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Activation of group III metabotropic glutamate receptors is neuroprotective in cortical cultures

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Abstract

(*RS*)-α-Methyl-4-phosphonophenylglycine (MPPG) and (*S*)-α-methyl-3-carboxyphenylalanine (M3CPA), two novel preferential antagonists of group III metabotropic glutamate (mGlu) receptors, antagonized the neuroprotective activity of L-2-amino-4-phosphonobutanoate (L-AP4) or L-serine-*O*-phosphate in mice cultured cortical cells exposed to a toxic pulse of *N*-methyl-D-aspartate. In contrast, MPPG did not influence the neuroprotective activity of the selective group II mGlu receptors agonist, (2_S,$1'_R$,$2'_R$,$3'_R$)-2-(2,3-dicarboxycyclopropyl) glycine (DCG-IV). These results indicate that activation of group III mGlu receptors exerts neuroprotective activity against excitotoxic neuronal death. At least one of the two major group III mGlu receptor subtypes, i.e. $mGlu_4$ receptor, is expressed by cultured cortical neurons, as shown by immunocytochemical analysis with specific polyclonal antibodies.

Keywords: Metabotropic glutamate receptor; Excitotoxicity; Cortical neuron

1. Introduction

Metabotropic glutamate (mGlu) receptors form a family of at least 8 subtypes, which have been classified into three groups, based on sequence homology, pharmacological profile of activation and transduction pathways (reviewed in Nakanishi, 1994; Pin and Duvoisin, 1995). Group I includes $mGlu_1$ and $mGlu_5$ receptors, which are coupled to polyphosphoinositide (Pi) hydrolysis in transfected cells and are preferentially activated by quisqualate and (*S*)-3,5-dihydroxyphenylglycine (DHPG). Group II includes $mGlu_2$ and $mGlu_3$ receptors, which are negatively linked to adenylyl cyclase and are potently activated by (2_S,$1'_R$,$2'_R$,$3'_R$)-2-dicarboxycyxlopropyl) glycine (DCG-IV). Members of group III ($mGlu_{4,6\text{-}8}$ receptors) are also negatively linked to adenylyl cyclase in transfected cells and are selectively activated by L-2-amino-4-phosphonobutanoate (L-AP4) or by its analogue, L-serine-*O*-phosphate. The neuroprotective activity of group II mGlu receptor agonists has been recently documented (Bruno et al., 1994, 1995; Buisson and Choi, 1995; Buisson et al., 1996). L-AP4 and L-serine-*O*-phosphate can also partially protect cultured neurons against degeneration induced by excitotoxins (Bruno et al., 1995), β-amyloid peptide (Copani et al., 1995) or nitric oxide (Maiese et al., 1995). Although these effects have been related to the activation of group III mGlu receptors, conclusive evidence is lacking. L-AP4 and L-serine-*O*-phosphate exert multiple actions in brain tissue. They are, for example, good substrates for the Ca^{2+}/Cl^--dependent glutamate transporter (Monaghan et al., 1983; Fagg and Foster, 1983), and inhibit mGlu receptor agonist-stimulated PI hydrolysis in a non-competitive fashion (Nicoletti et al., 1986; Schoepp and Johnson, 1988). We now report that two novel group III mGlu receptor antagonists selectively prevent the neuroprotective action of L-AP4 and L-serine-*O*-phosphate in cultured cortical neurons, and that these neurons express the $mGlu_4$ receptor protein. This supports the view that group III mGlu receptors behave as neuroprotective receptors in the CNS.

