PARTICIPATION OF THE GABAERGIC SYSTEM ON THE GLUTAMATE RELEASE OF FRONTAL CORTEX SYNAPTOSOMES FROM WISTAR RATS WITH EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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Abstract—We previously found that the glutamate release was decreased in synaptosomes from rat cerebral cortex during the development of experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis. Various other reports have shown a deficit in the expression of proteins associated with GABAergic neurotransmission in the neocortex of patients with multiple sclerosis and it was also demonstrated that the activation of GABA$_A$ receptors leads to an inhibition of glutamate release. Now, in order to evaluate the events that may affect the neuronal function in EAE synaptosomes, we analyzed the participation of the GABAergic system in glutamate release and in the flunitrazepam-sensitive GABA$_A$ receptor density. This revealed alterations in the GABAergic system of the frontal cortex synaptosomes from EAE animals. GABA induced a decrease in the 4-aminopyridine-evoked glutamate release in control synaptosomes which was abolished by picrotoxin, a GABA$_A$ receptor antagonist. In contrast, synaptosomes from EAE rats showed a loss in the inhibition of glutamate release mediated by GABA. Furthermore, the flunitrazepam-sensitive GABA$_A$ receptor density was decreased during the acute stage of the disease in synaptosomes from EAE rats. We also observed a loss of inhibition in the Ca^{2+}-dependent phosphorylation of synapsin I mediated by GABA in nerve terminals from EAE animals, which could explain the loss of GABAergic regulation on evoked glutamate release. The changes observed in the GABA$_A$ receptor density as well as the loss of GABAergic inhibition of glutamate release were partially reverted in cortical synaptosomes from recovered EAE animals. These results suggest that the decrease in the flunitrazepam-sensitive GABA$_A$ receptor density may explain the observed failure of GABAergic regulation in the glutamate release of synaptosomes from EAE rats, which might contribute to the appearance of clinical symptoms and disease progression. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

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As the CNS inflammation and neurodegeneration are two major pathological processes in multiple sclerosis (MS), the understanding of the pathology and mechanisms of CNS degeneration in this disease is essential for developing techniques to protect neural structures and functions in MS patients. In addition to the multiple demyelinated lesions disseminated throughout the white matter of the CNS, pathologic investigations have shown that neocortical neuronal and glial degeneration are frequent in MS and can contribute to clinical symptoms and disease progression (Kutzelnigg et al., 2005; Wegner et al., 2006). The main clinical and pathological features of MS can be reproduced in the inducible animal counterpart, experimental autoimmune encephalomyelitis (EAE). This experimental disease can be induced in susceptible animals by a single injection of CNS antigens homogenized in an adequate adjuvant. Wistar rats develop only a monophasic course (acute stage, 11–13 days post induction, dpi), characterized by ataxia and hind limb paralysis associated with weight loss and fecal and urinary incontinence. Affected animals show a spontaneous neurological improvement 2–4 days after the onset of the disease and regain the full ability to walk by 17–18 dpi (Slavin et al., 1996; Degano and Roth, 2000). Nevertheless, in spite of the well-defined histopathology, the events that contribute to neurological deficits and persistent disability in MS and EAE remain elusive.

Glutamate is the major excitatory amino acid transmitter within the CNS, with its signaling being mediated by a number of postsynaptic ionotropic and metabotropic receptors. The central role played by glutamate receptors in mediating excitotoxic neuronal death in stroke, epilepsy, trauma, and MS has been well established (Bolton and Paul, 2006). However, the glutamatergic release, crucial in information intake and processing within the brain (Vereker et al., 2000), has been poorly explored for this disease. Recently, we described alterations in the glutamate release in isolated nerve terminals of the cerebral cortex from EAE animals and found that the physiological exocytotic vesicular release was inhibited to a certain extent. Moreover, although this inhibition coincided with the onset of the clinical signs, the animals later totally recovered to have a glutamate release level similar to that of controls (Vilcaes et al., 2009).

GABA is the major inhibitory neurotransmitter balanced with glutamate in the CNS. GABA$_A$ receptors, a large and diverse family of Cl$^-$-permeable ion channels, mediate fast transmission at inhibitory GABAergic synapses and are critical for the development and coordina-

tion of the neuronal activity underlying the majority of physiological and behavioral processes in the brain (Jacob et al., 2008). These receptors are perfectly positioned to reduce excitability and inhibit the positive-feedback loop that constitutes excitotoxicity (Zhang et al., 2007). Previous studies have shown that the concentration of GABA and the glutamate decarboxylase activity in blood are reduced in EAE and MS (Gottesfeld et al., 1976; Demakova et al., 2003). Long et al. (2009) have recently demonstrated that nerve terminal GABA$_A$ receptors modulate phosphorylation of synapsin I by presynaptic Ca^{2+}/calmodulin-dependent signaling to inhibit glutamate release from rat neocortex. In fact, synapsins are presynaptic proteins expressed ubiquitously in all presynaptic nerve terminals in the CNS and are major synaptic vesicle-specific phosphoproteins that play multiple roles in neural development, synaptic transmission, and plasticity (Cesca et al., 2010). Related to this, we have previously described that the synapsin I Ca^{2+}-dependent phosphorylation was inhibited in synaptosomes from EAE animals, which in turn decreased the glutamate release (Vilcaes et al., 2009).

