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L-methionine enhances neuroinflammation and impairs neurogenesis: Implication for Alzheimer's disease

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ABSTRACT

The disruption of methionine (L-MET) metabolism has been linked with neurodevelopmental disorders such as autism and schizophrenia and neurodegenerative disorders such as Alzheimer's disorder. We previously showed that repeated administration to adult mice of methionine produced impairments of cognitive deficits. Considering the decreased neurogenesis and increased molecular inflammation hypotheses of cognitive deficits in Alzheimer's, we aimed to explore whether the methionine regimen that produced cognitive deficits is associated with altered neuroinflammation, neurogenesis, or neurodegeneration. We found that repeated administration of L-MET at a dose equivalent to two-fold of daily dietary intake for seven days enhanced the activation of microglia and inflammation in the brain, and decreased neurogenesis in the hippocampus without affecting degeneration. Furthermore, sub-chronic and chronic L-MET treatment of human neuroblastoma (SH-SY5Y) inhibited cell cycle progression, an effect that was reversed by decreasing removing L-MET from the medium. These results support a role for neuroinflammation and neurogenesis in mediating the mechanism through which L-MET induces cognitive deficits. The results also uncover L-MET restriction, neuroinflammation, and neurogenesis as potential preventive and/or therapeutic targets for mental disorders associated with cognitive disorders, including schizophrenia and Alzheimer's disease.

1. Introduction

L-Methionine (L-MET) is an essential amino acid that is a key component of one-carbon (C1) metabolism. It is required for the production of S-adenosyl methionine (SAM), the primary methyl donor in the body used in almost all methylation reactions, in which substrates can be ribonucleic acids, proteins, carbohydrates, phospholipids, and neurotransmitters (Loenen, 2006; Sharma et al., 1999; Trivedi and Deth, 2012; Zhao et al., 2001). Methylation is a universal reaction that plays a critical role in numerous biological processes and metabolic pathways involved in cell proliferation, differentiation, survival, and other cellular functions (Chen et al., 2013; Horvath et al., 2012; Kim et al., 2014a; Roidl and Hacker, 2014; Wu et al., 2010).

L-MET is also a precursor of homocysteine, a neurotoxic sulfur amino acid intermediate in the methylation, and a precursor of glutathione, a tripeptide protective antioxidant agent [12].

Disruption of the finely tuned metabolism of methionine has been associated with neurological and psychiatric disorders. For example, altered levels of L-MET and other C1 cycle metabolites have been reported in neurodevelopmental disorders such as autism and schizophrenia patients (Indika et al., 2021; Main et al., 2010; Onozato et al., 2020; Pasca et al., 2009; Puig-Alcaraz et al., 2015; Smythies et al., 1986; Strous et al., 2009; Tisato et al., 2021; Waligora et al., 2019; Zhu et al., 2021), as well as age-related neurodegenerative disorders such as Alzheimer's disease (AD) and vascular dementia (Deng et al., 2019; Fayez et al., 2019; Hooshmand et al., 2019; Hu et al., 2016; Koladiya et al.,

Abbreviations: L-MET, L-methionine; SAM, S-adenosyl methionine; SAL, Saline.

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2008; McCampbell et al., 2011; Pi et al., 2021; Ringman et al., 2012; Tapia-Rojas et al., 2015; Velazquez et al., 2020; Villa et al., 2009; Wang et al., 2021; Zuin et al., 2021). We previously showed that repeated administration to adult mice of methionine equivalent to double daily dietary intake produced various cognitive deficits that mimic those seen in schizophrenia and Alzheimer's disease, including impairments of working memory, object recognition, spatial memory, and contextual memory (Wang et al., 2015). However, the molecular mechanisms of L-MET in inducing these cognitive deficits were not investigated. Previous studies showed that a high-methionine diet in mice caused an increase in Aβ1–40 and Aβ1–42 levels as well as serum homocysteine, alongside alterations in the levels of 5-mC and AD-like cognitive deficits (Pi et al., 2021). High methionine was also found to increase neuronal degeneration and vascular dysfunction and impair short-term memory (Kalani et al., 2019; Nuru et al., 2018).

Considering the decreased neurogenesis and increased molecular inflammation hypotheses of cognitive deficits in Alzheimer's, it is tempting to explore whether neurogenesis and inflammation mechanisms mediate the cognitive-producing effects of methionine. Neuroinflammation, including microglial inflammation, may thus play an early causative role in modifying the stem cell niches for neurogenesis. Indeed, methionine restriction was proposed to delay aging through suppressing inflammation mechanisms.

The association between methionine and immuno-inflammatory activation was supported by that methionine restriction in a mouse model of multiple sclerosis reduces the ability of T cells to cause inflammation in the brain and spinal cord that delayed disease onset and slowed progression (Agbas and Moskovitz, 2009; Grimble, 2006), and that methionine affects the epigenetic reprogramming in CD4 T cells (Roy et al., 2020). Further, methionine restriction reduced histone H3K4 methylation that affected the promoters of genes responsible for the differentiation of T cells towards inflammatory Th17 cells. However, most studies have determined the effect of methionine metabolism on the functions of T cells, and less is known about its effect on innate immune cells that include microglia in the brain.

Since DNA methylation is a critical epigenetic factor that modifies the expression of genes involved in vital neural functions such as neurogenesis and differentiation (Boku et al., 2014; Kim et al., 2014b), L-MET levels fluctuations may affect the neurogenesis process. Indeed, our previous work showed that neurogenesis in the hippocampus was reduced in mice prenatally exposed to excessive L-MET (Alachkar et al., 2018). Recent evidence indicates that high levels of methionine may also affect neurogenesis due to DNA hypermethylation.

While the roles of high methionine diets in cognitive deficits, neurogenesis, and neuroinflammation have been discretely studied, it is not known whether the same methionine levels that produce cognitive deficits can alter neurogenesis and neuroinflammation. Therefore, the goal of the present study was to determine whether the repeated administration of methionine equivalent to twofold of daily dietary intake, which we demonstrated to produce cognitive deficits, is associated with altered neuroinflammation, neurogenesis, or neurodegeneration.

2. Materials and methods

2.1. Animals and drug administration

Male Swiss Webster mice, ages 8–11 weeks, were used. Mice were group-housed and maintained on a 12-h light/dark cycle (light on at 7:00 am) with food and water available ad libitum. L-methionine (L-MET, Sigma-Aldrich) was dissolved in saline. L-MET (750 mg/kg, 15 mg/kg, i.p.) was administered twice a day (9:00 am/3:00 pm) for consecutive seven days.

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine, and were performed in compliance with national and institutional guidelines for the care and use of laboratory animals.

2.2. Immune/inflammatory assays in L-MET treated brain

2.2.1. Flow cytometry of brain cells

Brains collected after L-MET treatment were treated with collagen for 1 h at 37 °C, homogenized, and myelin was removed via Percoll density gradient (25%) as described (Posel et al., 2016). The cells collected were then stained with viability dye 510 (BD Biosciences, San Jose, CA) to distinguish live from dead cells. Then the cells were stained with CD45, CD11b, and HLADR to identify microglia and acquired on BD FACS Celesta. The flow cytometry results were analyzed using FlowJo™ v10.8 Software (BD Life Sciences). Live cells were gated on CD45 to identify hematopoietic cells. Subsequently, CD45 gated cells were analyzed for the presence $CD11b^{+}$ cells to identify microglia (Fig. 1A). The expression of HLADR was determined on gated $CD11b^{+}$ cells.

2.2.2. Brain cytokine assay

We have established an assay to determine levels of inflammatory mediators in the brain (Markarian et al., 2021). Freshly dissected hemibrains (6 brains/group) were flash-frozen in liquid nitrogen. Subsequently, they were pulverized on dry ice and solubilized in PBS containing protease inhibitor cocktail. After centrifugation, the supernatants were collected and assayed for cytokines using a Magnetic Bead-Based Customized Kit (ThermoFisher Scientific). The levels of the mediators (IL-1β, IL-17, IL-21, IL-1α, IL-6, CCL-5, TNF-α, CCL-2, IL-18) were determined and normalized to the amount of the protein in the lysate.

