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Rapid Rise in Transcription Factor mRNAs in Rat Brain After Electroshock-Induced Seizures

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Abstract: Recent studies have demonstrated that several transcription factor genes are rapidly activated by neuronal stimulation. For example, we have found that prolonged and repeated seizure activity produced by administration of chemical convulsants induces a rapid and transient increase in mRNA levels of four immediate early genes in rat brain. These genes, *zif/268*, c-*fos*, c-*jun*, and *jun*-B, encode sequence specific DNA binding proteins thought to act as transcription regulatory factors. To ascertain whether a brief electrically induced seizure discharge of the type utilized in clinical electroconvulsive treatment is sufficient to induce a similar genomic response, we have examined the response of these mRNAs in rat brain following single and repeated electroshock-induced seizures. After electroshock, mRNA levels of each of these genes increase within 15 min, and all except c-*jun* return to near baseline levels within 4 h. Although this response is most prominent in granule cell neurons of the hippocampus, increases are also apparent in neocortex and pyriform cortex. The rapid mRNA response persists in animals receiving a chronic electroshock protocol similar to that used in clinical electroconvulsive therapy. Intrahippocampal infusion of the sodium channel antagonist tetrodotoxin blocks hippocampal mRNA responses without blocking seizures, indicating a role for electrical excitation in the electroshock-induced mRNA response. By contrast, pretreatment with anticonvulsants or selective NMDA antagonists, which reduce seizure intensity and block hindlimb extension, fails to alter mRNA responses, suggesting that seizure induction, rather than spread, is linked to these mRNA responses. Because electroshock induces robust, highly reproducible mRNA responses, it may be useful to study the neuronal genomic response to stimulation. **Key Words:** In situ hybridization—Hippocampus—Anticonvulsants—Early genes—c-*fos*—*zif/268*—c-*jun*—*jun*-B. **Cole A. J. et al.** Rapid rise in transcription factor mRNAs in rat brain after electroshock-induced seizures. *J. Neurochem.* **55,** 1920–1927 (1990).

Recent studies have focused attention on the rapid activation of transcription factor genes in stimulated neurons. For example, c-*fos* mRNA levels rise in spinal cord neurons after peripheral sensory stimulation (Hunt et al., 1987) and in brain after pentylenetetrazol (PTZ)-induced seizures (Morgan et al., 1987), kindling stimulation (Dragunow and Robertson, 1987), or lesion-induced seizures (White and Gall, 1987). c-*fos* is one member of a class of genes, referred to as immediate early genes (IEGs), that are rapidly induced by growth factors in cells in vitro (Greenberg and Ziff, 1984; Muller et al., 1984; Lau and Nathans, 1987; Rollins and Stiles, 1989). We and others have found that in addition to c-*fos*, three other IEG mRNAs, previously identified in fibroblasts or PC12 cells, are coordinately activated after systemic administration of PTZ or picrotoxin, convulsant agents that produce prolonged and repeated episodes of seizure discharge (Saffen et al., 1988; Sukhatme et al., 1988). Each of these genes, *zif/268* (Christy et al., 1988) [also termed *egr-1* (Sukhatme et al., 1987), NGFI-A (Milbrandt, 1987), Krox 24 (Lemaire et al., 1988)], c-*jun* (Maki et al., 1987), and *jun*-B (Ryder et al., 1988), like c-*fos*, code for sequence specific DNA binding transcription regulatory proteins (Curran and Franza, 1988; Nakabeppu et al., 1988; Christy and Nathans, 1989) and therefore may play a key role in coordinating long-term changes in neuronal activity (Goelet et al., 1986). Because electroconvulsive treatment (ECT) is a widely used psychiatric therapy effective in affective disorders (Weiner, 1989), we wondered whether a similar rapid mRNA response is induced by the brief seizure activity pro-

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Abbreviations used: ECT, electroconvulsive treatment; ES, electroshock; 5-HT_2, 5-hydroxytryptamine$_2$; ^{125}I-CYP, [^{125}I]iodocyanopindolal; IEG, immediate early gene; NMDA, *N*-methyl-D-aspartate; PTZ, pentylenetetrazole; SDS, sodium dodecyl sulfate; TTX, tetrodotoxin.

duced by this treatment. Accordingly, in the present study we examined the levels of these mRNAs in rats after single and repeated electroshock (ES). While these studies were underway, c-*fos* was found to be induced by a single ES in mice (Daval et al., 1989; Sonnenberg et al., 1989*a*). Our results in rat demonstrate that c-*fos*, as well as several other IEGs, are induced by either single or chronic ES in rat forebrain.

