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Activation of Metabotropic Glutamate Receptors Protects Cultured Neurons Against Apoptosis Induced by β-Amyloid Peptide

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SUMMARY

Prolonged exposure of cultured cortical cells or cultured cerebellar granule cells to the residue 25–35 fragment of β-amyloid peptide (βAP), $\beta AP_{(25-35)}$, induced neuronal apoptosis, as revealed by morphological analysis, fluorescent chromatin staining, and immunodetection of oligonucleosomes released from the nucleus into the cytoplasm. $\beta AP_{(25-35)}$-induced apoptosis was insensitive to ionotropic glutamate receptor antagonists but was substantially attenuated by the metabotropic glutamate receptor (mGluR) agonist (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid. The neuroprotective action of (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid was antagonized by (*RS*)-α-methyl-4-carboxyphenylglycine and was mimicked by (2*S*,1′*R*,2′*R*,3′*R*)-2-(2,3-dicarboxycyclopropyl)glycine (a selective agonist of mGluR2 and -3 subtypes) and by L-2-amino-4-phosphobutanoate and L-serine-*O*-phosphate (selective agonists of mGluR4, -6, and -7 subtypes). However, whereas all of these drugs behaved as neuroprotectants in cultured cortical cells, only L-2-amino-4-phosphobutanoate and L-serine-*O*-phosphate [and not (2*S*,1′*R*,2′*R*,3′*R*)-2-(2,3-dicarboxycyclopropyl)glycine] reduced $\beta AP_{(25-35)}$-induced apoptosis in cultured cerebellar granule cells. The neuroprotective activity of mGluR agonists may be related to their ability to inhibit membrane Ca^{2+} conductance, because drugs that block voltage-sensitive Ca^{2+} channels, such as nimodipine or Co^{2+}, could also attenuate $\beta AP_{(25-35)}$-induced apoptosis.

βAP is a 40–42-amino acid peptide that accumulates as insoluble extracellular deposits in amyloid plaques of Alzheimer's brain (1, 2). The evidence that βAP aggregates are toxic to cultured neurons (3–8) supports the hypothesis that formation of amyloid plaques is causally linked to the cytoskeletal destabilization and neuronal degeneration that occur in Alzheimer's disease (9, 10). βAP, or its active, residue 25–35 fragment (8) $\beta AP_{(25-35)}$, destabilizes intracellular Ca^{2+} homeostasis (7, 11), thus amplifying the toxicity of NMDA receptor agonists or other excitotoxins (11, 12). A possible synergism between βAP and endogenous excitotoxins in Alzheimer's brain helps to explain the early onset of degeneration in the hippocampus (13, 14), where neurons are densely innervated by glutamatergic fibers. βAP, however, can also induce degeneration of cultured neurons through a different process, which does not incorporate an excitotoxic component. This process develops within 24–48 hr and exhibits the typical morphological and biochemical features of apoptosis, including formation of membrane blebs, fragmentation and condensation of chromatin, and DNA cleavage into oligonucleosomes (15, 16). Although it is uncertain to what extent βAP-induced apoptosis occurs *in vivo*, this process may be relevant for the degeneration of scattered neurons in Alzheimer's brain (9, 13, 17). Hence, the identification of pharmacological agents that oppose βAP-induced apoptosis may furnish new tools for the experimental therapy of Alzheimer's disease. We have focused on mGluR agonists, which are known to protect cultured neurons against excitotoxic damage (18–21). The activity of mGluR agonists has been compared with that of the VSCC blockers nimodipine and Co^{2+} (22–24), which have been reported to attenuate βAP-induced toxicity in cultured neurons (25).

ABBREVIATIONS: βAP, β-amyloid peptide; VSCC, voltage-sensitive Ca^{2+} channel(s); L-SOP, L-serine-*O*-phosphate; ACPD, 1-aminocyclopentane-1,3-dicarboxylic acid; AP4, L-2-amino-4-phosphonobutanoate; MCPG, (*RS*)-α-methyl-4-carboxyphenylglycine; DNQX, 6,7-dinitroquinoxaline-2,3-dione; MK-801, 10,11-dihydro-5-methyl-5*H*-dibenzo[*a*,*c*]cyclohepten-5,10-imine; DCG-IV, (2*S*,1′*R*,2′*R*,3′*R*)-2-(2,3-dicarboxycyclopropyl)-glycine; L-CCG-I, L-2-carboxycyclopropylglycine; NMDA, *N*-methyl-D-aspartate; mGluR, metabotropic glutamate receptor; MS, medium stock; LDH, lactate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; GABA, γ-aminobutyric acid; DIV, days *in vitro*; ANOVA, analysis of variance; PLSD, protected least significant difference; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Experimental Procedures

Materials. βAP was purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). NMDA, $CoCl_2$, cycloheximide, and L-SOP were obtained from Sigma Chemical Co. (St. Louis, MO). (1*S*,3*R*)-ACPD, quisqualate, L-AP4, MCPG, and DNQX were purchased from Tocris Neuramin (Essex, UK). MK-801 was obtained from Research Biochemicals (Natick, MA). DCG-IV and L-CCG-I were synthesized and kindly provided by Dr. H. Shinozaki (Tokyo Metropolitan Institute for Medical Sciences, Tokyo, Japan) and Dr. R. Pellicciari (Institute of Pharmaceutical Chemistry, University of Perugia, Perugia, Italy), respectively. Nimodipine was a generous gift from Bayer S.p.A. (Wuppertal, Germany).

Cell culture. Mixed cortical cell cultures containing both neurons and astrocytes were prepared from fetal mice at 14–17 days of gestation, as described previously (26, 27). In brief, dissociated cortical cells were plated in 15-mm multiwell vessels (Falcon Primaria) on a layer of confluent glial cells (7–14 DIV), using, as a plating medium, Eagle's minimal essential medium (with Earle's salt, supplied glutamine-free; GIBCO) supplemented with 5% heat-inactivated horse serum, 5% fetal calf serum, 2 mM glutamine, and 21 mM glucose. After 3–5 DIV, proliferation of glial cells was halted by addition of 10 μM cytosine arabinoside. After an additional 3 days, cultures were shifted to a maintenance medium identical to the plating medium but lacking fetal calf serum (MS). Subsequent partial medium replacement was carried out twice each week. Cultures at 13–16 DIV were used.

Primary cultures of cerebellar granule cells were prepared from 8-day old rats, as described previously (28). In brief, dissociated cerebellar cells were suspended in basal Eagle's medium (GIBCO) containing 10% fetal calf serum, 2 mM glutamine, 0.05 mg/ml gentamicin, and 25 mM K^+ and were plated onto 35-mm Nunc Petri dishes that had been precoated with poly-L-lysine (10 μg/ml). Cytosine arabinoside (10 μM) was added to the cultures after 18 hr to inhibit proliferation of non-neuronal cells. Cultures at 7–9 DIV contained >90% granule cells, with about 3–5% GABAergic neurons and few glial and endothelial cells as contaminants (28, 29).

