

## (–)-PHCCC, a positive allosteric modulator of mGluR4: characterization, mechanism of action, and neuroprotection

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Received 25 February 2003; received in revised form 27 May 2003; accepted 23 June 2003

### Abstract

Group-III metabotropic glutamate receptors (mGluR4, -6, -7, and -8) modulate neurotoxicity of excitatory amino acids and beta-amyloid-peptide ( $\beta$ AP), as well as epileptic convulsions, most likely via presynaptic inhibition of glutamatergic neurotransmission. Due to the lack of subtype-selective ligands for group-III receptors, we previously utilized knock-out mice to identify mGluR4 as the primary receptor mediating neuroprotection of unselective group-III agonists such as L-AP<sub>4</sub> or (+)-PPG, whereas mGluR7 is critical for anticonvulsive effects.

In a recent effort to find group-III subtype-selective drugs we identified (+/–)-PHCCC as a positive allosteric modulator for mGluR4. This compound increases agonist potency and markedly enhances maximum efficacy and, at higher concentrations, directly activates mGluR4 with low efficacy. All the activity of (+/–)-PHCCC resides in the (–)-enantiomer, which is inactive at mGluR2, -3, -5a, -6, -7b and -8a, but shows partial antagonist activity at mGluR1b (30% maximum antagonist efficacy). Chimeric receptor studies showed that the binding site of (–)-PHCCC is localized in the transmembrane region.

Finally, (–)-PHCCC showed neuroprotection against  $\beta$ AP- and NMDA-toxicity in mixed cultures of mouse cortical neurons. This neuroprotection was additive to that induced by the highly efficacious mGluR1 antagonist CPCCOEt and was blocked by MSOP, a group-III mGluR antagonist. Our data provide evidence for a novel pharmacological site on mGluR4, which may be used as a target-site for therapeutics.

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**Keywords:** Positive modulation; Metabotropic glutamate receptors; Excitotoxicity;  $\beta$ -amyloid toxicity; Neuroprotection

### 1. Introduction

Metabotropic glutamate receptors (mGluRs) form a family of eight subtypes subdivided into three groups on the basis of sequence homology, pharmacological profile and transduction pathway. Group-I includes mGluR1 and -5, which are coupled to polyphosphoinositide hydrolysis; group-II includes mGluR2 and -3, which are coupled to G<sub>i</sub> proteins and negatively modulate adenylyl cyclase activity in recombinant cells; group-III includes mGluR4, -6, -7 and -8. All group-III subtypes

negatively modulate adenylyl cyclase activity in heterologous expression systems, but at least one of them (mGluR6) regulates a cGMP-specific phosphodiesterase in its native environment (i.e. in the ‘ON’ bipolar cells of the retina, Nawy and Jahr, 1990). The wide involvement of mGluRs in physiology and pathology has encouraged the search for subtype-selective agonists and antagonists which are used in experimental animal models of human pathology and, at the same time, may serve as templates for drug discovery. Examples are provided by a battery of potent and highly selective mGluR2/3 agonists (of which LY354740 and LY379268 are prototypic compounds), and by the phenylethynylpyridine series of non-competitive mGluR5 antagonists (e.g. MPEP; Gasparini et al., 1999b).

The pharmacology of group-III receptors is the least

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developed because of the requirement for an omega-phosphono group in current agonist compounds like L-AP4 or (+)-PPG (Thomsen et al., 1992; Gasparini et al., 1999a; Flor et al., 2002; Fig. 1). This limitation can be overcome by the search for molecules that behave as positive or negative allosteric modulators rather than interacting with the agonist binding site. Here we report (–)-N-Phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide ((–)-PHCCC; Fig. 1) as the first receptor subtype-selective positive modulator within group-III mGluRs; it shows enhancement of agonist effects at mGluR4 in the GTP $\gamma$ [<sup>35</sup>S] binding assay and in cellular cAMP and calcium assays, but it is inactive on human mGluR2, -3, -5a, -6, -7b and -8a. We also demonstrate that (–)-PHCCC can be used to selectively probe the function of mGluR4 in a neuronal system.

## 2. Methods

### 2.1. Stable mammalian cell lines for cloned mGluR subtypes

Generation, culture and pharmacological characterization of stable cell lines for human mGluR1b, -2, -3, -4a, -5a, -6, -7b, and -8a have been described recently

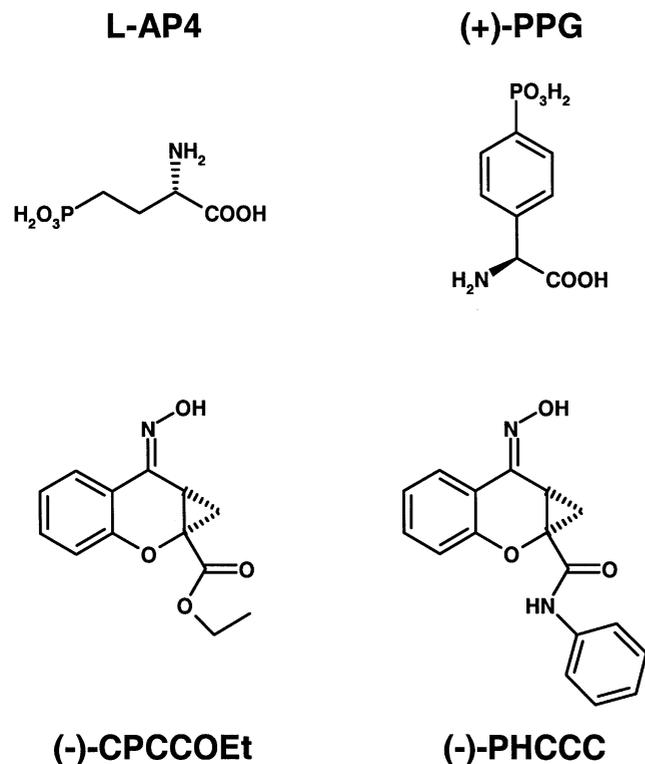


Fig. 1. Chemical structures of L-AP<sub>4</sub>, (+)-PPG, (–)-CPCCOEt, and (–)-PHCCC.

(Knöpfel et al., 1995; Flor et al., 1995, 1997; Gasparini et al., 1999a,b; Varney et al., 1999).

### 2.2. Membrane preparation for GTP $\gamma$ [<sup>35</sup>S] binding assays

Membranes were prepared from transfected cells using essentially the protocol described by Lazareno and Birdsall, 1993 and Gasparini et al., 1999b. The culture of transfected hmGluR2, -3, -4a, -6, -7b or -8a expressing cells was expanded to seed 30–60 cells per 15-cm dishes. The cells were harvested when they reached 80–95% confluency followed by homogenization in buffer A (20 mM HEPES, 10 mM EDTA, pH 7.4) using a Polytron and centrifugation at 39 000 g at 4 °C for 20 min. The pellet was resuspended in buffer B (20 mM HEPES, 0.1 mM EDTA, pH 7.4), homogenized using a Polytron and recentrifuged at 39 000 g at 4 °C for 20 min. The pellet was resuspended in buffer B at a protein concentration of 2 to 3 mg/ml, aliquoted, snap frozen on dry ice and stored at –70 °C.

