

# Selective Activation of mGlu4 Metabotropic Glutamate Receptors Is Protective against Excitotoxic Neuronal Death

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Activation of group III metabotropic glutamate receptors (mGluR4, mGluR6, mGluR7, and mGluR8) has been established to be neuroprotective *in vitro* and *in vivo*. To disclose the identity of the receptor subtype(s) that exert(s) the protective effect, we have used group III agonists in combination with mGluR4 subtype-deficient mice ( $-/-$ ). In cortical cultures prepared from wild-type ( $+/+$ ) mice and exposed to a toxic pulse of NMDA, the selective group III agonist (+)-4-phosphonophenylglycine [(+)-PPG] reversed excitotoxicity with an  $EC_{50}$  value of 4.9  $\mu$ M, whereas its enantiomer ( $-$ )-PPG was inactive. This correlated closely with the potency of (+)-PPG in activating recombinant mGluR4a. In cortical neurons from  $-/-$  mice, (+)-PPG showed no protection against the NMDA insult up to 300  $\mu$ M, whereas group I/II mGluR ligands still retained their protective activity. Classical group III agonists (L-2-amino-4-phosphonobutyrate and L-serine-O-phosphate) were also substantially neuroprotective against NMDA toxicity in  $+/+$  and heterozygous ( $+/-$ ) cul-

tures but were inactive in  $-/-$  cultures. Interestingly,  $-/-$  cultures were more vulnerable to low concentrations of NMDA and showed higher extracellular glutamate levels compared with  $+/+$  cultures.

We have also examined neurodegeneration induced by intrastriatal infusion of NMDA in wild-type or mGluR4-deficient mice. Low doses of (*R,S*)-PPG (10 nmol/0.5  $\mu$ l) substantially reduced NMDA toxicity in  $+/+$  mice but were ineffective in  $-/-$  mice. Higher doses of (*R,S*)-PPG were neuroprotective in both strains of animals. Finally, microdialysis studies showed that intrastriatal infusion of NMDA increased extracellular glutamate levels to a greater extent in  $-/-$  than in  $+/+$  mice, supporting the hypothesis that the mGluR4 subtype is necessary for the maintenance of the homeostasis of extracellular glutamate levels.

**Key words:** neurodegeneration; knock-out mice; cortical cultures; metabotropic glutamate receptors; gene targeting; excitotoxicity

Metabotropic glutamate receptors form a family of currently eight subtypes (mGluR1–GluR8), subdivided into three groups (I–III) on the basis of their amino acid sequence identities, pharmacological profiles, and signal transduction pathways (Nakanishi, 1994; Conn and Pin, 1997). Group III includes mGluR4, mGluR6, mGluR7, and mGluR8 subtypes, which are negatively coupled to adenylate cyclase in heterologous expression systems. In the mammalian brain, mGluR4, mGluR7, and mGluR8 are presynaptically localized and are thought to mediate presynaptic depression of glutamatergic synaptic potentials, most likely via inhibition of voltage-gated calcium entry and regulation of glutamate release (Trombley and Westbrook, 1992; Conn and Pin, 1997; Shigemoto et al., 1997). In contrast, mGluR6 appears to be exclusively expressed in retinal ON bipolar cells in which it couples to a cGMP-phosphodiesterase and amplifies visual transmission (Nakanishi, 1994). L-AP-4, L-serine-O-phosphate (L-SOP), (*R,S*)-4-phosphonophenylglycine [(*R,S*)-PPG], and close analogs are the only selective agonists known for group III mGluRs, with low micromolar potency ( $EC_{50}$  values of 0.1–7  $\mu$ M) at mGluR4, mGluR6, and mGluR8; mGluR7 can only be activated at concentrations higher than 100  $\mu$ M (Okamoto et al., 1994; Johansen et al., 1995; Conn and Pin, 1997; Flor et al., 1997; Wu et al., 1998; Gasparini et al., 1999a). Selective activation of group III mGluRs results in neuroprotection *in vitro*: agonists such as L-AP-4, L-SOP, and (*R,S*)-PPG promote survival of rat cerebellar granule cells and protect cultured cortical and cerebellar neurons against toxic insults, such as prolonged  $\beta$ -amyloid peptide exposure, transient ionotropic glutamate recep-

tor activation, or mechanical damage (Graham and Burgoyne, 1994; Copani et al., 1995; Bruno et al., 1996; Faden et al., 1997; Gasparini et al., 1999a). In addition to those *in vitro* studies, the group III agonist (*R,S*)-PPG was recently found neuroprotective and anticonvulsive *in vivo* (Gasparini et al., 1999a). However, L-AP-4, L-SOP, and (*R,S*)-PPG are not receptor subtype-specific drugs, and they share properties that cannot always be reconciled with the activation of group III mGluRs, such as the noncompetitive inhibition of excitatory amino acid-induced polyphosphoinositide hydrolysis in brain tissue (Nicoletti et al., 1986; Schoepp et al., 1990) and the binding to the  $Ca^{2+}/Cl^{-}$ -dependent glutamate transporter (Fagg et al., 1982; Gasparini et al., 1999a). Because of that, some concern remains on the role of group III mGluRs in neuroprotection.

Here we attempt to disclose the contribution of individual receptor subtypes to neuroprotection induced by group III agonists. Therefore, we used various group III agonists, including the two purified enantiomers of (*R,S*)-PPG [(+)-PPG and ( $-$ )-PPG], and we compared agonist activity at cloned receptors with neuroprotective effects in cortical cultures, which were prepared from wild-type ( $+/+$ ), heterozygous ( $+/-$ ), and mGluR4 subtype-deficient ( $-/-$ ) mice. We have also performed *in vivo* studies by injecting NMDA into the caudate nucleus of  $+/+$  or  $-/-$  mice.

## MATERIALS AND METHODS

**Chemical synthesis of (*R,S*)-PPG, (+)-PPG and ( $-$ )-PPG.** (*R,S*)-PPG was synthesized in four steps starting from 4-hydroxybenzaldehyde as described previously (Gasparini et al., 1999a). (+)- and ( $-$ )-PPG were purified from (*R,S*)-PPG as starting material; enantiomeric resolution was realized by chromatographic separation of the fully protected enantiomers. Details will be described elsewhere (F. Gasparini, manuscript in preparation).

**Breeding of wild-type (*CD-1*), heterozygous, and homozygous mGluR4-deficient mutant mice.** Two breeding pairs of homozygous mGluR4-

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deficient mice (Pekhltski et al., 1996) were shipped from the University of Toronto (Toronto, Canada) to Novartis Pharma AG (Basel, Switzerland), and mating procedures of homozygotes and CD-1 wild types were performed according to standard procedures. The genotype at the mGluR4 locus was confirmed by Southern blotting and multiple primer PCR performed on tail biopsy samples of parent mice (for a detailed description, see Pekhltski et al., 1996).