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2. Materials and methods

2.1. Characterization of $mGlu_4$ receptor antibodies

Polyclonal antibodies were raised in rabbits against a synthetic peptide corresponding to the following non-conserved carboxy-terminal amino-acid sequences (one-letter code): CLETPALATKQTYVTYTNHAI for $mGlu_4$ receptor and PAKKKYVSYNNLVI for $mGlu_7$ receptor. Antibodies were purified by immunoaffinity chromatography with peptide-coupled Affigel 10/15 (Bio-Rad). The specificity of the antibody was tested in human embryonic kidney (HEK) 293 cells transfected with either $mGlu_4$ and $mGlu_7$ receptors, as follows. HEK 294 cells grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum were transfected with 4 μg of plasmid DNA per dish (density = 5×10^{-5} cells) by calcium phosphate precipitation. Cell monolayers were harvested 48 h after transfection by a 10-min incubation on ice with 300 μl of lysis buffer (50 mM HEPES, pH 7.5; 150 mM NaCl; 1.5 mM $MgCl^2$, 1 mM EGTA; 10% glycerol; 1% Triton X-100; 1 mM phenylmethylsulphonyl fluoride; 0.5 μg/ml leupeptin; 1 μg/ml pepstatin; 1 μg/ml aprotinin). Twenty μg of protein from cell lysates was separated by electrophoresis on 8% sodium dodecyl sulphate-polyacrylamide gels and transferred to nitrocellulose membranes by standard procedures in 0.2 M phosphate buffer. The blots were blocked for 1 h with NETG buffer (150 mM NaCl; 5 mM EGTA; 50 mM Tris-HCl pH 7.4; 0.05% Triton X-100; 0.25% gelatine) and incubated for 1 h at room temperature with the primary and secondary antibodies. The primary antibodies were diluted 1 : 1000 in NETG buffer, the secondary antibody (peroxidase-coupled goat anti-rabbit, Bio-Rad) was diluated 1 : 1500. After each antibody incubation of 1 h, the blots were washed in NETG for 1 h. Detection was performed using the enhanced chemioluminescence (ECL) Western blotting analysis system (Amersham).

2.2. Preparation of cortical cell cultures

Mixed cortical cultures containing both neurons and glia were prepared from fetal mice at 14–16 days of gestation, as described previously (Rose et al., 1993). Briefly, dissociated cortical cells were plated in 15-mm multiwell vessels (Nunc) on a layer of confluent glial cells (7–14 days in vitro), using a plating medium of Eagle's minimal essential medium (MEM-Earle's salts, supplied glutamine free) supplemented with 5% heat-inactivated horse serum, 5% fetal calf serum, glutamine (2 mM) and glucose (final concentration 21 mM). Cultures were kept at 37°C in a humified 5% CO_2 atmosphere. After 3–5 days in vitro, non-neuronal cell division was halted by 3-day exposure to 10 μM cytosine arabinoside, and cultures were shifted to a maintenance medium identical to the plating medium but lacking fetal serum. Subsequent partial medium replacement was carried out twice per week. Only mature cultures (13–14 days in vitro) were used for the experiments.

2.3. Immunocytochemistry

Cortical cultures at 13–14 days in vitro were stained with $mGlu_4$ receptor antibodies. Cells were washed twice with phosphate-bufferd saline (PBS), fixed for 30 min 2% paraformaldehyde, washed three times with PBS and then permeabilized with 0.1% Triton X-100 in PBS for 10 min. Cells were then washed, blocked with serum and incubated with the primary antibody (1 : 1000) for 2 h at room temperature. After cells were washed three times, the secondary antibody (1 : 200) was added for 1 h. After the reaction with avidin-biotin-horseradish peroxidase (Vectastain ABC-Elite kit; Vector Labs, Burlingame, CA, USA), staining was developed by exposure to 0.5% diaminobenzidine/0.01% H_2O_2 (2–10 min).

2.4. Exposure to excitatory amino acids

Exposure to *N*-methyl-D-aspartate (NMDA) (10 min), in the presence or absence of mGlu receptor agonists and antagonists, was carried out in mixed cortical cultures at room temperature in a HEPES-buffered salt solution (HBSS) containing (in mM): NaCl, 120: KCl, 5.4; $MgCl_2$, 0.8; $CaCl_2$, 1.8; HEPES, 20; glucose, 15. After 10 min, the drugs were washed out and the cultures were incubated at 37°C for the following 24 h in medium stock (Eagle's minimal essential medium, supplemented with 15.8 mM $NaHCO_3$ and glucose up to 25 mM), and then assessed for neuronal injury.

2.5. Assessment of neuronal injury

Neuronal injury was estimated by examination of the cultures with phase-contrast microscopy at 100×, 24 h after the insult. Neuronal damage was quantitatively assessed in all experiments by estimation of dead neurons by Trypan blue staining. Stained neurons were counted from three random fields per well. Neuronal injury was also assessed by measuring the activity of lactate dehydrogenase (LDH) released from damaged or destroyed cells into the extracellular medium, as described in Koh and Choi (1987).

