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Glutamatergic Alterations in STZ-Induced Diabetic Rats Are Reversed by Exendin-4

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Abstract
Diabetes mellitus is a metabolic disorder that results in glucotoxicity and the formation of advanced glycated end products (AGEs), which mediate several systemic adverse effects, particularly in the brain tissue. Alterations in glutamatergic neurotransmission and cognitive impairment have been reported in DM. Exendin-4 (EX-4), an analogue of glucagon-like peptide-1 (GLP-1), appears to have beneficial effects on cognition in rats with chronic hyperglycemia. Herein, we investigated the ability of EX-4 to reverse changes in AGE content and glutamatergic transmission in an animal model of DM looking principally at glutamate uptake and GluN1 subunit content of the *N*-methyl-D-aspartate (NMDA) receptor. Additionally, we evaluated the effects of EX-4 on in vitro models and the signaling pathway involved in these effects. We found a decrease in glutamate uptake and GluN1 content in the hippocampus of diabetic rats; EX-4 was able to revert these parameters, but had no effect on the other parameters evaluated (glycemia, C-peptide, AGE levels, RAGE, and glyoxalase 1). EX-4 abrogated the decrease in glutamate uptake and GluN1 content caused by methylglyoxal (MG) in hippocampal slices, in addition to leading to an increase in glutamate uptake in astrocyte culture cells and hippocampal slices under basal conditions. The effect of EX-4 on glutamate uptake was mediated by the phosphatidylinositide 3-kinases (PI3K) signaling pathway, which could explain the protective effect of EX-4 in the brain tissue, since PI3K is involved in cell metabolism, inhibition of apoptosis, and reduces inflammatory responses. These results suggest that EX-4 could be used as an adjuvant treatment for brain impairment associated with excitotoxicity.

Keywords Astrocyte · Diabetes mellitus · Exendin-4 · Glutamate uptake · GLP-1

Introduction

Diabetes mellitus, characterized primarily by chronic hyperglycemia, is a metabolic disorder that results in glucotoxicity, including the formation of advanced glycated end products (AGEs), which can contribute to complications of DM [1]. AGEs are a diverse group of compounds that are generated by non-enzymatic glycation or glycoxidation of proteins, lipids, or nucleic acids through a series of reactions [2]. The main precursor of AGEs is methylglyoxal (MG) and, in normal situations, cells are protected from its toxicity by the glyoxalase system. This MG detoxification pathway is formed by the enzymes, glyoxalase 1 (GLO 1), and glyoxalase 2 (GLO 2) [3], which catalyze the conversion of MG to *D*-lactate [4]. DM is associated with several adverse effects in the brain, including increases in oxidative stress, inflammation, and changes in brain energy metabolism, possibly mediated by AGEs and their receptor, RAGE [5]. This pathway may cause impairment of astroglial function, which further compromises the CNS, leading to dysregulation of the extracellular glutamate concentration and a decrease in the antioxidant compounds produced by astrocytes [1, 6].

Glutamate is the main excitatory neurotransmitter in the central nervous system (CNS). Moreover, 40% of all synapses are glutamatergic [7]. During neuronal activity, glutamate is

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released to the synaptic cleft and acts at both ionotropic and metabotropic glutamate receptors to cause depolarization in the post-synaptic neuron [8]. *N*-methyl-D-aspartate (NMDA) receptors are a subtype of the ionotropic glutamate receptors, which play a central role in synaptic development, neuroplasticity, and differentiation. Furthermore, changes in NMDA receptors are related to cognitive impairment [9].

Astrocytes are the most abundant cells of the CNS and play a pivotal role in brain homeostasis, interacting with neurons at many levels, modulating ion and water homeostasis, as well as synaptic transmission, and regulating blood flow, protection from oxidative stress, and neurogenesis [10]. Astrocytes are enriched with Na^+-dependent glutamate transporters (mainly EAAT1/GLAST and EAAT2/GLT-1), which are responsible for rapidly removing extracellular glutamate from the synaptic cleft in order to maintain brain homeostasis and avoid excitotoxicity [7]. In turn, glutamate is converted to glutamine by glutamine synthetase, inside the astrocyte, and glutamine is captured by the glutamatergic neurons and reconverted into glutamate, which then acts on new synapses [11]. Extracellular glutamate accumulation activates a cascade of events including excessive stimulation of glutamate receptors, such as NMDA, leading to neurotoxicity [12].

Exendin-4 (EX-4) is a glucagon-like peptide-1 (GLP-1) agonist and is a well-established drug for treating type 2 diabetes mellitus (T2DM). Some studies have investigated a role for GLP-1 agonists in treating type 1 diabetes mellitus (T1DM), reporting benefits, including improved glycemic levels, increased beta cell proliferation, and a reduction in the apoptosis of beta cells [13]. Additionally, when administered peripherally, EX-4 can pass through the blood-brain barrier (BBB) and appears to have protective properties in the CNS [14]. Several studies have shown the beneficial effects of GLP-1 mimetics in CNS, which include amelioration of neurogenesis and cell repair, and abrogation of the inflammatory response, as well as improvements in cognitive impairment [15], suggesting a protective effect of this class of drugs against different CNS injuries.

Despite the well-established peripheral role of EX-4 in T2DM and the beneficial effects that this drug has been shown to exert in the CNS, few studies have demonstrated a protective role for EX-4 against CNS damage in T1DM. Considering the impairments in glutamatergic transmission that are associated with DM and the neuroprotective effects of EX-4 in several injuries, we investigated the ability of EX-4 to reverse the changes in glutamatergic transmission that occur in an animal model of DM and determine the direct effect of the drug on in vitro models. Additionally, we evaluated the role of EX-4 on alterations in peripheral metabolic parameters and the formation of AGEs caused by DM.

Materials and Methods

Material

Streptozotocin (STZ), EX-4, exendin 9-39 [EX (9-39)], MG, o-phenylenediamine (OPD), ponceau S, aminoguanidine hemisulfate salt (AG), and 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) were purchased from Sigma (St. Louis, MO, USA). Epsilon-*N*-carboxyethyl-L-lysine (CEL; a mixture of two diastereoisomers) was purchased from PolyPeptide Group (San Diego, CA, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM), and other materials for cell culture were purchased from Gibco BRL (Carlbad, CA, USA). Goat polyclonal anti-RAGE was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rat/mouse C-Peptide 2 enzyme-linked immunosorbent assay (ELISA) kit, mouse monoclonal anti-β-actin antibody (C4 clone), and mouse monoclonal anti-GluN1 (R1JHL clone) were obtained from Millipore (Darmstadt, Germany). The rabbit polyclonal anti-GLAST and anti-GLT-1 antibodies were purchased from ABCAM (Cambrigde, UK). The peroxidase-conjugated immunoglobulin (IgG) antibodies, ECL Western Blotting Detection Kit, and L-[2,3-^{3}H] glutamate were obtained from Amersham (Buckinghamshire, UK). The anti-AGE antibody (6D12) was purchased from Cosmo Bio (Tokyo, 395 Japan). All other chemicals were purchased from local commercial suppliers.

Experimental Design and Animals

A total of 87 male Wistar rats were obtained from our breeding colony for experiments, of which 70 were used for in vivo experiments (60 days old), 11 for ex vivo experiments (30 days old), and six for cell culture preparations (2 days old). Rats were maintained under a 12-h light/12-h dark cycle at a constant temperature of 22 ± 1 °C and had free access to commercial chow and water. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80–23), and all procedures were previously approved by the local Animal Care Ethical Committee (CEUA-UFRGS; project number 24076). All efforts were made to minimize animal suffering and reduce the number of animals used.

