Coexpression of neurotrophins and their receptors in neurons of the central nervous system

(in situ hybridization/brain-derived neurotrophic factor/brain damage/hippocampus/epilepsy)

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**ABSTRACT** Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are neuronal survival molecules which utilize the Trk family of tyrosine kinase receptors. Using double-label in situ hybridization, we demonstrate that mRNAs for BDNF and its high-affinity receptor TrkB are coexpressed in hippocampal and cortical neurons. Also, a large number of neurons in these areas coexpress NGF and BDNF mRNAs. Epileptic seizures lead to increased levels of both BDNF/TrkB and NGF/BDNF mRNAs in double-labeled cells. Our results show that individual neurons of the central nervous system can coexpress neurotrophins and their receptors and produce two neurotrophic factors. These factors could support neuronal survival after brain insults, not only via retrograde transport but also through autocrine mechanisms.

Neurotrophic factors are necessary for the normal development and maintenance of the peripheral and central nervous systems. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4 (NT-4) also named neurotrophin 5, belong to a family of structurally related neurotrophic molecules, the neurotrophins (1-12). Members of the Trk family of tyrosine kinase receptors—TrkA, TrkB, and TrkC, have been shown to encode essential components of the functional high-affinity receptors for NGF, BDNF, NT-3, and NT-4 (10, 12-21). NGF mediates its effect via TrkA (14-16), and BDNF and NT-4 via TrkB (10, 12, 13, 17-19), whereas NT-3 interacts mainly with the TrkC receptor but also binds to TrkA and TrkB receptors (21).

The neurotrophins are all expressed in neurons of the central nervous system, with the highest mRNA levels in the hippocampus (22, 23). NGF is the prototype of a target-derived neurotrophic molecule and promotes the survival of basal forebrain cholinergic neurons (24, 25). NGF is produced by hippocampal and cortical neurons, taken up in cholinergic nerve terminals, and transported retrogradely to the cell bodies (24, 25). Recently BDNF and NT-3 were reported to display distinct patterns of retrograde axonal transport in central neurons (26). In the brain, TrkA mRNA expression appears to be restricted to NGF-responsive cells (27, 28), whereas cells expressing TrkB and TrkC mRNAs are widely distributed (21, 28-30). Epileptic seizures markedly increase levels of mRNA and protein for NGF and BDNF (31-38), reduce NT-3 mRNA expression (34, 39), and lead to elevated levels of TrkB mRNA and protein (29). Similar changes are observed after cerebral ischemia and insulin-induced hypoglycemic coma in the absence of seizure activity (40). These findings have raised the possibility that the neurotrophins act locally and protect against neuronal necrosis after brain insults characterized by excessive glutamate release. Such a role would require that neurotrophins and their receptors are localized in the same or neighboring cells—i.e., that they function through autocrine or paracrine mechanisms. In fact, autocrine or paracrine stimulation of cholinergic neurons by NGF has been postulated to occur in the embryonic hippocampus (41) and in the adult striatum (41) and basal forebrain (42). In this study, we show, using double-label hybridization histochemistry, that mRNAs for BDNF and TrkB and for BDNF and NGF are coexpressed in neurons in the normal brain and that seizure activity induces increased expression of both mRNA species in the same cell.

**MATERIALS AND METHODS**

Adult male Sprague–Dawley rats were either subjected to recurrent seizures induced by electrical kindling stimulations in the hippocampus or injected with kainic acid (10 mg/kg, i.p.). For kindling, stainless steel electrodes (o.d., 0.25 mm) were implanted bilaterally in the ventral hippocampus by using a Kopf stereotaxic frame (43). One week later, animals were given 40 stimulations on the left side (400-μA, 10-Hz, 1-ms square-wave pulses for 10 s with 5-min intervals) (44). Electroencephalographic activity was continuously monitored and behavioral seizures were scored. Control rats were connected to the stimulating-recording device or injected with saline. Two and 3 hr after the last kindling stimulation and the injection of kainic acid or vehicle, respectively, animals were decapitated and the brains were immediately frozen on dry ice and analyzed by in situ hybridization histochemistry.

