

Heparin-Binding Epidermal Growth Factor-Like Growth Factor in Hippocampus: Modulation of Expression by Seizures and Anti-Excitotoxic Action

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The expression of heparin-binding epidermal growth factor-like growth factor (HB-EGF), an EGF receptor ligand, was investigated in rat forebrain under basal conditions and after kainate-induced excitotoxic seizures. In addition, a potential neuroprotective role for HB-EGF was assessed in hippocampal cultures. *In situ* hybridization analysis of HB-EGF mRNA in developing rat hippocampus revealed its expression in all principle cell layers of hippocampus from birth to postnatal day (P) 7, whereas from P14 through adulthood, expression decreased in the pyramidal cell layer versus the dentate gyrus granule cells. After kainate-induced excitotoxic seizures, levels of HB-EGF mRNA increased markedly in the hippocampus, as well as in several other cortical and limbic forebrain regions. In the hippocampus, HB-EGF mRNA expression increased within 3 hr after kainate treatment, continued to increase until 24 hr, and then decreased; increases occurred in the dentate gyrus granule cells, in the molecular layer of the dentate gyrus, and in and

around hippocampal pyramidal CA3 and CA1 neurons. At 48 hr after kainate treatment, HB-EGF mRNA remained elevated in vulnerable brain regions of the hippocampus and amygdaloid complex. Western blot analysis revealed increased levels of HB-EGF protein in the hippocampus after kainate administration, with a peak at 24 hr. Pretreatment of embryonic hippocampal cell cultures with HB-EGF protected neurons against kainate toxicity. The kainate-induced elevation of $[Ca^{2+}]_i$ in hippocampal neurons was not altered in cultures pretreated with HB-EGF, suggesting an excitoprotective mechanism different from that of previously characterized excitoprotective growth factors. Taken together, these results suggest that HB-EGF may function as an endogenous neuroprotective agent after seizure-induced neural activity/injury.

Key words: HB-EGF; hippocampus; neuroprotection; epilepsy; excitotoxicity; *in situ* hybridization; kainic acid; calcium

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a member of the epidermal growth factor (EGF) family of structurally related polypeptides that include EGF, transforming growth factor- α (TGF α), amphiregulin, and betacellulin (Higashiyama et al., 1991, 1992; Watanabe et al., 1994; Barnard et al., 1995). Each family member activates the EGF receptor (EGF-R), a 170 kDa transmembrane glycoprotein with intrinsic tyrosine kinase activity (Besner et al., 1992). HB-EGF and other EGF-R ligands are synthesized as membrane-anchored precursors that during proteolytic cleavage produce diffusible factors that mediate their effects on various cellular processes (Raab et al., 1994; Cook et al., 1995). HB-EGF and amphiregulin can be distinguished from other members of the EGF family by the presence of a heparin-binding domain, which interacts with membrane-bound heparin sulfate proteoglycans and may thereby

enhance EGF receptor activation. HB-EGF mRNA is expressed in brain (Abraham et al., 1993), and its precise cellular distribution is beginning to be elucidated (Opanashuk et al., 1995; Mishima et al., 1996). EGF-R mRNA and protein are expressed in brain where they are localized to many different cell populations, including neurons and astrocytes (Gomez-Pinilla et al., 1988; Faundez et al., 1992; Tucker et al., 1993; Seroogy et al., 1994, 1995; Kornblum et al., 1995, 1997a). Cultured embryonic neurons and glia from rodent brain also express EGF-R (Mazzone and Kenigsberg, 1994; Yamada et al., 1997).

Both TGF α and EGF promote survival and/or process outgrowth of cultured neurons from several brain regions (Morrison et al., 1987, 1988; Kornblum et al., 1990; Casper et al., 1991; Ferrari et al., 1991; Alexi and Hefti, 1993), indicating possible neurotrophic functions for EGF-R agonists. EGF promotes hippocampal long-term potentiation (Terlau and Seifert, 1989; Abe et al., 1991, 1992) and protects dopaminergic neurons against glutamate (Casper and Blum, 1995) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Park and Mytilineau, 1992; Schneider and DiStefano, 1995) toxicity, suggesting potential neuromodulatory and neuroprotective roles for EGF-R ligands. Because HB-EGF interacts with the EGF-R, it is reasonable to consider that HB-EGF serves roles overlapping those of EGF and TGF α .

Administration of the excitotoxin kainate to adult rodents induces seizures and a stereotyped pattern of neuronal degeneration involving limbic and cortical structures, with certain hip-

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pocampal neurons being particularly vulnerable (Nadler et al., 1978; Sperk et al., 1983; Ben-Ari, 1985). During and after seizures induced by kainate, the expression of several neurotrophic factors and cytokines increases, including NGF (Gall and Isackson, 1989; Gall et al., 1991), basic fibroblast growth factor (bFGF) (Humpel et al., 1993; Gall et al., 1994), brain-derived neurotrophic factor (BDNF) (Isackson et al., 1991; Dugich-Djordjevic et al., 1992), glial cell line-derived neurotrophic factor (GDNF) (Humpel et al., 1994), and tumor necrosis factor- α (TNF α) (Bruce et al., 1996). Such injury-responsive neurotrophic factors and cytokines may function to prevent neuronal death (for review, see Mattson and Scheff, 1994; Mattson and Lindvall, 1997). In the present study we examined the cellular expression of HB-EGF in hippocampus, cortex, and limbic structures under basal conditions and after kainate-induced seizures, and we tested the hypothesis that HB-EGF serves an excitoprotective role.

MATERIALS AND METHODS

Animals and kainate administration. Male and female Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) taken at postnatal day (P) 0, P7, P14, and P21, and adult ages (>60 d old, $n = 4$ for each age) were used to examine the *in situ* hybridization localization of HB-EGF mRNA in hippocampus during postnatal development. Rat pups taken at the day of birth (P0) and at P7 were deeply anesthetized by hypothermia and decapitated. Animals at the other ages were deeply anesthetized with sodium pentobarbital before decapitation. The brains were rapidly dissected out, quickly frozen over dry ice, and stored at -80°C until further processing. Adult (>60 d old) male Sprague Dawley rats weighing 200–250 gm were used for the kainic acid experiments. Kainate (Sigma, St. Louis, MO) was injected intraperitoneally at 12 mg/kg (dissolved in 0.9% saline, pH 7.0). Control animals received an equivalent volume of saline. After kainate administration, rats were monitored for convulsive activity for 8 hr and rated on a scale of 1–5 as described previously (Sperk et al., 1983). Behavioral changes were evident within 30 min of kainate administration and consisted of “wet-dog shakes” followed by staring spells, forehead-nodding, forelimb myoclonus, rearing, and excessive salivation. Only animals achieving at least a level of 3 (Sperk et al., 1983) were used for analyses. At various survival periods after kainate injection (3, 6, 12, 24, and 48 hr), rats ($n = 3$ –4 per time point) were deeply anesthetized with an overdose of sodium pentobarbital and decapitated. The brains were quickly removed and processed for mRNA or protein analyses as described below.

RNA isolation and Northern blot analysis. Hippocampi were rapidly removed and placed in Trizol reagent (Life Technologies BRL, Gaithersburg, MD), and tissue was homogenized using a Polytron homogenizer. The homogenate was extracted twice with phenol/chloroform, and RNA was precipitated with isopropanol. The RNA pellet was air-dried and dissolved in DEPC-treated water, and the RNA concentration was determined by measuring OD₂₆₀. RNA (20 μg) was separated by electrophoresis through a 1.2% formaldehyde-agarose gel containing ethidium bromide. RNA was then transferred to nylon membranes by vacuum transfer in $10\times$ SSC (0.3 M sodium citrate, 3.0 M NaCl); RNA integrity and transfer efficiency were visualized by UV light. Membranes were prehybridized in a solution containing $2\times$ SSC, $5\times$ Denhardt's solution, 10% dextran sulfate, 2% SDS, and the [^{32}P]-labeled probe at a concentration of 2×10^6 cpm/ml. After treatment with 1 $\mu\text{g}/\text{ml}$ ribonuclease A (RNase) at room temperature, membranes were sequentially washed in decreasing concentrations of SSC containing 0.25% SDS at 65°C . The final wash in $0.1\times$ SSC was performed at room temperature. Membranes were exposed to X-OMAT film (Kodak, Rochester, NY) for 5–7 d, and specific hybridization was visualized by autoradiography. Membranes were stripped and re probed with a [^{32}P]-labeled β actin probe (kindly provided by James Hyde, University of Kentucky, Lexington, KY). HB-EGF mRNA levels were quantified using

NIH Image software and normalized relative to actin mRNA levels. Control membranes were pretreated with 1 $\mu\text{g}/\text{ml}$ RNase for 30 min before hybridization or were hybridized with the [^{32}P]-labeled sense cRNA HB-EGF probe (transcribed with T7 RNA polymerase). No specific hybridization was observed in either case (data not shown). As a positive control, RNA was isolated from rat lung, an organ known to express HB-EGF mRNA (Abraham et al., 1993), and hybridized with both the antisense and sense HB-EGF cRNA probes. The 2.5 kb transcript was detected on films from membrane probed with the antisense cRNA probe, whereas membranes hybridized with sense probe were devoid of signal.