2.2.3. Brain qPCR

RNA was extracted from the brains of 6 L-MET and 6 saline treated mice using an RNA easy kit (Zymo Research, Irvine). After conversion to cDNA (ThermoFisher Scientific), qPCR was performed using specific primers for mouse NLRP3 and beta-actin.

2.3. Neurogenesis and neuronal survival

2.3.1. BrdU labeling

Using BrdU, a thymidine analog, which is incorporated into the cell during DNA synthesis, we studied the effect of L-MET on the proliferation and survival of newborn cells in the dentate gyrus of the hippocampus. 2.5-Bromodeoxyuridine (BrdU) (75 mg/kg, i.p.) was administered to saline- and L-MET-treated mice following two protocols. Protocol 1 was designed to investigate the effect of L-MET on neuronal survival. Mice received BrdU injections three times (every 4h) at day 0 (one day prior to the first injection of L-MET) and were killed 24 h after the last injection of L-MET/SAL. In protocol 2, the effect of L-MET treatment on neurogenesis was assessed. Mice received a single dose of BrdU (75 mg/kg, i.p.) 2 h after the last injection of L-MET/Sal on day 7 and were sacrificed 24 h after the BrdU injection.

2.3.2. Immunohistochemistry

Eight mice (4 treated with saline and 4 treated with L-MET) were deeply anesthetized with isoflurane and perfused intracardially with ice cold 40 ml saline, followed by 50 ml of 4% paraformaldehyde in phosphate buffer (PBS). Brains were removed, post-fixed in 4% paraformaldehyde overnight at 4 °C. Twenty μm coronal sections were cut at the level of the Dentate Gyrus of the hippocampus. Sections were blocked with 4% normal goat serum in PBS with 0.3% Triton X-100 for 60 min, then incubated with mouse anti-BrdU (1:500, ThermoFisher) for 24 h at room temperature. After three wash steps with PBS, sections were incubated with goat Alexa Fluor 568 anti-mouse (1:500, Life technologies) and DAPI for 1 h at room temperature. Sections were then washed with PBS three times and mounted with an Anti-fade solution. After staining, slides were analyzed using Zeiss microscope (Carl Zeiss,

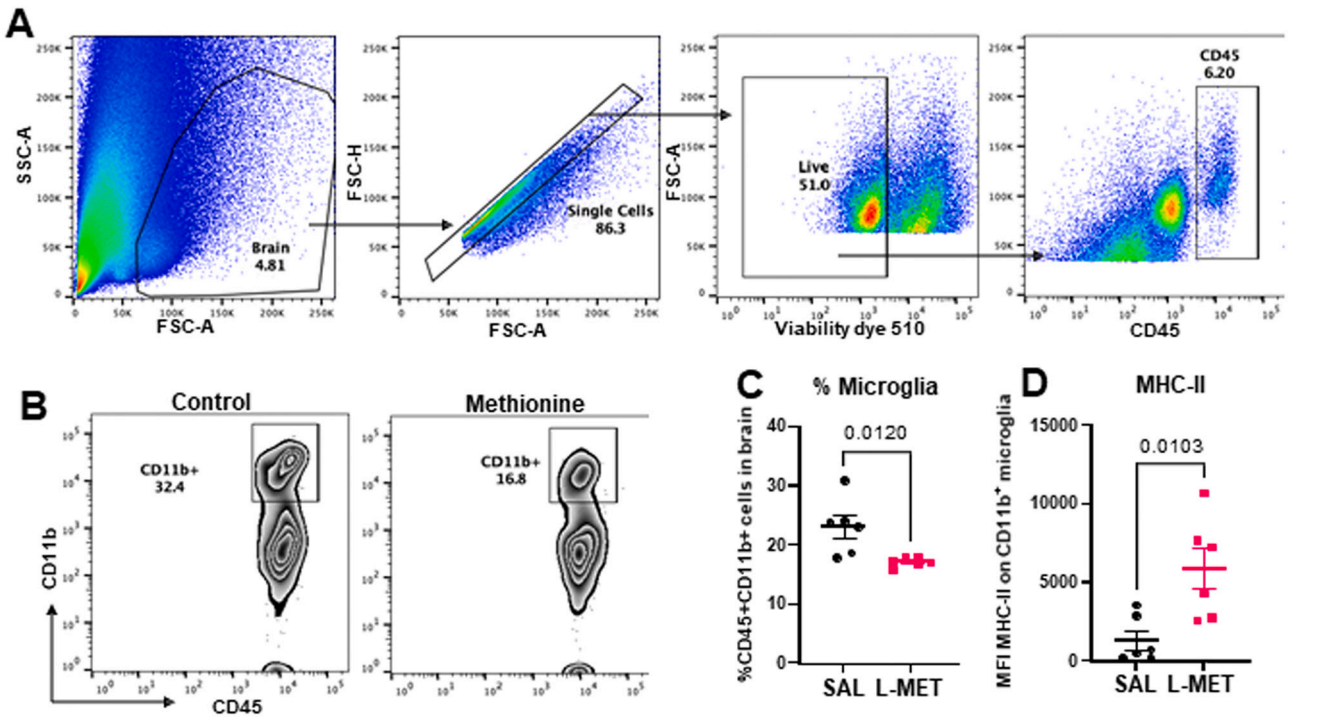

Fig. 1. L-MET affects numbers and activation of microglia in the brain. (A) Gating strategy for CD45+ immune cells in the brain. (B) Representative zebra plots of CD11b + microglia percentage in the brains of saline and L-MET treated mice. (C) Quantification of the same. (D) Mean fluorescence intensity (MFI) of MHC-II expression on CD11b gated microglia.

USA), and Zeiss LSM image browser software (Carl Zeiss, USA) was used for image acquisition and analysis. The number of BrdU positive cells was counted on three sections from each mouse.

2.4. Treatment of human neuroblastoma SH-SY5Y with L-MET

2.4.1. Cell culture

SH-SY5Y human neuroblastoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (50 U/ml), streptomycin (50 μg/ml), and maintained in a humidified, 5% CO2, 37 °C incubator.

2.4.2. MTT viability assay

Cell viability was evaluated as described previously (Datki et al., 2003), by measuring the reducing capacity of intracellular dehydrogenases through quantifying MTT reduction to the insoluble formazan.SH-SY5Y cells were cultured onto a 96-well plate at 20.000 cells/well. Cells were allowed to grow in DMEM-10%FBS for 24 h. Then, the culture medium was replaced with fresh DMEM-10%FBS, containing L-MET 5 mg/ml, and incubated for 24, 72, 168 h. MTT assay was performed as previously described (reference). The medium was removed, and cells were incubated with 100ul DMEM-10%FBS and 10 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 12 mM for 3 h at 37 °C, and the formazan crystals were dissolved in 100 μL of the SDS 10% in 0.01 N HCl. Absorbance was measured using a microplate reader (VERSAmax, Molecular Devices) at 570 nm. The data are expressed as a percentage of the absorbance in the control wells in which cells were incubated with medium only.

2.4.3. Neurite outgrowth

Immunofluorescent staining was used to analyze the expression of the neuronal marker NF-H. SH-SY5Y cells at 20.000 cells/well were cultured onto a 24-well plate. Cells were incubated with DMEM or L-MET (5 mg/ml) for various periods: 7 days incubation with DMEM; 4 days incubation with DMEM followed by 3 days incubation with L-MET; group 3: 2 days incubation with DMEM followed by incubation with L-MET for 5 days; group 4: 7 days incubation with L-MET. After 7 days, cells were washed with PBS, and fixed for 10 min in 4% paraformaldehyde at 4 °C. Cells were then blocked with 4% normal donkey serum in PBS with 0.1% Triton X-100 for 60 min, then incubated with chicken anti-NF-H (1:500, Aveslabs) for 24 h at 4 °C. After washing, cells were incubated with Alexa488 labeled anti-chicken and DAPI for 1 h at room temperature, followed by washing.

