Neurochemistry International 128 (2019) 175–185

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Modulation of extrasynaptic GABAergic receptor activity influences glutamate release and neuronal survival following excitotoxic damage to mouse spinal cord neurons

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ARTICLE INFO

Keywords:
Neuroprotection
Kainic acid
Spinal cord injury
Extrasynaptic GABA receptor
Excitotoxicity

ABSTRACT

Excitotoxic levels of released glutamate trigger a cascade of deleterious cellular events leading to delayed neuronal death. This phenomenon implies extensive dysregulation in the balance between network excitation and inhibition. Our hypothesis was that enhancing network inhibition should prevent excitotoxicity and provide neuroprotection. To test this notion, we used mouse organotypic spinal slice cultures and explored if excitotoxicity caused by the potent glutamate analogue kainate was blocked by pharmacological increase in $GABA_A$ receptor activity. To this end we monitored (with a biosensor) real-time glutamate release following 1 h kainate application and quantified neuronal survival 24 h later. Glutamate release evoked by kainate was strongly decreased by the allosteric $GABA_A$ modulator midazolam (10 nM) or the GABA agonist THIP (10 μM), leading to neuroprotection. On the contrary, much higher glutamate release was induced by the GABA antagonist bicuculline (20 μM) that inhibits synaptic and extrasynaptic $GABA_A$ receptors. Gabazine (20 μM), an antagonist of synaptic $GABA_A$ receptors, had no effect on glutamate release or neuroprotection. No effect was observed with the glycine antagonist strychnine or the glycine agonist L-alanine. These findings indicate that enhancement of GABA receptor activity was an effective tool to counteract excitotoxic death in spinal networks. In view of the potent activity by THIP, preferentially acting on extrasynaptic $GABA_A$ receptors, the present data imply a significant role for extrasynaptic $GABA_A$ receptors in sparing spinal cord neurons from injury.

1. Introduction

In the spinal cord massive release of the excitatory transmitter glutamate evoked by a lesion generates excitotoxicity, namely a pathophysiological process that largely expands the initial damage through free radical production and metabolic dysfunction (Ahuja et al., 2017). To achieve neuroprotection against this process, previous studies have focused on blocking excitatory glutamatergic transmission with drugs like riluzole or glutamate receptor antagonists (Ramer et al., 2014; Ulndreaj et al., 2017), yet the result has not been satisfactory (Cifra et al., 2012b; Sámano and Nistri, 2019). To identify novel potential targets for neuroprotection, our former studies had devised a model of neuronal excitotoxicity by applying the glutamate analogue kainate to *in vitro* spinal networks to destroy neurons with minimal harm to white matter which is, on the other hand, damaged by transient oxygen-glucose deprivation (Kuzhandaivel et al., 2011; Mazzone et al., 2010; Sámano and Nistri, 2019; Taccola et al., 2008). In particular, using spinal cord organotypic cultures, we were able to estimate damage induced by kainate by measuring real-time release of endogenous glutamate (via a specific biosensor), and evaluating cell losses (Mazzone and Nistri, 2011a).

This model prompted us to investigate if enhancing inhibition may effectively contrast excitotoxicity. Indeed, clues to the potential usefulness of enhancing inhibition were provided by the observation that general anaesthetics like methoxyflurane and propofol could be experimentally neuroprotective, though with different degree of effectiveness and cellular mechanisms (Bajrektarevic and Nistri, 2016; Kaur et al., 2016; Shabbir et al., 2015). Nonetheless, translating the

Abbreviations: ANOVA, analysis of variance; AU, arbitrary unit; BIC, bicuculline; DAPI, 4′,6-diamidino-2-phenylindole; DIV, days *in vitro*; DME/HIGH, Dulbecco's modified Eagle's medium high glucose; FCS, fetal calf serum; KA, kainate; GABA, gama-aminobutyric acid; GBZ, gabazine; MDZ, midazolam; NGF, nerve growth factor; NeuN, neuronal specific nuclear protein; PBS, phosphate-buffered saline; S100β, astroglial calcium-binding protein S100β; SCI, spinal cord injury; SEM, standard error of the mean; STRY, strychnine; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-c]-pyridin-3-ol

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https://doi.org/10.1016/j.neuint.2019.04.018
Received 16 January 2019; Received in revised form 8 April 2019; Accepted 30 April 2019
Available online 30 April 2019
0197-0186/

laboratory use of general anesthetics to *in vivo* injury might be difficult because these drugs may further depress compromised functions like respiration and blood pressure control.

Because GABA is the principal inhibitory neurotransmitter in the central nervous system (CNS) acting predominantly on $GABA_A$ receptors (Nistri and Constanti, 1979; Sivilotti and Nistri, 1991), up or downregulating such receptors strongly affects the maintenance of dynamic neuronal homeostasis (Le Magueresse and Monyer, 2013). $GABA_A$ receptors located on the postsynaptic membrane mediate synaptic inhibition, while those on the extrasynaptic membrane respond to ambient GABA and confer long-term excitability decrease (Baur et al., 2009; Sigel and Steinmann, 2012). As recently demonstrated in lampreys with complete spinal cord transection, GABA release is also an important factor to promote axonal regeneration mediated by $GABA_A$ and $GABA_B$ receptors via decreased caspase activation (Romaus-Sanjurjo et al., 2018; Sobrido-Cameán et al., 2018). Thus, potentiating the role of GABA might be a strategy for experimental spinal neuroprotection and is a development of an earlier proposal for the protective role of spinal inhibitory interneurons against motor degeneration (Ramírez-Jarquín et al., 2014).

To this end, using the *in vitro* model of excitotoxicity in organotypic spinal culture (Mazzone et al., 2010), we studied the effects of midazolam (a water soluble benzodiazepine) that binds to the benzodiazepine binding site on $GABA_A$ receptors (Rudolph and Knoflach, 2011) to allosterically potentiate them via increased opening frequency of the intrinsic chloride channel (Bounds and Nelson, 2017). Furthermore, we tested the effect of the GABA analogue THIP (4,5,6,7-tetrahydroisothiazolo-[5,4-c]pyridine-3-ol), an agonist preferentially acting on extrasynaptic α4βδ-$GABA_A$ receptors (Iversen, 2004) that are reported to be mainly responsible for controlling neuronal excitability changes and damage induced by reactive oxygen species in rat spinal cord slices (Ohashi et al., 2016). As a proof of principle for the role of $GABA_A$ receptors in excitotoxicity, we also examined the effects of gabazine, an antagonist of synaptic $GABA_A$ receptors, and bicuculline that has a broader antagonistic action (Krall et al., 2015), to find out if they could intensify the effect evoked by kainate. Finally, since glycine is also an important inhibitory neurotransmitter in spinal networks (Aprison and Werman, 1965; Davidoff et al., 1967; Prescott, 2015), we explored the effect of L-alanine (a glycine receptor agonist; Curtis et al., 1968; Schmieden and Betz, 1995), and strychnine, a glycine receptor antagonist (Curtis, 1969; Davidoff et al., 1969; Du et al., 2015). Since the complex mechanisms regarding GABA receptor pathophysiological role remain incompletely understood, we propose that drug pre-application protocols can provide an insight into mechanisms of action for neurotoxicity rather than indicating translational approaches to treat it. In addition, previous work has shown that delayed application of midazolam becomes rapidly ineffective as neuroprotectant (Gilby et al., 2005; Wu et al., 2018).

