Alterations in glutamate transport and group I metabotropic glutamate receptors in the rat brain during acute phase of experimental autoimmune encephalomyelitis

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Abstract
Experimental autoimmune encephalomyelitis (EAE) is an animal model that mimics many aspects of multiple sclerosis (MS). In MS the immune system attacks the white matter of the brain and spinal cord, leading to disability and paralysis. Neurons, oligodendrocytes and myelin are lost due to the release of cytotoxic cytokines, autoantibodies and toxic amounts of the excitatory neurotransmitter glutamate. This study was designed to determine the changes in: a) glutamate transport in nerve endings and astroglial fraction, b) level of excitatory amino acid transporters (EAATs) and c) level of group I metabotropic glutamate receptors (mGluR G I) protein in the acute phase of EAE (12 d.p.i. – day post immunization), in the peak of neurological deficits. We have found that glutamate uptake in synaptosomes and GPV fraction increases by about 30% and 15%, respectively, compared to controls. We also observed an increase in KCl-dependent glutamate release from synaptosomes and GPV fraction obtained from EAE rats by about 20%. Western blots analysis of protein expression shows elevation of group I metabotropic glutamate receptors (mGluR G I) and excitatory amino acid transporters (EAATs) in EAE rats during the acute phase of the disease (12 d.p.i.), when the level of proinflammatory cytokines (IL-1β, IL-6, TNF-α) rises. The results suggest that during the inflammatory conditions in the acute phase of EAE, disturbances in glutamate transport take place that may lead to the excitotoxicity.

Key words: EAE, cytokines, glutamate excitotoxicity, mGluRs.

Introduction
Multiple sclerosis (MS) is a chronic disabling autoimmune neurological disorder targeting the white matter of the central nervous system (CNS). The etiology of MS has not been fully elucidated yet, but it is believed that immunological mechanisms operate in disease initiation and progression [38]. In addition to the autoimmune attack, there is also a local inflammatory response leading to demyelination, oligodendrocyte death, axonal damage and even neuronal loss in the CNS [14,40]. Experimental autoimmune encephalomyelitis (EAE) is an animal model that mimics many aspects of multiple sclerosis (MS) and has

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been widely used to study the mechanisms of disease and therapeutic approaches to MS.

Myelin, oligodendrocytes and neurons are lost due to inflammation induced by infiltrating leukocytes. Released cytotoxic cytokines, and large amounts of the excitatory neurotransmitter glutamate lead to cell death. Recent experimental evidence indicates glutamate, the major excitatory neurotransmitter in the mammalian brain, as an important contributing factor in MS pathogenesis [15,24]. It was suggested that glutamate production by macrophages might be involved in axonal damage and oligodendrocyte pathology in MS lesions and amyotrophic lateral sclerosis (ALS) [16,27,32,43]. The disturbances in glutamatergic transmission were also observed in brains MS patients [15,41].

Glutamate transporters play an important role in glutamate homeostasis. They prevent neurons and other brain cells against excitotoxic damage when the level of extracellular glutamate in the synaptic cleft increases. Glutamate released to extracellular space is inactivated by taking up into glia and neurons in a process mediated by excitotoxic amino acid transporters (EAATs). Human most important glutamate transporters are: EAAT1, EAAT2 and EAAT3 which have different nomenclature in rats: GLAST, GLT-1 and EAAC1, respectively. In the rodent CNS neurons express mainly EAAC1, and GLT1 and GLAST are localized in astrocytes [9]. GLAST and GLT-1 glutamate transporters expression and function are positively regulated by the presence of extracellular glutamate [13,39]. As the main function of glutamate transporters is control the level of potentially toxic glutamate, dysfunction of this system may lead to the excitotoxic damage of neurons.

It was suggested that activation of glutamate receptors contributes to the process of cell death in chronic neurodegenerative disorders [16]. While ionotropic NMDA-, AMPA-, and kainate-type glutamate receptors (iGluRs) mediate fast synaptic transmission, metabotropic glutamate receptors (mGluRs) modulate neuronal excitability and transmitter release, so as synaptic plasticity, and memory function using variety intracellular second messenger systems. The excitotoxic hypothesis in neurodegenerative diseases, including MS, is confirmed by observed neuroprotection of anti-glutamatergic agents [24].

Recent studies have found a positive correlation between TNF-α levels and clinical course of MS [2,17,42,46]. Constitutive expression of IL-1β which is very low in normal brain, is rapidly up-regulated in multiple sclerosis lesions [11,18]. Also interleukin-6, a pleiotropic cytokine, is present during inflammation [1,18]. Participation and significance of IL-6 in MS/EAE in relation to immune process of the disease was confirmed and this cytokine seems to be important for the course of disease and the treatment [18,37].