2.6. Materials

N-methyl-D-aspartate (NMDA) and L-serine-*O*-phosphate were obtained from Sigma (St. Louis, MO, USA); L-AP4 was purchaed from Tocris Cookson (Bristol, UK); DCG-IV was kindly provided by Dr. H. Shinozaki (Tokyo Metropolitan Institute for Medical Sciences, Japan). (2_S,$1'_S$,$2'_S$)-2-methyl-2-(2'-carboxycyclopropyl) glycine (MCCG-I), (*RS*)-α-methyl-4-phosphonophenylglycine

(MPPG) and (*S*)-α-methyl-3-carboxyphenylalanine (M3CPA) were synthesized by Dr. D.E. Jane (University of Bristol, UK).

3. Results

3.1. Characterization of $mGlu_4$ receptor antibodies

In lysates from cells transfected with $mGlu_{4a}$ receptor cDNA, $mGlu_4$ receptor antibodies labeled a major band at about 100 kDa, which corresponds to the deduced molecular weight of the receptor, and an additional band of higher

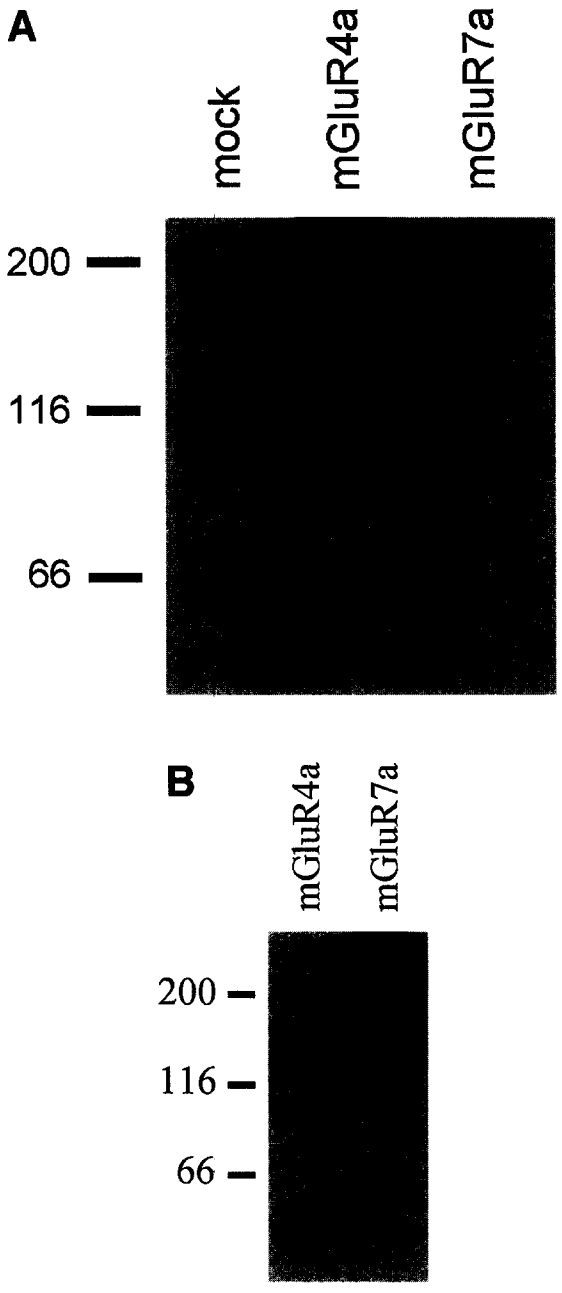

Fig. 1. Immunoblotting with purified mGluR4 (A) or mGluR7 (B) anbitodies in lysates from cells transfected with plasmids encoding mGluR4 and mGluR7 or with a control plasmid. Molecular weight markers are shown on the left side of each figure.

Table 1
Group III mGlu receptor antagonists reverse the protective effect of L-serine-*O*-phosphate against NMDA-induced toxicity

	% NMDA-induced toxicity (Trypan blue staining)		
		MPPG 30 μM	M3CPA 30 μM
NMDA 100 μM	100 ± 2	98 ± 5	95 ± 5
+ L-SOP 100 μM	54 ± 4	113 ± 12 [a]	81 ± 7 [a]

Values are the means ± S.E.M. of 8 individual determinations from two different experiments. [a] $P < 0.05$ vs. NMDA + L-SOP (ANOVA + Fisher PLSD test). L-SOP = L-serine-*O*-phosphate.

