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Transcriptional Program Coordination by *N*-Methyl-D-aspartate-Sensitive Glutamate Receptor Stimulation in Primary Cultures of Cerebellar Neurons

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SUMMARY

In primary cultures of rat cerebellar neurons, a brief stimulation of glutamate receptors results in coordinated activation of a programmed early gene response involving increases in the amount of c-*fos*, c-*jun*, *jun*-B, and *zif*/268 mRNAs. Each of these genes was induced to a different extent and showed a temporal pattern characterized by either a monophasic "early" response, occurring within 30 min of glutamate addition, or a biphasic response (c-*jun*), lasting for up to 6 to 8 hr after the initial stimulus. The early phase of the glutamate-induced gene expression was prevented by 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid, a highly selective isosteric antagonist of the *N*-methyl-D-aspartate (NMDA)-sensitive glutamate receptor (NMDA receptor). The second phase of the c-*jun* response was not blocked when the NMDA receptors were completely inhibited after the initial pulse of agonist or when the quisqualate-kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione was added, suggesting that a brief NMDA receptor stimulation triggers a cascade of events critical for the manifestation of the delayed c-*jun* expression. Furthermore, gel retardation assays demonstrated that NMDA receptor activation results in a prolonged increase in nuclear DNA-binding activity specific for the AP-1 transcriptional regulatory element. Protein immunoblot analysis showed that the composition of this nucleoprotein complex changes as a function of time, reflecting a cascade that involves an increased translation of Fos and several Fos-related proteins. The coordinated induction of several different transcription factors and the variations in transcriptional complex formation initiated by NMDA receptor stimulation may be a key mechanism in the orchestration of specific target gene expression that underlies various aspects of neuronal function, including plasticity responses.

Various types of transsynaptically modulated neuronal plasticity operate in the mammalian central nervous system (for review see Ref. 1). Although a large number of studies have attempted to elucidate the molecular events that follow transmitter receptor activation, the precise nature of the processes underlying adaptive changes in neuron-to-neuron signaling is not known. One proposal considers plastic changes in neuronal function as deriving from structural modifications requiring a coordinated change in gene expression (2).

Long term modifications of neuronal function, including learning, memory consolidation, or compensation and repair triggered by neuropathological processes such as seizure activity and excitotoxicity, are associated with the activation of glutamate receptors sensitive to NMDA (3–5). A sustained increase in cytosolic ionized free Ca^{2+} subsequent to NMDA receptor stimulation appears to be critical in triggering a cascade of intracellular events. The successive activation of Ca^{2+}-dependent protein kinases (most importantly protein kinase C and Ca^{2+}/calmodulin-dependent protein kinases) and other Ca^{2+}-dependent enzymes (6–9) might ultimately result in coordinated changes in gene expression, consisting of the transcription of a set of early response genes such as c-*fos*, c-*jun*, and c-*myc*. These genes encode nuclear proteins (for reviews see Refs. 10 and 11) that, by interacting with regulatory DNA elements of neuronal target genes, can function as third messengers, bringing about transcriptional activation or repression. Thus, the products of early response genes, acting as third messengers, may encode a specific nuclear language for the coordination of many aspects of neuronal function, including plasticity responses. These genomic responses may be part of a transcriptional program reminiscent of that operating during central nervous system development.

One such third messenger appears to be the transcription factor AP-1. This was first identified as a DNA-binding activity present in HeLa cell extracts that could recognize specific DNA sequences (AP-1 sites) found within the key regulatory regions

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; CPP, 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; AP-1, activator protein 1; FRA, Fos-related antigens; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; PCP, phencyclidine; MK-801, dibenzocycloheptenemine; kb, kilobase; N-CAM, neural cell adhesion molecule; CREB, cAMP response element-binding protein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

of several genes (12). Recent studies have revealed that this factor consists of several polypeptides encoded by structurally related genes belonging to the *fos* and *jun* family (11, 13, 14). The c-*fos*-encoded protein (Fos) forms a stable heterodimer with the protein product of c-*jun* (Jun) via a "leucine zipper" domain common to both proteins (15). The Jun protein, in the form of a homodimer, exhibits a low level of DNA-binding activity, whereas Fos, which is incapable of forming homodimers, does not bind to AP-1 sites in the absence of Jun (16). Thus, homo- or heterodimerization is a prerequisite for the DNA-binding activity of these proteins and the consequent formation of a functional complex. Furthermore, the leucine zipper and the adjacent basic amino acid domain responsible for specific DNA-binding activity are conserved in the family of Fos-related proteins (FRAs) and in the family of Jun-related proteins (JunB, JunD) (17–19). However, these families display a low level of sequence similarity to each other in this region. The FRAs can also complex with Jun and bind to AP-1 sites; similarly, Jun family members may form hetero- and homodimeric complexes exhibiting DNA-binding activity (19–21).

Accordingly, a small number of factors may generate a large diversity of transcriptional complexes due to, among other things, the temporal pattern and relative extent of their expression, thus mediating numerous coordination patterns of target gene expression. Eukaryotic promoters represent a complex arrangement of short DNA sequences that are recognized by a wide variety of specific DNA-binding proteins (for review see Ref. 22). Therefore, the final coordinated transcriptional program may depend upon a complex interplay between several classes of transcription factors, as well as on posttranslational modification (such as phosphorylation and glycosylation) of these factors.

In primary cultures of rat cerebellar granule cells, the activation of NMDA receptors results in an induction of c-*fos* that is associated with a parallel increase in nuclear Fos immunoreactivity (23, 24). These cultures consist of a homogeneous neuronal population expressing various excitatory amino acid receptor subtypes linked to metabolotropic and ionotropic effector systems (for review see Ref. 25). We now show that several early-inducible gene products may participate with Fos in a transcriptional program initiated by NMDA receptor activation. Receptor stimulation induces the expression of c-*fos*, c-*jun*, *jun*-B, and *zif*/268, the latter being a recently described early response gene encoding a "zinc finger" protein showing a DNA-binding specificity unrelated to the AP-1 site (26, 27). Each of these genes is induced to a different extent and shows a temporal pattern characterized by either a monophasic response (early phase) or a biphasic response (with both an early and a delayed phase). Moreover, a brief glutamate pulse given to primary cultures of cerebellar neurons results in a prolonged increase in the level of AP-1 DNA-binding activity and in a temporal change in the steady state composition of the AP-1 DNA-protein complex.

