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Glutamate Induces Apoptosis in Anterior Pituitary Cells through Group II Metabotropic Glutamate Receptor Activation

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Glutamate can induce neuronal cell death by activating ionotropic glutamate receptors (iGluRs) as well as metabotropic glutamate receptors (mGluRs). In the present study, we investigated whether glutamate induces apoptosis of cultured anterior pituitary cells from female rats. Glutamate (1 mM) significantly reduced the metabolic activity of viable cells and increased the percentage of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL)-positive cells and caspase-3 activity in anterior pituitary cells. The inhibitory effect of glutamate on the viability of anterior pituitary cells was not observed in the presence of [2S]-α-ethylglutamic acid (0.75 mM), a specific group II mGluR antagonist. Also, (2S,1′S,2′S)-2-(carboxycyclopropyl)glycine (LCCG-I; 0.75 mM), a specific group II mGluR agonist, reduced viability and increased the percentage of TUNEL-positive anterior pituitary cells. Group I and III mGluRs and iGluRs agonists failed to modify the metabolic activity of anterior pituitary cells. Glutamate and LCCG-I increased the percentage of TUNEL-positive lactotropes and somatotropes. The subunit mGluR2/3, belonging to group II mGluR, was localized in these cell types. Glutamate increased nitric oxide (NO) synthase (NOS) activity and inducible NOS expression in anterior pituitary cells. *N*-methyl-L-arginine (NMMA, 0.5 mM), a NOS inhibitor, potentiated the apoptotic effect of glutamate in anterior pituitary cells, indicating that NO may restrain glutamate-induced apoptosis. Incubation of anterior pituitary cells with a cAMP analog (N6, 2′-o-dibutyryladenosine 3′, 5′-cyclic monophosphate; 1 mM) attenuated the apoptosis induced by glutamate. Glutamate and LCCG-I decreased prolactin release from anterior pituitary cells. N6, 2′-o-dibutyryladenosine 3′, 5′-cyclic monophosphate reversed the inhibitory effect of glutamate on prolactin release, but NMMA failed to modify it. Our data show that glutamate induces apoptosis of lactotropes and somatotropes through group II mGluR activation, probably by decreasing cAMP synthesis. (*Endocrinology* 145: 4677–4684, 2004)

GLUTAMATE IS THE major excitatory neurotransmitter in the brain and an important amino acid of the intermediate metabolism. Glutamate binds to ionotropic receptors (iGluRs) and metabotropic receptors (mGluRs). The iGluRs possess intrinsic cation-permeable channels and include *N*-methyl-D-aspartate (NMDA), kainate (KA), and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (1). The mGluRs are a distinct group within a G protein-coupled receptor superfamily and have been classified into three groups on the basis of sequence homology and coupling to second-messenger systems (2). Group I (mGluR1 and mGluR5 subunits) stimulates inositol phosphate metabolism and mobilization of intracellular Ca^{2+}. Group II (mGluR2 and mGluR3) and group III (mGluR4 and mGluR6–8) are negatively coupled to adenylate cyclase.

Excessive stimulation of glutamate receptors can be neurotoxic, a phenomenon known as excitotoxicity that causes neuronal damage (3). Endogenous glutamate, by activating NMDA, AMPA, or mGluR1 receptors, may contribute to the acute brain damage after cerebral ischemia or traumatic brain injury and to chronic neurodegeneration in disorders such as Huntington's chorea, Alzheimer's disease, and Parkinson's disease (4). Serum glutamate levels, which are normally about 100 μM, increase in some pathologies such as migraine and amyotrophic lateral sclerosis (4). Besides its role as a neurotransmitter modifying neuronal and glial excitability, glutamate regulates proliferation, differentiation, and cell death in the central nervous system (CNS) (4). Activation of mGluRs can initiate neuroprotective mechanisms (5) as well as apoptotic events (6).

Glutamate is an important signal molecule in the hypothalamus and has been implicated in critical neuroendocrine events such as puberty and sexual behavior (7). The presence of different subtypes of glutamate receptors has been demonstrated in peripheral neural and nonneural tissues including the pituitary (3). The NMDA receptor subunit 1 was

Abbreviations: AIDA, [RS]-1-Aminoindan-1,5-dicarboxylic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AP-5, DL-2-amino-5-phosphonovaleric acid; CNS, central nervous system; dbcAMP, N6, 2′-o-dibutyryladenosine 3′, 5′-cyclic monophosphate; DNQX, 6,7-dinitroquinoxaline-2,3(1H,4H)-dione; EGLU, [2S]-α-ethylglutamic acid; FBS, fetal bovine serum; 3-HPG, [+]-α-amino-3-hydroxybenzeneacetic acid; iGluR, ionotropic glutamate receptor; iNOS, inducible nitric oxide synthase; KA, kainate; L-AP4, L(+)-2-amino-4-phosphonobutyric acid; LCCG-I, (2S,1′S,2′S)-2-(carboxycyclopropyl)-glycine; mGluR, metabotropic glutamate receptor; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NMDA, *N*-methyl-D-aspartate; NMMA, *N*-methyl-L-arginine; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; PRL, prolactin; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

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found in all the cell types of the anterior pituitary except corticotropes (8), whereas the subunit GluR1 of AMPA receptors was localized in gonadotropes (9), and GluR2 and GluR3 were localized in lactotropes and somatotropes (10, 11). The GluR6/7 subunit of KA receptors and group II mGluRs are present in the anterior pituitary, although the cell types expressing them have not been identified yet (12, 13). Glutamate can access the anterior pituitary from the median eminence via long portal veins, from the posterior pituitary via the short portal vessels, and from peripheral blood. Glutamate is known to be involved in the regulation of prolactin (PRL) and LH secretion (14).

In this study, we investigated the effect of glutamate on the viability of cultured anterior pituitary cells and determined the involvement of iGluRs and mGluRs in its effects. In the CNS, glutamate elicits many of its effects by activating nitric oxide (NO) synthase (NOS) (15). NOS isoforms are present in the anterior pituitary (16–18) where NO inhibits PRL secretion in a paracrine manner (19). Because NO produces cytotoxic as well as cytoprotective events, we examined the participation of NO in the action of glutamate on anterior pituitary cells. In addition, in view of cAMP involvement in the control of apoptotic events (20), we investigated the role of cAMP in glutamate effects. We also determined the effect of glutamate on PRL release from anterior pituitary cells and examined the involvement of cAMP and NO in this effect.

Materials and Methods

Animals

Adult intact female Wistar rats weighing 200–250 g were housed in a light- (12-h light, 12-h dark cycle) and temperature-controlled environment (20–25 C). Rats were fed laboratory chow and water *ad libitum* and were kept according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were killed by decapitation at random stages of the estrous cycle.

