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PAIN SENSITIVITY AND BEHAVIORAL INDICES IN MICE EXPOSED TO GLUTAMATE TOXICITY: NEUROPROTECTIVE EFFECTS OF VITAMIN SUPPLEMENTATION

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We examined the effects of glutamate toxicity induced by chronic (for 21 days) intraperitoneal (i.p.) introductions of monosodium glutamate (MSG, 4 mg/kg per day) into albino mice. It was found that such treatment significantly ($P < 0.05$) decreased the latencies of defensive motor responses in the hot plate and tail withdrawal tests, as compared with the respective index in the control group (injected with saline). This treatment also led to dramatic decreases ($P < 0.05$) in the intensities of locomotor activity and orientational/research phenomena (rearings) in the open field test; the number of grooming episodes also considerably decreased. Glutamate intoxication also provided significant ($P < 0.05$) decreases in the time spent in the open arms of the elevated plus maze and in the number of entries in these arms. Introductions of 200 mg/kg vitamin C, as well as of 20 mg/kg vitamin E, into MSG-treated mice significantly ($P < 0.05$) increased the response latencies in the hot plate and tail withdrawal tests; the respective values became even greater than those in the norm. Injections of both vitamins also partly normalized the values reflecting the intensity of locomotion and rearings and nearly completely normalized grooming behavior. These vitamins provided also clear trends toward normalization with respect to the anxiety indices in the elevated plus maze test. Thus, glutamate intoxication leads to the development of a hyperalgesic state, significant suppression of behavioral activities, and a significant increase in the anxiety level. Introductions of vitamins C and E known as effective antioxidants considerably moderate these negative shifts.

Keywords: glutamate intoxication, monosodium glutamate, pain thresholds, open field, elevated plus maze, anxiety.

INTRODUCTION

Glutamate is one of the most important excitatory neurotransmitters in the brain. Excessive activation of the brain by high amounts of glutamate has been reported to contribute to neuronal damage, in particular in the case of many neurological disorders (Alzheimer's, Parkinson's, Huntington's diseases, etc.) [1]. Excessive generation of free radicals is believed to be an important factor in the pathogenesis of these disorders. Glutamate possesses toxic effects provided it reaches the CNS in abnormally high concentrations. Glutamate toxicity emanates, in particular, through over-activation of N-methyl- D-aspartate (NMDA) receptors [2]. The toxicity of monosodium glutamate (MSG) has been reported to cause degeneration of the retina, fibers of the optic nerve, neurons in the arcuate nucleus, and other parts of the nervous system which are further believed to have emanated through oxidative stress [3]. Meanwhile, some studies further suggested the potent role of certain antioxidants in preventing the complications associated with diseases involving oxidative stress and inflammation [4].

Vitamins are components of food supplements, and some of them (in particular vitamins E and C) have been identified over the years to be potent antioxidants. Antioxidants function as active scavengers for the available free radicals within the organism's body. α-Tocopherol (vitamin E) is a fat-soluble compound, while ascorbic acid (vitamin C) is water-soluble. Both

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these vitamins are naturally found in many foods, including fruits, vegetables, wheat germ oil, cereals, meat, and so on. Vitamin supplements are used for treating vitamin deficiencies, which are at present relatively rare, but can occur in people with certain genetic disorders [5].

A number of clinical trials and a smaller number of supplementation studies investigated the linkage between α-tocopherol and the risk of cardiovascular diseases. The results from numerous clinical trials with α-tocopherol were largely negative from this aspect [6]. A mixed tocopherol supplement inhibited ADP-induced *ex-vivo* platelet aggregation and increased endothelial constructive nitric oxide synthase activation and nitric oxide release [7]. Also, it was shown that supplementation with α-tocopherol can significantly inhibit platelet functions in patients with type-II diabetes and hypercholesterolemia [8].

In addition**,** high doses of dietary vitamin C were shown to possess a mild protective effect on the risk of heart diseases [9]. The biochemical mechanism responsible for salubrious effects of vitamin C remains mostly uncertain, but it may be related to its ability to function as an antioxidant helping to attenuate oxidative stress [9].

Pain is a modality of sensation, which could be elicited by excessively intense mechanical, chemical, or thermal stimuli. Pain perception could be mediated by excitation of certain chemicals, like bradykinin, serotonin, histamine, potassium ions, prostaglandins, substance P, and so on. Apart from the pain-relieving drugs, various medicinal plant extracts have been reported to possess some analgesic properties [10,11]. But what about "common" antioxidants? Can they possess the same property aside scavenging free radicals? Therefore, the focus of our research effort was to investigate the effect of exposure of mice to MSG at its chronic low doses on some neurobehavioral activities and pain perception, as well as possible effects of vitamin supplementation in the respective situation.