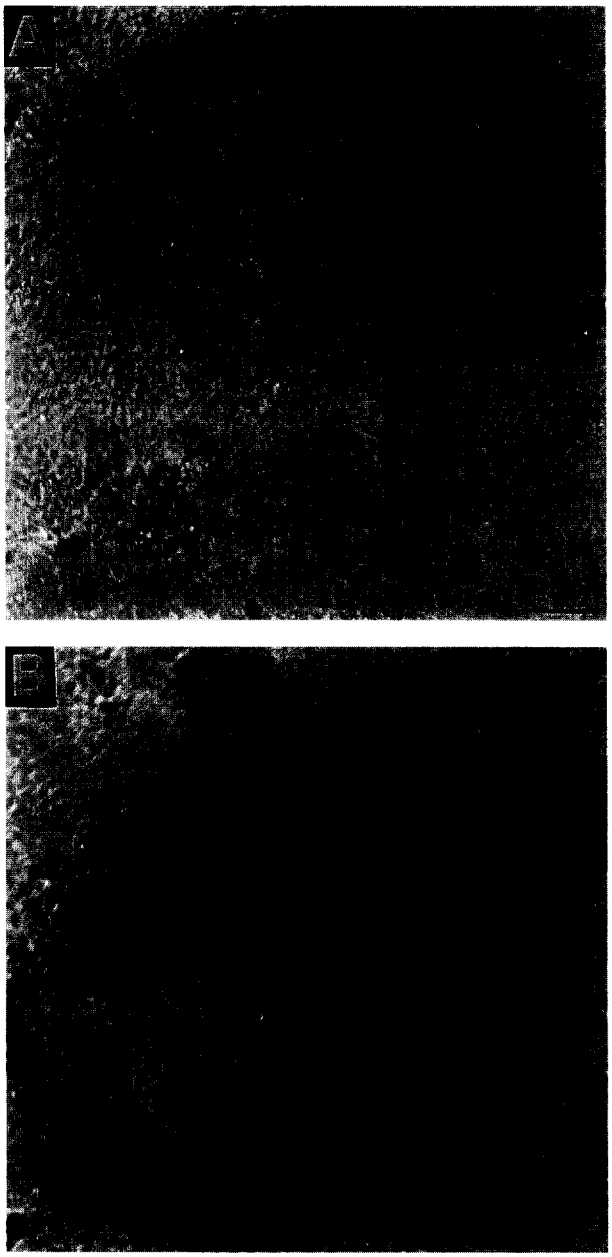

Fig. 2. Immunocytochemistry of mixed cortical cells stained with mGluR4 antibodies (B). Non-specific staining is shown for comparison in a sister culture not exposed to the mGluR4 antibody (A). Bar, 16 μm.

molecular weight, which probably represents a polymeric aggregate of the receptor. $mGlu_4$ receptor antibodies did not react with an protein in lysates from cells transfected with either $mGlu_{7a}$ receptor or a control plasmid (Fig. 1a). In contrast, lysates from cells transfected with $mGlu_{7a}$ (but not $mGlu_{4a}$) receptor cDNA were immunoreactive to $mGlu_7$ receptor antibodies (Fig. 1b).

3.2. Immunocytochemistry with $mGlu_4$ receptor antibodies in cortical neurons

Cultured cortical neurons were immunoreactive to $mGlu_4$ receptor antibodies, which appeared to stain both cell bodies and neurites (Fig. 2).

