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p53 induction is associated with neuronal damage in the central nervous system

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ABSTRACT **The p53 tumor-suppressor gene encodes a growth-regulatory protein that has been implicated in programmed cell death. To investigate the possible role of p53 in neuronal death, we studied p53 expression associated with excitotoxicity in the adult rat brain. Within hours of systemic administration of the glutamate analogue kainic acid, p53 mRNA levels were increased in neurons exhibiting morphological features of damage within kainate-vulnerable brain regions. A similar distribution was found for neurons exhibiting DNA damage as evidenced by *in situ* end-labeling of fragmented DNA. Pretreatment with the protein synthesis inhibitor cycloheximide prevented both kainate-mediated p53 induction and neuronal damage. The distinctive pattern of excitotoxin-mediated p53 expression suggests that p53 induction is a marker of irreversible injury in postmitotic cells of the central nervous system and could have functional significance in determining selective neuronal vulnerability.**

The p53 tumor-suppressor gene encodes a protein that is important in the regulation of cell proliferation (1). Accordingly, mutations in the p53 gene have been identified in numerous human cancers (2, 3). Wild-type p53 protein exhibits sequence-specific DNA-binding activity (4, 5) and modulates gene transcription (6, 7), suggesting that p53 regulates the expression of other genes involved in the cell cycle. Although the antiproliferative action of wild-type p53 is relatively well established, reports indicating that overexpression of p53 may accompany programmed cell death, or apoptosis, in cultured cells or during tumor regression (8–11) suggest additional functions for the gene product. Though the significance of these other observations remains unclear, it is possible that p53 overexpression in cells that have reached a certain stage of their proliferative potential could be linked with irreversible injury. Further, since wild-type p53 protein can act as a transcription factor, a relationship between p53 and the regulation of genes that promote cell death is also possible. In trying to identify genes involved in neuronal death, we and others (12, 13) have demonstrated a correlation between prolonged increased expression of the protooncogene c-*fos* and selective vulnerability to excitotoxin-mediated neuronal damage in the mammalian central nervous system, suggesting that persistent c-*fos* expression occurs in cells that are destined to die. In cultured cells, c-*fos* induction is commonly associated with proliferative responses and can be modulated by wild-type p53 (14). Taken together, these observations raised the possibility of a functional relationship between p53 and c-*fos* gene expression in the intact central nervous system. We therefore studied p53 expression associated with excitotoxicity to investigate whether p53 could participate in selective neuronal vulnerability. Here we report that kainate-mediated p53 induction occurs in neurons exhibiting morphological signs of damage. In addition, the protein synthesis inhibitor cycloheximide prevents both kainate-mediated p53 induction and neurodegeneration. Thus, p53 induction may be a marker of selective neuronal vulnerability and may possibly play a more direct role in excitotoxic cell death.

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MATERIALS AND METHODS

Animal Treatments and Tissue Preparation. Adult male Sprague–Dawley rats (200–250 g) received the glutamate analogue kainic acid (10 mg/kg of body weight) by subcutaneous injection and were decapitated under methoxyfluorane anesthesia 4, 8, or 16 hr following the onset of kainate-induced seizures. Control animals received either normal isotonic saline or were uninjected. Separate groups of animals received kainate with or without subcutaneous cycloheximide (2 mg/kg) 1 hr prior to kainate, and were killed 16 hr after seizure onset. Untreated animals served as controls. The brain was rapidly removed, immersed in isopentane at −20°C for 5 min, and stored at −70°C prior to sectioning. Cryostat sections (10 μm) were thaw-mounted onto chrome-alum gelatin-coated (300 Bloom, Sigma) slides and stored at −70°C.

RNA Probe Preparation. Sense or antisense p53 cRNA probes were transcribed from an appropriately linearized plasmid containing 0.95 kb of the coding region of mouse wild-type p53, kindly provided by G. Lozano (M.D. Anderson Cancer Center, Houston), corresponding to amino acids 13–330 of the published sequence (G. Lozano, personal communication; ref. 15). Transcription reaction mixtures (10 μl) contained 25 μM uridine 5′-[α-[^{35}S]thio]triphosphate (1300 Ci/mmol, NEN; 1 Ci = 37 GBq), 50–100 ng of linearized template, 40 mM Tris·HCl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 500 μM nonradioactive GTP, CTP, and ATP, 10 mM dithiothreitol, 20–40 units of RNasin (Promega), and 1 μl of T3 or T7 RNA polymerase (Stratagene) and were incubated for 1 hr at 37°C. This was followed by the addition of 16 μl of 40 mM Tris·HCl, pH 8.0/8 mM $MgCl_2$/2 mM spermidine/50 mM NaCl/10 mM dithiothreitol containing 10 units of RNase-free DNase (Promega), with incubation for 15 min at 37°C. Unincorporated nucleotides were removed by ethanol precipitation. The probe mass was estimated from the percent incorporation of labeled nucleotide as measured by acid-precipitable cpm. Specific activities averaged between 1 and 2 × 10^9 cpm/μg.

