

Induction of *c-fos* mRNA by Kindled Seizures: Complex Relationship with Neuronal Burst Firing

David M. Labiner,^{1,4} Linda S. Butler,^{1,4} Zhen Cao,^{1,4} David A. Hosford,^{1,4} Cheolsu Shin,^{1,4} and James O. McNamara^{1,2,3,4}

Departments of ¹Medicine (Neurology), ²Neurobiology, and ³Pharmacology, Duke University Medical Center, and ⁴Epilepsy Research Laboratory, Department of Veterans Affairs Medical Center, Durham, North Carolina 27710

Expression of the immediate-early gene *c-fos* has been advanced as a marker of neuronal activity in the adult nervous system. We sought to test the validity of *c-fos* mRNA expression as a marker of neuronal activity during seizures and to elucidate specific neurotransmitter receptors whose activation was necessary for seizure-evoked *c-fos* mRNA expression. We correlated *c-fos* mRNA expression, measured with *in situ* hybridization, with kindled seizure-induced firing of hippocampal dentate granule cells or substantia nigra pars compacta and pars reticulata neurons. We found that the occurrence of seizure-evoked synchronous action potentials during the seizure exhibited a perfect qualitative correlation with the presence of *c-fos* mRNA expression in the granule cells 30 min following the seizure (Fisher's exact test, $p = 0.002$). However, there was no quantitative correlation between the number of seizure-induced population action potentials and the magnitude of *c-fos* mRNA expression in the granule cells. In the substantia nigra, where neuronal populations have previously been demonstrated to exhibit synchronous firing during kindled seizures, no induction of *c-fos* mRNA was detected in either pars compacta or pars reticulata. Pretreatment with antagonists of the NMDA subtype of glutamate receptor selectively and markedly decreased seizure-induced *c-fos* mRNA expression in the dentate granule cells, despite increasing the number of granule cell population action potentials.

These findings illustrate the complexity of the relationship between *c-fos* induction and neuronal burst firing during kindled seizures. We caution that *c-fos* mRNA expression is not a simple marker of neuronal activity. We suggest that seizure-induction of *c-fos* mRNA expression may correlate better with sustained neuronal depolarization and subsequent rise of intracellular calcium evoked by multiple factors, one of which is NMDA receptor activation.

[Key words: *c-fos*, neuronal activity, kindling, epilepsy, seizure, NMDA, dentate granule cells, substantia nigra]

Received May 22, 1992; revised July 31, 1992; accepted Aug. 19, 1992.

We thank Drs. Douglas Bonhaus, Raymond Dingleline, James Eberwine, and J. Victor Nadler for helpful discussions. We are grateful to Lewis Rigsbee for the technical assistance and to Drs. J. I. Morgan and T. Curran (Roche Institute of Molecular Biology, Nutley, NJ) for providing the *c-fos* cDNA probe used in preliminary studies. This study was supported in part by grants from the National Institutes of Health (NS17771, NS25608, NS01232, NS08524) and the Veterans Administration.

Correspondence should be addressed to James O. McNamara, M.D., Duke University Medical Center, 401 Bryan Building, Box 3676, Durham, NC 27710. Copyright © 1993 Society for Neuroscience 0270-6474/93/130744-08\$05.00/0

c-fos, an immediate-early gene, undergoes dramatic and transient expression in response to a diversity of stimuli *in vitro* (Curran and Morgan, 1987; Morgan and Curran, 1989). The striking activation of *c-fos* expression in neurons by depolarization *in vitro* (Morgan and Curran, 1986) and by seizures *in vivo* (Morgan et al., 1987) led to its proposed use as a marker of neuronal activity in the mammalian nervous system (Sagar et al., 1988).

Seizures are characterized by a specific pattern of neuronal activity, namely, the synchronous firing, or burst firing, of populations of neurons in discrete pathways of the nervous system. The availability of a sensitive and reliable histochemical marker of this pattern of neuronal activity with cellular resolution would permit elucidating the networks activated during a seizure, and in turn facilitate pharmacologic and surgical efforts aimed at controlling medically refractory seizures.

Despite its attractiveness, the validity of *c-fos* expression as a marker of neuronal activity during a seizure has not been directly tested. The goals of this work were twofold: (1) to test the validity of *c-fos* mRNA expression as a marker of seizure-induced neuronal activity by correlating the activity of distinct populations of neurons during a seizure with *c-fos* mRNA expression induced in these neurons by a seizure, and (2) to elucidate specific neurotransmitter receptors whose activation is required for seizure induction of *c-fos* mRNA expression in these neurons.

We examined the seizure-evoked neuronal activity of the hippocampal dentate granule cells with field potential recordings *in vivo*, and we measured *c-fos* mRNA expression with *in situ* hybridization. The dentate granule cells were chosen because of the robust effect of seizures on *c-fos* expression in this group of neurons (Morgan et al., 1987) and because the population field potentials generated by the dentate granule cells have been extensively characterized electrophysiologically (Andersen et al., 1971; Lomo, 1971). We also examined *c-fos* mRNA expression in the substantia nigra, a nucleus in which burst-firing neuronal activity during kindled seizures has been previously characterized with single- and multiple-unit activity (Bonhaus et al., 1986, 1991).

Materials and Methods

Preparation and kindling of animals for correlation of dentate field potentials and c-fos expression

Adult male Sprague-Dawley rats (Charles River, Wilmington, DE) weighing at least 250 gm underwent stereotaxic implantation of a bipolar stimulating/recording electrode in the right amygdala under pentobar-

bital anesthesia using the following coordinates with instrument zero as reference: AP +3.0 mm, ML 4.8 mm, DV 2.3 mm below dura. A concentric bipolar electrode used for recording electroencephalographic activity (EEG) and dentate field potentials was placed in the dentate hilus of the left dorsal hippocampus. Characteristic extracellular field potentials of dentate granule cells following stimulation of the left angular bundle were used to optimize electrode placements as described previously (Shin et al., 1987). Following a postoperative recovery period of at least 7 d, stimulus trains (500 μ A, 1 msec biphasic rectangular pulses, 60 Hz, 1 sec duration) were administered once daily, 5 d per week, through the amygdala electrode until two class 5 seizures were evoked. Following a 1 week seizure-free period, single shocks were administered through the stimulating electrode in the left angular bundle to verify that the recordings of the left dentate exhibited the pattern typical of the field EPSP and population action potentials of the dentate granule cells (Shin et al., 1987). Thereupon, a stimulus train was administered through the amygdala electrode and a class 5 kindled seizure was evoked; dentate field potentials were recorded during the seizure. Each animal was killed 30 min following the seizure and brain sections were prepared for *in situ* hybridization as described below.

During the kindled seizure, electrographic seizure [afterdischarge (AD)] was recorded from the bipolar electrode in the right amygdala and from the concentric bipolar electrode in the contralateral (left) dentate hilus; field potentials were also recorded in the left dentate. These events were recorded on a Vetter-modified Panasonic video cassette recorder (model 420B). The analog recording was digitized and analyzed by Computer-scope (R.C. Electronics, Santa Barbara, CA). The recordings were analyzed by counting the number of population action potentials ("population spikes") and by measuring the amplitude of the largest population spike during the seizure. The presence or absence of population action potentials was ascertained without prior knowledge of *c-fos* expression.

