

# Immediate Early Gene Expression and Delayed Cell Death in Limbic Areas of the Rat Brain after Kainic Acid Treatment and Recovery in the Cold

Sharon Goodenough, Mark Davidson, Wenbin Chen, Alison Beckmann, Zac Pujic, Manabu Otsuki,<sup>1</sup> Izuru Matsumoto,<sup>2</sup> and Peter Wilce

*Alcohol Research Unit, Department of Biochemistry, The University of Queensland, St Lucia, Queensland, 4072, Australia*

**Systemic injection of kainic acid (KA) results in characteristic behaviors and programmed cell death in some regions of the rat brain. We used KA followed by recovery at 4°C to restrict damage to limbic structures and compared patterns of immediate early gene (IEG) expression and associated DNA binding activity in these damaged areas with that in spared brain regions. Male Wistar rats were injected with KA (12 mg/kg, ip) and kept at 4°C for 5 h. This treatment reduced the severity of behaviors and restricted damage (observed by Nissl staining) to the CA1 and CA3 regions of the hippocampus and an area including the entorhinal cortex. DNA laddering, characteristic of apoptosis, was first evident in the hippocampus and the entorhinal cortex 18 and 22 h after KA, respectively. The pattern of IEG mRNA induction fell into three classes: IEGs that were induced in both damaged and spared areas (*c-fos*, *fos B*, *jun B*, and *egr-1*), IEGs that were induced specifically in the damaged areas (*fra-2* and *c-jun*), and an IEG that was significantly induced by saline injection and/or the cold treatment (*jun D*). The pattern of immunoreactivity closely followed that of mRNA expression. Binding to the AP-1 and EGR DNA consensus sequences increased in all three regions studied. This study describes a unique modification of the animal model of KA-induced neurotoxicity which may prove a useful tool for dissecting the molecular cascade that ultimately results in programmed cell death.** © 1997 Academic Press

## INTRODUCTION

It is now widely recognized that programmed cell death (PCD) plays an important part in the modeling of

the developing nervous system (1, 34). Recently, PCD has been associated with several brain pathologies in the adult, including ischemia, neurodegenerative diseases, and epilepsy (12). Further, it has been suggested that excitotoxicity via activation of the ligand-gated ion channel type of glutamate receptors could play an important role in these disorders (16). These glutamate receptors have been classified as the *N*-methyl-D-aspartate (NMDA) and non-NMDA types. Kainic acid (KA), a cyclic mimetic of glutamate, is known to depolarize both pre- and postsynaptic cells by interaction with the non-NMDA type of receptor. Systemic administration of KA causes prolonged limbic seizures and irreversible neuronal cell damage in specific brain regions hours to days later (48). The KA-induced delayed neuronal cell death occurs, at least in part, via PCD (46).

The molecular cascade leading to PCD has been partially elucidated. Cell surface events activate second messenger systems, particularly calcium flux, which ultimately lead to increased activity of specific endonucleases and digestion of chromosomal DNA into fragments with sizes based on a 180-bp repeat. This intrachromosomal DNA cleavage is thought to be a hallmark of apoptosis, a type of PCD characterized by this DNA fragmentation and other distinct morphological changes (22). Oligosomal DNA cleavage has been demonstrated after KA injection in various brain regions using *in situ* end labeling and gel electrophoresis of extracted DNA (14, 36).

Of considerable interest is the link between cell surface events, the induction of protein synthesis, and DNA cleavage. In the central nervous system, basal expression of the immediate early genes (IEG)s coding for inducible transcription factors is low, but transcription is rapidly and transiently induced through the activation of a number of second messengers (23, 33). The DNA binding proteins encoded by these genes can thus serve as third messengers that dictate the response of the cell. Members of the Fos and Jun families of leucine zipper proteins form hetero- or homodimeric

<sup>1</sup> Current addresses: Department of Anaesthesiology, Fukushima Prefectural Medical School, 1 Hikarigoaka, Fukushima, 960-12, Japan.

<sup>2</sup> Current address: Department of Neuropsychiatry, Fukushima Prefectural Medical School, 1 Hikarigoaka, Fukushima, 960-12 Japan.

complexes that interact with the AP-1 consensus DNA binding sequence. The Egr family are zinc finger proteins which bind to a GC-rich DNA sequence. Expression of both gene families after a variety of stimuli including KA have been reported (12) and in several experimental systems an association with the molecular cascade leading to apoptosis has been suggested (16). However, their precise role in apoptosis remains equivocal.

The majority of studies on KA-induced neurotoxicity have used systemic administration to induce damage in many brain regions. KA-induced behaviors are temperature-dependent (2, 31) and in a previous study, we showed that postinjection recovery in the cold markedly reduces seizures and restricts KA-induced damage to the CA subfields of the hippocampus (HP) and the entorhinal cortex (ECTX) area, including the amygdaloid complex and the piriform cortex (PCTX) (31). We proposed that this model could prove a useful tool for dissecting the molecular mechanisms in the apoptotic cascade and delineate them from those changes more associated with excitation per se (31). We have now further characterized the KA-induced morphological changes and compared the induction of IEG expression preceding the onset of DNA fragmentation in damaged brain areas with that in an adjacent spared area.

## EXPERIMENTAL PROCEDURES

*Animal treatment and histological assessment of brain damage.* Male Wistar rats (300–350 g) were obtained from the Central Animal Breeding House, The University of Queensland. The animals were injected with KA [12 mg/kg intraperitoneally (ip), Sigma] and kept at 4°C for 5 h. One or 3 days after injection, animals were anesthetized with halothane before transcardial perfusion with 100 ml of 10 mM phosphate-buffered saline (PBS) followed by 100 ml of 4% (w/v) paraformaldehyde in PBS (PFA). The brain was removed, postfixed in PFA overnight, cryoprotected in 30% (w/v) sucrose in PBS for 3 days, embedded in OCT compound (Miles Inc.), and frozen. Forty-micrometer coronal sections were cut and stained for Nissl substance with thionin (10).

*DNA fragmentation assay.* Rats were treated with KA as described above and killed up to 24 h after injection. The HP, ECTX, and sensory cortex (SCTX) were frozen in liquid nitrogen. The DNA was extracted using a modification of the procedure previously described (20). Briefly, the tissues were homogenized in 10 vol of TNE buffer (10 mM Tris, 100 mM NaCl, 25 mM EDTA). Before incubation overnight at 50°C, 0.5 vol of 10% (w/v) SDS and 0.1 vol of 10 mg/ml proteinase K were added. After addition of 0.5 vol of 1 mg/ml heat-treated RNase and incubation for 1 h at 37°C, the DNA was purified by phenol/chloroform extraction fol-

lowed by ethanol precipitation. The resulting pellet was resuspended in 0.5 vol of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). The concentration of DNA was determined spectrophotometrically at 260 nm.