In situ hybridization. Coronal tissue sections through the forebrain were cut using a cryostat (14 μm thickness for the developmental studies, 10 μm thickness for the adult rat kainate treatment experiments) and stored at -20°C . The slide-mounted sections were processed for the localization of HB-EGF mRNA by *in situ* hybridization as described previously (Seroogy et al., 1993, 1994, 1995; Seroogy and Herman, 1997). The antisense HB-EGF cRNA was transcribed *in vitro* from a pBluescript vector containing a 258 bp fragment from the 5' end of the cloned rat cDNA using T3 RNA polymerase in the presence of [^{35}S]UTP. Hybridization was accomplished at 60°C for 18–20 hr in a solution containing the [^{35}S]-labeled HB-EGF cRNA probe at a concentration of 1×10^6 cpm/0.50 ml per slide. After post-hybridization ribonuclease treatment and washes in SSC, the distribution of hybridization was localized by film [β -Max Hyperfilm; Amersham, Arlington Heights, IL] 10 d exposures for the experimental/seizure series, 14 d exposures for the developmental series] and then emulsion [(Kodak NTB2) 4 week exposures for the experimental/seizure series, 6 week exposures for the developmental series] autoradiography. The differences in exposure times were chosen, on the one hand, to accentuate the normal developmental distribution of HB-EGF mRNA, and, on the other hand, to emphasize the effect between control and experimental/seizure-induced expression without overexposing the film at certain time points. Control sections were pretreated with ribonuclease A (45°C for 30 min) before hybridization with the antisense probe or were hybridized with the [^{35}S]-labeled sense sequence (transcribed using T7 polymerase). No specific labeling was observed in tissue processed under either control condition.

Western blot analysis. These methods were similar to those described in our previous studies (Cheng et al., 1995). Hippocampal homogenates were prepared as detailed elsewhere (Cheng et al., 1995) and assayed for protein content at 562 nm using a BCA kit (Pierce, Rockford, IL) according to the manufacturer's protocol. Proteins were separated by SDS-PAGE in 15% gel and then transferred electrophoretically to Hybond Membrane (Bio-Rad, Hercules, CA). The membranes were blocked in TBS containing 5.0% nonfat milk and 0.1% Tween-20, and then incubated overnight at 4°C in PBS containing primary antibody (goat polyclonal anti-HB-EGF antibody 197; provided by Judith Abraham, Scios Inc.) The membranes were then incubated for 1 hr at room temperature in TBS containing a peroxidase-conjugated rabbit anti-goat secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and processed further using a chemiluminescence detection system (ECL system; Amersham). Human recombinant HB-EGF (R&D Systems, Minneapolis, MN) and lung tissue were used as positive controls for the HB-EGF antibody, and BSA was used as a negative control. To verify equal protein gel loading, membranes were stripped and re probed with an anti-neurofilament (200 kDa) protein antibody (1:500 dilution; Sternberger Monoclonals, Lutherville, MD). Equivalent electrophoretic transfer of protein was verified on membranes reversibly stained with Ponceau S dye.

Hippocampal cell cultures and quantification of neuronal survival. Primary hippocampal cell cultures were established from embryonic rats (day 18 of gestation) as detailed elsewhere (Mattson et al., 1995). Cultures were maintained in Eagle's Minimum Essential Medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Life Technologies), 20 mM KCl, and 1 mM pyruvate. Experiments were performed in cultures that had been maintained for 6–10 d. In these cultures, ~90% of the cells are neurons, and the remaining cells are astrocytes, as judged by characteristic morphology and differential immunoreactivity with antibodies to neuron-specific (neurofilament, MAP2, and tau) and astrocyte-specific (glial fibrillary acidic protein and S-100 β) proteins (Mattson et al., 1993, 1995). Immediately before experimental treatment, the culture maintenance medium was replaced with Locke's solution containing (in mM): NaCl 154, KCl 5.6, CaCl₂ 2.3, MgCl₂ 1.0, NaHCO₃ 3.6, glucose 10, and HEPES buffer 5, pH 7.2. Cultures were pretreated for 16 hr with HB-EGF in Locke's solution before the addition of kainate

(Sigma). Neuronal survival was quantified by counting the number of viable neurons in premarked microscope fields before and at indicated time points after exposure to experimental treatments as described previously (Mattson et al., 1995).

Measurement of intracellular calcium levels. The intracellular free calcium concentration ($[Ca^{2+}]_i$) in individual neuronal cell bodies was quantified by ratiometric imaging of the calcium indicator dye fura-2 acetoxyethyl ester as described previously (Mark et al., 1995; Mattson et al., 1995). Cells were pretreated for 16 hr with 100 ng/ml HB-EGF or vehicle and then incubated at 37°C with fura-2 for 30 min. After rinsing, cultures were placed in HBSS supplemented with 10 mM glucose and 10 mM HEPES, pH 7.2. Cells were imaged using a Zeiss Atofluor system, which included a Zeiss Axiovert microscope with a 40× oil objective and an Atofluor-intensified CCD camera. The dye was excited at 340 and 380 nm, and images were taken of the emitted fluorescence after excitation at each wavelength. The method of Grynkiewicz et al. (1985) was used to calibrate the system and quantify ($[Ca^{2+}]_i$). The $[Ca^{2+}]_i$ in individual neuronal cell bodies was monitored at 7 sec intervals for 1 min before, and 5 min after, exposure to kainate. Values for peak $[Ca^{2+}]_i$ were determined and used for statistical comparisons between control cultures and those pretreated with HB-EGF.

RESULTS

Postnatal ontogeny of HB-EGF mRNA expression in rat hippocampus and forebrain

As determined by *in situ* hybridization, expression of HB-EGF mRNA was evident in hippocampal neurons at all ages examined (Fig. 1). From P0 to P7, labeling was distributed within all principle cell layers of the hippocampus, including stratum pyramidale fields CA1–CA3 and stratum granulosum of the dentate gyrus. However, by P14 and through adulthood, hybridization decreased substantially in the pyramidal cell layer to levels just slightly above background. In contrast, levels of HB-EGF mRNA appeared to slightly increase in stratum granulosum throughout development (Fig. 1). During development and in the adult, labeled cells were never detected in the molecular layers of the hippocampus or in the hilus of the dentate gyrus. In neocortex, hybridization for HB-EGF mRNA was localized to cell bodies in middle layers, with levels being greatest at P7 and then declining through adulthood (Fig. 1). The pattern of neocortical labeling appears to correspond to the region of somatosensory cortex. HB-EGF mRNA was also moderately expressed neonatally in other cortical areas, including entorhinal, piriform, and retrosplenial cortices; hybridization levels decreased with increasing age in these regions (data not shown). Moderate labeling for HB-EGF mRNA was also present neonatally in the tectum and within several thalamic regions, including the dorsolateral and medial geniculate nuclei and the ventrobasal complex (data not shown). Again, hybridization levels in these latter regions declined substantially throughout postnatal development to very low to undetectable levels in the adult. Labeling for HB-EGF mRNA was never localized to regions of white matter in the forebrain (i.e., corpus callosum, anterior commissure, internal and external capsules, lateral olfactory tract, optic tract, etc.) at any of the ages examined.

HB-EGF mRNA expression in hippocampus after kainate administration

To determine whether HB-EGF mRNA is modulated by neuronal activity/injury, kainate was administered to adult rats (12 mg/kg, i.p.) to induce seizures, and rats were killed 3, 6, 12, 24 and 48 hr later. We first assessed overall levels of HB-EGF mRNA in hippocampus by Northern blot analysis (Fig. 2). An increase in the 2.5 kb HB-EGF transcript was detected 3 hr after kainate administration, and the level of HB-EGF mRNA continued to increase through 6 and 12 hr, with peak expression occur-

