

Acute Administration of Methionine Affects Performance of Swiss Mice in Learning and Memory Paradigms

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Summary: Methionine, an essential amino acid, plays an essential role in the central nervous system CNS development. It serves as a crucial intermediate in the methylation, trans-sulfuration and amino-phosphorylation pathways, necessary for the synthesis of nucleic acids, phospholipids, hormones, neurotransmitters, antioxidants, polyamines, catecholamines and other biogenic amines. The effect of methionine on learning and memory in mice was investigated using Morris water maze (MWM), Elevated plus maze (EPM) and Y maze (YM). Animals were administered with distilled water (control), methionine (1,700mg/kg); folate (3mg/kg) or methionine (1700mg/kg) plus folate (3mg/kg) for 14 days. Escape latency and time spent in target quadrants; transfer latency and percentage spontaneous alternations were measured in the MWM, EPM and YM respectively. The animals were anaesthetized with inhalational chloroform and their brains subsequently harvested, homogenized and assayed for acetylcholinesterase 24 hours after the experiment. Folate significantly ($p < 0.05$) increased transfer latency (53.33 ± 12.62) as compared to control (20.1 ± 5.01) and reduced spontaneous alternations significantly (25.0 ± 8.9) when compared to control (44.33 ± 3.07). When folate was combined with methionine there was also a significant increase in transfer latency (43.0 ± 14.39) when compared with control (20.1 ± 5.01). Folate-methionine combination also significantly ($p < 0.05$) reduced spontaneous alternations (20.4 ± 8.4) as compared to the control (44.33 ± 3.07) much more than folate alone. Acetylcholinesterase activities in all groups were not statistically significant. It can be concluded that acute methionine administration has some benefits in memory enhancement. However, a short course folate supplementation impairs learning and working memory especially when combined with methionine which may be as a result of sudden overwhelming of the methylation cycle, leading to homocysteinemia which is pro-dementia.

Keywords: Methionine, Memory, Learning, MWM, EPM, Y-Maze

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INTRODUCTION

Memory is one of the earliest cognitive functions to show decline during the aging process. Due to growing stress and competition in our world, memory related problems are multiplying (Parle and Vasudevan, 2007; Sunita *et al.*, 2010). It is projected that by 2050, people aged 60 and over will account for 22% of the world's population with four-fifths living in Asia, Latin America or Africa. This translates to more cases of either pathologic (e.g Alzheimer's disease) or physiologic (e.g Age associated memory impairment) memory losses (WHO, 2013). It is pertinent to note that myriads of supplements including—caffeine, *Ginkgo biloba*, *Bacopamonniera* etc have been employed to reverse memory loss, no significant outcome has been achieved (Sunita *et al.*, 2010).

Methionine, an essential amino acid, is an important substrate in the brain's methylation cycle. Interference with its metabolism has been found to result in hyperhomocysteinemia which is pro-dementia (Miler,

2003). It is an indispensable dietary amino acid required for normal growth and development of humans; a substrate for protein synthesis and the main methyl group donor to both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) intermediates (Mohammad *et al.*, 2006; Deborah *et al.*, 2007). Methionine gets activated by reacting with ATP to form S-adenosylmethionine (SAME), which when decarboxylated, results in biosynthesis of polyamines necessary for cell proliferation and growth (Robert *et al.*, 2009). Also, methionine residues constitute an important antioxidant defense mechanism via formation of methionine sulfoxide with free radicals (Rodney, 1996) and production of potent antioxidants like glutathione and cystathione necessary for preventing cellular damage (Tor-Agbidye, 1998; Tor-Agbidye, 1999; Nkabyo, 2006). Chronic administration of methionine has been found to cause memory deficit in the Morris water maze task by the elevation of blood homocysteine levels in experimental animals. High homocysteine levels have

the potentials of causing vascular dementia either by direct cerebral vascular damage, over-activation of N-methyl-D-aspartate receptors or enhanced vulnerability of hippocampal neurons to excitotoxic insults (Rajeshkumar et al., 2008; Rajeshkumar et al., 2009). Similarly, chronic blood homocysteine (HCY) levels have also been shown to cause adult neurological disorders by excitotoxicity and generation of reactive oxygen species (ROS) (Louisa, 2004).

