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## **Delayed behavioral and neurochemical effects of glutamate antibodies in aging C57BL/6 mice**

T. V. Davydova<sup>1</sup>, M. A. Gruden<sup>2</sup>, V. S. Kudrin<sup>3</sup>, V. B. Narkevich<sup>3</sup>,  
L. A. Vetrile<sup>1</sup>, I. A. Zakharova<sup>1</sup>, R. D. E. Sewell<sup>4</sup>

<sup>1</sup>Research Institute of General Pathology and Pathophysiology, Moscow, Russia.

<sup>2</sup>P. K. Anokhin Research Institute of Normal Physiology, Moscow, Russia.

<sup>3</sup>V. V. Zakusova Research Institute of Pharmacology, Moscow, Russia.

<sup>4</sup>Cardiff School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff, UK.

A study of the delayed effect of intranasal administration of glutamate antibodies on mnemonic function and the concurrent tissue concentrations of neurotransmitters in the hippocampus and prefrontal cortex in aging C57BL/6 mice was performed. It was disclosed that an improvement in the development of the conditioned passive avoidance reflex following 14-day administration of glutamate antibodies persisted for 7 days after treatment was discontinued. In the hippocampus 7 days after cessation of glutamate antibody dosing, an increase in the content of dopamine and its metabolites, as well as a rise in the level of aspartic acid and taurine was documented. In the prefrontal cortex, glutamate antibodies elevated the concentration of dopamine without modifying the levels of other monoamine neurotransmitters although there was a simultaneous increase in the glutamate concentration. It was concluded that the changes observed in hippocampal and prefrontal cortical dopamine levels were associated with improved mnemonic function in the aged mice.

**Key words:** memory; antibodies to glutamate; neurotransmitters; mice

**Address for correspondence:** dav-ta@yandex.ru. T.V. Davydova

## **Abstract**

Employing a neuroimmunological approach to ameliorate age-related cerebral decline is a promising new direction in the prevention and treatment of cognitive impairment. It has the potential to extend an individual's active and creative quality of life. Of particular interest, is the mechanism of CNS immune regulation involving the production of autoantibodies to neurotransmitters, which is an important molecular link in the interaction of these systems. In this context, it has been shown that antibodies to neurotransmitters may act as endogenous protective substances involved in synergistic mechanisms in various disorders of the central nervous system [7].

In earlier studies, anti-amnesic properties of polyclonal monospecific antibodies to glutamate (Abs-Glu) have been identified in experimental models of cognitive impairment in neurodegenerative brain damage [1-3,9]. In experiments on aging C57BL/6 mice, it has been shown that intranasal administration of Abs-Glu improved conditioned passive avoidance response (CPAR) performance. At the same time, in the hippocampus of mice receiving Abs-Glu, the content of dopamine decreased, but the concentration of its metabolites increased. No effect of Abs-Glu on the metabolism of neurotransmitter amino acids was found. In the frontal cortex, Abs-Glu did not affect the metabolism of monoamine neurotransmitters, while the level of both excitatory and inhibitory amino acids increased, but not their ratio [5].

Specific cerebral structures are selected for neurochemical examination because of their involvement in functional remodeling in determining cognitive function (learning and memory formation). In acute behavioral laboratory animal studies, learning and memory is modeled based on the response to avoidance of an electrocutaneous stimulus (CPAR). However, the duration of the effects of Abs-Glu on behavioral and neurochemical parameters has not been subjected to examination up to now. Consequently, the aim of this investigation was to study the delayed effects of Abs-Glu on mnemonic functions as well as neurotransmitter levels in relevant brain structures (hippocampus and prefrontal cortex), in C57BL/6 mice during aging.

## MATERIALS AND METHODS

The study was performed using 12-month-old male C57BL/6 mice (n = 26) weighing  $32.4 \pm 2.1$  g, which were kept under standard vivarium conditions with free access to water and food and under a 12:12-hour light:dark regime. All animal procedures were carried out in compliance with Directive 2010/63 / EU of the European Parliament and the Council of the European Union of September 22, 2010, on the protection of animals used for scientific purposes, and the Rules of Good Laboratory Practice (Order of the Ministry of Health of the Russian Federation No. 199n dated April 1, 2016).

