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EFFECTS OF GLUTAMATE ANTIBODIES ON THE CEREBRAL EXPRESSION OF THE *Tnfrsf1*A GENE UNDER INDUCED SPATIAL AMNESIA BY PROINFLAMMATORY PROTEIN S100A9 FIBRILS IN AGING MICE

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Proinflammatory S100A9 protein is acknowledged as a promoter of inflammation-linked neurodegeneration and the *Tnfrsf1A* gene encodes the tumor necrosis factor receptor 1A that binds TNFα to function as a regulator of inflammation. We studied the outcome of chronic intranasal administration of *in vitro*-derived S100A9 fibrils alone or in combination with glutamate antibodies on the expression of the *Tnfrsf1A* gene in three brain structures of aging C57BL/6 mice under conditions of impaired spatial memory. A differential cerebral pattern of *Tnfrsf1A* gene activity and its modification by S100A9 fibrillar structures was observed. There was a S100A9 fibril generated suppression of *Tnfrsf1A* gene expression in the hippocampus and cerebellum, but it was increased in the prefrontal cortex. Subsequently, glutamate antibodies normalized the S100A9 aggregate augmented expression of the *Tnfrsf1A* gene in the prefrontal cortex, in contrast with the hippocampus and cerebellum, thereby affecting the TNF signaling pathway and preventing the development of inflammation.

Key words: S100A9, fibrils, *Tnfrsf1A* gene, glutamate antibodies, spatial memory, mice, aging.
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Alzheimer's disease (AD) which is characterized by cognitive impairment and the onset of dementia, is one of the most common causes of disability. Currently, there is both scientific and clinical interest in the study of inflammatory processes as the initiating phase of AD pathogenesis associated with impaired folding of a number of proteins, instigation of the amyloid cascade and the development of neurodegeneration [6]. In addition, among the molecular factors simultaneously involved in inflammatory reactions and the amyloid cascade, close attention has been paid to the proinflammatory protein S100A9, which is also considered as a differential biomarker of early cognitive deficit in AD. It has been shown that under certain conditions *in vivo* and *in vitro*, S100A9 protein may form toxic amyloidogenic oligomeric and fibrillar structures causing apoptotic cell death in the brain [13]. The pathogenic nature of fibrillar S100A9 aggregates is also confirmed by the fact that they may induce an increased release of hippocampal glutamate as well as central dopaminergic dysregulation, followed by behavioral impairment in two *in vivo* models: the

development of a conditioned passive avoidance response and long-term spatial memory in the Morris water maze in aging C57BL/6 mice [8,10].

Currently, there is insufficient experimental data on the interaction of S100A9 amyloidogenic aggregates with molecular factors that generate a long-term inflammatory response prompting an understanding of the molecular mechanisms leading to the development of chronic inflammation and neurodegeneration. Among such considerations is the study of a possible effect of S100A9 amyloid fibrils on the proinflammatory cytokine system, a component of which is tumor necrosis factor (TNF) and its receptors. TNF is produced by macrophages/monocytes during acute inflammation and is responsible for a wide range of signaling events in cells leading to apoptosis or necrosis. One member of the TNF receptor family is the membrane protein TNFRSF1A (tumor necrosis factor receptor superfamily, member 1A), which is involved in the regulation of the inflammatory process [9]. In this regard, possible modification of the impact of S100A9 amyloidogenic structures on the expression of receptor genes, in particular, the *Tnfrsf1A* gene, which expresses the TNF receptor Tnfrsf1A protein, is of undoubted interest.

In earlier reports, using experimental models of cognitive impairment following neurotoxicity to Aβ(25-35) or S100A9 fibrils, we exposed an antiamnestic property of polyclonal monospecific glutamate antibodies (Abs-Glu) [1, 2].

The aim of this study therefore, was to investigate the outcome of chronic intranasal administration of *in vitro*-created proinflammatory S100A9 fibrils, either by themselves or in combination with glutamate antibodies on the expression of the *Tnfrsf1A* gene. In this context the hippocampus, prefrontal cortex and cerebellum of aging C57BL/6 mice were studied under conditions of impaired spatial memory.