Experimental procedure and assessment of neuronal degeneration. $\beta AP_{(25\text{-}35)}$ was solubilized in sterile, doubly distilled water at an initial concentration of 2.5 mM and was stored frozen at −20°. Cortical cultures, shifted into MS, were exposed to βAP for different times (up to 48 hr) before the assessment of neuronal degeneration. mGluR agonists, VSCC blockers, or ionotropic glutamate receptor antagonists were all coapplied with βAP and maintained for the entire exposure time. To test the combined effect of βAP and an excitotoxic insult, we exposed cortical cultures to a submaximal concentration of $\beta AP_{(25\text{-}35)}$ (12.5 μM) for 24 hr. Cultures were then shifted into a HEPES-buffered salt solution containing 120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 20 mM HEPES, and 15 mM glucose, and NMDA (60 μM) was added for 10 min at room temperature. At the end of this incubation, cultures were returned to the MS and incubated for 20 hr before the assessment of neuronal degeneration. mGluR agonists were applied in combination with NMDA.

Cultured cerebellar granule cells at 5–6 DIV were shifted into a medium identical to the plating medium but lacking fetal calf serum. Three hours later, cultures were exposed to $\beta AP_{(25\text{-}35)}$, in the absence or presence of mGluR agonists and/or ionotropic glutamate receptor antagonists, for 48–72 hr. Neuronal degeneration was assessed by combining phase-contrast microscopy, fluorescent chromatin staining, photometric enzyme immunoassays for the determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes), and determination of the activity of LDH released from damaged neurons into the medium.

Fluorescent staining of nuclear chromatin. Cultured cells were fixed with methanol/acetic acid (3:1) for 30 min, washed three times in phosphate-buffered saline, and then incubated for 15 min at 37° with 0.4–0.8 μg/ml levels of the fluorescent nuclear dye Hoechst 33258. After a two-step wash with water, cells were viewed for nuclear chromatin morphology with a fluorescence microscope, using an oil-immersion objective. Apoptotic neurons were recognized by nuclear condensation and/or fragmented chromatin. In the phase-contrast analysis, apoptotic neurons were irregularly shaped, with shrunken cell bodies and/or distrophic neurites. The number of viable and apoptotic neurons was counted in three fixed fields/culture dish.

Immunodetection of oligonucleosomes. Mono- and oligonucleosomes released from the nucleus into the cytoplasm of apoptotic

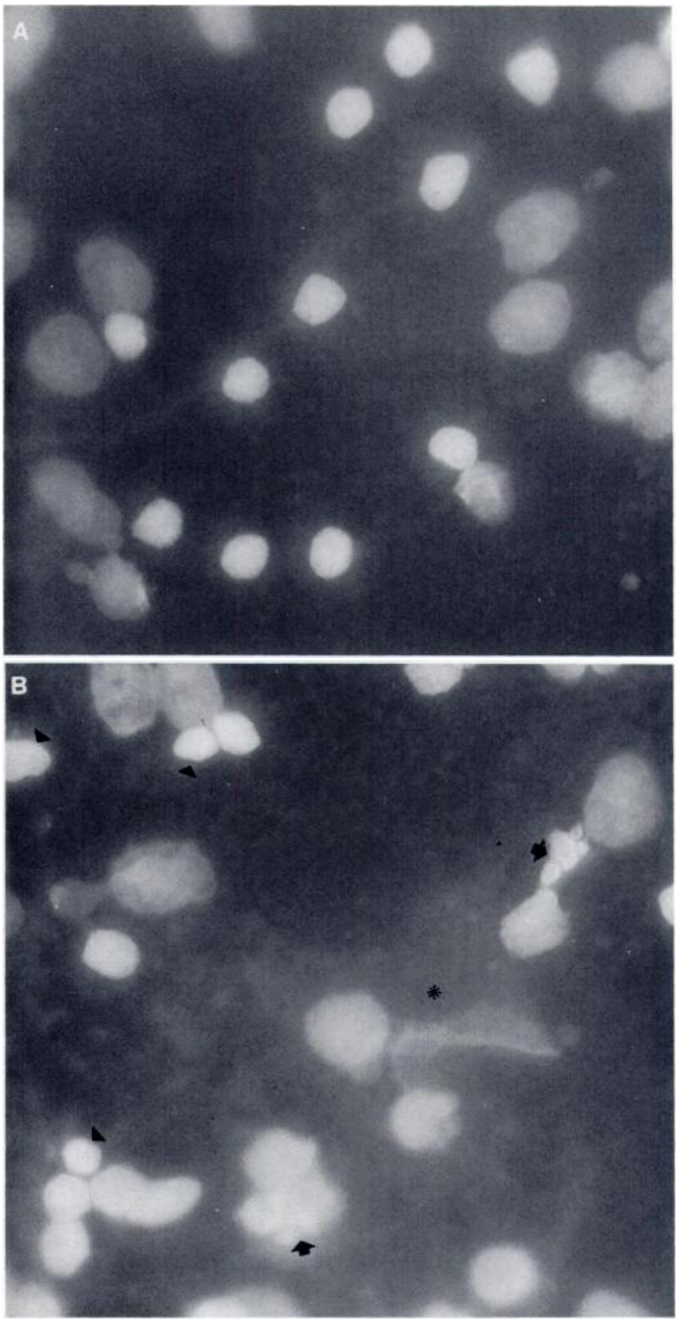

Fig. 1. Fluorescent chromatin staining of cultured cortical cells exposed for 48 hr to $\beta AP_{(25\text{-}35)}$ (25 μM). A, Control cells with regularly dispersed chromatin; B, examples of fragmentation (*arrows*) or condensation (*arrowheads*) of nuclear chromatin in neurons surrounded by aggregates of $\beta AP_{(25\text{-}35)}$ (*). Note that the larger nuclei of astrocytes do not show any morphological alteration of chromatin in cultures treated with $\beta AP_{(25\text{-}35)}$. In both A and B the incubation medium contained MK-801 (10 μM) and DNQX (30 μM).

neurons were detected by using a sandwich ELISA (Cell Death Detection ELISA; Boehringer Mannheim, Germany). The assay is based on the quantitative sandwich ELISA principle, using mouse monoclonal antibodies directed against DNA and histones, respectively. For sample preparation, cortical cultures were washed in phosphate-buffered saline and then scraped into 400 μl of cold buffer containing 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM dithiothreitol, pH 7.9. Cells were placed on ice for 10 min, added to Nonidet P-40 (0.1% final concentration), and vortex-mixed for 10 sec. Individual homogenates were centrifuged for 10 min with an Eppendorf microfuge at maximal speed. The supernatant was diluted to yield 13×10^3 cell equivalents/ml and used for immunodetection. The assay was performed as follows: (i) an antibody that reacts with the histones H1, H2A, H2B, H3, and H4 was fixed on the wall of a microtiter plate module provided with the kit; (ii) samples prepared as described above were added to the plate containing the immobilized anti-histone antibody; (iii) anti-DNA monoclonal antibodies conjugated to peroxidase were added, to allow their binding to the DNA part of nucleosomes; and (iv) after removal of unbound peroxidase conjugate, the amount of peroxidase retained in the immunocomplex was determined photometrically with 2,2′-azino-di(3-ethylbenzthiazoline sulfonate) as a substrate.

Measurement of LDH activity released from damaged cells into the medium. LDH activity was measured spectrophotometrically, as described previously (30).

