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L-Type Ca^{2+} Channels Are Essential for Glutamate-Mediated CREB Phosphorylation and *c-fos* Gene Expression in Striatal Neurons

Anjali Rajadhyaksha, Amy Barczak, Wendy Macías, Jean-Christophe Leveque, Susan E. Lewis, and Christine Konradi

Molecular and Developmental Neuroscience Laboratory and Department of Psychiatry, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts 02129

The second messenger pathways linking receptor activation at the membrane to changes in the nucleus are just beginning to be unraveled in neurons. The work presented here attempts to identify in striatal neurons the pathways that mediate cAMP response element–binding protein (CREB) phosphorylation and gene expression in response to NMDA receptor activation. We investigated the phosphorylation of the transcription factor CREB, the expression of the immediate early gene *c-fos*, and the induction of a transfected reporter gene under the transcriptional control of CREB after stimulation of ionotropic glutamate receptors. We found that neither AMPA/kainate receptors nor NMDA receptors were able to stimulate independently a second messenger pathway that led to CREB phosphorylation or *c-fos* gene expression. Instead, we saw a consecutive pathway from AMPA/kainate receptors to NMDA receptors and from NMDA receptors to L-type Ca^{2+} channels. AMPA/kainate receptors were involved in relieving the Mg^{2+} block of NMDA receptors, and NMDA receptors triggered the opening of L-type Ca^{2+} channels. The second messenger pathway that activates CREB phosphorylation and *c-fos* gene expression is likely activated by Ca^{2+} entry through L-type Ca^{2+} channels. We conclude that in primary striatal neurons glutamate-mediated signal transduction is dependent on functional L-type Ca^{2+} channels.

Key words: glutamate; NMDA; AMPA; kainate; L-type Ca^{2+} channels; CREB; c-fos

The striatum (caudate, putamen, and nucleus accumbens) has been implicated in movement disorders like Parkinson's disease and Huntington's disease (Vonsattel et al., 1985; DiFiglia, 1990; Langston, 1996). The striatum also plays a critical role in drug addiction (Pich et al., 1997; Volkow et al., 1997), schizophrenia (Buchsbaum et al., 1992; Siegel et al., 1993; Busatto and Kerwin, 1997; Heckers, 1997), and memory (Knowlton et al., 1996). Neuroplasticity of the striatum enables compensation for the loss of dopamine (Bernheimer et al., 1973; Neve et al., 1982; Calne et al., 1985; Burns, 1991; Hornykiewicz, 1993) and contributes to both the therapeutic potential of neuroleptics (Burt et al., 1977; Eastwood et al., 1994, 1997; Mijnster et al., 1996) and the addictive properties of drugs of abuse (Nestler et al., 1993; Self and Nestler, 1995; Hyman, 1996; Hyman and Nestler, 1996). Synaptic plasticity forms the basis of learning and memory and involves mechanisms such as protein phosphorylation and protein synthesis, leading to the strengthening of preexisting synapses and the establishment of new synaptic connections (Stanton and Sarvey, 1984; Deadwyler et al., 1987; Matthies, 1989; Nairn and Shenolikar, 1992; Schulman, 1995; Bailey et al., 1996). The examination of the mechanisms of gene regulation in the striatum is vital for our understanding of striatal plasticity, striatal function, and malfunction and for the treatment of disorders of the striatum.

The excitatory amino acid L-glutamate (glutamate) participates in processes from neuronal communication to plasticity and neuropathology via its interaction with ionotropic and metabotropic receptors (Greenamyre and Porter, 1994; Michaelis, 1998). Ionotropic glutamate receptors are classified into AMPA, kainate, and NMDA receptors (Hollmann and Heinemann, 1994; Schoepfer et al., 1994; Michaelis, 1998; Ozawa et al., 1998). AMPA/kainate receptors gate ion channels that promote Na^+ influx and, to a lesser extent, Ca^{2+} influx (Hollmann et al., 1991; Egebjerg and Heinemann, 1993; Kohler et al., 1993; Lomeli et al., 1994) and desensitize rapidly (Lomeli et al., 1994; Mosbacher et al., 1994).

NMDA-type glutamate receptor channels bind glycine and glutamate (Ascher and Nowak, 1987; Johnson and Ascher, 1987; Hollmann and Heinemann, 1994) and are permeable to Ca^{2+} (MacDermott et al., 1986; Connor et al., 1988) as well as Na^+ and K^+ ions (Ascher and Nowak, 1987; Kandel et al., 1991). At resting potential, NMDA receptors are blocked by Mg^{2+} (Mayer et al., 1984; MacDermott et al., 1986; Ruppersberg et al., 1993; Schoepfer et al., 1994). This Mg^{2+} block can be relieved by depolarization, which can be achieved by activation of AMPA/kainate receptors.

Another route for Ca^{2+} entry into striatal neurons is provided by voltage-operated Ca^{2+} channels (VOCCs) (Sanna et al., 1986; Thayer et al., 1986). The α1 subunit of VOCCs determines the properties of the Ca^{2+} channel (Hofmann et al., 1994; McCleskey, 1994; Walker and De Waard, 1998) and forms the pore through which Ca^{2+} ions enter the cell (Catterall, 1991; Walker and De Waard, 1998). Ca^{2+} channels that contain the α1C or α1D subunit are classified as dihydropyridine-sensitive L-type Ca^{2+} channels (Hofmann et al., 1994; McCleskey, 1994; Walker and De Waard, 1998). The L-type Ca^{2+} channel is activated by strong depolarization from relatively depolarized holding potentials and shows almost no inactivation by depolarization (Tsien et al., 1988). Therefore, it opens only after a strong stimulus and,

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Correspondence should be addressed to Dr. Christine Konradi, Laboratory of Molecular and Developmental Neuroscience, Massachusetts General Hospital, CNY 2510, Building 149, 13th Street, Charlestown, MA 02129.

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after opened, causes extensive Ca^{2+} influx (Catterall and Striessnig, 1992). These characteristics and the distribution of L-type Ca^{2+} channels on somata and at the base of dendrites (Westenbroek et al., 1990; Schild et al., 1995) provide a favorable setting to mediate gene regulation in the nucleus.

Ca^{2+}-stimulated second messenger pathways can activate the transcription factor cAMP response element–binding protein (CREB) (Sheng et al., 1991; Sun et al., 1994; Thompson et al., 1995; Bito et al., 1996; Deisseroth and Tsien, 1997). CREB constitutively binds with high affinity to the cAMP-responsive element (CRE) and becomes a transcriptional activator after phosphorylation of Ser^{133} (Montminy and Bilezikjian, 1987; Gonzalez and Montminy, 1989; Montminy et al., 1990). CREB has been linked to memory formation (Bourtchuladze et al., 1994; Yin et al., 1994), neuroplasticity (Murphy and Segal, 1997), and long-term potentiation (Impey et al., 1996). The immediate early gene *c-fos* is activated by CREB (Sheng et al., 1990). The promoter of the *c-fos* gene contains the cAMP and Ca^{2+}–responsive element (CaRE), which interacts with CREB (Sheng et al., 1990; Ghosh et al., 1994). The CaRE site integrates several second messenger pathways (Bonni et al., 1995; Ahn et al., 1998) and is one of the preeminent regulatory sites of the *c-fos* promoter (Robertson et al., 1995). Like CREB phosphorylation, *c-fos* is induced after NMDA receptor stimulation (Cole et al., 1989; Aronin et al., 1991; Lerea and McNamara, 1993; Dave and Tortella, 1994) and after L-type Ca^{2+} channel activation (Murphy et al., 1991; Misra et al., 1994).

We show here that in primary striatal cultures, glutamate via activation of NMDA receptors mediates CREB phosphorylation and gene expression via L-type Ca^{2+} channels.

MATERIALS AND METHODS

Drugs. NMDA, (±)AMPA hydrobromide, kainate (kainic acid), dizocilpine maleate [(+)MK 801 hydrogen maleate], (±)2-amino-5-phosphonopentanoic acid (APV), DNQX, 2,5-dimethyl-4-[2-(phenylmethyl)benzoyl]-1H-pyrrole-3-carboxylic acid methylester (FPL 64176), 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-3,4-dihydro-5H-2,3-benzodiazepine (GYKI 52466) hydrochloride, tetrodotoxin citrate (TTX), (±)verapamil hydrochloride, nifedipine, bicuculline, and picrotoxin were purchased from Research Biochemicals (Natick, MA), and L-glutamate was purchased from Sigma (St. Louis, MO). The Ser^{133} CREB antiserum (Ginty et al., 1993), the CREB antiserum, and the Fos antiserum were purchased from Upstate Biotechnology (Lake Placid, NY). The antiserum against the α1C Ca^{2+} channel was purchased from Alomone Labs (Jerusalem, Israel).

