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ORIGINAL PAPER

$GABA_A$ Modulation of S100B Secretion in Acute Hippocampal Slices and Astrocyte Cultures

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Abstract

Astrocytes are the major glial cells in brain tissue and are involved, among many functions, ionic and metabolic homeostasis maintenance of synapses. These cells express receptors and transporters for neurotransmitters, including GABA. GABA signaling is reportedly able to affect astroglial response to injury, as evaluated by specific astrocyte markers such as glial fibrillary acid protein and the calcium-binding protein, S100B. Herein, we investigated the modulatory effects of the $GABA_A$ receptor on astrocyte S100B secretion in acute hippocampal slices and astrocyte cultures, using the agonist, muscimol, and the antagonists pentylenetetrazol (PTZ) and bicuculline. These effects were analyzed in the presence of tetrodotoxin (TTX), fluorocitrate (FLC), cobalt and barium. PTZ positively modify S100B secretion in hippocampal slices and astrocyte cultures; in contrast, bicuculline inhibited S100B secretion only in hippocampal slices. Muscimol, per se, did not change S100B secretion, but prevented the effects of PTZ and bicuculline. Moreover, PTZ-induced S100B secretion was prevented by TTX, FLC, cobalt and barium indicating a complex $GABA_A$ communication between astrocytes and neurons. The effects of two putative agonists of $GABA_A$, β-hydroxybutyrate and methylglyoxal, on S100B secretion were also evaluated. In view of the neurotrophic role of extracellular S100B under conditions of injury, our data reinforce the idea that $GABA_A$ receptors act directly on astrocytes, and indirectly on neurons, to modulate astroglial response.

Keywords Astrocyte · $GABA_A$ receptor · PTZ · Bicuculline · Methylglyoxal · S100B secretion

Introduction

Astrocytes, the most abundant glial cells in brain tissue, envelop synapses and are able to sense and to respond to neuronal activity [1]. They are responsible for potassium and neurotransmitter clearance at the synaptic cleft [2–5], and also provide energy supplementation and antioxidant defense for neuronal activity [6–8]. Astrocytes supply neurons with glutamine for the synthesis of glutamic acid and γ-aminobutyric acid (GABA) in glutamatergic and GABAergic neurons, respectively [9]. Astrocytes are heterogeneous cell types that express a variety of neurotransmitter receptors [10], including ionotropic glutamate (e.g. *N*-methyl-D-aspartate) and $GABA_A$ receptors [11, 12].

GABA receptors are expressed in astrocytes, in both cell cultures and tissue slices, but the physiological significance of these receptors is not well understood and may be related to extracellular ion homeostasis and pH regulation [13]. Glial cells in adult and neonatal hippocampal slices exhibit an electrophysiological response to muscimol, a $GABA_A$ agonist, but not baclofen, a $GABA_B$ agonist [14]. Moreover, that study presents data to indicate that ionotropic GABA signaling in hippocampal glial cells from adult rats differs to that in slices from young animals and astrocytes in culture.

Impaired GABAergic signaling and astrogliosis have been reported in many neurological disorders, including

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amyotrophic lateral sclerosis [15] and seizures [16]. Indeed, antagonists of $GABA_A$ receptor, pentylenetetrazole (PTZ) and bicuculline, have been used to induce electrophysiological alterations and discharges in hippocampal slices [17, 18], but these compounds act at different sites. Bicuculline binds to the GABA site (competitive antagonism) while PTZ binds at a site closer to the chloride pore (non-competitive antagonism) [19].

Astrogliosis is characterized by increases in glial fibrillary acidic protein (GFAP) and/or S100B, two specific glial markers in the brain tissue. Interestingly, gabapentin, a general GABA agonist protects against streptozotocin-induced astrogliosis in the hippocampus, cerebral cortex and cerebellum [20]. Accordingly, the neuroprotective effect of theanine on ischemia-induced astrogliosis is prevented by bicuculline [21]. In addition, PTZ-induced seizure is accompanied by increased S100B in brain tissue and serum [22]. Moreover, it has been proposed that astrocytic $GABA_A$ receptors are reduced possibly through the overproduction of S100B in activated astrocytes [23].

S100B is a calcium-binding protein mainly produced (and secreted) by astrocytes in the central nervous system (CNS) [24]. Cerebrospinal fluid (CSF) and serum S100B levels have been used to indicate astroglial activation in several conditions of brain injury [25, 26]. Our group, observed a high S100B level in CSF in rat epilepsy model [27]. Several S100B secretagogues have been identified, including glutamate and cytokines [28, 29]. Some metabolites, such as methylglyoxal (MG) and ketone bodies, which are thought to exert effects on GABA receptors [30, 31], regulate S100B secretion [32, 33]. However, a direct effect of $GABA_A$ signaling on S100B secretion has yet to be demonstrated.

Herein, we investigated the specific effects of $GABA_A$-mediated signaling on astrocyte S100B secretion in acute hippocampal slices, using classical (PTZ and bicuculline), as well as new, putative (e.g. MG) modulators of $GABA_A$ receptors. $GABA_A$-mediated signaling also was studied in astrocyte cultures.

Materials and Methods

Materials

Poly-L-lysine, methylthiazolyldiphenyltetrazolium bromide (MTT), MG, anti-S100B (SH-B1), 4-(2-hydroxyethyl) piperazine-l-ethanesulfonic acid (HEPES), *o*-phenylenediamine (OPD), [3(4,5-dimethylthi-azol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT), muscimol, β-hydroxybutyrate and fluocitrate (FLC) were purchased from Sigma (Saint Louis, MO, USA). Fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM) and other materials for cell culture were purchased from Gibco. PTZ and bicuculline were purchased from TOCRIS (Bristol, United Kingdom). Polyclonal anti-S100B and anti-rabbit peroxidase-linked antibodies were purchased from DAKO (São Paulo, Brazil) and GE, respectively (Little Chalfont, United Kingdom). Tetrodotoxin (TTX) was from Abcam (Cambridge, MA, USA). The LDH kit assay was purchased from BioClin, Brazil.

