Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2015 | 135 | 714–726 doi: 10.1111/jnc.13291

ORIGINAL ARTICLE

Adenosine A_{2A} receptors permit mGluR5-evoked tyrosine phosphorylation of NR2B (Tyr1472) in rat hippocampus: a possible key mechanism in NMDA receptor modulation

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Abstract

A great body of evidence points toward a functional interaction between metabotropic glutamate 5 receptors (mGluR5) and NMDA receptors (NMDAR) that enhances synaptic plasticity and cognition. However, the molecular mechanism underlying this interaction remains unclear. Here, we show that co-activation of mGluR5 and NMDAR in hippocampal slices synergistically leads to a robust phosphorylation of NR2B (Tyr1472), which is Src kinase dependent and is enabled by endogenous adenosine acting on A_{2A} receptors. As it is well known, NR2B (Tyr1472) phosphorylation anchors NR2B-containing NMDARs to the surface of post-synaptic membranes, preventing their internalization. This is supported by our electrophysiological experiments showing that co-activation of mGluR5 and NMDARs robustly enhances NMDAR-dependent neuronal excitability recorded in CA1 hippocampal region, which temporally coincides with the robust increase in NR2B (Tyr1472) phosphorylation, depends on Src kinases and is also permitted by A_{2A} receptors. Thus, we strongly suggest that NR2B (Tyr1472) phosphorylation constitutes, at least to some extent, the molecular mechanism underlying the mGluR5-mediated enhancement of NMDAR-dependent responses, which is modulated by A_{2A} receptors. A better understanding of the molecular basis of mGluR5/NMDAR interaction would elucidate their role in synaptic plasticity processes as well as in pathological conditions.

Keywords: adenosineA_{2A}R, hippocampus, mGluR5, NR2B, phosphorylation, Src kinases.

J. Neurochem. (2015) **135**, 714–726.

The neuromodulatory actions of glutamate, the major excitatory neurotransmitter within the CNS, are mediated by activation of the metabotropic glutamate receptors (mGluRs), comprised of eight different subtypes (Sheffler *et al.* 2011). Among them, Group I mGluRs, including mGluR1 and mGluR5, are extensively expressed throughout the brain, having a predominantly post-synaptic distribution (Baude *et al.* 1993; Lujan *et al.* 1996; Homayoun and Moghaddam 2010; Sheffler *et al.* 2011). From these subtypes, mGluR5 are most abundant in corticolimbic areas responsible for controlling higher cognitive function, including the hippocampus (Ferraguti and Shigemoto 2006; Sheffler *et al.* 2011), a brain structure involved in processes of learning and memory (Eichenbaum 2004; Tse *et al.* 2007).

The mGluR5 plays important role in synaptic plasticity, including Long Term Potentiation (LTP) and Long Term Depression (LTD) (Anwyl 1999; Ayala *et al.* 2009; Sheffler *et al.* 2011). Using mGluR5 KO mice, it has been demonstrated that deletion of these subtypes results in short-term spatial memory deficits (Gray *et al.* 2009), a reduction in hippocampal CA1 LTP and impaired performance in cognitive tasks (Lu *et al.* 1997). Moreover, repeated treatment with mGluR5 antagonists impairs working and spatial memory (Manahan-Vaughan and Braunewell 2005), which supports a role for mGluR5 in learning and memory (Homayoun and Moghaddam 2010).

Received June 24, 2015; revised manuscript received July 23, 2015; accepted August 07, 2015.

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Abbreviations used: A_{2A}R, Adenosine A_{2A} receptors; ACSF, artificial cerebrospinal fluid; ADA, adenosine deaminase; CA1, Cornu Ammonis area 1; DMSO, Dimethyl-sulfoxide; EPSP, excitatory post-synaptic potential; Fv, fiber volley; mGluR5, metabotropic glutamate 5 receptors; NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptors; PS, population spike; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline.

An interesting implication of mGluR5 in cognition is mediated through its modulatory role by potentiating NMDAR-mediated evoked responses (Pisani *et al.* 1997; Anwyl 1999; Bortolotto *et al.* 1999; Tebano *et al.* 2005; Rebola *et al.* 2008). In particular, the mGluR5 are involved in the potentiation of NMDA-induced currents in the hippocampus (Doherty *et al.* 1997; Mannaioni *et al.* 2001). In addition, selective mGluR5 agonists potentiate the NMDAR-induced membrane depolarization in striatal neurons (Pisani *et al.* 1997). This interaction involves several components of intracellular signaling machinery including protein kinase C (Alagarsamy *et al.* 1999; Mao and Wang 2002a,b) and ERK1/2 (Yang *et al.* 2004). These kinase-dependent signal pathways modulate NMDAR-mediated responses by phosphorylation of NMDAR subunits (Chen and Roche 2007). Among these kinase signal transduction pathways, the protein tyrosine kinase pathway, including Src and Fyn kinases, is of great interest, so that its activation leads to potentiation of NMDAR-mediated currents (Wang and Salter 1994; Wang *et al.* 1996; Chen and Roche 2007; Rebola *et al.* 2008). One major phosphorylation site of Fyn kinase is the tyrosine residue 1472 of NR2B subunit (Nakazawa *et al.* 2001; Takasu *et al.* 2002). The phosphorylation of this tyrosine residue results in the inhibition of NR2B-mediated endocytosis and the stabilization of the NMDARs in the plasma membrane (Prybylowski *et al.* 2005; Lee 2006; Chen and Roche 2007; Lau and Zukin 2007). Accordingly, the level of Tyr-1472 phosphorylation is increased after induction of long-term potentiation (LTP) in the hippocampus, suggesting that Fyn-mediated phosphorylation of Tyr-1472 is involved in synaptic plasticity (Nakazawa *et al.* 2001). Moreover, mice carrying mutation on the NR2B tyrosine 1472 phosphorylation site show impaired fear learning and amygdaloid LTP (Nakazawa *et al.* 2006).

It has been demonstrated that in the striatum, mGluR5 interacts with adenosine A_{2A} receptors forming heteromeric complexes (Ferre *et al.* 2002), and the activation of A_{2A} receptors influences the mGluR5-dependent NMDA potentiation (Domenici *et al.* 2004). A similar functional interaction between A_{2A} and mGluR5 has been recently reported in the hippocampus as well, where A_{2A} and mGluR5s are co-localized and synergistically interact to modulate NMDAR-mediated effects (Tebano *et al.* 2005). Moreover, in mossy fiber synapses in the hippocampus, co-activation of A_{2A} and mGluR5s are essential for long-term potentiation of NMDAR-mediated component of Excitatory Postsynaptic Currents (EPSCs), depending on the activation of Src kinases (Rebola *et al.* 2008).

Taking the above into consideration, the aim of this study was to investigate the molecular mechanism by which mGluR5 interact with NMDARs, thus, potentiating their responses. To this end, we have examined rat hippocampal slices to determine whether the activation of mGluR5 by their selective agonist (RS)-2-Chloro-5-hydroxyphenylglycine (CHPG) would induce phosphorylation of NR2B subunit at tyrosine residue 1472. In order to investigate the functional role of this mGluR5- mediated phosphorylation of NR2B subunit, we have correlated it with the mGluR5-mediated potentiation of NMDAR-mediated responses in rat hippocampal slices using electrophysiological techniques. Furthermore, we have investigated the role of A_{2A} adenosine receptors in this mGluR5/NMDAR interaction.

Materials and methods

Animals and slice preparation

Adult male Wistar rats were used. The animals were obtained from the Animal Facility of the Medical School of the University of Patras. All experimental treatment and procedures were conducted in accordance with the European Communities Council Directive Guidelines (86/609/EEC, JL 358, 1, December, 12, 1987) for the care and use of Laboratory animals, and they have been approved by the Prefectural Animal Care and Use Committee (No: EL 13BIO04). In addition, all efforts have been made to minimize the number and the suffering of animals used. Animals were housed under controlled conditions of temperature (20–22°C), light-dark cycle (12/12 h), and free access to food and water. Hippocampal slices were prepared as previously described (Papatheodoropoulos *et al.* 2005). Briefly, after decapitation under deep anesthesia with diethyl ether, the brain was removed and placed in chilled (2–4°C) standard artificial cerebrospinal fluid containing 124 mM NaCl, 4 mM KCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM glucose, equilibrated with 95% O_2 and 5% CO_2 gas mixture at pH = 7.4. Then, the hippocampi were excised free and transverse slices 500–550 μm thick were prepared from the dorsal part of hippocampus using a McIlwain tissue chopper.