DM was induced in 36 rats by intraperitoneal injection of STZ (75 mg/kg in citrate buffer with an injection volume of 300 μL/kg body weight, pH 4.5). A schematic representation of the experimental protocol is shown in Fig. 1. Forty-four out of 48 rats became diabetic (as assessed by glycemic parameters measured 48 h after STZ administration). Eleven age-matched animals that received only vehicle (citrate buffer) were categorized as SHAM ($n = 11$) and 11 rats that received vehicle and 10 μg/kg EX-4 intraperitoneally once a day for 28 days were denominated the SHAM-EX-4 group ($n = 11$),

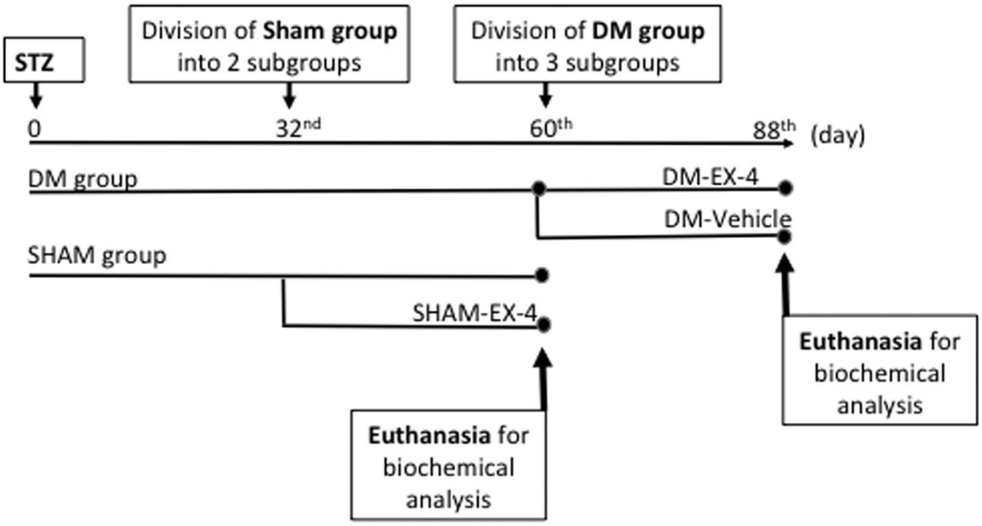

Fig. 1 Schematic representation of the in vivo experimental protocol. Adult Wistar rats were submitted to i.p. STZ administration (DM group) assumed as time 0. Two other groups received only vehicle/citrate (Sham group) or vehicle/citrate and EX-4 (Sham-EX-4 group). Sham-EX-4 received EX-4 for 28 days before euthanasia. Neurochemical and blood analyses were performed at 60 days afterwards. Only one third of the DM group was euthanized at 60 days. Two thirds of the group were subdivided and received EX-4 for 28 days (DM-EX-4) or vehicle/saline (DM vehicle) and were euthanized at 28 days for neurochemical and blood analyses

based on [16, 17]. The animals were then housed (3–4 per cage) for a period of 60 days. Notice that the SHAM-EX-4 group received EX-4 from the 32nd day to the 60th day and our original aim was to investigate the effect per se of EX-4 on glutamate uptake, which will also be investigated by direct EX-4 exposure in acute hippocampal slices and astrocyte cultures (see later on). Body weight and blood glucose levels were recorded every 2 weeks. Glycemia was measured with an Accu-Chek® Active Kit (Roche Diagnostics, Basilea, Switzerland), and the animals were considered diabetic if their blood glucose levels were more than 250 mg/dL at any time of the day.

Biochemical analyses were performed 60 days after STZ or vehicle injection. The DM group rats ($n = 33$) were randomly divided into three subgroups. One group was euthanized for biochemical analysis ($n = 11$) and the two others were assigned to the DM vehicle ($n = 11$) and DM-EX-4 ($n = 11$) groups. The DM-EX-4 animals received 10 μg/kg EX-4 intraperitoneally once a day for 28 days, whereas the DM-vehicle animals received an equal volume of saline solution. For biochemical analysis, 11 DM, 11 SHAM and 11 SHAM-EX-4 rats were used at 60 days. Finally, at 28 days after EX-4 treatment, 11 DM-EX-4 and 11 DM-vehicle rats were used for biochemical analysis. Six animals from each subgroup were used for glutamate uptake. Five animals were used for western blot assays. The numbers of animals used for each evaluated parameter are stipulated in the figure legends.

Cerebrospinal Fluid and Serum Samples

Animals were anesthetized using ketamine and xylazine (Syntec, São Paulo, Brazil) administered at doses of 75 and 10 mg/kg, respectively, and then positioned in a stereotaxic holder for collection of 100 μL (approximately) of cerebrospinal fluid **(**CSF) from the cisterna magna. The puncture was performed using an insulin syringe (1 mL) with a 31G needle (0.25 mm diameter, 6 mm length). Rats were then removed from the stereotaxic apparatus and placed on a flat surface, where approximately 3 mL of whole blood were obtained by intracardiac puncture using a 5 mL syringe and 21 G needle (0.80 mm diameter, 25 mm length) inserted into the intercostal space above the sternum. Serum was separated by centrifugation at 3200 rpm for 10 min. CSF and serum samples were frozen (− 80 °C) until further analysis [18].

C-Peptide Assay

Proinsulin C-peptide was assessed using a Rat/Mouse C-Peptide 2 ELISA kit, according to the manufacturer's recommendations.

AGE Measurement

AGEs were measured in the serum and CSF by ELISA, as previously described by Ikeda et al. [19] with some modifications. Briefly, the wells of a microtiter plate were coated overnight with 0.1 μg protein in 0.1 mL of 50 mM carbonate bicarbonate buffer (pH 9.6). The wells were washed with washing buffer (PBS containing 0.05% Tween 20) and then incubated for 3 h with 2% albumin. Subsequently, wells were washed again and incubated with 100 μL of anti-AGE (6D12) for 1 h. After washing, wells were incubated with 100 μL peroxidase-conjugated secondary antibody for 1 h. The reactivity of peroxidase was determined by incubation with OPD for 30 min. The reaction was stopped by the addition of 50 μL sulfuric acid (3 M). Absorbance measurements were taken at 492 nm. Results were calculated and expressed as a percentage of the control.

Preparation of Hippocampal Slices for Glyoxalase Activity Assays

Hippocampal slices were lysed and homogenized in sodium phosphate buffer, pH 7.4. Subsequently, slices were centrifuged at 13,000 rpm for 15 min at 4 °C and the supernatant was used for enzymatic activity and protein content measurements [20].

Glyoxalase 1 Activity Assay

GLO 1 activity was assayed according to Mannervick et al. [21] with some modifications. The assay was carried out in 96-well microplates using a microplate spectrophotometer (UV Star—Greiner). The reaction mixture (200 μL/well) contained 50 mM sodium-phosphate buffer pH 7.2, 2 mM MG and 1 mM glutathione (GSH) (pre-incubated for 30 min at room temperature). Protein from the sample (10–20 μg per well) was added to the buffer. The formation of S-(D)-lactoylglutathione was linear and monitored at 240 nm for 15 min at 30 °C. A unit of GLO 1 activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of S-(D)-lactoylglutathione per minute. Specific activity was calculated in milliunits per milligram of protein (mU/mg protein).