Cell morphology and the ratio of neurite extension to cell area were analyzed using Zeiss microscope (Carl Zeiss, USA) and ImageJ software (NIH, Bethesda, MD). Results are expressed as mean ± SEM of the values of 6 images from 3 cell cultures.

2.4.4. BrdU labeling in SY5Y

BrdU immunofluorescent staining was used to analyze the effect of L-MET treatment on the proliferation rate of SY5Y cells. SH-SY5Y cells at 20.000 cells/well were cultured onto a 24-well plate. Cells were incubated with DMEM or L-MET (5 mg/ml) for various periods: 7 days incubation with DMEM; 4 days incubation with DMEM followed by 3 days incubation with L-MET; group 3: 2 days incubation with DMEM followed by incubation with L-MET for 5 days; group 4: 7 days incubation with L-MET; group 5: 3 days incubation with L-MET followed by incubation with DMEM for 4 days. On day 7, cells were washed with PBS, incubated with BrdU in DMEM (10 μM) for four hours. Cells were then washed with PBS and fixed for 10 min in 4% paraformaldehyde at 4 °C. Cells were then blocked with 4% normal donkey serum in PBS with 0.1% Triton X-100 for 60 min, then incubated with mouse anti-BrdU antibody (1:500, ThermoFisher), and anti-chicken anti-NF-H antibody (1:500, Aveslabs) for 24 h at 4 °C. After washing, cells were incubated with goat Alexa Fluor 568 anti-mouse and Alexa488 labeled anti-chicken (1:500, Life technologies) and DAPI for 1 h at room temperature, followed by washing.

2.5. Statistical analysis

GraphPad Prism (GraphPad Software, Inc.) was used for statistical analysis. Data are presented as means ± S.E.M. Results were analyzed by *t*-test or ANOVA followed by the appropriate post hoc comparisons, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Microglia numbers and phenotype in the brain

High methionine levels are associated with neurological diseases such as AD (Pi et al., 2021; Tapia-Rojas et al., 2015). Still, its effect on microglia, the major innate immune cell type that is a significant perpetuator of AD pathogenesis, is unknown. Mice were injected for seven days with either saline or L-MET, and flow cytometry was used to assay the effect of L-MET treatment on the numbers and phenotype of microglia in the brain. Briefly, live brain cells were gated on CD45+ cells (Fig. 1A). CD45+ cells were further gated on CD11b + cells to identify microglia (Fig. 1B). L-MET reduced the percentages of microglia in the brain (Figs. 1B & C; $p < 0.05$; *t*-test) compared to controls. Next, we determined the effect of L-MET on microglia activation by assessing the expression of MHC-II on these cells. L-MET treatment led to significant activation ($p < 0.05$; t-test) of microglia as compared to saline treated mice (Fig. 1D).

3.2. The assay of inflammatory mediators in the brain

Increased activation of microglia suggests enhanced inflammation. Therefore, we assayed various cytokines and chemokines levels in the brain using multiplex. The levels of inflammatory cytokines, IL-1β, TNF-α, IL-18, IL-33 were significantly increased ($p < 0.05$; t-test) in the brains of L-MET treated mice compared to saline controls (Fig. 2A). In contrast to these cytokines, the level of CCL5 was significantly reduced in the L-MET group. The levels of other mediators, including IL-1α, CCL-2, IL-10, IL-6 were comparable between the two groups.

Interestingly, IL-1β, IL-18, and IL-33 are all inflammasome activation-related cytokines. NLRP3 is the major inflammasome found in the brain. Therefore, we determined whether L-MET has an effect on its expression using qPCR. As is evident from Fig. 2B, L-MET does not enhance the expression of NLRP3, indicating that the increased inflammasome-related cytokines are a consequence of enhanced activation of the receptor. Altogether, these data indicate an increase in inflammatory responses in the brain of L-MET treated mice.

3.3. Neurogenesis and cell survival

Neuroinflammation has been shown to affect neurogenesis. Therefore, BrdU-positive cells $BrdU^+$ were counted in the dentate gyrus in the brains of saline- and L-MET treated mice. In the first experiment, we investigated the effect of L-MET on neuronal survival. We found that the mean $BrdU^+$ cells number in L-MET-treated mice was not significantly different from that in the control group, 26.8 ± 1.9 cells in the L-MET treated group compared with 28.4 ± 1.8 cells in the saline treated group ($P > 0.05$ *t*-test, Fig. 3B,C). In the second experiment, we assessed the effect of L-MET on neurogenesis. There was a significant decrease in the $BrdU^+$ cells in the L-MET-treated mice 9.7 ± 1.2 compared with the saline group (23.9 ± 1.8) ($P < 0.001$, t-test, Fig. 3D,E).

3.4. L-MET alters cell proliferation, neuritogenesis, and cell morphology in SH-SY5Y

MTT test was used to assess the effect of L-MET on cell growth in SH-SY5Y. L-MET reduced cell growth, and this reduction increased with the L-MET treatment time. The cell viability decreased to 80 ± 4%, 71 ± 6, 46 ± 3 in cells treated with L-MET for periods of 1, 3, 7 days respectively, $P < 0.01$, 0.01, 0.001, two-way ANOVA followed by Bonferroni post-test (Fig. 4A,B). To determine whether the L-MET-induced decrease in cell growth is caused by increased cell death or decreased proliferation, we quantified the number of BrdU-positive cells treated for different times with L-MET. While L-MET treatment for 3, 5, and 7 days did not cause cellular death, it inhibited the proliferation of SY5Y cells measured by the percentage of DAPI positive cells that were BrdU positive on day 7 post-treatment ($F_{(4, 65)} = 8.972$, one-way ANOVA, Fig. 4C,

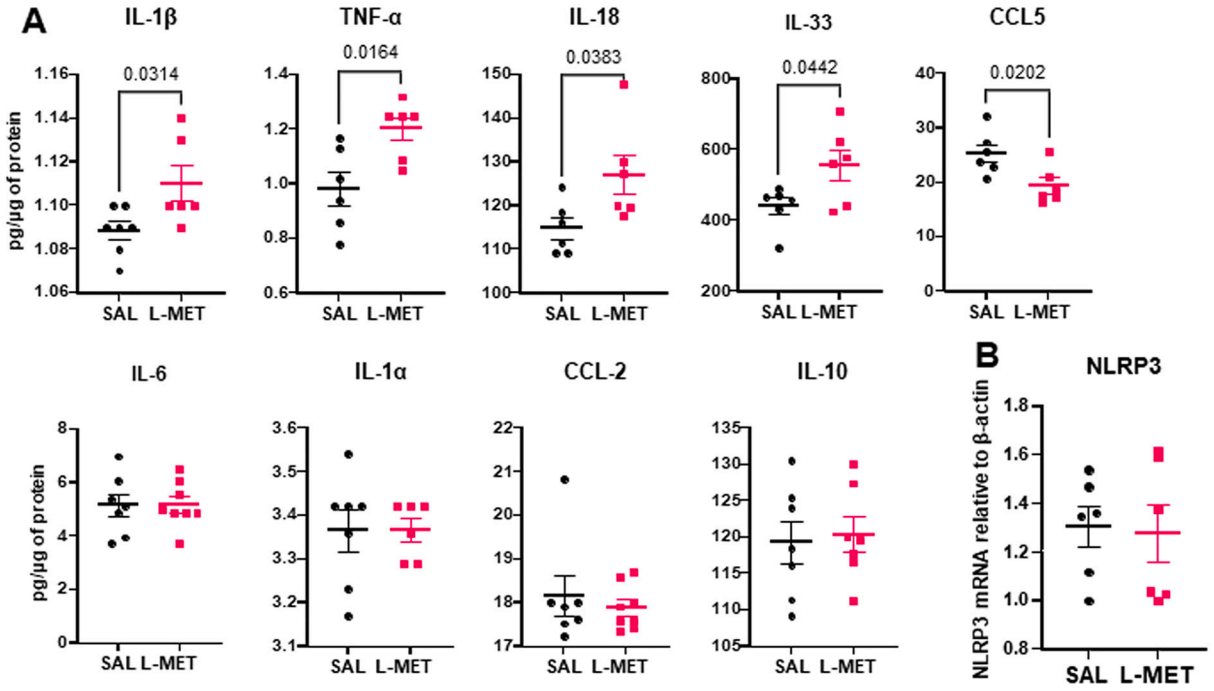

Fig. 2. L-MET enhances inflammatory cytokine production in the brain. (A) Dot plots depict the levels of cytokines and chemokines in the brains of saline and L-MET treated mice. The levels are represented as pg/ug/of brain protein. (B) Gene expression of NLRP3 relative β-actin assayed by qPCR.