Materials and Methods

Cell culture. Primary cultures of cerebellar granule cells were prepared from cerebella of 8-day-old rat pups (Sprague-Dawley), as previously described (28). Over 90% of the cells are glutamatergic granule cells, with minimal contamination (less than 3%) with glial and endothelial elements. The presence of inhibitory γ-aminobutyric acid-containing neurons (Purkinje, stellate, basket, and Golgi cells) amounts to about 5% of the total cell population (28). The experiments were performed on the 8th or 9th day of the cell culture.

Analysis of mRNA content. The different treatments of the granule cell monolayers were performed in tissue culture medium (basal Eagle's medium, GIBCO) containing 0.8 mM magnesium and supplemented with 10% fetal calf serum (GIBCO), 25 mM KCl, 2 mM glutamine, and 50 μg/ml gentamycin. The dishes were incubated at 37° in a humidified atmosphere (5% CO_2/95% O_2). At the indicated time points, the cells were lysed in guanidinium isothiocyanate and total RNA was prepared by gradient ultracentrifugation. Samples containing 20 μg of total RNA were electrophoresed through 1.1% agarose/2.2 M formaldehyde gels and transferred onto nitrocellulose membranes by capillary blotting. Hybridization of the blots to the different ^{32}P-labeled, randomly primed cDNA probes was performed for 16 to 24 hr at 42°, as described previously (24). Final washings were in 0.1 × SSC (1 × SSC buffer contains 0.015 M trisodium citrate and 0.15 M NaCl) with 0.1% sodium dodecyl sulfate for 1 hr at 55° for the c-*fos* probe, 50° for the c-*jun*, *jun*-B, and *zif*/268 probes, and 42° for the β-actin, N-CAM, and CREB probes. The filters were exposed to X-ray film (Kodak XAR-2) with an intensifying screen (Dupont Cronex Lightning Plus) at −70°. Before reprobing, the filters were incubated in 5 mM Tris·HCl, pH 8.0, 0.1 mM EDTA, 0.05% (w/v) sodium pyrophosphate, 0.1× Denhardt's solution (1 × contains 0.2% Ficoll, 0.2% polyvinylpyrollidone, 0.2% bovine serum albumin) for 1 hr at 65°.

Quantitation of the mRNA content and statistical analysis. The amount of RNA applied to the gels was determined by rehybridizing the stripped filters with a ^{32}P-labeled β-actin cDNA probe (24) and, in several cases, also with cDNA probes encoding CREB (29) and N-CAM (30). The hybridization signals were determined by scanning the appropriately exposed autoradiograms by laser densitometry (LKB Ultrascan 2202; Pharmacia, LKB). The mRNA content is expressed in arbitrary units, where 1 unit is defined as the peak densitometric area of the given mRNA hybridization signal divided by the corresponding peak densitometric area of the β-actin mRNA signal. Moreover, the densitometric analysis of the β-actin hybridization signal was compared with that corresponding to CREB and N-CAM, which consistently resulted in a time-dependent ratio very close to 1, indicating that in our primary cultures the β-actin signal is not regulated by our experimental manipulations and can, therefore, be taken as a reference signal. The results are expressed as the mean ± standard error of three or four independent experiments. Significant differences between hybridization signals were determined by Duncan's multiple range test (31).

Preparation of nuclear extracts and gel retardation assays. The granule cell monolayers were washed twice with ice-cold phosphate-buffered saline and collected by scraping. After centrifugation at 800 × *g* for 7 min, the pellet was resuspended in a buffer containing 10 mM HEPES (pH 7.9), 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EGTA, 1 mM EDTA, 0.32 M sucrose, 1 mM DTT, and the following protease inhibitors: 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine hydrochloride, 0.5 μg/ml leupeptin, and 1 μg/ml pepstatin. Nuclei were isolated by addition of NP-40 to the resuspended cell pellet, to a final concentration of 0.5%, followed by homogenization with a motor-driven Teflon pestle or a glass homogenizer (10 to 15 strokes) and centrifugation at 1000 × *g* for 10 min. The pellets were carefully resuspended and washed in the buffer described above, except that instead of sucrose it contained 20% glycerol. The nuclei were isolated by centrifugation as before, and the final nuclear pellet was resuspended in the glycerol buffer and subjected to salt extraction with 0.4 M KCl for 1 hr on a rotating platform. The nuclei were then transferred to Eppendorf microfuge tubes and collected by centrifugation (10,000 × *g* for 10 min). The supernatant was dialyzed overnight against a buffer containing 0.15 M KCl, 20 mM HEPES, pH 7.9, 0.1 mM EGTA, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and the protease inhibitors. The entire procedure was carried out at 4°. Protein concentration was determined by the method of Bradford (32).

A 30-base pair, double-stranded oligonucleotide corresponding to the AP-1 site from the α_1-antitrypsin gene was end-labeled with the Klenow

fragment of DNA polymerase I and both [^{32}P]dATP and [^{32}P]dGTP (33). The DNA-protein binding reactions were performed by incubating 800 ng of nuclear extract with approximately 5×10^4 cpm of the labeled AP-1 oligonucleotide for 30 min at room temperature, in the presence of 1 μg of poly(dI-dC), in 20 mM HEPES (pH 7.5), 40 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EGTA, 4% (w/v) Ficoll. The reaction mixture was subjected to electrophoresis on an 8% native polyacrylamide gel, in a buffer containing 22 mM Tris-borate (pH 7.4) and 5 μM EDTA. For specific and nonspecific competition studies, a 100-fold molar excess of unlabeled AP-1 probe or an AP-1-unrelated oligonucleotide probe was used, respectively. For quantitation, the autoradiograms were scanned by laser densitometry and the results were expressed as percentage of control (mean ± standard error of three independent experiments) and analyzed by Duncan's multiple range test (31).

Immunoblot analysis. After the incubation with 10 μM glutamate, the granule cells were washed with ice-cold phosphate-buffered saline, scraped, and centrifuged at 800 × *g* for 7 min. The pellet was resuspended in a buffer containing 10 mM Tris·HCl (pH 7.4), 10 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM $Na_2S_2O_4$, and the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 10 μM pepstatin A, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. The cells were then lysed with NP-40 for 5 min (final concentration, 0.5%) and the nuclear pellet was gently homogenized with a Teflon pestle and glass homogenizer (five to seven strokes) and centrifuged at 1000 × *g* for 10 min. The entire preparation of the nuclear extract was performed at 4°. After DNase treatment, protein aliquots (80 μg) were analyzed by 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Hybond membranes (Amersham), according to standard procedures. Immunoblotting was performed with affinity-purified polyclonal antibodies to Fos that were raised against a synthetic peptide corresponding to amino acids 127 to 152 of Fos (34, 35). Immunoreactive bands were detected by ^{125}I-labeled Protein A (0.1 μCi/ml). The appropriately exposed autoradiograms were scanned using a laser densitometer.