Primary Astrocyte Culture

Primary cortical astrocyte cultures from Wistar rats were prepared as previously described [22]. Procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local authorities. Briefly, the cerebral cortices of newborn Wistar rats were removed and mechanically dissociated in Ca^{2+}- and Mg^{2+}-free balanced salt solution, pH 7.4, which contained (in mM) 137 NaCl, 5.36 KCl, 0.27 Na_2HPO_4, 1.1 KH_2PO_4, and 6.1 glucose. The cortices were cleaned of meninges and mechanically dissociated via sequential passage through a Pasteur pipette. After centrifugation at 1400 rpm for 5 min, the pellet was resuspended in DMEM (pH 7.6) supplemented with 8.39 mM HEPES, 23.8 mM $NaHCO_3$, 0.1% amphotericin, 0.032% gentamicin, and 10% FBS. Approximately, 300,000 cells were seeded in each well of a 24-well plate and maintained in DMEM containing 10% FBS in 5% CO_2/95% air at 37 °C, allowed to grow to confluence, and used at 21 days in vitro. The medium was replaced by DMEM with 2.5% FBS in the presence of 10 nM EX-4 or vehicle for 1 h, 24 h, or 4 days.

Preparation and Incubation of Hippocampal Slices

The preparation of acute slices was performed as previously described [23]. Briefly, another 11 Wistar rats (30 days old), without any previous treatment, were killed by decapitation. The brains were removed and placed in cold saline medium with the following composition (in mM): 120 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgSO_4$, 25 HEPES, 1 KH_2PO_4, and 10 glucose, adjusted to pH 7.4 and previously aerated with O_2. The hippocampi were dissected and transverse slices of 0.3 mm were obtained using a McIlwain Tissue Chopper®. Slices were then transferred immediately into 24-well culture plates, each well containing 0.3 mL of physiological medium and only one slice. The medium was exchanged every 15 min with fresh saline medium at room temperature. Following a 120-min equilibration period, the medium was removed and replaced with medium or specific treatments (10 nM EX-4, 500 μM MG, or 1 mM CEL) for 60 min at 30 °C on a warming plate. EX (9–39) (200 nM, GLP-1 antagonist), AG (500 μM, antiglycation compound), Rp-cAMPS (20 μM, antagonist of cAMP), H-89 (10 μM, protein kinase A inhibitor), and LY294002 (10 μM, phosphatidylinositide 3-kinase inhibitor) were added 15 min before EX-4 exposure. Subsequently, the medium was collected and cell integrity was evaluated by measuring lactate dehydrogenase (LDH) activity. The glutamate uptake was assessed in the slices ($n = 6$) or the slices were collected for western blotting ($n = 5$).

Glutamate Uptake Assay

Glutamate uptake was measured as previously described [23] with some modifications. The hippocampal slices or astrocyte culture cells were incubated in Hank's balanced salt solution (HBSS) containing (in mM) 137 NaCl, 5.36 KCl, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$, 0.63 Na_2H-$PO.7H_2O$, 0.44 KH_2PO_4, 4.17 $NaHCO_3$, and 5.6 glucose, adjusted to pH 7.2. The assay was initiated by the addition of 0.1 mM L-glutamate and 0.66 or 0.33 μCi/ml L-[2,3-^{3}H] glutamate in hippocampal slices or astrocyte cells, respectively. The incubation was stopped after 5 min in hippocampal slices or 7 min in astrocyte cells by removal of the medium and rinsing the slices or cells twice with ice-cold HBSS. Slices or cells were then lysed in a solution containing 0.1 or 0.5 M NaOH, respectively. Sodium-independent uptake was determined using *N*-methyl-D-glucamine instead of sodium chloride. Sodium-dependent glutamate uptake was obtained by subtracting the non-specific uptake from the total uptake to obtain the specific uptake. Radioactivity was measured in a scintillation counter. Results were calculated as nmol/mg protein/min and were expressed as a percentage of the control.

Western Blotting

Equal amounts (30 μg) of proteins from each sample were boiled in a sample buffer [0.0625 M Tris–HCl pH 6.8, 2% (*w*/*v*) SDS, 5% (*w*/*v*) β-mercaptoethanol, 10% (*v*/*v*) glycerol, 0.002% (*w*/*v*) bromophenol blue] and electrophoresed on a 10% (*w*/*v*) SDS-polyacrylamide gel. The separated proteins were blotted onto a nitrocellulose membrane. Equal loading of each sample was confirmed with Ponceau S staining [24]. After incubating for 1 h at room temperature with the primary antibodies (anti-GluN1, anti-RAGE, anti-O-GlcNAc, anti-GLAST, anti-GLT-1, or anti-β-actin at 1:5000 dilution), the filters were washed and incubated with anti-mouse, anti-goat, or anti-rabbit peroxidase-conjugated immunoglobulin (IgG) (at 1:10000 dilution). The chemiluminescence signal was detected using an ECL Western Blotting Detection Kit from Amersham and captured using an ImageQuant LAS400 (GE).

Lactate Dehydrogenase (LDH) Activity Assay

The LDH assay was carried out using 200 μL of the incubation medium collected from treated slices using a commercial ultraviolet assay from Bioclin (Belo Horizonte, Brazil).

Protein Determination

Protein levels were measured by Lowry's method using bovine serum albumin as a standard [25].

Statistical Analysis

Parametric data from the experiments are presented as the mean ± standard error (SE) and were statistically evaluated by student's *t* test or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using PRISM 5.0 (GraphPad Software Inc., San Diego, CA, USA) assuming $p < 0.05$ as significant.

Results

Glutamatergic Neurotransmission Is Reversed by EX-4 in Diabetic Rats

Considering that a key role of astrocytes is providing energy metabolism for glutamatergic transmission, we investigated glutamate transport in hippocampal slices of diabetic rats. Glutamate uptake in hippocampal slices differed among the groups evaluated [F (2,14) = 10.84, $p = 0.0014$]. The diabetic rats presented a reduction of 64.1% in glutamate uptake, compared with SHAM (Fig. 2a). Glutamatergic metabolism was reevaluated in DM rats after EX-4 administration (or vehicle) and demonstrated a reversal of the impairment of glutamate uptake by DM (Fig. 2b). The rats that received EX-4 exhibited an increase of 68.3% in glutamatergic metabolism compared with diabetic rats that received vehicle solution ($p = 0.0005$).

EX-4 Improves the Impairment in GluN1 Caused by DM

In contrast to the alterations in glutamate uptake observed in diabetic rats, the protein levels of the major astrocyte glutamate transporter in the hippocampus, GLT-1, did not differ between the SHAM and DM groups (Fig. 3a; $p = 0.99$), or between the DM vehicle and DM-EX-4 subgroups (Fig. 3b; $p = 0.09$). Additionally, protein levels of the GLAST glutamate transporter did not differ between groups (data not shown). The GluN1 subunit of the NMDA receptor for glutamate was found to be reduced in the hippocampus of diabetic rats (Fig. 3c; $p = 0.03$). After treatment with EX-4 (or vehicle), the levels of this protein were increased in the DM-EX-4 subgroup compared with the DM-vehicle subgroup (Fig. 3d; $p = 0.008$), suggesting a recovery in glutamatergic transmission.

