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Title: 5-HT2A-mGlu2/3 receptor complex in rat spinal cord glutamatergic nerve endings: a 5-HT2A to mGlu2/3 signalling to amplify presynaptic mechanism of auto-control of glutamate exocytosis

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Abstract: Presynaptic mGlu2/3 autoreceptors exist in rat spinal cord nerve terminals as suggested by the finding that LY379268 inhibits the 15 mM KCl-evoked release of [3H]D-aspartate ([3H]D-Asp) in a LY341495-sensitive manner. Spinal cord glutamatergic nerve terminals also possess presynaptic release-regulating 5-HT2A heteroreceptors. Actually, the 15 mM KCl-evoked [3H]D-Asp exocytosis from spinal cord synaptosomes is reduced by the 5-HT2A agonist ( $\pm$ )DOI, an effect reversed by the 5-HT2A antagonists MDL11,939, MDL100907, ketanserin and trazodone (TZD). We investigated whether mGlu2/3 and 5-HT2A receptors colocalize and cross-talk in these terminals and if 5-HT2A ligands modulate the mGlu2/3-mediated control of glutamate exocytosis. Western blot analysis and confocal microscopy highlighted the presence of mGlu2/3 and 5-HT2A receptor proteins in spinal cord VGLUT1 positive synaptosomes, where mGlu2/3 and 5-HT2A receptor immunoreactivities largely colocalize. Furthermore, mGlu2/3 immunoprecipitates from spinal cord synaptosomes were also 5-HT2A immunopositive. Interestingly, the 100 pM LY379268-induced reduction of the 15 mM KCl-evoked [3H]D-Asp overflow as well as its inhibition by 100 nM ( $\pm$ )DOI became undetectable when the two agonists were concomitantly added. Conversely, 5-HT2A antagonists (MDL11,939, MDL100907, ketanserin and TZD) reinforced the release-regulating activity of mGlu2/3 autoreceptors. Increased expression of mGlu2/3 receptor proteins in synaptosomal plasmamembranes paralleled the gain of function of the mGlu2/3 autoreceptors elicited by 5-HT2A antagonists. Based on these results, we propose that in spinal cord glutamatergic terminals i) mGlu2/3 and 5-HT2A receptors colocalize and interact one each other in an antagonist-like manner, ii) 5-HT2A antagonists are indirect positive allosteric modulator of mGlu2/3 autoreceptors controlling glutamate exocytosis.

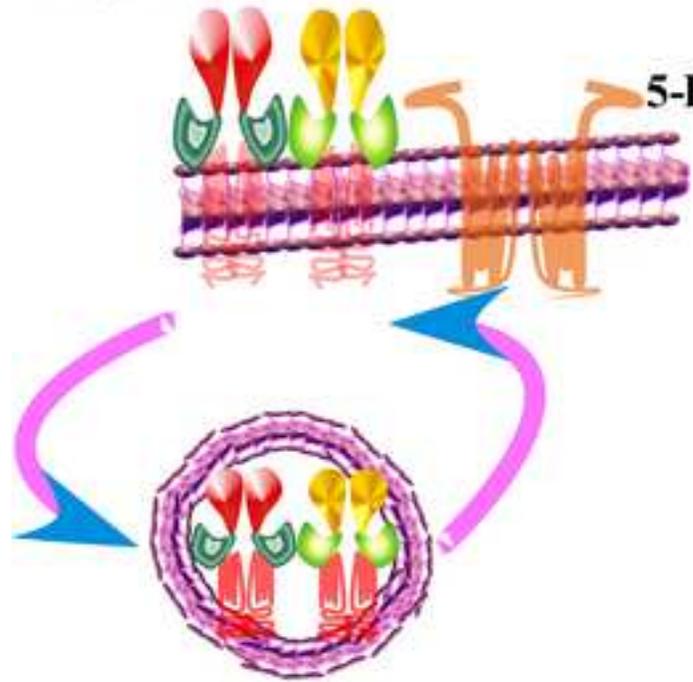
### *Highlights*

- spinal cord glutamatergic terminals possess inhibitory 5-HT<sub>2A</sub> heteroreceptors
- 5-HT<sub>2A</sub> heteroreceptors colocalize with inhibitory mGlu2/3 autoreceptors
- 5-HT<sub>2A</sub> and mGlu2/3 receptors cross talk in an antagonist-like fashion
- 5-HT<sub>2A</sub> antagonism reinforces the release-regulating activity of mGlu2/3Rs
- 5-HT<sub>2A</sub> antagonists are "*indirect positive allosteric modulators*" of mGlu2/3Rs

**A**

mGlu2/3Rs

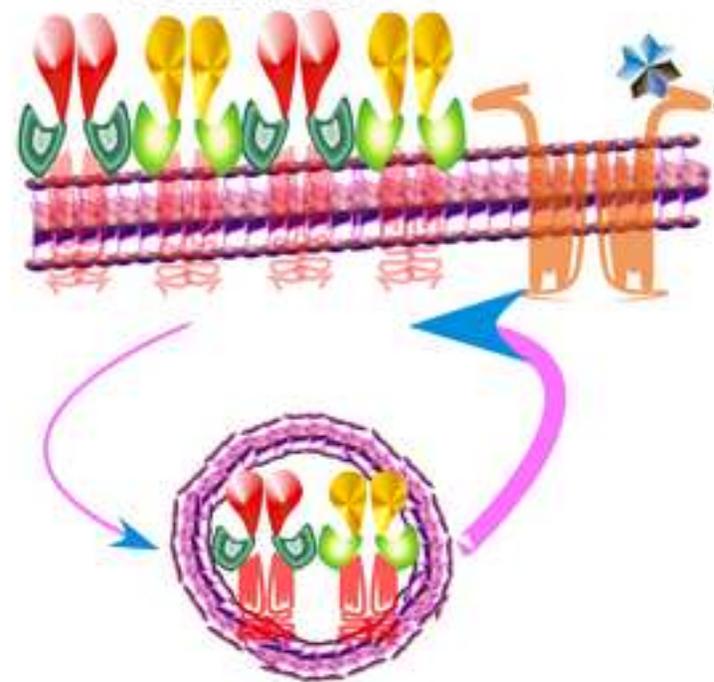
5-HT<sub>2A</sub>Rs



**B**

mGlu2/3Rs

5-HT<sub>2A</sub>Rs



 5-HT<sub>2A</sub> R antagonist

**5-HT<sub>2A</sub>-mGlu2/3 receptor complex in rat spinal cord glutamatergic nerve endings: a 5-HT<sub>2A</sub> to mGlu2/3 signalling to amplify presynaptic mechanism of auto-control of glutamate exocytosis**

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**A B S T R A C T**

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*Abbreviations:* metabotropic glutamate receptor type 2/3 (mGlu2/3); serotonin type 2A (5-HT<sub>2A</sub>); [<sup>3</sup>H]D-aspartate ([<sup>3</sup>H]D-Asp); central nervous system (CNS); excitatory postsynaptic currents (EPSCs); layer V pyramidal cells (L5P); Tris-buffered saline-Tween (t-TBS); vesicular glutamate transporters type 1 (VGLUT1); trazodone (TZD)

## ABSTRACT

Presynaptic mGlu2/3 autoreceptors exist in rat spinal cord nerve terminals as suggested by the finding that LY379268 inhibits the 15 mM KCl-evoked release of [<sup>3</sup>H]D-aspartate ([<sup>3</sup>H]D-Asp) in a LY341495-sensitive manner. Spinal cord glutamatergic nerve terminals also possess presynaptic release-regulating 5-HT<sub>2A</sub> heteroreceptors. Actually, the 15 mM KCl-evoked [<sup>3</sup>H]D-Asp exocytosis from spinal cord synaptosomes is reduced by the 5-HT<sub>2A</sub> agonist (±)DOI, an effect reversed by the 5-HT<sub>2A</sub> antagonists MDL11,939, MDL100907, ketanserin and trazodone (TZD). We investigated whether mGlu2/3 and 5-HT<sub>2A</sub> receptors colocalize and cross-talk in these terminals and if 5-HT<sub>2A</sub> ligands modulate the mGlu2/3-mediated control of glutamate exocytosis. Western blot analysis and confocal microscopy highlighted the presence of mGlu2/3 and 5-HT<sub>2A</sub> receptor proteins in spinal cord VGLUT1 positive synaptosomes, where mGlu2/3 and 5-HT<sub>2A</sub> receptor immunoreactivities largely colocalize. Furthermore, mGlu2/3 immunoprecipitates from spinal cord synaptosomes were also 5-HT<sub>2A</sub> immunopositive. Interestingly, the 100 pM LY379268-induced reduction of the 15 mM KCl-evoked [<sup>3</sup>H]D-Asp overflow as well as its inhibition by 100 nM (±)DOI became undetectable when the two agonists were concomitantly added. Conversely, 5-HT<sub>2A</sub> antagonists (MDL11,939, MDL100907, ketanserin and TZD) reinforced the release-regulating activity of mGlu2/3 autoreceptors. Increased expression of mGlu2/3 receptor proteins in synaptosomal plasmamembranes paralleled the gain of function of the mGlu2/3 autoreceptors elicited by 5-HT<sub>2A</sub> antagonists. Based on these results, we propose that in spinal cord glutamatergic terminals i) mGlu2/3 and 5-HT<sub>2A</sub> receptors colocalize and interact one each other in an antagonist-like manner, ii) 5-HT<sub>2A</sub> antagonists are indirect positive allosteric modulator of mGlu2/3 autoreceptors controlling glutamate exocytosis.

*Keywords:*

mGlu2/3 receptor

5-HT<sub>2A</sub> receptor

glutamate release

spinal cord

GPCR crosstalk

heterocomplex

## 1. Introduction

Abnormal glutamate exocytosis from nerve terminals alters synaptic plasticity, contributing to the etiopathogenesis of most of the central neurological disorders. In this context, drugs activating presynaptic, inhibitory, release-regulating receptors on glutamatergic nerve terminals could be therapeutic to disease progression, since they counteract hyperglutamatergicity participating to the restoration of glutamate transmission in the central nervous system (CNS). Among all the potential candidates, presynaptic metabotropic glutamate receptors (mGluRs) belonging to the second group, namely the mGlu2/3 autoreceptors, represent preferential targets for such a therapeutic approach (Nicoletti et al., 2011).

The existence and the role of presynaptic-release-regulating mGlu2/3 autoreceptors in selected regions of the CNS is well documented (Cartmell and Schoepp, 2000; Di Prisco et al., 2016 and references therein). New evidence showing that mGlu2/3 receptors can form intergroup dimers with co-localized mGluRs belonging to the third group (El Moustaine et al., 2012; Kammermeier, 2012; Yin et al., 2014), as well as heteromeric complexes with non-glutamatergic metabotropic receptors, however, has increased the complexity of the scenario. It is the case of 5-HT<sub>2A</sub> receptors, that were recently reported to associate to mGlu2/3 receptors in CNS (Delille et al., 2012,2013; Baki et al., 2016).

Activation of 5-HT<sub>2A</sub> receptors in the rodent medial prefrontal cortex were found to induce excitatory postsynaptic currents (EPSCs) in layer V pyramidal cells (L5P cells, Aghajanian and Marek, 1997,1999) that were blocked by AMPA receptor antagonists. This seemed compatible with the idea that 5-HT<sub>2A</sub> receptors exist in this CNS region and that their activation modulates the release of glutamate. More recently, the 5-HT<sub>2A</sub> receptors were shown to be physically linked and to functionally interact with mGlu2/3 receptors (Delille et al., 2012, 2013 and references therein). Surprisingly, an antagonist-like cooperation was found to bridge the two receptors (Marek et al., 2000), as activation of mGlu2/3 receptors reduced the 5-HT<sub>2A</sub> receptor-induced EPSCs, while

mGlu2/3 antagonist / negative allosteric modulators reinforced the 5-HT-mediated excitations of L5P cells.