Animals

Forty-five male *Wistar* rats, at postnatal day 30, were obtained from our breeding colony (Department of Biochemistry, UFRGS) and maintained under controlled light and environmental conditions (12 h light/12 h dark cycle at a constant temperature of 22 ± 1 °C). We focused on this animal age due to the developed and matured GABAergic neurotransmission in these rats [34].

Procedures were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and followed the regulations of the local animal house authorities and Committee of Animal Use of UFRGS (Project Number 24,472).

Preparation and Incubation of Hippocampal Slices

Animals were killed by decapitation, their brains were removed and placed in cold saline medium of 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. The hippocampi were dissected and transverse slices of 0.3 mm were obtained using a McIlwain Tissue Chopper. Slices were then transferred immediately to 24-well culture plates, each well containing 0.3 mL of physiological medium and only one slice. The medium was replaced every 15 min with fresh saline medium at room temperature. Following a 120 min equilibration period, the medium was removed and replaced with basal or specific treatments for 60 min at 30 °C on a warm plate [35].

Slices were incubated with the following treatments: muscimol (5, 10 and 20 µM), PTZ (5, 10 and 15 mM), bicuculline (5, 10 and 20 µM), MG (1, 10, 100 and 500 µM), β-hydroxybutyrate (1, 5 and 10 mM), high potassium (20 mM, adjusting the medium osmolarity by reducing NaCl), $CoCl_2$(1 mM), $BaCl_2$ (100 µM), TTX (1 µM), FLC was used at 100 µM and diluted in HCl 0.1 M. Experiments with FLC were always performed with a vehicle control. The mM and µM concentrations of PTZ and bicuculline, respectively, were chosen according to data in the literature and were able to induce bursts in hipocampal slices [18, 36], which were also able to induce changes in astroglial parameters [37, 38].

Astrocyte Cultures

Primary astrocyte cultures from Wistar rats were prepared as previously described [39]. Procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local authorities. Briefly, the cerebral cortices of newborn Wistar rats (1–2 days old) were removed and mechanically dissociated in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS), pH 7.2, containing (in mM) 137.93 NaCl, 2.66 KCl, 8.09 Na_2HPO_4, 1.47 KH_2PO_4, and 5.55 glucose. The cortices were cleaned of meninges and mechanically dissociated by sequential passages 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 B, 0.032% garamycin, and 10% fetal bovine serum. Approximately 300,000 cells were seeded in each well of 24-well plates, and maintained in DMEM containing 10% fetal bovine serum in 5% CO_2/95% air at 37 °C. Cells were then allowed to grow to confluence and used at 21 days in vitro. The medium was replaced by DMEM without fetal bovine serum in the absence or presence of PTZ (15 mM) or bicuculline (10 µM), co-incubated with FLC (100 µM) or muscimol (10 µM).

Lactate Dehydrogenase Assay

Slice integrity was evaluated by lactate dehydrogenase (LDH) activity using a commercial kit (BioClin, Brazil). The assay was performed according to the manufacturer's instructions. Results are expressed as percentages of the control.

MTT Reduction Assay

Slice cell viability was assayed using the colorimetric MTT method [40]. Slices were incubated with 0.5 mg/mL MTT for 30 min in 5% CO2/95% air at 37 °C. The formazan product generated during the incubation was solubilized in dimethyl sulfoxide (DMSO) and measured at 560 and 650 nm. The reduction of MTT was calculated by the following formula: (abs 560 nm) − (abs 650 nm). Results are expressed as percentages of the control.

S100B Measurement

S100B content in the incubation medium of hippocampal slices and the supernatant of astrocyte cultures was measured by an enzyme-linked immunosorbent (ELISA) assay [41]. Briefly, 50 µL of sample (previously diluted when necessary) plus 50 µL of Tris buffer were incubated for 2 h on a microtiter plate that was previously coated overnight with monoclonal anti-S100B (SH-B1) antibody. Polyclonal anti-S100 was added and incubated for 30 min, and then peroxidase-conjugated anti-rabbit antibody was added for a further 30 min, at 37 °C. The color reaction with OPD was measured at 492 nm. The standard S100B curve ranged from 0.002 to 1 ng/mL. S100B data (in ng/mL) were expressed as percentages of the controls.

Statistical Analysis

All results are expressed as mean ± standard error mean (SEM) and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. The level of statistical significance was set at $p < 0.05$. All analyses were performed using the Prism 5.0 (GraphPad).

Results

GABAergic Receptors Alter S100B Secretion in Acute Hippocampal Slices

Muscimol, an agonist of the $GABA_A$ receptor, did not affect S100B secretion [Fig. 1a]. However, two well-known $GABA_A$ antagonists, PTZ and bicuculline modify S100B secretion, in opposing directions [Fig. 1d, g]. PTZ increased S100B secretion at 15 mM [Fig. 1d, $F(3, 133) = 6.681$; $p = 0.0003$], while bicuculline, at 10 µM, decreased S100B secretion [Fig. 1g, $F(3, 111) = 5.162$; $p < 0.0001$]. These treatments did not affect cell viability (measured by MTT reduction assay) or integrity (LDH release assay) [Fig. 1, panels b, c, e, f, h and i].

Interestingly, co-incubation of muscimol with these $GABA_A$ antagonists prevented the alteration of S100B secretion; muscimol prevented the increment in extracellular S100B levels promoted by PTZ [Fig. 2a, $F(3, 116) = 8.640$; $p < 0.0001$] and also prevented the decrease inS100B secretion, induced by bicuculline [Fig. 2b, $F(3, 83) = 5.342$; $p = 0.0021$].