The aim of the present study was now to investigate the possible impairment of the GABAergic system and whether it regulates the glutamate release and phosphorylation of synapsin I in nerve terminals isolated from the frontal cortex of sick and recovered EAE rats.

EXPERIMENTAL PROCEDURES

Materials

Myelin was purified from bovine spinal cords as previously described (Degano and Roth, 2000). Complete Freund's adjuvant (CFA), GABA, glutamate dehydrogenase (EC 1.4.1.3), NADP$^+$, and 4-aminopyridine (4AP) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). [^{3}H]-flunitrazepam was purchased from New England Nuclear (Boston, MA, USA), Percoll was obtained from Pharmacia (Peapack, NJ, USA), diazepam from Hoffmann-LaRoche (Basel, Switzerland), and picrotoxin from Fluka (Steinhem, Germany). Synapsin I-specific antibody (AB1543P) and synapsin phosphorylation state P-site 1 (Ser-9) antibody (AB5881) were purchased from Chemicon International, Inc. (Temecula, CA, USA). All other chemicals were analytical grade reagents of the highest available purity.

Animals and EAE induction

45-day-old Albino rats from a Wistar strain inbred in our laboratory for 40 years were used. All experiments were performed in accordance with international and institutional guidelines for animal care, and the protocol was approved by the local institutional review committee for animal studies (Exp. No. 15-99-40426). Under ketamine/xilazin anesthesia (10 and 65 mg/kg, i.p., respectively), the active disease was induced by intradermal inoculation in both hind feet with 0.5 ml of an emulsion consisting of 0.25 ml saline solution and 0.25 ml CFA containing 8 mg bovine myelin (EAE group). Control animals received 0.5 ml of the same emulsion without any antigenic preparation (CFA group). About 85% of the animals from the EAE group developed a monophasic course (acute stage, 11–13 dpi), but later these affected animals showed a spontaneous neurological improvement by regaining their full ability to walk by 17–18 dpi. Animals were assessed daily for clinical signs of EAE and scored as follows: 0, no clinical expression of the disease; 1, flaccid tail; 2, hind limb weakness; 3, complete hind leg paralysis accompanied by urinary incontinence;

4, quadriparesis, moribund state, or death. Control and sick EAE animals were decapitated at 24–36 h after onset of the disease. Also, the CFA and EAE rats completely recovered from any clinical signs (EAErec) were sacrificed between 20 and 22 dpi. Since the results for the control CFA animals were similar, independent of the sampling day (14 or 20 dpi), these animals were processed together and designated as CFA group.

Preparation of cerebrocortical synaptosomes

The frontal cortex was isolated from CFA, sick EAE, and EAErec animals, and synaptosomes were purified on discontinuous Percoll gradients as described previously (Dunkley et al., 1988). These nerve terminals are subcellular membranous structures that are formed during the mild disruption of the brain tissue and retain the morphological features and chemical composition of the presynapses (Nicholls, 2003). Synaptosomes which sedimented between the 10 and 23% Percoll bands were collected and diluted in a final volume of 30 ml of HEPES buffer medium consisting of 140 mM NaCl, 5 mM KCl, 5 mM NaHCO$_3$, 1 mM MgCl$_2$, 1.2 mM Na$_2$HPO$_4$, 10 mM glucose, and 10 mM HEPES, pH 7.4, before centrifugation at 27,000×g for 10 min at 4 °C. The pellets thus formed were resuspended in 5 ml of HEPES buffer medium, and the protein content was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). After determination of total protein, the glutamate release and [^{3}H]-flunitrazepam binding assays were performed. For the glutamate release assay, the synaptosomal pellets were stored on ice and used within 3–4 h.

Glutamate release assay

Glutamate release from cerebrocortical synaptosomes was monitored online, using an assay employing exogenous glutamate dehydrogenase and NADP$^+$ to couple the oxidative decarboxylation of the released glutamate. Then, the generated NADPH was detected fluorometrically (Nicholls et al., 1987; Vilcaes et al., 2009). Briefly, synaptosomal pellets were resuspended in HEPES buffer medium and incubated in a stirred and thermostated cuvette maintained at 37 °C in a FluoroMax-P Horiba Jobin Yvon spectrofluorimeter. 1 mM NADP$^+$, 50 units/ml glutamate dehydrogenase, and 1.2 mM CaCl$_2$ were added after 3 min. After 5 min of incubation, 3 mM 4AP was added to stimulate the glutamate release. Where indicated, synaptosomes were incubated in the presence of GABA (500 μM) for 4 min or GABA (500 μM) plus picrotoxin (100 μM) for 10 min prior to the addition of 4AP. Traces were calibrated by the addition of 4 nmol of glutamate at the end of each assay. Data points were obtained at 1-s intervals. **AQ: 2**

