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ORIGINAL ARTICLE

Taurine-like GABA aminotransferase inhibitors prevent rabbit brain slices against oxygen–glucose deprivation-induced damage

Lorenzo Ricci · Massimo Valoti · Giampietro Sgaragli · Maria Frosini

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Abstract The activation of the GABAergic system has been shown to protect brain tissues against the damage that occurs after cerebral ischaemia. On the other hand, the taurine analogues (±)Piperidine-3-sulphonic- (PSA), 2-aminoethane phosphonic- (AEP), 2-(*N*-acetylamino) cyclohexane sulfonic-acids (ATAHS) and 2-aminobenzene sulfonate-acids (ANSA) have been reported to block GABA metabolism by inhibiting rabbit brain GABA aminotransferase and to increase GABA content in rabbit brain slices. The present investigation explored the neuroprotection provided by GABA, Vigabatrin (VIGA) and taurine analogues in the course of oxygen–glucose deprivation and reperfusion induced damage of rabbit brain slices. Tissue damage was assessed by measuring the release of glutamate and lactate dehydrogenase (LDH) during reperfusion and by determining final tissue water gain, measured as the index of cell swelling. GABA (30–300 μM) and VIGA (30–300 μM) significantly antagonised LDH and glutamate release, as well as tissue water gain caused by oxygen–glucose deprivation and reperfusion. Lower (1–10 μM) or higher concentrations (up to 3,000 μM) were ineffective. ANSA, PSA and ATAHS significantly reduced glutamate and LDH release and tissue water gain in a range of concentrations between 30 and 300 μM. Lower (0–10 μM) or higher (up to 3,000 μM) concentrations were ineffective. Both mechanisms suggest hormetic ("U-shaped") effects. These results indicate that the GABAergic system activation performed directly by GABA or indirectly through GABA aminotransferase inhibition is a promising approach for protecting the brain against ischemia and reperfusion-induced damage.

Keywords Taurine analogues · GABA · GABA aminotransferase inhibitors · Brain ischemia · Neuroprotection · Oxygen–glucose deprivation

Abbreviations

ACSF	Artificial cerebrospinal fluid
AEP	2-Aminoethane phosphonic acid
ANSA	2-Aminobenzene sulfonate
ATAHS	2-(*N*-acetylamino) cyclohexane sulfonic acid
GABA-AT	GABA aminotransferase (E.C.2.6.1.19)
PSA	(±)Piperidine-3-sulfonic acid
VIGA	4-Amino-5-hexenoic acid or γ-vinyl GABA

Introduction

Ischemic injury to cerebral tissues triggers complex biochemical cascades that ultimately lead to neuronal death. Excessive release of excitatory neurotransmitters occurs during the first phase of this process, stimulating "excitotoxic" conditions that hasten further brain depletion of oxygen and nutrients (Lau 2010). GABA and GABA agonists represent a physio-pharmacological means to inhibit cellular activity within the brain, possibly blunting ischemic injury (Green et al. 2000; Schwartz-Bloom and Sah 2001; Calabresi et al. 2003). Initially, endogenous GABA synthesis and release decrease after brain ischemia with consequent reduction in GABAergic transmission (Schwartz-Bloom and Sah 2001). Since glutamatergic and GABAergic systems work to counterbalance each other, the enhancement of GABAergic activity should antagonize the

L. Ricci · M. Valoti · G. Sgaragli · M. Frosini (✉)
Department of Neurosciences, Pharmacology Unit, University of Siena, Viale A. Moro 2 lotto C, 53100 Siena, Italy
e-mail: frosinim@unisi.it

excessive glutamatergic excitation. Thus, drugs that block GABA metabolism by inhibiting GABA aminotransferase (GABA transaminase, E.C.2.6.1.19) have neuroprotective potential (Schwartz–Bloom and Sah 2001; Costa et al. 2004; Ginsberg 2008). This approach might be promising especially in view of the increased bioavailability of GABA in brain tissues promoted by GABA aminotransferase inhibitors (Ricci et al. 2006). Previous studies have demonstrated that some structural taurine analogues such as (±)piperidine-3-sulfonic acid (PSA), 2-aminoethane phosphonic acid (AEP), 2-(*N*-acetylamino) cyclohexane sulfonic acid (ATAHS) and 2-aminobenzene sulfonate (ANSA) inhibit rabbit brain GABA aminotransferase with K_i values close to that of Vigabatrin (VIGA), a mechanism-based GABA aminotransferase inhibitor (Ricci et al. 2006).

