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RESEARCH PAPER

# Functional interactions between presynaptic NMDA receptors and metabotropic glutamate receptors co-expressed on rat and human noradrenergic terminals

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**Background and purpose:** Electrophysiological studies described potentiation of NMDA receptor function by metabotropic glutamate receptors (mGluRs) of group I occurring postsynaptically. Since release-enhancing NMDA receptors exist on noradrenergic terminals and group I mGluRs have recently been identified on these nerve endings, we have investigated if NMDA receptor-mGluR interactions also can occur at the presynaptic level.
**Experimental approach:** Rat hippocampus and human neocortex synaptosomes were labelled with [$^3$H]noradrenaline and superfused with mGluR agonists and antagonists. NMDA-evoked [$^3$H]noradrenaline release was produced by removal of external $Mg^{2+}$ or by simultaneous application of NMDA and AMPA in $Mg^{2+}$-containing solutions.
**Key results:** The mGluR1/5 agonist 3,5-DHPG, inactive on its own, potentiated both the release of [$^3$H]noradrenaline elicited by AMPA/NMDA/glycine and that evoked by NMDA/glycine following $Mg^{2+}$ removal. The effect of 3,5-DHPG on the AMPA/NMDA/glycine-induced release was insensitive to the mGluR1 antagonist CPCCOEt, but it was abolished by the mGluR5 antagonist MPEP; moreover, it was potentiated by the mGluR5 positive allosteric modulator DFB. When NMDA receptors were activated by $Mg^{2+}$ removal, both mGluR5 and mGluR1 contributed to the evoked release, the mGluR-mediated release being blocked only by CPCCOEt and MPEP in combination. Experiments with human neocortex synaptosomes show NMDA receptor-mGluR interactions qualitatively similar to those observed in rodents.
**Conclusions and implications:** Group I mGluRs, both of the mGluR1 and mGluR5 subtypes, co-localize with NMDA receptors on noradrenergic terminals of rat hippocampus and human neocortex. Depending on the mode of activation, NMDA receptors exert differential permissive roles on the activation of presynaptic mGluR1 and mGluR5.
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**Keywords:** mGluR1; mGluR5; NMDA receptor; AMPA receptor; noradrenaline release; synaptosomes; rat brain; human brain

**Abbreviations:** AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; CNS, central nervous system; CPCCOEt, 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester; DFB, 3,3′difluorobenzaldazine; 3,5-DHPG, (RS)-3,5-dihydroxyphenylglycine; MPEP, 2-methyl-6-(phenylethynyl) pyridine hydrochloride; mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate

## Introduction

Glutamate, the principal excitatory neurotransmitter in the central nervous system (CNS), exerts its actions through activation of a receptor family that includes ionotropic and metabotropic members. Ionotropic receptors are divided into *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainate receptors, which exist as multiple heteromers formed by co-assembly of different subunits (Dingledine *et al*., 1999). Metabotropic glutamate receptors (mGluRs) also are heterogeneous and constitute a subfamily of eight subtypes (mGluR1–mGluR8) subdivided into three groups (I, II and III) on the basis of structural homology, pharmacological profile and associated transduction pathways (Conn and Pin, 1997). There is ample evidence that glutamate receptors exhibit both post- and presynaptic localization; at the presynaptic level, they generally mediate control of neurotransmitter release (see, for reviews, MacDermott *et al*., 1999;

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Cartmell and Schoepp, 2000; Raiteri, 2006). The most extensively studied of the presynaptic mGluRs are those of group I that exist as release-enhancing autoreceptors on glutamatergic axon terminals (Herrero *et al*., 1992; Rodriguez-Moreno *et al*., 1998; Reid *et al*., 1999; Thomas *et al*., 2000; Fazal *et al*., 2003; Rodriguez *et al*., 2005; see for a review, Cartmell and Schoepp, 2000), where they mediate enhancement of glutamate exocytosis. Only recently, release-enhancing group I mGluRs were identified on isolated cholinergic (Marti *et al*., 2001; Feligioni *et al*., 2003) and noradrenergic (Longordo *et al*., 2006; Parodi *et al*., 2006) nerve endings. In all these types of nerve endings mGluR-mediated release could be observed only when depolarizing stimuli including 4-aminopyridine (Herrero *et al*., 1992; Reid *et al*., 1999), veratrine or (−) nicotine (Parodi *et al*., 2006) were co-applied. However, whether or not presynaptic group I mGluRs can be made functional under more physiological conditions by glutamate acting concomitantly at ionotropic receptors coexisting on the same nerve ending, has not been investigated.

Reciprocal interactions between mGluRs of group I and NMDA receptors have been the object of several investigations carried out in recombinant and in native systems, the latter involving receptors localized postsynaptically (Bleakman *et al*., 1992; Bruno *et al*., 1995; Doherty *et al*., 1997; Pisani *et al*., 1997; Alagarsamy *et al*., 1999, 2005; Skeberdis *et al*., 2001; Benquet *et al*., 2002; Heidinger *et al*., 2002). In contrast, very little is known about functional interactions between presynaptic ionotropic and metabotropic glutamate autoreceptors coexpressed on glutamatergic terminals and, particularly, on the possible interactions between presynaptic ionotropic and metabotropic glutamate heteroreceptors that may coexist on non-glutamatergic nerve endings.