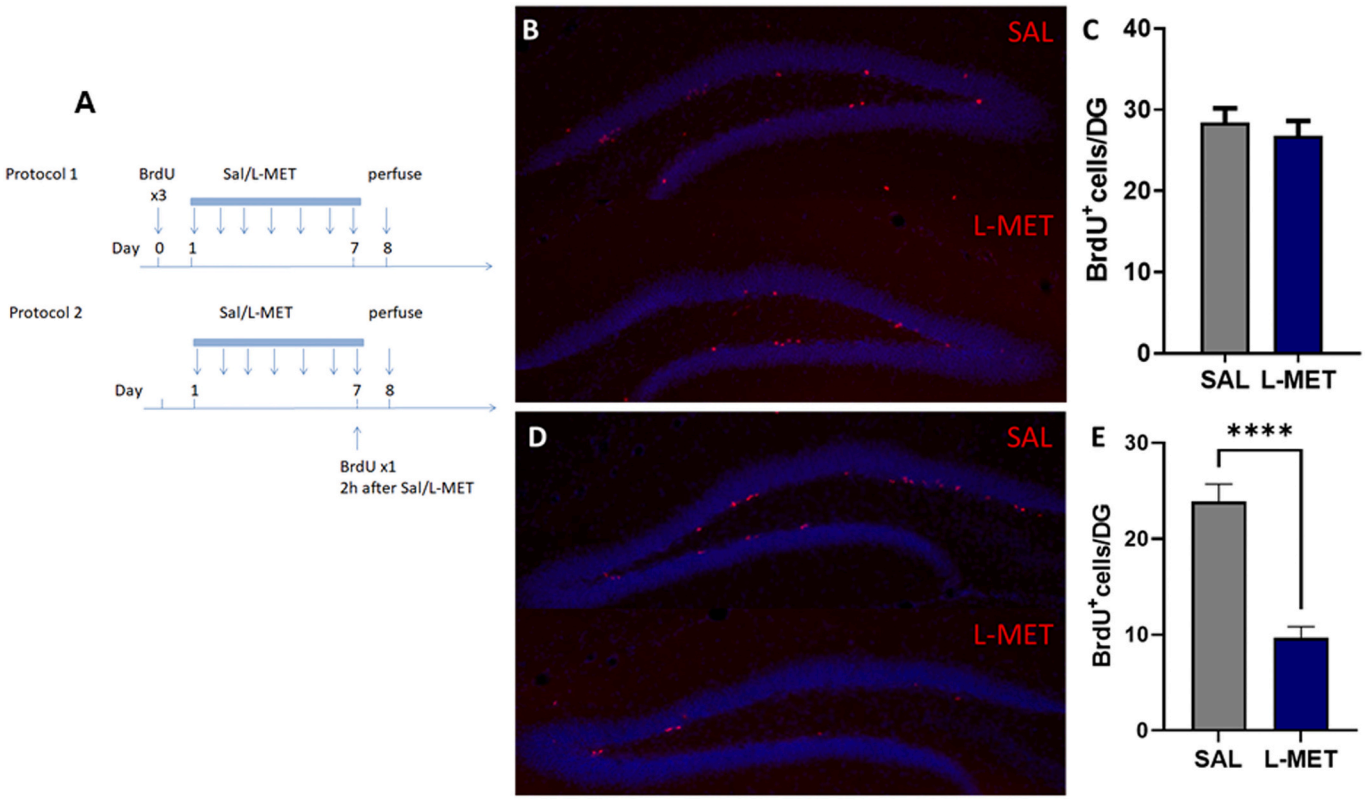

Fig. 3. L-MET decreases Neurogenesis (A, B) but not survival (C, D) of neurons in the dentate gyrus (DG). (A) Schematic of the two experimental designs. (B, C) Representative immunofluorescent images (B) and quantification of BrdU positive cells (red) (C) in the DG from mice treated with SAL/L-MET for 7 days and injected with BrdU on day 7, 24 h before animal were sacrificed (nuclei are stained with DAPI (blue)). (D, E) Representative immunofluorescent images (D) and quantification of BrdU positive cells (E) in the DG from mice treated with SAL/L-MET for 7 days of SAL/L-MET administration and injected with BrdU on day 0 of SAL/L-MET administration and sacrificed 8 days after BrdU injection. ****$P < 0.0001$ determined by Unpaired test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

D). The anti-proliferative effect of L-MET was reversed by its removal after three days and replacing it with DMED for 4 more days; $BrdU^+/DAPI^+$ ratio was similar to that in the control group ($P > 0.05$, one-way ANOVA followed by Tukey's post-test).

SH-SY5Y cells morphology examined by immunofluorescence showed that NF-H was present in control as well as L-MET-treated cells. However, the morphology of cells treated with L-MET resembled primary neurons more than control cells in terms of neurite processing and lengths. Also, the ratio of neurite extension to cell area was higher in L-MET treated cells, and this ratio increased with the treatment time (the intensity were 0.95 ± 0.08, 1.2 ± 0.07, 1.3 ± 0.1 in cells incubated with L-MET for 3, 5, 7 days out of 7 days respectively compared with 0.7 ± 0.04 (Fig. 5A,B).

4. Discussion

In the present study, we demonstrated that chronic L-MET treatment enhances the activation of microglia and inflammation in the brain and decreases neurogenesis in the hippocampus without affecting cell survival. Furthermore, sub-chronic and chronic methionine treatment inhibited cell cycle progression in human neuroblastoma (SH-SY5Y), an effect that was reversed by decreasing removing L-MET from the medium.

High methionine diet is associated with increased inflammation that is characteristic of neurodegenerative diseases like AD (Heneka et al., 2015; Pi et al., 2021; Tapia-Rojas et al., 2015). Nevertheless, the effect of L-MET on microglia has not been studied. We find that L-MET equivalent to double daily dietary intake decreases microglial numbers. One explanation could be activation induced cell death. Several reports indicate that activated microglia undergo cell death to limit immune and inflammatory responses involving them (Lee et al., 2001; Mayo et al., 2008). Initially, microglia become activated in response to injury or insult to the brain to protect neurons. However, prolonged activation is detrimental since activated microglia produce inflammatory cytokines and ROS that have been shown to accelerate the cognitive decline and pathology of dementia. In keeping with increased microglial activation, we also observed an enhancement in the levels of proinflammatory cytokines in the brain after L-MET treatment. IL-1β, IL18, and IL-33 are all products of inflammasome activation. Inflammasomes are innate immune sensors belonging to the family of NOD-like receptors (NLRs) present in many cells (Heneka et al., 2018). Rather than inducing transcriptional signaling, inflammasomes control post-translation events after cytosolic receptor triggering. The inflammasomes are large, multi-molecular signaling complexes that cleave caspase-1 to convert it from an inactive to an active form. Activated caspase-1 converts pro-forms of proinflammatory cytokines like IL-1β, IL-18, IL-33, etc., to secretory forms that are then released from the cells to act on other cells. In addition, activation of caspase-1 also leads to cell death via pyroptosis (Yu et al., 2021). There are several different types of inflammasomes. However, NLRP3 inflammasome is the most studied in the brain and is located predominantly in the microglia (Heneka et al., 2018). High methionine diet has been associated with increased NLRP3 activation in macrophages in a mouse model of atherosclerosis (Wang et al., 2017). Though we observed increased levels of inflammasome-associated cytokines, the expression of the NLRP3 gene was comparable between L-MET and saline groups, indicating increased activation of the inflammasome rather than induction of the gene. Our data suggest that L-MET induces neuronal inflammation that is often accompanied by cell death.

