Neuroprotection Afforded by GABA Against Oxygen-Glucose Deprivation-Induced Injury in Rat Cortical Brain Slices: An Hormetic Dose-Response Effect

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Abstract: The aim of the present investigation was to assess neuroprotection exerted by GABA (100 nM-100 mM) in rat cortical brain slices subjected to oxygen-glucose deprivation and reoxygenation. Neuronal injury and neuroprotection were assessed by measuring the release of glutamate and lactate dehydrogenase and tissue water content. Results demonstrate that GABA exerted neuroprotective effects according to a U-shaped, hormetic-like, concentration-response curve, with an efficacy window of 10-100 µM concentration. In order to verify whether GABA-neuroprotective effects were $GABA_A$- or $GABA_B$-mediated, Muscimol ($GABA_A$ agonist) and Baclofen ($GABA_B$ agonist) were tested. Results demonstrated that only Muscimol exerted neuroprotection according to a U-shaped, hormetic-like, concentration-response curve, while baclofen was ineffective. In conclusion, the hormetic response of GABA or $GABA_A$ agonists should be taken into consideration when designing experiments aimed at assessing neuroprotection by these agents against ischemia/reoxygenation injury.

Key words: Brain ischemia, GABA, hormesis, GABA agonist/antagonist, neuroprotection

INTRODUCTION

Ischemic injury to cerebral tissues triggers complex biochemical cascades that ultimately lead to neuronal death. Excessive excitatory neurotransmitter release occurs during this process, stimulating excitotoxic conditions that hasten depletion of oxygen and nutrients. Therefore, neuroprotective therapy goals are to antagonize the neuronal damage-promoting mechanisms and to strengthen the auto protective pathways in an attempt to prevent cells at risk from dying[1,2]. Gamma-aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter of human cerebrum. The application of drugs that promote neuronal inhibition mediated by GABA receptor, may be a strategy to prevent neuronal injury. Increasing GABA function might thus represent a beneficial therapeutic approach to acute ischemia for different reasons[3,4,5]. First, endogenous GABA synthesis and release are decreased after an ischemic insult with consequent reduction in GABAergic transmission[4]. Second, since glutamatergic and GABAergic system work by counterbalancing each the function of the other, the enhancement of GABAergic activity should antagonize the excessive glutamatergic excitation, which is the pivotal event leading to cell death. Thus, an abundance of literature deals with the putative neuroprotective potential of GABAergic compounds[4]. Many *in vitro* and in vivo data demonstrate efficacy in various neuropathological models: however, studies tend to be equivocal and clinical trials have so far been disappointing. Interestingly, a recent study performed in this laboratory, demonstrated that diazepam was neuroprotective in an *in vitro* model of brain ischemia (rat cortical brain slices subjected to oxygen-glucose deprivation and reoxygenation)[6]. On the other hand, its neuroprotective effects followed an U-shaped concentration-response curve typical of drugs that exhibit an hormetic-like effect and this could explain the failure of diazepam to protect brain tissues against the damage from ischemia/reoxygenation injury observed in some studies. The aim of the present work was to assess whether the pharmacological conditioning hormesis exhibited by the benzodiazepine receptor agonist diazepam is indeed possessed by GABA itself. This could help in designing experiments aimed at assessing whether such agent (s) may be successfully employed as neuroprotectants against ischemia/reoxygenation injury. Consequently, the degree of neuroprotection afforded by a wide range of GABA concentrations (100 nM-100 mM) in rat cortical brain slices subjected to oxygen-glucose deprivation

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(OGD) and reperfusion was studied. Tissue damage and protection were assessed by measuring the release of glutamate (GLU) and lactate dehydrogenase (LDH) into bathing artificial cerebrospinal fluid (ACSF) during reperfusion and by determining tissue edema at the end of the experiment.

MATERIALS AND METHODS

Animals and slices preparation: 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. Male Sprague-Dawley rats (7-10 weeks, 350-450 g; Charles River Italia, Calco, Italy) were sacrificed and the whole brain rapidly removed and placed in cold oxygenated 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-thickness coronal slices by using a manual chopper (Stoelting Co., Wood Dale, IL, USA). Afterward, 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[7].