The aim of the present investigation was to assess whether GABA, VIGA and the above-mentioned taurine analogues could counteract neuronal injury induced by oxygen–glucose deprivation and reperfusion by increasing GABA-dependent pathway activation in rabbit brain cortical slices. Tissue damage was assessed by measuring the release of glutamate and lactate dehydrogenase (LDH) into the bathing artificial cerebrospinal fluid (ACSF) during reperfusion. Tissue water gain, taken as an index of tissue edema (Hrabetová et al. 2002; MacGregor et al. 2003), was also determined at the end of the experiment.

Materials and methods

Materials

GABA, trizma® base, ascorbic acid, sodium pyruvate, β-nicotinamide adenine dinucleotide (NAD^+), β-nicotinamide adenine dinucleotide reduced form (NADH), glutamate, glutamate dehydrogenase (GDH), bovine serum albumine and ACSF components were acquired from Sigma-Aldrich Co. (St Louis, MO, USA). All the other chemicals were from standard local sources and of the highest grade commercially available. Reagents were dissolved in Milli-Q deionized water. Taurine analogues used in the present study are detailed in Table 1.

Animals

All experiments were performed in strict compliance with the recommendation of the EEC (86/609/CEE) for the care and use of laboratory animals and the protocols were approved by the Animal Care and Ethics Committee of the University of Siena, Italy. Adult male New Zealand rabbits (2.0–2.5 kg, Charles River Italia, Calco, Italy) were kept in large cages under a 12:12 h day–night cycle at 20°C ambient temperature. Drinking water and conventional laboratory rabbit food were available ad libitum. Before killing, animals were anaesthetised by intraperitoneal injection of xylazine chloride (10 mg kg^{-1}, Rompun® Vet., Bayer AG, Germany) and ketamine hydrochloride (35 mg kg^{-1}, Ketavet®, Parke Davis/Warner–Lambert, USA).

Slice preparation

After killing the whole brain was rapidly removed, chilled to 4°C and placed in ACSF (composition in mM: 120 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 1.0 NaH_2PO_4, 1.5 $CaCl_2$, 26 $NaHCO_3$, 11 glucose, saturated with 95% O_2 –5% CO_2, with a final pH of 7.4, osmolality 285–290 mOsmol). The cortex was dissected and cut into 400-μm thick slices by using a manual chopper (Stoelting Co., Wood Dale, IL, USA). Afterwards, slices were maintained in oxygenated ACSF enriched with 400 μM ascorbic acid for 1 h at room temperature to allow maximal recovery from slicing trauma (Brahma et al. 2000; Ricci et al. 2009).

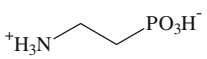
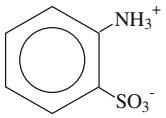
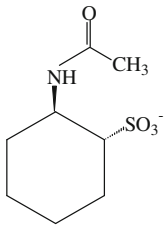
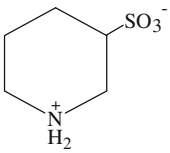
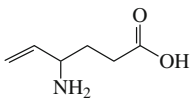
In vitro ischemia-like conditions

Cortical slices (≈4–5, total wet weight 50 ± 3.7 mg, $n = 10$) were placed in covered incubation flasks, containing 2 ml ACSF continuously bubbled with 95% O_2/5% CO_2 and incubated at 37°C for an additional period of 30 min. Afterwards, oxygen–glucose deprivation was carried out by incubating slices for 30 min into ACSF in which glucose was replaced by an equimolar amount of saccharose, and continuously bubbled with 95% N_2/5% CO_2. After the oxygen–glucose deprivation period, ischemic solution was replaced by fresh, oxygenated ACSF for an additional 90 min (reoxygenation). GABA, VIGA or taurine analogues were added to ACSF during the reperfusion period.

Assessment of neuronal injury

Neuronal damage was assessed quantitatively by measuring the amount of both glutamate and LDH released into the ACSF during 90 min of reperfusion. In particular, glutamate was measured fluorimetrically (excitation 366 nm; emission 450 nm) using the conversion of NAD^+ to NADH by glutamate dehydrogenase (Eilers et al. 1999), while LDH activity was determined spectrophotometrically by the rate of decrease in absorbance at 340 nm via the oxidation of NADH to NAD^+ using pyruvate as substrate (Gay et al. 1968). Tissue water gain (edema) was calculated as described by MacGregor et al. (2003). Briefly, at the end of experimental session, slices were weighed on pre-weighed pieces of aluminium foil (typically 2 × 2 cm), dried overnight at 95°C, and then reweighed. All water content data were referred to tissue dry weight