2. Materials and methods

2.1. Preparation and maintenance of spinal cord organotypic cultures

All experiments were carried out in accordance with the regulations of the Italian Animal Welfare act, (DL 27 / 1 / 92 n.116) following the European Community directives no. 86 / 609 93 / 88 (Italian Ministry of Health authorization for the local animal care facility in Trieste D 69 / 98-B), and approved by the ethics committee of Scuola Internazionale Superiore di Studi Avanzati (SISSA), Trieste.

The experiments were conducted with spinal cord organotypic slice cultures prepared from embryos (at 13 days of gestation) obtained from pregnant C57BL/6 mice and GAD67-GFP mice (Chattopadhyaya et al., 2004), kindly provided by Prof. E. Cherubini, EBRI, Rome, Italy, as previously described (Cifra et al, 2012a). Briefly, 18 mice were used in this study, namely 12 timed-pregnant C57BL/6J and 6 GAD67-GFP mice that were killed by slowly raising levels of CO_2. In each mouse 4–6 fetuses were delivered by caesarian section and were pooled together to obtain on average 40 slices. Slices were then embedded in a plasma clot, cultured on glass coverslips and introduced into individual tubes that undergo continuous slow rotation until use (Gahwiler et al., 1997). Slices were maintained for 22 days *in vitro* (DIV) in a medium containing Dulbecco's Modified Eagle medium with high glucose (DME/HIGH, 82%), sterile water for tissue culture (8%), nerve growth factor (NGF, 5 ng/ml), fetal calf serum (FCS, 10%; Invitrogen, Carlsbad, CA, USA), at controlled osmolarity (300 mOsm) and pH 7.35, in accordance with standard procedures (Spenger et al., 1991; Streit et al., 1991). DME/HIGH, penicillin and streptomycin were purchased from Euroclone (Devon, UK). NGF was obtained from D.B.A. Italia (Segrate, Italy), while the other reagents were purchased from Sigma-Aldrich, Milan, Italy. Fig. 1 shows examples of typical spinal cord organotypic slice cultures with staining for neurons (NeuN positive cells in green) and astrocytes (S100β positive cells in red) after 22 DIV in two region of interest (ROIs) indicated by white boxes, namely dorsal and ventral ones.

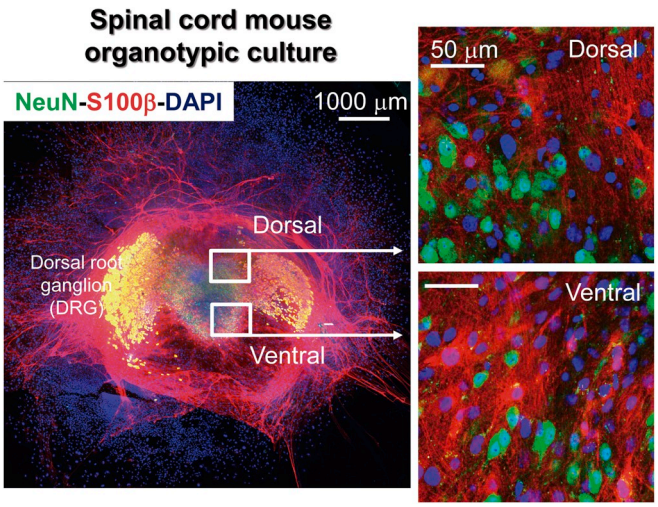

Fig. 1. Mouse spinal cord organotypic culture. Example of 22 DIV slice with two dorsal and ventral ROIs. Inset shows typical neuronal staining NeuN (green) restricted to the spinal cord tissue region, S100β (red) for glial staining, and DAPI (blue) used for general nuclear staining.

2.2. Protocols for excitotoxic injury and neuroprotection

Excitotoxic insult was produced by applying kainate (100 μM, Ascent Scientific, Cambridge, UK) for 1 h in complete medium in accordance with our previous report (Mazzone et al., 2010). Fig. 2 summarizes the experimental protocol used for the current study for the following eight groups: control, kainate alone, kainate after pre-treatment for 15 min with midazolam (MDZ, 0.01 μM, Hameln Pharmaceuticals, Hameln, Germany); bicuculline (BIC, 20 μM, Hello Bio, Bristol, UK); gabazine (GBZ, 20 μM, Santa Cruz Biotechnology, Santa Cruz, CA, USA); strychnine (STRY, 0.4 μM, Tocris Bioscience, Bristol, UK); THIP (10 μM, Tocris Bioscience) and L-alanine (6 mM, Sigma). Although bicuculline is reported to antagonize recombinant 5-HT3 serotonin (Sun and Machu, 2000), and nicotinic receptors (Demuro et al., 2001; Rothlin et al., 1999) expressed by oocytes, and to be a weak blocker of NMDA receptors of cultured neurons (Wright and Nowak, 1992), its selectivity for $GABA_A$ receptors of the mammalian spinal cord has been previously shown (Curtis et al., 1971; Long et al., 1989).

In each experiment control preparations were untreated sister cultures maintained *in vitro* for 24 h. Some cultures were treated only with BIC, GBZ, STRY, THIP or L-alanine, and subjected to the same experimental procedures. Drug concentrations used in the present study were selected on the basis of previous experiments (Bajrektarevic and Nistri,