***In Situ* Hybridization.** Frozen brain sections were fixed in 4% paraformaldehyde/1× phosphate-buffered saline (pH 7.0) for 30 min, washed several times in phosphate-buffered saline, sequentially transferred through 0.1 M triethanolamine (pH 8.0) and 0.1 M triethanolamine/0.25% acetic anhydride, dehydrated through a graded series of ethanol solutions, and air dried. The p53 cRNA probe at 0.2 μg/ml in

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hybridization buffer [50% formamide/4× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0)/0.1% Ficoll/ 0.1% polyvinylpyrrolidone/0.1% bovine serum albumin/1% sodium dodecyl sulfate/10% dextran sulfate/0.1 M dithio-

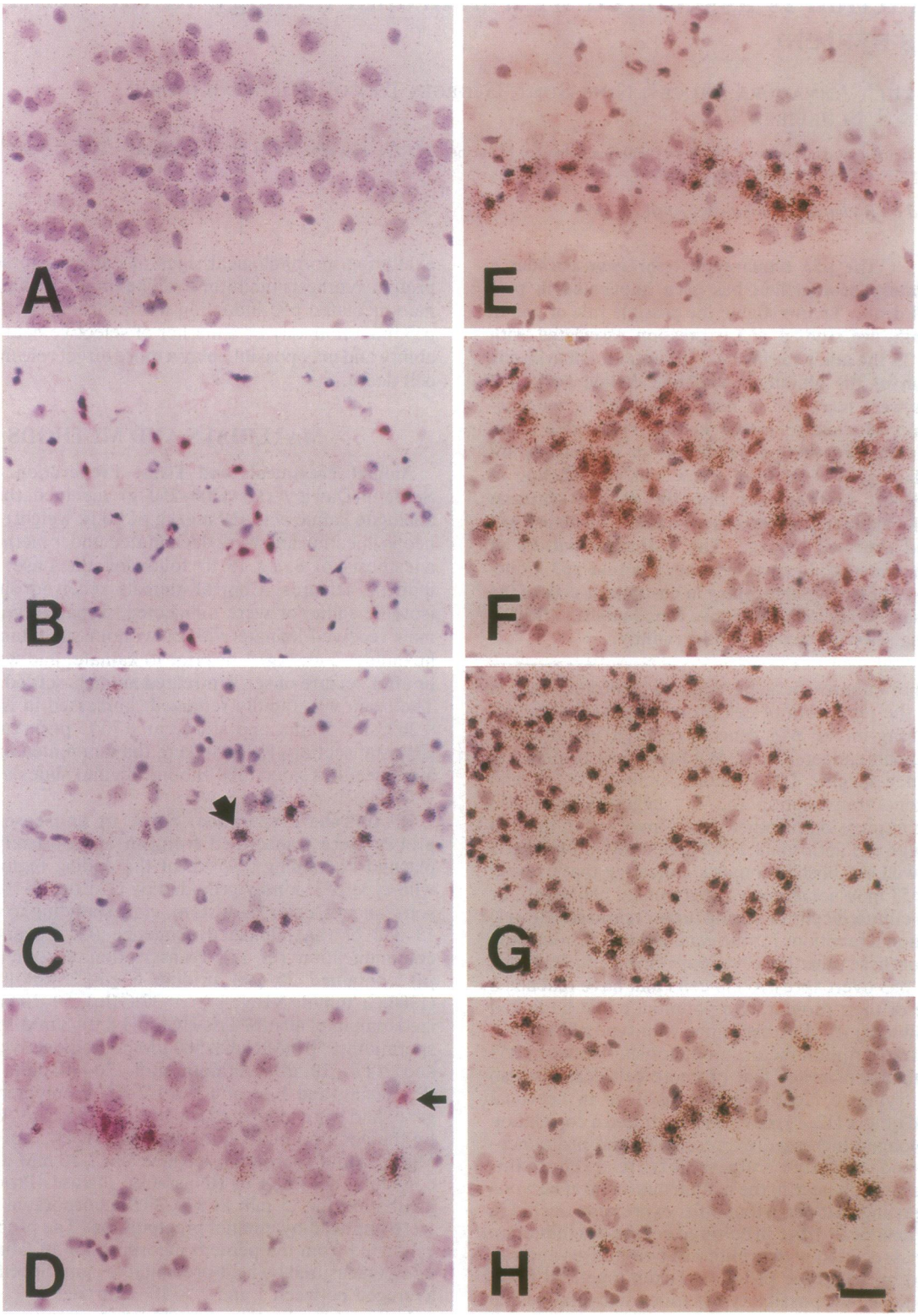

FIG. 1. Cellular expression pattern of p53 mRNA in the adult rat brain following kainic acid-induced seizures. *In situ* hybridization was performed as described. (*A*) Basal level of p53 expression in the hippocampal CA3 region of an untreated rat showing homogeneous distribution of silver grains over pyramidal cells. (*B*) Hybridization with a sense RNA probe in the amygdala 16 hr after kainate-induced seizure onset. Note cells with small, condensed nuclei, eosinophilic cytoplasm, and diffuse pattern of silver grains. (*C–H*) Hybridization with an antisense probe in vulnerable brain regions at 4 hr (*C*) ($n = 7$), 8 hr (*F*) ($n = 8$), and 16 hr (*D–H*) ($n = 10$) following kainate-induced seizure onset. (*C*) Piriform cortex. Note accumulation of grains over specific cells with nuclear condensation and eosinophilic cytoplasm (arrow). (*D*) CA1 region (dorsal hippocampus) showing several injured pyramidal neurons with increased p53 mRNA, as well as an injured, p53-negative cell (arrow). (*E*) CA3 region, dorsal hippocampus. (*F*) CA3 region, ventral hippocampus. (*G*) Amygdala. (*H*) Thalamus. Sections were counterstained with hematoxylin and eosin. (Bar = 35 μm.)