Table 1 Compounds used in the present study

Compound (abbreviation)	Structure	Origin
2-Aminoethane phosphonic acid (AEP)		Sigma-Aldrich
2-Aminobenzene sulfonate (ANSA)		Sigma-Aldrich
2-(*N*-acetylamino) cyclohexane sulfonic acid (ATAHS)		Our own synthesis (according to Freifelder et al. 1964)
(±)Piperidine-3-sulfonic acid (PSA)		Our own synthesis (according to Cordero et al. 2002)
γ-Vinyl GABA (VIGA)		Sabril® (Camillo Corvi S.p.A., Italy)

The purity of synthesised compounds was shown to be above 95% by ^{1}H NMR or high-performance liquid chromatography (HPLC)

(DW), which is assumed to be constant under conditions of water gain (Cserr et al. 1991).

Data analysis

For each experimental setting, brain slices derived from at least four rabbits were used. Data are reported as mean ± SEM and "*n*" is defined as the number of samples. For glutamate content and LDH activity of ACSF, data are expressed as nmol/mg wet tissue and U/mg wet tissue, respectively. One unit (U) of LDH activity is defined as that which gives rise to one micromole of lactate in one minute.

Statistical analysis was performed using one-way ANOVA followed by Tukey–Kramer post test (GraphPad Software, San Diego, CA, USA). In all comparisons, the level of statistical significance (*P*) was set at 0.05.

Results

Rabbit cortical slices incubated in ACSF for 120 min (control conditions, CTRL) were found to release 0.36 ± 0.04 nmol/mg tissue ($n = 16$) and 5.76 ± 0.98 U/mg prot. ($n = 10$) of glutamate and LDH, respectively into the medium (Fig. 1a, b). After 120 min incubation in ACSF, water content of control slices was 9.02 ± 0.21 g H_20 (g dry weight)$^{-1}$ (Fig. 1c), i.e. a value slightly higher, on average by about 4.0%, than the basal water content determined after 1-h recovery period at room temperature, as previously observed in rat brain slices (MacGregor et al. 2003; Ricci et al. 2009).

After 30 min of oxygen–glucose deprivation followed by 90 min of reperfusion, a significant increase in glutamate and LDH release, as well as slices water gain was observed (Fig. 1). GABA (1–3,000 μM) antagonised oxygen–glucose deprivation and reperfusion-induced effects. This antagonism, however, followed an "U-shaped" concentration–response curve, suggesting an hormetic behaviour. In particular, in the range of concentration between 30 and 300 μM, GABA significantly reverted glutamate and LDH release, while it was ineffective at lower (1–10 μM) or at higher (1,000–3,000 μM) concentrations. 300 μM GABA proved to be the most effective concentration, as glutamate and LDH values were close to those of control slices. As for tissue edema, GABA was effective between 30 and 1,000 μM, with 100 μM being the most active concentration. Lower (1–10 μM) or higher (3,000 μM)

