Metabotropic Glutamate Receptors Modulate the NMDA- and AMPA-Induced Gene Expression in Neocortical Interneurons

Group I metabotropic glutamate receptors (mGluRs) can be colocalized with ionotropic glutamate receptors in postsynaptic membranes. We have investigated whether mGluRs alter the gene transcription induced by N-methyl-D-aspartate (NMDA) and (S)-α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid (AMPA) receptors in rat neocortical γ-aminobutyric acid (GABA) interneurons. In cultures of dissociated interneurons, the mGluR1 antagonists LY367385 and MPEP reduced the increase in phosphorylation of the transcription factor CREB induced by NMDA as well as the expression of the proenkephalin (PEnk) gene. In contrast, they enhanced the AMPA-induced CREB phosphorylation and PEnk gene expression. Stimulation of the mGluR1s was due to network activity that caused the release of endogenous glutamate and could be blocked by tetrodotoxin. In organotypic cultures of neocortex, endogenous glutamate enhanced the PEnk gene expression by acting on NMDA and AMPA receptors. These effects were modulated via mGluR5s. In patch-clamp experiments and in biochemical studies on receptor density, stimulation of mGluR1s acutely affected NMDA receptor currents but had no long-term effect on NMDA receptor density at the cell surface. In contrast, stimulation of mGluR5s decreased the density of AMPA receptors located at the cell surface. Our results suggest that mGluR5s regulate the glutamate-induced gene expression in neocortical interneurons in a physiologically relevant manner.

Keywords: CREB, GluR1 subunit, glutamate, MPEP, NR1 subunit

Introduction

Changes in the cytosolic concentration of Ca++ regulate the neuronal expression of numerous genes and thus contribute to neuroplasticity (Ghosh and others 1994; Bitto and others 1996; Impey and others 1998; Bading 2000). Glutamate released from presynaptic terminals can enhance the intracellular Ca++ levels by activating ionotropic receptors of the N-methyl-D-aspartate (NMDA) and (S)-α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid (AMPA) type. Also metabotropic glutamate receptors may be involved.

According to their pharmacological properties, sequence homologies, and signal transduction pathways, metabotropic glutamate receptors have been divided into groups I, II, and III (Conn and Pin 1997; Schoepf and others 1999). Metabotropic glutamate receptor 1 (mGluR1) and mGluR5, the members of group I, exist in several spliced forms (mGluR1α, β, c, and d as well as mGluR5α and b) and can increase phosphatidylinositide hydrolysis. They are mainly found in the postsynaptic membrane, whereas mGluRs of groups II and III seem to be presynaptic (Conn and Pin 1997). mGluR1 and mGluR5 are the subjects of this study and will be summarily addressed as mGluR5s.

In projection neurons from hippocampus, striatum, and neocortex, ionotropic glutamate receptors and mGluRs interact. Thus, mGluR1 agonists such as (RS)-3,5-dihydroxyphenylglycine (DHPG), (RS)-2-chloro-5-hydroxyphenylglycine (CHPG), or (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) potentiate NMDA receptor currents (Doherty and others 1997, 2000; Awad and others 2000; Mannaini and others 2001; Pisani and others 2001; Benquet and others 2002; Heidinger and others 2002). The signal transduction pathways responsible include IP3-induced Ca++ release as well as tyrosine kinases, such as Src/Pyk2, which phosphorylate the NR2A/B receptor subunits (Benquet and others 2002; Heidinger and others 2002, Kotecha and others 2003). In striatal projection neurons, NMDA receptors and mGluRs synergistically activate signal transduction pathways. Thus, simultaneous application of NMDA and DHPG can activate mitogen-activated protein (MAP) kinases and the transcription factor CREB (Mao and others 2002a). This activation may be independent of an NMDA receptor-mediated increase in cytosolic Ca++ concentration (Yang and others 2004). Taken together, the present data obtained with projection neurons show that mGluR1 agonists enhance the response of NMDA receptors. The effect of mGluR1 agonists on AMPA receptors is opposite, that is, they attenuate the responses to AMPA by reducing AMPA receptor density (Snyder and others 2001; Xiao and others 2001; Zhou and others 2002; Huang and others 2004).

Also, interneurons express ionotropic glutamate receptors as well as mGluRs (Masu and others 1991; Porter and others 1998; Lopez-Bendito and others 2002; Goldberg and others 2003; Ferraguti and others 2004). In rat neocortex, fast-spiking (FS) interneurons express mGluR1 and respond to the agonist ACPD with accelerated discharges (Bandrowski and others 2001). These basket cells are GABAergic and contain parvalbumin and somatostatin and make up the majority of interneurons (Markram and others 2004).

In the present study, we have investigated in rat neocortical GABAergic interneurons whether mGluRs change the gene expression induced by stimulation of NMDA or AMPA receptors. In dissociated neurons from postnatal neocortex, we examined the phosphorylation state of the transcription factor CREB at Ser133, which is phosphorylated in a Ca++-dependent manner and plays an essential role in neuronal gene expression and processes like learning and memory (Deisseroth and others 1996, 2003; West and others 2002; Pittenger and Kandel 2003). We stimulated CREB phosphorylation by adding NMDA or AMPA in the absence or presence of mGluR1 antagonists. To evaluate later changes in gene expression, we measured the expression of the proenkephalin (PEnk) gene, which is expressed in neocortical interneurons (Taki and others 2000). Its expression is regulated in a Ca++-dependent manner (Hyman and others 1994; Hamelin and others 2002; Hahm and others 2003). Because the neocortical enkephalin gene expression is enhanced by
stimulation of NMDA as well as AMPA receptors (Just and others 1998), it was possible to examine the interactions of mGluRIs with both ionotropic receptors in their effects on the same gene. Finally, we used organotypic slices of neocortex and striatum to examine whether glutamate regulated the gene expression also in the tissue context by acting simultaneously on ionotropic and metabotropic receptors.

In the cultured neocortical interneurons, endogenous glutamate enhanced the NMDA-induced gene expression by acting via mGluRIs. In contrast, stimulation of mGluRIs by endogenous glutamate decreased the CREB phosphorylation and PENk gene expression induced by the activation of AMPA receptors. In organotypic cultures of neocortex, mGluRIs modulated the gene expression in the same manner, suggesting that they played a physiologically relevant role.

Materials and Methods

Drugs and Plasmids

NMDA, AMPA, kainic acid ((2S,3R,4R)-carboxy-4-(1-methylthienyl)-3-pyrrolidinecarboxylic acid), 6-cyano-7-nitroquinoloxaline-2,3-dione (CNQX), DHPG, (S(+)-2-amino-4-carboxy-2-methylbenzenecarboxylic acid (LY367385), and 2-methyl-6-(phenylethyl)pyridine (MPEP) hydrochloride were from Tocris (Köln, Germany). Phantothxin 433 Tris (trifluoroacetate) salt (PhTX-133), dizocilpine ((+)-MK-801 hydrogen maleate), 1-(+)-aminophenyl-1-ethyl-7,8-methylendioxy-5H-2,3-benzodiazepine hydrochloride (GYK) 52466 hydrochloride, tetrodotoxin (TTX), picrotoxin, and strychnine were from Sigma (Taufkirchen, Germany). Nimodipine (1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinecarboxylic acid-2-methoxethyl-1methylethyl-ester) was from Calbiochem/Merck (Darmstadt, Germany). The PENk cDNA was a gift from Dr Steven Sabol (National Institutes of Health, Bethesda, MD). The cyclophilin cDNA was obtained from Dr Mi (University of Freiburg, Freiburg, Germany).

Cell and Slice Cultures

Primary high- and low-density cultures of dissociated neocortical cells enriched in interneurons were prepared from brains of 1- or 2-day-old Wistar rats. Neocortical tissue was carefully dissected, minced, and then incubated for 10 min with trypsin (Sigma). Enzymatic digestion was stopped by adding fetal calf serum (Biochrom, Berlin, Germany). Cells were dissociated by trituration followed by centrifugation. Finally, cells were taken up in the incubation medium (Neurobasal A medium supplemented with B27; Invitrogen, Karlsruhe, Germany), pH 7.3, and seeded on coverslips precoated with poly-n-lysine and laminin (Sigma). Cells were incubated without a proliferation inhibitor for 10 days in a humidified atmosphere at 37°C. The incubation medium was replaced every third day.