Primary striatal cultures. Primary striatal cultures were prepared as described previously, with minor modifications (Konradi et al., 1996; Rajadhyaksha et al., 1998). Striata were dissected under a stereomicroscope from 18-d-old Sprague Dawley rat fetuses. Tissue was resuspended in 2 ml of defined medium [50% F12/DMEM and 50% DMEM (Life Technologies, Gaithersburg, MD) with the following supplements per liter of medium: 4 gm of dextrose, 1× B27, 10 ml of penicillin–streptomycin liquid (Life Technologies), and 25 mM HEPES]. The tissue was mechanically dissociated with a fire-narrowed Pasteur pipette; the cells were resuspended in defined medium to 10^6 cells/ml and plated in six-well plates (Costar, Cambridge, MA) at 2×10^6 cells/well. Plates were pretreated with 2 ml of a 1:500-diluted sterile solution of polyethylenimine in water for 24 hr, washed twice with sterile water, coated with 2.5% serum-containing PBS solution for at least 4 hr, and aspirated just before plating. All experiments were performed with cells 6–8 d in culture and repeated at least once in an independent dissection. As determined by HPLC analysis, glutamate levels in the medium on the day of the experiments ranged from 1 to 5 μM. The neuron to astroglia ratio was below 25:1, as established by immunocytochemical staining with the glial fibrillary acid protein (Dako, Carpinteria, CA) and counterstaining with 1% cresyl violet.

Defined salt solutions. To have comparable parameters, none of the defined salt solutions contained sodium bicarbonate. Sodium bicarbonate was replaced by *N*-methyl-D-glucamine (45 mM). Control salt solutions contained 110 mM NaCl, 2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 400 μM glycine, 45 mM *N*-methyl-D-glucamine, 0.5% phenol red, 3 gm/l dextrose, and 20 mM HEPES-KOH. In experiments without Na^+, NaCl was replaced by 110 mM *N*-methyl-D-glucamine. In experiments without Ca^{2+} or without Mg^{2+}, either ion was left out. Salt solutions were adjusted with HCl to pH 7.2. In experiments that used NMDA as an agonist, 10 μM glutamate was added 18 hr before the experiment. All salt solutions were added 18 hr before the experiment to avoid false results because of media change. Neurons were carefully monitored for neuronal death.

Immunoblots. Primary rat striatal cultures were harvested in boiling sample buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.025% bromophenol blue). Cell lysates were sonicated and centrifuged for 10 min. Equal volumes of the lysates were loaded on 12% polyacrylamide gels for phospho-CREB and CREB immunoblots or on 8% gels for Fos and α1C Ca^{2+} channel–subtype immunoblots. Protein was transferred to polyvinylidene fluoride membrane (Immobilon-P; 0.45 mm; Millipore, Bedford, MA) and blocked in blocking buffer (5% nonfat dry milk in PBS and 0.1% Tween 20) for 1 hr. The blots were incubated in primary antibody (1:1000 anti-Ser^{133}-phospho-CREB or anti-CREB; 1:10,000 anti-Fos; or 1:500 anti-α1C) for 2 hr followed by three washes for 10 min in blocking buffer. This was followed by a 1 hr incubation in goat anti-rabbit horseradish peroxidase–linked IgG (Vector Laboratories, Burlingame, CA) at a dilution of 1:3000. Blots were washed three times for 10 min in blocking buffer, developed with the Renaissance detection system (Dupont NEN, Wilmington, DE), and exposed to autoradiographic film (Kodak, Rochester, NY). Kaleidoscope-prestained standards (Bio-Rad, Hercules, CA) were used for protein size determination. Phospho-CREB and CREB bands were detected at the 43 kDa standard. The Fos band was detected at 60 kDa.

Calcium phosphate transfections. Transfection of primary striatal neurons was performed following the protocol of Xia et al. (1996). Embryonic day 18 striatal neurons were transfected on 4 d *in vitro* (DIV). The DNA/calcium phosphate precipitate was prepared by mixing the DNA in 250 mM $CaCl_2$ with an equal volume of 2× HEPES-buffered saline (0.14 mM NaCl, 0.025 mM HEPES, and 0.7 μM Na_2HPO_4). The precipitate was allowed to form for 1 hr at room temperature. Fifteen minutes before addition of the DNA mixture, the conditioned culture medium was removed from the cells and replaced with 1.5 ml of F12/DMEM (Life Technologies). The conditioned media were kept under 5% CO_2. The DNA mixture (100 μl) was added dropwise to each well of a six-well plate and rocked gently. Plates were incubated for 80 min in a 5% CO_2 incubator. After 80 min the cells were shocked with 500 μl of 2% DMSO in F12/DMEM for 2 min and washed twice with 1.5 ml of F12/DMEM. The conditioned media were added back to the cells, and the plates were incubated in a 5% CO_2 incubator at 37°C. For all transfections, 6 μg of total DNA was used per well (35 mm) of a six-well plate. Forty-eight hours after transfection, cells were treated with the respective drugs for 6 hr. Media were aspirated, and plates were quick-frozen on liquid nitrogen and stored at −80°C.

3xCRE-luciferase *construct.* A DNA sequence containing three CRE sequences (TGACGTCA) was fused to a minimal Rous sarcoma virus promoter (enhancerless) and was inserted into the pA3Pluc vector (Maxwell et al., 1989) 5′ of a luciferase reporter gene.

Luciferase assay. The luciferase assay was performed using the Promega luciferase assay kit (Madison, WI). Cells were lysed in 150 μl of 1× cell culture lysis reagent (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-*N,N,N′,N′*-tetra-acetic acid, 10% glycerol, and 1% Triton X-100); 100 μl of the lysate was used for the luciferase reporter assay. Luciferase activity was measured for a period of 10 sec using a luminometer (EG & G Berthold, Oak Ridge, TN), and light intensity was expressed as relative light units.

Antisense experiments. Antisense sequences were targeted to the translation initiation sites. Both antisense and scrambled antisense oligonucleotides were synthesized by the Massachusetts General Hospital core DNA synthesis facility. The first and last three nucleotides were phosphorothioate modified to avoid degradation of the oligonucleotides and also to reduce the cytotoxic effect observed with fully phosphorothioate-modified oligonucleotides. Striatal cultures were treated according to the method of Bito et al. (1996) with a mixture of 5 μM α1C (GGCCCGAATCATTGTGACTCCAGT) and 5 μM α1D (TCATCATCATCATCATCATCCACG) antisense oligonucleotides for L-type Ca^{2+} channel antisense experiments or a mixture of 5 μM NR1 (TGCTCATGAGCTCCGGGCACAGCG), 5 μM NR2A (CAATCTGCCCATGGTCG-

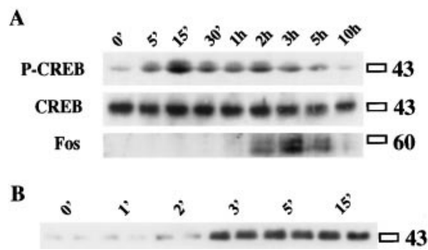

Figure 1. Glutamate induces Ser 133 CREB phosphorylation and Fos protein in primary striatal cultures. *A*, Immunoblots of rat primary striatal cultures were treated with glutamate (50 μM) for 5 min to 10 hr (*times* indicated *above* the *blots*). *P-CREB*, CREB was phosphorylated 5 min after the addition of glutamate and remained phosphorylated for at least 5 hr. *CREB*, An antiserum that is indifferent to the state of phosphorylation of CREB showed that the protein levels of CREB were not changed. *Fos*, Fos protein was induced 2–5 hr after the addition of glutamate. CREB and Ser 133 CREB had a molecular weight of ~43 kDa, whereas Fos protein was slightly below the 60 kDa protein marker. *B*, Primary striatal cultures were exposed to glutamate (50 μM) for 1–15 min, at which time glutamate was removed from the cells. All cells were harvested 15 min after the addition of glutamate. Immunoblots were developed with Ser 133 CREB antiserum (Ginty et al., 1993). All treatments are shown in duplicates.