Results

Toxicity of $\beta AP_{(25-35)}$ in cultured cortical neurons. The occurrence of apoptotic degeneration in cultured cortical neurons exposed to $\beta AP_{(25-35)}$ has been recently shown by DNA laddering and electron microscopic analysis (15, 16). To demonstrate the same phenomenon in mixed cortical cultures, we combined phase-contrast microscopy, nuclear chromatin staining with the fluorescent dye Hoechst 33258, and an ELISA that allows the detection of oligonucleosomes released from the nucleus into the cytoplasm. The latter method has been used for the assessment of the neuroprotective activity of mGluR agonists. Phase-contrast analysis of

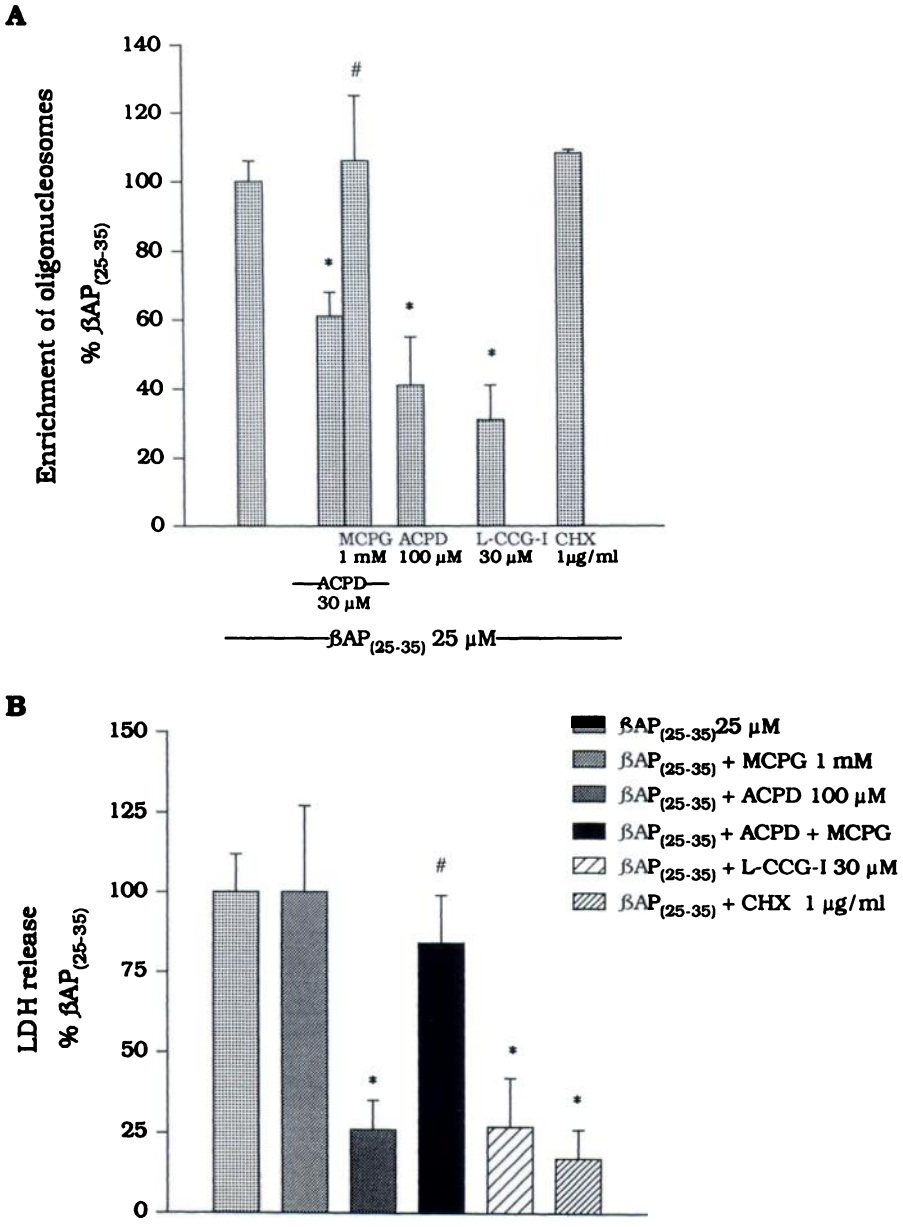

Fig. 2. A, Immunodetection of oligonucleosomes in cultures exposed for 48 hr to $\beta AP_{(25-35)}$ (25 μM), in the absence or presence of mGluR agonists or antagonists or cycloheximide (*CHX*). B, Measurement of LDH release under the same conditions as in A. In A, values (means ± standard errors) are expressed as percentages of the $\beta AP_{(25-35)}$ effect and were calculated from six to 28 determinations in two to four individual experiments. In a typical experiment, absolute values were 734 ± 58 and 1326 ± 84 milliunits of absorbance (A_{405nm}/A_{490})/1300 cell equivalents (six determinations) in control cultures and in cultures treated with βAP(25-35), respectively. Cycloheximide applied for 48 hr in control cultures induced a nonsignificant increase in oligonucleosome release, which was 32 ± 12% of the value observed with $\beta AP_{(25-35)}$ (12 determinations). In B, values (means ± standard errors) are expressed as percentages of the $\beta AP_{(25-35)}$ effect and were determined in medium collected from parallel cultures treated as in A. The medium was collected 48 hr after the addition of $\beta AP_{(25-35)}$. All experiments were performed in the presence of 10 μM MK-801 and 30 μM DNQX. Basal LDH values ranged from 20 to 75 milliunits of absorbance/min/well in most experiments. In two experiments, however, basal LDH values were <10 milliunits of absorbance/min/well. Stimulation of LDH release by $\beta AP_{(25-35)}$ ranged from about 90 to 180% above basal levels in different experiments. (1*S*,3*R*)-ACPD, L-CCG-I, and cycloheximide (all applied for 48 hr) did not affect the basal LDH activity in cultures that were not treated with $\beta AP_{(25-35)}$. Values of LDH activity were 88 ± 7, 95 ± 3, and 104 ± 11% of control in response to (1*S*,3*R*)-ACPD (100 μM), L-CCG-I (100 μM), and cycloheximide (1 μg/ml), respectively (six to 18 determinations). * and #, $p < 0.05$ (one-way ANOVA plus Fisher PLSD test), compared with βAP alone (*) or βAP plus 30 μM (1*S*,3*R*)-ACPD (#).