METHODS

Chemicals. Normal saline and ascorbic acid were obtained from DANA drugs (Nigeria), α-tocopherol from May and Baker Pharmaceutical (India), and 99% monosodium glutamate (MSG) from Ajinomoto Inc. (Japan).

Experimental Design. Male albino mice (body mass 18-22 g) were obtained from the Animal house of the Faculty of Basic Medical Sciences, Obafemi Awolowo University, Ile-Ife, Nigeria. The animals were kept at room temperature (28 ± 1°C, 70–80% humidity, and 12/12 h light-dark cycle) in the animal holding unit of the laboratory of the Department of Physiological Sciences of the above Faculty for one week before the commencement of the study. The experiments were conducted in a quiet testing room with overhead fluorescent lights, between 7a.m. and 3p.m. at an ambient temperature of 27-28°C, to avoid the confounding variable of the circadian rhythm. The animals were further divided into four groups, six animals in each. Group 1 was administered with 10 ml/kg of normal saline during 21 days, group 2 received 4 mg/kg of MSG only within the same period, group 3 was administered with 4 mg/kg of MSG chronically and 200 mg/kg of vitamin C at the end of the above period, and group 4 was served with 4 mg/kg of MSG and 20 mg/kg of vitamin E. All drugs were administered via i.p. injections with minimum possible traumatization of the animals.

Hot Plate Test. The original paradigm of Eddy and Leimbach [12], partly modified according to [13], was used; a hot-plate apparatus (Ugo Basile, Italy) was adopted. The hot plate temperature was maintained at 52 ± 0.5°C, and the cut-off interval of 60 sec was imposed to avoid significant tissue damage. The pain sensitivity was evaluated by the response latency for either paw licking or jumping on the hot plate. The latency was measured with 15-min-long intervals for the next one hour, and the average was calculated.

Tail Flick Test. Pain reflexes in response to intense thermal stimulation of the tail were measured using a tail immersion method [14]. Each animal was gently held with the distal one-third portion of the tail immersed in water at a temperature of 55 ± 0.1°C after pre-treatment with saline (control), MSG, and MSG in combination with vitamins. The tail flick latency was recorded with a stop watch, and the intragroup averages were calculated.

Open Field Test. Each mouse was placed in an open field box (68×68×45 cm) equipped with a 20 MP videocamera. Observations over a period of 30 min were recorded for the assessment [10]. The intensities of spontaneous behavioral phenomena of the mice were measured, namely of locomotion, rearing, and grooming. The open field box was cleaned with 70% alcohol and allowed to dry before introducing each animal into the box, in order to remove any olfactory

cue that might have been left by the previous animal. Locomotion was characterized by the number of floor units crossed within the observation period with all paws. The number of times where the animal stood on its hindlimbs or leaned on the wall of the observation box was counted as rearing events (characterizing orientation/research motor activities). The frequency of grooming was counted as the number of times where the animal cleaned its body, face, or pubic area with its forepaws (an index related to the emotional state).

Elevated Plus Maze Test. Each animal of groups 3 and 4 after 21-day-long administration of MSG received the vitamin treatments. Thirty min later, each mouse was placed at the center of the elevated plus maze set with both closed and open arms having equal dimensions (50×10 cm) and rose to a height of 50 cm [11]. The behavioral indices were evaluated by recording the time spent in the open arms within a period of 5 min and number of entries into these arms within the above period.

Statistical Analysis. Numerical values are expressed below as means ± s.e.m. The data were analyzed using one-factor analysis of variance (ANOVA) followed by the *post-hoc* analysis using the Student–Newman–Keul's multiple comparison test. Intergroup differences were considered significant at P values less than or equal to 0.05.

RESULTS

Hot Plate Test. As was found, the mean latency of a defensive reflex response shown in this test by the MSG group (isolated injections of glutamate) was about six times shorter than the respective index in the control group 1. This fact indicated that MSG treatment resulted in the development of the state of intense hyperalgesia. Injections of both tested vitamins into MSG-treated animals exerted dramatic effects on the examined index. The mean latencies of either paw liking or jumping on the plate were not only normalized; the respective values even significantly exceeded the control ones. The mean latency in the MSG+vit.C group was longer than in the control by 70%, while an increment of the respective value in the MSG+vit.E group was 19%; in both cases, $P < 0.05$ (in fact, $P < 0.001$ for the MSG+vit.C) in comparison with the group solely treated with MSG. (Fig. 1A).