***In vitro* ischemia-like conditions:** Cortical slices (4-5, total wet weight 33.6±2.6 mg, n = 10) were then placed in covered incubation flasks, containing 2ml ACSF continuously bubbled with 95% O_2/5% CO_2 and incubated at 37°C for an additional period of 30 min. Afterwards, OGD was carried out by incubating slices for 30 min (or for 5, 15, 30 or 60 min in the time-dependent induced injury experiments, see below) into ACSF in which glucose had been replaced by an equimolar amount of saccharose and continuously bubbled with 95% N_2/ 5% CO_2. After the OGD period, this solution was replaced by fresh, oxygenated ACSF for an additional 90 min period (reoxygenation). In drug-treated slices, OGD was followed by reoxygenation with ACSF containing GABA, muscimol, or baclofen.

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-reoxygenation period. In particular, glutamate concentration was determined fluorimetrically (excitation 340 nm; emission: 460 nm) using the conversion of NAD^+ to NADH by glutamate

dehydrogenase[8] while LDH activity was determined spectrophotometrically by the rate of decrease in absorbance at 340 nm *via* the oxidation of NADH to $NAD^{+[9]}$. Tissue water gain (edema) was calculated as (wet weight - dry weight)(dry weight)$^{-1}$ and given as g H_2O (g dw)$^{-1}$ as described by MacGregor *et al.*[10].

Data Analysis: All the experiments were performed by using brain slices derived from at least 4 different rats. Data are reported as mean±S.E.M. and n is defined as the number of cluster of slices. For glutamate and LDH activity, data are expressed as nmol mg^{-1} wet tissue and U mg^{-1} wet tissue, respectively. One unit (U) of LDH activity is defined as that which gives rise to the formation of one micromole of lactate in one minute.

Statistical analysis was performed by using one-way ANOVA followed by post-hoc Dunnett test (GraphPad INSTAT v3.00, GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSION

Effects of different time periods of OGD/reperfusion on glutamate- and LDH- release and tissue edema in rat cortical slices: In order to establish the time period of OGD that induced maximal damage to the brain tissue, cortical slices were subjected to 5, 15, 30 or 60 min of OGD followed by 90 min of reperfusion. Rat cortical slices incubated in ACSF for 120 min (control conditions, CTRL) released into the medium 0.31±0.02 nmol mg^{-1} tissue (n = 8) and 4.1±0.6 U mg^{-1} prot. (n = 17) of GLU and LDH, respectively. After 120 min incubation in ACSF, water gain of CTRL slices was 9.93±0.4 g H_2O (g dry weight)$^{-1}$ (n = 16): this value was about 4.0% higher than that measured after 1 h recovery period at room temperature[10]. As shown in Fig. 1, the release of glutamate and LDH, as well as water gain, increased linearly with the duration of OGD. When ischemia-like conditions were limited to 5 min, statistically significant changes were observed only for glutamate and LDH release (+53 and +138% versus CTRL, respectively) while tissue edema was not modified. On the contrary, 15, 30 and 60 min of OGD followed by 90 min of reoxygenation caused a more pronounced neuronal injury. In particular, the highest glutamate- and LDH-release were observed at 30 min (+107% and +340% versus control values, respectively) which were accompanied by a huge edema (+142% versus control). However, when the duration of OGD was increased up to 60 min, the amount of glutamate and LDH released into ACSF as well as tissue water gain were very close to those observed after 30 min. Therefore, in all subsequent experiments, OGD of cortical slices was limited to 30 min.
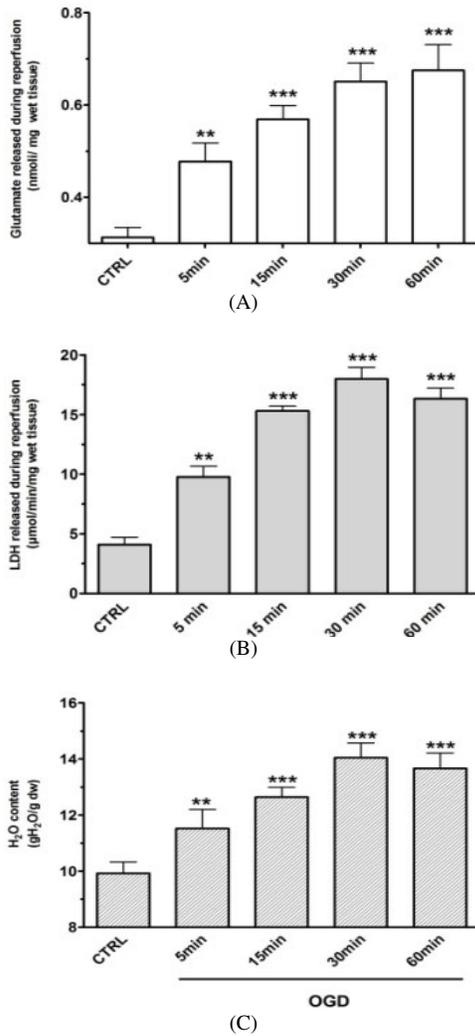
Fig. 1: Effects of different time periods of OGD and reperfusion on glutamate- and LDH- release and tissue edema. Rat cortical slices were incubated in artificial cerebrospinal fluid for 120 min (control conditions, CTRL) or exposed to 5, 15, 30 and 60 min of OGD followed by 90 min of reoxygenation. OGD/reoxygenation-induced release of glutamate, LDH and tissue water content are reported in panel A , B and C respectively. Data are means±S.E.M. of 4 different experiments. Statistical analysis was performed by using ANOVA followed by post-hoc Dunnett test. **p<0.01, ***p<0.001 vs CTRL
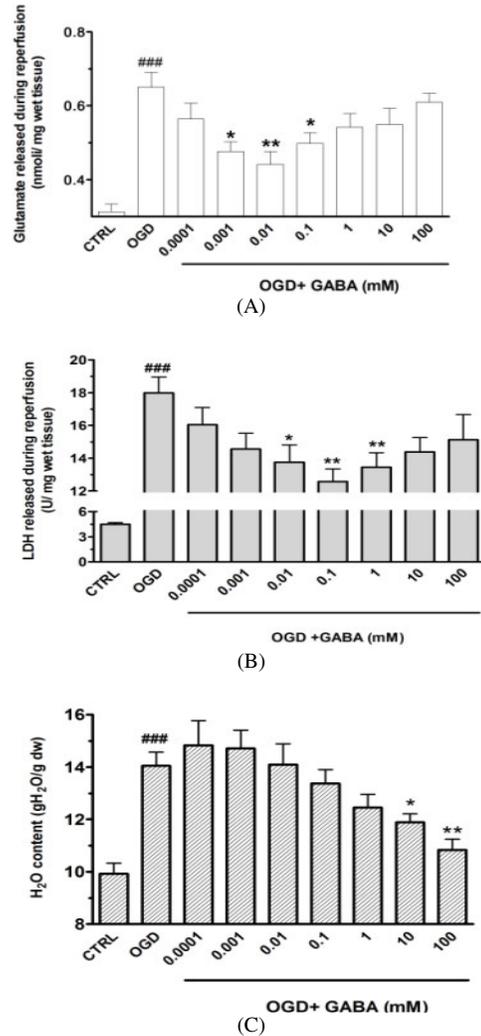
Fig. 2: Effect of GABA on OGD and reperfusion – induced release of glutamate (panel A), LDH (panel B) and tissue edema (panel C) in rat cortical slices. Slices were incubated in artificial cerebrospinal fluid for 120 min (control conditions, CTRL) or exposed to 30 min of OGD followed by 90 min of reoxygenation (OGD). GABA (100 Nm-100 mM) was added during reoxygenation phase. Data are means±S.E.M. of 5 different experiments. Statistical analysis was performed by using ANOVA followed by post-hoc Dunnett test. *p<0.05, **p<0.01, vs OGD, ### p<0.001 vs CTRL

