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Differential regulation of mRNAs for nerve growth factor, brain-derived neurotrophic factor, and neurotrophin 3 in the adult rat brain following cerebral ischemia and hypoglycemic coma

(*in situ* hybridization/brain damage/hippocampus)

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ABSTRACT **_In situ_ hybridization was used to study expression of mRNAs for members of the nerve growth factor (NGF) family in the rat brain after 2 and 10 min of forebrain ischemia and 1 and 30 min of insulin-induced hypoglycemic coma. Two hours after the ischemic insults, the level of brain-derived neurotrophic factor (BDNF) mRNA was markedly increased in the granule cells of the dentate gyrus, and at 24 h it was still significantly elevated. NGF mRNA showed a pronounced increase 4 h after 2 min of ischemia but had returned to a control level at 24 h. Both 2 and 10 min of ischemia caused a clear reduction of the level of mRNA for neurotrophin 3 (NT-3) in the dentate granule cells and in regions CA2 and medial CA1 of the hippocampus 2 and 4 h after the insults. The increase of BDNF mRNA could be partially blocked by the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist NBQX but was not influenced by the *N*-methyl-D-aspartate (NMDA) receptor antagonist MK-801. Both NBQX and MK-801 attenuated the decrease of NT-3 mRNA after ischemia. One and 30 min of hypoglycemic coma also induced marked increases in BDNF and NGF mRNA in dentate granule cells with maximal levels at 2 h. If the changes of mRNA expression lead to alterations in the relative availability of neurotrophic factors, this could influence functional outcome and neuronal necrosis following ischemic and hypoglycemic insults.**

Neurotrophic factors are critically involved in the development and maintenance of both the peripheral and central nervous system (1). The best characterized neurotrophic molecule is nerve growth factor (NGF), and ample evidence exists that basal forebrain cholinergic neurons innervating hippocampus, olfactory cortex, and neocortex depend on target-derived NGF for trophic support (2–4). Recently, molecular clones have been isolated and characterized for three other members of the same gene family, brain-derived neurotrophic factor (BDNF), hippocampus-derived neurotrophic factor/neurotrophin 3 (NT-3), and neurotrophin 4 (5–13). NGF, BDNF, and NT-3 mRNAs are expressed in neurons in the brain, with the highest levels in the hippocampus (6, 8, 10, 14–16). However, the distribution of cells expressing BDNF, NGF, and NT-3 mRNA in the adult rat hippocampus is unique for each factor (10, 17). Outside the hippocampus, BDNF mRNA has the most widespread distribution and has been found in many brain regions including neocortex, piriform cortex, and amygdala (10, 15, 17, 18). The functions of BDNF and NT-3 in the central nervous system are not yet known but BDNF has been shown to increase the survival in culture of retinal ganglion cells (19, 20), basal forebrain cholinergic neurons (21), and ventral mesencephalic dopaminergic neurons (22, 23).

During brain development, the neurotrophic factors of the NGF family display distinct stage-specific and tissue-specific patterns of expression. The highest level of NT-3 mRNA is found shortly after birth, whereas maximal expression of BDNF and NGF mRNAs is observed 2 and 3 weeks postnatally, respectively (8, 16). The different neurotrophic factor mRNAs are transiently expressed in several brain regions during development (16, 24–27). In the adult central nervous system, recurrent seizures induced by a unilateral electrolytic lesion of the dentate gyrus hilus cause bilateral increases of NGF mRNA in the dentate gyrus granule cells 3 h after the lesions and also at later time points in the pyramidal cell layers of the olfactory cortex and neocortex (28). Intraventricular, intracerebral, or systemic administration of kainic acid also causes increased expression of NGF (29–31) and BDNF mRNA (29, 30) in hippocampus and neocortex. Furthermore, brief focal hippocampal seizures (lasting ≈1 min), evoked by electrical kindling stimulation, lead to a marked increase of BDNF and NGF mRNA expression in the dentate gyrus (32). With repeated seizures, the mRNAs for both factors also increase in the parietal and piriform cortices and BDNF mRNA in the pyramidal layer of the hippocampus and in the amygdaloid complex.