Cryostat sections (14 μm) through the hippocampus were first hybridized to a 35S-labeled NGF or TrkB cRNA probe and then to a digoxigenin-11-dUTP-labeled BDNF cRNA probe, which had been prepared as follows: A 456-bp PCR fragment including primers and restriction site and encompassing nucleotides 1135–1515 in the rat trkB sequence (45) and a 762-bp fragment of the 3′ exon of the rat NGF gene (46) encompassing nucleotides 334–1095 were subcloned in plasmid pBluescript KS (Stratagene). The plasmid was linearized and the cloned DNA was transcribed in vitro by using T3 (antisense) or T7 (sense) RNA polymerase in the presence of [α-35S]UTP (Amersham, >1000 Ci/mmol; 1 Ci = 37 GBq). A 243-bp partial cDNA for rat BDNF (47) encoding amino acids 75–155 in prepro-BDNF was subcloned in pBSKS, linearized, and transcribed in vitro with T3 (antisense) or T7 (sense) RNA polymerases and labeled with digoxigenin DNA-labeling mixture (Boehringer Mannheim), containing digoxigenin-11-dUTP. Some sections were hybridized to a BDNF cRNA probe labeled with [α-35S]UTP.

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Abbreviations: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3.

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Fig. 1. Expression of NGF, BDNF, and TrkB mRNAs in coronal sections subjected to the double-label in situ hybridization procedure using 35S-labeled and digoxigenin-conjugated cRNA probes. Hybridization was performed using 35S-labeled NGF (A–C), TrkB (D–F, J, K, and N–P), and BDNF (L and M) antisense and TrkB sense (G–I) RNA probes and digoxigenin-conjugated BDNF antisense (A–K) and sense (N–P) RNA probes. Darkfield photomicrographs of x-ray film (A–I and L–N) or autoradiographic emulsion (O) show the hybridization to the 35S-labeled NGF (A–C), TrkB (D–F, N, and O), BDNF (L and M) antisense and TrkB sense (G–I) cRNA probes. Brightfield photomicrographs show hybridization to the digoxigenin-conjugated BDNF antisense (J and K) and sense (P) cRNA probes. A, D, G, J, and L are from controls; B, E, H, K, M, and N–P are from kainic acid-treated brains, and C, F, and I are from kindled brains. Kainic acid- and kindling-induced seizures caused increased expression of NGF mRNA in the dentate gyrus (compare B and C with A), of TrkB mRNA in the dentate gyrus, hippocampal pyramidal layer, piriform cortex, and neocortex (compare E and F with D), and of BDNF mRNA in dentate gyrus, hippocampal pyramidal layer (compare K and M with J and L), piriform cortex, and neocortex. Control hybridization to the 35S-labeled TrkB sense and the digoxigenin-conjugated BDNF antisense cRNA probes did not show any radioactive labeling (compare G–I with D–F), and control hybridization to the 35S-labeled TrkB antisense and the digoxigenin-conjugated BDNF sense cRNA probes (N–P) did not show any nonradioactive labeling (compare P with J and K). Arrowheads in O and P indicate borders of dentate gyrus granule-cell layer. Abbreviations: dg, dentate granule-cell layer; h, dentate gyrus hilus; CA1–CA3, hippocampal pyramidal layer. [Bar = 3.6 mm (for A–I and L–N), 330 μm (for J and K), and 645 μm (for O and P).]
In the first step, the sections were thawed onto poly(L-lysine)-coated slides (50 μg/ml). After fixation with 3% formaldehyde in phosphate-buffered saline (PBS) for 5 min, sections were rinsed once with PBS and twice with deionized and autoclaved water and were placed for 15 min in 0.2 M HCl. Sections were then rinsed twice with PBS and immersed in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 20 min. After two washes in PBS, sections were dehydrated and air-dried, and 80 μl of hybridization buffer (50% (vol/vol) deionized formamide/0.33 M NaCl/20 mM Tris-HCl, pH 7.5/1 mM EDTA/0.1 M dithiothreitol/10% (wt/vol) dextran sulfate/5× Denhardt’s solution with yeast tRNA at 0.5 mg/ml and synthetic poly(A) DNA at 0.1 mg/ml) containing 35S-labeled cRNA probe at 2.5 × 106 cpm/ml was applied per section. After hybridization overnight at 42°C, slides were washed four times (15 min each) in × SSC (standard saline citrate) at 55°C, cooled to room temperature, briefly dipped in deionized and autoclaved water, and air-dried.