Effect of GABA on OGD and reperfusion-induced glutamate and LDH release and tissue edema in rat cortical slices: As shown in Fig. 2 (panels A and B),

GABA (100 nM-100 mM) significantly reduced oxygen/glucose deprivation- and reoxygenation-induced glutamate and LDH release. These effects,

however, followed a U-shaped concentration-response curve, typical of a chemical postexposure conditioning hormesis effect[11]. In particular, in the range of concentrations between 1 µM and 1 mM, GABA significantly antagonised glutamate and LDH release, while at lower (10 nM-1µM) or higher (10-100 mM) concentrations it did not reduce significantly both parameters. The most effective GABA concentrations turned out to be 10 and 100 µM which were capable to decrease the release of glutamate to 0.44±0.03 nmol/mg tissue (p<0.01, n = 9) and that of LDH to 12.6±0.7 U mg^{-1} proteins (p<0.01, n = 11-16), respectively. GABA significantly antagonised tissue water gain in a concentration-dependent fashion being 10 and 100 mM the most effective concentrations. Oxygen/glucose deprivation- and reoxygenation-induced water gain, in fact, was reduced by 50% (p<0.05, n = 20) and by 78.8% (p<0.01, n = 10), respectively (Fig. 2, panel C).

Effect of Muscimol and Baclofen on OGD and reperfusion-induced glutamate and LDH release and tissue edema in rat cortical slices: To verify whether GABA effects where GABA$_A$-or GABA$_B$-mediated, the GABA$_A$ receptor agonist muscimol and the GABA$_B$ receptor agonist baclofen were used. Both agonists by themselves, as well as GABA, did not modify glutamate and LDH release and tissue edema in control conditions (data not shown). Interestingly, muscimol (1 nM-100 µM) reduced OGD-induced glutamate and LDH release, as well as tissue edema (Fig. 3). These effects, as already shown for GABA, followed an U-shaped concentration-response curve, typical of an hormetic behaviour. In particular, muscimol in the range of 10 nM-1 µM concentrations, significantly reduced OGD/reperfusion-induced release of GLU, while lower (1 nM) or higher (10-100 µM) concentrations were ineffective (Fig. 3, panel A). At 10 nM and 100 nM concentrations, muscimol reduced glutamate release to 0.45±0.03 nmol mg^{-1} tissue (p<0.01, n = 10) and 0.40±0.03 nmol mg^{-1} tissue (p<0.01, n = 19), respectively. Similarly, also LDH release was reduced to 13.4±0.7 U mg^{-1} prot (p<0.01, n = 18) and 14.3±0.8 U mg^{-1} prot (p<0.05, n = 16), by 100 nM and 1000 nM muscimol, respectively (Fig. 3, panel B). In addition, muscimol significantly reduced tissue water gain induced by OGD/reperfusion (Fig. 3, panel C). Edema was reduced to 11.7±0.3 g H$_2$O g^{-1} dry weight (p<0.05, n = 10) by 10 µM muscimol while lower (1 nM-1µM) or higher (50-100 µM) concentrations did not significantly affect tissue edema.