From the data obtained in the different seizure models, it has been hypothesized that the synthesis of NGF and BDNF in the brain is regulated by neuronal activity and stimulated by glutamate release (29, 32). The present study was designed to explore the possibility that the expression of mRNAs for NGF, BDNF, and NT-3 can be altered in response to ischemic and hypoglycemic insults which, similar to epileptic seizures, lead to an increase in glutamate release and to an increase in the intracellular cytosolic calcium concentration. Neurotrophin mRNA levels were measured after both brief periods of ischemia (2 min) and hypoglycemic coma (1 min), which cause no brain damage, and longer insults (10 min of ischemia and 30 min of hypoglycemic coma), which give rise to neuronal necrosis (33, 34). We report here that transient forebrain ischemia and insulin-induced hypoglycemic coma in rats rapidly cause marked increases in the levels of mRNAs for NGF and BDNF but lead to reduced expression of NT-3 mRNA.

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Abbreviations: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin 3; EEG, electroencephalogram; ANOVA, analysis of variance; NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.

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MATERIALS AND METHODS

Animals and Experimental Groups. Male Wistar rats (Mollegaard's Breeding Center, Copenhagen), were fasted overnight. Anesthesia was induced with 3.5% halothane in N_2O/O_2 (70:30) and the animals were intubated and artificially ventilated with the halothane concentration lowered to 1.0–1.5%. Arterial and venous catheters were inserted for blood pressure recording, blood sampling, and drug infusions. Needle electrodes were placed in the temporalis muscles for electroencephalographic (EEG) measurements, and a rectal temperature probe was inserted for measurement of body temperature. In the animals to be used for ischemia experiments, loose ligatures were placed around the isolated common carotid arteries, and in the hypoglycemic animals, a small craniotomy was made to allow for registration of the cortical DC potential.

After surgery, the halothane concentration was lowered to 0.3%, the animals were heparinized, and vecuronium bromide (Norcuron; Organon Teknika) was given i.v. as a muscle relaxant.

In a first experiment, 20 animals were subjected to 2 min of forebrain ischemia; divided into five groups; and decapitated after 10 min, 30 min, 2 h, 4 h, or 24 h of recovery, respectively ($n = 4$ in each group). Ten rats served as sham-operated controls and were decapitated after the corresponding recovery periods ($n = 2$ in each group). Four rats were subjected to 10 min of ischemia and sacrificed after 30 min or 2 h ($n = 2$ in each group). One sham-operated rat was included in each group. A separate group of 3 animals was implanted with recording electrodes into the hippocampus and parietal cortex. One week thereafter, an EEG was recorded during ischemia of 2 ($n = 2$) or 10 ($n = 1$) min duration and in the subsequent 2-h recovery period. In a second experiment, 2 min of ischemia was induced in 12 animals, which were given MK-801 ($n = 4$; 1 mg/kg i.p. 30 min before ischemia), NBQX ($n = 4$; 30 mg/kg i.p. 15 and 5 min before and 10 min after ischemia), or corresponding vehicle ($n = 2 + 2$). Four sham-operated animals were given either MK-801 ($n = 2$) or NBQX ($n = 2$) as described above. All animals were killed after 2 h. In a third experiment, 8 animals were subjected to insulin-induced hypoglycemic coma during 1 ($n = 4$) or 30 min ($n = 4$) and were sacrificed after 30 min (2 rats from both the 1- and 30-min group) or 2 h ($n = 2 + 2$) recovery. One sham-operated rat was matched to each of the four experimental groups. After decapitation, the brains were immediately frozen on dry ice and analyzed by *in situ* hybridization histochemistry.

Induction of Ischemia. Ischemia was induced by bilateral common carotid artery occlusion, combined with hypotension (mean arterial blood pressure, 50 mmHg) by exsanguination (35). After 2 or 10 min, circulation was restored by removal of the occluding clasps and reinfusion of blood. Anesthesia was then discontinued, and animals that regained spontaneous respiration were extubated. The sham-operated animals were treated identically but the common carotid arteries were not occluded and blood pressure was only briefly reduced to 80 mmHg in order to maintain autoregulation of cerebral blood flow.

