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GABAergic Agonists Modulate the Glutamate Release from Frontal Cortex Synaptosomes of Rats with Experimental Autoimmune Encephalomyelitis

Nicolás Fernández Hurst, Natalí L. Chanaday[§] and German A. Roth[*]

Centro de Investigaciones en Química Biológica de Córdoba (CIQUIBIC, UNC-CONICET), Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, X5000HUA Córdoba, Argentina

German A. Roth

Abstract: Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease that mimics many of the clinical and pathological features of multiple sclerosis. We have previously described a significant diminution in the GABAergic regulation of glutamate release from synaptosomes of EAE rats isolated during the acute stage of the disease. In order to explore the possible metabolic pathways responsible for this alteration, in this work we evaluate the direct effect of different GABAergic agonists on the glutamate release and concomitant synapsin I phosphorylation in synaptosomes from the frontal cortex of control and EAE animals. The results show that GABA as well as the GABA receptor agonists Muscimol ($GABA_A$ agonist) and Baclofen ($GABA_B$ agonist) caused a decrease in glutamate release in control rats paralleled by a similar reduction in synapsin I phosphorylation. Meanwhile synaptosomes from EAE animals are responsive only to Baclofen with respect to nontreated EAE synaptosomes, since glutamate release from the synaptosomes treated with Muscimol was similar to that observed in EAE rat synaptosomes which was already reduced as consequence of the disease. In the case of the benzodiazepines Diazepam and Clonazepam ($GABA_A$ allosteric agonists), both of them induced a reduction in glutamate release in synaptosomes from the CFA rats, effect that was only observed in synaptosomes of EAE rats treated with Clonazepam. In all cases both benzodiazepines showed a higher effect on synapsin I phosphorylation than in glutamate release. These results indicate that the extent of GABAergic modulation of presynaptic terminals depends on the type of agonist employed and this regulation is altered in the frontal cortex during the acute phase of EAE with respect to control animals.

Keywords: Autoimmunity, multiple sclerosis, synapsins, benzodiazepines, glutamate release, GABA

1. INTRODUCTION

Glutamate is the most abundant neurotransmitter in the mammalian brain and mediates fast excitatory neurotransmission [1], meanwhile γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter balanced with glutamate in the central nervous system (CNS) [2]. Both, the inhibitory neurotransmitter GABA and the excitatory neurotransmitter glutamate are deregulated in the pathophysiology of stroke, epilepsy, neuropathic pain and multiple sclerosis [3, 4]. An inhibition of excessive glutamate release or an enhancement of the GABAergic neurotransmission could explain the therapeutic effects of diverse drugs clinically used as medication [5]. Among them benzodiazepines enhance GABAergic neurotransmission mediated by the central benzodiazepine receptor (CBR) which is part of the $GABA_A$ receptor-ion channel complex [6]. However, the benzodiazepines have an alternative site of binding named peripheral benzodiazepine receptor (PBR) or translocator protein (TSPO), expressed in abundance in endocrine organs, skin, heart, lung, liver and spleen, and to a lesser extent in the CNS where it is associated to glial cells [7, 8]. The anticonvulsant drug Clonazepam exhibits a high selectivity and affinity for the CBR, but has only very low affinity for the PBR. In addition a number of synthetic benzodiazepines termed mixed-type ligands can bind to the CBR and PBR and include the widely prescribed compounds Midazolam, Diazepam and Flunitrazepam [9]. Muscimol is a potent $GABA_A$ agonist, activating the receptor for GABA by binding to the same site on the $GABA_A$ receptor complex as GABA itself, as opposed to other GABAergic drugs such as barbiturates and benzodiazepines which bind to separate regulatory sites [10]. Conversely, Baclofen is a derivative of GABA and it is a selective agonist for the $GABA_B$ receptors belonging to a class of metabotropic G-protein-coupled receptors that modulate excitatory neurotransmission [11].