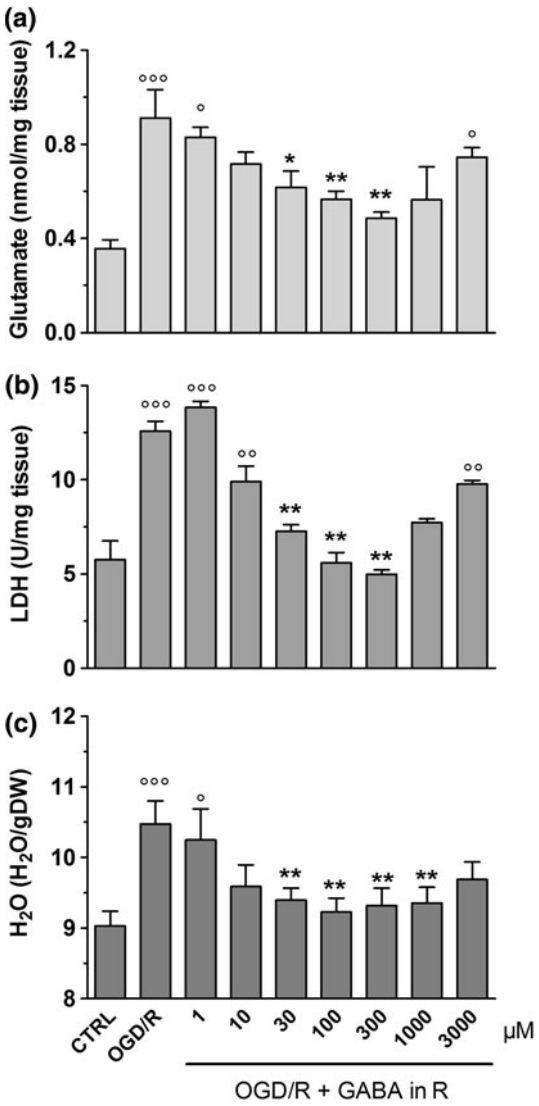


Fig. 1 Effect of GABA on oxygen–glucose deprivation and reoxygenation (OGD/R)-induced release of glutamate (**a**), LDH (**b**) and on tissue water content (**c**) in rabbit brain cortical slices. Slices were incubated in ACSF for 120 min (control conditions, CTRL) or subjected for 30 min to oxygen–glucose deprivation followed by 90 min of immersion in normally oxygenated artificial cerebrospinal fluid (reoxygenation). Different concentrations of GABA (1–3,000 μM) were added to the reoxygenation buffer. Data are mean ± SEM of at least four different experiments. $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$, $^{\circ\circ\circ}P < 0.01$ versus CTRL; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus OGD

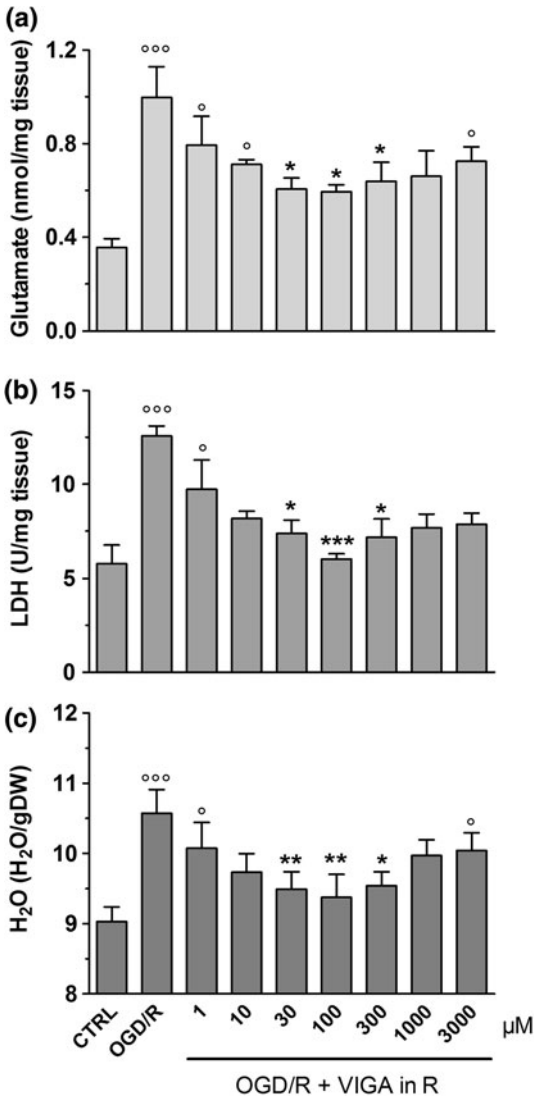


Fig. 2 Effect of Vigabatrin (VIGA) on oxygen–glucose deprivation and reoxygenation (OGD/R)-induced release of glutamate (**a**), LDH (**b**) and on tissue water content (**c**) in rabbit brain cortical slices. Slices were incubated in ACSF for 120 min (control conditions, CTRL) or subjected for 30 min to oxygen/glucose deprivation followed by 90 min of immersion in normally oxygenated artificial cerebrospinal fluid (reoxygenation). Different concentrations of VIGA (1–3,000 μM) were added to the reoxygenation buffer. Data are mean ± SEM of at least four different experiments. $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$, $^{\circ\circ\circ}P < 0.01$ versus CTRL; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus OGD

GABA concentrations had no significant effects on the prevention in tissue water gain (Fig. 1c).

As shown in Fig. 2, VIGA (1–3,000 μM) antagonised oxygen–glucose deprivation and reperfusion-induced effects in a GABA-like fashion. Both drugs, in fact, exhibited the same efficacy window of concentrations (30–300 μM) and maximal effects occurred at 100 μM VIGA.