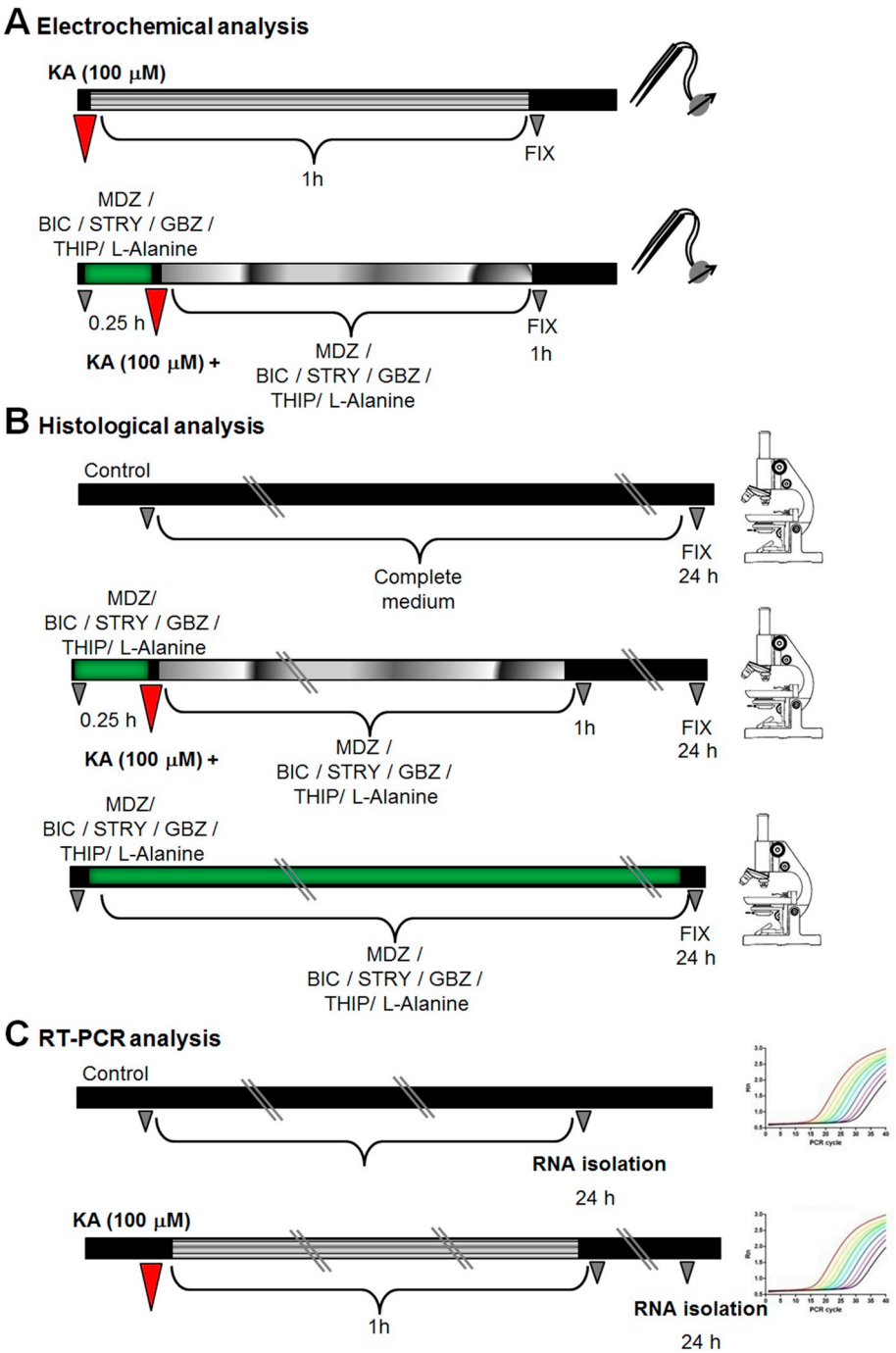

Fig. 2. Schematic representation of the experimental protocols used for detecting excitotoxic glutamate release, neurotoxicity and its pharmacological modulation. Excitotoxicity was produced on mouse spinal cord organotypic slices (at 22 DIV) by kainate (KA) application for 1 h, followed by careful wash with complete medium, and testing at 24 h. Cultures were pre-treated for 15 min with midazolam (MDZ, 0.01 μM), bicuculline (BIC, 20 μM), gabazine (GBZ, 20 μM), strychnine (STRY, 0.4 μM), THIP (10 μM), or L-alanine (6 mM) and then KA was applied for 1 h for electrochemical (A) and histological (B) studies.

2016; Ghezzi et al., 2017; Sivilotti and Nistri, 1991). In particular, our test concentration of midazolam (10 nM) is reported to depress by approximately 50% the network activity of organotypic neuronal cultures (Balk et al., 2017) and accords with the average plasma concentration of midazolam during human anaesthesia/sedation (Crevoisier et al., 1983; Greenblatt et al., 1989). Slices were used for real-time glutamate release (Fig. 2 A), or washed thrice, left in complete medium to recover for 24 h in standard condition, and later immunostained to study cell survival (Fig. 2 B).

2.3. Electrochemical real-time glutamate release

Real-time glutamate release was measured in accordance with our previous report (Mazzone and Nistri, 2011b) by using a specific biosensor obtained from Sarissa Biomedical Ltd (Coventry, UK) coupled to a multichannel potentiostat to integrate the redox reaction current (Pinnacle Technology Inc., Lawrence, KS, USA). Slices were recorded while kept in basal extracellular solution containing (mM): NaCl, 152; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10; glucose, 10, pH 7.4; 300–320 mOsm; Sigma. In brief, the system comprises a pair of electrodes, namely one glutamate biosensor and one null sensor (for assessing non-

specific electroactive interfering agents released from the slice). Both electrodes were placed at each side of the ventral fissure. The average sensor sensitivity to glutamate *in vitro* is reported to be linearly related to glutamate concentration in the 0.1–100 μM range (http://www.sarissa-biomedical.com/products-GLU.php, Gourine et al., 2008). In the present study, the specificity and stability of each glutamate sensor were validated by using a calibration curve (0.5–100 μM) and a test application of 50 μM glutamate before and after each experimental session. The control value for glutamate release was determined once the sensor was placed on the slice for a few min. Signals were processed with PAL software (V1.5.0; Pinnacle Technology Inc.) for subsequent off-line analysis.

2.4. Slice immunostaining and cell counting

Full details of this procedure have previously been published (Cifra et al, 2012a; Mazzone et al., 2017). In brief, cultures were fixed in 4% paraformaldehyde for 60 min at room temperature and stored in phosphate buffer saline (PBS) until use. Slices were blocked with fetal calf serum (FCS, 3%), bovine serum albumin (BSA, 3%) and Triton X-100 (0.3%) in PBS for 1 h at room temperature, followed by overnight incubation at 4 °C in blocking solution containing the following antibodies: NeuN for neurons (1:250, Millipore, Billerica, MA, USA) and S100β (1:750, Dako, Glostrup, Denmark) for astrocytes. Primary antibodies were visualized using the corresponding secondary fluorescent antibody (1:500 dilution; Invitrogen). To visualize cell nuclei, slices were incubated in 1 μg/ml solution of 4′, 6-diamidino-2-phenylindole (DAPI). To visualize GABAergic interneurons GAD67-GFP organotypic slice cultures were fixed as described before and the same immunostaining protocol for NeuN was followed by using the corresponding secondary fluorescent antibody. The number of NeuN or GAD 67 positive cells was obtained by counting images with a FV300 confocal microscope (Olympus Optical, Tokyo, Japan) or Zeiss Axioskop2 microscope (Zeiss Axioskop2, Carl Zeiss MicroImaging, Thornwood, NY, USA). NeuN positive cells were counted with Image J software (http://imagej.nih.gov/ij) in the dorsal and ventral ROIs and normalized by the ROI size (μm^2) to obtain cell density.