DNA (1 µg) was labeled by incubation for 15 min at 37°C in 10 mM Tris (pH 7.5) containing 5 mM MgCl<sub>2</sub>, 0.5 µCi of [ $\alpha$ -<sup>32</sup>P]dCTP (Bresatec, Australia), and 5 U of Klenow polymerase (Bresatec, Australia) (39). The reaction was terminated by the addition of EDTA (final concentration 10 mM) and the unincorporated nucleotides were removed by chromatography on a Sephadex G-50 column (Pharmacia) followed by ethanol precipitation. The DNA pellet was resuspended in TE buffer and loaded on a 2.3% (w/v) agarose gel. The gel was electrophoresed for 2 h at 50 V, dried for 4 h at 60°C, and exposed to Kodak X-OMAT X-ray film. DNA from apoptotic B thymocytes showing a positive DNA ladder with ethidium bromide-stained DNA (supplied by Dr. K. Arai, Queensland Institute of Medical Research, Herston, QLD) acted as a positive control.

*Northern hybridization.* Extraction of RNA and Northern hybridization were performed essentially as described elsewhere (30). Briefly, total RNA was extracted from frozen tissue using the guanidine isothiocyanate/phenol method (8). RNA was fractionated by electrophoresis in denaturing 1% (w/v) agarose-formaldehyde gels (41), transferred to Hybond N nylon membranes (Amersham) by capillary transfer using 10× salt/sodium citrate buffer (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), and fixed by baking at 80°C for 2 h and irradiation with ultraviolet light (125 mJ/cm<sup>2</sup>). cDNA probes were obtained from the following sources: *c-fos*, T. Curran, Roche Centre, U.S.A.; *fos* B, R. Bravo, Princeton University, U.S.A.; *fra-2*, D. Cohen, ANU, Australia; *c-jun*, I. M. Verma, Salk Institute, U.S.A.; *jun* B, ATTC; *jun* D, ATTC; *egr-1*, D. Nathans, Johns Hopkins University School of Medicine, U.S.A.; and glyceraldehyde phosphate dehydrogenase (GAPDH), ATTC. The membrane was prehybridized for 20 min in Rapid Hybridisation Solution (Amersham) at 65°C before addition of <sup>32</sup>P-labeled cDNA probes and hybridization for 2 h at 65°C. Membranes were washed to a final stringency of 0.2× SSPE (1× SSPE is 0.15 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub> · H<sub>2</sub>O, 1 mM EDTA), 0.1% (w/v) SDS at 65°C and exposed to Kodak X-OMAT X-ray film for between 6 h and 5 days at -70°C. After exposure, the membranes were stripped by washing in 1% (w/v) SDS in water at 100°C and the consistency of loading and transfer was determined by the hybridization of a [<sup>32</sup>P]GAPDH cDNA probe. Autoradiographs were analyzed using an imaging densitometer (Bio-Rad Model GS-670). After adjusting the signal for variation in the level of GAPDH mRNA, results were expressed relative to appropriate mRNA at 0 h. Statistical significance was assessed using a one-way analysis of variance (ANOVA) followed by the Student–Newman–Keul's mul-

tiple comparison test. For post hoc comparisons *P* values <0.05 were considered significant.

**Immunohistochemistry.** Immunohistochemistry was performed as described previously (30). Samples were processed as free-floating sections (40  $\mu$ m). After washing in PBST (PBS containing 0.2% (w/v) Triton X-100), sections were incubated in 50% (v/v) ethanol/0.9% (v/v) hydrogen peroxide for 30 min and then in 3% (w/v) bovine serum albumin (BSA) in PBS for 1 h before addition of primary antibodies for 48 h at 4°C. Monoclonal antibodies to c-Fos, Fos B, Jun B, and Jun D (Santa Cruz) and Egr-1 and c-Jun (30) were used at dilutions in 1% (w/v) BSA/PBS of 1:500 and 1:35,000 respectively. After washing in PBST, immunoreactivity was detected with a biotinylated secondary antibody and an avidin-horseradish peroxidase complex (ABC, Vector Laboratories) and visualized with 0.02% (w/v) diaminobenzidine, 0.1% (w/v) hydrogen peroxide, 0.01% (w/v) cobalt chloride, and 0.01% (w/v) nickel ammonium sulfate in PBS. The reaction was terminated by dilution with water and the sections were mounted on microscope slides, cleared with xylene, and coverslipped. Immunoreactivity (IR) in the various brain regions was assessed using the criteria previously described (11).

**Electrophoretic mobility shift assay (EMSA).** After KA injection, the rats were decapitated, and brains were dissected and snap frozen in liquid nitrogen. Nuclear proteins were extracted by the method of Beckmann *et al.* (3). Briefly, brain tissue was homogenized in 5–10 vol of homogenization buffer [100 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.7% Nonidet P-40, 0.5 mM DTT, 10% (w/v) glycerol, 10  $\mu$ g/ml leupeptin, 5  $\mu$ g/ml aprotinin, 0.5 mM PMSF in 20 mM Hepes, pH 7.9] and centrifuged for 10 min at 2000g. The pellet was washed in a further 10 vol of homogenization buffer and centrifugation repeated. The pellet was incubated for 30 min at 4°C on a rocker in 2 vol of high-salt buffer [500 mM KCl, 0.5 mM EDTA, 25% (w/v) glycerol, 0.5 mM DTT, 0.5 mM PMSF in 20 mM Hepes, pH 7.9]. Nuclear debris was removed by centrifugation at 14,000g for 30 min at 4°C and the supernatant stored in aliquots at –70°C.

Double-stranded oligonucleotides containing either the consensus binding motif for Egr (7) or the AP-1 consensus binding motif were synthesized at the Centre for Molecular and Cellular Biology of The University of Queensland or obtained from Promega (Australia). After end labeling with [<sup>32</sup>P]dATP (41), the products were separated on a 20% native polyacrylamide gel in TBE (89 mM Tris–borate, 2 mM EDTA) and the uppermost band was excised, eluted into 330 mM sodium acetate (pH 7.9), and purified by phenol/chloroform extraction. Following ethanol precipitation, the probe was resuspended in 1 mM EDTA in 10 mM Tris (pH 7.4). For binding reactions, 10  $\mu$ g of nuclear protein was preincubated with 1  $\mu$ g poly(dI:dC) (Boehringer Mann-

heim) for 10 min at 25°C in either 100 mM KCl, 5 mM MgCl<sub>2</sub>, 5 mM EDTA, 1 mM DTT, 5% (w/v) glycerol in 10 mM Tris (pH 7.5) for AP-1 or 100 mM KCl, 0.2 mM ZnCl<sub>2</sub>, 3 mM DTT, 3 mg/ml BSA, 5% (w/v) glycerol in 10 mM Tris (pH 7.5) for Egr before incubation for a further 20 min at 25°C in the presence of labeled DNA. DNA–protein complexes were resolved by 4% (AP-1) or 6% (Egr) nondenaturing polyacrylamide gel electrophoresis in TBE.