Tetrodotoxin and Fluorocitrate Prevent the S100B Secretion Induced by PTZ, but do not Modify the Effect of Bicuculline

In an attempt to clarify which cells were involved in the effect induced by PTZ or bicuculline, we evaluated the effects of the co-incubation of these compounds with TTX, a sodium channel blocker (virtually absent in astrocytes), or fluorocitrate (FLC), an inhibitor of aconitase (predominantly uptaken by astrocytes). As expected, FLC caused a decrease in S100B secretion [Fig. 3a, $F(2, 51) = 5.016$; $p = 0.0103$], while no direct effect was observed with TTX. The PTZ-induced increment in S100B was prevented by co-incubation

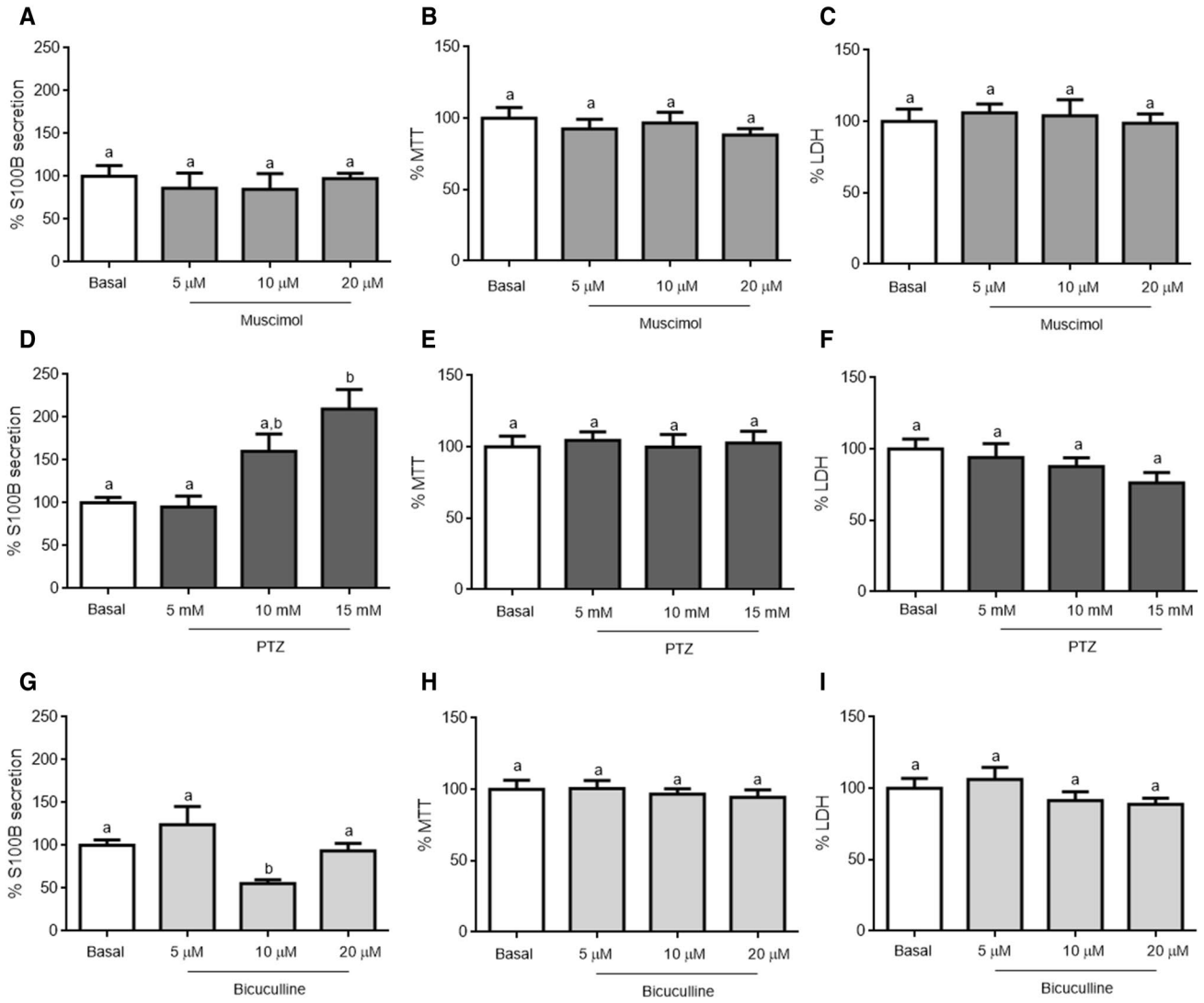

Fig. 1 Effects of GABAergic agonist and antagonists on S100B secretion in hippocampal slices. The $GABA_A$ agonist, muscimol, did not affect S100B secretion (**a**). The $GABA_A$ antagonists, PTZ and bicuculline, altered S100B secretion in opposing directions (**d, g**). These treatments did not affect slice viability (MTT) or integrity (LDH) (**b, c, e, f, h, i**). Values are expressed as means ± SEM, of 6–8 animals per group; independent experiments were performed in triplicate, assuming the control value as 100%. Data were analyzed by ANOVA, followed by Tukey's test. Bars without a common letter differ significantly, assuming $p < 0.05$

with FLC or TTX [Fig. 3b, F (3, 101) = 7.931; $p < 0.0001$]. However, co-incubation with FLC or TTX did not decrease the S100B secretion induced by bicuculline [Fig. 3c, F (3, 87) = 19.69; $p < 0.0001$].

Cobalt and Barium Ions Prevent the S100B Secretion Induced by PTZ, but do not Alter the Effect of Bicuculline

In order to expand the understanding the effects of the antagonists $GABA_A$ receptors on S100B secretion, we co-incubated PTZ and bicuculline with cobalt ($CoCl_2$ 1 mM), a non-selective calcium channel blocker, and barium ($BaCl_2$ 100 μM), a general potassium channel blocker. Cobalt and barium co-incubation prevented the increased S100B secretion that was induced by PTZ [Fig. 4a, F (5, 88) = 7.542; $p < 0.0001$]. However, the effects of barium and cobalt were not observed when co-incubated with bicuculline [Fig. 4b, F (5, 85) = 5.900; $p < 0.0001$]. Furthermore, only cobalt per se decreased S100B secretion.

Methylglyoxal-Induced S100B Secretion is Prevented by TTX

We subsequently measured the effect of two physiological metabolites MG and β-hydroxybutyrate on S100B secretion.