Moreover, the taurine analogues PSA, ANSA and ATAHS reverted the ischemia-like damage according to an "U" shaped concentration–response curve. All the compounds tested, in fact, exhibited the "efficacy window" of 30–300 μM but possessed different degree of efficacy. The most interesting compound turned out to be PSA since it was able to completely revert cell damage. As shown in Fig. 3, glutamate and LDH levels in ACSF after reperfusion phase were close to control values in the presence of 100 and 300 μM PSA (Fig. 3a, b). In addition, 30–100 μM PSA significantly antagonised tissue

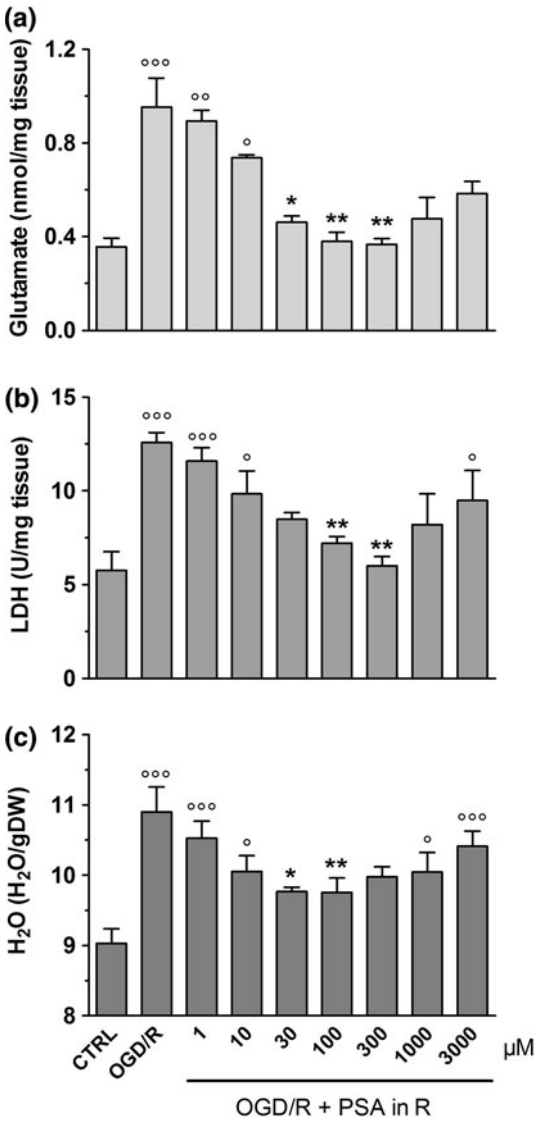


Fig. 3 Effect of (±)piperidine-3-sulfonic acid (PSA) on oxygen–glucose deprivation and reoxygenation (OGD/R)-induced release of glutamate (**a**), LDH (**b**) and on tissue water content (**c**) in rabbit brain cortical slices**.** Slices were incubated in ACSF for 120 min (control conditions, CTRL) or subjected for 30 min to oxygen/glucose deprivation followed by 90 min of immersion in normally oxygenated artificial cerebrospinal fluid (reoxygenation). Different concentrations of PSA (1–3,000 μM) were added to the reoxygenation buffer. Data are mean ± SEM of at least four different experiments. $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$, $^{\circ\circ\circ}P < 0.01$ versus CTRL; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus OGD