Results

Coordinated regulation of early response gene expression in primary cerebellar neurons following a glutamate pulse. In the present study we have demonstrated by Northern blot analysis that a protocol consisting of a pulse treatment (24) of primary cerebellar granule cells with 10 μM glutamate, followed 30 min later by 1 μM PCP, an NMDA receptor antagonist, resulted in a rapid increase in the steady state amount of c-*fos* mRNA. The amount of c-*fos* mRNA reached a peak at 30 min, returned to the basal level after 2 hr (Fig. 1A, Table 1), and thereafter remained unchanged for up to 12 hr after the addition of glutamate. The same treatment failed to alter the expression of the mRNA for the structural protein β-actin, similar to previously published results obtained in primary cultures of cerebellar neurons (24). Also, when compared with the β-actin hybridization signal, the expression of genes encoding both CREB and N-CAM was not altered by the glutamate treatment (Table 1). These observations allowed us to use the β-actin signal to monitor and correct for variations in the amounts of RNA loaded on the gels. Using intravital staining with fluorescein diacetate/propidium iodide (36), we documented that from 90 to 95% of the neurons were viable through the time frame studied following the glutamate treatment.

Because the functionally active AP-1 transcription complex is composed of stable heterodimers of the protein products of the c-*fos* and c-*jun*-related gene families, we investigated whether c-*jun* and the c-*jun*-related *jun*-B are activated simultaneously with c-*fos* when the neurons are stimulated with glutamate. Glutamate treatment elicited a significant increase in the steady state amount of *jun*-B mRNA within 30 min, with the amount returning to the basal level within 1 hr (Fig. 1B, Table 1). The induction showed a monophasic pattern similar to that observed with c-*fos* (Fig. 1A). In contrast, glutamate elicited a biphasic induction of c-*jun* expression, which clearly showed a rapid and transient early phase with an initial maximal stimulation at 30 min; this was followed by a longer lasting, delayed phase of c-*jun* induction, which began at 3 hr after glutamate addition and persisted for an additional 4 to 6 hr (Fig. 1B, Table 1). The same blot was rehybridized with the cDNA probes encoding CREB (29) and N-CAM (30). After correction for loading differences using the corresponding β-actin hybridization signals, the mRNA contents of CREB and N-CAM expressed in arbitrary units were virtually unchanged for up to 12 hr following the glutamate treatment (Table 1). The increase in c-*jun* expression was attributable to an increase in the amounts of two mRNA species, of 3.4 and 2.7 kb, consistent with the two different polyadenylation signals present in the gene (37). The relative extent of the increase in *jun*-B mRNA was similar to that of c-*fos*, whereas the basal c-*jun* expression was higher and the extent of induction less dramatic.

zif/268, the murine homolog of the NGFI-A and *egr*-1 genes (38, 39), belongs to a different subclass of early-inducible genes. We investigated whether *zif*/268 mRNA is present in cerebellar neurons and, if so, whether it is regulated by glutamate stimulation. As shown in Fig. 2, the basal expression of *zif*/268 was considerably lower than that of either c-*fos* or c-*jun*. Glutamate stimulation led to a rapid and transient increase in the 3.3-kb *zif*/268 mRNA species. The time course of this induction was very similar to that of the c-*fos* and *jun*-B mRNAs (Table 1).

The diversity of the time courses of the expression of different early-inducible genes activated by glutamate indicates that the responses can be grouped into at least two classes, that is, an "early" and a "delayed" phase of induction.

Pharmacology of the glutamate-mediated early-inducible gene expression. Because cerebellar granule cells contain various excitatory amino acid receptor subtypes, we investigated whether the induction of c-*fos*, c-*jun*, *jun*-B, or *zif*/268 expression was the consequence of the preferential activation of a specific subclass of glutamate receptors. The granule cells were incubated with 10 μM glutamate in the absence or presence of 10 μM CPP, a potent and highly selective isosteric antagonist of NMDA receptors (40). As shown in Fig. 3, the stimulatory action of glutamate on c-*fos*, c-*jun*, and *jun*-B expression was virtually completely blocked, whereas an incomplete inhibition of *zif*/268 expression was observed with this competitive antagonist. Comparable results were obtained with the noncompetitive NMDA receptor antagonist PCP (data not shown). Thus, in our neuronal cultures, the early phase of the glutamate-induced expression of these genes would appear to be regulated preferentially by stimulation of the NMDA-sensitive receptor subtype.

The unique delayed phase of the c-*jun* mRNA induction raised several questions concerning how this might be mediated. We investigated whether the protracted phase was the consequence of a cascade of events triggered by the initial glutamate pulse or whether it was due to other mechanisms that might involve the activation of other glutamate receptor subtypes by the glutamate spontaneously secreted from neurons or the