Drugs

Fetal bovine serum (FBS) was obtained from GenSa (Buenos Aires, Argentina). [RS]-1-Aminoindan-1,5-dicarboxylic acid (AIDA) [2S]-α-ethylglutamic acid (EGLU), L(+)-2-amino-4-phosphonobutyric acid (L-AP4), (2S,1′S,2′S)-2-(carboxycyclopropyl)glycine (LCCG-I), AMPA, and [+]-α-amino-3-hydroxy-benzeneacetic acid (3-HPG) were obtained from Tocris Cookson Inc. (Ballwin, MO). All terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) reagents were obtained from Roche Molecular Biochemicals (Mannheim, Germany), and RT-PCR reactives were purchased from Invitrogen Corporation (Carlsbad, CA), unless otherwise specified. NMDA, KA, 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX), DL-2-amino-5-phosphonovaleric acid (AP-5), glutamate (L-glutamic acid), N6, 2′-o-dibutyryladenosine 3′, 5′-cyclic monophosphate (dbcAMP), *N*-methyl-L-arginine (NMMA), digitonin, and all other media and supplements were obtained from Sigma Chemical Co. (St. Louis, MO). Dowex AG 50W-X8 resin was purchased from Bio-Rad Laboratories (Hercules, CA), and L-[^{14}C]arginine (specific activity, 11.26 GBq/mmol) from Amersham Int. (Buckinghamshire, UK).

Anterior pituitary cell culture

Anterior pituitary glands (posterior pituitaries removed) were obtained within minutes after decapitation. The glands were washed several times with DMEM and cut into small fragments. Sliced fragments were dispersed enzymatically by successive incubations in DMEM supplemented with 3 mg/ml BSA, containing 2.5 mg/ml trypsin (type I from bovine pancreas), 1 mg/ml deoxyribonuclease II (type V from bovine spleen), and 1 mg/ml trypsin inhibitor (type II-S from soybean). The cells were finally dispersed mechanically by extrusion through a Pasteur pipette in Krebs buffer without Ca^{2+} and Mg^{2+}. Dispersed cells were washed twice and suspended in DMEM supplemented with 10 μl/ml MEM amino acids, 5.6 μg/ml amphotericin B, 25 μg/ml gentamicin, and 2 mM glutamine (DMEM-S). Cell viability as assessed by trypan blue exclusion was above 90%. Anterior pituitary cells were cultured for 72 h (37 C, 5% CO_2-95% O_2 in air) in DMEM-S with 10% FBS. After the culture period, the medium was replaced with fresh DMEM-S with 1% FBS alone or plus test substances and incubated for 24 h. At the end of the incubation period, the cells were processed, and the media were aspirated and stored at −20 C until assayed for PRL determination.

Metabolic activity determination

The metabolic activity of viable cells was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, cells were washed twice and incubated for 4 h in 100 μl Krebs buffer plus 50 μg of MTT reagent dissolved in 10 μl PBS at 37 C. The developed crystals were dissolved in 100 μl of 0.04 N HCl in isopropanol, and the OD was read in a microplate spectrophotometer at a wavelength of 595 nm.

Microscopic determination of DNA fragmentation by TUNEL method

After the culture period, cells were fixed with 4% formaldehyde in PBS for 30 min and permeabilized by microwave irradiation (21). DNA strand breaks were labeled with digoxigenin-deoxyuridine triphosphate using terminal deoxynucleotidyl transferase (0.18 U/μl) according to the manufacturer's protocol (Roche Molecular Biochemicals). The incorporation of nucleotides into the 3′-OH end of damaged DNA was detected with an antidigoxigenin-fluorescein antibody and visualized in a fluorescence microscope (Axiophot; Carl Zeiss, Jena, Germany).

Immunofluorescent identification of anterior pituitary cells

Anterior pituitary cells were identified by indirect immunofluorescence staining. Cultured cells were fixed as described earlier and incubated with 10% normal donkey serum in PBS with 2% BSA for 30 min. Then the slides were incubated with primary antibodies (provided by the National Hormone and Pituitary Program, Torrance, CA) [guinea pig antirat PRL (NHPP-IC, 1:2500), guinea pig antirat GH (NHPP-IC, 1:2000), guinea pig antirat β-LH (NHPP-IC-2, 1:5000), guinea pig antirat ACTH (NHPP-IC-1, 1:400), and guinea pig antirat β-TSH (NHPP-IC, 1:500)] in PBS with 0.5% BSA, 1% blocking reagent, 1% normal sheep serum, and 1% normal donkey serum for 1 h. After rinsing, slides were incubated for 1 h with donkey antiguinea pig rhodamine-conjugated secondary antibody (Chemicon International Inc., Temecula, CA) at 1:200 dilution in the same buffer. Control slides were incubated with normal guinea pig serum instead of primary antibody.

Double immunofluorescent labeling of anterior pituitary cells

Cultured cells were fixed as described earlier and incubated with 5% normal sheep serum and 5% normal donkey serum in PBS for 1 h. Then the slides were incubated with rabbit antirat mGluR2/3 antibody (1:75), which was kindly donated by Dr. Robert Wenthold (National Institute on Deafness and Other Communication Disorders, National Institutes of Health, Bethesda, MD), and guinea pig antibodies against each pituitary hormone as described earlier in PBS with 0.5% normal sheep serum and 0.5% normal donkey serum for 1 h. The slides were rinsed and incubated with a mixture of secondary antibodies (sheep antirabbit fluorescein-conjugated 1:200 and donkey antiguinea pig rhodamine-conjugated 1:200) in PBS with 0.5% normal sheep serum and 0.5% normal donkey serum for 1 h. Control slides were incubated with normal rabbit serum and guinea pig serum instead of primary antibodies.

Caspase-3 activity assay

Anterior pituitary cells (3×10^6) were homogenized in lysis buffer [50 mM Tris/HCl, pH 7.4; 1 mM EDTA; 10 mM EGTA; 40 μM digitonin; and 1 mM phenylmethylsulfonylfluoride (Alexis Corporation, Lausen, Swit-

zerland)] and centrifuged (14,000 rpm, 20 min). The supernatant was added to the reaction mixture (100 mM HEPES, pH 7.4; 1 mM EDTA; 10 mM dithiothreitol; 1 mM phenylmethylsulfonylfluoride; and 25 μg of DEVD-PNA, a colorimetric substrate from Alexis Corporation) and incubated at 37 C for 120 min. The OD was measured in a microplate spectrophotometer at a wavelength of 415 nm. Protein concentration in aliquots of supernatant were determined by a colorimetric method (Protein assay; Bio-Rad Laboratories). Caspase activity was expressed as OD per nanogram of protein.

NOS activity determination

NOS activity was determined by conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline, using a modification of the Bredt and Snyder method (15). Briefly, cultured cells were incubated for 24 h in the presence of glutamate (1 mM). Then the cells were washed twice with Krebs buffer containing 20 mM HEPES (pH 7.4) and incubated in the same buffer with 0.1 μCi L-[U-^{14}C]arginine. After a 30-min incubation at 37 C, the cells were washed in cold Krebs buffer containing 20 mM HEPES, 2 mM EDTA, 0.2 mM EGTA, and 2 mM Nω-nitro-L-arginine methyl ester hydrochloride and immediately sonicated in the same buffer. Aliquots of sonicate were applied to 1.5-ml columns of Dowex AG 50W-X8 in 20 mM HEPES (pH 7.4) loaded with 20 μl of 100 mM L-citrulline. L-[^{14}C]citrulline was eluted with 3 ml of distilled water, and the radioactivity was quantified by liquid scintillation spectrometry of the flow-through.

