Methionine Supplementation Enhances Learning and Rescues Depression Through Epigenetic Regulation of Hippocampal $Bdnf$

By
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Methionine Supplementation Enhances Learning and Memory Through Epigenetic Regulation of *Bdnf*

Pascal Ibrahim

**ABSTRACT**

Brain-derived neurotrophic factor (BDNF) is essential for synaptic plasticity and learning and memory formation. It has been previously shown that the expression of BDNF is epigenetically regulated through histone methylation. Methionine, an amino acid obtained from the diet, is the precursor of S-adenosylmethionine (SAM), which is the major methyl donor in most methylation reactions. In this study, the effect of methionine supplementation on BDNF expression was assessed. Methionine treatment (100 μM) induced BDNF promoter I (pI) and coding mRNA expression in primary neuronal cell cultures. Treatment also activated Tropomyosin-related Kinase B (TrkB) signaling. Learning and memory were assessed using the Morris Water Maze. 6-Week-old C57BL/6 male mice intraperitoneally injected with a combination of 1.55175 mg/Kg methionine and 80 μg/Kg folic acid (FA), which allows regeneration of methionine, exhibited improved learning. Additionally, mice treated with methionine showed enhanced short-term memory. Inhibition of TrkB signaling led to a loss of this positive effect. These results suggest that methionine intake could enhance learning and memory possibly through modifying methylation marks in the brain and that this enhancement is mediated through BDNF, which is upregulated upon methionine treatment.

**Keywords:** Methionine, Methylation, BDNF, Learning, Memory, Folic acid, High Protein Diet.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures</td>
<td>IX</td>
</tr>
<tr>
<td>List of abbreviations</td>
<td>X-XI</td>
</tr>
<tr>
<td>1. Literature Review</td>
<td>1-16</td>
</tr>
<tr>
<td>1.1 Neurotrophins</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1 Bdnf Gene Structure and Expression</td>
<td>2-3</td>
</tr>
<tr>
<td>1.1.2 BDNF Signaling</td>
<td>4-5</td>
</tr>
<tr>
<td>1.1.3 BDNF and Memory Formation</td>
<td>6</td>
</tr>
<tr>
<td>1.2 Epigenetics and the Diet</td>
<td>7</td>
</tr>
<tr>
<td>1.3 Histone and DNA Methylation</td>
<td>8</td>
</tr>
<tr>
<td>1.4 High Protein Diet Induces BDNF</td>
<td>9-10</td>
</tr>
<tr>
<td>1.5 The Candidate: Methionine</td>
<td>11</td>
</tr>
<tr>
<td>1.5.1 Methionine Metabolism</td>
<td>11-12</td>
</tr>
<tr>
<td>1.5.2 Folic Acid in the Brain</td>
<td>13</td>
</tr>
<tr>
<td>1.5.3 Methyltransferases in the Brain</td>
<td>14</td>
</tr>
<tr>
<td>1.5.4 Demethylases and α-ketoglutarate in the Brain</td>
<td>15</td>
</tr>
<tr>
<td>1.5.5 Methionine and SAM in the Brain</td>
<td>16</td>
</tr>
<tr>
<td>1.5.6 BDNF and Methylation</td>
<td>17</td>
</tr>
<tr>
<td>1.6 Aim of this Study</td>
<td>18</td>
</tr>
<tr>
<td>2. Materials and Methods</td>
<td>19-23</td>
</tr>
<tr>
<td>2.1 Cell Culture and Treatment</td>
<td>19</td>
</tr>
<tr>
<td>2.2 RNA Extraction and Real time PCR</td>
<td>19-20</td>
</tr>
<tr>
<td>2.3 Animal housing and treatments</td>
<td>20</td>
</tr>
<tr>
<td>2.4 Morris water maze</td>
<td>20-21</td>
</tr>
<tr>
<td>2.5 Chronic social defeat stress paradigm</td>
<td>21-22</td>
</tr>
<tr>
<td>2.5 Protein extraction</td>
<td>22</td>
</tr>
</tbody>
</table>
3. Results

3.1 Treatment of primary neuronal cultures with methionine induces Bdnf expression and TrkB signaling

3.2 Intra-peritoneal injection of methionine enhances spatial learning and memory

3.3 Methionine enhances learning and memory in a BDNF-dependent manner

3.4 Methionine rescues mice from social stress by restoring the lost BDNF signaling

3.5 Methionine enhances learning and memory in a BDNF-dependent manner

4. Discussion

5. References
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1: <em>Bdnf</em> gene structure, various transcripts, and polypeptide.</td>
<td>3</td>
</tr>
<tr>
<td>Figure 2: Summary of BDNF signaling through TrkB receptor.</td>
<td>5</td>
</tr>
<tr>
<td>Figure 3: Relative quantification of expression of Bdnf pI and coding mRNA extracted from hippocampi of mice under different conditions as measured by real-time RTPCR.</td>
<td>10</td>
</tr>
<tr>
<td>Figure 4: Overview of the methionine metabolism cycle involved in methylation.</td>
<td>12</td>
</tr>
<tr>
<td>Figure 5: Schematic representation of the Morris Water Maze setup used for our experiment.</td>
<td>21</td>
</tr>
<tr>
<td>Figure 6: Schematic representation of the CSDS paradigm.</td>
<td>22</td>
</tr>
<tr>
<td>Figure 7: Methionine induces Bdnf expression and TrkB signaling in primary cortical neurons.</td>
<td>25-26</td>
</tr>
<tr>
<td>Figure 8: α-ketoglutarate induces <em>Bdnf</em> pI expression in primary cortical neurons.</td>
<td>27</td>
</tr>
<tr>
<td>Figure 9: Methionine enhances mice spatial learning and memory performance in the Morris Water Maze.</td>
<td>29</td>
</tr>
<tr>
<td>Figure 10: α-ketoglutarate shows a trend in enhancing spatial learning and memory performance in Morris Water Maze.</td>
<td>30</td>
</tr>
<tr>
<td>Figure 11: Methionine rescues social defeat stress in a BDNF dependent manner.</td>
<td>31-32</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

