Interleukin 3 Prevents Delayed Neuronal Death in the Hippocampal CA1 Field

By Tong-Chun Wen,* Junya Tanaka,‡ Hui Peng,* Junzo Desaki,* Seiji Matsuda,* Nobuji Maeda,‡ Hiroko Fujita,* Kohji Sato,* and Masahiro Sakanaka*

*From the *Department of Anatomy and the ‡Department of Physiology, Ehime University School of Medicine, Shigenobu, Ehime, 791-0295 Japan*

Summary

In the central nervous system, interleukin (IL)-3 has been shown to exert a trophic action only on septal cholinergic neurons in vitro and in vivo, but a widespread distribution of IL-3 receptor (IL-3R) in the brain does not conform to such a selective central action of the ligand. Moreover, the mechanism(s) underlying the neurotrophic action of IL-3 has not been elucidated, although an erythroleukemic cell line is known to enter apoptosis after IL-3 starvation possibly due to a rapid decrease in Bcl-2 expression. This in vivo study focused on whether IL-3 rescued noncholinergic hippocampal neurons from lethal ischemic damage by modulating the expression of Bcl-x_L, a Bcl-2 family protein produced in the mature brain. 7-d IL-3 infusion into the lateral ventricle of gerbils with transient forebrain ischemia prevented significantly hippocampal CA1 neuron death and ischemia-induced learning disability. TUNEL (terminal deoxynucleotidyltransferase–mediated 2′-deoxyuridine 5′-triphosphate-biotin nick end labeling) staining revealed that IL-3 infusion caused a significant reduction in the number of CA1 neurons exhibiting DNA fragmentation 7 d after ischemia. The neuroprotective action of IL-3 appeared to be mediated by a postischemic transient upregulation of the IL-3R α subunit in the hippocampal CA1 field where IL-3Rα was barely detectable under normal conditions. In situ hybridization histochemistry and immunoblot analysis demonstrated that Bcl-x_L mRNA expression, even though upregulated transiently in CA1 pyramidal neurons after ischemia, did not lead to the production of Bcl-x_L protein in ischemic gerbils infused with vehicle. However, IL-3 infusion prevented the decrease in Bcl-x_L protein expression in the CA1 field of ischemic gerbils. Subsequent in vitro experiments showed that IL-3 induced the expression of Bcl-x_L mRNA and protein in cultured neurons with IL-3Rα and attenuated neuronal damage caused by a free radical–producing agent $FeSO_4$. These findings suggest that IL-3 prevents delayed neuronal death in the hippocampal CA1 field through a receptor-mediated expression of Bcl-x_L protein, which is known to facilitate neuron survival. Since IL-3Rα in the hippocampal CA1 region, even though upregulated in response to ischemic insult, is much less intensely expressed than that in the CA3 region tolerant to ischemia, the paucity of IL-3R interacting with the ligand may account for the vulnerability of CA1 neurons to ischemia.

Key words: interleukin 3 • transient forebrain ischemia • DNA fragmentation • receptor • Bcl-x_L

Interleukin (IL)-3, identified as a T cell–derived cytokine, affects the proliferation of hematopoietic progenitor cells at early stages of their differentiation (1). It also possesses a potent macrophage-activating action which is associated with T cell–dependent immune responses (2, 3) and stimulates the differentiation of stem cells and various cell types originating in bone marrow (4, 5). A single receptor for IL-3 has been postulated (6), and IL-3 acts on cells possibly through binding to the surface receptor. The receptor is composed of α and β subunits; the α subunit is responsible for binding of IL-3, and the ligand-activated α subunit is associated with the β subunit, which transmits signals across the plasma membrane (7).

Recent studies have demonstrated the possibility that IL-3 functions in the central nervous system. Frei et al. (8, 9) found that IL-3 stimulates the growth and proliferation of microglial cells in vitro. Moroni and Rossi (10) demonstrated that IL-3 facilitates significantly the survival of sensory neurons and stimulates the formation of the neural network in vitro. Kamegai et al. (11) also showed that IL-3 promotes the process extension of cultured cholinergic

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neurons without affecting somatostatin release, glutamate decarboxylase activity, or 2′,3′-cyclic nucleotide 3′-phosphodiesterase activity and rescues axotomized cholinergic neurons from degeneration, suggesting a protective effect of IL-3 specifically on cholinergic neurons. However, the mechanism(s) by which IL-3 supports neurons has not yet been determined. Moreover, it remains unsolved whether or not IL-3 exhibits a trophic action on neuronal cells other than septal cholinergic neurons in vivo. Since IL-3R and its associated antigens are distributed in a variety of brain regions, including the hippocampus, despite unknown localization of the ligand in the brain (7, 12–14) IL-3 is expected to exert a central action in brain areas apart from the septum containing cholinergic neurons.

Upon withdrawal of IL-3, the erythroleukemic cell line TF-1 has been shown to enter apoptosis as a result of decreased production of Bcl-2 mRNA and protein (15). Furthermore, the expression of Bcl-2 protein is known to attenuate oxygen free radical cytotoxicity (16, 17). Since Bcl-x_L is an apoptosis-inhibiting agent of the Bcl-2 family (18–20) and is expressed mainly in the mature brain (21, 22), we speculated that IL-3 exhibited neurotrophic action by inducing Bcl-x_L expression. In this in vivo study, we investigated whether IL-3 rescued hippocampal CA1 neurons from lethal ischemic damage, using the gerbil forebrain ischemia model. This animal model exhibits an invariable CA1 neuron damage and abnormal behavior 7 d after ischemia, and thus appears to be suitable for studying the neurotrophic actions of peptide growth factors in vivo (23–25). Although excitotoxins, including *N*-methyl-d-aspartate and kainic acid, can also be used to damage hippocampal CA1 and/or CA3 neurons (26, 27), the excitotoxin-induced neuronal degeneration is not necessarily identical to ischemic neuronal death, which is caused by multiple factors such as free radicals, excess extracellular glutamate, hypoxia, and glucose deficiency (28–31), and it may be a rather artificial model for neuron death compared with the gerbil ischemia model. Therefore, we chose the gerbil ischemia model as a tool to examine the central action of IL-3. In ischemic gerbils with or without intracerebroventricular IL-3 infusion, CA1 neuron density, volume of the CA1 pyramidal cell layer, learning ability, CA1 synapse number, DNA fragmentation of CA1 neurons, and local expressions of IL-3Rα and Bcl-x_L were investigated. Subsequently, the culture experiments concentrated on whether or not IL-3 enhanced neuronal survival through a receptor-mediated induction of Bcl-x_L expression and protected neurons against oxidative injury induced by $FeSO_4$. The latter in vitro experiment was based on the notion that oxygen free radicals are, at least in part, responsible for delayed neuronal death in the hippocampal CA1 field (28, 32–34).

Materials and Methods

In Vivo Ischemia Study

Osmotic Minipump Implantation. Male Mongolian gerbils weighing 70–80 g (~12 wk of age) were used in the in vivo study. The following experiments were conducted in accordance with the Guide for Animal Experimentation at Ehime University School of Medicine. The animals were anesthetized with 1.5% halothane in a 4:3 mixture of nitrous oxide and oxygen and placed in a stereotaxic apparatus. An osmotic minipump (model 2001; Alza Corp., Palo Alto, CA) was implanted subcutaneously into the back of each animal, and a needle from the minipump was placed in the left lateral ventricle at the point 1.5 mm anterior, 1.0 mm lateral, and 2.7 mm ventral to bregma as illustrated in the atlas of Thiessen and Yahr (35).

Preischemic Infusion of IL-3. Recombinant murine IL-3 (PeproTech, Inc., Rocky Hill, NJ) was dissolved in 0.05 M PBS containing 0.1% BSA (Sigma Chemical Co., St. Louis, MO). IL-3 (64 or 320 ng/d) or vehicle was infused for 7 d into the left lateral ventricles of normothermic gerbils exposed to 3-min forebrain ischemia ($n = 6$–8 in each group). The molar concentrations of infused IL-3 were similar to those of the other growth factors examined to date in the same gerbil ischemia model (23–25). Sham-operated animals received vehicle infusion ($n = 8$). The infusion was started 2 h before an ischemic insult as described elsewhere (23–25, 36, 37).

Postischemic Infusion of IL-3. To investigate the effect of postischemic treatment with IL-3 on delayed neuronal death, 5.3 or 26.5 ng of IL-3 in 2 μl of vehicle was injected into the left lateral ventricle through a Hamilton syringe immediately after 3-min forebrain ischemia, and then IL-3 (64 or 320 ng/d) was continuously infused for 7 d into the cerebral ventricles as described above ($n = 8$ in each group). In control experiments, ischemic animals received vehicle infusion ($n = 8$).

Occlusion of the Common Carotid Arteries. Occlusion of the common carotid arteries was performed as described previously (38). In brief, both common carotid arteries were exposed through a ventral midline incision and separated carefully from the adjacent veins and nerves while the gerbil was anesthetized as described above. Immediately after the termination of inhalation anesthesia, the common carotid arteries were clamped for 3 min with aneurysm clips.

During forebrain ischemia, brain temperature has been shown to fall differently in individual animals, thereby affecting the number of viable CA1 neurons after ischemia (38, 39). To avoid the effect of unstable brain temperature on ischemic neuronal loss, we kept brain and rectal temperatures at 37.0 ± 0.2°C while clamping the common carotid arteries (23–25, 36–39). This enabled us to induce an invariable neuronal damage in the hippocampal CA1 field even after a 3-min ischemic insult (23–25, 36, 37) and to evaluate accurately the in vivo effects of IL-3 on delayed neuronal death.

Passive Avoidance Task. 7 d after forebrain ischemia, the gerbils were trained in a conventional step-down passive avoidance apparatus that was divided into a safe platform and a foot-shock chamber with a stainless steel grid floor (40). Each animal was placed initially on the safe platform, but if the gerbil stepped down onto the grid floor, it received a foot shock. After repeated movements between the platform and the grid, the gerbil eventually stayed on the platform. This training session lasted 300 s. 24 h later, the gerbil was again placed on the safe platform while the shock generator was turned off, and the response latency, i.e., the time until it stepped down onto the grid floor, was measured. This test session also lasted 300 s. Each animal received only one training session and only one test session (23–25, 36, 37).