2.5. Real-time PCR expression levels

The expression levels of GAD67 was evaluated in C57BL/6 mouse spinal cord organotypic cultures by real-time PCR using specific primers in accordance with our previous report (Mazzone et al., 2017) with 18S and β-actin as housekeepers and GAD67 controls as previously reported (Trifonov et al., 2014). Briefly, the total RNA was isolated using Tri Reagent solution according to the manufacture's protocol (Invitrogen). RNA samples were quantified in a spectrophotometer at 260 nm. Retrotranscription using 1 μg of total RNA was performed with an iScript cDNA Synthesis Kit (BIORAD Cat. No 170–8891) according to the manufacture's suggestions. The reaction was run in a thermocycler at 25 °C for 5 min, 42 °C for 45 min, 85 °C for 5 min. The reaction was performed in the iQ Real Time PCR Thermal Cycler (BIO RAD) using the fluorescent dye SYBR Green (SYBR Green Supermix kit IQTM; BIORAD). The analysis of the results was performed using iCycle iQ Real Time PCR Detection System (BIORAD) program. mRNA samples were calibrated to obtain similar amplification of the 18S and β-actin as housekeeping mRNA. Cycling parameters were determined, and calculations for relative mRNA transcript levels were performed using the comparative CT method (ΔCT; Pfaffl, 2001) between cycle thresholds of different reactions, normalized to the housekeeping gene. Melting curve analysis was performed to assess product specificity.

2.6. Statistics

This study comprised 18 series of different organotypic culture preparations from 12 pregnant C57BL/6J or 6 GAD67-GFP mice. On average, at least 4–6 fetuses for each mouse in each preparation were delivered by caesarean section and pooled together to obtain the slices cultures (on average 40 slices). Data were expressed as mean ± S.E.M where n = number of slices (organotypic cultures slices from 3 to 12 independent preparations). Details of number of slices or immunohistochemistry, electrochemical glutamate release and real-time PCR expression levels in each group are indicated in Figure legends. Statistical analysis was carried out with SigmaStat (SigmaStat 3.1, Systat Software, Chicago, IL, USA): in detail, after applying the normality and the equal variance test (Brown-Forsythe) test in each case, values were analyzed with one-way ANOVA for multiple comparisons (with the Tukey-Kramer post-hoc test). As directed by the software, non-parametric values were analyzed by Kruskal–Wallis one-way analysis of variance followed by the Dunn's method for multiple comparisons. When two groups were compared, the Student's t-test was used for parametric data, or the Mann-Whitney Rank Sum Test for non-parametric data was applied. Two groups of data were considered statistically different if $p \leq 0.05$.

3. Results

3.1. Glutamate release induced by kainate was inhibited by midazolam

The present study first investigated whether facilitation or inhibition of $GABA_A$ and GlyRs could modulate glutamate release stimulated by kainate and the associated excitotoxic stress. Fig. 3 indicates NeuN-positive GABAergic (GAD67-GFP) interneurons at 22 DIV in control condition (A, left) or after kainate treatment (A, right), and demonstrates how NeuN positive elements were largely decreased after excitotoxic stress (Fig. 3B, n = 6–25; *, #$p \leq 0.05$ vs each control). In control condition, very small variability in NeuN numbers was observed among slices (see low S.E.M values in Fig. 3 B): these data accord with our former study that provides a comparative description of the different cell types, including neurons, in spinal cord standard organotypic culture (Cifra et al, 2012a). We next investigated whether kainate-mediated excitotoxicity might impact the GAD67 gene expression 24 h later. GAD 67 was the predominant mRNAs, while no expression of the different splicing isoforms producing the shorter, enzymatically inactive GAD25 protein was observed (data not shown) in accordance with the very low level previously found in the mouse brain (Trifonov et al., 2014). When examining GAD67 expression 24 h after kainate, its mRNA levels were significantly reduced vs control (Fig. 3 C, n = 15 different slices for each experimental condition from 3 independent preparations, *$p \leq 0.005$).

Fig. 4A and B compares examples of extracellular glutamate levels (expressed as biosensor oxidation current) induced by 100 μM kainate (applied for 60 min; see Mazzone et al., 2010) in standard solution or in the presence of midazolam (10 nM; applied 15 min before kainate). When midazolam was preapplied, the effect of kainate was blocked even if this benzodiazepine *per se* did not change the basal level of glutamate (basal mean value prior to kainate: 0.33 ± 0.10 nA, 0.32 ± 0.11 nA for control and MDZ + KA, respectively, n = 6–7 slices, $p = 0.963$). On average, strong depression of the extracellular glutamate signal was detected (on average from 0.85 ± 0.09 to 0.24 ± 0.06 nA, for kainate and MDZ + KA, respectively, Fig. 4 C, n = 6–7 slices, *$p \leq 0.01$).

3.2. Midazolam contrasted neurotoxicity

We next enquired whether inhibition of glutamate levels might have been sufficient to prevent late onset of neuronal death observed 24 h after kainate application (see protocols in Fig. 2; Mazzone et al., 2010). To find out any midazolam neuroprotection we imunostained neurons with the selective nuclear marker NeuN (Fig. 4D–E) with two distinct approaches. First, we observed significant preservation of neuronal numbers in the ventral and dorsal regions when midazolam was

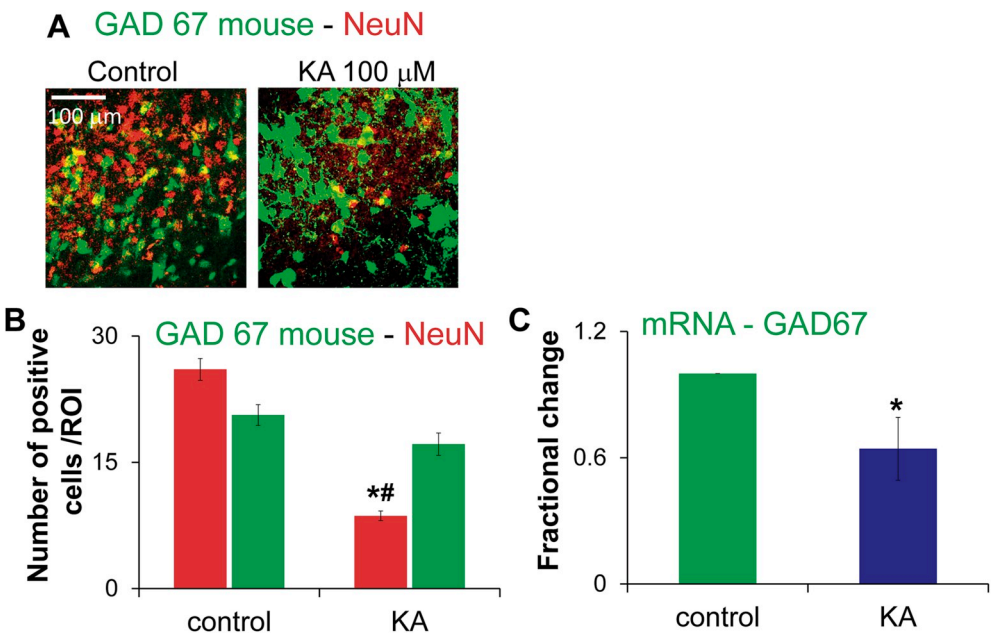

Fig. 3. Quantification of GAD67 positive cells and GAD67 mRNA after kainate application. (A) Example of NeuN and GAD 67 signal (NeuN red, GAD67 green) in control condition (left) or after kainate (KA) application (right) to spinal cord organotypic cultures (22 DIV). (B) Histograms showing the number of GAD67 positive cells (green columns) or NeuN-positive cells (red columns) 24 h in control condition or after application of KA for 1 h, n = 6–25 slices, *p ≤ 0.05 vs control wildtype; #p ≤ 0.05 vs KA GAD 67 mouse. (C) GAD67 mRNA expression detected with RT-PCR. Ordinate: fractional change with respect to control condition. Experimental protocols as in (B). Cultures treated with KA demonstrated changes in the levels of GAD67 mRNA transcript, (n = 15 different slices for each experimental condition from 3 independent preparations, *p ≤ 0.005 vs control).