Central cholinergic system plays a major role in regulation of cognitive functions and inhibition of acetylcholinesterase leads to increased levels of brain acetylcholine (Dinesh and Kumar, 2012). Dementia illnesses (e.g Huntington's disease) has been proven to result from loss of acetylcholine-secreting neurons in the thinking areas of the cerebral cortex. Measuring levels of brain acetylcholinesterase would be a good way of knowing the brain acetylcholine levels which is a good parameter for assessing memory functions (Guyton and Hall, 2011).

To the best of our knowledge, there are paucity of data on the effect of acute administration of methionine on learning and memory in normal healthy subjects. We employed multiple mazes for a more reliable result, considering the fact that it is unsafe to rely on any one assay in making a claim regarding the psychological basis of a behavioral phenotype (Laurence, 2004).

The present study aimed at investigating the effect of methionine and folate supplementation on learning and memory in rodents. It simply seeks to know the effect of an essential amino acid like methionine (known to mediate various cellular reactions) on the learning and memory functions of an apparently normal brain. This is in a bid to proffer long-term prophylactic solutions to memory impairment that may occur in later life.

MATERIALS AND METHODS

Chemicals and Drugs

Methionine (Neros pharmaceuticals {Vietnam} Batch number: 11007CX, Registration number: VD5202-08, NAFDAC number: A4-0546). Folic acid (Pharmacy of Saint Luke's Anglican Hospital Wusasa, Zaria, NAFDAC number 040169)

Animals

A total of 20 Male Swiss mice weighing between 15-25g(6-8weeks) were obtained from the Animal House, Federal College of Animal Health Facility, National Veterinary Research Institute, Vom, Plateau State, Nigeria. They were housed in standard polypropylene cages in the Animal House Facility, of the Department of Human Physiology, Faculty of Medicine, Ahmadu Bello University Zaria, Nigeria under normal environmental temperature and were fed with standard

pellet diet and water, *ad libitum*. They were allowed to acclimatize to the laboratory environment for one week before the commencement of the experiment. They were randomly divided into four groups of 5 animals each (Normal control, methionine treated, methionine plus folate and folate only group). Methionine was administered orally at a dose of 1700mg/kg (Rajeshkumar, 2009) and folate was at 3mg/kg (Shin et al., 1999) for 14 days. The animals were tested in the various mazes between day 11 and 14 and were euthanized on day 15 with subsequent craniotomy for acetylcholinesterase assessment.

MAZES

14 days Supplementation of Methionine and Folate in the Morris Water Maze (MWM) experiment

MWM was first described by Morris (Morris, 1981). It was used to assess visuo-spatial memory, reference memory and learning which involves using extra maze cues to find location of a hidden escape platform (Villarreal et al., 2002). The maze constructed out of a circular plastic tank was filled with water at room temperature to a depth of 14 cm to enable the animal swim unhindered. A sealed cylindrical plastic container submerged 1cm below the water surface in the escape quadrant served as the escape platform. The animals were subjected to a 3-day acquisition training phase with starting points changed sequentially as shown in Table 1.

Table 1. Sequence table for acquisition training in MWM

DAY 1	Q1	Q2	Q3	Q4
DAY 2	Q2	Q3	Q4	Q1
DAY 3	Q3	Q4	Q1	Q2

The time (escape latency) to locate the hidden platform after 120seconds of exploration on each day was noted. Reduction in escape with training is a positive index of learning and memory recall. In case an animal fails to locate the hidden platform it was assisted to the platform and allowed to stay there for 30 seconds to build cohesive visuospatial memory and appropriate representation of the pool. The fourth day being the last day (the probe phase), each animal was given a single trial. They were released into the pool from Q4 (where the hidden platform has always been) but this time without the hidden platform to explore for 120 seconds. The total time spent in Q4 in search of the removed platform was measured. Prolonged time spent in the target quadrant in search of the absent platform was an index of positive memory recall.