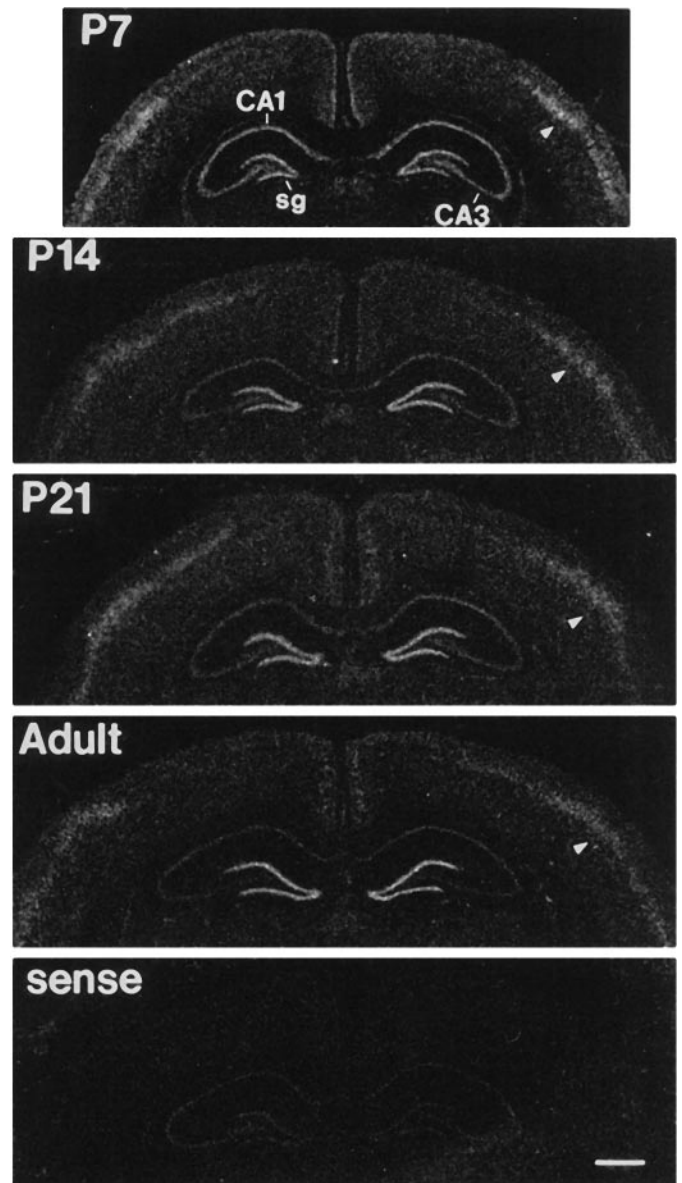


Figure 1. Expression of HB-EGF mRNA in developing hippocampus. Autoradiograms showing the *in situ* hybridization localization of HB-EGF mRNA in coronal sections through the hippocampus at the indicated postnatal day ages. Note that at P7, labeling is present throughout hippocampal fields CA1 and CA3 and dentate gyrus stratum granulosum (sg), whereas in older (P14, P21, Adult) rats, HB-EGF mRNA expression is substantially reduced in the pyramidal cell layer relative to the stratum granulosum. In neocortex, HB-EGF cRNA hybridization is prominent in the middle layers (arrowheads), with levels gradually declining from P7 to adulthood. The bottom panel (sense) demonstrates the lack of hybridization obtained with the control sense cRNA probe in a section through the adult hippocampus. Film exposure, 14 d; section thickness, 14 μ m. Scale bar, 1000 μ m.

ing at the 24 hr time point. HB-EGF mRNA levels were declining toward control levels at 48 hr after kainate administration (Fig. 2).

In situ hybridization analyses demonstrated an extensive increase in HB-EGF mRNA within the hippocampus, as well as in other limbic and cortical structures, after kainate treatment (Fig. 3; see summary in Table 1). In the hippocampus, there was a pronounced increase in HB-EGF cRNA hybridization in the

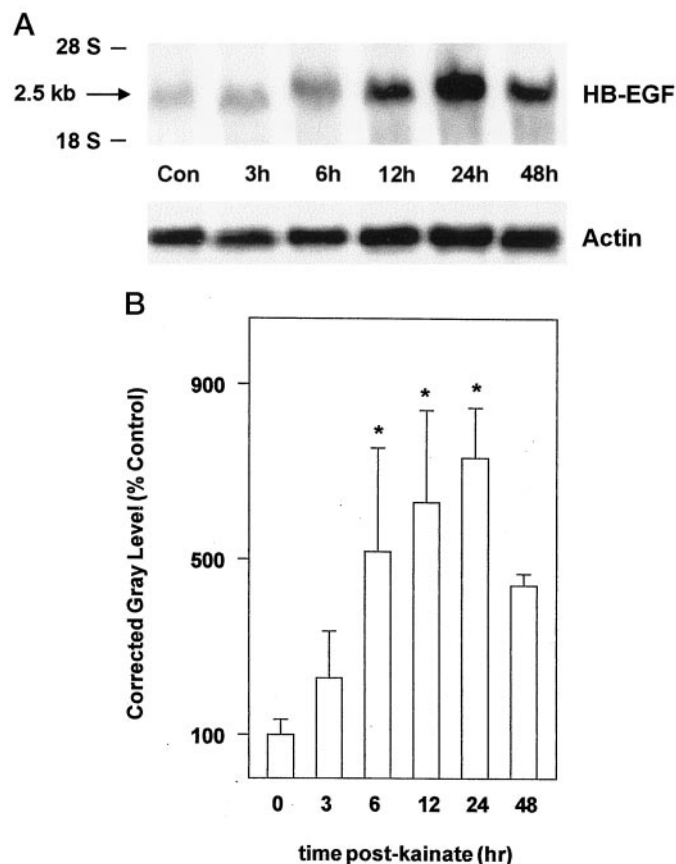


Figure 2. *A*, Northern blot analysis of HB-EGF mRNA levels in adult rat hippocampus at various survival times (3–48 hr) after kainate administration. Total RNA (20 μ g/lane) was separated on a 1.2% agarose gel, transferred to nylon membrane, and hybridized with an antisense 32 P-cRNA HB-EGF probe (*top*). Membranes were stripped and reprobed with a 32 P-labeled β actin cRNA to control for equal loading of RNA (*bottom*). HB-EGF mRNA was detected by autoradiography with an exposure time of 5 d. Levels of the 2.5 kb transcript corresponding to HB-EGF mRNA were elevated 3 hr after kainate treatment and continued to increase through the 24 hr time point. By 48 hr, HB-EGF mRNA levels were declining. *Con*, Control. *B*, Densitometric analysis of HB-EGF mRNA levels at various times after kainate administration. HB-EGF mRNA levels were normalized relative to β actin mRNA levels. Data are expressed as mean \pm SEM of determinations made in three rats per time point and were analyzed by one-way ANOVA with Duncan *post hoc* analysis. * $p < 0.05$ compared with the control (0 hr) value.

stratum granulosum of the dentate gyrus that was evident at 3 hr, reached maximal levels at 6 hr, and declined to control levels by 48 hr after kainate administration (Figs. 3, 4). By the 6 hr time point, increased expression was no longer restricted to the dentate granule cells such that labeled perikarya were now scattered in the hilus and molecular layer of the dentate gyrus (Fig. 4C). The densely labeled cells were most numerous in these regions between 12 and 24 hr after kainate treatment (Figs. 3D,E, 4D); by 48 hr, HB-EGF cRNA-hybridizing cells were no longer detected in the dentate hilus and molecular layer (Fig. 4E), as in control brains.

Between 12 and 24 hr after kainate administration, HB-EGF cRNA hybridization was prominently elevated in perikarya within and around stratum pyramidale of the hippocampus (Fig. 3D,E). In and adjacent to region CA3, labeling was highest at 12 hr and declined to control levels by 48 hr. Cells expressing HB-EGF mRNA were observed within region CA1 and were

scattered in the molecular layers surrounding CA1 (strata oriens and radiatum) by 12 hr after kainate administration (Figs. 3D, 5C). However, at later survival periods, the labeled cells were located in successively closer proximity to the pyramidal cell layer of CA1 (Fig. 5D) such that by 48 hr, expression was restricted to cells dispersed within or along the edge of the CA1 cell layer (Figs. 3F, 5E).

HB-EGF mRNA expression in extra-hippocampal regions after kainate administration

Injection of kainate induced the expression of HB-EGF mRNA in several forebrain limbic and cortical regions outside of the hippocampus (Table 1). In the neocortex, hybridization signal was elevated in middle layers within 3 hr of kainate administration (Figs. 3B, 6B) and peaked between 12 and 24 hr, at which time HB-EGF cRNA-hybridizing cells appeared to encompass nearly all cortical laminae (Figs. 3D,E, 6C,D). However, by the 48 hr time point, labeled cells were again absent from the neocortex (Figs. 3F, 6E). Kainate treatment increased HB-EGF mRNA levels in piriform cortex within 3 hr of administration (Fig. 6), with highest levels of expression observed at the 12 and 24 hr time points (Fig. 6). In addition to the piriform cortex, transient elevations in HB-EGF mRNA expression were found in other olfactory-related areas, including the entorhinal cortex, amygdaloid nuclei, olfactory tubercle, and anterior olfactory nuclei after kainate treatment (Table 1). Kainate administration also induced dense hybridization for HB-EGF mRNA in numerous perikarya of the lateral septal nuclei, with peak expression found between 12 and 24 hr after injection (Fig. 6). Transient increases in HB-EGF cRNA hybridization were also noted in several thalamic and hypothalamic nuclei (Table 1). Finally, mainly at the 24 and 48 hr time points, perikarya densely labeled for HB-EGF mRNA surrounded prescribed areas of the perirhinal, piriform, and entorhinal cortices and amygdala, as well as several thalamic regions (Figs. 3, 6; Table 1).