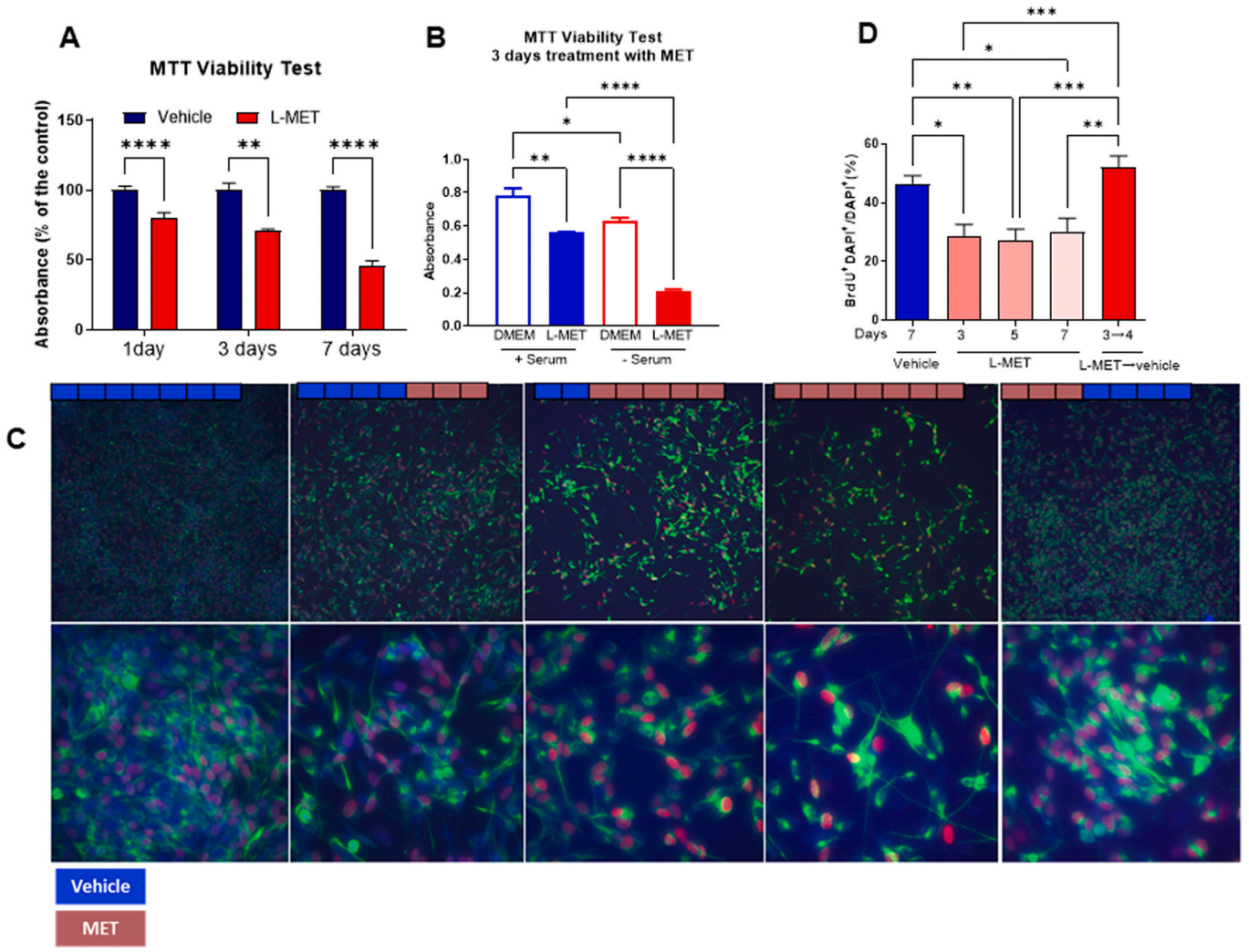

Fig. 4. L-MET treatment of SH-SY5Y causes reduction in reduction in cell proliferation, which is reversed by removal of L-MET (A) MTT viability test, results are expressed as a percentage of the absorbance in the control cell incubated with medium. (B) MTT viability test on day 3 in serum free conditions, results are expressed as a percentage of the absorbance in the control cell incubated with medium. (C) Representative immunofluorescent image of SH-SY5Y treated for different time with L-MET and incubated with BrdU for 4 h on the day 7. BrdU (red), NF-H (green), and DAPI (blue), (D) Ratio of BrdU+/DAPI+, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

One example is the reduced numbers of microglia in our experiments. Nucleic acids from dying cells may be acting as a trigger for the infiammasome in L-MET mice, while this is not the case for control mice. It has been shown that following traumatic brain injury, dying cells activate microglia (Kabba et al., 2018; Lee et al., 2019).

NLRP3 activation in microglia is observed in several neurodegenerative disorders, including AD and Parkinson's disease, and is associated with increased inflammation and disease pathogenesis(Heneka et al., 2018). NLRP3 and the cytokines produced due to its activation have also been reported to have a profound effect on neurogenesis. The activation of NLRP3 inflammasome was reported to aggravate PSCI by enhancing activation of microglia and impairment of neurogenesis in the hippocampus (Li et al., 2020). The cytokines IL1β, IL-18, and IL-33 all have important functions in the brain(Borsini et al., 2015). The cognate receptors for IL-1β and IL-18 are expressed by many brain cell types. These receptors initiate signaling cascades that can contribute to neuronal injury and death. For instance, activated microglia are considered the major source of IL-18. IL-18 produced acts in an autocrine manner to induce the production of inflammatory cytokines TNF-α and IL-1β from microglia (Felderhoff-Mueser et al., 2005). Furthermore, IL-18 also induces the expression of FAS ligand (Fas-L) on microglia, oligodendrocytes, and astrocytes which enhances Fas-mediated neuronal apoptosis during inflammation (Bossaller et al., 2012). Increased IL-18 production due to a high methionine diet may therefore be responsible for the observed reduction in microglia numbers in the brain (Fig. 1C). Similar to IL-18, several studies indicate that IL-1β is also a major player in the loss of neurogenesis and the depression-like behavior caused by stress (Koo and Duman, 2008; Koo et al., 2010). Koo et al. demonstrated that blocking IL-1β reverses stress-induced impairment of neurogenesis and behavioral deficits (Koo and Duman, 2009). In contrast to IL-1β and IL-18, IL-33 has been shown to have both beneficial and detrimental effects in brain pathologies. IL-33 is reported to protect neurons by inhibiting cell death and inflammation in AD and other diseases (Gao et al., 2017). However, IL-33 has also been reported to promote an influx of inflammatory cells in the brain in multiple sclerosis (Jafarzadeh et al., 2016).

