THE EFFECTS OF METHIONINE-ENRICHED AND VITAMINS (FOLATE, PYRIDOXINE AND COBALAMINE)-DEFICIENT DIET ON EXPLORATORY ACTIVITY IN RATS - A BRIEF REPORT

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EFEKTI METIONINOM-OBOGAĆENE I VITAMINIMA (FOLATI, PIRIDOKSIN I KOBALAMIN) DEFICIJENTNE DIJETE NA EKSPLORATIVNU AKTIVNOST PACOVA-KRATAK IZVEŠTAJ

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ABSTRACT

The aim of this study was to evaluate the impact of increased homocysteine levels induced by methionine nutritional overload (twice as standard) and deficiency of the vitamins folate, pyridoxine and cobalamine, which plays an important role in homocysteine metabolism in anxiety-related behaviour, expressed by means of exploratory activity in rats. Twenty-three male Wistar albino rats (4 weeks old, 100±15 g body weight) were divided into three groups: control (n=8), methionine-enriched (Meth+, 7.7 g of methionine/kg chow, n=7) and methionine-enriched vitamin-deficient (Meth+Vit-, 7.7 g of methionine/kg chow, deficient in folate, pyridoxine and cobalamine - 0.08, 0.01 and 0.01 mg/kg, n=8). All animals had free access to food and water for 30 days. Behavioural testing was performed using the elevated plus maze (EPM) test. Standard parameters for vertical exploratory activity, the number of rearings and the number of head-dippings, as well as the total exploratory activity (summarizing overall exploratory activity in the EPM) were significantly reduced following 30 days of methionine nutritional overload ($p<0.05$, $p<0.05$ and $p<0.01$, respectively). A methionine-enriched diet coupled with a reduction in some B vitamins resulted in a more pronounced decline in exploratory drive observed in the EPM test compared to the control ($p<0.01$). The decline in total exploratory activity associated with vitamin deficiency was significant compared to the Meth+ group ($p<0.05$). The results of this study highlight the important role of homocysteine in the modulation of exploratory activity in rats. Decreased exploratory drive induced by both a methionine-enriched and vitamin-deficient diet could be attributed to an anxiogenic effect of hyperhomocysteinemia.

Keywords: *methionine, homocysteine, folate, pyridoxine, cobalamine, exploratory activity, anxiety, rats*

SAŽETAK

Cilj ovog istraživanja je bio ispitivanje uticaja povišenih nivoa homocisteina, uzrokovanih povećanim sadržajem metionina u hrani (dvostruko u odnosu na standard), kao i deficijencijom vitamina (folati, piridoksin, kobalamin) koji imaju značajnu ulogu u metabolizmu homocisteina, na nivo anksioznosti koji se iskazuje u ponašanju. Dvadeset tri Wistar albino pacova muškog pola (4 nedelje starosti, 100±15g telesne mase) je podeljeno u 3 grupe: kontrola (n=8), metioninom obogaćena (Meth+, 7,7 g metionina/kg hrane, n=7) i metioninom obogaćena i deficijentna u sadržaju vitamina (Meth+Vit-, 7,7 g metionina/kg hrane, deficijentna u sadržaju folata, piridoksina i kobalamina - 0,08, 0,01 i 0,01 mg/kg, n=8). Sve životinje su imale slobodan pristup hrani i vodi tokom 30 dana. Bihejvioralno testiranje je sprovedeno u testu uzdignutog krstastog lavirinta (UKL). Standardni parametri vertikalne eksplorativne aktivnosti, broj uspravljanja i broj naginjanja, kao i ukupna eksplorativna aktivnost (sumacija sveukupne eksplorativne aktivnosti u UKL), su bili značajno smanjeni nakon 30 dana povećanog unosa metionina ($p<0,05$, $p<0,05$ i $p<0,01$). Metioninom obogaćena ishrana, udružena sa smanjenjem pojedinih B vitamina, je uzrokovala dodatno smanjenje eksplorativne aktivnosti u UKL testu u odnosu na kontrolu ($p<0,01$). Smanjenje ukupne eksplorativne aktivnosti uzrokovano dodatnom deficijencijom vitamina je bilo značajno u odnosu na Meth+ grupu ($p<0,05$). Rezultati ove studije naglašavaju značajnu ulogu homocisteina u modulaciji eksplorativne aktivnosti kod pacova. Smanjena eksplorativna aktivnost uzrokovana metioninom obogaćenom ishranom i deficijencijom vitamina u hrani se može smatrati anksiogenim efektom hiperhomocisteinemije.