'*In vitro*' stimulation of mGluR5/NMDAR under pharmacological manipulation

Hippocampal slices were incubated in warm (± 35°C) artificial cerebrospinal fluid solution and constantly oxygenated with 95% O_2–5% CO_2 for at least 60 min before drug application. The slices were classed in the following drug combinations: (i) control slices incubated with saline, (ii) slices incubated with the selective mGluR5 receptor agonist CHPG (Sigma, St Louis, MO, USA) (15, 30, 50, 100, 300, and 500 μM), (iii) slices co-incubated with the selective mGluR5 receptor agonist CHPG (15 μM) and the NMDAR selective agonist, NMDA (Sigma) (5 μM), (iv) slices incubated with NMDA (5 μM), (v) slices incubated with the selective mGluR5 receptor agonist CHPG (15 μM and 50 μM) in the presence of the selective inhibitor of Src family tyrosine kinases, PP1 (Tocris) (30 μM), (vi) slices co-incubated with the selective mGluR5 receptor agonist CHPG (15 μM) and the NMDAR selective agonist, NMDA (5 μM) in the presence of the selective inhibitor of Src family tyrosine kinases, PP1 (30 μM), (vii) slices incubated with the selective inhibitor of Src family tyrosine kinases, PP1 (30 μM), (viii) slices incubated with the selective antagonist of A_{2A} adenosine receptors, ZM241385 (Tocris) (1 μM), (IX) slices incubated with the selective mGluR5 receptor agonist CHPG (50 μM and 500 μM) in the presence of the selective antagonist

of A_{2A} adenosine receptors, ZM241385 (Tocris) (1 μM), (X) slices co-incubated with the selective mGluR5 receptor agonist CHPG (15 μM) and the NMDAR selective agonist, NMDA (5 μM) in the presence of the selective antagonist of A_{2A} adenosine receptors, ZM241385 (1 μM), (XI) slices incubated with the selective agonist of A_{2A} adenosine receptors, CGS21680 (Tocris) (30 nM), (XII) slices co-incubated with the selective mGluR5 receptor agonist CHPG (50 μM) in the presence of the adenosine deaminase (ADA) (Roche Molecular Biochemicals, Indianapolis, IN, USA) (10 μg/mL), XIII) slices co-incubated with the selective mGluR5 agonist CHPG (50 μM), the selective agonist of A_{2A} adenosine receptors, CGS21680 (30 nM) in the presence of the ADA (10 μg/mL). The drugs were applied for 5 min and the slices were instantly frozen in isopentane (−50°C) and stored at −80°C until use. In the experiments performed in the presence of the selective inhibitor of the Src family tyrosine kinases, PP1 (30 μM) (Hanke *et al.* 1996; Rebola *et al.* 2008), the selective antagonist of A_{2A} adenosine receptors, ZM241385 (Tocris) (1 μM) (Cunha *et al.* 1997; Rebola *et al.* 2008), and the ADA (10 μg/mL) (Dell'anno *et al.* 2013), the PP1, the ZM241385 and the ADA were applied 30 min prior to the incubation of slices with the drug combinations.

Western blotting

The experiments were performed as previously described (Brooks-Kayal *et al.* 2001). Briefly, the slices were rapidly microdissected at 4°C and then solubilized in 100 μL 1% sodium dodecyl sulfate v/w with 4 μL Sigma Phosphatase Inhibitor Cocktail I, 4 μL Sigma Phosphatase Inhibitor Cocktail II, 4 μL Sigma Protease Inhibitor Cocktail, sonicated, and boiled for 10 min. Protein concentration was determined for each sample by using the bovine serum albumin protein kit (Pierce, Rockford, IL, USA) and spectrophotometry. Duplicated samples (50 μg of total protein) were separated on 7% polyacrylamide gel and then transferred to nitrocellulose. After 1 h of blocking in 10% non-fat dried milk at 21°C, the nitrocellulose was incubated overnight at 4°C with the following antibodies: rabbit anti-PhosphoNR2B (ser1303) polyclonal antibody (1 : 750, Millipore Corporation, Bedford, MA, USA) and rabbit anti-PhosphoNR2B (tyr1472) polyclonal antibody (1 : 500, Cell Signaling Technology, Beverly, MA, USA). Total amounts of proteins were detected using rabbit anti-NR2B polyclonal antibody (1 : 1000, Millipore) diluted in 10% non-fat dried milk, after the stripping of the membrane using the Re-Blot Plus Mild Solution (Millipore) for approximately 15 min at 21°C. Molecular masses were determined by comparison with pre-stained protein molecular weight marker standards from Biomol. The blot was rinsed with Tris-buffered saline-Tween and then incubated with goat anti-rabbit horseradish peroxidase-linked IgG (1 : 2500, Cell Signaling Technology) for 1 h at 21°C, followed by the Enhanced Chemiluminescence detection system (Millipore). Blots were reprobed with anti-tubulin mouse monoclonal antibody (1 : 20 000; Sigma) rinsed with Tris-buffered saline-Tween, and then incubated with anti-mouse antibody and normalized to verify equivalent protein loading. Luminescence from the blots was detected by exposing the membranes to Fuji-Hyperfilm for 30 s–7 min, to ensure that we were operating within the linear range of the film, followed by digital scanning of the developed film in transparency mode. The scanned image of the membranes and band intensities were calibrated and quantified using NIH ImageJ software (version 1.34; National Institutes of Health, Bethesda, MD, USA). For each animal, the values of the phosphorylated levels of each protein examined were normalized with the respective total levels. Each experiment was performed two to four times.

Electrophysiology, data processing and Analysis

After their preparation, hippocampal slices were immediately transferred and maintained to an interface type recording chamber at a constant temperature of 31 ± 0.5°C, perfused with standard medium (with superfusion rate of 1.6–1.8 mL/min) and continuously humidified with a mixed gas containing 95% O_2 and 5% CO_2. Slices were left to equilibrate for at least 1 h before recordings were started. In some experiments, slices containing only the CA1 field (CA1 minislices) were prepared by making three knife cuts in intact slices, at Schaffer collaterals, at the level of the hippocampal fissure and at the CA1-subiculum border. Evoked field potentials consisting of pre-synaptic fiber volley (Fv), excitatory post-synaptic potential (EPSP), and population spike (PS) were simultaneously recorded from the stratum radiatum (EPSP, Fv) and stratum pyramidale (PS) of CA1 field of individual slices using carbon fiber electrodes (diameter 7 μm, Kation Scientic, Minneapolis, USA). The two recording electrodes were positioned in the stratum radiatum and pyramidale of the CA1b field. Stimulation pulses were delivered to Schaffer collaterals using a bipolar platinum/iridium electrode (25 μm wire diameter, at an inter-wire distance of 100 μm, World Precision Instruments, USA). Potentials were acquired using a Neurolog amplifier (Digitimer Limited, UK), they were band-pass filtered at 0.5 Hz–2 kHz, digitized at 10 kHz, and stored in a computer disk using the CED 1401-plus interface and the Signal6 software (Cambridge Electronic Design, Cambridge, UK) for off-line analysis. Only slices which displayed stable EPSP and PS for at least 10 min were selected for further experimentation. EPSP was quantified by the maximum slope of its rising phase; Fv was quantified by its amplitude measured as the difference between the peak negative voltage and the baseline; PS was quantified by its amplitude measured as the length of the projection of the negative peak on the line connecting the two positive peaks of the PS waveform. In instances where secondary PSs appeared in the response, we measured the sum of the amplitudes of all PSs (Swearengen and Chavkin 1987). In all instances, measures of EPSP and Fv were obtained from recordings made in stratum radiatum while measures of PS were made on recordings from st. pyramidale. The EPSP/Fv ratio was used to quantify synaptic effectiveness while the PS/EPSP ratio was used as an index of post-synaptic excitability. Baseline responses were evoked by stimulation intensity that produced an EPSP 50–60% of the maximum and a corresponding half-maximum PS, based on input/output curves. Input/output curves between stimulation intensity and response were routinely made in control and drug conditions. However, under conditions of strong drug-induced suppression of the synaptic response input/output curves could not be made. Comparisons between control and drug conditions were made using similar EPSPs in control and drug conditions.