Using the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis, we have previously described a diminution on Ca^{+2}-dependent glutamate release in isolated nerve terminals from the frontal cortex of rats with EAE, which was coincident with the onset of the clinical signs [12, 13]. Furthermore, we observed that, in contrast to controls, synaptosomes from EAE rats isolated from frontal cortex showed a loss in the modulation of the

*Address correspondence to this author at the Departamento de Química Biológica -CIQUIBIC, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, Córdoba X5000HUA, Argentina; Tel/Fax: +54-351-5353855, Ext. 3418;
E-mail: garoth@fcq.unc.edu.ar

[§]Present address: Department of Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX 75390-9111, USA.

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glutamate release mediated by GABA and a decreased flunitrazepam sensitive-$GABA_A$ receptor density [14]. These results indicate that both glutamatergic and GABAergic neurotransmission are impaired in symptomatic EAE animals, and could contribute to clinical signs and disease progression. Administration of Diazepam to rats after EAE active induction leads to the induction of functional tolerance. Beyond the effects of Diazepam on the autoimmune, inflammatory response, we also observed that Diazepam treatment prevented the previously described reduction in glutamate release in the frontal cortex synaptosomes from EAE animals [15]. These data suggest that an endogenous inhibitory GABAergic system within the immune system is involved at least in part in the Diazepam effect on EAE and indicate that increasing GABAergic activity potently ameliorate EAE. At this regard, we described that Diazepam has a direct inhibitory effect on the activation of T lymphocytes involved in the disease development [16].

On the other hand, synapsins are expressed ubiquitously in all presynaptic nerve terminals in the CNS and they are major synaptic vesicle-specific phosphoproteins that play multiple roles in neural development, synaptic transmission and plasticity [17]. It has been described that $GABA_A$ receptors presented in nerve terminals modulate phosphorylation of synapsin I by presynaptic Ca^{2+} [18]. In addition, we have previously described that the synapsin I Ca^{2+}-dependent phosphorylation is inhibited in synaptosomes from EAE animals, which in turn decreased glutamate release [12].

In order to achieve a better understanding of the mechanism of action of GABA in the development of EAE, the effect of GABA agonists was studied. Therefore, the impact of various agents that can modulate GABA action by different kind of receptors was studied *in vitro* on glutamate release and synapsin I phosphorylation in synaptosomes from the frontal cortex of control and EAE animals.

2. MATERIALS AND METHODS

2.1. Reagents

Myelin was purified from bovine spinal cords as previously described [19]. Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-1,4-2H-benzodiazepin-2-one, Valium) 5 mg/ml and Clonazepam (5-(2-chlorophenyl)-7-nitro-2,3-dihydro-1,4-benzodiazepin-2-one, Rivotril) 2.5 mg/ml were from Roche International Limited (Montevideo, Uruguay). GABA, Muscimol (3-hydroxy-5-aminomethyl-isoxazole), Baclofen (β-(aminomethyl)-4-chlorobenzenepropanoic acid), complete Freund's adjuvant (CFA), bovine serum albumin, glutamate dehydrogenase (EC 1.4.1.3), $NADP^+$ and 4-aminopyridine (4-AP) were from Sigma-Aldrich Co. (St. Louis, MO, USA). Percoll was purchased from GE Healthcare Bio-Sciences (Pittsburgh, PA, USA). Synapsin I phospho-site 3 (Ser 603) rabbit pAb (RU19) was generously provided by Dr. Paul Greengard (Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, NY, USA). Neuronal Class III β-Tubulin (TUJ1) mouse monoclonal antibody (MMS-435P) was from Covance, Inc. (Princeton, NJ, USA). The rest of the chemicals were analytical grade reagents of the highest purity available.

2.2. EAE Induction and Experimental Groups

Forty-five-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 ethical committee for animal studies (Res. 832/2015). Animals were anesthetized with a mixture of xylazine and ketamine (10 and 65 mg/kg respectively, ip) and the active disease was induced by intradermal inoculation in both hind feet with 8 mg of whole bovine myelin in 0.5 ml of an emulsion consisting of 0.25 ml saline solution and 0.25 ml CFA (EAE Group). In the same experiment, other group of rats received 0.5 ml of the same emulsion without any antigenic preparation (CFA group) as controls. Animals were weighted, and assessed daily for clinical signs of EAE and scored as indicated [19]. Animals were sacrificed by decapitation when the rats from the EAE group showed the clinical signs (acute period, score 2), 24-48 h after onset.