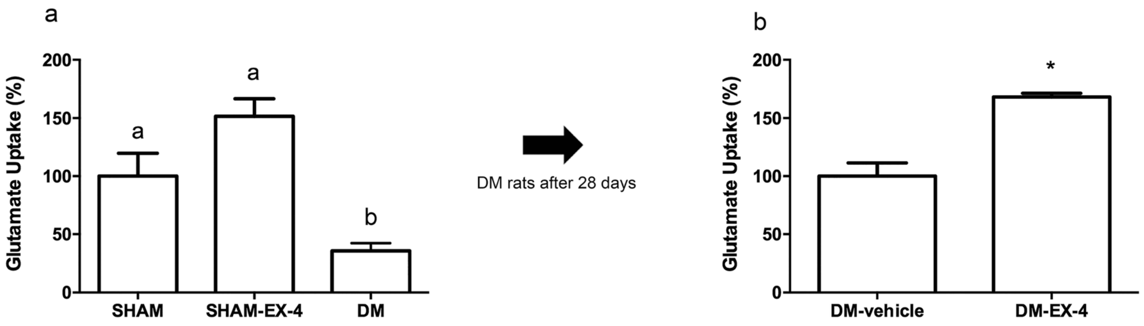

Fig. 2 Glutamatergic neurotransmission is reversed by EX-4 in diabetic rats. Glutamate uptake in hippocampal slices revealed a decrease in DM rats compared to SHAM and SHAM-EX-4 rats (**a**). The DM-EX-4 group showed a recovery in glutamate uptake compared to the DM-vehicle subgroup (**b**). Results depict means ± SEM, $n = 6$, bars without a common letter statistically differ assuming $p < 0.05$ from SHAM and SHAM-EX-4, $^*p < 0.05$ significantly different from DM-EX-4 by one-way ANOVA followed by Tukey's post hoc test

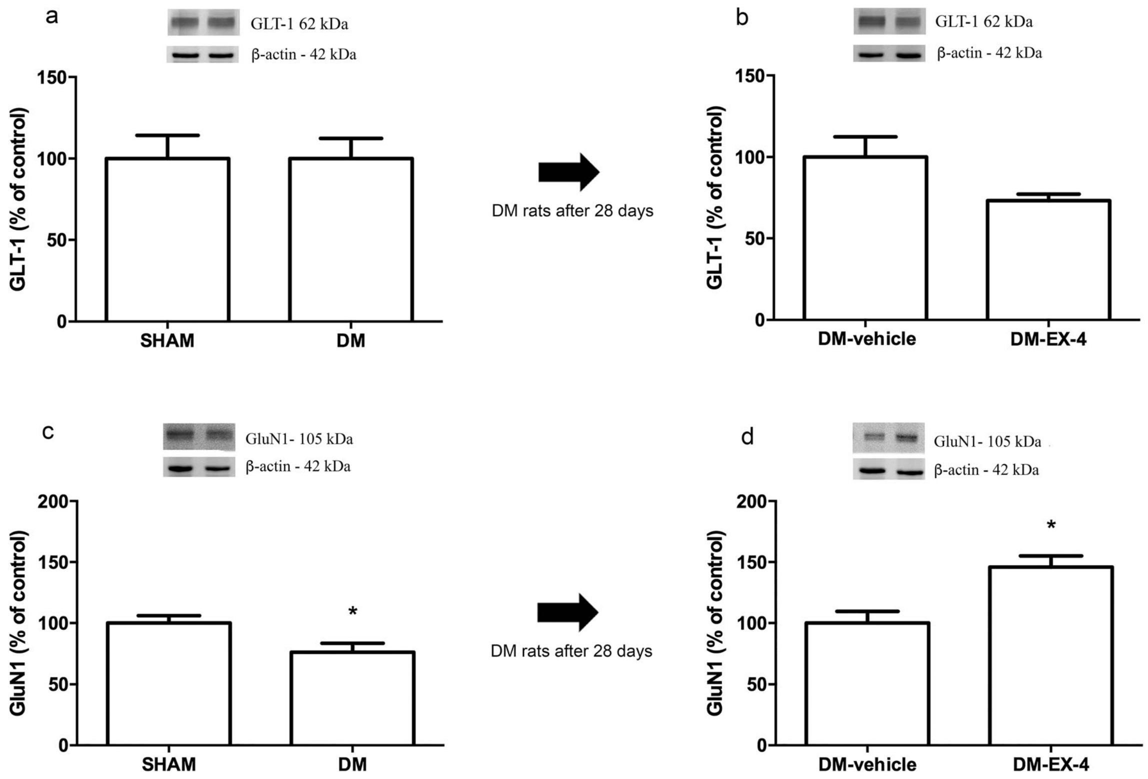

Fig. 3 EX-4 improves the impairment in GluN1 caused by DM. Western blotting demonstrated no alterations in protein levels of GLT-1 (**a**), but showed a decrease in GluN1 protein levels (**c**) in diabetic rats, compared with the SHAM. After EX-4 treatment, the GLT-1 content continued unchanged (**b**), but the GluN1 content was recovered in DM-EX-4, compared with DM-vehicle (**d**). Representative western blots for each protein are presented above each respective graph. The results are normalized to β-actin and represent the means ± SEM, $n = 5$, $*p < 0.05$ significantly different from SHAM or DM vehicle as determined by unpaired student's *t* test

The STZ-Induced DM Increase in O-GlcNAcylation Is Not Reverted by EX-4

Since post-translational modifications involving the addition of N-acetyl-glucosamine (GlcNAc) to the hydroxyl residues of serine and threonine forming *O*-linked β-*N*-acetylglucosamine (O-GlcNAc) are dependent on glucose flux via the hexosamine biosynthetic pathway (which plays a role in DM), we assessed O-GlcNAc alterations in the hippocampal tissue of diabetic rats. Significant differences were observed among the groups evaluated [F (2,12) = 4.72, $p = 0.03$]; the DM group presented a 26.4% increase in O-GlcNAc, compared with SHAM. However, the O-GlcNAc of the SHAM-EX-4 group was not significantly different to those of the other groups (Fig. 4a). The O-GlcNAc of the DM vehicle and DM-EX-4 subgroups did not differ statistically (Fig. 4b; $p = 0.10$).

EX-4 Increases Glutamate Uptake in Astrocytes and Hippocampal Slices

In order to investigate whether the alterations in glutamate uptake were attributable to a direct effect of EX-4 on astrocytes, we added 10 nM EX-4 to primary astrocyte cell cultures and measured glutamate uptake at 1 h, 24 h, or 4 days after addition (Fig. 5). EX-4 increased glutamate uptake by 38% at 1 h (Fig. 5a; $p = 0.001$) and 117.8% at 24 h (Fig. 5b; $p = 0.01$), but no effect was observed at 4 days (Fig. 5c; $p = 0.06$). We also evaluated the direct effect of EX-4 on glutamate uptake in acute hippocampal slices and observed an increase of 42.3% in hippocampal slices incubated for 1 h with EX-4 (Fig. 5d; $p = 0.002$).

MG-Impaired Glutamatergic Transmission Is Reversed by EX-4

As AGE content increases and glutamate uptake is modulated in diabetic rats, we evaluated the effects of MG (a reactive aldehyde that is involved in AGE formation) and CEL (an AGE derived from MG) on acute hippocampal slices. MG significantly decreased glutamate uptake by 47.8% [F (2,31) = 4.51, $p = 0.02$] (Fig. 6a) and reduced levels of the GluN1 subunit of NMDA by 44.3% [F (2,10 = 23.54, $p = 0.0002$] (Fig. 6b) in hippocampal slices. Slices exposed to CEL did not present any alterations in either glutamate uptake (Fig. 6a) or in levels of the GluN1 subunit (Fig. 6b).