MATERIALS AND METHODS

ECT

Electrically induced convulsions were produced in male Sprague–Dawley rats (175–225 g) by standard techniques (Swinyard, 1972). Following attachment of saline-soaked earclips, ES consisting of a 1-s, 100-Hz, 85-mA stimulus of 0.5-ms square-wave pulses was delivered using a UGO Basile ECT unit, Model 7801. At these settings, except for those pretreated with anticonvulsants, all animals had generalized tonic–clonic convulsions with hindlimb extension. In some experiments, designed to assess effects of less intense stimuli, the current was reduced to 10 or 35 mA. Following ES, animals were killed by decapitation and the brains processed for in situ hybridization or Northern blot analysis.

Intracerebral injections

Rats were anesthetized with chloral hydrate (400 mg/kg i.p.) and placed in a stereotaxic apparatus. Injections of tetrodotoxin (TTX) (2 μl of 1 m*M* TTX in 0.9% NaCl infused over 30 min) into the dorsal hippocampus were performed through a Hamilton syringe attached to a broken tip glass microelectrode lowered 3 mm below the dura at 3 mm posterior and 2 mm lateral to bregma, according to the atlas of Paxinos and Watson (1982). In preliminary experiments, extracellular field potential recordings were performed as previously described by Cole et al. (1989) to confirm that this drug treatment was sufficient to block the synaptically induced electrical responses of granule cells to ipsilateral perforant path stimulation.

DNA and RNA probes

Murine *zif/268* (gift from B. Christy), rat NGFI-A (gift from J. Milbrandt), and human c-*fos* (gift from Y. Nakebeppu) cDNA sequences were cloned in Bluescript M13+ (Stratagene, La Jolla, CA, U.S.A.). Murine *jun*-B and c-*jun* cDNA sequences (gift from K. Ryder) were cloned in p-Gem2 (Promega Biotec, Madison, WI, U.S.A.). For RNA probes, plasmids containing full-length or nearly full-length cDNAs for each of the IEGs were linearized with appropriate restriction enzymes to produce either antisense or sense transcripts. ^{35}S-labelled RNA sense or antisense probes were prepared from cDNA sequences using T7 or SP6 RNA polymerase (Promega Biotech) and uridine 5′-(α-[^{35}S]thio)triphosphate (New England Nuclear) essentially as described by Melton et al. (1984). ^{32}P-labelled DNA probes were prepared using random primer initiated synthesis with the Klenow fragment of DNA polymerase (Feinberg and Vogelstein, 1983). *zif/268* and NGFI-A probes were used interchangeably in these studies and gave similar results.

In situ hybridization

In situ hybridization was performed as previously described by Saffen et al. (1988). Frozen 10-μm thick sections were mounted on gelatin-coated glass slides, and stored desiccated at −20°C. Prior to hybridization, sections were fixed with freshly prepared, depolymerized 4% (wt/vol) paraformaldyhyde, acetylated with 0.5% (vol/vol) acetic anhydride in 0.1 *M* triethanolamine dissolved in 0.9% (wt/vol) NaCl adjusted to pH 8.0, and delipidated. Hybridization was performed in a moist chamber under unsealed dimethyldichlorosilane (Atomergic Chemetals)-treated coverslips at 56°C using 5 × 10^5–1 × 10^6 cpm of probe for each slide in 50 μl of hybridization buffer containing 50% vol/vol formamide, 10 m*M* dithiothreitol, 600 m*M* NaCl, 1× Denhardts (1× Denhardt's contains Ficoll, polyvinylpyrrolidone, and bovine serum albumin each at 0.02% wt/vol), 1 m*M* EDTA, 0.2 mg/ml of yeast tRNA, and 10% vol/vol dextran. After overnight incubation, slides were washed in 2× SSC (1× SSC contains 150 m*M* NaCl, 15 m*M* sodium citrate adjusted to pH 7.0), treated with 10 μg/ml of RNase A (Worthington) in 2× SSC at 30°C, air-dried, and apposed to audioradiographic film (Ultrofilm, LKB Gaithersburg) at room temperature for 1–5 days.

To ensure specificity of in situ hybridization, wash steps described above were based on empirically determined melt conditions. No hybridization of sense strand probes was detected. RNase A pretreatment of tissue sections blocked hybridization of nick-translated cDNA probes. ^{35}S-riboprobe complementary to the 3′ noncoding region of *zif/268* gave results identical to those obtained using full-length antisense probes. All in situ hybridization studies were done using control and experimental brains comounted in the same tissue block to minimize differences in handling of tissue sections, including freeze–thaw cycles and section thickness. Optical densities (OD values) of representative autoradiograms were measured over the granule cell layer of the dentate gyrus (Loats Image Analysis System).