Baclofen was added to the reoxygenation buffer in a concentration range between 0.1 and 100 µM. The GABA$_B$ agonist, however, was not effective in reducing
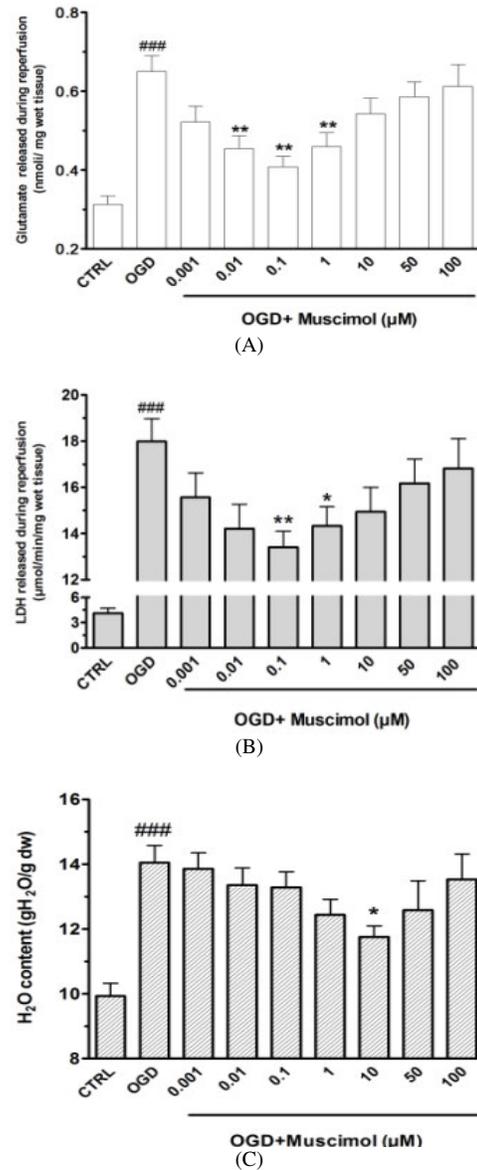

(A)

(B)

(C)

Fig. 3: Effect of Muscimol on OGD and reperfusion-induced release of glutamate (panel A), LDH (panel B) and tissue edema (panel C) in rat cortical slices. Slices were incubated in artificial cerebrospinal fluid for 120 min (control conditions, CTRL) or exposed to 30 min of OGD followed by 90 min of reoxygenation (OGD). Muscimol (1 Nm-100 µM) was added during the reoxygenation phase. Data are means±S.E.M. of 5 different experiments. Statistical analysis was performed by using ANOVA followed by post-hoc Dunnett test. *p<0.05, **p<0.01, vs OGD, ### p<0.001 vs CTRL
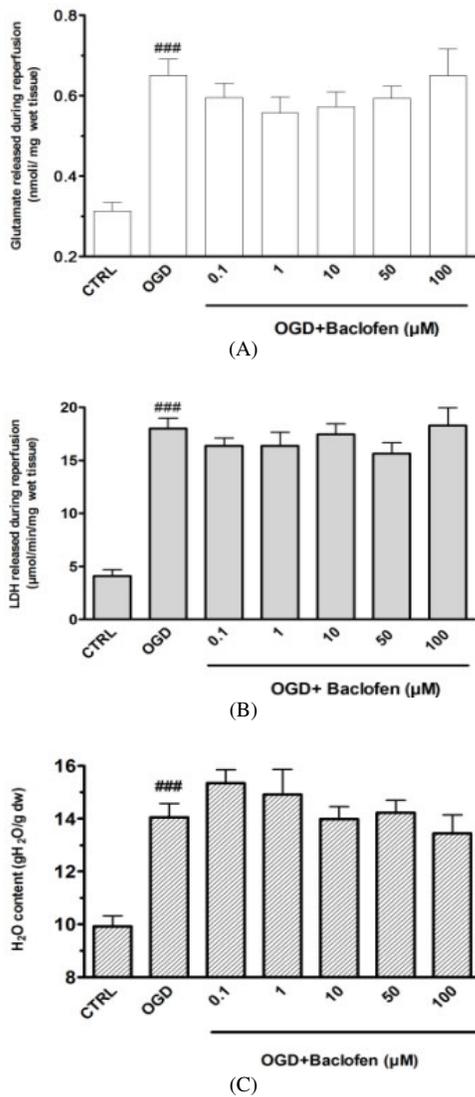
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Fig. 4: Effect of Baclofen on OGD and reperfusion-induced release of glutamate (panel A), LDH (panel B) and tissue edema (panel C) in rat cortical slices. Slices were incubated in artificial cerebrospinal fluid for 120 min (control conditions, CTRL) or exposed to 30 min of OGD followed by 90 min of reoxygenation (OGD). Baclofen (0.1 µM-100 µM) was added during the reoxygenation phase. Data are means±S.E.M. of 4 different experiments. Statistical analysis was performed by using ANOVA followed by post-hoc Dunnett test. ### p<0.001 vs CTRL

OGD and reperfusion-induced glutamate and LDH release and tissue water gain (Fig. 4).