In order to evaluate whether the effects of MG on glutamate uptake and GluN1 content involved glycation processes and

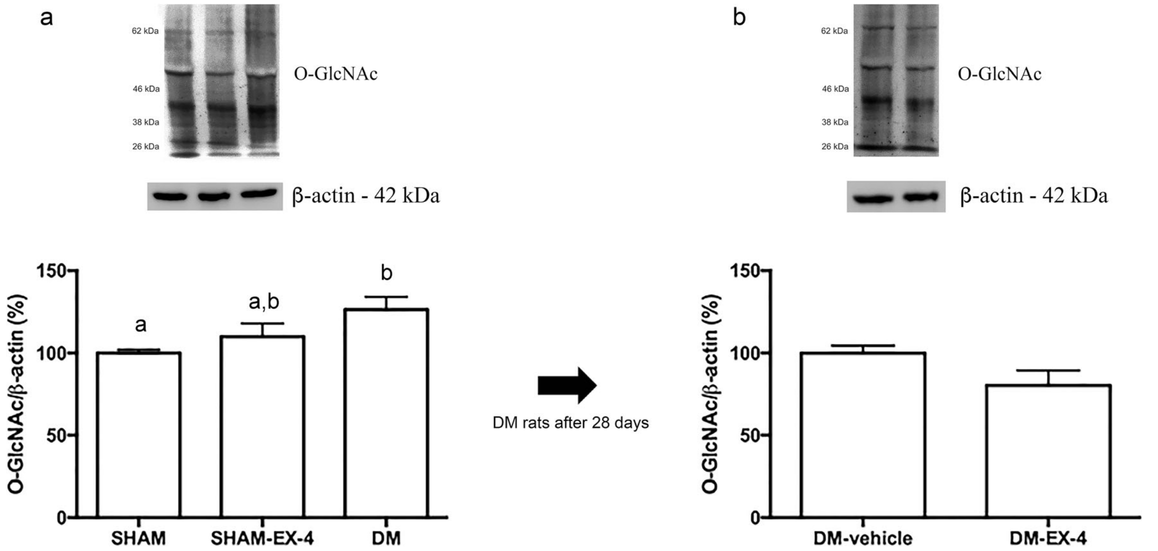

Fig. 4 Increased O-GlcNAcylation caused by DM is not reverted by EX-4. Western blotting showed an augment in *O*-GlcNAc content in DM rats, compared with SHAM (**a**). After EX-4 for 28 days, the DM-vehicle and DM-EX-4 subgroups demonstrated no alterations (**b**). The results are normalized to β-actin and represent the means ± SEM, *n* = 5, bars without a common letter statistically differ assuming $p < 0.05$ by one-way ANOVA followed by Tukey's post hoc test

the effect of EX-4 on these alterations, the slices were incubated concomitantly with MG and aminoguanidine (AG) or EX-4. The decreases in glutamate uptake [F (4,40) = 7.635, *p* = 0.0001] (Fig. 6c) and in GluN1 content [F (4,19) = 7.74,

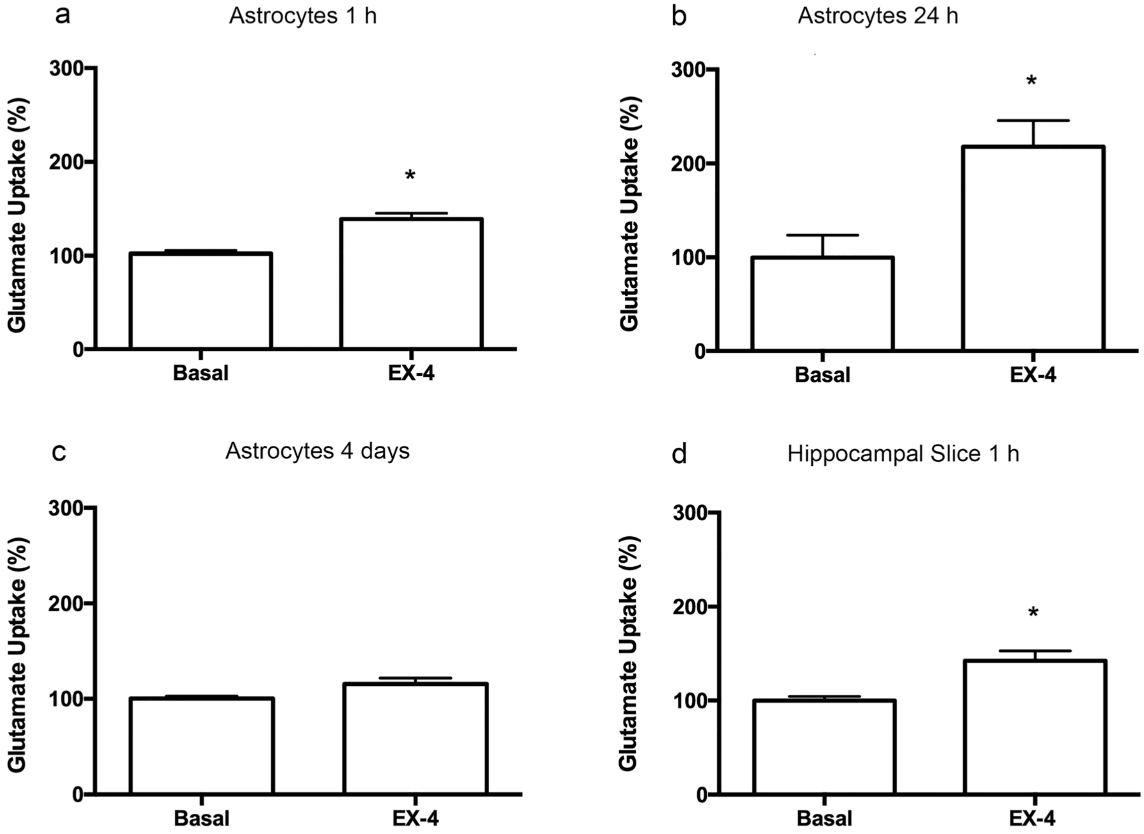

Fig. 5 EX-4 increases glutamate uptake in astrocytes and hippocampal slices. Cultured astrocyte cells treated with EX-4 (10 nM) revealed an increase in glutamate uptake following incubation for 1 h (**a**) or 24 h (**b**). Cells treated for 4 days did not present alterations (**c**). Acute hippocampal slices treated with EX-4 (10 nM) for 1 h showed an increase in glutamate uptake compared with basal (**d**). Data are shown as the means ± SEM of six independent experiments performed in triplicate. $^{*}p < 0.05$ significantly different from basal as determined by unpaired student's *t* test