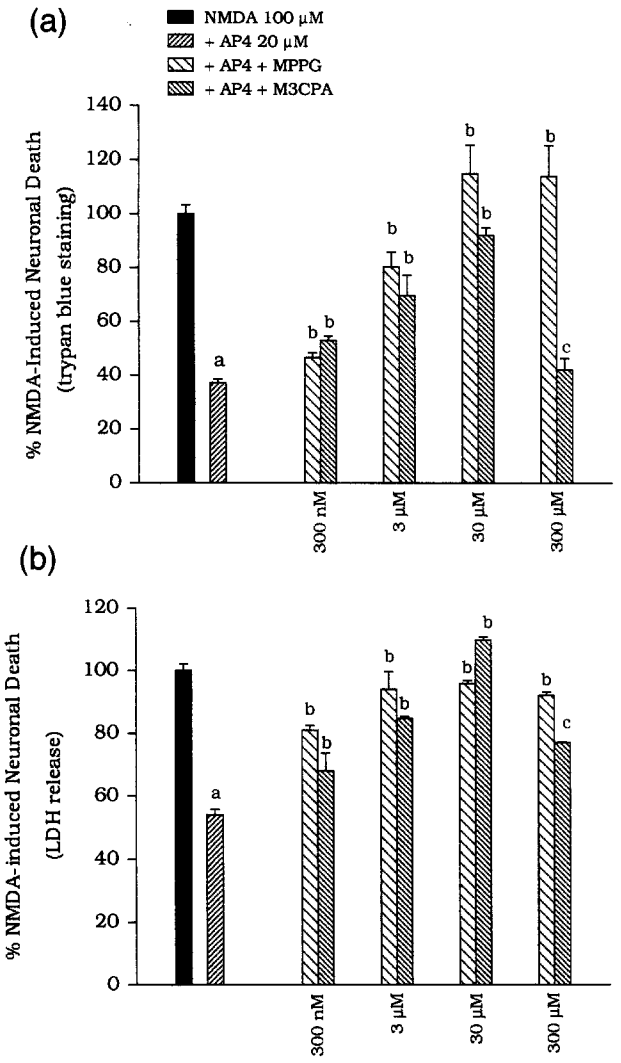

Fig. 3. MPPG or M3CPA antagonize the neuroprotective effect of L-AP4 in a concentration-dependent fashion. (A) Data refer to the number of cells stained with Trypan blue (mean ± S.E.M.) and are expressed as percentage of NMDA-induced neuronal death. Values were calculated from 8–12 individual determinations (2 or 3 independent experiments). (B) Extracellular LDH activity was measured in parallel in the same experiment. [a] $P < 0.05$ vs. NMDA; [b] $P < 0.05$ vs. NMDA + AP4; [c] $P < 0.05$ vs. NMDA + AP4 + M3CPA 30 μM (one-way ANOVA + Fisher PLSD test). Neither MPPG or M3CPA influenced per se NMDA toxicity at any concentration tested.

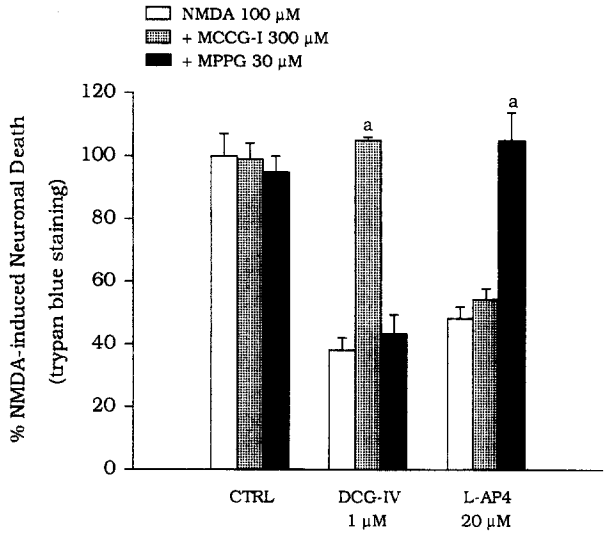

Fig. 4. Influence of MCCG-I or MPPG on the neuroprotective activity of DCG-IV or L-AP4 against NMDA toxicity. Values refer to the number of dead neurons, as stained by Trypan blue and were calculated from 8 individual determinations (2 independent experiments). [a] $P < 0.05$ (one-way ANOVA + Fisher PLSD test), if compared with the respective values obtained in the absence of antagonists.

3.3. Toxicity studies

A 10-min exposure to maximal concentrations of NMDA (> 150 μM) induced necrotic death in about 90% of cortical neurons, as indicated by both Trypan blue staining and LDH release. In our experiments, we used a submaximal concentration of NMDA (100 μM), which produced the death of 75–80% of neurons (here indicated as 100% of NMDA toxicity). As expected (Bruno et al., 1995), L-AP4 partially protected cortical neurons against NMDA toxicity in a concentration-dependent fashion, with an apparent EC_{50} value of 1 μM (not shown). Two novel group III mGlu receptor antagonists, MPPG and M3CPA, were challenged against a maximally effective concentration of L-AP4 (20 μM). Neither MPPG nor M3CPA (up to 300 μM) influenced per se neuronal viability or NMDA-induced neuronal death (not shown). Both drugs instead antagonized the neuroprotective effect of L-AP4 in a concentration-dependent fashion (IC_{50} between 1 and 3 μM for both MPPG and M3CPA) (Fig. 3a,b). At high concentrations, however, M3CPA was less effective in antagonizing neuroprotection by L-AP4 (although this tendency was more evident by using Trypan blue staining rather than LDH release for the detection of neuronal toxicity) (Fig. 3). Both MPPG and M3CPA (the latter at 30, but not 100 μM) also antagonized the neuroprotective effect of L-serine-*O*-phosphate (Table 1). Finally, we challenged the neuroprotective action of DCG-IV (1 μM) or L-AP4 (20 μM) with the selective class II mGlu receptor antagonist, MCCG-I or with MPPG. As expected, the neuroprotective activity of DCG-IV was antagonized by MCCG-I (300 μM), but not by MPPG at a concentration (30 μM) that