In contrast to inflammasome-related cytokines, we observed a decrease in the production of chemokine, CCL-5, in mice treated with L-MET. The constitutive expression of CCL5 and its receptor has been observed in neurons and glia (Lanfranco et al., 2017), where it has been reported to enhance survival and development of neurons (Sorce et al., 2010; Sorce et al., 2011; Tripathy et al., 2010). Therefore, L-MET-induced decreased CCL-5 and increased IL-1β may be partially involved in the observed impaired neurogenesis. Indeed, the decreased hippocampal neurogenesis in aging has been shown to occur via epigenetic

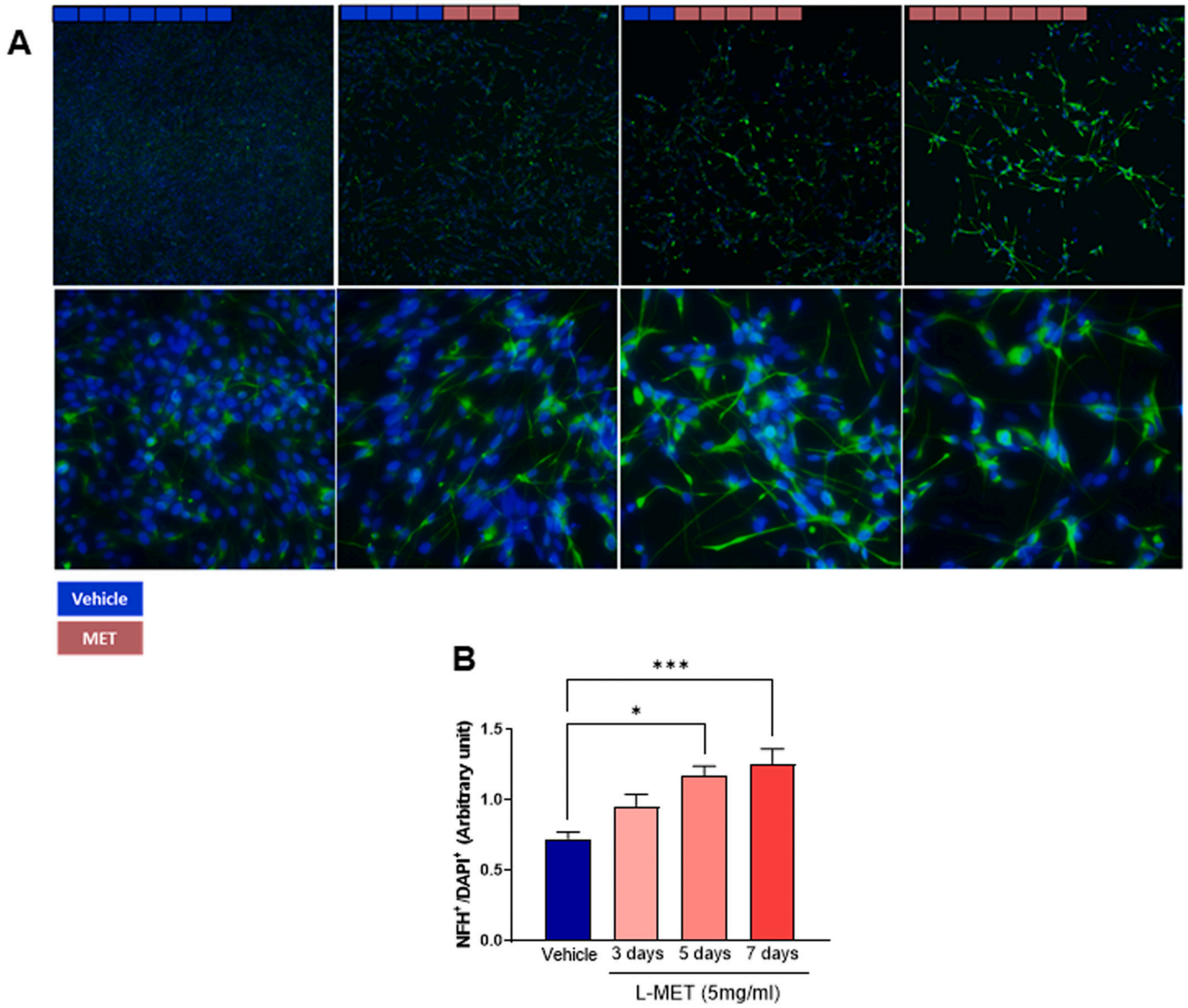

Fig. 5. L-MET treatment of SH-SY5Y increases neurite extension. (A) Representative immunofluoresent image of SH-SY5Y treated for different time with L-MET and stained with neuronal marker NF-H (green) and DAPI (blue), (B) Ratio of Neurite Extension/cell area, $*P < 0.05$, $***P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

modifications, particularly DNA methylation, accompanied by the activation of microglia and astrocytes and the augmented expression of IL-1β (Covic et al., 2010; Kuzumaki et al., 2010a; Kuzumaki et al., 2010b). Further, SAM, a universal methyl donor, was shown to inhibit neurogenesis in the hippocampus (Carrasco et al., 2014). Interestingly, L-MET treatment did not affect the number of born cells in the hippocampus or SY5Y, indicating no effect of L-MET on cell survival. These findings support that L-MET is not an apoptotic agent, and therefore the behavioral responses to its chronic administration do not simply reflect its toxic effects on the brain. Interestingly, the growth of SY5Y returned to the normal rate four days after removing excess L-MET. This finding is of particular clinical relevance as it suggests that the mechanisms through which L-MET produces removal of L-MET inhibit proliferation are reversible. While neurites' outgrowth has been shown to correlate with increased neural differentiation and enhanced memory, the high neurite extension/cell area ratio in the L-MET-treated SY5Y cells may reflect a lower rate of cell division in L-MET-treated cells compared to the control cells. The lower rate of cell proliferation gives cells more space and time to grow their neurites.

Altogether, we demonstrate that chronic administration of L-MET equivalent to double daily dietary enhances the activation of microglia and inflammation in the brain and decreases neurogenesis. We showed that the removal of the excessive L-MET can reverse the effects of neurogenesis. Our results support a role for neuroinflammation and neurogenesis in mediating the mechanism through which L-MET induces cognitive deficits. While our results establish the causal relationship between L-MET and neurogenesis, and between L-MET and neuroinflammation, they do not confirm whether reduced neurogenesis induced by of L-MET is mediated by neuroinflammation. This question is the focus of our ongoing research. The results also uncover L-MET restriction, neuroinflammation, and neurogenesis as potential preventive or therapeutic targets for mental disorders associated with cognitive disorders, including schizophrenia and Alzheimer's disease.

Author contributions

A. Agrawal and A. Alachkar conceived the idea, performed experiments and wrote the manuscript; SA performed flow cytometry; JS and

MB contributed to performing experiments; KN contributed to the manuscript writing.

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Declaration of Competing Interest

The authors declare no competing interests.