To quantify dead cells, propidium iodide (Sigma) was added to the culture medium at a concentration of 5 µg/ml 20 min before fixation with paraformaldehyde. To estimate the total cell number, 4',6-diamidino-2-phenylindole (DAPI) dilactate (15 µg/ml) was added to the cells for 15 min. Lactate dehydrogenase (LDH) activity in the incubation medium was measured with a Cytotoxicity Detection Kit (LDH) (Roche Applied Science, Mannheim, Germany).

Slice cultures were prepared from brains of 2-day-old Wistar rats. Coronal slices (275 µm thick) of the frontoparietal neocortex with the adjacent neostriatum were cut with a McIlwain tissue chopper. They were incubated on translucent membranes (Millipore, Eschborn, Germany) at 37°C in a humidified atmosphere (5% CO₂) (Stoppini and others 1991; Heimrich and Frotscher 1994). The incubation medium consisted of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered minimum essential medium (50%), Hank’s balanced salt solution (25%), and heat-inactivated horse serum (25%) (Invitrogen). It was replaced three times a week. The slices were incubated for 5 days. Then, the actual experiments were started by adding the drugs to the incubation medium. Three days later, the slices were fixed for in situ hybridization.

Northern Blot and In Situ Hybridization

For northern blot hybridization, dissociated neocortical neurons were seeded in petri dishes (0.1 cm²) precoated with poly-n-lysine and laminin. Total RNA was extracted with 7.6 M guanidine hydrochloride as described previously (Cheley and Anderson 1984) and separated over a denaturing 1.25% agarose/66% formaldehyde gel. The running buffer contained 20 mM 4-morpholinopropanesulfonic acid (MOPS), 8 mM NaOAc, and 1 mM ethylenediaminetetraacetic acid (EDTA). The RNA bands were electroblotted onto a nylon membrane (Roche Applied Science). The membranes were cohybridized with digoxigenin-labeled antisense probes for PENk and cyclophilin mRNA at 65°C overnight and then washed with 0.2x sodium chloride/sodium citrate buffer (SSC) at 65°C (Höltte and Kessler 1990). The digoxigenin-labeled cRNAs were generated in vitro transcription with use of the T77T3 18U vector (Pharmacia, Freiburg, Germany) (Höltte and Kessler 1990; Theodoridou and others 1994). The cRNA for PENk was 935 nucleotides in length and complementary to the coding region of PENk mRNA (Yoshikawa and others 1984). The cyclophilin cRNA contained 510 bp and was complementary to the complete coding region of the cyclophilin mRNA (Haendler and others 1994). Digoxigenin-labeled mRNA hybrids were detected and quantified according to the applications manual "DIG DNA labeling and detection" (Roche Applied Science).

In situ hybridization, cell or slice cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 4 h. After prehybridization for 4 h at 65°C, they were hybridized overnight at 65°C with the sense or antisense probe (concentration 200 ng/ml) and then washed in 0.2x SSC at 65°C. For detection of PENk mRNA, the antisense probe described above was used, whereas the corresponding sense probe was used for hybridization controls. To enhance tissue penetration and reduce nonspecific background, the probes were alkali hydroyzed to an average length of 100-200 bases. This antisense probe detected a single specific band of 1.4 kb in a filter hybridization assay performed under identical conditions (Theodoridou and others 1994) and was tested by in situ hybridization of adult rat caudate–putamen. Digoxigenin-labeled mRNA hybrids were detected as described above. Color development was performed in the dark with substrate solution of nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate (Roche Applied Science). Cells used for in situ hybridization were additionally immunostained for β-tubulin III and DAPI (15 µg/ml) (Molecular Probes, Leiden, Netherlands), added to the cells for 15 min.

Quantification of PENk Gene Expression

In experiments with primary cultures, each group consisted of 3 or more coverslips. Cells containing visible amounts of PENk mRNA were counted in randomly chosen areas (0.4 mm²) of the respective coverslips. Each experiment was repeated at least once. In experiments with slice cultures, experimental groups consisted of 3 slices, Positive cells were counted in randomly chosen areas (0.4 mm²). Each experiment was repeated at least once. Within 1 experiment, the mean number of positive cells of the group showing the most positive neurons was taken as 100%, and the numbers of all groups were expressed as the percentage of this mean value.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde and 4% sucrose in PBS (pH 7.4) for 20 min at room temperature and washed three times with PBS. Then, they were treated for 20 min with PBS containing 0.1% (v/v) Triton X-100 and 4% normal goat serum. For immunocytochemistry of glutamic acid decarboxylase 65/67 (GAD65/67), cells were not treated with normal goat serum but additionally incubated in 100% methanol at −20°C for 10 min and washed three times with PBS–Twee (0.1% (v/v) for 5 min as previously described (Tominaga and others 2005). For labeling of surface AMPA and NMDA receptors, unspecific binding was blocked under nonpermeabilizing conditions. Then, the cells were incubated in working dilutions of primary antibodies at 4°C overnight. The primary antibodies were mouse anti-β-tubulin III (1:1000) (Sigma), mouse anti-enkephalin (MAB350) (1:200), mouse anti-NR1 (MAB363) (1:2000), rabbit anti-calbindin D-28K (1:1000), rabbit anti-GAD65/67 (1:2000) (Chemicon, Temecula, CA), rabbit anti-phospho-CREB (Ser-133) (1:500) (Cell Signaling Technology Inc., Beverly, MA), rabbit anti-GluR1 against an extracellular epitope (aa 271–285) of rat GluR1 subunit.

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CaCl$_2$ 1.3, MgCl$_2$ 1, HEPES 10, and glucose 11. The solution had an ionic composition of 140 mM NaCl, 1.25 mM NaH$_2$PO$_4$, 26 mM NaHCO$_3$, 0.8 mM MgCl$_2$, 1.8 mM CaCl$_2$, and 10 mM dextrose; saturated with 95% O$_2$ and 5% CO$_2$). Then, the cells were rinsed with tris-buffered saline (TBS) to quench the biotinylation reaction. The remaining supernatant was incubated with 600 μg/ml of 50% neutravidin agarose (Pierce Chemical Company) for 3 h at 4 °C and washed four times with TBS buffer. Agarose-bound proteins were taken up in 20 μl of SDS sample buffer and boiled.

**Analysis of Immunocytochemical Data**

Cells were cultured by using a BioRad MRC 1024 (version 3.2) confocal system with a krypton–argon laser and a Zeiss (Oberkochen, Germany) Axiosvert135 microscope. Images were obtained by using Laser sharp2.1T software and processed with the MRC 1024 software. Single z-stack micrographs were obtained with identical settings of the confocal system within each experiment. The average intensities of enkephalin immunostaining were measured in 12 randomized 250-μm$^2$ fields per coverslip as supplied by the histogram function of MRC 1024 software. Six coverslips originating from 2 different experiments were used. pCREB: only the perikarya of interneurons were analyzed. The average intensities of pCREB staining of 12 randomized chosen interneurons were measured as supplied by the histogram function. Six coverslips originating from 2 different experiments were used. GluR1: the perikarya and neurites of interneurons were analyzed. The average intensities of GluR1 staining of 8–12 randomized chosen interneurons were measured as supplied by the histogram function; 6 coverslips originating from 2 different experiments were used.

**Biotinylation Assay for Surface Receptors**

Ten-day-old cultures of postnatal neocortical neurons were used. The biotinylation procedure was performed as described previously (Chung and others 2000; Snyder and others 2001). After pretreatment with drugs for 24 h, the culture dishes were placed on ice and washed twice with ice-cold artificial cerebrospinal fluid (ACSF): 124 mM NaCl, 5 mM KCl, 1.25 mM NaH$_2$PO$_4$, 26 mM NaHCO$_3$, 0.8 mM MgCl$_2$, 1.8 mM CaCl$_2$, and 10 mM dextrose; saturated with 95% O$_2$ and 5% CO$_2$. Then, the cultures were incubated for 30 min on ice with ACSF that contained 1 mg/ml sulfo-NHS-LC-biotin (Pierce Chemical Company, Rockford, IL). After rinsing with Tris-buffered saline (TBS) to quench the biotin reaction, cells were lysed in 100 μl of modified radioimmuno precipitation assay (RIPA) buffer (1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholic acid, 50 mM NaPO$_4$, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mg/ml leupeptin). The homogenates were centrifuged at 14 000 rpm for 30 min at 4 °C. An aliquot (15%) of the supernatant was removed to measure total GluR1 or NR1. The remaining supernatant was incubated with 60 μl of 50% neutravidin agarose (Pierce Chemical Company) for 3 h at 4 °C and washed four times with RIPA buffer. Agarose-bound proteins were taken up in 20 μl of SDS sample buffer and boiled. Quantitative western blots were performed on both total and biotinylated (surface) proteins using rabbit anti-GluR1 C-terminal (1:1000) and Alexa fluor 488 goat anti-rabbit IgG (Molecular Probes) were used as second antibodies.