In the second step, 100 μl of hybridization buffer containing 50 ng of digoxigenin-11-dUTP-labeled BDNF cRNA probe was applied per section. After hybridization overnight at 37°C, slides were washed twice in 1× SSC at 48°C, placed for 30 min at 37°C in 0.5 M NaCl/10 mM Tris-HCl, pH 8.0/1 mM EDTA with RNase A at 10 μg/ml, and washed twice in 1× SSC at 48°C and in 0.5× SSC and 0.1× SSC at 60°C. Slides were cooled to room temperature, rinsed twice with buffer 1 (100 mM Tris-HCl, pH 7.5/150 mM NaCl, incubated with 5% normal sheep serum in buffer 1 for 1 hr (150 μl per section), and then incubated for 5 hr at room temperature with 1% normal sheep serum, 0.3% Triton X-100, and alkaline-phosphatase-conjugated anti-digoxigenin serum (Boehringer Mannheim; 1:500) in buffer 1. Sections were washed twice for 10 min in buffer 1 and for 5 min in buffer 3 (100 mM Tris-HCl, pH 9.5/100 mM NaCl/50 mM MgCl2). They were then incubated overnight at room temperature in the dark in buffer 3 containing nitroblue tetrazolium salt (0.34 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (0.18 mg/ml) (Boehringer Mannheim). The sections were rinsed twice in buffer 2 and immersed in buffer 4 (10 mM Tris-HCl/1 mM EDTA, pH 8.0) for 6–8 hr and then in ×1 SSC overnight at room temperature. On the next day the slides were briefly rinsed in distilled water and air-dried, and sections were apposed to β-max x-ray film (Amersham). When films had been developed, photos of the distribution of digoxigenin-positive cells were taken under the microscope and the sections were then dipped in Ilford K5 emulsion and exposed for 8 weeks.

RESULTS

The specificity of the hybridization reaction was inferred from the following observations. (i) In situ hybridization of sections with a digoxigenin-labeled BDNF sense probe after hybridization to a 35S-labeled TrkB antisense probe gave rise to radioactive labeling of TrkB mRNA (Fig. 1 A and O) but did not result in any specific labeling of BDNF mRNA (Fig. 1P). Conversely, hybridization to a 35S-labeled TrkB sense probe and subsequent hybridization to a digoxigenin-labeled BDNF antisense probe showed no specific TrkB mRNA signal (Fig. 1 G–I). (ii) Identical regional distribution of NGF and TrkB mRNAs, as assessed with radiolabeled probes, was seen before and after hybridization to the digoxigenin-labeled BDNF cRNA probe (Fig. 1A–F). Similarly, nonradioactive hybridization to the BDNF cRNA probe (Fig. 1 J and K) was not changed by hybridization to 35S-labeled NGF or TrkB cRNA probe, and regional expression of BDNF mRNA was similar with both 35S- and digoxigenin-labeled probes (Fig. 1 J–M). (iii) The distribution, density, and mRNA levels of cells which hybridized to the various probes in the present double-labeling procedure in intact rats and after kindling and kainic acid (Fig. 1 A–F, L, and M) were identical to what has been described previously with single labeling techniques (22, 23, 28, 29, 35, 48–50).

In the control brains, BDNF and NGF mRNAs were colocalized in scattered neurons in the dentate gyrus granule-cell layer and hilus (Fig. 2A), in the pyramidal layer of the hippocampal CA1–CA3 regions, and in piriform cortex (Fig. 2C). All NGF mRNA-positive cells also expressed BDNF mRNA, whereas >95% of BDNF mRNA-positive cells in these regions were not labeled for NGF mRNA. No clearly double-labeled neurons were observed in the neocortex. Kainic acid-induced and kindled seizures markedly increased NGF mRNA expression in the dentate gyrus and piriform cortex and BDNF mRNA levels in dentate gyrus, hippocampal CA1–CA3 regions, piriform cortex, amygdala, and neocortex (Fig. 1 A–C and J–M). Following these treatments, >95% of BDNF mRNA-containing neurons in the dentate gyrus (Fig. 2B) and piriform cortex (Fig. 2D) exhibited NGF mRNA labeling, and the levels of both mRNAs within double-labeled cells were markedly higher than in controls. Also after seizures, all NGF mRNA-containing cells in dentate gyrus and piriform cortex expressed BDNF mRNA, while all labeled neurons in neocortex and amygdala contained BDNF mRNA alone.