### 2.3. GTP $\gamma$ [<sup>35</sup>S] binding assay

Membrane fractions were diluted in assay buffer (20 mM HEPES, 10 mM MgCl<sub>2</sub>, 100 mM NaCl, 2 mM EGTA, 20  $\mu$ M GDP, pH 8.0), homogenized briefly using a Polytron and incubated for 10 min at 30 °C. Following pre-incubation, assay-mixtures were prepared in 96-well microtiter plates. After optimization of various parameters, the composition of the assay mixtures in a final volume of 200  $\mu$ l per well was as follows: 20 mM HEPES, 10 mM MgCl<sub>2</sub>, 100 mM NaCl, 2 mM EGTA, 20  $\mu$ M GDP, pH 8.0, 15–35  $\mu$ g membrane protein (pre-treated as described above), 1.5 mg wheat germ agglutinin SPA beads (Amersham, Freiburg, Germany), 0.05–0.2 nM GTP $\gamma$ [<sup>35</sup>S] (Amersham, Freiburg, Germany), and the test compounds (agonists, modulators, and/or antagonists) at the appropriate concentrations. Non-specific binding was measured in the presence of unlabelled GTP $\gamma$ S (Sigma) in excess (10  $\mu$ M). The samples were incubated at room temperature for 40–60 min (with shaking) before the SPA beads were sedimented by centrifugation at 200 g for 10 min at room temperature. The plates were then counted in a Packard TopCount (Packard, Switzerland).

### 2.4. Cyclic AMP assay

Measurements of cyclic AMP accumulation were performed essentially as previously described using CHO cell lines stably expressing individual mGluR-subtypes (Flor et al., 1995, 1997).

## 2.5. Calcium measurements

Functional activation of  $G_q$ -coupled receptors was assayed by means of fluorometric measurements of changes in intracellular free calcium concentration  $[Ca^{2+}]_i$  as described by Flor et al. (1996) based on the calcium sensitive dye fura-2 (Grynkiewicz et al., 1985). Briefly, COS-1 cells were transiently transfected 1 day before and grown on glass cover slips. On the day of the assay, the cover slips were incubated at room temperature for 60 min in a HEPES buffered saline (Life Technologies/GibcoBRL, Basel, Switzerland) containing 10 g/ml fura-2/AM (Molecular Probes Inc., Eugene, OR, USA), 5 l/ml DMSO and 0.5% pluronic (Molecular Probes Inc., Eugene, OR, USA). Glass cover slips carrying the dye loaded cells were mounted into a perfused cuvette in a fluorescence spectrophotometer (Hitachi F-4500, Japan). Changes in  $[Ca^{2+}]_i$  were monitored by measuring the ratio of fura-2 fluorescence (510 nm) excited alternatively (1.6 Hz) at 340 and 380 nm. Thus, an increase of the fluorescent response ratio  $F_{340}/F_{380}$  corresponds to an increase in  $[Ca^{2+}]_i$  (Grynkiewicz et al., 1985).

## 2.6. Mixed cortical cultures

Mixed cortical cell cultures containing both neurons and astrocytes were prepared from fetal mice at 14–16 days of gestation, as described previously (Rose et al., 1992). Briefly, dissociated cortical cells were plated in 15 mm multiwell vessels (Falcon Primaria, Lincoln Park, NJ, USA) on a layer of confluent astrocytes (7–14 days in vitro), using a plating medium of MEM-Eagle's salts (supplied glutamine-free) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, glutamine (2 mM), and glucose (final concentration 21 mM). Cultures were kept at 37 °C in a humidified 5%  $CO_2$  atmosphere. After 3–5 days in vitro, non-neuronal cells division was halted by 1–3 days exposure to 10  $\mu$ M cytosine arabinoside, and cultures were shifted to a maintenance medium identical to plating medium but lacking fetal serum. Subsequent partial medium replacement was carried out twice a week. Only mature cultures (13–14 days in vitro) were used for the experiments.

## 2.7. Glial cell cultures

Glial cell cultures were prepared as described previously (Rose et al., 1992) from postnatal mice (1–3 days after birth). Dissociated cortical cells were grown in 15-mm multiwell vessels (Falcon Primaria) using a plating medium of MEM-Eagle's salts supplemented with 10% fetal bovine serum, 10% horse serum, glutamine (2 mM), and glucose (final concentration 21 mM). Cultures were kept at 37 °C in a humidified  $CO_2$  atmosphere until

they reached confluence (13–14 days in vitro). Confluent cultures were then used as a support for mixed cultures.

## 2.8. Exposure to excitatory amino acids and $\beta$ AP

Brief exposure to NMDA 100  $\mu$ M (10 min), in the presence or absence of (+/–)-, (+)- and (–)-PHCCC was carried out in mixed cortical cultures at room temperature in a HEPES-buffered salt solution containing (in mM): 120 NaCl, 5.4 KCl, 0.8  $MgCl_2$ , 1.8  $CaCl_2$ , 20 HEPES, 15 glucose. After 10 min the drugs were washed out, and cultures were incubated at 37 °C for the following 24 h in medium stock (MS) (MEM-Eagle's supplemented with 15.8 mM  $NaHCO_3$  and glucose <25 mM).  $\beta$ AP<sub>(25-35)</sub> was solubilized in sterile water at an initial concentration of 2.5 mM and was stored frozen at –20 °C for at least 1 week prior to use. Neurodegeneration induced by  $\beta$ AP (25  $\mu$ M) for 24–48 h was done in the presence of 10  $\mu$ M 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 10  $\mu$ M MK-801 as described by Copani et al. (1995).

## 2.9. Assessment of neuronal injury

Neuronal injury was estimated in all experiments by examination of cultures with phase-contrast microscopy at 100- to 400-fold magnification, 1 day after the insult. Neuronal damage was assessed quantitatively by trypan blue staining. Stained neurons were counted from three random fields per well.

## 2.10. Materials

Tissue culture reagents were from Sigma (Buchs, Switzerland or Milano, Italy) or Life Technologies/GibcoBRL (Basel, Switzerland). NMDA, DNQX and MK-801 were purchased from Tocris (Bristol, U.K.). (+/–)-, (+)- and (–)-PHCCC were synthesized at Novartis Pharma AG (Gasparini et al., manuscript in preparation).  $\beta$ AP<sub>(25-35)</sub> was obtained from Bachem Feinchemikalien AG (Bubendorf, Switzerland).