Histopathological Study of Hippocampal CA1 Region. 1 h after the passive avoidance experiments, each animal was anesthetized with pentobarbital, and the osmotic minipump was disconnected from

the needle placed in the left lateral ventricle. Bromphenol blue was injected through the needle to ascertain the infusion of IL-3 or vehicle into the cerebral ventricles. The animals were perfused transcardially with 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4; reference 38). A brain region including the dorsal hippocampus from 0.5 to 1.5 mm posterior to bregma was excised and kept in the same fixative overnight at 4°C. Four serial coronal sections (50 μm thick at the level of 1.0–1.2 mm posterior to bregma) from the brains of gerbils infused with IL-3 or vehicle before ischemia were cut with a microslicer (Dosaka EM Co., Ltd., Kyoto, Japan). The remaining dorsal hippocampus and the dorsal hippocampus of gerbils infused with IL-3 or vehicle after ischemia were embedded in paraffin, and 5-μm serial frontal sections were cut and stained with 0.1% cresyl violet. All neurons with intact morphological appearance along 1 mm linear length of the hippocampal CA1 field in six serial paraffin sections (1.20–1.23 mm posterior to bregma) were counted in each animal. The volume of the pyramidal cell layer along 1.5 mm linear length of the hippocampal CA1 field was also measured in the six paraffin sections from each animal (measuring system MS3000; Mitsubishi Chemical Corp., Tokyo, Japan). For electron microscopy, the 50-μm sections were postfixed with 1% osmium tetroxide for 30 min, dehydrated with a graded series of ethanol, transferred to propylene oxide, and embedded in epoxy resin. The strata moleculare, radiatum, pyramidale, and oriens of the CA1 field were identified in semithin sections stained with 1% toluidine blue, and ultrathin sections 70-nm thick were cut with a Reichert-Jung ultramicrotome (C. Reichert Optische Werke AG, Vienna, Austria) and mounted on single-slot (2 × 0.5 mm) grids coated with formvar film. They were subjected to dual staining with uranyl acetate and lead citrate, and examined with a transmission electron microscope (model H-12A; Hitachi Ltd., Tokyo, Japan). Electron micrographs of the central area ($15\ \mu m \times 18.75\ \mu m = 280\ \mu m^2$) of each stratum were taken, and intact synapses with thick apposed membranes and synaptic vesicles in the area were counted. The fine structure of the stratum pyramidale in the CA1 field was also observed in ischemic gerbils with or without IL-3 infusion.

In Situ Detection of DNA Fragmentation (TUNEL Staining). To analyze quantitatively the changes in the nuclear chromatin of the hippocampal CA1 neurons as revealed by electron microscopy, we conducted in situ detection of DNA fragmentation using TUNEL (terminal deoxynucleotidyltransferase–mediated 2′-deoxyuridine 5′-triphosphate-biotin nick end labeling)[1] staining. Animals infused with IL-3 (64 or 320 ng/d) or vehicle (n = 6–8 in each group) before ischemia were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) under pentobarbital anesthesia after passive avoidance experiments. The number of neurons and the volume of the pyramidal cell layer of the hippocampal CA1 region in each animal were determined as described above, and two additional paraffin sections from each animal were processed for TUNEL staining to estimate the number of degenerating CA1 neurons after 7 d infusion of IL-3 or vehicle. In brief, the sections were deparaffinized in xylene and in a graded series of ethanol; incubated with 20 μg/ml of proteinase K (Sigma Chemical Co.) in 0.05 M PBS for 15 min; incubated with equilibration buffer (In Situ Apoptosis Detection kit; Oncor Inc., Gaithersburg, MD) for 5 min, and then incubated in a mixture of terminal deoxynucleotidyltransferase and reaction buffer containing digoxigenin-2′-deoxyuridine 5′-triphosphate-biotin in a humidified chamber for 1 h at 37°C; washed in wash buffer for 10 min and incubated with anti-digoxigenin peroxidase for 1 h at room temperature; and rinsed three times in PBS for 5 min each, and exposed to 0.05% diaminobenzidine and 0.02% hydrogen peroxide. All TUNEL-positive neurons along 1 mm linear length of the CA1 field in the two serial coronal sections were counted, and the mean number of positive neurons was calculated in each animal.

Immunohistochemical Analysis of Ischemic Hippocampal CA1 Neurons with an Anti–IL-3R Antibody. At 1, 2, 4, and 7 d after 3-min ischemia, the gerbils with or without IL-3 infusion were anesthetized with pentobarbital and perfused transcardially with 4% paraformaldehyde (n = 4 in each group). The brains were excised and immersed overnight in 0.1 M phosphate buffer (pH 7.4) containing 30% sucrose at 4°C. Serial frozen sections 30 μm thick were cut with a cryostat and processed for immunohistochemistry with an affinity-purified rabbit antibody against IL-3Rα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). In brief, the sections were washed with 0.1 M PBS for 30 min and incubated with the IL-3Rα antibody diluted 1:100 with PBS containing 1% normal swine serum and 0.1% Triton X-100 for 48 h at 4°C; incubated with biotinylated anti–rabbit IgG and peroxidase-conjugated streptavidin (DAKO A/S, Glostrup, Denmark) overnight at 4°C; and subjected to a modified version of the cobalt-glucose oxidase-diaminobenzidine intensification method (41). In control experiments, cryosections were incubated with the IL-3Rα antibody, which had been adsorbed with an excess of the homologous antigen (Santa Cruz Biotechnology, Inc.), and were processed as described above. Some sections were directly incubated with the second antibody without any preincubation.

Immunoblot Analysis of the Hippocampal CA1 Field with IL-3Rα and Bcl-x_L Antibodies. Homogenates of the hippocampal CA1 field were obtained from sham-operated and ischemic gerbils 1, 2, and 4 d after 3-min ischemia (n = 4 in each group). They were solubilized in a sample solution containing 2% SDS, and an equal amount of protein (40 μg) in the homogenates was electrophoresed in individual lanes using 6% polyacrylamide gel in the Laemmli's buffer system (42). Protein concentration was determined by BCA protein assay reagent (Pierce Chemical Co., Rockford, IL) with BSA as a standard. The electrophoretic bands were transferred to nitrocellulose sheets (43) and immunoblotted with the IL-3Rα antibody or with a mouse mAb against Bcl-x_L protein (Transduction Laboratories, Inc., Lexington, KY). Anti–rabbit or anti–mouse IgG coupled with alkaline phosphatase (Promega Corp., Madison, WI) was used for the second immunoreaction. The immunoreactive bands were visualized as described elsewhere (44). Prestained molecular weight markers were purchased from Bio-Rad Laboratories (Richmond, CA). Immunoblot analysis with the Bcl-x_L antibody was also conducted using homogenates of the ischemic hippocampal CA1 field treated with IL-3. The immunoblot analyses were repeated three times.

In Situ Hybridization Histochemistry for Detection of Bcl-x_L mRNA. To detect gerbil Bcl-x_L mRNA, we determined a DNA sequence specific for gerbil Bcl-x_L using reverse transcription (RT)-PCR. Based on this data, an oligonucleotide probe was designed and synthesized (Pharmacia Biotech AB, Uppsala, Sweden). The probe (5′-GGTGGTCATTCAGGTAGGTGGCCATCCAAC TTGCG-3′) corresponds to the bases 498–532 of mouse Bcl-x_L mRNA. Computer-assisted homology searches (GenBank and

[1]*Abbreviations used in this paper:* MAP2, microtubule-associated protein 2; RT, reverse transcription; TBS, Tris-buffered saline; TUNEL, terminal deoxynucleotidyltransferase–mediated 2′-deoxyuridine 5′-triphosphate-biotin nick end labeling.

EMBL updates, December 1997) showed that the probe shared no homology with any sequences contained in the gene bank. 1, 2, 4, and 7 d after 3-min ischemia, the gerbils were anesthetized with pentobarbital and decapitated. The brains were excised and immediately frozen on powdered dry ice. Serial sections 15-μm thick were cut on a cryostat, thaw-mounted onto polysilane-coated slides, and stored at −80°C until use. The sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 15 min at room temperature, rinsed three times (5 min each) in 4× SSC (pH 7.2; 1× SSC contained 0.15 M sodium chloride and 0.015 M sodium citrate), and dehydrated through a graded series of ethanol. The sections were then treated with chloroform for 5 min and immersed in 100% ethanol twice for 5 min each. Hybridization was performed by incubating the sections with a buffer (4× SSC, 50% deionized formamide, 0.12 M phosphate buffer, pH 7.2, Denhardt's solution, 2.5% tRNA, 10% dextran sulfate) containing [α-^{35}S]dATP (37–55.5 TBq/mmol; NEN Research Products, Boston, MA)–labeled probes for 24 h at 41°C (1–2 × 10^7 dpm/ml, 0.3 ml/slide). After hybridization, the sections were rinsed in 1× SSC (pH 7.2) for 10 min, followed by rinsing three times in 1× SSC at 55°C for 20 min each. The sections were then dehydrated through a graded series of ethanol and exposed at room temperature for 2 d using an image analyzer (model BAS-3000; Fuji Photo Film Co., Tokyo, Japan), followed by coating with NBT-5 emulsion (Eastman Kodak Co., Rochester, NY) diluted 1:1 with water. They were exposed to the emulsion at 4°C for 4 wk in a tightly sealed dark box, developed in D-19 developer (Eastman Kodak Co.), and counterstained with thionin solution. Relative changes in Bcl-x_L mRNA expression were quantified by determining, with the NIH image analysis system, the ratio of the optical density in the ischemic CA1 field to that in the control (sham-operated) CA1 field.

In Vitro Culture Experiments

Cortical and Hippocampal Neuron Cultures. The cerebral cortex and hippocampus of 17-d-old rat embryos were aseptically dissected out. Cortical and hippocampal neurons were dissociated from the tissues as described elsewhere (44). The dissociated cells were seeded on 24-well plastic plates (Corning Glass Works, Corning, NY) coated with poly-l-lysine at a density of 3–5 × 10^4 cells/cm^2. The cells were cultured at 37°C in DMEM (Iwaki Glass Co., Ltd., Tokyo, Japan) supplemented with 10% FCS (GIBCO BRL, Gaithersburg, MD) under an atmosphere of 5% CO_2 and nearly 100% humidity.