Preparation and kindling of animals for analysis of drug effects on seizure-evoked *c-fos* expression

To simplify investigation of drug on seizure-induced *c-fos* mRNA expression, kindled seizures evoked by hippocampal stimulation were studied because these are associated with a massive, bilateral induction of *c-fos* mRNA expression in the hippocampal dentate granule cells in every animal. To prepare these animals, a stainless steel bipolar stimulating/recording electrode was implanted in the right dorsal hippocampus under pentobarbital anesthesia (50 mg/kg, i.p.); the stereotaxic coordinates, using bregma as a reference, were AP -3.2, ML +2.1, and DV -3.1 from dura (nose bar at 0). An additional electrode was secured to skull screws for use as a reference in EEG recordings. After a postoperative recovery of at least 1 week, animals were stimulated twice daily (1 sec trains of 1 msec biphasic rectangular pulses delivered at a frequency of 60 Hz) with a current intensity sufficient to evoke an AD. Stimulations were administered until the animals exhibited at least six class 4 or 5 seizures (Racine, 1972), the last three being consecutive. The generalized seizure threshold was subsequently determined by once-daily stimulations as previously described (McNamara et al., 1984), except that the current intensities used were 30 μ A above and below the threshold. After a stimulation-free interval of at least 1 week, animals were used in one of the experimental protocols described below.

Kindled animals received an intraperitoneal injection of either vehicle or drug prior to a stimulus-evoked seizure. The stimulations were 30 μ A above the generalized seizure threshold and 2 sec in duration. Animals were killed by decapitation 30 min after the evoked seizure; the brains were rapidly dissected and frozen in 2-methylbutane in a dry ice/methanol bath.

Determination of drug effects on kindled seizure-evoked granule cell population action potentials

To characterize drug effects on the electrophysiologic events in the right dentate gyrus during seizures evoked by left hippocampal stimulation, additional animals were prepared as follows. A bipolar electrode to be used for kindling stimulations was implanted in the left hippocampus using stereotaxic guidance as above. In addition, a concentric bipolar electrode used for recording EEG and dentate granule cell field potentials was placed in the dentate hilus of the right dorsal hippocampus using electrophysiologic guidance with stimulation of the right angular bundle as described previously (Shin et al., 1987). These animals received kindling stimulations and their seizure thresholds were determined as de-

scribed for the experiments examining drug effects. In order for each animal to serve as its own control, seizures were evoked after treatment with either vehicle (H_2O , 1.0 ml/kg) or MK-801 (1.0 mg/kg). Two of the animals initially received vehicle, and the other two initially received MK-801. After receiving the initial treatment and a seizure, the generalized seizure threshold was determined again. Following a 1 week stimulation-free period, the second treatment was administered. Prior to each seizure the field potential response in the right dentate evoked by right angular bundle stimulation (0.1 Hz) was recorded as previously described (Shin et al., 1987). EEG and dentate field potentials were recorded and analyzed without knowledge of drug treatments; the methods were as described in the experiments correlating seizure-induced *c-fos* expression with granule cell activity.

c-fos in situ hybridization

Oligonucleotide probes. The oligonucleotide sequence comprised 50 bases, 50% of which were deoxyguanosine and deoxycytidine. The sequence utilized, nucleotides 270-319 of rat *c-fos* (Curran et al., 1987), was not significantly homologous to other sequences cataloged in the rodent gene bank library accessed through the Baylor College of Medicine Molecular Biology Information Resource. Radiolabeled oligonucleotide probes were prepared with a 3'-terminal transferase reaction and ^{32}P -dATP (6000 Ci/mmol; Du Pont-New England Nuclear, Wilmington, DE) to yield a specific activity of $1.6-6.0 \times 10^9$ dpm/ μ g (1-4 mol of ^{32}P -dATP per mole of oligonucleotide). The *c-fos* oligonucleotide sequence was validated by demonstrating that it hybridized to a single 2.2 kilobase band in a Northern blot containing RNA extracted from rat hippocampus following an AD evoked by angular bundle stimulation; a nick-translated rat *c-fos* cDNA probe hybridized to the same band.

In situ hybridization. *In situ* hybridization was performed with 10- μ m-thick frozen sections that were thaw-mounted on autoclaved gelatin-coated slides. The slide-mounted sections were fixed for 30 min at room temperature in phosphate-buffered saline (PBS; 10 mM NaCl, 1.6 mM NaH_2PO_4 , 8.4 mM Na_2HPO_4 , pH 7.0) containing 4% paraformaldehyde, followed by graded ethanol dehydration (70% for 5 min, 95% for 5 min, 100% for 5 min). The sections were prehybridized for 4 hr at 37°C in a solution containing 50% formamide (Bethesda Research Labs, Gaithersburg, MD), 2 \times NTE (300 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0), 1 \times Denhardt's solution, 500 μ g/ml yeast tRNA (Sigma), 500 μ g/ml salmon sperm DNA (Sigma), and 0.05% sodium pyrophosphate. The sections were hybridized for 16 hr at 37°C in a similar solution with the following alterations: 100 μ g/ml tRNA, 100 μ g/ml DNA, and 75,000 cpm/ μ l of ^{32}P -labeled oligonucleotide probe. Nonspecific hybridization of the radiolabeled probe to sections was determined by including a 10 \times concentration of unlabeled probe in the prehybridization solution and a 100 \times concentration in the hybridization solution. Equivalent amounts of nonspecific hybridization were obtained with a 100 \times concentration of unlabeled probe in the prehybridization solution and only labeled probe in the hybridization solution. Following hybridization, sections were washed in 1 \times NTE, 0.05% sodium pyrophosphate solution for 20 min at room temperature followed by 3 hr in a solution containing 10 mM Tris, 75 mM NaCl, and 0.05% pyrophosphate at 37°C. The sections were then dehydrated with ethanol solutions (5 min each in 50% ethanol containing 300 mM ammonium acetate, 70% ethanol, and 95% ethanol, and 10 min in 100% ethanol). After air drying, the slides were mounted in x-ray cassettes with intensifying screens and exposed to Beta-max hyperfilm (Amersham, Arlington Heights, IL) for 72 hr at -70°C. The film was developed in D-19 (Kodak, Rochester, NY) for 5 min at 18°C, washed briefly in 1% acetic acid, and fixed with Kodak Rapid Fixer for 5 min at 18°C.

These hybridization experiments were performed two additional times on nearby sections from each brain region of these animals, and similar results were consistently obtained.

Optical density measurements. Optical density (OD) determinations were made using an Amersham RAS/R1000 Image Analysis System (Loats Associates, Inc., Westminster, MD). Each film contained sections from vehicle- and drug-treated animals. All sections on a single film were hybridized on the same day with the same preparation of radiolabeled probe. To permit comparisons of data from different films, OD measurements from each film were normalized by assigning a value of 1.0 to the OD of the infrapyramidal blade of the dentate granule cell layer to the vehicle-treated animal with the highest absorbance. Mean OD values (total) were obtained for each brain. Nonspecific hybridization values for all of the sections from a film were averaged and subtracted from each total value to determine specific hybridization values.