**Electrophysiology**

Whole-cell recordings from rat cortical neurons were performed in low-density cultures incubated for 10–14 days. Current- and voltage-clamp experiments were made with an extracellular patch clamp (EPC-9) amplifier (HEKA, Lambrrecht, Germany) at room temperature (20–24 °C). The external bath solution contained (in mM) NaCl 162, KCl 2.4, CaCl$_2$ 1.3, MgCl$_2$ 1, HEPES 10, and glucose 10. The solution had an osmolarity of ~320 mosm/L and a pH value of 7.3. Action potential waveforms of rat cortical neurons were recorded in the fast current-clamp mode of the EPC-9. In these experiments, the pipette solution contained (in mM) potassium glutonate 144, MgCl$_2$ 3, HEPES 10, ethylene glycol-bis(2-aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA) 0.2, Mg-adenosine triphosphate (ATP) 4, and Li-guanosine triphosphate (GTP) 0.3 (~290 mosm, pH 7.2 with KOH). Filled patch pipettes had a resistance of ~2 MΩ. Prior to the start of the current-clamp experiments, the largest somatic diameter of all neurons was measured under phase contrast optics. The resting membrane potential was measured just after passing to the whole-cell configuration. All membrane potentials were corrected for a liquid junction potential of 16 mV. The membrane potential was then adjusted to ~70 mV by continuous current injections. The input resistance ($R_I$) of the cells was determined by hyperpolarizing current pulses inducing voltage shifts <15 mV. The analysis of the waveform of the first 2 action potentials was performed on spikes elicited by short, just suprathreshold depolarizing current pulses (80 ms, sampling rate 62.5 kHz, filter 20.8 kHz). Action potential amplitude and action potential duration were measured from the onset of the fast spike upstroke to the peak and at half amplitude, respectively. The after-hyperpolarization (AHP) was measured as the difference between the spike onset and the voltage minimum following the action potential peak. The time to peak of AHP was the time difference between the spike onset and this voltage minimum. Maximum steady-state firing rate and adaptation of action potential firing were measured from discharges elicited by 800-ms duration depolarizing current pulses (sampling rate 20 kHz, filter 6.7 kHz), which were increased in strength (10-pA increments) until spike failure occurred. Instantaneous spike frequencies ([1/inter spike interval] × 100) were measured by determining the times at which the rising phase of the action potentials crossed a fixed threshold potential. The maximum steady-state firing rate was then computed as the average of instantaneous frequency for the last 3 intervals of those trains of action potentials elicited by the 3 current strengths before that which caused spike failure. To assess spike frequency adaptation (A), the instantaneous discharge frequencies between the first 2 spikes ($f_{200}$) and 400 ms after the beginning of the discharge ($f_{400}$) were measured. Adaptation was then calculated according to: $A_{200} = f_{200} / f_{400}$, and the reported $A_{200}$ was an average of the $A_{200}$ values measured from the traces used to calculate the maximum steady-state firing frequency. NMDA- and AMPA-induced transmembrane currents were measured in neurons voltage clamped at a holding potential of ~70 mV. In these experiments, Mg$^{2+}$ was omitted from the bath solution, which also contained glycine (10 μM), TTX (0.3 μM), picROTOXIN (100 μM), and strychnine (2 μM). Patch pipettes had a resistance of 1–5 MΩ when filled with the intracellular solution (in mM: CsCl 95, CaCl$_2$ 1, MgCl$_2$ 6, HEPES 10, EGTA 11, ATP potassium salt 4, potassium creatine phosphate 20, and 50 μM phosphocreatine kinase; pH 7.2 with CsOH; ~290 mosm). In these experiments, no corrections were made for a small liquid junction potential of 5 mV. During experiments, series resistance did not change significantly and was 17.0 ± 1.4 MΩ and 18.7 ± 1.5 MΩ ($n = 108$) at the start and end of the trials, respectively, thus indicating stable membrane conditions. Current membrane currents were evoked by focal pressure application (DAD-12; Adams and List, New York, NY) of either NMDA (30 μM) or AMPA (30 μM). They were filtered at 3 kHz and digitized at 1 kHz (EPC-9). For statistical evaluation, peak currents amplitudes were measured (PULSE software, HEKA).

**Statistical Analysis**

Results are expressed throughout as means ± standard error of mean (SEM). $n$ shows the number of areas counted in the coverslips of at least 2 separate experiments. Immunocytochemical and electrophysiological data were evaluated by Kruskal–Wallis test and Mann–Whitney $U$-test. $p < 0.05$ was the accepted level of significance.

**Results**

**Cultured Neocortical Interneurons Are Mainly GABAergic**

As shown by β-tubulin III immunostaining (Fig. 1B), our high-density cultures of postnatal neocortical cells contained a dense network of neurons. In our experiments, we counted between 700 and 870 neurons per counting area (0.4 mm$^2$) (see also Supplementary Table 1, A). Approximately 86% of these neurons also expressed GAD65/67, indicating that they were GABAergic inhibitory interneurons (Fig. 1A and Supplementary Table 1, A). The calcium-binding proteins calretinin, calbindin, and parvalbumin mark subpopulations of GABAergic interneurons, although with partial overlaps (Gonchar and Burkhalter 1999; Markram and others 2004). In our immunocytochemical analysis (Fig. 1B), we did not consider the possible coexpression of

mGluRs and Gene Expression in Neocortical Interneurons · Lindemeyer and others
NMera Enhances the Expression of the PENk Gene in Cultured GABAergic Interneurons

Under basal conditions, a negligible number of the neurons expressed the PENk gene when examined with in situ hybridization for PENk mRNA (Fig. 1A). NMera (8 μM) applied for 24 h induced a visible increase in the amount of PENk mRNA in approximately 150 of 700 (21%) interneurons. Typically, this staining was perinuclear and was not observed after hybridization with a sense probe (Fig. 1A). A total of 95 ± 1.2% of the neurons that responded to NMera with a visibly enhanced PENk mRNA content expressed GAD65/67 (n = 10) (Fig. 1A).

When added to the incubation medium for 24 h, NMera exerted a concentration-dependent effect. The lowest effective concentration was 4 μM. NMera (8 μM) produced the strongest effect (Fig. 1C, lower panel), although this concentration was far below the 50% effective concentration value of 28 μM reported in electrophysiological studies (Nörenberg and Meyer 2003). At NMera concentrations of 16 μM or 32 μM, only a few neurons with visible amounts of PENk mRNA were found. In these cultures, the occurrence of granular debris indicated pronounced cell damage and death. Therefore, we quantified the number of dead cells by analyzing the cellular uptake of propidium iodide (Fig. 1C, upper panel). Compared with controls, 16 μM and 32 μM NMera increased cell death by a factor of 7.4 and 14.6, respectively. In contrast, 8 μM NMera did not increase the number of propidium iodide-positive cells.

In addition, we measured the concentration of LDH in the cultures, the occurrence of granular debris indicated pronounced cell damage and death. Therefore, we quantified the number of dead cells by analyzing the cellular uptake of propidium iodide (Fig. 1C, upper panel). Compared with controls, 16 μM and 32 μM NMera increased cell death by a factor of 7.4 and 14.6, respectively. In contrast, 8 μM NMera did not increase the number of propidium iodide-positive cells. In addition, we measured the concentration of LDH in the cultures, which is released by damaged cells. Compared with controls, the apparent LDH release was not enhanced (Supplementary Table 1, B). Also, the number of GAD65/67-containing neurons was not changed by NMera treatment (Supplementary Table 1, A).

To study the time course of the NMera-induced PENk gene expression, NMera (8 μM) was added to the culture medium for 8, 16, or 24 h. To normalize the effects of the shorter incubation periods, the number of PENk mRNA-positive cells found after the 24-h exposition to NMera was set at 100% (± 7.5%). Incubation with NMera for 16 or 8 h raised the number of positive cells only to 78.0 ± 6.2% or 14.7 ± 2.4%, respectively (n = 50). To study whether NMera induced an initial effect that then needed up to 24 h for its full development, we incubated the cells with 8 μM NMera for a period of 2, 4, 8, or 16 h.