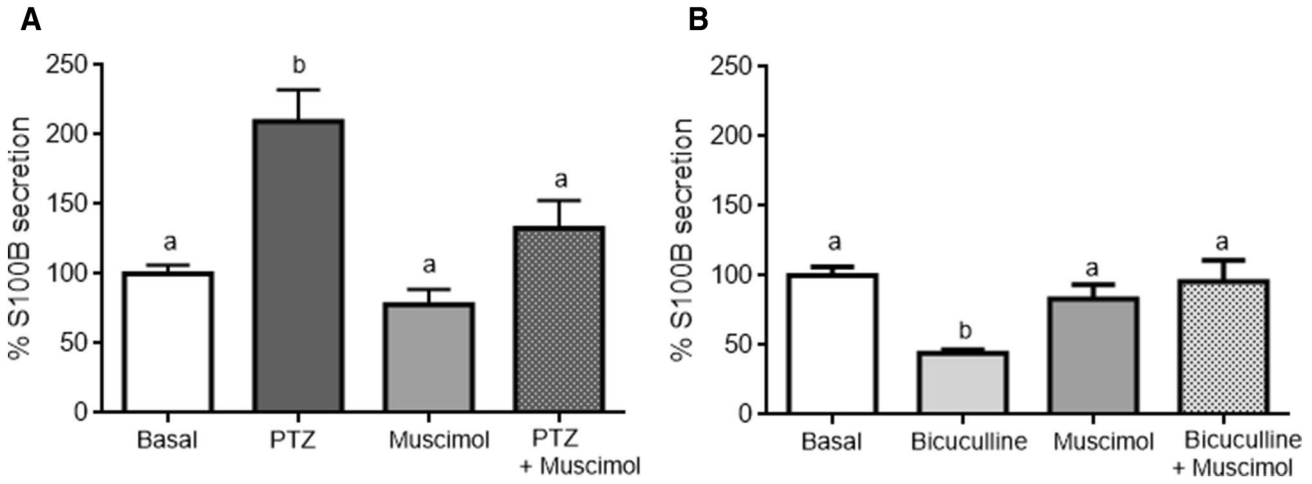

Fig. 2 Muscimol prevents the effects of GABAergic antagonists on S100B secretion in hippocampal slices. Muscimol (10 μM) prevented the PTZ-stimulated increase in S100B secretion (PTZ, 15 mM) (**a**) and the decrease in S100B levels induced by bicuculline (10 μM) (**b**). Values are expressed as means ± SEM, of 6–8 animals per group; independent experiments were performed in triplicate, assuming the control value as 100%. Data were analyzed by ANOVA, followed by the Tukey's test. Bars without a common letter differ significantly, assuming $p < 0.05$. Cell viability was not altered by different treatments (See Supplementary Fig. S1)

These compounds are putative agonists of GABA receptors [30, 42]. MG, at 10 μM, increased S100B secretion [Fig. 5a, $F(4, 102) = 4.541; p < 0.0001$]. However, β-hydroxybutyrate, at different concentrations (between 1 and 10 mM), was unable to alter S100B secretion [Fig. 5b, $F(3, 103) = 1.908$; $p = 0.1330$].

Since PTZ caused an increment in S100B secretion prevented by TTX, we investigated whether TTX would prevent the augmentation induced by methyglyoxal. Indeed, co-incubation with TTX (1 μM) also prevented the augmentation of S100B secretion induced by MG [Fig. 6a, $F(2, 78) = 14.37$; $p < 0.0001$]. In order to determine whether another well-characterized modulator of S100B, high-K^+ medium [35], also was affected by TTX, we measured S100B secretion in high-K^+ medium containing TTX. The prevention of high-K^+ medium-mediated alterations in S100B secretion was confirmed [Fig. 6b, $F(2, 64) = 6.602$; $p = 0.0025$].

PTZ and Bicuculline Modulate S100B Secretion in Astrocyte Cultures

Finally, we examined the effect of GABAergic agonists on astrocyte cultures. PTZ increased S100B secretion in primary astrocyte cultures, while muscimol prevented the effect of PTZ, just as occurred in hippocampal slices [Fig. 7a, $F(3, 26) = 3.739; p = 0.0234$]. Notably, as observed in hippocampal slices, muscimol per se had no effect on S100B secretion. On the other hand, in contrast to assays carried out with slices, bicuculline had no effect on S100B secretion in astrocyte cultures [Fig. 7b, $F(3, 29) = 0.5252$; $p = 0.8515$].

FLC evoked a decrease in S100B secretion in astrocytes and, expectedly, abrogated the increment caused by PTZ [Fig. 7c, $F(3, 27) = 8.705$; $p = 0.0003$]. However, unexpectedly, bicuculline reverted the effect of FLC on S100B secretion [Fig. 7d, $F(3, 26) = 5.507$; $p = 0.0046$].

Discussion

Several studies have demonstrated the importance of astrocytes in neuronal activity [1, 43]; these cells express the GABA transporter [44], $GABA_A$ receptors [45] and all protein machinery for GABA synthesis and release [46]. S100B is a calcium-binding protein predominantly synthesized and secreted by astrocytes in brain. This protein is involved in several intracellular and extracellular mechanisms [24]. In the extracellular space, S100B causes changes in neuron and glial cells and these effects depend upon the S100B concentration [47]. S100B has been postulated as an astrocyte modulator of neuronal synaptic plasticity [48] and its release is affected by several secretagogues, including cytokines and neurotransmitters such as glutamate and dopamine [29, 35, 49, 50]. Our present results show that $GABA_A$ signaling altered S100B secretion in hippocampal slices and astrocyte cultures.