Recent studies have shown that glutamate excitotoxicity may be a component of EAE pathology. The aim of the present study is to analyze glutamate transport in different cell fractions, the expression of glutamate transporters: GLAST, GLT-1, EAAC1 and selected metabotropic glutamate receptors (mGluR G I). Additionally, levels of cytokines (IL-1β, IL-6 and TNF-α) expression were assessed in EAE brain homogenates in acute phase of disease (at 12 days post immunization) when the peak of neurological deficits was observed.

Materials and Methods

Animals

Female Lewis rats weighing 190-200g were used throughout the study. All procedures were carried out in accordance with ethical guidelines for care and use of laboratory animals and were approved by the Local Care of Experimental Animals Committee. During the experiment, animals were fed a standard laboratory diet R-ZV 1324 (SSNIFF, Germany). After experiments rats were decapitated and brains were rapidly removed. Tissues were then frozen in liquid nitrogen and stored at –70°C for immunoblots. Glutamate uptake and release assays were done on freshly isolated synaptosomal or GPV fractions.

Immunisation procedures

To induce experimental autoimmune encephalomyelitis (EAE) rats were immunized subcutaneously in both hind feet with inoculums containing guinea pig spinal cord homogenate emulsified in Freund’s complete adjuvant containing 5.5 mg/ml Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI).

Rats were housed in environmentally controlled conditions and were permitted free access to food and water. Body weight and neurological deficits de
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Preparation of synaptosomal fraction

Synaptosomes were isolated according to the method of Booth and Clark [5], with centrifugation in discontinuous Ficoll gradient (7%, 12%) at 99,000 g. The synaptosomal pellet was washed once in Krebs-Ringer buffer at pH 7.4 (140 mM NaCl, 5 mM KCl, 10 mM Tris-HCl, 1.4 mM MgSO₄, and 1 mM Na₂HPO₄) and suspended in the above buffer to obtain protein concentration of about 5 mg/ml. As demonstrated previously, the synaptosomes obtained by this procedure were highly pure and well maintained energy metabolism; therefore they can be considered as a good model for nerve endings [5,33]. Synaptosomes were used for [3H] glutamate transport measurements, and for expression of neuronal glutamate transporter (EAAC1) using immunoblots technique.

Preparation of glial fraction

Glial plasmalemmal vesicles fractions (GPV) were obtained using a technique by Daniels and Vickroy [10]. Briefly, brain was homogenized in 30 ml of isolated medium (0.32 M sucrose, and 1 mM EDTA) and centrifuged at 1,000 g for 10 min. The supernatant was diluted using SEDH medium (0.32 M sucrose, 1 mM EDTA, 0.25 mM dithiothreitol and 20 mM HEPES, pH 7.4) and centrifuged at 5,000 g for 15 min. After several additional fractionations, the material was centrifuged in a three-step discontinuous Percoll gradient (20% : 10% : 6%) for 10 min at 33,500 g. The layer between 0% and 6% Percoll was collected to obtain the glial plasmalemmal vesicles fraction (GPV) used for further examinations. Measurements of [3H] glutamate transport and the expression of astroglial glutamate transporters (GLAST and GLT-1) were done.

[3H] Glutamate transport assay

Synaptosomal and GPV fractions were used for the measurement of Na⁺-dependent [3H] glutamate uptake and KCl-dependent release of accumulated amino acid. Radioactive glutamate accumulation was performed according to the filtration method described by Divac et al. [12]. Aliquots of fraction were added to the buffer containing 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose and 20 mM HEPES, pH 7.4. Isoosmolar concentrations of choline chloride were used instead to NaCl to measure Na⁺-independent uptake. Na⁺-dependent uptake was calculated as a difference between total and Na⁺-independent uptake. The assay was initiated by addition of [³H]-glutamate (f.c. 5 μM; 45 Ci/nmol). Uptake was quenched by filtration under vacuum through glass filters (Whatman GF/B) at several times points. Filters were washed in ice-cold buffer and soaked in 1 ml of 10% Triton X-100 for 10 min. Radioactivity trapped on filters was then measured in the liquid scintillation counter (Wallack 1409). In the case of release, 50 mM KCl was used at a maximum of the uptake curves (4 min) and liberated radioactivity was assayed after 6 min. In order to prevent the conversion of glutamate to α-ketoglutarate, AOAAs as an inhibitor of AAT (aspartate aminotransferase) [30].