Northern blots

Total cellular RNA was isolated from rat hippocampus using the acid guanidinium–phenol extraction procedure of Chomczynski and Sacchi (1987). RNA was separated by electrophoresis through a 1% agarose–formaldehyde gel, transferred to nitrocellulose membranes, and probed essentially as described by Linzer and Nathans (1983). In brief, filters were prehybridized in a sealed bag containing 50% vol/vol formamide, 4× SSC, 4× Denhardt's solution, 0.1% wt/vol sodium dodecyl sulfate (SDS), 0.1% wt/vol sodium pyrophosphate, and 100 μg/ml of sheared salmon sperm DNA. Labelled probe was added to a final concentration of 1 × 10^6 cpm/ml, and filters were hybridized overnight at 42°C. Filters were washed in four changes of 2× SSC with 0.1% wt/vol SDS at room temperature, and then in four changes of 0.1× SSC with 0.1% SDS at 50°C, air dried, and exposed to x-ray film (Kodak XAR) at −70°C with an intensifying screen for up to a week.

Receptor radioligand autoradiography

Frozen 10-μm thick tissue sections were mounted on gelatin-coated glass slides and stored desiccated at −20°C. β-Adrenoreceptor autoradiography was performed as described by Bigeon and Israeli (1986). β-Adrenoreceptors were labelled with [^{125}I]iodocyanopindolal (^{125}I-CYP) (New England Nuclear; sp act, 2,200 Ci/mmol). Nonspecific binding was assessed by coincubation with 10^{-6} *M* timolol. 5-Hydroxytryptamine$_2$ (5-HT$_2$) receptor autoradiography was performed as described by Bigeon et al. (1986). 5-HT$_2$ receptors were labeled with [^{3}H]ketanserin (New England Nuclear; sp act, 77 Ci/mmol). Nonspecific binding was assessed by coincubation with 10^{-6} *M* mianserine.

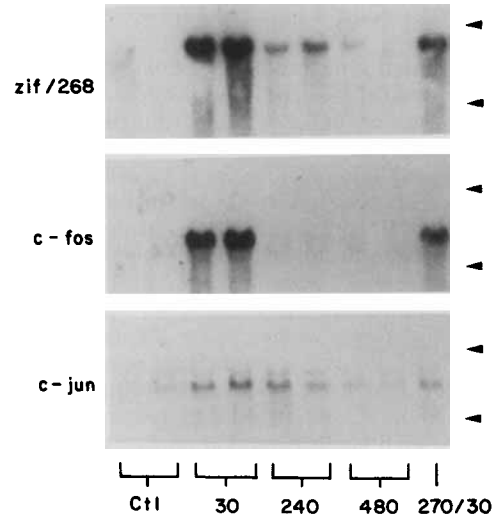

FIG. 1. Blot hybridization analysis of *zif/268*, c-*fos*, and c-*jun* mRNAs after maximal ES. Each lane contains 15 μg of total RNA, isolated from hippocampi of rats killed at the indicated time (min) after maximal ES. RNA in the lane at the far right was isolated from rats that received a second ES 30 min prior to killing and 4 h after the first ES. The *jun*-B results are similar to those of c-*fos*.

RESULTS

Blot hybridization analysis of brain RNA

Total cellular RNA from hippocampus isolated from animals killed 30 min, 4 h, or 8 h after ES (85 mA) was analyzed for the presence of *zif/268*, *jun*-B, c-*fos*, and c-*jun* mRNAs. As shown in Fig. 1, 30 min after ES, levels of each of the mRNAs were increased in hippocampus compared to untreated animals. By 4–8 h after ES, levels of mRNA had returned to near baseline levels. Previous studies indicate that after PTZ-induced seizures, subsequent readministration of PTZ 4 h later, which causes repeated seizures, induces a much smaller rise in c-*fos* mRNA (Morgan et al., 1987). We checked for the possibility of a similar refractory period by repeating ES 4 h after the initial treatment. Responses for each of the IEG mRNAs were moderately attenuated 30 min after a second seizure in comparison with those seen 30 min after a single seizure (Fig. 1, lane 9).

In situ hybridization

To examine the anatomic distribution of each of the IEG transcripts, we performed in situ hybridization.