threitol with yeast transfer RNA (250 μg/ml), poly(A) (25 μg/ml), and poly(C) (25 μg/ml) was applied to the sections for 3 hr at 50°C. After hybridization the sections were placed in 4× SSC/20 mM dithiothreitol for 15 min at room temperature, rinsed in 4× SSC for 2 min, and incubated with RNase A (20 μg/ml) in 10 mM Tris·HCl, pH 8.0/0.5 M NaCl/1 mM EDTA for 30 min at 37°C. Sections were washed in 2× SSC/20 mM 2-mercaptoethanol for 2 hr at room temperature followed by 0.1× SSC for 1 hr at 60°C. The sections were dehydrated and air dried, coated with Kodak NTB-2 photographic emulsion, exposed at 4°C for 1 week, developed, and counterstained with hematoxylin and eosin.

***In Situ* End Labeling.** Two recently reported protocols (16, 17) were modified and performed as follows. Paraformaldehyde-fixed sections were dehydrated, air-dried, and incubated with 0.02 mM dATP, dCTP, and dGTP, 13 μM dTTP, 7 μM digoxigenin-11-dUTP (Boehringer Mannheim), and DNA polymerase I (Boehringer Mannheim) at 10 units/100 μl at 37°C for 2 hr. The reaction was stopped by addition of 20 mM EDTA (pH 8.0). Sections were incubated at room temperature overnight with an alkaline phosphatase-conjugated digoxigenin antibody diluted 1:200 in maleate blocking solution (Genius System, Boehringer Mannheim). Colorimetric detection with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate was performed with the Genius System by the manufacturer's protocol. In some experiments sections were counterstained with hematoxylin and eosin. Labeled DNA was not observed in sections from untreated animals or following incubation without the antibody (data not shown).