2.3. Preparation of Synaptosomes and Glutamate Release Assay

Frontal cortex, defined as the frontal region of the isocortex from Bregma 1.0, was isolated from each rat and synaptosomes purified on discontinuous Percoll gradient as described previously [12]. Synaptosomes that sedimented between the 10 and 23% Percoll layers were collected and diluted to a final volume of 30 ml with HEPES buffer medium (140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 10 mM glucose, and 10 mM HEPES, pH 7.4) before centrifugation at 27,000 x *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).

For glutamate release assays the synaptosomal pellets were stored on ice and used within 3 to 4 h. Glutamate release from synaptosomes was monitored on-line, using an assay that employs exogenous glutamate dehydrogenase and $NADP^+$ to couple the oxidative decarboxylation of the released glutamate. Then, the generated NADPH was detected fluorometrically [12, 20].

2.4. Immunoblot Analysis

Synaptosomal samples were resuspended in HEPES buffer medium, 1.2 mM $CaCl_2$ was added and incubated at 37°C for 2 min. Then, the different GABA agonists tested were added and incubated 5 min further. After that 4-AP was added to evoke Ca^{2+}-dependent synapsin I phosphorylation concomitant to the glutamate release. Past 2 min incubation, aliquots were solubilized in sample buffer, and equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrotransferred onto nitrocellulose membranes [19]. Immunoblotting was done with 1:2,000 dilution of the synapsin I phosphorylation state-specific antibody, P-site 3 antibody. Immunoreactive bands were detected by infrared probe-labeled secondary antibodies followed by scanning in an Odyssey imager (LI-COR Inc., Lincoln, NE, USA).

Fluorescence intensity was quantified using GelAnalyzer 2010a software. Phosphorylated synapsin I content was normalized for βIII-tubulin content (1:2,000 dilution). Tubulin shows a ubiquitous distribution, validating its use as control.

2.5. Statistical 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). Whenever ANOVA indicated significant effects (p<0.05), a pair-wise comparison of means respect to control group was carried out (Dunnett's multiple comparison test). In all cases, the assumptions of the analysis of variance (homogeneity of variance and normal distribution) were attained. In all statistical analysis a p<0.05 was considered to represent a significant difference between groups.

3. RESULTS

3.1. Effect of GABA and its Agonists on Isolated Synaptosomes

Synaptosomes isolated from CFA or sick EAE rats were incubated for 5 min in the presence of 500 μM GABA, 500 μM Muscimol (specific $GABA_A$ receptor agonist) or 500 μM Baclofen (specific $GABA_B$ receptor agonist). Then, the release of glutamate was evoked by addition of 4-AP (Fig. **1**). In control nerve terminals this release reached 21.22 ± 1.09 nmol glutamate/mg protein/7 min. Synaptosomes from EAE rats showed a significant decrease in the total release of glutamate evoked by 4-AP (17.47 ± 1.17 nmol/mg protein/7 min, -17.67% respect to the CFA group). Addition of GABA or its agonists Muscimol or Baclofen significantly reduced glutamate release in synaptosomes from CFA rats (-29.24%, - 21.05%, - 36.30%, respectively) in comparison with the value in non-treated CFA synaptosomes. Glutamate release from synaptosomes from EAE rats was only significantly diminished respect to the non-treated EAE synaptosomes when they were treated with Baclofen (-31.28%, p<0.01). These results indicate that GABA and Muscimol ($GABA_A$ agonist) reduces glutamate release in CFA synaptosomes, but not in the EAE ones. Baclofen ($GABA_B$ agonist) in turn, decreases glutamate release in both CFA and EAE synaptosomes.