We also calculated the paired-pulse facilitation of EPSP (at 50 ms) which was measured by the ratio between the second and the first response (i.e., EPSP2/EPSP1). Measures were made on individual responses. Comparisons of the parameters between different conditions were made after calculating the mean values of three responses.

Drugs

In electrophysiology, the following drugs were used: the NMDAR agonist *N*-Methyl-D-aspartic acid (NMDA, 50 μM); the NMDAR antagonist 3-((R)-2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP, 10 μM); the selective mGluR5 agonist (*RS*)-2-Chloro-5-hydroxyphenylglycine sodium salt (CHPG, 50 μM); the selective mGluR5 antagonist 3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine hydrochloride (MTEP, 200 μM); the selective A_{2A}R antagonist 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino] ethyl)phenol (ZM 241385, 1 μM), 1-(1,1-Dimethylethyl)-3-(1-naphthalenyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (PP1, 30 μM), 4-[2-[[6-Amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride (CGS21 680, 30 nM).

Drugs were purchased from Tocris Cookson Ltd, UK (NMDA, CPP, CHPG, 2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), ZM 241385), Roche (ADA), as well as from Sigma-Aldrich, Germany (NMDA and CHPG). Drugs were first prepared as stock solutions and then dissolved in standard medium and bath-applied to the tissue. Stock solutions of NMDA, CPP, CHPG, MPEP, and ADA were prepared in distilled water, whereas stock solutions of ZM 241385, CGS21680, PP1 were prepared in Dimethyl-sulfoxide (DMSO) at such a concentration that when diluted for bath-application, the final volume of DMSO was lower than 0.05%. Stock solutions in water were maintained at 4°C while solutions in DMSO were prepared in aliquots and kept at −20°C. Stock solutions were diluted in standard medium to the desired concentrations on the day of the experiment.

Statistics

For the electrophysiological experiments, the paired t-test and the independent t-test, as well as the non-parametric Wilcoxon test and Mann–Whitney *U*-test, were used for comparisons between related and independent groups of values, respectively. The values of various parameters are expressed as mean ± SEM and throughout the text '*n*' indicates the number of slices used in the analysis.

For the western blotting experiments, statistical analysis used ANOVA followed by Tukey *post hoc* test. The values of the various parameters are expressed as means ± SEM values (number of animals, $N = 8$) per group of representative western blots.

Results

Stimulation of mGluR5 induces phosphorylation of NR2B subunit of NMDAR at Tyr1472, but not at Ser1303

Since it has been indicated that mGluR5 activation enhances the NMDA-mediated effects in hippocampus (Anwyl 1999; Bortolotto *et al.* 1999; Tebano *et al.* 2005), we investigated the effects of the *in vitro* mGluR5 stimulation on the phosphorylation level of NR2B subunit of NMDARs in hippocampal slices. Our results showed that the basal levels of phosphorylation at Tyr1472 of NR2B subunit were hardly detectable. However, *in vitro* incubation of hippocampal slices with the selective agonist of mGluR5s, CHPG induced a dose-dependent increase in the phosphorylation level of NR2B subunit of NMDAR at the residue Tyr1472 (44% by 30 μM CHPG and 77% by 50 μM CHPG compared to basal levels, Fig. 1a), bringing the phosphorylation state at a plateau level, showing no further increase by either 100 μM or 300 μM CHPG (Fig. 1a). However, using 500 μM CHPG, the phosphorylation state increased significantly further (Fig. 1a). This phenomenon seems to be site specific,

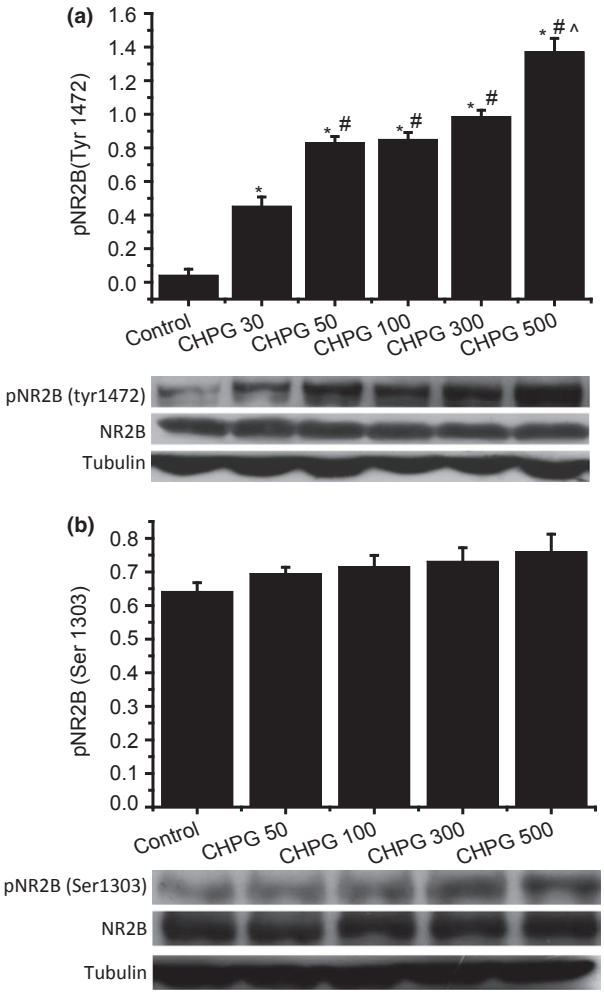

Fig. 1 Stimulation of mGluR5 induces phosphorylation of NR2B subunit at Tyr1472. Western blot analysis of the phosphorylation level of (a) NR2B (Tyr1472) subunit and (b) NR2B (Ser1303) subunit of NMDAR, normalized with the respective total protein levels in dorsal hippocampal slices in different animal groups, according to the drug treatment: [control], [CHPG 30], [CHPG 50], [CHPG 100], [CHPG 300], [CHPG 500]. Tubulin was used as loading control. Incubation of hippocampal slices with the selective agonist of mGluR5 receptor, CHPG, at different doses, induced a dose-dependent increase in the phosphorylation level of the NR2B (Tyr1472), but not of the NR2B (Ser1303) subunit of NMDAR. Results are shown in means ± SEM values (number of animals, $N = 8$) per group of representative western blots. $^{*}p < 0.05$ significantly different compared with the control group. $^{\#}p < 0.05$ significantly different compared with the [CHPG 30] group. $^{\wedge}p < 0.05$ significantly different compared with all other drug treated groups. Statistical analysis used ANOVA followed by Tukey *post hoc* test.