**Preparation of cultured cortical cells from wild-type, heterozygous, and homozygous mGluR4-deficient mice.** Mixed cultures of cortical cells containing both neurons and astrocytes were prepared from fetal mice (14–16 d of gestation), as described previously (Bruno et al., 1996, 1997), and were used 13–14 d after plating.

**Assessment of NMDA toxicity in culture.** Mixed cortical cultures were exposed to 100  $\mu\text{M}$  NMDA for 10 min at room temperature in a HEPES-buffered salt solution. After extensive washing, cultures were incubated for 22–24 hr at 37°C in MEM–Eagle's buffer (Life Technologies, Basel, Switzerland) supplemented with 25 mM NaHCO<sub>3</sub> and 21 mM glucose. Neuronal toxicity was examined by phase-contrast microscopy and quantitated after staining with trypan blue. Stained neurons were counted from three random fields per well. Lactate dehydrogenase (LDH) release into the medium was also measured either as described previously (Bruno et al., 1996) or by using a commercially available kit (Promega, Madison, WI).

**D-[<sup>3</sup>H]Aspartate uptake in cortical cultures.** Mixed cortical cultures derived from wild-type or mGluR4-deficient mice were washed twice in Locke's solution and exposed for 15 min to 60 nCi/well of D-[2,3-<sup>3</sup>H]aspartate (specific activity, 20 Ci/mmol; Amersham Pharmacia Biotech, Milano, Italy) in the absence or presence of group III mGluR agonists. At the end of the exposure, cultures were washed three times in Locke's solution and lysed in 0.5 M NaOH. The lysate was counted by scintillation spectrometry.

**Determination of extracellular L-glutamate levels.** Mixed cultures of cortical neurons were prepared, and excitotoxicity was performed as described above. Analysis of glutamate was performed by precolumn derivatization with *o*-phthalaldehyde and mercaptoethanol, followed by HPLC with fluorescence detection. Culture medium was collected at the end of the NMDA exposure (100  $\mu\text{M}$  in +/+ culture and 60  $\mu\text{M}$  in –/– culture) in the presence or absence of L-AP-4 (100  $\mu\text{M}$ ), (R,S)-PPG (100  $\mu\text{M}$ ), and (R,S)- $\alpha$ -methyl-4-phosphonophenylglycine (MPPG) (100  $\mu\text{M}$ ). One hundred microliter sample aliquots were diluted with 0.1 N HCl and mixed with equal volumes of fluorescent reagent. The mixture was kept at room temperature for 1 min to derivatize the sample before being injected into the column by a 200  $\mu\text{l}$  loop. The system used an autosampler 507 (Beckman Instruments, Fullerton, CA), a programmable solvent module 126 (Beckman Instruments), an analytical reverse phase C-18 column at 30°C (Ultrasphere ODS 3  $\mu\text{m}$  Spherical, 80 Å pore, 2  $\times$  250 mm; Beckman Instruments), an RF-551 spectrofluorimetric detector (Shimadzu, Tokyo, Japan), and a computer running a Gold Nouveau software (Beckman Instruments). The excitation and emission wavelengths were set at 360 and 450 nm, respectively. The mobile phase consisted of (A) 50 mM sodium phosphate, pH 7.2, containing 10% methanol and (B) 50 mM sodium phosphate, pH 7.2, containing 70% methanol, at a flow rate of 0.3 ml/min. Both buffers were filtered through a 0.45  $\mu\text{m}$  filter and degassed under vacuum for 5 min. Gradient elution consisted of 98% A and 2% B initially for 16 min, was then increased to 98% B over 1 min, maintained for 12 min to elute other substances, and then returned to the initial conditions before running the next sample. From peak areas, culture medium concentrations of glutamate were calculated by the use of external standards.

**Stable mammalian cell lines for cloned mGluR subtypes and pharmacological assay for cloned group III mGluRs.** Generation, culture, and pharmacological characterization of stable cell lines for human (h) mGluR4a, mGluR6, mGluR7b, and mGluR8a have been described previously (Flor et al., 1995b, 1996, 1997; Laurie et al., 1997; Gasparini et al., 1999). Measurements of cAMP accumulation was performed essentially as described previously (Flor et al., 1995a,b, 1997).

**Assessment of in vivo neuronal injury.** CD-1 wild types or homozygous mGluR4-deficient mice weighing 24–28 gm were used for all the experiments. Mice were injected, under ketamine (100 mg/kg, i.p.) plus xylazine (10 mg/kg, i.p.) anesthesia, with NMDA (50 nmol/0.5  $\mu\text{l}$ ) or NMDA (50 nmol/0.5  $\mu\text{l}$ ) plus (R,S)-PPG (10 or 100 nmol/0.5  $\mu\text{l}$ ) in a stereotaxic frame. The site of injection was the left anterior striatum with the following coordinates: 0.6 mm anterior to the bregma, 1.7 lateral from the midline, and 3.5 mm ventral from the surface of skull, according to the atlas of Franklin and Paxinos (1997). After surgery, mice were housed in separate cages in a temperature-controlled environment on a 12 hr light/dark cycle, with access to water and food *ad libitum*. The animals were allowed 7 d to develop an excitotoxic striatal neuronal death induced by NMDA. In animals injected with NMDA or NMDA plus (R,S)-PPG, neuronal injury was assessed by performing histological analysis. Serial frontal sections including the whole caudate nucleus were Nissl-stained and examined by phase-contrast microscopy.

**Microdialysis in freely moving animals.** CD-1 wild types or homozygous mGluR4-deficient mice weighing 24–28 gm were implanted with microdialysis intracerebral guides (CMA/7 Guide Cannula; CMA Microdialysis, Stockholm, Sweden), under ketamine (100 mg/kg, i.p.) plus xylazine (10 mg/kg, i.p.) anesthesia, in a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, CA). The site of implantation was the left striatum [coordinates: 0.6 mm anterior to the bregma, 1.7 mm lateral to the midline,