Ključne reči: *metionin, homocistein, folati, piridoksin, kobalamin, eksplorativna aktivnost, anksioznost, pacovi*

ABBREVIATIONS

BHMT - betaine-homocysteine methyl-transferase
CBS - cystathionine-beta-synthase
CNS - central nervous system
CSE - cystathionine-gamma-lyase
EPM - elevated plus maze
Hcy - homocysteine
SAM - S-adenosylmethionine
TEA - total exploratory activity

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INTRODUCTION

Homocysteine (Hcy) is a non-protein-forming, sulfur-containing, non-essential amino acid. Increased Hcy levels have been associated with a diversity of diseases, including cardiovascular diseases (1, 2), malignancies (3), birth defects and pregnancy complications (4, 5), as well as with central nervous system (CNS) dysfunction (6, 7).

Hcy is synthesized by transmethylation of the (essential) diet-derived amino acid methionine. Its metabolism may be represented as the intersection of two metabolic pathways: remethylation and transsulfuration (8). In the remethylation pathway, Hcy is recycled to methionine under the catalysis of methionine syntase or betaine-homocysteine methyltransferase (BHMT). In the transsulfuration pathway, Hcy is condensed with serine, forming cystathionine (by enzyme cystathionine-beta-synthase, CBS), which is subsequently hydrolized to cysteine (by enzyme cystathionine-gammalyase, CSE), essential for the formation of glutathione, or finally decomposed to taurine (9-11).

Folic acid (B_9), pyridoxine (B_6) and cobalamine (B_{12}) are crucially involved in processes important for homocysteine metabolism. B_6-dependent enzymes CBS and CSE are basically involved in the transsulfuration pathway, as previously described (12). Folate and vitamin B_{12} are involved in the remethylation reactions of Hcy back to methionine to complete the methyl cycle (folate/B_{12}-dependent remethylation). Folate, in the form of coenzyme N-5-methyl tetrahydrofolate, can donate a methyl group to Hcy in a reaction catalyzed by the vitamin B_{12}-dependent enzyme methionine synthase. The remethylation reactions of Hcy to methionine may also include folate/B_{12}-independent remethylation, which involves betaine-homocysteine methyltransferase (BHMT). Therefore, vitamins B_9 and B_{12} play an important role in Hcy content within the cell and, subsequently, in plasma level circulation (12).

Hyperhomocysteinemia represents a risk factor for numerous disorders affecting the CNS, such as neurological cognitive deficit, mental retardation, demyelination (13, 14), Alzheimer's disease (15), Parkinson's disease (16), stroke (17) and schizophrenia (18). The strong causal connectivity between elevated Hcy levels and this diversity of CNS disorders may be found in some differences in Hcy metabolism that are specific for brain tissue. It has been reported that transmethylation of Hcy in the brain is limited exclusively to the folate/B_{12}-dependent pathway due to lack of BHMT in brain tissue. The other metabolic pathway of Hcy, transsulfuration, in brain tissue is also limited due to lack of CSE (also observed in adipose tissue). Therefore, the metabolic processes that can limit Hcy accumulation in brain tissue are limited only to folate/B_{12}-dependent remethylation, which may explain the higher vulnerability of the CNS to increased Hcy levels (19). In addition, the methylation reactions that are critical for the proper synthesis of serotonin, other monoamine neurotransmitters and catecholamines (20), which play an important role in mood regulation, largely rely on B vitamin cofactors (B_6, B_9 and B_{12}).

Numerous clinical trials have provided additional information on the importance of the factors that may affect Hcy metabolism in the genesis of various mood disorders. Therefore, it has been reported that patients with depression had lower serum levels of folate and vitamin B_{12} (21, 22). Additionally, numerous anxiety disorders are related to impaired one-carbon metabolism (23-26).

The aim of this study was to evaluate the impact of increased Hcy levels induced by methionine nutritional overload, as well as by deficiency in vitamins (folate, pyridoxine and cobalamine) that play an important role in homocysteine metabolism in anxiety-related behaviour, expressed by means of exploratory activity in rats.