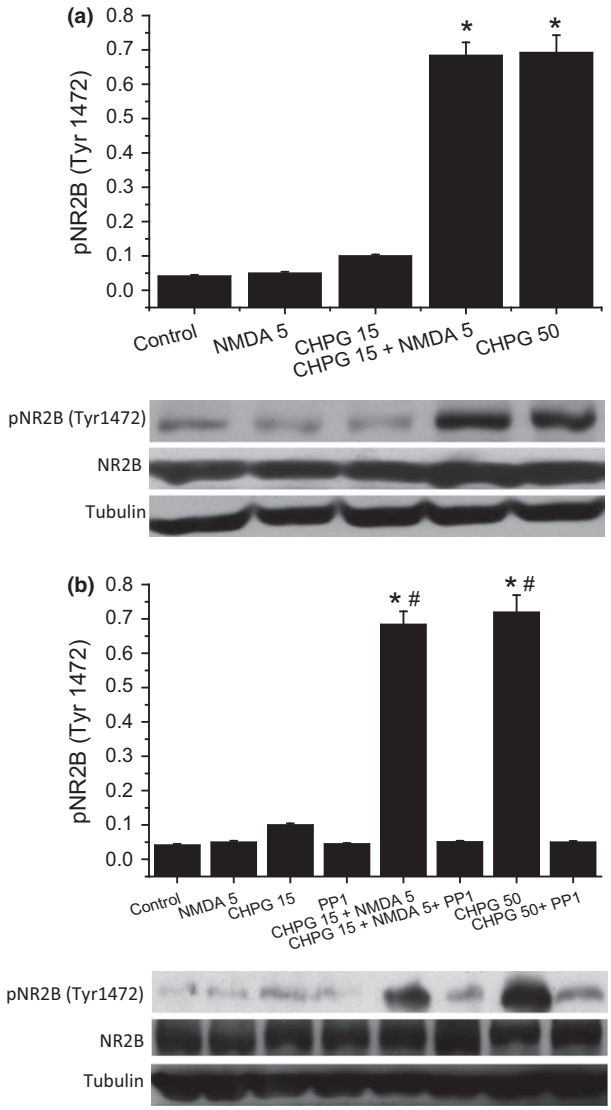

Fig. 2 Synergistic interaction between mGluR5 and NMDAR strongly increases the phosphorylation level of the NR2B (Tyr1472), which involves the Src kinases. (a) Synergistic interaction between mGlu5 and NMDARs, as detected by western blot analysis of NR2B (Tyr1472) phosphorylation normalized with the respective total protein levels in dorsal hippocampal slices in different animal groups, according to drug treatment: [control], [NMDA 5], [CHPG 15], [CHPG 15 + NMDA 5], [CHPG 50]. Tubulin was used as loading control. The co-incubation of hippocampal slices with ineffective doses of the mGluR5 agonist CHPG (15 μM) and NMDAR selective agonist, NMDA (5 μM) induces a robust increase in the NR2B (Tyr1472) phosphorylation compared to basal levels. Results are shown in means ± SEM values (number of animals, $N = 8$) per group of representative western blots. $^{*}p < 0.05$ significantly different compared with the control group. Statistical analysis used ANOVA followed by Tukey *post hoc* test. (b) The mGluR5- and the mGluR5/NMDAR-induced NR2B (Tyr1472) phosphorylation involves the Src family kinases. Western blot analysis of NR2B (Tyr1472) phosphorylation normalized with the respective total protein levels in dorsal hippocampal slices in different animal groups, according to the drug treatment: [control], [NMDA 5], [CHPG 15], [PP1], [CHPG 15 + NMDA 5], [CHPG 15 + NMDA 5 + PP1] [CHPG 50], [CHPG 50 + PP1]. Tubulin was used as loading control. The incubation of hippocampal slices by CHPG (50 μM) and CHPG (15 μM)/NMDA (5 μM) in the presence of PP1 (30 μM) (selective inhibitor of Src kinases) completely abolished the mGluR5- and the mGluR5/NMDAR-induced NR2B (Tyr1472) phosphorylation. Results are shown in means ± SEM values (number of animals, $N = 8$) per group of representative western blots. $^{*}p < 0.05$ significantly different compared with the control group. $^{\#}p < 0.05$ significantly different compared with the [CHPG 15 + NMDA 5 + PP1] and [CHPG 50 + PP1] groups. Statistical analysis used ANOVA followed by Tukey *post hoc* test.

since *in vitro* stimulation of mGluR5 by their selective agonist, CHPG, at any concentration used (50–500 μM), failed to increase the phosphorylation levels of the residue Ser1303 of NR2B subunit of NMDAR compared to the control levels in the hippocampal slices (Fig. 1b).

Synergistic interactions between mGluR5 and NMDARs

Previous studies from our Lab have shown that synergistic interaction between dopamine D1 and NMDARs enhances the phosphorylation levels of NMDAR's subunits in the hippocampus (Sarantis *et al.* 2009, 2012). Considering this in conjunction with how the mGluR5s 'set the tone' of NMDAR-mediated neurotransmission (Alagarsamy *et al.* 1999), we wondered whether a similar synergy between mGluR5 and NMDARs exists. Our results showed that *in vitro* incubation of hippocampal slices with the mGluR5-selective agonist CHPG at the concentration of 15 μM, failed to increase the phosphorylation level of the NR2B subunit (Tyr1472) of NMDARs compared to control levels (Fig. 2a). The same result was observed, when the hippocampal slices were incubated only with NMDA, the selective agonist of the NMDARs, at the concentration of 5 μM. Interestingly, the co-incubation of hippocampal slices with the ineffective doses of the mGluR5 agonist CHPG (15 μM) and NMDAR-selective agonist, NMDA (5 μM), induced a robust increase in the phosphorylation state of the NR2B subunit (Tyr1472) compared to basal levels (Fig. 2a), reaching the plateau levels seen before by using 50 μM CHPG (Fig. 2a). This indicates a strong synergy between mGluR5 and NMDARs.

Phosphorylation of NR2B (Tyr 1472) subunit by mGluR5 activation or mGluR5/NMDAR co-activation requires activation of Src family kinases

Previous studies have shown that the Tyr1472 residue of NR2B subunit is phosphorylated by Fyn kinase, which is a member of Src family kinases (Nakazawa *et al.* 2001). To investigate whether Src family kinases are required for the CHPG and/or CHPG/NMDA-induced phosphorylation at Tyr1472 of NR2B subunit, we incubated the hippocampal slices with CHPG (50 μM) and CHPG (15 μM)/NMDA (5 μM) combination in the presence of the selective inhibitor of Src family kinases, PP1 (30 μM). Our results showed that

phosphorylation of NR2B at Tyr1472 either by CHPG (50 μM) alone or by co-application of 15 μM CHPG and 5 μM NMDA was completely abolished in the presence of PP1 (Fig. 2b). This observation showed that the intracellular signal pathway activated by stimulation of mGluR5 alone or by the co-stimulation of mGluR5 with NMDARs, involves the Src family kinases, such as Fyn kinase.

The synergistic effects of NMDAR and mGluR5 co-activation on post-synaptic excitability coincided in time with the phosphorylation of NR2B (Tyr1472)

In order to study the relationship between the mGluR5-induced modulation of NMDAR and the electrophysiological correlates of this modulation, we examined the phosphorylation of NR2B at the site Tyr1472 in homogenates of hippocampal slices containing only the CA1 field (i.e., CA1 minislices). Extracellular measures from these minislices were obtained before and during the application of a drug cocktail containing the agonists of NMDAR and mGluR5 NMDA (50 μM), and CHPG (50 μM), respectively. In order to accurately examine the temporal relationship between the electrophysiological effects and NR2B phosphorylation, we used five groups of CA1 minislices. The minislices of the first group were superfused with standard medium (control group) while the minislices of the other four groups were superfused with medium containing the drug cocktail NMDA-CHPG (drug groups) for progressively increasing time. Specifically, the CA1 minislices of the four drug groups were superfused with the drug cocktail for 1, 2, 3–5, and 6–7 min, respectively. At the specified time point, the minislices were taken and instantly frozen in isopentane (−50°C) and stored at −80°C until use for the western blotting experiments.

Measures of post-synaptic excitability and synaptic transmission represented by the PS/EPSP and EPSP/Fv ratios, respectively, were continuously obtained from all groups of minislices. As shown in Fig. 3, no significant drug effects could be detected during the first 2 min of drug perfusion either in the two electrophysiological indexes (PS/EPSP, EPSP/Fv) or in the phosphorylation levels. Remarkably, robust drug effects occurred during the next minutes in all measures. In particular, we observed multifold enhancement in the post-synaptic excitability (by 559 ± 193%, $n = 6$, Wilcoxon test, $p < 0.05$) and in the amount of NR2B (Tyr1472) phosphorylation during the 3–5 min of drug perfusion (Fig. 3a and b). It is striking that the enhancement in post-synaptic excitability was virtually coincident with the strong increase in the phosphorylation of the NR2B (Tyr1472) subunit that occurred during the 3–5 min of drug application (Fig. 3a and b). The above phenomena were accompanied by a significant reduction in the synaptic transmission by 36.3 ± 4.1% (Wilcoxon test, $p < 0.05$, $n = 6$) during the 3–5 min of drug perfusion (Fig. 3c). Paired-pulse facilitation was not significantly affected (it changed by 5.4 ± 6.0%, $n = 5$, Wilcoxon test, $p > 0.05$). Furthermore, during the 6–7 min of drug application the PS/EPSP ratio strongly declined (by 80.23 ± 19.8% compared with control values, Wilcoxon test, $p < 0.05$, $n = 6$)