Table 1. Relation between seizure indices and seizure-induced *c-fos* expression in amygdala-stimulated animals

| Animal | Right amygdala ADD (sec) | Left dentate ADD (sec) | Forelimb clonus (sec) | Population spikes/seizure | <i>c-fos</i> mRNA (10 ⁻²¹ mol/mm ²) |
|--------|--------------------------|------------------------|-----------------------|---------------------------|--|
| 1 | 122 | 84 | 91 | 139 | 2.1 |
| 2 | 81 | 41 ^a | 66 | 491 ^a | 1.1 |
| 3 | 84 | 27 | 65 | 1108 | 2.4 |
| 4 | 172 | 143 | 58 | 1140 | 3.5 |

This table presents electrophysiologic and behavioral features of seizures evoked by amygdala stimulation and the seizure-evoked *c-fos* expression. AD duration (ADD) was recorded from the electrode in the right amygdala and also from the bipolar electrode in the left dentate. The duration of forelimb clonus was determined by behavioral observation. The number of population action potentials was counted in off-line analysis of recorded seizures from three animals as described in Materials and Methods. *c-fos* mRNA content (10⁻²¹ mol/mm²) in the infrapyramidal blade of the left dentate was measured as described in Materials and Methods.

^a Animal 2 clearly exhibited abundant population action potentials during the final seizure, but technical difficulties precluded tabulation of numbers; this same animal exhibited 491 population action potentials during the 41 sec dentate AD immediately prior to the final one.

Drugs

Drugs and chemicals were purchased from Sigma Chemical Co., St. Louis, MO, except for MK-801, which was provided by Merck, Sharp and Dohme Research Lab, Rahway, NJ, and CGP-37849, which was provided by Ciba-Geigy Ltd., Basel, Switzerland.

Drug doses were based upon studies that documented antagonism of receptor-mediated effects in the CNS after intraperitoneal administration to adult rats. These included antagonists of the NMDA receptor (MK-801; McNamara et al., 1988; Olney et al., 1989), β -adrenergic receptor (propranolol; Mezey et al., 1983), muscarinic cholinergic receptor (scopolamine; de la Garza et al., 1987), and μ -opiate receptor (naloxone; Cottrell et al., 1988). The doses of CGP-37849 were selected on the basis of our preliminary experiments examining doses effective in inhibiting tonic hindlimb extension of electroshock seizures in rats.

Results

Correlation of seizure-induced *c-fos* expression in dentate granule cells with dentate field potentials recorded during kindled seizures

Dentate field potentials were recorded during kindled seizures, the animals were killed 30 min thereafter, and *c-fos* expression was examined in the dentate granule cells with *in situ* hybridization. Stimulation of the amygdala elicited class 5 behavioral seizures and ADs in EEG recordings from the bipolar electrode in the left dentate hilus in all 11 animals. Only 4 of the 11 animals exhibited bursts of population action potentials ("population spikes") in the dentate field potential recordings during the seizures (compare Fig. 1A,B) (Somjen et al., 1985; Stringer et al., 1991). Robust *c-fos* mRNA expression was apparent in the dentate granule cells of the left hippocampus in all four of the animals exhibiting bursts of population action potentials and was absent in all seven animals in which population action potentials were not detected during the kindled seizure (Fig. 2A,B). This correlation between the presence of population action potentials during the seizures and *c-fos* mRNA expression was highly significant (Fisher's exact test, $p = 0.002$).

Analysis of the electrophysiologic and behavioral features of the seizures among these 11 animals disclosed several interesting features. The duration of the seizures in the group with *c-fos* expression exceeded the group without *c-fos* expression [seconds of AD duration (ADD) in the right amygdala, ADD in the left dentate, duration of forelimb clonus, respectively: *c-fos* present,

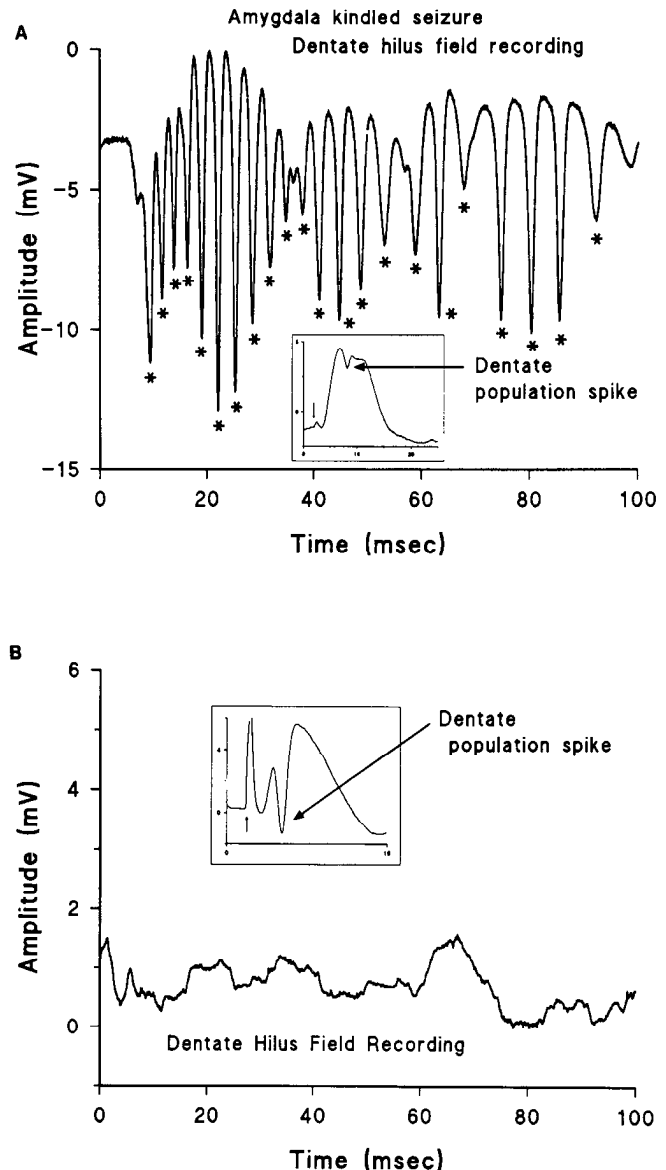


Figure 1. Dentate hilar field potential recordings during seizures evoked by right amygdala stimulation. These representative tracings were obtained from an animal exhibiting burst firing of dentate granule cells (A) and an animal without this pattern (B). Both animals exhibited a class 5 seizure with AD durations recorded from the amygdala and dentate, respectively, of 172 sec and 143 sec (A), and 50 sec and 15 sec (B). The insets show the characteristic upgoing EPSP with sharp negative population spike produced by angular bundle stimulation before the seizure was induced (vertical arrows point to stimulation artifact). During part of the kindled seizure, dentate granule cells fire in synchrony as evidenced by multiple population action potentials labeled with asterisks.