Western blot analysis

For Western blots, fractions (synaptosomal and GPV) or brain homogenates prepared in 50 mM phosphate buffer (pH 7.4) containing 10 mM EGTA, 10 mM EDTA, 0.1 mM PMSF and 100 mM NaCl in the presence of protease inhibitor cocktail (1 μg/ml leupeptin, 0.1 μg/ml pepstatin and 1 μg/ml aprotinin) were subjected to SDS-polyacrylamide gel electrophoresis according to Laemmli [20] towards the expression of metabotropic glutamate receptors group I (mGluR 1a, mGluR 5), excitatory amino acids transporters (GLAST, GLT-1, EAAC1) and cytokines (IL-1β, IL-6, TNF-α). Separated proteins (20-100 μg) were then transferred into nitrocellulose membrane. Blots were incubated first with respective primary monoclonal or polyclonal antibodies against: mGluR 1a and mGluR 5 (Sigma) (1 : 1000); GLAST, GLT-1, EAAC1 (Santa Cruz Biotechnology Inc.) (1 : 500); IL-1β (Santa Cruz Biotechnology Inc.) (1 : 500), IL-6 (R&D Systems) (1 : 250), TNF-α (Chemicon International) (1 : 250) and then with secondary antibodies conjugated with HRP.
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Fig. 1A-B. Na⁺-dependent [³H]-glutamate uptake into synaptosomes (A) and GPV fraction (B) obtained from control and EAE rat brains in the acute phase of disease (12 d.p.i.). Results are mean values ±SD from five separate experiments performed in duplicate; *p < 0.05 significantly different from control group (Student’s t-test).

(Sigma) at dilution from 1 : 4000 to 1 : 6000. Bands were visualized with the chemiluminescence ECL kit (Amersham). Densitometric analysis was performed using UltraScan TMXL (Pharmacia).

Protein assay

Protein concentration in homogenates was measured according to the method Lowry [21] using bovine albumin as a standard.

Statistical analysis

The results of experiments are expressed as a mean ± S.D. from five independent animals. Statistical analysis was performed using the Student’s t-test to compare differences between control and EAE group. P < 0.05 was considered significant.

Results

The rate of radioactive glutamate uptake into synaptosomal fraction was significantly enhanced in EAE rats by about 30% (Fig. 1A). The similar increase by about 15 % of glutamate accumulation in astroglial GPV fraction was observed (Fig. 1B). Stimulated release of glutamate was changed within the similar range in both fractions compared to the respective control values. In the case of synaptosomes we observed 25% increases in the liberation of previously accumulated [³H] glutamate (Fig. 2A) whereas in GPV fraction it rose by about 20% (Fig. 2B).

The major components of astrocytic and neuronal glutamate uptake systems are sodium-dependent transporters of high affinity towards glutamate. Thus, we analyzed the expression of the two astroglial glutamate transporters (GLAST and GLT-1) and neuronal transporter – EAAC1 in GPV and synaptosomal fraction, respectively. Significant increase of immunoreactivity in EAE rats could be observed only for GLT-1 and EAAC1 transporters. In the case of GLT-1 (Fig. 3B) relative expression of protein quantified by densitometric analysis and calculated against β-actin (as an internal standard) was different from the control by about 40%. The expression of EAAC1 protein rose by about 35% (Fig. 3C). Instead, we did not observed significant changes in the expression of GLAST protein compared to the control (Fig. 3A). The patterns of expression of both examined metabotropic glutamate receptors mGluR 1a (Fig. 4A) and mGluR 5 (Fig. 4B) were similar. Overexpression of both receptors’ protein was revealed in EAE rat brain’s homogenates, the level of which exceeded the respective controls by about 40% and 60%.

To confirm the existence of inflammatory conditions in the acute symptomatic phase of EAE, we exa-
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Fig. 2A-B. [³H]-Glutamate release from synaptosomes (A) and GPV fraction (B) after depolarization. 50 mM KCl was used at a maximum of the uptake curves (6 min) and radioactivity was assayed after 6 min. Results are mean values ±SD from five separate experiments performed in triplicate; *p < 0.05 significantly different from spontaneous release control group (Student’s t-test).

Fig. 3A-C. Representative immunoblots showing the expression of excitatory amino acid transporters (EAATs) protein in brain fractions prepared from control and EAE rats in the acute phase of disease (12 d.p.i.). GLAST (A) and GLT-1 (B) are expressed on astroglial GPV fraction and EAAC1 (C) on synaptosomal fraction, respectively. Graphs present the results of densitometric analysis of four independent immunoblots, each done from distinct brain; *p < 0.05 compared with respective control (Student’s t-test).
mined the expression of proinflammatory cytokines – IL-1β, IL-6 and TNF-α. In control animals, cytokines were not detectable, whereas the marked increase of all examined cytokines was observed in brain homogenates obtained from EAE rats. We noticed a significant (p < 0.001) elevation of IL-1β expression which exceeded control value by about 200% (Fig. 5A). The similar rate of increase of relative protein concentration (150% control) was visible in the case of TNF-α (Fig. 5C). The most intensive immunoreactivity we noticed for IL-6. The comparison with control values (Fig. 5B) revealed the 600% increase of protein’s level.