Whether the mGlu2/3- 5-HT<sub>2A</sub> receptor-receptor interaction also occurs in other regions was not so far investigated, although presynaptic release-regulating mGlu2/3 autoreceptors controlling glutamate exocytosis are widely express in CNS (Cartmell and Schoepp, 2000). In particular, we recently characterized by a pharmacological point of view the mGlu2/3 autoreceptors controlling glutamate exocytosis in spinal cord nerve endings (Di Prisco et al., 2016; Olivero et al., 2017). The possibility that these terminals also possess presynaptic release-regulating 5-HT<sub>2A</sub> heteroreceptors which could functionally couple to mGlu2/3 autoreceptors has not been analyzed so far.

In an attempt to fill the gap, our study aimed i) at investigating the existence and the functions of presynaptic release-regulating 5-HT<sub>2A</sub> heteroreceptors in spinal cord glutamatergic nerve endings, and then ii) at determining whether these receptors functionally cross-talk with presynaptic mGlu2/3 autoreceptors. The results described in the present research demonstrate that mGlu2/3 and 5-HT<sub>2A</sub> receptors colocalize on spinal cord nerve terminals and functional cross-talk in an antagonist-like manner to control glutamate exocytosis. Our findings suggest new therapeutic approaches to contain hyperglutamatergicity in this CNS region.

## 2. Material and Methods

### 2.1. *Animals*

Adult rats (female and male, strain Sprague Dawley) were obtained from Charles River (Calco, Italy) and were housed in the animal facility of DIFAR, Section of Pharmacology and Toxicology (authorization n° 484 of 2004, June, 8<sup>th</sup>). The experimental procedures were in accordance with the European legislation (European Communities Council Directive of 2010/63/EU) and the ARRIVE guidelines, and they were approved by the Italian Ministry of Health (DDL 26/2014 and previous legislation; protocol number 867/2016-PR).

### 2.2. *Preparation of synaptosomes*

Rat spinal cord purified synaptosomes were prepared as previously described (Musante et al., 2011). Synaptosomes were resuspended in a physiological solution with the following composition (mM): NaCl, 140; KCl, 3; MgSO<sub>4</sub>, 1.2; CaCl<sub>2</sub>, 1.2; NaH<sub>2</sub>PO<sub>4</sub>, 1.2; NaHCO<sub>3</sub>, 5; HEPES, 10; glucose, 10; pH 7.2-7.4.

### 2.3. *Experiments of transmitter release*

Synaptosomes were incubated for 15 min at 37 °C in a rotary water bath in the presence of [<sup>3</sup>H]D-aspartate ([<sup>3</sup>H]D-Asp, f.c.: 50 nM). Identical portions of the synaptosomal suspensions were layered on microporous filters at the bottom of parallel thermostated chambers in a Superfusion System (Raiteri et al., 1974; Summa et al., 2013); Ugo Basile, Gemonio, Varese, Italy).

Synaptosomes were transiently (90 s) exposed, at  $t = 39$  min, to high KCl containing medium (Di Prisco et al., 2012) in the absence or in the presence of agonists. When indicated, antagonists were added 20 min before agonists. Fractions were collected as follow: two 3-min fractions (basal release), one before ( $t = 36-39$  min) and one after ( $t = 45-48$  min) a 6-min fraction ( $t$

= 39-45 min; evoked release). Fractions collected and superfused synaptosomes were measured for radioactivity.

The amount of radioactivity released into each superfusate fraction was expressed as percentage of the total radioactivity. The KCl-induced overflow was estimated by subtracting the neurotransmitter content into the first and the third fractions collected (basal release, b1 and b3) from that in the 6-min fraction collected during and after the depolarization pulse (induced release, b2). The effect of agonists/antagonists is expressed as percentage of the KCl-induced overflow of tritium observed in the absence of receptor agonists and antagonists (percent of control).

#### *2.4. Immunoblotting*

Rat spinal cord purified synaptosomes were lysed in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris, 1% Triton X-100, protease inhibitors, pH 8.0) and quantified for protein content. Samples were boiled for 5 min at 95°C in SDS-PAGE loading buffer and then separated by SDS-7.5% PAGE (20-10 µg/lane) and transferred onto PVDF membranes. Membranes were incubated for 1 h at room temperature in Tris-buffered saline-Tween (t-TBS: 0.02 M Tris, 0.150 M NaCl, and 0.05% Tween 20), containing 5% (w/v) non-fat dried milk and then probed with rabbit anti-mGlu2/3 (1:2000), rabbit anti-5-HT<sub>2A</sub> (1:500) and mouse anti-β-actin (1:5000) antibodies overnight at 4°C. After extensive washes in t-TBS, membranes were incubated for 1h at room temperature with appropriate horseradish peroxidase-linked secondary antibodies (1:20000). Images were acquired using the Alliance LD6 images capture system (Uvitec, Cambridge, UK) and analysed with UVI-1D software (Uvitec, Cambridge, UK).

#### *2.5. Biotinylation*

The amount of mGlu2/3 receptors proteins in synaptosomal plasmamembranes was evaluated by performing surface biotinylation and subsequent immunoblot analysis (Salamone et

al., 2014). Briefly, purified synaptosomes were divided into 2 aliquots: the first aliquot was incubated for 20 min with 100 nM MDL11,939 or 1 nM trazodone at 37°C with mild shaking (T), while the other one was kept as control (C). Synaptosomes (T and C) were then treated with sulfo-NHS-SS-biotin (2 mg/ml) in PBS/Ca-Mg of the following composition (mM): 138 NaCl, 2.7 KCl, 1.8 KH<sub>2</sub>PO<sub>4</sub>, 10 Na<sub>2</sub>HPO<sub>4</sub>, 1.5 MgCl<sub>2</sub>, 0.2 CaCl<sub>2</sub>, pH 7.4 for 1 h at 4 °C and then incubated in PBS/Ca-Mg with 100 mM glycine for 15 min at 4 °C to quench the reaction. Biotinylated synaptosomes were then lysed in RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, protease inhibitors). Samples (100 µg) were incubated with NeutrAvidin agarose beads for 1 h at room temperature under shaking. Beads were added to the biotinylated synaptosomes to pull down the biotinylated proteins, as well as to non-biotinylated synaptosomes, to evaluate the specificity of neutravidin pull-down (B). After extensive washes, samples were boiled for 5 min at 95 °C in SDS-PAGE loading buffer to separate biotinylated proteins from the beads. Eluted fractions were analyzed through immunoblot assay (see section Immunoblotting). The immunoreactivity of mGlu2/3 receptors was monitored in the total lysate (L), in control and in antagonist-pretreated biotinylated synaptosomes (respectively C and T) and in the streptavidin pull-down of the non-biotinylated synaptosomal lysate (B). β-actin, a cytosolic protein, was used as control to evaluate the specificity of biotinylation reaction.

## *2.6. Co-immunoprecipitation*

Rat spinal cord purified synaptosomes were lysed in ice-cold lysis buffer having the following composition: 140 mM NaCl, 20 mM Tris, 0.5% Triton X-100, protease inhibitors, pH 7.4. Protein A Dynabeads were incubated with anti-mGlu2/3 antibody (1:500) diluted in PBS containing 0.02% Tween 20 (t-PBS) for 10 min at room temperature. Synaptosomal lysate (200 µg) was added to antibody-bound Protein A Dynabeads (I.P.), as well as to beads without antibody (negative control, B). After an incubation for 25 min at room temperature under shaking, beads were washed three times in t-PBS and then resuspended in SDS-PAGE loading buffer. Samples

were boiled at 95°C for 5 min in order to elute proteins from beads and subjected to Western Blot analysis (see section Immunoblotting). mGlu2/3 receptors were detected with rabbit anti-mGlu2/3 antibody (1:2000) and 5-HT<sub>2A</sub> receptors were detected with rabbit anti-5-HT<sub>2A</sub> antibody (1:250).

### *2.7. Immunocytochemical analysis in mouse cortical and spinal nerve terminals*

For immunocytochemical analysis, spinal cord synaptosomes were fixed with 2% paraformaldehyde for 15 min, permeabilized with 0.05% Triton X-100 PBS for 5 min and incubated with the following primary antibodies: rabbit anti-mGlu2/3 receptor (1:1000), mouse anti-5-HT<sub>2A</sub> receptor (1:200) and guinea pig anti-vesicular glutamate transporters type 1 (VGLUT1; 1:500). After extensive washing, synaptosomes were incubated for 1 h at room temperature with the following antibodies: donkey anti-rabbit AlexaFluor-488 and goat anti-guinea pig AlexaFluor-633 (1:1000 both, confocal analysis aimed at identifying colocalization of mGlu2/3 receptor and VGLUT1 proteins), with donkey anti-mouse AlexaFluor-488 and goat anti-guinea pig AlexaFluor-633 (1:1000 both, confocal analysis aimed at identifying colocalization of 5-HT<sub>2A</sub> receptor and VGLUT1 proteins), with donkey anti-rabbit AlexaFluor-488 with goat anti-mouse AlexaFluor-633 (1:1000 both, confocal analysis aimed at identifying colocalization of mGlu2/3 and 5-HT<sub>2A</sub> receptor proteins). Synaptosomes were then applied onto coverslips (Musante et al., 2008b).

## 2.8. Confocal microscopy and colocalization

Fluorescent images acquisition was performed by a six-channel Leica TCS SP2 confocal microscope equipped with the LCS software package. Images were taken through a plan-apochromatic oil immersion objective 63x/NA1.4. Light collection configuration was optimized according to the combination of chosen fluorochromes, and sequential-channel acquisition was performed to avoid cross-talk phenomena. The quantitative estimation of colocalized proteins was performed as already described (Summa et al., 2011; Zappettini et al., 2014).

## 2.9. Calculations and statistical analysis

Multiple comparisons were performed with analysis of variance (ANOVA) followed by Dunnett's test or Newman Keuls multiple-comparisons test, as appropriate; direct comparisons were executed by Student's *t*-test. Data were considered significant for  $P < 0.05$  at least. Experiments were always carried out to quantify the effects of antagonists alone on the 15 mM KCl-evoked release of [<sup>3</sup>H]D-Asp.

## 2.10. Chemicals

[2,3-<sup>3</sup>H]D-Asp (specific activity 11.3 Ci/mmol) was from Perkin Elmer (Boston, MA, USA). LY379268 and LY341495 were purchased from Tocris Bioscience (Bristol, UK). (±)-1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride, (±)-2,5-Dimethoxy-4-iodoamphetamine hydrochloride ((±)DOI), MDL11,939, ketanserin, MDL100907, trazodone, mouse anti-β-actin, horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were from Sigma (Milan, Italy). Sulfo-NHS-SS-biotin, Tris (2-carboxyethyl)phosphine hydrochloride (TCEP), Neutravidin agarose beads were purchased from Pierce Thermo Scientific (Rockford, IL, USA). Western blotting detection system was purchased from GeHealthcare (Italy). Rabbit anti-mGlu2/3 was from Novus Biologicals (Littleton CO, USA), rabbit anti-5-HT<sub>2A</sub> antibody was from

Immunostar (Hudson, WI, USA), mouse anti-5-HT<sub>2A</sub> receptor was from Santa Cruz Biotechnology (Dallas, Texas, USA), guinea pig anti-vesicular glutamate transporters type 1 (VGLUT1) were from Millipore Corporation, (Billerica, MA, USA), Protein A Dynabeads were from Invitrogen, (Life Technologies, Milano, Italy). Donkey anti-rabbit AlexaFluor-488, goat anti guinea pig AlexaFluor 633, donkey anti-mouse AlexaFluor-488 and goat anti-mouse AlexaFluor-633 were from Life Technologies Corporation, (Carlsbad, CA, USA).