115 ± 21, 74 ± 26, 70 ± 7; *c-fos* absent, 54 ± 8, 45 ± 10, 38 ± 4]. However, considerable overlap existed between the two groups in that five of the seven animals without *c-fos* expression exhibited longer AD duration in the left dentate than the shortest AD duration in the left dentate of the group with *c-fos* expression (27 sec in animal 3, Table 1). The intensity of *c-fos* mRNA expression varied threefold among the four animals in which *c-fos* was induced, suggesting that *c-fos* expression may be a graded response once it reaches a minimal threshold for expression. However, there was no linear relation between the number of population action potentials and the magnitude of

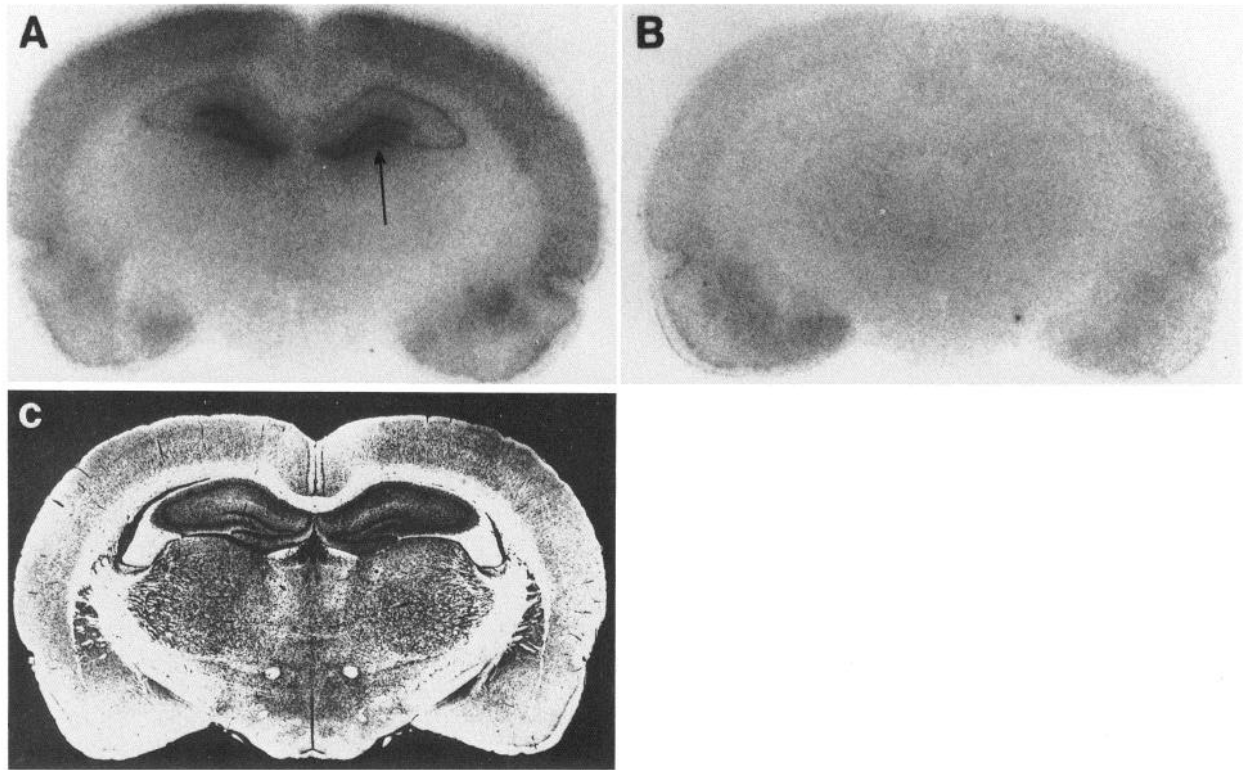


Figure 2. Total binding of oligonucleotide probe (*c-fos*) to sections of brain from animals portrayed in Figure 1 exhibiting the presence (*A*) or absence (*B*) of population action potentials in the left dentate recordings during seizures evoked by right amygdala stimulation. Note the robust *c-fos* mRNA expression bilaterally in the dentate granule cell layer of the animal presented in *A*, in contrast to the nearly absent expression of *c-fos* mRNA in the dentate granule cell layer of the animal presented in *B*. Note also the moderate expression of *c-fos* mRNA in the piriform cortex ipsilateral to the kindling electrode in *B*. Hybridization in the presence of excess unlabeled oligonucleotide, in an adjacent section from the brain used in *A*, resulted in a homogeneous pattern of binding similar to that of *B* (not shown). *C* is a Nissl-stained coronal section of rat brain at a level similar to *A* and *B*. OD measurements were made in the infrapyramidal blade of the dentate granule cell layer as described in Materials and Methods. Scale bar, 5 mm.

c-fos expression, in that nearly equivalent amounts of *c-fos* mRNA were detected despite a sevenfold difference in number of population action potentials (compare animals 1 and 4, Table 1).

Comparison of kindled seizure-induced *c-fos* mRNA expression in the dentate gyrus and in the substantia nigra

Absence of correlation between the number of population action potentials and the magnitude of seizure-induced *c-fos* mRNA expression in the granule cells led us to examine this relationship in additional populations of neurons. Using both single- and multiple-unit recordings, we had demonstrated the presence of nearly synchronous burst firing of substantia nigra pars reticulata neurons during kindled seizures in 9 of 12 animals (Bonhaus et al., 1986, 1991). We therefore determined whether *c-fos* mRNA expression was induced in substantia nigra neurons by kindled seizures in a separate group of four animals. To include a positive control internal to these animals, *c-fos* mRNA expression was measured in the dentate granule cells as well. Three of the four animals exhibited population action potentials in the dentate hilus recordings during the kindled seizures and *c-fos* mRNA expression in the dentate granule cell layer (Table 2, Fig. 3). The fourth animal exhibited neither population action potentials in the dentate hilus during the kindled seizure nor *c-fos* mRNA expression in the dentate granule cells. None of these four animals exhibited detectable *c-fos* mRNA expression in either substantia nigra pars reticulata or pars compacta (Fig. 3). Ad-

ditionally, *c-fos* was not detected in the substantia nigra in any of eight animals following kindled seizures evoked by hippocampal stimulation (vehicle group of Mk-801 experiment, Table 3), despite robust expression in the dentate granule cells.

Effects of neurotransmitter receptor antagonists on seizure-induced *c-fos* mRNA expression in dentate granule cells

To determine whether activation of specific neurotransmitter receptors is required for seizure induction of *c-fos* mRNA expression, we examined the effects of several receptor antagonists on the seizure-induced *c-fos* expression. We hypothesized that activation of the NMDA subtype of glutamate receptor during the seizure induced the expression of *c-fos* mRNA. The rationale

Table 2. Comparison of *c-fos* induction in dentate granule cells (DGC) and in substantia nigra (SN)

| Animal | Clonic seizure (sec) | Seizure class | Total AD duration (sec) | Population spikes in DGC | <i>c-fos</i> in DGC | <i>c-fos</i> in SN |
|--------|----------------------|---------------|-------------------------|--------------------------|---------------------|--------------------|
| 1 | 57 | 5 | 112 | + | + | - |
| 3 | 64 | 5 | 134 | + | + | - |
| 4 | 49 | 5 | 77 | + | + | - |
| 5 | 49 | 5 | 116 | - | - | - |

+, Present; -, absent.