was maximally effective against L-AP4. In contrast, the neuroprotective action of L-AP4 was insensitive to MCCG-I (Fig. 4).

4. Discussion

L-AP4, a close structural analogue of glutamate, has long been known to depress excitatory synaptic transmission (Koerner and Cotman, 1981; Davies and Watkins, 1982; Baskys and Malenka, 1991; Rainnie and Shinnick-Gallagher, 1992) and to reduce glutamate release (Jones and Roberts, 1990; Vasquez et al., 1995). L-AP4 and its analogue L-serine-*O*-phosphate have been shown to partially protect cultured cortical neurons against excitotoxic degeneration (Bruno et al., 1995). The recent progress in the pharmacology of mGlu receptors now makes it possible to establish whether this neuroprotective effect is mediated by the activation of group III mGlu receptors. A series of compounds bearing an α-methyl group have been described as preferential antagonists of group III mGluRs. We used MPPG and M3CPA (Kemp et al., 1994; Roberts et al., 1994; Roberts, 1995) rather than α-methyl-AP4 (Jane et al., 1994), because the latter exhibits low potency and mimics the action of L-AP4 in inhibiting forskolin-stimulated cAMP formation in rat cortical slices (Kemp et al., 1994; Gottesman et al., 1995; Roberts, 1995). MPPG is an analogue of (*RS*)-α-methyl-4-carboxymethylphenylglycine (MCPG), in which the substitution of a carboxyl by a phosphonate group in position 4 lowers the affinity of the compound for group I and II mGluRs and substantially enhances the affinity for group III (Jane et al., 1995; Roberts, 1995). MPPG binds to and activates recombinant human $mGlu_4$ receptor expressing cells (M.A. Tones, P.J. Flor, R. Kuhn and T. Knoepfel, personal communication). Both MPPG and M3CPA substantially antagonized the neuroprotective effect of L-AP4 in cultured cortical neurons, and their apparent IC_{50} values fell in the same range as those reported in electrophysiological experiments (Jane et al., 1995). M3CPA, however, showed a tendency to have a bell-shaped concentration-response curve, because it appeared to be less effective at 300 than at 30 μM. The action of MPPG and M3CPA was specific for L-AP4 and L-SOP, because they did not influence the neuroprotective action of the group II mGlu receptor agonist, DCG-IV. In contrast, MCCG-I, a selective antagonist of group II mGlu receptors (Jane et al., 1994), antagonized the action of DCG-IV, but was inactive when used to challenge L-AP4. Taken together, these results indicate that the neuroprotective effect of L-AP4 and L-SOP in cultured cortical neurons was mediated by the activation of group III mGlu receptors. We have therefore studied the expression of $mGlu_4$ receptors in mixed cortical cultures. Cortical neurons were immunopositive for $mGlu_4$ receptors, and immunostaining was present on both neuronal cell bodies and neurites, as one can expect based on the putative presynaptic location of $mGlu_4$ receptors. It is conceivable that cortical cultures express also $mGlu_7$ receptors, as a high expression of $mGlu_7$ receptor mRNA is observed in neuronal cells throughout the cerebral cortex (Okamoto et al., 1994; Saugstad et al., 1994). However, a major involvement of $mGlu_4$ receptors is suggested by the high potency exibited by L-AP4 ($EC_{50} = 1$ μM) as a neuroprotectant, according to what is observed in transfected cells, where L-AP4 interacts with $mGlu_4$ receptors in the high nanomolar range, but interacts with $mGlu_7$ receptors at much higher concentrations (reviewed in Pin and Duvoisin, 1995).

In conclusion, the present results provide clear-cut pharmacological evidence that activation of class III mGlu receptors protects cultured cortical neurons against excitotoxic death. Class III mGlu receptors therefore become a putative target for the design of novel neuroprotective agents.

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