Induction of Hypoglycemic Coma. All animals were injected i.p. with 2 international units of insulin per kg (Actrapid; Novo-Nordisk) 30 min before the operation (36). The decrease in blood glucose was closely followed by repeated measurements, and EEG and dc potential were recorded continuously. A sudden, nonreversible negative shift of the dc potential was regarded as time zero for the onset of hypoglycemic coma. After a comatose period of 1 or 30 min, glucose was infused (100 mg/ml) to restore normal blood glucose levels. In the sham groups, glucose (100 mg/ml) was infused throughout the experiment to maintain normal blood glucose levels.

***In Situ* Hybridization.** Fresh-frozen rat brains were sectioned coronally (14 μm) at a level including the dorsal hippocampus and processed for *in situ* hybridization as described (10). Four oligonucleotides, specific for BDNF, NGF, NT-3, and c-fos mRNA were used for *in situ* hybridizations. The rat BDNF mRNA antisense oligonucleotide is complementary to nucleotides 650–699 in the sequence of mouse BDNF (15), and the rat NGF- and NT-3-specific oligonucleotides are complementary to nucleotides 869–918 of rat NGF (37) and nucleotides 667–716 of rat NT-3 (8), respectively. For detection of rat c-fos mRNA, a 48-mer oligonucleotide complementary to nucleotides 543–590 in the sequence of Curran *et al.* (38) was used. The oligonucleotides were labeled at the 3′ end with deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate by using terminal deoxyribonucleotidyltransferase (IBI) to a specific activity of $\approx 1 \times 10^9$ cpm/μg. Hybridization was performed as described (10) using 10^7 cpm of respective probe per ml. After hybridization, the sections were exposed to x-ray film followed by coating in Kodak NTB-3 photoemulsion (diluted 1:1 in water), exposed for 5–6 weeks at −20°C, developed, fixed, and counterstained with cresyl violet.

Levels of neurotrophin mRNA were quantified by computerized image analysis using Image software (Wayne Rasband, National Institute of Mental Health) and a Macintosh IIs computer as described (32). The mean value of 12 measurements from the dentate gyrus in each animal was used for statistical analysis.

RESULTS

Expression of Neurotrophin and c-fos mRNAs After Forebrain Ischemia. Levels of mRNA for NGF, BDNF, and NT-3 were studied by *in situ* hybridization following forebrain ischemia induced by bilateral common carotid artery occlusion and simultaneous arterial hypotension. Rats were sacrificed 10 min, 30 min, 2 h, 4 h, and 24 h after 2 min of ischemia and 30 min and 2 h after 10 min of ischemia. Increased levels of mRNA for BDNF could be observed bilaterally over dentate gyrus granule cells starting at 30 min after 2 min of ischemia. The highest expression of BDNF mRNA was seen 2 h after the insult (Figs. 1 *A* and *B*, 2, and 3 *A* and *B*) when quantitative image analysis of autoradiograms showed a 316% increase compared to sham-operated animals [$P < 0.001$, one-way analysis of variance (ANOVA); for the statistical analysis, sham-operated animals from the five different postischemic recovery periods were pooled into one control group]. The level then tapered off, although 24 h after the insult it was still 80% higher than those measured in controls (Fig. 2). After 10 min of forebrain ischemia, maximal expression of BDNF mRNA in the dentate gyrus (160% increase) was seen 2 h after the insult (data not shown). Increased levels of mRNA for NGF in the dentate gyrus granule cells were detected 2 h after 2 min of ischemia and the highest expression (219% increase) was seen at 4 h ($P < 0.005$, one-way ANOVA; Figs. 1 *C* and *D*, 2, and 3 *C* and *D*). Twenty-four hours after the insult, NGF mRNA expression had returned to the control level. No increase in NGF mRNA was seen 30 min or 2 h after a 10-min period of ischemia. NT-3 mRNA expression was reduced in dentate gyrus granule cells and in the CA2 and medial CA1 regions of the hippocampus (where NT-3 mRNA is normally expressed) 2 and 4 h after a 2-min period of ischemia (Figs. 1 *E* and *F*, 2, and 3 *E* and *F*) and 2 h after 10 min of ischemia. The maximal reduction in the dentate gyrus (61%) was seen 4 h after 2 min of ischemia ($P < 0.005$, one-way ANOVA). At 24 h, NT-3 mRNA had returned to control levels. The increase of BDNF and NGF mRNA and the decrease of NT-3 mRNA were due to a

change both in the number of dentate granule cells displaying detectable levels and in the expression in individual neurons (Fig. 3). No changes in mRNA for BDNF and NGF were detected outside the dentate gyrus after ischemia.