## 3. Results

### 3.1. (–)-PHCCC positively modulates mGluR4 in a $GTP\gamma^{35}S$ binding assay

Stimulation of  $GTP\gamma^{35}S$  binding is widely used to assess functional activity of G-protein coupled receptors (Lazareno and Birdsall, 1993; Kowal et al., 1998; Urwyler et al., 2001). The saturating concentration of 10  $\mu$ M L-AP<sub>4</sub> stimulated  $GTP\gamma^{35}S$  binding on membranes from CHO cells stably expressing human mGluR4a (hmGluR4a; Flor et al., 1995) approximately two-fold above the basal activity measured in the absence of

agonist (Fig. 2A–C). This effect is mediated by mGluR4 because it is not seen in untransfected CHO cells or cells transfected with mGluR2 (not shown). The compounds (+/–)-PHCCC and (–)-PHCCC (Fig. 1) were found to substantially increase the effects of sub-maximal and saturating L-AP<sub>4</sub> on membranes from hmGluR4a-expressing cells, while showing comparatively low partial activity when applied in the absence of L-AP<sub>4</sub>; the (+)-enantiomer of PHCCC was inactive in the presence and in the absence of L-AP<sub>4</sub> (Fig. 2). The positive modulation of GTPγ[<sup>35</sup>S] binding on hmGluR4a by 3 μM and 10 μM of (–)-PHCCC was completely blocked by the group-III mGluR-selective antagonists CPPG and MSOP (Fig. 3A). In order to determine half-maximally effective concentrations (EC<sub>50</sub>s) of (–)-PHCCC, we performed concentration-response curves in the absence and presence of constant L-AP<sub>4</sub> concentrations. Without L-AP<sub>4</sub>, (–)-PHCCC showed an EC<sub>50</sub> of >30 μM; at 0.2 and 0.6 μM of L-AP<sub>4</sub>, the EC<sub>50</sub> of (–)-PHCCC was approximately 6 μM; and in the presence of 10 μM L-AP<sub>4</sub>, the EC<sub>50</sub> of (–)-PHCCC decreased to 3.8 μM (Fig. 3B).

To characterize the positive modulatory effects of (–)-PHCCC on hmGluR4a in more detail, concentration–response curves for L-glutamate at different fixed concentrations of (–)-PHCCC were done (Fig. 4). The compound (–)-PHCCC showed a dual mechanism on mGluR4-expressing membranes, displaying an increase of both, L-glutamate potency and maximal efficacy (see plot and table in Fig. 4).

### 3.2. Profiling of (–)-PHCCC against all eight cloned metabotropic glutamate receptors

Before we addressed the activity of (–)-PHCCC at all cloned mGluRs, binding assays at 28 different nervous system targets were conducted. No considerable radioligand displacement at any of the sites was found at 1 μM and 10 μM of (–)-PHCCC; this included a selection of receptors for adrenaline, dopamine, GABA, histamine, acetylcholine, opiates, serotonin, and substance P plus selected neurotransmitter re-uptake sites (data not shown).

As shown in Fig. 5A, the positive modulation of (–)-PHCCC is selective for mGluR4: when tested in the presence of approximately EC<sub>20</sub> of an agonist, GTPγ[<sup>35</sup>S] binding is markedly stimulated by (–)-PHCCC only on hmGluR4a-expressing membranes while no stimulating effect is seen on membranes from hmGluR2- or hmGluR7b-expressing cells (Fig. 5A). Also, no significant antagonist activity of 10 μM (–)-PHCCC was observed at hmGluR2 or -7b when tested against EC<sub>90</sub> of an agonist; we have also tested (–)-PHCCC in the GTPγ[<sup>35</sup>S] binding assay on hmGluR3- hmGluR6- and hmGluR8a-expressing membranes and up to 10 μM (–)-PHCCC did not show any positive modulatory or antagonist activity at those receptors (data not shown). How-

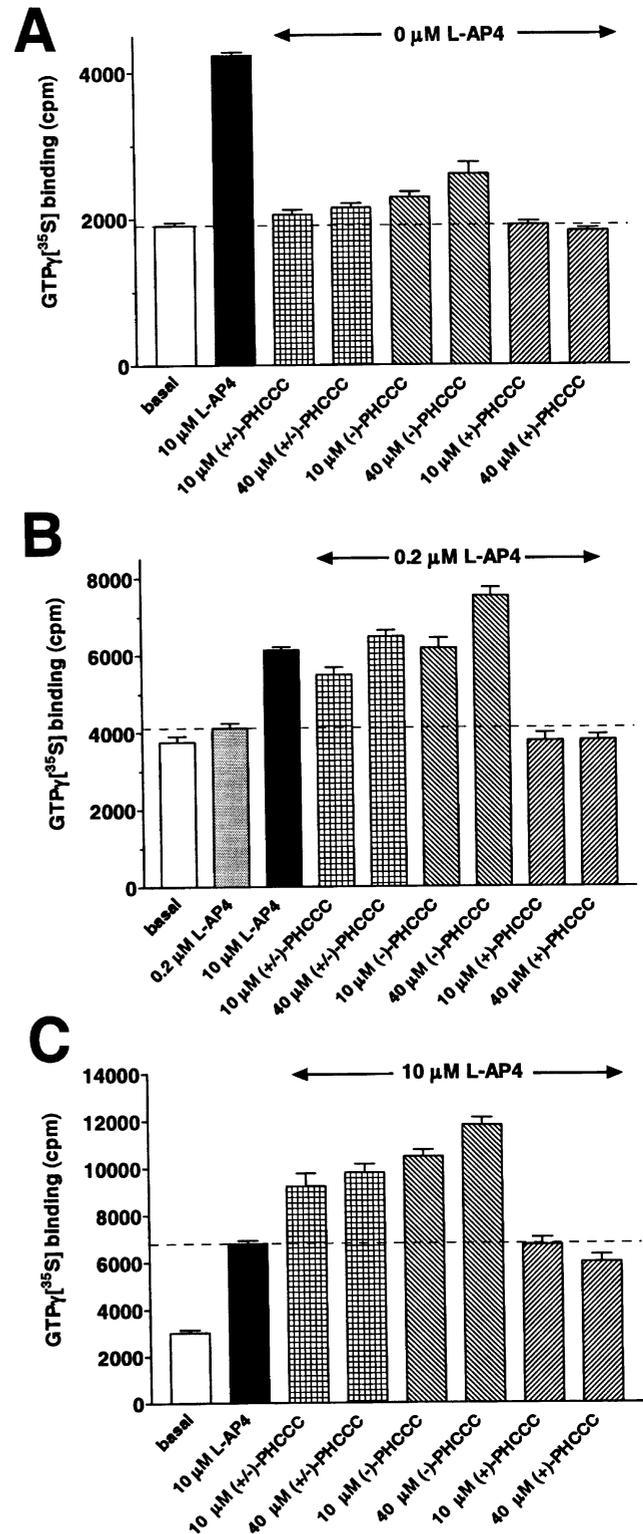


Fig. 2. Potentiation of the L-AP<sub>4</sub>-induced stimulation of GTPγ[<sup>35</sup>S] binding via hmGluR4a. The effects of the (+/–)-, (+)-, and (–)-PHCCC on GTPγ[<sup>35</sup>S] binding to membranes from hmGluR4a stably transfected CHO cells were measured in the absence (A), and in the presence of a low (0.2 μM, B) and a high (10 μM, C) concentration of L-AP<sub>4</sub>. The data shown are from a typical experiment, performed in triplicate, and expressed as mean counts per minute (cpm) values with S.E.M.