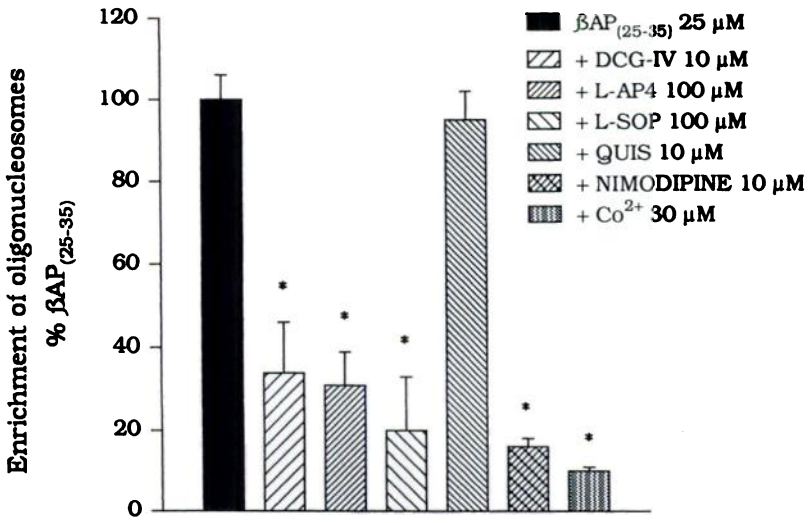

Fig. 3. Attenuation of $\beta AP_{(25-35)}$-induced apoptosis in cultured cortical cells by agonists of class II or III mGluRs or inhibitors of VSCC. All drugs were coincubated with $\beta AP_{(25-35)}$ and 10 μM MK-801 plus 30 μM DNQX. Values are expressed as in Fig. 2 and were calculated from six to 12 individual determinations (two independent experiments). *, $p < 0.05$ (one-way ANOVA plus Fisher PLSD test), compared with $\beta AP_{(25-35)}$ alone. *QUIS*, quisqualate.

TABLE 1

Necrotic degeneration induced by the combination of $\beta AP_{(25-35)}$ and NMDA, in the absence or presence of mGluR agonists

For estimation of LDH activity, the incubation medium was collected 20 hr after the NMDA pulse. $\beta AP_{(25-35)}$ was applied to the cultures 24 hr before NMDA. Values are means ± standard errors of six determinations.

	LDH release
	milliunits of absorbance/min/well
Control	4.5 ± 3.7
$\beta AP_{(25-35)}$ (12.5 μM)	21 ± 2
NMDA (60 μM)	37 ± 6.8
$\beta AP_{(25-35)}$ + NMDA	66 ± 2
$\beta AP_{(25-35)}$ + NMDA + DCG-IV (5 μM)	72 ± 15
$\beta AP_{(25-35)}$ + NMDA + L-SOP (100 μM)	87 ± 22

cortical cultures exposed to $\beta AP_{(25-35)}$ (25 μM) showed neurons with shrunken and irregularly shaped cell bodies and degenerating neurites. No neuronal swelling was observed throughout the incubation with $\beta AP_{(25-35)}$ (data not shown). Astrocytes did not show any morphological sign of toxicity (see also Ref. 16). Staining with Hoechst 33258 showed fragmentation and condensation of chromatin in neurons of cultures treated with $\beta AP_{(25-35)}$. Chromatin staining of astrocytes did not differ between control and $\beta AP_{(25-35)}$-treated cultures (Fig. 1). All of these morphological features typical of apoptotic degeneration developed within 24–48 hr of incubation with $\beta AP_{(25-35)}$ and were not influenced by the presence of the ionotropic glutamate receptor antagonists MK-801 (10 μM) and DNQX (30 μM) (data not shown). The amount of oligonucleosomes released from the nucleus into the cytoplasm was almost doubled in cultures exposed to $\beta AP_{(25-35)}$ (see the legend to Fig. 2A), and substantial increases in LDH release were observed only with incubation times of >24 hr, when cell lysis was completed (Fig. 2B; see also Ref. 16). In agreement with previous results (16), the protein synthesis inhibitor cycloheximide (1 μg/ml) delayed the lysis of cells exposed to $\beta AP_{(25-35)}$, as reflected by a reduced release of LDH at 48 hr after addition of the peptide (Fig. 2B). However, cycloheximide did not reduce the amount of oligonucleosomes detected by the ELISA in cultures exposed to $\beta AP_{(25-35)}$ (Fig. 2A).

To study the influence of mGluR activation on βAP-induced toxicity, we coapplied $\beta AP_{(25-35)}$ with mGluR agonists in the presence of 10 μM MK-801 and 30 μM DNQX (added to avoid any secondary activation of ionotropic glutamate receptors). The mixed mGluR agonist (1*S*,3*R*)-ACPD attenuated $\beta AP_{(25-35)}$-induced apoptosis without preventing the formation of βAP aggregates in cultures (data not shown). The neuroprotective action of (1*S*,3*R*)-ACPD was antagonized by MCPG [coapplied with (1*S*,3*R*)-ACPD and $\beta AP_{(25-35)}$] and mimicked by L-CCG-I (Fig. 2), which also behaves as a mixed mGluR agonist (31). Neither (1*S*,3*R*)-ACPD nor L-CCG-I, when applied for 48 hr, attenuated spontaneous neuronal degeneration in control cultures (see the legend to Fig. 2).

DCG-IV (10 μM), L-AP4 (100 μM), and L-SOP (100 μM), which selectively activate mGluR subtypes negatively linked to adenylyl cyclase (32–38), protected cortical neurons against $\beta AP_{(25-35)}$-induced apoptosis, whereas quisqualate (10 μM) was inactive (Fig. 3). The protective action of mGluR agonists against apoptosis was mimicked by drugs that inhibit VSCC, such as Co^{2+} (30 μM) or nimodipine (10 μM) (Fig. 3).

We also studied the influence of mGluR agonists on toxicity induced by a 10-min pulse with NMDA in cultures exposed for 24 hr to 12.5 μM $\beta AP_{(25-35)}$. This combination resulted in extensive necrotic degeneration, characterized by initial neuronal swelling and LDH release, which was substantial at 2–5 hr after the NMDA pulse. Neither DCG-IV nor L-SOP protected cortical neurons against toxicity induced by the combination of $\beta AP_{(25-35)}$ and NMDA (Table 1).

Toxicity of $\beta AP_{(25-35)}$ in cultured cerebellar granule cells. $\beta AP_{(25-35)}$ also induced apoptosis when added to primary cultures of cerebellar granule cells, although the number of apoptotic neurons after 48 hr of exposure was smaller than that in cultures of cortical neurons. The high level of homogeneity of cultured cerebellar granule cells and the large cell nuclei allowed a reliable microscopic count of neurons bearing chromatin fragmentation or condensation (Fig. 4). $\beta AP_{(25-35)}$-induced apoptosis in cerebellar granule cells was insensitive to MK-801 (10 μM) and DNQX (30 μM) (data not shown), which were therefore routinely included in the incubation medium.