Two classical $GABA_A$ antagonists, PTZ and bicuculline, positively and negatively, respectively, modulate S100B secretion in hippocampal slices. The effect of PTZ was also observed in astrocyte cultures. Muscimol, an agonist of $GABA_A$ receptors, did not affect S100B secretion, but prevented the effect observed with PTZ or bicuculline. Bicuculline has been known as a prototypic antagonist of $GABA_A$ receptor [51] and binds to the GABA-binding domain, reduces chloride conductance and

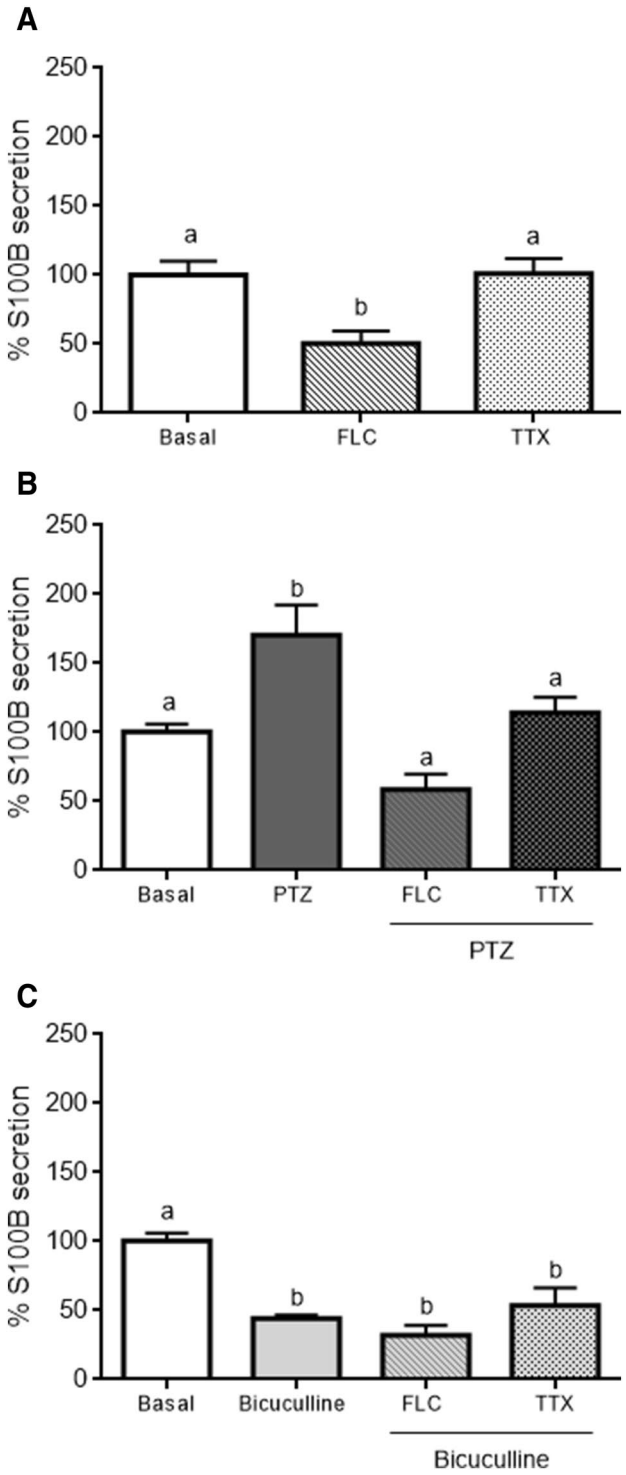

Fig. 3 TTX and FLC prevented PTZ-induced, but not bicuculline-induced, alterations in S100B secretion from hippocampal slices. FLC decreased S100B secretion (**a**). Co-incubation of TTX (1 µM) or FLC (100 µM) (**b, c**) prevented the increase in S100B caused by PTZ (15 mM) (**b**); however, these compounds did not affect bicuculine-stimulated (10 µM) S100B secretion (**c**). Values are expressed as means ± SEM, of 6–8 animals per group; independent experiments were performed in triplicate, assuming the control value as 100%. Data were analyzed by ANOVA, followed by Tukey's test. Bars without a common letter differ significantly, assuming $p < 0.05$. Cell viability and integrity were not altered by different treatments (See Supplementary Fig. S2)

affects calcium-activated potassium channels [52]. PTZ is a bicyclic tetrazole derivative compound that binds close to the chloride pore, as does picrotoxin, and blocks the passage of chloride and, to a lesser extent, potassium and sodium conductance promoting a depolarizing scenario and seizure developmental [19, 51–53].

The mechanism of S100B secretion is unknown, but involves cAMP signaling and/or internal Ca^{2+} mobilization [54, 55]. S100B secretion is negatively regulated by elevated extracellular levels of glutamate [50, 56] and appears to be dependent on glutamate transporters [28]. It is possible that glutamate and GABA transporters in astrocytes increase intracellular Na^+ concentrations, consequently increasing Ca^{2+} through Na^+/Ca^{2+} exchange [57]. Moreover, the high-K^+ environment resulting from neural activity, which stimulates the astrocytic Na/K-ATPase also increases cAMP (via soluble adenylyl cyclase) [58]. However, despite potential increments in Ca^{2+} and cAMP, there is a decrease in S100B secretion in both conditions. Therefore, at this moment, the mechanisms by which glutamate, high-K^+ or GABA antagonists regulate S100B secretion, based on ionic changes caused by neurotransmitter transporters and ionotropic channels at the astrocytic membrane, remain unclear.

It is conceivable that PTZ (at 15 mM) and bicuculline (at 10 µM) have direct effects on different $GABA_A$ sites in astrocytes, which may explain these distinct effects. However, the effect of PTZ was prevented by TTX, suggesting that the PTZ-induced S100B increment is, at least in part, dependent on neuronal activity and therefore the GABA receptor would be in neurons. In this case, some substances mobilized by neurons could mediate the S100B release by astrocytes. In contrast, bicuculline reduced S100B secretion and this effect was not affected by TTX, indicating an effect that was independent of neuronal activity. Furthermore, cobalt and barium, non-selective inhibitors of calcium and potassium channels, respectively, were only able to prevent the action of PTZ on secretion of S100B in acute hippocampal slice. Previous study suggested that bicuculline may have another mechanism of action [59]. In addition to being a $GABA_A$ antagonist, this drug can block the slow afterhyperpolarization through calcium-activated potassium channel. However, our results showed that the decreased effect of S100B secretion by bicuculline was not changed by co-incubation of cobalt and barium. Therefore, based on these findings we suggest that, in hippocampal slices, PTZ indirectly mobilizes S100B from astrocytes through the $GABA_A$ receptor in neurons, as well as by ionic conductance of calcium and potassium. Apparently, bicuculline mobilizes S100B directly through $GABA_A$ in astrocytes. Consistent with this hypothesis, in the hippocampus in a model of epilepsy, PTZ has been shown to induce an increase in CSF and serum levels of S100B [22, 60].