NOS RT-PCR

Total RNA from cultured anterior pituitary cells was extracted from tissues using TRIZOL (GibcoBRL, Gaithersburg, MD) according to the manufacturer's protocol. Five micrograms of total RNA were reverse transcribed with Superscript Rnase H Reverse Transcriptase (Invitrogen) in 20 μl reaction, using 0.2 μg of Oligo $(dT)_{12–18}$ as primer. cDNA amplification was performed with 1 μl of cDNA as template in 50 μl PCR reaction containing $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate sense and antisense primers, and *Taq* DNA polymerase in the buffer provided by the manufacturer. Temperature cycles always had an initial denaturation at 94 C for 10 min and a final extension period of 7 min at 72 C. Inducible NOS (iNOS), neuronal NOS (nNOS), and β-actin primers were purchased from Transgenomics Inc. (Omaha, NE). Amplifications were performed with a UNO II thermocycler (Biometra, Göttingen, Germany). The number of cycles and the template input for PCR were determined empirically within the linear range of amplification.

Amplification of iNOS cDNA was performed with 1.5 mM $MgCl_2$, 50 pmol of each primer, and 2.5 U of *Taq* DNA polymerase. Temperature cycles were 94 C, 58 C, and 72 C steps, 30 sec each. The primers for iNOS, the number of cycles, and the product size were as follows: sense, 5′-TAGAAACAACAGGAACCTACCA-3′; antisense, 5′-ACAGGGGTGATGCTCCCGGACA-3′; 33 cycles; and 907 bp. Amplification of nNOS was performed with 2.5 mM $MgCl_2$, 50 pmol of each primer, and 2.5 U of *Taq* DNA polymerase. Temperature cycles were 94 C, 66 C, and 72 C steps, 30 sec each. The primers for nNOS, number of cycles, and product size were as follows: sense, 5′-GAATACCAGCCTGATCCATGGAACACC-3′; antisense, 5′-TCCAGGAGGGTGTCCACCGCATGCC-3′; 32 cycles; and 599 bp. The primers for β-actin and product size were as follows: sense, 5′-ACCACAGCTGAGAGGGAAATCG-3′; antisense, 5′-AGAGGTCTTTACGGATGTCAACG-3′; and 281 bp.

Ten microliters of each reaction were analyzed on 1.5% agarose gels, stained with ethidium bromide, and visualized using UV light. RT-PCR products were analyzed quantitatively using SCION Image software (Scion Corporation, Frederick, MD). Results were normalized to the internal control β-actin. Values were expressed as relative increments of respective controls. Experiments always included non-reverse-transcribed RNA samples as negative controls.

PRL RIA

PRL was measured by a double-antibody RIA, with reagents provided by the National Hormone and Pituitary Program. Rat PRL-RP-3 was used as the standard, and NIDDK-anti-rPRL-S-9 was used as antiserum. Cross-reactivity with other pituitary hormones was negligible. The sensitivity of the assay was 0.1 ng/ml. The intra- and interassay coefficients of variation were less than 10%. Hormone concentration was expressed as nanograms per well.

Statistical analysis

Data were expressed as mean ± SEM and were analyzed by the Student's *t* test, one sample *t* test, one-way ANOVA followed by Student-Newman-Keuls multiple comparisons test, or two-way ANOVA with interaction terms. Differences with a $P < 0.05$ were considered statistically significant.

The experiments were performed at least twice. The number of apoptotic cells (identified by the TUNEL method) was analyzed in duplicate slides for each cell preparation from at least two independent experiments. Re-

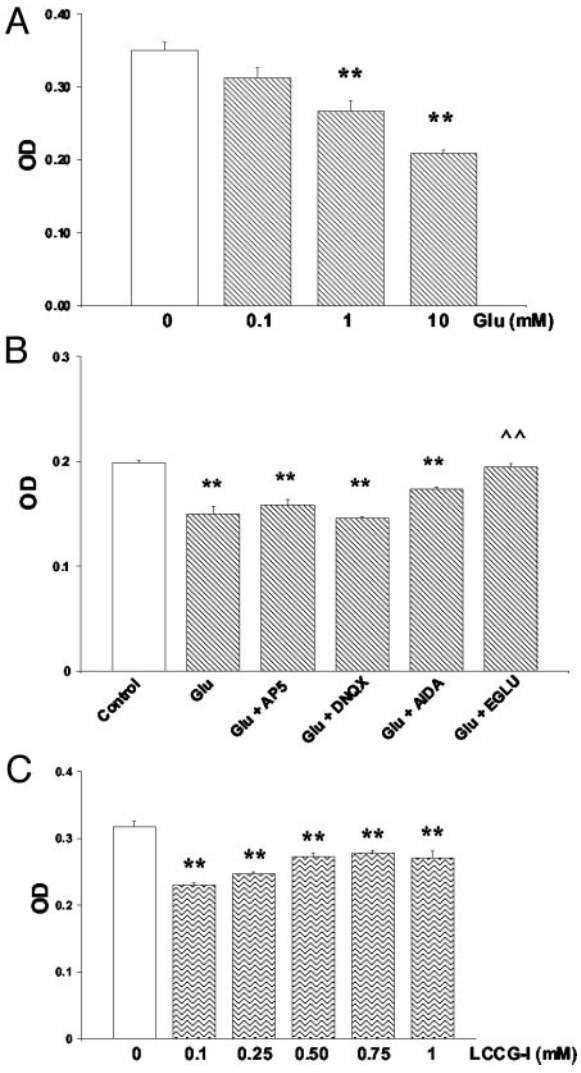

FIG. 1. Effect of glutamate (A), a group II mGluR antagonist, EGLU (B), and LCCG-I (C) on the metabolic activity of anterior pituitary cells. Anterior pituitary cells were cultured in DMEM-S (10% FBS) for 3 d. Then the medium was replaced by DMEM-S (1% FBS) containing different concentrations of glutamate (Glu) or LCCG-I or 1 mM glutamate and 0.75 mM of AP5 (NMDA antagonist), DNQX (KA-AMPA antagonist), AIDA (group I antagonist), and EGLU (group II antagonist) for 24 h. Cell viability was assessed by MTT assay. Each column represents the mean ± SE of six to eight wells in one of three independent experiments. A and C, Data were evaluated by one-way ANOVA followed by Dunnett's test. **, $P < 0.01$ *vs.* control. B, Data were evaluated by one-way ANOVA followed by Student-Newman-Keuls multiple comparisons test. **, $P < 0.01$ *vs.* control; ^, $P < 0.01$ *vs.* 1 mM glutamate.

sults were expressed as the percentage of apoptotic cells of the total number of cells counted for each specific condition. Differences between proportions were analyzed by the χ^2 test with 95% confidence.

