation. Med Sci (Paris) 23:311-315.

Melnikov AA, Gartenhaus RB, Levenson AS, Motchoulskaia NA, Levenson Chernokhvostov VV (2005) MSRE-PCR for analysis of gene-specific DNA methylation. Nucleic Acids Res 33:e93.

Miller CA, Sweatt JD (2007) Covalent modification of DNA regulates memory formation. Neuron 53:857-869.

Niculescu MD, Zeisel SH (2002) Diet, methyl donors and DNA methylation: interactions between dietary folate, methionine and choline. J Nutr 132:2333S-2335S.

O'Leary CJ, Bradford D, Chen M, White A, Blackmore DG, Cooper HM (2015) The Netrin/RGM receptor, Neogenin, controls adult neurogenesis by promoting neuroblast migration and cell cycle exit. Stem Cells 33:503-514.

Preston AR, Eichenbaum H (2013) Interplay of hippocampus and prefrontal cortex in memory. Curr Biol 23:R764-773.

Qureshi IA, Mehler MF (2010) Emerging role of epigenetics in stroke: part 1: DNA methylation and chromatin modifications. Arch Neurol 67:1316-1322.

Reeves WB, Kwon O, Ramesh G (2008) Netrin-1 and kidney injury. II. Netrin-1 is an early biomarker of acute kidney injury. Am J Physiol Renal Physiol 294:F731-738.

Smyth A, Dehghan M, O'Donnell M, Anderson C, Teo K, Gao P, Sleight P, Dagenais G, Probstfield JL, Mente A, Yusuf S, Ontarget, Investigators T (2015) Healthy eating and reduced risk of cognitive decline: A cohort from 40 countries. Neurology 84:2258-2265.

Sorensen LB, Damsgaard CT, Dalskov SM, Petersen RA, Egelund N, Dyssegaard CB, Stark KD, Andersen R, Tetens I, Astrup A, Michaelsen KF, Lauritzen L (2015) Diet-induced changes in iron and n-3 fatty acid status and associations with cognitive performance in 8-11-year-old Danish children: secondary analyses of the Optimal Well-Being, Development and Health for Danish Children through a Healthy New Nordic Diet School Meal Study. Br J Nutr 114:1623-1637.

Sudduth TL, Powell DK, Smith CD, Greenstein A, Wilcock DM (2013) Induction of hyperhomocysteinemia models vascular dementia by induction of cerebral microhemorrhages and neuroinflammation. J Cereb Blood Flow Metab 33:708-715.

Sudduth TL, Weekman EM, Brothers HM, Braun K, Wilcock DM (2014) beta-amyloid deposition is shifted to the vasculature and memory impairment is exacerbated when hyperhomocysteinemia is induced in APP/PS1 transgenic mice. Alzheimers Res Ther 6:32.

Sun H, Le T, Chang TT, Habib A, Wu S, Shen F, Young WL, Su H, Liu J (2011) AAV-mediated netrin-1 overexpression increases peri-infarct blood vessel density and improves motor function recovery after experimental stroke. Neurobiol Dis 44:73-83.

Tian XF, Xia XB, Xiong SQ, Jiang J, Liu D, Liu JL (2011) Netrin-1 overexpression in oxygen-induced retinopathy correlates with breakdown of the blood-retina barrier and retinal neovascularization. Ophthalmologica 226:37-44.

Wang W, Reeves WB, Ramesh G (2008) Netrin-1 and kidney injury. I. Netrin-1 protects against ischemia-reperfusion injury of the kidney. Am J Physiol Renal Physiol 294:F739-747.

Waterland RA (2006) Assessing the effects of high methionine intake on DNA methylation. J Nutr 136:1706S-1710S.

Zhang J, Cai H (2010) Netrin-1 prevents ischemia/reperfusion-induced myocardial infarction via a DCC/ERK1/2/eNOS s1177/NO/DCC feed-forward mechanism. J Mol Cell Cardiol 48:1060-1070.

Zhuo JM, Portugal GS, Kruger WD, Wang H, Gould TJ, Pratico D (2010) Diet-induced hyperhomocysteinemia increases amyloid-beta formation and deposition in a mouse model of Alzheimer's disease. Curr Alzheimer Res 7:140-149.

P-Reviewer: Ramezani M; C-Editors: Zhao M, Li CH; T-Editor: Liu XL