### 3. Results

#### *3.1. Presynaptic release-regulating 5-HT<sub>2A</sub> heteroreceptors exist in rat spinal cord glutamatergic nerve endings*

Spinal cord nerve endings from rats were preloaded with [<sup>3</sup>H]D-Asp, a non-metabolizable glutamate analogue routinely used as a marker for the endogenous aminoacid in release studies (Grilli et al., 2004; Di Prisco et al., 2016, 2012). The transient exposure of synaptosomes to a mild depolarizing stimulus (15 mM KCl) elicits a significant release of the preloaded [<sup>3</sup>H]D-Asp, that occurs in a Ca<sup>2+</sup> dependent, exocytotic-like manner. Actually, lowering the external concentration of calcium ions in the superfusion medium drastically reduces the tritium overflow [15 mM KCl / 1.2 mM CaCl<sub>2</sub>: 1.58 ± 0.23; 15 mM KCl / 0.1 mM CaCl<sub>2</sub>: 0.19 ± 0.10, *n* = 4 experiments run in triplicate (i.e. three superfusion chambers for each experimental condition) results expressed as KCl-induced overflow, *P* < 0.01].

The effect of the selective 5-HT<sub>2A</sub> agonist (±)DOI on the 15 mM KCl-evoked release of [<sup>3</sup>H]D-Asp from rat spinal cord synaptosomes was investigated. (±)DOI inhibited the [<sup>3</sup>H]D-Asp exocytosis evoked by 15 mM KCl in a concentration-dependent manner (Fig 1A). A significant reduction of tritium exocytosis (-34.12 ± 4.20 %) was observed when 100 nM (±)DOI was added concomitantly to the depolarizing stimulus. The inhibitory effect relied on the activation of presynaptic 5-HT<sub>2A</sub> heteroreceptors, as suggested by the efficacy of the 5-HT<sub>2A</sub> antagonist MDL11,939 (Fig. 1A) to prevent the (±)DOI-induced reduction of tritium exocytosis. At the concentration applied, the antagonist failed to modify on its own the 15 mM KCl-evoked release of preloaded [<sup>3</sup>H]D-Asp (not shown).

The existence of the 5-HT<sub>2A</sub> receptors in spinal cord nerve terminals was confirmed by Western blot analysis, which showed a 5-HT<sub>2A</sub>- immunopositive component in the synaptosomal lysate, with a mass compatible with the homomeric assembly of the receptors (Fig. 1B).

This observation indicates that spinal cord synaptosomes possess 5-HT<sub>2A</sub> receptors, but does not permit to define whether the glutamatergic terminals express these proteins. To address this point, spinal cord glutamatergic particles were identified in confocal microscopy by using a selective antibody recognizing the VGLUT1. An important percentage of VGLUT1-positive particles ( $78 \pm 5 \%$ , Fig. 2, panel a, red) was immunopositive for the 5-HT<sub>2A</sub> receptor protein (Fig. 2, panel b, green), confirming the existence of these receptors in spinal cord glutamatergic presynaptic terminals (Fig. 2, panel c, yellow, merge).

### *3.2. Presynaptic release-regulating LY379268-sensitive mGlu2/3 autoreceptors exist in rat spinal cord glutamatergic nerve endings.*

The existence of presynaptic inhibitory mGlu2/3 receptor subtypes in spinal cord glutamatergic terminals is largely described in the literature (Cartmell and Schoepp, 2000 and references therein; Di Prisco et al., 2016). To the best of our knowledge, however, the effect of LY379268 on glutamate release from rat spinal cord synaptosomes was not so far investigated. Experiments were therefore carried out to fill this gap.

LY379268 inhibited in a concentration-dependent manner the 15 mM KCl-evoked release of [<sup>3</sup>H]D-Asp (Fig. 3A). The agonist efficiently reduced the [<sup>3</sup>H]D-Asp overflow (apparent EC<sub>50</sub> =  $7.36 \pm 3.49$  pM; maximal inhibition:  $54.32 \pm 5.8 \%$ , reached at 1 nM) and the inhibitory effect was prevented by the mGlu2/3 broad spectrum antagonist LY341495 (1-10 nM). At the concentration applied, the antagonist failed to modify on its own the 15 mM KCl-evoked release of preloaded [<sup>3</sup>H]D-Asp (not shown).

Western blot analysis confirmed the presence of mGlu2/3 receptor protein in the spinal cord synaptosomal lysate. An antibody raised against the mGlu2/3 receptor protein recognized a component with a mass compatible with the dimeric assembly of the receptor (Fig. 3B). Inasmuch, confocal microscopy showed that VGLUT1-positive glutamatergic particles (Fig. 4, panel a, red)

are immunopositive for mGlu2/3 staining (Fig. 4, panel b, green). About  $77 \pm 6$  % of the VGLUT1 positive particles were also positive for mGlu2/3 receptor (Fig. 4, panel c, merge, yellow).

### *3.3. Presynaptic release-regulating mGlu2/3 autoreceptors and 5-HT<sub>2A</sub> heteroreceptors functionally interact in rat spinal cord glutamatergic nerve endings*

Figure 5A shows that LY379268 (0.1 nM) and ( $\pm$ )DOI (100 nM) inhibited the 15 mM KCl-evoked release of [<sup>3</sup>H]D-Asp from spinal cord synaptosomes ( $-28.5 \pm 7.1$  and  $-27.3 \pm 4.3$ , respectively). When concomitantly added, however, the agonists failed to reduce significantly the 15 mM KCl-evoked [<sup>3</sup>H]D-Asp release. These observations suggest that 5-HT<sub>2A</sub> and mGlu2/3 receptors influence one each other, nulling their efficacy to control glutamate exocytosis.

### *3.4. Presynaptic release-regulating mGlu2/3 autoreceptors and 5-HT<sub>2A</sub> heteroreceptors colocalize and physically interact in rat spinal cord glutamatergic nerve endings*

The functional cross-talk described above suggests that mGlu2/3 and 5-HT<sub>2A</sub> receptors colocalize and functionally cross talk in spinal cord glutamatergic nerve terminals. This hypothesis was confirmed by confocal microscopy showing that a significant percentage of mGlu2/3-immunopositive particles (Fig. 5B, panel b green) is also immunopositive for 5-HT<sub>2A</sub> (Fig. 5B, panel a, red), as highlighted by the merge staining (Fig. 5B, panel c, yellow). Confocal analysis revealed that about  $67 \pm 4$  % of the mGlu2/3 positive particles was positive for 5-HT<sub>2A</sub> immunostaining and, conversely, that about  $59 \pm 6$  % of the 5-HT<sub>2A</sub> positive particles was positive for mGlu2/3 immunostaining.

Furthermore, in mGlu2/3 receptor immunoprecipitates from spinal cord synaptosomes a band corresponding to the dimeric form of the mGlu2/3 protein ( $\sim 200$  kDa, Fig. 5C, mGlu2/3 i.p., W.B.: mGlu2/3) as well as a band with a mass corresponding to the homomeric assembly of the 5-HT<sub>2A</sub> protein (Fig. 5C,  $\sim 60$  kDa, mGlu2/3 i.p., W.B.: 5-HT<sub>2A</sub>) was recognized. To note,

precipitation of synaptosomal lysate with Protein A Dynabeads alone did not yield any specific bands at the above-indicated masses (Fig. 5C).

### *3.5. The 5-HT<sub>2A</sub> receptor antagonist MDL11,939 favours the mGlu2/3 autoreceptor-mediated presynaptic control of [<sup>3</sup>H]D-Asp release in rat spinal cord glutamatergic nerve endings*

The impact of selective 5-HT<sub>2A</sub> antagonist on the LY379268-mediated inhibition of [<sup>3</sup>H]D-Asp exocytosis was evaluated. Synaptosomes were superfused for 20 min with a medium containing the 5-HT<sub>2A</sub> antagonist MDL11,939 (100 nM) and then they were transiently exposed (90 seconds) to 15 mM KCl-containing solution in the absence or in the presence of LY379268 (1 and 3 pM, i.e., those concentrations unable to modify significantly the 15 mM KCl-evoked [<sup>3</sup>H]D-Asp exocytosis from spinal cord synaptosomes, see Fig. 3A). A large, significant inhibition of glutamate exocytosis was observed in synaptosomes pre-exposed to the 5-HT<sub>2A</sub> antagonist and then depolarized with the 15 mM KCl medium containing 1 or 3 pM LY379268 (Fig. 6A). The inhibitory effects were significantly higher than that elicited by the mGlu2/3 agonist in synaptosomes that were not exposed to the 5-HT<sub>2A</sub> antagonist. Differently, when added at 100 pM, LY379268 elicited a significant reduction of the [<sup>3</sup>H]D-Asp exocytosis ( $-33 \pm 10$ ) that was not modified by 100 nM MDL11,939 ( $-44 \pm 6$ , Fig. 6A).

### *3.6. Blockade of presynaptic release-regulating 5-HT<sub>2A</sub> heteroreceptors with MDL11,939 alters the expression of mGlu2/3 autoreceptor protein in rat spinal cord synaptosomal plasmamembranes*

The surface levels of mGlu2/3 subunit proteins in the plasmamembranes of spinal cord synaptosomes exposed to MDL11,939 for 20 min was quantified. Figure 6B shows that exposure of synaptosomes to MDL11,939 caused a significant increase (Fig. 6C) of the biotin-tagged mGlu2/3 receptor protein (Fig. 6B, lane T) in synaptosomal plasma membranes when compared to control (Fig. 6B, lane C).

### *3.7. MDL100907, ketanserin and trazodone favours the mGlu2/3 autoreceptor-mediated presynaptic control of preloaded [<sup>3</sup>H]D-Asp in rat spinal cord glutamatergic nerve endings*

The effect of the selective 5-HT<sub>2A</sub> antagonist MDL100907 on the release of preloaded [<sup>3</sup>H]D-Asp from spinal cord synaptosomes was also investigated. The exposure of isolated nerve endings (for 20 min before the depolarizing stimulus) to the antagonist prevented the 100 nM (±)DOI-induced inhibition of the 15 mM KCl-evoked release of [<sup>3</sup>H]D-Asp in a concentration-dependent fashion (Fig. 7A). Inasmuch, 100 nM MDL100907 (i.e., the concentration of antagonist causing the maximal reversion of the (±)DOI-induced inhibition of the 15 mM KCl-evoked release of [<sup>3</sup>H]D-Asp, Fig. 1A) unveiled a LY379268-mediated inhibition of [<sup>3</sup>H]D-Asp exocytosis that could not be observed in untreated synaptosomes (Fig. 7B). Similarly, ketanserin (1-10 nM) efficiently prevented the 100 nM (±)DOI-induced inhibition of the 15 mM KCl-evoked release of [<sup>3</sup>H]D-Asp from rat spinal cord synaptosomes (Fig. 8A), but disclosed the inhibitory control of presynaptic mGlu2/3 autoreceptors on [<sup>3</sup>H]D-Asp exocytosis when synaptosomes were exposed to 3 pM LY379268 (Fig. 8B).