CCACTTA), and 5 μM NR2B (ACTCTGCGCTGGGCTTCATCTTCA) antisense oligonucleotides for the NMDA receptor experiment. Oligonucleotides were added 30 min after cells were plated and daily thereafter until 4 DIV. Twenty-four hours after the last addition, cells were treated with the respective agonists. For scrambled antisense DNA controls, approximately every third and fourth nucleotide of the antisense constructs were exchanged (α1C, GACCTGAGTTACTGGCATATCCGC; α1D, TAAACCTCTCCATTCTCGTACACA; NR1, TGCCTAGTAGCTCGCGGACCAGGC; NR2A, ACATCGTCATCGCGCTGATCTCAC; and NR2B, TATGCCTCGGCGTTCAGCTCCATT).

c-fos *Northern blot analysis.* Medium was aspirated, and striatal neurons were lysed in 500 μl of lysis buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, and 0.5% NP-40). After a 5 min incubation on ice, lysates were transferred into microcentrifuge tubes and centrifuged for 2 min at 14,000 rpm at 4°C; the supernatant was transferred, and SDS was added to a final concentration of 0.2%. Cells were extracted with phenol, followed by a chloroform extraction and ethanol precipitation. RNA was size-separated on a 1.2% denaturing agarose gel (1 M paraformaldehyde) in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (20 mM MOPS, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA), electroblotted onto a nylon membrane (GeneScreen; DuPont, Billerica, MA), and hybridized with a *c-fos* riboprobe (Riboprobe system; Promega). Northern blots were analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), with the IP lab imaging software. Expression of cyclophilin mRNA was used as a loading control (Danielson et al., 1988).

Statistical analyses. Autoradiographic films were scanned with the Hewlett Packard Scan Jet. Because of the narrow range of film (approximately one order of magnitude), the data obtained for immunoblots are not comparable with the data obtained for *c-fos* Northern blots, which were analyzed with a PhosphorImager that has a range of five orders of magnitude. Because the data are compiled of many different Northern blots and immunoblots, they had to be normalized to internal, untreated controls. Thus, the data are semiquantitative and are not based on absolute numbers. Data were analyzed with one-way ANOVAs. The Tukey–Kramer honestly significant difference was used to analyze differences between the groups, whereas the Dunnett's test was used for comparisons of treatment groups with controls. The JMP computer program (SAS Institute, Cary, NC) was used for data analysis.

RESULTS

Glutamate induces CREB phosphorylation and Fos protein expression in primary striatal cultures

Glutamate (50 μM) induced Ser 133 CREB phosphorylation in rat primary striatal cultures within 5 min of treatment (Fig. 1*A*, *P-CREB*). Ser 133 CREB phosphorylation peaked at 15 min and returned to basal levels 10 hr after glutamate was added. When immunoblots were reprobed with a CREB antiserum that is indiscriminate to the state of CREB phosphorylation, no treatment-mediated regulation was seen (Fig. 1*A*, *CREB*). In most blots, the CREB antiserum and the Ser 133 CREB antiserum revealed a double band slightly >40 kDa, but some variability in the resolution of the double band and/or staining of the upper band was observed with either antiserum. Because the two bands had identical patterns of regulation, these variations were not a problem. A temporal analysis of Fos protein levels demonstrated an increase between 2 and 5 hr after the onset of glutamate treatment (Fig. 1*A*, *Fos*). In a different time course, cultures were exposed to glutamate for limited times (Fig. 1*B*), refed with medium lacking glutamate, and harvested 15 min after glutamate treatment had begun. A 3 min exposure to glutamate yielded maximum levels of CREB phosphorylation, comparable with treatment for the entire 15 min (Fig. 1*B*; see also Fig. 6*D* for a time course similar to that of Fig. 1*A*). In all subsequent experiments agonists were present for the entire treatment period, which was 15 min in experiments that examined Ser 133 CREB phosphorylation.

Activation of ionotropic glutamate receptors and L-type Ca^{2+} channels induces CREB phosphorylation and gene expression

To analyze the contribution of different ionotropic glutamate receptors to Ser 133 CREB phosphorylation, we used agonists specific for each class and found that NMDA (50 μM), AMPA (50 μM), and kainate (50 μM) all induced CREB phosphorylation (Fig. 2*A*). Higher concentrations of these agonists did not induce more CREB phosphorylation (data not shown). The medium contained Mg^{2+} (2 mM) as well as ambient glutamate levels (2–5 μM, as determined by HPLC measurement). The ambient glutamate levels supported the effect of NMDA on CREB phosphorylation in the presence of Mg^{2+} (see below). Ser 133 CREB phosphorylation was also induced by the L-type Ca^{2+} channel agonist FPL 64176 (20 μM; Fig. 2*A*). The phosphorylation of Ser 133 CREB coincided with *c-fos* gene expression in all stimulus paradigms used (Fig. 2*B*). A luciferase construct under the transcriptional control of three CRE enhancer elements, *3xCRE-luciferase* (Fig. 2*C*), was transfected into primary striatal cultures and was activated by all agonists, comparable with *c-fos* mRNA (Fig. 2*B*) and Ser 133 CREB phosphorylation (Fig. 2*A*).

Glutamate-mediated CREB phosphorylation and gene expression require functional NMDA receptors, Ca^{2+}, and Na^{+}

MK 801 (1 μM) and APV (100 μM), noncompetitive and competitive antagonists of the NMDA receptor, respectively, blocked glutamate (see Fig. 10*A*)-, NMDA-, AMPA-, and kainate (all 50 μM)-induced CREB phosphorylation (Fig. 3*A*). Expression of the endogenous *c-fos* gene (see Figs. 3*B*, 10*C*) and the *3xCRE-luciferase* construct (Fig. 3*C*) by ionotropic glutamate receptor agonists was also blocked by MK 801.

Glutamate- and NMDA-mediated CREB phosphorylation was dependent on extracellular Ca^{2+} ions. Removal of Ca^{2+} from the medium prevented CREB phosphorylation (Fig. 4*A*). Glutamate receptor–mediated CREB phosphorylation was also dependent on Na^{+} ions (Fig. 4*B*). In Na^{+}-free medium, glutamate could not induce CREB phosphorylation (Fig. 4*B*), even in the absence of the NMDA-blocking ion Mg^{2+}. However, influx through voltage-operated Na^{+} channels was not necessary for glutamate- or FPL 64176–mediated CREB phosphorylation, because the Na^{+} channel blocker TTX had no effect (Fig. 4*C*).

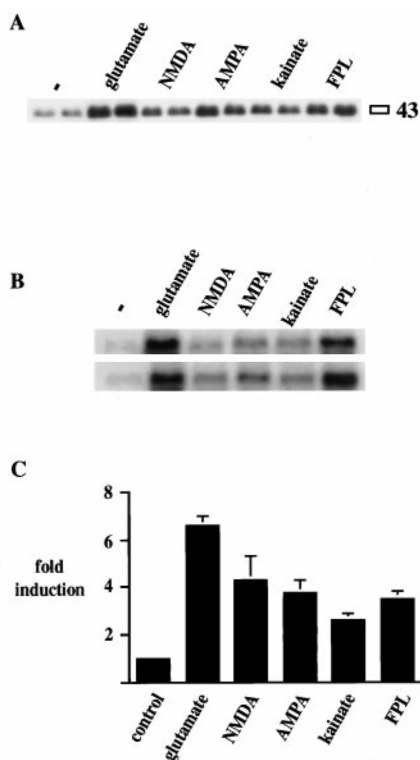

Figure 2. Activation of ionotropic glutamate receptors and of L-type Ca^{2+} channels induces CREB phosphorylation, *c-fos* gene expression, and the *3xCRE-luciferase* construct in primary striatal cultures. *A*, Cultures were exposed to glutamate (50 μM), NMDA (50 μM), AMPA (50 μM), kainate (50 μM), and FPL 64176 (*FPL*; 20 μM) and harvested 15 min after the addition of each drug. Immunoblots were developed with the Ser^{133} CREB antiserum. All drugs induced CREB phosphorylation to varying degrees. Treatments are shown in duplicates from a representative experiment that was repeated four times. *B*, Cultures were exposed to glutamate (50 μM), NMDA (50 μM), AMPA (50 μM), kainate (50 μM), and FPL 64176 (20 μM) and harvested 40 min after the addition of each drug. Northern blots were developed with a *c-fos* riboprobe. All drugs induced *c-fos* mRNA to varying degrees. Duplicate treatments are shown in two separate blots. The experiment was repeated twice. *C*, Primary striatal cultures were transfected with a *3xCRE-luciferase* construct and treated with glutamate (50 μM), NMDA (50 μM), AMPA (50 μM), kainate (50 μM), and FPL 64176 (20 μM) for 6 hr. Cells were harvested, and luciferase activity was measured. The average fold induction of luciferase activity (± SEM) over control levels is shown ($n = 10$ for each treatment). − indicates control without agonist.