Since presynaptic NMDA receptors were found on rat hippocampal (Pittaluga and Raiteri, 1990; Raiteri *et al*., 1992) and rat and human neocortical (Fink *et al*., 1990, 1992, Pittaluga *et al*., 1996) noradrenergic nerve terminals, we have investigated if presynaptic NMDA receptors and group I mGluRs can interact to modulate noradrenaline release from rat and human noradrenergic nerve endings. Our results show that both mGluR1 and mGluR5 can mediate enhancement of noradrenaline release provided that coexisting NMDA receptors are activated. Moreover, depending on the mode of NMDA receptor activation, only mGluR5 or both mGluR5 and mGluR1 can exhibit releasing activity.

## Methods

*Rat brain tissue samples*

Adult male rats (Sprague–Dawley; 200–250 g) were housed at constant temperature (22±1°C) and relative humidity (50%) under a regular light–dark schedule (light on from 0700 to 1900 h). Food and water were freely available. The animals were killed by decapitation and hippocampi were rapidly dissected at 0–4°C. The experimental procedures were approved by the Ethical Committee of the Pharmacology and Toxicology Section, Department of Experimental Medicine, University of Genoa, in accordance with the European legislation (European Communities Council Directive of 24 November 1986, 86/609/EEC) and with the NIH Guide for the Care and Use of Laboratory Animals.

*Human brain tissue samples*

Samples of human cerebral cortex were obtained from informed and consenting patients undergoing neurosurgery, each on a different day, to reach deeply seated tumours. The samples represented parts of temporal ($n=7$) lobes obtained from two women and five men (aged 30–70 years). Immediately after removal, the tissue was placed in a physiological salt solution at 2–4°C and synaptosomes were prepared within 90 min. The experimental procedures were approved by the Ethical Committee of the Pharmacology and Toxicology Section, Department of Experimental Medicine, University of Genoa.

*Preparation of synaptosomes*

Crude synaptosomes were prepared according to Raiteri *et al*. (1984). Briefly, brain tissues were homogenized in 40 volumes of 0.32 M sucrose, buffered at pH 7.4 with phosphate (final concentration 0.01 M). The homogenate was first centrifuged at 1000 *g* for 5 min, to remove nuclei and cellular debris, and synaptosomes were then isolated by centrifugation at 13 000 *g* for 20 min. The synaptosomal pellet was resuspended in a physiological medium having the following composition: 125 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 1 mM $NaH_2PO_4$, 22 mM $NaHCO_3$ and 10 mM glucose (pH 7.2–7.4) (gassed with 95% $O_2$ and 5% $CO_2$).

*Release experiments*

Synaptosomes were selectively labelled with [$^3$H]noradrenaline (final concentration: 30–50 nM) in the presence of the serotonin transporter blocker 6-nitroquipazine (0.1 $\mu$M) and the dopamine transporter blocker GBR 12909 (0.1 $\mu$M) to avoid false labelling of serotonergic and dopaminergic nerve terminals, respectively. Incubation was performed at 37°C, for 15 min, in a rotary water bath and in an atmosphere of 95% $O_2$ and 5% $CO_2$.

After the labelling period, release experiments were performed in a multichamber superfusion system (Ugo Basile, Comerio, Varese, Italy). Identical portions of the synaptosomal suspensions were layered on microporous filters at the bottom of up to 24 parallel superfusion chambers maintained at 37°C (Raiteri and Raiteri, 2000). The amount of synaptosomes retained on each filter (2.5 cm diameter) had been calculated to constitute less than a monolayer (Raiteri *et al*., 1986). Synaptosomes were superfused at 0.5 ml $min^{-1}$ with standard physiological solution aerated with 95% $O_2$ and 5% $CO_2$, at 37°C. The system was first equilibrated during 38 min of superfusion; subsequently, eight consecutive 1-min fractions were collected. Synaptosomes were exposed to agonists at the end of the first fraction collected ($t=39$ min) until the end of the superfusion, while antagonists were added 8 min before agonists. When indicated, the medium was replaced, at $t=20$ min, with a medium from which $Mg^{2+}$ ions were omitted, to permit NMDA receptor activation. The radioactivity in the

collected fractions and in superfused synaptosomes was counted.

*Calculation and statistics*

The amount of radioactivity released into each superfusate fraction was expressed as a percentage of the total synaptosomal tritium content at the start of the fraction collected (fractional efflux). Drug effects were expressed as percent increase over basal release and were calculated as the ratio between the percentage of tritium released into the fraction, where the maximal releasing effect was observed and that in the first fraction collected; this ratio was compared with the corresponding ratio obtained under control conditions (no drug added). Analysis of variance was performed by ANOVA followed by the Newman Keuls multiple comparison test. Data were considered significant at $P<0.05$ at least. Appropriate controls with antagonists and inhibitors were always run in parallel.