Finally, TZD (0.1-1 nM) prevented the inhibitory effect exerted by 100 nM (±)DOI on the 15 mM KCl-evoked [<sup>3</sup>H]D-Asp exocytosis (Fig. 9A). The drug also unveiled the LY379268-mediated control of glutamate exocytosis when the agonist was added at concentration per se inactive (3 pM, Fig. 9B). The expression of mGlu2/3 receptor proteins in synaptosomal plasmamembranes was increased in TZD-treated synaptosomal plasmamembranes (Fig. 9C and D). Ketanserin, MDL100907, MDL11,939 and TZD did not significantly- affected the [<sup>3</sup>H]D-Asp exocytosis from spinal cord synaptosomes.

### *3.8. Antagonists acting at 5-HT receptor(s) other than the 5-HT<sub>2A</sub> receptor subtype do not favour the mGlu2/3 autoreceptor-mediated presynaptic control of preloaded [<sup>3</sup>H]D-Aspartate in rat spinal cord glutamatergic nerve endings*

In order to evaluate whether 5-HT receptor subtypes other than the 5-HT<sub>2A</sub> ones could affect the mGlu2/3-mediated control of glutamate overflow, experiments were carried out to investigate the impact of WAY100635 (a selective 5-HT<sub>1A</sub> antagonist), SB224289 (a selective 5-HT<sub>1B</sub> antagonist) and SB714786 (a selective 5-HT<sub>1D</sub> antagonist) on the LY379268-mediated inhibition of the 15 mM KCl-evoked [<sup>3</sup>H]D-Asp exocytosis. We focused on these compounds since their cognate receptors have a presynaptic location in CNS and control transmitter exocytosis (Maura et al., 1986, 1993, 1998; Matsumoto et al., 1992; Dergacheva et al., 2011). Table 1 shows that WAY100635 and SB224289 failed to affect significantly the 15 mM KCl-evoked [<sup>3</sup>H]D-Asp exocytosis from rat spinal cord synaptosomes, while SB714786 significantly inhibited it. Furthermore, WAY100635 and SB224289 did not significantly modify the 15 mM KCl / 3 pM LY379268-induced of [<sup>3</sup>H]D-Asp exocytosis (Table 1), while SB714786 significantly increased it. Further studies are required to investigate the molecular events accounting for the latter effect.

## **Discussion**

The first result of this study is that spinal cord glutamatergic nerve endings are endowed with presynaptic release-regulating 5-HT<sub>2A</sub> heteroreceptors. This conclusion relies on functional observations showing that the 5-HT<sub>2A</sub> agonist (±)DOI concentration-dependently inhibited [<sup>3</sup>H]D-aspartate exocytosis, and that the 5-HT<sub>2A</sub> antagonists MDL11,939, MDL100907, ketanserin and TZD efficiently prevented the (±)DOI-induced control of glutamate exocytosis. These results were obtained by using an experimental approach, the “up-down superfusion of a synaptosomal monolayer”, widely recognised as a method of choice to monitor transmitter release and its regulation by presynaptic receptors. The continuous up-down superfusion of the synaptosomal monolayer assures the rapid removal of any endogenous substances released by superfused particles, limiting the indirect effects due to the presence of the biophase. In these experimental conditions, the finding that agonist(s) acting at the receptor A cause significant changes to transmitter outflow that are abolished by the concomitant application of antagonist(s) binding the same receptor (as indeed observed in our case) implies that the superfused particles possess presynaptic release-regulating receptors of the A subtype (Raiteri and Raiteri, 2000; Pittaluga, 2016). The conclusion that presynaptic release-regulating 5-HT<sub>2A</sub> heteroreceptors exists in spinal cord glutamatergic nerve endings is further supported by immunochemical analysis showing the expression of the protein in synaptosomal plasmamembranes and it is confirmed by confocal microscopy unveiling a diffused 5-HT<sub>2A</sub> staining in VGLUT1 positive particles.

The presynaptic location of the 5-HT<sub>2A</sub> receptors deserves a brief comment. Actually, despite the general consensus that 5-HT<sub>2A</sub> receptors preferentially locate postsynaptically (Aghajanian and Marek, 1997), the existence of 5-HT<sub>2A</sub> receptor proteins on glutamatergic axon terminals in CNS was proposed on the basis of results from electron microscopic analysis (Huang and Pickel, 2003) and of functional data in literature proving the existence of presynaptic release-regulating 5-HT<sub>2A</sub> receptors (Marcoli et al., 2001; Wang et al., 2006).

The inhibitory activity of the presynaptic 5-HT<sub>2A</sub> heteroreceptors also requires some discussion. The existence of presynaptic 5-HT<sub>2A</sub> receptors inhibiting glutamate exocytosis has been already reported in literature (Marcoli et al., 2001; Wang et al., 2006). This conclusion, however, contrasts the data from electrophysiological studies suggesting that 5-HT<sub>2A</sub> receptors preferentially favour glutamate exocytosis (Aghajanian and Marek, 1997; Marek et al., 2000). Two are the main explanations that could be proposed to reconcile these contradictory observations. One explanation considers that the presynaptic inhibitory 5-HT<sub>2A</sub> heteroreceptors on glutamatergic nerve endings represent a small percentage of the entire receptor population, that hardly can emerge in electrophysiological studies. Alternatively, the possibility exists that, at the presynaptic level, 5-HT<sub>2A</sub> heteroreceptors could switch from a facilitatory to an inhibitory mode of action depending on the depolarizing status of the synaptosomal plasmamembrane, as already observed for other receptors (Musante et al., 2008a; Di Prisco et al., 2012).

In the last decade, evidence have been provided proving the functional interaction bridging mGlu2/3 and 5-HT<sub>2A</sub> receptors in the prefrontal cortex of mammals (Delille et al., 2013; Wischhof and Kock, 2016). Besides 5-HT<sub>2A</sub> receptors, rat spinal cord glutamatergic terminals also are endowed with mGlu2/3 autoreceptors. The second main finding of our research is that presynaptic release-regulating mGlu2/3 and 5-HT<sub>2A</sub> receptors functionally interact in this CNS region.

Again, this conclusion was first proposed on the basis of results from release studies, showing that the inhibitory effect elicited by either LY379268 (0.1 nM, i.e., the concentration causing a submaximal inhibition of [<sup>3</sup>H]D-aspartate exocytosis) or (±)DOI (100 nM, the concentration eliciting the maximal inhibition of the 15 mM KCl-evoked release of [<sup>3</sup>H]D-aspartate) was almost abrogated when the two agonists were added concomitantly. The loss of function of both agonists is best interpreted by assuming the co-localization and the functional cross-talk of the two receptors. Actually, if localized on different nerve terminals or, alternatively, if present on the same terminals but functionally uncoupled, the concomitant activation of the two receptors should lead to an inhibitory effect more pronounced than that triggered by the activation

of each one (Longordo et al., 2006, Luccini et al., 2007, Musante et al., 2008b; Pittaluga, 2016). This was not however our case, since the concomitant activation of the two receptors nulled the presynaptic release-regulating activity of the two agonists. The proposed receptor-receptor functional interaction is further supported by results from confocal microscopy showing a large colocalization of mGlu2/3 and 5-HT<sub>2A</sub> receptor proteins in spinal cord synaptosomal particles and by immunoprecipitation studies, demonstrating that mGlu2/3 immunoprecipitates from spinal cord synaptosomal plasmamembranes are also positive for 5-HT<sub>2A</sub> receptor immunostaining.

One possible explanation for the loss of function of the two agonists when concomitantly applied is that the binding of one agonist (i.e. (±)DOI) at its orthosteric site might cause structural changes to the colocalized receptor (the mGlu2/3 receptor in our case), influencing the binding of the other agonist (i.e. LY379268) at its binding site (as already proposed to occur in the cortex, Baki et al., 2016). Well in line with this hypothesis are data in literature showing that, in binding studies, a significant reduction of the affinity of mGlu2/3 agonist is observed in cultured cortical neurones concomitantly exposed to agonist acting at colocalized 5-HT<sub>2A</sub> receptor (González-Maeso et al., 2008).

If this is the case, antagonist(s) acting at one receptor should be expected to favor the action of orthosteric agonist(s) acting at the colocalized receptor. Accordingly to expectation, the third finding of our study was that concentration of LY379268 that were unable to inhibit [<sup>3</sup>H]D-aspartate exocytosis became efficacious when synaptosomes were pre-exposed to low concentration of MDL11,939, of MDL100907, TZD or ketanserin. The magnification of the LY379268-mediated control of glutamate exocytosis was evident when the agonist was added at low, almost ineffective, concentrations, but became less pronounced when the agonist was added at higher, submaximal, concentrations. Further studies are required to better address the latter observation. Last but not least, the pivotal role of 5-HT<sub>2A</sub> receptors in controlling the mGlu2/3-mediated functions was proven by the fact that antagonists acting at other presynaptic 5-HT receptor subtypes (namely the

5-HT<sub>1A</sub>, 1B and 1D) did not mimic 5-HT<sub>2A</sub> antagonists in controlling LY379268-mediated modulation of glutamate exocytosis.

To conclude, by combining the data from the functional studies with those obtained in immunocytochemistry and western blot analysis, we propose that, in rat spinal cord glutamatergic terminals, 5-HT<sub>2A</sub> heteroreceptors and mGlu2/3 autoreceptors physically associate and undergo functional adaptation when concomitantly activated. As already described to occur in the cortex (González-Maeso et al., 2008), the two receptors couple one each other in an antagonist-like manner, since blockade of one receptor favors the releasing activity of the other one.

Owing to identify a mechanism accounting for the 5-HT<sub>2A</sub> antagonist-induced facilitation of the mGlu2/3 receptor-mediated control of glutamate exocytosis, we hypothesized that drugs acting at 5-HT<sub>2A</sub> receptors could alter the insertion of mGlu2/3 receptors in synaptosomal plasmamembranes, as already observed to occur for other colocalized receptors (Pittaluga et al., 2005,2006; Grilli et al., 2009; Salamone et al., 2014; Zappettini et al., 2014). Accordingly to this view, the last finding of the present study is that exposure of synaptosomes to MDL11,939 and TZD causes a significant increase in the amount of mGlu2/3 receptor proteins in the plasmamembranes of synaptosomes. The increased expression of the mGlu2/3 receptor protein could be consistent with a rapid changes in the number (and maybe in the subunit composition) of presynaptic mGlu2/3 autoreceptors. This observation is particularly intriguing since it indirectly implies that i) a ready-releasable pool of mGlu2/3 receptor proteins exists in spinal cord glutamatergic nerve endings, ii) mGlu2/3 receptor proteins could traffic in-out terminal plasmamembranes, iii) antagonists acting at co-localized non-glutamatergic receptors can modulate these rapid in-out movements, controlling the insertion of mGlu2/3 receptors in plasmamembranes.

To note, at the spinal cord level, molecules acting as *indirect modulators* of mGlu2/3 receptors were reported to upregulate the expression of group II mGlu receptors through epigenetic mechanisms that favor the overexpression of mGlu2 receptor subunits. It is the case of N-acetyl cysteine, which causes a rapid and robust increase of mGlu2 receptor expression by inhibiting

histone deacetylase-mediated processes (Bernabucci et al., 2012) or of L-acetyl carnitine (Chiechio et al., 2002), which increases the expression of mGlu2 receptor subunits. Our results do not allow to conclude whether the gain of function of mGlu2/3 receptors elicited by 5-HT<sub>2A</sub> antagonists in spinal cord terminals could relies on an epigenetic phenomenon. The rapid onset of the event (20 minutes of exposure of synaptosomes to the 5-HT<sub>2A</sub> antagonist), however, seems incompatible with an epigenetic phenomenon. Furthermore, whether isolated nerve endings possess the machinery for the epigenetic control of mGlu2/3 expression has not so far been investigated. Further studies are required to address this point. Nonetheless, whatever the mechanisms accounting for the changes in the expression of the mGlu2/3 receptor proteins in synaptosomal plasmamembranes, our observations provide the first demonstration that 5-HT<sub>2A</sub> antagonists act as “*indirect positive allosteric modulator*” of presynaptic release-regulating mGlu2/3 autoreceptors in rat spinal cord.