IL-3 Treatment. On day 2 of culture, the culture medium was replaced with serum-free DMEM containing 0–15 ng/ml of IL-3, 20 mM Hepes, 0.45% glucose, 5 μg/ml pig insulin (Boehringer Mannheim, Mannheim, Germany), 5 μg/ml human transferrin (Boehringer Mannheim), 5 ng/ml sodium selenite (Boehringer Mannheim), 25 nM progesterone (Sigma Chemical Co.), and 0.1% BSA. The concentrations of IL-3 chosen in these in vitro studies were similar to those used in the study of Kamegai et al. (11). The cultures were maintained for 3 d.

Immunocytochemical Staining of IL-3Rα in Cultured Neurons. On day 4 of culture with or without IL-3 treatment, the neurons were rinsed and fixed with 4% paraformaldehyde in PHEM buffer (60 mM Pipes, 25 mM Hepes, 1 mM EDTA, and 2 mM $MgCl_2$, pH 6.9; reference 45) and processed for immunocytochemical staining with the IL-3Rα antibody. In brief, the cultured neurons were washed with 20 mM Tris-buffered saline (TBS) and incubated for 30 min at 37°C with the primary antibody diluted 1:100 with TBS containing 1% normal goat serum and 0.02% Triton X-100. After rinsing with TBS containing 0.02% Triton X-100, the neurons were incubated with alkaline phosphatase–conjugated goat anti–rabbit IgG (Promega Corp.) for 1 h at 37°C. The IL-3Rα immunoreactivity was visualized as described elsewhere (44).

Microtubule-associated Protein 2 Staining of Cultured Neurons. One third of the cultured neurons were fixed with PHEM buffer containing 4% paraformaldehyde. They were then processed for immunohistochemical staining with an mAb against microtubule-associated protein 2 (MAP2), which is known to be a specific neuronal marker (Sternberger Monoclonals Inc., Baltimore, MD). In brief, the cultured neurons were washed with TBS for 30 min and incubated with the MAP2 antibody diluted 1:1,000 with TBS containing 1% normal goat serum and 0.3% Triton X-100 for 48 h at 4°C; incubated with biotinylated anti–mouse IgG and peroxidase-conjugated streptavidin (DAKO A/S) overnight at 4°C; and subjected to a modified version of the cobalt-glucose oxidase-diaminobenzidine intensification method (41). MAP2-positive neurons in four randomly selected fields (1 mm^2 per field) of individual cultures were counted.

Immunoblot Analysis of Cultured Neurons. The cells from each well not processed for MAP2 immunohistochemical staining were solubilized in a sample solution containing 2% SDS. For MAP2 immunoblot, the final volume was adjusted to 100 μl, and 15 μl of the sample was electrophoresed into each lane using 6% polyacrylamide gel in the Laemmli's buffer system (42). For Bcl-x_L immunoblot, the final concentration of protein was adjusted to 50 μg/ml, and 20 μl of the sample was electrophoresed. The electrophoretic bands were immunoblotted with the anti-MAP2 or Bcl-x_L antibody. For quantitative evaluation, the immunoreactive bands were subjected to densitometric analysis with a combination of Adobe Photoshop and the NIH image analysis system (46).

Analysis of Bcl-x_L mRNA Expression in Cultured Neurons. Total RNA was extracted from cortical neurons cultured with 0–10 ng/ml of IL-3 using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan), and was treated with DNase. Oligo dT primers together with 3 μg of DNase-treated total RNA and Moloney murine leukemia virus reverse transcriptase (GIBCO BRL) were used to obtain single-strand DNA. PCR was conducted using Taq polymerase (TaKaRa, Tokyo, Japan). The following conditions were used for PCR amplification: cDNA products of the reverse transcription reaction were denatured for 2 min at 94°C before 20 cycles (for β-actin) or 25 cycles (for Bcl-x_L) at 94°C for 1.5 min, 55°C for 1.5 min, and 72°C for 2 min. The RT-PCR products were separated on 3% agarose gel and visualized with ethidium bromide. Quantification of the PCR bands was done using the NIH image analysis system. The following pairs of oligonucleotides corresponding to certain sequences within the coding regions of Bcl-x_L and β-actin genes were used as primers: rat Bcl-x_L primers, 5′ primer (5′-GTAGTGAATGAACTCTTTCGGGAT-3′) and 3′ primer (5′-CCAGCCGCCGTTCTCCTGGATCCA-3′); and rat β-actin primers, 5′ primer (5′-AGAAGAGCTATGAGCTGCCTGACG-3′) and 3′ primer (5′-TACTTGCGCTCAGGAGGAGCAATG-3′).

$FeSO_4$-induced Oxidative Damage. To introduce oxidative damage to cultured neurons from cortex or hippocampus, freshly prepared 10 or 90 μM $FeSO_4$ solution, respectively, was added to the medium on day 3 of culture with or without IL-3 pretreatment (47–51). The cortical neuron cultures were maintained for 2 h and the hippocampal neuron cultures for 24 h at 37°C. MAP2 staining and MAP2 immunoblot analysis were then conducted as described above.

Statistics

All experiments were done blindly with respect to experimental group. The two-tailed Mann-Whitney U-test was used to evaluate the effects of IL-3 in vivo. Statistical analyses for the in vitro culture experiments and in situ hybridization histochemistry were conducted using analysis of variance followed by Fisher's post hoc test (PLSD). All data were represented as mean ± SD.

Results

Effects of IL-3 on CA1 Neuronal Density, Volume of the CA1 Pyramidal Cell Layer, and Response Latency In Vivo. We first investigated CA1 neuronal density, volume of the CA1 pyramidal cell layer, and response latency in sham-operated and ischemic gerbils with vehicle infusion to ascertain that they were significantly reduced by a 3-min ischemic insult. The mean CA1 neuronal density and volume of the pyramidal cell layer along 1.5 mm linear length of the hippocampal CA1 field in sham-operated animals were 248.7 ± 12.4 cells/mm and 0.390 ± 0.02 mm^3/per section, respectively, and those of 3-min ischemic gerbils infused with vehicle alone were 127.3 ± 31.5 cells/mm and 0.268 ± 0.03 mm^3/per section, respectively. The mean response latency in sham-operated animals was 230.8 ± 40.5 s, and that in vehicle-infused ischemic gerbils was 124.5 ± 28.8 s. There were significant differences in CA1 neuronal density (U = 0, P <0.01), volume of the CA1 pyramidal cell layer (U = 0, P <0.01), and response latency (U = 0,