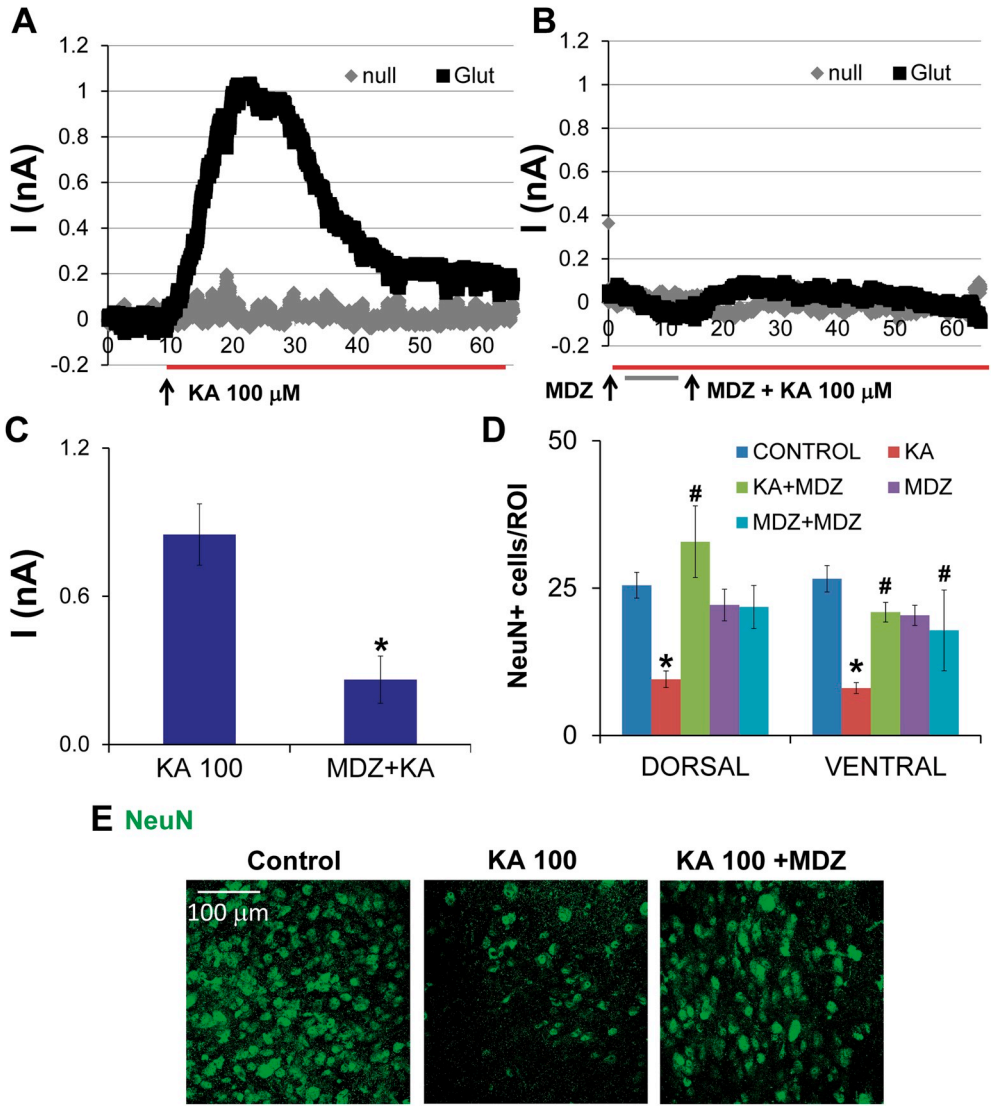

Fig. 4. Real-time release of glutamate from organotypic cultures of the mouse spinal cord during application of kainate (KA). Examples of timecourse of endogenous glutamate release detected by biosensor: the null and glutamate sensors were positioned in the ventral area with reference to the ventral fissure. Cultures were treated with KA (applied within arrows) for 1 h (A) or pretreated with midazolam for 15 min and then co-treated with KA for 1 h (B) while glutamate release was continuously recorded. Abscissas indicate time (min) while ordinates show glutamate oxidation current. (C) Histograms show average timecourse for the redox current of glutamate continuously monitored throughout the experiment (n = 6–7 slices, *p ≤ 0.01). (D) Histograms showing the number of NeuN positive cells counted 24 h after the application of KA for 1 h, in control condition or with MDZ (0.01 μM), n = 3–11 slices, *p < 0.05 vs control, #p < 0.05 vs KA. (E) Examples of neuronal staining (NeuN in green) in dorsal region of mouse spinal cord organotypic cultures at 22 DIV following protocols as above.