Results

Effect of glutamate on the metabolic activity of anterior pituitary cells

To study the effect of glutamate on anterior pituitary cell viability, we determined the metabolic activity of these cells by MTT assay. Glutamate at 1 mM or 10 mM concentrations significantly decreased the metabolic activity of viable anterior pituitary cells (Fig. 1A).

To investigate which type of glutamate receptors was involved in the inhibitory effect of glutamate on the metabolic activity of anterior pituitary cells, we determined the effect of different agonists and antagonists of glutamate receptors. In the presence of EGLU, a group II mGluR antagonist, glutamate failed to decrease the metabolic activity of anterior pituitary cells (Fig. 1B). On the contrary, AP-5 (NMDA antagonist), DNQX (KA-AMPA antagonist), and AIDA (group I mGluR antagonist) did not modify the effect of glutamate on metabolic activity (Fig. 1B). LCCG-I, a specific group II mGluR agonist, decreased metabolic activity at all concentrations tested (Fig. 1C). None of the other agonists, including iGluR agonists (NMDA, KA, and AMPA), group I mGluR agonist ([+]-α-amino-3-hydroxy-benzeneacetic acid), and group III mGluR agonist (L-AP4), modified the metabolic activity of anterior pituitary cells (Table 1).

Effect of glutamate on apoptosis of anterior pituitary cells

To determine whether the decrease in metabolic activity induced by glutamate was produced by cell death, we determined the percentage of anterior pituitary apoptotic cells by the TUNEL method. Both glutamate (1 mM) and LCCG-I (0.75 mM) significantly increased the percentage of TUNEL-positive total anterior pituitary cells (Fig. 2A), lactotropes (Fig. 2B), and somatotropes (Fig. 2C). A TUNEL-positive lactotrope and a somatotrope showing nuclear apoptotic morphology are shown in Fig. 3A. In addition, glutamate increased caspase-3 activity of anterior pituitary cells (control, 0.265 ± 0.027 OD/ng protein; glutamate, 0.390 ± 0.035 OD/ng protein; $P < 0.05$).

Localization of group II mGluR receptors in anterior pituitary

By double immunocytochemistry, we detected the presence of group II mGluR in lactotropes and somatotropes (Fig. 3B) but not in corticotropes, gonadotropes, and thyrotropes (data not shown).

Effect of glutamate on NOS activity and gene expression in anterior pituitary cells

Glutamate increased NOS activity as determined by L-[^{14}C]arginine to L-[^{14}C]citrulline conversion in anterior pituitary cells (Fig. 4A). Also, glutamate increased the expression of iNOS mRNA (Fig. 4B) but not nNOS in these cells (data not shown).

Role of NO and cAMP in the glutamate-induced apoptosis of anterior pituitary cells

Because glutamate increased NOS activity and iNOS expression, we examined whether NO is involved in the apoptotic effect of glutamate. NMMA (0.5 mM), a NOS inhibitor, failed *per se* to modify the percentage of TUNEL-positive anterior pituitary cells (Fig. 5A) but potentiated glutamate-induced apoptosis in these cells (Fig. 5A).

Because group II mGluR activation decreases cAMP levels (2), we investigated the effect of a cAMP analog, dbcAMP, on the apoptotic effect of glutamate. dbcAMP (1 mM) *per se* did not modify the percentage of TUNEL-positive anterior pituitary cells but significantly reduced glutamate-induced apoptosis in these cells (Fig. 5B).

Effect of glutamate on PRL release from anterior pituitary cells

Glutamate and LCCG-I, at all tested concentrations, significantly decreased PRL release from anterior pituitary cells (Table 2). All the other glutamate receptor agonists studied failed to modify PRL release, except KA, which increased it (Table 2). NMMA failed to modify basal PRL release and the inhibitory effect of glutamate on this release (Fig. 6A). dbcAMP did not modify basal PRL release but completely reversed the inhibitory effect of glutamate on PRL release (Fig. 6B).

Discussion

Our results indicate that glutamate induces apoptosis of anterior pituitary cells. Glutamate decreased the viability of anterior pituitary cells in culture and increased the percentage of TUNEL-positive cells. Morphological nuclear features of apoptosis and activation of caspase-3 in anterior pituitary cells induced by glutamate support the idea that glutamate induces cell death by apoptosis (3).

TABLE 1. Effect of glutamate receptor agonists on the metabolic activity of anterior pituitary cells

Concentration (mM)	OD				
	Ionotropic receptor agonist			Metabotropic receptor agonist	
	NMDA	KA	AMPA	3-HPG	L-AP4
0	0.350 ± 0.011	0.297 ± 0.017	0.188 ± 0.012	0.268 ± 0.026	0.210 ± 0.010
0.1	0.350 ± 0.009	0.310 ± 0.016	0.185 ± 0.004	0.296 ± 0.019	0.220 ± 0.018
1	0.349 ± 0.004	0.312 ± 0.021	0.183 ± 0.006	0.311 ± 0.024	0.186 ± 0.016
10	0.344 ± 0.008	0.337 ± 0.017	0.178 ± 0.005	0.230 ± 0.028	0.185 ± 0.009

Anterior pituitary cells were cultured in DMEM-S (10% FBS) for 3 d. Then the medium was replaced by DMEM-S (1% FBS) containing the different tested agonists for 24 h. Cell viability was assessed by MTT assay. Values represent mean ± SE of seven to eight wells in one of three independent experiments. Data were evaluated by one-way ANOVA.