Figure 1. NMera increases the expression of the PENk gene in cultured neocortical interneurons. (A) Upper panel: PENk mRNA after in situ hybridization with an antisense probe in controls and in cells treated with 8 μM NMera for 24 h. After hybridization with a sense probe, no positive cells were found. Lower panel: staining of cells with a GAD65/67 antibody after PENk mRNA in situ hybridization. Arrows indicate the same cells. Cultures were treated with 8 μM NMera for 24 h. Scale bar = 10 μm. (B) Cells were treated with NMera (8μM) for 24 h. In situ hybridization for PENk mRNA was performed first, and then additional immunostaining for the neuronal marker β-tubulin III as well as the calcium-binding proteins calretinin, parvalbumin, or calbindin was performed. Pictures show the same cells after the respective immunostaining on the left side and after in situ hybridization on the right side. Arrows indicate the same cells;
Subsequently, the incubation medium was replaced with medium free of NMDA, and the cells were further incubated up to 24 h. Again, the number of positive cells counted after a 24-h incubation with NMDA was set at 100% (±5.3%). Transient incubation with NMDA for 12, 6, or 3 h raised the number of positive cells only to 44.5 ± 3.2%, 17.6 ± 2.1%, or 4.4 ± 1%, respectively (n = 50). Based on these results, we performed the subsequent experiments by adding 8 μM NMDA to the incubation medium for 24 h.

Dizocilpine (100 nM), which blocks the ion pore of the NMDA receptor in a use-dependent manner (Foster and Wong 1987), had no effect on the Penk gene expression when used alone (Fig. 1D). It prevented the effect of 8 μM NMDA on the Penk gene expression, confirming the involvement of NMDA receptors. In contrast, CNQX, which blocks AMPA and kainate receptors, had no effect on the increase in gene expression induced by NMDA. It was also without any effect when applied alone (Fig. 1D). To study whether voltage-gated L-type Ca²⁺ channels (L-VGCCs) mediated the effects of NMDA on gene expression, we blocked the channels with nimodipine (2 μM). This treatment abolished the effect of NMDA (Fig. 1D).

**mGluRI Antagonists Decrease the Effect of NMDA Receptors on Penk Gene Expression**

To study the role of metabotropic glutamate receptors in NMDA-induced Penk gene expression, we blocked mGluR1 and mGluR5 with the competitive agonist LY367385 and the noncompetitive antagonist MPEP, respectively (Gasparini and others 1999; Herman and others 1999). When used alone, LY367385 as well as MPEP diminished the NMDA-induced increase in the Penk gene expression in a concentration-dependent manner (Table 1). MPEP (128 nM) reduced it by 30%, whereas LY367385 (80 μM) reduced it by 54%. Combined application of both inhibitors did not have a stronger effect. After combined treatment with NMDA and both mGluRI antagonists, 95.9 ± 0.8% (n = 20) of the neurons with visible Penk mRNA were GAD65/67 positive, indicating that also under these conditions the gene was predominantly expressed in inhibitory interneurons. When applied in the absence of NMDA, both inhibitors had no effect on the Penk gene expression (Table 1). To exclude that the mGluR antagonists decreased gene expression by increasing cell death, we counted the total number of neurons. Compared with controls, neither NMDA nor its combination with the mGluR antagonists changed this number (Supplementary Table 1, A). In addition, we measured the uptake of propidium iodide and the apparent LDH release. The antagonists increased neither the number of propidium iodide–positive cells nor the apparent LDH release (Supplementary Table 1, B), confirming that cell damage was not involved.

Because the mGluR antagonists inhibited the NMDA-induced gene expression in our high-density cultures, we hypothesized that the mGluRs were activated by endogenous glutamate released from the neuronal network by the excitatory stimulus. To test this hypothesis, we used TTX to block voltage-gated Na⁺ channels and thus interrupt the network activity. Simultaneous application of 1 μM TTX reduced the effect of NMDA on the Penk gene expression by approximately 50% (Table 2, A). In the presence of TTX, the combination of LY367385 and MPEP no longer reduced the NMDA-induced gene expression (Table 2, B). However, the mGluR agonist DHPG (90 μM) (Ito and others 1992) reversed the reduction in NMDA-induced gene expression caused by TTX (Table 2, B). This effect of DHPG was mediated by mGluRs because it was prevented by MPEP plus LY367385. In contrast, DHPG did not change the effect of NMDA in the absence of TTX. Taken together, these results suggested that NMDA stimulated the release of endogenous glutamate, which then acted on mGluRs and thus synergistically affected the NMDA receptor action on Penk gene expression.

![Image](https://academic.oup.com/cercor/article-abstract/16/11/1662/320292)

**Table 2**

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<td>DHPG (10 μM) (6 h)</td>
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<td>DHPG (10 μM) (12 h)</td>
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Note: A—cultures were treated for 24 h with the drugs at the concentrations shown. The average number of neurons found with in situ hybridization for Penk mRNA after treatment with 8 μM NMDA was set at 100%. Mean ± SD (n = 30–40); * = significant difference (P < 0.05) to controls, ** = significant difference (P < 0.05) to NMDA-treated cells.

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**Table 1**

<table>
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<th>Penk mRNA-positive neurons (%) of NMDA</th>
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<tr>
<td>Control</td>
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<tr>
<td>NMDA (8 μM)</td>
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<td>NMDA + LY367385 (1.25 μM)</td>
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<td>LY367385 (80 μM) + MPEP (128 nM)</td>
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Note: Cultures were treated for 24 h with the drugs at the concentrations shown. The average number of neurons found with in situ hybridization for Penk mRNA after treatment with 8 μM NMDA was set at 100%. Mean ± SD (n = 30–40); * = significant difference (P < 0.05) to controls, ** = significant difference (P < 0.05) to NMDA-treated cells.

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**mGluRs and Gene Expression in Neocortical Interneurons**

Lindemeyer and others
mGluRI Antagonists Increase the Effect of AMPA Receptors on PEnk Gene Expression

In rat neocortex, stimulation of not only NMDA but also AMPA receptors induces the expression of the PEnk gene (Just and others 1998). Next, we studied whether mGluRs also affected the gene expression induced by AMPA. In our neocortical cultures, AMPA exerted a concentration-dependent effect on the number of neurons that contained visible amounts of PEnk mRNA (Fig. 2A, lower panel). Because 95 ± 1.4% of these neurons were positive for GAD65/67 (n = 20), inhibitory interneurons expressed the gene. AMPA was ineffective at concentrations of 0.5 and 1 μM but increased the expression at concentrations of 2 and 4 μM. The increase caused by the latter concentration amounted to approximately 25% of the effect induced by 8 μM NMDA. At a concentration of 8 μM, AMPA only weakly stimulated the PEnk gene expression but increased the number of propidium iodide-positive cells by a factor 18.7, suggesting cytotoxic effects (Fig. 2A, upper panel). However, 4 μM AMPA increased neither the number of propidium iodide-positive cells nor the apparent LDH release (Supplementary Table 1, B). Also, the number of GAD65/67-positive neurons remained unchanged (Supplementary Table 1, A). The increase in PEnk gene expression induced by 4 μM AMPA was not affected by the NMDA receptor antagonist dizocilpine but was blocked by 20 μM CNQX (Fig. 2B), which blocks AMPA as well as kainate receptors. It was also blocked by 20 μM GYKI 52466 (Fig. 2B). This noncompetitive antagonist of AMPA/kainate receptors is 45-fold more potent at AMPA than at kainate receptors (Dai and others 2001). To investigate whether AMPA receptors with Ca"++ conductivity were involved (Hollmann and others 1991; Jonas and others 1994), we used the polyamine toxin PhTX-433, which is known to selectively block Ca"++-permeable receptors lacking the GluR2 subunit (Waslburn and Dingledine 1996; Toth and McBain 1998). When used at concentrations of 2, 10, or 50 μM, PhTX-433 did not affect the increase in PEnk gene expression caused by AMPA (Fig. 2B). In contrast, blocking L-VGCCs with nimodipine (2 μM) prevented the effect of 4 μM AMPA on the gene expression (Fig. 2B).