Tail Flick Test. In the group injected with MSG, the mean latency of tail removal from hot water in this test was more than 2.5 times shorter than the respective index in the control. In the groups treated with MSG but also injected with high doses of vitamins, significant increases in the latencies were observed. In group MSG+vit.C, the respective value

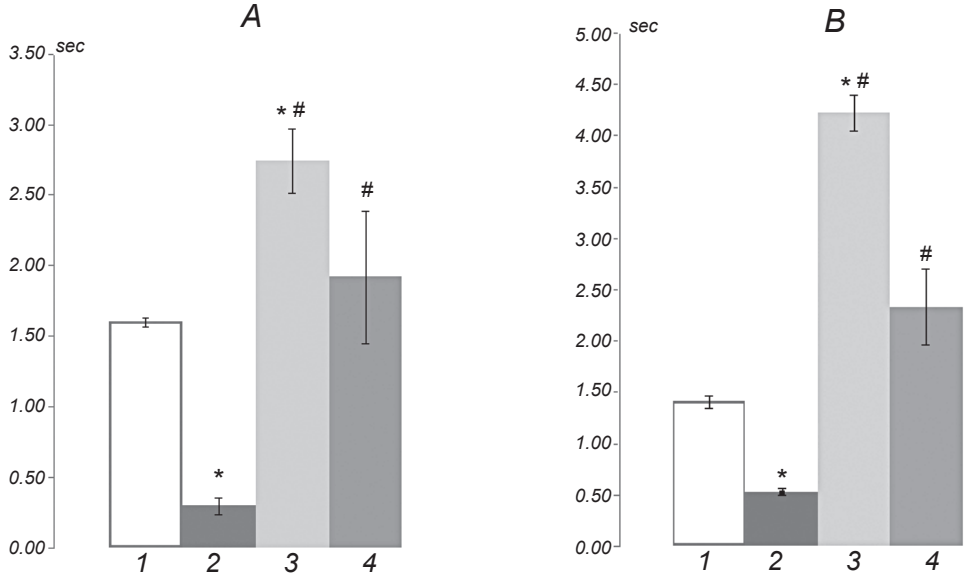


Fig. 1. Diagrams of the mean response latencies (M ± s.e.m., sec) shown by mice of different experimental groups in the hot plate test (A) and tail flick test (B). Groups: 1) control, 2) solely treated for 21 days with monosodium glutamate, MSG (4 mg/kg); 3) treated with MSG + 200 mg/kg vitamin C, and 4) treated with MSG + 20 mg/kg vitamin E. * Difference is significant ($P < 0.05$) in comparison with the control; # the same ($P < 0.05$) in comparison with group 2 (treated with MSG only).

Р и с. 1. Діаграми середніх значень латентних періодів відповідей (*M ± s.e.m.*, с) у мишей різних експериментальних груп в умовах тесту «гаряча платівка» (*A*) та відсмикування хвоста (*B*).

was three times greater than in the norm and more than eight times greater than in the MSG group. In group MSG+vit.E, the mean latency of tail withdrawal was by 66% longer than in the norm (Fig. 1B).