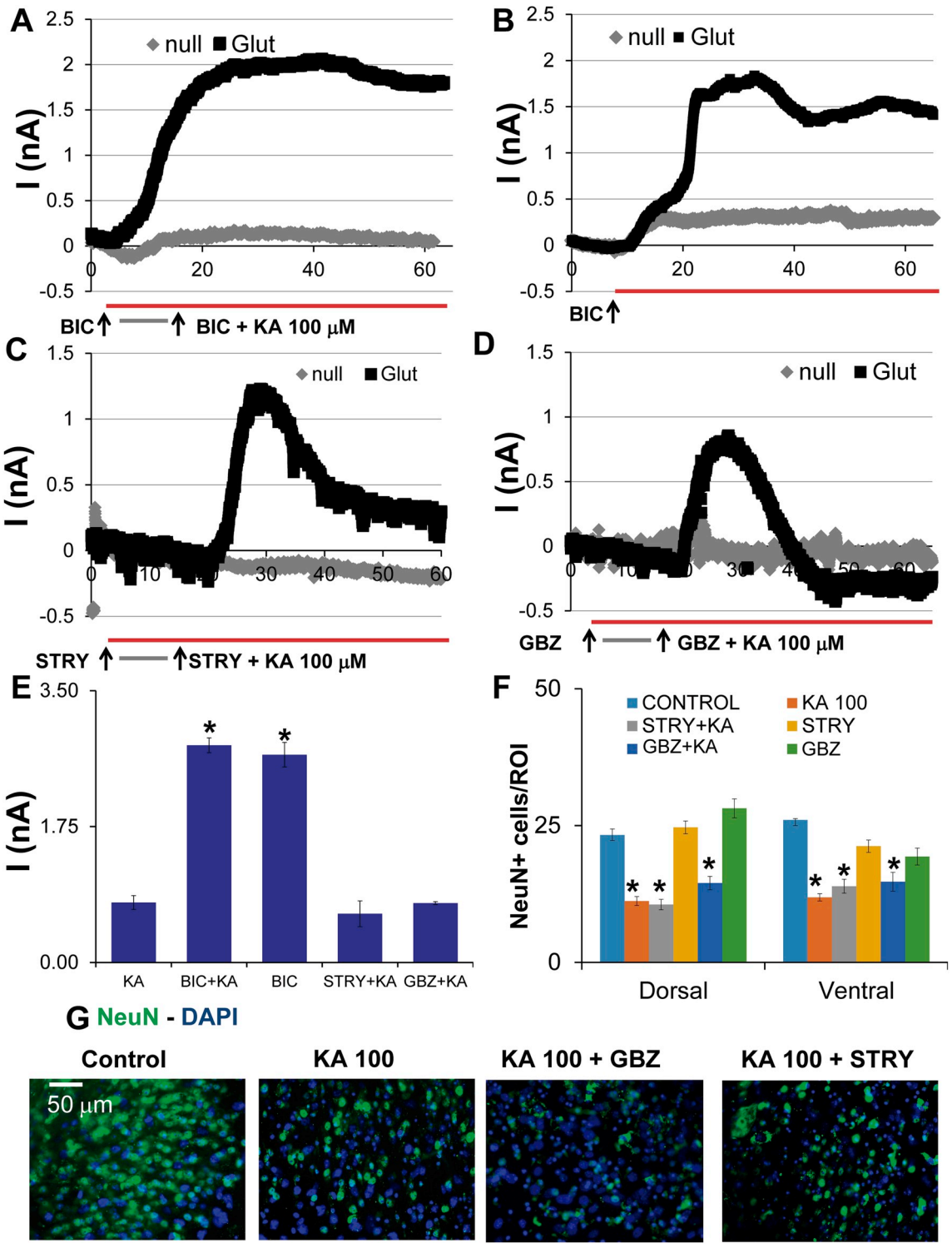

Fig. 5. Endogenous glutamate release after treatment with bicuculline (BIC), strychnine (STRY), or gabazine (GBZ) for 15 min and then co-treated with KA for 1 h. (A) Examples of timecourse of endogenous glutamate release after BIC (A) pre-applied before KA, BIC only (B), STRY (C) or GBZ (D). (E) Histograms show average of glutamate current for slices treated with KA for 1 h, or slices treated with BIC (n = 5–10, *p ≤ 0.05), STRY (n = 9–10, p = 0.072), or GBZ (n = 8–10, p = 0.450, *t*-test) for 15 min and then co-treated with KA for 1 h (n = 5–8, *p ≤ 0.01), or with BIC only (n = 3–6, *p ≤ 0.05). (F) Histograms showing the number of NeuN positive cells counted at 24 h, after the application of KA for 1 h, n = 3–7 slices, *p < 0.05 vs control. (G) Examples of neuronal staining (NeuN green, and DAPI blue) in ventral region of mouse spinal cord organotypic cultures at 22 DIV following protocols as above.

preapplied for 15 min prior to kainate and maintained for 1 h followed by full washout of both drugs. Second, similar results were obtained when midazolam application persisted for the following 24 h after kainate (Fig. 4D, n = 3–11, *p ≤ 0.05 vs control, #p ≤ 0.05 vs MDZ alone). To sum up, neuroprotection was detected when midazolam was preapplied, while this benzodiazepine *per se* was not neurotoxic. Fig. 4E shows representative images staining for NeuN under control condition, after kainate treatment, after kainate application plus midazolam, or midazolam alone. The data indicate that neuronal staining was well preserved except that in the case of kainate application. In our former

studies we have analyzed the complex cell-death mechanisms induced by kainate (Kuzhandaivel et al., 2011). We have shown that, after kainate treatment, neurons mainly die because of hyperactivation of poly(ADP-ribose)polymerase-1(PARP1) enzyme with subsequent DNA damage and mitochondrial energy collapse (Kuzhandaivel et al., 2010; Mazzone and Nistri, 2011).

3.3. Bicuculline strongly enhanced glutamate release

Fig. 5A–B shows examples of extracellular glutamate signal when bicuculline (20 μM) was applied together with kainate or alone. In either case, a strong increase in glutamate level was detected (Fig. 5 E), suggesting that the effect of bicuculline was not simply additive to the one of kainate. This observation is in line with our previous report indicating that bicuculline largely enhanced the lesional effect of kainate (Bajrektarevic and Nistri, 2016). On the contrary, neither gabazine nor strychnine significantly affected the glutamate signal evoked by kainate (Fig. 5C–E). Histological analysis of dorsal and ventral ROIs performed after 24 h showed significant reduction in the number of neurons evoked by kainate without further change in the presence of gabazine or strychnine (Fig. 5F–G).

3.4. Modulation by δ-subunit-containing $GABA_A$ receptors of excitotoxic stress

To investigate the contribution by δ-subunit $GABA_A$ receptors, we studied the effect of THIP on glutamate signals evoked by kainate. Fig. 6 A, C shows that THIP pre-application prior to excitotoxic stress (induced by kainate) significantly inhibited glutamate release. On average, the THIP pre-treatment yielded a smaller peak amplitude (0.45 ± 0.08 nA; $n = 9$; $p \leq 0.05$), and a delayed time to peak (40.32 ± 2.26 min; $n = 9$; $p \leq 0.005$) of the glutamate signal in comparison to the one observed with kainate (Figs. 4 A, 0.70 ± 0.13 nA, peak time 26 ± 2.96 min; $n = 10$). On the contrary, L-alanine, an agonist preferential for glycine receptors, significantly increased the glutamate signal elicited by kainate (1.23 ± 0.11 nA; $n = 4$; $p \leq 0.01$, Fig. 6 B, C). NeuN staining confirmed large neuroprotection by THIP and unaltered neuronal loss by L-alanine when these drugs were applied together with kainate (Fig. 6 D, E), suggesting that activation of δ-subunit $GABA_A$ receptors was instrumental to spare neurons from excitotoxicity.

4. Discussion

The principal finding of the present report is that pharmacological enhancement of $GABA_A$ receptors was an efficacious tool to counteract excitotoxic death in spinal networks *in vitro*. This effect appeared to be, at least in part, mediated by extrasynaptic $GABA_A$ receptors and involved inhibition of glutamate release.