[^{3}H]-flunitrazepam binding assay

The specific binding of [^{3}H]-flunitrazepam (85 Ci/mmol) was measured by a filtration technique (Cid et al., 2008). Binding was carried out in the presence of radioligand at final concentrations of 0.5, 1, 2, 3, 4, 5, 8, and 9 nM, at 4 °C. Each assay was performed in triplicate using 1-ml aliquots containing 0.3 mg of proteins from the synaptosomal fractions. Nonspecific binding was measured in the presence of 10 μM diazepam. After 60 min of incubation, samples were filtered under vacuum through Whatman GF/B filters using a Brandel M-24 filtering manifold. Samples were washed three times with 4 ml of ice-cold Tris–HCl buffer (50 mM, pH 7.4) and the radioactivity was measured using an LKB-1214-RackBeta counter at 60% efficiency. The values Kd and Bmax were obtained by nonlinear regression using the equation for hyperbola (one binding site): $Y = Bmax*X/(Kd+X)$, where Bmax is the maximal binding, and Kd is the concentration of ligand required to reach half-maximal binding.

Immunoblot analysis

Synaptosomal samples were resuspended in HEPES buffer medium, 1.2 mM $CaCl_2$ was added, and samples were incubated at 37 °C for 2 min with stirring. This was followed by a further incubation with 3 mM 4AP for 5 min in order to stimulate Ca^{2+}-dependent synapsin I phosphorylation concomitant to the glutamate release. Where indicated, synaptosomes were incubated in the presence of GABA (500 μM) for 4 min or GABA (500 μM) plus picrotoxin (100 μM) for 10 min prior to the addition of 4AP. Aliquots were rapidly solubilized in sample buffer, and equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis and then electrotransferred onto nitrocellulose membranes (Degano and Roth, 2000; Vilcaes et al., 2009). Immunoblotting was performed at a 1:500 dilution of the synapsin phosphorylation state-specific antibody to P-site 1 and at a 1:1000 dilution of synapsin I-specific antibody for detected total synapsin I. The immunoreactive bands in the immunoblot were detected by infrared probe-labeled secondary antibodies and the fluorescence was then analyzed by the Odyssey scanner with the fluorescence intensity being quantified by the GelPro analyzer software. The phosphorylated synapsin I and total synapsin I content were normalized for tubulin content, determined using the mouse anti-βIII-tubulin antibody TUJ 1 (1:2500).

Data analysis

The results were expressed as the mean±SEM. The levels of glutamate were analyzed using the one-way or two-way analysis of variance (ANOVA). The Bmax and Kd values of the $GABA_A$ receptor density were analyzed using a one-way ANOVA. The phosphorylation of synapsin I is shown as the percentage of p-syn/tubulin with respect to control, and these values were analyzed using a one-way ANOVA. Whenever the ANOVA indicated significant effects ($P<0.050$), a pairwise comparison of means by Newman–Keuls test (NK) was carried out. In all cases, the assumptions of the analysis of variance (homogeneity of variance and normal distribution) were verified. In all the statistic analyses a $P<0.050$ was considered to represent a significant difference between groups.

RESULTS

Inhibition of glutamate release of frontal cortex synaptosomes in EAE animals

The glutamate release evoked by 4AP of frontal cortex synaptosomes from control (CFA group), sick EAE animals with a clinical score of 2–3 (EAE group), and EAE rats totally recovered (score 0) from clinical signs (EAErec group) was studied. 4AP is a potassium channel blocker which has been shown to destabilize the plasma membrane potential of the synaptosomes, resulting in an increase in the cytoplasmic free Ca^{2+} concentration through the opening of voltage-gated Ca^{2+} channels, thus allowing them to fire spontaneous action potentials capable of triggering the exocytotic release of glutamate (Tibbs et al., 1989). This repetitive firing induced by 4AP, in fact mimics more closely the physiological mechanism of terminal depolarization than other inductors. In the present study, in the control nerve terminals from CFA rats, this release rate reached 26.3±2.0 nmol glutamate/mg protein/7 min in the presence of 1.2 mM $CaCl_2$. However, in the synaptosomes from sick EAE rats, as we previously reported (Vilcaes et al., 2009), the total release of glutamate evoked by 4AP was reduced to 18.9±1.0 nmol/mg protein/7 min. Finally,
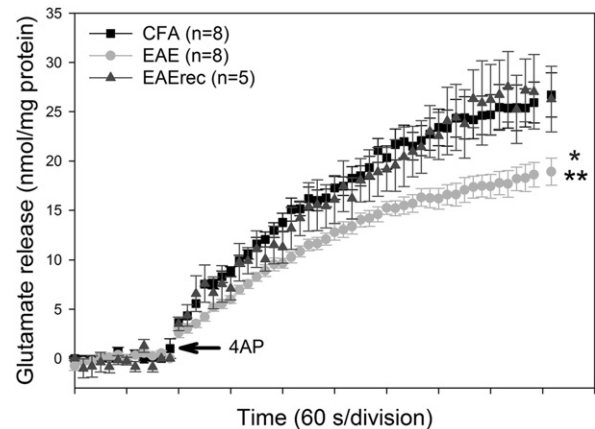