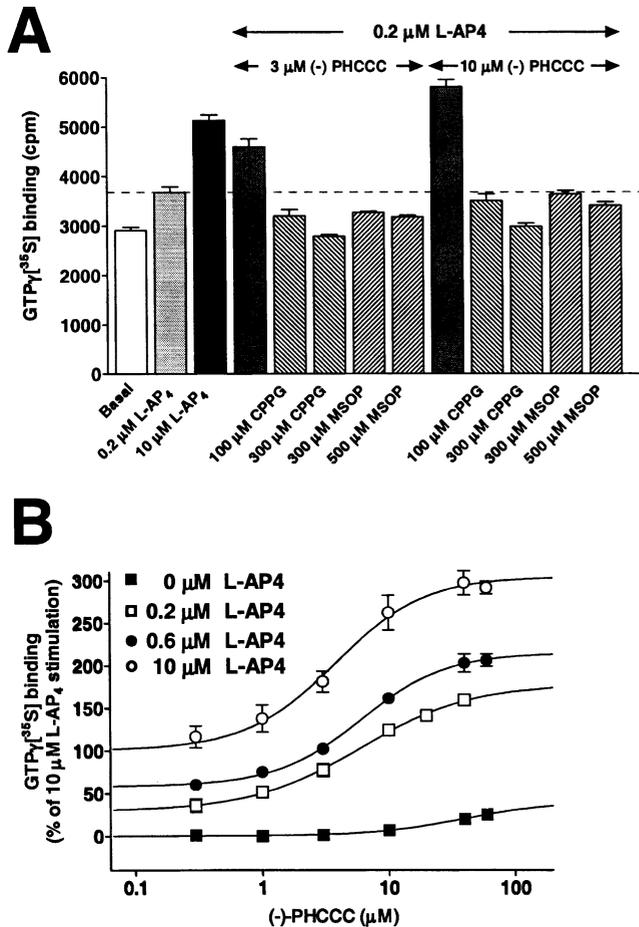


Fig. 3. (A) Effect of group-III antagonists on the (-)-PHCCC potentiation of the L-AP<sub>4</sub>-induced stimulation of GTP $\gamma$ <sup>35</sup>S] binding at hmGluR4a. The effects of (-)-PHCCC on GTP $\gamma$ <sup>35</sup>S] binding to membranes from hmGluR4a stably transfected CHO cells were measured in the absence and in the presence of the group-III antagonists MSOP and CPPG. The data shown are from a typical experiment, performed in triplicate, and expressed as mean cpm values with S.E.M. (B) Concentration–response curves for (-)-PHCCC in the GTP $\gamma$ <sup>35</sup>S] binding assay on hmGluR4a in the absence and in the presence of different L-AP<sub>4</sub> concentrations. The data are quadruplicate determinations with S.E.M. and all values were normalized to the control stimulation of 10 μM L-AP<sub>4</sub> (set to 100%).

ever, (-)-PHCCC also positively modulated the negative coupling of hmGluR4a to adenylate cyclase: in the presence of 5 μM L-glutamate, (-)-PHCCC amplified the inhibition of forskolin-stimulated cAMP accumulation with an EC<sub>50</sub>-value of 2.8 μM, while there was no amplification seen in the same assay conducted with hmGluR6-expressing cells (Fig. 5B); in addition, PHCCC was also tested for activity in the cAMP assay at hmGluR2 and hmGluR7b and no modulating effect was observed (data not shown).

A recent report by Annoura et al. (1996) demonstrated antagonist activity of (+/-)-PHCCC at rat mGluR1a in a calcium assay; the antagonist efficacy of (+/-)-PHCCC was reported to be 60% as compared to 90% for CPCCOEt (see chemical structure in Fig. 1). Fig. 6

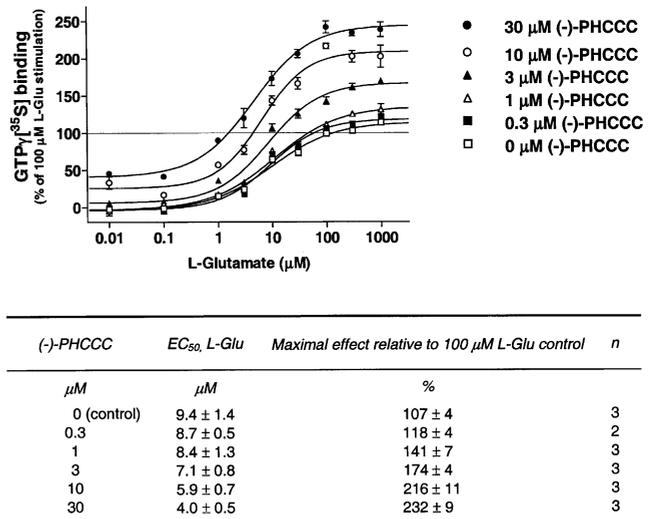


Fig. 4. Concentration–response curves for L-glutamate in the GTP $\gamma$ <sup>35</sup>S] binding assay in the absence and in the presence of (-)-PHCCC (0.3 μM, 1 μM, 3 μM, 10 μM, and 30 μM). The data shown are from a typical experiment, performed in triplicate, and expressed as mean % of 100 μM L-glutamate stimulation (set to 100%); values with S.E.M. The calculated parameters (averaged from three experiments, one of which is shown as plot) describing the different concentration–response curves are given in the table.

shows assessment of (-)-PHCCC's activity on hmGluR1b and hmGluR5a using a fluorimetric calcium assay. We found no positive modulatory and no antagonist activity of (-)-PHCCC on hmGluR5-expressing cells. However, (-)-PHCCC could antagonize hmGluR1b, although its efficacy was substantially lower than that of the related drug, CPCCOEt. When tested up to 30 μM, (-)-PHCCC produced a maximal inhibition of hmGluR1b of only 30% (IC<sub>50</sub> value: 3.4 μM), as compared with 80% of inhibition produced by CPCCOEt (Fig. 6A,B); see also Litschig et al. (1999) for the inhibition of hmGluR1b by CPCCOEt.

It has been demonstrated in the past that the known agonists of metabotropic glutamate receptors bind to the large N-terminal extracellular domain of the receptors, while non-competitive antagonists were found to interact with an allosteric binding site located in the C-terminal transmembrane region (for review see Pin et al., 1999). This prompted us to address the location of the binding site for PHCCC on hmGluR4a. We decided to use constructs of wild-type hmGluR4a in comparison with an hmGluR4/1b chimera, which contains 519 amino acids derived from the N-terminal extracellular domain of hmGluR4a and the remaining C-terminal portion of hmGluR1b comprising the entire transmembrane region (Tones et al., 1995). The activity of (-)-PHCCC on the hmGluR4a and hmGluR4/1b receptors was examined with the fluorimetric calcium assay (Fig. 7). (-)-PHCCC markedly enhanced calcium signal amplitudes on hmGluR4a (co-transfected with the promiscuous G-protein Gα16) when co-applied with sub-maximal or maxi-

