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Adenosine A_{2A} receptors and metabotropic glutamate 5 receptors are co-localized and functionally interact in the hippocampus: a possible key mechanism in the modulation of *N*-methyl-D-aspartate effects

M. T. Tebano,* A. Martire,* N. Rebola,† R. Pepponi,* M. R. Domenici,* M. C. Grò,* M. A. Schwarzschild,‡ J. F. Chen,§ R. A. Cunha† and P. Popoli*

**Department of Drug Research and Evaluation, Istituto Superiore di Sanità, Rome, Italy*

†*Center for Neuroscience of Coimbra, Institute of Biochemistry, Faculty of Medicine, University of Coimbra, Coimbra, Portugal*

‡*MassGeneral Institute for Neurodegenerative Disease, Boston, Massachusetts, USA*

§*Molecular Neuropharmacology Laboratory, Department of Neurology, Boston University School of Medicine, Boston, Massachusetts, USA*

Abstract

Hippocampal metabotropic glutamate 5 receptors (mGlu5Rs) regulate both physiological and pathological responses to glutamate. Because mGlu5R activation enhances NMDA-mediated effects, and given the role played by NMDA receptors in synaptic plasticity and excitotoxicity, modulating mGlu5R may influence both the physiological and the pathological effects elicited by NMDA receptor stimulation. We evaluated whether adenosine A_{2A} receptors (A_{2A}Rs) modulated mGlu5R-dependent effects in the hippocampus, as they do in the striatum. Co-application of the A_{2A}R agonist CGS 21680 with the mGlu5R agonist (RS)-2-chloro-s-hydroxyphenylglycine(CHPG) synergistically reduced field excitatory postsynaptic potentials in the CA1 area of rat hippocampal slices. Endogenous tone at A_{2A}Rs seemed to be required to enable mGlu5R-mediated effects, as the ability of CHPG to potentiate NMDA effects was antagonized by the selective A_{2A}R antagonist ZM 241385 in rat hippocampal slices and cultured hippocampal neurons, and abolished in the hippocampus of *A_{2A}R* knockout mice. Evidence for the interaction between A_{2A}Rs and mGlu5Rs was further strengthened by demonstrating their co-localization in hippocampal synapses. This is the first evidence showing that hippocampal A_{2A}Rs and mGlu5Rs are co-located and act synergistically, and that A_{2A}Rs play a permissive role in mGlu5R receptor-mediated potentiation of NMDA effects in the hippocampus.

Keywords: adenosine A_{2A} receptors, hippocampus, metabotropic glutamate 5 receptors, *N*-methyl-D-aspartate.

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In the hippocampus, group I metabotropic glutamate receptors (mGluRs, which include the mGlu1R and mGlu5R subtypes) regulate synaptic plasticity and spatial learning (reviewed in Anwyl 1999; Balschun *et al*. 1999; Bortolotto *et al*. 1999; Balschun and Wetzel 2002), contribute to the generation of epileptiform activity (Sacaan and Schoepp 1992), and modulate excitotoxic processes (Attucci *et al*. 2002). Because mGlu5R is the most abundant group I mGluR in the hippocampus (Romano *et al*. 1995; Lujan *et al*. 1996, 1997; Shigemoto *et al*. 1997), this subtype is thought to play a predominant role. One of the most noteworthy effects of mGlu5R activation is an enhancement of NMDA-mediated effects (reviewed in Anwyl 1999; Bortolotto *et al*. 1999) and,

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Address correspondence and reprint requests to P. Popoli, Department of Drug Research and Evaluation, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. E-mail: patrizia.popoli@iss.it

Abbreviations used: ACSF, artificial cerebrospinal fluid; A_{2A}R, adenosine A_{2A} receptor; cAMP, cyclic AMP; CHPG, (RS)-2-chloro-s-hydroxyphenylglycine; DARPP-32, dopamine- and cAMP-regulated phosphoproten of mr 32,000; DHPG, RS3,5-dihydroxyphenylglycine; fEPSP, field excitatory postsynaptic potential; KO, knockout; LDH, lactate dehydrogenase; mGluR, metabotrophic glutamate receptor; MPEP, 2-methyl-6-(phenylethyny) pyridine hydrochloride; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PKA, protein kinase A; PMSF, phenylmethylsulfonyl fluoride; PPS, paired-pulse stimulation; PSD, postsynaptic density; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline with Tween 20; vGluT, vesicular glutamate transporter.

in general, it is well accepted that mGlu5Rs 'set the tone' of NMDA receptor-mediated neurotransmission (Alagarsamy *et al*. 1999). Given the key role of NMDA receptors in both synaptic plasticity (Collingridge and Bliss 1995) and excitotoxicity (Rothman and Olney 1995), modulating mGlu5R may help regulate both the physiological and the pathological effects elicited by NMDA receptor stimulation in the hippocampus. Indeed, hippocampal long-term potentiation is blocked in rats by the selective mGlu5R antagonist 2-methyl-6-(phenylethyny) pyridine hydrochloride (MPEP) (e.g. Balschun and Wetzel 2002), and reduced in mice lacking mGlu5Rs (Lu *et al*. 1997). In another brain region, the striatum, mGlu5Rs are under the tight control of adenosine A_{2A} receptors (A_{2A}Rs). Indeed, A_{2A}Rs and mGlu5Rs have been reported to interact in models of Parkinson disease (Popoli *et al*. 2001; Coccurello *et al*. 2004), and to synergistically modulate GABA (Diaz-Cabiale *et al*. 2002) and glutamate release (Rodrigues *et al*. 2005). Furthermore, the existence of A_{2A}R–mGlu5R heteromeric complexes in striatal membranes and a synergistic interaction between the two receptors in inducing c-*fos* expression has been reported (Ferre *et al*. 2002; Fuxe *et al*. 2003). Very recently, it has been shown that the state of activation of striatal A_{2A}Rs influences some mGlu5R-dependent effects, such as dopamine-and cAMP-regulated phosphoproten of Mr 32,000 (DARPP-32) phosphorylation (Nishi *et al*. 2003) and NMDA potentiation (Domenici *et al*. 2004).

Although A_{2A}Rs are most abundant in the striatum, they are also present in the hippocampus, where they modulate synaptic transmission and excitotoxicity (reviewed in Cunha 2005). However, unlike the striatum, nothing is known about the possible co-localization of A_{2A}Rs and mGlu5Rs in the hippocampus, nor whether A_{2A}Rs can modulate hippocampal mGlu5Rs, thus influencing NMDA-mediated responses.

The primary aim of the present study was to explore the hypothesis that A_{2A}Rs can regulate mGlu5R-mediated effects in the hippocampus. Having found a clear functional A_{2A}R–mGlu5R interaction in electrophysiological and cell culture experiments, we then confirmed the hypothesis that hippocampal A_{2A}Rs exert a permissive role on mGlu5R-mediated effects (namely the potentiation of NMDA responses) by using mice lacking A_{2A}Rs. The interaction between A_{2A}Rs and mGlu5Rs was further strengthened by the finding of their co-localization in hippocampal synapses.

Materials and Methods

Animals

Male and pregnant female Wistar rats (2–3 months old) obtained either from Harlan-Nossan (Udine, Italy) or from Charles River (Barcelona, Spain), and *$A_{2A}R$* knockout (KO) and wild-type (WT) mice (2–4 months old, see below) were used. The animals were kept under standardized temperature, humidity and lighting conditions, and had free access to water and food. All animal procedures were carried out according to the European Community Guidelines for Animal Care, DL 116/92, application of the European Communities Council Directive (86/609/EEC).

Preparation and maintenance of slices

The animals were decapitated under ether anesthesia, and brains were quickly removed from the skull. Transverse hippocampal slices (400 μm thick) were cut with a McIlwain tissue chopper. Slices were maintained at room temperature (22–25°C) in artificial cerebrospinal fluid (ACSF) containing 126 mM NaCl, 3.5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$ and 11 mM glucose (pH 7.3) saturated with 95% O_2 and 5% CO_2. After incubation in ACSF for at least 1 h, a single slice was transferred to a submerged recording chamber and continuously superfused at 32–33°C with ACSF at rate of 2.7–3 mL/min. The drugs were added to this superfusion solution. Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded through a glass microelectrode filled with 2 M NaCl (pipette resistance 2–5 MΩ) placed in the stratum radiatum of the CA1 area. A bipolar twisted NiCr-insulated electrode (50 μm outer diameter), placed in the stratum radiatum, was used to stimulate the Schaffer collaterals. Stimulation was delivered every 20 s (square pulses of 100-μs duration at a frequency of 0.05 Hz) and every three consecutive responses were averaged. Signals were acquired with a DAM-80 AC differential amplifier (WPI Instruments, Woltham, MA, USA) and analyzed with the LTP program (Anderson and Collingridge 2001). At least 10 min of stable baseline recording preceded drug application. To allow comparisons between experiments, in each experiment the slope values were normalized, taking the average of the values obtained over the 10-min period immediately before applying the test compound as 100%. The washout period lasted at least 30 min.

In order to establish a possible involvement of presynaptic mechanisms in the effects of A_{2A}R and mGlu5R agonists, a series of experiments was performed under a protocol of paired-pulse stimulation (PPS), in which the Schaffer fibers were stimulated twice with an interpulse interval of 50 ms. Under control conditions, such a protocol normally elicits a condition of paired-pulse facilitation, in which the response elicited by the second stimulus (R2) is greater than that elicited by the first stimulus (R1). The degree of paired-pulse facilitation is quantified by the R2/R1 ratio and a modification of this ratio is an indication of a presynaptic action on neurotransmitter release (Schulz *et al*. 1994). Data were expressed as mean ± SEM from *N* slices.

Lactate dehydrogenase (LDH) release from cultured hippocampal neurons

Neurons were isolated from hippocampi dissected from E17 Wistar rat embryos. Pregnant rats were anesthetized with ether, decapitated and the fetuses were collected and rapidly decapitated. After removal of the meninges, the hippocampi were collected in Hank's balanced salt solution (HBSS) and dissociated. Hippocampal cells were then washed in HBSS and resuspended in Neurobasal medium supplemented with 0.5 mM L-glutamine, 2% B-27 supplement and gentamicin (50 μg/mL) (referred as complete medium). Aliquots of $2–3 \times 10^5$ cells were placed in 24-well culture plates coated with poly-L-lysine (5 μg/mL) and maintained at 37°C in humidified air with 5% CO_2.