**BDNF**: Brain-derived neurotrophic factor

**SAM**: S-adenosylmethionine

**pI**: Promoter I

**TrkB**: Tropomyosin-related kinase B

**pTrkB**: Phosphorylated tropomyosin-related kinase B

**NGF**: Nerve growth factor

**LTP**: Long term potentiation

**CNS**: Central nervous system

**aa**: Amino acid

**p75NTR**: p75 neurotrophin receptor

**PLCγ**: Phospholipase C γ

**PI3K**: Phosphatidylinositol 3-kinase

**MAPK**: Mitogen-activated protein kinase

**DAG**: Diacylglycerol

**IP3**: Inositol trisphosphate

**cAMP**: Cyclic adenosine monophosphate

**CREB**: cAMP response-element binding protein

**CaM**: Calmodulin

**CamK**: Calmodulin kinase

**ERK**: Extracellular signal-regulated kinase

**AKT**: Protein kinase B

**H3K4, H3K9 and H3K27**: Histone 3 lysine residue 4, 9 and 27 respectively

**MeCP**: Methyl cytosine-binding proteins

**HF**: High fat

**HP**: High protein

**CTRL**: Control

**RTPCR**: Reverse transcription polymerase chain reaction

**MET**: Methionine
MAT: Methionine adenosyl transferase
ATP: Adenosine triphosphate
SAH: S-adenosyl homocysteine
THF: Tetrahydrofolate
DHF: Dihydrofolate
SET: Su(var)3-9, Enhancer-of-zeste and Trithorax
DNMT: DNA methyl transferase
FA: Folic acid
KMT: Lysine methyl transferase
KCNA: Potassium voltage-gated channel
ZIF: Zinc finger protein
MLL: Myeloid/lymphoid or mixed-lineage leukemia
H3K4me3: H3K4 trimethylation
NMDA: N-Methyl-D-aspartic acid
H3K9me2: H3K9 dimethylation
KDM: Histone lysine demethylase
E17: Embryonic day 17
PBS: Phosphate buffered saline
Fwd: Forward
Rev: Reverse
Q: Quadrant
SDS-PAGE: Sodium-dodecyl sulfate polyacrylamide gel electrophoresis
EDTA: Ethylenediaminetetraacetic acid
TBS: Tris-buffered saline
PVDF: Polyvinylidene difluoride
ECL: Enhanced chemiluminescence
DIV: Days in vitro
CSDS: Chronic social defeat stress
AKG: α-ketoglutarate
Tet: Ten-eleven translocation
PHF: Plant homeodomain finger
Chapter 1

Literature Review

1.1 Neurotrophins

Neurotrophins are dimeric signaling proteins in the nervous system, and the family of neurotrophins includes nerve growth factor (NGF), neurotrophins 3 and 4, and brain-derived neurotrophic factor (BDNF) (Autry & Monteggia, 2012). During development, they are present in limiting amounts and function as survival factors which ensure that the number of surviving neurons matches the number required for appropriate target innervation. They are responsible for axon growth, dendrite pruning, cell fate decisions, and neurotransmitter and ion channel expression. In the adult nervous system, they continue to regulate neuronal survival, but they also play a role in synaptic function and plasticity and long term potentiation (LTP). The fact that the mRNA of at least some neurotrophins is upregulated in an activity-dependent manner also suggests that they are crucial for the mature brain (Thoenen, 1995; Lewin & Barde, 1996; Huang & Reichardt, 2001). BDNF is the most characterized neurotrophin in terms of its relevance to central nervous system (CNS) diseases, and it has become of great interest to target this neurotrophin to treat such disorders (Autry & Monteggia, 2012).
1.1.1 *Bdnf* Gene Structure and Expression

The rodent *bdnf* gene contains eight 5’ untranslated exons (I-VIII) and one protein coding 3’ exon (IX), each preceded by a promoter. Alternative splicing results in several *Bdnf* transcripts, all of which contain one of the eight 5’ exons spliced to exon IX that encodes the BDNF protein since there is a splicing donor site at the 3’ end of each of the first eight exons (Zheng et al., 2012). The presence of multiple promoters plays a role in tissue-specific expression of the BDNF transcripts in different brain regions and nonneural tissues (Aid et al., 2007, Autry & Monteggia, 2012). Exon I or promoter I (pI) expression in particular has been found to be activity–dependent, for an increase in mRNA levels is observed after kainic acid treatment (Timmusk et al., 1993). Moreover, an increase in pI expression has been observed in the hippocampus after fear conditioning (Lubin, Roth & Sweatt, 2008), which suggests that it could be the promoter involved in memory formation processes. BDNF is first synthesized as a precursor protein called prepro-BDNF (249 amino acids (aa) long), in which the “pre” peptide of the protein constitutes a signal that leads to the sequestration of the formed polypeptide to the rough endoplasmic reticulum. Once docked, the signal peptide is directly cleaved off. The resulting pro-BDNF (231 aa) is transferred to the Golgi apparatus and further processing takes place. Finally the pro-domain is cleaved by a pro-protein convertase called furin, which is a Ca\(^{2+}\) - dependent serine proteinase, to give mature BDNF (118 aa). Furin is ubiquitously expressed in all tissue types, but it is also highly expressed in the hippocampus and cortex (Autry & Monteggia, 2012; Lessman et al., 2003).
Figure 1: Bdnf gene structure, various transcripts, and polypeptide. (A) The Bdnf gene consists of eight non-coding exons and one coding exon. Promoters are differentially activated to produce the various transcripts. White boxes represent untranslated regions, while the grey boxes represent coding regions. (B) BDNF is synthesized as prepro-BDNF, which is sequentially cleaved into pro-BDNF and then mature BDNF. The scale represents amino acid positions.
1.1.2 BDNF Signaling

Pro-BDNF has different binding activity and thus activates intracellular signaling pathways distinct from those activated by mature BDNF. In fact, pro-BDNF binds to the low affinity p75 neurotrophin receptor p75NTR, which is a distant member of the tumor necrosis factor receptor family. p75NTR is believed to be involved in apoptosis, and signaling through this receptor is important for selecting which neurons survive during development (Huang & Reichardt, 2001). On the other hand, mature BDNF is the ligand for TrkB receptor, and upon binding, the receptor dimerizes and autophosphorylates. Phosphorylated and thus activated TrkB (pTrkB) activates at least three signal transduction pathways: the phospholipase C γ (PLCγ) pathway, the phosphatidylinositol 3-kinase (PI3K) pathway, and the mitogen-activated protein kinase (MAPK) pathway (Autry & Monteggia, 2012). The PI3K and MAPK pathways regulate neuronal survival and growth, respectively, and they are involved in the long-lasting effects on transcription regulation. The activation of PLCγ leads to the cleavage of phosphatidylinositol 4,5-bisphosphate into diacylglycerol (DAG) and inositol trisphosphate (IP₃). In turn, IP₃ leads to the release of Ca²⁺ which is responsible for the rapid synaptic and ion channel changes that occur. However, all three pathways converge at the transcription factor cAMP response-element binding protein (CREB), which mediates BDNF function through activating gene transcription (Autry & Monteggia, 2012).
Figure 2: Summary of BDNF signaling through TrkB receptor. The binding of BDNF triggers autophosphorylation of TrkB and subsequent activation of downstream cascades. PI3K activates AKT which mediates survival, while MAPK activates ERK which mediates growth. The production of IP3 through the activation of PLCγ leads to the release of Ca\(^{2+}\) which activates calmodulin kinase (CamK) and mediates synaptic plasticity. The three pathways converge at CREB to induce gene expression. CaM, calmodulin; ERK, extracellular signal-regulated kinase; AKT, protein kinase B.
1.1.3 BDNF and Memory Formation