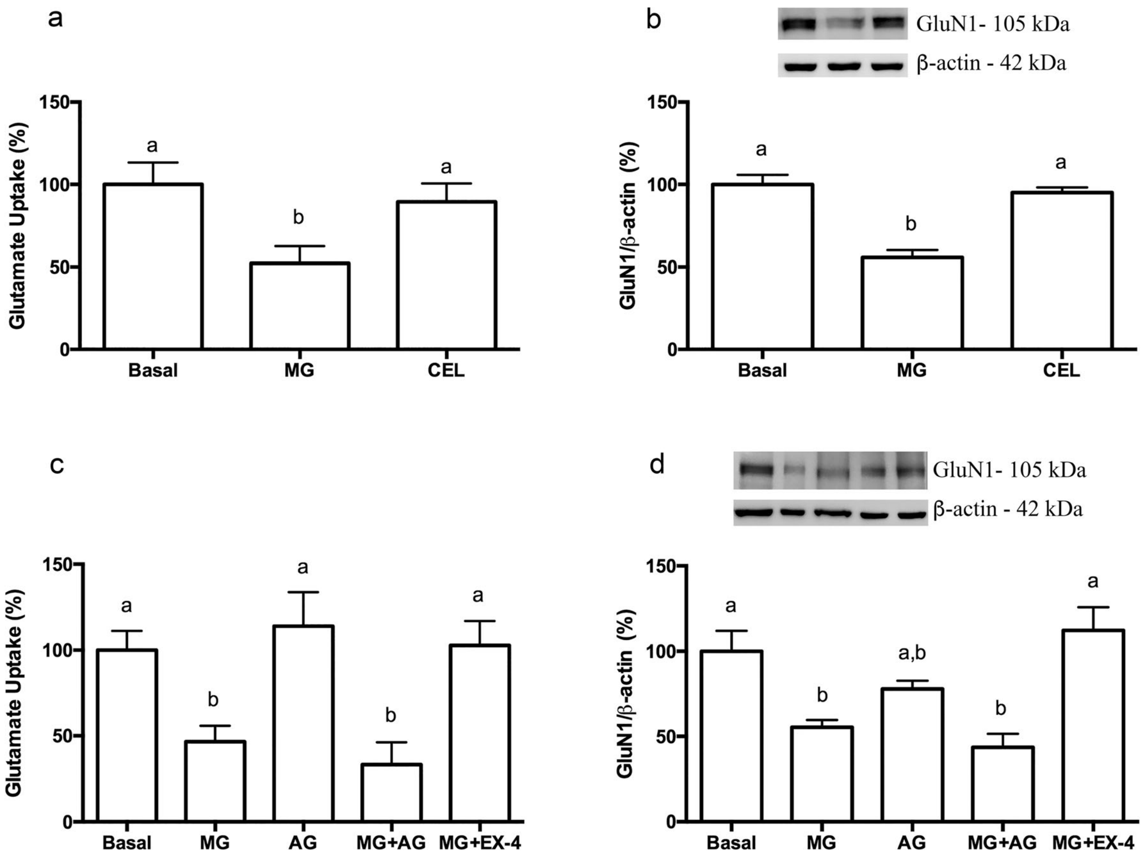

Fig. 6 MG-impaired glutamatergic transmission is reversed by EX-4. Acute hippocampal slices were incubated with EX-4 (10 nM), MG (500 μM), or CEL (1 mM). The AG (500 μM, antiglycation compound) was added 15 min before EX-4 exposure. Slices incubated with MG, but not CEL, presented decreased glutamate uptake (**a**) and GluN1 content (**b**). Co-incubation with EX-4 and MG abolished the effects observed with MG in glutamate uptake (**c**) and GluN1 (**d**). Representative western blots for each protein are presented above each respective graph and the results are normalized to β-actin. Data are shown as the means ± SEM of six independent experiments performed in triplicate. Bars without a common letter statistically differ assuming $p < 0.05$ by one-way ANOVA followed by Tukey's post hoc test

$p = 0.0007$] (Fig. 6d) induced by MG were reversed by EX-4. Co-incubation of MG with AG did not prevent changes in glutamate uptake or GluN1 content. Cell integrity was not affected by the basal or treatment conditions as evaluated by measuring extracellular LDH activity (data not shown).

EX-4 Improves Glutamatergic Transmission through the PI3K Pathway

With the purpose of evaluating whether the effects of EX-4 on glutamate uptake were dependent upon the binding of the agonist to the GLP-1 receptor, we incubated hippocampal slices concomitantly with EX-4 and an antagonist of the GLP-1 receptor, EX (9–39). Importantly, the increase in glutamate uptake induced by EX-4 was abolished by co-incubation with EX (9–39) [F (3,26) = 4.764, $p = 0.009$] (Fig. 7a), suggesting that the EX-4-induced increase in glutamate uptake is mediated by its binding to the GLP-1 receptor.

Several intracellular signaling pathways are involved in the effects of EX-4, such as protein kinase A (PKA) and phosphatidylinositide 3-kinase (PI3K). To characterize further the role of these pathways in the effects of EX-4 on glutamate uptake, we used pharmacological inhibitors of these signaling proteins: H-89 (a PKA inhibitor) and Rp-cAMPS (a cAMP inhibitor). Glutamate uptake was increased in slices treated with EX-4 and H-89 [F (3,26) = 7.96, $p = 0.0006$] or EX-4 and Rp-cAMPS [F (3,23) = 9.067, $p = 0.0004$] (Fig.7b, c, respectively), suggesting that PKA and cAMP are not involved in the mechanism by which EX-4 exerts its effects on glutamate uptake. Subsequently, we incubated the slices with EX-4 and LY294002 (a PI3K inhibitor). LY294002 inhibited the EX-4-induced increase in glutamate uptake [F (3,30) = 4.310, $p = 0.01$], indicating that the effect of EX-4 in glutamate uptake is mediated by PI3K pathway activation (Fig. 7d).

EX-4 Does Not Alter Peripheral Metabolic Parameters in the Diabetes Model

Measurement of peripheral parameters demonstrated that the DM rats exhibited higher glycemia than age-matched SHAM rats ($p < 0.0001$) (Table 1). The body weight of DM rats was 35% lower than that of the controls at 60 days after STZ, and