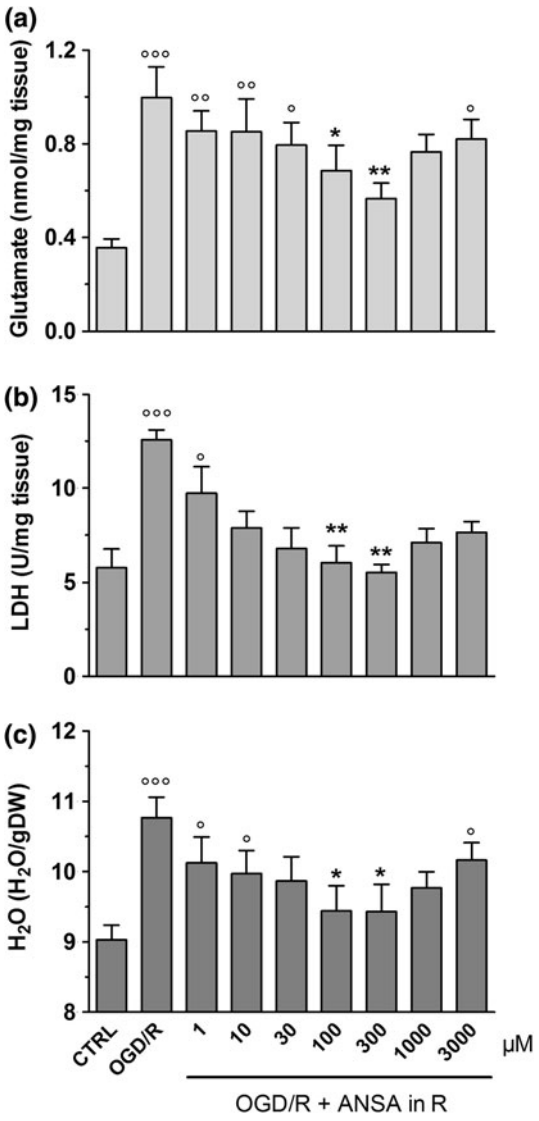


Fig. 4 Effect of 2-aminobenzene sulfonate (ANSA) on oxygen–glucose deprivation and reoxygenation (OGD/R)-induced release of glutamate (**a**), LDH (**b**) and on tissue water content (**c**) in rabbit brain cortical slices**.** Slices were incubated in ACSF for 120 min (control conditions, CTRL) or subjected for 30 min to oxygen/glucose deprivation followed by 90 min of immersion in normally oxygenated artificial cerebrospinal fluid (reoxygenation). Different concentrations of ANSA (1–3,000 μM) were added to the reoxygenation buffer. Data are mean ± SEM of at least four different experiments. $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$, $^{\circ\circ\circ}P < 0.01$ versus CTRL; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus OGD

edema as the tissue water content was similar to pre-ischemic values (Fig. 3c).

Moreover, both ANSA and ATHAS were very effective in preventing oxygen–glucose deprivation and reperfusion damage in brain slices. ANSA, in fact, at 100 and 300 μM concentration gave rise to a 67% reduction in glutamate release and tissue edema (Fig. 4a, c respectively) while fully antagonising LDH release (Fig. 4b). At 30 μM concentration, ATAHS almost suppressed the over-release of both glutamate and LDH (Fig. 5a, b), while concentrations between 30 and 300 μM were able to antagonize tissue water gain in a concentration-dependent fashion. Lower (1–10 μM) or higher (1,000–3,000 μM) ATAHS concentrations were ineffective (Fig. 5c). In contrast, AEP affected the parameters under investigation in a rather complicated way. As

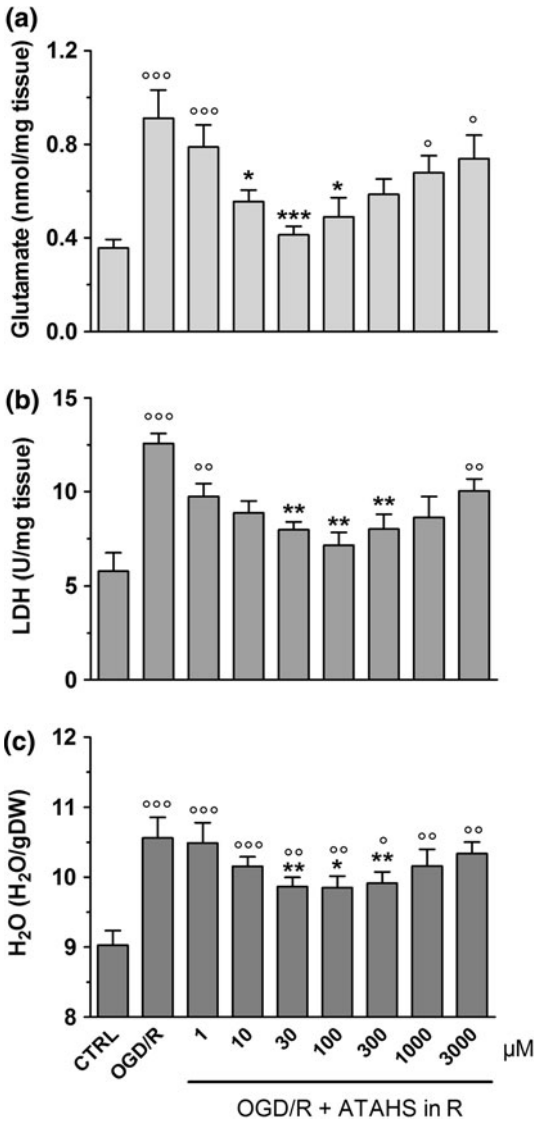


Fig. 5 Effect of 2-(*N*-acetylamino) cyclohexane sulfonic acid (ATAHS) on oxygen–glucose deprivation and reoxygenation (OGD/R)-induced release of glutamate (**a**), LDH (**b**) and on tissue water content (**c**) in rabbit brain cortical slices**.** Slices were incubated in ACSF for 120 min (control conditions, CTRL) or subjected for 30 min to oxygen/glucose deprivation followed by 90 min of immersion in normally oxygenated artificial cerebrospinal fluid (reoxygenation). Different concentrations of ATAHS (1–3,000 μM) were added to the reoxygenation buffer. Data are mean ± SEM of at least four different experiments. °$P < 0.05$, °°$P < 0.01$, °°°$P < 0.01$ versus CTRL; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus OGD