It is well known today that BDNF plays an essential role in learning and memory. In the mature brain, BDNF mediates synaptic plasticity, learning and memory processes, and may support long-term potentiation (LTP) (Autry & Monteggia, 2012). A simple alteration in the structure of BDNF can have grave effects. For example, a single nucleotide polymorphism in the \textit{bdnf} gene which leads to a substitution of the valine at residue 66 with a methionine has been associated with smaller hippocampal volumes and poor performance on hippocampal-dependent memory tasks in humans (Szeszko et al., 2005; Egan et al., 2003). The same polymorphism has been associated with impaired digital working memory, spatial localization, and episodic memory (Gong et al., 2009; Egan et al., 2003). Indeed, BDNF mediates important hippocampal functions. A study has shown that an increase of BDNF in the ventral hippocampus can support memory extinction when rats are subjected to fear conditioning (Rosas-Vidal et al., 2014). Another study showed that long term memory storage persists due to a BDNF-dependent phase that occurs in the rat hippocampus 12 hours after an associative learning task (Bekinschtein et al., 2007). Short term spatial memory was also improved in rats receiving intra-hippocampal administration of BDNF before Morris water maze testing (Cirulli et al., 2004). Furthermore, several studies have found that BDNF/TrkB signaling is linked to the NMDA receptor 2B through the tyrosine kinase Fyn. Since NMDA receptors are involved in LTP, this signaling pathway enhanced spatial learning in the radial maze, and when a tyrosine inhibitor was introduced, this enhancement was lost (Mizuno et al., 2003; Yamada & Nabeshima, 2003). This evidence displays the importance of BDNF signaling in hippocampal-dependent memory formation.
1.2 Epigenetics and the Diet

The term ‘epigenetics’ is defined as the study of mitotically and/or meiotically heritable changes in gene expression that do not entail a change in the DNA sequence itself (Egger et al., 2004; Dupont et al., 2009). Some of these changes are induced by environmental factors, while others are programmed (Lardenoije et al., 2015). The diet is an important factor through which the environment can mediate its effects on the epigenome (Jaenisch & Bird, 2003). There is increasing evidence that the consumption of bioactive dietary compounds can induce epigenetic modifications that would reverse abnormal gene activation or silencing. For this reason, it would be interesting to consider using these bioactive dietary compounds to target specific pathological epigenetic alterations as a form of therapy for “epigenetic” diseases, such as those of CNS. Since the term “dietary” implies that the compounds are absorbed and subsequently enter cells easily, they could be administered to a patient in ways similar to any other medication without any delivery problems. They could also be supplemented in the diet as an enhancement for the main treatment.
1.3 Histone and DNA Methylation

Epigenetic mechanisms include X-chromosome inactivation, autosomal imprinting, DNA methylation and hydroxymethylation, chromatin remodeling, non-coding RNA regulation, post-translational histone modifications, and others (Dupont et al., 2009; Lardenoije et al., 2015). Histones include H2A, H2B, H3, and H4, where two of each form an octamer around which the DNA double helix is wrapped, and H1 which associates with the linker DNA. Among the most characterized histone modifications are methylations of lysine residues on H3 and H4. Methylation of lysine residues may lead to either transcriptional repression as in the case of H3K9 and H3K27, or transcriptional activation as in the case of H3K4 found at promoters of active genes. Methylation can also be monomeric, dimeric, or trimeric, which dictates the effect of the modification on transcription as well (Egger et al., 2004; Dupont et al., 2009). To add to that, covalent modifications of DNA, particularly the methylation of the C5 position of cytosine residues, have been implicated in epigenetic transcriptional silencing. Such methylation at promoters may achieve silencing by attracting methyl cytosine-binding proteins such as MeCP1 and MeCP2 that prevent the binding of specific transcription factors or bind to chromatin remodelers that will in turn lead to gene silencing (Dupont et al., 2009; Niculescu & Zeisel, 2002).
1.4 High Protein Diet Induces BDNF

Our lab has previously shown that when mice are allowed to voluntarily exercise, *Bdnf* expression is upregulated in the hippocampus, the center of learning and memory (El Hayek et al., under review). We also showed that when mice are fed a high protein, casein-based diet, hippocampal BDNF is also increased, and this effect is additive to exercise. In addition, our lab has identified molecules, such as beta-hydroxybutyrate (Sleiman et al., 2016) and lactic acid (El Hayek et al., under review), that induce *Bdnf* expression in the hippocampus. When lactic acid was administered through intraperitoneal injections, it promoted learning and memory in a BDNF-dependent manner. This is of great significance for the development of therapeutics for disorders in which memory formation is impaired or in which BDNF signaling is impaired. These notions suggest that peripheral delivery of certain compounds can have an effect on *Bdnf* gene expression in the brain, and this effect is most probably due to an epigenetic mechanism. At this point, what remains is to identify the specific component of the high protein diet that is responsible for the induction observed in *Bdnf* expression in the hippocampus.
Figure 3: Relative quantification of expression of *Bdnf* pl and coding mRNA extracted from hippocampi of mice under different conditions as measured by real-time RTPCR. High protein diet induces *Bdnf* pl and coding mRNA expression, and the effect is additive when exercise is accompanied with the diet. CTRL, control; HF, high fat; HP, high protein.
1.5 The Candidate: Methionine

Methionine is an essential sulfur-containing amino acid and is of high biological importance due to its involvement in the one-carbon metabolism cycle. It has been chosen as the candidate molecule through which a high protein diet mediates Bdnf induction, and the reasons will be discussed below.