MATERIALS AND METHODS

Animals and treatment

The dietary protocols were performed on 23 male Wistar albino rats (4 weeks old, 100±15 g body weight). Rats were housed in standard environmental conditions (12/12 h light/dark cycle, 23±1 °C) in polycarbonate cages (2 animals per cage). The rats were randomly divided into three groups: control (n=8), methionine-enriched (Meth+, n=7) and methionine-enriched vitamin-deficient (Meth+Vit-, n=8). The animals in the control group were fed with standard chow, whereas the animals in the two other groups were fed with commercial methionine-enriched chow (7.7 g of methionine/kg) combined with variable vitamin B content (Mucedola SRL., Milan, Italy). Methionine content in enriched diets was twice as high compared to that in standard chow (3.85 g of methionine/kg, (27). The Meth+ group received methionine-enriched chow with regular vitamin B content (folate, pyridoxine and cobalamine: 2.0, 70.0 and 0.03 mg/kg, respectively). The Meth+Vit- group was fed with the methionine-enriched chow that was deficient in folate, pyridoxine and cobalamine (0.08, 0.01 and 0.01 mg/kg, respectively). All animals had free access to food and water. Applied dietary protocols lasted for 30 days.

Behavioural testing

Behavioural testing was performed 24 h following the completion of dietary pretreatment. Rats were transported in their home cages to the testing room (approx. at 9 a.m.) and allowed to acclimate 1 hour prior to testing. Testing was performed in the elevated plus maze (EPM), as the EPM test is commonly considered a standard for measuring anxiety levels in rodents (28). EPM consisted of two opposite open arms (50x20 cm) and two opposite enclosed arms (50x20x30 cm), elevated 100 cm from the floor. Each rat was placed in the centre of the elevated plus maze facing the open arm and was allowed 5 minutes of free exploration. The following parameters of exploratory activity in the EPM test were estimated: the number of rearings, the number of head-dippings and the total exploratory activity (TEA). The number of rearings and the number of

head-dippings are considered indicators of anxiety level (28, 29) because exploratory drive is in conflict with fear of the unknown, i.e., anxiety (30). To estimate overall exploratory activity in the EPM test, we used a recently proposed parameter—total exploratory activity (31)—which includes patterns of exploratory activity observed in different zones of the EPM (closed and open arms). TEA is calculated as the sum of the numbers of rearings and head-dippings during 5 minutes of testing in the EPM. EPM tests were recorded by digital camera, mounted above mazes at the appropriate height, under proper conditions of silence and illumination. Video files were analysed using Ethovision software XT 12, Noldus Information Technology, the Netherlands.

All research procedures were carried out in accordance with the European Directive for the welfare of laboratory animals No: 2010/63/EU and Principles of Good Laboratory Practice (GLP). The protocol for the current study was approved by the Ethics Committee for experimental animal wellbeing of the Faculty of Medical Sciences of the University of Kragujevac, Serbia (No: 01-11794).

RESULTS

Both the methionine-enriched diet and the methionine-enriched, folate-, vitamin B_6- and B_{12}-deficient diet resulted in significant decline in exploratory activity in the EPM test by means of the number of rearings ($F=9.011$, $df=2$), the number of head-dippings ($F=10.851$, $df=2$), and total exploratory activity ($F=19.779$, $df=2$).

As shown in Fig. 1, increased methionine intake for one month induced significant decreases in vertical exploratory activity in the EPM test, expressed as the number of rearings, compared to the control ($p<0.05$). The methionine-enriched diet coupled with the total restriction of folate, vitamin B_6 and vitamin B_{12} intake also reduced the number of rearings in the EPM test compared to the control ($p<0.01$), but this additional decline in exploratory activity was not significant when compared to the group on the methionine-enriched diet.

The prolonged intake of methionine-enriched chow also reduced the other pattern of vertical exploratory activity in the EPM test, the number of head-dippings (Fig. 2), compared to the control ($p<0.05$). Folate, vitamin B_6 and B_{12} deficiency, simultaneously applied with the methionine-enriched diet, resulted in an even greater reduction in the number of head-dippings when compared to the control group ($p<0.01$). However, such a decrease in this behavioural pattern was not significant compared to the effects of the methionine-enriched diet with sufficient folate, vitamin B_6 and B_{12} intake.