Blocking mGluR1 or mGluR5 with LY367385 plus MPEP enhanced the effect of AMPA on the PEnk gene expression by approximately 40% or 70%, respectively (Fig. 2C). Combined application of both antagonists had no additional effect. Also, after cotreatment with AMPA and the mGluRI antagonists, 95.8 ± 0.8% of the neurons containing PEnk mRNA were GAD65/67 positive. The cotreatment affected neither the apparent LDH release of the cells (Supplementary Table 1, B). Compared with controls, (2 μM) were applied 30 min prior to AMPA (4 μM). The average number of positive neurons found with in situ hybridization after treatment with 4 μM AMPA was set at 100%. Mean ± SEM (n = 30–40); * = significant difference (P < 0.05) to controls; ** = significant difference (P < 0.05) to neurons treated with AMPA alone. (C) mGluRI antagonists facilitate the AMPA-induced increase in the PEnk gene expression. Neurons were treated for 24 h with AMPA (4 μM), DHPG (90 μM), LY367385 (80 μM), or MPEP (128 nM). The average number of positive neurons found with in situ hybridization after treatment with AMPA was set at 100%. Mean ± SEM (n = 30–40); * = significant difference (P < 0.05) to controls; ** = significant difference (P < 0.05) to neurons treated with AMPA alone. (D) mGluRs do not modulate the increase in the PEnk gene expression caused by activation of L-VGCCs with KC. Neurons were treated for 24 h with KCi (25 mM), nimodipine (2 μM), CNOX (20 μM), PhTX-433, and nimodipine (100 mM), LY367385 (80 μM), or MPEP (128 nM). The average number of positive neurons found with in situ hybridization after treatment with KCi alone was set at 100%. Mean ± SEM (n = 30); * = significant difference (P < 0.05) to controls, ** = significant difference (P < 0.05) to neurons treated with KCi alone.

Figure 2. AMPA increases the number of neurons that contain visible amounts of PEnk mRNA after in situ hybridization. (A) AMPA changes the PEnk gene expression in a concentration-dependent manner. Neurons were treated for 24 h with AMPA at the concentrations shown. The average number of PEnk mRNA-positive neurons found with in situ hybridization after treatment with 4 μM AMPA was set at 100% (n = 30–40). The ratio of propidium iodide- and DAPI-positive cells is shown in the upper panel (n = 20–30); mean ± SEM; * = significant difference (P < 0.05) to controls. (B) Ca"++-impermeable AMPA receptors mediate the PEnk gene expression via L-VGCCs. GYKI 52466 (20 μM), dizocilpine (100 nM), CNOX (20 μM), PhTX-433, and nimodipine (2 μM) were applied 30 min prior to AMPA (4 μM). The average number of positive neurons found with in situ hybridization after treatment with 4 μM AMPA was set at 100%. Mean ± SEM (n = 30–40); * = significant difference (P < 0.05) to controls; ** = significant difference (P < 0.05) to neurons treated with AMPA alone. (C) mGluRI antagonists facilitate the AMPA-induced increase in the PEnk gene expression. Neurons were treated for 24 h with AMPA (4 μM), DHPG (90 μM), LY367385 (80 μM), or MPEP (128 nM). The average number of positive neurons found with in situ hybridization after treatment with AMPA was set at 100%. Mean ± SEM (n = 30–40); * = significant difference (P < 0.05) to controls; ** = significant difference (P < 0.05) to neurons treated with AMPA alone. (D) mGluRs do not modulate the increase in the PEnk gene expression caused by activation of L-VGCCs with KC. Neurons were treated for 24 h with KCi (25 mM), nimodipine (2 μM), CNOX (20 μM), PhTX-433, and nimodipine (100 mM), LY367385 (80 μM), or MPEP (128 nM). The average number of positive neurons found with in situ hybridization after treatment with KCi alone was set at 100%. Mean ± SEM (n = 30); * = significant difference (P < 0.05) to controls, ** = significant difference (P < 0.05) to neurons treated with KCi alone.

Figure 2. AMPA increases the number of neurons that contain visible amounts of PEnk mRNA after in situ hybridization. (A) AMPA changes the PEnk gene expression in a concentration-dependent manner. Neurons were treated for 24 h with AMPA at the concentrations shown. The average number of PEnk mRNA-positive neurons found with in situ hybridization after treatment with 4 μM AMPA was set at 100% (n = 30–40). The ratio of propidium iodide- and DAPI-positive cells is shown in the upper panel (n = 20–30); mean ± SEM; * = significant difference (P < 0.05) to controls. (B) Ca"++-impermeable AMPA receptors mediate the PEnk gene expression via L-VGCCs. GYKI 52466 (20 μM), dizocilpine (100 nM), CNOX (20 μM), PhTX-433, and nimodipine (2 μM) were applied 30 min prior to AMPA (4 μM). The average number of positive neurons found with in situ hybridization after treatment with 4 μM AMPA was set at 100%. Mean ± SEM (n = 30–40); * = significant difference (P < 0.05) to controls; ** = significant difference (P < 0.05) to neurons treated with AMPA alone. (C) mGluRI antagonists facilitate the AMPA-induced increase in the PEnk gene expression. Neurons were treated for 24 h with AMPA (4 μM), DHPG (90 μM), LY367385 (80 μM), or MPEP (128 nM). The average number of positive neurons found with in situ hybridization after treatment with AMPA was set at 100%. Mean ± SEM (n = 30–40); * = significant difference (P < 0.05) to controls; ** = significant difference (P < 0.05) to neurons treated with AMPA alone. (D) mGluRs do not modulate the increase in the PEnk gene expression caused by activation of L-VGCCs with KC. Neurons were treated for 24 h with KCi (25 mM), nimodipine (2 μM), CNOX (20 μM), PhTX-433, and nimodipine (100 mM), LY367385 (80 μM), or MPEP (128 nM). The average number of positive neurons found with in situ hybridization after treatment with KCi alone was set at 100%. Mean ± SEM (n = 30); * = significant difference (P < 0.05) to controls, ** = significant difference (P < 0.05) to neurons treated with KCi alone.
AMPA alone or combined with the mGluRI antagonists did not change the total number of neurons (Supplementary Table 1, A), confirming that cell damage or even death were negligible. In contrast to the antagonists, the agonist DHPG had no additional effect on the gene expression caused by AMPA (Fig. 2C).

To confirm that the changes found with in situ hybridization indeed reflected those in gene expression, we used northern blot hybridization as a biochemical measurement of Penk mRNA. The increase in Penk mRNA caused by NMDA was indeed reduced by the mGluRI antagonists (Fig. 3A). Northern blot hybridization also showed that the mGluR antagonists enhanced the AMPA-induced Penk gene expression (Fig. 3A).

The Effect of mGluRIs on Penk Gene Expression Is Linked to Ionotropic Glutamate Receptors

In striatal projection neurons, DHPG has been shown to increase Penk gene expression independent of ionotropic glutamate receptors (Mao and Wang 2001). To find out whether activation of mGluRs also directly elevated the Penk gene expression in neocortical interneurons, we used the agonist DHPG. At concentrations of 10 or 90 nM, DHPG slightly increased the number of neurons with visible Penk mRNA. Whereas we found 1 neuron per counting area in controls, 3 or 4 were positive when DHPG (10 μM) was applied for 12 or 24 h, respectively (Table 2, C). At a concentration of 90 nM, DHPG had similar effects. In contrast, NMDA elevated the number of Penk mRNA-containing neurons by a factor of 30–40 and caused a more intense staining per neuron.