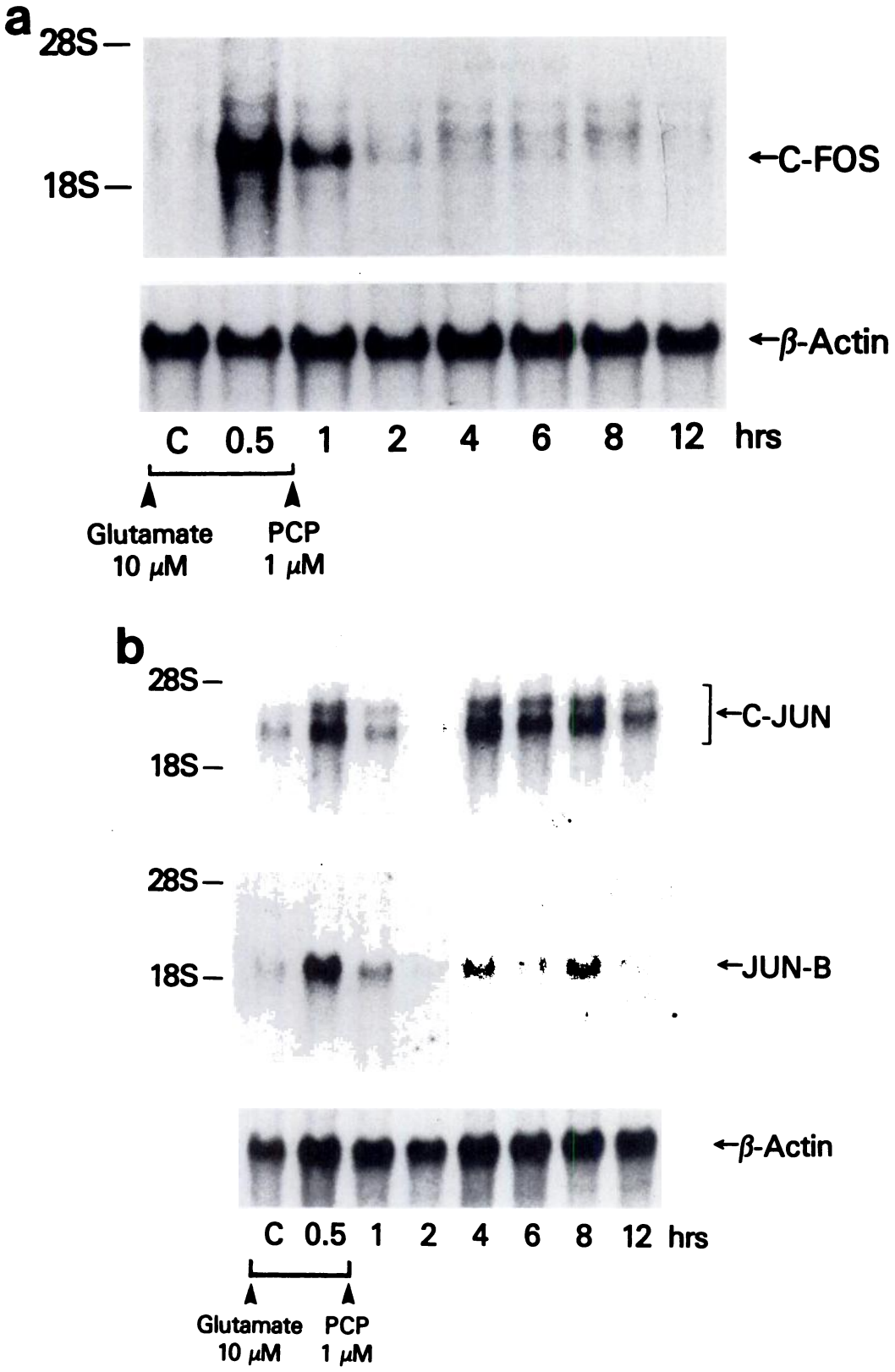

Fig. 1. Differential temporal induction of c-*fos*, *jun*-B, and c-*jun* genes by a glutamate pulse in primary cultures of cerebellar granule cells. A, Time course of c-*fos* mRNA induction. Granule cell monolayers were treated with 10 μM glutamate for 30 min before the addition of 1 μM PCP. Total RNA was prepared at the indicated times. Samples (20 μg) of total RNA were analyzed by the Northern blot technique. The positions of 28 S and 18 S ribosomal RNAs, as well the 2.2-kb c-*fos* and 1.8-kb β-actin mRNAs, are indicated. B, Time course of c-*jun* and *jun*-B mRNA induction. The granule cells were treated as described in A and total RNA was prepared at the indicated times. Northern blots containing 20 μg of total RNA were probed with random-primed c-*jun* and *jun*-B cDNA probes. *Arrows*, positions of the 3.4- and 2.7-kb c-*jun* mRNAs, the 2.1-kb *jun*-B mRNA, and the β-actin mRNA. *C*, control.

neuronal glutamate released following the initial depolarization. The primary neuronal cultures were exposed to glutamate for 30 min. Specific blockers of NMDA-sensitive (1 μM PCP or 0.5 μM MK 801) or of quisqualate-kainate-sensitive glutamate receptors (2 μM CNQX) were then added (for review see Ref. 41) and the incubations were continued for the time intervals indicated in Fig. 4. RNA was isolated and subjected to Northern analysis, and the relative levels of c-*fos* and c-*jun* mRNA, after correction for the respective β-actin signal, were expressed in arbitrary units. Glutamate in the absence of any antagonists increased the amount of c-*jun* mRNA by 30 min, which, after a small decrease, increased again and remained elevated for the

TABLE 1

Quantitation of the changes in mRNA contents of different early response genes following glutamate stimulation in primary cultures of cerebellar granule cells

The treatment of the cultures is described in the legend to Fig. 1A. The relative amounts of different mRNAs are expressed in arbitrary units, using β-actin as a reference signal (for details, see Materials and Methods). Each data point represents the mean ± standard error of three or four independent experiments.

	mRNA content							
	Control	0.5 hr	1 hr	2 hr	4 hr	6 hr	8 hr	12 hr
				units				
c-*fos*	1	5.2 ± 0.6[a]	1.6 ± 0.2[a]	1.0 ± 0.1	0.9 ± 0.2	0.8 ± 0.1	0.9 ± 0.1	1.0 ± 0.2
c-*jun*	1	2.2 ± 0.2[a,b]	1.5 ± 0.4	0.4 ± 0.1	2.4 ± 0.6[a,b]	2.2 ± 0.4[a,b]	1.9 ± 0.3[a,b]	1.3 ± 0.2
jun-B	1	4.4 ± 0.4[a]	1.3 ± 0.2	0.9 ± 0.2	1.1 ± 0.1	1.0 ± 0.1	0.9 ± 0.2	0.9 ± 0.1
zif/268	1	7.6 ± 0.6[a]	4.1 ± 0.5[a]	1.2 ± 0.2	1.0 ± 0.2	1.1 ± 0.2	1.0 ± 0.1	1.0 ± 0.1
CREB[c]	1	0.9	0.8	0.8	0.8	0.9	1.3	1.1
N-CAM[c]	1	1.0	0.8	0.9	0.8	0.8	0.9	1.0

[a] Significantly different ($p < 0.05$) from control.
[b] Significantly different ($p < 0.05$) from the 2-hr value by Duncan's multiple range test (31).
[c] The amount of CREB and N-CAM mRNAs indicated were measured using the nitrocellulose blot shown in Fig. 1B. Similar values were obtained by rehybridizing the blot shown in Fig. 1A.