Since some studies have reported that coactivation of $GABA_A$ and $GABA_B$ receptor is required for neuroprotection when counteracting *in vitro* ischemia-like conditions[12] we added 1 µM baclofen to a neuroprotective (0.1 µM) or an ineffective (10 µM) muscimol concentration (Fig. 5). Both drugs were added to the ACSF used during reoxygenation period. Results demonstrated that the addition of baclofen did not modify the effects elicited by muscimol alone.

The data in the present study provide further support to the hypothesis that, by increasing GABAergic transmission, it is possible to counteract the neuronal death subsequent to an ischemic insult[3,4,5]. However, it was also found that GABA-mediated neuroprotection during energy deprivation was essentially mediated by $GABA_A$-, while $GABA_B$ - receptors were not involved.

In the presence of exogenous GABA, neuroprotective effects similar to those obtained with muscimol were observed. GABA may exert neuroprotection not only by directly inhibiting neurons[4], but also by exerting an inhibitory influence on glutamate-mediated neuronal activity[13]. Consequently, it is conceivable that the neuroprotection (in terms of reduced glutamate-and LDH-release as well as cell swelling) observed after GABA and muscimol treatment could depend on a reduction in glutamate-induced neurotoxicity. In agreement with this hypothesis, it has been shown that GABA and muscimol prevent NMDA-, AMPA-, or kainate-induced neuronal death in mouse cortical cell cultures[14,15]. In the present study, however, the effect of both GABA and muscimol were not concentration-dependent, but resembled a chemical postexposure conditioning hormetic effect[11]. This hormetic trend might be explained by different mechanisms. Cerebral ischemia is accompanied by disruption of neuronal ionic homeostasis, including significant increases in intracellular Na^+, Cl^- and Ca^{2+}, as well as increases in extracellular K^+ (for a review, see reference 4). Previous studies indicate that during oxygen-glucose deprivation in rat hippocampal neurons intracellular Cl^- concentrations rise probably because of an enhanced activity of the Cl^- cotransporter NKCC-1[16,17]. Reoxygenation causes intracellular Cl^- to recover, but is followed by a second prolonged rise in intracellular Cl^-[17]. It is conceivable that an elevated increase in GABA and muscimol levels at synaptic cleft during reoxygenation might cause an excessive activation of $GABA_A$ receptors, which aggravates the overload of Cl^- in neurons[15] as suggested by the present study where high concentrations of these drugs were used. The major consequence of the excessive rise in
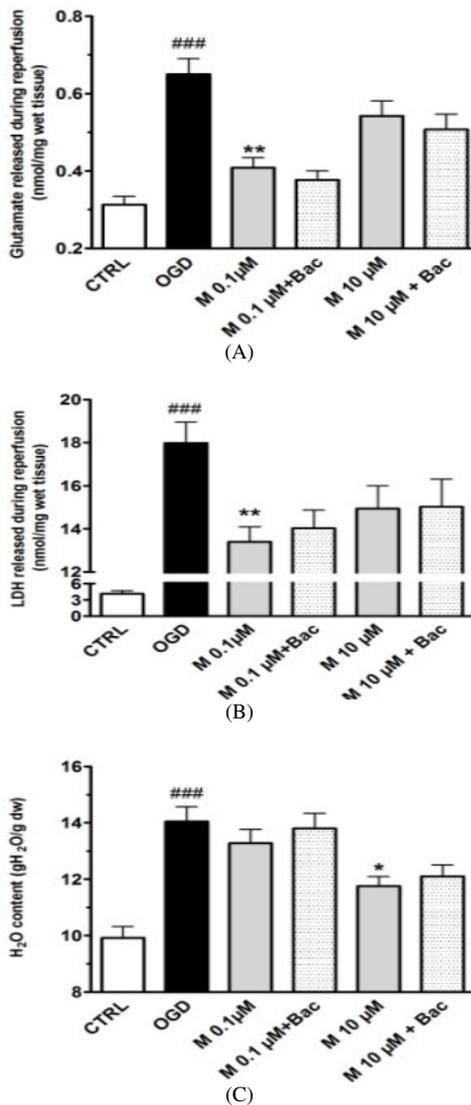
(A)