Fig. 1. 4-aminopyridine (4AP)-evoked glutamate release from rat frontal cortex synaptosomes. The synaptosomes were purified from control (CFA), sick EAE animals (EAE), and EAE rats recovered from clinical signs (EAErec), and the glutamate release was determined as indicated in Experimental procedures. Results are means±SEM of the indicated number of independent experiments calculated at each time point (1 s); however, for clarity data points are only represented at 10-s intervals including standard errors. Glutamate release from EAE rats was significantly different in EAErec and CFA animals, * $P<0.05$; ** $P<0.01$, two-way ANOVA, respectively.

the glutamate release of synaptosomes from EAErec animals induced by 4AP was similar to that of the control preparations (26.2±3.0 nmol/mg protein/7 min) (Fig. 1).

Loss of GABAergic inhibition of the glutamate release of synaptosomes from EAE animals

To analyze the participation of the GABAergic system on the glutamate release, synaptosomes from CFA, EAE, and EAErec animals were incubated in the presence of GABA (500 μM) for 4 min, which was then followed by the addition of 3 mM 4AP in order to trigger release. The statistical analysis by one-way ANOVA of the glutamate release in CFA animals revealed a significant effect of the presence of GABA ($F(2,9)=18.599$, $P<0.001$). As shown in Fig. 2, there was a decrease in the glutamate release rate in the presence of GABA from 34.1±4.9 to 20.6±2.4 nmol/mg/7 min. To investigate if the inhibition of 4AP-evoked glutamate release was indeed mediated by $GABA_A$ receptors, we incubated the synaptosomes with GABA plus picrotoxin, and demonstrated that control glutamate release by GABA was almost completely reversed by 100 μM picrotoxin (31.0±4.3 nmol/mg/7 min). Interestingly and at variance with CFA synaptosomes, the presence of GABA did not modify the glutamate release levels in EAE synaptosomes for all conditions analyzed. In addition, the presence of GABA plus picrotoxin on the incubation system did not alter the glutamate release levels in the cortical synaptosomes from sick EAE rats. However, as similarly shown for CFA synaptosomes, GABA induced an inhibition of the glutamate release in EAErec synaptosomes with respect to synaptosomes without GABA (30.1±1.4−23.6±2.1 nmol/mg/7 min), with the inhibition produced by GABA being reversed by picrotoxin (30.0±1.9 nmol/mg/7 min).
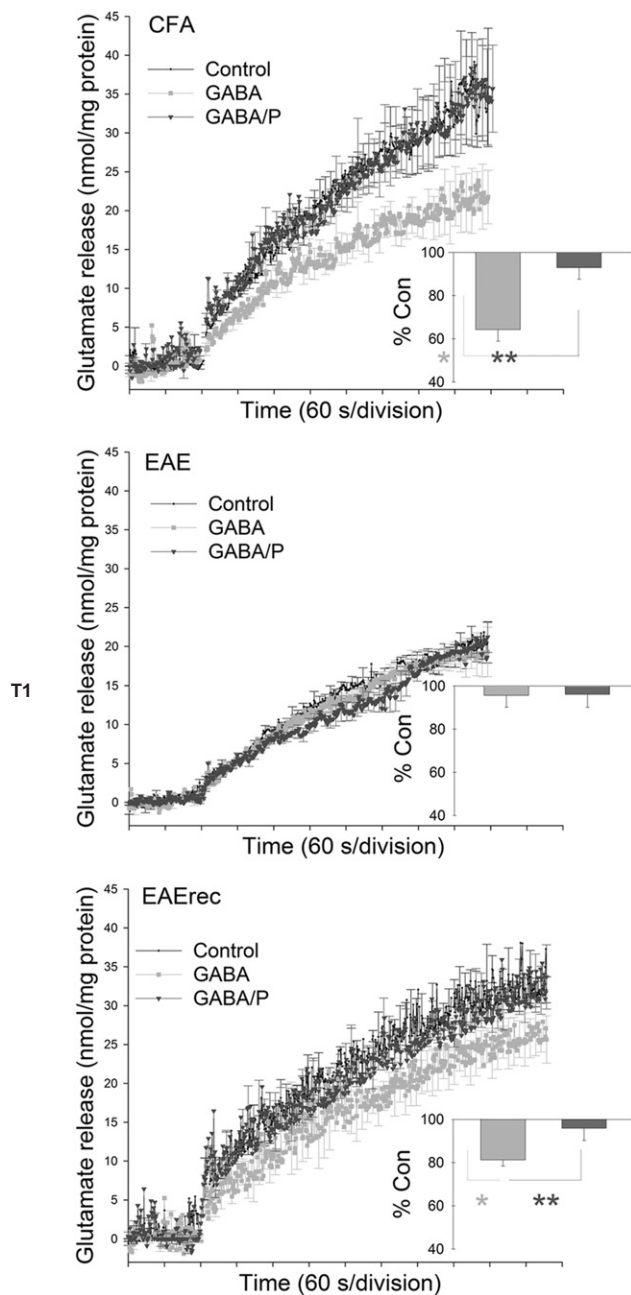
Fig. 2. Loss of the inhibition of glutamate release mediated by GABA$_A$ receptor in frontal cortex synaptosomes. The synaptosomes from control (CFA), sick EAE animals (EAE), and EAE rats recovered from clinical signs (EAErec) were incubated in the presence of GABA (500 μM) or GABA plus picrotoxin (P, 100 μM). Glutamate release was evoked by the addition of 4-aminopyridine (4AP) and assayed by online fluorometry. In all cases, the inset quantifies both the reduction of 4AP-evoked glutamate release by GABA (% control 8 min after 4AP addition) and the occlusion of the GABA-induced decrease of 4AP-evoked glutamate release (% control 5 min after 4AP addition) by picrotoxin. Data are expressed as means±SEM from four different synaptosomal preparations. GABA had no effect on the glutamate release in synaptosomes from EAE animals, but induced a decrease in the 4AP-evoked glutamate release in synaptosomes from EAErec animals and CFA with respect to control condition, with GABA/P eliminating the inhibitory effect of the glutamate release induced by GABA, * $P<0.05$ and *** $P<0.001$, respectively (NK test).