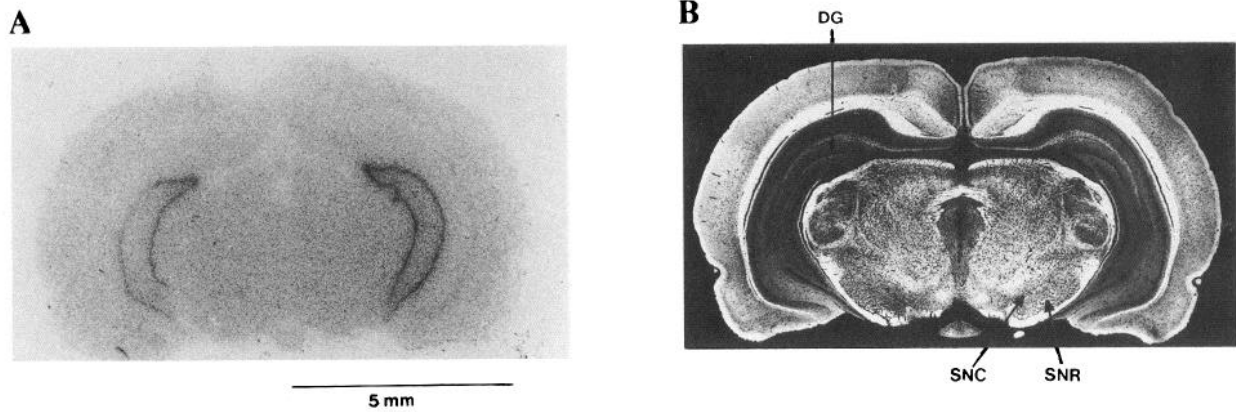


Figure 3. *A*, Total binding of oligonucleotide probe (*c-fos*) to coronal section of brain from animal kindled from the right amygdala at the level of substantia nigra and ventral hippocampus. This animal had population action potentials recorded in the dentate hilus in the dorsal hippocampus during the fully kindled seizure. Thirty minutes later, the animal was killed and the brain was isolated and processed for *in situ* hybridization. *B*, Nissl-stained section shown for anatomic localization of structures: *DG*, dentate gyrus; *SNC*, substantia nigra pars compacta; *SNR*, substantia nigra pars reticulata. Scale bar, 5 mm.

for this hypothesis was twofold: (1) the principal excitatory afferents of the dentate granule cells from the entorhinal cortex (Andersen et al., 1966; Matthews et al., 1976) use glutamate as a transmitter (White et al., 1977; Keller et al., 1991), and (2) activation of either the α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) or NMDA subtype of glutamate receptor is sufficient to induce *c-fos* mRNA expression in isolated dentate gyrus neurons (Lerea et al., 1992).

Pretreatment of animals with CGP-37849, a competitive antagonist of the NMDA subtype of glutamate receptor (Fagg et al., 1990), markedly inhibited the seizure-induced expression of *c-fos* mRNA. The inhibition was dose dependent, with a 3 mg/kg dose producing a 70% inhibition of *c-fos* expression contralateral and 66% ipsilateral to the stimulating electrode (Table 3). Similarly, the uncompetitive NMDA receptor antagonist MK-801 (Wong et al., 1986) reduced seizure-induced expression of *c-fos* mRNA by approximately 50% in the dentate granule cells bilaterally (Fig. 4, Table 3). The inhibitory effects of the NMDA receptor antagonists were selective inasmuch as antagonists of μ -opiate (naloxone), muscarinic cholinergic (scopolamine), and β -adrenergic (propranolol) receptors did not attenuate seizure-induced *c-fos* mRNA expression (Table 3). Doses of the antagonists used above had been shown to be effective in the CNS (see Materials and Methods).

The possibility that NMDA antagonists inhibited seizure-

induced *c-fos* expression by suppressing seizure duration or seizure-induced bursts of population action potentials of the dentate granule cells was tested electrophysiologically. Pretreatment with MK-801 did not shorten seizure duration recorded on EEG from a hippocampal electrode (ADD: vehicle, 92 ± 14 sec; MK-801, 89 ± 17 sec), nor did it decrease the number of population action potentials recorded in dentate granule cells during the seizure (Fig. 5). Unexpectedly, MK-801 increased the number of population action potentials recorded in the dentate hilus during seizures in each of four animals studied (number of population action potentials/seizure: vehicle, 297 ± 114 ; MK-801, 849 ± 80 ; $p < 0.05$).

Discussion

Three principal findings emerge from this study. (1) The occurrence of synchronous firing of dentate granule cells during a seizure correlated with the presence of *c-fos* mRNA expression in these neurons 30 min following a seizure. The magnitude of *c-fos* mRNA expression was not correlated with the number of population action potentials. (2) In the substantia nigra, no induction of *c-fos* mRNA expression was detected in either pars compacta or pars reticulata neurons, neuronal populations previously demonstrated to exhibit synchronous firing during kindled seizures. (3) Antagonists of the NMDA subtype of gluta-

Table 3. Relative OD values (see Materials and Methods) for *c-fos* mRNA for vehicle- and drug-treated groups

| Drug (<i>N</i>) | mg/kg | Ipsilateral vehicle | Ipsilateral drug-treated | Contralateral vehicle | Contralateral drug-treated |
|---------------------|-------|---------------------|--------------------------|-----------------------|----------------------------|
| (+)MK-801 (8) | 1.0 | 0.347 ± 0.019 | $0.171 \pm 0.048^{**}$ | 0.373 ± 0.021 | $0.176 \pm 0.051^*$ |
| CGP-37849 (5) | 2.0 | 0.392 ± 0.024 | 0.383 ± 0.053 | 0.370 ± 0.024 | 0.329 ± 0.051 |
| CGP-37849 (5) | 3.0 | 0.287 ± 0.031 | $0.098 \pm 0.027^*$ | 0.272 ± 0.032 | $0.081 \pm 0.027^*$ |
| Naloxone·HCl (3) | 1.0 | | 0.544 ± 0.084 | | 0.441 ± 0.045 |
| Scopolamine·HCl (3) | 2.0 | 0.355 ± 0.043 | 0.426 ± 0.139 | 0.346 ± 0.026 | 0.396 ± 0.148 |
| Propranolol·HCl (3) | 2.5 | | 0.368 ± 0.109 | | 0.399 ± 0.160 |

Values are mean \pm SEM; *N* refers to number of animals receiving a given treatment. All comparisons are between drug- and vehicle-treated animals. Sham stimulation of the angular bundle or basomedial amygdala in similar experiments failed to induce *c-fos* expression above its constitutive level; in these animals, there was no detectable constitutive expression above nonspecific background. * and ** refer to $p < 0.01$ and $p < 0.05$, respectively (Mann-Whitney *U* test; Conover, 1980).