3.2. Effect of Allosteric Agonists of GABA on Synaptosomes

The effect of allosteric agonists of $GABA_A$ receptor, Clonazepam, with high affinity for CBR and very low affinity for the PBR, and Diazepam, a mixed-type ligand was evaluated (Fig. **2**). Addition of these benzodiazepines at concentrations of 43 and 172 μM significantly reduced glutamate release in synaptosomes from CFA as well as EAE rats respect to non-treated CFA synaptosomes. However, glutamate release from synaptosomes from EAE rats was significantly decreased only by Clonazepam at the same concentrations respect to the non-treated EAE synaptosomes (- 39.21% and - 68.40%, respectively). Synaptosomes from EAE rats treated with Diazepam showed a glutamate release diminished but similar to that of the nontreated EAE synaptosomes where glutamate release is already reduced with respect to control animals. Therefore, both drugs reduced in a concentration dependent manner the release of glutamate from synaptosomes isolated from CFA as well as EAE animals, although the effect of Diazepam on glutamate release was significant only in CFA synaptosomes. The

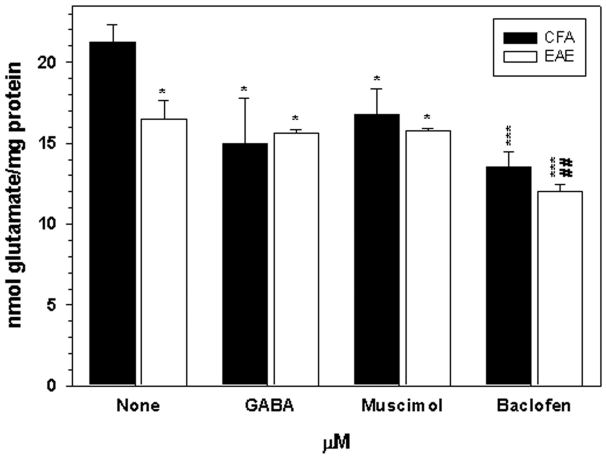


Fig. (1). 4-AP-evoked total glutamate release from rat frontal cortex synaptosomes. Each experiment consisted of synaptosomes purified from control (CFA) and EAE animals incubated with GABA (500 μM), Muscimol (500 μM) or Baclofen (500 μM) before that glutamate release was evoked by the addition of 3 mM 4-aminopyridine and assayed by on-line fluorometry. Results represent means ± S.E.M. of four independent experiments. Significant differences respect to non-treated CFA control are indicated by *p<0.05, ***p<0.001, and differences with respect to non-treated EAE group by ##p<0.01.

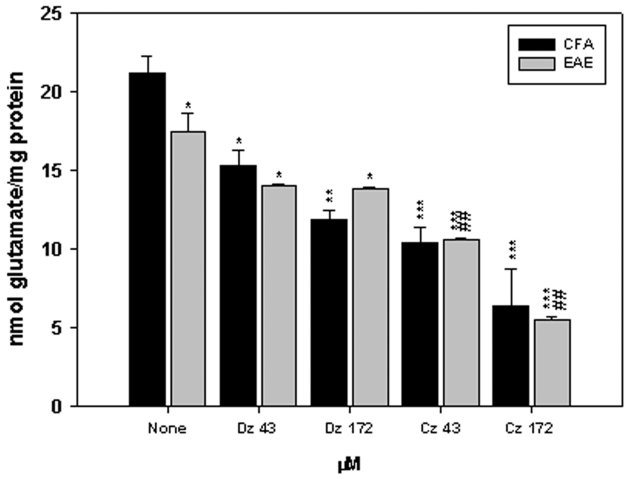


Fig. (2). 4-AP-evoked total glutamate release from rat frontal cortex synaptosomes. Synaptosomes were purified from CFA and EAE animals and incubated with Diazepam (Dz) or Clonazepam (Cz) at the indicated concentrations before glutamate release was evoked by the addition of 3 mM 4-aminopyridine and assayed by on-line fluorometry. Results represent means ± S.E.M. of four independent experiments. Significant differences respect to non-treated CFA group are indicated by *p<0.05, **p<0.01, ***p<0.001, and differences respect to non-treated EAE group by ##p<0.01.

inhibition of glutamate release induced by Clonazepam is in accordance with previous reports that indicate a higher affinity of Clonazepam for the $GABA_A$ receptor. In addition these results might suggest that the effect of benzodiazepines on synaptosomes is mediated by the CBR and not by the PBR, since the anticonvulsant drug Clonazepam exhibits a high selectivity and nanomolar affinity for CBR, but has only a very low affinity for the PBR [21].