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## Legend to the figures

**Fig. 1.** ( $\pm$ )DOI concentration-dependently inhibits the 15 mM KCl-evoked [ $^3$ H]D-Asp overflow from rat spinal cord nerve terminals: antagonism by MDL11,939. **A)** Synaptosomes were exposed to 15 mM KCl in the presence or in the absence of ( $\pm$ )DOI. When indicated, MDL11,939 was added 20 min before stimulus. Results are expressed as percentage of the 15 mM KCl-induced [ $^3$ H]D-Asp overflow (% of control). Data are the means  $\pm$  SEM of six to ten experiments run in triplicate (three superfusion chambers for each experimental condition). \*  $P < 0.05$  versus the 15 mM KCl-induced tritium overflow; #  $P < 0.05$  versus the 15 mM KCl / 100 nM ( $\pm$ )DOI-induced tritium overflow. **B)** 5-HT<sub>2A</sub> receptor proteins exist in rat spinal cord synaptosomes. Synaptosomal lysates were immunoblotted and probed with anti-5-HT<sub>2A</sub> and anti  $\beta$ -actin antibodies. The figure shows a representative Western blot of six analysis carried out in different days. Protein weight is expressed in kDalton.

**Fig. 2.** 5-HT<sub>2A</sub> receptor proteins exist in VGLUT1-positive synaptosomal particles isolated from the rat spinal cord. Glutamatergic synaptosomes were identified as VGLUT1-immunopositive particles (red, panel **a**) and they were analysed for the 5-HT<sub>2A</sub> receptor protein content (green, panel **b**; yellow, merge, panel **c**). The figure shows representative images of eight independent experiments carried out in different days.

**Fig. 3.** **A)** LY379268 concentration-dependently inhibits the 15 mM KCl-evoked [ $^3$ H]D-Asp overflow from rat spinal cord nerve terminals: antagonism by LY341495. Synaptosomes were exposed to 15 mM KCl in the presence or in the absence of LY379268. When indicated, LY341495 was added concomitantly to LY379268. Results are expressed as percentage of the 15 mM KCl-induced [ $^3$ H]D-Asp overflow (% of control). Data are the means  $\pm$  SEM of eight to twelve experiments run in triplicate \*  $P < 0.05$  versus the 15 mM KCl- induced tritium overflow; \*\*  $P <$

0.01 versus the 15 mM KCl- induced tritium overflow; <sup>#</sup>  $P < 0.05$  versus the 15 mM KCl / 0.1 nM LY379268-induced tritium overflow. **B)** mGlu2/3 receptor proteins exist in rat spinal cord synaptosomes. Synaptosomal lysates were immunoblotted and probed with anti-mGlu2/3 antibody and with anti  $\beta$ -actin antibodies. The figure shows a representative Western blot of six analysis. Protein weight is in kDalton.

**Fig. 4.** mGlu2/3 receptor proteins are present in VGLUT1 positive synaptosomal particles isolated from the rat spinal cord. Confocal microscopy unveiled a significant colocalization of VGLUT1 (red, panel **a**) and mGlu2/3 (green, panel **b**) immunopositivities (yellow, merge, panel **c**). The figure shows representative images of seven independent experiments carried out in different days.

**Fig. 5.** Presynaptic release-regulating mGlu2/3 autoreceptors and 5-HT<sub>2A</sub> heteroreceptors physically associate and functionally cooperate to control glutamate exocytosis from glutamatergic nerve endings isolated from the rat spinal cord. **A)** Effects of LY379268 (0.1 nM) and of ( $\pm$ )DOI (100 nM) alone or concomitantly added on the 15 mM KCl- induced [<sup>3</sup>H]D-Asp overflow from rat spinal cord nerve terminals. Results are expressed as percentage of the 15 mM KCl-induced [<sup>3</sup>H]D-Asp overflow (% of control). Data are the means  $\pm$  SEM of five experiments run in triplicate. \*  $P < 0.05$  versus the 15 mM KCl- induced tritium overflow; <sup>°</sup>  $P < 0.05$  versus the 15 mM KCl / 0.1 nM LY379268-induced tritium overflow; <sup>§</sup>  $P < 0.05$  versus the 15 mM KCl / 100 nM ( $\pm$ )DOI-induced tritium overflow. **B)** Confocal microscopy unveiled a significant colocalization of 5-HT<sub>2A</sub> (red, **a**) and mGlu2/3 (green, **b**) immunopositive signals in rat spinal cord (yellow, merge, **c**). The figure shows representative images of nine independent experiments carried out in different days. **C)** mGlu2/3 receptor protein were immunoprecipitated from spinal cord synaptosomal lysates (I.P.) and immunoprecipitates were analysed for their content in mGlu2/3 and 5-HT<sub>2A</sub> receptor proteins. Synaptosomal lysate were also precipitated with Protein A Dynabeads in the absence of the mGlu2/3 antibody (lane B). Synaptosomal lysate (lane L) were also used as control of Western blot

analysis. The figure shows a representative Western blot of ten analysis. Protein weight is in kDalton.

**Fig. 6.** The 5-HT<sub>2A</sub> receptor antagonist MDL11,939 favours the mGlu2/3 autoreceptor-mediated presynaptic control of preloaded [<sup>3</sup>H]D-Asp from glutamatergic nerve endings isolated from the rat spinal cord. **A)** Effect of MDL11,939 on the release of preloaded [<sup>3</sup>H]D-Asp elicited by 15 mM KCl in the presence of LY379268. Results are expressed as percentage of the 15 mM KCl-induced [<sup>3</sup>H]D-Asp overflow (% of control). Data are the means ± SEM of eight experiments run in triplicate. \* *P* < 0.05 versus the 15 mM KCl-induced tritium overflow; \*\* *P* < 0.01 versus the 15 mM KCl-induced tritium overflow; # *P* < 0.05 versus the 15 mM KCl in the presence of LY379268 (concentration as indicated). **B)** Representative Western blot of mGlu2/3 receptor protein surface density in spinal cord terminals. The Western blot compares total synaptosomal lysates (lane L), synaptosomal membranes that were not treated with biotin and were subject to streptavidin pull-down (lane B), synaptosomal membranes incubated with biotin and subject to streptavidin pull-down (lane C) and MDL11,939 pre-treated synaptosomal membranes incubated with biotin and subject to a streptavidin pull-down (lane T). The blot is representative of six different experiments carried out in different days with synaptosomal preparations from different rats. **C)** Changes in mGlu2/3 receptor protein surface density in MDL11,939-treated (grey bar) synaptosomal plasmamembranes when compared to untreated particles. \*\* *P* < 0.01 versus control.

**Fig. 7.** Effect of MDL100907 on the release of preloaded [<sup>3</sup>H]D-Asp elicited by 15 mM KCl in the presence 5-HT<sub>2A</sub> (**A**) and mGlu2/3(**B**) agonists. Results are expressed as percentage of the 15 mM KCl-induced [<sup>3</sup>H]D-Asp overflow (% of control). Data are the means ± SEM of seven (**A**) to ten (**B**) experiments run in triplicate. \* *P* < 0.05 versus the 15 mM KCl-induced tritium overflow; # *P* < 0.05 versus the 15 mM KCl-induced tritium overflow in the presence of (±)DOI (concentration as indicated);

**Fig. 8.** Effect of ketanserin on the release of preloaded [<sup>3</sup>H]D-Asp elicited by 15 mM KCl in the presence 5-HT<sub>2A</sub> (A) and mGlu2/3(B) agonists. Results are expressed as percentage of the 15 mM KCl-induced [<sup>3</sup>H]D-Asp overflow (% of control). Data are the means ± SEM of seven (A) to ten (B) experiments run in triplicate. \* *P* < 0.05 versus the 15 mM KCl-induced tritium overflow; \*\* *P* < 0.01 versus the 15 mM KCl-induced tritium overflow; # *P* < 0.05 versus the 15 mM KCl-induced tritium overflow in the presence of (±)DOI (concentration as indicated)..

**Fig. 9.** Effects of trazodone (TZD) on the release of preloaded [<sup>3</sup>H]D-Asp elicited by 15 mM KCl in the presence 5-HT<sub>2A</sub> (A) and mGlu2/3(B) agonists. Results are expressed as percentage of the 15 mM KCl-induced [<sup>3</sup>H]D-Asp overflow (% of control). Data are the means ± SEM of six experiments for each experimental conditions run in triplicate. \* *P* < 0.05 versus the 15 mM KCl-induced tritium overflow; # *P* < 0.05 versus the 15 mM KCl/ 3 pM LY379268-induced tritium overflow. C) Representative Western blot of mGlu2/3 receptor protein surface density in spinal cord terminals. The Western blot compares total synaptosomal lysates (line L), synaptosomal membranes that were not treated with biotin and were subject to a streptavidin pull-down (line B), synaptosomal membranes incubated with biotin and subject to a streptavidin pull-down (line C) and trazodone pre-treated synaptosomal membranes incubated with biotin and subject to a streptavidin pull-down (line T). The blots are representative of six different experiments carried out with synaptosomal preparations from different rats. D) Quantification of the changes in mGlu2/3 receptor protein surface density in trazodone-treated (grey bar) synaptosomal plasmamembranes when compared to untreated ones. \*\* *P* < 0.01 versus respective control.