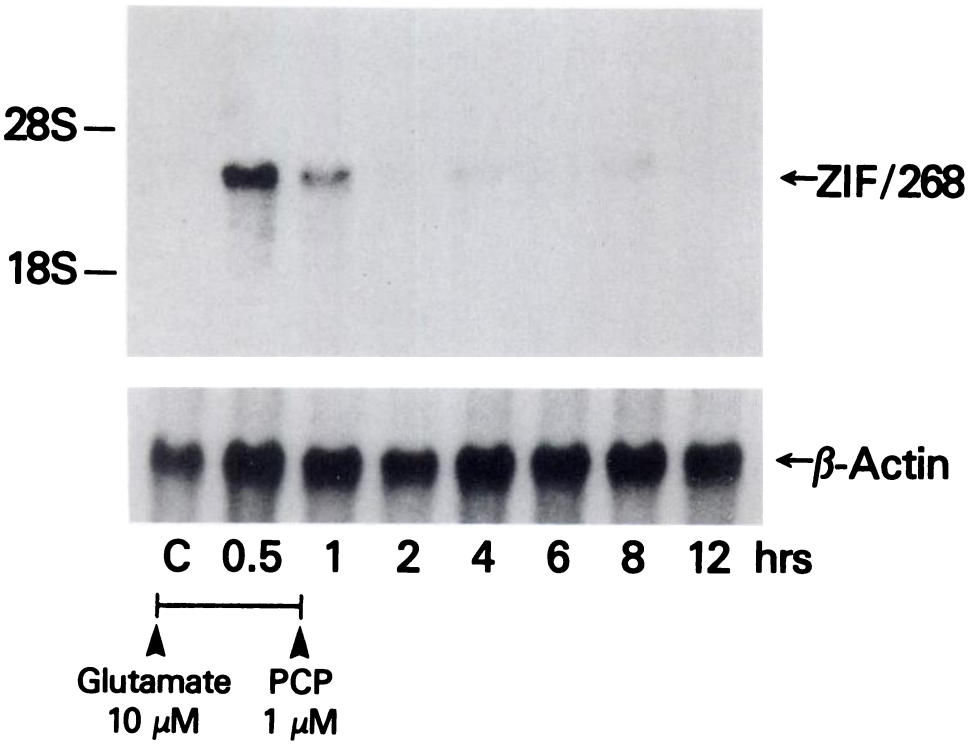

Fig. 2. Increase in the amount of *zif*/268 mRNA after a pulse stimulation of granule cells with glutamate. Primary cultures of cerebellar granule cells were treated with 10 μM glutamate for 30 min before the addition of 1 μM PCP, as described in the legend to Fig. 1. The blot presented in Fig. 1B was stripped and hybridized to the random-primed cDNA probe corresponding to *zif*/268. The positions of ribosomal RNAs and the 3.3-kb *zif*/268 mRNA are indicated. *C*, control.

7-hr period studied (Fig. 4, A and B). This pattern of c-*jun* mRNA induction was not altered significantly by the addition of the quisqualate-kainate receptor antagonist CNQX (Fig. 4A).

By the following experiments, we ruled out the possible involvement of NMDA receptors in the late phase of the c-*jun* induction. The application of either PCP or MK 801 after the 30-min exposure to glutamate resulted in a decrease in the amount of c-*jun* mRNA (Fig. 4B). That is, following the early phase peak at 30 to 45 min, the amount of c-*jun* mRNA declined by 2 hr to a level below that observed in the absence of any treatment. After 4 to 7 hr, the signal response surpassed the initial basal level of expression (Fig. 4B). The reduced amount of c-*jun* mRNA present 2 hr after the addition of glutamate was comparable to that obtained when the cultures were pretreated for 15 min with PCP before the addition of glutamate (Fig. 4B). Following PCP pretreatment, the subsequent addition of 10 μM glutamate failed to significantly increase the amount of c-*jun* mRNA. To verify that neither PCP nor MK 801 had lost its pharmacological potency during the long term incubation of the cultures, we treated granule cells for 6 to 8 hr with either 1 μM PCP or 0.5 μM MK 801 and then challenged them with glutamate. This procedure resulted in a significant decrease in the basal level of c-*jun* expression, and 10 μM glutamate failed to alter the amount of c-*jun* mRNA (data not shown). The analysis of c-*fos* mRNA under the same experimental conditions showed that PCP and MK 801, added 30 min after glutamate, accelerated the return of the signal to the basal level, whereas CNQX was without effect (Fig. 4C). A similar pattern was observed with the *jun*-B mRNA (data not shown). These observations suggest that a brief glutamate stimulus triggers a cascade of events critical for the manifestation of the delayed c-*jun* expression and that this expression is not the consequence of a persistent stimulation of glutamate receptors.

Prolonged increase in specific AP-1 DNA-binding activity in cerebellar neurons after a glutamate pulse.

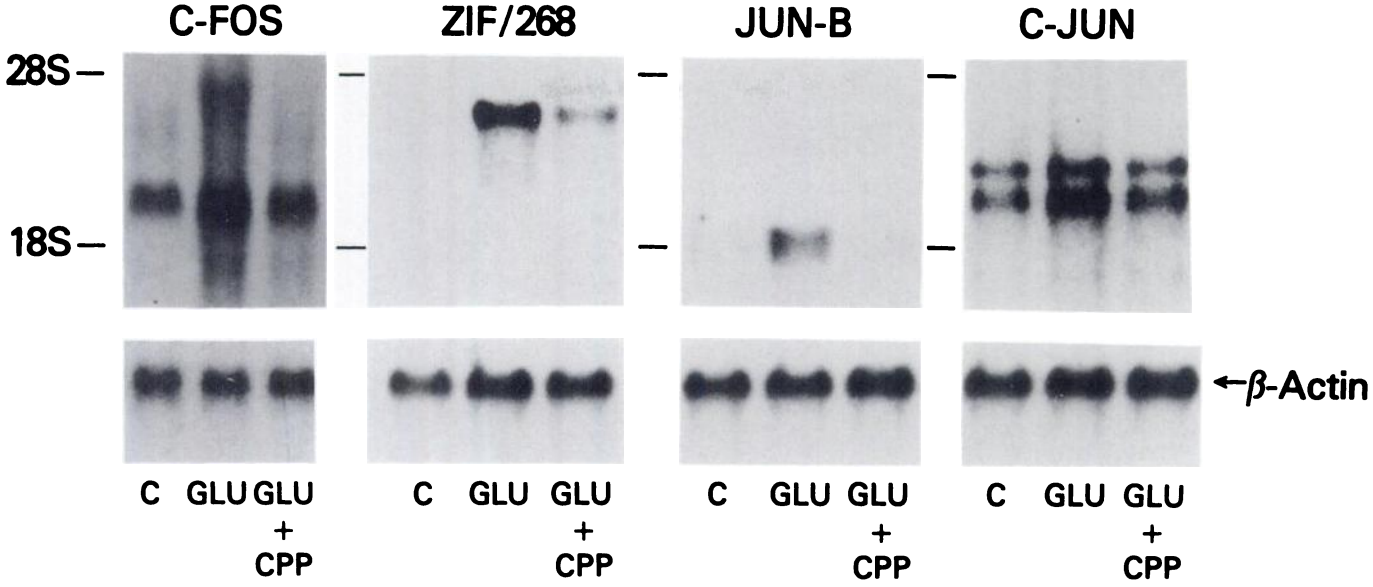

Fig. 3. CPP antagonizes the early phase of the immediate genomic response after glutamate (*GLU*) treatment. The granule cells were incubated for 30 min with 10 μM glutamate, in the absence or presence of the highly selective NMDA receptor antagonist CPP (10 μM). Total RNA was prepared and subjected to Northern analysis with random-primed cDNA probes for c-*fos*, *jun*-B, *zif*/268, and c-*jun*. *C*, control.