Relationships between kainate-induced neuronal degeneration and HB-EGF mRNA localization

Systemic administration of kainate results in a characteristic pattern of neuronal cell loss, which in the hippocampus involves regions CA1 and CA3 strata pyramidale, as well as hilar neurons (Nadler et al., 1978; Sperk et al., 1983; Ben-Ari, 1985). As expected, this pattern of cell loss was observed in the present study. At the 48 hr survival time, HB-EGF mRNA-labeled cells were consistently present in the proximity of vulnerable regions of hippocampus (Figs. 3, 5). In addition, degenerating regions of amygdala, piriform cortex, and related areas of cortex and thalamus were encircled by HB-EGF cRNA-hybridizing cells 24–48 hr after kainate administration (Figs. 3, 6, 7). An example of this hybridization pattern can be seen in Figure 7 where HB-EGF mRNA-expressing perikarya are lining the medial edge of the amygdala, a structure that has undergone extensive cell loss 48 hr after kainate treatment.

HB-EGF protein expression in hippocampus after kainate administration

Western blot analysis was used to examine alterations in HB-EGF protein levels in hippocampus at various survival times after kainate treatment. Consistent with previous studies (Ono et al., 1994; Kim et al., 1995; Davis et al., 1996), HB-EGF protein was identified as four species, with apparent molecular weights ranging from 14 to 27 kDa (Fig. 8); none of the four bands were present when the primary antibody was preincubated with excess

Table 1. Relative hybridization densities^a of HB-EGF mRNA in rat forebrain after kainate-induced seizures

Brain region	Time after kainate injection					
	0	3 hr	6 hr	12 hr	24 hr	48 hr
Frontal cortex	–	+	+	++	++	–
Cingulate cortex	–	+	+	++	+/-	–
Entorhinal cortex*	+/-	+	+	+	++	++
Perirhinal cortex*	+/-	+	+	++	++	++
Piriform cortex*	+	+	++	+++	+++	++
Olfactory tubercle	+/-	+	++	++	++	+/-
Anterior olfactory nuclei	–	+	+	+	++	+/-
Caudate-putamen	–	–	–	+/-	+/-	–
Lateral septal nucleus	–	+	++	+++	++++	+
Amygdaloid complex*	–	+/-	+	+	++	++
Dentate gyrus GCL	++	++++	++++	+++	++	++
Hippocampal CA1 region*	+/-	+/-	+	++++	++++	++
Hippocampal CA3 region*	+/-	+/-	+	+++	++	+/-
Medial thalamic nuclei*	–	–	+	++	+	++
VPM	+/-	+	+	++	++	+/-
VPL	+/-	+	+	++	++	+/-
Medial geniculate nucleus	+/-	+	+	++	++	+/-
Anterior hypothalamic nucleus	–	–	++	++	+	–
VM hypothalamic nucleus	–	–	++	++	+	–

GCL, Granule cell layer; VM, hypothalamic nucleus, ventromedial hypothalamic nucleus; VPL, ventroposterolateral thalamic nucleus; VPM, ventroposteromedial thalamic nucleus.

^a Qualitative estimates of the relative hybridization densities of HB-EGF mRNA as determined by *in situ* hybridization with a ³⁵S-labeled cRNA probe. Hybridization densities were scored and agreed on by at least two independent observers according to the following rating scale: +++++, highest density; +++, high density; ++, moderate density; +, low density; +/-, low density, inconsistently above background; –, no hybridization, background density. Asterisks indicate regions in which hybridization associated with cells surrounding fields of neural degeneration persisted at the 24 and 48 hr post-kainate time points, as discussed in Results.

(50 ng) recombinant human HB-EGF (data not shown). Levels of the 14 and 27 kDa isoforms increased after 3 hr and peaked at 24 hr after kainate treatment. Levels of HB-EGF protein declined but were still above control levels 48 hr after kainate administration (Fig. 8). By 72 hr after kainate treatment, expression of the 14 kDa HB-EGF isoform remained elevated.

HB-EGF protects hippocampal neurons against kainate toxicity

Exposure of cultures to kainate (10–50 μ M for 12 hr) resulted in concentration-dependent decreases in neuronal survival (Fig. 9). Approximately 40 and 85% of rat hippocampal neurons degenerated after exposure to 10 and 50 μ M kainate, respectively. Neuronal survival was increased in a concentration-dependent manner after pretreatment with HB-EGF. When cultures were pretreated with HB-EGF (10–100 ng/ml) for 16 hr, there was a dramatic reduction in kainate-induced neuronal damage when compared with cultures not receiving growth factor. Shorter pretreatments with HB-EGF (2–6 hr exposures to 100 ng/ml HB-EGF) did not protect neurons against kainate toxicity (data not shown). The protection against kainate toxicity afforded by HB-EGF was sustained for at least 48 hr (Fig. 9B), indicating that HB-EGF prevented neuronal death rather than just delaying the death. Calcium influx has previously been shown to play an important role in neuronal injury induced by kainate (Choi, 1987; Giusti et al., 1995). To determine whether HB-EGF protects against neurotoxicity by suppressing the kainate-induced rise in $[Ca^{2+}]_i$, calcium levels were measured in cultured hippocampal neurons by ratiometric imaging of the fluorescent calcium indicator dye fura-2. Cultures were pretreated with 100 ng/ml HB-EGF for 16 hr and then challenged with 50 μ M kainate for 5 min. After exposure to kainate the $[Ca^{2+}]_i$ in neurons was increased to

~350 nM compared with 100 nM $[Ca^{2+}]_i$ in neurons exposed to vehicle (Fig. 10). The elevation in $[Ca^{2+}]_i$ in response to kainate was not attenuated by pretreatment with HB-EGF.

DISCUSSION

The principal findings of the present study reveal that HB-EGF mRNA and protein are dramatically upregulated in the hippocampal formation after seizure-induced neuronal activity/injury. Sustained HB-EGF expression was also induced in forebrain areas vulnerable to degeneration after seizures. Moreover, our *in vitro* data indicate that HB-EGF is neuroprotective for hippocampal neurons against kainate toxicity, with an excitoprotective mechanism different from that of previously characterized excitoprotective trophic factors. Taken together, these results provide the first evidence that a member of the EGF family may play a role in neuroprotection and in the longer-term neuropathological changes associated with seizures.

The regional and temporal patterns of HB-EGF mRNA expression in response to kainate-induced seizures exhibited features similar to those described previously for other neurotrophic factors. Accordingly, HB-EGF mRNA was robustly expressed in several limbic, cortical, and subcortical regions after kainate administration. The earliest alterations in HB-EGF mRNA levels occurred in hippocampus, followed by a more gradual and widespread induction in cortical and septal areas. In hippocampus, HB-EGF mRNA was rapidly induced in neurons of the dentate gyrus granule cell layer followed by a more protracted upregulation in the CA1–CA3 regions and molecular layers. The alterations in hippocampal HB-EGF mRNA were accompanied by a corresponding elevation in HB-EGF protein; both mRNA and protein levels appeared to peak between 12 and 24 hr after