METHODS

The investigation was carried out on 36 male C57BL/6 mice aged 12 months and weighing 31.4 ± 4.2 g. (FGBUN NCBMT FMBA farm"Stolbovaya", Russia). Mice were housed 3-5 animals per home cage on a 12/12 hour light/dark cycle with free access to food and water. All animal procedures were performed in compliance with the requirements set out in the directive of the European Parliament and the Council of the European Union (2010/63 / EU of September 22, 2010), as well as being in accordance with the rules approved by the Commission on Bioethics of the Federal State Budgetary Scientific Institution "Anokhin Institute of Normal Physiology". In advance of the study, all animals were habituated to the conditions of the vivarium for two weeks.

Detailed protocols for the *in vitro* preparation of S100A9 fibrillar structures and purified polyclonal monospecific Abs-Glu have been described previously [10]. The animals were divided into 3 treatment groups, which were inoculated daily intranasally alternately in each nostril for 14 days: group 1 - normal saline vehicle, 8 μL (n = 12); group 2 - a solution of S100A9 fibrils (15.0 μg in 8 μL = 0.48 mg/kg; n = 12); group 3 - simultaneously alternating treatment with S100A9 fibrillar solution in one nostril (15.0 μg in 4 μL) and Abs-Glu solution in the other nostril (in a volume of 4 μL, at a dose of 250 μg/kg, n = 12). Behavioral experiments on learning, formation and testing of spatial memory on the Morris water maze model (Columbus Instruments, USA) were carried out 24 hours after the end of each 14-day group treatment schedule according to the protocol described earlier [10]. Behavioral data are presented in detail in [10] and validate the formation of long-term spatial memory. Twenty-four h after the end of the behavioral experiments, all animals were killed and hippocampal, prefrontal cortical and cerebellar brain structures were dissected on a cold plate (+ 4°C) Subsequently, real-time PCR was performed on samples of the brain structures and the expression of the *Tnfrsf1A* gene was determined according to a previously described protocol [11]. *Tnfrsf1A* expression in the cerebral structures of age-matched native mice was taken as the control level when calculating the expression level in the control and experimental groups [12].

The statistical significance of the differences between groups was evaluated using the nonparametric Mann-Whitney U-test. Correlation analysis was performed using Spearman's rank correlation coefficient (r). Data are presented as mean values ± standard error. Differences were considered statistically significant at $p \leq 0.05$ level.

RESULTS AND DISCUSSION

S100A9 protein fibrils produced *in vitro* are characterized by a helical morphology, reaching several hundred nanometers in length. Their amyloid nature has been confirmed by a multifold increase in the fluorescence of thioflavin-T and immunoreactivity with A11 antibodies [10]. We have previously shown that chronic intranasal administration of S100A9 fibrils to aging mice impaired the formation of long-term spatial memory in the Morris water maze and this finding was also associated with a dysregulation of neurochemical processes in the brain [10]. As a corollary to this earlier study, the possibility of diminishing the amnesia caused by S100A9 fibrils using Abs-Glu was examined in the present study. It can be assumed that one of the molecular mechanisms underlying the improvement of memory in experimental aging mice after administration of Abs-Glu is the binding of excess cerebral

glutamate and an improved synchronization of neurochemical systems in various brain regions [10].

In the gene expression experiments, it was shown that in the group of aging mice treated with saline, the relative expression of the *Tnfrsf1A* gene was 37.7, 5.7 and 86.4 relative units calculated from the expression level of this gene in native animals in the hippocampus, prefrontal cortex, and cerebellum, respectively (Fig. 1, 2, 3).