As shown in Figures 1D and 2B, L-VGCCs were essential for the NMDA- and AMPA-induced Penk gene expression. Because mGluRs can modulate these channels (Sayer and others 1992; Sahara and Westbrook 1993; Mao and Wang 2002b), we examined whether the mGluRs antagonists affected the Penk gene expression by acting on these channels. To activate L-VGCCs independent of glutamate receptors, we used KCl-mediated membrane depolarization (Fig. 2D). KCl (25 mM) applied for 24 h increased the number of neurons showing visible Penk mRNA. Its effect was comparable with that of NMDA (8 μM). The increase was independent of NMDA or AMPA receptor activation as shown by the lack of effects of the respective antagonists. However, it was blocked by nimodipine (2 μM), indicating that L-VGCCs indeed mediated the KCl-induced gene expression. Neither the mGluR antagonists LY367385 plus MPEP nor the agonist DHPG changed the Penk gene expression induced by high extracellular K+ concentrations (Fig. 2D).

mGluRI Antagonists Change the Neuronal Levels of Enkephalin Peptide

Next, we studied by immunocytochemistry whether the mGluRI antagonists changed the content of enkephalin peptide in neocortical neurons. After treatment of the cultures with NMDA (8 μM) for 24 h, immunostaining was performed. After treatment with the drugs for 24 h, immunocytochemistry was performed. Upper panel: labeled neurites in controls and after treatment with NMDA or NMDA plus LY367385 and MPEP (scale bar = 10 μm). Lower panel: the staining intensity in neurites treated with NMDA was set at 100%. Mean ± SEM (n = 6); * = significant difference (P < 0.05) to controls; ** = significant difference (P < 0.05) to neurites treated with NMDA or AMPA alone. [C] Immunocytochemical analysis of CREB phosphorylation at Ser-133 after treatment for 45 min with NMDA (8 μM), AMPA (4 μM), LY367385 (80 μM), and MPEP (128 nM). Upper panel: labeled nuclei (scale bar = 5 μm) after treatment with the agents; lower panel: quantification. Staining intensity found after treatment with NMDA was set at 100%. Mean ± SEM (n = 6); * = significant difference (P < 0.05) to controls; ** = significant difference (P < 0.05) to neurons treated with AMPA or NMDA, respectively.
antibody against enkephalin showed positive neuronal extensions (Fig. 3B). Thus, the neurons not only transcribed the gene but also generated the peptide. The increase in enkephalin-like immunoreactivity caused by NMDA was reduced by LY367385 and MPEP (Fig. 3B). The minor increase in PEnk mRNA caused by AMPA (4 μM) alone did not lead to a detectable rise in enkephalin immunoreactivity. In the presence of the mGluR antagonists, AMPA caused a significant elevation (Fig. 3B).

**mGluRI Antagonists Affect the Phosphorylation of the Transcription Factor CREB**

Among other genes, the transcription factor CREB regulates the expression of the PEnk gene (Hyman and others 1994; Bito and others 1996). When phosphorylated at Ser-133, CREB binds to the CRE-2 site of the PEnk gene promoter and activates the transcription (Kobierski and others 1999). Next, we studied whether the interaction of mGluRIs with NMDA and AMPA receptors also changed the phosphorylation state of CREB. Compared with controls, NMDA applied for 45 min increased the nuclear fluorescence by approximately 150% (Fig. 3C). Combined application of LY367385 and MPEP reduced the effect of NMDA by approximately 68%. In contrast, AMPA applied for 45 min increased the fluorescence by 58% compared with controls. LY367385 plus MPEP nearly tripled the effect of AMPA (Fig. 3C). Taken together, these data showed that stimulation of mGluRIs by endogenous glutamate modulated the efficacy of NMDA and AMPA to induce the phosphorylation of CREB at Ser-133.

**Stimulation of mGluRIs Enhances the NMDA-Induced Ion Currents**

Next, we studied whether the mGluRIs also modulated the currents induced by stimulation of ionotropic receptors in neocortical interneurons. We found 3 types of neurons in our cultures that differed in their morphology, that is, pyramidal-like, multipolar, and bipolar neurons (Supplementary Fig. 1A). The 12 neurons selected for their pyramidal-like morphology had the following typical properties. They displayed broad action potentials. The second spike was decreased in amplitude but enhanced in duration. The neurons had slow and low-amplitude AHPs. In response to strong depolarizing currents, they fired trains of action potentials with a spike frequency adaptation at relatively moderate maximum steady-state firing rates (Supplementary Fig. 1B and Supplementary Table 2). Taken together, these cultured pyramidal-like neurons had electrophysiological properties comparable with those of the regularly spiking pyramidial projection neurons studied in rat neocortical slices (McCormick and others 1985; Kawaguchi 1993; Cauli and others 1997, 2000).

Based on their morphology, we took 37 neurons for non-pyramidal neurons, that is, 26 multipolar and 11 bipolar neurons. These neurons were similar to pyramidal-like cells in their soma diameter or resting membrane potential but differed in their responses to current injection (Supplementary Fig. 1 and Supplementary Table 2). In both multipolar and bipolar neurons, we observed 2 different groups of responses (Supplementary Fig. 1C). The neurons of the first group (n = 21; 56.8% of all analyzed neurons; 15 multipolar, 6 bipolar) fired action potentials of a short duration, which were followed by fast and pronounced AHPs. The second spike was unchanged in amplitude and width. These neurons responded to suprathreshold stimuli with high-frequency discharges and low-frequency adaptation (Supplementary Fig. 1CD and Supplementary Table 2). Compared with pyramidal-like neurons, these neurons differed significantly in the spike amplitude reduction of the second spike, spike duration, amplitude and speed of AHPs, maximum steady-state firing rate, and frequency adaptation (Supplementary Table 2). The properties of these neurons were similar to those of the FS class of parvalbumin-containing GABAergic cortical interneurons in neocortical slices (McCormick and others 1985; Kawaguchi 1993, 1995; Kawaguchi and Kubota 1993; Cauli and others 1997, 2000; Erisir and others 1999). They only differed in the lower maximum steady-state firing rate (present study ~30 Hz vs. ~100 Hz [Cauli and others 1997; Erisir and others 1999]). This group of interneurons apparently represented in our culture the FS interneurons found in neocortical tissue.

The second group of neurons (n = 16; 43.2% of all analyzed neurons; 11 multipolar, 5 bipolar) significantly differed from pyramidal-like neurons in their higher input resistance (Ri), stronger spike amplitude reduction for the second action potential, as well as the greater extent of spike frequency adaptation (Supplementary Fig. 1BE and Supplementary Table 2). Compared with the FS cells, they had a significantly higher Ri, smaller and broader action potentials, as well as slower AHPs of smaller amplitude (Supplementary Fig. 1CE and Supplementary Table 2). In addition, they had a more pronounced spike amplitude reduction and spike broadening between the first and second action potential. Also the maximum firing rate was slower and the spike frequency adaptation was more pronounced than that observed in the FS cells (Supplementary Fig. 1E and Supplementary Table 2). Thus, the properties of these neurons resembled those of the regular-spiking non-pyramidal class of GABAergic interneurons in neocortical slices (Kawaguchi 1995; Cauli and others 1997, 2000; Erisir and others 1999). The cultured neurons only differed in their maximum firing rate, which was considerably lower than in their counterparts in vivo (present study 11 Hz vs. 25–42 Hz [Cauli and others 1997; Erisir and others 1999]). According to these data, not only the bipolar but also most, if not all, multipolar neurons seemed to be interneurons.

In our patch-clamp experiments on the effects of mGluRIs, we studied only nonpyramidal neurons but did not select for a certain type of interneurons. The superfusion technique used in the low-density cultures made it impossible to examine the effect of endogenous glutamate with mGluRI antagonists. Because the mGluRI agonist DHPG had been shown to enhance the NMDA-induced PEnk gene expression in the absence of endogenous glutamate (see Table 2, B), we used DHPG (90 μM) together with NMDA (30μM) or AMPA (30μM). When NMDA (4AC) or AMPA (4BD) were pressure applied to rat cortical neurons 11 times over a period of 20 min, the evoked currents remained stable. The inward current amplitudes obtained after the 11th application of NMDA or AMPA amounted to 92.4 ± 1.9% and 95.5 ± 1.9% of the first amplitude of the respective series (n = 9 each). When DHPG was applied for 6 min, the NMDA-evoked peak currents were significantly increased (Fig. 4AC). Compared with the respective time-matched control (n = 9), DHPG applied for 4 min enhanced the currents by 36.7 ± 10.8% (range 7.8-118.0%; n = 12) (Fig. 4AC, II). In contrast, DHPG did not change the AMPA-induced peak currents (Fig. 4BD). After 4 min, they were -0.3 ± 2.1% (range -19.5% to 9.6%; n = 12) of the time-matched control (n = 9) (Fig. 4BD, II).
In a second set of experiments, we studied the long-term effects of glutamate receptor agonists. We treated the cultures for 24 h with NMDA (8 μM) or AMPA (4 μM) in the absence or presence of DHPG (90 μM). Then, we measured within the subsequent 5 h the peak current amplitudes in response to a single pressure application (2 s) of NMDA (30 μM) or AMPA (30 μM) and compared them with those obtained from untreated neurons. Pretreatment with NMDA did not affect NMDA
(30 μM)-induced transmembrane currents (Fig. 4E). Peak current amplitudes were $-446 \pm 61$ pA ($n = 12$) and $-393 \pm 62$ pA ($n = 7$) under control conditions and after pretreatment with NMDA, respectively. Pretreatment with DHPG alone, however, significantly enhanced NMDA (30 μM)-induced currents ($-703 \pm 50$ pA; $n = 7$), whereas the current response after combined pretreatment with DHPG and NMDA did not significantly differ from that of controls ($-570 \pm 45$ pA; $n = 7$; Fig. 4E).