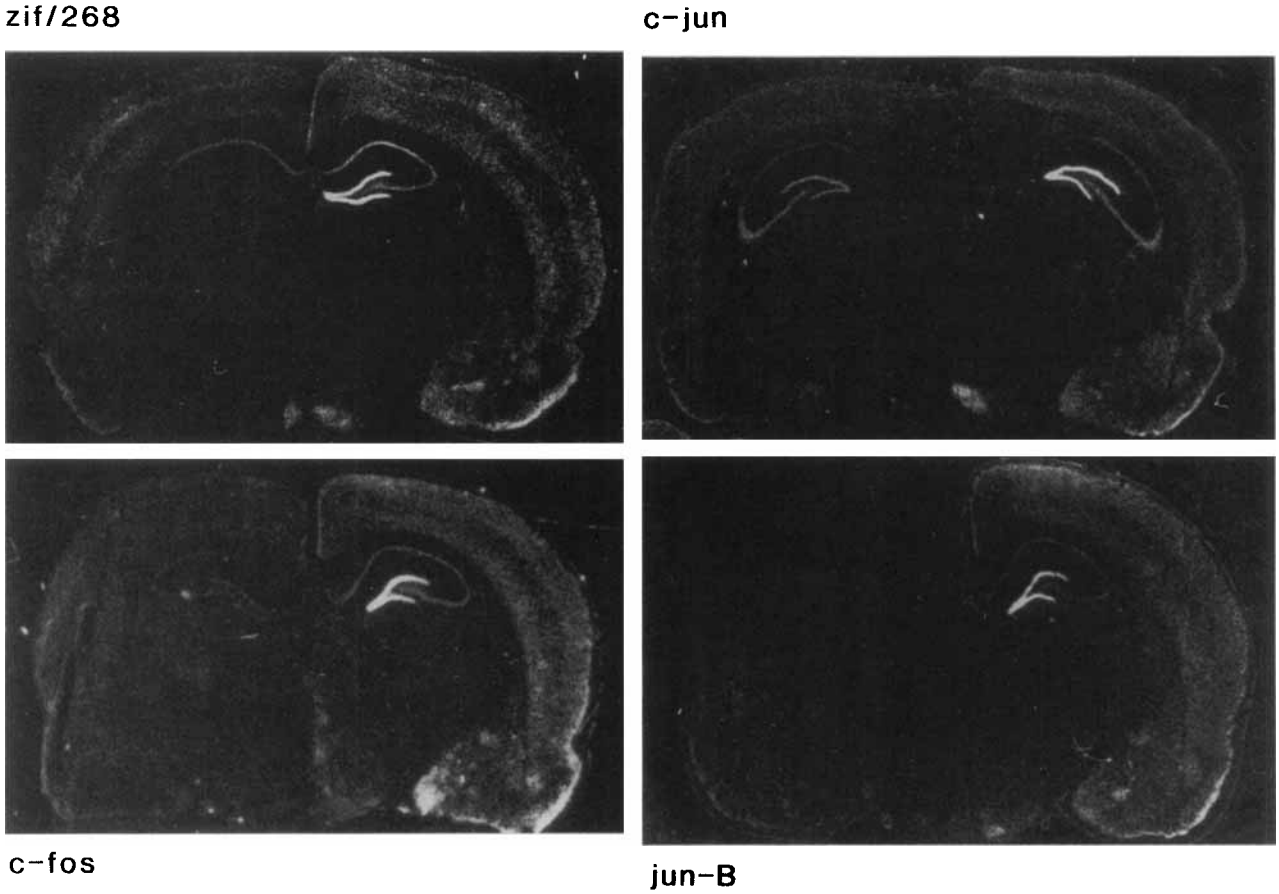

FIG. 2. Response of *zif/268*, c-*jun*, c-*fos*, and *jun*-B mRNAs in rat brain after maximal ES. In each panel, the left hemisphere is taken from an animal subjected to sham ES. The right hemisphere is taken from an animal killed 30 min after maximal ES. ES typically produced an increase in OD of ≥100% between stimulated and control granule cells.

As shown in Fig. 2, maximal levels of expression for each of the mRNAs were found in the granule cell layer of the dentate gyrus. In addition, there were significant mRNA increases noted throughout the neocortex, entorhinal cortex, and pyriform cortex. Light microscopic resolution of *jun*-B and *zif/268* probe hybridization in the hippocampus and neocortex indicates that enhanced hybridization is associated with neurons based on the topographic distribution and morphology of cells underlying exposed photographic grains (data not shown).

To characterize further the time course of the mRNA response, brains were harvested 15, 30, 60, 90, 120, and 240 min and 24 h after ES and mRNA levels assayed in hippocampal granule cells by in situ hybrid-