(B)

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Fig. 5: Effects of muscimol (M) or muscimol+baclofen
(M+Bac) on OGD/reoxygenation-induced
release of glutamate (Panel A), LDH (Panel B)
and tissue edema (Panel C) in rat cortical slices.
Drugs were added to the reoxygenation buffer
after 30 min of OGD at the following
concentrations: Muscimol 0.1 µM or 10 µM,
Baclofen, 1 µM. Data are means±S.E.M. of 4
different experiments. Statistical analysis was
performed by using ANOVA followed by post-
hoc Dunnett test. *p<0.05, **p<0.01, vs OGD,
p<0.001 vs CTRL

intracellular Cl⁻ is that the normal hyperpolarizing
GABA$_A$ responses become depolarizing[18], thus
contributing to neuronal hyperexcitability and

damage[19]. Furthermore, even under conditions when
equilibrium potential for Cl⁻ does become more positive
relative to the resting membrane potential, GABA$_A$-
linked chloride channels mediates a shunting inhibition
that suppresses excitation[20]. We can speculate that in
the case of elevated GABA and muscimol
concentrations, this type of inhibition could not operate,
thus further leading to neuronal damage. Finally, at the
basis of the hormetic GABA- or muscimol-mediated
effect may be the excessive activation of GABA$_A$
receptors, which could provoke a desensitisation of the
same receptors, thus causing depolarisation and damage
to neurons[15].

While there is convincing evidence that GABA$_A$-
mimetic drugs are neuroprotective in several animal
models of cerebral ischemia[4], less clear is the
protective role played by GABA$_B$ receptors. In the
CNS, these receptors are localized on both pre- and
post-synaptic neurons and their activation increases K⁺
channel conductance resulting in hyperpolarization of
the neuronal membrane directly or, post-synaptically,
through an intervening GTP-binding protein, which
inhibits adenylyl cyclase. In presynaptic neurons,
GABA$_B$ receptors partly regulate the dynamics of
cytosolic Ca²⁺ and the release of many
neurotransmitters[21].

The results of the present study clearly
demonstrated that the activation of GABA$_B$ receptors by
baclofen, did not result in neuroprotection. These data
are in agreement with those already reported in the
literature[15,22,23,24], although some authors reported a
neuroprotective role of this GABA$_B$ agonist[25,26]. The
reason for this discrepancy is still debated and may
depend on differences in severity of challenges
associated with each experimental model. Furthermore,
any neuroprotective effect of baclofen most likely
involves inhibition of pre-synaptic release of glutamate,
as excessive levels of glutamate and glutamatergic
activity have been reported to occur in the most
vulnerable regions of the brain following global
ischemia[2]. The present results, however, demonstrated
that baclofen did not modify glutamate release.
Consequently, the lack of neuroprotection in terms of
LDH release and tissue edema could arise from the
failure of baclofen, to reduce ischemia-induced
glutamate release. Finally, to further explore the
potential role of coactivation of GABA$_A$ and GABA$_B$
receptors in GABA-mediated protection, cortical slices
were treated simultaneously with muscimol and
baclofen. Results demonstrated that the addition of
baclofen did not modify muscimol neuroprotective
effects, further confirming no involvement of GABA$_B$
receptors in GABA neuroprotection in this brain
ischemia/reperfusion experimental model.

In conclusion, the hormetic-like dose-response curve of GABA as well as that of many GABA mimetics such as muscimol (present study), diazepam[6] and GABA-transaminase inhibitors[12,27] should be taken into consideration when designing experiments aimed at assessing whether such agent(s) may be successfully employed as neuroprotectants against ischemia/reoxygenation injury.

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