1.5.1 Methionine Metabolism

In cells, the enzyme methionine adenosyl-transferase (MAT) adds an adenosine group from adenosine triphosphate (ATP) to the sulfur group of methionine in order to form S-adenosylmethionine (SAM). The adjacent methyl group would subsequently be activated, and SAM would thus have the ability to donate its methyl group to form S-adenosylhomocysteine (SAH) (Tapia-Rojas et al., 2015). When SAH loses the adenosine group, it is converted to homocysteine, high levels of which would cause homocystinuria (Finkelstein, 1974). Homocystinuria can cause epigenetic alterations due to induction of oxidative stress (Kalani et al., 2013). Homocysteine can be converted to cystathionine and then cysteine in the presence of the vitamin B6, and cysteine can be utilized to form glutathione (Finkelstein, 1974; Waterland, 2017). An alternative path for homocysteine is to regenerate methionine through methylation by the methyl donor betaine, which is obtained from dietary choline. Another interesting way to regenerate methionine is through the folate cycle, in which vitamin B12 aids in catalyzing the reaction between homocysteine and 5-methyltetrahydrofolate to produce methionine and tetrahydrofolate (THF). THF can be reconverted to 5-methyltetrahydrofolate in the presence of serine and B6 through the intermediate 5,10-methylenetetrahydrofolate (Waterland, 2017). FA can also be converted to THF through the intermediate dihydrotetrafolic acid (DHF) (Bailey & Gregory, 1999). Since FA can be obtained through the diet, it represents a feasible way to increase the levels of methionine, while avoiding the increase in the levels of toxic homocysteine.

Protein methyltransferases catalyze the transfer of the methyl group of SAM to either lysine or arginine residues of proteins. The vast majority of lysine
methyltransferases contain a conserved SET domain, which forms the active site next to the SAM binding pocket (Kaniskan, Konze & Jin, 2014). All the known DNA methyltransferases also recruit SAM in their methylation processes (Poh, Wee & Gao, 2016), and they include DNA methyltransferase (DNMT) 1, DNMT3a, and DNMT3b in mammals (Bestor, 2000). SAM thus acts as a cofactor for DNA and histone methyltransferases. All in all, this suggests that methionine and folic acid, both dietary compounds, might modify the activity of methyltransferases if supplemented at certain doses.

(Adapted from Waterland, *The Journal of Nutrition*, 2017)

**Figure 4:** Overview of the methionine metabolism cycle involved in methylation. Adenosine is added to methionine to give SAM, which in turn acts as the methyl donor in a reaction catalyzed by a methyltransferase. Once the substrate is methylated, SAM is converted to SAH and then homocysteine, which can enter the folate cycle to regenerate methionine. Folic acid may also aid in methionine regeneration by entering the folate cycle.
1.5.2 Folic Acid in the Brain

Knowing that FA can regenerate methionine and lower the levels of homocysteine, it most probably has beneficial effects on the brain due to the enhancement of methylation reactions, including those involved in epigenetic regulation. Indeed, several studies reflect the importance of FA in the brain. Animal models have been used for this purpose. For instance, FA reverses the epigenetic modifications caused by homocystinuria in brains of mice heterozygous for cystathione-beta synthase fed with methionine (Kalani et al., 2013). Intraperitoneal injections of 80 μg/Kg of FA also improve early functional recovery from traumatic brain injury in piglets (Naim et al., 2010). Memory improvements in particular have been mediated by FA. Folate deficiency has been associated with cognitive decline in elderly people (Durga et al., 2007). Many trials have been performed in humans to examine the effect of folate supplementation on this cognitive decline, and results showed that when the subjects were supplemented with folate, their memory was improved, while their cognitive decline was ameliorated (Durga et al., 2007; Fioravanti et al., 1997). The positive effect of folate supplementation was mostly pronounced in those with the most severe folate deficiency as well (Fioravanti et al., 1997). Moreover, FA supplementation improved short and long-term memory in healthy rats subjected to a passive avoidance task (Shooshtari, Moazedi & Parham, 2012). Another study showed that a single treatment of homocysteine impaired rats’ performance in short and long-term memory tasks and reduced BDNF levels in their hippocampi. When rats were pretreated with FA, these deficits were inhibited (Matte et al., 2009). All these findings support the notion that FA administration could indeed have a positive effect on the brain, particularly on memory formation, in addition to the fact that it can boost the effect of methionine.
1.5.3 Methlytransferases in the Brain

Methyltransferases are implicated in several brain functions. For instance, KMT2A and KMT2B regulate memory formation by affecting H3K4 methylation at distinct genomic regions, and the loss of KMT2A results in changes similar to a model for Alzheimer’s disease (Kerimoglu et al., 2017). A study by Gupta et al. (2010) found that H3K4 and H3K9 methylation patterns change during fear conditioning, and that mice that were deficient in the H3K4-specific methyltransferase KMT2A, also known as $Mll$, exhibited impaired contextual fear conditioning. The study also found that H3K4 tri-methylation at promoters such as Zif268 and $bdnf$ increased with fear learning. Another methyltransferase, SETDB1, was found to regulate affective and motivational behaviors by altering NMDA receptor composition through repressive chromatin remodeling and histone methylation (Jiang et al., 2010). DNA methylation also plays important roles in the brain. A study by Zhao et al. (2017) showed that DNMT3a may contribute to neuropathic pain by repressing a potassium voltage-gated channel, KCNA2 in the dorsal root ganglion. This evidence suggests that methionine might be relevant in brain function by activating methyltransferases and thus modulating methylation patterns. Since methylation is an important phenomenon in the brain, one can assume that not only the histone and DNA methylases play a role, but also the histone and DNA demethylases. Some demethylases are Fe(II) and α-ketoglutarate dependent (Xiao et al., 2012), which implies that supplementation of the metabolite α-ketoglutarate might activate certain demethylases and affect brain function as well.
1.5.4 Demethylases and $\alpha$-ketoglutarate in the Brain

$\alpha$-Ketoglutarate (AKG) is involved in four different pathways in cells: amino acid synthesis, energy metabolism, ammonia detoxification, and dioxygenation (Xiao et al., 2012). The latter function is catalyzed by Fe(II)/AKG-dependent dioxygenases which use AKG as a cosubstrate (Xiao et al., 2012). These dioxygenases include the ten-eleven translocation (Tet) family of DNA hydroxylases as well as the Jumonji C domain-containing histone demethylases, both of which are essential for epigenetic regulation of gene expression through their demethylation activity (Xiao et al., 2012). There have been several studies highlighting the importance of these enzymes in the brain. For example, a study by Zhang et al. (2013) demonstrated that the DNA hydroxylase Tet1, which eventually induces DNA demethylation, upregulates adult hippocampal neural progenitor cell proliferation, and knocking out this enzyme leads to poor learning and memory. The study also showed that Tet1 maintains the expression of certain genes by inhibiting their methylation, knowing that DNA methylation is a repressive process (Zhang et al., 2013). Another study found that the histone demethylase PHF8 is involved in serotonin signaling, and a loss of this enzyme promotes resilience to anxiety and depression-like behavior (Walsh et al., 2017). This suggests that activating certain demethylases could have positive effects on the brain, and since AKG is a metabolite and cosubstrate for these enzymes, AKG supplementation might be a feasible way of achieving this. In fact, AKG was found to aid in the inhibition of seizures and of the resulting mitochondrial damage induced by excitotoxicity (Yamamoto & Mohanan, 2003). Therefore, examining the effect of AKG supplementation on BDNF expression would be interesting.
1.5.5 Methionine and SAM in the Brain