*Chemicals*

1-[7,8–$^3$H]noradrenaline (specific activity 39 Ci mmol$^{-1}$) was from Amersham Radiochemical Centre (Amersham, UK). NMDA, (RS)-3,5-dihydroxyphenylglycine (3,5-DHPG), AMPA, 2-methyl-6-(phenylethynyl) pyridine hydrochloride (MPEP), 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt), 3,3′difluorobenzaldazine (DFB) were obtained from Tocris Cookson (Bristol, UK). Glycine was purchased from Sigma Chemical Co (St Louis, MO, USA). The following compounds were gifts: 6-nitroquipazine maleate from Duphar (Amsterdam, The Netherlands), GBR 12909 from Gist-Brocades (Delft, The Netherlands), clonidine from Boehringer Ingelheim (Florence, Italy).

## Results

*Interactions between ionotropic and mGluR1s mediating noradrenaline release in rat hippocampus*

AMPA receptors and NMDA receptors mediating noradrenaline exocytosis are present on noradrenergic terminals (Fink *et al*., 1990; Pittaluga and Raiteri, 1990; Wang *et al*., 1991; Malva *et al*., 1994; Pittaluga *et al*., 2006). The possible interactions between presynaptic mGluRs of group I and presynaptic ionotropic glutamate receptors were first studied by monitoring [$^3$H]noradrenaline release from rat hippocampal synaptosomes during exposure to the group I mGluR agonist 3,5-DHPG and to agonists at AMPA receptors and NMDA receptors.

Activation of presynaptic AMPA receptors by 100 $\mu$M AMPA elicited [$^3$H]noradrenaline release (40.3 $\pm$ 4.3%; $n=4$; expressed as percent increase over basal). When 100 $\mu$M 3, 5-DHPG was coapplied with AMPA, the release of the $^3$H-catecholamine remained unchanged (42.3 $\pm$ 6.4%; $n=4$).

We then investigated the possibility that activation of NMDA receptors affected the function of presynaptic group I mGluRs. Activation of NMDA receptors requires removal of $Mg^{2+}$ ions from the receptor channel, which can be achieved by two main methods: (i) by omitting external $Mg^{2+}$ from the solutions (as it is generally performed experimentally); (ii) by concomitant activation of coexisting AMPA and NMDA receptors (this probably occurs physiologically), in the presence of external $Mg^{2+}$.

AMPA receptors and NMDA receptors, originally identified on noradrenergic nerve terminals, were subsequently found to coexist on the same terminals where activation of AMPA receptors permits activation of NMDA receptors in the presence of physiological concentrations of extracellular $Mg^{2+}$ (Raiteri *et al*., 1992). Thus, in our experimental setup, functional interactions between NMDA receptors and mGluRs can be investigated by monitoring [$^3$H]noradrenaline release from noradrenergic terminals on which NMDA receptors are activated either by AMPA/NMDA/glycine in $Mg^{2+}$-containing solutions or by NMDA/glycine following $Mg^{2+}$ removal.

The release of [$^3$H]noradrenaline evoked by AMPA/NMDA/glycine in $Mg^{2+}$ (1.2 mM)-containing medium was significantly potentiated by adding 100 $\mu$M 3,5-DHPG (Figure 1a; $F(3, 11)=6.89$; $P<0.01$). This effect could be observed with 0.1 $\mu$M glycine, but not with 1 $\mu$M of the NMDA receptor coagonist. As illustrated in Figure 1b, addition to the $Mg^{2+}$-free medium of 3,5-DHPG (10–100 $\mu$M) concentration-dependently potentiated the release of [$^3$H]noradrenaline