Every 4 days, 0.5 mL medium was removed and replaced by the same volume of fresh complete medium. Assays were done on 12–14-day-old cultures. At the time of the experiment, culture medium was removed and substituted with an appropriate volume of Neurobasal medium supplemented with gentamicin (50 μg/mL). Cultured cells were then exposed to NMDA (300 μM) and/or CHPG (500–1000 μM) for 60 min. ZM 241385 (30 nM) or MPEP (30 μM) were applied 15 min before and then co-applied with NMDA and/or CHPG. Following exposure to the drugs, the culture medium was removed and replaced with fresh complete medium. Cultures were then returned to the incubator and cellular damage was evaluated 24 h later by measuring the amount of LDH released into the medium using a cytotoxicity detection kit (Roche Diagnostic, Indianapolis, IN, USA). Results are expressed as a percentage of control (100%), and represent mean ± SEM values of 3–4 independent experiments, assayed in triplicate.

Experiments in *$A_{2A}R$* KO mice

$A_{2A}R$ KO mice were generated as described previously (Chen *et al*. 1999) to produce the near congenic (N6) line used here. *$A_{2A}R$* KO mice (*$A_{2A}R$*–/–) and their WT littermates (*$A_{2A}R$*+/+) were generated by cross-breeding heterozygous *$A_{2A}R$* mice (*$A_{2A}R$*+/–). They were genotyped on the basis of PCR analysis performed on DNA isolated from tail samples. PCR amplification products were routinely fractionated through 2% agarose gels and stained with ethidium bromide. Photographs of the gels were made by using incident ultraviolet light to record the results of electrophoresis. In each experiment, age- and sex-matched *$A_{2A}R$* KO mice and WT littermates were used.

The experimental procedure described for rats was used for electrophysiological recordings in mice hippocampal slices.

To verify whether changes in mGlu5R density occurred in the hippocampus of *$A_{2A}R$* KO versus WT mice, western blot experiments were performed. Proteins for mGlu5R analysis were extracted from previously frozen hippocampal tissues by homogenization in cold buffer containing 0.32 M sucrose, 10 mM HEPES, 0.1 mM EGTA, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL leupeptin and 10 μg/mL aprotinin (pH 7.4). Homogenates were centrifuged at 500 *g* for 20 min and the resulting supernatant was centrifuged at 20 000 *g*. Pellets were resuspended in ice-cold 25 mM Tris-HCl buffer (pH 7.4) containing 1 mM PMSF, and an aliquot was used for protein determinations (Bio-Rad DC protein assay; Bio-Rad, Hercules, CA, USA). Samples were resuspended in Laemmli sample buffer, boiled for 5 min at 90°C and resolved by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) using 10% gels (Laemmli 1970). For western blot analysis, proteins (5, 10 or 20 μg) were separated by SDS–PAGE (10% gels) and transferred overnight to nitrocellulose paper (Shleicher & Schuell BioScience, Dassel, Germany) at 35 V. Blots were washed with TBST buffer (20 mM Tris, pH 7.5, 0.05% Tween 20, 150 mM NaCl) and blocked with TBST containing 1% bovine serum albumin for 1 h. Blots were then incubated at room temperature with the primary antibody, a rabbit anti-mGlu5R antibody (1 : 1000 dilution; Upstate Biotechnology, Lake Placid, NY, USA) for 1 h. After extensive washes in TBST, the immunoreactive bands were detected by incubation with alkaline phosphatase-conjugated secondary antibodies (Promega Corporation, Madison, WI, USA) and revealed by Western blue substrate for alkaline phosphatase (Promega).

Subcellular distribution of $A_{2A}R$ and mGlu5R immunoreactivity in cultured hippocampal neurons

Immunocytochemistry in the coverslip-mounted neurons was carried out as described previously (Rebola *et al*. 2005). Briefly, the cultures were washed twice with 1 mL phosphate-buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 20 mM Na_2HPO_4, 1.5 mM KH_2PO_4) kept at 37°C during 10 min, and fixed with 4% paraformaldehyde with 4% sucrose for 30 min at 37°C. Coverslip-mounted cells were then permeabilized with 0.2% Triton X-100 for 2 min at room temperature (22–25°C) and non-specific binding subsequently blocked with 3% bovine serum albumin for 30 min at room temperature. Cells then incubated for 1 h at room temperature with mouse anti-$A_{2A}R$ (1 : 500 dilution; Upstate Biotechnology), rabbit anti-mGlu5R receptor antibody (1 : 1000 dilution; Upstate Biotechnology), rabbit or mouse anti-synaptophysin antibody (1 : 200 dilution; Sigma, Sintra, Portugal) and guinea pig anti-vesicular glutamate transporter (vGluT) type 1 (1 : 5000; Chemicon, Temecula, CA, USA) and guinea pig vGluT2 (1 : 5000; Chemicon), and then washed three times with 200 μL PBS for 5 min. Incubation with secondary antibody, an AlexaFluor-598 (green)-labeled goat anti-guinea pig IgG antibody (1 : 200 dilution; Amersham, Little Chalfont, UK), AlexaFluor-598 (red)-labeled goat anti-mouse IgG antibody (1 : 200 dilution; Amersham), AlexaFluor-488 (green)-labeled goat anti-rabbit IgG antibody (1 : 200 dilution; Amersham) or AlexaFluor-488 (green)-labeled goat anti-mouse IgG antibody (1 : 200 dilution; Amersham), was conducted for 1 h at room temperature. We confirmed that none of the secondary antibodies produced any signal in preparations from which the corresponding primary antibody was omitted. After three washing periods of 5 min with 200 μL PBS, the cells were mounted using a Prolong Antifade kit (Amersham) and, after drying, were visualized in a Zeiss Axiovert 200 fluorescence microscope (Obertcochen, Germany) equipped with a cooled camera (Coolsnap Photometrics, Tucson, AZ, USA) or with a Bio-Rad 600 confocal microscope (Hercules, CA, USA) and analyzed with MetaFluor 4.0 software (Molecular Devices, Downingtown, PA, USA).

Subsynaptic distribution of $A_{2A}R$s and mGlu5Rs in rat hippocampus

Separation of the presynaptic active zone, postsynaptic density (PSD) and non-synaptic fractions from hippocampal nerve terminals was carried out as initially described by Phillips *et al*. (2001) with minor modifications (see Rebola *et al*. 2003). Briefly, the hippocampi from eight male Wistar rats were homogenized at 4°C with a Teflon–glass homogenizer in 15 mL isolation solution (0.32 M sucrose, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM PMSF). The concentration of sucrose was raised to 1.25 M by addition of 75 mL 2 M sucrose and 30 mL 0.1 mM $CaCl_2$, and the suspension was divided into 10 ultracentrifuge tubes. The homogenate was overlaid with 8 mL of a solution containing 1.0 M sucrose and 0.1 mM $CaCl_2$, and with 5 mL homogenization solution and centrifuged at 100 000 *g* for 3 h at 4°C. Synaptosomes were collected at the 1.25/1.0 M sucrose interface, diluted 1 : 10 in cold 0.32 M sucrose containing 0.1 mM $CaCl_2$ and pelleted by centrifugation at 15 000 *g* for 30 min at 4°C. The pellets were resuspended in 1 mL 0.32 M sucrose with 0.1 mM $CaCl_2$ and a small sample was stored for western blot analysis. Synaptosomes were then diluted 1 : 10 in cold 0.1 mM $CaCl_2$ and an equal volume of 2 × solubilization buffer (2% Triton X-100, 40 mM Tris, pH 6.0) was added to the suspension. The membranes were

incubated for 30 min on ice with mild agitation and the insoluble material (synaptic junctions) was pelleted by centrifugation at 40 000 *g* for 30 min at 4°C. The supernatant (extra-synaptic fraction) was decanted, and proteins were precipitated with six volumes of acetone at −20°C and recovered by centrifugation at 18 000 *g* for 30 min at −15°C. The synaptic junction pellet was washed in solubilization buffer (pH 6.0) and resuspended in 10 volumes of a second solubilization buffer (1% Triton X-100, 20 mM Tris but at pH 8.0). This increase in pH allows the dissociation of the extracellular matrix that maintains the presynaptic active zone tightly bound to the PSD (Phillips *et al*. 2001). Hence, the active zone is solubilized whereas the PSD is essentially preserved because the amount of detergent is not enough for its solubilization (Phillips *et al*. 2001). After incubation for 30 min on ice with mild agitation, the mixture was centrifuged (18 000 *g* for 30 min at −15°C) and the supernatant (presynaptic fraction corresponding to the active zone) processed as described for the extra-synaptic fraction. The protease inhibitor PMSF (1 mM) was added to the suspension in all extraction steps. The pellets from the supernatants and the final insoluble pellet (postsynaptic fraction) were solubilized in 5% SDS and the protein concentration determined by the bicinchoninic acid protein assay. The samples were added to an equal volume of 2 × SDS–PAGE sample buffer before freezing at −20°C. As reported previously (Rebola *et al*. 2003), this fractionation procedure allows an effective separation (over 90% efficiency) of markers of the presynaptic (containing syntaxin or SNAP25), postsynaptic (containing PSD-95 or NMDA receptor subunits) and non-active zone (containing synaptophysin) fractions, and can be used to determine the subsynaptic distribution of mGluRs by western blot analysis (e.g. Rebola *et al*. 2003; Rodrigues *et al*. 2005).

Western blot analysis was carried out using 20–140 μg of each protein fraction, obtained as described above. Samples were loaded on to SDS–polyacrylamide gels (7.5%) and transferred on to polyvinylidene difluoride membranes. The membranes were then blocked for 1 h at room temperature in 5% low-fat milk in Tris-buffered saline medium with 0.1% Tween 20 (Sigma) before being probed with primary antibodies raised against the A_{2A}R (1 : 500) and mGlu5R (1 : 1000), applied overnight at 4°C. Detection was performed using alkaline phosphatase-conjugated secondary antibodies goat anti-rabbit IgG (1 : 20 000 dilution; Amersham) or rabbit anti-goat IgG (1 : 5000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoblots were visualized using ECF detection reagent (Amersham) and a VersaDoc 3000 (Bio-Rad).