There has been ample work on methionine and SAM in the brain. The administration of methionine can increase the levels of SAM in the brain because MAT is usually not fully saturated with methionine (Rubin, Ordonez & Wurtman, 1974). Several studies have reported effects on the brain when methionine or SAM were administered. For example, a study by Parrish et al. (2015) reported that methionine supplementation via injections increased bdnf DNA methylation and decreased bdnf transcripts in the epileptic hippocampus, which ameliorated memory impairments associated with temporal lobe epilepsy, through the action of methyltransferases. Another study by Wright et al. (2015) showed that methyl supplementation attenuates cocaine-seeking behavior in a DNA methylation-dependent manner. Moreover, methionine was found to have a protective effect against free radical damage of rat brain synaptosomes by inhibiting lipid peroxidation, membrane damage, and glutathione system alterations when oxidative stress was induced (Slyshenkov et al., 2002). The antidepressant effects of SAM have been previously investigated through clinical trials as well (Mischoulon et al., 2013; Benelli et al., 1999). Ischemia induces polar lipid alterations, and injections of SAM corrected these by increasing brain phosphatidylcholine and choline plasmalogen due to its role in methylation reactions (Trovarelli et al., 1983). The above data highlights the potential in peripheral administration of methionine as a tool to modify methylation marks in the brain.
1.5.6 BDNF and Methylation

Interestingly, *Bdnf* expression is epigenetically regulated by histone and DNA methylation and demethylation. In a mouse model for Huntington’s disease, Vashishtha et al. (2013) reported a reduction in H3K4 trimethylation (H3K4me3) at the *bdnf* locus. N-Methyl-D-aspartic acid (NMDA) receptor stimulation induces a decrease in H3K9 dimethylation (H3K9me2) at the transcription start site of rat *Bdnf* (Tian et al., 2009), and H3K27me3 demethylation at *bdnf* promoters (Palomer et al., 2016). Fear conditioning is associated with increased H3K4me3 and DNA demethylation at *Bdnf* promoter 1 (Gupta et al., 2010). Global inhibitors of DNA methyltransferases modify DNA methylation at the *Bdnf* promoter in the adult brain (Lubin, 2011). The histone demethylase KDM5C interacts with a transcription factor involved in the regulation of BDNF, and mutations in KDM5C have been linked to epilepsy and X-linked mental retardation (Lubin, 2011). The *Bdnf* promoter is found to be hypermethylated in major depressive disorder patients (Lopez et al., 2012). Moreover, patients having a history of antidepressant treatment exhibited lower H3K27 methylation at *Bdnf* promoter IV (Lopez et al., 2012). These observations suggest that histone and DNA methylation play an important role in the epigenetic regulation of the *Bdnf* gene.
### 1.6 Aim of this Study

In this study, we hypothesized that peripheral delivery of methionine at proper doses can modify methylation patterns in the brain, which might affect BDNF expression and thus have implications on learning and memory formation. We aimed to test this hypothesis by examining whether methionine can indeed induce BDNF expression in neurons, whether it can enhance spatial learning and memory in the Morris water maze, and whether this enhancement is BDNF-dependent.
2.1 Cell culture and treatment

Immature primary cortical neurons were obtained from C57BL/6 mice at embryonic day 17 (E17) as previously described (Ratan, Murphy, & Baraban, 1994b, 1994a). Mature cortical neurons were maintained in Neurobasal media (Invitrogen) supplemented with B27, and Glutamax (Invitrogen). Primary neurons were isolated as described and 1 million cells were plated in each well. On Day 6, cells were treated with different concentrations of methionine (Sigma) or dimethyl α-ketoglutarate which is cell permeable and is cleaved into α-ketoglutarate once in the cell (Sigma) for 16 hours (overnight). Both methionine and dimethyl α-ketoglutarate were prepared as 200 mM stock in PBS and used at final concentrations of 100 μM, 500 μM, 1 mM, 2 mM, 5 mM and 20 mM in the wells.

2.2 RNA extraction and Real Time PCR

Total RNA was prepared from primary cortical neurons using the Rneasy Plus Mini RNA extraction kit (Qiagen) according to the manufacturer’s protocol. cDNA was prepared by Reverse transcription using QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer’s protocol. Real-time PCRs were performed using SYBR® Green Master Mix (BioRad).

Primer sequence (5’-3’):

\textit{Gapdh:}
\begin{align*}
\text{Fwd:} & \quad 5’- \text{CTCTCTGCTCCTCCCTGTTC} \\
\text{Rev:} & \quad 5’- \text{CCGACCTTCACCATTTTGTC}
\end{align*}
2.3 Animal housing and treatments

Adult male C57BL/6 mice, 6-7 weeks of age, were housed in cages, maintained on a 12-hour light-dark cycle, and provided with food and water *ad libitum*. The mice were divided into groups according to the treatment received through daily intraperitoneal injections: saline, methionine (1.55175 mg/Kg), FA (80 μg/Kg), a combination of methionine (1.55175 mg/Kg) and FA (80 μg/Kg), and dimethyl α-ketoglutarate (dose). Animal care and use was in accordance with the guidelines set by the National Institutes of Health and the Lebanese Ministry of Health.

2.4 Morris water maze

The mice were subjected to behavioral testing 4 hrs (for saline, MET, FA, MET+FA) or 30 mins (for dimethyl α-ketoglutarate) after injection time. The Morris water maze was performed as previously described (Morris, 1984). Briefly, a pool was filled with clear water and divided into 4 equal quadrants (Q1, Q2, Q3, and Q4). A platform was placed in the center of Q1, and visual cues were distributed around the pool. Mice were introduced into the water on day 1 to get accustomed to the set up. From day 2 through day 6, the platform was hidden using white paint. This resembles the learning phase in which mice were expected to depend on their spatial memory to navigate through the maze and find the platform. Each mouse was given three 1-min trials and tracked using ANY-maze Video Tracking System. The latency to reach the platform was measured. On day 7, the platform was removed, and the time the mice spent in Q1 was measured. This resembles the memory test. Mice were sacrificed at the
end of the experiment. Brains were dissected on dry ice, and the hippocampi were collected and stored at -80 °C for later analysis.