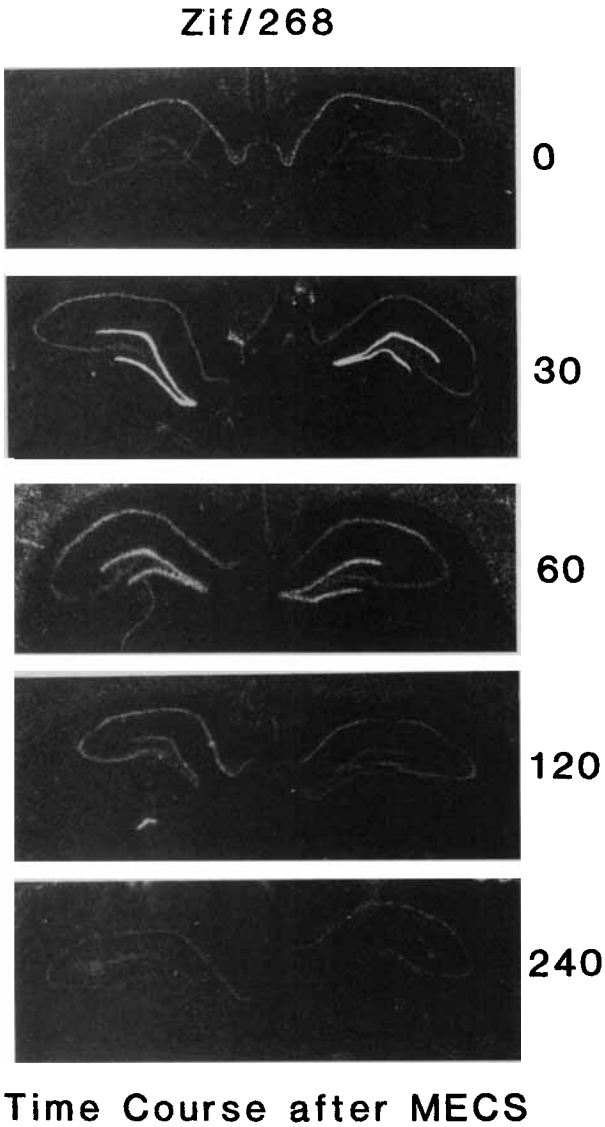

FIG. 3. Time course of *zif/268* mRNA in hippocampus harvested at indicated time points in minutes after maximal ES. Each panel contains hippocampi from two animals.

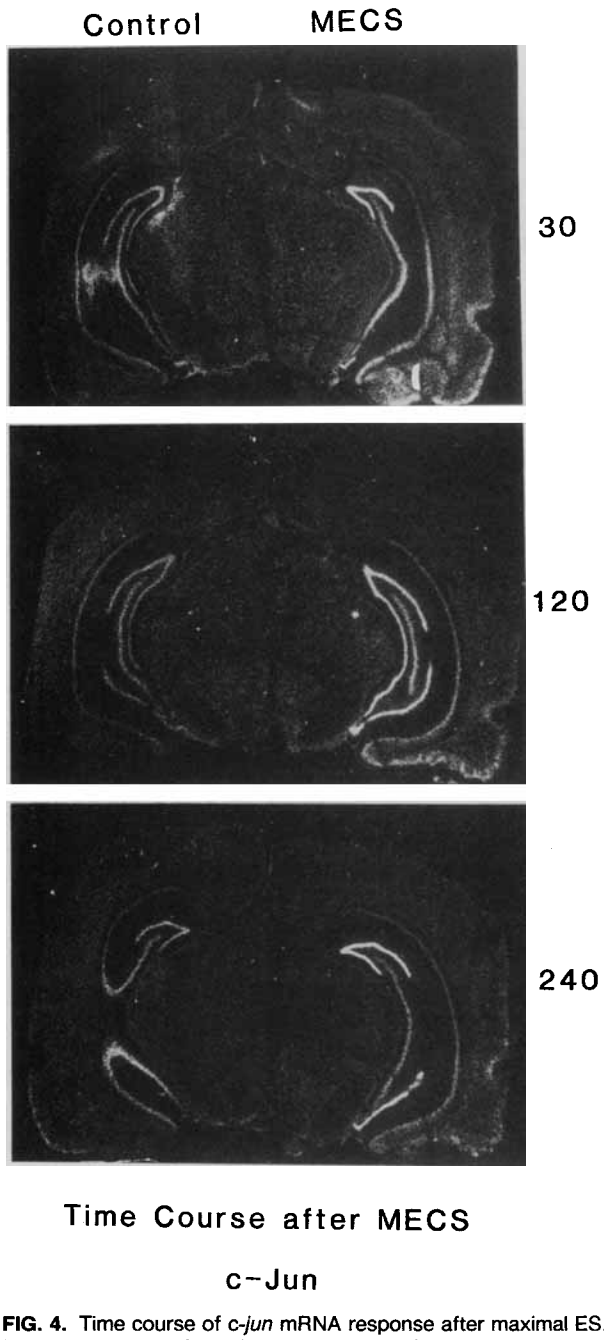

FIG. 4. Time course of c-*jun* mRNA response after maximal ES. In each panel, the left hemisphere was taken from a control animal; the right hemisphere was harvested at indicated time after maximal ES in minutes.

ization. mRNA levels of each of the IEGs were maximally increased within 30 min of treatment. c-*fos*, *jun*-B, and *zif/268* returned to basal levels within 4 h (Fig. 3). c-*jun* mRNA remained increased in hippocampal granule cells 4 h after ES (Fig. 4), but returned to basal by 24 h.