and 2.5–5.5 mm ventral from the surface of skull, according to the atlas of Franklin and Paxinos (1997)]. After surgery, mice were housed in separate cages in a temperature-controlled environment on a 12 hr light/dark cycle, with access to water and food *ad libitum*, and were allowed to recover for 4 d before the experiment. On the evening before the experiment, a probe was inserted into the intracerebral guide, after removing a dummy, and mice were transferred to a plastic bowl cage with a moving arm (CMA/120 System for Freely Moving Animals; CMA Microdialysis) with access to water and food *ad libitum*. Concentric vertical microdialysis probes 2 mm long and 0.24 mm in outer diameter having a cuprophane membrane with a molecular cutoff of 6000 Da (CMA/7 Microdialysis Probe; CMA Microdialysis) were used. The probes were perfused continuously with artificial CSF (ACSF), at a flow rate of 1.5  $\mu\text{l}/\text{min}$ , using a microinjection pump (Bioanalytical Systems Inc., Congleton, UK). The ACSF contained (in mM): 150 NaCl, 3 KCl, 1.7 CaCl<sub>2</sub>, and 0.9 MgCl<sub>2</sub>. This solution was not buffered, and the pH was typically 6.5. On the following morning, 30  $\mu\text{l}$  (20 min) consecutive perfusate sample fractions were continuously collected by a fraction collector (CMA/142 Microfraction Collector; CMA Microdialysis). After four sample fractions, used to determine the basal levels of glutamate, NMDA (37.5 mM) was perfused for 7 min. When needed, (R,S)-PPG (5 mM) was perfused for the 20 min preceding the NMDA infusion. Sample fractions of perfusate were collected for the next 2 hr. Analysis of glutamate was performed by HPLC with fluorescence detection.

**Statistics.** Significant differences were estimated using the two-tailed Dunnett's *t* test by comparing multiple test–drug groups with one control group (Dunnett, 1964). Values of  $2p < 0.05$  were considered statistically significant.

**Materials.** Tissue culture reagents were from Life Technologies and Sigma (Buchs, Switzerland). Forskolin, 3-isobutyl-1-methylxanthine, and L-SOP were obtained from Sigma. L-AP-4, NMDA, DNQX, and (+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine maleate (MK-801) were purchased from Tocris Cookson (Anawa Trading SA, Zürich, Switzerland). 2-Methyl-6-(phenylethynyl)-pyridine (MPEP) and 7-hydroxyimino-cyclopropan[b]chromen-1 $\alpha$ -carboxylic acid ethyl ester (CPCCOEt) were synthesized at Novartis Pharma AG (Gasparini et al., 1999b; Litschig et al., 1999). All other chemicals were of reagent grade and were obtained from Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), or Sigma.

## RESULTS

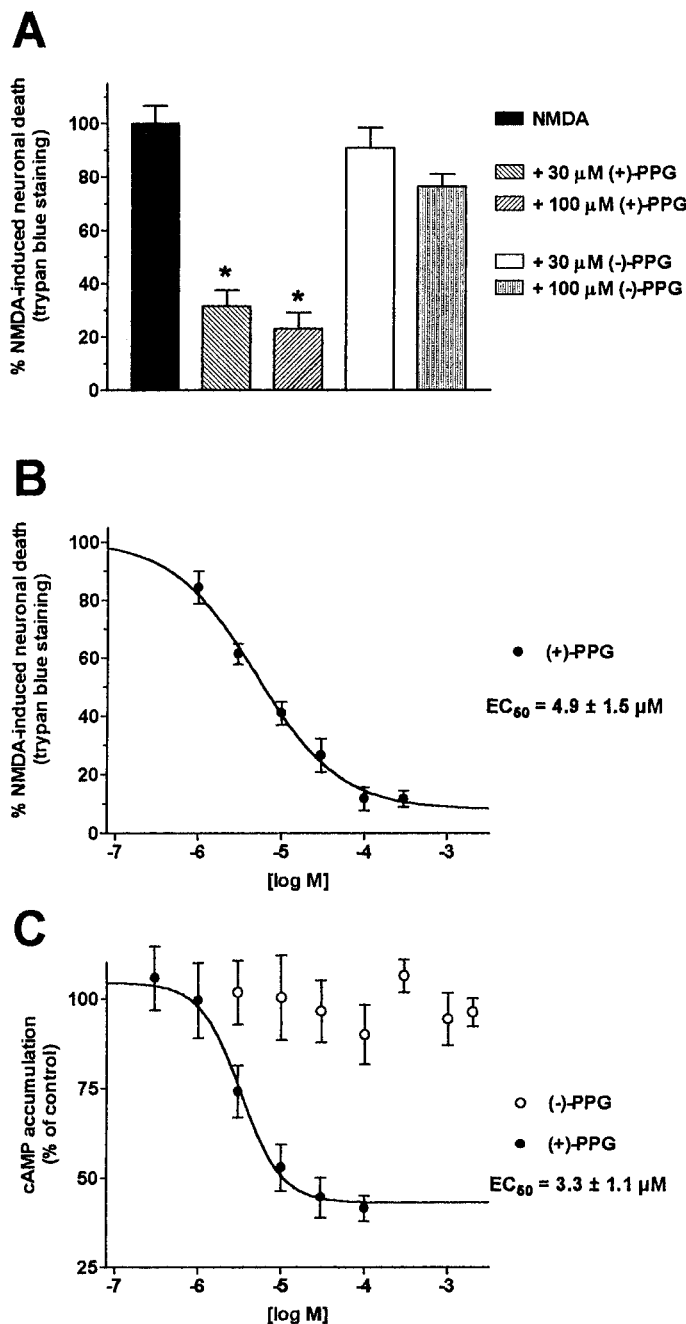
### *In vitro* studies

#### Neuroprotective activity of the active isomer of (R,S)-PPG in cultured cortical cells

(R,S)-PPG activates mGluR4, mGluR6, mGluR7, and mGluR8 and is neuroprotective against excitotoxic death (Gasparini et al., 1999). To provide a stronger pharmacological evidence that activation of group III mGluRs is neuroprotective, we prepared the pure (+) and (–) stereoisomers of PPG and compared their activities at heterologously expressed mGluR4 with their effects on NMDA toxicity. Toxicity was induced by exposing mixed cultures of cortical cells to a 10 min pulse with NMDA. As shown in Figure 1A, only the (+) isomer of PPG was neuroprotective, whereas the (–) isomer was inactive up to 100  $\mu\text{M}$ . The calculated EC<sub>50</sub> value for (+)-PPG-evoked neuroprotection was essentially identical to the EC<sub>50</sub> value of the compound to inhibit cAMP formation in Chinese hamster ovary (CHO) cells expressing recombinant human mGluR4a (Fig. 1B,C), but it was substantially different from the reported potency of (R,S)-PPG at hmGluR7b- or mGluR8a-expressing cells (Gasparini et al., 1999). (–)-PPG had no activity at recombinantly expressed mGluR4 up to 2000  $\mu\text{M}$  (Fig. 1C). These observations confirmed the protective role for group III mGluRs and suggested that mGluR4 rather than mGluR7 or mGluR8 mediates the protective activity of group III mGluR agonists in cultured cortical cells (Bruno et al., 1995; Gasparini et al., 1999).