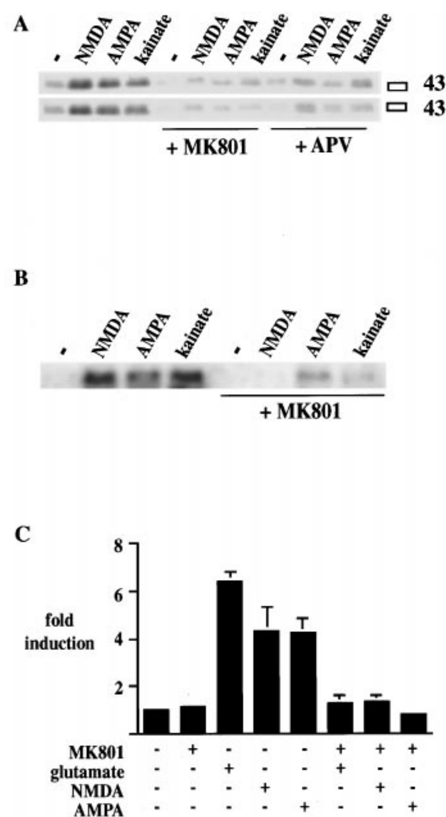

Figure 3. NMDA antagonists block ionotropic glutamate receptor–mediated Ser^{133} CREB phosphorylation, *c-fos* gene expression, and the induction of the *3xCRE-luciferase* construct in primary striatal cultures. *A*, Ser^{133} CREB phosphorylation mediated by NMDA (50 μM), AMPA (50 μM), and kainate (50 μM) is blocked by pretreatment for 20 min with the NMDA antagonists MK 801 (1 μM) and APV (100 μM). Duplicate treatments are shown in two separate blots. The experiment was repeated four times. (See also Fig. 10.) *B*, *c-fos* gene induction mediated by NMDA (50 μM), AMPA (50 μM), and kainate (50 μM) is blocked by pretreatment for 20 min with the NMDA antagonist MK 801 (1 μM). Duplicate treatments are shown in two separate blots. The experiment was repeated four times. (See also Fig. 10.) *C*, Induction of the *3xCRE-luciferase* construct after treatment for 6 hr with glutamate (50 μM), NMDA (50 μM), and AMPA (50 μM) is blocked by pretreatment for 20 min with the NMDA antagonist MK 801 (1 μM; $n = 6$ for each treatment). The block of induction by MK 801 is significant in all groups. Data are the average ± SEM. − indicates control without agonist.

Glutamate-mediated CREB phosphorylation and gene expression are supported by AMPA/kainate receptors

The role of AMPA/kainate receptors in glutamate-mediated gene expression was investigated with the AMPA/kainate receptor inhibitors GYKI 52466 (50 μM) and DNQX (100 μM; Fig. 5). Because DNQX can bind the glycine site of the NMDA receptor (Patel et al., 1990) and thus has inhibitory properties for NMDA and AMPA/kainate receptors, we used the more specific AMPA/kainate inhibitor GYKI 52466 in experiments that differentiated inhibition of the NMDA receptor from that of AMPA/kainate receptors. Both inhibitors partially blocked glutamate (50 μM)- and NMDA (50 μM)-mediated CREB phosphorylation and *c-fos* gene expression (DNQX not shown) and completely blocked AMPA (50 μM)- and kainate (50 μM)-mediated CREB phosphorylation and *c-fos* gene expression (Fig. 5*A*,*B*). FPL 64176 (20 μM)–mediated CREB phosphorylation or *c-fos* gene expression was not affected by GYKI 52466 (see Figs. 5*A*,*B*, 10*B*,*D*) or DNQX (data not shown). In transfection assays with the *3xCRE-luciferase* construct, glutamate-and AMPA-mediated induction was blocked by DNQX (Fig. 5*C*).

AMPA/kainate receptors relieve the Mg^{2+} block of the NMDA receptor

The role of AMPA/kainate receptors in NMDA-mediated CREB phosphorylation was further revealed in experiments that examined the effect of Mg^{2+} (Fig. 5*D*). With ambient glutamate (2–5 μM), NMDA induced CREB phosphorylation despite the presence of Mg^{2+} (2 mM; Fig. 5*D*, *+Mg*$^{2+}$*/+glutamate*). This phosphorylation was blocked by the AMPA/kainate antagonist GYKI 52466 (50 μM). In the absence of glutamate, NMDA (50 μM) did not induce CREB phosphorylation in the presence of Mg^{2+} (Fig. 5*D*, *+Mg*$^{2+}$*/−glutamate*). In the absence of Mg^{2+}, NMDA-induced CREB phosphorylation was independent of AMPA/kainate receptors because it was insensitive to GYKI 52466 and did not require ambient glutamate (Fig. 5*D*, *−Mg*$^{2+}$*/*

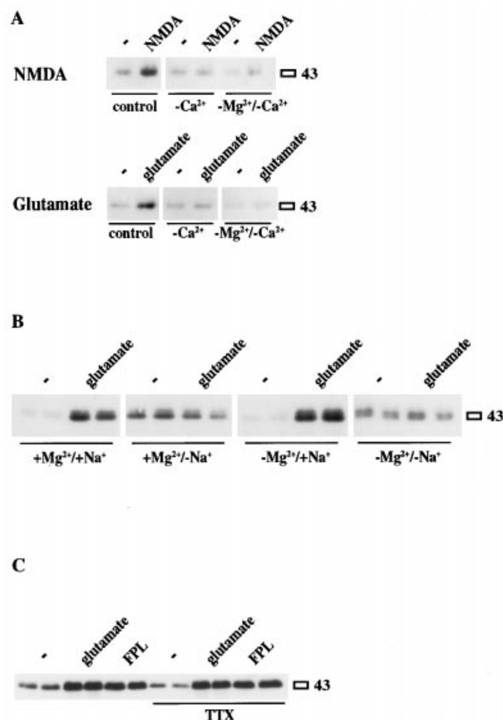

Figure 4. The role of Na^+, Mg^{2+}, and Ca^{2+} in Ser^{133} CREB phosphorylation mediated by ionotropic glutamate receptors. Primary striatal cultures were switched to defined salt solutions (see Materials and Methods) and treated 24 hr later as indicated. Immunoblots were developed with the Ser^{133} phospho-CREB antiserum. *A*, Ca^{2+} is necessary for NMDA- and glutamate-mediated phosphorylation of Ser^{133} CREB. In medium without Ca^{2+} ($-Ca^{2+}$) neither NMDA (50 μM; *top*) nor glutamate (50 μM; *bottom*) can induce Ser^{133} CREB phosphorylation. This dependency persists in Mg^{2+}-free medium. A representative experiment that was repeated twice is shown. *B*, Na^+ is necessary for glutamate-mediated phosphorylation of Ser^{133} CREB. In medium without Na^+ ($-Na^+$) glutamate (50 μM) cannot induce Ser^{133} CREB phosphorylation. This dependency persists in Mg^{2+}-free medium. Treatments are shown in duplicates from a representative experiment that was repeated twice. *C*, Na^+ channels are not involved in Ser^{133} CREB phosphorylation. The voltage-operated Na^+ channel blocker TTX (2 μM) does not block glutamate (50 μM)- or FPL 64176 (20 μM)–mediated CREB phosphorylation. Treatments are shown in duplicates from a representative experiment that was repeated three times. − indicates control without agonist.

−glutamate). These data suggest that AMPA/kainate receptors are necessary for removal of the Mg^{2+} block of NMDA receptors.

L-type Ca^{2+} channels are necessary for glutamate-mediated CREB phosphorylation and gene expression

The role of L-type Ca^{2+} channels in CREB phosphorylation and gene expression stimulated by ionotropic glutamate receptors was examined with the L-type Ca^{2+} channel blocker nifedipine (Fig. 6). Nifedipine (20 μM) blocked CREB phosphorylation induced by glutamate, NMDA, AMPA, or kainate (all 50 μM) or by the L-type Ca^{2+} channel agonist FPL 64176 (20 μM; see Figs. 6*A*,*D*, 10*A*,*B*). The block of glutamate receptor–mediated CREB phosphorylation was also observed with another L-type Ca^{2+} channel antagonist, verapamil (data not shown). Likewise, induction of *c-fos* gene expression by ionotropic glutamate receptor agonists (50 μM) and by FPL 64176 (20 μM) was blocked by nifedipine (20 μM; see Figs. 6*B*, 10*C*,*D*). In transfection experiments with the *3xCRE-luciferase* construct, nifedipine (20 μM) inhibited glutamate- and FPL 64176–induced increases in luciferase expression (Fig. 6*C*). Because a transient phosphorylation of CREB in the presence of nifedipine may not have been detected after 15 min, we harvested cultures between 1 and 15 min after glutamate stimulation in the presence or absence of nifedipine (20 μM). Nifedipine blocked glutamate-mediated CREB phosphorylation at all times examined (Fig. 6*D*; note that this time course is different from the time course in Fig. 1*B*, because the cells were harvested immediately after treatment). Because neurons in culture synapse onto each other, we examined whether the GABA antagonists bicuculline (Fig. 6*E*) or picrotoxin (data not shown) affect the inhibitory potency of nifedipine on glutamate-mediated Ser^{133} CREB phosphorylation. Neither antagonist stimulated CREB phosphorylation, nor did either affect the potency of nifedipine. In line with the excitatory properties of GABA in early development (Cherubini et al., 1991), both antagonists slightly inhibited CREB phosphorylation after glutamate treatment.