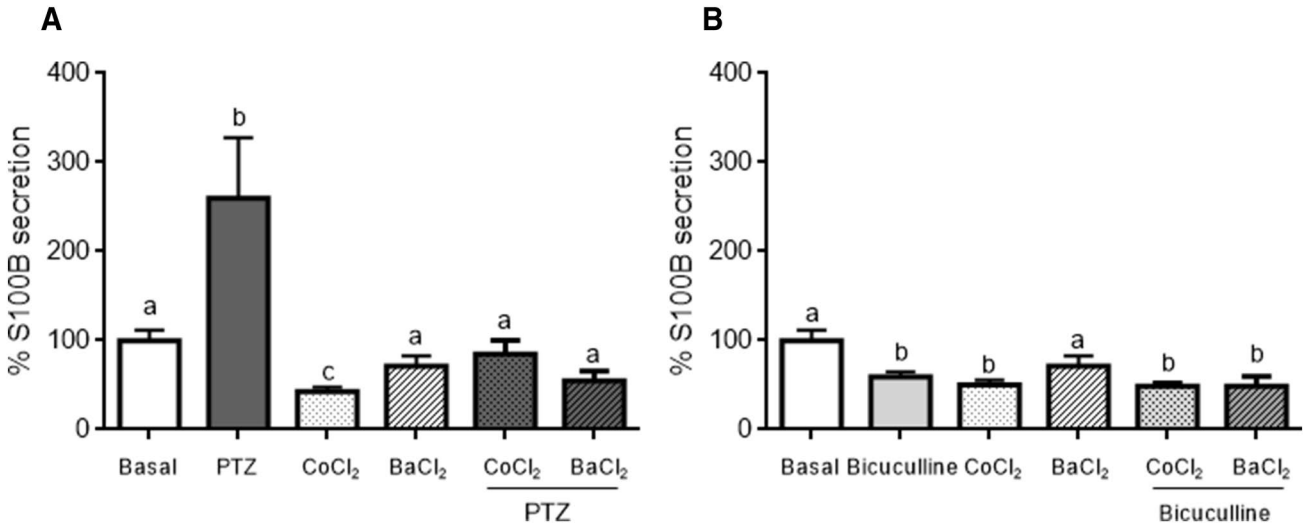

Fig. 4 Cobalt and barium prevented PTZ-induced, but not bicuculline-induced, alterations in S100B secretion from hippocampal slices. Co-incubation of $CoCl_2$ (1 mM) or $BaCl_2$ (100 µM) prevented the increase in S100B caused by PTZ (15 mM) (**a**); however, these compounds did not affect bicucculine-stimulated (10 µM) S100B secretion (**b**). Values are expressed as means ± SEM, of 6–8 animals per group; independent experiments were performed in triplicate, assuming the control value as 100%. Data were analyzed by ANOVA, followed by Tukey's test. Bars without a common letter differ significantly, assuming $p < 0.05$. Cell viability was not altered by different treatments (See Supplementary Fig. S3)

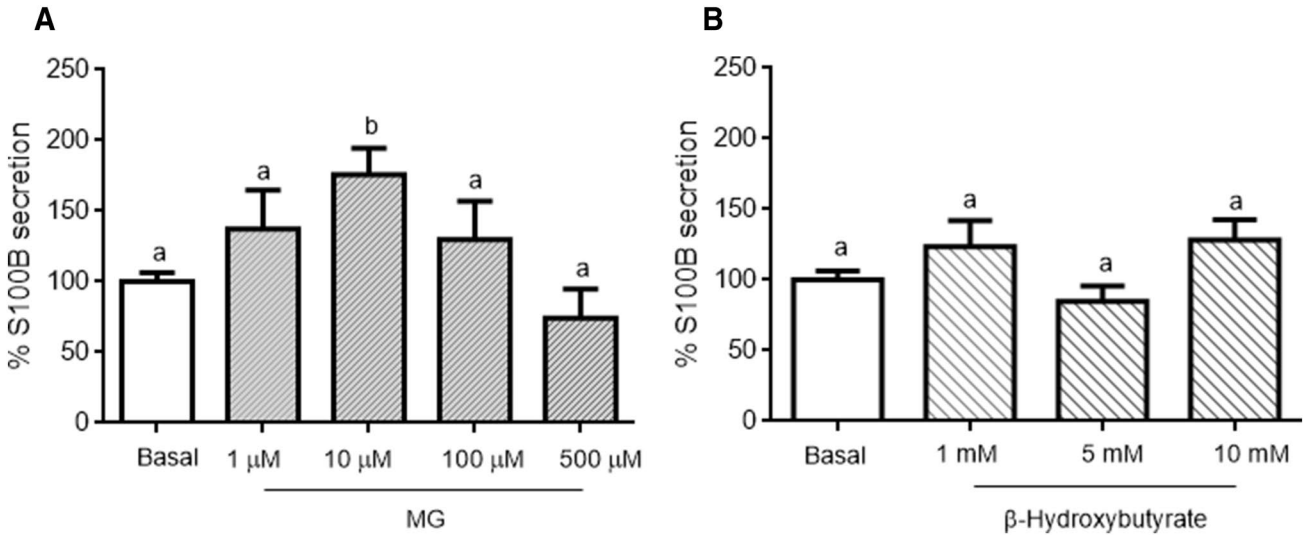

Fig. 5 Methyglyoxal (MG), but not β-hydroxybutyrate, increased S100B secretion from hippocampal slices. Effects of MG (**a**) and β-hydroxybutyrate (**b**) on S100B secretion. Values are expressed as means ± SEM, of 6–8 animals per group; independent experiments were performed in triplicate, assuming the control value as 100%. Data were analyzed by ANOVA, followed by Tukey's test. Bars without a common letter differ significantly, assuming $P < 0.05$. Cell viability and integrity were not altered by different treatments (See Supplementary Fig. S4)