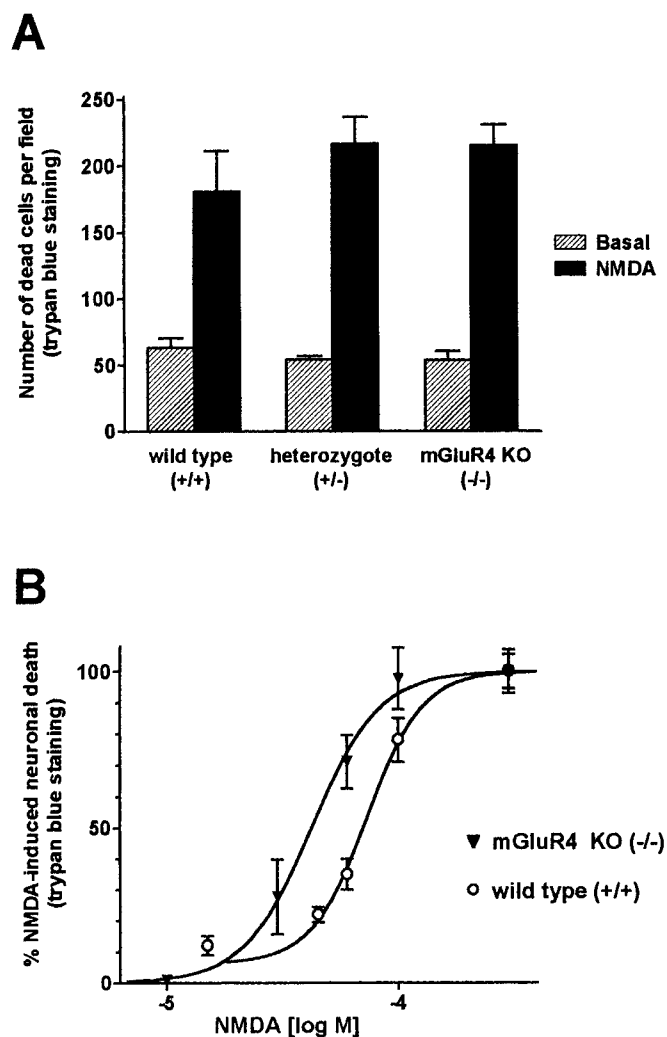
#### Lack of neuroprotective activity of group III mGluR agonists in cultures from mGluR4-deficient mice

To test the above hypothesis, we have prepared mixed cortical cultures from mGluR4-deficient (–/–) embryonic mice (Pekhltski et al., 1996). The lack of functional mGluR4 was confirmed by Southern blotting and multiple primer PCR performed on tail biopsy samples of parent mice (for a detailed description, see Pekhltski et al., 1996; data not shown). When microscopically examined at 13 d *in vitro* (i.e., when toxicity experiments are usually performed), cultures from –/– mice showed a normal morphology of neuronal cell bodies, although the neuritic connections between clusters of neu-



**Figure 1.** Neuroprotective activity of the two purified PPG enantiomers and correlation with agonist activity at mGluR4. *A*, Neuronal degeneration in mixed cortical cultures (CD-1 wild-type mice) was induced by 100  $\mu$ M NMDA (resulting in ~80% of maximal NMDA toxicity; set to 100%) and assessed by trypan blue staining. Statistically significant neuroprotection by (+)-PPG is indicated by asterisks ( $2p < 0.01$ ; Dunnett's *t* test;  $n = 6$ ; 2 independent experiments). *B*, Concentration–protection relationship of (+)-PPG against NMDA (100  $\mu$ M)-induced degeneration in mixed cortical cultures assessed by trypan blue staining. The value measured with 100  $\mu$ M NMDA alone was taken as 100%. Mean  $\pm$  SEM values from three independent experiments ( $n \geq 7$ ) are shown. *C*, Concentration–response curves for inhibition of forskolin (10  $\mu$ M)-stimulated cAMP accumulation by (+)- and (–)-PPG at CHO cells stably expressing human mGluR4a (Flor et al., 1995). Each data point represents the mean  $\pm$  SEM values from two independent experiments;  $n \geq 4$ . To determine the  $EC_{50}$  values, sigmoidal curves were fit using the GraphPad Prism program (GraphPad Software, Inc., San Diego, CA).

rons were less pronounced than in wild-type (+/+) cultures. Neuronal viability under basal conditions, as well as toxicity in response to high concentrations of NMDA (100  $\mu$ M), were not significantly different between cultures prepared from +/+, heterozygous (+/-),



**Figure 2.** NMDA-induced neuronal toxicity in mixed cortical cultures from wild-type (+/+), heterozygous (+/-), and mGluR4 knock-out (KO; -/-) mice. *A*, The number of dead cells per microscopic field (250 $\times$  magnification) was determined after trypan blue staining. Means  $\pm$  SEM from at least five independent determinations per group are shown; NMDA was applied at 100  $\mu$ M. Experimental procedures for obtaining the basal values differed from NMDA values only by omitting the toxin. *B*, Concentration–response relationships for NMDA-induced neurotoxicity in mixed cortical cultures from wild-type and mGluR4 knock-out mice. The maximal extent of toxicity, obtained with 300  $\mu$ M NMDA, was set to 100%; each experiment was performed three times;  $n \geq 8$ . Sigmoidal curves were fit using the GraphPad Prism program (GraphPad Software, Inc.).

or -/- mutant mice (Fig. 2*A*). However, the potency of NMDA in inducing neuronal toxicity was slightly higher in cultures from -/- than in cultures from +/+ mice ( $EC_{50}$  values of  $42 \pm 2$  and  $73 \pm 4$   $\mu$ M, respectively; means  $\pm$  SEM) (Fig. 2*B*), suggesting that cortical neurons from mGluR4-deficient mice are more vulnerable to excitotoxic damage. That endogenous activation of mGluR4 regulates neuronal vulnerability is strengthened by the observation that low/intermediate concentrations of NMDA (30 or 60  $\mu$ M) produced a greater extent of neuronal death in +/+ cultures when coincubated with MPPG (Table 1), a drug that preferentially antagonizes group III mGluRs (Jane et al., 1996).