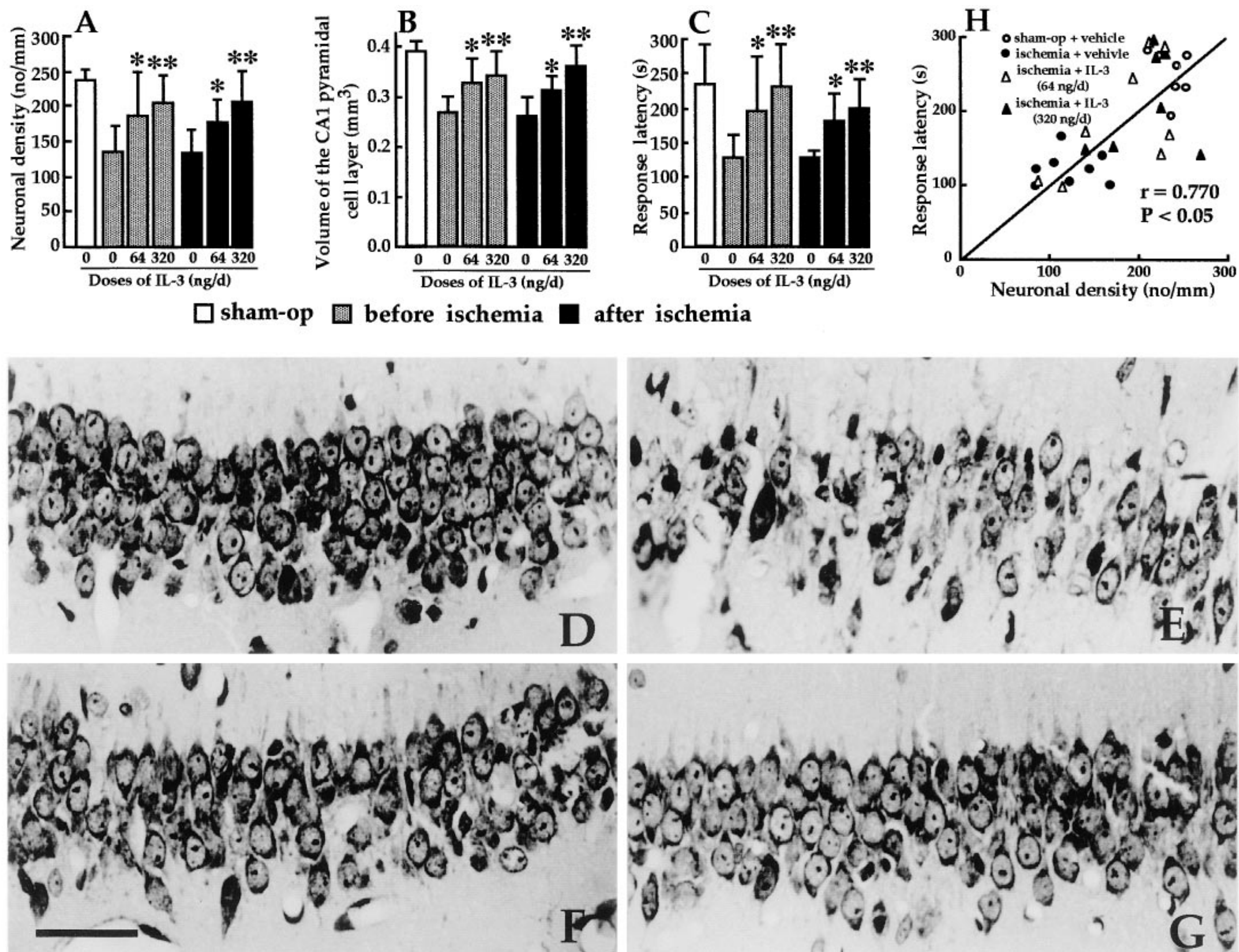

Figure 1. (*A* and *B*) Effects of intracerebroventricular IL-3 infusion on CA1 neuronal density (*A*) and volume of the CA1 pyramidal cell layer (*B*) in ischemic gerbils. The infusion of IL-3 started 2 h before or just after 3-min ischemia, and continued for 7 d. Significant dose-dependent increases in CA1 neuronal density and volume of the CA1 pyramidal cell layer were noted in IL-3–infused ischemic gerbils compared with vehicle-infused ischemic animals. (*C*) Effect of IL-3 infusion on response latency in the passive avoidance task. The infusion of IL-3 significantly prolonged the response latency in a dose-dependent manner in ischemic gerbils compared with vehicle infusion. Each column in *A*–*C* represents mean ± SD (n = 6–8). *P <0.05, **P <0.01, Significantly different from the corresponding vehicle-infused ischemic group (statistical significance tested by the two-tailed Mann-Whitney U-test). (*D*–*G*) Photomicrographs of the hippocampal CA1 field: sham-operated animal infused with vehicle (*D*); ischemic animal infused with vehicle (*E*); ischemic animal infused with 64 ng/d of IL-3 (*F*); ischemic animal infused with 320 ng/d of IL-3 (*G*). Note that the infusion of IL-3, starting 2 h before 3-min ischemia, rescued a significant number of hippocampal CA1 pyramidal neurons. Sections were stained with 0.1% cresyl violet. Bar = 100 μm. (*H*) Positive correlation between the response latency in the passive avoidance task and the neuronal density of the hippocampal CA1 field, as evaluated by Pearson product–moment correlation analysis.

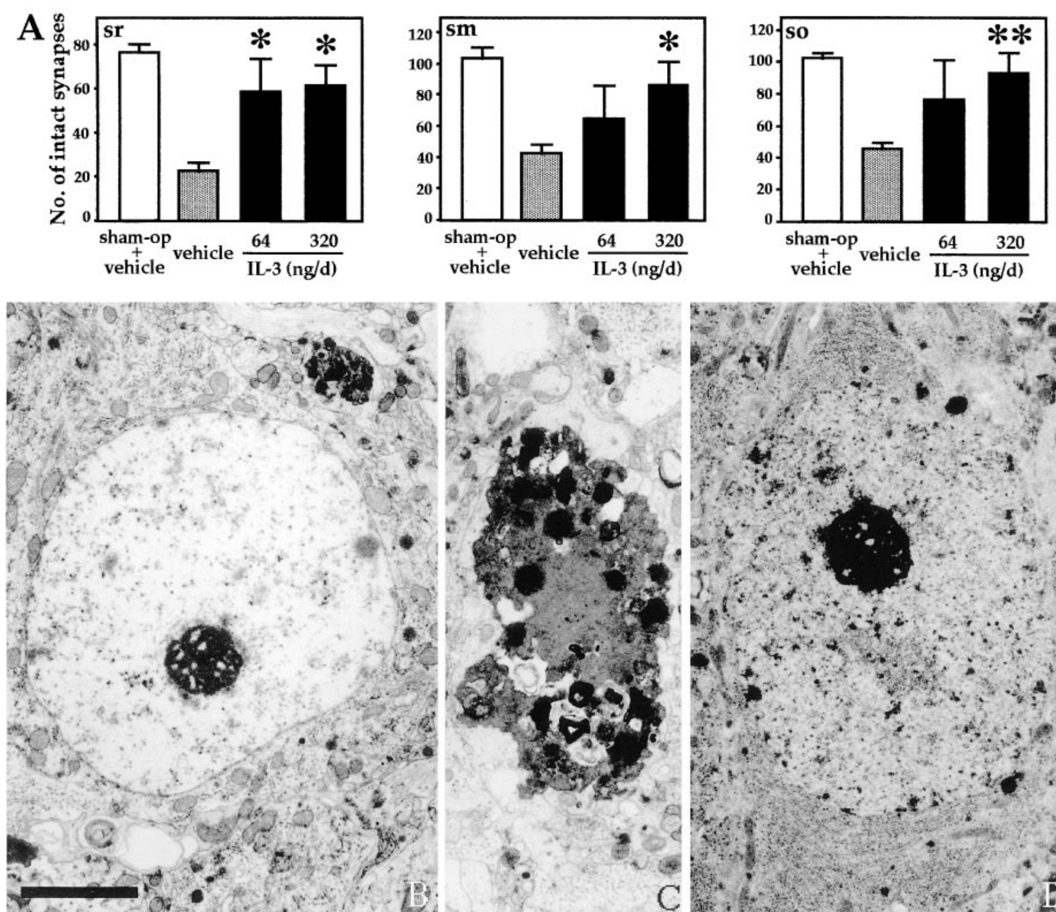

Figure 2. (*A*) Effect of IL-3 on the number of intact synapses in the three strata of the hippocampal CA1 region. The infusion of IL-3 or vehicle was started 2 h before 3-min ischemia, and continued for 7 d. Intact synapses in the strata radiatum (*sr*), moleculare (*sm*), and oriens (*so*) of the hippocampal CA1 region of ischemic gerbils with vehicle infusion were less numerous than in the strata of sham-operated (*sham-op*) animals, and the decrease in intact synapse number in the ischemic hippocampus was prevented significantly when IL-3 (64 or 320 ng/d) was infused into the lateral ventricle for 7 d. Each column represents mean ± SD (n = 6–8). *P <0.05, **P <0.01, Significantly different from the ischemic group with vehicle infusion (statistical significance tested by the two-tailed Mann-Whitney U-test). (*B–D*) Electron micrographs of the soma of ischemic hippocampal CA1 neurons treated with vehicle (*B* and *C*) or 320 ng/d of IL-3 (*D*). Note that the hippocampal CA1 pyramidal neurons in the vehicle- but not IL-3–treated ischemic gerbils were in the course of nuclear chromatin fragmentation and/or condensation (*C*), and that the cell nucleus of a vehicle-treated ischemic neuron at an early stage of degeneration (*B*) had an irregular euchromatin with electron density lower than that of an intact nuclear euchromatin (*D*). Bar = 5 μm.

P <0.01) between the two groups (Fig. 1, *A–C*). In histological sections, the CA1 region of ischemic gerbils exhibited a marked decline in viable neurons compared with the CA1 field of sham-operated animals (Fig. 1, *D* and *E*).

We next investigated whether IL-3 treatment prevented the ischemia-induced decreases in CA1 neuronal density and volume of the CA1 pyramidal cell layer. Hippocampal CA1 neurons in ischemic gerbils infused with IL-3, starting 2 h before ischemia, outnumbered significantly those in ischemic gerbils infused with vehicle (64 or 320 ng/d of IL-3 versus vehicle in ischemic gerbils: U = 20, P <0.05, or U = 6, P <0.01, respectively [Fig. 1, *A*, *F*, and *G*]). The volume of the CA1 pyramidal cell layer per section was also significantly larger in the IL-3–treated than in vehicle-treated ischemic gerbils (64 or 320 ng/d of IL-3 versus vehicle in ischemic gerbils: U = 12, P <0.05, or U = 1, P <0.01, respectively [Fig. 1 *B*]).

The prevention by IL-3 infusion of ischemic neuronal damage in the hippocampal CA1 field was further reinforced by the results of passive avoidance tests. The infusion of IL-3, starting 2 h before ischemia, caused a significant dose-dependent prolongation in response latency in the step-down passive avoidance task (64 or 320 ng/d of IL-3 versus vehicle in ischemic gerbils: U = 20, P <0.05, or U = 4, P <0.01, respectively [Fig. 1 *C*]). The results of passive avoidance experiments correlated well with the neuronal density of the hippocampal CA1 region in sham-operated and ischemic gerbils infused with vehicle or IL-3 (r = 0.770, P <0.05 [Fig. 1 *H*]).

The effect of postischemic treatment with IL-3 on delayed neuronal death was also investigated. IL-3 infusion in a dose of 64 or 320 ng/d for 7 d, starting just after 3-min ischemia, also prevented the ischemia-induced decreases in the number of CA1 neurons and volume of CA1 field (64

or 320 ng/d of IL-3 versus vehicle in ischemic gerbils: U = 10, P <0.05, U = 8.5, P <0.05; or U = 7, P <0.01, U = 0.5, P <0.01, respectively) and caused a significant prolongation in response latency compared with that of vehicle-treated ischemic gerbils (64 or 320 ng/d of IL-3 versus vehicle in ischemic gerbils: U = 10, P <0.05, or U = 5, P <0.01, respectively [Fig. 1, *A–C*]).

Effects of IL-3 on the Number of Synapses and Fine Structures of Pyramidal Neurons in the Hippocampal CA1 Field. In line with the results of the light microscopic observations and passive avoidance task, electron microscopy showed that intact synapses within the stratum moleculare, stratum radiatum, and stratum oriens of the hippocampal CA1 region were more numerous in IL-3– than in vehicle-treated ischemic gerbils (64 ng/d of IL-3 versus vehicle in the stratum radiatum of ischemic animals: U = 12, P <0.05; 320 ng/d of IL-3 versus vehicle in the individual strata of ischemic animals: U = 7, P <0.05; U = 8, P <0.05; U = 5, P <0.01 [Fig. 2 *A*]). Under light microscopy, vehicle-treated hippocampal CA1 neurons surviving 7 d after 3-min ischemia seemed to be intact (Fig. 1 *E*). However, a careful observation of the neurons with an electron microscope revealed that significant numbers of neurons were in the course of nuclear chromatin fragmentation and/or condensation to different degrees (Fig. 2, *B* and *C*); the nuclei of vehicle-treated ischemic neurons at early stages of degeneration had an irregular euchromatin with low electron density compared with intact nuclear euchromatin (Fig. 2 *B*). On the other hand, most of the surviving pyramidal neurons in the CA1 field of IL-3–treated gerbils retained normal morphological features even 7 d after ischemia (Fig. 2 *D*). These findings suggest that IL-3 facilitates the survival of hippocampal neurons loaded with ischemic insult in vivo.