The total exploratory activity in the EPM, expressed by means of the sum of the number of rearings and the number of head-dippings, was confirmed to be a more sensitive marker of exploratory activity in the EPM test, as it covers both patterns of vertical exploratory activity (Fig. 3). Therefore, both applied dietary protocols resulted in significant reductions in the total exploratory activity compared to the control ($p<0.01$). Moreover, unlike the indi-

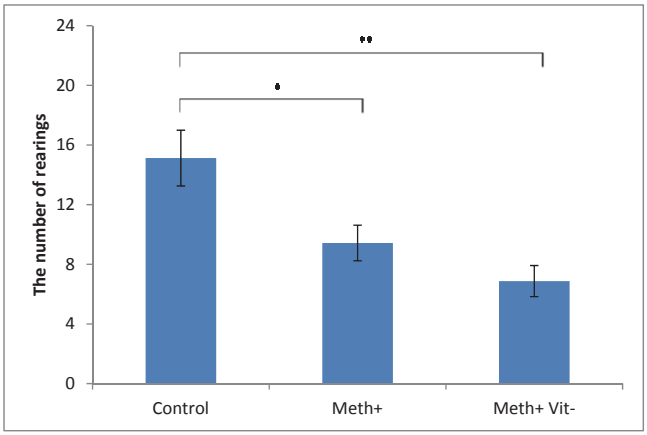

Figure 1 The number of rearings (Mean ± SEM, *denotes a significant difference $p<0.05$, **denotes a significant difference $p<0.01$). Control: control group, Meth+: methionine-enriched diet group, Meth+Vit-: methionine-enriched vitamin-deficient diet group.

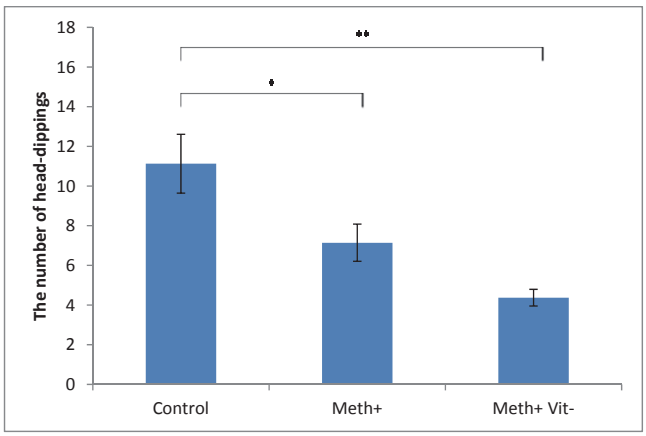

Figure 2 The number of head-dippings (Mean ± SEM, *denotes a significant difference $p<0.05$, **denotes a significant difference $p<0.01$). Control: control group, Meth+: methionine-enriched diet group, Meth+Vit-: methionine-enriched vitamin-deficient diet group.

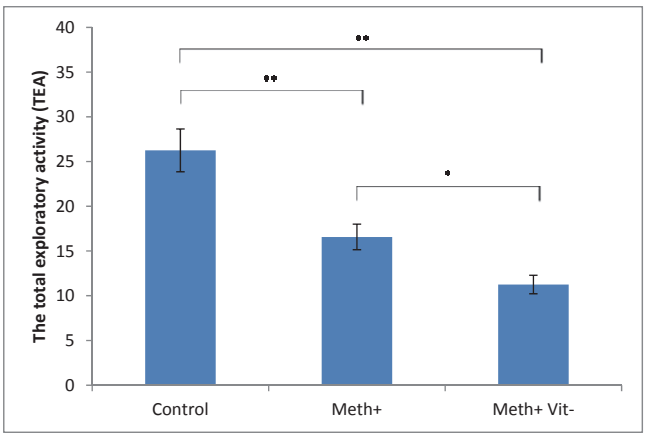

Figure 3 The total exploratory activity (Mean ± SEM, *denotes a significant difference $p<0.05$, **denotes a significant difference $p<0.01$). Control: control group, Meth+: methionine-enriched diet group, Meth+Vit-: methionine-enriched vitamin-deficient diet group.

vidual markers of exploratory activity in the EPM test, the total exploratory activity revealed that total restriction in folate, vitamin B_6 and B_{12} accompanied with methionine-enriched diet significantly diminished total exploratory activity compared to the group with adequate folate, vitamin B_6 and B_{12} intake ($p<0.05$).