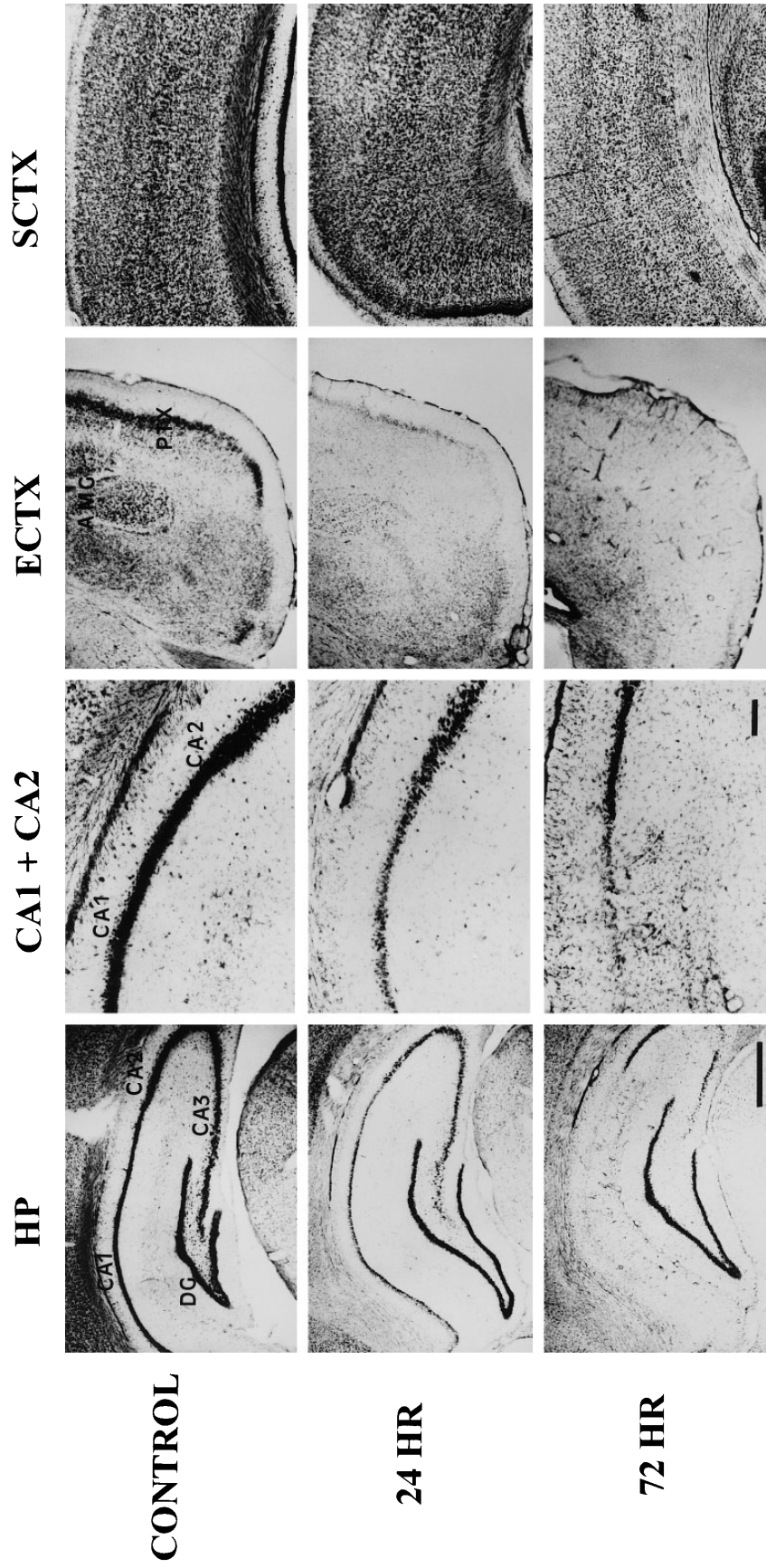
## RESULTS

**Animal behaviors.** In a previous report (31), we noted that animals recovered at 4°C for 5 h after 12 mg/kg KA had increased survival and displayed a markedly reduced intensity of behavior compared to animals kept at room temperature. At 4°C there was a phase of motionless staring initially, which was closely followed by a series of recurrent wet dog shakes. After the first hour, hypersalivation occurred with concurrent forepaw clonus, rearing, and occasional falling for a further 4 h. No myoclonic jerks, wild running seizures, tonic–clonic convulsions, or mortality were observed in any animals.

**Neuronal cell damage and DNA fragmentation after kainic acid injection.** Nissl-stained sections of brain regions from KA-treated rats are represented in Fig. 1. Systemic KA injection produced severe cell loss in the CA1 and CA3 regions of the HP and the ECTX region, which includes the PCTX and amygdala. Cell loss was detected 24 h after KA administration and was extensive at 72 h. No damage to cells within the SCTX was detected at any time. DNA fragmentation with a characteristic oligosomal repeat pattern was evident in the HP 18, 20, and 22 h after KA (Fig. 2). No laddering was observed at 0 h or in saline-treated animals sampled at similar times (data not shown). There was a distinct difference in the time of onset of fragmentation in the ECTX when compared to that in the HP (Fig. 2). Onset of fragmentation occurred sharply between 20 and 22 h after KA, suggesting that the timing of the apoptotic process may differ between brain regions. Fragmentation of DNA from the SCTX was not seen at any time of sample even after overexposure of the X-ray film.

**Induction of IEG expression after KA.** Although the induction of IEGs in response to KA administration has been extensively studied (19, 20, 29), the role of the IEG protein products in PCD remains equivocal. We therefore compared the induction of IEG expression in the two affected area with that in the undamaged SCTX. Figure 3 shows representative Northern blot analyses. Figures 4 and 5 are graphical representations of this IEG mRNA expression after KA administration adjusted for changes in GAPDH mRNA.

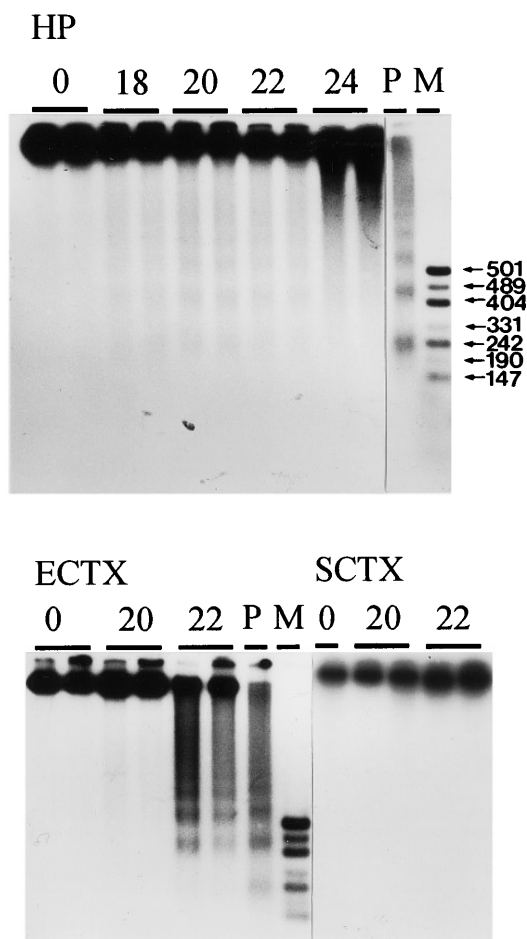
Essentially, the pattern of expression of the IEG fell into three classes: IEGs that were induced in both