**Table 1.**

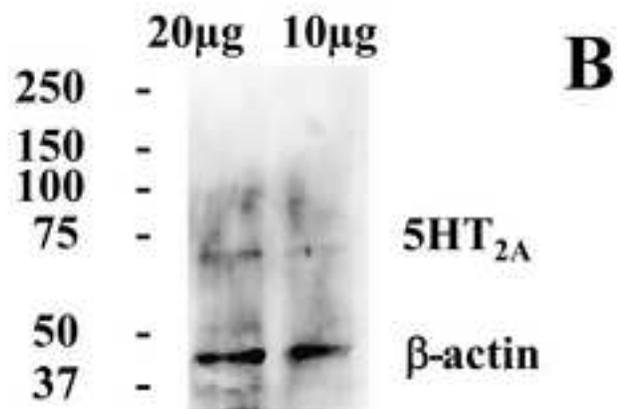
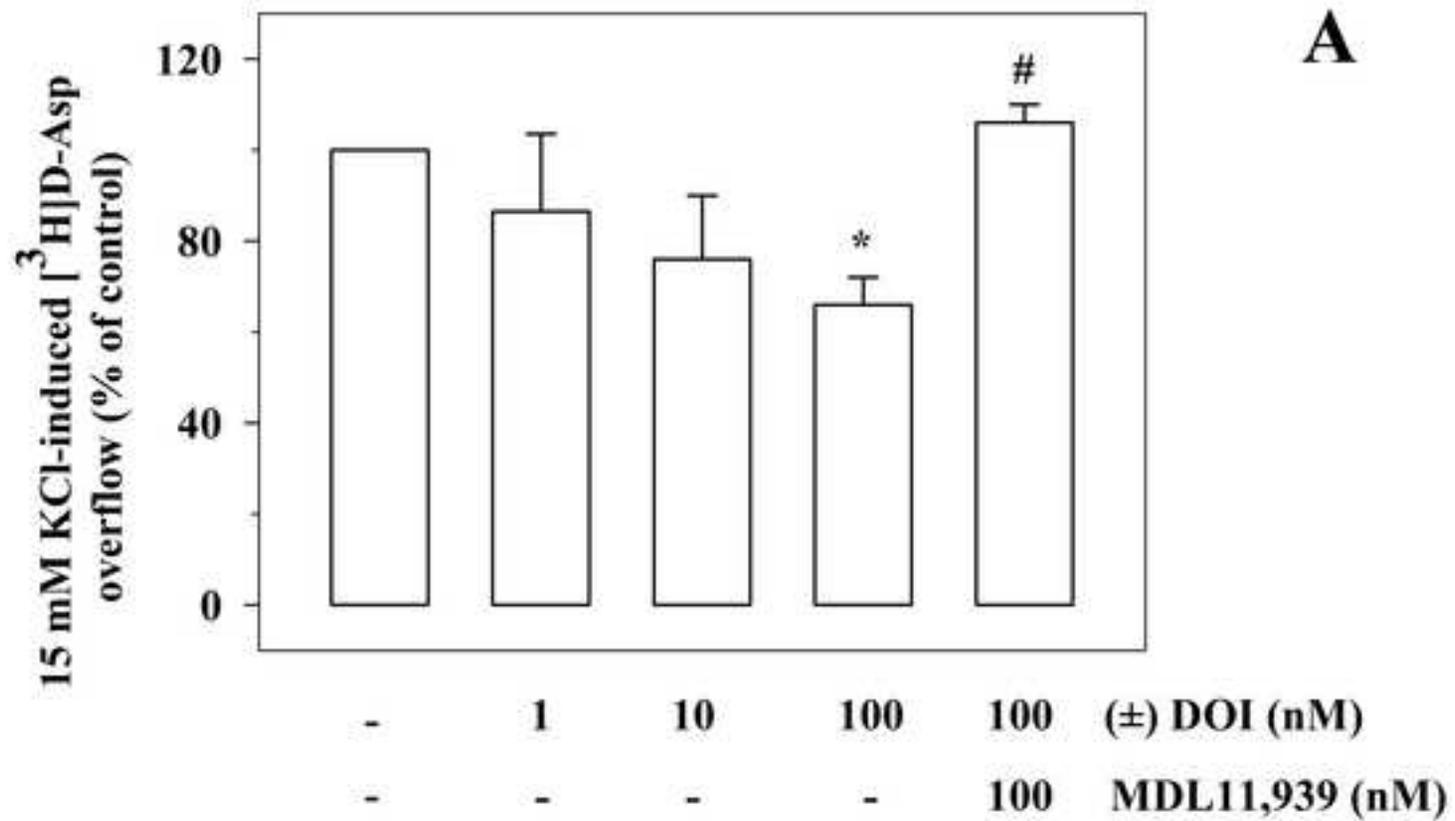
**5-HT receptor(s) other than 5-HT<sub>2A</sub> receptor subtypes does not mimic TZD in controlling the mGlu2/3 receptor-induced presynaptic activity**

	Control	+ LY379268 (3pM)
15 mM KCl	100	76 ± 16
WAY100635 (100 nM)	85 ± 22	97 ± 12
SB224289 (1 nM)	92 ± 10	114 ± 22
SB714786 (100 nM)	80 ± 4*	122 ± 6 <sup>#</sup>

Synaptosomes were exposed in superfusion to the following compounds: WAY100635 (a quite selective 5-HT<sub>1A</sub> antagonist), SB224289 (a selective 5-HT<sub>1B</sub> antagonist) and SB714786 (a selective 5-HT<sub>1D</sub> antagonist) and then they were transiently depolarized with 15 mM KCl-containing medium in the absence (control) or in the presence of the mGlu2/3 agonist, the compound LY379268 (3 pM). Results are expressed as percent residual of the KCl-evoked overflow of tritium. Data originate from 5 experiments run in triplicate (three superfusion chambers for each experimental condition). \*  $P < 0.05$  versus control; <sup>#</sup>  $P < 0.05$  versus 15 mM KCl plus LY379268 (3 pM).