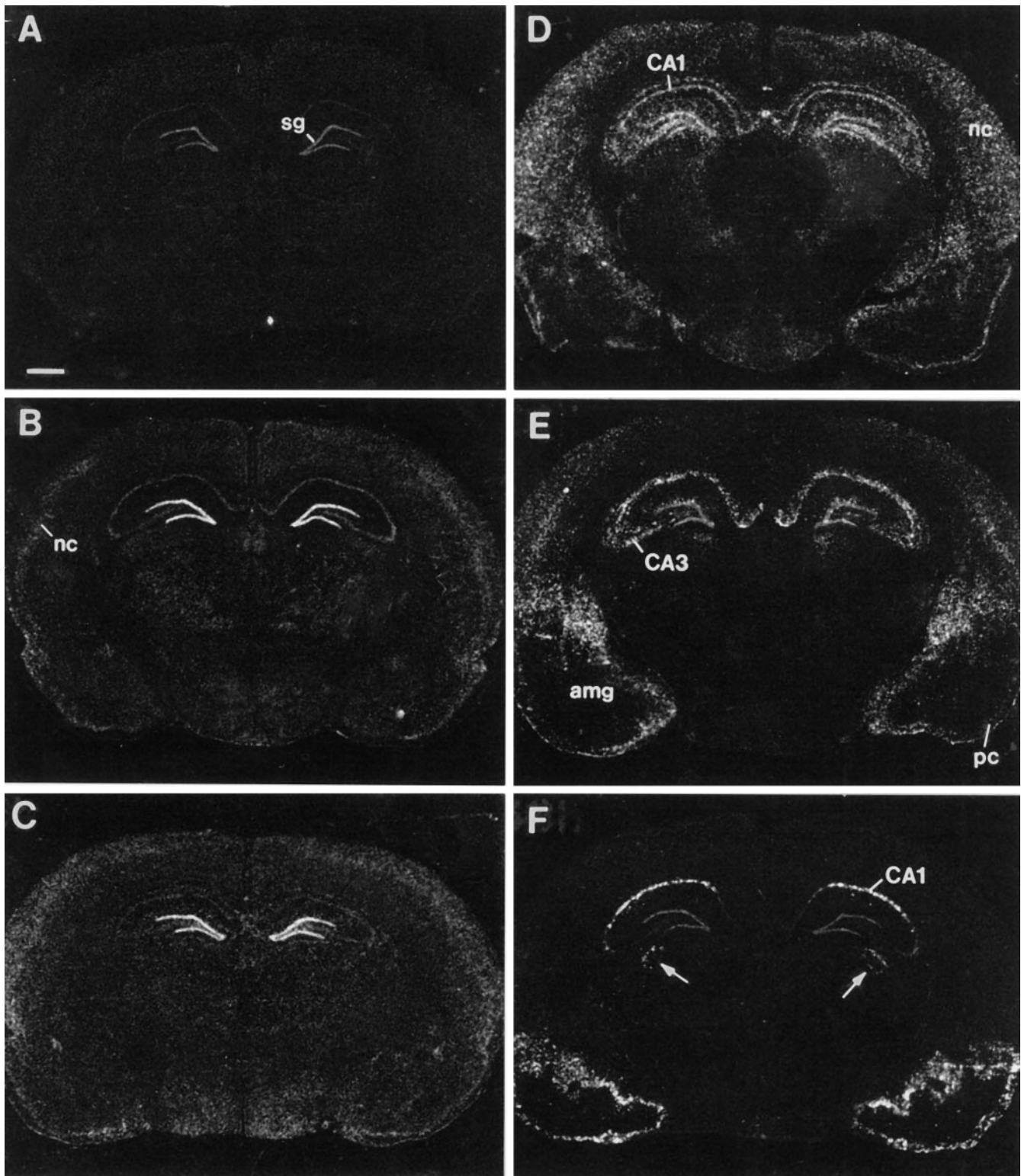


Figure 3. Kainate-induced increase of HB-EGF mRNA expression in hippocampus. Autoradiograms demonstrating the *in situ* hybridization localization of HB-EGF mRNA in coronal sections through the hippocampus in a saline-injected control rat (*A*) and in rats killed 3 (*B*), 6 (*C*), 12 (*D*), 24 (*E*), and 48 hr (*F*) after kainate administration. Expression of HB-EGF mRNA is upregulated in the granule cell layer [stratum granulosum (*sg*)] of the dentate gyrus 3–6 hr after kainate treatment (*B*, *C*) but then declines to control levels by 48 hr (*F*). Kainate treatment induces prominent hybridization in and around the pyramidal cell layers of hippocampal subfields *CA1* and *CA3* at the 12 and 24 hr time points (*D*, *E*); by 48 hr, labeling is restricted to the *CA1* region in the hippocampus (*F*). In neocortex (*nc*), HB-EGF mRNA expression increases from 3 to 12 hr after kainate treatment (*B–D*) and then decreases to control levels by 48 hr (*E*, *F*). Note that hybridization signal is also present in and around areas of kainate-induced cell loss at the 24 and 48 hr time points (*E*, *F*), including regions of the dorsal lateral thalamus (arrows in *F*), amygdaloid complex (*amg*), and piriform cortex (*pc*). Film exposure, 10 d; section thickness, 10 μm . Note that because of thinner sectioning and shorter exposure time, the control section in *A* cannot be directly compared with the normal adult section in Figure 1. Scale bar, 1000 μm .

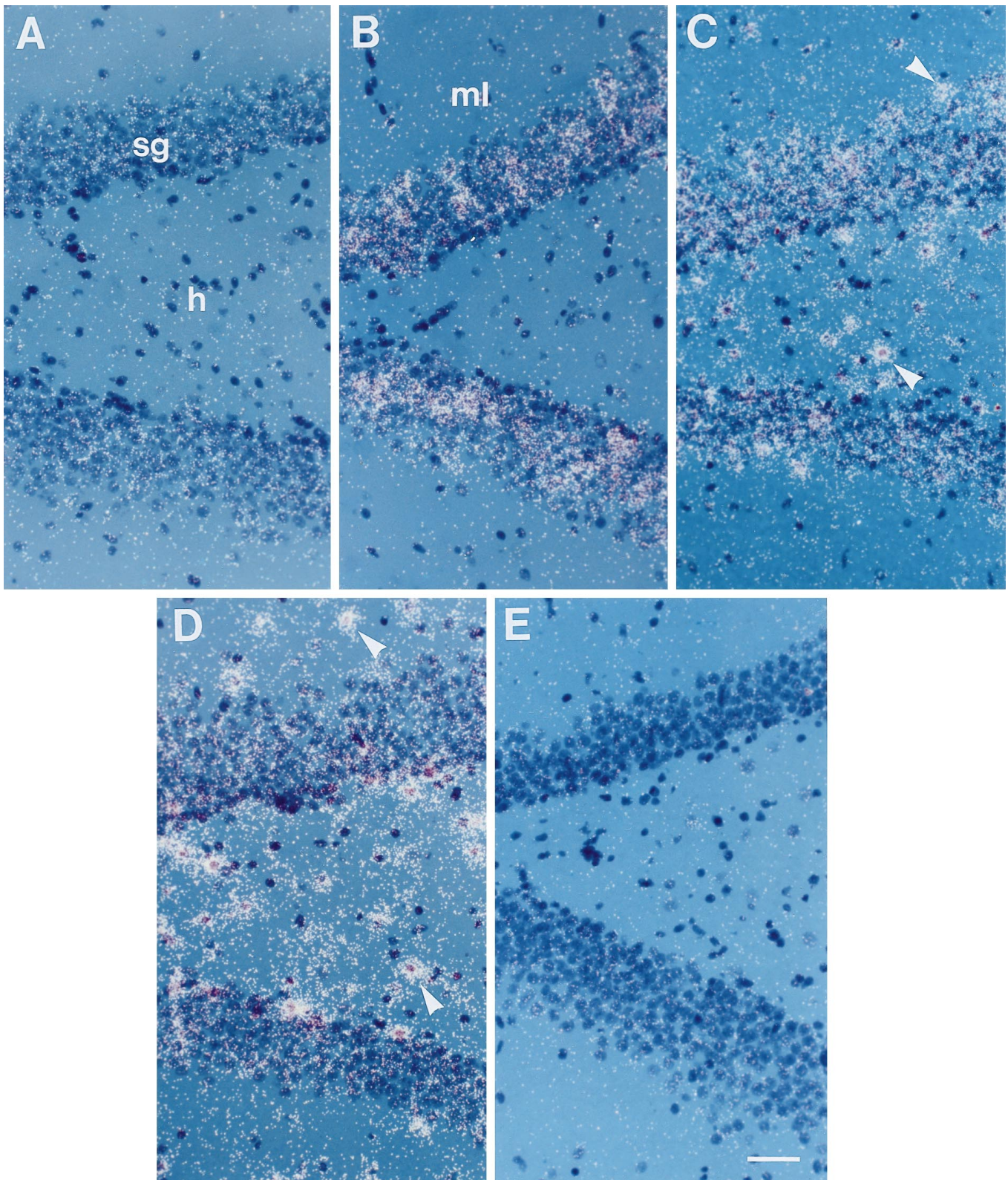


Figure 4. Kainate-induced increase of HB-EGF mRNA in dentate gyrus. Dark-field photomicrographs showing the cellular distribution of HB-EGF mRNA in sections through the dentate gyrus in a saline-injected control rat (*A*) and in rats killed 3 (*B*), 6 (*C*), 24 (*D*), and 48 hr (*E*) after kainate administration. Note that kainate treatment initially increased HB-EGF cRNA hybridization in the granule cell layer [stratum granulosum (*sg*)] at the 3 hr time point (*B*). However, from 6 to 24 hr after kainate injection, numerous labeled cells (*arrowheads* indicate several examples) were distributed throughout the hilus (*h*) and molecular layer (*ml*) (*C*, *D*). By 48 hr, cellular expression of HB-EGF mRNA was barely detectable within the dentate gyrus (*E*). Scale bar, 50 μ m.