Table 1. The flunitrazepam-sensitive GABA$_A$ receptor density in synaptosomes from EAE rats

Group	Bmax (fmol/mg protein)	Decrease (%)	Kd (nM)
CFA	1054±75	—	2.13±0.53
EAE	591±68*,#	44	1.81±0.52
EAErec	834±57*	21	1.73±0.15

Binding maximum of [3H]-flunitrazepam in frontal cortex synaptosomes from control (CFA group), sick EAE animals (EAE) and EAE rats recovered from clinical signs (EAErec). Each value of Kd represents the mean±SEM of values (n=4) obtained by nonlinear regression of experimental data from saturation curves. No significant differences were observed in the Kd values.
* $P<0.050$ compared to CFA rats.
# $P<0.050$ compared to EAErec rats (NK test).

The flunitrazepam-sensitive GABA$_A$ receptor density was decreased in synaptosomes from EAE animals

In order to investigate a possible cause for the absence of GABA regulation of the glutamate release observed in sick EAE rats, we evaluated the density of the GABA$_A$ receptor in synaptosomes from CFA, EAE, and EAErec animals using a flunitrazepam-sensitive GABA$_A$ receptor-binding assay. A one-way ANOVA (Table 1) revealed a significant effect of the disease on Bmax values, with the Bmax in synaptosomes from EAE rats being significantly lower than in CFA rats (56% of the control). Although the Bmax from EAErec animals was 834 fmol/mg (79% of the control), which was higher than the EAE group, this was still lower than the CFA group. In addition, no significant differences in Kd values were observed (Table 1). These results mean that there were lower binding sites for flunitrazepam in the EAE rats but the affinity for flunitrazepam was similar to that of control animals.

Loss of GABAergic regulation on Ca^{2+}-dependent phosphorylation of synapsin I in nerve terminals from EAE animals