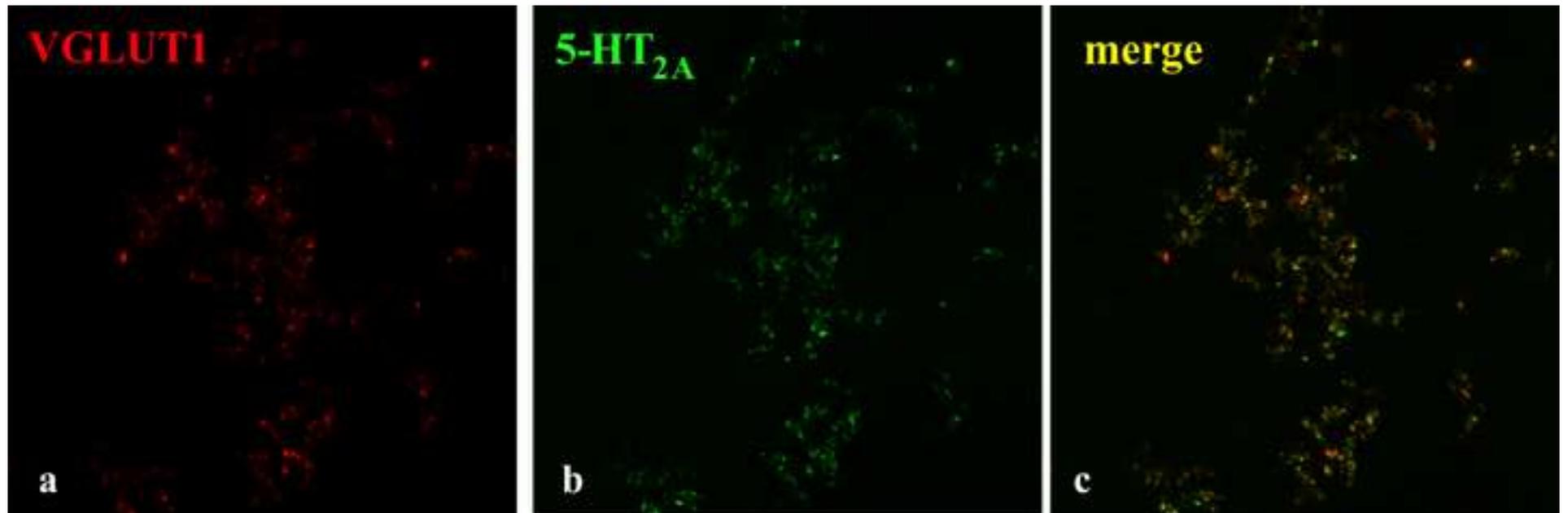
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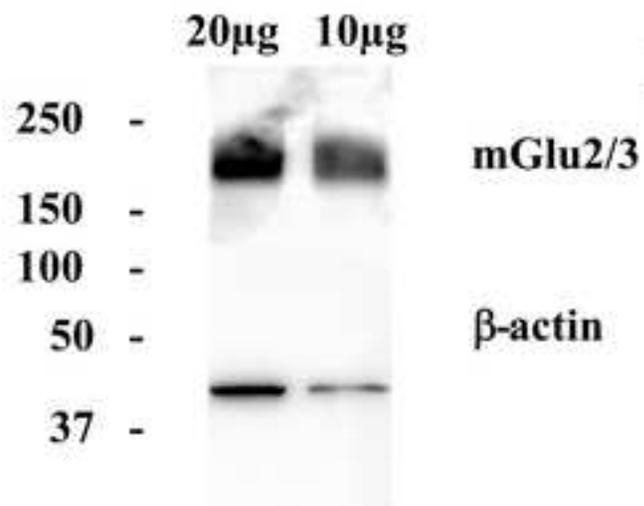
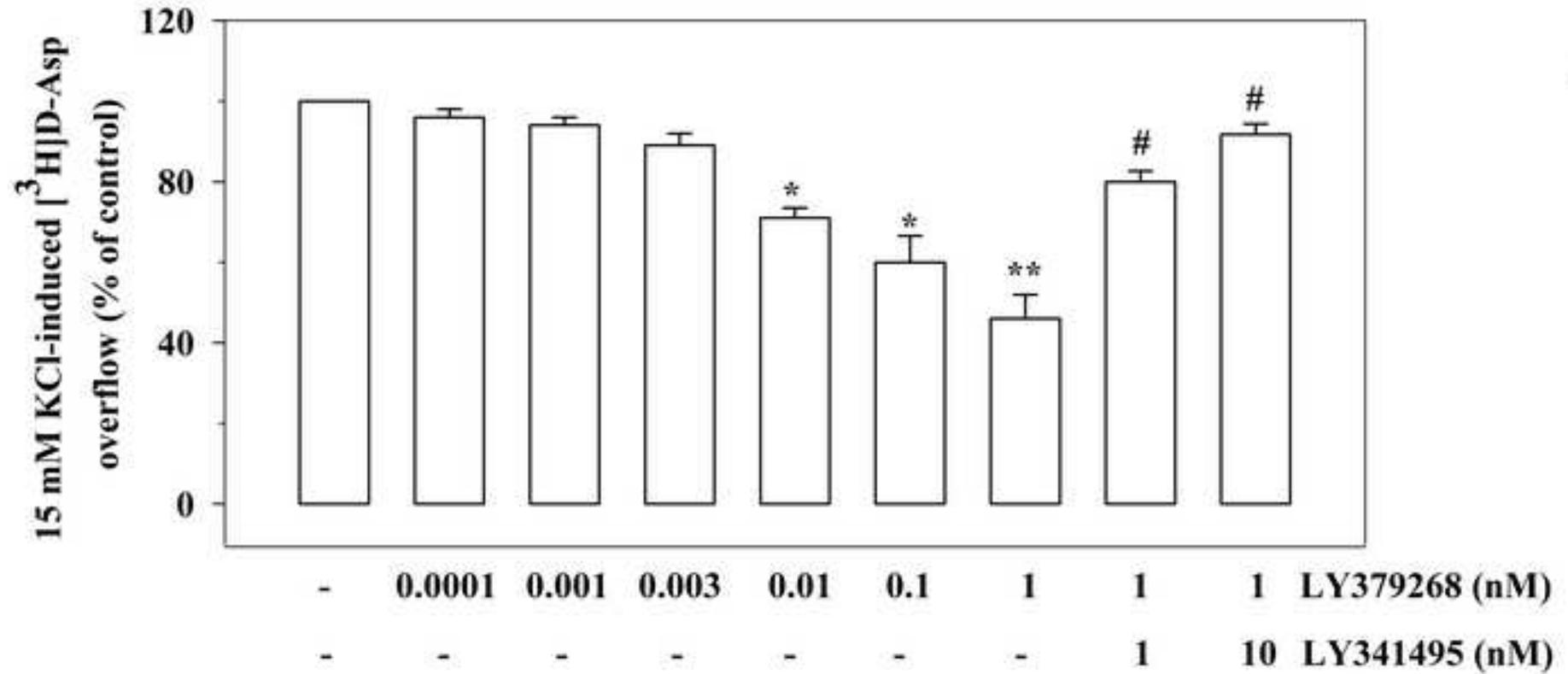
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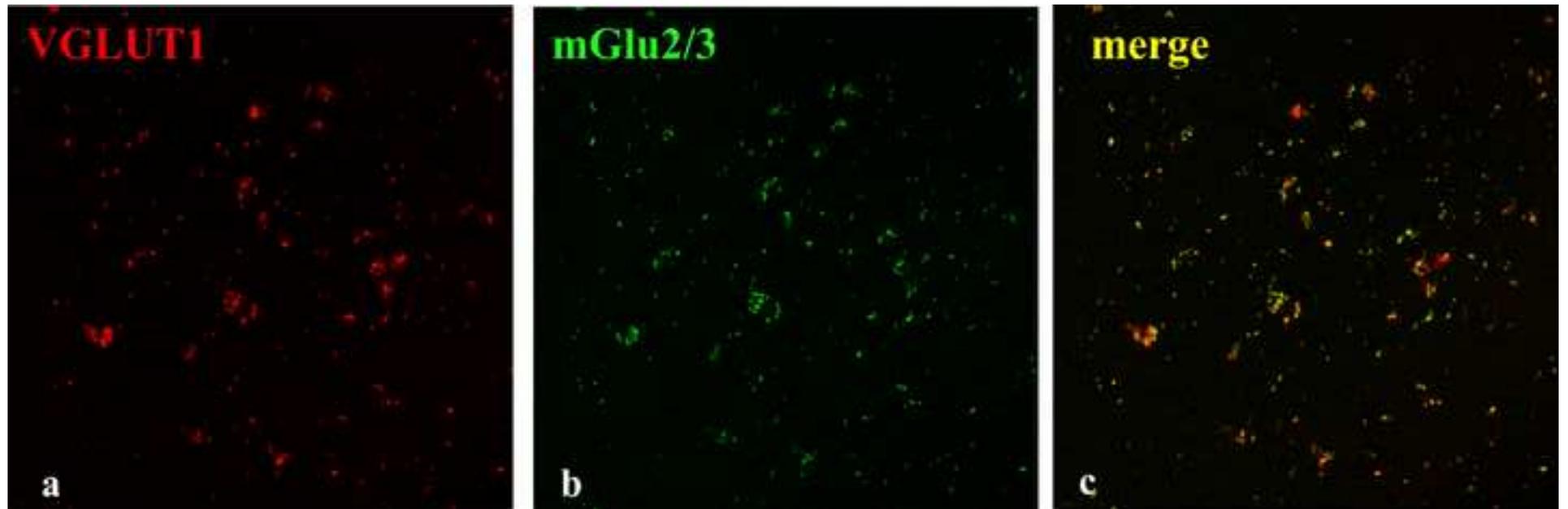
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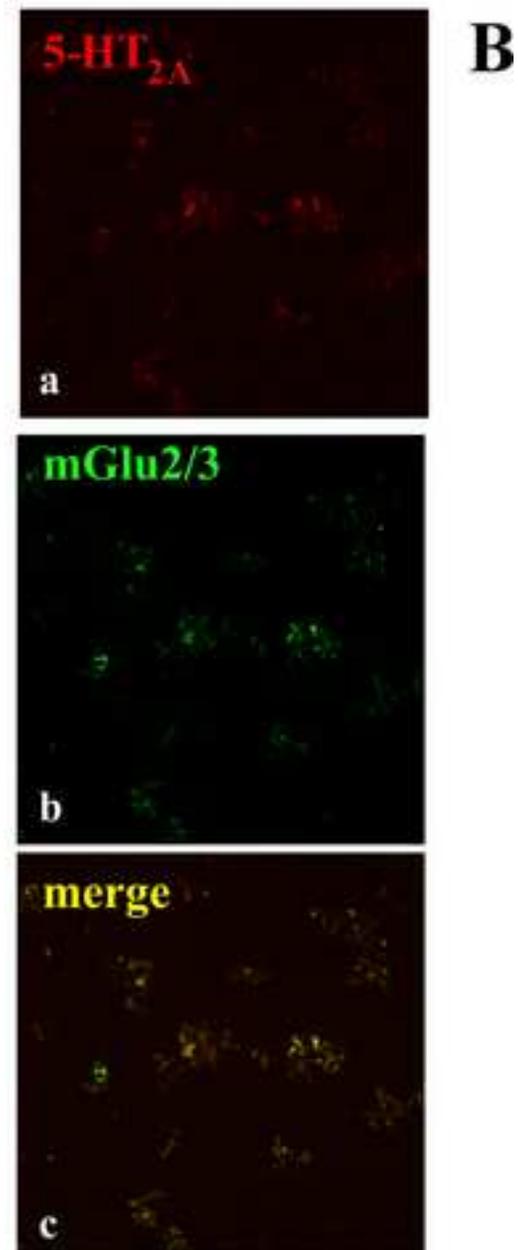
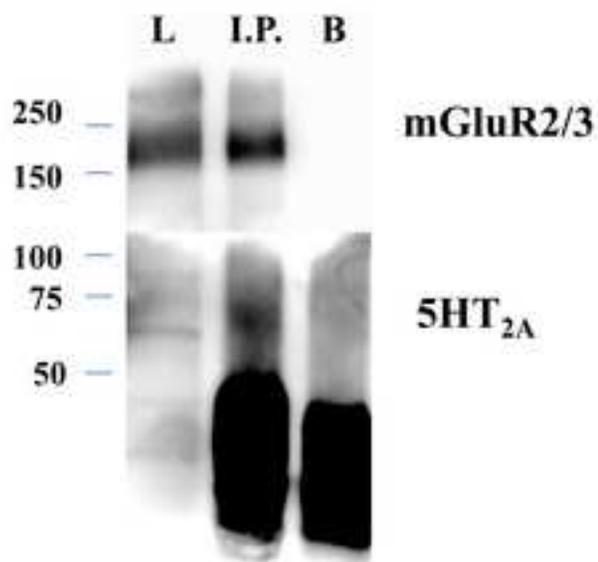
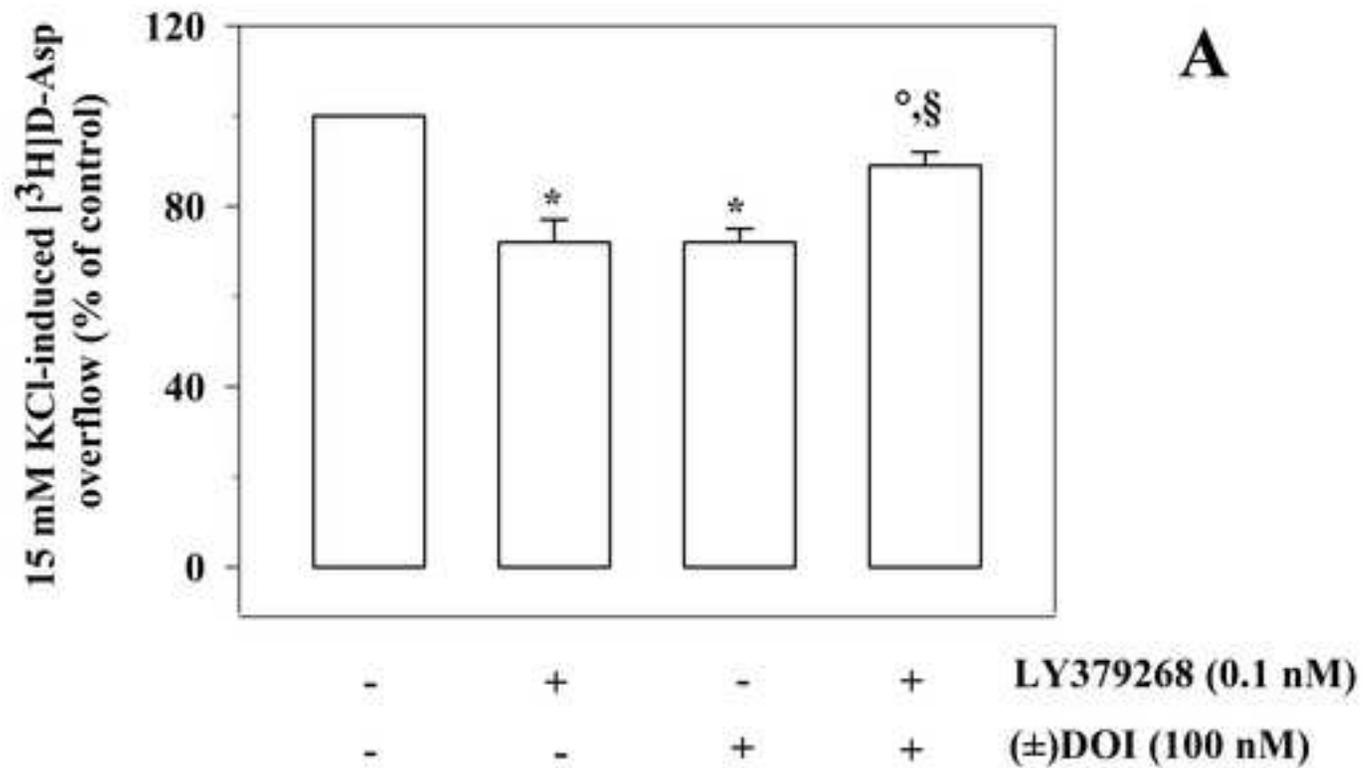
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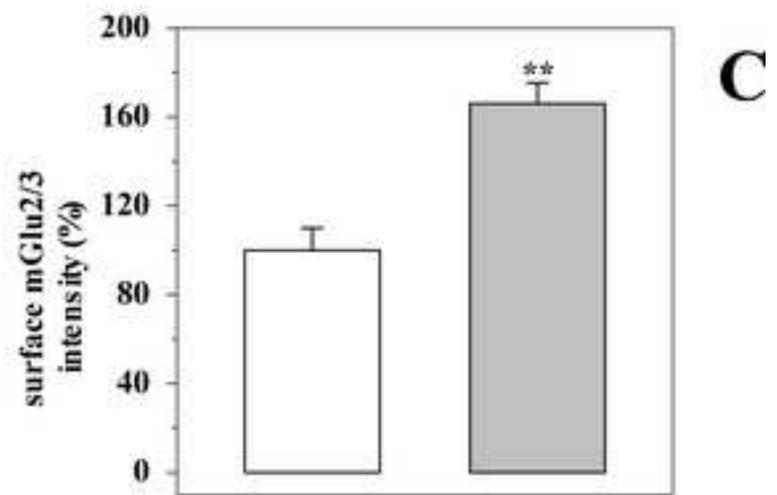
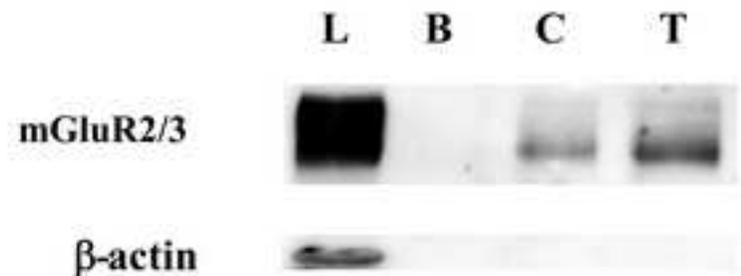
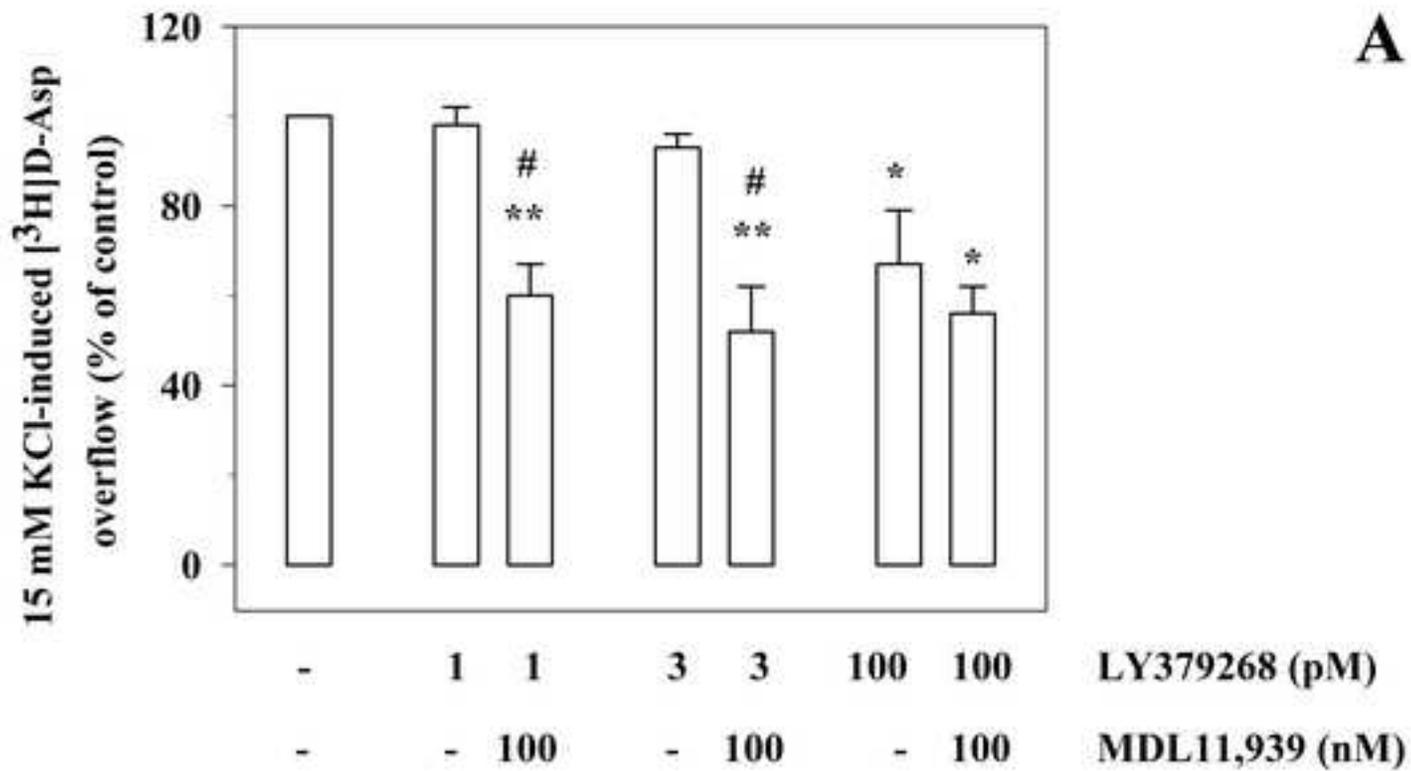