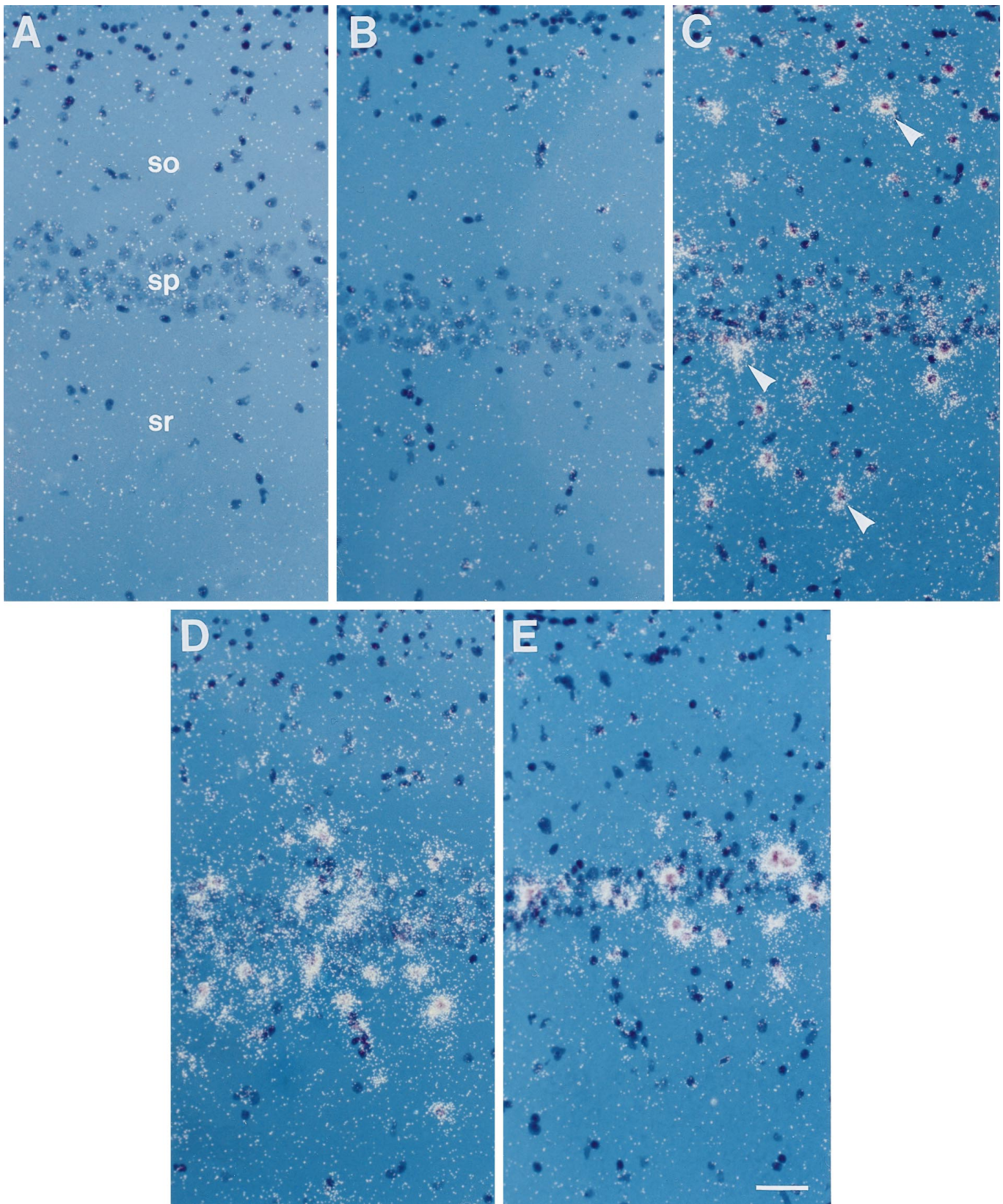


Figure 5. Kainate-induced increase of HB-EGF mRNA in hippocampal region CA1. Dark-field photomicrographs show the autoradiographic localization of HB-EGF cRNA *in situ* hybridization in the CA1 region of hippocampus in a saline-injected control rat (*A*) and in rats killed 3 (*B*), 12 (*C*), 24 (*D*), and 48 hr (*E*) after kainate administration. Note that by 12 hr after kainate injection, labeling for HB-EGF mRNA was induced in stratum pyramidale (*sp*) and particularly within numerous cells scattered throughout the stratum oriens (*so*) and stratum radiatum (*sr*) molecular layers (*C*, arrowheads indicate examples of labeled somata). By 24 hr, densely labeled cells were situated in close proximity to the pyramidal cell layer (*D*). At 48 hr after kainate treatment, perikarya expressing HB-EGF mRNA were intermittently dispersed within and along the edges of CA1 stratum pyramidale (*E*). Scale bar, 50 μ m.

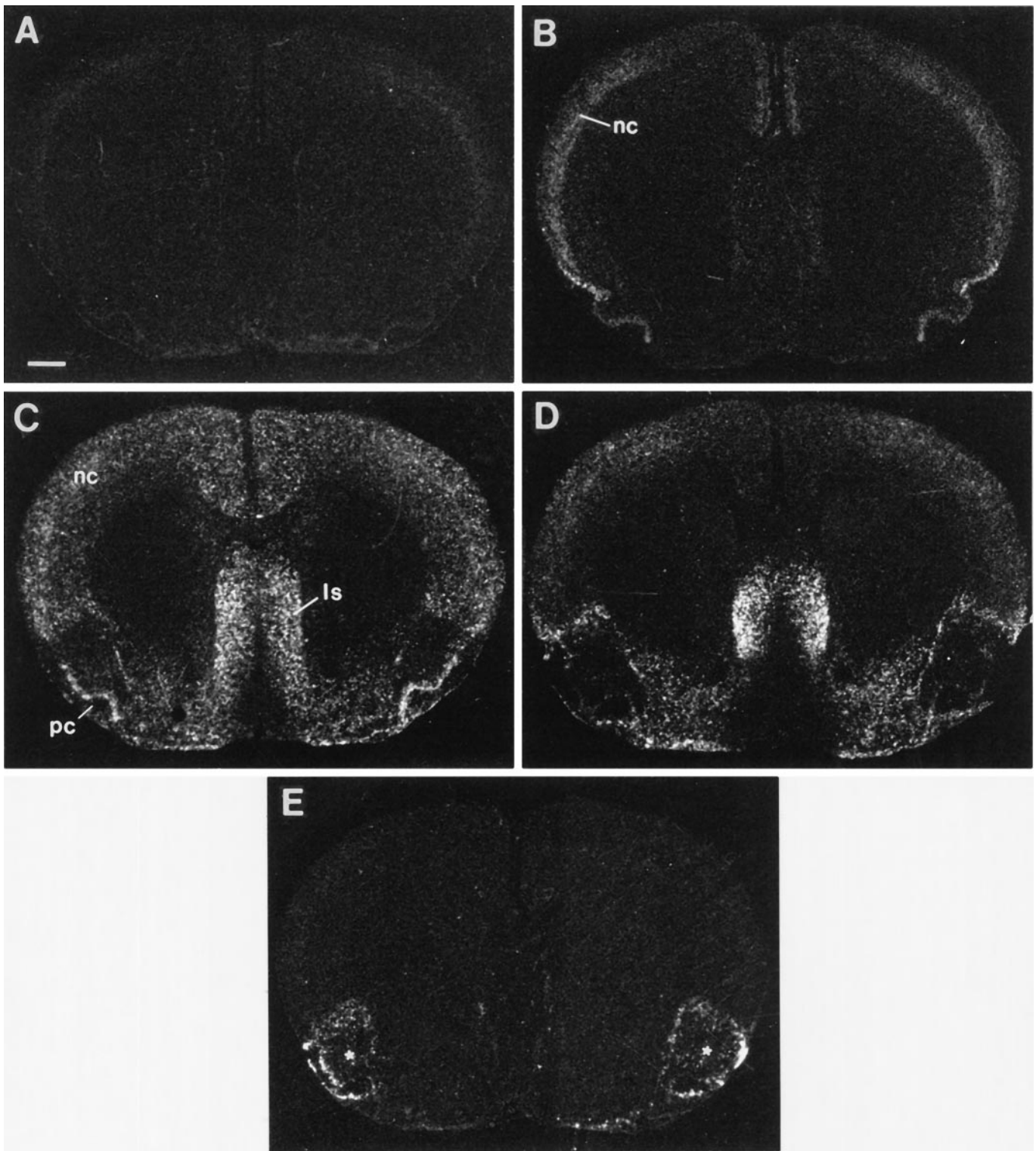


Figure 6. Kainate-induced expression of HB-EGF mRNA in septum and cortex. Autoradiograms demonstrating the regional distribution of HB-EGF cRNA hybridization in coronal sections through the septal nuclei in a saline-injected control rat (*A*) and in rats killed at 3 (*B*), 12 (*C*), 24 (*D*), and 48 hr (*E*) after kainate administration. Expression of HB-EGF mRNA is induced in the lateral septal nuclei (*ls*), neocortex (*nc*), and piriform cortex (*pc*) by 3 hr after kainate treatment (*B*), with peak expression levels found between 12 and 24 hr after kainate treatment (*C*, *D*). By 48 hr (*E*), labeling is restricted to scattered cells within the lateral septum and piriform cortex and to degenerating areas of perirhinal cortex (*asterisks*). Film exposure, 10 d; section thickness, 10 μm . Scale bar, 1000 μm .