Discussion

Pathological studies have indicated that MS is an immunomediated disorder of the central nervous system (CNS) which is characterized by inflammation, demyelination and oligodendroglial cell death accompanied by axonal damage. These features of the disease are also expressed in experimental autoimmune encephalomyelitis (EAE) – an animal model that mimics SM and is widely used to study the mechanisms of this pathology. It is known that cytokines play important role in pathogenesis of MS/EAE [18,37], however an implication of glutamergic component and excitotoxic mechanisms of cell death are also suggested [8,23,28,43]. The inflammatory factors like IL-1β, IL-6, and TNF-α are implicated in destructive processes leading to the neuronal cell death. Previous studies showed that all of them are involved in pathological mechanisms connected with MS/EAE [7,18,34,36]. In our study we did confirm the existence of inflammatory microenvironment in brains of rodents subjected to EAE. The expression of all examined proinflammatory cytokines (i.e. IL-1β), IL-6, and TNF-α was significantly enhanced.

Inflammatory conditions may influence glutamatergic pathways. Recent evidence suggest that IL-1β can functionally interact with ionotropic and metabotropic glutamate receptors mGluRs [3,23], while TNF-α may influence GLT-1 expression and the rate of glutamate taken up by this transporter [31]. The involvement of glutamatergic receptor-mediated excitotoxicity in pathological changes observed in patients suffering from MS and in EAE rodents was reported, as evidenced by beneficial effects of glutamate receptors antagonist [16,32]. Our intension was to check if under inflammatory conditions, in the peak of symptomatic phase of EAE, there are any functional and quantitative changes in main glutamate transporters responsible for maintaining of proper extracellular glutamate concentration. We here provided evidence that mainly, GLT-1, but also neuronal EAAC1 transporter are significantly overexpressed. This suggests the protective role of transporter systems against elevated glutamate. Similarly, the enhanced function of glutamate uptake reflects activation of mechanism controlling glutamate homeostasis. What is interesting another astrogial
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Glutamate transporter GLAST was not overexpressed. These observations are consistent with our previous results [26] and those of Ohgoh [28] who noticed dramatic increase of EAAC1 expression in the spinal cord of rats subjected to EAE. Although the upregulation of astroglial and neuronal glutamate transporters system is evident, simultaneously the stimulated release of glutamate is enhanced in both fractions. This observation leads to the suggestion that glutamatergic transmission can be impaired. When released from presynaptic terminals, glutamate activates ionotropic (NMDA, AMPA and KA) and metabotropic receptors (mGluRs). Then, it must be taken up from the synaptic cleft by the transporter systems, to prevent overstimulation of the receptors and subsequent cell death. It has been generally accepted that acute excitotoxic degeneration of neurons, evoked by glutamate, is mediated by NMDA receptors, whose activation leads to pathological increases in the intracellular Ca^{2+} concentrations [4,8,35]. However, evidence supports also a role of glutamatergic receptors in pathogenesis of MS [29,32]. Since the role of ionotropic glutamate receptors in the pathological conditions under EAE conditions is evident, we investigate the expression of selected metabotropic receptors of this amino acid. We tested the expression of group I mGluR because there are some results demonstrating that apart from NMDA receptors, also these receptors may participate in neurotoxicity during SM [15], rat brain ischemia [22], and acute homocysteine-induced toxicity in vitro [45] accelerating NMDA-induced cell death. Over the recent years the group I mGluRs have been extensively studied in experimental animals and results of these studies lead to an appreciation of their importance in the CNS. They are involved in controlling of glutamate level and its transmission via interaction of G protein with K^{+} channel. Activation of mGluR G I leads to the simultaneous activation of K^{+} channel by PLC kinase and to the controlling of neurons activation [6,44]. Thus, our observation of enhanced expression of both mGluR 1a and mGluR 5 may suggest disturbances in glutamate metabolism in synapse during acute phase of EAE.

In conclusion, our findings confirm the involvement of glutamatergic component into the pathological events which take place in the rat brain during the acute, inflammatory phase of EAE. The results of enhanced release and overexpressed mGluRs suggest the impairment of glutamatergic transmission that can lead to the elevation of extracellular glutamate. The existence of such elevation is confirmed by the enhancement of glutamate uptake system and the overexpression of glutamate transporters. These

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**Fig. 5A-C.** Expression of IL-1β (A), IL-6 (B) and TNF-α (C) in brain homogenates obtained from control and EAE rats. Results of densitometric analysis of Western blots (n = 5) are presented in the graph; *p < 0.01 (Student’s t-test).
changes are of protective nature and may reflect the compensatory adjustment against elevated glutamate. Whether this compensation is efficient enough to prevent excitotoxicity, needs further study.

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