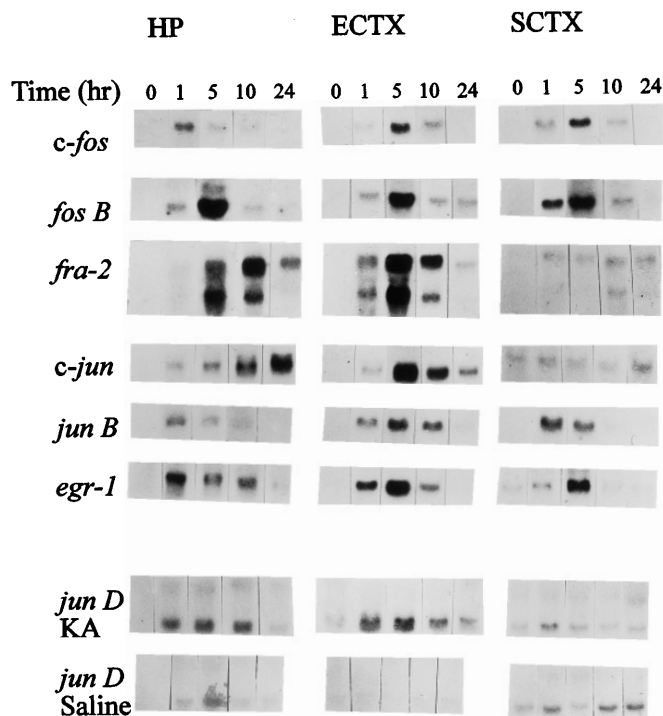
**FIG. 1.** Localized damage induced by ip injection of 12 mg/kg kainic acid in the hippocampus (HP), the entorhinal cortex (ECTX), and the sensory cortex (SCTX). Sections were stained using thionin. Bar, 200  $\mu$ m.

damaged and spared areas, those IEGs that were selectively induced in areas of damage, and an IEG that was significantly induced by saline injection and/or the cold treatment.

In all three brain regions studied, the expression of *c-fos* and *fos B* increased transiently, peaking around 5 h after KA injection and returning to basal levels by 24 h. No change in expression of these IEGs was detected in response to saline injection (data not included). In contrast, the levels of *fra-2* and *c-jun* mRNA did not change in the SCTX after KA injection. The expression of these IEGs appeared to increase only in the brain regions that subsequently exhibited DNA fragmentation. In the ECTX, levels of *fra-2* and *c-jun* mRNA were maximal at 5 h and remained elevated at 24 h. In the HP the pattern of induction of these two genes was essentially similar, being maximal at 10 h and remaining significantly enhanced at 24 h. Expression of *fra-2*



**FIG. 2.** Fragmentation of DNA extracted from the hippocampus (HP), entorhinal cortex (ECTX), and the sensory cortex (SCTX) after kainic acid injection (12 mg/kg, ip). After extraction the DNA was end labeled with [ $^{32}$ P]dCTP using a Klenow fragment, displayed on agarose gels, and autoradiographed. P, positive control. M, molecular weight marker.



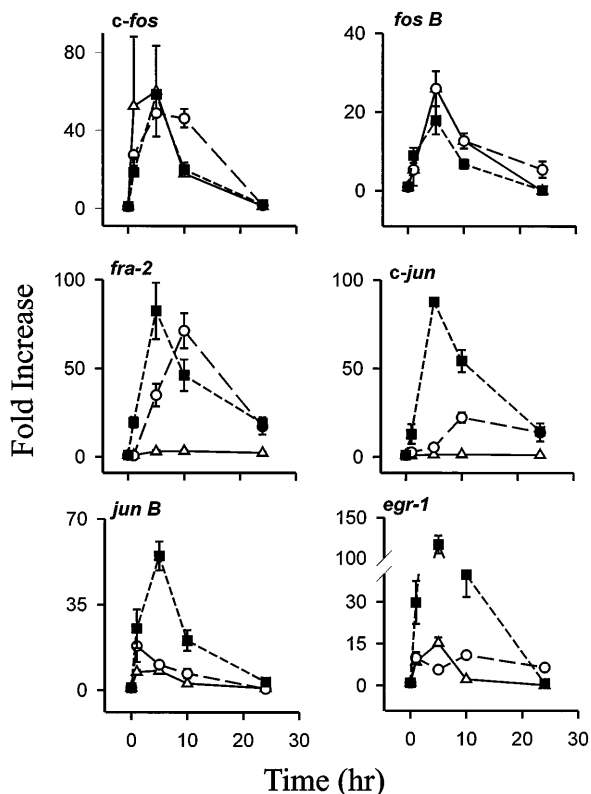
**FIG. 3.** Immediate early gene expression in the hippocampus (HP), entorhinal cortex area (ECTX), and sensory cortex (SCTX) after kainic acid treatment detected by Northern hybridization. Bands are representative at each time point.

and *c-jun* was not changed in response to saline injection (data not included).

An increase in *jun B* and *egr-1* expression was observed in all three brain regions after KA injection. KA treatment stimulated an early transient increase in mRNA in the SCTX and the HP. In contrast, there was a vigorous increase in the level of both *jun B* and *egr-1* mRNA, in the ECTX. Saline injection did not stimulate expression of these IEGs (data not included).

The expression of *jun D* after KA and saline administration is shown in Fig. 5. The basal level of *jun D* mRNA in the SCTX was relatively high compared to that of the other IEGs but no induction above this level was observed after either saline or KA treatment. Increased mRNA was evident in the HP 1 h after both KA and saline administration. The induction observed after saline injection was transient, returning to basal levels by 24 h, whereas KA administration caused a protracted pattern of gene expression that remained elevated at 24 h. A significant difference in *jun D* mRNA levels between the two treatments was only seen 5 h after injection, with gene expression increasing sixfold after KA injection and twofold after saline. Induction of *jun D* in the ECTX only occurred following KA treatment which resulted in elevated mRNA levels at 1 to 24 h after injection.

To determine if the changes in IEG mRNA expression



**FIG. 4.** Relative IEG expression in the HP (open circle), ECTX (solid square), and SCTX (open triangle) after quantitation using image densitometry. Results are expressed as fold increase of basal expression adjusted for expression of GAPDH mRNA content (mean  $\pm$  SEM from four animals).

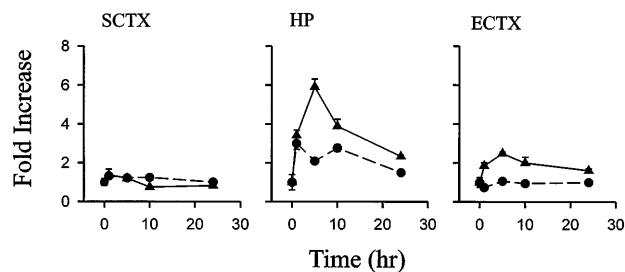
were mimicked by equivalent changes in protein expression, immunohistochemistry was performed. Figure 6 shows representative IR of proteins 5 h after KA treatment and Table 1 summarizes IR at various time points after KA administration. At 3 h after injection c-Fos IR and Fos B IR were induced in all areas studied. The level of c-Fos IR approached basal at 13 h in all hippocampal regions but remained elevated in levels II to IV of the SCTX and in the PCTX and ECTX. Weak staining was detected in these regions at 24 h. Similarly, Fos B IR was maximal 5 h after injection. Weak IR was still observed at 24 h in the CA1, CA3, and DG subfields of the HP and in levels II to IV of the SCTX, while stronger staining was detected in the PCTX and ECTX. Interestingly, an unusual pattern of Fos B staining could be observed in the molecular layers of the DG of the HP at 5 h but not at 24 h. This staining appears to be cytoplasmic and is similar to the results of Gass *et al.* (15). C-Jun IR was strongly induced in all subfields of the HP, being elevated above baseline by 3 h and remaining elevated 24 h after injection in the damaged subfields. A similar pattern was evident in the ECTX. Weak induction occurred in levels II to IV of the SCTX, albeit above a high basal level of expression.