S100B secretion has a close relationship with energetic metabolism [61]. Therefore, two compounds from energetic metabolic pathways that potentially alter GABA signaling were evaluated. MG, an endogenous by product of glycolysis, produced by the non-enzymatic cleavage of dihydroxyacetone phosphate, is an active glycation compound in hyperglycemic conditions (see Thornalley [62] for a review). This compound putatively binds to the $GABA_A$ receptor (competing with GABA) and, at 1–10 µM, it reduces seizure susceptibility [30, 63]. Under our experimental conditions, S100B secretion was increased by MG (a $GABA_A$ agonist, at 10 µM), and by PTZ (a GABA antagonist). On the

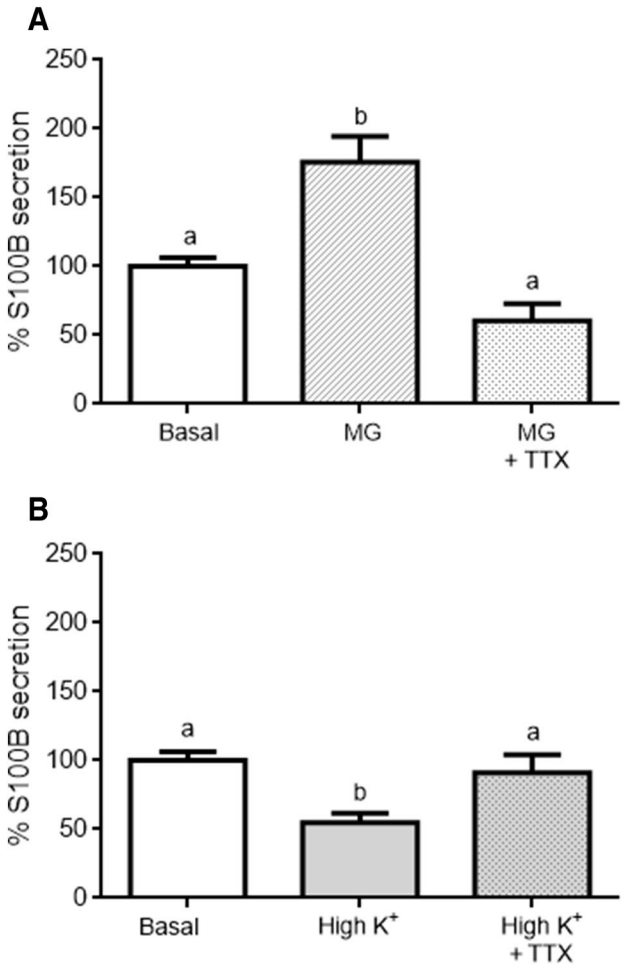

Fig. 6 TTX prevents the effects of S100B secretion modulators in hippocampal slices. TTX (1 μM) prevented the S100B levels augmentation promoted by MG (10 μM) (**a**). TTX (1 μM) also prevented the decrease in S100B levels caused by high potassium concentration (20 mM) (**b**). Values are expressed as means ± SEM, of 6–8 animals per group; independent experiments were performed in triplicate, assuming the control value as 100%. Data were analyzed by ANOVA, followed by Tukey's test. Bars without a common letter differ significantly, assuming $p < 0.05$. Cell viability was not altered by different treatments (See Supplementary Fig. S5)

other hand, elevated concentrations of MG (e.g. 0.5 mM), as found in pathological conditions, decreased S100B secretion in hippocampal slices of adult rats [32]. However, the physiological and pathological role of MG is still unclear [64]. Nevertheless, our data for MG reinforce the idea that GABA signaling affects S100B secretion. Moreover, it is of note that TTX prevented this effect, suggesting that, as occurred for PTZ, this effect was dependent upon neuronal activity. The main ketone body, β-hydroxybutyrate, also reduces seizure susceptibility by altering GABA metabolism [65] and $GABA_B$ receptors [66]. We did not find changes in S100B secretion in acute hippocampal slices incubated with β-hydroxybutyrate, although a decrease in S100B levels has been reported in the CSF of rats submitted to a ketogenic diet [67, 68] and an increase in the medium of astrocyte cultures incubated with β-hydroxybutyrate [33].

Another relevant finding of this study was that PTZ, but not bicuculline, altered S100B secretion in astrocyte cultures. It is possible that astrocytes in cultures, which are isolated from neurons, express different GABA receptors depending on the medium [69]. Nevertheless, as in the hippocampal slice assay, PTZ increased S100B secretion and muscimol prevented this increment in astrocyte cultures. For these comparisons, it is important to take into account the type of cell preparation and the age of the animal for interpretation of results, due to the developmental changes of the expression of $GABA_A$ in neurons [34] and glial cells [23, 70]. For example, in rats, $GABA_A$ activation causes a depolarization in immature but not adult neurons in the hippocampus. However, results in hippocampal slices, prepared from neonatal or adult rats, indicate that glial cells display qualitatively similar responses to GABA agonists [70].