Drugs

CHPG, MPEP, CGS 21680, ZM 241385, forskolin, cyclopenthyladenosine (CPA) and KT 5720 were obtained from Tocris Cookson (Northpoint, UK); NMDA was from RBI (Natik, MA, USA) and bicuculline was from Sigma-Aldrich (Milan, Italy).

Results

A_{2A}R activation facilitates CHPG-induced effects in rat hippocampal slices

As shown in Fig. 1(a and c), the selective mGlu5R agonist CHPG (500 μM over 10 min) did not significantly affect the fEPSP recorded in the CA1 area ($p > 0.05$). Only at concentration of 1 mM did CHPG induce a reduction in the fEPSP slope (− 21.8 ± 4% of basal, $N = 13$; $p < 0.005$ versus baseline, Wilcoxon signed rank test) that completely recovered after washout. This effect was significantly reduced by the selective mGlu5R antagonist MPEP (30 μM) (− 7.1 ± 1.5% of basal, $N = 4$; $p < 0.01$ vs. 1 mM CHPG, Mann–Whitney *U*-test) (Fig. 1c). MPEP (30 μM) by itself did not affect synaptic transmission.

We then investigated whether the co-activation of A_{2A}Rs and mGlu5Rs had a synergistic effect on synaptic transmission, as reported to occur in the striatum (Popoli *et al*. 2001; Domenici *et al*. 2004; Rodrigues *et al*. 2005). Co-application of the selective A_{2A}R agonist CGS 21680 (50 nM) and CHPG (500 μM) elicited a significant reduction in the fEPSP slope (− 25.0 ± 5.4%, $N = 6$; $p < 0.03$ vs. CHPG or CGS 21680 alone, Mann–Whitney *U*-test; $p < 0.005$ vs. baseline, Wilcoxon signed rank test) (Figs 1b and c), whereas neither CGS 21680 (50 nM) nor CHPG (500 μM) alone affected synaptic transmission (Fig. 1c). The selective A_{2A}R antagonist ZM 241385 (100 nM) abolished the synergistic effect resulting from the co-activation of A_{2A}Rs and mGlu5Rs (data not shown), whereas ZM 241385 had no effect on its own, as reported previously (Cunha *et al*. 1997). MPEP (30 μM) also prevented the fEPSP slope reduction induced by CGS 21680 + CHPG (500 μM) (data not shown). These data indicate that activation of A_{2A}Rs facilitates mGlu5R receptor-mediated effects.

In order to explore the mechanisms responsible for the A_{2A}R–mGlu5R interaction, we investigated the possible involvement of the cyclic AMP (cAMP)–protein kinase A (PKA) pathway, the canonical transduction mechanism of A_{2A}Rs (Fredholm *et al*. 2003). To this end, we tested whether the adenylyl cyclase activator forskolin also potentiated the effect of CHPG. As shown in Fig. 2(a), application of forskolin (30 μM) plus CHPG failed to significantly modify the fEPSP slope (− 5.2 ± 2.4%, $N = 5$, $p > 0.05$ vs. baseline). Moreover, application of the PKA inhibitor KT 5720 (1 μM, added 10 min before and then along with CGS and CHPG) did not influence the fEPSP slope reduction with respect to that observed with CGS 21680 + CHPG (mean fEPSP slope 84.2 ± 1.8%, $N = 4$) (Fig. 2a). Forskolin (30 μM) and KT 5720 (1 μM) did not influence the fEPSP slope on their own (data not shown).

We next investigated whether the interaction between A_{2A}Rs and mGlu5Rs involved GABAergic transmission, which has been reported to be controlled by both A_{2A}Rs (Cunha and Ribeiro 2000a) and mGlu5Rs (Mori and Gerber 2002). We observed that CGS 21680 (50 nM) and CHPG (500 μM) still inhibited the fEPSP slope in the presence of bicuculline (10 μM) (− 32.2 ± 8.5%, $N = 5$; $p > 0.05$ vs. CGS + CHPG in the absence of bicuculline) (Fig. 2a). This suggests that GABAergic transmission is not involved in the

synergistic reduction of the fEPSP slope induced by the co-administration of CGS 21680 and CHPG.

We tested whether the synergistic effect resulting from the co-activation of A_{2A}Rs and mGlu5Rs involved presynaptic control of glutamate release. This was evaluated by testing whether the co-administration of CGS 21680 and CHPG affected PPS. However, co-application of CGS 21680 (50 nM) and CHPG (500 μM) did not modify the R2/R1 value (1.44 ± 0.07) compared with that of the control (1.38 ± 0.06, $N = 5$) (Fig. 2b). Finally, in order to check the sensitivity of the PPS protocol to changes in presynaptic neurotransmitter release, we tested whether the adenosine A_1 receptor agonist CPA, a known inhibitor of presynaptic neurotransmitter release, was able to increase the R2/R1 ratio. As expected, CPA (50 nM) significantly increased the R2/R1 value with respect to control (1.89 ± 0.09 vs. 1.45 ± 0.07, $N = 5$; $p < 0.05$). Thus, the failure of CGS 21680 + CHPG to influence the PPS protocol makes it unlikely that the synergism between A_{2A}Rs and mGlu5Rs in the control of glutamatergic transmission has a presynaptic locus.

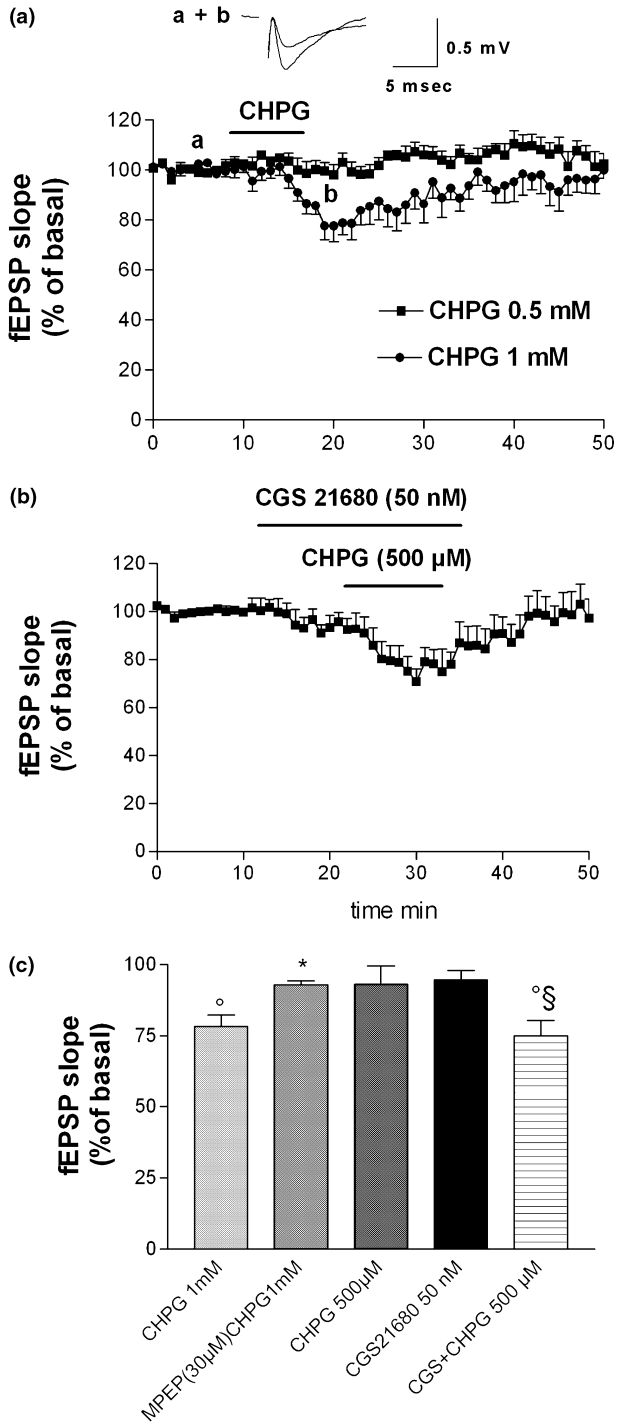

Fig. 1 Stimulation of A_{2A}Rs and mGlu5Rs synergistically reduces the fEPSP slope in rat hippocampal slices. Superfusion of rat hippocampal slices with the selective mGlu5R agonist CHPG (500 μM) did not significantly modify the fEPSP slope, whereas CHPG (1 mM) induced a significant decrease in fEPSP slope that recovered after washing (a, c). This effect was reduced significantly by the selective mGlu5R antagonist MPEP (30 μM) (c). Co-application of concentrations of CGS 21680 (50 nM) and CHPG (500 μM) that were ineffective when each drug was applied alone significantly reduced the fEPSP slope (b, c). The graphs in (a) and (b) represent the average time course of changes in fEPSP slope derived from 6–13 experiments. Inserts in (a) show representative fEPSP traces obtained in one of the individual experiments in control conditions and in the presence of 1 mMCHPG. In (a) and (b) values are mean ± SEM percentage of baseline values and the period of drug application is indicated by the horizontal bars. (c) Histograms show the mean ± SEM of the fEPSP slope, expressed as a percentage of baseline, at the end of the superfusion period. °$p < 0.005$ versus baseline (Wilcoxon signed rank test); *$p < 0.01$ versus 1 mM CHPG (Mann–Whitney U-test); §$p < 0.03$ versus CHPG and CGS 21680 alone (Mann–Whitney U-test).