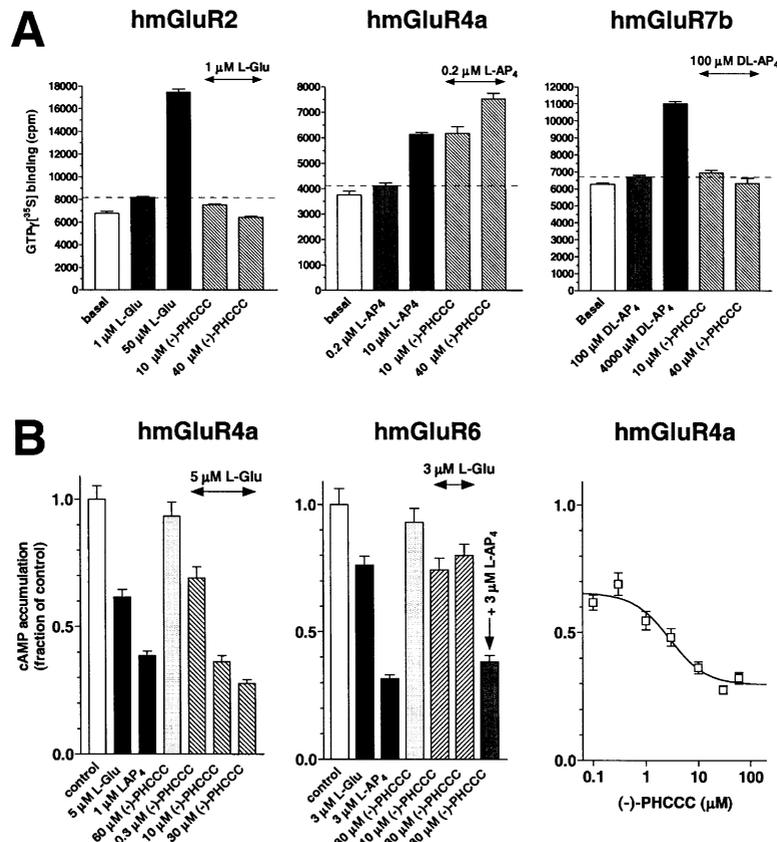


Fig. 5. Effect of (-)-PHCCC on groups-II and -III mGluRs. (A) Influence of (-)-PHCCC on the agonist-induced stimulation of GTP[<sup>35</sup>S] binding via hmGluR2, -4a and -7b stably expressed in CHO cells. The effects of (-)-PHCCC on GTP[<sup>35</sup>S] binding to membranes from human mGluR stably transfected CHO cells were measured in the presence of 1 μM L-glutamate, 0.2 μM L-AP<sub>4</sub> and 100 μM DL-AP<sub>4</sub> in case of hmGluR2, -4a and -7b, respectively. The data shown are taken from a typical experiment, performed in triplicate, and expressed as mean counts per minute (cpm) values with S.E.M. (B) Inhibition of forskolin-stimulated cAMP accumulation in CHO cells expressing hmGluR4a or hmGluR6 receptors. All values are given as fraction of control and were pooled from at least four measurements obtained in two independent experiments, and expressed as means with S.E.M.

mal effective concentrations of L-glutamate; on the other hand, (-)-PHCCC decreased L-glutamate-induced calcium signals by approximately 50% on the hmGluR4/1b chimeric receptor (Fig. 7A,B), indicating that receptor modulation by (-)-PHCCC critically depends on the identity of the transmembrane domains. We also constructed hmGluR1/4a chimeras, with fusion sites at similar positions as the hmGluR4/1b chimera described above. Unfortunately, hmGluR1/4a-transfected cells did not show any L-glutamate-induced calcium signals, even when co-transfected with the promiscuous G-protein Gα16 (data not shown).

### 3.3. Neuroprotection by (-)-PHCCC against NMDA or β-amyloid toxicity

We used mixed cultures of cortical cells to examine whether PHCCC is neuroprotective by interacting with native mGluR4. In these cultures, mGluR4 mediates the neuroprotective activity of L-AP<sub>4</sub>, (+)-PPG or other group-III mGluR agonists (Bruno et al., 2000). Cultures were challenged with 100 μM NMDA for 10 min

(according to the paradigm of 'fast' excitotoxicity), and neuronal death was assessed 24 h later. Addition of L-AP<sub>4</sub> during the NMDA pulse reduced the extent of neuronal death, consistently with previous reports (Bruno et al., 1996, 2000). (-)-PHCCC also reduced neuronal death when applied at concentrations of 30–100 μM, whereas the (+)-isomer was inactive (Fig. 8A). Neuroprotection was abrogated by the group-III mGluR antagonists, MSOP or MPPG (Fig. 8B), suggesting that (-)-PHCCC acted by enhancing the endogenous activation of mGluR4. Although inhibition of mGluR1 by (-)-PHCCC might have contributed to neuroprotection, this possibility was excluded by the additive effect of CPCCOEt and (-)-PHCCC (Fig. 8C). To further confirm that neuroprotection by (-)-PHCCC was mediated by group-III mGluRs, we applied (-)-PHCCC in combination with the agonist L-AP<sub>4</sub>. In Table 1, values in parentheses refer to the expected values of NMDA toxicity if the actions of L-AP<sub>4</sub> and (-)-PHCCC were additive. Note that (-)-PHCCC and L-AP<sub>4</sub> (at least at 10 or 100 μM) acted synergistically/more than additive in protecting against NMDA toxicity (Table 1).

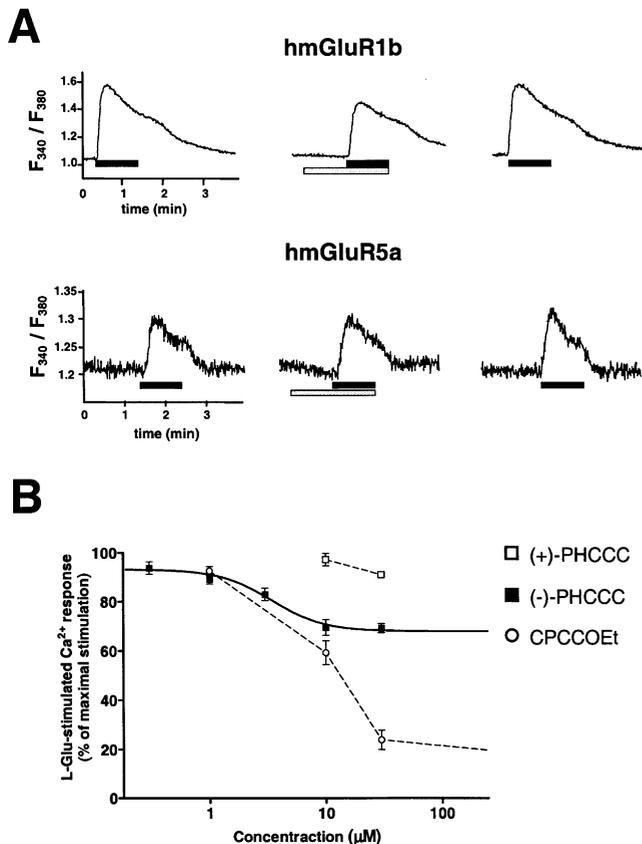


Fig. 6. Effect of (-)-PHCCC on group-I mGluRs. (A) Changes in the cytoplasmic calcium ion concentrations,  $[Ca^{2+}]_i$ , in COS-1 cells transiently expressing cloned hmGluR1b or hmGluR5a receptors. An increase in the fluorescent response ratio  $F_{340}/F_{380}$  corresponds to an elevation in  $[Ca^{2+}]_i$ . Three consecutive calcium measurements per receptor-subtype are shown: application of L-glutamate alone, followed by co-application of L-glutamate and (-)-PHCCC and again, following wash-out, application of L-glutamate alone. The duration of the applications is indicated by bars (black: L-glutamate; grey: 30  $\mu M$  (-)-PHCCC). 1000  $\mu M$  and 5  $\mu M$  of L-glutamate have been used in case of hmGluR1b and hmGluR5a, respectively. (B) Concentration-response curves for inhibition of L-glutamate-induced  $[Ca^{2+}]_i$  by CPCCOEt (non-competitive mGluR1 antagonist), (+)-PHCCC, and (-)-PHCCC in cells transiently expressing hmGluR1b. Results are normalized as a percentage of stimulation observed with 1000  $\mu M$  L-glutamate (set to 100%). Between successive recordings with different concentration of CPCCOEt, (+)-PHCCC and (-)-PHCCC, an interval of at least 10 min was allowed for wash-out and recovery. Measurements were performed at least three times in separate transfection experiments; means with S.E.M. are shown.