14 Days Supplementation of Methionine and Folate in the Elevated Plus Maze (EPM) Experiment

The Elevated plus maze for mice (Lister, 1987) which have also been employed in studying learning and retention in experimental animals was also used. The maze consisted of two perpendicular open arms and

closed arms. The open and closed arms were connected by a central platform and raised 45 cm above the floor. The entire experiment lasted two days as described by Jiro (1990) and Dinesh *et al* (2012). On the first day (after completing the day-3 acquisition trial on the MWM) each animal was placed at the end of an open arm facing away from the central platform. The time taken (transfer latency) for the animal to move from the open arm to the enclosed arm within the space of 90seconds was measured. This process was repeated the next day (1 hour after completing probe trial on the MWM experiment). If the animal fails to enter the closed arm on the first day, it was gently assisted into one of the closed arms and allowed to remain there for 20seconds to enable it integrate memory. After each trial the maze was wiped with a cloth dipped in 70% ethyl alcohol and allowed to dry, to prevent subsequent animals from getting any clue. The transfer latency for Day 1 was designated T1 and that of Day 2 T2. A short transfer latency on Day 2 was taken as a measure of good memory recall.

14 days Supplementation of Methionine and Folate in the Y- Maze experiment

The Y-maze as described by Reddy (Reddy, 1998) was also used to assess working and spatial memory. It consisted of three identical arms A, B and C at 120° to each other. This test was based on the innate preference of animals to explore an arm that has not been previously explored (Swonger and Rech, 1972; Drew, 1973; Hughes, 2004). Approximately 1 hour after completing the day-2 EPM experiment, trial 1 for the Y-maze experiment was commenced. One of the arms of the maze was blocked with a shutter, allowing for a 5-min exploration of only two arms. After a 30 min delay, trial 2 was commenced in similar manner, this time with all three arms open for another 5-minutes exploration. Trial 2 took advantage of the innate tendency of mice to explore novel unexplored areas (the previously blocked arm). After each trial, the maze was wiped with a cloth dipped in 70% ethyl alcohol and allowed to dry, to remove any olfactory clue. The number of maximum spontaneous alternations (total number of arms entered minus two) and actual alternations (i.e ABC, ACB, BAC, BCA, CAB, OR CBA). Percentage alternation was therefore calculated thus:

$$\text{percentage alternations} = \frac{\text{actual alternations}}{\text{maximum alternations}} \times 100$$

High percentage alternations were considered as positive index of memory recall.

Brain preparation and Acetylcholinesterase (Ache) activity

The animals were sacrificed 24 hours after the last experiment by cervical dislocation under light anaesthesia with chloroform vapour. Following craniotomy, brain homogenization was done Methionine in Learning and Memory

according to Luigi *et.al* (1968) with slight modifications. The brains of the animals were gently homogenized with a small pestle and mortar using 0.1 M sodium phosphate buffer at pH of 8. The tissue concentration was approximately 20mg of tissue per ml of buffer. The homogenates were cold centrifuged.

Samples were transferred into plain sterilized bottles and taken to Chemical Pathology Department, Ahmadu Bello University Teaching Hospital, Shika, Zaria-Nigeria for biochemical analysis. Acetylcholinesterase activities were determined spectrophotometrically using the Ellman method (Ellman *et al.*, 1961). The Ellman method for assaying enzymes is based on the reaction between thiols and chromogenic 5'5'-dithiobis-2-nitrobenzoic acid (DTNB) as it measures the formation of the yellow ion of 5'-thio-2-nitrobenzoic acid (TNB). Cholinesterase activity is measured indirectly by quantifying the concentration of TNB ion formed (i.e a product of substrate hydrolysis by cholinesterase). Absorbance was measured at a wavelength of 412mp using a spectrophotometer. Rates were calculated as follows:

$$R = \frac{(5.74 \times 10^4)AA}{C}$$

R = Rate, in moles of substrate hydrolyzed per minute per gram of tissue, AA= Change in absorbance per minute, C= Original concentration of tissue (mg/ml)

Statistical Analysis

Data obtained from the study were expressed as mean ± SEM. The differences between the groups were analyzed by One-way analysis of variance (ANOVA) followed by Dunnett post hoc test for multiple comparisons using SPSS statistical tool version 22. Values of p < 0.05 were considered significant.

RESULTS

Escape latency in MWM

There was found no statistically significant difference across the groups as seen in Fig 1. The average escape latency decreased consistently in all groups.