Synapsin I is a member of a family of neuron-specific phosphoproteins associated with the cytoplasmic surface of small synaptic vesicles. In adult synapses, synapsin tethers synaptic vesicles to the actin cytoskeleton in a phosphorylation-dependent manner and through this mechanism regulates the proportion of vesicles in the nerve terminal that are available for neurotransmitter release (Jovanovic et al., 1996). To study whether the mechanism by which GABA regulates the 4AP-evoked glutamate release involves the phosphorylation of synapsin I, we monitored the effects of GABA$_A$ receptor activation on the presynaptic Ca^{2+}/calmodulin-dependent kinase (CaMK) I/synapsin I signaling pathway. For this purpose, we used a phosphorylation state-specific antibody which recognizes synapsin I only when phosphorylated by CaMK I at Ser-9 (anti-P-site 1 synapsin I antibody). To detect the activity of GABA$_A$ receptors localized to nerve terminals in the rat neocortex which were independent of the large postsynaptic pool of these receptors, synaptosomes were isolated using a well-established procedure. Then, we tested whether activation of GABA$_A$ receptors with the agonist GABA induced
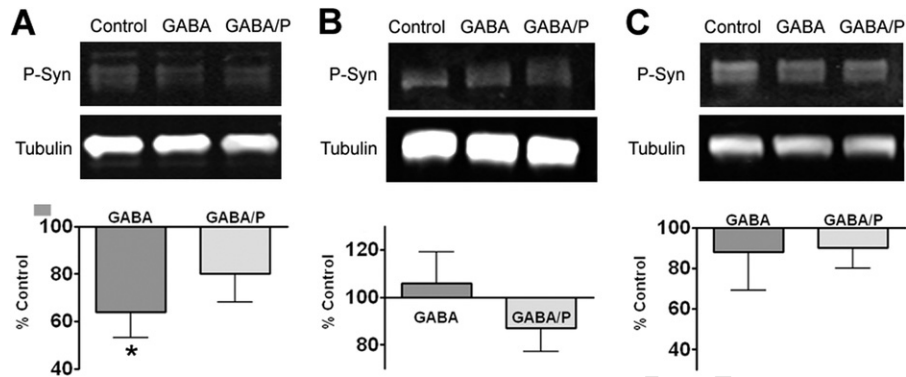
Fig. 3. Loss of the inhibition of phosphorylation of synapsin I mediated by GABA$_A$ receptor in frontal cortex synaptosomes. Immunodetection of phosphorylated synapsin I at P-site 1 (% of the control) in purified neocortical synaptosomes stimulated by 4AP from CFA (A), sick EAE (B), and EAErec rats recovered from clinical signs (C). GABA had no effect on the phosphorylation of synapsin I in synaptosomes from EAE ($n=7$) and EAErec ($n=5$) animals, but induced a decrease in the 4AP-evoked phosphorylation of synapsin I in synaptosomes from CFA rats ($n=5$) with respect to control condition, * $P<0.050$ (NK test). GABA/P eliminated the inhibitory effect of the phosphorylation of synapsin I induced by GABA in synaptosomes from CFA animals.

changes in the CaMK I-dependent signaling in nerve terminals from CFA, EAE, and EAErec animals stimulated by Ca^{2+} and 4AP (Fig. 3) or with Ca^{2+} alone (Fig. 4). In synaptosomes from CFA rats stimulated by 4AP, a one-way ANOVA revealed a decrease in P-site 1 phosphorylation of synapsin I in the presence of GABA, with a 64±11% activity of control synaptosomes. To investigate if the observed regulation of presynaptic CaMK II/synapsin I signaling by ambient GABA was mediated by GABA$_A$ receptors, synaptosomes were incubated with GABA plus picrotoxin and we observed a partial recovery of the phosphorylation of synapsin I (80±12%) (Fig. 3A). Statistical analysis of the P-site 1 phosphorylation of synapsin I levels in EAE animals did not reveal a significant effect of the presence of GABA (106±13%) or GABA plus picrotoxin (87±10%) with respect to control (Fig. 3B). Moreover, analysis of the P-site 1 phosphorylation of synapsin I levels in EAErec animals did not show any significant differences between control and in presence of GABA (8±19%) or GABA plus picrotoxin (90±10%) (Fig. 3C). Finally, neither GABA nor GABA plus picrotoxin affected the amount of total synapsin I in the presence of 4AP in CFA, EAE, or EAErec animals (data not shown).

We also tested whether the activation of GABA$_A$ receptors in the presence of Ca^{2+} but in the absence of 4AP induced changes in the CaMK I-dependent signaling in nerve terminals from the different groups. No significant differences were observed with the treatments (GABA or GABA plus picrotoxin) in the CFA, EAE, or EAErec groups. Furthermore, the statistical analyses of the immunocontent of total synapsin I in the frontal cortex synaptosomes in the absence of 4AP did not reveal a significant effect of the treatments in any of the groups studied, and the addition of GABA or GABA plus picrotoxin did not affect the amount of total synapsin I for CFA, EAE, or EAErec animals (data not shown).

DISCUSSION

The present report describes for the first time a loss of the reduction of glutamate release mediated by GABA$_A$ receptors in frontal cortex synaptosomes from EAE animals during the acute stage of the disease. In synaptosomes from the CFA group, GABA induced a decrease in the 4AP-evoked glutamate release with respect to control condition (64% of the control) and picrotoxin partially abol-
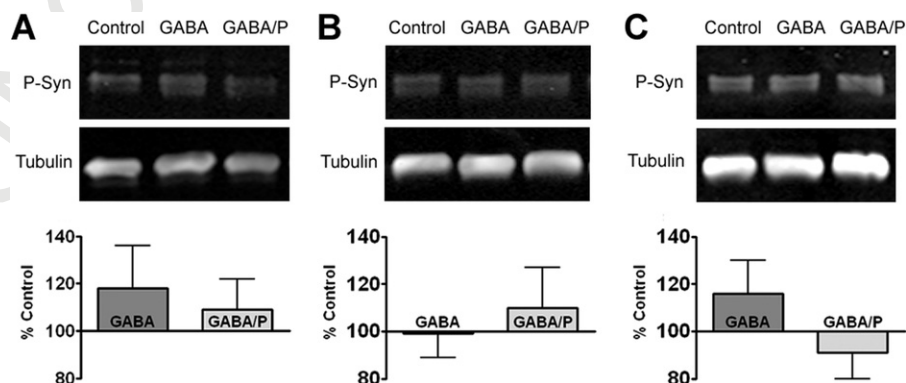