References

Agbas, A., Moskovitz, J., 2009. The role of methionine oxidation/reduction in the regulation of immune response. Curr Signal Transduct Ther. 4, 46–50.
Alachkar, A., Wang, L., Yoshimura, R., Hamzeh, A.R., Wang, Z., Sanathara, N., et al., 2018. Prenatal one-carbon metabolism dysregulation programs schizophrenia-like deficits. Mol. Psychiatry 23, 282–294.
Boku, S., Toda, H., Nakagawa, S., Kato, A., Inoue, T., Koyama, T., et al., 2014. Neonatal maternal separation alters the capacity of adult neural precursor cells to differentiate into neurons via methylation of retinoic acid receptor gene promoter. Biol. Psychiatry.
Borsini, A., Zunszain, P.A., Thuret, S., Pariante, C.M., 2015. The role of inflammatory cytokines as key modulators of neurogenesis. Trends Neurosci. 38, 145–157.
Bossaller, L., Chiang, P.I., Schmidt-Lauber, C., Ganesan, S., Kaiser, W.J., Rathinam, V.A., et al., 2012. Cutting edge: FAS (CD95) mediates noncanonical IL-1beta and IL-18 maturation via caspase-8 in an RIP3-independent manner. J. Immunol. 189, 5508–5512.
Carrasco, M., Rabaneda, L.G., Murillo-Carretero, M., Ortega-Martinez, S., Martinez-Chantar, M.L., Woodhoo, A., et al., 2014. Glycine N-methyltransferase expression in the hippocampus and its role in neurogenesis and cognitive performance. Hippocampus. 24, 840–852.
Chen, Y., Ozturk, N.C., Zhou, F.C., 2013. DNA methylation program in developing hippocampus and its alteration by alcohol. PLoS One 8, e60503.
Covic, M., Karaca, E., Lie, D.C., 2010. Epigenetic regulation of neurogenesis in the adult hippocampus. Heredity. 105, 122–134.
Datki, Z., Juhasz, A., Galfi, M., Soos, K., Papp, R., Zadori, D., et al., 2003. Method for measuring neurotoxicity of aggregating polypeptides with the MTT assay on differentiated neuroblastoma cells. Brain Res. Bull. 62, 223–229.
Deng, Y., Marsh, B.M., Moskovitz, J., 2019. Increased levels of protein-methionine sulfoxide in plasma correlate with a shift from a mild cognitive impairment to an Alzheimer's disease stage. Innovations in clinical neuroscience. 16, 29–31.
Fayez, A.M., Elnoby, A.S., Bahnasawy, N.H., Hassan, O., 2019. Neuroprotective effects of zafirlukast, piracetam and their combination on L-methionine-induced vascular dementia in rats. Fundam. Clin. Pharmacol. 33, 634–648.
Felderhoff-Mueser, U., Schmidt, O.I., Oberholzer, A., Buhrer, C., Stahel, P.F., 2005. IL-18: a key player in neuroinflammation and neurodegeneration? Trends Neurosci. 28, 487–493.
Gao, Y., Luo, C.L., Li, L.L., Ye, G.H., Gao, C., Wang, H.C., et al., 2017. IL-33 provides neuroprotection through suppressing apoptotic, Autophagic and NF-kappaB-mediated inflammatory pathways in a rat model of recurrent neonatal seizure. Front. Mol. Neurosci. 10, 423.
Grimble, R.F., 2006. The effects of sulfur amino acid intake on immune function in humans. J. Nutr. 136, 1660S–5S.
Heneka, M.T., Carson, M.J., El Khoury, J., Landreth, G.E., Brosseron, F., Feinstein, D.L., et al., 2015. Neuroinflammation in Alzheimer's disease. Lancet Neurol. 14, 388–405.
Heneka, M.T., McManus, R.M., Latz, E., 2018. Inflammasome signalling in brain function and neurodegenerative disease. Nat. Rev. Neurosci. 19, 610–621.
Hooshmand, B., Refsum, H., Smith, A.D., Kalpouzos, G., Mangialasche, F., von Arnim, C. A.F., et al., 2019. Association of Methionine to homocysteine status with brain magnetic resonance imaging measures and risk of dementia. JAMA Psychiatr. 76, 1198–1205.
Horvath, S., Zhang, Y., Langfelder, P., Kahn, R.S., Boks, M.P., van Eijk, K., et al., 2012. Aging effects on DNA methylation modules in human brain and blood tissue. Genome Biol. 13, R97.
Hu, Q., Teng, W., Li, J., Hao, F., Wang, N., 2016. Homocysteine and Alzheimer's disease: evidence for a causal link from Mendelian randomization. J. Alzheimer's Dis. : JAD. 52, 747–756.
Indika, N.R., Deutz, N.E.P., Engelen, M., Peiris, H., Wijetunge, S., Perera, R., 2021. Sulfur amino acid metabolism and related metabotypes of autism spectrum disorder: a review of biochemical evidence for a hypothesis. Biochimie. 184, 143–157.
Jafarzadeh, A., Mahdavi, R., Jamali, M., Hajghani, H., Nemati, M., Ebrahimi, H.A., 2016. Increased concentrations of Interleukin-33 in the serum and cerebrospinal fluid of patients with multiple sclerosis. Oman Med J. 31, 40–45.
Kabba, J.A., Xu, Y., Christian, H., Ruan, W., Chenai, K., Xiang, Y., et al., 2018. Microglia: housekeeper of the central nervous system. Cell. Mol. Neurobiol. 38, 53–71.
Kalani, A., Chaturvedi, P., Kalani, K., Kamat, P.K., Chaturvedi, P., 2019. A high methionine, low folate and vitamin B6/B12 containing diet can be associated with memory loss by epigenetic silencing of netrin-1. Neural Regen. Res. 14, 1247–1254.
Kim, M., Park, Y.K., Kang, T.W., Lee, S.H., Rhee, Y.H., Park, J.L., et al., 2014a. Dynamic changes in DNA methylation and hydroxymethylation when hES cells undergo differentiation toward a neuronal lineage. Hum. Mol. Genet. 23, 657–667.
Kim, M., Park, Y.K., Kang, T.W., Lee, S.H., Rhee, Y.H., Park, J.L., et al., 2014b. Dynamic changes in DNA methylation and hydroxymethylation when hES cells undergo differentiation toward a neuronal lineage. Hum. Mol. Genet. 23, 657–667.
Koladiya, R.U., Jaggi, A.S., Singh, N., Sharma, B.K., 2008. Ameliorative role of atorvastatin and Pitavastatin in L-methionine induced vascular dementia in rats. BMC Pharmacol. 8, 14.
Koo, J.W., Duman, R.S., 2008. IL-1beta is an essential mediator of the antineurogenic and anhedonic effects of stress. Proc. Natl. Acad. Sci. U. S. A. 105, 751–756.
Koo, J.W., Duman, R.S., 2009. Evidence for IL-1 receptor blockade as a therapeutic strategy for the treatment of depression. Curr. Opin. Investig. Drugs 10, 664–671.
Koo, J.W., Russo, S.J., Ferguson, D., Nestler, E.J., Duman, R.S., 2010. Nuclear factor-kappaB is a critical mediator of stress-impaired neurogenesis and depressive behavior. Proc. Natl. Acad. Sci. U. S. A. 107, 2669–2674.
Kuzumaki, N., Ikegami, D., Imai, S., Narita, M., Tamura, R., Yajima, M., et al., 2010a. Enhanced IL-1beta production in response to the activation of hippocampal glial cells impairs neurogenesis in aged mice. Synapse. 64, 721–728.
Kuzumaki, N., Ikegami, D., Tamura, R., Sasaki, T., Niikura, K., Narita, M., et al., 2010b. Hippocampal epigenetic modification at the doublecortin gene is involved in the impairment of neurogenesis with aging. Synapse. 64, 611–616.
Lanfranco, M.F., Mocchetti, I., Burns, M.P., Villapol, S., 2017. Glial- and neuronal-specific expression of CCL5 mRNA in the rat brain. Front. Neuroanat. 11, 137.
Lee, J., Hur, J., Lee, P., Kim, J.Y., Cho, N., Kim, S.Y., et al., 2001. Dual role of inflammatory stimuli in activation-induced cell death of mouse microglial cells. Initiation of two separate apoptotic pathways via induction of interferon regulatory factor-1 and caspase-11. J. Biol. Chem. 276, 32956–32965.
Lee, S.W., de Rivero Vaccari, J.P., Truettner, J.S., Dietrich, W.D., Keane, R.W., 2019. The role of microglial inflammasome activation in pyroptotic cell death following penetrating traumatic brain injury. J. Neuroinflammation 16, 27.
Li, Y.Q., Chen, J.X., Li, Q.W., Xiao, Z.J., Yuan, T., Xie, Z.H., 2020. Targeting NLRP3 inflammasome improved the neurogenesis and post-stroke cognition in a mouse model of photothrombotic stroke. Neuroreport. 31, 806–813.
Loenen, W.A., 2006. S-adenosylmethionine: jack of all trades and master of everything? Biochem. Soc. Trans. 34, 330–333.
Main, P.A., Angley, M.T., Thomas, P., O'Doherty, C.E., Fenech, M., 2010. Folate and methionine metabolism in autism: a systematic review. Am. J. Clin. Nutr. 91, 1598–1620.
Markarian, M., Krattli Jr., R.P., Baddour, J.D., Alikhani, L., Giedzinski, E., Usmani, M.T., et al., 2021. Glia-selective deletion of complement C1q prevents radiation-induced cognitive deficits and Neuroinflammation. Cancer Res. 81, 1732–1744.
Mayo, L., Jacob-Hirsch, J., Amariglio, N., Rechavi, G., Moutin, M.J., Lund, F.E., et al., 2008. Dual role of CD38 in microglial activation and activation-induced cell death. J. Immunol. 181, 92–103.
McCampbell, A., Wessner, K., Marlatt, M.W., Wolffe, C., Toolan, D., Podtelezhnikov, A., et al., 2011. Induction of Alzheimer's-like changes in brain of mice expressing mutant APP fed excess methionine. J. Neurochem. 116, 82–92.
Nuru, M., Muradashvili, N., Kalani, A., Lominadze, D., Tyagi, N., 2018. High methionine, low folate and low vitamin B6/B12 (HM-LF-LV) diet causes neurodegeneration and subsequent short-term memory loss. Metab. Brain Dis. 33, 1923–1934.
Onozato, M., Uta, A., Magarida, A., Fukuoka, N., Ichiba, H., Tsujino, N., et al., 2020. Alterations in methionine to homocysteine ratio in individuals with first-episode psychosis and those with at-risk mental state. Clin. Biochem. 77, 48–53.
Pasca, S.P., Dronca, E., Kaucsar, T., Craciun, E.C., Endreffy, E., Ferencz, B.K., et al., 2009. One carbon metabolism disturbances and the C677T MTHFR gene polymorphism in children with autism spectrum disorders. J. Cell. Mol. Med. 13, 4229–4238.
Pi, T., Wei, S., Jiang, Y., Shi, J.S., 2021. High methionine diet-induced Alzheimer's disease like symptoms are accompanied by 5-Methylcytosine elevated levels in the brain. Behav. Neurol. 2021, 6683318.
Posel, C., Moller, K., Boltze, J., Wagner, D.C., Weise, G., 2016. Isolation and flow cytometric analysis of immune cells from the ischemic mouse brain. J. Vis. Exp. 53658.
Puig-Alcaraz, C., Fuentes-Albero, M., Calderon, J., Garrote, D., Cauli, O., 2015. Increased homocysteine levels correlate with the communication deficit in children with autism spectrum disorder. Psychiatry Res. 229, 1031–1037.
Ringman, J.M., Fithian, A.T., Gylys, K., Cummings, J.L., Coppola, G., Elashoff, D., et al., 2012. Plasma methionine sulfoxide in persons with familial Alzheimer's disease mutations. Dement. Geriatr. Cogn. Disord. 33, 219–225.
Roidl, D., Hacker, C., 2014. Histone methylation during neural development. Cell Tissue Res. 356, 539–552.
Roy, D.G., Chen, J., Mamane, V., Ma, E.H., Muhire, B.M., Sheldon, R.D., et al., 2020. Methionine metabolism shapes T helper cell responses through regulation of epigenetic reprogramming. Cell Metab. 31 (250–66), e9.
Sharma, A., Kramer, M.L., Wick, P.F., Liu, D., Chari, S., Shim, S., et al., 1999. D4 dopamine receptor-mediated phospholipid methylation and its implications for mental illnesses such as schizophrenia. Mol. Psychiatry 4, 235–246.
Smythies, J.R., Alarcon, R.D., Morere, D., Monti, J.A., Steele, M., Tolbert, L.C., et al., 1986. Abnormalities of one-carbon metabolism in psychiatric disorders: study of methionine adenosyltransferase kinetics and lipid composition of erythrocyte membranes. Biol. Psychiatry 21, 1391–1398.
Sorce, S., Bonnefont, J., Julien, S., Marq-Lin, N., Rodriguez, I., Dubois-Dauphin, M., et al., 2010. Increased brain damage after ischaemic stroke in mice lacking the chemokine receptor CCR5. Br. J. Pharmacol. 160, 311–321.
Sorce, S., Myburgh, R., Krause, K.H., 2011. The chemokine receptor CCR5 in the central nervous system. Prog. Neurobiol. 93, 297–311.