Nuclear protein complexes containing both Jun and Fos have been shown to bind to the consensus AP-1 recognition sequence T(T/G)AGTCA. We, therefore, investigated whether the glutamate-induced increase of c-*fos* and *jun* mRNAs resulted in an increased formation of functional AP-1 DNA-binding complexes. Using the paradigm described in Fig. 1, nuclear extracts were prepared at various times after glutamate treatment of the neuronal cultures and tested for DNA-binding activity in gel retardation assays with a synthetic oligonucleotide containing the AP-1 consensus sequence from the α_1-antitrypsin gene (33). As shown in the representative autoradiogram (Fig. 5A), nuclear extracts from cerebellar granule cells exhibited a single gel-retarded band, which corresponded to the DNA-bound protein complex. Compared with nonstimulated cells, glutamate treatment of cells elicited a significant increase in DNA-binding activity after 1 hr of treatment. The binding activity showed a biphasic character; it declined by 2 hr and increased again by 4 and 6 hr after glutamate addition (Fig. 5B). The bands were specific for the AP-1 oligonucleotides, because unlabeled specific oligonucleotide present in a 100-fold molar excess interrupted the formation of gel-shifted bands, whereas oligonucleotides containing unrelated sequences did not (data not shown).

Different temporal induction of Fos and FRAs after a glutamate pulse. The AP-1 DNA-binding activity shown in Fig. 5 occurred somewhat in parallel with the observed biphasic pattern of c-*jun* expression (Fig. 1B). However, the amount of c-*fos* mRNA decays to near basal levels within 2 hr after the glutamate stimulus (Fig. 1A). These observations prompted us to examine the temporal pattern of the appearance and disappearance of the Fos protein. Immunoblot analysis was performed on nuclear extracts prepared from glutamate-treated cerebellar neurons, using an affinity-purified antibody raised against the conserved M peptide region of Fos (34). A 5-fold increase in the amount of the M_r 55,000 Fos protein was seen within the first hour after the stimulus and progressively declined during the next 4 hr (Fig. 6). However, between 2 and 6 hr, the amounts of two Fos-related immunoreactive proteins of M_r 46,000 and 35,000 increased by 2.5- to 3-fold. Immunocytochemical analysis also indicated that there is an increased nuclear Fos-like immunoreactivity persisting 6 to 8 hr after glutamate treatment (data not shown).

Discussion

We have demonstrated that, in primary cultures of cerebellar neurons, a brief stimulation of NMDA receptors leads to a cascade of genomic events. This coordinated transcriptional activation displays characteristic early and delayed phases of induction. The participation of different subclasses of early-inducible genes, with different DNA-binding structural motifs, is evident. The induced mRNAs are translated and the post-translationally modified proteins, after returning to the nucleus, form functionally active DNA-binding complexes. The neurotransmitter pulse causes a prolonged biphasic increase in AP-1-specific DNA-binding activity in the primary neuronal cultures. The composition of the AP-1 transcriptional regulatory complex appears to change with time as a result of the sequential appearance and disappearance of Fos and Fos-related proteins. Our data provide insights as to how glutamate, through nuclear third messengers, may orchestrate the expression of specific target genes in neurons.

Prolongation of the glutamate signal in the nucleus. A brief glutamate pulse evoked a rapid and transient monophasic induction of c-*fos*, *jun*-B, and *zif*/268 mRNAs. However, we found a unique temporal pattern of c-*jun* expression. After a transient early induction, there was a prolonged increase in the amount of c-*jun* mRNA, lasting for 6 to 8 hr. The fact that the amounts of β-actin, CREB, and N-CAM mRNAs were not increased by glutamate indicates that the early-inducible gene response is specific. The primary neuronal cultures we use consist of a homogeneous neuron population, about 90% of which express the various glutamate receptor subtypes. Moreover, we have previously demonstrated that stimulation of these neuronal cultures with glutamate or phorbol esters elicits an increase of Fos immunoreactivity in 80 to 90% of the cells