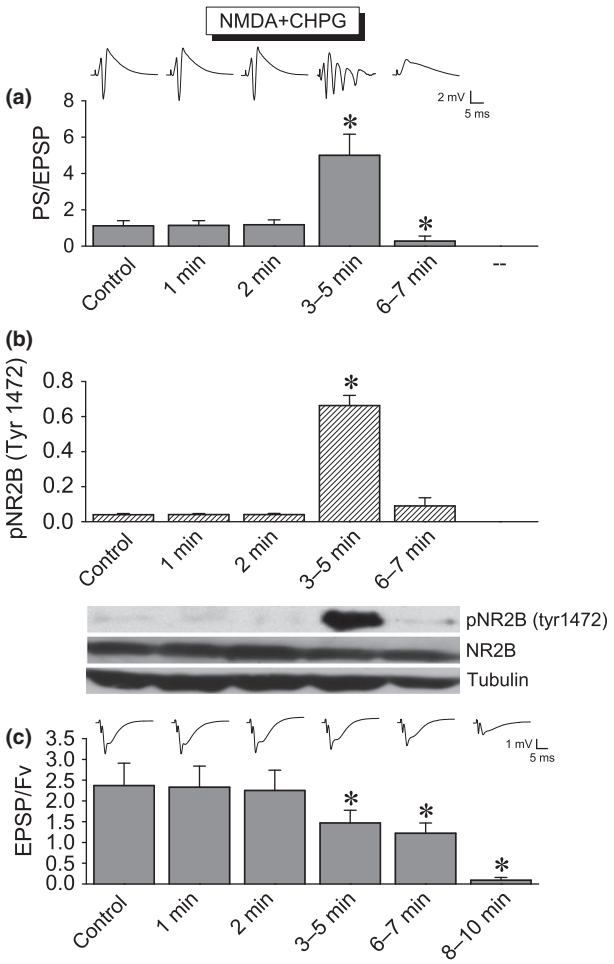

Fig. 3 Temporal coincidence between molecular and electrophysiological actions of NMDAR/mGluR5 synergy. Time course of the effects of co-activation of NMDAR and mGluR5 on: (a) the post-synaptic excitability [PS/excitatory post-synaptic potential (EPSP)], (b) the phosphorylation levels of NR2B (Tyr1472) and (c) the synaptic transmission (EPSP/Fv). Experiments were performed in CA1 minislices. Measures of electrophysiological parameters were obtained before and during co-application of 50 μM NMDA and 50 μM CHPG. Measures of the three parameters were made on the same CA1 minislices. Representative examples of field recordings from the st. radiatum and st. pyramidale are shown on the top of the collective plots for PS/EPSP and EPSP/Fv, respectively. Representative examples of phosphorylation of NR2B (Tyr1472) are shown in the corresponding collective plot. The drug-induced increase in post-synaptic excitability was expressed by the remarkable appearance of multiple PSs despite the decline in the synaptic transmission (EPSP/Fv). Note the temporal coincidence of the effects of co-activation of NMDARs and mGluR5 on the neuronal excitability and the phosphorylation of NR2B (Tyr1472). Asterisks denote statistically significant differences compared with control values at $*p < 0.05$ (Wilcoxon test and ANOVA with Tukey *post hoc* test).

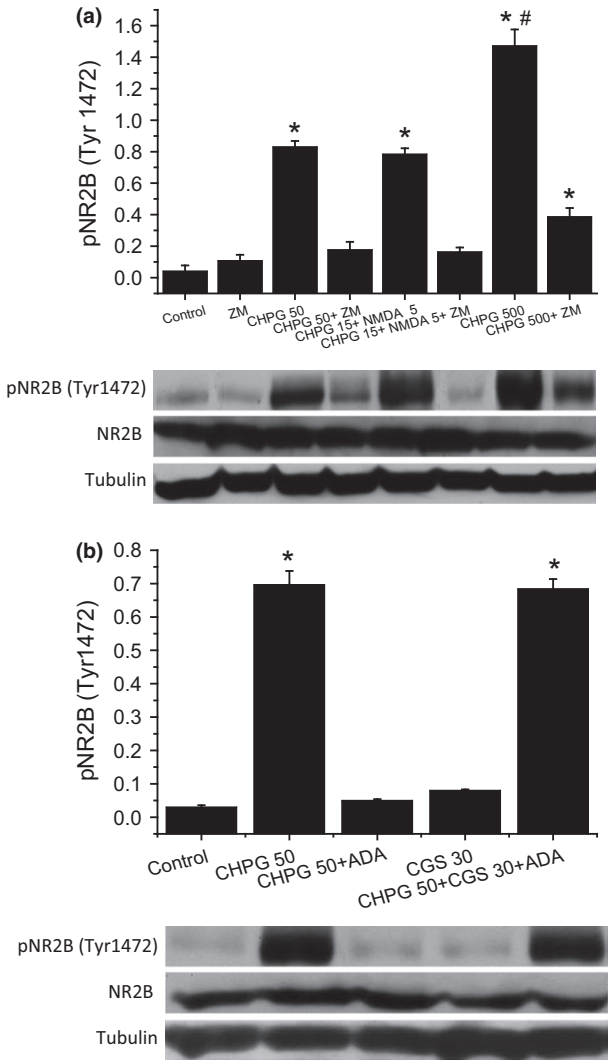

Fig. 4 Adenosine A_{2A}Rs enable the phosphorylation of NR2B (Tyr1472) induced by mGluR5/NMDAR activation. (a) Western blot analysis of NR2B (Tyr1472) phosphorylation normalized with the respective total protein levels in dorsal hippocampal slices in different animal groups, according to the drug treatment: [control], [ZM], [CHPG 50], [CHPG 50 + ZM], [CHPG 15 + NMDA 5], [CHPG 15 + NMDA 5 + ZM], [CHPG 500], [CHPG 500 + ZM]. Tubulin was used as loading control. Co-incubation of hippocampal slices with CHPG (50 μM) and ZM241385 (1 μM) (A_{2A}Rs selective antagonist) or CHPG (15 μM)/ NMDA (5 μM) and ZM241385 (1 μM) completely abolished the CHPG- and CHPG/NMDA- induced phosphorylation of the NR2B (Tyr1472). Results are shown in means ± SEM values (number of animals, $N = 8$) per group of representative western blots. $^{*}p < 0.05$ significantly different compared with the control group. $^{\#}p < 0.05$ significantly different compared with the [CHPG 50] group. Statistical analysis used ANOVA followed by Tukey *post hoc* test. (b) Western blot analysis of NR2B (Tyr1472) phosphorylation normalized with the respective total protein levels in dorsal hippocampal slices in different animal groups, according to the drug treatment: [control], [CHPG], [CHPG+adenosine deaminase (ADA)], [CGS], [CHPG+ CGS+ ADA]. Tubulin was used as loading control. Enzymatic degradation of endogenous adenosine by adenosine deaminase (ADA, 10 mg/mL) completely abolishes the CHPG (50 μM)- induced NR2B (Tyr1472) phosphorylation. Note that application of the selective agonist of A_{2A} adenosine receptor, CGS21680 (30 nM), totally reverses the above effect. Results are shown in means ± SEM values (number of animals, $N = 8$) per group of representative Western blots. $^{*}p < 0.05$ significantly different compared with the control group. Statistical analysis used ANOVA followed by Tukey *post hoc* test.

concomitantly with an abrupt reduction in the phosphorylation level (Fig. 3a and b). The synaptic transmission continued to progressively decline during the period of abrupt changes in post-synaptic excitability and phosphorylation, reaching a maximum at 8–10 min of drug application. Specifically, EPSP/Fv declined by 96.24 ± 2.4% compared with control values (Wilcoxon test, $p < 0.05$, $n = 6$) (Fig. 3c). Paired-pulse facilitation did not significantly display any further change (7.6 ± 13.9%, $n = 4$, Wilcoxon test, $p > 0.05$). It should be noted that measures of excitability during the period of profound suppression in the synaptic transmission (i.e., 8–10 min) could not be obtained, since it was not possible to evoke a PS, not even with strong stimulation.