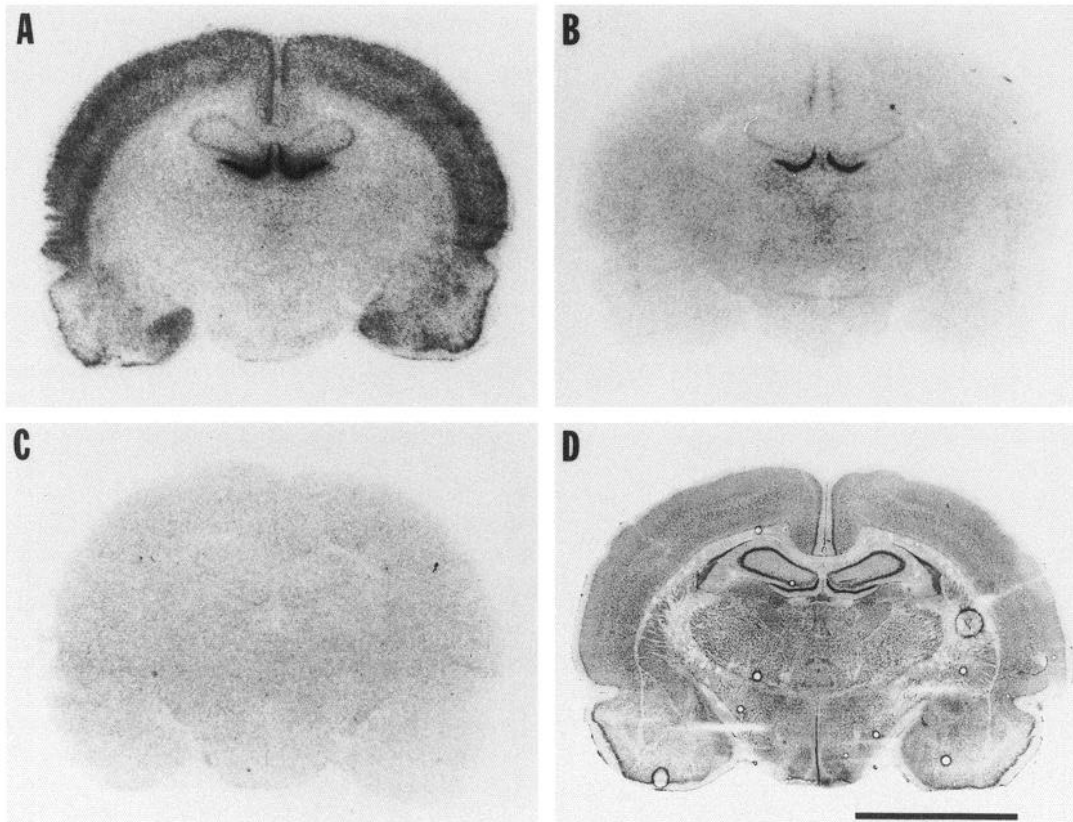


Figure 4. *A* and *B*, Total binding of oligonucleotide probe (*c-fos*) to sections of brain from animals pretreated with vehicle (*A*) or 1.0 mg/kg MK-801 (*B*) prior to a stimulus-evoked seizure. Seizures evoked by hippocampal stimulation elicited a rapid and dramatic induction of *c-fos* mRNA bilaterally in the hippocampal formation, pyriform cortex, and layers 3 and 5/6 of neocortex (*A*). By contrast, the level of constitutive expression of *c-fos* mRNA in the unstimulated brain was virtually undetectable (not shown). OD measurements of infrapyramidal blade of dentate granule cell layer (in *B*) revealed a 60% reduction in *c-fos* expression in this MK-801-treated animal compared to the vehicle-treated animal. *C*, Hybridization in the presence of excess unlabeled oligonucleotide, in an adjacent section from the brain used in *A*, resulted in a homogeneous pattern of binding. *D*, Nissl-stained coronal section of rat brain at a level similar to *A*–*C*. OD measurements were made in the infrapyramidal blade of the dentate granule cell layer as described in Materials and Methods. Scale bar, 5 mm.

mate receptor selectively and markedly inhibited seizure-induced expression of *c-fos* mRNA in dentate granule cells despite unexpectedly increasing the number of population action potentials by almost threefold.

Validity of c-fos mRNA expression as a marker of neuronal burst firing during kindled seizures

These correlative studies of seizure-induced firing and *c-fos* mRNA expression illustrate the complexity of the relationship between neuronal activity and the induction of *c-fos*. Importantly, neuronal activity is defined here as a specific pattern of activity that represents the cellular hallmark of a seizure, namely, the sustained repetitive synchronous firing of populations of neurons (henceforth referred to as epileptiform burst firing). Certainly, *c-fos* expression is *not* simply a quantitative or even a qualitative marker of this firing in *all* CNS neurons. In the dentate granule cells, a striking qualitative correlation was observed between the synchronous firing of granule cells and *c-fos* mRNA expression; this firing pattern of the dentate granule cells was initially described during kindled seizures by Somjen et al. (1985) and later by Stringer et al. (1991), who coined the term maximal dentate activation for this pattern. However, the epileptiform burst firing was not correlated with seizure induction of *c-fos* mRNA expression in neurons of the substantia nigra. No detectable *c-fos* mRNA expression was found in substantia

nigra pars compacta or pars reticulata neurons in any of four animals undergoing class 5 kindled seizures evoked by amygdala stimulation or in eight animals by hippocampal stimulation. We previously demonstrated that individual substantia nigra pars compacta and pars reticulata neurons fire in bursts during amygdaloid kindled seizures evoked in five of seven animals in a paralyzed, ventilated preparation (Bonhaus et al., 1986). We confirmed and extended these analyses by demonstrating a nearly synchronous burst firing pattern of populations of substantia nigra pars reticulata neurons in four of five freely moving animals during amygdaloid kindled seizures (Bonhaus et al., 1991). Although the measurements of substantia nigra unit activity and *c-fos* mRNA expression were not performed in the same animals, it seems likely that *c-fos* mRNA expression is not induced in substantia nigra neurons despite the presence of epileptiform burst firing during a kindled seizure.

Further complexity is introduced in the dentate granule cells with respect to quantitative considerations in that the magnitude of *c-fos* mRNA expression, once induced, did not correlate with the number of population action potentials (Table 1). Additionally, as discussed below with hippocampal kindled animals, pretreatment with NMDA receptor antagonists produced a 50–70% reduction of seizure-induced *c-fos* mRNA expression in the dentate granule cells despite a threefold increase of the number of population action potentials.