Pretreatment with AMPA for 24 h significantly decreased the AMPA (30 μM)-induced transmembrane currents (Fig. 4F). Peak current amplitudes were $-143 \pm 17$ pA ($n = 12$) and $-45 \pm 10$ pA ($n = 7$) under control conditions and after pretreatment with AMPA, respectively. In addition, AMPA (30 μM)-evoked currents were also significantly depressed when cultures were pretreated with DHPG alone ($-63 \pm 14$ pA) or with the combination of DHPG and AMPA ($-28 \pm 4$ pA; $n = 7$ each; Fig. 4F).

According to these data in our cultured neocortical interneurons, simultaneous stimulation of NMDA receptors and mGluRIs acutely enhanced the response of NMDA receptors but did not induce persistent changes. In contrast, simultaneous stimulation of AMPA receptors and mGluRIs had no acute effect on the AMPA receptor activity but caused a long-term decrease in their activity.

**mGluRI Antagonists Modulate the Number of AMPA Receptors at the Cell Surface**

In pyramidal neurons, even short-term application of GluR1 agonists can change the membrane density of AMPA receptors (see Introduction). Therefore, we studied whether mGluRI antagonists applied for 24 h affected the density of the ionotropic receptors in the membranes of our cultured interneurons. For these experiments, we used receptor immuno-cytochemistry as well as the biotinylation procedure. When high-density cultures were treated for 24 h with 4 μM AMPA alone, the fluorescence of immunolabeled GluR1 subunits at the neuronal surface was decreased by 60%, whereas the total number of GluR1 subunits in the neurons remained unchanged (Fig. 5A). In the presence of LY367385 (80 μM) plus MPEP (128 nM), however, AMPA reduced the fluorescence only by 40%. Biotinylation of surface receptors and the subsequent quantification of GluR1 subunits by western blotting confirmed that AMPA reduced the density of GluR1 subunits in the cell membrane (Fig. 5B). Combined treatment with LY367385 and MPEP prevented the AMPA-induced reduction in the membrane density of GluR1 subunits. To confirm the specificity of the biotinylation procedure for membrane proteins, we also analyzed the strictly intracellular protein β-tubulin III, which was indeed not detected at the cell surface (Fig. 5D). Finally, we studied whether NMDA affected the number of NMDA receptors at the cell surface. Treatment of the cultures for 24 h with NMDA (8 μM) in the absence or presence of LY367385 plus MPEP did not affect the number of NR1 subunits of NMDA receptors at the cell surface detected by biotinylation (Fig. 5C). In an additional experiment, we studied the effect of NMDA in the absence or presence of LY367385 plus MPEP on the NR1 subunit number after immunolabeling but did not find any changes (data not shown). Taken together, the data suggested that endogenous glutamate contributed to the regulation of AMPA receptor number in the membrane by acting via mGluRIs.

**mGluRI Antagonists Change the PEnk Gene Expression in Slice Cultures of Neocortex**

In organotypic cultures of rat neocortex, the PEnk gene expression is caused by glutamate released from neurons under basal conditions. Glutamate acts via NMDA and AMPA receptors
We used neocorticostriatal slice cultures to study whether mGluRIs modulated the expression of the Penk gene also in neurons in the tissue context (Fig. 6).

In the neocortex, 4 μM dizocilpine and 40 μM GYKI 52466 inhibited the expression of the Penk gene by 72% and 39%, respectively (Fig. 6A,B), confirming the involvement of NMDA and AMPA receptors in neocortical Penk gene expression. Also, when applied together, the antagonists reduced the expression by 70%. When used in combination, the mGluR antagonists LY367385 (100 μM) and MPEP (5 μM) decreased by 23% the number of neocortical neurons that visibly expressed the gene (Fig. 6A,B). When we blocked the NMDA receptors with dizocilpine to study the AMPA receptors, LY367385 plus MPEP strongly enhanced the expression of the Penk gene, that is, by 125%, compared with dizocilpine alone (Fig. 6A,B). In contrast, LY367385 plus MPEP reduced by 33% the expression observed in the presence of GYKI 52466 when AMPA receptors were blocked but NMDA receptors were still active (Fig. 6A,B). When dizocilpine and GYKI 52466 were used simultaneously, LY367385 and MPEP no longer changed the expression (Fig. 6A,B). These results suggested that in the neocortical part of the slice cultures endogenous glutamate acted on mGluRIs and thereby enhanced the NMDA receptor–induced but antagonized the AMPA receptor–induced Penk gene expression.

In the striatal part of the cocultures, dizocilpine reduced the Penk gene expression by 84%, whereas the AMPA receptor blocker GYKI 52466 had no effect (Fig. 6A,C). This finding was in agreement with our previous observation that mainly NMDA receptors...
receptors mediate striatal PEnk gene expression (Just and others 1999). The mGluR antagonists decreased by 52% the PEnk gene expression (Fig. 6C).

Discussion

In the present study, we have examined whether mGluRs modulate the PEnk gene expression induced in neocortical GABAergic interneurons by the activation of NMDA and AMPA receptors. In cultures of dissociated interneurons as well as in organotypic cultures, endogenous glutamate modulated the expression of the PEnk gene by acting via mGluRs. mGluRI antagonists reduced the effect of NMDA receptor stimulation but enhanced that of AMPA receptor stimulation. The phosphorylation and thus activation of the transcription factor CREB were regulated in a similar manner. Our additional observation that the mGluRI antagonists diminished the downregulation of AMPA receptors induced by the continuous presence of AMPA over 24 h suggests that glutamate released from nerve endings can regulate via mGluRs the density of AMPA receptors. According to our data, mGluRs may play in neocortical interneurons a physiologically relevant role in the gene expression controlled by endogenous glutamate.

Due to the use of postnatal tissue and the cultivation procedure, our cultures were enriched in interneurons. Approximately 86% of the neurons were immunolabeled for GAD65/67, indicating that they were GABAergic interneurons. We also found Ca**/binding proteins in the cultured interneurons. Their cellular distribution was similar to that previously described for neocortical tissue (Gonchar and Burkhalter 1999; Markram and others 2004). The majority of labeled neurons expressed parvalbumin, although calretinin and calbindin were present in smaller populations. After stimulation of PEnk gene expression, GAD65/67 was expressed in approximately 96% of the neurons containing PEnk mRNA. Treatment of the cultures with NMDA, AMPA, and the mGluRI antagonists did not change this ratio, indicating that the excitatory inputs did not induce the PEnk gene expression in additional neuronal populations. Most of these neurons also contained parvalbumin, although calbindin- or calretinin-positive neurons were also observed. Apparently, several types of GABAergic interneurons were able to express the enkephalin gene when strongly stimulated. This speculation is in line with our previous finding in neocortical slice cultures that the removal of the GABAergic inhibitory tone by the GABA_A receptor antagonist bicuculline increases the PEnk gene expression in a large number of neurons (Mörl and others 2002).

In the neocortical interneurons, the mGluRII antagonists LY367385 and MPEP reduced the NMDA-stimulated PEnk gene expression, measured as mRNA and enkephalin peptide concentration. In contrast, the antagonists enhanced the effect of AMPA on PEnk gene expression. LY367385 and MPEP were effective at concentrations known to specifically block mGluR1 and mGluR5, respectively (Gasparini and others 1999; Hermans and others 1999), suggesting that both receptor subtypes were involved. The mGluR1 and mGluR5 may have been expressed in different types of neurons (see also Lopez-Bendito and others 2002), as was the PEnk gene. Taken together, these findings suggest that mGluRs can modulate in neocortical interneurons the gene expression mediated by NMDA and AMPA receptors. NMDA increased the PEnk gene expression when AMPA/kainate receptors were inhibited with CNQX. On the other hand, AMPA enhanced the PEnk gene expression in the presence of the NMDA receptor blocker dizocilpine. These data suggested that the respective NMDA and AMPA receptors were not functionally linked. Whether they were expressed in the same interneurons remains open.

