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5-HT_{2A}-mGlu2/3 receptor complex in rat spinal cord glutamatergic nerve endings: A 5-HT_{2A} 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 inhibited the 15 mM KCl-evoked release of [^{3}H]D-aspartate ([^{3}H]D-Asp) in a LY341495-sensitive manner. Spinal cord glutamatergic nerve terminals also possess presynaptic release-regulating 5-HT_{2A} heteroreceptors. Actually, the 15 mM KCl-evoked [^{3}H]D-Asp exocytosis from spinal cord synaptosomes was reduced by the 5-HT_{2A} agonist (±)DOI, an effect reversed by the 5-HT_{2A} antagonists MDL11,939, MDL100907, ketanserin and trazodone (TZD). We investigated whether mGlu2/3 and 5-HT_{2A} receptors colocalize and cross-talk in these terminals and if 5-HT_{2A} 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_{2A} receptor proteins in spinal cord VGLUT1 positive synaptosomes, where mGlu2/3 and 5-HT_{2A} receptor immunoreactivities largely colocalize. Furthermore, mGlu2/3 immunoprecipitates from spinal cord synaptosomes were also 5-HT_{2A} immunopositive. Interestingly, the 100 pM LY379268-induced reduction of the 15 mM KCl-evoked [^{3}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_{2A} 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_{2A} antagonists. Based on these results, we propose that in spinal cord glutamatergic terminals i) mGlu2/3 and 5-HT_{2A} receptors colocalize and interact one each other in an antagonist-like manner, ii) 5-HT_{2A} antagonists are indirect positive allosteric modulator of mGlu2/3 autoreceptors controlling glutamate exocytosis.

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, because they counteract hyperglutamatergicity participating to the restoration of glutamate transmission in the central nervous system (CNS). Among all the potential candidates, presynaptic

Abbreviations: mGlu2/3, metabotropic glutamate receptor type 2/3; 5-HT_{2A}, serotonin type 2A; [^{3}H]D-Asp, [^{3}H]D-aspartate; CNS, central nervous system; EPSCs, excitatory postsynaptic currents; L5P, layer V pyramidal cells; t-TBS, Tris-buffered saline-Tween; VGLUT1, vesicular glutamate transporters type 1; TZD, trazodone.

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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_{2A} receptors, which were recently reported to associate to mGlu2/3 receptors in CNS (Delille et al., 2012, 2013; Baki et al., 2016).

Activation of 5-HT_{2A} 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_{2A} receptors exist in this CNS region and that their activation modulates the release of glutamate. More recently, the 5-HT_{2A} 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_{2A} 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_{2A} 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_{2A} heteroreceptors which could functionally couple to mGlu2/3 autoreceptors has not been analysed 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_{2A} 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_{2A} 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, 8th). 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; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 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 [^{3}H]D-aspartate ([^{3}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_{2A} (1:500) and mouse *anti*-β-actin (1:5000) antibodies overnight at 4 °C. After extensive washes in t-TBS, membranes were incubated for 1 h at room temperature with appropriate horseradish peroxidase-linked secondary antibodies (1:20,000). 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_2PO_4, 10 Na_2HPO_4, 1.5 $MgCl_2$, 0.2 $CaCl_2$, pH 7.4 for 1 h at 4 °C and then