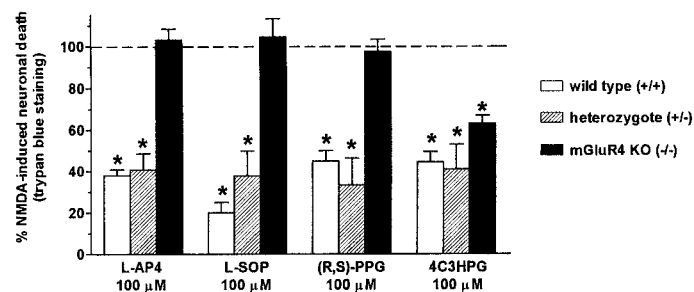
We examined the effect of various mGluR-selective compounds on NMDA toxicity in cortical cultures from the three different mouse genotypes. As shown in Figure 3, the group III agonists L-AP-4, L-SOP, and (*R,S*)-PPG (all at 100  $\mu$ M) were substantially neuroprotective against NMDA toxicity in +/+ or +/- cultures (55–80% protection in all cases), but they were all inactive in -/- cultures. The pure enantiomer (+)-PPG tested against NMDA toxicity in -/- cultures showed no significant neuroprotection up



**Table 1. NMDA toxicity in wild-type mouse cortical cultures in the absence (control) or presence of MPPG (100  $\mu$ M)**

Treatment	Number of dead cells (trypan blue staining)	
	Control	MPPG
Basal	24 $\pm$ 8	31 $\pm$ 3
NMDA (30 $\mu$ M)	77 $\pm$ 7	144 $\pm$ 13*
NMDA (60 $\mu$ M)	139 $\pm$ 5	177 $\pm$ 5*
NMDA (100 $\mu$ M)	185 $\pm$ 3	217 $\pm$ 9

Values are means  $\pm$  SEM of four to six independent determinations. \* indicates  $2p < 0.01$  versus the respective control values.



**Figure 3.** Protection against NMDA-induced neuronal toxicity in mixed cortical cultures from wild type (+/+), heterozygous (+/-), and mGluR4 knock-out (KO; -/-) mice. Neuronal degeneration in wild-type and heterozygous cultures was induced by 100  $\mu$ M NMDA. In knock-out cultures, 60  $\mu$ M NMDA was used (resulting in  $\sim$ 80% of maximal NMDA toxicity for each genotype). This level of neuronal death was set to 100%. Statistically significant neuroprotection by the different mGluR ligands is indicated by asterisks ( $2p < 0.01$ ; Dunnett's  $t$  test;  $n \geq 9$ ; 3 independent experiments).

to 300  $\mu$ M ( $2p > 0.05$ , Dunnett's  $t$  test,  $n = 6$  for each concentration tested). In contrast, (*S*)-4-carboxy-3-hydroxyphenylglycine (4C3HPG) (a mixed group I antagonist/group II mGluR agonist), showed significant neuroprotection in +/+, +/-, and -/- genotypes (Fig. 3). Similarly, the selective group I mGluR antagonists CPCCOEt (Litschig et al., 1999) and MPEP (Gasparini et al., 1999b), as well as the NMDA receptor antagonist MK-801, were all effective as neuroprotective agents in cultures from mGluR4-deficient mice (Table 2).

#### Determination of extracellular L-glutamate levels in cultured cortical cells

Searching for a mechanism responsible for the increased vulnerability of -/- cultures to NMDA toxicity, we measured extracellular glutamate levels because NMDA-stimulated glutamate release contributes to excitotoxic damage (Monyer et al., 1992) and presynaptic group III mGluRs are known to inhibit glutamate release. Interestingly, the basal levels of glutamate (i.e., levels measured in cultures incubated for 10 min in serum- and glutamine-free medium stock but without NMDA) were more than fourfold higher in -/- than in +/+ cultures. The toxic pulse with NMDA increased extracellular glutamate levels to the same extent (approximately twofold over the respective basal levels) in both cultures (Table 3). L-AP-4 or (*R,S*)-PPG (both at 100  $\mu$ M) coapplied with NMDA reduced extracellular glutamate levels in both +/+ and -/- cultures but did not normalize the difference in basal glutamate levels between the two cultures. Application of L-AP-4 or (*R,S*)-PPG with NMDA in -/- cultures never reduced extracellular glutamate below the levels found in +/+ cultures treated with NMDA alone (Table 3).

#### Measurement of D-[<sup>3</sup>H]aspartate uptake in cultures

To exclude that the difference in extracellular glutamate between cultures from wild-type and knock-out mice was attributable to changes in the activity of glutamate transporters, we have mea-

sured the uptake of D-[<sup>3</sup>H]aspartate (a common substrate for all known glutamate transporters) in culture. Time-dependent studies showed that D-[<sup>3</sup>H]aspartate was at plateau after 20 min, when 25–30% of radioactivity was incorporated into the cells. When assessed by HPLC, all intracellular radioactivity coeluted with authentic D,L-aspartate, confirming that D-[<sup>3</sup>H]aspartate was entirely taken up as such and was not metabolized intracellularly. Membrane binding of D-[<sup>3</sup>H]aspartate to NMDA receptors did not contribute to the overall cellular radioactivity, because the intracellular amount of D-[<sup>3</sup>H]aspartate did not differ when incubations were performed in the presence of 1 mM NMDA or 1 mM of the competitive NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid. In contrast, D-[<sup>3</sup>H]aspartate uptake was reduced by  $\sim$ 75% when cultures were incubated in the presence of the two inhibitors of the Na<sup>+</sup>-dependent glutamate transporter, L-trans-pyrrolidine-2,4-dicarboxylic acid and L-threo-3-hydroxyaspartic acid (both at 0.5 mM) (data not shown). As shown in Table 4, there was no substantial difference in D-[<sup>3</sup>H]aspartate uptake between cultures prepared from wild-type and mGluR4-deficient mice. Table 4 also shows that group III agonists did not affect D-[<sup>3</sup>H]aspartate uptake in both types of cultures.

#### In vivo studies

##### Assessment of NMDA toxicity in the striatum of wild-type and mGluR4-deficient mice

To examine the role for mGluR4 in regulating excitotoxic death *in vivo*, we assessed NMDA toxicity in the caudate nucleus, which receives extensive glutamatergic innervation from the cerebral cortex. A single unilateral infusion of low doses of NMDA (50 nmol/0.5  $\mu$ l) in the caudate nucleus produced the same extent of neuronal degeneration in wild-type and mGluR4-deficient mice. However, the two groups of animals substantially differed when NMDA was combined with 10 nmol of (*R,S*)-PPG. In knock-out mice, this dose of (*R,S*)-PPG did not produce any detectable neuroprotection, and the extension of the lesion was similar to that observed after infusion of NMDA alone (789  $\pm$  159 vs 835  $\pm$  122  $\mu$ m along the anteroposterior axis; means  $\pm$  SEM;  $n = 4$ ). In contrast, the extension of the lesion was markedly reduced in wild-type mice treated with NMDA plus 10 nmol of (*R,S*)-PPG (149  $\pm$  82  $\mu$ m) compared with mice treated with NMDA alone (802  $\pm$  79  $\mu$ m; means  $\pm$  SEM;  $n = 4$ ) (Fig. 4). At higher doses (100 nmol), (*R,S*)-PPG continued to be protective in +/+ mice (extension of the lesion, 286  $\pm$  78  $\mu$ m) but was also partially protective in -/- mice (extension, 470  $\pm$  35  $\mu$ m; means  $\pm$  SEM;  $n = 4$ ).