Effects of IL-3 on the Number of TUNEL-positive Neurons in the Hippocampal CA1 Field. TUNEL staining revealed that many TUNEL-positive neurons were present in the hippocampal CA1 field of 3-min ischemic gerbils with vehicle infusion 7 d after ischemic insult (Fig. 3 *A*), suggesting that irreversible neuronal degeneration was in progress at this period as deduced from the electron microscopic findings (Fig. 2, *B* and *C*). The 7-d infusion of IL-3 not only prevented delayed neuronal death in the hippocampal CA1 field 7 d after ischemia, but also reduced the number of TUNEL-positive neurons which were in the course of a

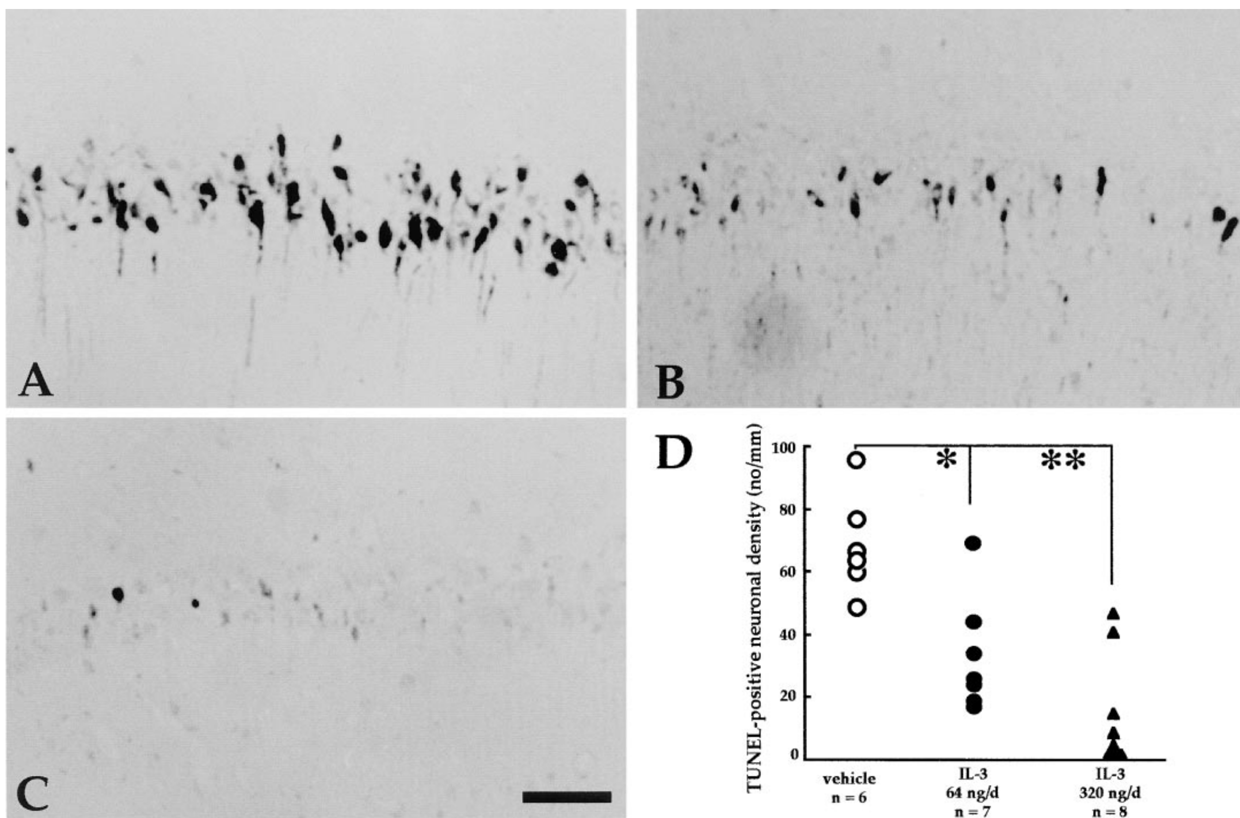

Figure 3. (*A–C*) Photomicrographs of TUNEL-positive neurons in the hippocampal CA1 field of ischemic gerbils after 7-d infusion of vehicle or IL-3: vehicle (*A*); 64 ng/d of IL-3 (*B*); 320 ng/d of IL-3 (*C*). The infusion was started 2 h before 3-min ischemia, and continued for 7 d. Note that there were many TUNEL-positive cells in the hippocampal CA1 field of ischemic gerbils with vehicle infusion. Bar = 100 μm. (*D*) The number of TUNEL-positive neurons in the hippocampal CA1 field of ischemic gerbils. The number of TUNEL-positive neurons in the hippocampal CA1 field of IL-3 (64 or 320 ng/d)-infused ischemic gerbils was less than in vehicle-infused ischemic animals. *P <0.05; **P <0.01, Significantly different from the vehicle-infused ischemic group (statistical significance tested by the two-tailed Mann-Whitney U-test).

more delayed degeneration (Fig. 3, *B* and *C*). The count of TUNEL-positive cells in the vehicle-infused ischemic gerbils indicated that without IL-3 treatment, nearly one half of the CA1 neurons surviving 7 d after ischemia undergo a further degeneration within a few days (Figs. 1 *A* and 3 *D*). IL-3 treatment precluded the late onset of ischemia-induced neuronal degeneration in a dose-dependent manner (64 or 320 ng/d of IL-3 versus vehicle: $U = 4$, $P < 0.05$, or $U = 0$, $P < 0.01$, respectively [Fig. 3 *D*]).

Demonstration of IL-3Rα in the Hippocampal CA1 Field. Although we assumed that IL-3 infused into the left lateral ventricle reached the hippocampus to rescue ischemic CA1 neurons through its binding to the local receptors, immunohistochemical analysis using the IL-3Rα antibody showed only scattered positive staining in the hippocampal CA1 field of sham-operated gerbils (Fig. 4 *A*), despite an intense staining in the hippocampal CA3 field, which is known as a site tolerant to ischemia (Fig. 4 *B*). Expecting that IL-3Rα might be abundantly expressed in the hippocampal CA1 field of ischemic but not sham-operated gerbils with vehicle infusion, we investigated the temporal profile of IL-3Rα expression in the CA1 field during 1–7 d after 3-min ischemia. Occlusion of the common carotid arteries induced a significant increase in IL-3Rα immunoreactive neurons at 2 and 4 d after ischemia (Fig. 4, *C* and *D*). The enhanced immunoreactivity of IL-3Rα began to decline 7 d after ischemia (Fig. 4 *E*). The IL-3Rα immunoreactions in the hippocampal CA1 field of ischemic gerbils were completely abolished by adsorbing the primary antibody with the homologous antigen (Fig. 4 *F*). Immunoblot analysis showed a weak but distinct constitutive expression of IL-3Rα with a molecular mass of 70 kD in the hippocampal CA1 field of sham-operated gerbils (Fig. 4 *G*). The receptor expression in the field increased at 2 and 4 d after ischemia (Fig. 4 *G*). Thus, the transient upregulation of IL-3Rα expression in a population of hippocampal CA1 neurons after