RESULTS

Regional Distribution and Time Course of Kainate-Mediated p53 Induction. To evaluate changes in gene expression, we used *in situ* hybridization with emulsion autoradiography for cellular localization of p53 mRNA. In untreated animals, basal levels of p53 expression were evident in several brain regions, including the hippocampus (Fig. 1*A*). A sense cRNA probe and high-stringency conditions in all experiments were employed to rule out the possibility that this was nonspecific background (Fig. 1*B*). As early as 4 hr after onset of kainate-induced seizures, elevated levels of p53 mRNA were observed in cells that simultaneously exhibited cytoplasmic eosinophilia and nuclear condensation, morphological features of cell damage (18) (Fig. 1*C*). From 4 through 16 hr following seizure onset, increasing numbers of damaged cells with increased p53 mRNA were observed in the hippocampus (CA1 and CA3 pyramidal cells), amygdala, piriform cortex, and thalamus (Fig. 1 *D–H*). By 16 hr, almost all of the visibly damaged cells had irregular, pyknotic nuclei consistent with irreversible injury (18). In the hippocampus, the location of the cells exhibiting increased p53 expression indicated that they were neurons and not glial elements. Quantitative measures of p53 mRNA abundance revealed a significant difference in p53 expression in damaged compared with untreated control hippocampal pyramidal cells (Table 1). Similar results were obtained when comparing damaged with nondamaged pyramidal cells from the same section (data not shown). In contrast, p53 expression remained unchanged in neurons that are characteristically resistant to kainate such as dentate gyrus granule and CA2 pyramidal neurons (data not shown). Although the majority of damaged cells exhibited p53 induction (Table 2), a small percentage of cells that appeared injured did not contain increased p53 mRNA (Fig. 1*D*). These results demonstrate that increased p53 gene expression occurs within several hours of excitotoxin administration and exhibits a distinctive pattern of regional and cellular localization. That is, p53 induction occurs only in regions that have been well documented to be vulnerable to kainate toxicity (19, 20), and, within these regions, in cells with morphological features of damage.

Table 1. Effect of various treatments on p53 expression in hippocampal pyramidal cells

Treatment	Grains per 500 μm^2
Control	18.0 ± 1.3^{NS}
Kainate	$64.0 \pm 8.5^{*}$
Cycloheximide/ kainate	$19.0 \pm 2.6^{**}$

Rats received kainic acid (10 mg/kg, s.c.) with or without cycloheximide pretreatment (2 mg/kg, s.c.) and were sacrificed 16 hr after seizure onset. Untreated animals served as controls. *In situ* hybridization with a radiolabeled p53 antisense RNA and emulsion autoradiography were performed as described. Eighty cells from both the CA3 and CA1 pyramidal cells subfields were analyzed in each group (20 cells per rat, four rats per group). In the kainate-treated group only those cells exhibiting cytoplasmic eosinophilia and nuclear condensation (i.e., features of cell damage) were included. The number of grains per unit area was computed with a BioQuant system IV image analysis system (Nashville, TN), Newvicon video camera (Dage–MTI, Michigan City, IN), and Olympus BH-2 microscope. The background grain density, computed by taking the mean of 12 grain density measurements within a 0.01-mm^2 area of adjacent neuropil, was subtracted from each measurement. Each value represents the mean ± SEM. Statistical analysis was performed with Student's *t* test. *, $P < 0.001$ (kainate vs. control); **, $P < 0.004$ (kainate vs. cycloheximide/kainate); NS, not significant (control vs. cycloheximide/kainate).

Cycloheximide Treatment. Pretreatment with the protein synthesis inhibitor cycloheximide protects vulnerable neurons against kainate-induced cell death (12). Cycloheximide also blocks p53 accumulation in cultured cells exposed to DNA-damaging agents (21, 22). Therefore, if p53 expression is important in kainate-mediated excitotoxicity, then kainate-mediated p53 expression might be similarly reduced by cycloheximide pretreatment. In rats treated subcutaneously with cycloheximide 1 hr before kainate there were no effects on the time to onset, duration, or intensity of kainate-induced seizures. However, with cycloheximide pretreatment we did not observe any cell with cytoplasmic eosinophilia, condensed, pyknotic nuclei, or increased p53 expression up to 24 hr after seizure onset. This was corroborated by quantitative analysis, which revealed a statistically significant difference in p53 mRNA abundance in pyramidal cells of animals treated with kainate alone compared with those receiving cycloheximide and kainate (Table 1).