After KA treatment, Egr-1 IR was evident in all hippocampal regions and in the ECTX. The outer layers of the SCTX were also particularly immunoreactive. We obtained weak IR with the Jun B antibody. Jun D IR was also weak but distinct increases in IR were evident in the DG and CA2 regions of the HP. IR was also present in both the ECTX and layers II and III of the SCTX.

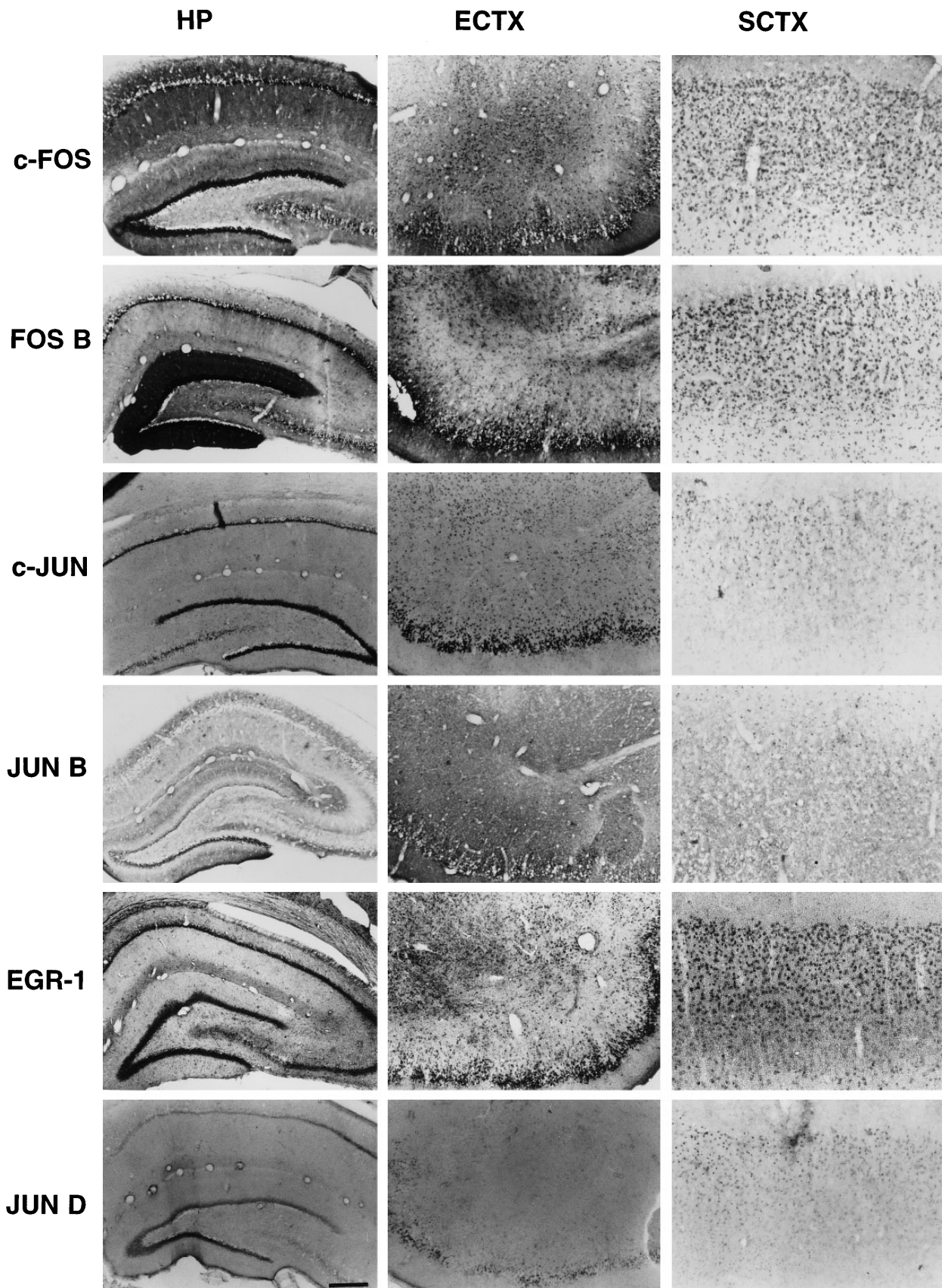
**DNA binding activities after KA treatment.** DNA binding to the AP-1 and Egr consensus oligonucleotides after KA injection is shown in Fig. 7. The sequence specificity of these activities has been shown previously (4). There was a marked increase in AP-1 DNA binding activity after 3 h, which persisted to 18 h in the SCTX, the HP, and the ECTX. Saline injection did not change AP-1 DNA binding activity. Egr DNA binding activity was resolved into a prominent slow-migrating band (indicated by the arrow in Fig. 7) shown by supershift assays to be Egr-1 (4) and a faster migrating, weaker, sequence-specific but unidentified activity (lower band, Fig. 7). A transient elevation of binding activity of Egr-1 was evident at 3 h in all three regions. This increase was more marked in the HP and ECTX compared to that in the SCTX. No change was observed in the faster migrating complex after treatment. There was no change in this activity after saline. There was, however, a modest increase in Egr-1 DNA binding activity after saline injection at 3 h in the ECTX but not in the other regions.

## DISCUSSION

Several studies have reported that hypothermia is effective in reducing behaviors, EEG pattern, brain damage, and mortality after KA treatment (2, 27). The protective mechanisms of the cold treatment have not been well characterized. Brain hypothermia markedly inhibits the release of glutamate and other neurotransmitters, delays the decrease in neuronal ATP levels, and reduces calcium accumulation after ischemia (6).



**FIG. 5.** Relative *jun D* mRNA expression in the HP, ECTX, and SCTX after KA injection (triangles) or saline injection (circles). Autoradiographs were quantitated using image densitometry. Results are expressed as fold increase of basal expression adjusted for expression of GAPDH mRNA content (mean  $\pm$  SEM from four animals).



**FIG. 6.** Photomicrographs of coronal sections showing the hippocampus (HP), entorhinal cortex (ECTX), and sensory cortex (SCTX) stained using immunohistochemistry. Sections are stained using antibodies directed against the specific IEGs and are taken 5 h after kainic acid administration. Bar, 400  $\mu$ m.