Polyclonal monospecific Abs-Glu were raised according to a previously described protocol [6] involving a standard scheme for immunization of male Chinchilla rabbits with a conjugate of glutamate and a BSA carrier protein. Antibodies in the form of a  $\gamma$ -globulin fraction from blood serum of immunized rabbits were precipitated with ammonium sulfate ( $(\text{NH}_4)_2\text{SO}_4$ ) at 40% saturation, followed by dialysis. This was performed by affinity chromatography using CNBr-activated Sepharose 4B (Sigma) as a sorbent with BSA immobilized on it and the  $\gamma$ -globulin fraction was purified from the admixture of antibodies to BSA, lyophilized, and stored at 40 ° C. Purified polyclonal Abs-Glu were used in experiments at a titer of  $1: 1024 \pm 1: 16$ . The animals were divided into two groups (13 animals each). Control mice received daily intranasal saline in a volume of 4.0  $\mu\text{l}$  while treated animals received purified polyclonal Abs-Glu at a dose of 250  $\mu\text{g} / \text{kg}$ , dissolved in 4.0  $\mu\text{l}$  of saline administered to each nostril daily for 14 days.

Seven days after discontinuation of intranasal treatment in both animal groups, the formation of memory processes was assessed in CPAR test, which was carried out in a chamber consisting of two compartments: light ( $15.5 \times 15.5 \times 19$  cm) and dark ( $9 \times 9 \times 17$  cm) compartments with a built-in floor consisting of metal rods each with a diameter of 0.3 cm and a distance of 0.9 cm distance apart. The compartments were connected to each other by a gate in the common wall and it was equipped with a guillotine door. CPAR was

developed according to a previously standardized method [9]. The latent period (LP) for the transition to the dark compartment was recorded on the first day during training and on the second day during testing. The observation period for each animal was 300 s (from the moment the gate was opened on the days of training and testing). The degree of memorization of an applied low-level electric shock by animals was determined by the difference in the LP of the animal's transition to the dark chamber during the development of the passive avoidance reaction and 24 h after training on the day of testing ( $\Delta$ LP, s). At the end of the CPAR test, according to the study protocol, all animals were decapitated, the brains were removed in ice and samples of the hippocampus and prefrontal cortex were dissected. Subsequently, the content of monoamine neurotransmitters was determined in the hippocampus and prefrontal cortex structures: dopamine (DA), serotonin (5-HT), norepinephrine (NA) and DA metabolites (DOPAC, HVA, 3-MT) in addition to 5-HT and its metabolite (5-HIAA). Using the content and ratio of the metabolites, indices of monoamine metabolism were evaluated. The concentrations of neurotransmitter amino acids were also determined: aspartic acid, glutamate, glycine, taurine and gamma aminobutyric acid (GABA). The neurotransmitter content was determined by HPLC with electrochemical detection (HPLC / ED) on an LC-304T chromatograph (Bioanalytical Systems, Inc.) equipped with a Rheodyne 7125 injector with a loop volume for applying samples of 20  $\mu$ L [2,9]. The concentration of monoamines in the experimental samples was calculated by the "internal standard" method, based on the ratio of the peak areas in the standard mixture against the experimental sample, and expressed in nmol/g tissue. The content of neurotransmitter amino acids was determined by HPLC/ED according to an established standard method [9]. A solution of a mixture of aspartic and glutamic acids, taurine, and GABA at a concentration of 0.1  $\mu$ mol / ml in 0.1 N HClO<sub>4</sub> was used as a standard mixture for the calibration of the column with the sorbent. The concentrations of neurotransmitter amino acids were expressed in mmol/g tissue.

The data obtained were statistically processed using Statistica 7.0

software (StatSoft, Inc.) using intergroup comparison by the Mann-Whitney U test. Data are presented as Mean  $\pm$  SEM. Statistical significance when testing null hypotheses was taken as  $p = 0.05$ .

## RESULTS AND DISCUSSION

In the aging mice, 7 days after discontinuation of 14-day intranasal administration of Abs-Glu, an improvement in memory was observed in the conditioned passive avoidance reaction (CPAR) test. This was manifested by a 2-fold increase in dark chamber entry latency on the 2nd day of CPAR testing. The  $\Delta$ LP in the Abs-Glu treated animals was  $226.8 \pm 42.6$  s compared to  $105.0 \pm 41.1$  s in the control group ( $p < 0.023$ ). At the same time, analysis of the latency of the transition to the dark chamber on the first day of training did not reveal significant differences between the groups:  $19.2 \pm 1.7$  and  $15.2 \pm 2.2$  s in the Abs-Glu experimental and control groups respectively. Thus, 7 days after the discontinuation of Abs-Glu, the improvement in CPAR was retained and it was comparable to that expressed on the first day after Abs-Glu dosing [5]. It could be hypothesized that one of the molecular mechanisms underlying the improvement of memory in mice after administration of Abs-Glu is attributable to remodeling of neurochemical systems in different regions of the brain. Thus, in the hippocampus 7 days after Abs-Glu treatment cessation, the tissue DA level significantly increased by 103.6% and the content of its metabolites, HVA and 3-MT also increased by 116.5 and 50%, respectively (Table 1). Arising from these findings, it might be postulated that activation of catechol-O-methyl-transferase enzyme had been developed and an enhanced DA utilization had given rise to augmented levels of its metabolites. Administration of Abs-Glu for 14 days followed by 7-day withdrawal boosted DA synthesis in the prefrontal cortex by 61%, but there was no significant change in the levels of its metabolites (Table 1). In contrast to the hippocampus, a 27.6% decrease in 5-HT level was found in the prefrontal cortex but there was no alteration in the level of its metabolite, 5-HIAA. Administration of Abs-Glu did not significantly affect serotonin metabolism nor the concentration of noradrenaline