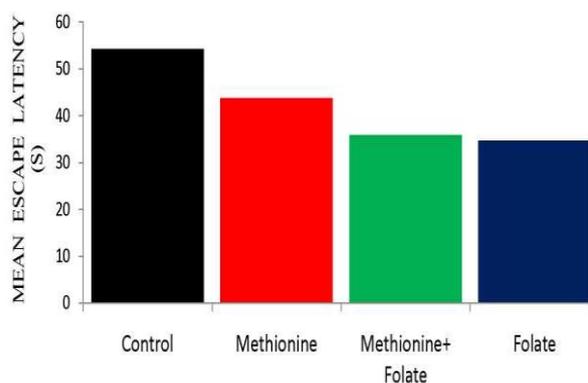


Fig 1. Effect of 14 days supplementation with methionine and folate on mean escape latency in MWM.

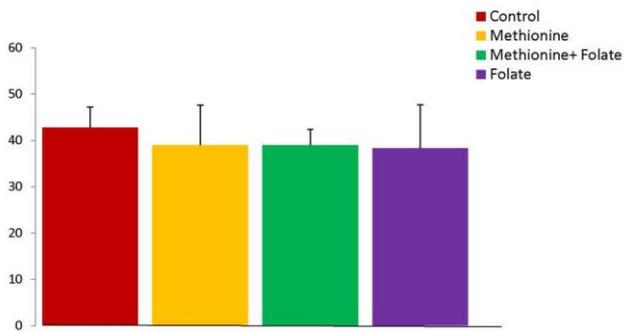


Fig 2. Effect of 14 days supplementation with methionine and folate on mean time spent in target quadrant in MWM.

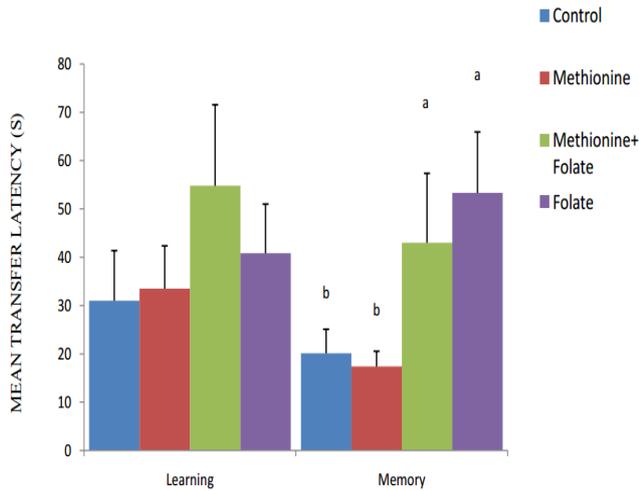


Fig 3. Effect of 14 days supplementation with methionine and folate on mean transfer latency in EPM. ^asignificant increase in transfer latency, ^bsignificant decrease in transfer latency.

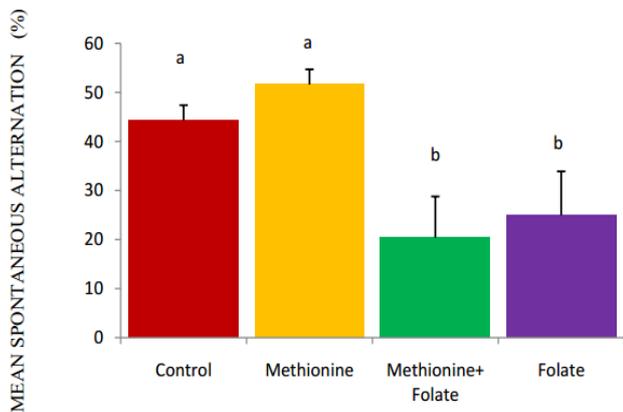


Fig 4. Effect of 14 days supplementation with methionine and folate on mean spontaneous alternation in Y-Maze. ^asignificant increase in mean spontaneous alternations, ^bsignificant decrease in mean spontaneous alternations

Mean time spent in target quadrant in MWM

The mean time spent in target quadrant was similar across groups. There was no statistical difference between the methionine groups compared with the control. There was also no difference between the other groups and the control group as seen in Fig 2.