An antisense oligonucleotide approach was used to confirm these results. A knockdown of L-type Ca^{2+} channels with antisense oligonucleotides against the α1C and α1D subunits of Ca^{2+} channels diminished glutamate- and FPL 64176–induced CREB phosphorylation significantly (Fig. 7*A*,*B*), supporting a role for L-type Ca^{2+} channels in glutamate-mediated CREB phosphorylation. With an antibody against α1C, a decrease in α1C protein was detected in cultures treated with antisense oligonucleotides (Fig. 7*A*, *α1C*). No change in total CREB protein was seen (Fig. 7*A*, *CREB*).

FPL 64176–mediated CREB phosphorylation and gene expression are independent of functional ionotropic glutamate receptors

The NMDA antagonist MK 801 (1 μM) did not block FPL 64176 (20 μM)–mediated CREB phosphorylation (see Figs. 8*A*, 10*B*), even at concentrations submaximal for the stimulation of CREB phosphorylation (Fig. 8*B*). Likewise, the AMPA/kainate antagonists GYKI 52466 (50 μM; see Figs. 5*A*, 10*B*) or DNQX (100 μM; data not shown) had no effect on FPL 64176–mediated CREB phosphorylation, nor did a combination of NMDA and AMPA/kainate receptor antagonists [MK 801 (1 μM) and DNQX (100 μM); Fig. 8*C*]. Knockdown of NMDA receptors with antisense oligonucleotides had no effect on FPL 64176 (20 μM)–mediated CREB phosphorylation but reduced CREB phosphorylation after NMDA (50 μM) treatment (Fig. 7*C*,*D*). A combination of antisense oligonucleotides against NR1, NR2A, and NR2B was used in these experiments, and NR1 protein was reduced in antisense-treated cultures (Fig. 7*C*, *NR1*). NR1 is a necessary component of all NMDA receptors (Hollmann and Heinemann, 1994), and NR2A and NR2B are the most prevalent NR2 subtypes in the striatum (Standaert et al., 1994; Landwehrmeyer et al., 1995; Mutel et al., 1998). *c-fos* gene expression activated by L-type Ca^{2+} channel agonists was also unaffected by the AMPA/kainate antagonist GYKI 52466 (50 μM; see Figs. 5*B*, 10*D*) and the NMDA antagonist MK 801 (1 μM; Fig. 8*D*). Combinations of FPL 64176 and AMPA or of FPL 64176 and NMDA were unaffected by MK 801 or by GYKI 52466 (Fig. 9), although these combinations were blocked by the L-type Ca^{2+} channel antagonist nifedipine. Moreover, Ser^{133} CREB phosphorylation induced by a combination of the glutamate receptor agonists AMPA and NMDA was significantly blocked by nifedipine (20 μM), MK 801 (1 μM), and GYKI 52466 (50 μM; Fig. 9). A comparison of the inhibitory properties of MK 801, GYKI

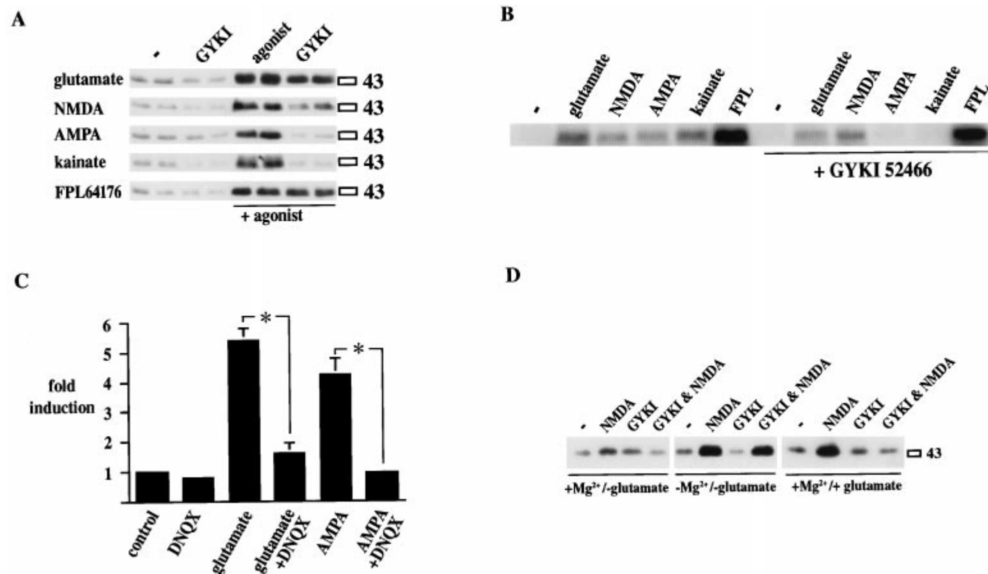

Figure 5. The AMPA/kainate antagonists GYKI 52466 and DNQX block ionotropic glutamate receptors to varying degrees, but they do not block L-type Ca^{2+} channels in primary striatal cultures. *A*, Ser^133^ CREB phosphorylation mediated by AMPA (50 μM) or kainate (50 μM) was fully blocked by GYKI 52466 (*GYKI*; 50 μM). Glutamate (50 μM)- and NMDA (50 μM)-mediated CREB phosphorylation was partially blocked by GYKI 52466, whereas FPL 64176 (20 μM)–mediated CREB phosphorylation was not blocked by GYKI 52466. Treatments are shown in duplicates. (See also Fig. 10.) *B*, *c-fos* gene expression induced by AMPA (50 μM) or kainate (50 μM) was fully blocked by GYKI 52466 (50 μM), whereas *c-fos* gene expression induced by glutamate (50 μM) or NMDA (50 μM) was partially blocked by GYKI 52466. *c-fos* gene expression induced by FPL 64176 (20 μM) was not blocked by GYKI 52466. The experiment was repeated three times. (See also Fig. 10.) *C*, DNQX (100 μM) reduced glutamate (50 μM)-mediated induction and blocked AMPA (50 μM)-mediated induction of the *3xCRE-luciferase* construct ($n = 6$). *Asterisks* mark statistically significant differences between groups that are linked in the graph. Data are the average ± SEM. *D*, Mg^{2+} prevents phosphorylation of Ser^{133} CREB by NMDA (50 μM) in the absence of AMPA/kainate activity. In glutamate-free medium, NMDA (50 μM) has little effect on CREB phosphorylation in the presence of Mg^{2+} (2 mM) but readily induces CREB phosphorylation in the absence of Mg^{2+} (compare *+Mg^2+^/−glutamate* with *−Mg^2+^/−glutamate*). Ambient glutamate levels (2–5 μM) are sufficient to prevent Mg^{2+} (2 mM) from blocking NMDA-mediated CREB phosphorylation (compare *+Mg^2+^/−glutamate* with *+Mg^2+^/+glutamate*). In the presence of Mg^{2+} (2 mM) the AMPA/kainate receptor antagonist GYKI 52466 (50 μM) blocks NMDA (50 μM)-mediated CREB phosphorylation but has little effect in the absence of Mg^{2+} (compare all three conditions). A representative experiment that was repeated twice is shown. Note that *+glutamate* indicates the exposure to 10 μM glutamate 24 hr before the experiment until the conclusion of the experiment; *−glutamate* indicates a change to glutamate-free medium 24 hr before the experiment until the conclusion of the experiment. Small amounts of metabolic glutamate (in the nanomolar range) released during the 24 hr may be present in the medium and explain the slight increase of CREB phosphorylation in the *+Mg^2+^/−glutamate* condition. − indicates control without agonist.

52466, and nifedipine on glutamate- and FPL 64176–activated CREB phosphorylation and *c-fos* gene expression is shown in Figure 10.