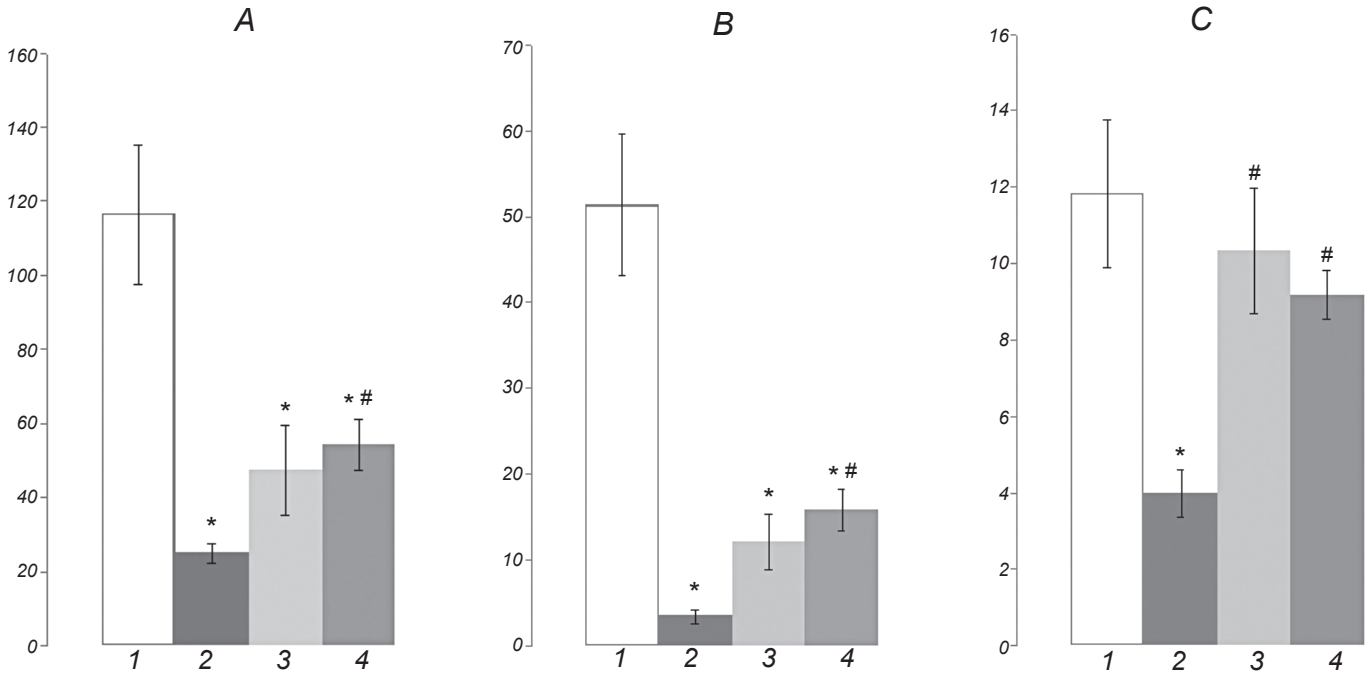
Thus, chronic injections of relatively small doses of MSG into mice resulted in dramatic shortening of the latencies in both tests characterizing the thermal pain sensitivity, i.e., in the development of the hyperalgesia state. Supplementation with both tested vitamins not only provided normalization of the pain sensitivity, but resulted in the development of the state of relative hypoalgesia.

Open Field Test. In this test, the state of glutamate toxicity provided significant suppression of locomotor activity. The number of crossed squares in the MSG group was about five times smaller than the respective number in the control group. Supplementation with both vitamins provided a clear trend toward normalization of this index, but its values remained to be much smaller than those in the norm (40 and 47% in the MSG+vit.C and MSG+vit.E groups, respectively; Fig. 2A).

Manifestations of orientational/research activities (rearings) were suppressed in the MSG group most dramatically; such events were observed, on average, 15 times more rarely than in the control group. Supplementation with vitamins C and E provided significant (severalxfold) increases in this index, but the respective figures remained much smaller than in the norm (24 and 31% in the MSG+vit. C and MSG+vit.E, respectively; Fig. 2B).

The frequency of grooming episodes in mice subjected to glutamate toxicity was also significantly lower, as compared with the norm; the number of the respective episodes within the observation period was, on average, three times smaller. Vitamin supplementation provided an intense trend toward increases in this index, and the latter was nearly fully normalized. The respective mean figures in groups MSG+vit.C and MSG+vit.E corresponded to 87 and 77% of the control value, respectively (Fig. 2C).

Elevated Plus Maze Test. In the group treated only with MSG, the mean time spent by mice in the open arms of the respective set was, on average, by an order of magnitude smaller than the respective value in the control group. Supplementation with vitamin C provided an about eightfold increase in the mentioned index, as compared with that in the "pure" MSG group,



F i g. 2. Diagrams of the mean intensities of locomotion (A), rearing (B), and grooming (C) shown by mice of different groups in the open field test. Vertical scales) Numbers of the respective behavioral events within the observation period. Other designations are the same as in Fig. 1.

Р и с. 2. Діаграми середніх значень інтенсивності локомоції (*A*), частоти стійок (*B*) та частоти грумінгу (*C*) у мишей різних груп у тесті відкритого поля.

and the respective figure reached about 67% of the mean control value. Supplementation with vitamin E provided complete normalization of the time spent in the open arms, as compared to the control (Fig. 3A).