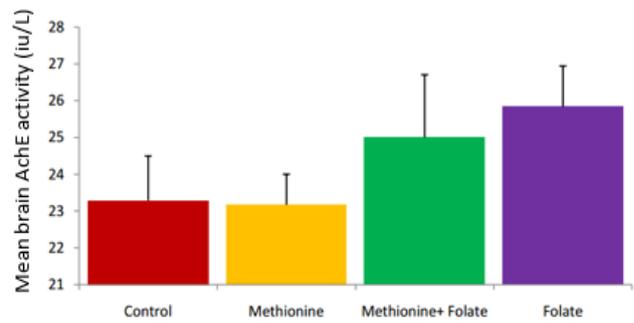


Fig 5. Effect of 14 days supplementation with methionine and folate on mean brain acetylcholinesterase activity.

Mean transfer latency in EPM

The group with methionine plus folate and that with folate alone had a significant reduction in memory recall when compared to the methionine and control group. The methionine-only group had a better performance in both learning and memory as evidenced by their shorter transfer latencies as shown in fig 3.

Percentage spontaneous alternations in Y-Maze

The methionine plus folate and the folate-only groups showed a statistically significant decrease in spontaneous alternations compared with the control as shown in Fig 5. The methionine group had the highest mean percentage spontaneous alternation but not statistically significant when compared with the control.

Acetylcholinesterase activity

There is no statistically significant difference in the brain acetylcholinesterase activity in all groups as shown in Fig 5.

DISCUSSION

The present study investigated the effect of 14 days methionine and folate supplementation on learning and memory paradigms in Swiss mice. Morris Water Maze, Elevated Plus Maze and Y Maze tests employed in this study, are widely accepted models for evaluating learning and memory in experimental animals (Rajeshkumar et al., 2008; Brian et al., 2009; Robert, 2004; Dinesh et al., 2012).

The other groups had a reduction in escape latency compared to control but not statistically significant. Time spent in target quadrant increased proportionately across groups indicating good memory retrieval in all groups. This agrees with the work of Rajeshkumar et al (2008) where a similar downward trend in escape latency time (ELT) was seen in methionine-treated rats during the acquisition trials (Rajeshkumar et al., 2008). A study by Cao et al (2008), agrees to some extent with this work, where they demonstrated both electrophysiologically and by Morris water maze test that S-adenosyl methionine

administered for a period of 20-22 days improved impaired learning ability induced by lead (Cao *et al.*, 2008). Similar results could be seen in the work of Sheryl *et al* (2008) where short-term administration of S-adenosyl methionine improved working memory in transgenic mice (Sheryl *et al.*, 2008). However, on the contrary, the work of Rajeshkumar *et al* showed that methionine administered at a similar dose of 1.7 g/kg/p.o significantly reduced time spent in target quadrant during the probe trial indicating memory impairment. This could possibly be due to the fact that they administered methionine for 32 days which was more than twice the time of exposure to methionine in this experiment. So, such chronic exposure may have resulted in the vascular dementia they found in their animals resulting from high homocysteine following chronic levels of methionine that overwhelms the brain methylation cycle (Rajeshkumar *et al.*, 2009). A similar but remote study by Wikken *et al* (1998) also revealed that individuals at risk of coronary artery disease have reduced ability to metabolize homocysteine when their methylation pathway is stressed by administering loading dose of methionine (Wikken *et al.*, 1998). The age of the animals used in this study may also support the contrasting result, seeing that previous studies involved administration of methionine to adult and aged rats resulted in memory impairment. Still in support of the effect of age variation, Dasarathy *et al* (2010) propounded that young human subjects (neonates) have more efficient transsulfuration and transmethylations processes due to their high demand of glutathione from methionine metabolism (Dasarathy *et al.*, 2010).