Adenosine A_{2A}R are required for the mGluR5-evoked NR2B (Tyr1472) phosphorylation in rat hippocampus

In order to investigate the regulatory process by which stimulation of mGluR5s induces the NR2B tyrosine phosphorylation, we investigated the role of adenosine A_{2A} receptors, since it has been demonstrated that A_{2A}Rs and mGluR5s are co-localized and functionally interact in the rodent hippocampus (Tebano *et al.* 2005). For this reason, we co-incubated hippocampal slices with CHPG in the presence of the selective antagonist of A_{2A} adenosine receptors ZM241385. We found that co-incubation of hippocampal slices with 1 μM ZM241385 and 50 μM CHPG completely abolished the CHPG-induced phosphorylation of the NR2B (Tyr1472) subunit (Fig. 4a). Similar results were obtained when we co- incubated hippocampal slices with 15 μM CHPG and 5 μM NMDA in the presence of 1 μM ZM241385. ZM241385 totally abolished the CHPG/NMDA- induced phosphorylation at tyr-1472 of NR2B subunit, indicating that A_{2A} adenosine receptors facilitate this phosphorylation (Fig. 4a). However, co-incubation of slices with 1 μM ZM241385 and 500 μM CHPG resulted in a significant decrease in the phosphorylation level of NR2B (Tyr1472) without bringing it to the control levels (Fig. 4a).

In order to further investigate the involvement of A_{2A} receptors in the CHPG- induced phosphorylation of NR2B subunit, we examined the effect of the mGluR5 stimulation on the NR2B subunit phosphorylation under enzymatic degradation of endogenous adenosine, achieved by ADA. Our results showed that *in vitro* co-incubation of hippocam-

pal slices with 50 μM CHPG in the presence of 10 μg/mL ADA completely abolished the CHPG- induced phosphorylation of the NR2B (Tyr1472) subunit of NMDAR (Fig. 4b). Interestingly, the co-addition of the selective agonist of A_{2A} adenosine receptors, CGS21680 (30 nM), in the above drug combination (CHPG/ADA), induced a robust increase in the phosphorylation level of the Tyr1472 NR2B subunit of NMDAR compared to the basal levels (Fig. 4b), reaching the plateau levels of 50 μM CHPG (Fig. 4b). The above results indicate a permissive role of adenosine A_{2A} receptors on mGluR5 receptor-mediated effects on the phosphorylation at the residue Tyr1472 of NR2B subunit of NMDAR.

The synergistic effect of mGluR5/NMDARs co-activation on post-synaptic excitability, phosphorylation of NR2B (Tyr1472) subunit, and synaptic transmission, depends on Src family kinases and is permitted by adenosine A_{2A} receptors

As shown in Fig. 5a, activation of NMDARs by 50 μM NMDA produced a significant increase in post-synaptic excitability (43.84 ± 12.25% maximum increase in the PS/EPSP ratio) observed at 3–5 min of drug application (Wilcoxon test, $p < 0.05$, $n = 5$). In addition, NMDA significantly reduced the excitatory synaptic transmission (EPSP/Fv) producing a maximum effect at 8–10 min from the beginning of drug application by 25.32 ± 2.2% (Wilcoxon test, $p < 0.05$, $n = 6$) (Fig. 5c). This reduction was accompanied by a small but significant increase in paired-pulse facilitation (5.7 ± 1.4%, $n = 5$, Wilcoxon test, $p < 0.05$). Activation of mGluR5s by 50 μM CHPG did not affect either PS/EPSP ($n = 5$, $p > 0.05$) or EPSP/Fv ($n = 5$, $p > 0.05$). However, co-activation of NMDARs and mGluR5s by NMDA (50 μM) and CHPG (50 μM) produced an abrupt and very large increase in the PS/EPSP ratio by 676 ± 161% (Wilcoxon test, $p < 0.05$, $n = 5$) during the first 3–5 min of drug application (Fig. 5a). Strikingly, the effects of co-activation of NMDARs and mGluR5 on the post-synaptic excitability appeared in parallel with a large increase in the phosphorylation of NR2B (Tyr1472) subunit (Fig. 5b). Furthermore, the co-activation of NMDARs and mGluR5s produced a robust reduction in EPSP/Fv by 88.46 ± 6.2% (Wilcoxon test, $p < 0.01$, $n = 9$) measured 8–10 min from the beginning of the drug cocktail application (Fig. 5c). Thus, the effects of co-activation of NMDARs and mGluR5s by far exceeded the actions of only NMDAR's activation on both post-synaptic excitability (PS/EPSP, Wilcoxon test, $p < 0.01$) and synaptic transmission (EPSP/Fv, Wilcoxon test, $p < 0.01$). The synergistic effects of co-application of NMDA and CHPG on post-synaptic excitability and phosphorylation were completely prevented by the selective antagonist of mGluR5 MTEP (200 μM) (Fig. 5a and b). The reduction in EPSP/Fv ratio by 29.05 ± 2.2% (Wilcoxon test, $p < 0.05$, $n = 5$) produced during co-application of NMDA and CHPG in the presence of MTEP actually reached only the effects of NMDAR's activation (Fig. 5c). MTEP alone did not produce any significant effect in either PS/EPSP or EPSP/Fv. In addition, the antagonist of NMDARs CPP (10 μM) abolished the actions of CHPG/NMDA drug cocktail in both PS/EPSP (−9.7 ± 4.7%, $n = 4$, measured to 3–5 min from drug application) and EPSP/Fv (−1.75 ± 1.9%, $n = 4$, measured at 10–12 min of drug application) (data not shown). Taking into account the co-localization of mGluR5 and A_{2A}R in the hippocampus, as well as the permissive role of A_{2A}R on the mGluR5/NMDAR synergy (Tebano *et al.* 2005), we examined the effects of NMDA/CHPG drug cocktail under the blockade of A_{2A}Rs by ZM 241385. As shown in Fig. 5, ZM 241385 (1 μM) completely prevented the synergistic effects of NMDAR-mGluR5 co-activation on PS/EPSP, phosphorylation of NR2B (Tyr1472), and EPSP/Fv. More specifically, co-activation of NMDARs and mGluR5s by 50 μM NMDA and 50 μM CHPG in the presence of ZM 241385 reduced the CHPG/NMDA-evoked PS/EPSP levels almost to those that were observed when only NMDAR was activated (Wilcoxon test, $p < 0.05$, $n = 6$), and abolished the phosphorylation of NR2B (Tyr1472) subunit (Fig. 5b). Moreover, the co-activation of NMDARs and mGluR5s in the presence of ZM 241385 reduced EPSP/Fv by 32.4 ± 8.6% (Wilcoxon test, $p < 0.05$, $n = 5$). In order to clarify whether phosphorylation of NR2B (at Tyr 1472) underlies the electrophysiological effects induced by the NMDA-CHPG drug cocktail, we applied the selective Src kinase inhibitor PP1. We found that PP1 completely blocked the synergistic effects of the co-activation of NMDARs and mGluR5 on PS/EPSP, phosphorylation of NR2B (Tyr1472), and EPSP/Fv. More specifically, co-application of NMDA (50 μM) with CHPG (50 μM) in the presence of PP1 (30 μM) virtually prevented the synergistic effects of mGluR5-NMDAR co-activation on both PS/EPSP and EPSP/Fv rendering them similar to those observed with NMDAR's activation (Wilcoxon test, $p < 0.05$, $n = 6$ for both parameters) and abolished the phosphorylation of NR2B (Tyr1472) subunit (Fig. 5b).