4.1. Midazolam neuroprotection

Benzodiazepines primarily act by potentiating $GABA_A$ receptors (Haefely, 1978). Midazolam shares this effect with the additional experimental advantage of being fast-acting and water soluble (Pieri, 1983). Using organotypic spinal cultures, we have demonstrated that kainate strongly and rapidly increases extracellular levels of glutamate leading to excitotoxicity (Mazzone and Nistri, 2011b), an effect blocked by riluzole that facilitates glutamate uptake and provides neuroprotection (Mazzone and Nistri, 2011a). The present data showed that enhancing $GABA_A$ receptor activity with midazolam was sufficient to block glutamate release, while the broad spectrum $GABA_A$ antagonist bicuculline largely increased it, and also augmented kainate neurotoxicity (Bajrektarevic and Nistri, 2016). Indeed, it is clear that 24 h after kainate application GABAergic interneurons were decreased in number in accordance with the lower ability to express the GAD 67 gene. Since GABAergic interneurons are likely making synaptic contacts with a large number of neurons, it is not expected that there would be a linear correlation between loss of NeuN stained neurons and the number of GAD 67 positive interneurons. Thus, because loss of GABAergic interneurons appeared to be a facet of neurotoxicity, we next enquired if enhanced $GABA_A$ receptor activity might confer neuroprotection. To address this question, in the present report we applied midazolam prior to kainate because previous work has shown that delayed application of midazolam becomes rapidly ineffective as neuroprotectant (Gilby et al., 2005; Wu et al., 2018) even if it is acknowledged that drug preapplication can point to mechanisms of action for neurotoxicity rather than providing translational approaches to treat it.

It was noteworthy that pharmacological modulation of glycine receptors with the agonist L-alanine or the antagonist strychnine failed to change neurotoxicity, again consistent with a preferential role of GABAergic mechanisms to suppress the action of kainate. On the assumption that organotypic slices can preserve the basic cytoarchitecture of the spinal cord (Cifra et al, 2012a), we suspect that the differential distribution of spinal $GABA_A$ and glycine receptors within the neuronal networks is a contributor to the apparent lack of effect by glycinergic agents on glutamate release (Takazawa and MacDermott, 2010). Since in immature networks GABA evokes neuronal depolarization with inhibitory function (Bracci et al., 1996; Streit, 1996; Tscherter et al., 2001) because of the associated strong shunting conductance observed in the isolated spinal cord of the rat (Jean-Xavier et al., 2007; Marchetti et al., 2002), it seems likely that neuroprotection might be obtained by upregulating GABAergic activity to decrease neuronal excitability in discrete network neurons. The role of glycine, however, remains more complex. In fact, while this neutral amino acid has long been recognized as an important inhibitory neurotransmitter in the spinal cord (Werman et al., 1968), its action as co-agonist at NMDA receptors (Llano et al., 1988; Thomson, 1990) might actually facilitate glutamate excitotoxicity (Regan and Choi, 1991). Lack of glycine neuroprotection in our experimental model might allude to a balance between its effects pro and against excitotoxicity.

4.2. Neuroprotection target for midazolam

In the current report on spinal neurons we observed neuroprotective effects of midazolam at concentrations shown to be efficacious to potentiate $GABA_A$ receptor currents, whereas within the brainstem network this phenomenon was not observed (Ghezzi et al., 2017). Analogous observations have recently been reported for rat retinal cells (Ulbrich et al., 2016). Furthermore, *in vivo* midazolam is apparently not neuroprotective against rabbit ischemic brain injury (Kochhar et al., 1991), although it is quite effective to control pharmacologically-induced seizures (Shih et al., 2003; Skovira et al., 2012). This intriguing discrepancy between brain and spinal networks raises several issues. First, it is unlikely that the benzodiazepine-sensitivity of $GABA_A$ receptors is broadly different between brain and spinal neurons (Pieri, 1983) since, for example, GABA-evoked electrophysiological responses of brainstem neurons (Ghezzi et al., 2017) are as sensitive to midazolam as spinal neurons of very different vertebrate species (Leah et al., 1983; Nistri and Berti, 1983). Second, if the $GABA_A$ receptor pharmacology is broadly similar, perhaps the cellular distribution of such receptors within the network becomes an important factor. Although we did not study the discrete cellular expression of GABA receptors in organotypic cultures, one clue was provided by the observation that gabazine, an antagonist of synaptic $GABA_A$ receptors, did not change the neurotoxic effects of kainate stemming from glutamate release. This result led us to examine the role of bicuculline that inhibits synaptic and extrasynaptic receptors. Indeed, in the presence of bicuculline, large glutamate release occurred and neurotoxicity ensued. These data, therefore, seemingly suggest that extrasynaptic $GABA_A$ receptors were preferentially targeted by midazolam in spinal cord slices in culture. This notion is