Table 2. Percent injured hippocampal neurons with increased p53 mRNA or DNA damage at various times after seizure onset

	% injured neurons		
	4 hr	8 hr	16 hr
Increased p53 mRNA	93.3	96.0	96.7
DNA damage	70.7	80.6	82.6

Rats received kainic acid (10 mg/kg, s.c.) and were sacrificed at the specified times. *In situ* hybridization and emulsion autoradiography using an ^{35}S-labeled p53 antisense RNA probe, or *in situ* end labeling, were performed on frozen coronal brain sections as described. Sections were counterstained with hematoxylin and eosin. Three hundred were counted per time point, 150 each for evaluation of p53 expression or DNA damage (four rats per group). For p53 expression, only those pyramidal neurons exhibiting cytoplasmic eosinophilia and nuclear condensation were included. Cells in both the dorsal and ventral hippocampus were considered positive for increased p53 expression if there was an accumulation of silver grains over the nucleus and cytoplasm. Focal accumulations of silver grains were not observed in control or noninjured cells, over which the grains were homogeneously distributed (see Fig. 1*A*). For the evaluation of DNA damage, pyramidal cells with cytoplasmic eosinophilia were assessed for the presence of labeled DNA.

Labeling of Damaged DNA. Recent evidence indicates that intracellular levels of p53 protein increase in response to various DNA-damaging agents (21–23). To verify the presence of DNA damage in the central nervous system following kainate treatment, we employed *in situ* end labeling to detect fragmented DNA (16, 17). As early as 4 hr and up to 16 hr following the onset of kainate-induced seizures, labeled cells were evident only in kainate-vulnerable regions and clearly overlapped the distribution of injured cells expressing increased p53 mRNA (Fig. 2). Conversely, when injured cells—i.e., neurons with cytoplasmic eosinophilia and nuclear condensation—were assessed, the majority were positive for DNA damage (Table 2).

DISCUSSION

This report demonstrates a direct relationship between the cellular expression pattern of tumor suppressor p53 in the mammalian central nervous system and neuronal vulnerability. The results clearly indicate that increased p53 expression following excitotoxic seizure activity occurs in neuronal populations susceptible to excitotoxin-mediated cell death and strongly support a role for p53 induction as a marker of irreversible neuronal injury. Since cycloheximide pretreatment did not alter kainate-induced seizure activity, our findings show that seizure activity alone is not sufficient to promote p53 gene activation and that additional factors are probably involved in the regulation of kainate-mediated p53 gene expression. Further, whether cycloheximide protects against kainate-mediated excitotoxicity through protein synthesis inhibition remains to be determined, as cycloheximide can produce other effects, such as hypothermia (24).

The observation that some neurons appeared morphologically damaged without any overt increase in p53 mRNA content suggests that the initial injury occurred prior to p53 gene activation. However, additional studies are needed to precisely define the temporal relationships between p53 induction, the occurrence of DNA damage, and the appearance of morphological changes. Likewise, whether increased p53 expression plays a direct functional role in excitotoxicity remains to be determined. Although p53 might not be causally related to neuronal death, our results extend current views concerning the relationship between p53 expression and DNA damage (25) to the intact mammalian central nervous system and suggest that p53 induction in postmitotic neurons is associated with mechanisms of cell death instead of recovery. In contrast to regulatory mechanisms in cultured cells (21–23), kainate-mediated p53 expression in the mature central nervous system most likely occurs at the level of gene transcription. Further, the relatively early appearance of kainate-induced DNA damage suggests that the biochemical events predisposing to cell death occur rather soon after excitotoxin administration and that kainate-mediated neuronal death shares some of the mechanistic pathways of apoptosis.

The cellular expression pattern of p53 has several features in common with that of c-*fos* following kainate administration, in that both genes are induced for relatively prolonged periods in vulnerable brain regions and that increased expression is prevented by cycloheximide pretreatment. This