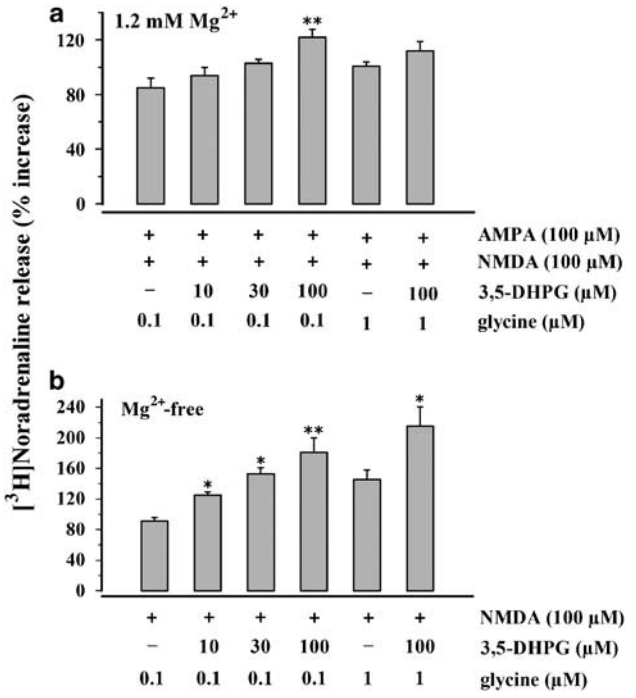


**Figure 1** Effects of 3,5-DHPG on the NMDA-evoked release of [$^3$H]noradrenaline from rat hippocampal nerve endings. Synaptosomes were exposed in superfusion to 3,5-DHPG, AMPA and NMDA plus glycine at the end of the first superfusion fraction collected ($t=39$ min). (**a**) Superfusion in the presence of $Mg^{2+}$ ions. (**b**) Superfusion in the absence of $Mg^{2+}$ ions. Results are expressed as percent increase over basal release; in each panel, data are means $\pm$ s.e.m. of three to six experiments run in triplicate (three superfusion chambers for each experimental condition). *$P<0.05$ versus respective control; **$P<0.01$ versus respective control. Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; 3,5-DHPG, (RS)-3,5-dihydroxyphenylglycine; NMDA, *N*-methyl-D-aspartate.

provoked by NMDA/glycine. The effects observed in $Mg^{2+}$-free conditions clearly differ from those in the presence of 1.2 mM $Mg^{2+}$. In particular: (i) the potentiation of the NMDA/glycine-evoked release was already significant when 3,5-DHPG was added at 10 $\mu$M; moreover, the potentiation observed in $Mg^{2+}$-free solution with 100 $\mu$M of the mGluR1/5 agonist was much higher than that in $Mg^{2+}$-containing medium; (ii) the potentiation of the NMDA/glycine-evoked release produced by 100 $\mu$M 3,5-DHPG occurred in the presence of both 0.1 and 1 $\mu$M glycine (Figure 1b; $F(5, 25) = 7.17$; $P<0.01$ and $P<0.05$, respectively).

*Pharmacology of the mGluRs involved*

The effects of AMPA/NMDA/glycine plus 3,5-DHPG in $Mg^{2+}$-containing solutions and of NMDA/glycine plus 3,5-DHPG in $Mg^{2+}$-free solutions were tested against selective group I mGluR antagonists and the results are shown in Figure 2. In the presence of 1.2 mM of $Mg^{2+}$, the release of [$^3$H]noradrenaline provoked by AMPA/NMDA/glycine plus 3,5-DHPG was insensitive to the selective mGluR1 antagonist CPCCOEt (5 $\mu$M); in contrast, the component of the evoked release mediated by 3,5-DHPG was abolished by 1 $\mu$M of the selective mGluR5 antagonist MPEP (Figure 2a, $F(3, 8) = 9.42$; $P<0.05$). In $Mg^{2+}$-free medium, neither 5 $\mu$M CPCCOEt nor 1 $\mu$M MPEP was able to inhibit significantly the release evoked by NMDA/glycine plus 3,5-DHPG. However, the component of the evoked release produced by 3,5-DHPG was attenuated when the mGluR1 and the mGluR5 antagonists were added in combination (Figure 2b; $F(4, 25) = 10.42$; $P<0.001$). Notably, CPCCOEt and MPEP failed to affect significantly the release of [$^3$H]noradrenaline induced by AMPA/NMDA/glycine in presence of $Mg^{2+}$ (Figure 3a; $F(2, 9) = 1.079$; n.s.) or that evoked by NMDA/glycine in absence of $Mg^{2+}$ (Figure 3b, $F(3,12) = 0.93$; n.s.). At the concentration applied, both antagonists did not affect the spontaneous release of noradrenaline (data not shown, but see also Longordo *et al*., 2006; Parodi *et al*., 2006).

To confirm the involvement of mGluR5 in the AMPA/NMDA/glycine-evoked release of [$^3$H]noradrenaline, the effect of DFB, a molecule reported to act as a selective positive allosteric modulator of human and rat recombinant mGluR5 (O'Brien *et al*., 2003), was tested. DFB was added together with a concentration of 3,5-DHPG (30 $\mu$M), that did not enhance significantly the release of [$^3$H]noradrenaline