The neuroprotective activity of (-)-PHCCC was also tested in mixed cultures of cortical cells exposed to 25  $\mu M$   $\beta$ -AP<sub>(25-35)</sub> for 48 hours. We performed all experiments in the presence of 10  $\mu M$  DNQX and 10  $\mu M$  MK-801 to limit the excitotoxic component mediated by the endogenous glutamate. This model is known to be sensitive to neuroprotection by group-III mGluR agonists (Copani et al., 1995; Gasparini et al., 1999a; present data). (-)-PHCCC was neuroprotective against  $\beta$ -AP toxicity at concentrations lower than those required for

protection against NMDA toxicity (Fig. 9A). Neuroprotection was stereoselective (Fig. 9A), was antagonized by MSOP (Fig. 9B), and was clearly synergic with that produced by L-AP4 (Fig. 9C).

#### 4. Discussion

Activation of group-II and group-III mGluRs is neuroprotective in a variety of experimental models of acute or chronic neurodegenerative disorders (reviewed by Bruno et al., 2001 and Flor et al., 2002). While neuroprotection by group-II mGluRs is mediated—at least in part—by a mechanism of glial-neuronal interaction (Bruno et al., 1997; 1998; Kingston et al., 1999; D’Onofrio et al., 2001; Matarredona et al., 2001; Venero et al., 2002), neuroprotection by group-III receptors does not involve a glial component (Bruno et al., 1997; Copani et al., 1995) and mainly depends on the inhibition of glutamate release (Bruno et al., 2000). The use of the group-III selective agonist PPG, combined with mGluR4 knock-out mice led to the conclusion that activation of mGluR4 is highly protective against neuronal death induced by excitotoxins or  $\beta$ -amyloid peptide, even if a role for mGluR7 or -8 cannot be totally excluded (Gasparini et al., 1999a; Bruno et al., 2000).

Although mGluR4 emerges as a promising drug target, the pharmacological use of agonists is limited by a number of factors, including competition with endogenous glutamate and induction of adaptation mechanisms at receptor level. Electron microscopy studies have shown that mGluR4 and -7 are located in the central portion of presynaptic terminals, in close vicinity to the active zone of neurotransmitter release (Shigemoto et al., 1997). Hence, it is likely that mGluR4 is easily saturated by the endogenous glutamate, particularly under conditions that are associated with an enhanced glutamate release, such as epilepsy, brain ischemia or head trauma (Bruno et al., 2001). In addition, agonist activation may result in mGluR4 receptor desensitization (De Blasi et al., 2001; Iacovelli et al., personal communication), a process possibly leading to development of pharmacodynamic tolerance. The identification of positive allosteric modulators for mGluR4 may offer a strategy to overcome these limitations. Allosteric modulators of mGluRs were first identified with the serendipitous finding that the compound 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate (CPCCOEt) acts as a selective mGluR1 antagonist (Annoura et al., 1996), although it does not interact with the glutamate binding site at the amino-terminal domain of the receptor (Litschig et al., 1999). 2-Methyl-6-(phenylethynyl)pyridine (MPEP), and its precursors, SIB-1893 and SIB-1757, act as non-competitive mGluR5 antagonists interacting in the transmembrane domain of the receptor (Gasparini et al., 1999b; Varney et al., 1999; Pagano et al., 2000). A new and yet

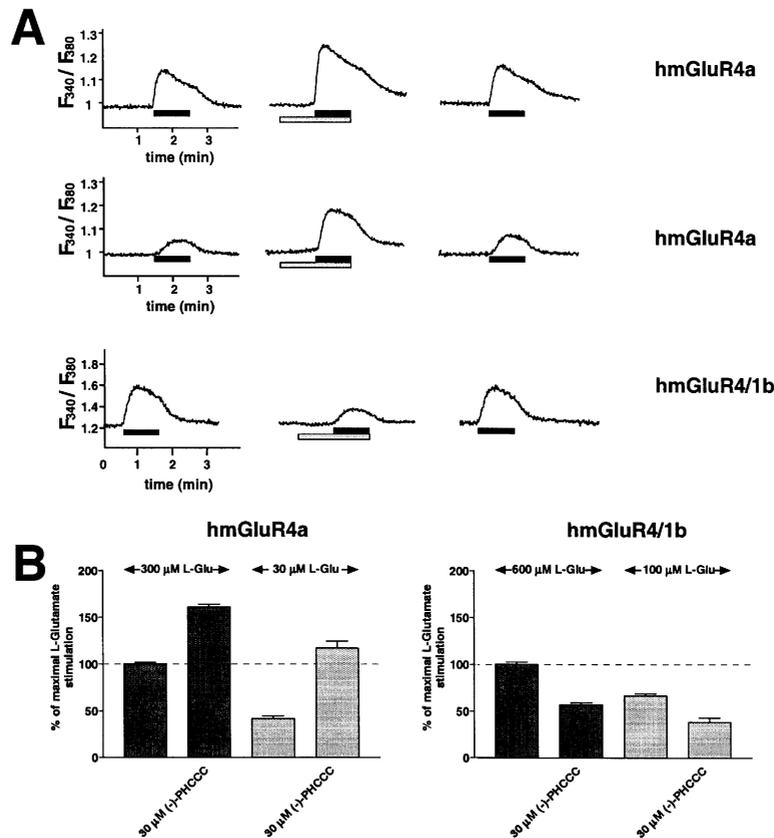


Fig. 7. (A) Influence of (-)-PHCCC on cytoplasmic calcium ion concentrations,  $[Ca^{2+}]_i$ , in COS-1 cells transiently expressing cloned hmGluR4a, co-transfected with the promiscuous G-protein  $G\alpha_{16}$ , or the hmGluR4a/1b chimera. An increase in the fluorescent intensity ratio  $F_{340}/F_{380}$  corresponds to an elevation in  $[Ca^{2+}]_i$ . Per row, three consecutive calcium measurements are shown: application of L-glutamate alone, co-application of L-glutamate and (-)-PHCCC and a final application of L-glutamate alone. The duration of the applications is indicated with bars (black: L-glutamate; grey: 30  $\mu$ M (-)-PHCCC). In the upper row, (hmGluR4a) an L-glutamate concentration of 300  $\mu$ M was used; in the middle row, (also hmGluR4a) 30  $\mu$ M was used; and in the lower row, (hmGluR4a/1b) 600  $\mu$ M L-glutamate has been applied. (B) Quantification and statistics of the experiments shown above. Each bar represents calcium signal amplitudes (mean values  $\pm$  S.E.M.) of at least eight measurements of two independent transfections. The data was normalized: responses of 300  $\mu$ M L-glutamate at hmGluR4a and 600  $\mu$ M L-glutamate in case of hmGluR4a/1b were taken as control and set to 100%.