##### Determination of extracellular glutamate levels in freely moving wild-type or mGluR4-deficient mice

We have performed microdialysis studies in wild-type and mGluR4 knock-out mice to extend the analysis of glutamate release to an *in vivo* model. The extracellular fluid was collected from the left caudate nucleus both under basal conditions and after infusion of NMDA through the microdialysis probe. In contrast to what was observed in cultured cortical cells, the basal extracellular levels of glutamate did not differ between wild-type and mGluR4 knock-out mice. However, NMDA infusion led to a much greater increase in glutamate levels in -/- than in +/+ mice (more than 10-fold vs twofold to threefold, respectively) (Fig. 5). We have also tested the effect of (*R,S*)-PPG infused at concentrations of 5 mM in the microdialysis probe. At this concentration, (*R,S*)-PPG reduced NMDA-stimulated glutamate release in both +/+ and -/- mice (Fig. 5). We did not extend the study to further concentrations of (*R,S*)-PPG.

#### DISCUSSION

Metabotropic glutamate receptor subtypes mediate distinct, facilitatory (group I subtypes) or inhibitory (group II and group III subtypes) actions on acute or chronic neurodegenerative processes. Drugs interacting with mGluR subtypes are expected to influence both the induction and progression of neuronal degeneration without blocking the efficiency of fast excitatory synaptic transmission.

**Table 2. NMDA-induced neuronal death in the presence of different mGluR ligands and NMDA receptor antagonists applied to mixed cortical cultures prepared from mGluR4 knock-out (KO,  $-/-$ ) or wild-type ( $+/+$ ) mice**

Compound	(Read-out)	% of NMDA-induced neuronal death	
		mGluR4 KO ( $-/-$ )	Wild-type ( $+/+$ )
L-AP-4	(trypan blue staining)	103 $\pm$ 5	38 $\pm$ 3
	(LDH release)	94 $\pm$ 8	41 $\pm$ 5
<i>(R,S)</i> -PPG	(trypan blue staining)	98 $\pm$ 6	45 $\pm$ 5
	(LDH release)	82 $\pm$ 12	55 $\pm$ 5
4C3HPG	(trypan blue staining)	63 $\pm$ 4	45 $\pm$ 5
	(LDH release)	56 $\pm$ 9	36 $\pm$ 3
CPCCOEt	(LDH release)	64 $\pm$ 16	79 $\pm$ 5
MPEP	(LDH release)	5 $\pm$ 5	32 $\pm$ 6
MK-801	(trypan blue staining)	27 $\pm$ 4	12 $\pm$ 7
	(LDH release)	18 $\pm$ 8	15 $\pm$ 3

L-AP-4, *(R,S)*-PPG, and 4C3HPG were applied at a final concentration of 100  $\mu$ M and CPCCOEt, MPEP, and MK-801 at 30, 3, and 10  $\mu$ M, respectively. All compounds were coapplied with the NMDA pulse and were maintained in the medium for the following 24 hr. Values are means  $\pm$  SEM of six to nine independent determinations.

**Table 3. Extracellular glutamate levels in mGluR4 knock-out (KO,  $-/-$ ) and wild-type ( $+/+$ ) mice cultures treated with NMDA and/or group III mGluR agonists or antagonists**

Treatment	L-Glutamate (nM)	
	mGluR4 KO ( $-/-$ )	Wild-type ( $+/+$ )
Basal	267 $\pm$ 41	57 $\pm$ 5
L-AP-4 (100 $\mu$ M)	167 $\pm$ 18*	68 $\pm$ 3
<i>(R,S)</i> -PPG (100 $\mu$ M)	n.d.	63 $\pm$ 9
MPPG (100 $\mu$ M)	n.d.	93 $\pm$ 7*
NMDA (60 or 100 $\mu$ M)**	470 $\pm$ 34*	152 $\pm$ 21*
NMDA plus L-AP 4	210 $\pm$ 25#	59 $\pm$ 13#
NMDA plus <i>(R,S)</i> -PPG	150 $\pm$ 21#	73 $\pm$ 8#

Values are means  $\pm$  SEM of 6–13 independent determinations. \* indicates  $2p < 0.05$  versus the respective basal values. # indicates  $2p < 0.05$  versus the respective NMDA values; \*\* 60  $\mu$ M was used for  $-/-$  and 100  $\mu$ M for  $+/+$  cultures. n.d., Not determined.

**Table 4. Group III mGluR agonists do not differentially affect D-[ $^3$ H]aspartate uptake in mGluR4 knock-out (KO,  $-/-$ ) and wild-type ( $+/+$ ) mice cultures**

Treatment	Counts per minute/dish	
	mGluR4 KO ( $-/-$ )	Wild-type ( $+/+$ )
Basal	26970 $\pm$ 436	28734 $\pm$ 537
L-AP-4 (100 $\mu$ M)	27507 $\pm$ 294	27829 $\pm$ 542
<i>(R,S)</i> -PPG (10 $\mu$ M)	26447 $\pm$ 665	27981 $\pm$ 875
<i>(R,S)</i> -PPG (100 $\mu$ M)	27613 $\pm$ 604	27172 $\pm$ 465

Values are means  $\pm$  SEM of four independent determinations.

For these reasons, mGluR subtypes may be activated or inhibited without producing severe side effects typical for drugs interacting with ionotropic glutamate receptors (Nicoletti et al., 1996; Wood, 1998). The role of postsynaptic group I mGluRs in neurodegeneration is still controversial, although mixed mGluR1/5 receptor antagonists were reported to be protective in *in vitro* and *in vivo* models of excitotoxic or hypoxic–ischemic neuronal death (Strasser et al., 1998; Bruno et al., 1999; Pellegrini-Giampietro et al., 1999). In contrast, agonists of group II and group III mGluRs were found to be neuroprotective but through different mechanisms. Protection by group II mGluR agonists *in vitro* requires the presence of astrocytes, involves a novel form of glial–neuronal interaction, and may be mediated by the release of growth factors such as TGF- $\beta$  (Bruno et al., 1997, 1998). *In vivo*, LY354740 (Monn et al., 1997), a