Ability of CHPG to potentiate NMDA effects requires the activation of A_{2A}Rs

ZM 241385 reduces the potentiating effects of CHPG on NMDA-induced fEPSP reduction in rat hippocampal slices and NMDA-induced LDH release in hippocampal neurons

As shown in Figs 3(a and d), superfusion of rat hippocampal slices with NMDA (8 μM for 10 min) caused a reversible reduction in the fEPSP slope (− 26.8 ± 2.3% of baseline, $N = 20$), as observed previously (Nikbakht and Stone 2001). This effect was strongly potentiated (− 76.9 ± 4.5%, $N = 6$; $p < 0.05$ vs. NMDA alone, Wilcoxon signed rank test) (Figs 3a and d) when 500 μM CHPG, a concentration devoid of effects on its own, was co-applied with NMDA. Such a potentiation was actually due to the co-application of CHPG, as in preliminary control experiments we had established that two consecutive applications of NMDA alone did not result in an increased response to the second application (M. T. Tebano and A. Martire, unpublished results). The selective mGlu5R antagonist MPEP (30 μM) completely abolished the

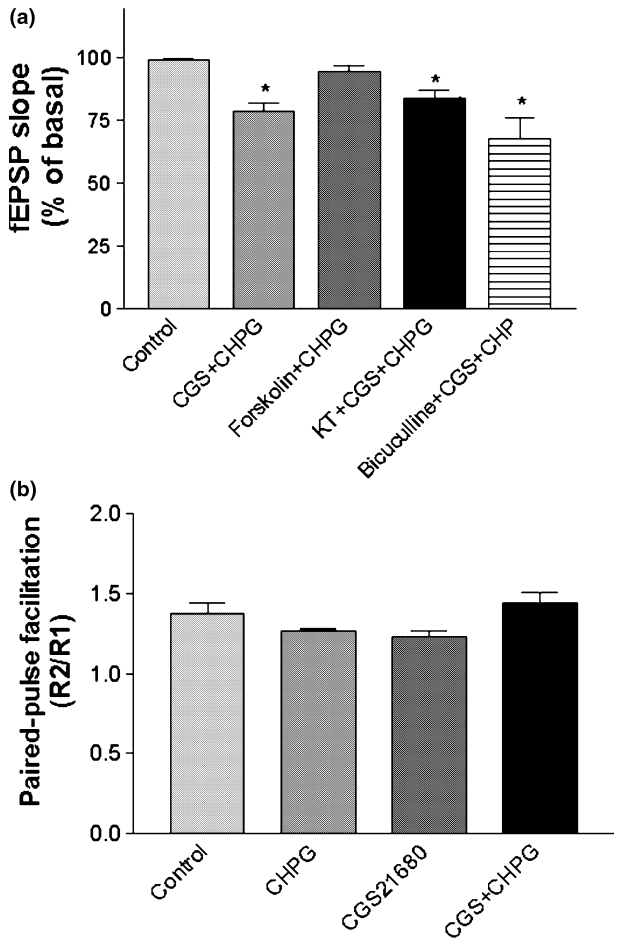

Fig. 2 Synergism between A_{2A}Rs and mGlu5Rs in the control of fEPSPs in rat hippocampal slices does not appear to involve PKA activation, GABAergic neurotransmission or presynaptic mechanisms. (a) Histograms show that inhibition of the fEPSP slope caused by the simultaneous application of CGS 21680 (50 nM) and CHPG (500 μM) was not mimicked by the co-application of CHPG (500 μM) together with the adenylyl cyclase activator forskolin (30 μM) instead of CGS 21680. Moreover, the PKA inhibitor KT 5720 (1 μM) did not prevent inhibition of the fEPSP slope induced by CGS 21680 + CHPG. Application of bicuculline (10 μM), which prevents GABAergic transmission, failed to modify the synergistic reduction in the fEPSP slope induced by CGS 21680 + CHPG. Values are mean ± SEM of five experiments. *$p < 0.05$ versus baseline (Wilcoxon signed rank test). (b) Application of CGS 21680 and CHPG, alone or in combination, did not modify the paired pulse facilitation ratio (R2/R1) in comparison to that in control (i.e. no added drugs). Values are mean ± SEM of five experiments.

CHPG-induced potentiation of NMDA effects (− 19.1 ± 6.9%; $N = 3$; $p < 0.03$ vs. NMDA + CHPG, Mann–Whitney *U*-test) (Figs 3b and d). Interestingly, the selective A_{2A}R antagonist ZM 241385 (30 nM) also significantly reduced the ability of CHPG to potentiate the effect of NMDA (− 50.1 ± 8.3%, $N = 8$; $p < 0.05$ vs. NMDA + CHPG, Mann–Whitney *U*-test) (Figs 3c and d). When given alone, neither MPEP nor ZM 241385 modified basal synaptic transmission or the NMDA-induced inhibition of the fEPSP slope (data not shown).

To evaluate whether the A_{2A}R–mGlu5R interaction also played a role in the modulation of NMDA-induced toxicity, primary cultures of rat hippocampal neurons were studied. Incubation of hippocampal neuronal cultures for 1 h with 300 μM NMDA induced a significant increase in LDH release with respect to basal levels (190.2 ± 32.4%, $N = 4$; $p < 0.01$, Mann–Whitney *U*-test) (Fig. 4). CHPG alone did not modify LDH release up to a concentration of 1 mM (100.9 ± 3.2%, $N = 3$). However, when co-applied with NMDA, CHPG (1 mM) significantly potentiated the NMDA-induced LDH release (312.4 ± 60.1%, $N = 4$; $p < 0.05$ vs. NMDA alone, Mann–Whitney *U*-test) (Fig. 4). The potentiating effect of CHPG on the NMDA-induced LDH release was prevented by MPEP (30 μM). Pretreatment with MPEP for 15 min before administration of CHPG + NMDA was able to restore the same degree of LDH release as induced by NMDA alone (174.2 ± 5.5%, $N = 3$; $p < 0.05$ vs. NMDA + CHPG, Mann–Whitney *U*-test) (Fig. 4). Similarly, ZM 241385 (30 nM) also abolished the ability of CHPG to potentiate NMDA-induced LDH release (160.8 ± 20.9%, $N = 4$; $p < 0.02$ vs. NMDA + CHPG, Mann–Whitney *U*-test) (Fig. 4). When applied alone, neither MPEP nor ZM 241385 influenced basal or NMDA-induced LDH release (data not shown).

Ability of CHPG to potentiate NMDA effects is lost in the hippocampus of $A_{2A}R$ KO mice

To further test the hypothesis that hippocampal A_{2A}Rs might play a permissive role in mGlu5R-dependent effects, the ability of CHPG to potentiate NMDA-induced fEPSP slope reduction in hippocampal slices was compared in $A_{2A}R$ KO and WT mice.

As observed in rat hippocampal slices, application of NMDA (8 μM during 10 min) in WT mice significantly depressed the fEPSP slope (− 33.3 ± 8.5%, $N = 8$; $p < 0.05$ vs. baseline, Wilcoxon signed rank test), an effect that was fully reversed after 30 min of washout (Figs 5a and c). An identical effect of NMDA was observed in $A_{2A}R$ KO mice (− 29.3 ± 6.6%, $N = 8$; $p < 0.05$ vs. baseline, Wilcoxon signed rank test) (Figs 5b and c). As CHPG (500 μM) depressed fEPSP on its own in mouse hippocampal slices (M. R. Domenici and A. Martire, unpublished results), a concentration of 300 μM was used. Ten minutes of subsequent co-administration of CHPG (300 μM) and NMDA (8 μM) produced a marked and significant potentiation of the NMDA-induced reduction in fEPSP slope in WT mice (− 46.4 ± 8.0%, $N = 8$; $p < 0.05$ vs. NMDA alone, Wilcoxon signed rank test) (Figs 5a and c), again in a manner similar to that observed in rat hippocampal slices. Likewise, the potentiating effect of CHPG was blocked either by MPEP (30 μM) (− 24.0 ± 2.3%, $N = 7$; $p < 0.02$ vs.

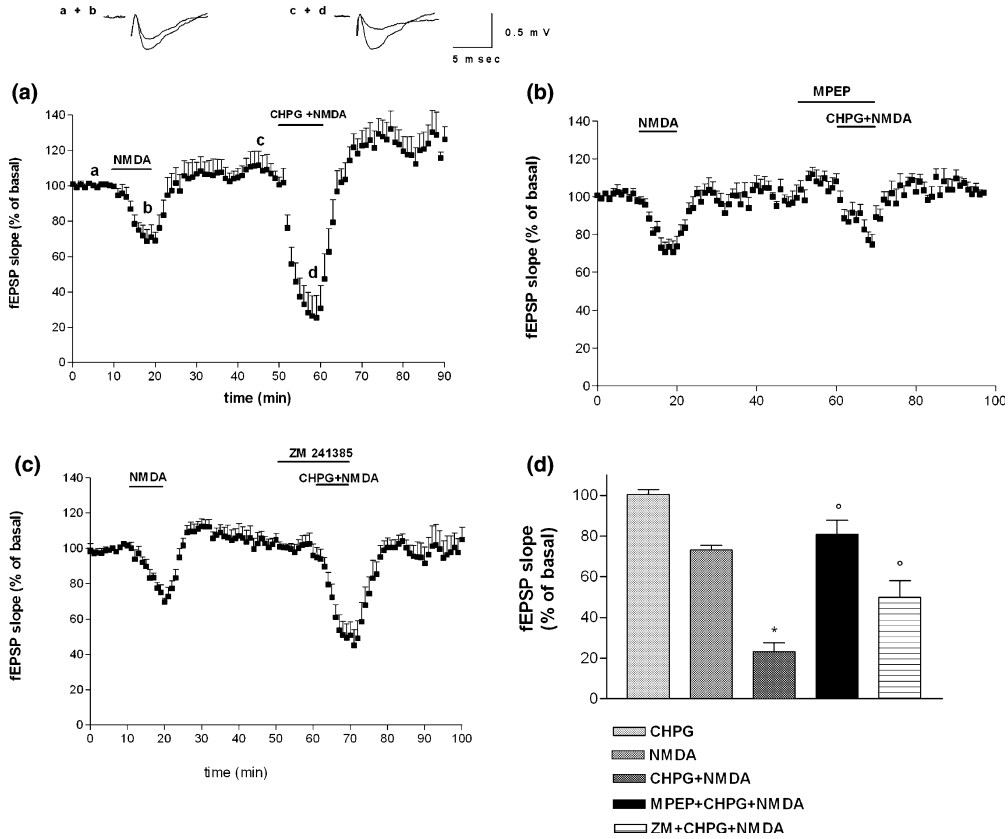

Fig. 3 Both the mGlu5R antagonist MPEP and the adenosine A_{2A} receptor antagonist ZM 241385 prevent CHPG-induced potentiation of NMDA effects in rat hippocampal slices (a–c). Time-course of changes in fEPSP slope recorded in rat hippocampal slices. A 10 min superfusion period with NMDA (8 μM) reduced the fEPSP slope, an effect that was potentiated by the co-application of 500 μM CHPG (a, d). Each point/bar represents the mean ± S.E.M. of 6–20 experiments, except for the group MPEP + CHPG + NMDA (N = 3). Representative fEPSP traces recorded in control conditions and in the presence of tested drugs are recorded in panel A. The CHPG-induced potentiation of NMDA effects was abolished by 30 μM MPEP (b,d) and significantly attenuated by 30 nM ZM 241385 (c, d). For clarity, the lack of effects of 30 μM MPEP or 30 nM ZM 241385 alone on fEPSP slope are not presented. $^*p < 0.05$ versus NMDA (Wilcoxon signed rank test); °p < 0.05 versus CHPG + NMDA (Mann–Whitney *U*-test).