Under conditions of confirmed spatial amnesia induced by S100A9 fibrils, the expression of the *Tnfrsf1A* gene in the hippocampus was decreased by 67.8% in comparison with the saline control (Fig. 1). Concomitantly, S100A9 fibrils induced not only an impairment of spatial memory, but also a pronounced increase in the expression of the *Tnfrsf1A* gene by 91.3% in the prefrontal cortex (Fig. 2). In an earlier study, an increase in the expression of the *S100A9* gene in microglial cells of the temporal cortex, both in genetic and sporadic cases of AD has been reported and accompanied by its colocalization with Aβ(1-42) peptide demonstrating initiation of inflammation in this area [13]. In the cerebellum of aging animals under the amnesic conditions induced by S100A9 fibrils in our study, the expression of the *Tnfrsf1A* gene in was suppressed by 53.2% (Fig 3) which was comparable to that observed in the hippocampus (Fig 1). The data obtained indicate that cerebral structures have their own specific level of *Tnfrsf1A* gene expression which is likely to be associated with the creation of a prerequisite density of TNF receptors as components of the cytokine/receptor/cell interaction *in vivo*. In addition, the ability of a certain level of receptor gene expression has been shown to change the nature and type of this interaction [3], which reflects the plasticity of the brain [4]. It is also possible that a specific genomic network influences the functioning of the brain as a whole [5].

The data on changes in the cerebral activity of the *Tnfrsf1A* gene following combined treatment with S100A9 toxic fibrils and Abs-Glu, which as shown earlier, restored the navigation skill disturbed by S100A9 aggregates, were analyzed. Consequently, in our present study it was disclosed that concurrent administration of S100A9 fibrils and Abs-Glu did not restore the level of expression of *Tnfrsf1A* in the hippocampus and cerebellum to that observed in the saline treated control group (Fig. 1, 3). However, in the prefrontal cortex, a pronounced reversal of S100A9 fibril elevated *Tnfrsf1A* expression (up to 53% in comparison with saline) was observed after the S100A9 aggregate treatment combination with Abs-Glu (Fig. 2). An increase in the cerebral expression of the TNF gene itself occurs during inflammation in AD [7]. Hence, changes in *Tnfrsf1A* gene activity, in response to the action of S100A9 fibrils, indicate a specific sensitivity of the TNF/TNFRSF1A receptor

system at the gene expression level to integrative factors of inflammation and the amyloid cascade.

Correlation analysis revealed the following relationships between behavioral parameters on the day of long-term memory testing and indicators of *Tnfrsf1A* gene expression: in control - between gene activity in the hippocampus and total distance traveled (r = 0.894427; ρ - 0.040519), as well as movement speed (r = 0.894427; ρ - 0.040519); in the cerebellum - with the speed of movement (r = -0.894427; ρ - 0.040519) and the total distance covered (r = -0.894427; ρ - 0.040519).

In the S100A9 fibril treated animal group, a negative correlation was found between the expression of the *Tnfrsf1A* gene in the hippocampus and the duration of stay in the quadrant where the platform was absent (r = -0.894427; ρ - 0.040519), in the cerebellum - a similar negative correlation was documented (r = -0.974679; ρ - 0.004818) while in the prefrontal cortex, there was a positive correlation (r = 0.894427; ρ - 0.040519) between the same indicators. In mice that received S100A9 fibrils in conjunction with Abs-Glu, a negative relationship was found between the hippocampal expression of the *Tnfrsf1A* gene and the duration of stay in the quadrant where the platform was absent (r = -0.894427; ρ - 0.040519). Moreover, a positive relationship was evident between the same parameters in the prefrontal cortex (r = 0.894427; ρ - 0.040519). At the same time, the emergence of a new relationship was observed in the cerebellum between the expression of the *Tnfrsf1A* gene and the total residence time of mice in the quadrant where the platform was located.

Thus, it was found that chronic intranasal administration of S100A9 fibrils led to a differential effect on the expression of the *Tnfrsf1A* gene in cerebral structures. Accordingly, in the hippocampus and cerebellum, there was a decrease in *Tnfrsf1A* expression compared to the control while in the prefrontal cortex expression of this gene was increased Furthermore, Abs-Glu reversed and normalized the S100A9 aggregate augmented expression of the *Tnfrsf1A* gene in the prefrontal cortex, thereby affecting the TNF signaling pathway potentially preventing the development of inflammation. The findings carry prospective significance for the development of new treatments for AD.