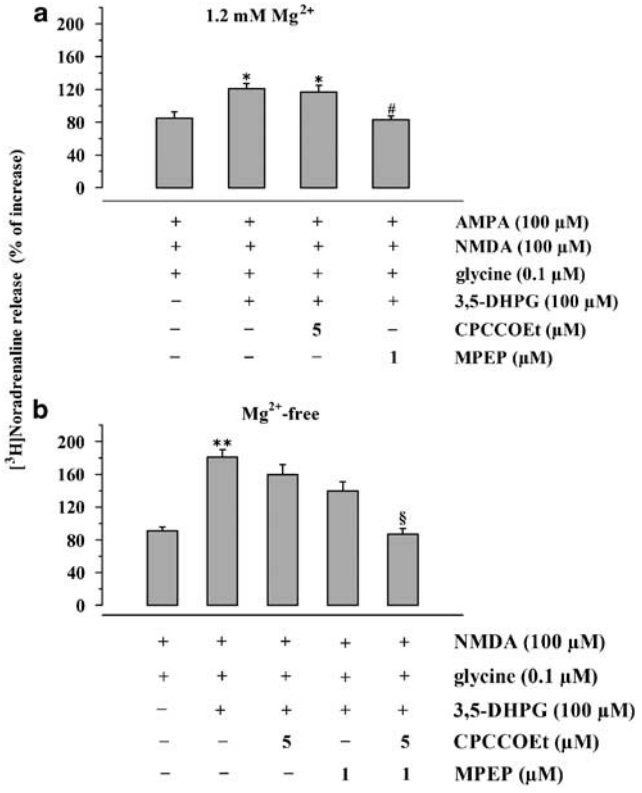


**Figure 2** Effects of group I mGluR antagonists on the 3,5-DHPG potentiation of the [$^3$H]noradrenaline release evoked by NMDA receptor activation in $Mg^{2+}$-containing or $Mg^{2+}$-free medium. Agonists were added at $t = 39$ min of superfusion. Antagonists were present from 8 min before agonists. (**a**) Superfusion in the presence of $Mg^{2+}$ ions. (**b**) Superfusion in the absence of $Mg^{2+}$ ions. Results are expressed as percent increase over basal release; data are means ± s.e.m. of three to six experiments run in triplicate. *$P<0.05$ versus AMPA/NMDA/glycine; **$P<0.001$ versus NMDA/glycine; $^{\#}P<0.05$ versus AMPA/NMDA/glycine/3,5-DHPG; $^{\S}P<0.05$ versus NMDA/glycine/3,5-DHPG. Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; 3,5-DHPG, (RS)-3,5-dihydroxyphenylglycine; mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate.

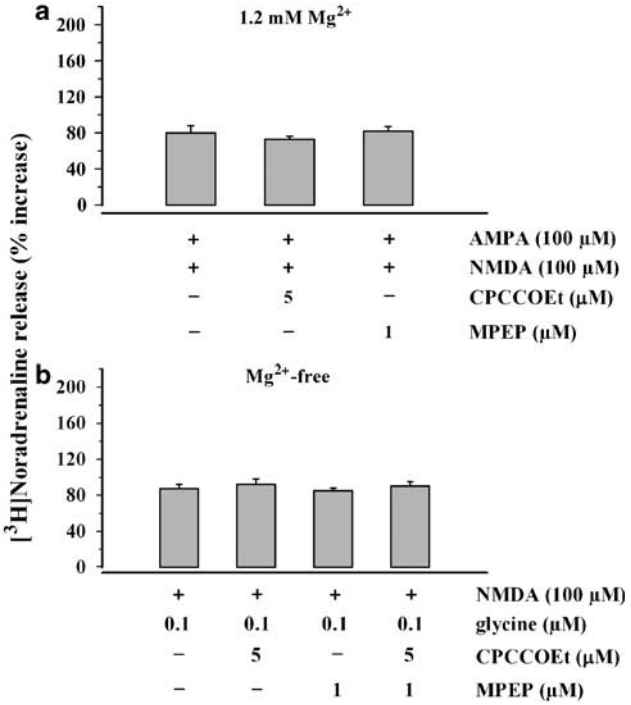


**Figure 3** Effects of group I mGluR antagonists on the [$^3$H]noradrenaline release evoked by NMDA receptor activation in $Mg^{2+}$-containing or $Mg^{2+}$-free medium. Agonists were added at $t = 39$ min of superfusion. (**a**) Superfusion in the presence of $Mg^{2+}$ ions. (**b**) Superfusion in the absence of $Mg^{2+}$ ions. Results are expressed as percent increase over basal release; data are means ± s.e.m. of four experiments run in triplicate. Abbreviations: mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate.

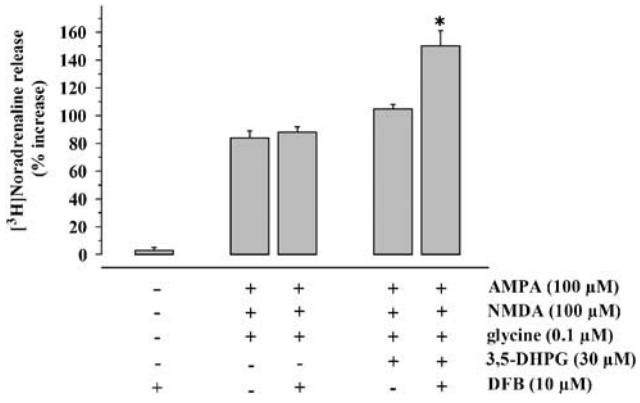


**Figure 4** Effects of DFB on the release of [$^3$H]noradrenaline induced by AMPA/NMDA in absence or in presence of 3,5-DHPG from rat hippocampal nerve endings. Superfusion was carried out in the presence of $Mg^{2+}$ ions. Synaptosomes were exposed to 3,5-DHPG, AMPA and NMDA at the end of the first fraction collected. DFB was added concomitantly with agonists. Results are expressed as percent increase over basal release; data are means ± s.e.m. of three experiments run in triplicate. *$P<0.01$ versus 100 $\mu$M AMPA + 100 $\mu$M NMDA + 0.1 glycine + 30 $\mu$M 3,5-DHPG. Abbreviations: AMPA, $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole propionate; DFB, 3′difluorobenzaldazine; 3,5-DHPG, (RS)-3,5-dihydroxyphenylglycine; NMDA, *N*-methyl-D-aspartate.