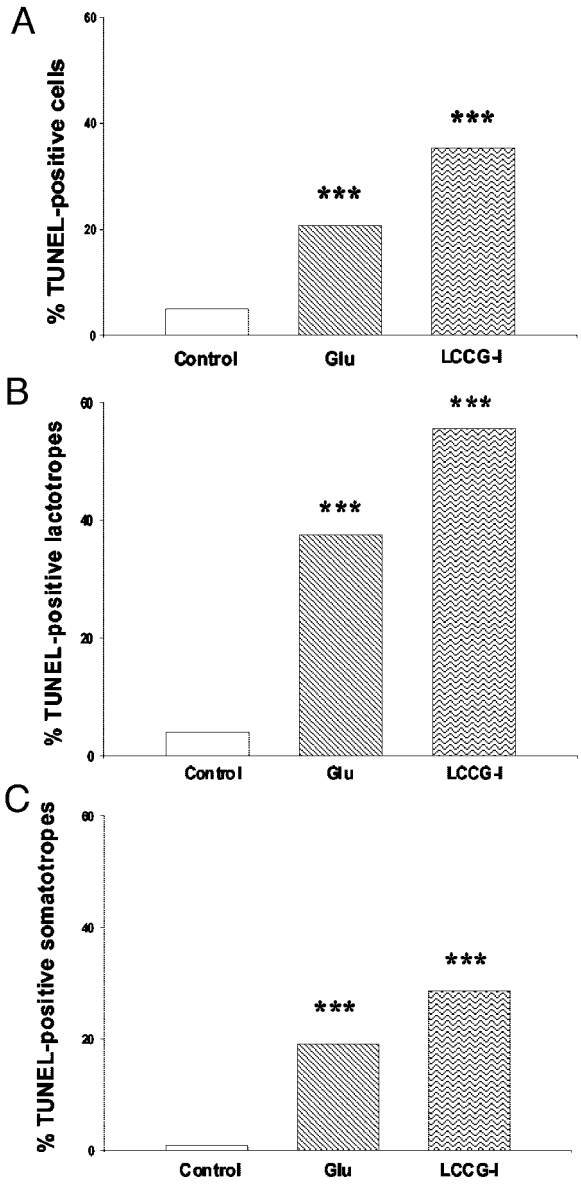

FIG. 2. Apoptotic effect of glutamate and LCCG-I on total anterior pituitary cells (A), lactotropes (B), and somatotropes (C). Anterior pituitary cells were cultured in DMEM-S (10% FBS) for 3 d. Then the medium was replaced by DMEM-S (1% FBS) containing 1 mM glutamate (Glu) or 0.75 mM LCCG-I for 24 h. Data were evaluated by χ^2 test. Each *column* represents the percentage of TUNEL-positive cells of the total number of cells from three independent experiments (A, anterior pituitary cells, n > 2000; B, lactotropes, n > 400; C, somatotropes, n > 300). ***, $P < 0.001$ *vs.* control.

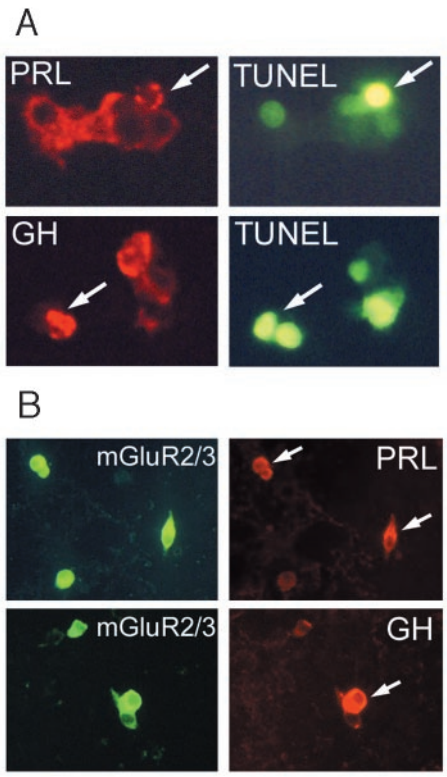

FIG. 3. A, TUNEL-positive lactotropes and somatotropes. Representative anterior pituitary cells showing immunoreactivity for PRL or GH and nuclear condensation by the TUNEL method (*green*); magnification, ×500. B, Immunoreactivity for group II mGluRs in anterior pituitary cells. Anterior pituitary cells were cultured in DMEM-S (10% FBS) for 3 d. Then the medium was replaced by DMEM-S (1% FBS) for 24 h. Immunoreactivity for mGluR2/3 is shown at *left*. The *arrows* show positive cells for mGluR2/3, which are also positive for PRL (*top*) or GH (*bottom*); magnification, ×250.

Glutamate has a dual effect on cell viability in the CNS. Low concentrations of glutamate exert trophic effects and promote neuronal survival and synapse formation, whereas high concentrations are neurotoxic (22). Glutamate neurotoxicity is mediated by calcium signaling (23) and depends on the animal species, developmental stage of the animal, type of agonist, duration of exposure to the agonist, and the cellular expression of the GluR subtypes (23). When the stress is severe, it leads to necrotic cell death, but when it is less severe, apoptosis may be the consequence (23).

Almost every glutamate receptor subtype has been implicated in mediating excitotoxic cell death (24). Different receptor subtypes regulate different cascades of signal transduction leading to apoptosis (25–27). Stimulation of mGluR may initiate both neuroprotective and neurotoxic events in the CNS (5, 6). Activation of group II mGluR has been shown to be either protective (28–32) or ineffective (5). On the other hand, recent reports indicate that group II mGluR agonists are cytotoxic (33, 34). The cytotoxic effects of glutamate have been restricted to the CNS, but the presence of glutamatergic receptors has recently been described in several peripheral tissues including the pituitary (3). Our present data show that glutamate exerts cytotoxic action on the anterior pituitary gland, suggesting that glutamate cytotoxicity can affect peripheral tissues and also brain tissues.

In this study, we show that the proapoptotic action of glutamate is exerted through group II mGluR because glutamate did not reduce cell viability in the presence of EGLU, a specific group II mGluR antagonist. Also, LCCG-I, a specific group II mGluR agonist, decreased the viability of anterior pituitary cells in culture.

Neither iGluR agonists (NMDA, AMPA, and KA) nor their antagonists nor the group I and III mGluR agonists (3-HPG and L-AP4) affected the viability of anterior pituitary cells, suggesting that the cytotoxic action of glutamate may be

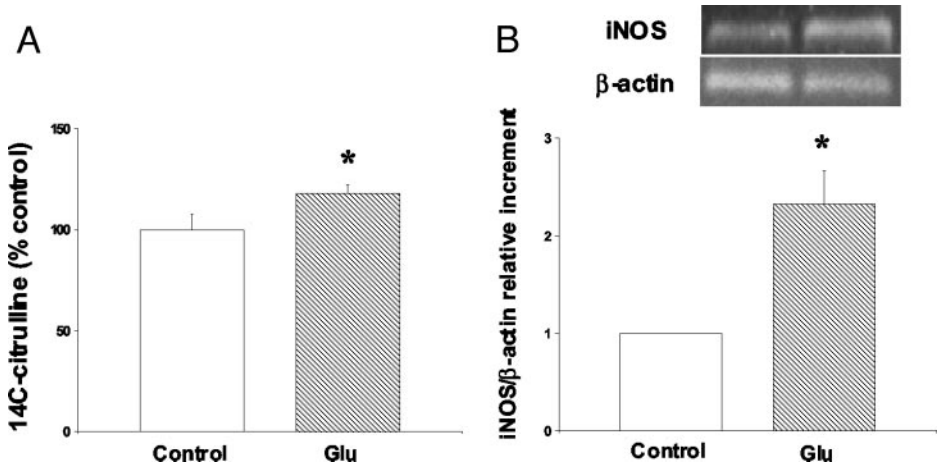

FIG. 4. Effect of glutamate on NOS activity and iNOS gene expression in anterior pituitary cells. Anterior pituitary cells were cultured in DMEM-S (10% FBS) for 3 d. Then the medium was replaced by DMEM-S (1% FBS) containing 1 mM glutamate (Glu) for 24 h. A, Control NOS activity was taken as 100% of activity. Each *column* represents the mean ± SE of six wells in one of two independent experiments. Data were evaluated by Student's *t* test.*, $P < 0.05$ *vs.* control. B, *Columns* represent the relative increase in iNOS mRNA (normalized to β-actin) in respect to control group of three independent experiments. Data were evaluated by one sample *t* test. *, $P < 0.05$ *vs.* control.