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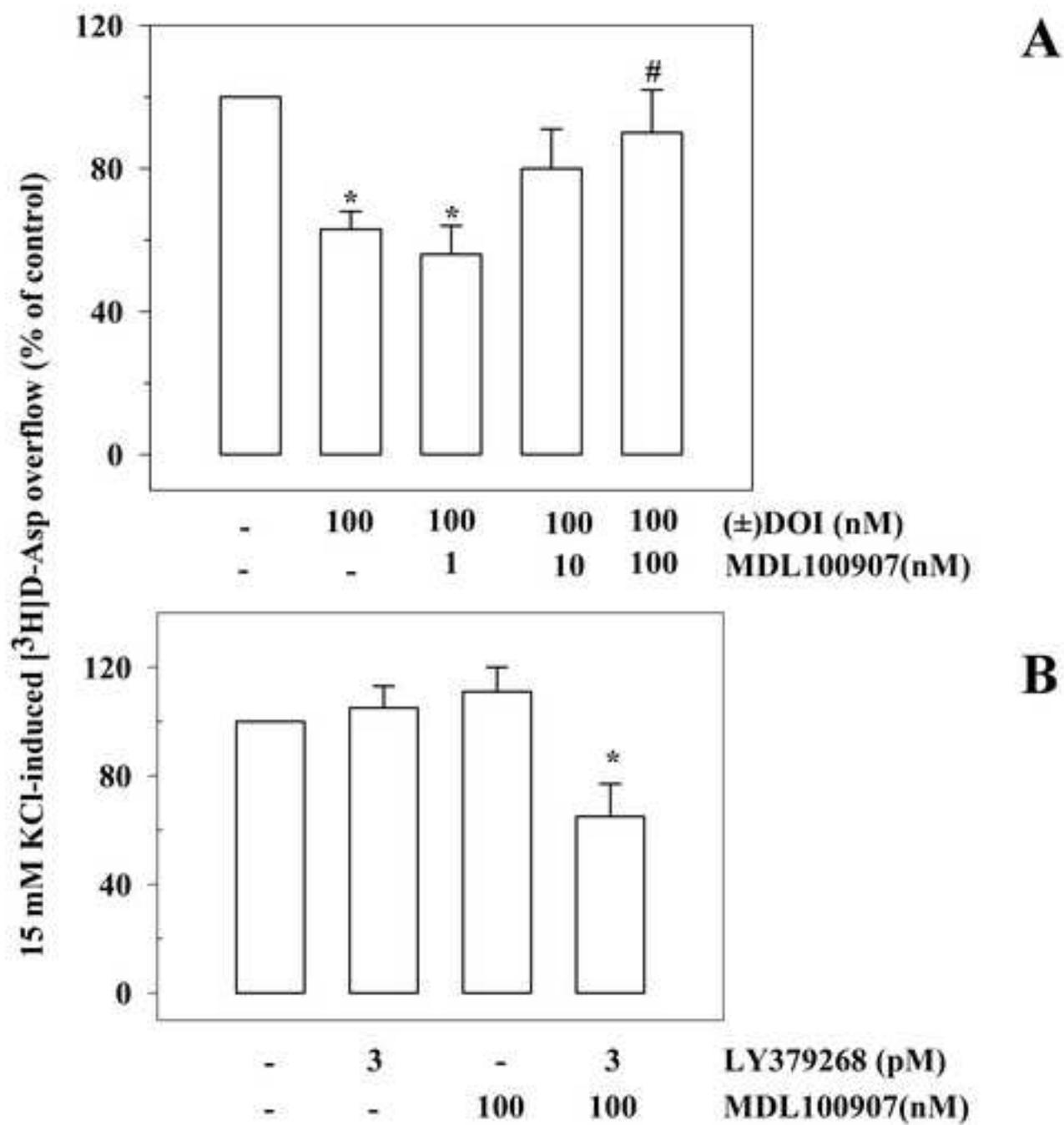


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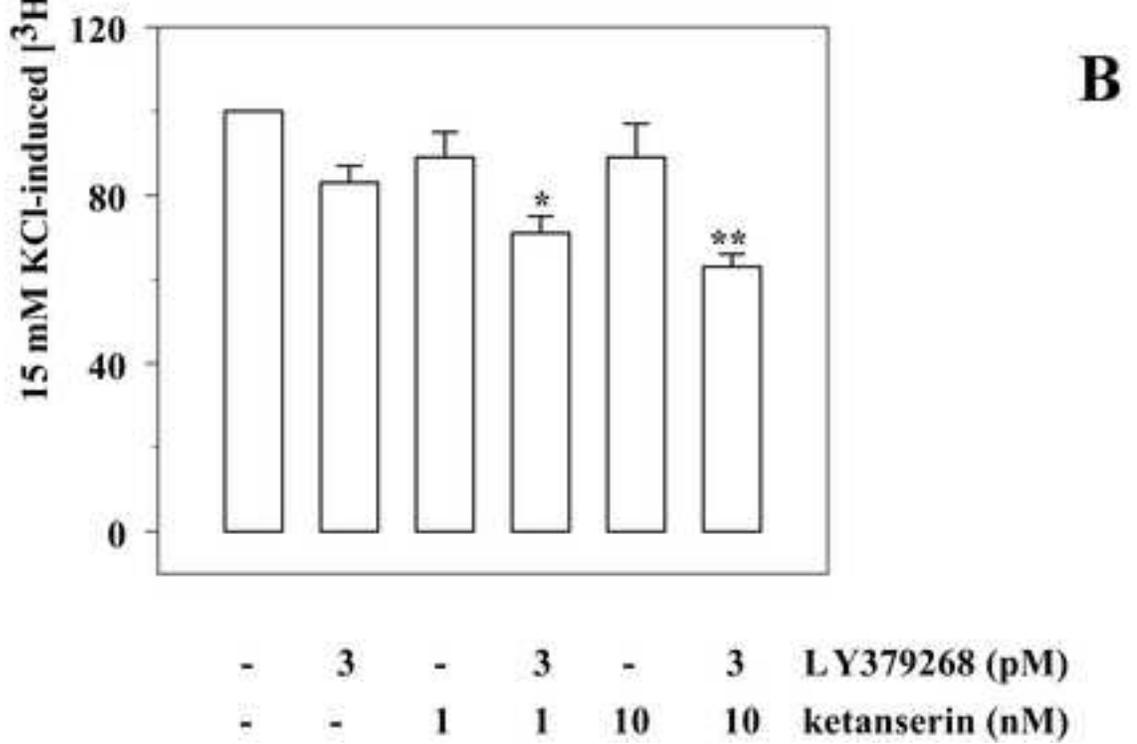
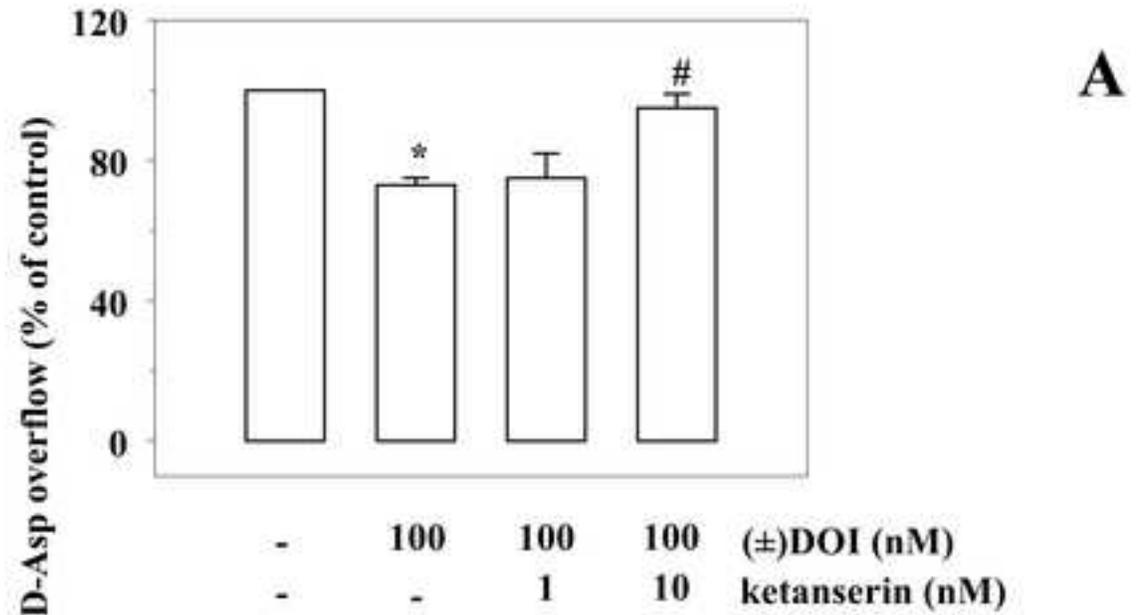
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