Increased levels of c-fos mRNA were observed in the dentate gyrus 10 min after 2 min of ischemia with maximal expression at 30 min (Fig. 1 *G* and *H*). At this time point, increased levels were also seen in the pyramidal layer and in the perirhinal cortex and parietal cortex. Two hours after the ischemic insult, c-fos mRNA was lower in the latter regions, but a more pronounced increase was found throughout the pyramidal layer of the hippocampus. The increase in c-fos mRNA had virtually disappeared in most regions after 4 h, and after 24 h c-fos mRNA levels were the same as in sham-operated animals.

To explore the possibility that the changes in mRNA for neurotrophins and c-fos seen after ischemia are secondary to insult-induced seizure activity, EEG recordings from deep electrodes implanted into the parietal cortex and hippocampus were made during 2- and 10-min periods of ischemia and throughout a 2-h recovery phase. Although single spikes and waves were seen, no seizure activity was observed during or after ischemia.

Effects of Glutamate Receptor Antagonists on Ischemia-Induced Changes in Neurotrophin mRNAs. In a separate experiment, the effects of the noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist MK-801 and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist NBQX on changes in BDNF, NGF, and NT-3 mRNA in the dentate gyrus were analyzed 2 h after a 2-min period of ischemia. The increase in BDNF mRNA expression after ischemia was reduced by 19% after treatment with NBQX (Fig. 4; $P < 0.05$, one-way ANOVA; animals receiving vehicle injections were pooled into a single group for statistical analysis). MK-801 had no significant effect on the increase in BDNF mRNA expression seen after ischemia ($P > 0.05$; Fig. 4). No significant change in the level of NGF mRNA was found after either MK-801 or NBQX ($P > 0.05$; Fig. 4). The insult-induced decrease in NT-3 mRNA was attenuated by 84% in MK-801-treated rats and by 67% in NBQX-treated rats as compared to vehicle-injected animals ($P < 0.05$, one-way ANOVA; Fig. 4). Neither MK-801 nor NBQX had any effect on the expression of mRNA for neurotrophins in sham-operated animals (data not shown).

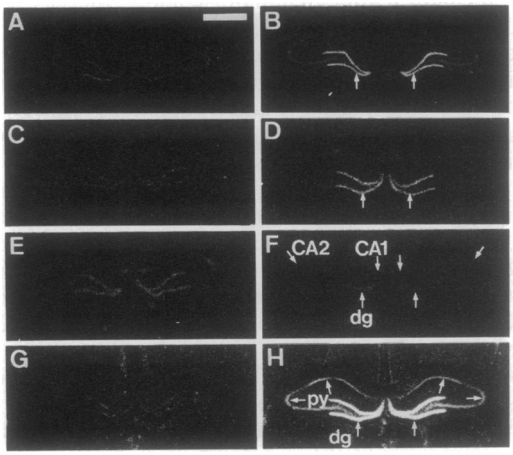

FIG. 1. Dark-field photographs of autoradiograms showing expression of mRNAs for neurotrophic factors and c-fos in the dorsal hippocampus after 2 min of forebrain ischemia (*B*, *D*, *F*, and *H*) or a sham operation (*A*, *C*, *E*, and *G*). Coronal sections were hybridized for BDNF mRNA (2 h after ischemia; *A* and *B*), NGF (4 h after ischemia; *C* and *D*), NT-3 (4 h after ischemia; *E* and *F*), or c-fos mRNA (30 min after ischemia; *G* and *H*). Arrows in *B* and *D* indicate increases in BDNF and NGF mRNA expression, respectively, in the dentate gyrus following ischemia. In *F*, arrows indicate the decrease in NT-3 mRNA levels seen in the dentate gyrus (dg) granular cell layer and medial CA1 and CA2 regions after ischemia. Arrows in *H* mark the ischemia-induced increase in c-fos mRNA in the dentate gyrus and throughout the hippocampal pyramidal (py) cell layer. (Bar = 160 μm.)