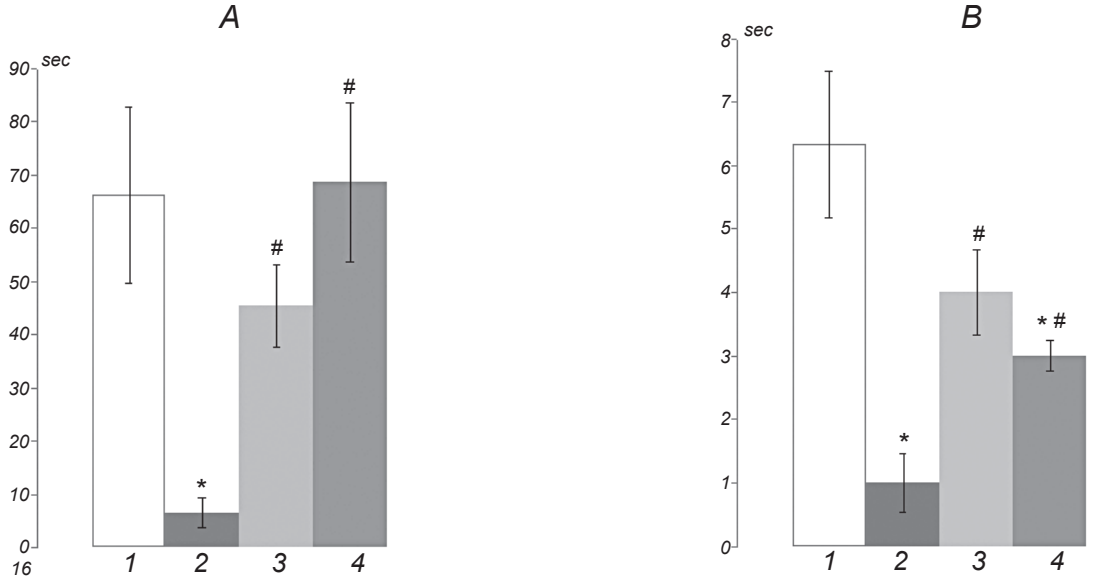
A quite comparable pattern of changes was observed with respect to another index measured in the elevated plus maze test, namely number of entries into the open arms. This index in the MSG group was more than six times smaller than in the norm. Supplementation with vitamins C and E provided severalfold increases in the respective value; in the two "supplemented" groups, the mean numbers of entries into the open arms were equal to 63 and 48% of the control value (Fig. 3B).

DISCUSSION

The results of the hot plate and tail withdrawal tests demonstrated that chronic (21 days) treatment of mice with relatively low doses of MSG resulted in the development of the state of intense hyperalgesia; the latencies of motor responses in these tests in the respective experimental group were several times shorter than those in the control group (injected with saline). The above tests are based on the action of phasic high-intensity thermal stimuli, and the respective responses are mediated by central mechanisms [15]. Supplementation with both tested vitamins highly significantly prolonged the reaction times in both hot plate and tail flick tests, and the respective values were found to be greater not only in comparison with those in the MSG group, but even in comparison with the control one (Fig. 1).

The hot plate is a specific nociceptive/antinociceptive test where opioidergic agents may exert the pain-suppressing activities through the respective spinal receptors [15]. The tail immersion test allows one to suppose that MSG may be not solely centrally acting; it also acts peripherally. The generated pain is likely to be modulated via κ-opioid receptors [16]. Significant prolongation of the onset of tail flick and of the reaction time on the hot plate after injections of rather high doses of vitamins C and E demonstrated that both vitamins can provide rather intense analgesic effects, in addition to their well-known action against oxidative stress.

Locomotion, rearing, and grooming activities are standard parameters measured under open field conditions. All these behavioral phenomena are controlled in rodents by the CNS [17]. These indices can be significantly modulated (either elevated or suppressed) by many drugs interacting with various neurotransmitter systems in the organism. Variations in the level of brain glutamate could result in



F i g. 3. Diagrams of the mean values of the time (sec) spent in the open arms (A) and numbers of entries into these arms (B) within the observation period in the elevated plus maze test. Other designations are the same as in Figs. 1 and 2.