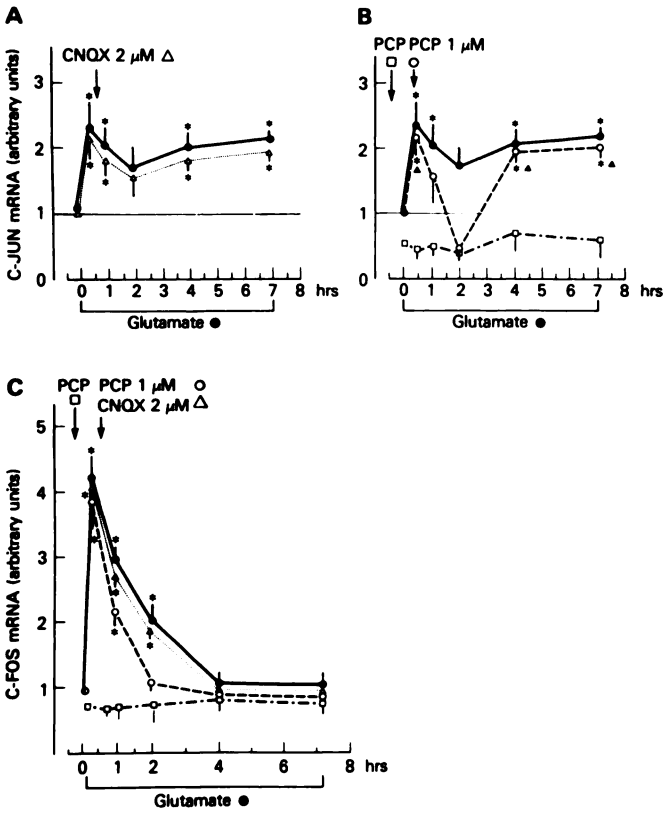

Fig. 4. Differential effects of glutamate receptor antagonists on c-*jun* and c-*fos* mRNA expression. A, The primary neuronal cultures were exposed to glutamate (10 μM) (added at time 0). After 30 min, 2 μM CNQX was added and the incubation was continued for the indicated time periods. The cells were harvested and RNA was prepared for Northern analysis with a c-*jun* probe. The amount of mRNA, after correction for the respective β-actin signal, is expressed in arbitrary units, as described in Materials and Methods. Each *point* represents the mean ± standard error of three independent experiments. ●, Glutamate alone; △, glutamate plus CNQX. B, The expression of c-*jun* mRNA was analyzed in cells treated with glutamate alone (●) and in cells to which PCP (1 μM) was added either 30 min after (○) or 15 min before (□) glutamate addition. Each *point* represents the mean ± standard error of three experiments (glutamate alone, glutamate plus PCP) or mean and range of two independent experiments (PCP pretreatment). C, The expression of c-*fos* mRNA was analyzed under the same experimental conditions. ●, Glutamate alone; △, CNQX or ○, PCP added 30 min after glutamate; □, PCP added 15 min before glutamate. *Points* represent the mean ± standard error of three independent experiments except for the PCP pretreatment, where the data represent the mean and range of two separate experiments. *, Significantly different ($p < 0.05$) from control; ▲, significantly different from the 2-hr value, by Duncan's multiple range test.

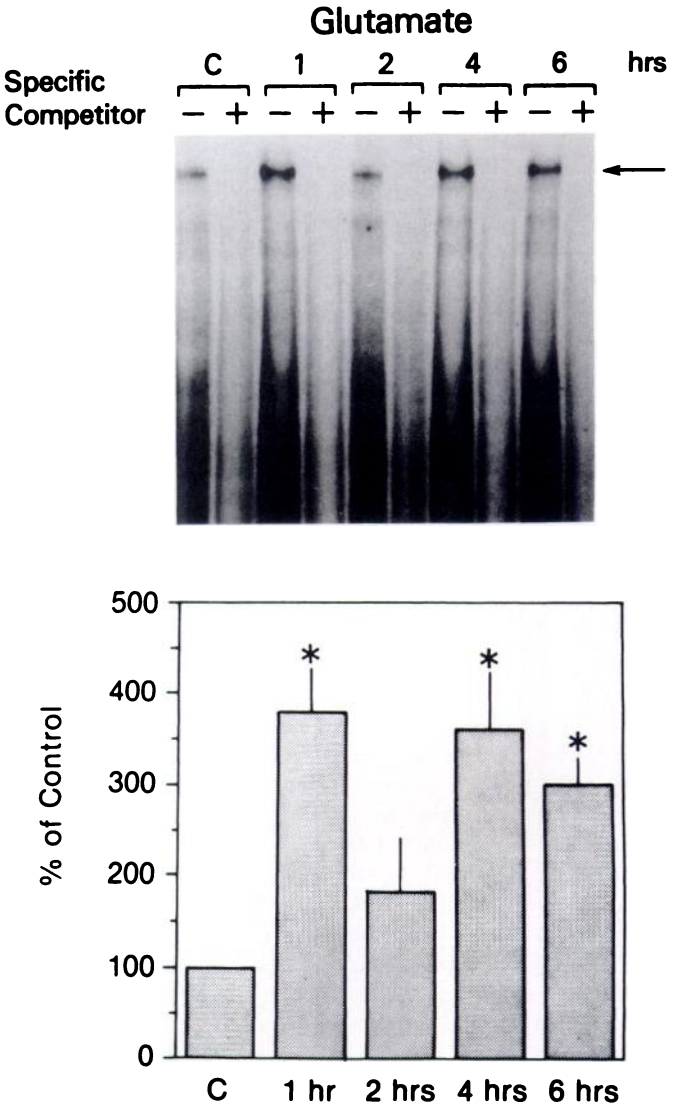

Fig. 5. Prolonged increase in AP-1-binding activity of granule cells after pulse treatment with glutamate. Gel retardation assays were performed with nuclear extracts prepared from glutamate-treated primary cultures of cerebellar granule cells. Glutamate (10 μM) was added at time 0, followed by 1 μM PCP 30 min later. Nuclear extracts were prepared at 1-, 2-, 4-, and 6-hr time points. A, Representative retardation assay. − and +, absence and the presence, respectively, of a 100-fold molar excess of unlabeled AP-1 oligonucleotide. *Arrow*, the AP-1-specific DNA-binding activity. B, Quantitation based on densitometric measurement, where the data are expressed as mean ± standard error of three independent experiments. *, Significantly different ($p < 0.05$) from control, by Duncan's multiple range test. *C*, control.

within 40 to 50 min (24, 42). Thus, we may assume that these cells exhibit a synchronous response to receptor stimulation. Therefore, the induction patterns of various early-inducible genes appropriately characterize the time frame of the genomic response elicited by glutamate receptor stimulation.

Chemically evoked seizure activity and hippocampal long term potentiation, as well as sensory stimulation of the spinal cord, rapidly induce the expression of several early-inducible genes (43–47). The relative extent to which each mRNA is induced apparently depends on the type of stimulation applied (48), but in each case the increase is transient, returning to control levels within 1–2 hr. According to the only long term study published thus far, metrazol-induced convulsions in mouse brain result in a protracted expression of c-*jun* mRNA and concomittant AP-1-binding activity (46). However, the results of these *in vivo* experiments are difficult to interpret with regard to their relation to transmitter receptor stimulation. Moreover, a prolonged induction pattern can depend on the temporal sequence of activation of different subsets of neurons, including neuronal networks connecting distinct brain areas.