To determine whether seizures are necessary for the ES stimulus to induce IEG mRNA increases, we examined the intensity of ES required to elevate mRNA

levels in hippocampus. In situ autoradiograms obtained from animals treated with ES performed with 10-mA, 35-mA, and 85-mA current pulses were compared. The 85-mA ES stimulus reproducibly induces a tonic–clonic seizure with hindlimb extension. The 35-mA ES stimulus induces a brief (<5 s) tonic seizure without hindlimb extension, whereas a 10-mA stimulus induces brief behavioral arrest only during the stimulus. A reproducible increase in mRNA levels is seen only after 85-mA ES, but not 35- or 10-mA ES (Fig. 5A and B).

Effects of anti-epileptic drugs and *N*-methyl-D-aspartate (NMDA) antagonists

The c-*fos* mRNA increases induced by PTZ are blocked by prior treatment with diazepam, a benzodiazepine anticonvulsant that blocks PTZ-induced seizures (Morgan et al., 1987). ES seizures are modulated by anticonvulsants and the ES model has been of major importance in the development and screening of potential anticonvulsants (Swinyard, 1972). To determine whether changes in IEG mRNA levels following ES are blocked by anticonvulsants, we pretreated a group of animals with phenytoin (50 mg/kg, n = 4), phenobarbital (50 mg/kg, n = 4), carbamazepine (30 mg/kg, n = 4), or diazepam (10 mg/kg, n = 4). One hour later, animals received ES (85 mA), and 30 min later were killed. Whereas each of these agents completely blocked hindlimb extension, none of the drugs tested significantly altered the mRNA response in the hippocampus as assayed by in situ hybridization.

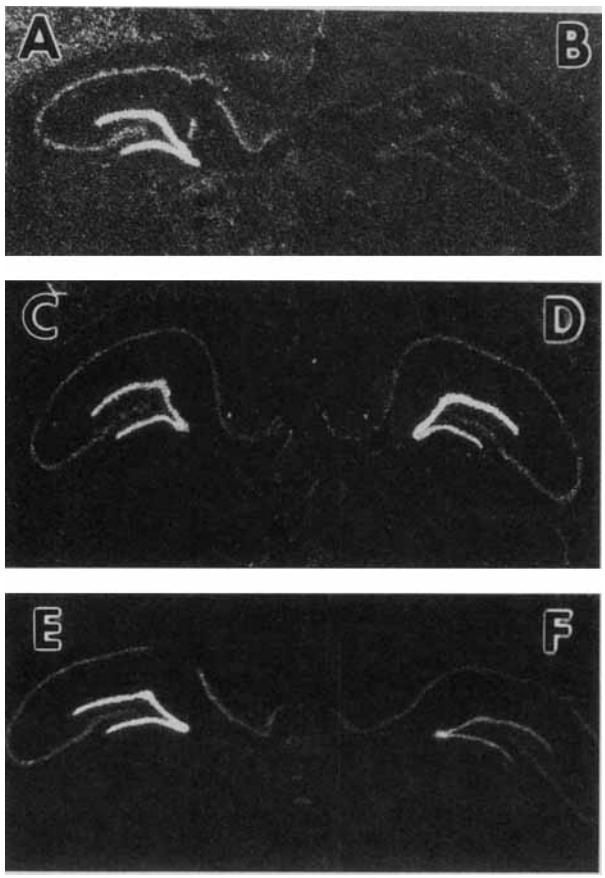

FIG. 5. Responses of *zif/268* mRNA 30 min after ES: effect of stimulus intensity, MK-801, and pretreatment with intrahippocampal TTX. **A:** After 85 mA ES. **B:** After 35 mA ES. **C:** After pretreatment with the noncompetitive NMDA antagonist MK-801 (10 mg/kg i.p. 120 min before ES). **D:** After saline injection. **E, F:** After intrahippocampal injection of TTX (2 μl of 1 m*M* TTX in 0.9% NaCl) into right hippocampus (F). c-*jun*, *jun*-B, and c-*fos* gave similar results.

In recent studies, we have demonstrated that *zif/268* mRNA levels are increased in dentate gyrus granule cells after high-frequency synaptic stimulation via perforant pathway afferents (Cole et al., 1989). This rise in *zif/268* mRNA is blocked by NMDA-type glutamate receptor antagonists, indicating a role for this receptor in synaptic regulation of IEG mRNA levels in brain. To determine whether NMDA receptors are involved in ES-induced IEG mRNA increases, we examined the effects of NMDA antagonists on the ES mRNA response. MK-801 is a noncompetitive NMDA receptor antagonist (Wong et al., 1986) that is centrally active following systemic administration (intraperitoneally) and blocks NMDA-dependent responses in the hippocampus at a dose of 1 mg/kg (Abraham and Mason, 1988). MK-801 also blocks ES-induced hindlimb extension (ED_{50} 0.3 mg/kg) (Clineschmidt et al., 1982). CGS-19755 is a competitive NMDA receptor antagonist (Murphy et al., 1987) that is centrally active after systemic administration (Lodge et al., 1988) and has anticonvulsant actions (ED_{50} 3.8 mg/kg) (Lehmann et al., 1988; Bennett et al., 1989). Neither high-dose MK-801 (10 mg/kg) nor CGS-19755 (30 mg/kg) has a significant effect on mRNA levels of the four IEGs in hippocampus harvested 30 min after ES, although both drugs block hindlimb extension (Figs. 5C and D, and 6).