The folate-treated mice and the folate plus methionine-treated group in the EPM experiment had significantly prolonged transfer latency when compared with the methionine and the control groups. This is an indication of suppressed short term/working memory. No study could be found that employed methionine in assessing learning and memory using specifically the elevated plus maze. Even though the results of this study tend to contradict some research findings that associate folate with improved cognitive function especially memory (Matteet *et al.*, 2009; Jane *et al.*, 2007; Linda *et al.*, 1999; Huang *et al.*, 2007). Some other studies however have shown that folic acid supplementation does not affect cognitive function. In a human trial, where 195 people aged 70 years and older with no or moderate cognitive impairment received either of folic acid, folic acid plus Vitamin B12 or a placebo for 24 weeks; there was no improvement in cognitive function (Eussen *et al.*, 2006). Similarly, another long term study found greater cognitive decline in people with a high intake of folic acid. The researchers therefore remarked that these findings were “unexpected” and called for further studies (Morris *et al.*, 2005). In support of the

homocysteine pathway for memory impairment in folate deficiency, some studies claim that folic acid reduces high homocysteine levels but other studies propose the reverse (Malinow *et al.*, 1999). Possibly, folate is being converted to more methionine within the methylation cycle, thus resulting in hypermethioninemia and ultimately homocysteinemia, which may have resulted in the poor performance noticed in both the folate and folate-methionine groups.

The Y-maze gave a similar result with the EPM experiment. The folate only and folate- methionine groups performed poorly (reduced percentage spontaneous alternations) at p value <0.05 when compared with the methionine-only and control groups, signifying memory impairment by these supplementations. The methionine-only group had a comparatively better performance when compared to the control group though not statistically significant. This contradicts the work of Zhang *et al* (2012) where folate prevented impairment in Y-maze performance in animals demented from middle cerebral artery occlusion. It however tends to agree with the work of Carla *et al* (2007) where they discovered that folate supplementation had only very minimal benefit in apolipoproteinE deficient mice lacking B-vitamins when tested on a T-maze; with no benefit at all in a water maze experiment. Again to the best of our knowledge no work has been found which investigated the effect of methionine on working memory using the Y-maze. Similar explanation as in the EPM experiment can be proffered i.e. more folate results in hypermethioninemia leading to homocysteinemia and subsequently poor memory in the folate and folate-methionine groups.

The minimal positive effect of methionine could possibly be attributed to its sulfur component which has similar activity as other sulfur-containing amino acids like cysteine and glutathione which has been well established as potent antioxidants; protecting neural cells not only from oxidant damage and apoptosis but also from progression of the pathology of dementia illnesses (Andrew, 2006; Andrew *et al.*, 2010).

Acetylcholine is considered to be one of the important neurotransmitters involved in the regulation of cognitive functions. Cognitive dysfunction has been shown to be associated with impaired cholinergic transmission and the facilitation of central cholinergic transmission resulting in improved memory. Moreover, selective loss of cholinergic neurons in certain brain parts appeared to be a characteristic feature of senile dementia (Dinesh *et al.*, 2012). Brain acetylcholinesterase activity in this study remained stable in all groups. This contradicts the finding of Rajeshkumar *et al* in which chronic administration of methionine at 1.7g/kg p.o led to a rise in brain

acetylcholinesterase activity. The possible explanation of this discrepancy could be as a result of the differences in the duration of exposure to methionine, where in this study, it was more a less an acute exposure. To support this assertion, Franciella et al (2007) in their work showed that acute administration of methionine did not alter cerebral cortex AChE activity, rather only chronic experimental hypermethioninemia caused cognitive dysfunction and an increase of AChE activity that might be related, at least in part, to the neurological problems presented by hypermethioninemic patients (Franciella et al., 2007).

In conclusion, this study employed the use of multiple neurobehavioural models in assessing the possible effect of acute methionine administration on learning and memory paradigms in Swiss mice. Methionine was found to possess minimal benefits on learning and memory which may be attributable to its sulfur component which is neuroprotective. Acute folate supplementation proved counterproductive when the EPM and Y-maze were employed as test models; more so when combined with methionine. Possible overwhelming of the brain methylation cycle, leading to increased homocysteine (which is pro-dementia) could be an explanation. Further work will involve investigating the influence of graded doses of methionine on learning and memory. Also, assaying other brain neurotransmitters like dopamine, glutamate as well as neurotrophic factors such as brain derived neurotrophic factor (BDNF) in a bid to establishing possible mechanism(s) of action.

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