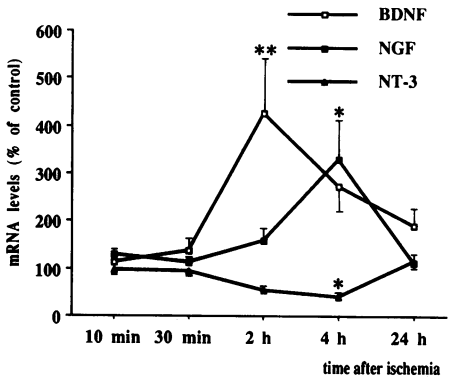

FIG. 2. Time course of changes in neurotrophin mRNA expression in the dentate gyrus after 2 min of forebrain ischemia as quantified by computerized image analysis of autoradiograms. Values are expressed relative to sham-operated animals (means ± SEM). *, $P < 0.005$; **, $P < 0.001$ compared to sham-operated animals (one-way ANOVA).

Expression of Neurotrophin mRNAs after Hypoglycemic Coma. Expression of mRNAs for NGF, BDNF, and NT-3 was studied 30 min and 2 h after 1- and 30-min periods of insulin-induced hypoglycemia. Two hours after 1 min of hypoglycemic coma, a 152% increase in BDNF mRNA expression was observed in dentate gyrus granule cells (Fig.

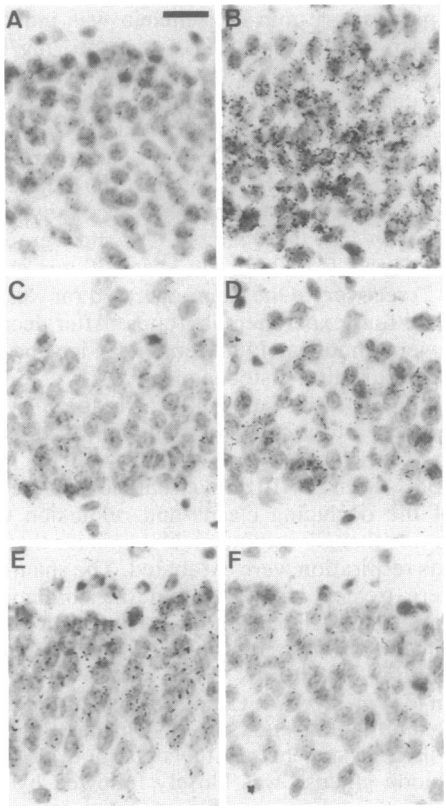

FIG. 3. Bright-field photomicrographs of coronal sections through the dentate gyrus granular cell layer hybridized for BDNF mRNA (*A* and *B*), NGF mRNA (*C* and *D*), or NT-3 mRNA (*E* and *F*) 2 h after 2 min of forebrain ischemia (*B*, *D*, and *F*) or a sham operation (*A*, *C*, and *E*). (Bar = 20 μm.)

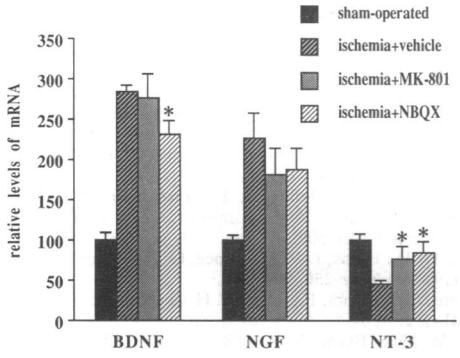

FIG. 4. Effects of pretreatment with MK-801 or NBQX on the expression of BDNF, NGF, and NT-3 mRNA 2 h after a 2-min period of ischemia. *, $P < 0.05$ compared to vehicle-injected animals (one-way ANOVA).