unpublished study shows that SIB-1893 and to a lesser extent MPEP (at high concentrations of 10  $\mu$ M and 50  $\mu$ M, respectively) enhance activation of mGluR4, but not mGluR2 (Mathiesen et al., 2003). Knoflach et al. (2001) identified a series of compounds that enhance responses mediated by mGluR1. These compounds, of which Ro 67-7476, Ro 01-6128 and Ro 67-4853 are the prototypes, act as positive allosteric modulators of mGluR1, interacting with a site that only partially overlaps with the CPCCOEt binding site (Knoflach et al., 2001). This was followed by the design of positive allosteric modulators of mGluR2 by Eli Lilly laboratories (Baez et al., 2002; Britton et al., 2002; Johnson et al., 2002). The use of compound LY508869 as a probe led to the identification of transmembrane regions III and V, and, particularly, of the Asn735 residue of TM5 as the critical site for the enhancement of mGluR2 activity (Baez et al., 2002). Here, we have identified with the compound (-)-PHCCC the first positive allosteric modulator selective for a group-III mGluR—namely mGluR4. PHCCC is a

close structural analogue of CPCCOEt, which is likely to account for the partial inhibition of mGluR1-mediated responses initially found by Annoura et al. (1996) and confirmed by ourselves. However, the importance of this component in the overall pharmacology of (-)-PHCCC may be little because, at least in the neuroprotection assay, the effect of (-)-PHCCC was additive with that produced by the conventional mGluR1 antagonist CPCCOEt and because neuroprotection by PHCCC was antagonized by the group-III mGluR antagonist MSOP.

The enhancing effect of (-)-PHCCC was selective for the mGluR4 subtype, as shown by the assessment of  $GTP\gamma^{35S}$  binding and signal transduction of mGluRs (inhibition of cAMP formation and elevation of calcium responses with a promiscuous G protein). Under all circumstances, (-)-PHCCC was highly effective in potentiating mGluR4 responses induced by the agonists, L-AP4 or L-glutamate, and was nearly devoid of activity when applied alone, with the exception of a partial increase in  $GTP\gamma^{35S}$  binding on hmGluR4a expressing

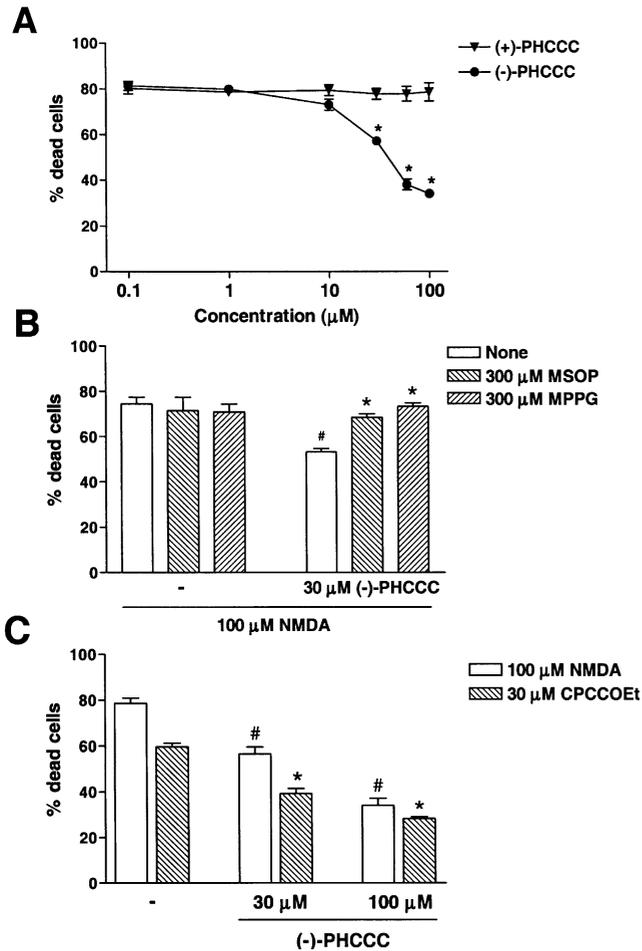


Fig. 8. Protection of cultured cortical neurons against NMDA-induced neuronal degeneration by (-)-PHCCC. (A) Neuronal degeneration in mixed cortical cultures was induced by 100 μM NMDA in the presence of the (-) and (+) isomers of PHCCC, and was assessed by trypan blue staining. Values are mean ± S.E.M. of 12 individual determinations. \**p* < 0.05 (one-way ANOVA + Fisher's PLSD) vs. NMDA alone. (B) The group-III mGluR antagonists, MSOP and MPPG, reverse the neuroprotective effect of (-)-PHCCC. Values are mean ± S.E.M. of 12 individual determinations. #*p* < 0.05 vs. NMDA alone; \**p* < 0.05 vs. NMDA + (-)-PHCCC (one-way ANOVA + Fisher's PLSD). (C) The additive effect of (-)-PHCCC and CPCCOEt in evoking neuroprotection suggests two different sites of action. Values are mean ± S.E.M. of 12 individual determinations. ##*p* < 0.05 (one-way ANOVA + Fisher's PLSD) vs. the corresponding values obtained in the absence of (-)-PHCCC (# is vs. NMDA alone and \* is vs. NMDA + CPCCOEt).

membranes. The use of hmGluR4/1b chimeras clearly showed that the transmembrane regions of the receptor are critical for the action of (-)-PHCCC, as already shown for CPCCOEt and other non-competitive ligands of mGluRs (see above). Therefore, (-)-PHCCC is likely to act via an allosteric site. Interestingly, (-)-PHCCC increases L-glutamate site ligand potency and intrinsic efficacy. Such dual effects of allosteric compounds are taken into account in a recently described extension of the two-state model of receptor activation (Hall, 2000; Parmentier et al., 2002). Accordingly, an enhancing

Table 1  
Combined effect of L-AP4 and (-)-PHCCC against NMDA toxicity

Treatment	% dead cells	+ (-)-PHCCC, 30 μM
NMDA, 100 μM	78 ± 1.3	56 ± 1.5
+L-AP4, 1 μM	72 ± 1.3	53 ± 0.94* (52)
+L-AP4, 10 μM	52 ± 1.2	32 ± 1.6* (37)
+L-AP4, 100 μM	39 ± 1.1	21 ± 0.78* (28)
		+ (-)-PHCCC, 10 μM
NMDA, 100 μM	73 ± 1.01	71 ± 1.5
+L-AP4, 10 μM	46 ± 0.94	38 ± 0.87* (45)

Values are expressed as % of dead cells and were calculated from eight individual determinations. The predicted values of a possible additive effect between L-AP4 and (-)-PHCCC are shown in parenthesis. \**p* < 0.05 (one-way ANOVA + Fisher's PLSD) vs. NMDA + L-AP4.

effect on the maximal response accompanied by a left-shift in the orthosteric agonist's concentration-response-curve is indicative of positive activation cooperativity ( $\delta > 1$ ; see Hall, 2000) between the allosteric and the orthosteric ligand. The allosteric two-state model of Hall (2000) also predicts intrinsic activation of a receptor with the allosteric ligand (agonist-like effects), which is consistent with our observation that (-)-PHCCC slightly increases GTP $\gamma$ [<sup>35</sup>S] binding in the absence of L-glutamate site ligands. Presently, we cannot know whether a certain level of constitutive, non ligand-induced, mGluR4 activation is required for (-)-PHCCC's partial agonist-like action.