To help ensure that mRNA increases are not simply the result of systemic metabolic changes associated with seizures, we monitored effects of focal injections of the fast sodium channel blocker TTX into the hippocampus. When sufficient TTX was injected into the hippocampus to block synaptically induced action potentials, *zif/268* mRNA increases following ES (85 mA) were dramatically reduced in the TTX-injected hippocampus relative to the noninjected side (Fig. 5E and F).

Effects of chronic ES treatment

To examine the effect of chronic ES, we treated animals with ES once each day for a period of 10 days. Brains were harvested 30 min, 45 min, 60 min, 2 h, 4 h, and 24 h after the last treatment. The mRNA responses after chronic ECT maintain a similar time course to that seen after a single treatment, that is, maximal induction by 30 min and return to baseline levels by 4 h after the final treatment for c-*fos*, *zif/268*, and *jun*-B. As observed after a single ES, c-*jun* mRNA levels remain above basal levels at 4 h, but return to baseline by 24 h. We find the maximal IEG mRNA

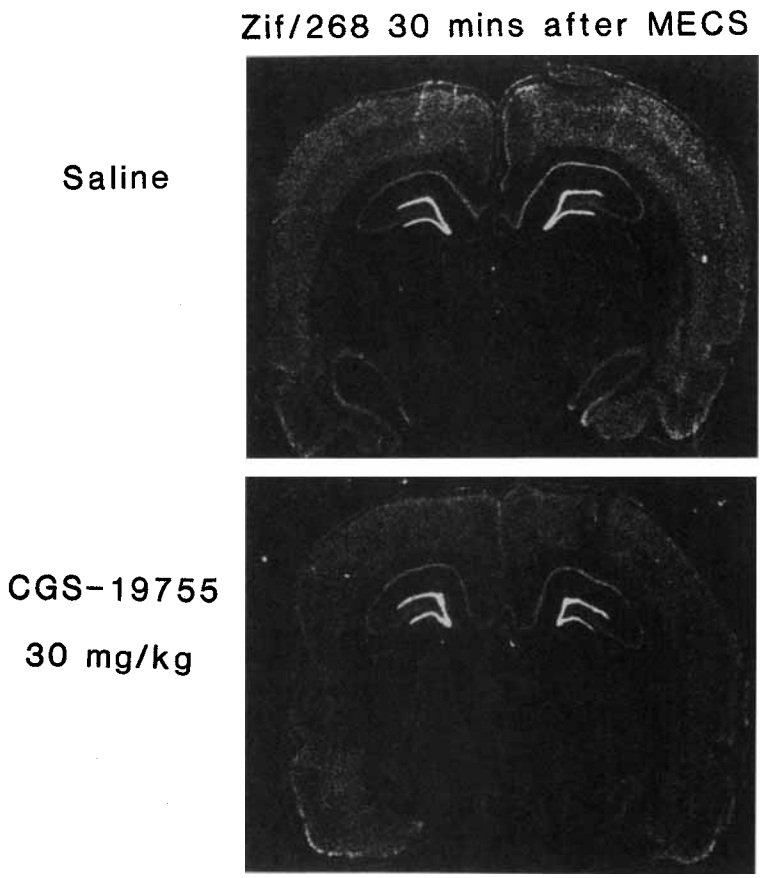

FIG. 6. *zif/268* mRNA in hippocampus 30 min after maximal ES. Upper panel is from an animal injected with saline 2 h before ES, whereas the lower animal received CGS-19755 (30 mg/kg i.p.) 2 h before maximal ES. c-*jun*, *jun*-B, and c-*fos* show a similar response.

response in dentate gyrus after chronic ECT to be attenuated by 20–30% for each of the four genes examined when compared to the mRNA response in dentate gyrus of animals killed after a single seizure (two-way analysis of variance: $p < 0.02$).

Chronic ES has been shown to increase 5-HT_2 (Kellar et al., 1981) and decrease β-adrenergic receptor density (Bergstrom and Kellar, 1979) in rat forebrain. To ensure that the ES treatments performed were comparable to those used in these receptor binding studies, we checked whether these animals displayed the expected changes in receptor density. Accordingly, 24 h after the last treatment, animals were killed and the brains prepared for ^{125}I-CYP and [^{3}H]ketanserine autoradiography to assess 5-HT_2 and β-adrenergic receptor levels, respectively. We found the expected changes in receptor density in these animals and also observed that daily treatment with MK-801 (3 mg/kg, i.p.) 2 h before each ES did not affect the receptor density changes (data not shown).