Figure 5: Schematic representation of the Morris Water Maze setup used for our experiment. During the first day, mice are habituated to the setup. The platform is visible and the water is clear. Days 2 to 6 represent the learning phase where the water is opaque and the platform is invisible. The last day is the memory test where the platform is removed.

2.5 Chronic social defeat stress (CSDS) paradigm

This paradigm utilizes BALB/c mice as aggressors. Aggressors were singly caged one week prior to the experiment, and three days before the experiment, they were screened for aggression. A C57BL/6 screener mouse was introduced into the cage, and if the aggressor initiated an aggressive attack within the first minute for two days, it was used. C57BL/6 mice were divided into four groups: a group receiving intraperitoneal saline injections (control), a group receiving methionine injections (1.55175 mg/Kg) (methionine), a group receiving saline injections and subjected to defeat (control defeat), and a group receiving methionine injections and subjected to defeat (methionine defeat). Each mouse was caged with an aggressor, and a perforated plexi glass separator divided the cage into two equal parts. This allowed for sensory interaction between the experimental mouse and the aggressor. For the groups that underwent defeat, four hours after they were injected, they were placed in direct contact with the aggressor for 5-10
minutes and were returned to their zone. This was repeated for 10 consecutive days, and on each day the aggressors were switched so that the mice wouldn’t get accustomed to one aggressor. On the eleventh day, the social interaction test was performed. It consisted of a three-chambered apparatus in which each of the two peripheral ones contained an enclosed chamber. The experimental mouse was placed in the apparatus for 5 minutes to habituate, and then a social stimulus was introduced in one of the enclosed chambers. The experimental mouse was tracked for 10 minutes using ANY-maze Video Tracking System.

**Figure 6:** Schematic representation of the CSDS paradigm. Mice are injected daily for 10 days. 4 hours after the injection, defeat groups are subjected to physical stress for 5-10 mins. On the 11th day, the SI test is performed.

### 2.6 Protein extraction

Total cell or hippocampal proteins were prepared by lysis in RIPA buffer (1 % Triton X-100, 1 % SDS, 50 mM Tris-Cl, 500 mM NaCl, and 1 mM EDTA; pH 7.4) in the presence of 1:100 protease inhibitors (Sigma), 1:1000 of the proteasome inhibitor MG-132 (Sigma) and 1:100 of phosphatase inhibitors (Sigma) followed by benzonase nuclease (Sigma) digestion for 15 minutes. Samples were centrifuged and the supernatant was collected and used for Western blot or stored at -80 °C.


2.7 Western blot analysis

Samples were heat-blocked in Laemmli buffer and loaded on a 10% gel for SDS-PAGE. Semi-dry transfer to a PVDF membrane (Bio-Rad) was performed using TransBlot Turbo Transfer System (Bio-Rad). The membranes were then blocked in blocking buffer composed of Bovine serum albumin dissolved in TBS-Tween20 (TBS-T). Antibodies against TrkB (Moses V. Chao Lab), pTrkB (Moses V. Chao Lab), and β-Actin (AC-74, Sigma) were diluted 1:1000, 1:500, and 1:5000 respectively in blocking buffer and added to the membranes which were then incubated overnight at 4 °C. The membranes were washed using TBS-T and then incubated with Secondary antibodies diluted at 1:1:5000 blocking buffer for 90 mins. Membranes were washed again in TBS-T, and bands were detected by chemiluminescence on ChemiDoc (Bio-Rad) using Clarity Western ECL Substrate (Bio-Rad). Results were analyzed using ImageJ software.

2.8 Statistical analysis

Unpaired t-test was used to measure statistical significance of the results. *: p < 0.05, **: p < 0.01, ***: p < 0.001, and ****: p < 0.0001.
Chapter 3

Results

3.1 Treatment of primary neuronal cultures with methionine induces Bdnf expression and TrkB signaling

Since a high protein diet rich in methionine leads to the induction of the bdnf gene expression in the hippocampus, we wanted to assess whether methionine induces bdnf expression in primary neuronal cultures. Primary neuronal cells (DIV6) were treated with 100 μM of methionine. RNA was extracted 16 hours later and Real Time RTPCR was performed. We found that the methionine treatment significantly induced BDNF p1 expression ($p=0.000498$; t-test) as well as coding mRNA expression ($p=0.060505$; t-test) (Fig. 7A and 7B). We were next interested in understanding whether methionine treatment activates the BDNF/TRKB signaling. For that purpose, we performed western blots on proteins extracted from the primary neuronal cultures treated with 100 μM methionine or vehicle. We observed a significant increase in the phosphorylation and thus activation of TrkB ($p=0.031221$; t-test) (Fig. 7C and 7D), the receptor of BDNF. Taken together, our results show that methionine treatment induces Bdnf expression and BDNF/TRKB signaling in primary neuronal cells.
A. Rel. Braf quantitation

B. Rel. Braf coding quantitation

C. Western blot images of Trk and Actin expression under Ctrl and MET conditions.
Figure 7. Methionine induces Bdnf expression and TrkB signaling in primary cortical neurons. (A) Real time quantification of Bdnf promotor I mRNA extracted from primary neuronal cell cultures treated with 100 μM of methionine. (B) Real time quantification of Bdnf coding mRNA extracted from primary neuronal cell cultures treated with 100 μM of methionine. (C) Representative blots of TrkB and pTrkB along with the loading control actin for extracts of primary cortical neurons treated with PBS (Control) and 100 μM methionine (MET). (D) Ratio of the relative quantification of pTrkB to TrkB as measured by ImageJ software. (n = 3 for Control, n = 3 for methionine; *: p < 0.05, ****: p < 0.0001)

3.2 Treatment of primary neuronal cultures with dimethyl α-ketoglutarate induces bdnf expression

Since α-ketoglutarate is a cofactor for histone demethylases, and demethylation has emerged as an important epigenetic mechanism that occurs in the brain, we also tested whether treatment with this metabolite induces bdnf expression. Primary neuronal cells (DIV6) were treated with 500 μM of dimethyl α-ketoglutarate. RNA was extracted 16 hours later and Real Time RTPCR was performed. We found that this dose significantly increased bdnf pI mRNA expression in the hippocampus (p=0.022347815, t-test), but not bdnf coding mRNA (p=0.251613205, t-test) (Fig. 8A and 8B). This result suggests that demethylation might also play a role in bdnf expression and thus function in learning and memory as well.
Figure 8. α-ketoglutarate induces Bdnf pI expression in primary cortical neurons. (A) Real time quantification of Bdnf promotor I mRNA extracted from primary neuronal cell cultures treated with 500 μM of dimethyl α-ketoglutarate. (B) Real time quantification of Bdnf coding mRNA extracted from primary neuronal cell cultures treated with 500 μM of dimethyl α-ketoglutarate. (n=2 for control, n=2 for α-ketoglutarate, *: p < 0.05)