Discussion

In this study, we show for the first time that stimulation of mGluR5 induces a dose-dependent elevation in the phosphorylation level of NR2B subunit of NMDAR at Tyr 1472 in rat hippocampus. This phenomenon is site specific, since it occurs at Tyr-1472 and not at Ser-1303 residue of NR2B subunit. Moreover, a synergistic interaction between mGluR5 and NMDARs exists, since co-activation of both receptors with inactive doses of their selective agonists (CHPG and NMDA, respectively) leads to a robust increase in NR2B (Tyr 1472) phosphorylation. The mGluR5, as well as the mGluR5/NMDAR-mediated Tyr 1472 phosphorylation, seems to be permitted by the activation of A2A adenosine receptors, since blockade of these receptors or

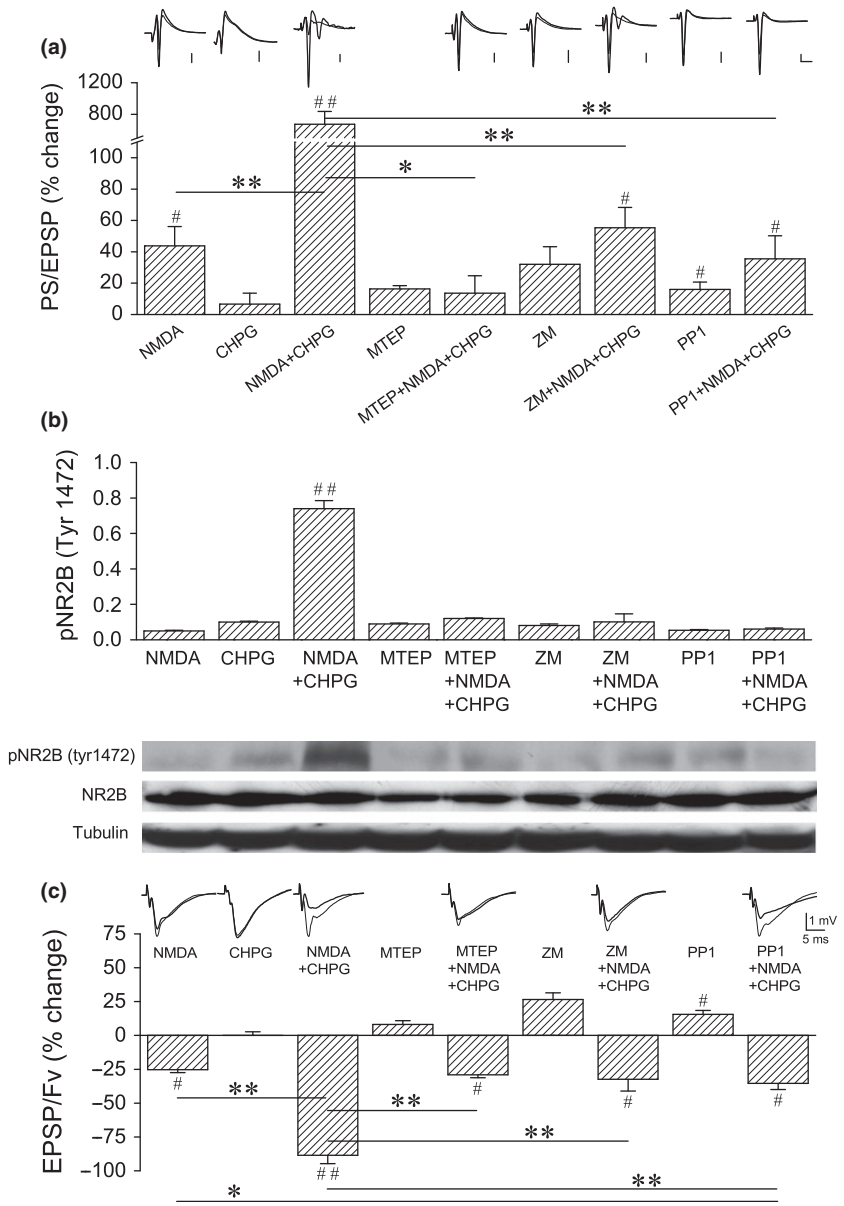

Fig. 5 The effects of co-activation of NMDAR and mGluR5 on the post-synaptic excitability [PS/excitatory post-synaptic potential (EPSP)], on the phosphorylation of the NR2B (Tyr1472) and on the excitatory synaptic transmission (EPSP/Fv) outscored the effects of only NMDAR's activation and required the activity of adenosine A_{2A}Rs, and the Src family kinases. Histograms of the effects of various pharmacological conditions on the: (a) PS/EPSP (b) phosphorylation of NR2B (Tyr1472) and (c) EPSP/Fv are shown. Examples of field potential recordings obtained before and during drug application (thin and thick line traces, respectively) are shown on the top of the columns of the PS/EPSP and EPSP/Fv histograms. Scale bars for recordings shown in (a and c): 1 mV, 5 ms. Measures of electrophysiological parameters were obtained during the time period when the NMDA-CHPG drug cocktail had maximal effects. Note that the increase in the post-synaptic excitability (PS/EPSP) was expressed as an increase in the amplitude of PS and/or the appearance of additional PSs. Asterisks denote statistically significant differences at $^{*}p < 0.05$ or $^{**}p < 0.01$ (Wilcoxon test). Statistically significant differences in the drug effects are indicated by diesis at $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$ (Mann–Whitney *U* test and ANOVA with Tukey *post hoc* test). Blockade of mGluR5 by 200 μM MTEP, blockade of A_{2A}Rs by 1 μM ZM 241385 and inhibition of Src family kinases by 30 μM PP1 occluded the potentiating effect of mGluR5 activation on the NMDA-induced responses.

removal of the endogenous adenosine abolishes the evoked phosphorylation in both cases. The intracellular signal pathway underlying the mGluR5, as well as the mGluR5/NMDAR-mediated Tyr 1472 phosphorylation involves Src family kinases, such as Fyn kinase, since their inhibition by PP1 (selective inhibitor of Src family kinases) results in the abolition of the induced phosphorylation. As shown in CA1 minislices the CHPG/NMDA-evoked robust increase in

neuronal excitability (measured by the PS/EPSP ratio) coincided temporally with the increase in the NR2B (Tyr 1472) phosphorylation. Furthermore, co-activation of the two receptors produced a gradual, yet strong, reduction in synaptic transmission, which was not, however, accompanied by any considerable change in paired-pulse facilitation as has been also previously shown (Tebano *et al.* 2005). These observations indicate that the NR2B (Tyr 1472) phosphorylation underlies, at least in part, the mGluR5-evoked potentiation of NMDAR-dependent responses. This is further supported by the concomitant abolishment of synergistic effects of CHPG/NMDA on PS/EPSP and NR2B (Tyr 1472) phosphorylation in the presence of either PP1 or ZM241385 (selective A_{2A}R antagonist). These results indicate that the coincident enhancing effects of mGluR5/NMDA co-activation on PS/EPSP and of NR2B (tyr1472) phosphorylation are mediated through Src family kinases and that they are actually under the permissive role of A_{2A} receptors.

Our results have shown that stimulation of mGluR5 by its selective agonist CHPG evoked a dose-dependent increase in the phosphorylation level of NR2B subunit at Tyr1472 in rat hippocampus, which is abolished in the presence of Src family tyrosine kinases inhibitor. The mGluR5s are coupled to G_q/G_{11} family of G-proteins and their stimulation leads to the elevation of intracellular levels of Ca^{2+} and activation of Protein Kinase C (PKC) kinase (Conn and Pin 1997; Nicoletti *et al.* 2011), which can activate Src family tyrosine kinases (Lev *et al.* 1995; Takagi *et al.* 2012).

According to our results, co-stimulation of mGluR5 and NMDARs by inactive doses of their selective agonists (CHPG 15 μM and NMDA 5 μM, respectively) induces a robust increase in the phosphorylation level of NR2B (Tyr1472), indicating a synergistic interaction between mGluR5 and NMDARs at the level of this specific phosphorylation. This synergy is also dependent on Src tyrosine kinases, since their inhibition by PP1 results in the complete abolition of the CHPG/NMDA- induced phosphorylation. The synergistic interaction between mGluR5 and NMDARs, seen in our study, could likely be explained by the fact that at the excitatory glutamatergic synapses in hippocampus, mGluR5s are located perisynaptically at the periphery of post-synaptic densities, where synaptic NMDARs are found (Baude *et al.* 1993; Lujan *et al.* 1996; Lan *et al.* 2001; Lau and Zukin 2007). Furthermore, mGluR5s are linked via synaptic scaffolding and adaptor proteins, such as PSD-95, Shank, and Homer with NMDARs, activating protein kinases (for example, Src tyrosine kinases) and other downstream signaling pathways, in the post-synaptic elements (Tu *et al.* 1999; Lau and Zukin 2007; Nicoletti *et al.* 2011), modulating NMDAR functions (Collett and Collingridge 2004).