3.3. Effect of GABA and $GABA_A$ Agonists on Synapsin I Phosphorylation

When synapsin I phosphorylation at P-site 3 was evaluated (Fig. **3**), it was observed that phosphorylation in the resting synaptosomes was almost null and increased along with the release of glutamate induced by 4-AP. GABA had an inhibitory effect on this phosphorylation, since it reduced it by 30% in the case of CFA and by 20% in EAE group. Muscimol reduced phosphorylation in a similar way to GABA. Benzodiazepines (Diazepam and Clonazepam) almost completely reduced synapsin I phosphorylation in a dose dependent manner and in a much greater degree than GABA and Muscimol (Fig. **3**, Table **1**).

Table 1. Phosphorylation of synapsin I in frontal cortex synaptosomes.

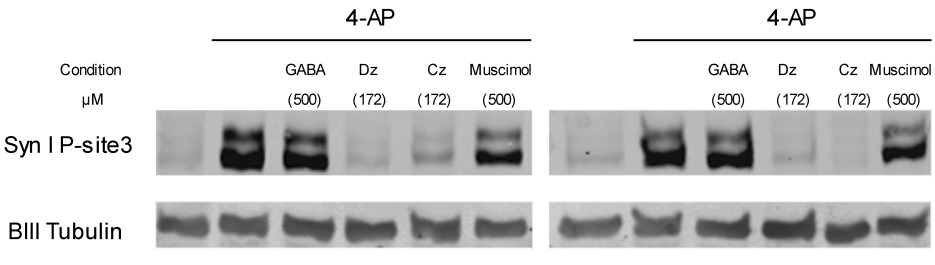
µM	Diazepam	Clonazepam
None	0.61 (9.4%)	0.61 (9.4%)
None/4-AP	6.46 (100%)	6.46 (100%)
43	2.60 (40.2%)	1.98 (30.6%)
86	1.14 (17.6%)	1.52 (23.6%)
172	0.58 (9.0%)	0.95 (14.7%)

Synaptosomes were purified from CFA animals and treated with Diazepam or Clonazepam at the indicated concentrations before the addition of 3 mM 4-aminopyridine (4-AP) to stimulate Ca^{2+}-dependent synapsin I phosphorylation concomitant to the glutamate release. For each sample, phosphorylated synapsin I at P-site 3 levels were normalized to βIII-tubulin and relativized to the correspondent control sample.

Depending on the treatment and the experimental group, a correlation between glutamate release and synapsin I phosphorylation could be observed (Table **2**). When synaptosomes were incubated in the presence of GABA, Muscimol, or Baclofen and then stimulated with 4-AP, in the CFA group glutamate release decreased (-29.3%, -21.2% and -36,3%, respectively) concomitantly with synapsin phosphorylation at site-3 (-30.6%, -21,0% and -37.6%, respectively). This correlation is not observed in synaptosomes isolated from EAE rats where phosphorylation was decreased with minor changes in the release of glutamate. Conversely, treatment with Diazepam and Clonazepam partially inhibited glutamate release in the CFA (-43.7% and -69.7%, respectively) and EAE rats (-20.5% and -68.4%, respectively), whereas synapsin I phosphorylation was almost completely prevented in both animal groups.

4. DISCUSSION

Previous work from our group showed an impaired Ca^{2+}-dependent glutamate release in frontal cortex synaptosomes from rats with EAE, which was associated with a failure in synapsin I phosphorylation and a loss of GABAergic regulation [12-14]. Taking into account that in many pathologies of the CNS, excitatory and inhibitory neurotransmission are altered, when we examined the glutamate modulation by the main (endogenous) inhibitory neurotransmitter GABA, we observed that glutamate release as well as Synapsin I phosphorylation were altered in EAE animals respect to control, with a concomitant reduction of Flunitrazepam-sensitive $GABA_A$ receptor density [14]. Since functional changes and atrophy in frontal cortex may play a crucial role in MS progression, in the present work we took advantage of EAE similitude in order to explore the molecular mechanisms underlying synaptic alterations in these pathologies. Herein we demonstrate that 4-AP-evoked glutamate release from isolated synaptosomes can be negatively modulated by GABA through $GABA_A$ (Muscimol) as well as $GABA_B$ (Baclofen) receptors (Fig. **1**). This modulation is observed in synaptosomes from CFA rats, but