The early phase of the genomic response, which includes a simultaneous activation of c-*fos*, c-*jun*, *jun*-B, and *zif*/268, is virtually completely inhibited by CPP, a specific competitive NMDA receptor antagonist. This result is consistent with the previous pharmacological characterization of glutamate-induced c-*fos* expression (24). It is important to consider the

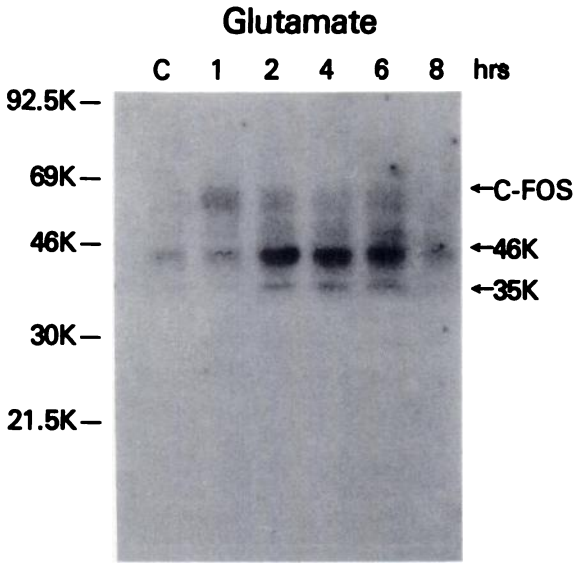

Fig. 6. Temporal alteration in the amounts of Fos and FRAs after glutamate treatment. Nuclear extracts were prepared from glutamate-stimulated neurons at the indicated times, and immunoblot analysis was performed. The positions of the M_r 55,000 Fos protein, the M_r 46,000 FRA, and the M_r 35,000 FRA, as well the molecular weight markers, are indicated. Densitometric analysis of the blot yielded the following results, expressed as percentage of control for the M_r 55,000, 46,000, and 35,000 proteins, respectively: 1 hr, 560%, 100%, and 120%; 2 hr, 300%, 240%, and 250%; 4 hr, 160%, 280%, and 300%; 6 hr, 200%, 260%, and 230%; and 8 hr, 80%, 70%, and 80%. This experiment was repeated twice with similar results. *C*, control.

mechanism and the functional significance of the delayed phase of the c-*jun* expression. Prior treatment of the cultures with the highly selective noncompetitive NMDA receptor antagonists PCP and MK-801 prevents the glutamate-mediated increase of c-*jun* mRNA during both the early and the delayed phases. However, when these inhibitors are added 30 min after glutamate, the initial induction of c-*jun* mRNA is followed by a decrease below the basal level, whereas the second phase of the c-*jun* increase is not altered. One of the possibilities as to why PCP lowers the basal level of c-*jun* mRNA expression is that it antagonizes the effect of the endogenous glutamate present in the cultures. In additional experiments, we excluded the possibility that PCP had lost its pharmacological potency when added to the cultures for a protracted period of time. On the other hand, the quisqualate-kainate receptor antagonist CNQX failed to affect the biphasic nature of c-*jun* expression. These results indicate that both components of the biphasic increase in c-*jun* mRNA are determined by the initial stimulation of the NMDA receptor. It is possible that the delayed phase of the c-*jun* response results from the stimulation of other nonglutamate receptors that become activated by an endogenous substance (e.g., a neuropeptide) released by glutamate. However, an appealing alternative is that the second phase of the response depends on genomic events triggered by the initial glutamate pulse. That is, the AP-1 complex, produced as a result of the early increase in the contents of c-*fos* and *jun* mRNAs, might serve as a positive transcriptional regulator acting on the AP-1 site present in the 5′ flanking region of c-*jun*. This hypothesis is supported by a recent report describing the existence of this positive regulatory loop; the transcriptional rate of c-*jun* was found to be stimulated by its own product (49). Therefore, the receptor-mediated signal would be prolonged in the nucleus by a genomic switch that converts early responses into further cascades of gene expression. This biphasic induction of nuclear third messengers observed *in vitro* represents a new additional level of complexity in the regulation of target gene expression.

Temporal alteration in the amount and composition of the AP-1 transcriptional complex after short term glutamate stimulation. The AP-1-binding site is a common constituent of both positive and negative transcriptional elements of several viral and cellular genes (14, 50, 51). In primary cultures of rat cerebellar granule cells, a brief stimulation of NMDA receptors elicits a prolonged biphasic increase of AP-1-specific DNA-binding activity, as demonstrated by gel retardation assays. It appears that, in addition to the Fos and Jun proteins, several FRAs may contribute to the persistent increase in AP-1-binding activity elicited by the brief stimulation of glutamate receptors. This is supported by experiments showing that certain FRAs cooperate with Jun and bind to the AP-1 site (20, 51). We have shown that the early phase of the increased DNA-binding activity occurred in parallel with an increase in the amounts of c-*jun*, *jun*-B, and c-*fos* mRNAs, whereas during the second phase only the amount of the c-*jun* mRNA remained elevated. Furthermore, the protein immunoblot analysis of nuclear extracts prepared from the stimulated neurons indicated an early rapid increase in the amount of M_r 55,000 Fos, which progressively declined between 2 and 6 hr after stimulation. This was concomitant with an increase in the contents of two FRAs starting at 2 hr after stimulation and lasting for up to 6 to 7 hr. These FRA bands are unlikely to reflect Fos processing products, because the M_r 55,000 Fos protein was undetectable in control cells, which contained the M_r 46,000 and 35,000 proteins. A similar temporal and molecular pattern of Fos and FRA induction was detected in brain after metrazol-induced convulsion and in serum-stimulated fibroblasts (20, 46, 52). The recognition of FRAs by the polyclonal antibody to Fos is attributable to amino acid sequence homology between FRAs and Fos. That is, the antibody was raised against a region of the DNA-binding domain of Fos (34) that is conserved among the Fos family members. We may infer that, during the initial phase of the response to glutamate receptor stimulation, predominantly Fos-Jun and Fos-JunB heterodimers with high DNA-binding affinity are formed. The composition of the AP-1 complex may gradually shift to the lower affinity Jun-Jun homodimer, whereas in the delayed phase the heterodimerization between the different FRAs and Jun may be prominent. We do not exclude, however, the possibility that other as yet unidentified proteins contribute to the final composition of the transcriptional complex. Because many variations of AP-1 binding sites exist (12, 53), these protein complexes may precisely regulate subsets of target genes containing variants of the AP-1 site. On the other hand, based on their differing affinity, the different complexes may compete with each other for the same binding site, resulting in variations in the efficacy of transcriptional regulation as a result of their specific intrinsic activity.

A temporal change in the composition of the AP-1 transcriptional complex, as a result of the combinatorial pattern of early response gene expression, could enable a relatively small set of proteins to mediate a flexible and precise neuronal response to receptor activation. Moreover, interplay with additional nuclear proteins (e.g., different classes of transcription factors,

cell-specific transcriptional modulators) greatly increases the significance and versatility of this signaling system.

In primary cultures of neurons, NMDA receptor stimulation leads to a coordinated transcriptional induction of a series of early response genes. The encoded nuclear third messenger proteins interact with their recognition sequences in DNA to mediate a change in the expression of the associated genes. By identifying and characterizing these genes, we might begin to understand the genomic language used by neurons following receptor stimulation by neurotransmitters, and more importantly neuromodulators, that underlie long term adaptive changes in neuronal functions.

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