in the hippocampus (Table 1). However, in the prefrontal cortex, intranasal Abs-Glu induced a decline in DA metabolism indicated by the respective 39% and 17% reductions in DOPAC/DA and HVA/DA ratios (Table 2). The upward trend in DA level in both cerebral structures may be a key contributory factor underlying the positive influence on memory formation instigated by Abs-Glu treatment.

It should be noted that there was a mixed effect of intranasal administration of Abs-Glu on neurotransmitter amino acid concentrations in the two cerebral structures studied. Thus, in the hippocampus, the content of aspartic acid and taurine increased by 229.4% and 360.7%, respectively. Aspartic acid is an excitatory neurotransmitter that stimulates NMDA receptors, but less so than glutamate. Nevertheless, the weak stimulation of NMDA receptors caused by aspartic acid has a neurotrophic effect in opposition to glutamate which has a stronger excitotoxic effect. Furthermore, the increased taurine content found in the hippocampus has previously been shown to enhance the formation of memory in the passive avoidance reaction [11]. In accordance with this, one of the cellular mechanisms underlying the improvement of memory in aging mice under the influence of Abs-Glu may be ascribed to stimulation of neurogenesis by taurine [8], which would be conducive to strengthening the memory trace in the CPAR model. Analysis of neurochemical data in the prefrontal cortex showed that under the influence of Abs-Glu, the concentration of glutamate increased by 39% (Fig. 1), leading to NMDA receptor activation and an improvement in memory formation. Considering the fact that memory improvement has been documented under the action of an immune factor, namely Abs-Glu, and it has also been shown [4] that glutamate receptors expressed in lymphocytes are involved in the activation of this type of cell, then glutamate can be considered as a neuroimmunomodulator linking the immune and nervous systems. It is likely therefore that excess glutamate in the prefrontal cortex stimulates NMDA receptors that are directly involved in the formation of spatial memory.

In consequence of delayed testing of mnemonic function in aging mice after 14-day Abs-Glu dosing, not only has an improvement in CPAR performance been disclosed, but this enhancement is also retained beyond the period of antibody treatment. This observation is attended by neurochemical alterations in specific brain structures. Hence, in the hippocampus and prefrontal cortex, the neurochemical changes 7 days after discontinuation of antibody administration and during the early stages of treatment, were associated with divergent activity on the DA-ergic system. The role of the DA-ergic system in regulating learning and memory has been well studied [10] and it could be postulated that hippocampal and prefrontal cortical dopamine levels were associated with improved mnemonic function in the aged mice.

Additionally, in the prefrontal cortex, the stimulatory action of Abs-Glu on serotonin and glutamate levels might be explained by a cooperative effect as well as the development of the passive avoidance reaction based on the elaboration of fear of the prospect of punishment. Thus, when administered to aging mice, Abs-Glu exert a protective effect, preventing age-related changes in memory in mice, which may be a compensatory brain response to the fear experienced by animals when placed in an experimental chamber.

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**Table 1.** The content of monoamine neurotransmitters and their metabolites (nmol/g tissue) in the hippocampus and prefrontal cortex of aging C57BL/6 mice 7 days after cessation of 14-day intranasal administration of Abs-Glu. (Values represent Mean  $\pm$  SEM).