TABLE 1

Representation of the Relative IEG Protein Expression in Each of the Brain Regions Studied at Time Points after Kainic Acid Injection

Hours	Hippocampus				Cortex		
	CA1	CA2	CA3	DG	SCTX	PCTX	ECTX
<b>c-FOS</b>							
0	-	-	-	-	-	-	-
3	++	++	++	++	++	++	++
5	++	++	++	++	+++	+++	+++
13	+	+	+	+	+++	+++	+++
24	+	+	+	-	+	+	+
<b>FOS B</b>							
0	-	-	-	-	-	-	-
3	++	++	++	++	++	+++	++
5	+++	+++	+++	+++	+++	+++	+++
13	++	+	+	++	++	++	+++
24	+	-	+	+	+	++	++
<b>c-JUN</b>							
0	-	-	-	-	-	-	-
3	++	++	+++	+++	++	+++	++++
5	+++	+++	++++	++++	++++	+++	++++
13	++	++	++	++	+	+++	+++
24	+	+	+	+	-	++	++
<b>JUN B</b>							
0	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-
5	+	+	+	+	++	+	+
13	-	-	-	-	-	+	+
24	-	-	-	-	-	-	-
<b>EGR-1</b>							
0	-	-	-	-	-	-	-
3	++	+	+	+	+	++	+++
5	+++	+++	+++	+++	++	+++	+++
13	+	+	+	+	+	++	++
24	-	-	-	-	-	-	-
<b>JUN D</b>							
0	-	-	-	-	-	-	-
3	+	+	+	-	+	-	-
5	++	++	+	+	+	++	++
13	-	-	-	-	+	+	+
24	-	-	-	-	-	-	-

Note. Staining is graded as none (-), weak (+), moderate (++), strong (+++), and very strong (++++).

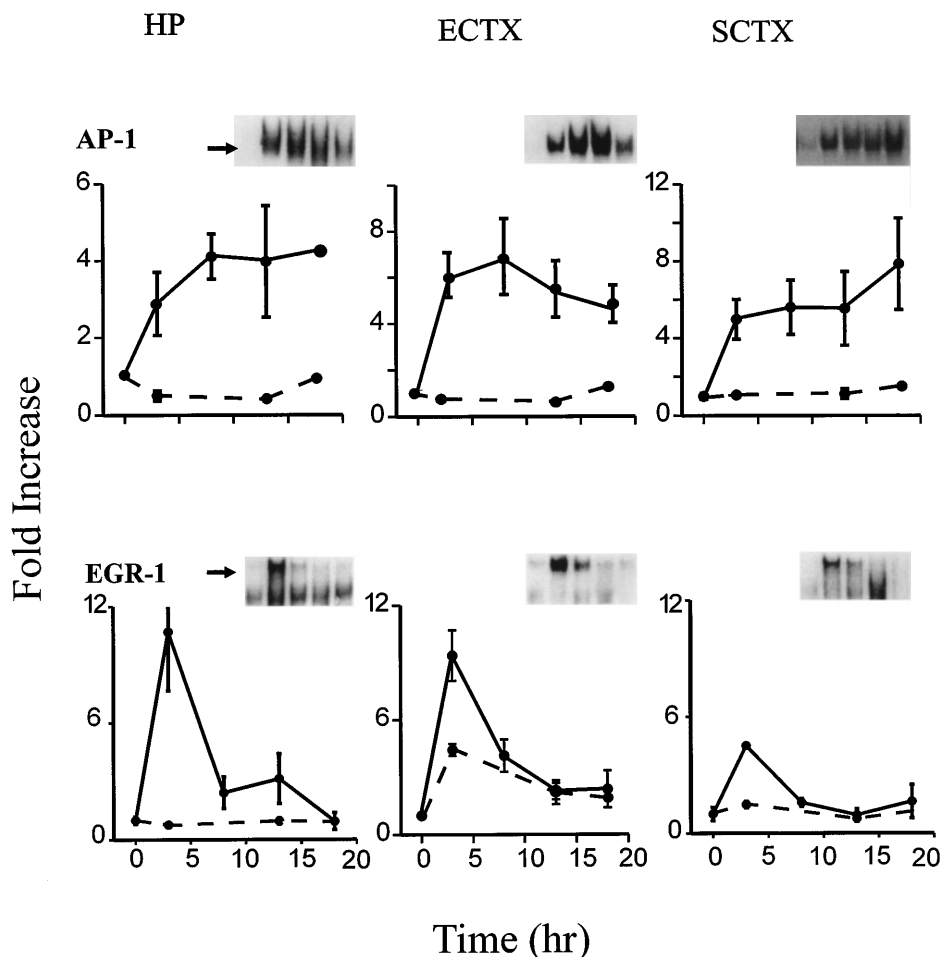
The latter action has been suggested as a mechanism by which hypothermia diminishes neuronal injury (32). A similar mechanism may account for the neuroprotective effect of cold treatment in the current study. Alternatively, the stress of hypothermia could influence IEG expression. Several studies have demonstrated that glucocorticoids potentiate and adrenalectomy reduces KA-induced seizures and neurodegeneration (25, 43). Glucocorticoids also potentiate age-related neurodegeneration (24, 42). In contrast, an inhibitory action of glucocorticoids on IEG expression has been demonstrated in rat brain *in vivo* after treatments including KA (26). Further, glucocorticoids attenuate KA-induced AP-1 DNA binding activity but this may ultimately

enhance neurotoxicity by reducing protective and adaptive functions of the AP-1 complex (49). During cold recovery, the stress hormone levels may increase but predictably their effect would be to enhance rather than inhibit KA-induced cell damage. Observation of damage in the three areas after KA injection revealed selective cell loss similar to that seen after KA treatment followed by recovery at room temperature (20). We conclude that the selective sensitivity of the various regions of the limbic structures to KA is unaffected by the cold treatment but the action of KA on the SCTX is markedly inhibited by some as yet uncharacterized mechanism.

Coupled with the cell loss in the HP and ECTX was the appearance of a characteristic oligosomal ladder-like pattern of DNA degradation. Such a pattern is thought to arise from fragmentation of chromatin by specific endonucleases and is believed to be diagnostic of PCD. In this study, the ladder pattern was not seen in the SCTX but has been observed in this region by other researchers after injection of a similar dose of KA followed by recovery at room temperature (14, 20). Our data suggest that the DNA fragmentation pattern seen in the SCTX by the other investigators may be closely associated with the intense prolonged seizure activity characteristic of recovery at room temperature from KA treatment. These behaviors were dramatically reduced in animals injected with KA and placed at 4°C for 5 h.

There has been considerable debate on the association between expression of the various IEGs and the apoptotic cascade. Proteins of the Fos and Jun families associate to form a complex that recognizes the AP-1 DNA consensus sequence and may influence the architecture of the surrounding DNA (33). Variation in the protein composition of the complex may ultimately determine the fate of a stimulated cell either for adaptation and repair or to PCD by altering the binding of other transcription factors. Therefore, it is important to consider not only the overall binding activity but also the expression of the genes coding for each of the possible components. Although binding activity to the AP-1 consensus sequence was elevated in all three regions studied with an essentially similar time course, the complex may represent a different combination of proteins.