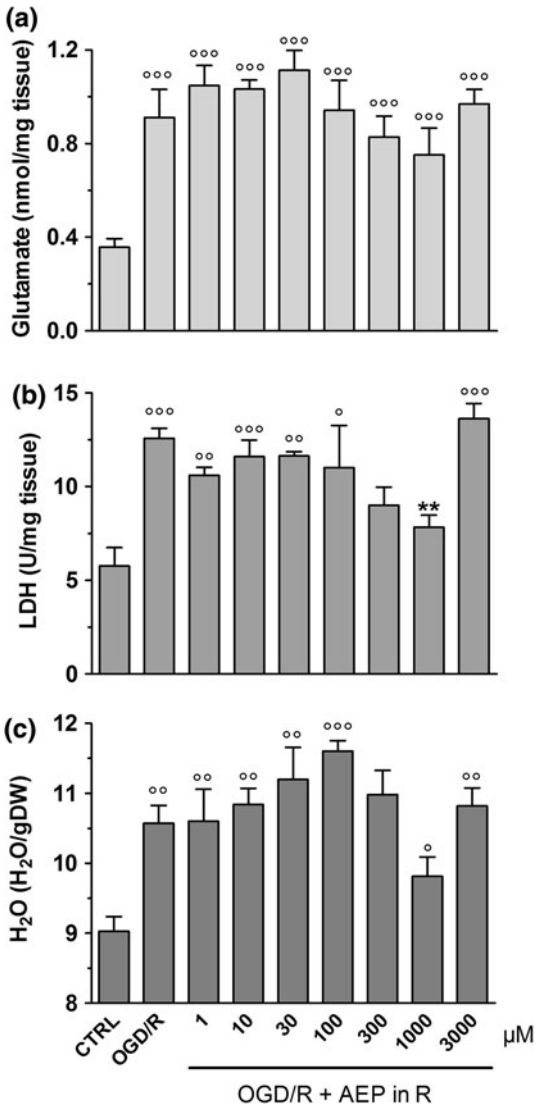


Fig. 6 Effect of 2-aminoethane phosphonic acid (AEP) on oxygen–glucose deprivation and reoxygenation (OGD/R)-induced release of glutamate (**a**), LDH (**b**) and on tissue water content (**c**) in rabbit brain cortical slices**.** Slices were incubated in ACSF for 120 min (control conditions, CTRL) or subjected for 30 min to oxygen/glucose deprivation followed by exposure to 90 min of reoxygenation. Different concentrations of AEP (1–3,000 μM) were added to reoxygenation buffer. Data are mean ± SEM of at least four different experiments. °$P < 0.05$, °°$P < 0.01$, °°°$P < 0.01$ versus CTRL; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus OGD

shown in Fig. 6, low concentrations (1–30 μM) increased glutamate release up to a value of 1.1 ± 0.10 nmol/mg tissue ($n = 5$) while concentrations between 100 and 1,000 μM caused a gradual decline of glutamate content in ACSF. Similarly, LDH released into ACSF was reduced only at 1,000 μM concentration (Fig. 6b) while at concentrations between 1 and 300 μM, AEP slightly enhanced tissue water gain (Fig. 6c).

Finally, GABA, VIGA and taurine analogues did not modify glutamate and LDH release as well as tissue water gain under control conditions (data not shown).

Discussion

In the present study, the potential neuroprotective effects of GABA and some GABA aminotransferase inhibitors