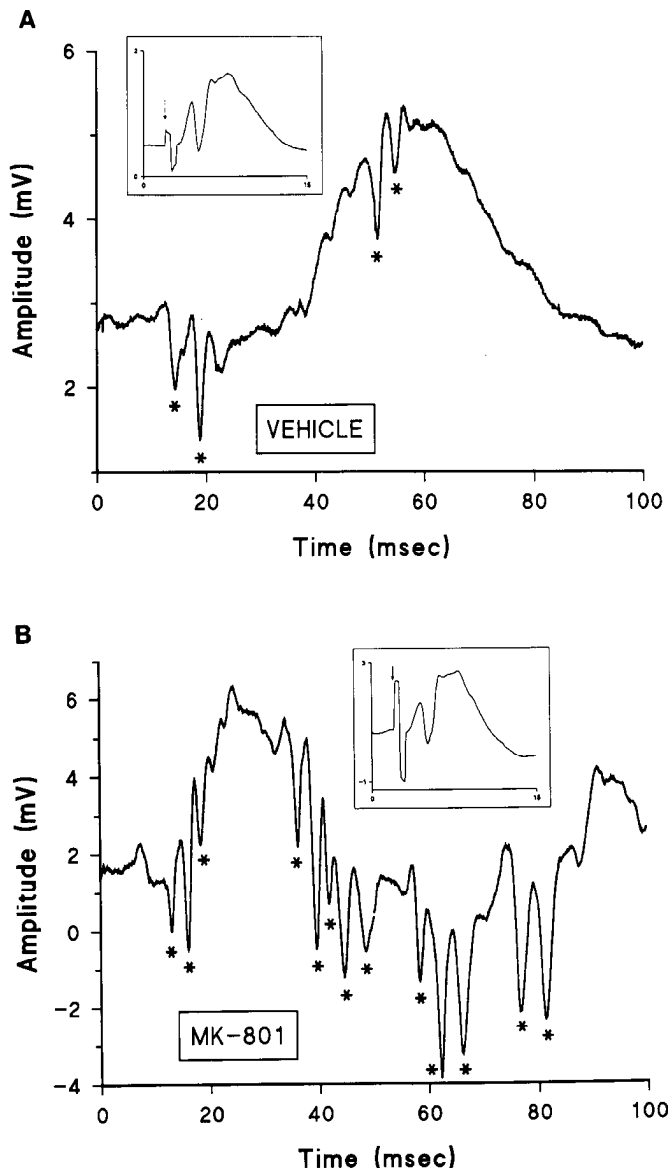


Figure 5. Field potential recordings from the dentate hilus, contralateral to the stimulated hippocampus. In this representative animal, vehicle injection prior to stimulus-evoked seizure (*A*) was followed 3 weeks later by MK-801 injection prior to stimulus-evoked seizure (*B*). AD duration was 129 sec and 116 sec during the vehicle-treated and MK-801-treated seizures, respectively. The insets show the characteristic upgoing EPSP with sharp negative population spike produced by angular bundle stimulation following administration of vehicle or drug but before the seizure was induced (vertical arrows point to stimulation artifact). During part of the kindled seizure, dentate granule cells fire in synchrony as evidenced by multiple population action potentials labeled with asterisks (number of population action potentials: vehicle, 345; MK-801, 991 in this particular example). This increase in the number of population action potentials with MK-801 was unexpected, but a previous *in vitro* study in CA1 may provide some clue (Dingledine et al., 1987). Thus, reduction of the calcium influx by NMDA antagonists may result in reduced activation of calcium-dependent potassium channel, leading to diminished inhibition, explaining at least in part the observed increase in the number of population action potentials.

Mechanisms governing seizure-induced expression of *c-fos* mRNA: a hypothesis

Understanding the mechanisms by which a seizure induces *c-fos* mRNA expression in the dentate granule cells may clarify the

relation between *c-fos* mRNA expression and epileptiform burst firing in other neuronal populations. *In vitro* studies together with the *in vivo* studies presented here lead us to suggest that kindled seizures trigger *c-fos* mRNA expression in the dentate granule cells by the synaptic activation of glutamate receptors on granule cell dendrites, which in turn leads both directly and indirectly to a sustained depolarization and rise of intracellular calcium. Analyses of mechanisms activating *c-fos* mRNA expression in individual dentate gyrus neurons cultured from 4-d-old rat pups and maintained *in vitro* disclose that activation of either NMDA or AMPA subtypes of glutamate receptors is sufficient to induce robust *c-fos* mRNA expression; activation of *c-fos* expression by either receptor requires extracellular calcium (Lerea et al., 1992). Likewise, activation of voltage-sensitive calcium channels by potassium depolarization is sufficient to induce *c-fos* mRNA expression in a calcium-requiring manner in these neurons. Increases of extracellular glutamate during a seizure (Dodd et al., 1980; Peterson et al., 1983) lead us to suggest that the kindled seizure results in synaptic release of glutamate (White et al., 1977), presumably from entorhinal afferents by virtue of their massive input to the granule cells (Andersen et al., 1966; Matthews et al., 1976), thereby activating both NMDA and AMPA receptors on granule cell dendrites (Keller et al., 1991). Subsequently, as suggested by data from the *in vitro* study (Lerea et al., 1992), transmembrane flux of Ca^{2+} through both the NMDA receptor and voltage-activated calcium channels results in an increased concentration of intracellular Ca^{2+} , triggering a signaling cascade that culminates in transcriptional activation of *c-fos*. The selective inhibition of seizure-induced *c-fos* mRNA expression by antagonists of NMDA but not three other neurotransmitter receptors is consistent with this interpretation. We suggest that seizure induction of *c-fos* mRNA may correlate better with sustained neuronal depolarization induced by NMDA receptor activation and other mechanisms than with neuronal burst firing itself. Lack of a straightforward method to measure depolarization of multiple neuronal populations during seizures in awake, freely moving animals precludes direct testing of this idea at present.

This line of reasoning may also provide an explanation for the unexpected increase in the number of population action potentials with MK-801. A previous *in vitro* study in CA1 using a competitive antagonist, D-aminophosphonovalerate, showed increased number of action potentials in a burst, despite reduced depolarization (Dingledine et al., 1987). The increased number of action potentials may arise from a reduction of calcium influx through the NMDA channel and reduced activation of calcium-dependent potassium channels, thereby permitting enhanced sustained firing. Alternatively, the reduction of depolarization may limit sodium channel inactivation.

We suspect that the residual 30–50% of *c-fos* mRNA expression induced by seizures in the presence of NMDA antagonists is due to activation of AMPA receptors and/or activation of voltage-dependent calcium channels, particularly in light of the increased number of population action potentials recorded in the presence of MK-801. The future availability of structurally distinct and highly selective AMPA receptor antagonists that can be administered systemically may allow this possibility to be addressed. We cannot exclude potential contributions from either incomplete blockade of NMDA receptors or activation of the metabotropic subtype of the glutamate receptor, with resultant release of Ca^{2+} from intracellular stores.

These explanations fail to elucidate why the neurons of the

substantia nigra do not express *c-fos* mRNA even though the majority of seizures evoked here are almost certainly associated with an epileptiform burst firing pattern of these neurons. Substantia nigra neurons appear to have the ability to express *c-fos*, since pentylentetrazol (PTZ) seizure induced modest Fos-lacZ expression in the substantia nigra in a transgenic mouse strain (Smeyne et al., 1992). Likewise, pilocarpine-evoked status epilepticus is associated with induction of Fos-like immunoreactivity in the substantia nigra (L. E. A. M. Mello, personal communication; Mello et al., 1991). It is possible that seizures evoked by PTZ or pilocarpine activate the neurons of substantia nigra more intensely than a single kindled seizure. Alternatively, these chemically induced seizures may induce *c-fos* through different mechanisms. Moreover, nigral neurons may have intrinsic properties, such as calcium buffering capacity, distinct from those of dentate granule cells. Further studies of the substantia nigra are needed to explain these differences.