CHPG + NMDA, Mann–Whitney *U*-test) (data not shown) or by ZM 241385 (30 nM) (− 25.1 ± 5.4%, $N = 5$; $p < 0.05$ vs. CHPG, Mann–Whitney *U*-test) (data not shown). In contrast, in slices from *$A_{2A}R$* KO mice, CHPG (300 μM) failed to potentiate the fEPSP slope reduction induced by NMDA (− 38.4 ± 7.4%, $N = 8$; $p > 0.05$ vs. NMDA alone) (Figs 5b and c). When given alone, CHPG (300 μM) did not modify basal synaptic transmission. The impairment of CHPG-mediated potentiation of NMDA responses did not appear to depend on a reduced density of mGlu5Rs in the hippocampus of *$A_{2A}R$* KO mice. Indeed, the density of mGlu5Rs was not modified in the hippocampus of *$A_{2A}R$* KO compared with WT mice, as evaluated by western blot analysis Fig. 5c.

Subcellular distribution of A_{2A}R and mGlu5R immunoreactivity in cultured hippocampal neurons

The synergistic effect achieved after stimulating A_{2A}Rs and mGlu5Rs raises the possibility that these two receptors are co-localized in the hippocampus. We decided to evaluate by immunocytochemistry the distribution of both receptors in cultured hippocampal neurons using antibodies against the A_{2A}R and mGlu5R. As observed previously (Rebola *et al.* 2005), the A_{2A}R was highly localized in synapses in hippocampal neurons (Fig. 6a). The mGlu5R had a much broader distribution, being present all over the neurons, namely in the cell body, axons, dendrites and synapses (Fig. 6b).

Co-localization studies with synaptophysin (located in synaptic vescicles and considered to be a marker of nerve terminals or synaptic contacts) and vGluT1 and vGluT2 (markers of glutamatergic synapses) indicated that the mGlu5R was present in synaptic contacts (although also elsewhere in neurons) and, in particular, in glutamatergic synapses (Figs 6d–f). The A_{2A}R was found to be highly concentrated in synaptic contacts (co-located with synaptophysin; see Rebola *et al.* 2005) and was present in glutamatergic synapses (co-located with vGluT1 and

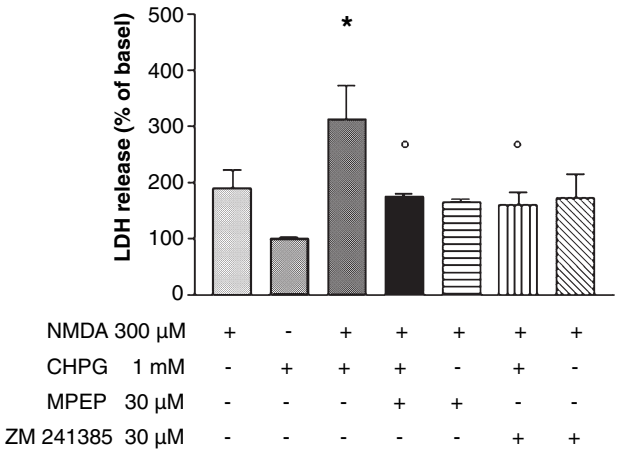

Fig. 4 Blockade of either A_{2A}Rs or mGlu5Rs prevents CHPG from potentiating NMDA-induced LDH release from cultured hippocampal neurons. Application of NMDA (300 µM for 60 min) to rat hippocampal neurons induced a significant increase in LDH release. The mGlu5R agonist CHPG (1 mM) had no effect on its own, but it significantly potentiated NMDA-induced LDH release. The mGlu5R antagonist MPEP (30 µM) and the A_{2A}R antagonist ZM 241385 (30 nM) had no effect by themselves but prevented CHPG from potentiating the release of LDH. Values are mean ± SEM of 3–4 independent experiments, assayed in triplicate. *$p < 0.05$ versus NMDA alone, °p < 0.05 versus CHPG + NMDA (Mann–Whitney *U*-test).

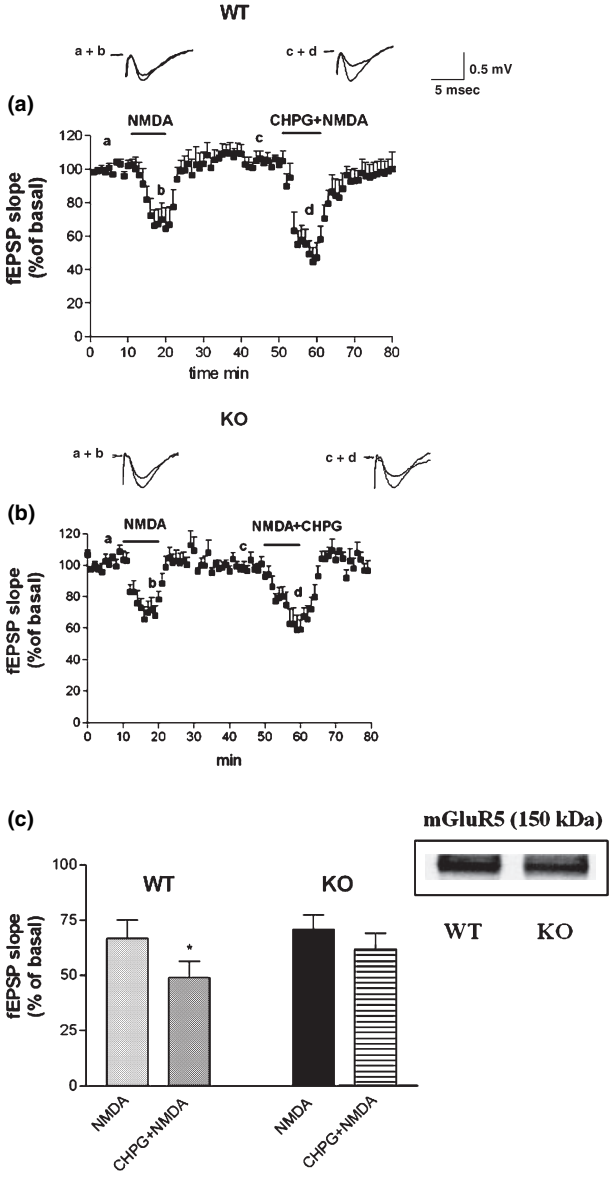

Fig. 5 Ability of CHPG to potentiate the effects of NMDA is abolished in the hippocampus of *$A_{2A}R$* KO mice. (a, b) Representative traces showing time course of changes in fEPSP slope recorded in mice hippocampal slices from (a) WT and (b) *$A_{2A}R$* KO mice. Values are mean ± SEM. (c) Histogram shows mean ± SEM values from eight experiments. In hippocampal slices from WT (a, c) and KO (b, c) mice application of 8 µM NMDA induced a very similar reduction in fEPSP slope. CHPG (300 µM) significantly potentiated this effect in WT mice (a, c) but not in KO mice (b, c). No changes in mGlu5R expression were revealed by western blotting of hippocampus from KO and WT mice (c). *$p < 0.05$ versus NMDA alone (Wilcoxon signed rank test).

VGluT2) (Figs 6g–i). Double immunocytochemical labeling with anti-A_{2A}R and anti-mGlu5R antibodies revealed co-localization of these two receptors mostly in synaptic contacts, where the A_{2A}R was concentrated (Fig. 6c).

Subsynaptic distribution of A_{2A}Rs and mGlu5Rs in rat hippocampus

The resolution of the above immunocytochemical studies did not allow discrimination between presynaptic and postsynaptic localization of either receptor. For example, under our experimental conditions, we observed co-localization of anti-synaptophysin (a presynaptic marker) and anti-PSD-95 (a marker of PSDs) immunoreactivities (data not shown). Thus, we decided to investigate the relative distribution of A_{2A}Rs and mGlu5Rs in the presynaptic active zone and in the PSD of hippocampal synapses. For this purpose, we used a pH-based fractionation of synaptic contacts into their main constituents (Phillips *et al*. 2001), namely the PSD, the active zone (a presynaptic specialization lining the PSD) and a presynaptic non-active zone fraction (that mostly includes the majority of presynaptic constituents apart from the active zone). As observed in Fig. 7, the mGlu5R was mostly located in the PSD but there was also some immunoreactivity present at the presynaptic active zone and extra-active zone fractions. Quantification of the relative density of mGlu5R immunoreactivity in the three fractions in three different separations from different groups of rats revealed that mGlu5R immunoreactivity was most abundant in the PSD fraction (61.3 ± 4.3% of the total immunoreactivity, $N = 3$) but was also present in the presynaptic active zone (25.0 ± 2.3% of total immunoreactivity, $N = 3$) and had a lower relative abundance in the extra-active zone fraction of