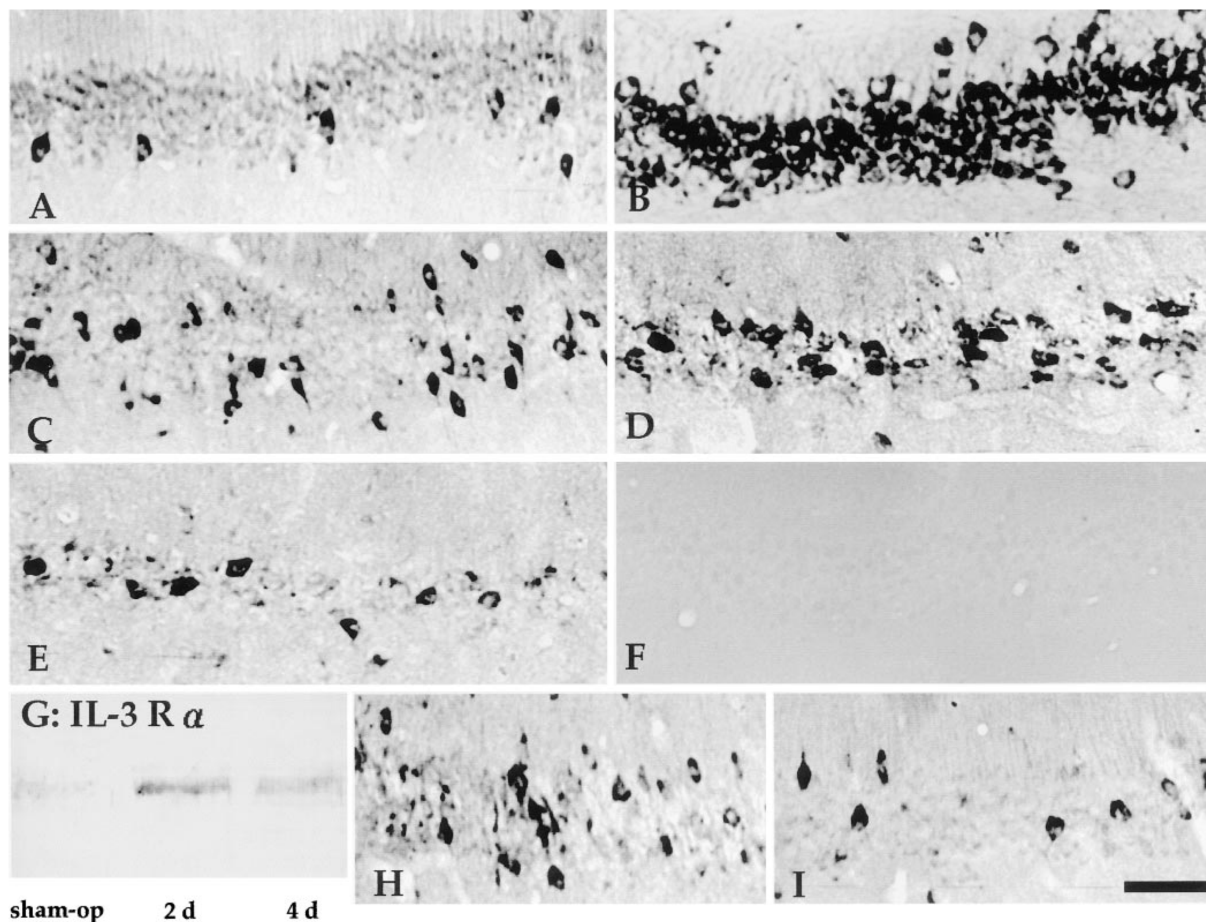

Figure 4. (*A–F*) Photomicrographs of IL-3Rα–immunoreactive neurons in the dorsal hippocampus: CA1 field of a sham-operated animal with vehicle infusion (*A*); CA3 field of a sham-operated animal with vehicle infusion (*B*); CA1 field of vehicle-treated animals 2 (*C*), 4 (*D*), and 7 (*E*) d after ischemia; CA1 field of a sham-operated animal stained with the immunoadsorbed primary antibody (*F*). Note a significant increase in IL-3Rα–immunoreactive neurons in the hippocampal CA1 field 2 (*C*) and 4 d (*D*) after ischemia. The CA3 field was more intensely labeled with the IL-3Rα antibody than was the CA1 field at any period examined. (*G*) Immunoblot analysis of IL-3Rα with a molecular mass of ~70 kD in the CA1 field of sham-operated (*sham-op*) and ischemic gerbils. 40 μg of protein was loaded onto each lane. Ischemic insult appeared to increase IL-3Rα expression 2 and 4 d after ischemia. (*H*) CA1 field of an IL-3–infused animal 2 d after ischemia. (*I*) CA1 field of an IL-3–infused animal 4 d after ischemia. Note that IL-3 infusion caused an apparent decline in IL-3Rα–immunoreactive CA1 neurons 4 but not 2 d after ischemia. Bar = 100 μm.

ischemic insult may have made it easy for cerebroventricularly infused IL-3 to act on the neurons. The infusion of IL-3 in ischemic gerbils did not affect IL-3Rα immunoreactivity in the CA1 field (Fig. 4 *H*), except that 320 ng/d of IL-3 infusion caused a decline in IL-3Rα–immunoreactive CA1 neurons at 4 d after ischemia (Fig. 4 *I*) compared with immunoreactive CA1 neurons in vehicle-treated ischemic animals (Fig. 4 *D*). This finding may reflect downregulation of IL-3R in the CA1 field of ischemic gerbils infused with the ligand.

Expression of Bcl-x_L in the Hippocampal CA1 Field. If binding to the receptor upregulated transiently after ischemia, centrally infused IL-3 should transmit signals in favor of neuronal survival, leading to the generation of neuroprotective agents in the CA1 neurons. Among Bcl-2 family proteins, Bcl-x_L protein, which suppresses apoptosis, is known to be expressed in the mature central nervous system, and Bcl-x_S protein, which facilitates apoptosis, is barely detectable in the adult brain (21, 22). Based on the finding that IL-3 precludes apoptotic death of an erythroleukemic cell line by inducing Bcl-2 protein (15), we speculated that Bcl-x_L might be a candidate for the neuroprotective agents induced by IL-3 treatment.

In sham-operated gerbils, Bcl-x_L mRNA was weakly and evenly expressed in pyramidal neurons of the CA1-4 fields and in dentate granule cells (Fig. 5 *A*). Forebrain ischemia of 3-min duration caused a selective increase in Bcl-x_L mRNA expression in the hippocampal CA1 field of gerbils at 1, 2, and 4 d after ischemic insult (Fig. 5, *B* and *C*). Quantitative analysis showed that relative amount of Bcl-x_L mRNA increased significantly in the CA1 field at 1, 2, and 4 d after ischemia, and thereafter declined to the control level (Fig. 5 *D*). No significant changes were observed in the other regions of the hippocampus. The expressions of Bcl-2 and Bax mRNAs were not affected by 3-min ischemia (data not shown).

In the hippocampal CA1 field of sham-operated gerbils, Bcl-x_L protein with a molecular mass of approximately 29 kD was constitutively expressed (Fig. 5 *E*). Forebrain ischemia of 3-min duration caused an apparent decline in Bcl-x_L content in the hippocampal CA1 field of gerbils treated with vehicle 1 d after ischemic insult, despite the transient upregulation of Bcl-x_L mRNA expression at the same period (Fig. 5 *E*). These findings suggest that translation of Bcl-x_L mRNA is suppressed in the hippocampal CA1 field. On the other hand, IL-3 infusion prevented the decrease in Bcl-x_L protein expression 1 d after ischemia (Fig. 5 *E*). There was also a slight increase in Bcl-x_L protein expression in the IL-3–treated hippocampal CA1 region 2 d after ischemia.

Neurotrophic Effect of IL-3 on Cultured Cortical and Hippocampal Neurons. The above in vivo studies suggest that IL-3 prevents delayed neuronal death in the hippocampal CA1 field through a receptor-mediated expression of Bcl-x_L protein. To ascertain this speculation in culture experiments, we investigated (*a*) whether IL-3 facilitated the survival of cortical and hippocampal neurons, (*b*) whether cultured cortical and hippocampal neurons expressed IL-3Rα, and (*c*) whether IL-3 treatment induced the expression of Bcl-x_L mRNA and protein in the cultured neurons. Treatment of cultured cortical or hippocampal neurons with IL-3 for 3 d significantly increased the number of surviving neu-

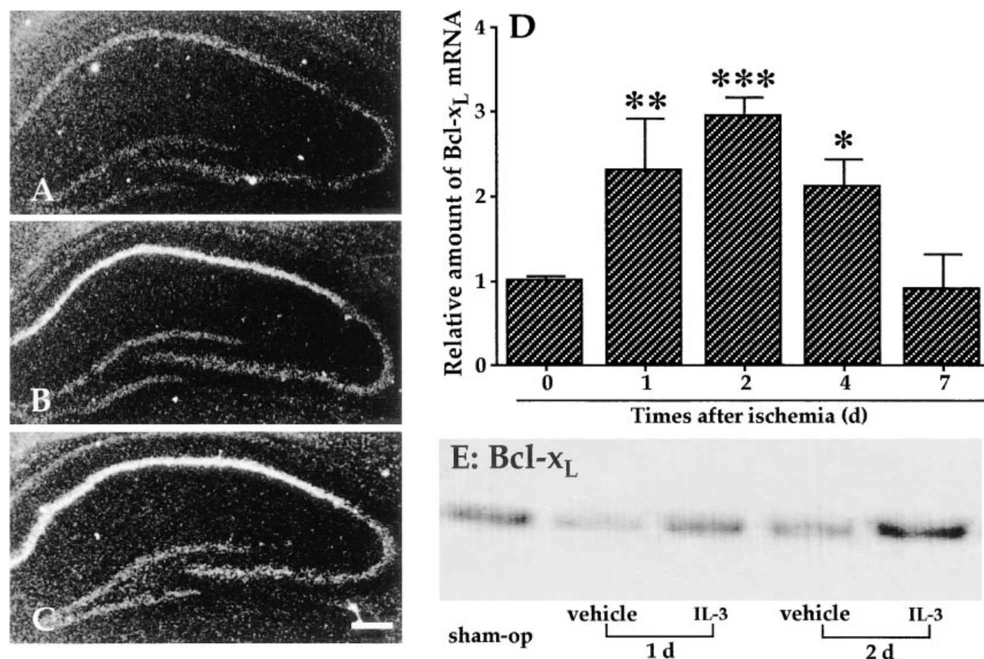

Figure 5. (*A–C*) Dark-field photomicrographs showing Bcl-x_L mRNA expression in the hippocampus of sham-operated and 3-min ischemic gerbils: hippocampus of a sham-operated animal (*A*); hippocampus 1 (*B*) and 2 (*C*) d after ischemia. Note a selective increase in Bcl-x_L mRNA expression in the hippocampal CA1 field 1 and 2 d after ischemia. Bar = 200 μm. (*D*) Time course of Bcl-x_L mRNA expression in the pyramidal cell layer of the CA1 field in 3-min ischemic gerbils. Relative amounts to the sham-operated control are expressed as mean ± SD. $^*P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, Significantly different from the control (statistical significance tested by analysis of variance followed by Fisher's post hoc test). (*E*) Immunoblot analysis of Bcl-x_L protein in the CA1 field of sham-operated (*sham-op*) and ischemic gerbils. Note a marked decline in Bcl-x_L protein expression in the vehicle-treated CA1 field 1 d after ischemia, and that IL-3 treatment prevented the decrease in Bcl-x_L protein expression in the CA1 field of ischemic gerbils 1 and 2 d after ischemia.