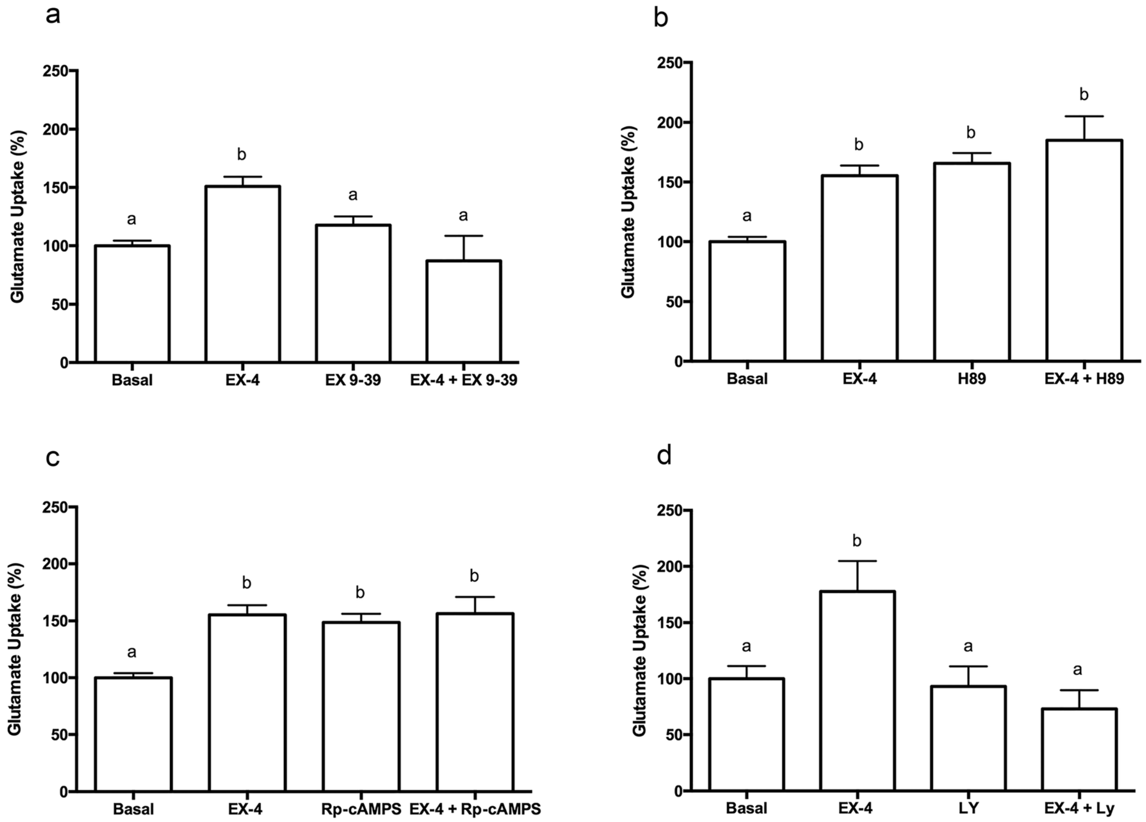

Fig. 7 EX-4 improves the glutamatergic transmission through the PI3K pathway. Acute hippocampal slices were incubated with EX (9–39) (200 nM), Rp-cAMPS (20 μM), H-89 (10 μM), or LY294002 (10 μM) for 15 min before EX-4 (10 nM) exposure. EX (9–39) abolished the effect of EX-4 in slices (**a**), suggesting that EX-4 mediates its effects by its binding to the GLP-1 receptor. H-89 (**b**) or Rp-cAMPS (**c**) did not modulate the effect of EX-4. LY294002 reverted the effects of EX-4 (**d**) suggesting that EX-4 acts on glutamate uptake via the PI3K pathway. Data are shown as the means ± SEM of six independent experiments performed in triplicate. Bars without a common letter statistically differ assuming $p < 0.05$ by one-way ANOVA followed by Tukey's post hoc test

EX-4 administration did not prevent this reduction in weight (data not shown). C-peptide was significantly lower in the DM group than in the SHAM group ($p < 0.0001$). After 28 days of EX-4 administration to the DM group, glycemia determinations did not differ between the DM-vehicle and DM-EX-4 subgroups ($p = 0.13$). Confirming insulin deficiency, the DM vehicle and DM-EX-4 subgroups presented no detectable concentrations of serum C-peptide.

AGEs and RAGE Are Altered in Diabetic Rats

To assess glucotoxicity, we determined AGE content in the serum and CSF of diabetic rats (Table 1). Increased AGE levels were observed in the serum ($p < 0.0001$) and CSF ($p = 0.03$) of the DM group was compared with the SHAM group. Moreover, RAGE levels were augmented in hippocampal slices from DM rats compared with SHAM rats ($p = 0.04$).

Table 1 EX-4 does not recover peripheral metabolic parameters, hippocampal RAGE content, and hippocampal GLO1 activity in STZ-induced DM

		SHAM	DM	DM vehicle	DM-EX-4
Peripheric parameters	Glycemia (mg/dL)	94.3 ± 4.9	477.4 ± 34.0*	439.5 ± 24.5	497.4 ± 26.71
	C-peptide (pM)	154 (123–180)	0 (0–1.5)*	0 (0–1.5)	0 (0–1.5)
	AGEs serum (%)	100.0 ± 3.3	140.8 ± 9.3*	100.0 ± 4.0	120.0 ± 4.2*
Central parameters	AGEs CSF (%)	96.2 (96–101)	131.6 (126–152)*	100.0 ± 7.6	89.48 ± 11.22
	RAGE (%)	100.0 ± 18.0	151.8 ± 8.5*	100.0 ± 13.5	124.3 ± 18.87
	GLO 1(%)	100.0 ± 10.1	131.8 ± 9.9*	100.0 ± 7.6	95.2 ± 2.9

Glycemia, C-peptide level (serum), AGEs content (serum and CSF), RAGE, and GLO 1 (hippocampus tissue) were evaluated in the DM ($n = 33$) and SHAM ($n = 11$) groups at 60 days after STZ induction. Randomly derived from DM, DM-vehicle ($n = 11$) and DM-EX-4 ($n = 11$) subgroups are shown in the right columns. Sham and DM groups were compared at 60 days after STZ (two left columns) by student's *t* test. DM vehicle and DM-EX-4 were compared 28 days after EX-4 administration (two right columns), also by student's *t* test assuming $p < 0.05$

Levels of the GLO 1 enzyme were determined in the hippocampus to evaluate the function of the glyoxalase system, accordingly, increased GLO 1 levels were observed in the DM group were compared with the SHAM group ($p = 0.03$).

Intriguingly, after 28 days of EX-4 administration to the DM group, we observed an increase in AGE serum concentrations in the DM-EX-4 subgroup compared with the DM-vehicle subgroup ($p = 0.003$). AGE concentrations in the CSF ($p = 0.48$) and RAGE levels in the hippocampus did not differ between the DM vehicle and DM-EX-4 subgroups ($p = 0.31$); furthermore, GLO 1 activity did not differ between the subgroups ($p = 0.66$).

Discussion

A previous study by our group demonstrated the reversal of the cognitive dysfunction that occurs in STZ-induced diabetes in rats by EX-4 [26]. Since this reversal could not be attributed entirely to biochemical and functional alterations in the brain barrier, we evaluated the neuroprotective effects of EX-4 on changes in glutamatergic transmission in diabetic rats. Moreover, we evaluated the effects of EX-4 directly on astrocyte culture cells and in acute hippocampal slices exposed to MG. Several reports have confirmed the cognitive deficit observed in the STZ-induced DM model commonly associated with hippocampal damage; importantly, EX-4 seems to recover this deficit [14, 27].

In agreement with our previous study [6], we found a decrease in glutamate uptake and GluN1 content in the hippocampi of diabetic rats. EX-4 was able to reverse the alterations in the glutamatergic system, but had no effects on other parameters evaluated (see Table 1). Moreover, the SHAM group treated with EX-4 exhibited an increase in glutamate uptake compared to the DM group indicating that EX-4 has neuroprotective effects even under basal conditions.

The peripheral parameters evaluated in the present study, blood glucose and C-peptide, were in agreement with those reported in the literature [6, 28, 29] and validate the model of STZ-induced DM. It is well established that the chronic hyperglycemia found in DM is directly related to the formation and accumulation of AGEs in both serum and tissues [6, 30]. Accordingly, we found an increase in AGE levels in the serum and CSF of diabetic rats, as well as elevated hippocampal levels of RAGE and GLO 1, the key enzyme that detoxifies MG. It is probable that the elevation in levels of the GLO 1 enzyme, observed in our diabetic rats, represents a compensatory mechanism in an attempt to detoxify AGE accumulation in the CNS [31]. However, this increase in GLO 1 was not enough to avoid the high levels of AGEs in diabetic animals. Moreover, we observed an increase in the post-translational modification of serine and threonine residues of proteins as determined by *O*-GlcNAc in the hippocampi of diabetic rats. Accordingly, increased *O*-GlcNAcylation is related to glucose toxicity in a number of metabolic diseases including DM [32]. Unfortunately, the increment of protein *O*-GlcNAcylation in this diabetic model was not reversed by EX-4 administration. However, considering the growing interest in protein *O*-GlcNAcylation in DM and other neurodegenerative diseases (see [33] for review), we further analyzed the effect per se of EX-4 on this parameter (see Fig. 4a). Protein *O*-GlcNAcylation in SHAM-EX-4 did not differ in the SHAM and DM groups; this finding may reflect the fact that the measurement is a mean value and the occurrence of specific alterations in determined proteins should not be ruled out.