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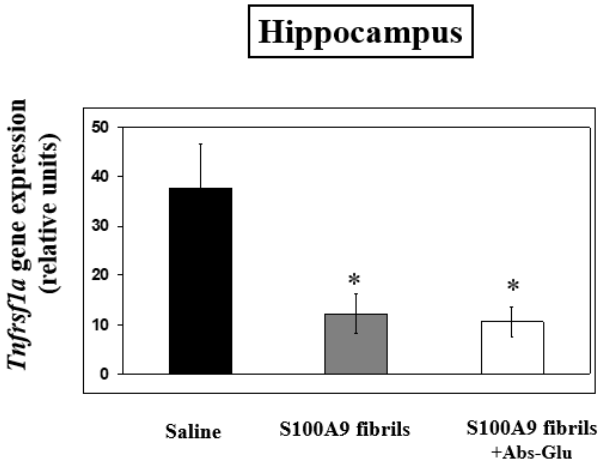

Fig. 1. Expression level of the *Tnfrsf1A* gene in the hippocampus of aging C57BL/6 mice that received saline intranasally for 14 days (control group), S100A9 fibrils or S100A9 fibrils in combination with glutamate antibodies (S100A9 fibrils + Abs–Glu).

*Note: the ordinate is the relative level of Tnfrsf1A gene expression in relative units for each animal treatment group. *p≤0.05 compared to control*.

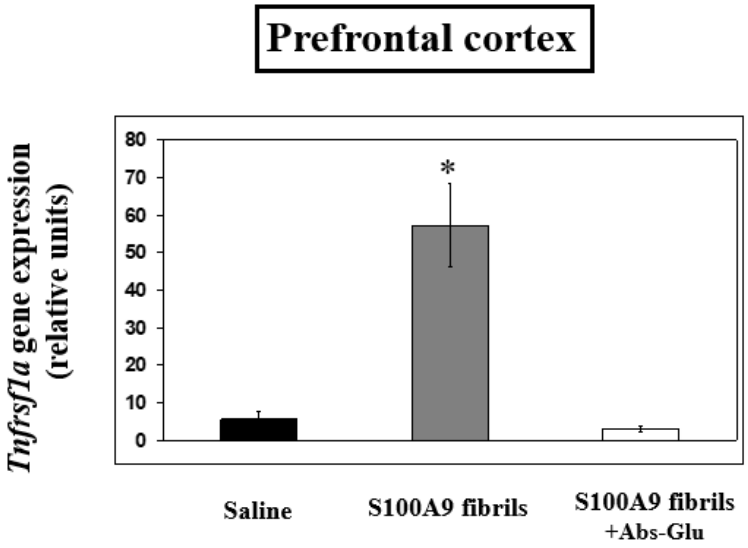

Fig. 2. Expression level of the *Tnfrsf1A* gene in the prefrontal cortex of aging C57BL/6 mice that received saline intranasally for 14 days (control group), S100A9 fibrils or S100A9 fibrils in combination with glutamate antibodies (S100A9 fibrils + Abs–Glu).

*Note: the ordinate is the relative level of Tnfrsf1A gene expression in relative units for each animal treatment group. *p≤0.05 compared to control*.

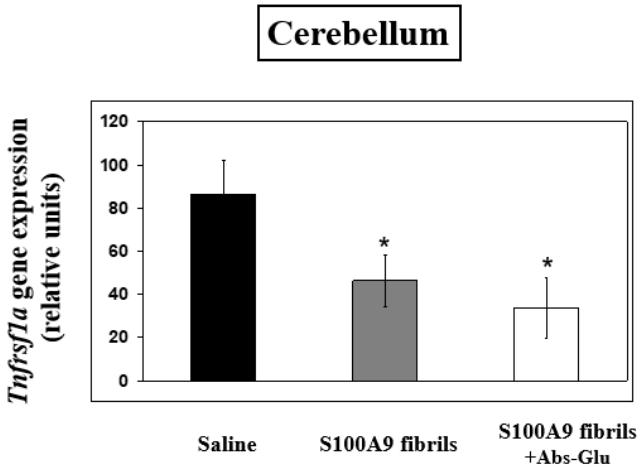

Fig. 3. Expression level of the Tnfrsf1A gene in the cerebellum of aging C57BL/6 mice that received saline intranasally for 14 days (control group), S100A9 fibrils or S100A9 fibrils in combination with glutamate antibodies (S100A9 fibrils + Abs–Glu).

*Note: the ordinate is the relative level of Tnfrsf1A gene expression in relative units for each animal treatment group. *p≤0.05 compared to control*.