DISCUSSION

The main finding of this study is that ES induces a brief but rapid increase in mRNA levels of four immediate early genes. Each of these IEGs, *zif/268*, c-*jun*, *jun*-B, and c-*fos* encode known or presumed transcription regulatory proteins, suggesting that their transcription after ES may be important in regulating a cascade of subsequent genomic activation (Morgan and Curran, 1989). These findings provide a conceptual framework for understanding how brief episodes of neuronal stimulation such as ES can elicit long-term changes in neuronal activity, morphology, and receptor distribution.

ES-induced mRNA responses are similar in several ways to PTZ- or picrotoxin-induced seizures. Responses to both types of stimuli display a rapid and brief rise that is most prominent in hippocampus and neocortex. ES offers several advantages over convulsant-induced seizures for examining the response of IEGs in brain, including the brief and discrete nature of the stimulus, its high degree of reproducibility, and ease of delivery. These characteristics suggest that ES may provide a useful system in which to screen for activation of other IEGs in brain. In a previous study examining induction of IEGs by PTZ, we reported that mRNA levels for these four genes displayed similar time courses. More detailed in situ studies performed with ES clearly demonstrate that c-*jun* levels remain near maximal at 4 h, whereas the other three have largely returned to basal by 2 h. The delayed drop in c-*jun* agrees with the findings of Sonnenberg et al. (1989*b*). Our previous findings after PTZ-induced sei-

zures (Saffen et al., 1988) may reflect the marked variability in seizure intensity and duration observed following PTZ injection in rats. Another consideration is that of these four genes, the c-*jun* response is smallest in amplitude; thus in the picrotoxin and PTZ models which exhibit substantial variability in response magnitude, the c-*jun* signal at late time points may have been underestimated.

Experiments were performed to assess the mechanism of ES-induced IEG mRNA increases. The ES stimulus appears to increase IEG mRNA levels by inducing seizures because less intense ES stimuli that fail to induce seizures do not cause IEG mRNA increases. In contrast to studies of ES in mice (Daval et al., 1989), we detected no change of c-*fos* mRNA associated with sham ES which may represent a species difference in stress response. The finding that local infusion of TTX into the dentate gyrus does block ES-induced increases in mRNA suggests that increased electrical discharge induced by ES is necessary for activation of these genes, as opposed, for example, to possible transient hypoxia or changes in cerebral blood flow.

Anticonvulsant drugs that act at the γ-aminobutyric acid–benzodiazepine receptor complex to block initiation of PTZ-induced seizures have been shown to block increases in c-*fos* mRNA and protein after PTZ administration. The results of this study in which anticonvulsant drugs that reduce motor manifestations of ES seizures have no apparent effect on mRNA levels following ES suggest that rises in mRNA levels after ES are mediated by early events in the development of ES-induced seizures and not by propagation of electrical stimulation to "down-stream" neuronal structures. This finding may be of clinical relevance in understanding the long-term effects of chronic epileptic discharge in the form of partial seizures, which often continue even in the face of anticonvulsant therapy that is sufficient to block generalized attacks.

In recent studies we have shown that *zif/268* mRNA levels are increased in dentate gyrus granule cells after in vivo synaptic stimulation via perforant pathway afferents that induces long-term potentiation. In that paradigm, the mRNA response is blocked by the NMDA receptor antagonists MK-801 (1.0 mg/kg) and CGS-19755 (10 mg/kg). In contrast, after ES mRNA responses in the same cell populations are not blocked by even higher doses of the same drugs. This finding suggests that at least two distinct pathways mediate the mRNA response in hippocampus, one that involves NMDA receptors, and one that does not. Alternatively, the stimulus for IEG mRNA increases due to ES and long-term potentiation might differ only in the amount of excitatory transmitter released by the stimulus. Lack of effect of the noncompetitive NMDA receptor antagonist MK-801 at high doses, however, supports the premise that ES-induced IEG mRNA increases are independent of NMDA receptor activation.

Repeated electrical activation of neurons can induce changes in neuronal phenotype, such as alterations in neurotransmitter receptor number and distribution (Kellar et al., 1981; Yeh et al., 1989), that may be relevant to the clinical efficacy of ECT and to the pathogenesis of chronic epilepsy. Our studies indicate that repeated ES stimuli remain effective at inducing IEG mRNA responses. Furthermore, both ES-induced IEG mRNA responses and some neurotransmitter receptor alterations appear to be mediated by NMDA receptor-independent mechanisms. Accordingly, rapid activation of these IEGs, which appear to encode transcription factors, may be involved in coordinating long-term neuronal changes following repeated seizures.

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