Р и с. 3. Діаграми середніх значень часу (с), проведеного у відкритих рукавах (*A*), та кількості заходів у такі рукави (*B*) у межах періоду спостереження в тесті піднятого лабіринту.

significant modifications of horizontal (locomotion) and vertical (rearing) behavioral activities [18]. In our study, chronic intoxication with MSG dramatically reduced the intensities of all behavioral phenomena, locomotion, orientational/research behavior, and grooming, in experimental mice compared with the saline-treated (control) group. As is believed, MSG is responsible for intensification of the central excitatory activity. Thus, the results of our study look as paradoxical to a certain extent (both horizontal and vertical activities in the open field were intensely suppressed). Probably, the reason for this situation can be easily explained if we take into consideration the results of the elevated plus maze test. Similar trends in significant reductions in the time of open arm exploration and frequency of open arm entries in the group treated with MSG alone show that chronic action of this agent provides an intense rise in the level of anxiety. Thus, the reduction in spontaneous motor activities in the open field test is likely to result from apprehension and fear of the animal in the novel environment. A negative action of sustained chronic doses of MSG on the emotiogenic cerebral systems is probably related to its possible influence on the dopaminergic cerebral system and/or other systems using monoamines [20].

Both vitamins tested were found to noticeably attenuate intensification of anxiety developed due to chronic introduction of MSG doses used. These antioxidants significantly ($P < 0.05$) increased both open arm exploratory time and number of entries in these arms in the elevated plus maze test. Vitamin E showed somewhat stronger effects form this aspect; it fully normalized the value of the open arm stay. With respect to the number of entries, its effect was not so strong. At present, it is difficult to decide what vitamin is more effective in a particular paradigm. The reason for the difference probably cannot be fully accounted to the intensity with which these antioxidants neutralize available free radicals produced under conditions of glutamate intoxication.

In summary, it is reasonable to expect that hypersensitivity in pain perception, as well as excessive anxiety caused by chronic doses of MSG can rather successfully be curtailed by concurrent consumption of vitamins E and C; it is obvious that such supplementation needs much recognition to the dosage.

All procedures performed in studies involving animals were in accordance with the international ethic norms and with the statements of the local Ethic Commitees.

The authors of this communication, T. S. Adu, D. C. Ajonijebu, and O. S. Akinsomosoye, confirm the absence of any conflict related to the comercial or financial interests, relation with organizations or person in any way involved in the research, and interrelations of the co-authors.

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БОЛЬОВА ЧУТЛИВІСТЬ ТА ПОВЕДІНКОВІ ПОКАЗНИКИ У МИШЕЙ ІЗ ГЛУТАМАТНОЮ ІНТОКСИКАЦІЄЮ: НЕЙРОПРОТЕКТОРНІ ВПЛИВИ ВІТАМІНІВ

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Р е з ю м е

Ми вивчали ефекти, індуковані глутаматною інтоксикацією у білих мишей (щоденне внутрішньоочеревинне введення натрію(1) глутамату, MSG, 4 мг/кг на добу протягом 21 дня). Виявилося, що такі ін'єкції призводили до істотного ($P < 0.05$) зменшення латентних періодів захисних моторних реакцій у тестах «гаряча платівка» та відсмикування хвоста порівняно з аналогічними значеннями в контрольній групі (введення фізіологічного розчину). Зазначені введення MSG також викликали драматичне зменшення ($P < 0.05$ у всіх випадках) інтенсивності локомоторної активності та частоти орієнтаційно-дослідницьких феноменів (стійок) під час тестування у відкритому полі; частота епізодів грумінгу також значно зменшувалася. Глутаматна інтоксикація також зумовлювала істотні зменшення ($P < 0.05$) значень часу, проведеного у відкритих рукавах припіднятого лабіринту, та кількості заходів у такі рукави. Ін'єкції 200 мг/кг вітаміну С або 20 мг/кг вітаміну Е мишам із глутаматною інтоксикацією призводили до істотних збільшень ($P < 0.05$) латентних періодів відповідей у тестах «гаряча платівка» та відсмикування хвоста; відповідні значення ставали навіть більшими порівняно з нормою. Ін'єкції обох вітамінів також частково нормалізували показники, що відбивали інтенсивність локомоції та дослідницьких феноменів, і майже цілком нормалізували грумінгову поведінку. Вказані вітаміни також забезпечували виразні тенденції до нормалізації показників рівня тривожності в тесті піднятого лабіринту. Отже, глутаматна інтоксикація призводить до розвитку стану гіпералгезії, істотного пригнічення поведінкової активності, а також значного підвищення рівня тривожності. Уведення вітамінів С та Е – ефективних антиоксидантів – значно зменшує ці негативні зрушення.

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