Addition of (1*S*,3*R*)-ACPD [200 μM, coapplied with $\beta AP_{(25-35)}$] attenuated $\beta AP_{(25-35)}$-induced apoptosis in cultured cerebellar

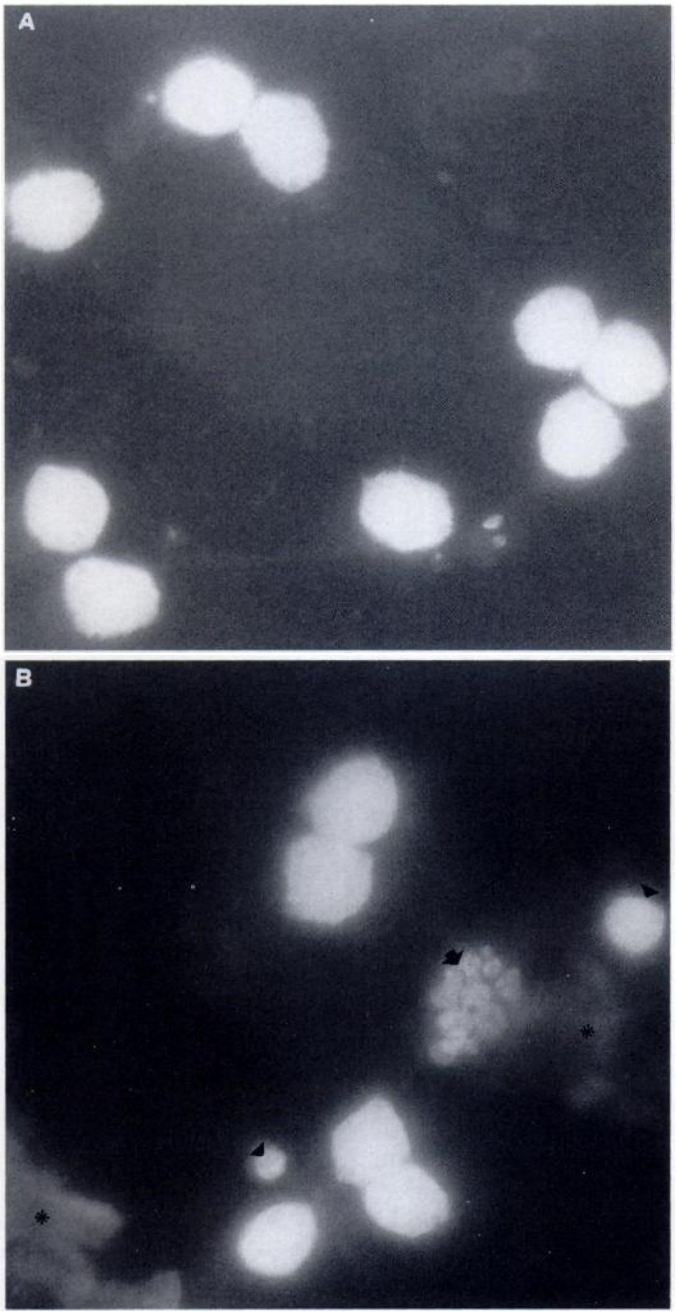

Fig. 4. Nuclear chromatin staining of cultured cerebellar granule cells exposed for 48 hr to 25 μM $\beta AP_{(25-35)}$. A, Control granule cells with regularly dispersed nuclear chromatin; B, examples of chromatin fragmentation (*arrow*) or condensation (*arrowheads*) in granule cells surrounded by aggregates of $\beta AP_{(25-35)}$ (*). In both A and B, the incubation medium contained MK-801 (10 μM) and DNQX (30 μM).

granule cells. The action of (1*S*,3*R*)-ACPD was mimicked by L-AP4 or L-SOP (both at 100 μM), whereas DCG-IV (10 μM) was inactive (Fig. 5).

Discussion

The evidence that aggregates of βAP or its active fragment, $\beta AP_{(25-35)}$, are toxic to cultured neurons has encouraged the search for drugs that act as specific neuroprotective agents in Alzheimer's disease. When βAP is applied to cultures in combination with an excitotoxin, neuronal toxicity progresses rapidly, with the typical features of necrotic degeneration (neuronal swelling and lysis of cell membranes, accompanied by an early leakage of LDH into the medium). This process is sensitive to ionotropic glutamate receptor antagonists (11, 12). When βAP is applied to cultures in the absence of an excitotoxic insult, toxicity has a slow progression and neuronal degeneration follows an apoptotic pathway (15, 16). Electron microscopic analysis of cultured cortical neurons exposed to βAP or to $\beta AP_{(25-35)}$ shows spheriform protrusion (or "blebs") of the plasma membrane, as well as fragmentation and condensation of nuclear chromatin (16), all features that are typical of apoptotic degeneration (39). DNA analysis by agarose gel electrophoresis reveals the presence of oligonucleosome-size fragments (16), which are a hallmark of apoptosis (39). βAP-induced apoptosis is preceded by a substantial increase in c-*jun* mRNA (40) and is reported to be attenuated by inhibition of protein synthesis with cycloheximide (16). This suggests that some of the intracellular events that are involved in βAP-induced apoptosis require the synthesis of specific proteins. βAP-induced apoptosis is resistant to ionotropic glutamate receptor antagonists (Ref. 16 and present data) and, in Alzheimer's brain, may provide an insidious mechanism whereby βAP present in amyloid plaques promotes the degeneration of scattered neurons regardless of their innervation. The identification of pharmacological agents that attenuate βAP-induced apoptosis may therefore have important implications for the experimental therapy of Alzheimer's disease. Knowing that mGluR agonists protect cultured neurons against excitotoxic degeneration (18–21), we tested their efficacy in mixed cortical cultures exposed to $\beta AP_{(25-35)}$. This model was selected because it has been widely used for the evaluation of neuronal toxicity and maintains the physiological interplay between neurons and astrocytes. The occurrence of neuronal apoptosis in mixed cortical cultures exposed to $\beta AP_{(25-35)}$ was indicated by the following observations: (i) the absolute lack of neuronal swelling at any stage of the degenerative process; (ii) the presence of chromatin fragmentation and condensation, revealed by the fluorescent dye Hoechst 33258; (iii) the lack of temporal correlation between morphological abnormalities and LDH release, which suggests that membrane integrity is preserved until the late stages of neuronal degeneration; and (iv) the immunodetection of an increased amount of oligonucleosomes released from the nucleus into the cytoplasm. We also studied the toxic effects of $\beta AP_{(25-35)}$ in the presence of cycloheximide, because new protein synthesis is generally required for the induction of apoptosis, although there are numerous cases in which protein synthesis inhibitors do not protect against apoptotic death (41). Addition of cycloheximide reduced the extent of cell lysis after 48-hr exposure to $\beta AP_{(25-35)}$. However, cycloheximide did not reduce the formation of oligonucleosomes, a process that is generally considered as an early marker of apoptotic death. One possible explanation is that protein synthesis is not required for chromatin fragmentation but is involved in one of the processes that lead to cell shrinkage and eventually lysis in response to βAP.