EX-4 is a well-established drug for treating T2DM patients, but studies have shown its beneficial effects in cases of T1DM and neurodegenerative diseases [34, 35]. Glycemic control by GLP-1 analogs seems to be dependent on insulin production by beta cells. In fact, hyperglycemia was attenuated by GLP-1 analogs in STZ-induced models in newborn rats (e.g., [36]) or in the type 2 diabetic model (e.g., [37]). Studies report a recovery in glycemia in patients with T1DM, with detectable levels of C-peptide, indicating that these patients present a residual pancreatic beta cell function [38]. Our diabetic animals did not have detectable levels of C-peptide suggesting that EX-4 was unable to recover glycemia. Moreover, the activity of the GLO 1 in hippocampal slices, as well as the levels of AGEs in the serum and CSF, were also not improved by EX-4. Therefore, based on glycemia and C-peptide levels, we can rule out an insulin-mediated effect of EX-4 in our observations. Indeed, it is important to mention that Ex-4 has other metabolic effects, independent of insulin, such as the paradoxical acute increment of glycemia following activation of the sympathetic system [39] and central negative-modulatory effect on appetite [40], which were not the focus of the present study. In addition, beyond its metabolic effects, GLP-1 and analogs, including EX-4, are able to improve long-term potentiation [41] and stimulate hippocampal neurogenesis [42] supporting their use in neurodegenerative diseases including DM and Alzheimer's disease [43, 44].

Exposure to AGEs may cause changes in the glutamatergic activity in a RAGE-dependent manner, whereby AGE/RAGE pathway activation may cause an inflammatory response with the release of pro-inflammatory cytokines (IL-1β, TNF-α) [45] and, thus, a reduction in glutamate uptake [46]; alternatively, this alterations may occur in a RAGE-independent manner [47]. Despite the decrease in glutamate uptake, we found no changes in glutamate transporter (GLT-1 and GLAST) levels in astrocytes. Although glutamate transporter expression was not altered, high-glucose levels can lead to glycation and consequent inactivation of these transporters [48].

Regardless of the mechanism by which it occurs, decreased glutamate uptake can result in high-extracellular levels of glutamate and, subsequently, excitotoxicity. Accordingly, the GluN1 subunit of the NMDA receptor, which is negatively regulated by chronic excitotoxicity [49] was decreased in our diabetic rats. The combination of excitotoxicity and alterations in glutamate receptor expression could cause glutamatergic dysfunction and, finally, cognitive deficit in DM [50].

Astrocytes play an important role in glutamatergic transmission since extracellular glutamate captured by astrocytes is converted to glutamine by glutamine synthetase (enzyme exclusively expressed by astrocytes). Glutamine is exported from astrocytes and then imported into neurons for conversion back to glutamate [51]. Considering the neuroprotective effects of EX-4 in glutamatergic transmission and the importance of astrocytes in the glutamate-glutamine cycle, we evaluated the direct effect of EX-4 on astrocyte culture cells and on acute hippocampal slices, which contain functional and interactive neurons and astrocytes [23]. EX-4 was able to increase basal glutamate uptake in both culture astrocyte cells and acute hippocampal slices; moreover, this compound reverted the decrease in GluN1 content and glutamate uptake caused by MG in acute hippocampal slices demonstrating the protective effect of EX-4 against a brain insult.

It has been shown that GLP-1 agonists are able to reduce inflammatory mediators, such as IL-1β and NF-κB, in hyperglycemic situations [52]. Moreover, GLP-1 agonists may reduce AGE-induced reactive oxygen species accumulation [53]. Both situations could improve glutamate uptake by astrocytes, avoiding excitotoxicity, and restore the GluN1 content. In addition, GLP-1 agonists may act directly on neurons and astrocytes improving their functions [54], which may explain the effect of EX-4 in astrocytes and slices under basal conditions. MG can form AGEs by glycation reactions with different molecules, such as lysine, when forming CEL. AG, an anti-glycation compound, can prevent AGE formation from MG [55]. Importantly, CEL and AG did not demonstrate any effect on glutamate uptake or GluN1 content and, therefore, did not affect glutamate transmission. As such, we can postulate that the alterations in MG were provoked by its direct effect.

GLP-1 receptors are widely expressed in the CNS, predominantly in neurons and microglia, however studies have shown GLP-1 receptor expression in astrocytes [56], mainly under inflammatory conditions [15]. GLP-1 analogs cross the BBB, a property that is vitally important for use in the treatment of CNS degenerative disorders [57]. Moreover, due to the fact that the GLP-1 receptors are present throughout the brain, the GLP-1/GLP-1R signaling axis is considered to be an important target for overcoming CNS disorders [56, 58]. Several studies have shown that GLP-1 agonists exert their neuroprotective activity through many pathways. Activation of the GLP-1R stimulates adenylyl cyclase and increases cAMP levels. Moreover, the PI3K pathway is involved in GLP-1 agonist effects [59]. The activation of these downstream kinases can mediate growth factor signaling and effects such as the induction of the expression of genes linked to increased cell metabolism, inhibition of apoptosis, and inhibition of inflammatory responses [15]. In order to understand the signaling pathway responsible for the effects of EX-4 in glutamatergic transmission, hippocampal slices were incubated with PKA, cAMP, or PI3K antagonists. Only the slices incubated with the PI3K inhibitor abolished the effects of EX-4, suggesting that the improvements in glutamatergic transmission caused by EX-4 in acute hippocampal slices were due to the PI3K activation.

Akt acts as a central mediator of PI3K signaling and the PI3K/Akt signaling pathway regulates various cellular functions in the CNS, such as cell proliferation, apoptosis, metabolism, and survival [60]. Activated Akt can interact with a spectrum of molecules to exert anti-inflammatory and anti-apoptotic effects [61], suggesting that the activation of the PI3K pathway could mediate the protective effects of EX-4 against damage caused by AGEs or other inflammatory mediators. Consistent with this hypothesis, EX-4 was able to reduce inflammatory hippocampal responses in a model of dementia induced by STZ [62] and GLP-1 analogs have been proposed as novel anti-inflammatory and immunomodulatory compounds [52]. Herein, we found the activation of hippocampal PI3K signaling by EX-4 in diabetic rats, but further data will be necessary to identify downstream signaling molecules to explain the changes in glutamate uptake/metabolism and glutamatergic transmission.

In summary, this study demonstrated changes in glutamatergic transmission in diabetic rats and in hippocampal slices exposed to MG. EX-4 was able to recover changes in glutamate uptake and GluN1 content in both cases, although it did not decrease the content of AGEs in DM rats. In hippocampal slices, the effects of EX-4 occurred via PI3K, which could explain the protective effect of EX-4 in the CNS. Moreover, EX-4 exerted a direct effect on astrocytes demonstrating a possible role for these cells in the neuroprotection exerted by EX-4.

Conclusions

Our results indicate the ability of EX-4, a GLP-1 agonist, to reverse impairments in glutamatergic transmission, particularly glutamate uptake and GluN1 content in diabetic rats. Moreover, these effects of EX-4 were not related to changes in glycemia, since EX-4 did not improve glycemia or AGE formation in STZ-induced diabetes. EX-4 has been shown to directly improve glutamatergic transmission in astrocytes and hippocampal slices mediated via the PI3K pathway. These results suggest that EX-4 could be an adjuvant treatment in brain damage related to excitotoxicity.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Statement of Informed Consent All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80–23), and all procedures were previously approved by the local Animal Care Ethical Committee (CEUA-UFRGS; project number 24076). All efforts were made to minimize animal suffering and reduce the number of animals used.

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