3.3 Intra-peritoneal injection of methionine enhances spatial learning and memory

Since the injection of 1.55175 mg/Kg methionine has been shown to cause epigenetic changes in the brain (Wright et al., 2015), and since methionine induced BDNF/TrkB signaling in cell culture, we hypothesized that this dose might cause
changes in the epigenetic regulation of the Bdnf gene in the hippocampus, enhancing spatial learning and memory. We also administered injections of methionine combined with 80 μg/Kg FA (Naim et al., 2010), since an increase in FA can potentially regenerate methionine and thus enhance the effect, as well as (dose) of dimethyl α-ketoglutarate, for its effect on bdnf pI expression and demethylase function. For this purpose, we used the Morris Water Maze, which is a paradigm used to assess spatial learning and memory. It consists of a pool that is filled with water and divided into four equal quadrants, one of which contains a platform. On the first day, C57BL/6 mice are introduced into the clear water in order to get accustomed to the maze. Each mouse is given three trials, one minute each trial. The platform is visible on that day, and mice would naturally try to find a way out of the water by reaching this platform. After that and for 5 consecutive days, coloration is added to the water to make it opaque. The platform is no longer visible, and the mice would then depend on their spatial learning skills to find the platform. The latency to reach the platform is recorded every day. One day after the learning phase is over, the memory test is performed in which the platform is removed, mice are introduced into the maze for one minute, and the time spent by each mouse in the target quadrant that contained the platform is recorded. This test assesses short-term memory. A week later, the same test is performed to assess long-term memory. Six-week old mice were divided into four groups: a group receiving daily injections of saline, one receiving 1.55175 mg/Kg of methionine, one receiving 80 μg/Kg FA, one receiving 1.55175 mg/Kg of methionine and 80 μg/Kg FA, and one receiving (dose) of dimethyl α-ketoglutarate. Four hours after methionine injection or 30 mins after α-ketoglutarate injection, the testing was performed. Results obtained indicated enhanced spatial learning (Fig. 9A) and short-term memory (Fig. 9B) for the group receiving methionine injections. This suggests that methionine enhances spatial learning and memory possibly by inducing BDNF expression in the hippocampus. Results also show a slight additive effect for FA in the learning process. As for the α-ketoglutarate, we observed a significant enhancement of learning and memory on day 3, but eventually the treated mice performed similar to control mice by the end of the experiment (Fig. 10).
Figure 9. Methionine enhances mice spatial learning and memory performance in the Morris Water Maze. (A) The graph shows the average latency of each group to reach the platform per day. Mice receiving the combination treatment show the best enhancement of spatial learning skills. (B) The graph shows the average time spent in the target quadrant by each group. This is a reflection of short-term memory retention. The methionine receiving group showed a slight improvement in short-term memory. (n = 30 for Control, n = 33 for methionine, n = 8 for FA, n = 9 for methionine and FA combination; *: p < 0.05)
Figure 10. α-ketoglutarate shows a trend in enhancing spatial learning and memory performance in Morris Water Maze. (A) The graph shows the average latency of each group to reach the platform per day. Mice receiving the treatment show a significant enhancement on day 3 but eventually perform similar to the control group during the last days. (n = 13 for Control, n = 14 for α-ketoglutarate; *: p < 0.05)

3.4 Methionine rescues mice from social stress by restoring the lost BDNF signaling

Since we showed that methionine can induce bdnf expression and enhance spatial learning and memory, and since low levels of BDNF have been previously implicated in depression (Lopez et al., 2012), we aimed to test whether methionine can rescue mice in the CSDS paradigm, which is an animal model for depression. BALB/c mice which are naturally aggressive are used to induce social stress in the experimental mice. The mice were divided in to three groups: Control, Defeat (both receiving saline injections), and Defeat + methionine (receiving 1.55175 mg/Kg methionine injections). The defeat groups were put in direct contact with the aggressors every day for 10 days. On the 11th day, they were subjected to the social interaction test, where mice are allowed to wander freely in a three-compartment chamber, in which only one contains a social stimulus. The more time they spend in the chamber containing the stimulus, the less stressed they are. The time they spent in that chamber was considered the
interaction time, whereas the time they spent in the opposite chamber was considered the no interaction time. Our results showed a significant decrease in the interaction time and a significant increase in the no interaction time for the defeat animals when compared to the control, indicating that the social defeat was successful (Fig. 11A). Moreover, mice treated with methionine showed a significant reversal of the phenotype observed with the defeat group, indicating that the methionine treatment rescued the mice from social defeat stress (Fig. 11A). To check whether this effect was BDNF dependent, we performed a western blot on hippocampal tissues extracted from these mice after behavioral testing. We checked for BDNF signaling by probing for pTrk and Trk and calculating the ratio. Interestingly, we observed a significant decrease in the phosphorylation of TrkB receptor for the defeat group (p=0.019006; t-test), and a reestablishment of the phosphorylation levels for the group treated with methionine (Fig. 11B and 11C). This indicates that the effect of methionine is most probably through the induction of BDNF, further supporting our hypothesis.
Figure 11. Methionine rescues social defeat stress in a BDNF dependent manner. (A) The graph shows the interaction and no interaction times for each group. Methionine treatment restores the normal phenotype (n=17 for control, n=11 for DEF, n=20 for DEF + MET; * : p < 0.05, **** : p < 0.0001). (B) Western blot for hippocampal proteins extracted from the mice. A decrease in TrkB activation is observed with defeat animals, and this activation is restored with methionine treatment. (C) Quantification of western blot. (n=3 for control, n=3 for DEF, n=3 for DEF+MET)
3.5 Methionine enhances learning and memory in a BDNF-dependent manner

In order to determine whether the positive effect of methionine on learning and memory is BDNF-dependent, we are currently repeating the water maze. But this time, we plan to inhibit BDNF-signaling with the TrkB inhibitor Cyclotraxin-b.
Chapter 4
Discussion