DISCUSSION

Glutamate mediates CREB phosphorylation and gene expression in primary striatal cultures in a Ca^{2+}- and Na^{+}-dependent manner

Glutamate was a potent inducing agent of Ser^{133} CREB phosphorylation in primary striatal cultures. Phosphorylation of Ser^{133} CREB was dependent on Ca^{2+} and Na^{+} and reached maximum levels after a 3 min exposure to glutamate. The subsequent expression of Fos protein was observed between 1 and 5 hr. The timing of induction of CREB phosphorylation and Fos protein expression is in agreement with regulation of the *c-fos* gene by Ser^{133} phospho-CREB. Glutamate treatment had no effect on the levels of CREB protein, consistent with the notion that CREB activates gene expression after it is phosphorylated on Ser^{133} and not via an increase in its protein levels. Because the *c-fos* promoter can also be regulated by regulatory elements other than CREB, we examined the regulation of a transfected reporter gene, luciferase, under the exclusive control of three copies of a consensus CREB-binding site (CRE). The regulation of the *3xCRE-luciferase* reporter construct by ionotropic glutamate receptor agonists was comparable with the regulation of the endogenous *c-fos* gene.

AMPA/kainate receptors activate the NMDA receptor by relieving the Mg^{2+} block

AMPA/kainate receptor agonists induced CREB phosphorylation and *c-fos* gene expression by an NMDA receptor–dependent mechanism. In glutamate-containing medium, NMDA antagonists blocked AMPA- and kainate-mediated Ser^{133} CREB phosphorylation and *c-fos* gene expression, demonstrating the need for functional NMDA receptors. In the presence of Mg^{2+}, AMPA/kainate receptors provided the depolarization that enabled the NMDA receptor to open in response to glutamate and glycine. In fact, the ability of the NMDA receptor to mediate CREB phosphorylation in Mg^{2+}-containing medium was significantly hampered when AMPA/kainate receptors were blocked. Our data suggest that a preexisting interaction of the NMDA receptor with ambient glutamate and glycine levels caused channel opening after the relief of the Mg^{2+} block by AMPA/kainate receptors. In glutamate-free medium, AMPA/kainate receptor agonists could not induce Ser^{133} CREB phosphorylation (data not shown). NMDA receptors induced CREB phosphorylation in glutamate-free medium only in the absence of Mg^{2+} but depended on glutamate (and AMPA/kainate receptors) in the presence of Mg^{2+}.

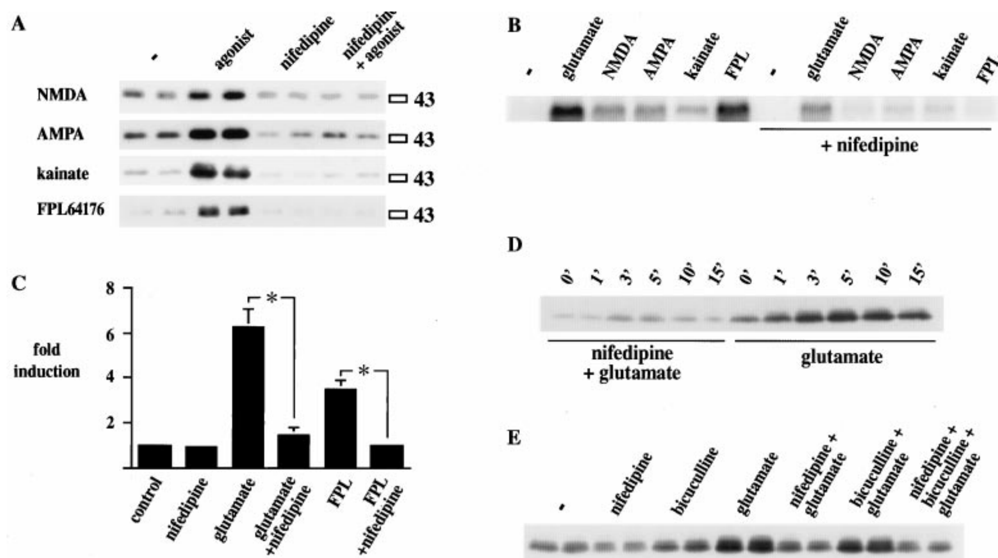

Figure 6. L-type Ca^{2+} channel blockers inhibit ionotropic glutamate receptor–mediated CREB phosphorylation in primary striatal cultures. *A*, Ser^{133} CREB phosphorylation after treatment with NMDA (50 μM), AMPA (50 μM), kainate (50 μM), and FPL 64176 (20 μM) is blocked by the L-type Ca^{2+} channel antagonist nifedipine (20 μM). All treatments are shown in duplicates. The experiment was repeated once. (See also Fig. 10.) *B*, *c-fos* mRNA induced by glutamate (50 μM), NMDA (50 μM), AMPA (50 μM), kainate (50 μM), and FPL 64176 (20 μM) is inhibited by nifedipine (20 μM). The experiment was repeated three times. (See also Fig. 10.) *C*, Nifedipine (20 μM) inhibited glutamate (50 μM)- and FPL 64176 (20 μM)–mediated induction of the *3xCRE-luciferase* construct ($n = 6$). *Asterisks* mark statistically significant differences between groups that are linked in the graph. Data are the average ± SEM. *D*, Nifedipine (20 μM) blocked glutamate (50 μM)-mediated CREB phosphorylation independent of time of exposure to glutamate. Cultures were exposed to glutamate for the *times* indicated *above* the *blots* and harvested immediately after exposure. Pretreatment with nifedipine (20 min) blocked glutamate-mediated CREB phosphorylation at all times. The experiment was repeated three times. *E*, The GABA receptor antagonist bicuculline (100 μM) did not prevent the inhibitory action of nifedipine (20 μM) on glutamate (50 μM)-mediated CREB phosphorylation. Preincubation with bicuculline did not increase glutamate-mediated CREB phosphorylation, nor did it affect the inhibitory action of nifedipine on glutamate-mediated CREB phosphorylation. − indicates control without agonist.

Figure 7. Knockdown of the L-type Ca^{2+} channel reduces CREB phosphorylation after treatment of cultures with glutamate or with FPL 64176. Knockdown of the NMDA receptor reduces CREB phosphorylation only after treatment with NMDA (50 μM). Striatal cultures were exposed to antisense oligonucleotides (*AS*) against the α1 subunits of the L-type Ca^{2+} channel (α1C and α1D) (*A, B*) or against the NMDA receptor (NR1, NR2A, and NR2B) (*C, D*) for 5 d and treated with glutamate (50 μM), NMDA (50 μM), or FPL 64176 (20 μM). In control experiments, sister cultures were treated with scrambled antisense oligonucleotides (*SAS*). *A*, Immunoblots developed with the Ser^{133} CREB antiserum (*P-CREB*), an antiserum against the α1C subtype of the L-type Ca^{2+} channel (*α1C*), or an antiserum against CREB (*CREB*). The antisense treatment reduced the α1C subtype of the L-type Ca^{2+} channel but not the levels of total CREB protein. Glutamate (50 μM)- and FPL 64176 (20 μM)–mediated CREB phosphorylation was partially blocked by the antisense treatment. *B*, Average induction of CREB phosphorylation (± SEM) after treatment with glutamate (50 μM) or FPL 64176 (20 μM) in cultures exposed to *SAS* (control) or *AS* ($n = 4$). The reduction in CREB phosphorylation in the presence of antisense oligonucleotides was statistically significant for glutamate-and FPL 64176–treated cultures (marked by *asterisks*). *C*, Immunoblots developed with the Ser^{133} CREB antiserum (*P-CREB*), an antiserum against the NR1 subtype of the NMDA receptor (*NR1*), or an antiserum against CREB protein (*CREB*). Levels of NR1 protein were reduced, whereas levels of total CREB protein were unaffected. The antisense treatment partially blocked NMDA (50 μM)-mediated CREB phosphorylation but did not affect FPL 64176 (20 μM)–mediated CREB phosphorylation. *D*, Average induction of CREB phosphorylation (± SEM) after treatment with NMDA (50 μM) or FPL 64176 (20 μM) in cultures exposed to *SAS* (control) or *AS* ($n = 4$). The reduction in CREB phosphorylation in the presence of antisense oligonucleotides was statistically significant for NMDA-treated cultures (marked by *asterisks*).

Two findings support the hypothesis that AMPA/kainate receptors mediate gene expression by removing the Mg^{2+} block of NMDA receptors; in the absence of extracellular NMDA or glutamate or when NMDA receptors were blocked, AMPA/kainate receptor stimulation could not induce CREB phosphorylation. Furthermore, in the absence of Mg^{2+}, AMPA/kainate receptors were irrelevant for NMDA receptor–mediated phosphorylation of Ser^{133} CREB.