We also found that bicuculline protected against the effect of FLC in astrocyte cultures, in contrast to results obtained in hippocampal slices. It is well known that FLC inhibits the Krebs cycle and consequently oxidative phosphorylation in the respiratory chain, predominantly in astrocytes, as these cells uptake this compound more actively. In other words, FLC (depending on the concentration) causes a kind of hypoxia only in astrocytes. Several studies suggest that dysfunction of ionotropic $GABA_A$ contributes to excitotoxicity and that $GABA_A$ activation improves astrocyte cell survival [71]. Interestingly, $GABA_A$ activation protected against glucose and oxygen deprivation and bicuculline aggravated the damage in the cerebral cortex, but not the hippocampus. In fact, bicuculline protected hippocampal cells exposed to glucose and oxygen deprivation [72]. In support of this observation, a recent in vivo study using 2-deoxy-2-[(18)F] fluoro-β-D-glucose showed that bicuculline changes glucose flow, causing a widespread hypermetabolism throughout the brain tissue [73]. Therefore, we suggest that the antagonism of $GABA_A$ signaling (by bicuculline and PTZ), could, at least in astrocyte cultures, neutralize the effect of FLC. However, this interesting effect demands further investigation.

Conclusions

This is the first study that, to our knowledge, connects $GABA_A$ signaling to S100B secretion. PTZ induced S100B secretion in hippocampal slices and astrocyte cultures, while bicuculline inhibited S100B secretion and only in hippocampal slices. Muscimol prevented the effects of PTZ and bicuculline. Moreover, this PTZ-induced S100B secretion was prevented by TTX, FLC, cobalt and barium, indicating a complex $GABA_A$ communication between

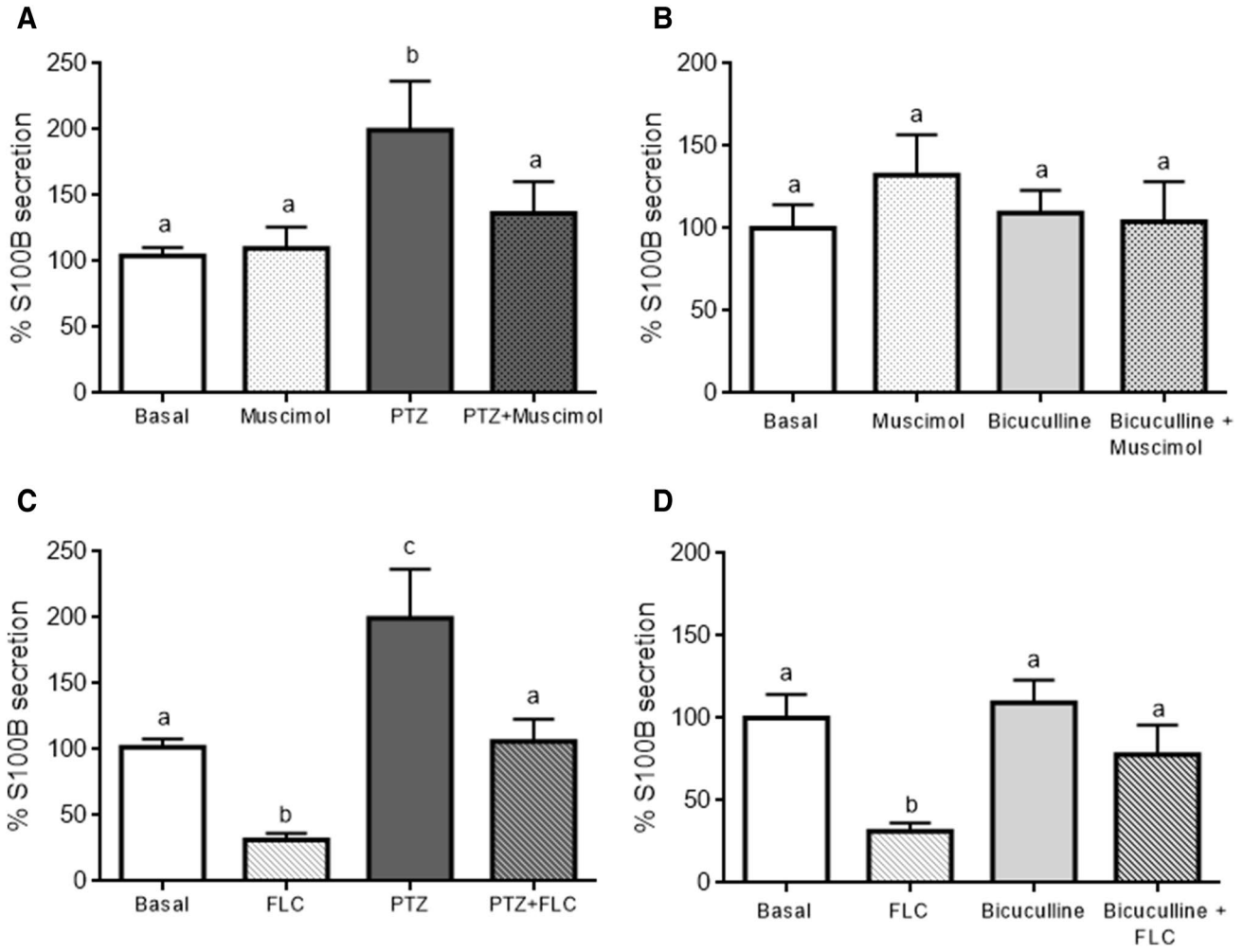

Fig. 7 Effects of GABAergic modulators on S100B secretion in astrocyte cultures. PTZ (15 mM) increased S100B secretion from astrocytes (**a, b**). FLC (100 µM) and muscimol (10 µM) prevented the effect of PTZ (**a, b**). However, bicuculline (10 µM) did not affect S100B secretion (**c, d**). The FLC-mediated reduction in S100B was prevented by bicuculline (**c**). Values are expressed as means ± SEM of 6 independent experiments performed in triplicate, assuming the control value as 100%. Data were analyzed by ANOVA, followed by Tukey's test. Bars without a common letter differ significantly, assuming $p < 0.05$. Cell viability was not altered by different treatments (See Supplementary Fig. S6)

astrocytes and neurons. In addition, MG, a putative modulator of the $GABA_A$ receptor, derived from the glycolytic pathway, also induced S100B secretion that could be blocked by TTX. Considering the neurotrophic role of extracellular S100B under conditions of injury, our data further suggest that the $GABA_A$ receptors act directly on astrocytes, and indirectly on neurons, to modulate astroglial response.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interests.

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