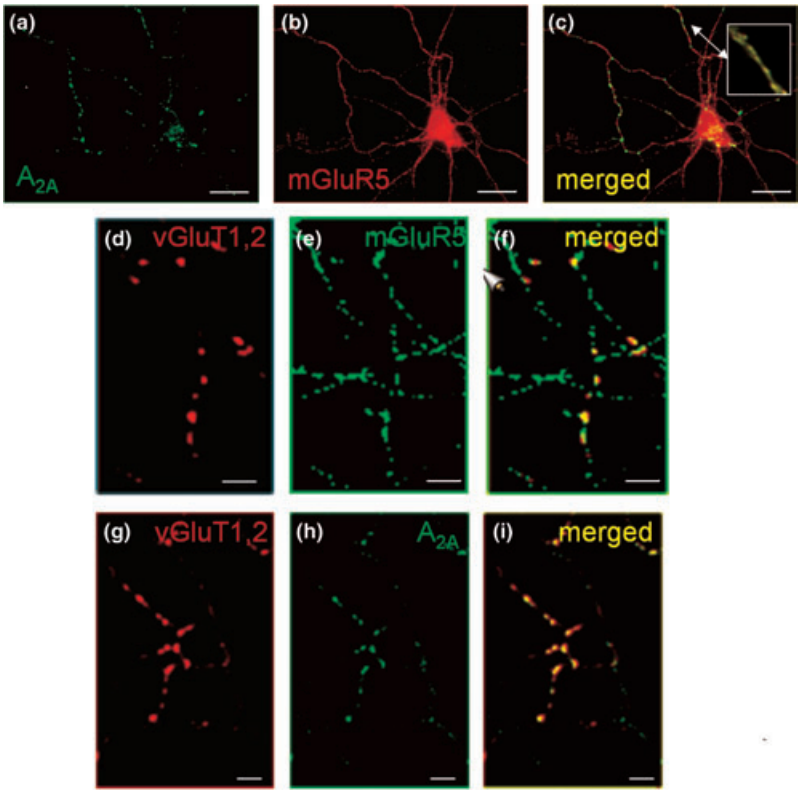

Fig. 6 Co-localization of A_{2A}Rs and mGlu5Rs in rat hippocampal neurons in culture. (a–c) Immunocytochemical identification of A_{2A} Rs (a) and mGlu5Rs (b) and merged image (c) illustrate the partial co-localization of the two receptors (yellow color); the insert in (c) corresponds to an amplification (6-8 times) of the region indicated by the arrow, clearly illustrating the co-localization of both receptors in neuronal branches. (d–e) Immunocytochemical identification of vGluT1 and vGluT2 (d; markers of glutamatergic nerve terminals) and mGlu5Rs (e) and the merged image (f) illustrate the partial co-localization of mGlu5Rs in glutamatergic nerve terminals (yellow color). (g–i) Immunocytochemical identification of vGluT1 and vGluT2 (g) and A_{2A}Rs (h) and the merged image (i) illustrate the partial co-localization of A_{2A}Rs in glutamatergic nerve terminals (yellow color). These images are representative of three different fields per coverslip, in experiments carried out 3–4 times using different hippocampal neurons prepared from different groups of rat embryos. Scale bars are 50 μm in (a)-(c) and 10 μm in (d)–(i).

hippocampal nerve terminals (13.7 ± 3.6% of total immunoreactivity, $N = 3$). The A_{2A}R was enriched in the presynaptic active zone fraction (~60%) but was also present at the PSD. Quantification of the relative density of A_{2A}R immunoreactivity in the three fractions in three different separations from different groups of rats revealed that A_{2A}R immunoreactivity was most abundant in the presynaptic active zone fraction (56.2 ± 3.3% of total immunoreactivity, $N = 3$) but was also present in the PSD (35.2 ± 2.7% of total immunoreactivity, $N = 3$) and had a lower relative abundance in the extra-synaptic fraction of hippocampal nerve terminals (8.6 ± 1.8% of total immunoreactivity, $N = 3$).

As also shown in Fig. 7, we validated this subsynaptic fractionation by confirming over 90% separation in the three subsynaptic fractions of the PSD marker PSD-95, presynaptic active zone marker syntaxin or SNAP-25, and extra-synaptic marker synaptophysin (present in synaptic vesicles; hence this protein is a presynaptic marker but is not located in the active zone, which is a restricted zone of the presynaptic boutton). In particular, we confirmed previously obtained data showing that NMDA receptor subunits were highly enriched in the PSD fraction and nearly absent in the presynaptic or extra-synaptic fractions (see Rebola *et al.* 2003) (Fig. 7). Thus, although A_{2A}Rs and mGlu5Rs were also co-localized in the presynaptic active zone, their ability to control NMDA-mediated effects may result from a postsynaptic interaction, as the PSD was the only subsynaptic compartment that contained all three receptors, i.e. A_{2A}Rs, mGlu5Rs and NMDA receptors.

Discussion

The present study demonstrated, for the first time, that A_{2A}Rs and mGlu5Rs are co-localized and functionally interact in the rodent hippocampus.

A synergism between A_{2A}Rs and mGlu5Rs was demonstrated by the finding that, in rat hippocampal slices, co-application of ineffective concentrations of CGS 21680 and CHPG (50 nM and 500 μM respectively) significantly reduced the fEPSP slope. Furthermore, selective antagonists of either mGlu5Rs (MPEP) or A_{2A}Rs (ZM 241385) prevented this synergistic effect, providing a pharmacological demonstration for the involvement of both receptors. This finding strictly reproduces our previous results in the striatum (Domenici *et al.* 2004; Rodrigues *et al.* 2005), a brain area in which the occurrence of functional interactions between A_{2A}Rs and mGlu5Rs has been reported in several models (see Introduction).

In order to investigate the mechanisms underlying the A_{2A}R–mGlu5R interaction, we tested the possible involvement of GABAergic transmission. The inability of bicuculline to modify the synergism between A_{2A}Rs and mGlu5Rs suggests that this interaction does not involve GABAergic neurotransmission. This is in agreement with previous

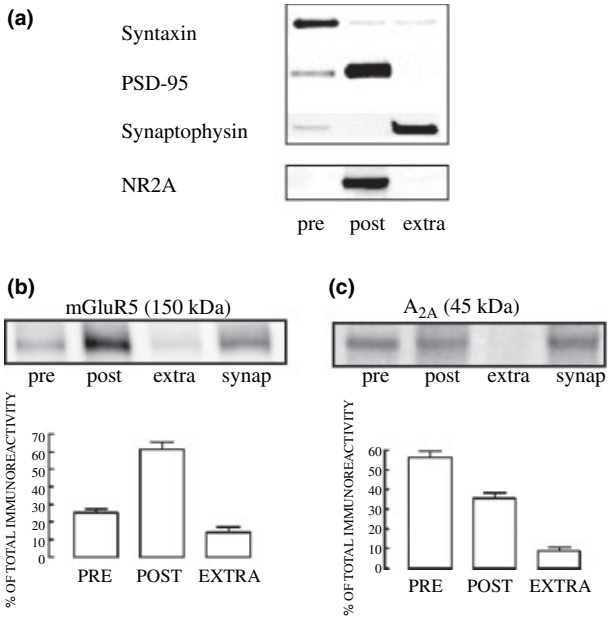

Fig. 7 Subsynaptic distribution of mGlu5Rs (b) and A_{2A}Rs (c) in the rat hippocampus. Hippocampal nerve terminals were prepared (synap) and further fractionated to obtain fractions enriched in the presynaptic active zone (pre), the PSD (post), in nerve terminals outside the active zone (extra). These fractions were over 90% pure, as illustrated by the ability to recover the immunoreactivity for syntaxin in the presynaptic active zone fraction, PSD95 in the PSD fraction and synaptophysin (a protein located in synaptic vesicles) in the extra-active zone fraction (a). One NMDA receptor subunit (NR2A) was nearly confined to the PSD (a). (b) Representative western blot showing mGlu5R immunoreactivity, corresponding to a 150-kDa band, in the four fractions and histogram showing average mGlu5R immunoreactivity in each fraction. Values are mean ± SEM of three experiments using fractions prepared from different groups of rats. (c) Representative western blot showing A_{2A}R immunoreactivity, corresponding to a 45-kDa band, in the four fractions and histogram showing average A_{2A}R immunoreactivity in each of the fractions. Values are mean ± SEM of three experiments using fractions prepared from different groups of rats.

reports showing that GABA receptor blockade did not influence CGS 21680-mediated effects in hippocampal slices (Lopes *et al*. 2002) and that, unlike the mGlu1R subtype of group I mGluRs, the mGlu5R subtype predominantly affects glutamatergic rather than GABAergic transmission (Battaglia *et al*. 2001). It can therefore be concluded that the interaction between A_{2A}Rs and mGlu5Rs might occur at glutamatergic synapses. Accordingly, the studies carried out in cultured hippocampal neurons allowed us to conclude that A_{2A}Rs and mGlu5Rs are co-localized in synapses, and in particular in glutamatergic synapses, although they did not allow discrimination between a presynaptic or postsynaptic localization. In electrophysiological experiments, we found that the application of CGS 21680 and CHPG did not influence the ratio of fEPSP responses under a protocol of PPS, which is an index of presynaptic neurotransmitter release. This finding suggests that, although both A_{2A}Rs (Lopes *et al*. 2002; Marchi *et al*. 2002; but see Nikbakht and Stone 2001) and mGlu5Rs (Fazal *et al*. 2003; Wang and Sihra 2004; Rodrigues *et al*. 2005) might control the evoked release of glutamate presynaptically, the interaction between these two receptors observed in the present study is unlikely to occur presynaptically. The most likely hypothesis is that the interaction between A_{2A}Rs and mGlu5Rs has a postsynaptic locus, in accordance with the known postsynaptic effects of both A_{2A}Rs (Li and Henry 1998; O'Kane and Stone 1998) and mGlu5Rs (Mannaioni *et al*. 2001). This possibility is reinforced by the predominant postsynaptic localization of mGlu5Rs, in particular in the hippocampus (Lujan *et al*. 1996, 1997; Shigemoto *et al*. 1997; present results), and by the present finding that one of the main consequences of the interaction between A_{2A}Rs and mGlu5Rs is the control of NMDA receptor-mediated effects, which is likely to occur postsynaptically, because the PSD was the only neuronal compartment shown to contain all three receptors.