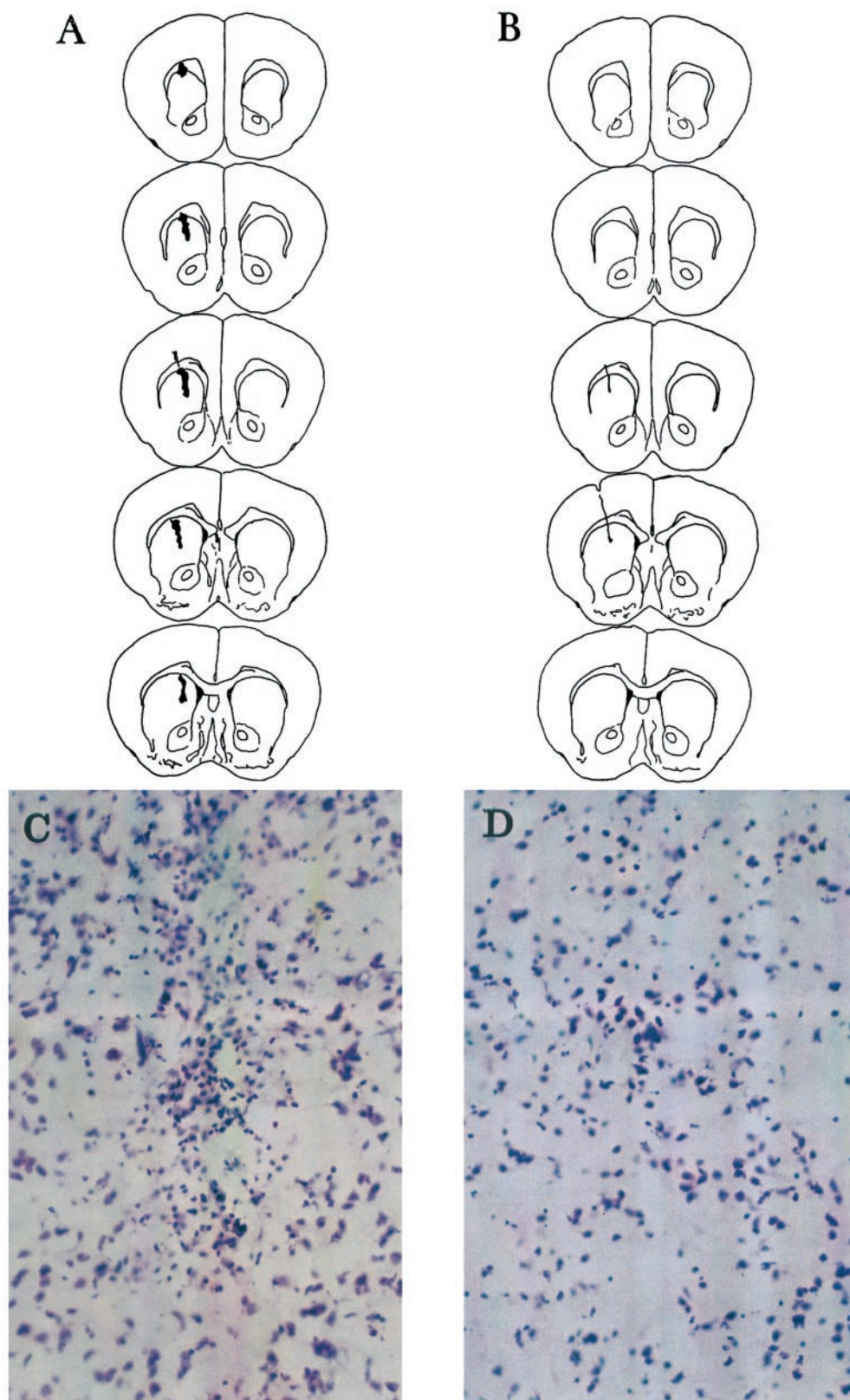
systemically active group II mGluR agonist, showed potential anti-parkinsonian properties (Konieczny et al., 1998).

Group III agonists are equally protective in mixed cortical cultures (containing astrocytes and neurons) and in cultures of pure neurons. Therefore, this neuroprotection is not expected to be mediated by astrocyte factors, but rather involves one or more receptor subtypes expressed on neuronal structures. Here, we have searched for the identity of the neuroprotective group III mGluR subtype using mixed cortical cultures. This model was particularly suitable for our study because cortical neurons of mixed cultures express all currently known group III mGluR subtypes (Faden et al., 1997). A possible role in neuroprotection for one or more group III mGluR subtype(s), most likely with low micromolar affinity for the known agonists, was initially suggested by the use of the novel phenylglycine derivative *(R,S)*-PPG (Gasparini et al., 1999a). We have separated the stereoisomers of *(R,S)*-PPG and found that all protective activity is harbored in the (+) isomer. (+)-PPG was neuroprotective against NMDA toxicity with an  $EC_{50}$  value of  $\sim$ 5  $\mu$ M. This value coincides with that found for the activation of recombinant mGluR4 but differs by at least 25-fold from the potency of *(R,S)*-PPG at mGluR7 and mGluR8 (Gasparini et al., 1999a). In the present study, we provide strong evidence for a critical role of mGluR4 in mediating neuroprotection via the use of mixed cortical cultures prepared from mGluR4 subtype-deficient mice ( $-/-$ ) in which all group III agonists (i.e., L-AP-4, *(R,S)*-PPG, and L-SOP) failed to protect against NMDA toxicity. This was in contrast to the protective effects of group III agonists in wild-type and heterozygous neurons and did not reflect a general refractoriness of  $-/-$  knock-out neurons to mechanisms of protection, because the group II agonist 4C3HPG and the group I antagonists CPCCOEt and MPEP (Gasparini et al., 1999b; Litschig et al., 1999) retained their protective activity in knock-out cultures.

We have extended the study to an *in vivo* model of excitotoxic degeneration by unilaterally injecting NMDA plus *(R,S)*-PPG into the caudate nucleus of wild-type or mGluR4-deficient mice. This brain region has been selected because it receives an extensive glutamatergic innervation from the cerebral cortex. Low doses of *(R,S)*-PPG (10 nmol), which may preferentially activate mGluR4 over mGluR7 receptors, were neuroprotective in  $+/+$  mice but were totally inactive in  $-/-$  mice. In contrast,  $-/-$  mice were partially sensitive to higher doses of *(R,S)*-PPG (100 nmol), which are expected to recruit mGluR7. The prominent role for mGluR4 in mediating agonist-induced neuroprotection is consistent with the evidence that this particular receptor subtype contributes substantially to the high-affinity binding of [ $^3$ H]L-AP-4 in many regions of mouse brain (Thomsen and Hampson, 1999).