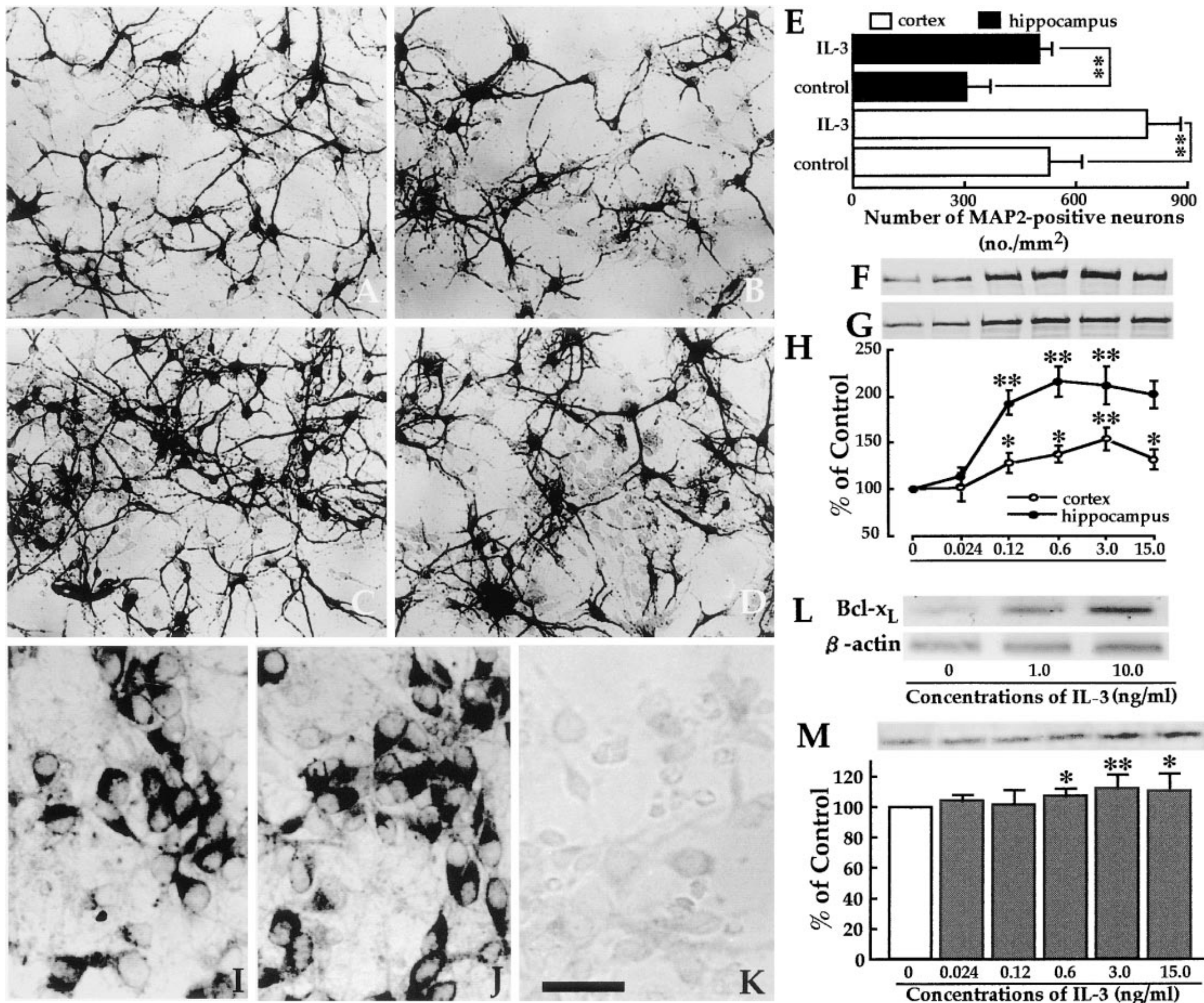

Figure 6. (*A–D*) Photomicrographs of MAP2-positive neurons in cultures: cortical (*A*) and hippocampal (*B*) neuron cultures without IL-3; cortical (*C*) and hippocampal (*D*) neuron cultures treated with 3.0 ng/ml of IL-3. Note that IL-3 significantly increased the number of MAP2-positive neurons. Bar = 200 μm. (*E*) Number of MAP2-positive neurons in cultures. The MAP2-positive neurons in the cultures treated with 3.0 ng/ml of IL-3 were more numerous than in the corresponding cultures without IL-3 (*control*). (*F–H*) MAP2 immunoblot analysis of IL-3–treated cortical (*F*) and hippocampal (*G*) neurons. Neurons cultured with 0–15.0 ng/ml of IL-3 were immunoblotted with an mAb against MAP2. MAP2-positive bands were observed in neurons without IL-3 treatment (lane *1* in *F* and *G*). MAP2-immunoreactive bands increased in intensity in cultures with 0.024–15.0 ng/ml of IL-3 (lanes *2–6* in *F* and *G*). Densitometric analysis of MAP2-immunoreactive bands showed that IL-3 enhanced the survival and possibly the neurite extension of cortical and hippocampal neurons in a dose-dependent manner (*H*). The data were obtained from four separate cultures and were expressed as a percentage of the corresponding control culture without IL-3. (*I–K*) Photomicrographs of IL-3Rα–immunoreactive neurons in cultures: cortical neuron culture (*I*), hippocampal neuron culture (*J*), and cortical neuron culture stained with the immunoadsorbed primary antibody (*K*). Note that IL-3Rα immunoreactions were abolished by adsorbing the primary antibody with the homologous antigen (*K*). Bar = 100 μm. (*L*) Semiquantitative RT-PCR analysis of Bcl-x_L mRNA in cortical neurons cultured in the presence of 0, 1, or 10 ng/ml of IL-3. β-Actin was also amplified as an internal control from each sample. (*M*) Immunoblot analysis of Bcl-x_L protein in cultured cortical neurons. IL-3 at concentrations of 0.6–15.0 ng/ml apparently induced Bcl-x_L expression in the cultured neurons. The data were obtained from five separate cultures and were expressed as a percentage of the corresponding control culture. Each value in *E*, *H*, and *M* indicates mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$, Significantly different from the corresponding control value (statistical significance tested by analysis of variance followed by Fisher's post hoc test).

rons compared with the corresponding control culture without IL-3 treatment. MAP2-positive cortical and hippocampal neurons in the IL-3–treated cultures were more numerous than in cultures without IL-3 treatment (Fig. 6, *A–E*). Subsequent immunoblot analysis showed that the MAP2-immunoreactive bands of cultured cortical and hippocampal neurons treated with 0.024–15.0 ng/ml of IL-3 (lanes *2–6* in Fig. 6, *F* and *G*) were more intense than those of the control cultures (lane *1* in Fig. 6, *F* and *G*). Densitometric analysis of the MAP2-immunoreactive bands revealed that IL-3 at concentrations of 0.12–15.0 ng/ml enhanced significantly the survival of cultured neurons in a dose-dependent manner (Fig. 6 *H*). Immunostaining of cultured cortical and hippocampal neurons with the IL-

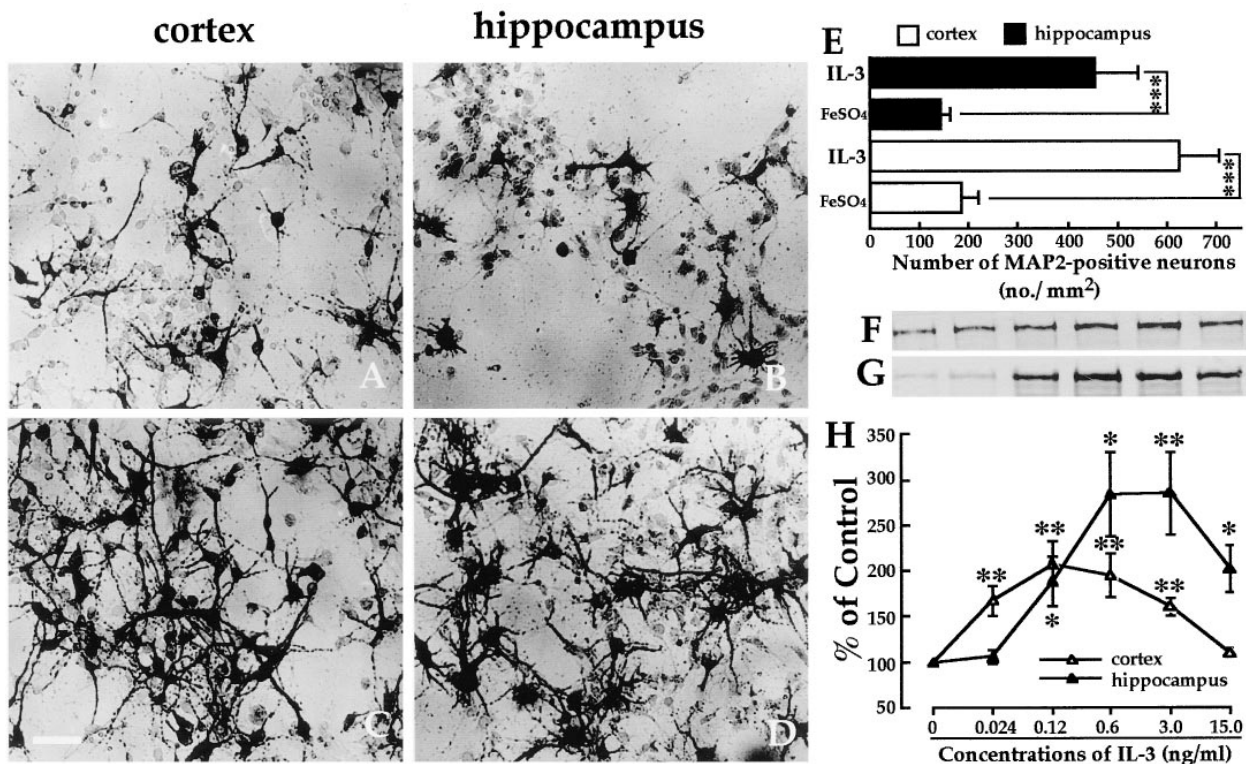

Figure 7. (*A–D*) Photomicrographs of MAP2-positive neurons in cultures exposed to $FeSO_4$: cortical (*A*) and hippocampal (*B*) neurons in cultures treated only with $FeSO_4$; cortical (*C*) and hippocampal (*D*) neurons in cultures pretreated with 3.0 ng/ml of IL-3 for 3 d, then treated with $FeSO_4$. Note that IL-3 treatment significantly increased the number of MAP2-positive neurons. Bar = 200 μm. (*E*) Number of MAP2-positive neurons in cultures exposed to $FeSO_4$. The MAP2-positive neurons in the cultures treated with 3.0 ng/ml of IL-3 were more numerous than in the corresponding control cultures without IL-3 (*FeSO4*). (*F–H*) MAP2 immunoblot analysis of IL-3–treated cortical (*F*) and hippocampal (*G*) neurons exposed to $FeSO_4$. Samples cultured for 3 d with 0–15.0 ng/ml of IL-3 and then treated with $FeSO_4$ were immunoblotted with an mAb against MAP2. Neurons cultured with $FeSO_4$ but without IL-3 pretreatment showed extremely weak immunoreactive bands (lane *1* in *F* and *G*). In neurons treated with 0.12–3.0 ng/ml of IL-3 and then with $FeSO_4$, the intensity of MAP2-immunoreactive bands significantly increased even if $FeSO_4$ existed in the cultures (lanes *3–5* in *F* and *G*). The densitometric analysis of MAP2-immunoreactive bands showed that IL-3 protected the cultured neurons against $FeSO_4$-induced oxidative injuries in a dose-dependent manner (H). The data were obtained from four separate cultures and were expressed as a percentage of the corresponding control culture. Each value in *E* and *H* indicates mean ± SD. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, Significantly different from the corresponding control value (statistical significance tested by analysis of variance followed by Fisher's post hoc test).