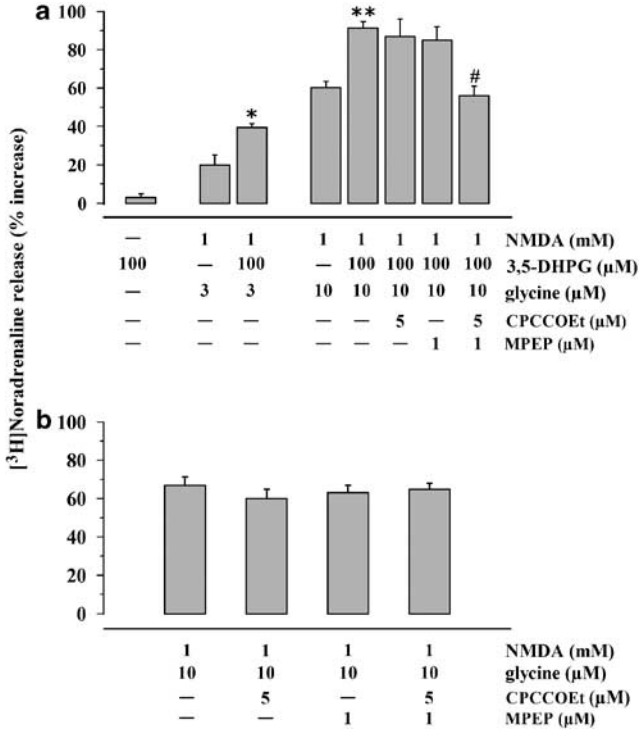


**Figure 5** Effects of 3,5-DHPG and group I mGluR antagonists on the NMDA-evoked release of [$^3$H]noradrenaline from human neocortical nerve endings. (**a**) Effects of group I mGluR antagonists on the NMDA/glycine/3,5-DHPG- induced release of tritium. (**b**) Effects of group I mGluR antagonists on the NMDA/glycine-induced release of tritium. Superfusion was carried out in the absence of $Mg^{2+}$ ions. Synaptosomes were exposed to 3,5-DHPG and to NMDA/glycine at the end of the first fraction collected. Antagonists were present from 8 min before agonists. Results are expressed as percent increase over basal release; data are means ± s.e.m. of four to six experiments run in triplicate. *$P<0.01$ versus NMDA/glycine; **$P<0.001$ versus NMDA/glycine; $^{\#}P<0.001$ versus NMDA/glycine/3,5-DHPG. Abbreviations: 3,5-DHPG, (RS)-3,5-dihydroxyphenylglycine; mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate.

evoked by AMPA/NMDA/glycine ($F(4,10)=90.35$; n.s.; $n=3$). Figure 4 shows that, in the presence of 10 $\mu$M DFB, 30 $\mu$M 3,5-DHPG significantly potentiated the [$^3$H]noradrenaline release evoked by AMPA/NMDA/glycine ($F(4, 10)=90.35$; $P<0.01$; $n=3$). DFB (10 $\mu$M) had no effect, on its own, either on the basal or on the AMPA/NMDA/glycine-evoked release of [$^3$H] noradrenaline (Figure 4, $F(4,10)=90.35$; n.s.; $n=3$). Concentrations of DFB higher than 10 $\mu$M could not be tested because of the low solubility of the compound.

*Studies with human neocortex noradrenergic nerve endings*

Human neocortical noradrenergic nerve endings possess release-enhancing NMDA receptors (Fink *et al*., 1992; Pittaluga *et al*., 1996), whereas the presence of AMPA receptors has not been investigated. NMDA receptors located on human cortical noradrenergic terminals were found to be less sensitive to agonists than the rodent ones, probably owing to a better viability of rat synaptosomes when compared to human ones (see also Longordo *et al*., 2006). As illustrated in Figure 5a, addition of 100 $\mu$M 3,5-DHPG, inactive on its own, more than doubled the release of [$^3$H]noradrenaline evoked by NMDA/glycine (3 $\mu$M) in $Mg^{2+}$-free solution ($F(7, 26)=45.9$; $P<0.01$ and $P<0.001$, respectively). The figure also shows that 3,5-DHPG was still able to significantly potentiate (Figure 5a; $F(7, 26)=45.9$; $P<0.001$) the release of [$^3$H]noradrenaline elicited by NMDA plus 10 $\mu$M glycine, which was almost threefold higher than that observed with 3 $\mu$M of the NMDA receptor coagonist. Neither the mGluR1 antagonist CPCCOEt nor the mGluR5 antagonist MPEP affected significantly the release of [$^3$H]noradrenaline evoked by NMDA/glycine (10 $\mu$M) plus 3,5-DHPG. However, the mGluR-dependent component of the evoked [$^3$H]noradrenaline release was attenuated when the two antagonists were added in combination (Figure 5a; $F(7, 26)=45.9$; $P<0.001$). At the concentrations used, the antagonists failed to affect either the spontaneous or the NMDA/glycine-evoked release of [$^3$H]noradrenaline (Figure 5b; $F(3, 8)=0.56$; n.s.].