Strous, R.D., Ritsner, M.S., Adler, S., Ratner, Y., Maayan, R., Kotler, M., et al., 2009. Improvement of aggressive behavior and quality of life impairment following S-adenosyl-methionine (SAM-e) augmentation in schizophrenia. Eur. Neuropsychopharmacol. 19, 14–22.

Tapia-Rojas, C., Lindsay, C.B., Montecinos-Oliva, C., Arrazola, M.S., Retamales, R.M., Bunout, D., et al., 2015. Is L-methionine a trigger factor for Alzheimer's-like neurodegeneration?: changes in Abeta oligomers, tau phosphorylation, synaptic proteins, Wnt signaling and behavioral impairment in wild-type mice. Mol. Neurodegener. 10, 62.

Tisato, V., Silva, J.A., Longo, G., Gallo, I., Singh, A.V., Milani, D., et al., 2021. Genetics and epigenetics of one-carbon metabolism pathway in autism Spectrum disorder: a sex-specific brain epigenome? Genes. 12.

Tripathy, D., Thirumangalakudi, L., Grammas, P., 2010. RANTES upregulation in the Alzheimer's disease brain: a possible neuroprotective role. Neurobiol. Aging 31, 8–16.

Trivedi, M.S., Deth, R.C., 2012. Role of a redox-based methylation switch in mRNA life cycle (pre- and post-transcriptional maturation) and protein turnover: implications in neurological disorders. Front. Neurosci. 6, 92.

Velazquez, R., Ferreira, E., Winslow, W., Dave, N., Piras, I.S., Naymik, M., et al., 2020. Maternal choline supplementation ameliorates Alzheimer's disease pathology by reducing brain homocysteine levels across multiple generations. Mol. Psychiatry 25, 2620–2629.

Villa, P., Bosco, P., Ferri, R., Perri, C., Suriano, R., Costantini, B., et al., 2009. Fasting and post-methionine homocysteine levels in Alzheimers disease and vascular dementia. International journal for vitamin and nutrition research Internationale Zeitschrift fur Vitamin- und Ernahrungsforschung Journal international de vitaminologie et de nutrition. 79, 166–172.

Waligora, A., Waligora, S., Kozarska, M., Damasiewicz-Bodzek, A., Gorczyca, P., Tyrpien-Golder, K., 2019. Autism spectrum disorder (ASD) - biomarkers of oxidative stress and methylation and transsulfuration cycle. Psychiatr. Pol. 53, 771–788.

Wang, L., Alachkar, A., Sanathara, N., Belluzzi, J.D., Wang, Z., Civelli, O., 2015. A Methionine-Induced Animal Model of Schizophrenia: Face and Predictive Validity. Int. J. Neuropsychopharmacol. 18.

Wang, R., Wang, Y., Mu, N., Lou, X., Li, W., Chen, Y., et al., 2017. Activation of NLRP3 inflammasomes contributes to hyperhomocysteinemia-aggravated inflammation and atherosclerosis in apoE-deficient mice. Lab. Investig. 97, 922–934.

Wang, Q., Zhao, J., Chang, H., Liu, X., Zhu, R., 2021. Homocysteine and folic acid: risk factors for Alzheimer's disease-an updated Meta-analysis. Front. Aging Neurosci. 13, 665114.

Wu, H., Coskun, V., Tao, J., Xie, W., Ge, W., Yoshikawa, K., et al., 2010. Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. Science. 329, 444–448.

Yu, P., Zhang, X., Liu, N., Tang, L., Peng, C., Chen, X., 2021. Pyroptosis: mechanisms and diseases. Signal Transduct Target Ther. 6, 128.

Zhao, R., Chen, Y., Tan, W., Waly, M., Sharma, A., Stover, P., et al., 2001. Relationship between dopamine-stimulated phospholipid methylation and the single-carbon folate pathway. J. Neurochem. 78, 788–796.

Zhu, J., Hua, X., Yang, T., Guo, M., Li, Q., Xiao, L., et al., 2021. Alterations in gut vitamin and amino acid metabolism are associated with symptoms and neurodevelopment in children with autism Spectrum disorder. J. Autism Dev. Disord. https://doi.org/10.1007/s10803-021-05066-w e-pub ahead of print.

Zuin, M., Cervellati, C., Brombo, G., Trentini, A., Roncon, L., Zuliani, G., 2021. Elevated blood homocysteine and risk of Alzheimer's dementia: an updated systematic review and Meta-analysis based on prospective studies. J. Preven. Alzheimer's Dis. 8, 329–334.