3Rα antibody showed the presence of IL-3Rα in a large population of the neurons (Fig. 6, *I* and *J*). Pretreatment of the IL-3Rα antibody with the homologous antigen abolished the immunoreactions (Fig. 6 *K*). In contrast to the result of in vivo experiments showing a ligand-induced decrease in IL-3Rα–positive CA1 neurons 4 d after ischemia, we could not detect downregulation of IL-3Rα expression in cultured neurons in response to IL-3 treatment. This might be caused by a large number of CA3 neurons contained in the neuronal culture, because the CA3 neurons exhibited stable IL-3Rα expression.

To investigate the effect of IL-3 on Bcl-x_L expression, we first conducted RT-PCR under quantitative conditions using specific primers that amplify a 189-bp fragment of the rat Bcl-x_L mRNA. The PCR product showed the expected size, and its identity was confirmed by direct sequencing. Densitometric analysis showed that neurons cultured in the presence of 1 or 10 ng/ml of IL-3 exhibited Bcl-x_L mRNA expression approximately three or six times as much as control cultured neurons without IL-3 treatment, indicating that IL-3 upregulated Bcl-x_L mRNA expression in a dose-dependent manner (Fig. 6 *L*). Moreover, IL-3 at concentrations of 0.6–15.0 ng/ml significantly induced Bcl-x_L expression in the cultured neurons (Fig. 6 *M*).

Neuroprotective Effect of IL-3 on Cultured Neurons Exposed to $FeSO_4$. We investigated whether or not IL-3 attenuated the damage to cortical and hippocampal neurons by $FeSO_4$. Cortical and hippocampal neurons were cultured for 3 d without IL-3 treatment, then $FeSO_4$ was added to the culture medium. The cortical neurons were no longer visible within 2 h in the culture, and hippocampal neurons within 24 h in the culture. MAP2-immunoreactive neurons exposed to $FeSO_4$ (Fig. 7, *A* and *B*) were less numerous than in cultures without $FeSO_4$ treatment (Fig. 6, *A* and *B*). Pretreatment with IL-3 protected cortical and hippocampal neurons against lethal damage caused by $FeSO_4$; MAP2-positive neurons in the IL-3–treated cultures outnumbered those in cultures without IL-3 pretreatment (Fig. 7, *A–E*). However, the protective effect of IL-3 on the oxidative damage to neurons by $FeSO_4$ was not ob-

served when IL-3 and $FeSO_4$ were simultaneously added to the cultured medium (data not shown). This suggests that IL-3 protects cultured neurons through induction of intracellular antioxidant agents, including a Bcl-2 family protein(s), rather than by acting alone as a free radical scavenger. Subsequent immunoblot analysis showed that the MAP2 bands of neurons exposed to $FeSO_4$ without IL-3 pretreatment were very thin (lane *1* in Fig. 7, *F* and *G*). In contrast, intense MAP2 bands were detected in neurons in cultures treated with IL-3 at concentrations of 0.12–3.0 ng/ml before the exposure to $FeSO_4$ (lanes *3–5* in Fig. 7, *F* and *G*). The neuroprotective action of IL-3 at different concentrations was quantitatively evaluated by densitometric analysis of the immunoreactive bands (Fig. 7 *H*). Pretreatment with IL-3 protected significantly cultured neurons against $FeSO_4$-induced damage in a dose-dependent manner. The most effective concentration was 0.6–3.0 ng/ml for hippocampal and cortical neurons (Fig. 7 *H*). Thus, these in vitro studies suggest that IL-3 exerts a protective effect on cultured neurons through binding to the cell surface receptor, leading to the induction of Bcl-x_L protein, which possibly counteracts the neurotoxicity of free radicals.

Discussion

Among the subregions of the gerbil hippocampus, the CA1 field is the most vulnerable to ischemia (52), although the other subregions in rats exposed to intermittent stress or physiological concentrations of glucocorticoids are also easily damaged by an excitotoxin kainic acid and by brain ischemia (27, 53). The hippocampal CA3 field of the rat appears to be selectively susceptible to prolonged glucocorticoid exposure (54). In cases of brain ischemia, CA1 neurons with *N*-methyl-d-aspartate receptor exhibit sustained elevation of intracellular Ca^{2+} concentration as elicited by an ischemia-induced increase in extracellular glutamate concentration, and such excessive Ca^{2+} loading to the CA1 but not CA3 neurons is considered to trigger the activations of various enzymes and genes facilitating neuron death (30, 55–57). It is also plausible that transient forebrain ischemia modulates the production of peptide growth factors and certain cytokines, including IL-6 (58–61), thereby affecting ischemic damage to the CA1 neurons. We speculate that there are multiple endogenous mediators regulating neuronal death or survival in the ischemic brain, since a variety of drugs and peptide growth factors possibly with different action mechanisms have been shown to prevent delayed neuronal death in the hippocampal CA1 field (23–25, 36–38, 49). Nevertheless, apart from studies dealing with the disrupted calcium homeostasis in the ischemic CA1 region (30, 56, 57), no previous studies have addressed the question of why hippocampal CA1 neurons are selectively vulnerable to brain ischemia. In this immunohistochemical study using an IL-3Rα antibody, we noted that IL-3Rα expression was barely detectable in the hippocampal CA1 field of sham-operated gerbils and was apparently upregulated 2 and 4 d after 3-min ischemia. However, the IL-3Rα immunoreactivity in the hippocampal CA1 field, even though upregulated in response to ischemic insult, was still less intense than that in the hippocampal CA3 field, which is known as a site tolerant to ischemia. The paucity of IL-3Rα in the CA1 field appears to account, in part, for the selective vulnerability of hippocampal CA1 neurons to brain ischemia, provided that the ligand is produced equally in the CA1 and CA3 fields. Since there is no information on the localization of IL-3 in the brain tissue, a more sensitive IL-3 assay system will be needed to detect the central production of IL-3 in vivo.

Unlike CA3 pyramidal neurons, only a limited population of CA1 neurons was labeled with the IL-3Rα antibody even after ischemia. In support of this finding, the continuous cerebroventricular infusion of IL-3 rescued many but not all ischemic CA1 neurons, and prevented significantly but not completely the occurrence of ischemia-induced learning disability as revealed by the step-down passive avoidance task. The precise mechanism by which centrally infused IL-3 supports ischemic CA1 neurons in vivo is beyond the scope of this discussion. In the hippocampal CA1 region of vehicle-treated gerbils with 3-min ischemia, Bcl-x_L mRNA expression was upregulated at 1, 2, and 4 d after ischemia without leading to translation of Bcl-x_L protein. This suggests that the impaired translation of Bcl-x_L mRNA is, in part, responsible for neuron death in the ischemic hippocampal CA1 field. On the basis of our immunoblot analysis, IL-3 is likely to enhance the survival of ischemic CA1 neurons by stimulating the expression of Bcl-x_L protein, which is known to inhibit apoptotic neuron death in cultures (18, 20) and possibly in the mature brain (21, 22).

Under electron microscopy, we noticed that vehicle-treated CA1 neurons which appeared to be intact at the light microscopic level exhibited pathological changes in the nuclear chromatin 7 d after ischemia. This prompted us to conduct TUNEL staining in paraffin sections from IL-3– and vehicle-treated ischemic gerbils. In the conventional 5-min ischemia, where brain temperature is not kept at 37.0 ± 0.2°C during ischemia, TUNEL-positive CA1 neurons have been shown to peak 4 d after ischemic insult, and only a few are visible 7 d after ischemia, possibly due to the so-called delayed death of almost all CA1 neurons at this period (62). In contrast, in the present study, the vehicle-treated normothermic gerbils with 3-min ischemia exhibited many TUNEL-positive neurons in the hippocampal CA1 field even 7 d after ischemia. This finding suggests that the hippocampal CA1 field loaded with 3-min ischemia undergoes a progressive degeneration slower than with 5-min ischemia. Thus, this animal model with an episode of 3-min ischemia has a wider therapeutic window than the conventional 5-min ischemia model (52) and may be useful for the screening of neuroprotective agents. In this study, IL-3 treatment reduced dramatically the number of TUNEL-positive neurons in 3-min ischemic gerbils. This indicates that the slowly progressive degeneration of

hippocampal CA1 neurons in vehicle-treated ischemic gerbils is markedly inhibited by IL-3 even after the termination of its infusion. Thus, in situ detection of DNA fragmentation by TUNEL staining appears to be a reliable tool for assessing the effects of neuroprotective agents on the late degeneration of hippocampal CA1 neurons in ischemic gerbils. We speculate that a brief ischemic insult similar to 3-min ischemia in gerbils is occasionally loaded to the human brain, leading to sustained neuronal damage of the ischemic focus. If this is the case, continuous treatment with peptide growth factors (23–25, 36) or nonpeptide neuroprotective agents (37, 38, 49) will be needed to lessen slowly progressive ischemic neuronal damage.

In line with the results of the in vivo experiments, these in vitro studies demonstrated that IL-3 enhances the survival of cultured neurons possibly through binding to the cell surface receptor, and induces Bcl-x_L mRNA and protein expression in a concentration-dependent manner. IL-3 also attenuated neuronal damage caused by free radicals (possibly hydroxyl radicals), which are known to be overproduced during and after brain ischemia (28, 32–34).

In conclusion, IL-3 protects neurons against ischemia/reperfusion injury and oxidative stress through a receptor-mediated increase in Bcl-x_L expression, which suppresses neuronal cell death. IL-3 exerts a trophic action on hippocampal neurons in vivo and in vitro.

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Address correspondence to Masahiro Sakanaka, Department of Anatomy, Ehime University School of Medicine, Shigenobu, Ehime, 791-0295, Japan. Phone: 81-89-960-5235; Fax: 81-89-960-5239; E-mail: sakanaka@m.ehime-u.ac.jp

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