Fig. 4. Immunodetection of phosphorylated synapsin I at P-site 1 (% of the control) in frontal cortex synaptosomes not stimulated with 4AP from CFA (A), sick EAE (B) or EAErec rats (C). GABA and GABA/P had no effect on the phosphorylation of synapsin I in synaptosomes from CFA ($n=5$), EAE ($n=7$) or EAErec animals ($n=5$).

ished this effect on the glutamate release induced by GABA (80±12%). This lack of total recovery of synapsin I phosphorylation could be due to the fact that picrotoxin acts by stabilizing a nonconducting state of the $GABA_A$ ionophore following binding to an allosteric site rather than through a pore-occluding mechanism (open-channel blocking) (Korshoej et al., 2010). However, we cannot discard any influence of other GABA receptors on the observed results. Related to this, it has been previously described that $GABA_B$ receptors directly inhibit several subtypes of voltage-sensitive calcium channels in the prefrontal cortex (Chalifoux and Carter, 2011).

In agreement with our previous results (Vilcaes et al., 2009), we found a reduction in the glutamate release stimulated by 4AP in frontal cortex synaptosomes from EAE rats, concomitant with the acute stage of the disease. Furthermore, the animals that were completely recovered from the paralysis had a glutamate release level similar to that of CFA control animals. A major decrease was found in the flunitrazepam-sensitive $GABA_A$ receptor density in the frontal cortex synaptosomes from EAE animals during the acute stage of the disease. In fact, the central benzodiazepine receptor density can be used to express the $GABA_A$ receptor density, because the flunitrazepam-binding site is located in the α-subunit of the $GABA_A$ receptor (Primus and Kellog, 1991).

A recent work has suggested that MS may be associated with impairment of neuroinhibition as part of a more general progressive failure of neuronal connectivity (Manson et al., 2008). In particular, these authors have hypothesized that the impairment of deactivation is related to deficits of transcallosal connectivity and the GABAergic system, occurring with the progression of the pathology in MS patients. Manyam et al. (1980) reported that the levels of GABA in the cerebrospinal fluid of patients with MS are significantly lower than levels in control subjects. Dutta et al. (2006) demonstrated that both, the GABA receptor-related gene transcripts and the density of inhibitory interneuron processes were reduced in motor cortex samples from MS patients. Our results are also in agreement with a recent report (Rossi et al., in press) where the authors demonstrated irreversible alterations of GABA transmission in the striatum of EAE mice, and reported that the chronic persistence of proinflammatory cytokines was able to produce profound alterations in the electrophysiological network properties in cultured cortical neurons, which were, however, reverted by GABA administration. In the present report, we described a flunitrazepam-sensitive $GABA_A$ receptor density reduction of 44% in synaptosomes from EAE rats with respect to CFA controls, whereas this decrease in synaptosomes from EAErec animals was only of 21%. However, in spite of the fact that the flunitrazepam-sensitive $GABA_A$ receptor density and the GABAergic functional recovery correspond with the improvement shown from clinical signs, this recovery was not total.

The reduced central flunitrazepam receptor binding may also reflect a terminal degeneration (Rudolf et al., 2002) or receptor downregulation produced by inflammatory mediators (Bhatt et al., 2005; Lewitus et al., 2007). Recently, increasing GABAergic activity strongly ameliorated the manifestations of the EAE clinical course, through a mechanism probably involving a direct neuroprotective effect and an inhibitory action on the antigen-presenting cells and the resulting inflammatory response (Bhat et al., 2010). A benzodiazepine receptor binding decrease in neurodegenerative diseases has also been described in human patients using [^{11}C]Flumazenil Positron Emission Tomography (Ishibashi et al., 1998; Pinborg et al., 2001). In these studies, the authors observed a binding reduction in the cortical areas that was associated with neuronal loss. The combination of neuronal damage and deafferentiation may however have underlain some of the changes observed here.