structurally related to taurine has been investigated in an in vitro experimental model of brain ischemia and reperfusion. Results demonstrated that GABA and VIGA antagonised oxygen–glucose deprivation and reperfusion-induced damage according to an "U-shaped" concentration–response curve typical of an hormetic behaviour. This has already been described for both diazepam and taurine as neuroprotecting agents in the same experimental setting (Ricci et al. 2007, 2008, 2009). As previously discussed, a desensitisation of $GABA_A$ receptors may be involved in the hormetic GABA-mediated effect, thus causing depolarisation and damaging of the neurons (Muir et al. 1996). Furthermore, an elevated increase of GABA levels at the synaptic cleft obtained directly (by treating slices with GABA) or indirectly (by treating slices with VIGA) might cause an excessive activation of $GABA_A$ receptors, leading to an overload of chloride ions into the neurons (Muir et al. 1996; Pond et al. 2006; Galeffi et al. 2004; Allen et al. 2004). The major consequences of an excessive rise in intracellular Cl^- is that $GABA_A$ responses, which are normally hyperpolarizing, can become depolarizing (Thompson et al. 1989; Kaila et al. 1994) and this contributes to neuronal hyperexcitability and neuronal damage, as demonstrated by the present results at high (>300 μM) GABA and VIGA concentrations. Interestingly, Costa et al. (2004) found that in rat corticostriatal slices subjected to oxygen–glucose deprivation and reperfusion, GABA, VIGA and the GABA-uptake blocker tiagabine were neuroprotective in an hormetic fashion and their "efficacy window" (i.e. the interval of concentrations at which they exerted neuroprotection) coincided with that found in the present study. Finally, the neuroprotective effects of VIGA were observed at concentrations much higher than those of GABA. This can be explained by considering that a defined period of time is required to inhibit GABA aminotransferase thus allowing GABA levels to increase in brain slices up to values that correlate with neuroprotection, as previously reported (Ricci et al. 2006).

"U-shaped" concentration–response curves were also obtained with taurine analogues. PSA ANSA and ATAHS turned out to be the most interesting GABA aminotransferase inhibitors since they possessed an efficacy comparable to that of VIGA. Interestingly, these taurine analogues are reversible GABA aminotransferase inhibitors at variance with VIGA itself (Ricci et al. 2006). VIGA use for the treatment of epilepsy has been cautioned owing to its retinal toxicity, possibly originating from its irreversible mechanism of action (Sills et al. 2003) as well as to generate taurine deficiency (Jammoul et al. 2009). Consequently, reversible GABA aminotransferase inhibitors like PSA, ANSA and ATAHS may turn out to be of interest in the clinic for their neuroprotective properties against ischemia/reperfusion injury.

However, VIGA and PSA were neuroprotective at concentrations below K_i values for GABA aminotransferase inhibition, thus suggesting that their effects are more complex. VIGA, in fact, blocks GABA transporter GAT1 of mouse brain either at nanomolar or millimolar concentrations (Leach et al. 1996, Eckstein-Ludwig et al. 1999). PSA binds to $GABA_A$ receptors in the low micromolar range (Frosini et al. 2003a). Consequently, since activation of GABA inhibitory neurotransmission provides protection of cerebral tissues exposed to ischemia (Schwartz-Bloom and Sah 2001), the opportunity to achieve this goal with VIGA and PSA, which enhance GABA activity concurrently through different mechanisms, might prove useful. Finally, ATAHS, besides being a GABA aminotransferase inhibitor is considered to be a direct taurinergic agent (Frosini et al. 2003a, b). As the neuroprotection from ischemia and reperfusion-induced damage by taurine has been demonstrated (Ricci et al. 2009), the ability of ATAHS to stimulate taurine activity could contribute to its effects.

AEP behaved differently from the other taurine analogues tested. This can be explained by considering that it acts in vivo like a $GABA_B$ antagonist in a micromolar range (Frosini et al. 2003a, 2006). Since $GABA_B$ antagonists could exacerbate oxygen–glucose deprivation-induced injury (Kulinskii et al. 2000; Costa et al. 2004), $GABA_B$ antagonistic properties of AEP represent an overwhelming problem in the use of this compound as a neuroprotective agent.

The effects of VIGA and taurine analogues on tissue edema deserve a particular mention. The development of cerebral edema is a complex, stepwise process that stems from the cytotoxic edema of neuroglial cells (which do not require active blood flow) to the subsequent development of ionic and vasogenic edema once ischemic tissues are reperfused (Hrabetová et al. 2002; MacGregor et al. 2003). The degree of water gain in brain slices following oxygen–glucose deprivation and reperfusion mirrors the progressive development of brain edema in stroke patients (Sherman and Easton 1980; Bounds et al. 1981; Hacke et al. 1996; Davalos et al. 1999; Rosenberg 1999; Kasner et al. 2001; Steiner et al. 2001). Brain edema is a major contributing factor to morbidity and mortality in strokes (Kasner et al. 2001; Steiner et al. 2001). The factors that contribute to its formation include activation of glutamate receptors, mitochondrial dysfunction, an increased concentration of intracellular calcium and oxidative stress (MacGregor et al. 2003). To efficiently counteract ischemia-induced brain edema in stroke patients, a "cocktail" of drugs that antagonize each of the above-mentioned "steps" should be employed (White et al. 2000). The present results suggest that indirectly acting GABA agonists could support an effective and

rational strategy for the prevention and treatment of brain cytotoxic edema.

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