Group	None	4-AP	GABA	Dz	Cz	Musc
CFA	0.79 (8 %)	7.35 (100 %)	5.10 (70 %)	0.58 (8 %)	0.95 (12 %)	4.59 (62 %)
EAE	1.03 (17 %)	5.81 (100 %)	4.63 (80 %)	0.40 (7 %)	0,36 (6 %)	3.90 (67 %)

Fig. (3). Phosphorylation of synapsin I in frontal cortex synaptosomes. Synaptosomes were purified from control (CFA) and EAE animals and incubated with GABA, Diazepam (Dz), Clonazepam (Cz) or Muscimol (Musc) at the indicated concentrations before the addition of 3 mM 4-aminopyridine (4-AP) to stimulate Ca^{2+}-dependent synapsin I phosphorylation concomitant to the glutamate release. For each sample, phosphorylated synapsin I at P-site 3 levels were normalized to βIII-tubulin and relativized to the correspondent control sample.

Table 2. Correlation of glutamate release and synapsin phosphorylation.

Treatment	CFA Group		EAE Group	
	Glutamate Release	P-Synapsin	Glutamate Release	P-Synapsin
None	21.22 (100%)	7.35 (100%)	17.47 (100%)	5.81 (100%)
GABA (500 μM)	15.01 (70.7%)	5.1 (69.4%)	15.59 (89.2%)	4.63 (79.7%)
Muscimol (500 μM)	16.75 (78.9%)	4.59 (62.4%)	15.73 (90.0%)	3.9 (67.1%)
Baclofen (500 μM)	13.52 (63.7%)	5.81 (79.0%)	12.01 (68.7%)	n.d (-)
Diazepam (172 μM)	11.95 (56.4%)	0.58 (7.9%)	13.89 (79.5%)	0.4 (6.9%)
Clonazepam (172 μM)	6.44 (30.3%)	0.95 (12.9%)	5.52 (31.6%)	0.36 (6.2%)

The table summarizes the work; where can be observed that while there is a correlation among the glutamate release and synapsin phosphorylation in synaptosomes isolated from CFA animals when they were preincubated with each indicated GABAergic agonists and then stimulated with 4-AP. This correlation is not observed in synaptosomes isolated from EAE rats where phosphorylation is highly decreased with minor changes in the release of glutamate. As for benzodiazepines (Clonazepam and Diazepam), no correlation is observed between glutamate release and synapsin phosphorylation with changes noticeable in synaptosomes isolated from both CFA and EAE animals.

the effect of the specific agonists over synaptosomes from EAE rats was only observed with Baclofen. Meanwhile the glutamate release from the synaptosomes treated with Muscimol was similar to that observed in the nontreated synaptosomes from EAE rats where was already reduced as consequence of the disease. Activation of $GABA_A$ receptors results in a reduction of subsequent depolarization-evoked Ca^{2+} influx, leading to an inhibition of glutamate release. Therefore, it is possible that the loss of GABAergic modulation on glutamate release in EAE synaptosomes could have been mediated by a decrease of $GABA_A$ receptor density. On the other hand, Baclofen reduced glutamate release significantly in both CFA and EAE synaptosomes, which suggest that $GABA_B$ receptors are not altered during the onset of clinical signs of the disease. When we studied the effect of $GABA_A$ allosteric agonists (Diazepam and Clonazepam) we observe that both reduced glutamate release in a concentration dependent manner on synaptosomes from CFA rats. A similar effect was observed in EAE rats only with Clonazepam, meanwhile the synaptosomes from EAE rats treated with Diazepam showed a glutamate release diminished but similar to that of the nontreated EAE synaptosomes where glutamate release was already reduced. This could indicate that GABA receptors in nerve terminals from EAE rats seems to be reduced and/or insensitive to GABA itself or Muscimol but are able to respond to allosteric modulators (like Clonazepam). We cannot discard the possibility of benzodiazepines having a secondary, GABA-independent effect over synaptosomes, for example through direct membrane interactions or through TSPO on synaptosomal mitochondria. Clonazepam has very low affinity for PBR (TSPO), which is highly express in steroidiogenic tissue [22]. In the normal brain overall TSPO expression is low, but in the abnormal inflammatory or neurodegenerative brain, TSPO is mainly expressed in glia, some hypertrophic astrocytes, infiltrating macrophages, and at low levels in neurons [8]. Our results show that the effect induced on the glutamate release and synapsin I phosphorylation by Clonazepam is stronger than the brought about by Diazepam, therefore we could speculate that the effect of benzodiazepines in synaptosomes is mediated by the $GABA_A$ receptor.