*Mode of release of the AMPA/NMDA/mGluR5 receptor-evoked release of [$^3$H] noradrenaline*

To obtain information on the mode of release of [$^3$H]noradrenaline during activation of the AMPA/NMDA/mGlu5 receptor pathway, we evaluated the sensitivity of the evoked [$^3$H]noradrenaline release to clonidine, a drug able to activate $\alpha_2$-adrenoceptors present on noradrenergic terminals and to inhibit the evoked release of the catecholamine when it occurs by exocytosis (Langer, 1981; Starke, 1981). As shown in Figure 6, the release of [$^3$H]noradrenaline provoked in rat hippocampal nerve endings by AMPA/NMDA/3,5-DHPG (about 100% increase over basal) was about 90% inhibited by 0.1 $\mu$M clonidine ($df=8$, $t=12.59$, $P<0.001$; $n=5$), consistent with a vesicular exocytotic release.

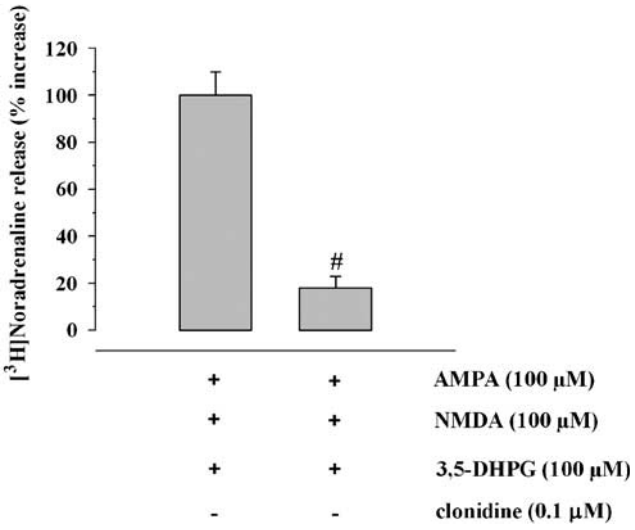


**Figure 6** Effects of the selective $\alpha_2$-adrenoceptor agonist clonidine on the release of [$^3$H]noradrenaline induced by AMPA, NMDA and 3,5-DHPG from rat hippocampal nerve endings. Superfusion was carried out in presence of $Mg^{2+}$ ions. Synaptosomes were exposed to 3,5-DHPG, AMPA and NMDA at the end of the first fraction collected. Results are expressed as percent increase over basal release; data are means$\pm$s.e.m. of five experiments run in triplicate. $^{\#}P<0.001$ versus respective control. Abbreviations: AMPA, $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole propionate; 3,5-DHPG, (RS)-3,5-dihydroxyphenylglycine; NMDA, *N*-methyl-D-aspartate.

## Discussion

It has often been shown that, during up–down perfusion of synaptosomal monolayers, the transmitters just released are quickly removed by the superfusion solution before they can feedback on targets of the releasing terminals or act on neighbouring particles, thus minimizing or excluding indirect effects. Accordingly, NMDA, added in $Mg^{2+}$-free solutions, releases [$^3$H]noradrenaline by directly acting at presynaptic NMDA receptors localized on noradrenergic axon terminals. This agonist which, in $Mg^{2+}$-containing solutions, is unable to evoke release of noradrenaline from synaptosomes, can however acquire activity if coapplied with AMPA, indicating that AMPA receptors and NMDA receptors coexist and interact on the same noradrenergic terminals (Raiteri *et al*., 1992). Similarly, if a group I mGluR agonist like 3,5-DHPG, inactive on its own, becomes able to release noradrenaline when added together with a mixture of AMPA and NMDA, one explanation is that three glutamate receptors, that is, mGluRs, AMPA receptors and NMDA receptors coexist and interact on the same noradrenergic terminal. Obviously, more direct (that is, morphological) evidences of receptor colocalization would be most convincing, but they are precluded by the extremely low density of noradrenergic terminals in CNS tissues.

The first finding of the present work is that presynaptic group I mGlu heteroreceptors of the mGluR5 subtype present on noradrenergic terminals are functional when terminals are exposed to AMPA plus NMDA, a condition mimicking the physiological stimulus represented by endogenously released glutamate. A second finding is that activation of NMDA receptors due to lack of external $Mg^{2+}$ ions permits activation of mGluRs of both mGluR5 and mGluR1 subtypes. Thus, activation of NMDA receptors permits group I mGluRs to become functional, consistent with the view that NMDA receptors colocalize with these mGluRs on noradrenergic axon terminals and positively couple to mGluRs. Unlike NMDA receptors, activation of AMPA receptors alone does not exert a permissive role on the function of group I mGluRs, suggesting that the two receptors types do not crosstalk on noradrenergic terminals. However, it has to be borne in mind that AMPA receptor activation permits NMDA receptor activation in presence of physiological $Mg^{2+}$ ions (Raiteri *et al*., 1992) and that co-activation of AMPA and NMDA receptors enables mGluR5 to augment the release elicited by AMPA/NMDA/glycine (Figure 1a). Thus, activation of AMPA receptors by endogenous glutamate might represent a prerequisite for the NMDA receptor-mediated activation of mGluR5 under physiological conditions.