Comparison of the IEGs expressed in the damaged and spared regions in the cold recovery paradigm indicates some clear differences. Only in the damaged areas was a distinct change in *c-jun* and *fra-2* mRNA expression detected along with strong and persistent c-Jun IR. In human leukemic cells, but not in mammary and cerebellar cells, induction of *c-jun* expression has been associated with the onset of apoptosis (9, 28, 40). In neuronal cells, suppression of c-Jun expression by antisense oligonucleotides or antibodies provides protection from apoptotic stimuli and expression of



**FIG. 7.** EMSAs showing the effect of KA treatment on the DNA binding activities of the transcription factors AP-1 and EGR-1. Autoradiographs were quantitated using image densitometry. Graphs show average binding activity after KA (solid line) or saline (broken line). Sequence-specific binding of Egr-1 is indicated by the arrow. Data are means  $\pm$  SEM from four animals. Inserts are representative of each time point.

negative mutant *c-jun* protects sympathetic neurons against NGF withdrawal-induced death (13, 18, 44). It is the persistent expression of *c-jun* that appears to be closely associated with subsequent PCD in a number of systems (12). Our data add further support to the concept of *c-Jun* expression as a critical step in PCD. We were unable to obtain Fra-2 IR with the antibodies available, but the selective increase in *fra-2* mRNA would also suggest an important role for this IEG product in damaged tissue. Pennypacker and colleagues have focused on Fras and have reported prolonged expression after KA in the hippocampus, although induction is particularly associated with adaptations in the surviving cells rather than preceding cell death (35).

Expression of *c-fos* is associated with PCD of cortical neurons during development and of olfactory neuroepithelia (17). Neurons vulnerable to various insults including KA injection have protracted expression of *c-fos* (37,

45, 46). Further, *c-fos* expression is transient after pentylenetetrazol-induced seizure activity which does not result in selective neuronal cell death (21). In our experiments, expression of *c-fos* mRNA was transient and Fos-IR was also not markedly elevated above basal levels in any regions at later times. Previous studies have noted an early rise in *c-fos* mRNA and *c-Fos* protein as a component of the AP-1 complex after various stimuli subsequently being replaced by other proteins eg FRA (20, 35, 47). Our data would support a possible role of *c-fos* in the apoptotic cascade at early times but not a selective expression in damaged tissue at later times.

A widespread induction of *jun B* and *fos B* mRNA has been previously reported in KA-treated animals recovered at room temperature (20). Prolonged expression of Fos B IR has also been reported after similar treatment (15). The prolonged nature of protein expression has been linked to long-term responses associated with

tissue regeneration. In our system, expression of both mRNAs was transient; however, Fos B IR remained high in the ECTX at later times. This may reflect the important role suggested for Fos B in the AP-1 complex at later times (20) in this tissue.

The induction of *egr-1* mRNA after KA administration has not previously been reported. However, protracted patterns of *egr-1* expression have been shown to occur in PC12 cells and sympathetic neurons undergoing nerve growth factor withdrawal-induced cell death (13, 18). Although, in the current study, expression of *egr-1* occurred in all regions, it was particularly strong in the ECTX. Egr DNA binding activity was also transiently induced in all regions but strongly in both the HP and ECTX. There is an apparent discrepancy in the magnitude of mRNA induction and the increase in Egr-1 DNA binding activity in the ECTX. The DNA binding properties of the Egr-1 protein are known to be influenced by several posttranslational mechanisms, including phosphorylation and oxidation state (5). We have noted distinct changes in oxidation state of several transcription factors in the ECTX after KA treatment (unpublished observations). A similar effect may have an important role in controlling the DNA interaction of the Egr-1 protein in the ECTX after KA.

A significant increase in *jun D* mRNA was observed in the HP up to 10 h after saline administration. It is possible that this was a result of the stress induced by saline and/or cold treatment. Ryabinin *et al.* (38) have shown increased *jun D* mRNA in the HP of restraint-stressed animals. The protracted expression of Jun D IR evident in the ECTX is consistent with a previous report that this protein is a component of the AP-1 complex at later times after KA treatment (20).

#### ACKNOWLEDGMENTS

This work was supported by the National Health and Medical Research Council of Australia in the form of a project grant. I.M. is a recipient of National Health and Medical Research Council Postdoctoral Fellowship.

#### REFERENCES

- Altman, J. 1992. Programmed cell death: The paths to suicide. *Trends Neurosci.* **15**: 278–280.
- Balchen, T., M. Berg, and N. H. Diemer. 1993. A paradox after systemic kainate injection in rats: lesser damage of hippocampal CA1 neurons after higher doses. *Neurosci. Lett.* **163**: 151–154.
- Beckmann, A. M., I. Matsumoto, and P. A. Wilce. 1994. Elevated Ap-1 DNA-binding activity in rat brain during ethanol withdrawal. *Alcohol Alcohol Suppl.* **2**: 225–229.
- Beckmann, A. M., I. Matsumoto, and P. A. Wilce. 1996. Immediate early gene-encoded transcription factors in rat brain during ethanol withdrawal. *Alcohol Clin Exp. Res.* **20**: 594.
- Beckmann, A. M., and P. A. Wilce. 1996. Egr transcription factors in the nervous system. *Neurochem. Int.*, in press.
- Busto, R., W. D. Dietrich, M. Y. Globus, and M. D. Ginsberg. 1989. Postischemic moderate hypothermia inhibits CA1 hippocampal ischemic neuronal injury. *Neurosci. Lett.* **101**: 299–304.
- Christy, B., and D. Nathans. 1989. DNA binding of the growth factor-inducible protein Zif268. *Proc. Natl. Acad. Sci. USA* **86**: 8737–8741.
- Chomczynski, P., and N. Sacchi. 1987. Single-step methods of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**: 156–159.
- Copani, A., V. Bruno, P. Dell'Albani, G. Battaglia, V. Barresi, A. Caruso, F. Nicoletti, and D. F. Condorelli. 1995. Growth conditions differentially affect the constitutive expression of primary response genes in cultured cerebellar granule cells. *Neurochem. Res.* **20**: 611–616.
- Davidson, M., P. A. Wilce, and B. C. Shanley. 1995. Increased NMDA-induced excitability during ethanol withdrawal: A behavioural and histological study. *Brain Res.* **674**: 91–96.
- Davidson, M., I. Matsumoto, B. C. Shanley, and P. A. Wilce. 1996. Fos and Jun as markers for ethanol-sensitive pathways in the rat brain. *Brain Res. Bull.* **39**: 177–184.
- Dragunow, M., and K. Preston. 1995. The role of inducible transcription factors in apoptotic nerve cell death. *Brain Res. Rev.* **21**: 1–28.
- Estus, S., W. J. Zaks, R. J. Freeman, M. Gruda, R. Bravo, and E. M. Johnson Jr. 1994. Altered gene expression in neurons during programmed cell death: Identification of c-jun as necessary for neuronal cell death. *J. Cell Biol.* **127**: 1717–1727.
- Filipkowski, R. K., M. Hetman, B. Kaminska, and L. Kaczmarek. 1994. DNA fragmentation in rat brain after intraperitoneal administration of kainate. *Neuroreport* **5**: 1538–1540.
- Gass, P., T. Herdegen, R. Bravo, and M. Kiessling. 1993. Spatiotemporal induction of immediate early genes in the rat brain after limbic seizures: Effects of NMDA receptor antagonist MK-801. *Eur. J. Neurosci.* **5**: 933–943.
- Glass, M., and M. Dragunow. 1995. Neurochemical and morphological changes associated with human epilepsy. *Brain Res. Rev.* **21**: 29–41.
- Gonzalez-Martin, C., I. De Diego, D. Crespo, and A. Fairen. 1992. Transient c-fos expression accompanies naturally occurring cell death in the developing interhemispheric cortex of the rat. *Development* **68**: 83–95.
- Ham, J., C. Babij, J. Whitfield, C. M. Pfarr, D. Lallemand, M. Yaniv, and L. Rubin. 1995. A c-jun dominant negative mutant protects sympathetic neurons against programmed cell death. *Neuron* **14**: 927–939.
- Jørgensen, M. B., F. F. Johansen, and N. H. Diemer. 1991. Post-ischaemic and kainic acid-induced c-fos protein expression in the rat hippocampus. *Acta Neurol. Scand.* **84**: 352–356.
- Kaminska, B., R. K. Filipowski, G. Zurkowska, W. Lason, R. Przewlocki, and L. Kaczmarek. 1994. Dynamic changes in the composition of the AP-1 transcription factor DNA-binding activity in the rat brain following kainate-induced seizures and cell death. *Eur. J. Neurosci.* **6**: 1558–1566.
- Kasof, G. M., A. Mandelzys, S. D. Malka, R. E. Hammer, T. Curran, and J. I. Morgan. 1995. Kainic acid-induced neuronal death is associated with DNA damage and a unique immediate-early gene response in c-fos-lacZ transgenic mice. *J. Neurosci.* **15**: 4238–4249.
- Kerr, J. F. R., A. H. Wyllie, and A. R. Currie. 1972. Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Br. J. Cancer.* **26**: 239.
- Kruijer, W., D. Schubert, and I. M. Verma. 1985. Induction of the protooncogene fos by nerve growth factor. *Proc. Natl. Acad. Sci. USA* **82**: 7330–7334.