DISCUSSION

Results of this study showed that dietary protocols designed to provide significant imbalance in Hcy metabolism can alter some specific behavioural patterns, such as exploratory activity in rats. On the other hand, exploratory activity obtained in the EPM test has been widely used as an indicator of anxiety (32-34). Because the reduction in exploratory activity has been interpreted as an augmented anxiety response in rodents (35, 36), our results confirmed the role of Hcy in maintaining anxiety state level.

Both standard parameters for vertical exploratory activity, the number of rearings (which mostly occur in the closed arms of the EPM) and the number of head-dippings (which occur exclusively in the open arms of the EPM), as well as the total exploratory activity (which summarizes overall exploratory activity in the EPM), were significantly reduced following 30 days of methionine nutritional overload. Although there are very few reports on the effects of high methionine intake on exploratory activity in rodents, our results correspond to previous research on the decreased number of rearings in the open field test following 30 days of nutritional overload with methionine (27). However, it seems that an acute increase in methionine-enriched food intake had no effect on exploratory drive, whereas a prolonged dietary protocol (5 and 10 mg/kg of L-methionine daily) resulted in significant decline in the number of rearings after 21 days (37). The proposed explanation for observed behavioural alterations was found in the fact that increased methionine intake induced lowering of S-adenosylmethionine (SAM, the compound that is involved in methylation cycle, affecting the formation of Hcy) in some specific brain regions that are responsible for the control of various brain functions (38). Methionine content in the applied diet was sufficient to produce an increase (4-5 folds) in serum Hcy levels (27). It is well known that the neurotoxic effects of hyperhomocysteinemia may be provoked directly by inducing DNA damage, resulting in increased apoptosis (39), as well as via N-methyl-D-aspartate and group I metabotropic glutamate receptors (40). Additionally, a methionine-enriched diet has been reported to significantly enhance the oxidative damage in different brain regions, such as the hippocampus, that have a crucial role in maintaining anxiety state levels (27). The reported alterations in brain oxidative status following methionine nutritional overload are mainly based on the reduction of antioxidant enzyme activity and depletion of glutathione levels (27). It has also been reported that increased reactive oxygen species production in specific brain regions is accompanied by increased anxiety levels in rodents (41) and in humans (42).

A methionine-enriched diet (for one month) accompanied with a reduction in some B vitamins (folate, pyridoxine and cobalamine) that are crucial for Hcy metabolism resulted in even more pronounced decline in exploratory drive observed in the EPM test compared to the control. Although the additional reduction in the number of rearings and the number of head-dippings was not significant comparing to methionine-enriched diet alone, diminishment of total exploratory activity (the most comprehensive indicator of exploratory drive in the EPM) revealed that deficiency in certain B vitamins can seriously inhibit exploratory activity in rats. There is no existing data on the impact of deficiency in the named vitamins (along with methionine overload) on the genesis of anxiety disorders by means of parameters of exploratory activity. However, our results are in line with previous reports of decreased exploratory drive in B vitamin (B_2, B_9, B_{12}, choline) deficiency in mice (43). The observed anxiogenic effect following B vitamin deficiency was achieved after 8 weeks of treatment and was accompanied by increased homocysteine plasma levels. Hyperhomocysteinemia, which may underlie the anxiogenic effect (such as that observed in this study), was also reported following dietary protocols with B_9 (44). Additionally, vitamin B_6 deficiency in rat chow resulted in hyperhomocysteinemia and enhanced lipid peroxidation accompanied by decreased SAM and glutathione levels in plasma and liver (45). Furthermore, the beneficial effects of B_{12} supplementation on hyperhomocysteinemia in aged rats have confirmed the importance of cobalamine in methionine metabolism in the brain (46). Although, numerous studies have pointed to the key role of hyperhomocysteinemia-induced neurotoxicity in various brain regions, recent research has presented the crucial possibility that hyperhomocysteinemia may be just a result of, rather than a cause of, at least some mood disorders, such as depression under chronic stress (47).

Based on reports that different patterns of exploratory activity can be considered reliable indicators of anxiety (48-50), we can also compare our results with clinical trials that analyse the connection between impaired one-carbon metabolism and anxiety disorders, such as obsessive-compulsive disorder (23, 24, 26).

In summary, the results of this study suggest the important role of Hcy in the modulation of exploratory activity in rats. Even more, because exploratory drive represents a reliable indicator of anxiety state level, it seems that future investigations considering protocols that can influence homocysteine metabolism (especially in the brain) may be a useful tool in the prevention and cure of some anxiety-related disorders.

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