One major mechanism by which NMDARs' trafficking is being regulated is the phosphorylation by protein tyrosine kinases, such as Fyn kinase, which is a member of Src tyrosine kinases (Lee 2006; Chen and Roche 2007). The NR2B subunit contains three major tyrosine phosphorylation sites, which are phosphorylated by the Fyn kinase, with Tyr1472 being the major phosphorylation site (Nakazawa *et al.* 2001; Takasu *et al.* 2002; Chen and Roche 2007). Tyr1472 of NR2B subunit belongs to YEKL amino acid sequence [Tyrosine (Y), Glutamic acid (E), Lysine (K), Leucine (L)] sequence, on which AP-2 adaptor binds, which mediates the internalization of NR2B subunit (Roche *et al.* 2001; Lavezzari *et al.* 2003; Prybylowski *et al.* 2005; Lee 2006). Phosphorylation of Tyr1472 by Fyn kinase prevents the binding of AP-2 adaptor to NR2B subunit, hence blocking the endocytosis of NR2B subunit and stabilizing it to the plasma membrane (Prybylowski *et al.* 2005; Lee 2006). Given that our results have shown that mGluR5 stimulation leads to the elevation of NR2B (Tyr1472) phosphorylation and that this effect is abolished by Src family kinases inhibitors, we can strongly suggest that mGluR5s contribute to the potentiation of NMDAR function by stabilizing them into the surface of the post-synaptic elements, preventing their endocytosis. This suggestion is supported by our electrophysiological data showing that co-activation of mGluR5 with NMDAR produces robust potentiating effects in cell excitability recorded from the CA1 region of the hippocampus, which temporally coincides with the appearance of the NR2B (Tyr1472) phosphorylation. The strong increase in post-synaptic excitability and the reduction in excitatory synaptic transmission induced after 3–5 min of perfusion by the NMDA-CHPG drug cocktail appear to have potentiating effects of NMDAR's action (Doherty *et al.* 1997; Anwyl 1999; Mannaioni *et al.* 2001; Tebano *et al.* 2005). Activation of NMDARs can significantly contribute to the post-synaptic depolarization in the CA1 principal cells, thus, enhancing their excitability (Forsythe and Westbrook 1988; Sah *et al.* 1989). The synergistic action between mGluR5 and NMDARs can further augment neuronal excitability (Mannaioni *et al.* 1999). Increase in excitability may also result from a reduction in inhibition. Actually, activation of NMDARs can lead to a reduction in $GABA_A$ receptor-mediated inhibition in pyramidal cells (Stelzer and Shi 1994). This effect can be enhanced by the synergistic action between mGluR5 and NMDARs that involves retrograde signaling mediated by endocannabinoids (Kano *et al.* 2009). This mechanism may also underlie the reduction in excitatory transmission (Kano *et al.* 2009).

The abrupt extinction of NR2B (Tyr1472) phosphorylation 6–7 min after receptor stimulation, which temporally coincides with the robust decline of NMDAR-dependent excitability, highlights this phosphorylation as a 'switch on' transient mechanism for modulating NMDAR-dependent responses. Furthermore, these mGluR5-evoked NMDA-dependent responses require the activation of Src tyrosine kinases, since their inhibition by PP1 results in a great reduction in the mGluR5/NMDA- induced increase in excitability, which coincides with the abolishment of

NR2B (Tyr1472) phosphorylation. According to the data mentioned above, we suggest that the mGluR5/NMDA-mediated phosphorylation of NR2B subunit at Tyr1472 could be importantly involved in the mGluR5-evoked potentiation of the NMDA-dependent responses (Mannaioni *et al.* 2001; Kotecha *et al.* 2003; Rebola *et al.* 2008).

Interactions between mGluR5 and NMDAR have been shown to be involved in synaptic plasticity and cognition, since mGluR5 knockout mice exhibit reduced NMDAR-mediated post-synaptic potentials in hippocampal pyramidal neurons, as well as reduced NMDAR-dependent synaptic plasticity and learning (Lu *et al.* 1997; Jia *et al.* 1998). Furthermore, spatial object recognition-related long-term depression in the hippocampus requires co-activation of mGluR5 with NMDAR (Goh and Manahan-Vaughan 2013). The molecular mechanism of Src family tyrosine kinases-dependent phosphorylation of NR2B (Tyr1472) induced by mGluR5/NMDAR synergy may underlie these cognitive effects in the hippocampus.

The functional interaction between mGluR5 and NMDAR has been documented in other brain structures as well, including striatum and cortex (Pisani *et al.* 1997; Ugolini *et al.* 1999; Attucci *et al.* 2001; Homayoun *et al.* 2004; Lecourtier *et al.* 2007; Homayoun and Moghaddam 2010). Thus, in the prefrontal cortex, the interaction between mGluR5 and NMDAR significantly ameliorates instrumental learning, working memory, and motor behaviors (Homayoun *et al.* 2004). These observations suggest that the synergy between these two receptors may be a widespread phenomenon in the brain.

Our results have shown that mGluR5 and mGluR5/NMDAR-induced phosphorylation in the NR2B (Tyr1472) subunit of NMDAR is licensed by A_{2A} adenosine receptors, since co-incubation of hippocampal slices with CHPG alone or CHPG/NMDA in the presence of ZM241385 (selective antagonist of A_{2A} adenosine receptor) results in the complete abolition of NR2B (Tyr1472)-induced phosphorylation. A similar effect was exhibited by the enzymatic degradation of the endogenous adenosine by ADA, indicating that the mGluR5/NMDAR-mediated phosphorylation is under the permissive control of A_{2A} receptors and thus the endogenous tone of A_{2A} receptors is required for the phosphorylation of the NR2B (Tyr1472) subunit. In accordance with the above, our electrophysiological data showed that pre-incubation of hippocampal slices with ZM241385 results in a significant reduction in the CHPG/NMDA-induced elevation in cell excitability, which coincides with the abolition of the phosphorylation of the NR2B (Tyr1472) subunit. This fact indicates that A_{2A} receptors have the permissive control of both the increase in excitability and the phosphorylation of the NR2B (Tyr1472) subunit, supporting our suggestion that NR2B (Tyr1472) phosphorylation is required for the mGluR5-mediated potentiation of NMDAR responses.

Our electrophysiological data are consistent with previous studies showing that stimulation of A_{2A} adenosine receptors facilitates mGluR5-induced NMDAR-dependent responses in rat hippocampal slices (Tebano *et al.* 2005) and is required for the long-term potentiation of NMDA-EPSCs in the CA3 region of the hippocampus (Rebola *et al.* 2008). Thus, the abolishment of CHPG/NMDA-induced phosphorylation of the NR2B (Tyr1472) subunit in hippocampal slices after the blockade of A_{2A} receptors, as seen in our study, could explain to some extent, and serve as the molecular basis of the permissive role of A_{2A} adenosine receptors on mGluR5-mediated NMDAR-dependent responses in rat hippocampus derived from our electrophysiological data. This suggestion is further supported by the fact that in hippocampal synapses, A_{2A} and mGluR5s are co-localized and act synergistically (Tebano *et al.* 2005).

In conclusion, our results have shown a tripartite mechanism involving A_{2A}/mGluR5/NMDARs, which must act synergistically in order to induce NR2B (Tyr1472) phosphorylation in rat hippocampus. We suggest that this mechanism constitutes (at least to some extent) the molecular mechanism underlying the mGluR5-evoked potentiation of NMDAR-dependent responses we see in CA1 hippocampal region for two reasons. First, the appearance of the two phenomena (Tyr1472 phosphorylation and increase in excitability in CA1 region) coincides temporally. Second, this mechanism is Src kinase dependent and requires the activation of A_{2A} receptors by endogenous adenosine. As hippocampal A_{2A} receptors need to be activated in order to elicit NR2B (Tyr1472) phosphorylation and the NMDA-potentiating effects of mGluR5, this emphasizes the role that A_{2A} receptors play as a fine-tuning modulatory system in the hippocampus (Sebastiao and Ribeiro 2000). Furthermore, since mGluR5/NMDAR interplay is essential for neuronal plasticity processes in the hippocampus and other brain areas, like the cortex, a better understanding of the molecular basis of this interaction will help us elucidate its role in learning and memory processes, as well as in pathological conditions, involving changes in NMDAR signaling.

Acknowledgements and conflict of interest disclosure

This research has been co-financed by the European Union (European Social Fund – ESF) and the Greek national funds through the Operational Program 'Education and Lifelong Learning' of the National Strategic Reference Framework (NSRF) – Research Funding Program: Thales. Investing in knowledge society through the European Social Fund. (MIS: 380342) Authors thank Dr Rodrigo A. Cunha for valuable discussions and suggestions carried out during the process of this work. None of the authors have any conflict of interest to disclose.

All experiments were conducted in compliance with the ARRIVE guidelines.

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