To study the influence of mGluR activation on $\beta AP_{(25-35)}$-induced apoptosis, we initially used the selective mGluR agonist (1*S*,3*R*)-ACPD, the active isomer of *trans*-ACPD. ACPD is known to protect cultured neurons against excito-

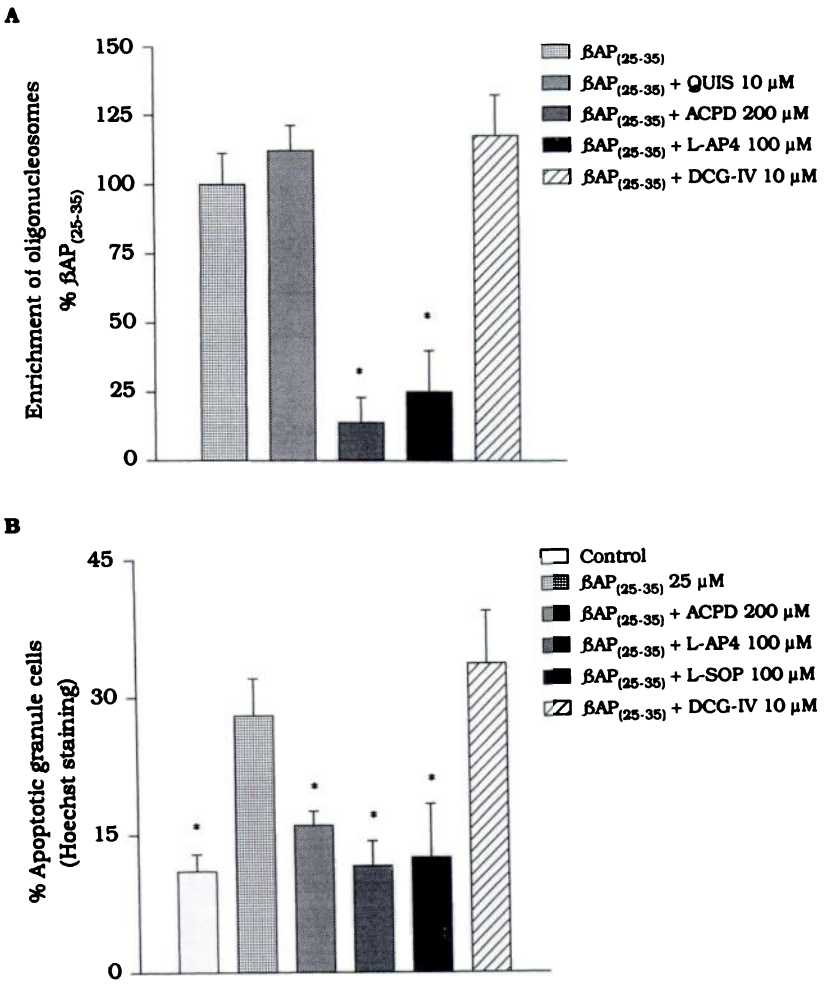

Fig. 5. $\beta AP_{(25-35)}$-induced apoptosis in cultured cerebellar granule cells treated with mGluR agonists. All drugs were coapplied with $\beta AP_{(25-35)}$ in the presence of MK-801 (10 μM) and DNQX (30 μM). The extent of apoptotic degeneration was quantitated either by detecting, by ELISA, the amount of oligonucleosomes released into the cytoplasm (A) or by counting the number of granule cells bearing chromatin abnormalities (B). Values are means ± standard errors of three to six individual determinations. *, $p < 0.05$ (one-way ANOVA plus Fisher PLSD test), compared with βAP alone. Basal and $\beta AP_{(25-35)}$-induced values in A were 75 ± 6 and 138 ± 16 milliunits of absorbance (A_{405nm}/A_{490nm})/1300 cell equivalents, respectively. *QUIS*, quisqualate.

toxic degeneration (20, 21), although it can also induce neuronal toxicity when locally infused into the rat hippocampus or corpus striatum (42–44). (1*S*,3*R*)-ACPD applied in combination with $\beta AP_{(25-35)}$ attenuated neuronal apoptosis, and its action was prevented by the mGluR antagonist MCPG (45). mGluRs form a family of at least seven subtypes, which, in transfected cells, are either coupled to polyphosphoinositide hydrolysis (mGluR1 and -5, class I) (31, 46, 47) or negatively linked to adenylyl cyclase (mGluR2 and -3, class II, and mGluR4, -6, and -7, class III) (31, 35–38). All of these subtypes are activated by (1*S*,3*R*)-ACPD (31). We therefore wondered which subtype or class of subtypes mediates the neuroprotective action of (1*S*,3*R*)-ACPD against βAP-induced apoptosis. The drugs we used to address this question were the following: (i) DCG-IV, which is highly selective for mGluR2 and -3 subtypes (32–34) and at high concentrations can also activate NMDA receptors (33), an action that was prevented by the inclusion of MK-801 in the incubation medium; (ii) L-CCG-I, which behaves as a mixed mGluR agonist but with preferential activity at mGluR2 and -3 subtypes (31); (iii) L-AP4 and L-SOP, which selectively activate mGlu4, -6, and -7 subtypes (36–38); and (iv) quisqualate, which, at the concentration we used (10 μM), activates mGluR1 and -5, with little or no activity at the other subtypes (35, 37).

In mixed cultures of cortical cells, quisqualate, L-CCG-I, or (1*S*,3*R*)-ACPD, but not DCG-IV, L-AP4, or L-SOP, stimulates polyphosphoinositide hydrolysis (21).[1] DCG-IV, L-CCG-I, L-AP4, L-SOP, and (1*S*,3*R*)-ACPD inhibit forskolin-stimulated cAMP formation in pure cortical neuronal cultures,[1] whereas in mixed cultures their effects are masked by the large response of astrocytes to forskolin (21).[1]

DCG-IV, L-CCG-I, L-AP4, or L-SOP, but not quisqualate, mimicked the protective action of (1*S*,3*R*)-ACPD against $\beta AP_{(25-35)}$-induced neuronal apoptosis in mixed cortical cultures. These results confirm the recent view that activation of class II or III mGluR subtypes protects cortical neurons against excitotoxic death (21, 48)[1] and that DCG-IV attenuates neuronal apoptosis induced by staurosporine or glucose deprivation (48). Neuroprotection, however, is limited by the strength of the toxic insult, because neither DCG-IV nor L-SOP attenuated neuronal toxicity induced by a combination of $\beta AP_{(25-35)}$ and NMDA, a condition that resulted in rapid and intense necrotic degeneration of cultured cortical neurons.

To establish whether the neuroprotective influence of mGluRs against βAP-induced apoptosis could be extended to other neuronal populations, we used cultured cerebellar granule cells, a model that is often utilized for the screening

[1] V. Bruno, G. Battaglia, A. Copani, R. G. Giffard, G. Raciti, G. Raffaele, H. Shinozaki, and F. Nicoletti. Activation of class II or III metabotropic glutamate receptors protects cultured cortical neurons against excitotoxic degeneration. Submitted for publication.

of neuroprotective agents (19, 49, 50) and has been shown to be sensitive to the toxic effects of $\beta AP_{(25-35)}$ (51). Cultured cerebellar granule cells represent a homogeneous population of glutamatergic neurons, which express at least five mGluR subtypes (mGluR1–5), although mGluR1 and -4 predominate over the others (52). Under our experimental conditions (cultures grown in the presence of 25 mM K^+ and shifted into serum-free medium at 5 DIV), $\beta AP_{(25-35)}$ induced apoptosis when applied to cultured cerebellar granule cells in the presence of ionotropic glutamate receptor antagonists. However, only 30% of the total cell population underwent apoptosis in response to $\beta AP_{(25-35)}$. This reflects an intrinsic resistance of cultured cerebellar granule cells, which is also reflected by the lower spontaneous apoptosis, compared with cortical cultures (compare the control values for oligonucleosomes given in the legends to Figs. 2A and 5A). $\beta AP_{(25-35)}$-induced apoptosis in cultured cerebellar granule cells was attenuated by (1*S*,3*R*)-ACPD or by the mGluR4/6/7 agonists L-AP4 and L-SOP but not by quisqualate or by the mGluR2/3 agonist DCG-IV. This is consistent with the evidence that cerebellar granule cells express large amounts of mGluR4 mRNA both in culture (52) and in the intact cerebellum (35), whereas mGluR2 and -3 mRNAs are expressed to a much lower extent.