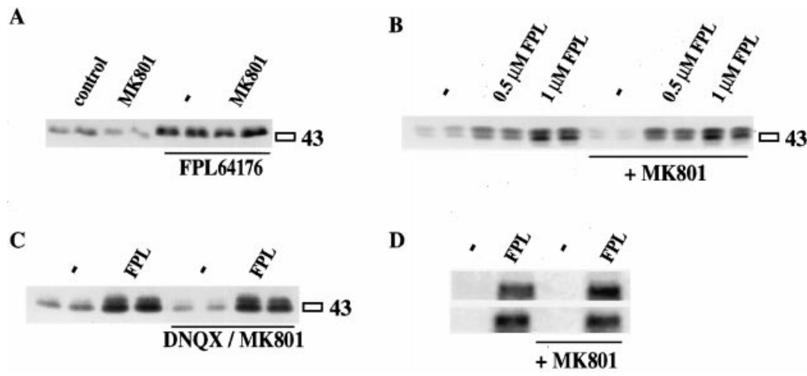

Figure 8. Ionotropic glutamate receptor antagonists cannot block FPL 64176–mediated Ser^{133} CREB phosphorylation in primary striatal cultures. *A*, The NMDA antagonist MK 801 (1 μM) does not block FPL 64176 (20 μM)–mediated CREB phosphorylation. All treatments are shown in duplicates. The experiment was repeated four times in duplicates. (See also Fig. 9.) *B*, Lower concentrations of FPL 64176 (0.5 and 1 μM) are also not blocked by MK 801. All treatments are shown in duplicates. *C*, A mixture of DNQX (100 μM) and MK 801 (1 μM) does not block FPL 64176 (20 μM)–mediated CREB phosphorylation. All treatments are shown in duplicates. The experiment was repeated twice. *D*, *c-fos* gene expression induced by FPL 64176 (20 μM) is not blocked by MK 801 (1 μM). Duplicate treatments are shown in two separate blots. (See also Fig. 9.) − indicates control without agonist.

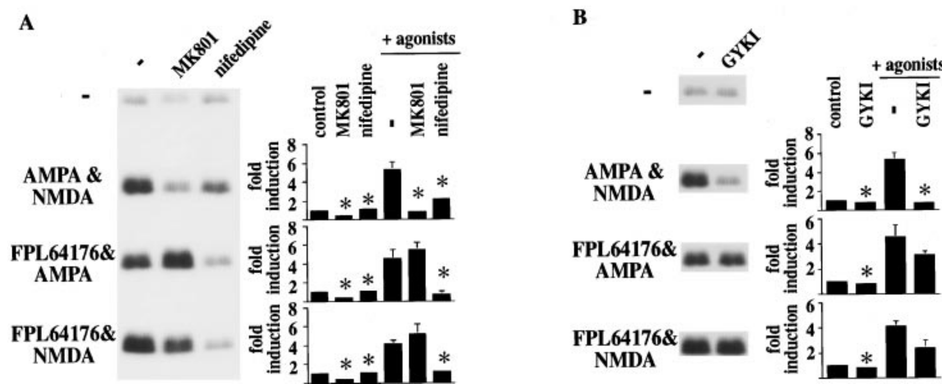

Figure 9. The induction of Ser^{133} CREB phosphorylation by ionotropic glutamate receptor agonists together with the L-type Ca^{2+} channel agonist FPL 64176 is inhibited by nifedipine but not by ionotropic glutamate receptor antagonists. Primary striatal cultures were treated with AMPA (50 μM) and NMDA (50 μM), FPL 64176 (20 μM) and AMPA (50 μM), or FPL 64176 (20 μM) and NMDA (50 μM). *A*, *B*, *Left*, Immunoblots with the Ser^{133} phospho-CREB antiserum are shown. *Right*, The *bar graphs* show the average fold induction of Ser^{133} CREB phosphorylation (± SEM) of six experiments. *Asterisks* mark statistically significant differences from the group treated with the agonist mixture. *A*, The induction of CREB phosphorylation was significantly blocked by the L-type Ca^{2+} channel antagonist nifedipine (20 μM) in all treatments but was not blocked by the NMDA antagonist MK 801 (1 μM) whenever FPL 64176 was part of the mixture. *B*, The induction of CREB phosphorylation was not blocked by the AMPA/kainate antagonist GYKI 52466 (50 μM) in the presence of FPL 64176. − indicates control without agonist.

L-type Ca^{2+} channels are essential for glutamate-mediated CREB phosphorylation and gene expression in primary striatal cultures

Ionotropic glutamate receptors induced CREB phosphorylation and *c-fos* gene expression with the assistance of L-type Ca^{2+} channels. Antagonists of L-type Ca^{2+} channels were able to reduce significantly the induction of CREB phosphorylation and gene expression by all ionotropic glutamate receptor agonists, alone or in combination. The effect of L-type Ca^{2+} channel blockers was independent of GABA activity in the cultures, because the potency of the block was not changed by GABA antagonists. Antisense oligonucleotides against the two α1 subtypes of the L-type Ca^{2+} channel in the brain, α1C and α1D, significantly reduced CREB phosphorylation mediated by glutamate or FPL 64176, whereas antisense oligonucleotides against the NMDA receptor had no effect on FPL 64176–mediated CREB phosphorylation. Glutamate-mediated CREB phosphorylation was dependent on Na^{+} ions yet independent of TTX-sensitive Na^{+} channels. This finding suggests that Na^{+} influx specifically through ionotropic glutamate receptors may be a necessary step toward glutamate-mediated CREB phosphorylation. We hypothesize that Na^{+} influx through NMDA receptors is important to depolarize the neuron and to trigger the opening of L-type Ca^{2+} channels. Even though there was Ca^{2+} in the Na^{+}-free medium, it was not enough to stimulate an intraneuronal second messenger pathway and CREB phosphorylation. This suggests that Na^{+} and Ca^{2+} ions have different roles in the signal transduction pathway, Na^{+} via membrane depolarization viaNMDA receptors and Ca^{2+} via membrane depolarization via L-type Ca^{2+} channels and via stimulation of the intracellular second messenger pathway.

AMPA/kainate receptors, NMDA receptors, and L-type Ca^{2+} channels contribute consecutively to the same signal transduction pathway

One important question was whether signals from ionotropic glutamate receptors and L-type Ca^{2+} channels mediate *c-fos* gene expression by distinct calcium-signaling pathways, as has been suggested for other culture systems (Bading et al., 1993; Hardingham et al., 1997). It has been found that the two primary *cis*-acting regulatory elements of the *c-fos* promoter, the CRE and the serum response element (SRE), are regulated differentially depending on the mode of calcium entry. The CRE is regulated by nuclear calcium from the L-type calcium channels, and the SRE is regulated by cytoplasmic calcium from the NMDA receptor (Bading et al., 1993; Hardingham et al., 1997). However, in our cultures, NMDA receptor–induced *c-fos* gene expression was dependent on L-type Ca^{2+} channels. Comparable regulation of CREB phosphorylation and *c-fos* gene expression was observed. Furthermore, direct activation of the L-type Ca^{2+} channels with FPL 64176 induced *c-fos* gene expression. This suggests that in our striatal cultures, either the SRE is not active or the L-type Ca^{2+} channel is able to provide all the factors necessary for *c-fos* transcriptional activation, including the SRE. The pharmacological profile of the ionotropic glutamate receptor antagonists and of the L-type Ca^{2+} channel blockers allowed us to determine further that AMPA/kainate receptors, NMDA receptors, and