BDNF has gained massive popularity in research due to its importance in the CNS. In this study, we were particularly interested in its role in learning and memory. Finding a way to increase the levels of BDNF in the hippocampus specifically is a very much worthwhile task, as the therapeutic implications of this are great. There have been several previous attempts to deliver BDNF to the brain, but none have reached complete success yet. One way to deliver BDNF is through direct infusion into the brain via a minipump (Kim & Jahng, 2004). Although the infusion was found to be beneficial in Kim & Jahng’s (2004) study, it is not quite feasible to insert and keep a minipump in a patient’s head for a prolonged period of time, as this is an invasive measure. Another attempt was through development of BDNF mimetics that would be delivered systemically and mimic the action of BDNF in the brain (Massa et al., 2010). The problem with such molecules is that they require tedious work in order to be used as medication due to many factors that should be taken into consideration: Crossing the blood-brain barrier, side effects, specificity, etc. Gene therapy has also been used to deliver BDNF \textit{in vivo} using Adeno-associated virus (Connor et al., 2015). The use of viruses for delivery is risky because there is a possibility of triggering immune responses after introducing the virus. In addition, \textit{ex vivo} delivery of BDNF via cells transduced with a viral vector expressing BDNF has been attempted (Rejali et al., 2007). However, such cells are difficult to keep viable long enough to be introduced into the body (Rejali et al., 2007). Moreover, peripheral administration of the BDNF molecule itself hasn’t been feasible since BDNF crosses the blood-brain barrier by a saturable transport system, so high doses do not cross (Pan et al., 1998). The best way to increase BDNF levels in the brain is thus to peripherally introduce molecules that would induce the endogenous expression of \textit{Bdnf} in the brain or increase the activities that would naturally induce \textit{Bdnf} in the brain. Examples of such cases have been found in our lab. Mice subjected to physical exercise \textit{ad libitum} exhibited increased levels of BDNF in their
Hippocampi (Sleiman et al., 2016). We also previously showed that $\beta$-hydroxybutyrate from the periphery mediates this induction in the brain (Sleiman et al., 2016). Lactic acid is another molecule we found that is produced naturally by the body after exercise, and when administered peripherally, it can induce $Bdnf$ expression as well.

Since methionine is an amino acid, and folic acid is a vitamin, we assumed that they both could easily cross the blood-brain barrier to reach the brain, as they are essential for the metabolism of any cell. What is interesting about these two molecules is that they are also involved in methylation reactions in cells, many of which are epigenetic modifications. The dose of 1.55175 mg/Kg methionine has been previously tested on cocaine-seeking behaviors in rats (Wright et al., 2015). Interestingly, methionine treatment was found to prevent the induction of the immediate early gene, c-Fos, in a DNA methylation-dependent pattern (Wright et al., 2015). It is logical to hypothesize that this specific methionine treatment could be affecting epigenetic methylation marks at the $Bdnf$ gene as well since it is also an immediate early gene. The dose of 80 $\mu$g/Kg of FA was chosen, as it was found to enhance recovery from brain injury by enhancing motor function and increasing exploratory interest, learning and problem solving (Naim et al., 2010). Both doses already showed an effect on the brain in vivo and were thus used in a learning paradigm. On the other hand, a dose response was performed in primary cortical neurons in vitro. Although most of the methionine doses induced an increase in both pI and coding $Bdnf$ mRNA (data not shown), the final concentration of 100 $\mu$M methionine in the well gave the most consistent results (Fig. 7). Furthermore, the 1.55175 mg/kg dose corresponds to a concentration of approximately 104 $\mu$M in the blood, assuming a 20 g mouse has 2 mL of blood. This is interesting because the in vivo and in vitro concentrations are very similar. We must note here that although the $Bdnf$ induction was observed in cortical neurons while we are testing the hippocampus, this does not pose a problem for two reasons: first, this kind of neuronal culture usually has traces of hippocampal cells, as it is difficult to isolate them. Second, since there is an effect on learning, which is hippocampus-dependent process, we assume that the effect is similar in the hippocampus. The increase in the pTrk/Trk
ratio in vitro after methionine treatment suggests an activation of the BDNF signaling pathway, which is what we require. We then proceeded to the animal testing.

The Morris water maze is used to test for spatial learning and memory (Morris, 1984). It depends on the fact that mice naturally tend to find an escape from the water, so swimming is a stressful activity that would motivate them to find the platform. At first, they take time to reach the platform, but after multiple trials for several days, they get better and better (Morris, 1984). Thus, they would take less time to reach the platform, so the latency decreases. Most importantly, they use the visual cues set up with the maze in order to locate themselves, navigate through the maze, and find the platform. With time, they learn exactly where the platform is placed and swim directly to it (Morris, 1984). Fig. 8A shows that the group treated with a combination of MET and FA displays the best learning curve, in which the latency to reach the platform decreased the fastest. This result suggests that MET+FA treatment has an effect on spatial learning and memory, which are hippocampus-dependent. On the contrary, Fig. 8B is not in according with the results in Fig. 8A, with only a slight improvement of memory for the methionine treated group. The memory here is assessed by the time mice spend in the quadrant that had the platform. It depends on the fact that the animals remember that the way to escape was roughly in that location, also using the visual cues. An explanation for this is that methionine and folic acid might be affecting pathways involved in learning and not memory. They could be two different pathways. We also note here that the effect of MET alone in the learning phase was not very pronounced when compared to the FA and MET+FA combination groups. It might be that the FA is the one affecting the learning process in vivo, probably due to the many essential processes it is involved in. As for the α-ketoglutarate, although a 500 μM dose led to a slight increase in the bdnf pI mRNA expression, it did not increase the coding mRNA and did not enhance spatial learning and memory in the same manner as that of methionine and folic acid. This suggests that methylation is of greater importance than demethylation when it comes to learning and memory.
What supports our hypothesis even further is the rescue of the defeated phenotype when methionine is administered to animals subjected to social defeat (Fig. 11A). Since the phosphorylation levels of hippocampal TrkB are restored after the decrease in defeated animals due to methionine treatment (Fig. 11B), we can assume that the positive effect of methionine is mediated by BDNF. This emphasizes the importance of methionine and its potential as therapy for depression as well.

Based on our results, supplementation with bioactive dietary compounds can have an effect on the brain epigenome. This highlights the importance of the quality of our diet and its impact on our mental health. Methionine and folic acid are both available in the diet and are thus regular nutrients for us. However, our results are particularly important for those who exhibit learning and memory deficits or impaired BDNF signaling. Simple supplementation of these two nutrients whether through injections or through the diet could in fact ameliorate the symptoms resulting from this impairment. Not only does this have implications on learning and memory, but also on any other disorder involving BDNF. For instance, deregulated BDNF/TrkB signaling plays a role in predisposition to depression, drug abuse, the progression of anxiety disorders, the pathophysiology of schizophrenia and bipolar disorder, and suicidal behavior (Gupta et al., 2013). Therefore, it is important to further investigate the effects of methionine and folic acid in other paradigms, as well as elucidate the methyltransferases involved and the exact pathways that mediate their effects.
References