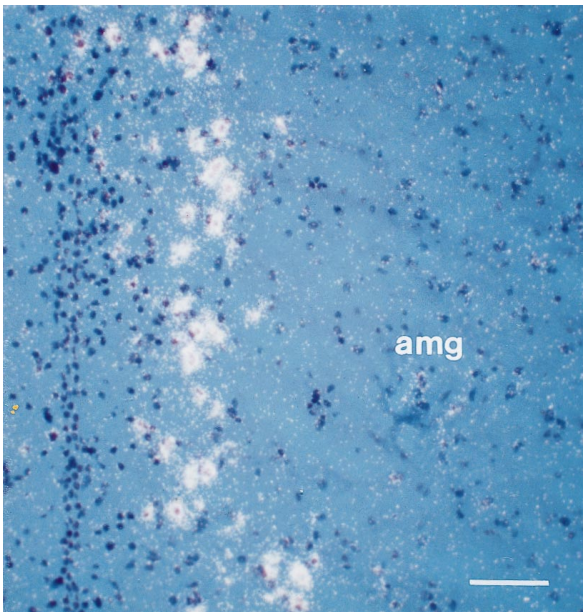


Figure 7. Kainate-induced expression of HB-EGF mRNA in extra-hippocampal regions of neuronal cell loss. Dark-field photomicrograph shows HB-EGF cRNA-hybridizing cells lining the medial edge of the amygdaloid complex (*amg*) in a rat killed 48 hr after kainate administration. Scale bar, 90 μ m.

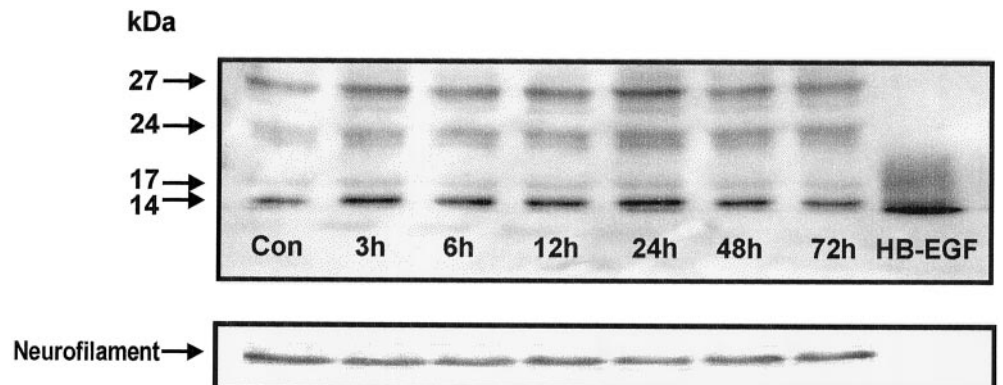
kainate injection. These findings demonstrate that HB-EGF expression is highly responsive to neuronal activity/injury consistent with roles in neuronal plasticity and injury responses.

The HB-EGF mRNA expression that occurred 24–48 hr after kainate injection in regions of neuronal degeneration (CA1 region of hippocampus, amygdala, piriform and entorhinal cortices) may represent expression by reactive astrocytes, activated microglia, or infiltrating macrophages. Indeed, HB-EGF was originally identified as a macrophage-derived secretory product (Higashiyama et al., 1991). The appearance of reactive astrocytes and infiltration of microglia has previously been connected with kainic acid-induced neuronal damage (Sperk et al., 1983; Marty et al., 1991; Sperk, 1994). Although microglia may participate in inflammatory responses and can phagocytose damaged cells, increasing data suggest that microglia also produce neurotrophic substances. For example, activated microglia produce both bFGF and TNF α , both of which have been shown to protect neurons against various insults (Mattson et al., 1989; Cheng and Mattson, 1991; Cheng et

al., 1994). Furthermore, it was recently reported that microglia express TGF- β 1 in the hippocampus, particularly in areas undergoing neurodegeneration after a systemic kainate injection (Morgan et al., 1993). These findings led to the hypothesis that recruitment of microglia and secretion of trophic factors may be important for early repair processes after neuronal injury. Similarly, HB-EGF induction after kainate-induced seizures may be indicative, in part, of activated microglia or astrocytes that respond to chemotactic factors released by dying neurons. In this view, the upregulation of HB-EGF is an adaptive response to neuronal activation and/or damage because induction continues during the period of neuronal degeneration.

The spatial and temporal increases in HB-EGF mRNA levels raise interesting questions regarding mechanisms of both gene and protein regulation. Modulation of HB-EGF mRNA expression was most likely stimulated by seizure-induced neuronal activity and/or cell death elicited by the kainate treatment, because rats that did not exhibit overt convulsive behavior failed to show elevated levels of HB-EGF mRNA or protein (L. A. Opanashuk and K. B. Seroogy, unpublished data). Regulation of some neurotrophic factors, for example BDNF, has previously been tightly linked to an appropriate balance between activation of GABA and glutamate receptors (Zafra et al., 1991). The varying kinetics of regional HB-EGF expression are suggestive of differential mechanisms of gene regulation. In some situations, HB-EGF has been designated as an immediate early gene (Polihronas et al., 1996) and might therefore serve functions similar to BDNF (Hughes et al., 1993; Lauterborn et al., 1996). This may be the case in the dentate gyrus granule cells because increases in HB-EGF protein were evident within 3 hr of kainate administration, a time period too short to permit *de novo* protein synthesis to take place before upregulation of the HB-EGF gene. Furthermore, levels of both the transmembrane and secreted forms of HB-EGF protein were moderately elevated 3 hr after treatment, suggesting that both transcriptional and post-translational mechanisms may be important in the processing of HB-EGF protein in the hippocampus. Alternative mechanisms may be responsible for the more protracted increases in HB-EGF mRNA. For example, other trophic molecules, peptides, or cytokines may mediate the induction of HB-EGF mRNA. In support of the latter possibility, HB-EGF gene expression was previously shown to be autoregulated (Barnard et al., 1994) and was also upregulated in response to TNF α , thrombin, and TGF α (Yoshizumi et al., 1992; Tan et al., 1994; Dlugosz et al., 1995). Regardless of the mechanisms involved, it still remains to be determined whether increases in

Figure 8. Western blot analysis of HB-EGF protein in rat hippocampus (*top panel*), in a saline-injected control rat (*Con*), and in rats killed at the indicated time points after kainate administration. Protein (100 μ g/lane) was separated by SDS-PAGE, transferred to Hybond (Amersham) membrane, and probed with a polyclonal antibody to HB-EGF (kindly provided by Judith Abraham, Scios Inc.). Proteins were visualized by enhanced chemiluminescence. Recombinant human HB-EGF (10 ng) was used as a positive control. Four bands corresponding to HB-EGF with apparent molecular weights of 27, 24, 17, and 14 kDa are detected in hippocampal extracts. Similar results were obtained in three different rats for each time point. Membranes were also probed with a monoclonal antibody for neurofilament protein (*bottom panel*) to ensure equivalent gel loading.



Similar results were obtained in three different rats for each time point. Membranes were also probed with a monoclonal antibody for neurofilament protein (*bottom panel*) to ensure equivalent gel loading.