The possible molecular mechanisms involved in the synergistic interaction between A_{2A}Rs and mGlu5Rs remain to be elucidated. Although the cAMP/PKA transduction pathway is considered the canonical transduction system operated by A_{2A}Rs (Fredholm *et al*. 2003), the inability of forskolin to reproduce the effects of CGS 21680, and the finding that KT 5720 did not inhibit the synergistic effect of CGS 21680 and CHPG, excludes the involvement of the cAMP/PKA pathway in the synergism between A_{2A}Rs and mGlu5Rs, in contrast to findings in striatal slices (Domenici *et al*. 2004). On the other hand, hippocampal A_{2A}Rs, besides coupling to the classical cAMP/PKA pathway (Okada *et al*. 2001; Rebola *et al*. 2002), also use a protein kinase C-dependent transduction pathway, in particular in the control of glutamatergic transmission (Cunha and Ribeiro 2000b; Lopes *et al*. 2002). However, we did not attempt to test whether protein kinase C might be involved in this potentiation of mGlu5R responses by A_{2A}Rs, as the manipulation of protein kinase C activity is also expected to directly affect the responses mediated by mGlu5Rs in the hippocampus (Benquet *et al*. 2002; Kotecha *et al*. 2003). Thus, at this stage, it can only be excluded that cAMP/PKA is involved and hypothesized that A_{2A}R stimulation facilitates mGlu5R-dependent effects through activation of the protein kinase C pathway.

We found that application of CHPG enhanced NMDA-mediated responses in hippocampal slices from rats and WT mice. This finding is in full agreement with some reports showing that CHPG and RS3,5-dihydroxyphenylglycine (DHPG) potentiated NMDA-induced depolarization in the rat hippocampus (Fitzjohn *et al*. 1996; Doherty *et al*. 1997), and is in line with several studies showing the ability of mGlu5Rs to increase NMDA responses in several other brain areas (Ugolini *et al*. 1999; Attucci *et al*. 2001; Pisani

et al. 2001; Domenici *et al*. 2004). In the present study, the ability of CHPG to potentiate NMDA-induced fEPSP reduction was prevented not only by MPEP (which confirms the selective involvement of mGlu5Rs in this effect of CHPG), but also by the $A_{2A}R$ antagonist ZM 241385, indicating that $A_{2A}Rs$ enable mGlu5R-mediated effects (namely, the potentiation of NMDA responses) in the hippocampus. This view was confirmed by the finding that CHPG was no longer able to potentiate the effects of NMDA effects in hippocampal slices from *$A_{2A}R$* KO mice. According to our western blot experiments, the reduced functional ability of CHPG in *$A_{2A}R$* KO mice does not appear to depend on changes in mGlu5R density. This finding is not surprising, because in rat hippocampal slices a single application of ZM 241385 (i.e. an 'acute' $A_{2A}R$ blockade, not implying changes in mGlu5R density) was enough to block the ability of CHPG to potentiate NMDA effects. Therefore, the present findings demonstrate that both the pharmacological blockade (use of ZM 241385 in rats and in WT mice) and the genetic inactivation (KO mice) of $A_{2A}Rs$ seriously impair the ability of hippocampal mGlu5Rs to potentiate NMDA responses. These results are in line with our recent results showing that the state of activation of $A_{2A}Rs$ influences mGlu5R-dependent NMDA potentiation in the striatum (Domenici *et al*. 2004). Thus, the ability of $A_{2A}Rs$ to control mGlu5R-dependent effects might be a general feature of $A_{2A}Rs$ in different brain regions, irrespective of their density (which is considerably greater in the striatum than in the hippocampus; Fredholm *et al*. 2003). This further emphasizes the role of $A_{2A}Rs$ as a fine-tuning modulatory system (i.e. modulator of other modulators) in the hippocampus (Sebastião and Ribeiro 2000).

The permissive role played by $A_{2A}Rs$ on mGlu5R-mediated effects also appears to be relevant in the modulation of NMDA-mediated neurotoxicity. We observed here that CHPG significantly potentiated NMDA-induced LDH release from cultured hippocampal neurons in a manner abolished by MPEP, whereas the mGlu5R ligands alone had no effect. Most importantly, we found that the NMDA potentiating effects of CHPG were fully prevented not only by MPEP but also by ZM 241385. This provides an important clue to our understanding of the surprising neuroprotective effect afforded by blockade of $A_{2A}Rs$ (pharmacological or genetic) in an extra-striatal region where this receptor is scarcely located (reviewed in Cunha 2005). It is important to note that ZM 241385 alone did not influence the NMDA effects, and that WT and *$A_{2A}R$* KO mice showed very similar responses to NMDA application, indicating that $A_{2A}R$ inactivation does not directly impair NMDA receptor-mediated effects.

In conclusion, the present results show that $A_{2A}Rs$ and mGlu5Rs are co-localized and interact functionally in the hippocampus. The stimulation of $A_{2A}Rs$ facilitates CHPG-induced effects (namely fEPSP reduction) and, even more interestingly, hippocampal $A_{2A}Rs$ need to be activated in order to elicit the NMDA potentiating effects of mGlu5Rs. These data suggest that $A_{2A}Rs$ might represent an interesting target for the development of new therapeutic strategies for disorders involving changes in NMDA receptor signaling, such as Alzheimer's disease, epilepsy and schizophrenia, where they might exert a neuroprotective effect similar to that recognized for striatal diseases (Chen *et al*. 2001; Popoli *et al*. 2002).

Besides its possible role in modulating excitotoxicity, the $A_{2A}R$–mGlu5R interaction might also be important as far as the physiological effects mediated by hippocampal NMDA receptors are concerned. Indeed, because hippocampal mGlu5Rs are highly involved in the modulation of NMDA-dependent plasticity (see Introduction), this interaction might represent a major mechanism in the regulation of learning and memory processes, an issue that requires further study.

Acknowledgements

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References

Alagarsamy S., Marino M. J., Rouse S. T., Gereau R. W. IV, Heinemann S. F. and Conn P. J. (1999) Activation of NMDA receptors reverses desensitization of mGlu5R in native and recombinant systems. *Nat. Neurosci.* **2**, 234–240.

Anderson W. W. and Collingridge G. L. (2001) The LTP Program: a data acquisition program for on-line analysis of long-term potentiation and other synaptic events. *J. Neurosci. Methods* **108**, 71–83.

Anwyl R. (1999) Metabotropic glutamate receptors: electrophysiological properties and role in plasticity. *Brain Res. Rev.* **29**, 83–120.

Attucci S., Carlà V., Mannaioni G. and Moroni F. (2001) Activation of type 5 metabotropic receptors enhances NMDA responses in cortical wedges. *Br. J. Pharmacol.* **132**, 799–806.

Attucci S., Clodfelter G. V., Thibault O., Staton J., Moroni F., Landfield P. W. and Porter N. M. (2002) Group I metabotropic glutamate receptor inhibition selectively blocks a prolonged Ca^{2+} elevation associated with age-dependent excitotoxicity. *Neuroscience* **112**, 183–194.

Balschun D. and Wetzel W. (2002) Inhibition of mGlu5R blocks hippocampal LTP *in vivo* and spatial learning in rats. *Pharmacol. Biochem. Behav.* **73**, 375–380.

Balschun D., Manahan-Vaughan D., Wagner T., Behnisch T., Reymann K. G. and Wetzel W. (1999) A specific role for group I mGluRs in hippocampal LTP and hippocampus-dependent spatial learning. *Learn. Mem.* **6**, 138–152.

Battaglia G., Bruno V., Pisani A., Centonze D., Catania M. V., Calabresi P. and Nicoletti F. (2001) Selective blockade of type-1 metabotropic glutamate receptors induces neuroprotection by enhancing gabaergic transmission. *Mol. Cell. Neurosci.* **17**, 1071–1083.

Benquet P., Gee C. E. and Gerber U. (2002) Two distinct signaling pathways upregulate NMDA receptor responses via two distinct metabotropic glutamate receptor subtypes. *J. Neurosci.* **22**, 9679–9686.

Bortolotto Z. A., Fitzjohn S. M. and Collingridge G. L. (1999) Roles of metabotropic glutamate receptors in LTP and LTD in the hippocampus. *Curr. Opin. Neurobiol.* **9**, 299–304.

Chen J. F., Huang Z., Ma J., Zhu J., Moratalla R., Standaert D., Moskowitz M. A., Fink J. S. and Schwarzschild M. A. (1999) A_{2A} adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. *J. Neurosci.* **19**, 9192–9200.

Chen J. F., Xu K., Petzer J. P., Staal R., Xu Y. H., Beilstein M., Sonsalla P. K., Castagnoli K., Castagnoli N. and jr and Schwarzschild M. A. (2001) Neuroprotection by caffeine and A_{2A} adenosine receptor inactivation in a model of Parkinson's disease. *J Neurosci.* **21**, RC 143.

Coccurello R., Breysse N. and Amalric M. (2004) Simultaneous blockade of adenosine A2A and metaboptropic glutamate mGlu5 receptors increase their efficacy in reversing Parkinsonian deficits in rats. *Neuropsychopharmacology* **29**, 1451–1461.

Collingridge G. L. and Bliss T. V. (1995) Memories of NMDA receptors and LTP. *Trends Neurosci.* **18**, 54–56.

Cunha R. A. (2005) Neuroprotection by adenosine in the brain: from A_1 receptor activation to A_{2A} receptor blockade. *Purinergic Signalling* **1**, 111–134.

Cunha R. A., Constantino M. D. and Ribeiro J. A. (1997) ZM241385 is an antagonist of the facilitatory responses produced by the A_{2A} adenosine receptor agonists CGS21680 and HENECA in the rat hippocampus. *Br. J. Pharmacol.* **122**, 1279–1284.

Cunha R. A. and Ribeiro J. A. (2000a) Purinergic modulation of [^{3}H]GABA release from rat hippocampal nerve terminals. *Neuropharmacology* **39**, 1156–1167.

Cunha R. A. and Ribeiro J. A. (2000b) Adenosine A_{2A} facilitation of synaptic transmission in the CA1 area of the rat hippocampus requires protein kinase C but not protein kinase A activation. *Neurosci. Lett.* **289**, 127–130.

Diaz-Cabiale Z., Vivò M., Del Arco A., O'Connor W. T., Harte M. K., Müller C. E., Martinez E., Popoli P., Fuxe K. and Ferré S. (2002) Metabotropic glutamate mGlu5 receptor-mediated modulation of the ventral striopallidal GABA pathway in rats: interactions with adenosine A2A and dopamine D2 receptors. *Neurosci. Lett.* **324**, 154–158.