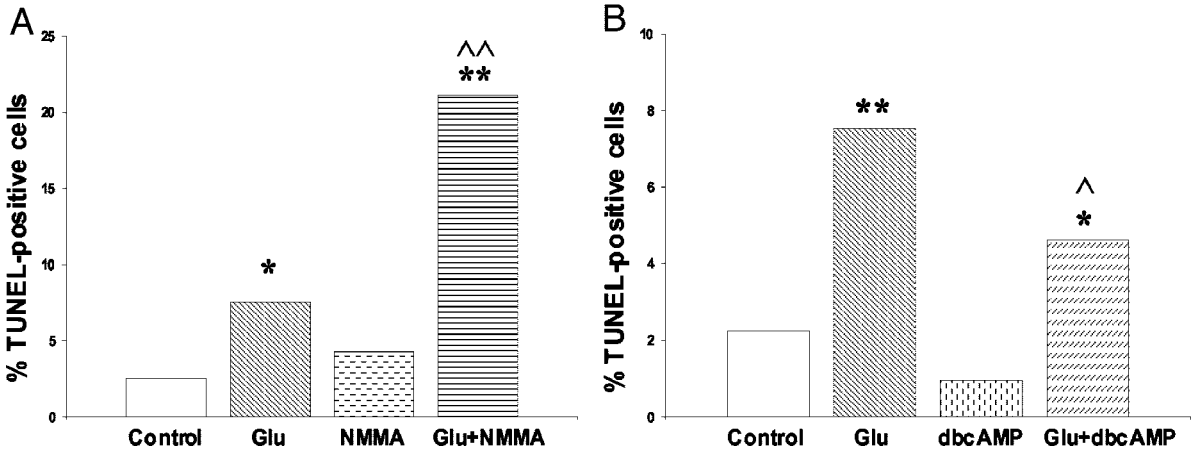

FIG. 5. Effect of NMMA (A) and dbcAMP (B) on glutamate-induced apoptosis of anterior pituitary cells. Anterior pituitary cells were cultured in DMEM-S (10% FBS) for 3 d. Then the medium was replaced by DMEM-S (1% FBS) containing 1 mM glutamate (Glu) and 0.5 mM NMMA (A) or 1 mM dbcAMP (B) for 24 h. Each *column* represents the percentage of TUNEL-positive cells of the total number of cells in each condition (n > 2000) of three independent experiments. Data were evaluated by χ^2 test. *, $P < 0.05$; **, $P < 0.01$ *vs.* respective control without Glu; ^^, $P < 0.01$ *vs.* respective control without NMMA (A); ^, $P < 0.05$ *vs.* respective control without dbcAMP (B).

TABLE 2. Effect of glutamate and glutamate receptor agonists on PRL release from anterior pituitary cells

Concentration (mM)	PRL (ng/well)					
	Glutamate	LCCG-I	NMDA	KA	3-HPG	L-AP4
0	602.57 ± 43.74	700.08 ± 74.48	692.18 ± 71.22	630.84 ± 50.23	745.50 ± 98.47	771.97 ± 56.52
0.1	232.09 ± 27.70[a]	464.26 ± 41.55[b]	787.32 ± 91.17	1229.90 ± 156.96[a]	—	—
0.75	—	295.61 ± 25.31[a]	—	—	—	—
1	184.45 ± 23.78[a]	—	782.76 ± 106.98	1305.26 ± 103.31[a]	641.06 ± 72.83	735.66 ± 89.63
10	189.06 ± 25.59[a]	—	601.62 ± 70.63	1872.40 ± 135.67[a]	611.41 ± 64.82	671.04 ± 40.60

Anterior pituitary cells were cultured in DMEM-S (10% FBS) for 3 d. Then the medium was replaced by DMEM-S (1% FBS) containing increasing concentrations of glutamate or agonists for 24 h. PRL concentration was determined by RIA. Values represent mean ± SE of six to eight wells in one of three independent experiments. Data were evaluated by one-way ANOVA followed by Dunnett's test.

[a] $P < 0.05$ *vs.* control.

[b] $P < 0.01$ *vs.* control.

exerted mainly through activation of group II mGluRs. The lack of effect of the iGluR agonists on the metabolic activity of viable cells may not result from the absence of these receptors in the anterior pituitary gland. NMDA receptor subunit 1 was localized in all the cell types of the anterior pituitary, except in corticotropes (8), and subunits of AMPA receptors were observed in gonadotropes, lactotropes, and somatotropes (9–11). Also, KA receptors were detected in unidentified cell types of the anterior pituitary (12).

In the present work, we localized the mGluR2/3 subunit, specific of group II mGluRs, in lactotropes and somatotropes but not in corticotropes, thyrotropes, or gonadotropes. Both glutamate and LCCG-I induced apoptosis in lactotropes and somatotropes, indicating that the cytotoxic action of gluta-

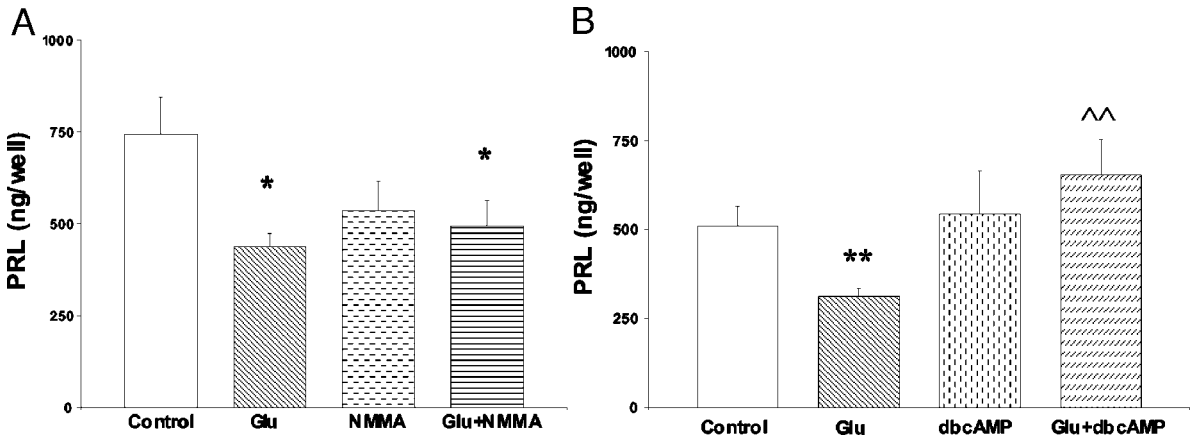

FIG. 6. Effect of NMMA (A) and dbcAMP (B) on the inhibitory effect of glutamate on PRL release from anterior pituitary cells. Anterior pituitary cells were cultured in DMEM-S (10% FBS) for 3 d. Then the medium was replaced by DMEM-S (1% FBS) containing different concentrations of glutamate (Glu) and 0.5 mM NMMA (A) or 1 mM dbcAMP (B) for 24 h. PRL concentration was determined by RIA. Each *column* represents the mean ± SE of six to seven wells in one of three independent experiments. Data were evaluated by two-way ANOVA with analysis of the interaction.*, $P < 0.05$; **, $P < 0.01$ *vs.* respective control without Glu; ^^, $P < 0.01$ *vs.* respective control without dbcAMP (B).

mate is mainly exerted on these cell types through activation of group II mGluR.