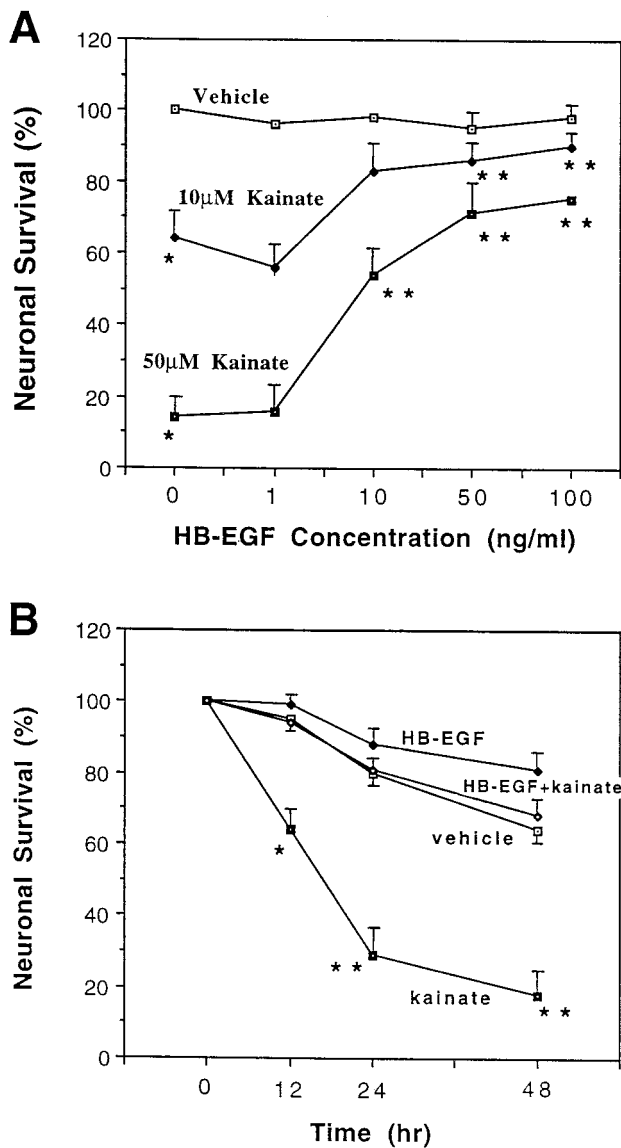


Figure 9. HB-EGF protects against kainate neurotoxicity in hippocampal cell cultures. *A*, Cultures were pretreated with 1–100 ng/ml human recombinant HB-EGF for 16 hr and were then exposed to either 10 or 50 μ M kainate. Neuronal survival was assessed 12 hr after kainate treatment. Values represent the mean \pm SEM of determinations made in five separate experiments. Pair-wise comparisons were made by ANOVA with Scheffé's *post hoc* analysis. * p < 0.01 compared with the value for vehicle-treated cultures not exposed to HB-EGF; ** p < 0.01 compared with values for cultures pretreated with either 0 or 1 ng/ml HB-EGF and then exposed to the same concentration of kainate. *B*, Cultures were pretreated with 100 ng/ml HB-EGF for 16 hr and then exposed to vehicle or 10 μ M kainate. Neuronal survival was assessed at the indicated time points after kainate administration. Values are the mean \pm SEM of determinations made in three separate experiments. Pair-wise comparisons were made by ANOVA with Scheffé's *post hoc* analysis. * p < 0.01, ** p < 0.001 compared with each of the other values at that time point.

HB-EGF mRNA are the result of increased gene transcription rates or message stabilization.

Several findings in the present study suggest that HB-EGF serves an excitoprotective role after seizures. First, levels of HB-EGF mRNA and protein were increased in hippocampus within 3–6 hr of kainate administration. Second, *in situ* hybridization analysis showed that HB-EGF was increased in cells in the

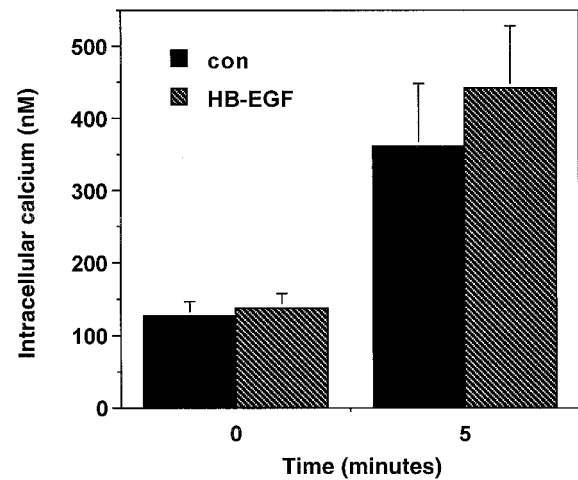


Figure 10. HB-EGF does not affect kainate-induced elevations in $[Ca^{2+}]_i$. Hippocampal cultures were pretreated with 100 ng/ml HB-EGF for 16 hr, and the $[Ca^{2+}]_i$ in neurons was then determined (time 0). Cells were then exposed to 50 μ M kainate for 5 min, and the $[Ca^{2+}]_i$ was again measured in the same neurons. Values are the mean \pm SEM of determinations made in four separate cultures (15–22 neurons per culture).

CA3 and CA1 pyramidal cell layers, cells susceptible to excitotoxic injury. Third, HB-EGF protected cultured hippocampal neurons against kainate toxicity. Collectively, these findings are analogous to previous studies demonstrating that neurotrophic factors induced by insults that include an excitotoxic component (e.g., ischemia) can protect neurons against excitotoxicity (for review, see Mattson, 1996). For example, bFGF, NGF, BDNF, platelet-derived growth factor, and TNF α are induced by seizures and other insults (Gall and Isackson, 1989; Gall et al., 1991, 1994; Isackson et al., 1991; Dugich-Djordjevic et al., 1992; Rocamora et al., 1992; Iihara et al., 1994; Lauterborn et al., 1994; Riva et al., 1994; Bruce et al., 1996); each factor can protect cultured hippocampal neurons against excitotoxic and metabolic insults (Mattson et al., 1989; Cheng and Mattson, 1991, 1994, 1995; Cheng et al., 1994). Administration of bFGF (Nozaki et al., 1993), GDNF (Martin et al., 1995), NGF (Shigeno et al., 1991), or BDNF (Beck et al., 1994) into the brains of rodents protects neurons against excitotoxic and/or ischemic injury, demonstrating their anti-excitotoxic activities *in vivo*. Although it remains to be established whether HB-EGF exhibits neuroprotective actions *in vivo*, its increased expression in response to seizures and its ability to protect cultured neurons against kainate toxicity are consistent with this possibility.

Our cell survival and calcium imaging analyses, in cultured hippocampal neurons exposed to kainate, suggest both similarities and differences in the excitoprotective actions of HB-EGF and other previously characterized neurotrophic factors. Pretreatment for 16 hr with HB-EGF was required for protection against kainate toxicity; shorter pretreatments (2–6 hr) were ineffective. This requirement for pretreatment is similar to bFGF (Mattson et al., 1989), BDNF (Cheng and Mattson, 1994), and TNF α (Cheng et al., 1994), which appear to act by altering gene expression. In particular, the neuroprotective mechanisms of bFGF, BDNF, and TNF α appear to involve modulation of the expression of gene products involved in regulation of calcium homeostasis and/or free radical metabolism (Cheng et al., 1994; Mattson et al., 1995). We reported previously that pretreatment of cultured hippocampal neurons with bFGF results in differential

modulation of calcium responses to activation of NMDA and AMPA/kainate receptors such that NMDA responses are reduced and AMPA/kainate responses enhanced (Cheng et al., 1995). In the present study we found that calcium responses to kainate were neither suppressed nor enhanced in hippocampal neurons pretreated for 16 hr with HB-EGF. Although considerable further work will be required to establish the excitoprotective mechanism of action of HB-EGF, it appears not to result simply from a suppression of kainate-induced elevation of $[Ca^{2+}]_i$. Another possible mechanism whereby HB-EGF might protect neurons is by suppressing accumulation of reactive oxygen species. Indeed, it was reported recently that EGF can prevent oxidative stress-induced cell death in cultured cortical neurons (Yamada et al., 1995).

In addition to the potential roles of HB-EGF in seizure responses, the normal postnatal expression of HB-EGF in rat forebrain, and in the cortex in particular, suggests possible roles for this factor in modulating processes such as cell survival, neurite outgrowth, and synaptogenesis. Levels of HB-EGF mRNA were maximal during the early postnatal period, a time during which synaptogenesis is occurring. Previous studies have shown that EGF promotes both cell survival and neurite outgrowth of cultured neocortical neurons (Morrison et al., 1987, 1988; Kornblum et al., 1990). Considering that levels of EGF mRNA and protein are relatively low in cortex during the early postnatal period (Fallon et al., 1984; Kudlow et al., 1989; Lazar and Blum, 1992), and that HB-EGF activates the EGF receptor [which is present at high levels during early brain development (Seroogy et al., 1994, 1995; Kornblum et al., 1997a)], it would not be unlikely that endogenous HB-EGF could regulate neurite outgrowth and synaptogenesis *in vivo*. Trophic actions on both neurons and glia are also suggested by preliminary *in vitro* data showing that HB-EGF induces the proliferation of astrocytes and pluripotent precursor cells and increases the survival of neurons derived from the cortex of perinatal rodents (Kornblum et al., 1997b) and induces the tyrosine phosphorylation of EGF-R in primary cultures of rat cerebral astrocytes (Elenius et al., 1997).

Consistent with a role for HB-EGF as a developmental and/or maintenance factor, EGF-R has been detected in both neonatal and adult hippocampus and neocortex (Gomez-Pinilla et al., 1988; Faundez et al., 1992; Tucker et al., 1993; Okano et al., 1996; Seroogy, unpublished data). Expression of HB-EGF persists in the rat neocortex, albeit at relatively low levels, and in hippocampal dentate gyrus in the adult brain. Because of the comparatively low levels of EGF in the adult brain, our data suggest important roles for HB-EGF in both the normal and injured adult CNS. It should be noted that in contrast to our data, Mishima et al. (1996) recently reported widespread expression of HB-EGF in most neuronal and glial cells throughout the normal adult rat brain. Our data strongly suggest a more restricted expression of HB-EGF in adult brain consistent with actions on a more limited set of neural populations.

Although little is known about the function of HB-EGF in the CNS, findings in this study implicate various potential roles for HB-EGF in response to limbic seizure activity. Considering that HB-EGF expression has been closely associated with wound healing and tissue repair in many tissues (Marikovsky et al., 1993), it could be predicted that this trophic molecule participates in similar responses in the injured nervous system. Overall, the present findings suggest that HB-EGF serves as an injury-inducible maintenance/neuroprotective factor involved in early

cellular responses and tissue remodeling events associated with seizures in the mammalian CNS.

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