5 *A*, *B*, and *J*). After 30 min of hypoglycemia, BDNF mRNA levels were increased by 213%. Both 1 and 30 min of hypoglycemia induced 79% and 103% increases in NGF mRNA in the dentate gyrus, respectively (Fig. 5 *D–F* and *J*). No significant change in the expression of NT-3 mRNA could be found after hypoglycemia (Fig. 5 *G–J*). Increases in mRNAs for both NGF and BDNF were most pronounced 2 h after the insult. Similar to ischemia, the changes in mRNA expression after hypoglycemia were confined to the dentate gyrus granule cells.

DISCUSSION

The present data demonstrate (*i*) that brief periods of ischemia and insulin-induced hypoglycemic coma lead to rapid changes of gene expression for neurotrophic factors of the NGF family in the granule cells of the dentate gyrus; (*ii*) that there is a differential regulation of the mRNAs for NGF, BDNF, and NT-3 after these insults, with BDNF mRNA showing a more marked increase than NGF mRNA, and with NT-3 mRNA exhibiting a significant decrease; and (*iii*) that these changes of mRNA levels can be slightly attenuated but not completely blocked by antagonists at glutamate receptors. The activity-dependent increase of gene expression for neurotrophic factors in seizures is probably mediated via non-NMDA glutamate receptors (29, 32) since it can be completely blocked *in vivo* by NBQX (32) and also *in vitro* by CBQX (29), whereas MK-801 has no effect (29, 32). It seems unlikely that the postischemic changes of mRNA levels are seizure evoked since during the insult and in the 2-h recovery period we did not observe any seizure activity in the hippocampus, which agrees well with the findings of Buzsaki *et al.* (39). This conclusion is further supported by the results obtained after hypoglycemia, which does not predispose to postinsult seizure activity. However, similar to seizures, ischemia and hypoglycemia lead to glutamate release and an increase in intracellular calcium (40, 41). The widely distributed increase of c-fos mRNA expression, previously demonstrated in pentylenetetrazol- and kainic-acid-induced seizures (42, 43) and observed here after brief periods of ischemia, can probably be attributed to rapid influx of calcium ions (44). The involvement of calcium in the activity-dependent regulation of BDNF mRNA has been proposed by Zafra *et al.* (29), and calcium ions could trigger changes in gene expression for neurotrophic factors in these three pathological conditions. The routes of entry and sites of accumulation of calcium ions in the cells, critical to induce these genomic events, are not yet known. Hypoglycemia and ischemia are, in contrast to seizures, associated with energy deprivation, which may lead to higher intracellular calcium levels, supposedly through ion influx via multiple pathways and release from intracellular stores (40, 45, 46). Blockade of NMDA or non-NMDA receptors would therefore be expected to have less influence on the increase of intracellular calcium in ischemia/hypoglycemia than in seizures. The discrete effect of NBQX on the increase of BDNF mRNA in ischemia, as compared to that found in our previous study with kindled seizures (32), is in good agreement with this hypothesis. However, expression of NGF and BDNF mRNA is most likely not regulated by intracellular calcium levels alone since—e.g., in the CA1 region where influx of calcium ions rapidly occurs in ischemia (45, 46), BDNF and NGF mRNA levels were unchanged.

Whether the altered expression of NGF, BDNF, and NT-3 mRNAs following ischemia and hypoglycemia leads to corresponding changes in the levels of the trophic factor proteins remains to be elucidated. NGF content has previously been reported to be increased in the hippocampus 5 days after hypoxic injury (47), but no data are yet available on changes in BDNF or NT-3. The neurotrophic factors could induce plastic responses in specific neuronal systems leading to synaptic reorganization. NGF can stimulate sprouting of cholinergic axons in the adult rat cerebral cortex and hippocampus (8, 48). Furthermore, in rats subjected to repeated seizures by electrical kindling stimulations, which lead to increased levels of mRNA for NGF and BDNF (32), sprouting of mossy fibers and formation of aberrant synaptic contacts have been found in the hippocampus (49–52). Increased production of neurotrophic factors might also constitute a protective mechanism ameliorating brain damage after ischemia and hypoglycemia, particularly neuronal necrosis of the delayed type, which develops over several days