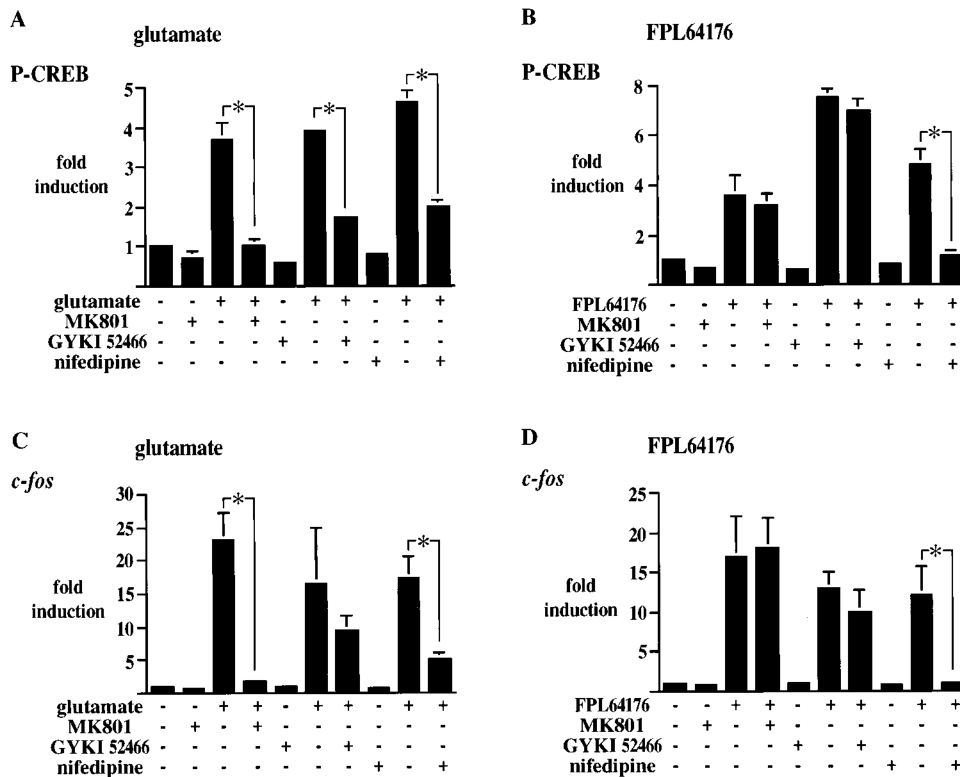

Figure 10. Average fold induction of Ser^{133} CREB phosphorylation and *c-fos* gene expression mediated by glutamate or FPL 64176 and inhibited by MK 801, GYKI 52466, or nifedipine. Each antagonist treatment was compared with the agonist treatment within the same blot. Different antagonists were run in separate blots. Levels of induction after agonist treatment were not identical in all blots and are presented in *individual bars* for each antagonist. Data are the average ± SEM. *Asterisks* mark statistically significant differences between groups that are linked in the graph. *A*, Glutamate (50 μM)-mediated CREB phosphorylation was significantly reduced after pretreatment with MK 801 (1 μM; $n = 6$), GYKI 52466 (50 μM; $n = 2$), or nifedipine (20 μM; $n = 4$). *B*, FPL 64176 (20 μM)–mediated CREB phosphorylation was unaffected by MK 801 (1 μM; $n = 9$) and by GYKI 52466 (50 μM; $n = 2$) but was blocked by nifedipine (20 μM; $n = 10$). *C*, Glutamate (50 μM)-mediated *c-fos* gene expression was significantly reduced after pretreatment with MK 801 (1 μM; $n = 10$) or nifedipine (20 μM; $n = 4$). The reduction after pretreatment with GYKI 52466 (50 μM; $n = 4$) was not significant. *D*, FPL 64176 (20 μM)–mediated *c-fos* gene expression was unaffected by MK 801 (1 μM; $n = 8$) and by GYKI 52466 (50 μM; $n = 4$) but was blocked by nifedipine (20 μM; $n = 6$).

L-type Ca^{2+} channels contribute consecutively to Ser^{133} CREB phosphorylation (see below).

The sequence of events leading from glutamate receptor activation to gene expression

AMPA/kainate receptor channels open after interaction with glutamate and permit Na^+ entry at the synapse (Kandel et al., 1991). The resulting local depolarization removes the Mg^{2+} block of the NMDA receptor, which permits the NMDA receptor to respond to extracellular glutamate and glycine. Opening of the NMDA receptor channel causes Na^+ and Ca^{2+} influx. Unlike the AMPA/kainate receptor channel that desensitizes rapidly, NMDA receptor channels have long opening times (Ascher and Nowak, 1987; Gasic and Hollmann, 1992). Therefore, NMDA receptors can trigger the opening of L-type Ca^{2+} channels that open during strong depolarization (Tsien et al., 1988). The activation of L-type Ca^{2+} channels promotes Ca^{2+} entry along the dendrites and at the cell body (Westenbroek et al., 1990; Schild et al., 1995). Second messengers activated by Ca^{2+} translocate to the nucleus and phosphorylate CREB (Deisseroth et al., 1998) (Fig. 11).

Our results suggest an important role for L-type Ca^{2+} channels in neuroplasticity of the striatum and confirm previous reports about the involvement of L-type Ca^{2+} channels in NMDA-mediated plasticity and toxicity (Weiss et al., 1990; Westenbroek et al., 1990; Chetkovich et al., 1991; Sucher et al., 1991; Aroniadou et al., 1993). Under our experimental conditions, NMDA receptors initiated a signal transduction pathway but did not initiate a significant intraneuronal second messenger pathway, either alone or together with AMPA/kainate receptors. Depolarization of L-type Ca^{2+} channels played a crucial role in the activation of an intraneuronal second messenger pathway.

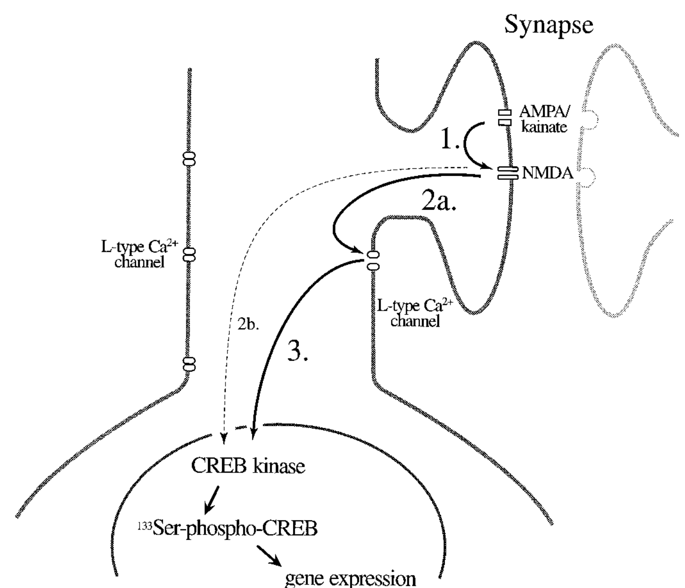

Figure 11. Model of the interaction of AMPA/kainate receptors, NMDA receptors, and L-type Ca^{2+} channels in striatal Ser^{133} CREB phosphorylation and gene expression. *1*, The activation of AMPA/kainate receptors causes Na^+ influx and a local depolarization that relieves the Mg^{2+} block of the NMDA receptor. *2a*, Activation of the NMDA receptor via ligand binding and depolarization leads to Na^+ influx as well as Ca^{2+} influx. Unlike the AMPA/kainate receptors, NMDA receptors do not rapidly desensitize and allow for a depolarization that is strong enough to trigger the opening of L-type Ca^{2+} channels. *3*, L-type Ca^{2+} channels allow for Ca^{2+} influx and activation of a kinase pathway that translocates to the nucleus to phosphorylate Ser^{133} CREB. *2b*, A signal transduction cascade originating at NMDA receptors that is independent of L-type Ca^{2+} channels is negligible for CREB phosphorylation or *c-fos* gene expression in primary striatal cultures.

Although the supportive role of AMPA/kainate receptors for NMDA receptors is in agreement with previous findings in hippocampal culture (Bading et al., 1995), other findings differ. In hippocampal cultures NMDA receptors and L-type Ca^{2+} channels seem to contribute to independent, parallel pathways rather than to the same pathway (Bading et al., 1993, 1995). Like in hippocampal cultures, L-type Ca^{2+} channels in the striatum activate the CRE and function independently of NMDA receptors. But although we do not exclude a direct pathway from NMDA receptors to the SRE in the striatum, this pathway in itself is not enough to mediate *c-fos* gene expression. This difference may be attributed to intrinsic differences between both types of neurons or to the different neurotransmitters released in either culture. Hippocampal neurons are mostly glutamatergic and express very high levels of glutamate receptors (Jarvis et al., 1987; Miyoshi et al., 1991; Sakurai et al., 1991). Striatal cultures are primarily GABAergic and express much lower levels of glutamate receptors (Jarvis et al., 1987; Miyoshi et al., 1991; Sakurai et al., 1991). Because neurons in culture synapse onto each other, hippocampal neurons excite each other after activation, whereas GABA in striatal neurons, dependent on the level of maturity (Cherubini et al., 1991), may be excitatory or inhibitory. To avoid trans-synaptic effects in hippocampal cultures, Na^{+} channels are often blocked with TTX. We repeated some of our experiments in the presence of TTX but observed results comparable with those of experiments without TTX (data not shown). Thus, there are fundamental differences in glutamate-mediated gene expression in neurons of both brain areas.

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