Interestingly, cultures from mGluR4  $-/-$  mice could be effectively damaged by low concentrations of NMDA, which produced

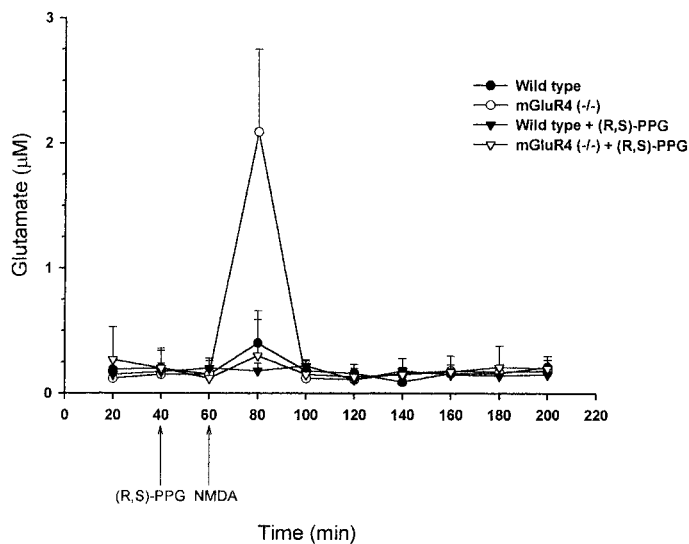




**Figure 4.** Serial sections across the extension of the caudate nucleus from a representative mGluR4 knock-out (*A*) or wild-type (*B*) mouse locally injected with NMDA (50 nmol) plus PPG (10 nmol). Necrotic areas are in black. Microphotographs at the injection site of the  $-/-$  and  $+/+$  mouse are shown in *C* and *D*, respectively. Note the absence of reactive gliosis in *D*. NMDA alone in  $+/+$  or  $-/-$  mice produced a lesion virtually identical to that shown in *A* (i.e., in  $-/-$  mice treated with NMDA plus 10 nmol PPG).

small effects in cultures from  $+/+$  mice. Searching for a reason responsible for the increased vulnerability of  $-/-$  cultures, we have focused on extracellular levels of glutamate because of the following reasons: (1) mGluR4 is known to be, at least primarily, presynaptically located and its activation may inhibit glutamate release (Conn and Pin, 1997; Shigemoto et al., 1997); (2) cultured cortical cells respond to a toxic NMDA pulse with an enhanced release of

glutamate, which in turn contributes to the development of excitotoxic neuronal death (Monyer et al., 1992; Bruno et al., 1995); and finally (3) inhibition of glutamate release is a validated mechanism for drugs of potential use in neurodegenerative disorders, such as riluzole (Doble, 1999). We have found a striking difference in the regulation of extracellular steady-state glutamate concentrations between  $+/+$  and mGluR4  $-/-$  cultures. Basal glutamate levels in



**Figure 5.** Extracellular levels of glutamate in the striatum of wild-type (+/+) and mGluR4-deficient (-/-) freely moving mice. Animals were perfused with NMDA (37.5 mM) in the presence or absence of (R,S)-PPG (5 mM). Values are mean  $\pm$  SEM of three animals.

-/- cultures were at least four times as high as levels in +/+ cultures and, in some experiments, they were even higher than in wild-type cultures exposed to NMDA. The higher glutamate levels in -/- cultures may be a direct consequence of the lack of mGluR4 because (1) no changes in glutamate transport (measured as D-[<sup>3</sup>H]aspartate uptake) were found between +/+ and -/- cultures, and (2) pharmacological inhibition of group III mGluRs by MPPG increased glutamate levels in +/+ cultures. In -/- cultures, (R,S)-PPG and L-AP-4 reduced both basal and NMDA-stimulated glutamate release, but only partially, leaving abnormally high levels of extracellular glutamate. This partial reduction may reflect the recruitment of mGluR7 or mGluR8 by group III agonists. mGluR8 is a better candidate because it accounts for most of the residual [<sup>3</sup>H]L-AP-4 binding in the brain of mGluR4 knock-out mice (Thomsen and Hampson, 1999). We speculate that one of the functions of mGluR4 is to maintain extracellular glutamate levels below a "toxic" threshold during synaptic activity. Elevated extracellular glutamate levels may contribute to NMDA toxicity by activating ancillary receptors, such as AMPA receptors or group I mGluRs (Table 2, neuroprotection by CPCCOEt and MPEP). In +/+ cultures, group III mGluR agonists might exert part of their protective activity by reducing NMDA-stimulated glutamate release, thus eliminating an amplifying component of NMDA toxicity. In -/- cultures, the higher basal levels of glutamate may well account for the increased vulnerability to NMDA, and the inability of agonists to reduce glutamate levels below a critical threshold (which in this model may be set at 150–160 nM) may contribute to explain the lack of neuroprotection. The assessment of extracellular glutamate levels in freely moving animals by microdialysis confirmed a critical role for mGluR4 in regulating extracellular glutamate levels. Accordingly, in mGluR4 -/- mice, intrastriatal NMDA infusion induced a greater increase in extracellular glutamate than in +/+ mice. In this particular model, basal glutamate levels did not significantly differ between the two strains of mice, perhaps because of compensatory mechanisms that might have occurred during the development of -/- mice. The reduction of glutamate release observed with high doses of (R,S)-PPG in microdialysis studies may again reflect the activation of mGluR7 or mGluR8. We decided not to titrate (R,S)-PPG to disclose a possible difference between the two strains of mice because the amount of (R,S)-PPG diffusing from the probe to the tissue cannot be accurately estimated. Comparative studies in mGluR4-, mGluR7-, and mGluR8-deficient mice (or studies in double knock-out mice) should be performed to determine the relative contribu-

tion of these receptor subtypes in neurodegeneration–neuroprotection and in the regulation of glutamate release. Although we favor the hypothesis that activation of mGlu4 receptors is neuroprotective by maintaining ambient glutamate concentrations below a critical threshold, other mechanisms that are only tangentially related to glutamate release could also be relevant. Similar to other group III mGluRs (Saugstad et al., 1996, 1997; Corti et al., 1998), mGluR4 receptors positively couple to inwardly rectifying K<sup>+</sup> channels in amphibian oocytes (Sharon et al., 1997). L-AP-4 can also activate K<sup>+</sup> channels in CNS neurons (Holmes et al., 1996; Dutar et al., 1999). Through this mechanism, activation of mGluR4 might affect neuronal sensitivity to excitotoxic damage independently of changes in glutamate release.

In conclusion, our results support the neuroprotective activity of group III mGluRs and indicate that, both in *in vitro* and *in vivo* models, neuroprotection is primarily mediated by mGluR4. This encourages studies aiming at synthesizing mGluR4-selective agonists, which may have a high chance of success in experimental models of acute and chronic neurodegenerative disorders.

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