On the other hand, since 4-AP-induced glutamate release involves Ca^{2+}/calmodulin kinase activation and the subsequent phosphorylation of synapsin I at site 3, which in turn modulates the neurotransmitter release by reversibly tethering the synaptic vesicles to the actin cytoskeleton, we investigated the regulation of synapsin I phosphorylation in synaptosomes stimulated by 4-AP. Our results show that the presence of GABA or its agonists decreased synapsin I P-site 3 phosphorylation in CFA and EAE animals. Synapsin I phosphorylation at sites 1 and 3 has a negative effect on synapsin I association to synaptic vesicles and on the kinetics of synaptic vesicles turnover [23]. Based on these results, there is a correlation between glutamate release and phosphorylation of synapsin I in frontal cortex synaptosomes isolated from control animals when GABA or Muscimol were added, but this correlation is partially lost in synaptosomes from EAE rats. Furthermore, in both EAE and CFA synaptosomes, although there is a correlation between glutamate release and phosphorylation of synapsin I on the action exerted by benzodiazepines, the magnitude of the effect on synapsin I phosphorylation is much higher than the reduction of glutamate release. Glutamate release induced by 4-AP has two components, one dependent and one independent of Ca^{2+} influx. The Ca^{2+}-dependent release is also dependent on synapsin I phosphorylation and the presynaptic molecular machinery, while the Ca^{2+}-independent release is a product of the reversal action of plasma membrane glutamate transporters [24]. This independent component comprises about 30% of total released glutamate [12, 13]. Taking that into account, if we discount the 30% of glutamate that was released independently of Ca^{2+} influx, the benzodiazepines effect over Ca^{2+}-dependent release the magnitude of the effect are closer to the results obtained for synapsin I. On the other hand, glutamate release can also occur in the absence of the synapsin proteins [25], and that can also explain why we still measure glutamate release when synapsin phosphorylation was completely abolished. Linking together this background and our results, the absence of effect of GABA and Muscimol over glutamate release in EAE group, while they effectively downregulate synapsin I phosphorylation, might be indicating that the measured release occurred independently of synapsin I pathway in sick animals.

CONCLUSION

We illustrated that EAE synaptosomes do not respond to $GABA_A$ agonists significantly. Even so, benzodiazepines are

effective reducing glutamate release and synapsin I phosphorylation, which suggest that these are more potent agonists or act through a different presynaptic pathway, possibly unaffected during EAE. On the other hand, we noticed that Baclofen reduce glutamate release in CFA and EAE synaptosomes, suggesting that $GABA_B$ receptors are not altered in the acute stage of the disease. Therefore, we can hypothesize that one way of GABAergic modulation on glutamate release is *via* phosphorylation of synapsin I, but this would not be the only mechanism. From a therapeutic point of view, our results indicate that the allosterically modulation of GABA receptors might have a greater suppressor effect on EAE development than the treatment with direct agonists, supporting our previous studies and generating new perspectives for future research.

ABBREVIATIONS

4-AP = 4-Aminopyridine

CBR = Central benzodiazepine receptor

CFA = Complete Freund's adjuvant

CNS = Central nervous system

EAE = Experimental autoimmune encephalomyelitis

GABA = γ-Aminobutyric acid

PBR = Peripheral benzodiazepine receptor

TSPO = Translocator protein (18 kDa).

CONFLICT OF INTEREST

The authors confirm that this article content has no potential conflict of interest, including any financial, personal or other relationships with other people or organizations.

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