Accordingly, with the absence of mGluR2/3 in corticotropes, it was reported that LY341495 (a mGluR2/3 receptor antagonist) has no effect on ACTH secretion from isolated mouse anterior pituitaries (35). Johnson and Chamberlain (36) suggested that the activation of group II mGluR indirectly inhibits PRL secretion by stimulating tubero-infundibular dopaminergic neurons. The presence of mGluR2/3 in lactotropes is consistent with our functional data showing that LCCG-I decreases PRL release from the anterior pituitary gland after 4- (37) or 24-h incubation.

cAMP has been shown to play an important role in cell differentiation during embryo development (38). High levels of cAMP suppress apoptosis in several cell types such as neutrophils, natural killers, and macrophages (39). It has been reported that cAMP response element-binding protein and p53 participate in cell-protective mechanisms elicited by cAMP analogs (40). Bcl-2, a promoter of cell survival, is one of the main targets regulated by cAMP response element-binding protein (41). In addition, cAMP could promote cell survival by activating other protective pathways (42–44). We previously reported that LCCG-I reduces cAMP content in the anterior pituitary (37). Our present results show that a cAMP analog reduces the apoptotic effect of glutamate, suggesting that group II mGluR activation triggers apoptotic events in the anterior pituitary via cAMP.

NO is a free radical gas synthesized from L-arginine by NOS that mediates several actions of glutamate in the CNS (15). Activation of NMDA receptors results in an increase of NO via NOS-dependent pathways. However, activation of non-NMDA iGluRs or mGluRs may produce NO not only through the NOS-dependent pathways but also by a NOS-independent pathway resulting from the release of NO from endogenous deposits of nitrosothioles (45). NO can either protect cells from apoptotic death or mediate apoptosis depending on the cell type and condition (46). Long-term treatment of anterior pituitary cells with NO was reported to induce apoptosis, dependent on caspase-9 and caspase-3 (47). Antiapoptotic NO effects have been shown in a variety of cells and seem to be independent of the apoptotic stimulus (48). Several mechanisms, such as generation of ceramides, S-nytrosilation of caspases, and regulation of Bcl-2 and Bax expression, have been suggested to mediate cytoprotective actions of NO (48). Our results show that glutamate increased NOS activity and iNOS gene expression in the anterior pituitary but failed to modify nNOS gene expression. The apoptotic effect of glutamate in anterior pituitary cells was higher in the presence of NMMA, a NOS inhibitor, suggesting that NO may protect cells from glutamate-induced apoptosis.

We previously demonstrated that 1 mM glutamate induces an increase in PRL release after 4-h incubation (37). In this study, 1 mM glutamate decreased PRL release after 24 h, indicating that long exposure to glutamate induced inhibition of this hormone release in anterior pituitary cells.

Our previous results showed that LCCG-I decreased cAMP levels in anterior pituitary cells (38), whereas our present data show that a cAMP analog reduces the inhibitory effect of glutamate on PRL release in these cells, thus suggesting that inhibition of adenylate cyclase activity could be involved in this effect of glutamate. We previously demonstrated that NO inhibits PRL release from the anterior pituitary gland (19). Nevertheless, NOS inhibition did not modify the decrease of PRL release induced by glutamate, suggesting that NO may not mediate the inhibitory effect of glutamate on PRL release.

In summary, our data demonstrate the presence of group II mGluR in lactotropes and somatotropes and indicate that glutamate induces apoptosis through the activation of this receptor type probably via a decrease of cAMP synthesis. Because GluRs have a wide distribution in peripheral tissues, glutamate-like products that contaminate foods or are used as food additives could have a pathophysiological role in neural and nonneuronal tissues.