24. Landfield, P. W., and J. C. Eldridge. 1994. The glucocorticoid hypothesis of age-related hippocampal neurodegeneration: role of dysregulated intraneuronal calcium. *Ann. N. Y. Acad. Sci.* **746**: 308–326.
25. Lee, P. H., L. Grimes, and J. S. Hong. 1989. Glucocorticoids potentiate kainic acid-induced seizures and wet dog shakes. *Brain Res.* **480**: 322–325.
26. Li, X., L. Song, and R. S. Jope. 1992. Adrenalectomy potentiates immediate early gene expression in rat brain. *J. Neurochem.* **58**: 2330–2333.
27. Liu, Z., A. Gatt, M. Mikati, and G. L. Holmes. 1993. Effect of temperature on kainic acid-induced seizures. *Brain Res.* **631**: 51–58.
28. Marti, A., B. Jehn, E. Costello, N. Keon, G. Ke, F. Martin, and R. Jaggi. 1994. Protein kinase A and AP-1 (c-Fos/Jun D) are induced during apoptosis of mouse mammary epithelial cells. *Oncogene* **9**: 1213–1223.
29. Massamiri, T., M. Khrestchatsky, and Y. Ben-Ari. 1994. Induction of c-fos mRNA expression in an in vitro hippocampal slice model of adult rats after kainate but not  $\gamma$ -aminobutyric acid or bicuculline treatment. *Neurosci. Lett.* **166**: 73–76.
30. Matsumoto, I., J. Leah, B. Shanley, and P. A. Wilce. 1993. Immediate early gene expression in the rat brain during ethanol withdrawal. *Mol. Cell. Neurosci.* **4**: 485–491.
31. Matsumoto, I., M. Davidson, M. Otsuki, and P. A. Wilce. 1996. Decreased severity of ethanol withdrawal behaviours in kainic acid-treated rats. *Pharmacol. Biochem. Behav.* **55**: 371–378.
32. Mitani, A., F. Kadoya, and K. Kataoka. 1991. Temperature dependence of hypoxia-induced calcium accumulation in gerbil hippocampal slices. *Brain Res.* **562**: 159–163.
33. Morgan, J. I., and T. Curran. 1990. Inducible proto-oncogenes of the nervous system: Their contribution to transcription factors and neuroplasticity. *Prog. Brain Res.* **86**: 287–294.
34. Oppenheim, R. W. 1991. Cell death during development of the nervous system. *Annu. Rev. Neurosci.* **14**: 453–501.
35. Pennypacker, K. R., J. S. Hong, and M. K. McMillian. 1995. Implications of prolonged expression of fos-related antigens. *Trends Pharmacol. Sci.* **16**: 317–321.
36. Pollard, H., S. Charriaud-Marlangue, S. Cantagrel, A. Represa, O. Robain, J. Moreau, and Y. Ben-Ari. 1994. Kainate-induced apoptotic cell death in hippocampal neurons. *Neuroscience* **63**: 7–18.
37. Popovici, T., A. Represa, V. Crepel, G. Barbin, M. Beaudoin, and Y. Ben-Ari. 1990. Effects of kainic acid-induced seizures and ischemia on c-fos-like proteins in rat brain. *Brain Res.* **536**: 183–194.
38. Ryabinin, A. E., K. R. Melia, M. Cole, F. E. Bloom, and M. C. Wilson. 1995. Alcohol selectively attenuates stress-induced c-fos expression in the rat hippocampus. *J. Neurosci.* **15**: 721–730.
39. Rösl, F. 1992. A simple and rapid method for detection of apoptosis in human cells. *Nucleic Acids Res.* **20**: 5243.
40. Rubin, E., S. Kharbanda, H. Gunji, and D. Kufe. 1991. Activation of the c-jun protooncogene in human myeloid leukemia cells treated with etoposide. *Mol. Pharmacol.* **39**: 697–701.
41. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
42. Sapolsky, R. M. 1985. Glucocorticoid toxicity in the hippocampus: temporal aspects of neuronal vulnerability. *Brain Res.* **359**: 300–305.
43. Sapolsky, R. M., M. Armanini, D. Packan, and G. Tombaugh. 1987. Stress and glucocorticoids in aging. *Endocrinol. Metab. Clin. N. Am.* **16**: 965–980.
44. Scheliengensiepen, K., F. Wollnik, M. Kunst, R. Schlingensiepen, T. Herdegen, and W. Brysch. 1994. The role of Jun transcription factor expression and phosphorylation in neuronal differentiation, neuronal cell death, and plastic adaptations in vivo. *Cell. Mol. Neurobiol.* **14**: 487–505.
45. Schreiber, S. S., G. Tocco, I. Najm, R. F. Thompson, and M. Baudry. 1993. Cycloheximide prevents kainate-induced neuronal death and c-fos expression in adult rat brain. *J. Mol. Neurosci.* **4**: 149–159.
46. Smeyne, R. J., M. Vendrell, M. Hayward, S. J. Baker, G. G. Miao, K. Schilling, L. M. Robertson, T. Curran, and J. I. Morgan. 1993. Continuous c-fos expression precedes programmed cell death in vivo. *Nature* **363**: 166–169.
47. Sonnenberg, J. L., C. Mitchelmore, P. F. Macgregor-Leon, J. Hempstead, J. I. Morgan, and T. Curran. 1989. Glutamate receptor agonists increase the expression of fos, fra, and AP-1 DNA binding activity in the mammalian brain. *J. Neurosci. Res.* **24**: 72–80.
48. Sperk, G. 1994. Kainic acid seizures in the rat. *Prog. Neurobiol.* **42**: 1–32.
49. Unlap, T., and R. S. Jolpe. 1994. Diurnal variation in kainate-induced AP-1 activation in rat brain: influence of glucocorticoids. *Mol. Brain Res.* **28**: 193–200.