Although class II or III mGluR subtypes are negatively linked to adenylyl cyclase (31, 35–38), this mechanism is difficult to reconcile with the evidence that cAMP itself is neuroprotective (53). Both L-AP4 and mGluR2 agonists inhibit Ca^{2+} influx through VSCC in cultured neurons (23, 24). Inhibition of VSCC may contribute to the neuroprotective effect of mGluR agonists, because βAP is known to increase intracellular free Ca^{2+} concentrations in cultured neurons (7, 11). It is consistent with this hypothesis that drugs that inhibit VSCC, such as Co^{2+} or nimodipine, attenuate the toxic action of βAP (25) and here reduced the extent of $\beta AP_{(25-35)}$-induced apoptosis. In conclusion, the present results provide additional evidence for the existence of "neuroprotective" mGluR subtypes, which may therefore be potential targets for specific drugs in the experimental therapy of acute or chronic neurodegenerative disorders.

References

1. Glenner, G. G., and C. W. Wong. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.* **12**:885–890 (1984).
2. Masters, C. L., G. Simms, N. A. Weinman, G. Multhaup, B. L. McDonald, and K. Beyreuther. Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc. Natl. Acad. Sci. USA* **82**:4245–4249 (1985).
3. Pike, C. J., A. J. Walencewicz, C. G. Glabe, and C. W. Cotman. *In vitro* aging of β-amyloid protein causes peptide aggregation and neurotoxicity. *Brain Res.* **56**:311–314 (1991).
4. Cotman, C. W., C. J. Pike, and A. Copani. β-Amyloid neurotoxicity: a discussion of *in vitro* findings. *Neurobiol. Aging* **13**:587–590 (1992).
5. Mattson, M. P., and R. E. Rydel. β-Amyloid precursor protein and Alzheimer's disease: the peptide plot thickens. *Neurobiol. Aging* **13**:617–621 (1992).
6. Yankner, B. A., and J. Busciglio. *Beta* amyloid causes neurofibrillary degeneration in cultured human cortical neurons. *Neurology* **42** (Suppl. 42):304 (1992).
7. Mattson, M. P., S. W. Barger, B. Cheng, I. Liebergurg, V. L. Smith-Swintosky, and R. E. Rydel. β-Amyloid precursor protein metabolites and loss of neuronal Ca^{2+} homeostasis in Alzheimer's disease. *Trends Neurosci.* **16**:409–414 (1993).
8. Pike, C. J., D. Burdik, A. J. Walencewicz, C. G. Gable, and C. W. Cotman. Neurodegeneration induced by β-amyloid peptides *in vitro*: the role of peptide assembly state. *J. Neurosci.* **13**:1676–1687 (1993).
9. Katzman, R. Alzheimer's disease. *N. Engl. J. Med.* **314**:964–973 (1986).
10. Selkoe, D. J. The molecular pathology of Alzheimer's disease. *Neuron* **6**:487–498 (1991).
11. Mattson, M. P., B. Cheng, D. Davis, K. Bryant, I. Lieberburg, and R. E. Rydel. β-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. *J. Neurosci.* **12**:376–389 (1992).
12. Koh, J.-Y., L. Yang, and C. W. Cotman. β-Amyloid protein increases the vulnerability of cultured cortical neurons to excitotoxic damage. *Brain Res.* **533**:315–320 (1990).
13. Hyman, B. T., G. W. van Hoesen, A. R. Damasio, and C. L. Barnes. Alzheimer's disease: cell specific pathology isolates the hippocampal formation. *Science (Washington D. C.)* **225**:1168–1170 (1984).
14. Davies, D. C., A. C. Wilmott, and D. M. A. Mann. Senile plaques are concentrated in the subicular regions of the hippocampal formation in Alzheimer's disease. *Neurosci. Lett.* **94**:228–233 (1988).
15. Forloni, G., R. Chiesa, S. Smiroldo, L. Verga, M. Salmona, F. Tagliavini, and N. Angeretti. Apoptosis mediated neurotoxicity induced by chronic application of β-amyloid fragment. *NeuroReport* **4**:523–526 (1993).
16. Loo, D. T., A. Copani, C. J. Pike, E. R. Whittemore, A. J. Walencewicz, and C. W. Cotman. Apoptosis is induced by β-amyloid in cultured central nervous system neurons. *Proc. Natl. Acad. Sci. USA* **90**:7951–7955 (1993).
17. Spillane, J. A., P. White, M. J. Goodhart, R. H. A. Flack, D. M. Bowen, and A. N. Davison. Selective vulnerability of neurones in organic dementia. *Nature (Lond.)* **266**:558–559 (1977).
18. Koh, J.-Y., E. Palmer, and C. W. Cotman. Activation of the metabotropic glutamate receptor attenuates *N*-methyl-D-aspartate neurotoxicity in cortical cultures. *Proc. Natl. Acad. Sci. USA* **88**:9431–9435 (1991).
19. Pizzi, M., C. Fallacara, V. Arrighi, M. Memo, and P. F. Spano. Attenuation of excitatory amino acid toxicity by metabotropic glutamate receptor agonists and aniracetam in primary cultures of cerebellar granule cells. *J. Neurochem.* **61**:683–689 (1993).
20. Schwarz, R. D., G. J. Birrel, and F. W. Marcoux. Involvement of metabotropic glutamate receptors in glutamate-induced neurotoxicity using rat cerebrocortical cultures. *Funct. Neurol.* **VII** (Suppl. 4):50 (1993).
21. Bruno, V., A. Copani, G. Battaglia, R. Raffaele, H. Shinozaki, and F. Nicoletti. Protective effect of the metabotropic glutamate receptor agonist, DCG-IV, against excitotoxic neuronal death. *Eur. J. Pharmacol.* **25**:109–112 (1994).
22. Lester, R. A. J., and C. E. Jahr. Quisqualate receptor-mediated depression of calcium currents in hippocampal neurons. *Neuron* **4**:741–749 (1990).
23. Trombley, P. Q., and G. L. Westbrook. L-AP4 inhibits calcium currents and synaptic transmission via a G-protein-coupled glutamate receptor. *J. Neurochem.* **12**:2043–2050 (1992).
24. Fagni, L., P. Chavis, J. L. Bossu, J. Nooney, A. Feltz, and J. Bockaert. Two different metabotropic receptors modulate in opposite manner L-type Ca^{2+} channels in cultured cerebellar granule cells. *Funct. Neurol.* **VII** (Suppl. 4):19 (1993).
25. Weiss, J. H., C. J. Pike, and C. W. Cotman. Ca^{2+} channel blockers attenuate β-amyloid peptide toxicity to cortical neurons in culture. *J. Neurochem.* **62**:372–375 (1994).
26. Choi, D. W., M. A. Maulucci-Gedde, and A. R. Kriegstein. Glutamate neurotoxicity in cortical cell culture. *J. Neurosci.* **7**:357–368 (1987).
27. Rose, K., M. P. Goldberg, and D. W. Choi. Cytotoxicity in murine neocortical cell culture. *Methods Toxicol.* **1**:46–60 (1993).
28. Nicoletti, F., J. T. Wroblewski, A. Novelli, H. Alho, A. Guidotti, and E. Costa. The activation of inositol phospholipid metabolism as a signal transducing system for excitatory amino acids in primary cultures of cerebellar granule cells. *J. Neurosci.* **6**:1905–1911 (1986).
29. Thangnipon, W., A. Kingbury, M. Webb, and R. Balazs. Observation on rat cerebellar cells *in vitro*: influence of substratum, potassium concentration and relationship between neurons and astrocytes. *Dev. Brain Res.* **11**:177–189 (1983).
30. Koh, J.-Y., and D. W. Choi. Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J. Neurosci. Methods.* **20**:83–90 (1987).
31. Nakanishi, S. Molecular diversity of glutamate receptors and implications for brain function. *Science (Washington D. C.)* **258**:597–603 (1992).
32. Hayashi, Y., A. Momiyama, T. Takahashi, H. Ohishi, R. Ogawa-Meguro, R. Shigemoto, N. Mizuno, and S. Nakanishi. Role of metabotropic glutamate receptors in synaptic modulation in the accessory olfactory bulb. *Nature (Lond.)* **366**:687–689 (1993).
33. Ishida, M., T. Saito, K. Shimamoto, Y. Ohfune, and H. Shinozaki. A novel metabotropic glutamate receptor agonist: marked depression of monosynaptic excitation in the newborn rat isolated spinal cord. *Br. J. Pharmacol.* **10**:1169–1177 (1993).
34. Ohfune, Y., K. Shimamoto, M. Ishida, and H. Shinozaki. Synthesis of L-2-(2–3-dicarboxycyclopropyl)glycines: novel conformationally restricted analogues. *Bioorg. Med. Chem. Lett.* **3**:15–18 (1993).
35. Tanabe, Y., M. Masu, I. Ishii, R. Shigemoto, and S. Nakanishi. A family of metabotropic receptors. *Neuron* **8**:169–179 (1992).
36. Saugstadt, J. A., J. M. Kinzie, T. P. Segerson, and G. L. Westbrook. Characterization of a new metabotropic glutamate receptor (mGluR7) homologous to the AP4 receptor (mGluR4). *Soc. Neurosci. Abstr.* **19**:36.1 (1993).
37. Tanabe, Y., A. Nomura, M. Masu, R. Shigemoto, N. Mizuno, and S. Nakanishi. Signal transduction, pharmacological properties, and expres-