Doherty A. J., Palmer M. J., Henley J. M., Collingridge G. L. and Jane D. E. (1997) (*RS*)-2-Chloro-5-hydroxyphenylglycine (CHPG) activates $mGlu_5$, but not $mGlu_1$, receptors expressed in CHO cells and potentiates NMDA responses in hippocampus. *Neuropharmacology* **36**, 265–267.

Domenici M. R., Pepponi R., Martire A., Tebano M. T., Potenza R. L. and Popoli P. (2004) Permissive role of adenosine A_{2A} receptors on metabotropic glutamate receptor 5 (mGluR5)-mediated effects in the striatum. *J. Neurochem.* **90**, 1276–1279.

Fazal A., Parker F., Palmer A. M. and Croucher M. J. (2003) Characterisation of the actions of group I metabotropic glutamate receptor subtype selective ligands on excitatory amino acid release and sodium-dependent re-uptake in rat cerebrocortical minislices. *J. Neurochem.* **86**, 1346–1358.

Ferré S., Karcz-Kubicha M., Hope B. *et al*. (2002) Synergistic interaction between adenosine A_{2A} and glutamate mGlu5 receptors: implications for striatal neuronal function. *Proc. Natl Acad. Sci. USA* **99**, 11 940–11 945.

Fitzjohn S. M., Irving A. J., Palmer M. J., Harvey J., Lodge D. and Collingridge G. L. (1996) Activation of group I mGluRs potentiates NMDA responses in rat hippocampal slices. *Neurosci. Lett.* **203**, 211–213.

Fredholm B. B., Cunha R. A. and Svenningsson P. (2003) Pharmacology of adenosine A_{2A} receptors and therapeutic applications. *Curr. Top. Med. Chem.* **3**, 413–426.

Fuxe K., Agnati L. F., Jacobsen J. *et al.* (2003) Receptor heteromerization in adenosine A_{2A} receptor signaling: relevance for striatal function and Parkinson's disease. *Neurology* **61**, S19–S23.

Kotecha S. A., Jackson M. F., Al-Mahrouki A., Roder J. C., Orser B. A. and MacDonald J. F. (2003) Co-stimulation of mGluR5 and *N*-methyl-D-aspartate receptors is required for potentiation of excitatory synaptic transmission in hippocampal neurons. *J. Biol. Chem.* **278**, 27 742–27 749.

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Li H. and Henry J. L. (1998) Adenosine A2 receptor mediation of pre- and postsynaptic excitatory effects of adenosine in rat hippocampus *in vitro*. *Eur. J. Pharmacol.* **347**, 173–182.

Lopes L. V., Cunha R. A., Kull B., Fredholm B. B. and Ribeiro J. A. (2002) Adenosine A_{2A} receptor facilitation of hippocampal synaptic transmission is dependent on tonic A_1 receptor inhibition. *Neuroscience* **112**, 319–329.

Lu Y. M., Jia Z., Janus C., Henderson J. T., Gerlai R., Wojtowicz M. J. and Roder J. C. (1997) Mice lacking metabotropic glutamate receptor 5 show impaired learning and reduced CA1 long-term potentiation (LTP) but normal CA3 LTP. *J. Neurosci.* **17**, 5196–5205.

Lujan R., Nusser Z., Roberts J. D., Shigemoto R. and Somogyi P. (1996) Perisynaptic location of metabotropic glutamate receptors mGlu1R and mGluR5 on dendrites and dendritic spines in the rat hippocampus. *Eur. J. Neurosci.* **8**, 1488–1500.

Lujan R., Roberts J. D., Shigemoto R., Ohishi H. and Somogyi P. (1997) Differential plasma membrane distribution of metabotropic glutamate receptors mGluR1 alpha, mGluR2 and mGluR5, relative to neurotransmitter release sites. *J. Chem. Neuroanat.* **13**, 219–241.

Mannaioni G., Marino M. J., Valemnti O., Traynelis S. F. and Conn J. P. (2001) Metabotropic glutamate receptors 1 and 5 differentially regulate CA1 pyramidal cell function. *J. Neurosci.* **21**, 5925–5934.

Marchi M., Raiteri L., Risso F., Vallarino A., Bonfanti A., Monopoli A., Ongini E. and Raiteri M. (2002) Effects of adenosine A_1 and A_{2A} receptor activation on the evoked release of glutamate from rat cerebrocortical synaptosomes. *Br. J. Pharmacol.* **136**, 434–440.

Mori M. and Gerber U. (2002) Slow feedback inhibition in the CA3 area of the rat hippocampus by synergistic synaptic activation of mGluR1 and mGluR5. *J. Physiol.* **544**, 793–799.

Nikbakht M. R. and Stone T. W. (2001) Suppression of presynaptic responses to adenosine by activation of NMDA receptors. *Eur. J. Pharmacol.* **427**, 13–25.

Nishi A., Liu F., Matsuyama S., Hamada M., Higashi H., Nairn A. C. and Greengard P. (2003) Metabotropic mGlu5 receptors regulate adenosine A_{2A} receptor signalling. *Proc. Natl Acad. Sci. USA* **100**, 1322–1327.

O'Kane E. M. and Stone T. W. (1998) Interaction between adenosine A1 and A2 receptor-mediated responses in the rat hippocampus *in vitro*. *Eur. J. Pharmacol.* **362**, 17–25.

Okada M., Nutt D. J., Murakami T., Zhu G., Kamata A., Kawata Y. and Kaneko S. (2001) Adenosine receptor subtypes modulate two major functional pathways for hippocampal serotonin release. *J. Neurosci.* **21**, 628–640.

Phillips G. R., Huang J. K., Wang Y., Tanaka H., Shapiro L., Zhang M., Gordon R. E., Gawinowicz M. A., Zhao Y. and Colman D. R. (2001) The presynaptic particle web: ultrastructure, composition, dissolution, and reconstitution. *Neuron* **32**, 1–20.

Pisani A., Gubellini P., Bonsi P., Conquet F., Picconi B., Centonze D., Bernardi G. and Calabresi P. (2001) Metabotropic glutamate receptor 5 mediates the potentiation of *N*-methyl-D-aspartate responses in medium spiny striatal neurons. *Neuroscience* **106**, 579–587.

Popoli P., Pèzzola A., Torvinen M., Reggio R., Pintor A., Scarchilli L., Fuxe K. and Ferré S. (2001) The selective mGlu5 receptor agonist CHPG inhibits quinpirole-induced turning in 6-hydroxydopamine-lesioned rats and modulates the binding characteristics of dopamine D_2 receptors in the striatum: interaction with adenosine A_{2A} receptors. *Neuropsychopharmacology* **25**, 505–513.

Popoli P., Pintor A., Domenici M. R. *et al.* (2002) Blockade of sriatal adenosine A_{2A} receptor reduces, through a presynaptic mechanism, quinolinic acid-induced excitotoxicity: possibile relevance to neuroprotective interventions in neurodegenerative diseases of the striatum. *J. Neurosci.* **22**, 1967–1975.

Popoli P., Pintor A., Tebano M. T. *et al.* (2004) Neuroprotective effects of the mGlu5R antagonist MPEP towards quinolinic acid-induced striatal toxicity: involvment of pre and post-synaptic mechanisms and lack of direct NMDA blocking activity. *J. Neurochem.* **89**, 1479–1489.

Rebola N., Oliveira C. R. and Cunha R. A. (2002) Transducing system operated by adenosine A_{2A} receptors to facilitate acetylcholine release in the rat hippocampus. *Eur. J. Pharmacol.* **454**, 31–38.

Rebola N., Pinheiro P. C., Oliveira C. R., Malva J. O. and Cunha R. A. (2003) Subcellular localization of adenosine A_1 receptors in nerve terminals and synapses of the rat hippocampus. *Brain Res.* **987**, 49–58.

Rebola N., Canas P., Oliveira C. R. and Cunha R. A. (2005) Different synaptic and subsynaptic localization of adenosine A_{2A} receptors in the hippocampus and striatum of the rat. *Neuroscience* **132**, 893–903.

Rodrigues R. J., Alfaro T. M., Rebola N., Oliveira C. R. and Cunha R. A. (2005) Co-localization and functional interaction between adenosine A_{2A} and metabotropic group 5 receptors in glutamatergic nerve terminals of the rat striatum. *J. Neurochem.* **92**, 433–441.

Romano C., Sesma M. A., McDonald C. T., O'Malley K., van den Pol A. N. and Olney J. W. (1995) Distribution of metabotropic glutamate receptor mGluR5 immunoreactivity in rat brain. *J. Comp. Neurol.* **355**, 455–469.

Rothman S. M. and Olney J. W. (1995) Excitotoxicity and the NMDA receptor – still lethal after eight years. *Trends Neurosci.* **18**, 57–58.

Sacaan A. I. and Schoepp D. D. (1992) Activation of hippocampal metabotropic excitatory amino acid receptors leads to seizures and neuronal damage. *Neurosci. Lett.* **139**, 77–82.

Schulz P. E., Cook E. P. and Johnston D. (1994) Changes in paired-pulse facilitation suggest presynaptic involvement in long-term potentiation. *J. Neurosci.* **14**, 5325–5337.

Sebastião A. M. and Ribeiro J. A. (2000) Fine-tuning neuromodulation by adenosine. *Trends Pharmacol. Sci.* **21**, 341–346.

Shigemoto R., Kinoshita A., Wada E. *et al.* (1997) Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. *J. Neurosci.* **17**, 7503–7522.

Ugolini A., Corsi M. and Bordi F. (1999) Potentiation of NMDA and AMPA responses by the specific mGluR5 agonist CHPG in spinal cord motoneurons. *Neuropharmacology* **38**, 1569–1576.

Wang S. J. and Sihra T. S. (2004) Noncompetitive metabotropic glutamate5 receptor antagonist (E)-2-methyl-6-styryl-pyridine (SIB1893) depresses glutamate release through inhibition of voltage-dependent Ca^{2+} entry in rat cerebrocortical nerve terminals (synaptosomes). *J. Pharmacol. Exp. Ther.* **309**, 951–958.