The mGluR1/5 agonist 3,5-DHPG was unable, on its own, to elicit release. Since transmitter release mediated by group I presynaptic mGluRs requires a concomitant depolarization of nerve terminals (Herrero *et al*., 1992; Parodi *et al*., 2006; see Cartmell and Schoepp, 2000, for a review), one might reasonably assume that presynaptic mGluRs of group I need more cytosolic $Ca^{2+}$ in addition to that mobilized through the phosphatidylinositol pathway. However, our results suggest that the situation is more complex: in fact, although both presynaptic NMDA and AMPA receptors can stimulate $Ca^{2+}$ influx into nerve terminals (Pittaluga and Raiteri, 1990; Raiteri *et al*., 1992; Malva *et al*., 1994), only NMDA receptors, and not AMPA receptors, mediate activation of mGluRs, implying that the mechanism by which the level of $Ca^{2+}$ in the cytosol is raised may differentially affect mGluR function (see below).

AMPA receptors exert a permissive role on NMDA$\rightarrow$mGluR5 interactions in presence of $Mg^{2+}$ as supported by the results with selective antagonists as well as with DFB. This compound exhibited no releasing activity on its own; nor did it affect the release due to AMPA/NMDA/glycine, but it did strongly potentiate the effect of a slightly effective concentration of 3,5-DHPG on the AMPA/NMDA-evoked release of noradrenaline. Based on the results on DFB reported by O'Brien *et al*. (2003), the compound behaved as a positive allosteric modulator of mGluR5.

If activation of 'permissive,' AMPA receptors appears to be a prerequisite to the NMDA$\rightarrow$mGluR5 interaction in the presence of $Mg^{2+}$, mGluR1 can be recruited and contribute to noradrenaline release when NMDA receptors are activated by $Mg^{2+}$ removal, independently of AMPA receptors. The concomitant activation of mGluR1 and mGluR5 observed under these conditions possibly occurs because $Mg^{2+}$ deficiency allows activation of the majority of NMDA receptors present on the terminals, including those that are not adjacent to permissive AMPA receptors. The finding that the mGluR-mediated component of the release evoked in $Mg^{2+}$-free conditions can only be blocked by a combination of mGluR1 and mGluR5 antagonists, tends to exclude the possibility that the two receptors are on different noradrenergic terminals. However, they could coexist on the same nerve terminal on distinct regions of the plasma membrane

as NMDA receptors/AMPA receptors/mGluR5 and NMDA receptors/mGluR1 combinations, respectively, but share the same transducing mechanisms.

Whatever the picture, the activation of mGluR1 in $Mg^{2+}$-free solutions might become relevant to some pathological conditions linked to $Mg^{2+}$-deficiency. Significantly decreased concentration of $Mg^{2+}$ in plasma was found in epileptic children (Benga *et al*., 1985). Seizures are a recognized complication of HIV-1 infection and seropositive patients with hypomagnesaemia appear to be at increased risk for convulsive status epilepticus (Van Paesschen *et al*., 1995). $Mg^{2+}$ wasting also occurs in other segments of the population, including alcoholics and patients with acute myocardial infarction (see Tejero-Taldo *et al*., 2004). Of note, Figure 1 shows that the release evoked by NMDA/glycine/3,5-DHPG in $Mg^{2+}$-free medium ($\sim$210%) is much higher than that evoked by AMPA/NMDA/glycine/3,5-DHPG in presence of $Mg^{2+}$ ($\sim$120%), suggesting a relevant contribution of mGluR1. Excessive activation of mGluR1 has been related to neurotoxic events including maintenance of seizure discharges and ischaemic damage (Bruno *et al*., 2001; Lee *et al*., 2002; Pellegrini-Giampietro, 2003).

Although most of the studies on the mGluR-NMDA receptor interactions describe mGluR-induced potentiation of NMDA-mediated responses (Bleakman *et al*., 1992; Bruno *et al*., 1995; Doherty *et al*., 1997; Skeberdis *et al*., 2001), the reciprocal interaction, that is, regulation of mGluR responses by NMDA receptor activation has been the object of some reports (Luthi *et al*., 1994; Alagarsamy *et al*., 1999, 2005). Alagarsamy *et al*. (1999) showed that activation of NMDA receptors potentiated responses to activation of mGluR5 by reversing desensitization of this receptor possibly through activation of protein phosphatase 2B/calcineurin (Alagarsamy *et al*., 2005). Understanding the mechanisms by which activation of NMDA receptors permits mGluRs to contribute to noradrenaline release from noradrenergic axon terminals, including a reversal of their desensitization requires further investigations. It is important that similar interactions seem to occur in rat and in human brain, so that rat brain tissues could represent a useful model in future work.

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## Conflict of interest

The authors state no conflict of interest.

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