Determining the causes and consequences of altered GABAergic transmission in the cortical networks of EAE disease require knowledge of which subpopulations of GABAergic neurons are affected. There has been increasing interest in the roles of GABAergic neurons in the neocortex, including their role in behavior, memory, perception, and consciousness (González-Burgos et al., 2007; Möhler, 2009). Furthermore, cognitive deficits, major depression, and disorders of behavior have also been described in a relevant percentage of MS patients and EAE animals (Calabrese et al., 2010; Paparrigopoulos et al., 2010; Zipoli et al., 2010). It is possible that the GABAergic alterations observed in frontal cortex from EAE animals could be implicated in cognitive and behavioral deficits observed in EAE. Nevertheless, further studies still need to be carried out to shed light on this matter. However, extensive functional studies of specific neuron populations at the cellular and system levels in different brain regions have been hampered by the difficulty of identifying these neurons during experiments.

Since 4AP-glutamate release involves Ca^{2+}/calmodulin activation and the subsequent phosphorylation of synapsin I, which in turn modulates the neurotransmitter release by reversibly tethering the synaptic vesicles to the actin cytoskeleton, we investigated whether GABA regulated synapsin I phosphorylation in synaptosomes from EAE rats stimulated by 4AP. Our results showed that the presence of GABA decreased P-site 1 synapsin I phosphorylation in CFA animals. However, we found a lack of GABAergic inhibition of 4AP-evoked Ca^{2+}-dependent phosphorylation of synapsin I in nerve terminals from EAE animals. In this regards, we reported previously that the inhibition of glutamate release in cerebral cortex synaptosomes from EAE rats is concomitant with a decrease of synapsin I phosphorylation (Vilcaes et al., 2009).

Long et al. (2009) described an alternative type of synaptic inhibition mediated by $GABA_A$ receptors present on neocortical glutamatergic nerve terminals. Activation of $GABA_A$ receptors resulted in a reduction of subsequent depolarization-evoked Ca^{2+} influx, leading to an inhibition of glutamate release. These authors described that in cortical synaptosomes, GABA increased synapsin I phosphorylation via Ca^{2+}/calmodulin, using an antibody against P-site 3 (Ser-603) phosphorylation of synapsin. However,

when we evaluated the possible GABAergic modulation of P-site 1 synapsin I phosphorylation in the presence of Ca^{2+} alone, we did not observe any differences in the synapsin I phosphorylation in any of the groups studied. This apparent discrepancy may be attributed to the fact that two different phosphorylated sites on synapsin I were analyzed, which were phosphorylated/dephosphorylated in different ways (Jovanovic et al., 2001). Therefore, it is possible that the loss of GABAergic modulation on glutamate release could have been mediated by a decrease of $GABA_A$ receptor density with a subsequent change in the synapsin I phosphorylation via Ca^{2+}/calmodulin, which in our case led to a reduction of synapsin I phosphorylation at Ser-9 (P-site 1).

The abnormalities of flunitrazepam-sensitive $GABA_A$ receptor binding and GABAergic function in the cortical synaptosomes from EAE rats suggest that basal and/or compensatory changes in the GABAergic system were involved in the pathophysiologic mechanism of EAE. The decrease of flunitrazepam-sensitive $GABA_A$ receptor density in the frontal cortex synaptosomes might explain, at least in part, the loss of inhibition of glutamate release mediated by $GABA_A$ receptor found on frontal cortex synaptosomes from EAE animals. The activation of somatodendritic $GABA_A$ receptors, which are composed of synaptic and extrasynaptic ones, exhibits inhibitory actions by membrane shunting or hyperpolarization of postsynaptic neurons. However, it has been revealed that $GABA_A$ receptors are also expressed on presynaptic terminals, with their activation modulating neurotransmitter release from nerve terminals in a variety of brain regions (Jang et al., 2001; Turecek and Trussell, 2002), including the hippocampal mossy fibers (Jang et al., 2006; Nakamura et al., 2007). On the other hand, neocortical neuronal damage is frequent in MS and can contribute to clinical symptoms and disease progression (Kutzelnigg et al., 2005; Wegner et al., 2006; Chang et al., 2008; Frischer et al., 2009). It is also worth mentioning that lesions of the primary motor or of the prefrontal cortex of both primates and rodents produce a consistent constellation of symptoms that are strikingly similar across species as diverse as rats and humans (Kolb, 1984). This raises the possibility that the failure of the GABAergic regulation of the glutamate release of frontal cortex synaptosomes from EAE rats could be associated to impairments of motor control.

CONCLUSION

In summary, the changes observed in the EAE frontal cortex suggest a role plated by alterations of the GABAergic system in the EAE cortical pathology. The decrease of the $GABA_A$ receptor density in nerve terminals from this cortical region and the failure of GABAergic regulation on glutamate release and synapsin I phosphorylation in synaptosomes from EAE rats may have contributed to clinical symptoms and disease progression. These findings could also have implications for the neuronal and synaptic dysfunction in EAE and possibly in MS cortex. Further studies, however, are needed to determine whether GABAergic transmission modulation could be successful in MS therapy.

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