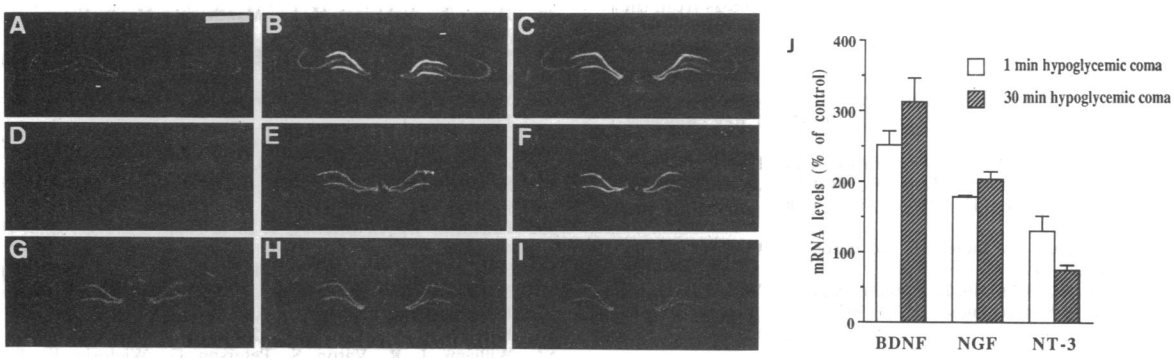

FIG. 5. Dark-field views of autoradiograms showing neurotrophin mRNA expression in the hippocampus after 1 min (*B*, *E*, and *H*) or 30 min (*C*, *F*, and *I*) of hypoglycemic coma. (*A*, *D*, and *G*) Sham-operated animals. Sections were hybridized to BDNF (*A–C*), NGF (*D–F*), or NT-3 (*G–I*) mRNA-specific probes 2 h after hypoglycemia. (Bar = 160 μm.) (*J*) Quantitative representation of neurotrophin mRNA expression in the dentate gyrus 2 h after 1 or 30 min of insulin-induced hypoglycemic coma. Values are expressed relative to sham-operated animals (means ± SEM).

after the insult (41, 53, 54). NGF is able to act as a rescue factor for axotomized basal forebrain cholinergic neurons (55–57). Similarly, BDNF increases the survival of several distinct neuronal populations *in vitro* (19–23). Although NT-3 can be expected to function as a trophic factor for specific central neurons *in vivo*, the role of this factor is at present not known. Interestingly, neuronal expression of NT-3 mRNA in the brain is not influenced either by kainic acid treatment or by kindled seizures (30, 32). Thus, the changes in NT-3 mRNA reported here document a possible regulation of this factor in response to altered physiological conditions in the brain.

The cells responsive to the presumed changes of NGF, BDNF, and NT-3 levels after ischemia and hypoglycemia are not known, but there are two main possibilities: First, both NGF and BDNF could influence long afferent systems such as cholinergic neurons from the basal forebrain. This is the classic target-mediated trophic action (1). Second, the neurotrophic factors could act locally, e.g., on the granule cells themselves in an autocrine or paracrine fashion. An autocrine stimulation by NGF has been postulated to occur in embryonic basal forebrain cholinergic neurons (24) and also for BDNF in stimulating neuronal survival in developing and possibly also in adult dorsal root ganglia (10, 58). It is interesting to note that the dentate granule cells, which showed rapid and marked changes of gene expression for neurotrophic factors after ischemia and hypoglycemic coma, are very resistant to ischemic damage (34, 41). Furthermore, the CA1 region, in which NGF and BDNF mRNA did not change, is most easily damaged in ischemia and hypoglycemia (34) and exhibits lower basal levels of BDNF mRNA than the CA3 region (10, 15, 17, 18). In addition, the level of NT-3 mRNA decreased in the CA1 region after the ischemic insults.

In conclusion, the changes of gene expression for neurotrophic factors described here might lead to a series of events affecting the survival, connectivity, and function of responsive neurons. If so, this could be important for the degree of neuronal necrosis and functional recovery after ischemic and hypoglycemic insults.

Note Added in Proof. In support of the hypothesis of a protective action of neurotrophins, Shigeno *et al.* (59) have reported that intraventricular injections of NGF ameliorate neuronal necrosis in the hippocampus after cerebral ischemia.

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