Upon stimulation, neither NMDA nor AMPA receptors directly enhanced the PEnk gene expression via the Ca**/entering through the respective receptor-gated ion channels. Instead, both types of receptors exerted their effects via L-VGCCs, confirming previous evidence (Rajadhyaksha and others 1999) (for a schematic presentation see Fig. 7). Activation of L-VGCCs by depolarization with KCl also enhanced the PEnk gene expression. This increase was not under the control of mGluRs. Moreover, stimulation of mGluRs with DHPG had no pronounced effect on PEnk gene expression in the absence of NMDA and AMPA. Taken together, these data indicated that the actions of mGluRs on PEnk gene expression were linked to the ionotropic receptors. Our findings are in contrast to previous results in striatal projection neurons, where DHPG can stimulate PEnk gene expression independent of NMDA or AMPA receptors (Mao and Wang 2001). Apparently, the action of mGluRs on PEnk gene expression is cell type specific.

At the concentrations used in our experiments, NMDA and AMPA are not known to stimulate mGluRs. However, neurons dissociated before seeding can form a network during the subsequent cultivation. Indeed, blockade of voltage-gated Na channels with TTX diminished the effect of NMDA on the PEnk gene expression (Just and others 2002). The mGluRI antagonists LY367385 and MPEP reduced the NMDA-stimulated PEnk expression, suggesting that mGluR antagonists are involved in the gene expression. In contrast, the stimulated mGluRs facilitate the response of NMDA to PEnk gene expression in the absence of NMDA and AMPA. Taken together, these data indicated that the actions of mGluRs on PEnk gene expression were linked to the ionotropic receptors. Our findings are in contrast to previous results in striatal projection neurons, where DHPG can stimulate PEnk gene expression independent of NMDA or AMPA receptors (Mao and Wang 2001). Apparently, the action of mGluRs on PEnk gene expression is cell type specific.
gene expression as well as prevented the inhibitory effect of the mGluR inhibitors. Under these conditions, the mGluRI agonist DHPG enhanced the effect of NMDA on gene expression. We considered these data as evidence that glutamate released from the cultured neurons stimulated mGluRs and thereby affected the gene expression (Fig. 7).

In our electrophysiological experiments, we established that the neurons selected for the experiments according to their morphology were indeed nonpyramidal. However, the type of interneurons patched was not determined. We prepared low-density cultures to be able to patch single nonpyramidal neurons. Because all agents were applied by superfusion, neuronal interactions were further reduced. To compensate for the resulting lack of endogenous glutamate, DHPG that enhanced within 2 min the NMDA-induced currents was applied, demonstrating the synergism of NMDA receptors and mGluRs in neocortical interneurons. When DHPG was applied together with NMDA for 24 h, however, it did not affect the current induced by the subsequent application of NMDA. Thus, the combined stimulation of NMDA receptors and mGluRs did not persistently change the response of NMDA receptors in the neocortical interneurons.

As in the studies on gene expression, we used high-density cultures of neocortical neurons for the biochemical experiments. In these experiments, all cells present in the culture were used so that it was impossible to distinguish between pyramidal neurons and interneurons. Because the latter type made up at least 86% of the total number, we assumed that our findings more or less reflected the changes in interneurons. NMDA applied for 24 h did not change the density of NR1 subunits at the cell surface. Our results confirmed and extended previous results showing that long-term application of NMDA alone does not affect NR1 subunit density (Läscher and others 1999; Ehlers 2000; Roche and others 2001). Also, the combined application of NMDA and mGluR antagonists did not change the density of NR1 subunits at the cell surface. This finding was complimentary to our electrophysiological data obtained with the agonist DHPG. When applied alone for 24 h, the mGluR inhibitors did not change the surface density of NR1 subunits. In contrast, the 24 h application of DHPG enhanced the subsequent electrophysiological effect of NMDA. The relevance of this finding has to be further investigated because application of DHPG for 1 h induces a decrease in surface NMDA receptors in hippocampal neurons (Snyder and others 2001).

Our finding that mGluR antagonists increased in our neocortical interneurons the stimulatory effect of AMPA on Penk gene expression reminds one of the previous evidence that mGluR agonist DHPG can suppress within minutes the AMPA receptor-mediated synaptic transmission (Doherty and others 1997; Fitzjohn and others 1999; Awad and others 2000). This reduction has been linked to the long-lasting removal of AMPA receptors from the postsynaptic membrane (Snyder and others 2001; Xiao and others 2001; ŽoÅ and others 2002; Mangiavacchi and Wolf 2004). In addition, AMPA itself can reduce the density of its receptor subunits at the neuronal surface. The rapid AMPA receptor endocytosis under basal conditions (t = 7-9 min) is further accelerated by 100 μM AMPA until a new steady state of exo- and endocytosis is established, which results in the reduced number of surface receptors (Carroll and others 1999; Lissin and others 1999; Lin and others 2000).

In our patch-clamp experiments, DHPG applied for 6 min did not affect AMPA-induced currents in the isolated interneurons but reduced the currents when applied for 24 h. The lack of effect of the short application of DHPG was in contrast to its inhibitory effect on the AMPA-induced field excitatory post-synaptic potentials (EPSPs) in hippocampal slices (Xiao and others 2001). The different conditions (acute slices vs. neuronal cultures) and DHPG concentrations (200 μM vs. 90 μM in our experiments) may be responsible for the discrepancy. In the high-density cultures used for the biochemical and morphological studies, network activity was possible. Here, application of AMPA for 24 h reduced by 60% the number of GluR1 subunits in the cell membrane measured with the immunolabeling as well as the biotinylation method. The mGluRI agonists diminished the reduction, suggesting that mGluRIs stimulated by AMPA-induced glutamate release contributed to the downregulation of these GluR1 subunits (Fig. 7). Whether all these changes observed in the biotinylation assays occurred only in inhibitory interneurons is doubtful in view of the fact that approximately 14% of the neurons did not stain for GAD65/67.

It is noteworthy that only after the blockade of the mGluRIs, stimulation of AMPA receptors induced a pronounced expression of the Penk gene. In contrast to NMDA receptors, which can induce the expression of numerous genes independent of L-VGCCs, AMPA receptors without Ca++ conductivity only change the expression of certain genes if they are linked to L-VGCCs. Apparently, the signal transduction pathways initiated by mGluRI can reduce the neuronal gene expression induced by AMPA receptors linked to L-VGCCs. Our finding may explain the fact that AMPA receptor stimulation of neuronal gene expression has been rarely reported.

Compared with cultures of dissociated neurons, organotypic cultures are much closer to the in vivo situation. In neocortico-striatal slice cultures, glutamate released from neurons under basal conditions increases the Penk gene expression via NMDA and AMPA receptors. Also in such neocortical cultures, the mGluRI antagonists reduced the effects of NMDA receptor stimulation and enhanced those of AMPA receptors. Pronounced effects were only observed when the mGluR antagonists were used in the presence of either dizocilpine or LYKI 52466 to restrict the effects to only 1 receptor type. These findings clearly showed that also within the tissue context endogenous glutamate modulated via mGluRs the gene expression induced by the stimulation of ionotropic receptors, suggesting a physiological role of these interactions. In the striatal tissue, the expression of the Penk gene is induced by endogenous glutamate via NMDA receptors only (Just and others 1999). Here, the mGluR antagonists only caused a pronounced inhibition of the Penk gene expression, confirming the synergistic effects of NMDA and metabotropic receptor agonists (Pisani and others 2001; Mao and Wang 2002b).

Not only the Penk gene expression but also the NMDA- and AMPA-induced phosphorylation of CREB was modulated by mGluRs. The transcription factor CREB is not only essential for the regulation of the Penk gene expression (Hyman and others 1994; Hamelink and others 2002; Hahn and others 2003) but is of great importance in the regulation of gene transcription and protein synthesis necessary for long-term memory processes and other cognitive functions (Deisseroth and others 1996, 2003; Pittenger and Kandel 2003). According to our results, endogenous glutamate can facilitate the effects of NMDA receptors and attenuate those of AMPA receptors via mGluRs.
in neocortical inhibitory interneurons. The opposite effects of
mGluRIs on NMDA and AMPA receptor activities make the final
outcome of the application of mGluRI agonists or antagonists
difficult to predict if the 2 ionotropic receptors have similar
actions. Studies on the use of such agents in neurological and
psychiatric diseases have to consider this complexity.

Supplementary Material

Supplementary material can be found at: http://www.cercor.
oxfordjournals.org/

Notes

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