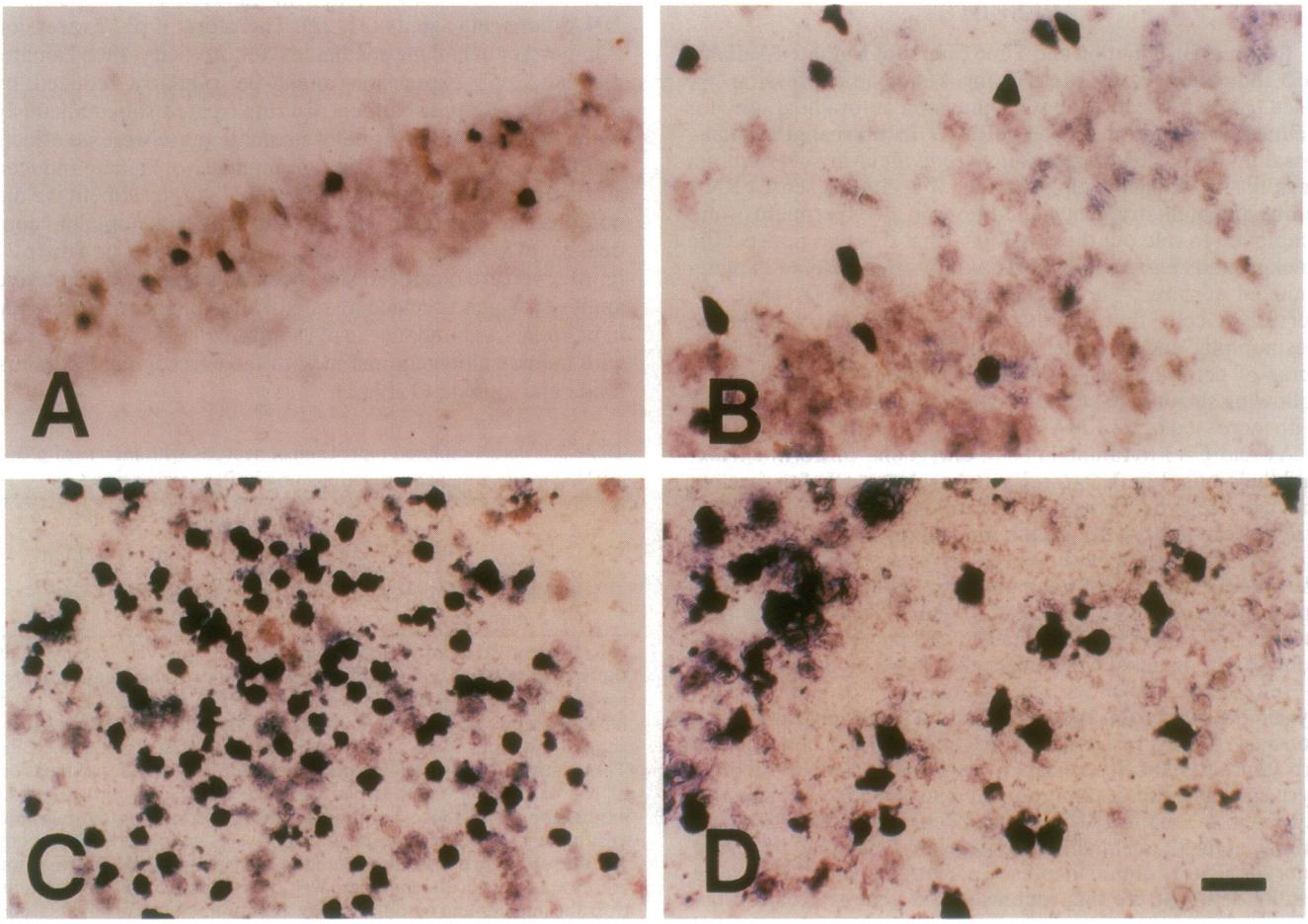

FIG. 2. DNA damage in vulnerable brain regions following kainate-induced seizures. *In situ* end labeling was performed on frozen brain sections as described. Sixteen hours after seizure onset, darkly labeled cells are evident in CA1 pyramidal region (*A*), piriform cortex (*B*), amygdala (*C*), and thalamus (*D*). (Bar = 35 μm.)

suggests that c-*fos* and p53 share similar functions and transcriptional regulatory pathways following excitotoxic stimulation *in vivo* and contrasts with the finding that wild-type p53 inhibits c-*fos* transcription in cultured cells (14). Observations that DNA-damaging agents can induce c-*fos* expression (26–28) add further support for a functional relationship between c-*fos* and p53.

Our findings are also consistent with the idea that p53 induction occurs in response to neuronal injury in a manner similar to the family of inducible heat shock proteins (29, 30). An association between the 72-kDa heat shock protein (hsp72) and p53 expression following cerebral ischemia has been suggested (31). Although the cellular localization of p53 immunoreactivity could not be identified, the authors speculated that p53 could be involved in ischemic cell death. In other studies, p53 protein was shown to regulate hsp70 gene expression (32) and to form stable complexes with hsp68 and heat shock protein-related cognate proteins (33) in cultured cells, providing some support for a functional interaction between p53 and heat shock proteins.

The present results suggest that similar molecular pathways exist for the regulation of cell growth and processes leading to cell death. Studies aimed at delineating the mechanisms that trigger p53 induction following excitotoxic injury should provide insight into the relationship between cell survival and cell death, as well as factors that contribute to selective neuronal vulnerability.

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