sion patterns of two rat metabotropic glutamate receptors, mGluR3 and mGluR4. *J. Neurosci.* **13**:1372–1378 (1993).
38. Okamoto, N., S. Hori, C. Akazawa, Y. Hayashi, R. Shigemoto, N. Mizuno, and S. Nakanishi. Molecular characterization of a new metabotropic glutamate receptor, mGluR7, coupled to inhibitory cyclic AMP signal transduction. *J. Biol. Chem.* **269**:1231–1236 (1994).
39. Wyllie, A. H., J. F. R. Kerr, and A. R. Currie. Cell death: the significance of apoptosis. *Int. Rev. Cytol.* **68**:251–306 (1980).
40. Anderson, A. J., B. J. Cimmings, C. J. Pike, D. T. Loo, and C. W. Cotman. Jun and Fos immunoreactivity in Alzheimer's brain and induction by β-amyloid in cultured neurons. *Soc. Neurosci. Abstr.* **19**:513.6 (1993).
41. Schwartzman, R. A., and J. A. Cidlowski. Apoptosis: the biochemistry and molecular biology of programmed cell death. *Endocr. Rev.* **14**:133–152 (1993).
42. McDonald, J. W., and D. D. Schoepp. The metabotropic excitatory amino acid receptor agonist 1*S*,3*R*-ACPD selectively potentiates *N*-methyl-D-aspartate-induced brain injury. *Eur. J. Pharmacol.* **21**:353–354 (1992).
43. Olney, J. W., M. T. Price, Y. Izumi, and C. Romano. Neurotoxicity associated with either suppression or excessive stimulation of mGluR function. *Funct. Neurol.* **VII** (Suppl. 4):38 (1993).
44. Schoepp, D. D., J. W. McDonald, R. A. Sacaan, C. R. True, J. P. Salhoff, J. P. Tizzano, and A. S. Fix. Mechanisms of *in vivo* metabotropic glutamate receptor mediated neuronal degeneration in the rat. *Funct. Neurol.* **VII** (Suppl. 4):50 (1993).
45. Eaton, S. A., D. E. Jane, P. L. S. J. Jones, R. H. P. Porter, P. C.-K. Pook, D. C. Sunter, P. M. Udvarhelyi, P. J. Roberts, T. E. Salt, and J. C. Watkins. Competitive antagonism of metabotropic glutamate receptors by (*S*)-4-carboxyphenylglycine and (*RS*)-α-methyl-4-carboxyphenylglycine. *Eur. J. Pharmacol.* **24**:195–197 (1993).
46. Abe, T., H. Sugihara, H. Nawa, R. Shigemoto, N. Mizuno, and S. Nakanishi. Molecular characterization of a novel metabotropic glutamate receptor, mGluR5, coupled to inositol phosphate/Ca^{2+} signal transduction. *J. Biol. Chem.* **267**:13361–13368 (1992).
47. Aramori, I., and S. Nakanishi. Signal transduction and pharmacological characteristics of a metabotropic glutamate receptor, mGluR1, in transfected CHO cells. *Neuron* **8**:757–765 (1992).
48. Buisson, A., S. P. Yu, and D. W. Choi. Effect of metabotropic glutamate receptor agonists on excitotoxic and apoptotic cell death in murine cortical cell culture. *Soc. Neurosci. Abstr.* **20**:198.5 (1994).
49. Favaron, M., H. Manev, H. Alho *et al.* Gangliosides prevent glutamate and kainate neurotoxicity in primary neuronal cultures of neonatal rat cerebellum and cortex. *Proc. Natl. Acad. Sci. USA* **85**:7351–7355 (1988).
50. Favaron, M., H. Manev, R. Siman *et al.* Down-regulation of protein kinase C protects cerebellar granule neurons in primary culture from glutamate-induced neuronal death. *Proc. Natl. Acad. Sci. USA* **87**:1983–1987 (1990).
51. Scorziello, A., O. Meucci, A. Avallone, C. Galli, S. Schinelli, M. Salmona, G. Forloni, and G. Schettini. β 25–35 amyloid fragment destabilizes [Ca^{2+}] homeostasis and induces neurotoxicity in cerebellar granule cells. *Soc. Neurosci. Abstr.* **20**:252.8 (1994).
52. Santi, M. R., S. Ikonomovic, J. T. Wroblewski, and D. R. Greyson. Absolute amounts of the mRNAs encoding metabotropic glutamate receptors in cerebellar granule neurons *in vitro*. *J. Neurochem.* **63**:1207–1217 (1994).
53. Mattson, M. P., and S. B. Kater. Intracellular messengers in the generation and degeneraton of hippocampal neuroarchitecture. *J. Neurosci. Res.* **21**:447–464 (1988).

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