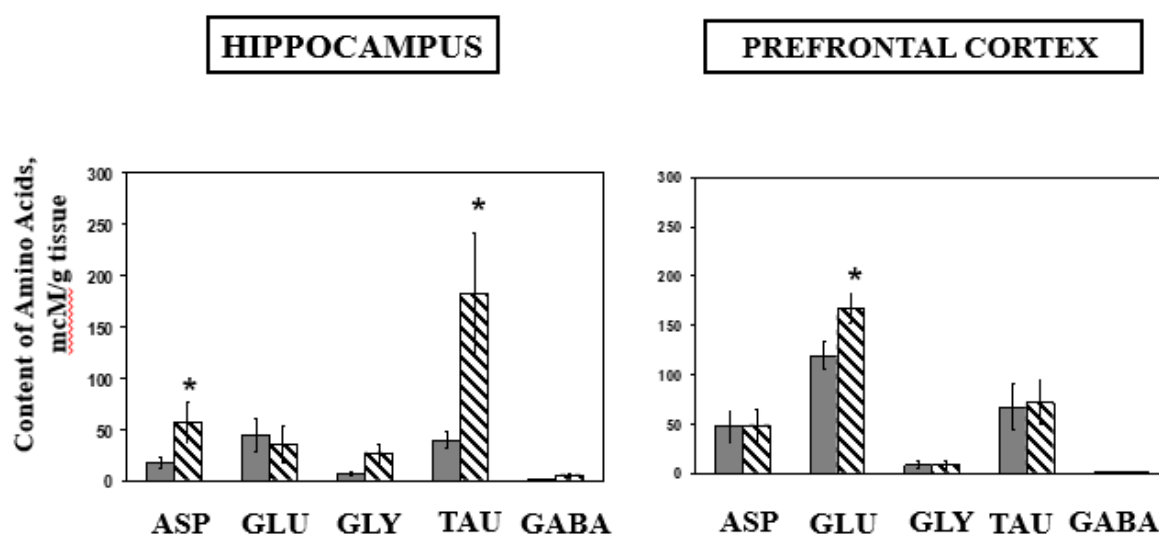
Group	NA	DA	DOPAC	HVA	3-MT	5-HT	5-HIAA
<b>Hippocampus</b>							
Control	4.81 $\pm$ 1.03	0.25 $\pm$ 0.07	0.10 $\pm$ 0.05	0.38 $\pm$ 0.18	0.10 $\pm$ 0.09	7.81 $\pm$ 0.23	9.69 $\pm$ 3.23
Abs-Glu	5.84 $\pm$ 1.05	0.50 $\pm$ 0.15*	0.13 $\pm$ 0.07	0.80 $\pm$ 0.26**	0.15 $\pm$ 0.06*	7.63 $\pm$ 0.29	9.14 $\pm$ 3.09
<b>Prefrontal cortex</b>							
Control	13.14 $\pm$ 6.05	0.77 $\pm$ 0.19	0.44 $\pm$ 0.19	1.58 $\pm$ 0.48	0.30 $\pm$ 0.17	20.30 $\pm$ 9.13	23.30 $\pm$ 3.21
Abs-Glu	13.90 $\pm$ 6.16	1.24 $\pm$ 0.7*	0.53 $\pm$ 0.17	1.63 $\pm$ 0.67	0.39 $\pm$ 0.19	14.88 $\pm$ 4.2*	22.40 $\pm$ 2.17

\*  $p < 0.05$ , \*\*  $p < 0.01$  compared to control.

**Table 2.** Indicators of metabolism of monoamine neurotransmitters (DA and 5-HT) and their metabolites (DOPAC, HVA and 5HIAA) expressed as metabolite/neurotransmitter ratios in the hippocampus and prefrontal cortex of aging C57BL/6 mice 7 days after cessation of 14-day intranasal administration of Abs-Glu. (Values represent Mean  $\pm$  SEM).

Group	DOPAC/DA	HVA/DA	5-HIAA/5-HT
<b>Hippocampus</b>			
Control	0.602 $\pm$ 0.18	2.12 $\pm$ 0.63	1.51 $\pm$ 0.17
Abs-Glu	0.59 $\pm$ 0.26	2.19 $\pm$ 0.91	1.49 $\pm$ 0.24
<b>Prefrontal cortex</b>			
Control	0.362 $\pm$ 0.12	1.897 $\pm$ 0.99	0.708 $\pm$ 0.69
Abs-Glu	0.217 $\pm$ 0.10*	1.57 $\pm$ 0.88*	0.836 $\pm$ 0.28*

\*  $p < 0.05$ , \*\*  $p < 0.01$  compared to control.



**Figure 1.**

The content of neurotransmitter amino acids in the hippocampus and prefrontal cortex in aging C57BL/6 mice 7 days after discontinuation of 14-day intranasal administration of Abs-Glu.

(Asp = Aspartic acid; Glu = Glutamic acid; Gly = glycine; Tau = taurine; GABA = gamma-aminobutyric acid). \*  $p < 0.05$  compared to control.