Kindled seizure induction of c-fos mRNA expression: implications

The fact that *c-fos* mRNA expression is not simply a marker of neuronal activity heightens the importance of elucidating the functional significance of its expression. These findings parallel the complex relationship between the photic induction of *c-fos* mRNA expression and neuronal activity identified in the suprachiasmatic nucleus of the hypothalamus (Kornhauser et al., 1990). Equivalence in light thresholds and timing of sensitivity of responses suggests that *c-fos* is a molecular component of the signaling cascade by which light entrains circadian rhythms (Kornhauser et al., 1990). By analogy, *c-fos* may be a molecular component of the signaling cascade by which seizures induce the lasting hyperexcitability of kindling or the emergence of spontaneous seizures in this model. Circumstantial evidence supports this idea in that two necessary conditions for kindling induction, the occurrence of electrical seizure and activation of NMDA receptors during the seizure, are also necessary for the full expression of *c-fos* mRNA in the kindling model.

References

- Andersen P, Holmqvist B, Voorhoeve PE (1966) Entorhinal activation of dentate granule cells. *Acta Physiol Scand* 66:448-460.
- Andersen P, Bliss TVP, Skrede KK (1971) Unit analysis of hippocampal population spikes. *Exp Brain Res* 13:208-221.
- Bonhaus DW, Walters JR, McNamara JO (1986) Activation of substantia nigra neurons: role in the propagation of seizures in kindled rats. *J Neurosci* 6:3024-3030.
- Bonhaus DW, Russell RD, McNamara JO (1991) Activation of substantia nigra pars reticulata neurons: role in the initiation and behavioral expression of kindled seizures. *Brain Res* 545:41-48.
- Conover WJ (1980) *Practical nonparametric statistics*, 2d ed. New York: Wiley.
- Cottrell GA, Nyakas C, Bohus B (1988) Hippocampal kindling-induced after-discharge and behavioural depression: immediate and long-term attenuation by opiate antagonists. *Eur J Pharmacol* 150:1-8.
- Curran T, Morgan JI (1987) Memories of *fos*. *Bioessays* 7:255-258.
- Curran T, Gordon MB, Rubino KL, Sambucetti LC (1987) Isolation and characterization of the *c-fos* (rat) cDNA and analysis of post-translational modifications *in vitro*. *Oncogene* 2:74-87.
- de la Garza R, Bickford-Wimer C, Hoffer BJ, Freedman R (1987) Heterogeneity of nicotine actions in the rat cerebellum: an *in vivo* electrophysiologic study. *J Pharmacol Exp Ther* 240:689-695.
- Dingledine R, Hynes MA, King KL (1986) Involvement of *N*-methyl-D-aspartate receptors in epileptiform bursting in the rat hippocampal slice. *J Physiol (Lond)* 380:175-189.
- Dodd PR, Bradford HF, Abdul-Ghani AS, Cox DWG, Continho-Netto J (1980) Release of amino acids from chronic epileptic and subepileptic foci *in vivo*. *Brain Res* 193:505-517.
- Fagg GE, Olpe HR, Pozza MF, Baud J, Steinmann M, Schmutz M, Portet C, Baumann P, Thedinga K, Bittiger H, Allgeier H, Heckendorn R, Angst C, Brundish D, Dingwall JG (1990) CGP 37849 and CGP 39551: novel and potent competitive *N*-methyl-D-aspartate receptor antagonists with oral activity. *Br J Pharmacol* 99:791-797.
- Keller BU, Konnerth A, Yaari Y (1991) Patch clamp analysis of excitatory synaptic currents in granule cells of rat hippocampus. *J Physiol (Lond)* 435:275-293.
- Kornhauser JM, Nelson DE, Mayo KE, Takahashi JS (1990) Photic and circadian regulation of *c-fos* gene expression in the hamster suprachiasmatic nucleus. *Neuron* 5:127-134.
- Lerea LS, Butler LS, McNamara JO (1992) NMDA and non-NMDA receptor-mediated increase of *c-fos* mRNA in dentate gyrus neurons involves calcium influx via different routes. *J Neurosci* 12:2973-2981.
- Lomo T (1971) Patterns of activation in a monosynaptic cortical pathway: the perforant path input to the dentate area of the hippocampal formation. *Exp Brain Res* 12:18-45.
- Matthews DA, Cotman C, Lynch G (1976) An electron microscopic study of lesion-induced synaptogenesis in the dentate gyrus of the adult rat. I. Magnitude and time course of degeneration. *Brain Res* 115:1-21.
- McNamara JO, Galloway MT, Rigsbee LC, Shin C (1984) Evidence implicating substantia nigra in regulation of kindled seizure threshold. *J Neurosci* 4:2410-2417.
- McNamara JO, Russell RD, Rigsbee L, Bonhaus DW (1988) Anticonvulsant and antiepileptogenic actions of Mk-801 in the kindling and electroshock models. *Neuropharmacology* 27:563-568.
- Mello LEAM, Tan AM, Finch DM (1991) C-FOS expression in pilocarpine-induced status epilepticus. *Soc Neurosci Abstr* 17:508.
- Mezey E, Reisine TD, Palkovits M, Brownstein MJ, Axelrod J (1983) Direct stimulation of β_2 -adrenergic receptors in rat anterior pituitary induces the release of adrenocorticotropin *in vivo*. *Proc Natl Acad Sci USA* 80:6728-6731.
- Morgan JI, Curran T (1986) Role of ion flux in the control of *c-fos* expression. *Nature* 322:552-555.
- Morgan JI, Curran T (1989) Stimulus-transcription coupling in neurons: role of cellular immediate-early genes. *Trends Neurosci* 12:459-462.
- Morgan JI, Cohen DR, Hempstead JL, Curran T (1987) Mapping patterns of *c-fos* expression in the central nervous system after seizure. *Science* 237:192-197.
- Olney JW, Labruyere J, Price MT (1989) Pathological changes induced in cerebrocortical neurons by phencyclidine and related drugs. *Science* 244:1360-1362.
- Peterson DW, Collins JF, Bradford HF (1983) The kindled amygdala model of epilepsy: anticonvulsant action of amino acid antagonists. *Brain Res* 275:169-172.
- Racine RJ (1972) Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroencephalogr Clin Neurophysiol* 32:281-294.
- Sagar SM, Sharp FR, Curran T (1988) Expression of *c-fos* protein in brain: metabolic mapping at the cellular level. *Science* 240:1328-1331.
- Shin C, Scialabba FA, McNamara JO (1987) Stimulation of substantia nigra pars reticulata enhances dentate granule cell excitability. *Brain Res* 411:21-27.
- Smeyne RJ, Schilling K, Robertson L, Luk D, Oberdick J, Curran T, Morgan JI (1992) Fos-lacZ transgenic mice: mapping sites of gene induction in the central nervous system. *Neuron* 8:13-23.
- Somjen GG, Aitken PG, Giacchino JL, McNamara JO (1985) Sustained potential shifts and paroxysmal discharges in hippocampal formation. *J Neurophysiol* 53:1079-1097.
- Stringer JL, Williamson JM, Lothman EW (1991) Maximal dentate activation is produced by amygdala stimulation in unanesthetized rats. *Brain Res* 542:336-342.
- White WF, Nadler JV, Hamberger A, Cotman CW, Cummins JT (1977) Glutamate as transmitter of hippocampal perforant path. *Nature* 270:356-357.
- Wong EHF, Kemp JA, Priestley T, Knight AR, Woodruff GN, Iversen LL (1986) The anticonvulsant Mk-801 is a potent *N*-methyl-D-aspartate antagonist. *Proc Natl Acad Sci USA* 83:7104-7108.