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References

1. **Dingledine R, Borges K, Bowie D, Traynelis S** 1999 The glutamate receptor ion channels. Pharmacol Rev 51:7–61
2. **Conn PJ, Pin JP** 1997 Pharmacology and functions of metabotropic glutamate receptors. Annu Rev Pharmacol Toxicol 37:205–237
3. **Gill S, Pulido O** 2001 Glutamate receptors in peripheral tissues: current knowledge, future research, and implications for toxicology. Toxicol Pathol 29:208–223
4. **Meldrum B** 2000 Glutamate as a neurotransmitter in the brain: review of physiology and pathology. J Nutr 130:1007S–1015S
5. **Vincent A, Maiese K** 2000 The metabotropic glutamate system promotes neuronal survival through distinct pathways of programmed cell death. Exp Neurol 166:65–82
6. **Wang Y, Qin ZH, Nakai M, Chen RW, Chuang DM, Chase TN** 1999 Co-stimulation of cyclic-AMP-linked metabotropic glutamate receptors in rat stratium attenuates excitotoxin-induced nuclear factor-κB activation and apoptosis. Neuroscience 94:1153–1162
7. **Dhandapani K, Brann D** 2000 The role of glutamate and nitric oxide in the reproductive neuroendocrine system. Biochem Cell Biol 78:165–179
8. **Bhat G, Mahesh V, Chu Z, Chorich L, Zamorano P, Brann D** 1995 Localization of the N-methyl-D-aspartate R1 receptor subunit in specific anterior pituitary hormone cell types of the female rat. Neuroendocrinology 62:178–186
9. **Petrusz P** 1994 The glutamate subunit GluR1 is expressed in gonadotrophs of the anterior pituitary and is regulated by gonadal feedback. Acta Biol Hung 45:387–397
10. **Login IS** 1990 Direct stimulation of pituitary prolactin release by glutamate. Life Sci 47:2269–2275
11. **Lindstrom P, Ohlsson L** 1992 Effect of *N*-methyl-D,L-aspartate on isolated rat somatotrophs. Endocrinology 131:1903–1907
12. **Hinoi E, Yoneda Y** 2001 Expression of GluR6/7 subunits of kainate receptors in rat adenohypophysis. Neurochem Int 38:539–547
13. **Petralia R, Wang Y, Niedzielski A, Wenthold R** 1996 The metabotropic glutamate receptors, mGluR2 and mGluR3, show unique postsynaptic, presynaptic and glial localizations. Neuroscience 71:949–976
14. **Brann D, Mahesh V** 1997 Excitatory amino acids: evidence for a role in the control of reproduction and anterior pituitary hormone secretion. Endocr Rev 18:678–700
15. **Bredt D, Snyder SH** 1989 Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. Proc Natl Acad Sci USA 86:9030–9033
16. **Kostic T, Andric S, Stojilkovic S** 2001 Spontaneous and receptor-controlled soluble guanylyl cyclase activity in anterior pituitary cells. Mol Endocrinol 15:1010–1022
17. **Qian X, Jin L, Lloyd R** 1998 Percoll density gradient-enriched populations of rat pituitary cells: interleukin 6 secretion, proliferative activity and nitric oxide synthase expression. Endocr Pathol 9:339–346
18. **Ceccatelli S, Hulting A, Zhang X, Gustafsson L, Villar M, Hokfelt T** 1993 Nitric oxide synthase in the rat anterior pituitary gland and the role of nitric oxide in regulation of luteinizing hormone secretion. Proc Natl Acad Sci USA 90:11292–11296
19. **Duvilanski B, Zambruno C, Seilicovich A, Pisera D, Lasaga M, Diaz MC, Belova N, Rettori V, McCann S** 1995 Role of nitric oxide in control of prolactin release by the adenohypophysis. Proc Natl Acad Sci USA 92:170–174
20. **Schwartzman R, Cidlowski J** 1993 Apoptosis: the biochemistry and molecular biology of programmed cell death. Endocr Rev 14:133–151
21. **Negoescu A, Lorimier P, Labat-Moleur F, Drouet C, Robert C, Guillermet C, Brambilla C, Brambilla E** 1996 In situ apoptotic cell labeling by the TUNEL method: improvement and evaluation on cell preparations. J Histochem Cytochem 9:959–968
22. **Zorumski CF, Thio LL** 1992 Properties of vertebrates glutamate receptors: calcium mobilization and desensitization. Prog Neurobiol 39:295–336
23. **Ankarcrona M** 1998 Glutamate induced cell death: apoptosis or necrosis? Prog Brain Res 116:265–272
24. **Sattler R, Tymianski M** 2001 Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. Mol Neurobiol 24:107–129
25. **Maiese K, Ahmad I, TenBroeke M, Gallant J** 1999 Metabotropic glutamate receptor subtypes independently modulate neuronal intracelullar calcium. J Neurosci Res 55:472–485
26. **Vincent AM, TenBroeke M, Maiese K** 1999 Metabotropic glutamate receptors prevent programmed cell death through the modulation of neuronal endonuclease activity and intracellular pH. Exp Neurol 155:79–94
27. **Ahlner J, Ljusegren ME, Grundstrom N, Axelsson KL** 1991 Role of nitric oxide and cyclic GMP as mediators of endothelium-independent neurogenic relaxation in bovine mesenteric artery. Circ Res 1991 68:756–762
28. **Matarredona ER, Santiago M, Venero JL, Cano J, Machado A** 2001 Group II metabotropic receptor activation protects striatal dopaminergic nerve terminals against MPP^+-induced neurotoxicity along with brain-derived neurotrophic factor induction. J Neurochem 76:351–360
29. **Kingston AE, O'Neill MJ, Bond A, Bruno V, Battaglia G, Nicoletti F, Harris JR, Clark BP, Monn JA, Lodge D, Schoepp DD** 1999 Neuroprotective actions of novel and potent ligands of group I and group II metabotropic glutamate receptors. Ann NY Acad Sci 890:438–449
30. **Buisson A, Choi DW** 1995 The inhibitory mGluR agonist, S-4-carboxy-3-hydroxyphenylglycine selectively attenuates NMDA neurotoxicity and oxygen-glucose deprivation-induced neuronal death. Neuropharmacology 34:1081–1087
31. **Bond A, O'Neill MJ, Hicks CA, Monn JA, Lodge D** 1998 Neuroprotective effects of a systemically active group II metabotropic glutamate receptor agonist LY354740 in a gerbil model of global ischaemia. Neuroreport 9:1191–1193
32. **Miyamoto M, Ishida M, Shinozaki H** 1997 Anticonvulsive and neuroprotective actions of a potent agonist (DCG-IV) for group II metabotropic glutamate receptors against intraventricular kainate in the rat. Neuroscience 77:131–140
33. **Taylor DL, Diemel LT, Cuzner ML, Pocock JM** 2002 Activation of group II metabotropic glutamate receptors underlies microglial reactivity and neurotoxicity following stimulation with chromogranin A, a peptide up-regulated in Alzheimer's disease. J Neurochem 82:1179–1191
34. **Venero JL, Santiago M, Tomas-Camardiel M, Matarredona ER, Cano J, Machado A** 2002 DCG-IV but not other group-II metabotropic receptor agonists induces microglial BDNF mRNA expression in the rat stratium. Correlation with neuronal injury. Neuroscience 113:857–869
35. **Scaccianoce S, Matrisciano F, Del Bianco P, Caricasole A, Di Giorgi Gerevini V, Cappuccio I, Melchiorri D, Battaglia G, Nicoletti F** 2003 Endogenous activation of group-II metabotropic glutamate receptors inhibits the hypothalamic-pituitary-adrenocortical axis. Neuropharmacology 44:555–561
36. **Johnson MP, Chamberlain M** 2002 Modulation of stress-induced and stimulated hyperprolactinemia with the group II metabotropic glutamate receptor selective agonist, LY379268. Neuropharmacology 43:799–808
37. **Pampillo M, Theas S, Duvilanski B, Seilicovich A, Lasaga M** 2002 Effect of ionotropic and metabotropic agonists and D-aspartate on prolactin release from anterior pituitary cells. Exp Clin Endocrinol Diabetes 110:138–144
38. **Defer N, Best-Belpomme M, Hanoune J** 2000 Tissue specificity and physiological relevance of various isoforms of adenylyl cyclase. Am J Physiol Renal Physiol 279:F400–F416
39. **Torgersen KM, Vaage JT, Levy FO, Hansson V, Rolstad B, Tasken K** 1997 Selective activation of cAMP-dependent protein kinase type I inhibits rat natural killer cell cytotoxicity. J Biol Chem 272:5495–5500
40. **von Knethen A, Brockhaus F, Kleiter I, Brune B** 1999 NO-evoked macrophage apoptosis is attenuated by cAMP-induce gene expression. Mol Med 5:672–684
41. **Wilson BE, Mochon E, Boxer LM** 1996 Induction of bcl-2 expression by phosphorylated CREB proteins during B-cell activation and rescue from apoptosis. Mol Cell Biol 16:5546–5556
42. **Barger SW, Mattson MP** 1996 Induction of neuroprotective κ B-dependent transcription by secreted forms of the Alzheimer's β-amyloid precursor. Brain Res Mol Brain Res 40:116–126
43. **Filippa N, Sable CL, Filloux C, Hemmings B, Van Obberghen E** 1999 Mechanism of protein kinase B activation by cyclic AMP-dependent protein kinase. Mol Cell Biol 19:4989–5000
44. **Li M, Wang X, Meintzer MK, Laessig T, Birnbaum MJ, Heidenreich KA** 2000 Cyclic AMP promotes neuronal survival by phosphorylation of glycogen synthase kinase 3β. Mol Cell Biol 20:9356–9363
45. **Yamada K, Nabeshima T** 1998 Modulation of nitric oxide production *in vivo* in the brain. Methods Find Exp Clin Pharmacol 20:601–605
46. **Brüne B** 2003 Nitric oxide: NO apoptosis or turning it ON? Cell Death Differ 10:864–869
47. **Velardez MO, Poliandri AH, Cabilla JP, Bodo CC, Machiavelli LI, Duvilanski BH** 2004 Long-term treatment of anterior pituitary cells with nitric oxide induces programmed cell death. Endocrinology 145:2064–2070
48. **Liu L, Stamler J** 1999 NO: an inhibitor of cell death. Cell Death Differ 6:937–942

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