To assess the effect of PHCCC at native mGluR4, we examined neurotoxicity in mixed cultures of mouse cortical cells challenged with NMDA or  $\beta$ AP<sub>(25-35)</sub>. In these cultures, the protective activity of group-III mGluR agonists against NMDA toxicity is clearly mediated by mGluR4, as shown by studies carried out with mGluR4 knock-out mice (Bruno et al., 2000). When cultures are switched from the growing medium into the HEPES-buffered solution used for toxicity experiments, extracellular glutamate concentrations are about 50–60 nM, but they rise to 150 nM during the 10 min pulse with NMDA (Bruno et al., 2000). These glutamate levels do not saturate group-III mGluRs (because cultures respond to L-AP4 or other agonists), but they are probably sufficient to partially activate native mGluR4 because NMDA toxicity is amplified by the group-III mGluR antagonist MPPG (Bruno et al., 2000). (-)-PHCCC applied alone protected cultured cortical neurons against NMDA toxicity. This effect was reversed by the competitive group-III antagonists MPPG and MSOP (Thomas et al., 1996), suggesting that the drug amplifies the endogenous activation of mGluR4. We could also show a synergism between (-)-PHCCC and L-AP4, although experiments were complicated by the substantial neuroprotection produced by either drug alone. (-)-PHCCC was also neuroprotective against  $\beta$ -amyloid tox-

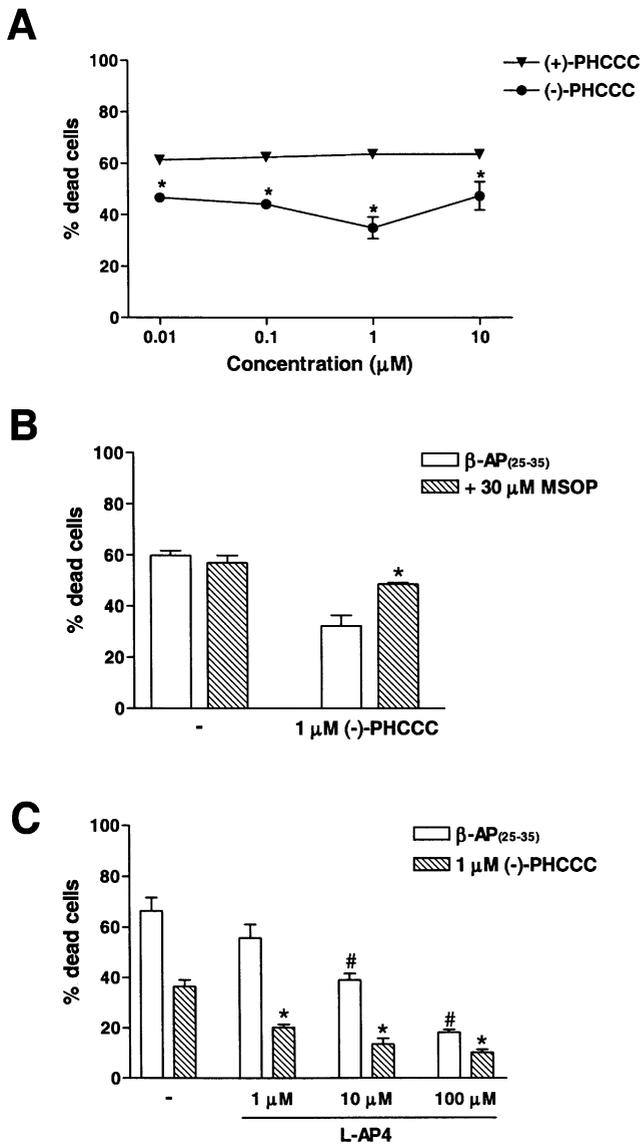


Fig. 9. Protection of cultured cortical neurons against  $\beta$ -AP-induced neuronal degeneration by (-)-PHCCC. (A) Concentration-dependent protection by (-)-PHCCC against  $\beta$ -AP-induced neurotoxicity. Neuronal degeneration was induced in mixed cortical cultures by a 48-exposure to 25  $\mu$ M  $\beta$ -AP in the presence of 10  $\mu$ M MK-801 and 10  $\mu$ M DNQX, and assessed by trypan blue staining. \* $p < 0.05$  (one-way ANOVA + Fisher's PLSD), if compared to  $\beta$ -AP alone. (B) Addition of MSOP (300  $\mu$ M) attenuates the protective effect of (-)-PHCCC against  $\beta$ -AP neurotoxicity. Values are means  $\pm$  S.E.M. from eight individual determinations. \* $p < 0.05$  (Student's *t*-test) vs. (-)-PHCCC alone. (C) Synergistic neuroprotection of L-AP4 and (-)-PHCCC against  $\beta$ -AP neurotoxicity. Values are means  $\pm$  S.E.M. from eight individual determinations. # $p < 0.05$  (one-way ANOVA + Fisher's PLSD) vs.  $\beta$ -AP alone; \* $p < 0.05$  vs.  $\beta$ -AP + (-)-PHCCC.

icity under conditions in which the endogenous excitotoxic component was minimized by the presence of MK-801 and DNQX. Remarkably, (-)-PHCCC showed a greater potency in protecting against  $\beta$ -amyloid toxicity than against NMDA toxicity, although the pharmacological profile of the response (antagonism by MSOP and synergism with L-AP4) was similar between the two

models. The most likely explanation is that mGluR4 activation is more efficient in protecting against a 'milder' neuronal death induced by  $\beta$ -amyloid in the presence of NMDA/AMPA receptor antagonists (apoptosis followed by secondary necrosis) than against the primary necrotic death induced by high concentrations of NMDA.

Group-III mGluRs have emerged as promising targets for drugs of potential use in the treatment of disorders, in which neurodegeneration includes an excitotoxic component (such as stroke, hypoglycaemic coma, brain trauma, Huntington's disease and status epilepticus) for their ability to inhibit glutamate release and to reduce NMDA responses via a postsynaptic mechanism (Bruno et al., 1996, 2000; Gasparini et al., 1999a; Lafon-Cazal et al., 1999; Martin et al., 1997; Maiese et al., 1995, 2000; Henrich-Noack et al., 2000; Sabelhaus et al., 2000). Activation of mGluR4 in particular has been found to protect cultured neurons against apoptosis by trophic deprivation (a process that contributes to the overall neuronal death in neurodegenerative disorders) through the stimulation of the phosphatidylinositol-3-kinase pathway (D'Mello et al., 1997; Iacovelli et al., 2002). In addition, mGluR4 has been implicated in the control of epileptic seizures because it is up-regulated in surgical specimen of patients with temporal lobe epilepsy (Lie et al., 2000), and is encoded by a gene that is found in a susceptible locus for juvenile myoclonic epilepsy (Wong et al., 2001). The recent evidence that glial mGluR4 inhibits the production of the chemokine RANTES (Besong et al., 2002) extends the involvement of these receptors to the control of neuroinflammatory disorders of the CNS. All these pathological conditions are possible therapeutic targets for positive allosteric modulators, such as (-)-PHCCC, which can exert their therapeutic efficacy even if the ambient glutamate saturates mGluR4. PHCCC can be a template for the design of novel drugs that are systemically active and lack interaction with mGluR1, although this property may further contribute to neuroprotection (Battaglia et al., 2001; Pellegrini-Giampietro et al., 1999a,b; Strasser et al., 1998; Bruno et al., 1999; Orlando et al., 2001).

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