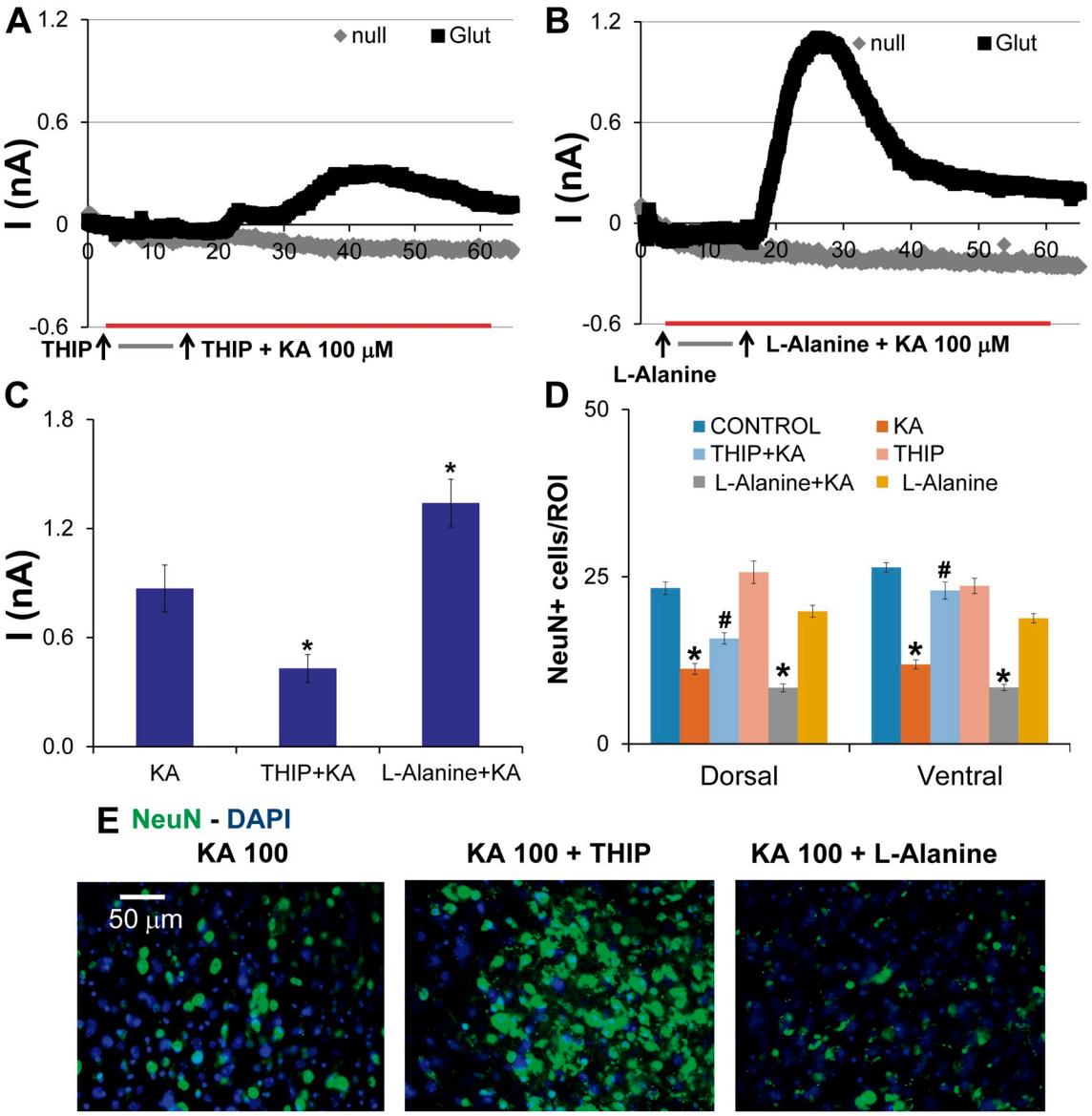

Fig. 6. Effect of THIP and L-alanine on glutamate release. Examples of timecourse traces of endogenous glutamate release from slices treated with THIP (A) or L-alanine (B) for 15 min and then co-treated with KA for 1 h. (C) Histograms show average glutamate current for slices treated with KA for 1 h or slices treated with THIP or L-alanine for 15 min and then co-treated with KA for 1 h (n = 9–10 slices, *p ≤ 0.05; n = 4–10 slices, *p ≤ 0.001). (D) Histograms showing the number of NeuN positive cells counted at 24 h after the application of KA for 1 h (n = 4–6 slices, *p < 0.05 vs control, #p < 0.05 vs KA). (E) Examples of neuronal staining (NeuN green and DAPI blue in ventral region) following protocols as above.

consistent with electrophysiological data on the presence of such receptors in about 2/3rd of spinal interneurons (Takahashi et al., 2006), the local expression of δ subunit-containing $GABA_A$ receptors (Takahashi et al., 2006) predominantly located at extrasynaptic sites to mediate tonic inhibition (Farrant and Nusser, 2005), and the report that midazolam, even in the presence of submicromolar concentration of gabazine (to selectively block synaptic currents), is more active on extra- than synaptic $GABA_A$ receptors in the spinal cord by potentiating GABA receptor affinity (Maeda et al., 2010).

Although it has recently been demonstrated that midazolam can have a protective effect against oxidative stress cytotoxicity using a motor neuron-like cell line (Li et al., 2018), our results are the first report that midazolam could protect spinal network neurons because it inhibited glutamate release by means of extrasynaptic $GABA_A$ receptor activation. Since no antagonists could change the glutamate baseline, this finding suggests that endogenous GABAergic mechanisms were *per se* insufficient to prevent the action of kainate.

It should be noted that experimental section of the spinal cord leads to delayed downregulation of the neuronal Cl^- extrusion system with ensuing hyperexcitability of the spinal networks below the lesion and emergence of spasticity (Boulenguez et al., 2010). Interestingly, this phenomenon is associated to enhanced synthesis of GABA, perhaps as a compensatory process (Tillakaratne et al., 2000), and regular exercise can reverse this pathological change and restore spinal inhibition (Côté et al., 2014). Globally, these results indicate that GABArgic mechanisms exert diverse effects (with a time-dependent fashion) on spinal networks subjected to experimental lesion.

4.3. Extrasynaptic GABA A receptors and neuroprotection

While distinct subunits confer different biological properties to $GABA_A$ receptors (Sigel and Steinmann, 2012; Sivilotti and Nistri, 1991), in the rat spinal cord there is ample GABA receptor heterogeneity (Bohlhalter et al., 1996) that comprises extrasynaptic receptors

preferentially containing the δ subunit, and mediating tonic inhibition (Takazawa and MacDermott, 2010) when such a subunit is expressed together with α6, α4 and/or α1 subunits (Shivers et al., 1989). Extrasynaptic receptors are excellent sensors for extracellular GABA due to their high affinity for GABA and slow rates of desensitization (Farrant and Nusser, 2005; Semyanov et al., 2004). As GABAergic tonic inhibition as well as δ-$GABA_A$ receptor subunits (Takahashi et al., 2006) are expressed by neurons in the dorsal horn, it is noteworthy that in this area there is the largest density of kainate receptors (Tolle et al., 1993). Hence, it seems likely that extrasynaptic $GABA_A$ receptors are strategically posed to modulate kainate-mediated excitation and toxicity as long as their activity is pharmacologically upregulated. In support of this proposal we observed that THIP, a synthetic GABA agonist preferentially acting on δ-subunit $GABA_A$ receptors (Iversen, 2004), was efficacious to inhibit release of glutamate evoked by kainate and to confer neuroprotection.

Conversely, on hippocampal CA1 pyramidal neurons, kainate is reported to facilitate the operation of extrasynaptic GABA receptors and to depress synaptic ones (Jiang et al., 2015): if this phenomenon is also occurring at the level of spinal networks, it seems feasible that inhibition of GABAergic transmission was the prevalent phenomenon and could not antagonize excitotoxicity.

In view of the substantial involvement of GABA-mediated tonic inhibition in the control of brain neuron excitability (Farrant and Nusser, 2005; Semyanov et al., 2004), future studies should address the issue of tonic inhibition in the long term evolution of spinal injury and associated functional changes like spasticity. In the mouse hippocampus there is already evidence for large downregulation of GABAergic tonic inhibition after brain trauma to account for delayed hyperexcitability including appearance of a seizure phenotype (Boychuk et al., 2016). Recently, two pharmacological studies of larval lampreys have shown that GABA promotes survival and axonal regeneration in descending neurons after complete spinal cord transection: this effect is mediated by $GABA_A$ and $GABA_B$ receptors and involves downregulation of caspases (Romaus-Sanjurjo et al., 2018; Sobrido-Cameán et al., 2018). Indeed, the first step for excitatory and inhibitory spinal circuit assembly and regrowth is the selection of appropriate pre and postsynaptic partners using cell recognition cues that implement the transmitter release machinery (Gamlin et al., 2018) and developmental receptor rearrangement (Takahashi, 2005).

5. Conclusions

These present study suggests that pharmacological enhancement of extrasynaptic GABAergic inhibition should dampen glutamate release to prevent excitotoxicity and provide neuroprotection. Further understanding of the complexities of GABA receptor pharmacology and development of selective and safe agonists might represent a translational target for the treatment of the very early stage of spinal cord injury.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgment

We thank Beatrice Pastore for the assistance with spinal cultures and mice genotyping. This study was supported by an intramural SISSA grant, CONICET and Regular Associate Scheme of the Abdus Salam International Centre for Theoretical Physics (ICTP).

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