The control brains showed coexistence of BDNF and TrkB mRNAs in many regions. Virtually all BDNF mRNA-expressing neurons in the dentate granule-cell layer and hilus (Fig. 3A), pyramidal layer of hippocampal CA1–CA3 regions, and piriform cortex (Fig. 3E) were also labeled for TrkB mRNA. A few TrkB mRNA-positive profiles, possibly glial cells, were present outside the principal neuronal layers in these regions. Also in neocortex (Fig. 3C) and amygdaloid complex, all BDNF mRNA-containing cells hybridized to the TrkB mRNA probe. Cells expressing only TrkB mRNA were

![Fig. 2. Brightfield photomicrographs showing hybridization to the 35S-labeled NGF and digoxigenin-conjugated BDNF cRNA probes in the dentate gyrus (A and B) and piriform cortex (C and D).](image-url)
in are probes in the dentate (4B), hippocampal (Fig. 3F and 4A), and the piriform cortex (E and F). Examples of cells coexpressing BDNF and TrkB mRNAs are seen in controls (A, C, and E), and with higher levels of both mRNA species, following kainic acid (B and F) or kindling (D). A cell in F expressing TrkB mRNA alone is indicated by arrowheads. (Bar = 16 μm.)

scarce (Fig. 3F) and in neocortex, for example, represented only about 3% of the labeled cells. Following kainic acid or kindling, the intensity of labeling for both BDNF and TrkB mRNAs within double-labeled neurons had increased markedly in the dentate granule-cell layer and hilus (Figs. 3B and 4B), hippocampal CA1–CA3 regions, piriform cortex (Figs. 3F and 4A), and neocortex (Fig. 3D).

**DISCUSSION**

Our data demonstrate that NGF and BDNF mRNAs can be synthesized by an individual neuron and that seizure activity leads to increased expression of both mRNA species in the same cell. The two neurotrophins produced by the same cell could have separate functional roles. NGF synthesized in a hippocampal or cortical neuron might act as a target-derived trophic factor for cholinergic neurons (24, 25), whereas BDNF in the same cell could provide local trophic support via an autocrine mechanism. The proposed similarities in the three-dimensional structure of the neurotrophins (51) may also suggest that NGF and BDNF could form heterodimeric complexes as a consequence of coexpression in the same cell. Receptor activation by such complexes may lead to hitherto unknown biological activities of the neurotrophins.

Supporting a role for NGF as a target-derived neurotrophic factor, mRNA for its high-affinity receptor, TrkA, has been found in basal forebrain cholinergic cell bodies but not in their hippocampal and cortical projection areas (27, 28). In contrast, the widespread coexpression of BDNF and TrkB mRNAs demonstrated here provides evidence that BDNF acts via an autocrine mechanism. Throughout the forebrain all BDNF mRNA-containing cells hybridized also to the TrkB probe. The TrkB probe used in this study detects messages encoding both truncated receptors lacking the tyrosine kinase domain and full-length TrkB receptors. Virtually all dentate granule cells and pyramidal neurons of the piriform cortex contain TrkB mRNA coding for the functional receptor (29). Since we found that the overall majority of neurons in these areas hybridized to both the BDNF and TrkB mRNA probes, it seems highly likely that BDNF mRNA is also coexpressed with mRNA for the functional TrkB receptor. The small portion of cells which hybridized to the TrkB mRNA probe alone often had a glial distribution, being localized in areas largely devoid of neurons.

If BDNF acts via an autocrine mechanism it is probably secreted and activates the TrkB receptor at the surface of the BDNF-synthesizing cell itself. A local effect of BDNF in the hippocampus is supported by data showing rapid induction of the c-fos gene in cultures of embryonic hippocampal neurons after addition of BDNF (52). Increased expression of BDNF and TrkB mRNAs in vivo occurs already after brief periods (1–2 min) of epileptic seizures, cerebral ischemia and hyperglycemic coma (34, 35, 40). If prolonged, these insults lead to neuronal necrosis in, for example, cortex and hippocampus (53). Our finding of a widespread increase of BDNF and TrkB mRNAs within the same neuron after kainic acid-induced and kindled seizures indicates that the rapid induction of BDNF

**Fig. 3.** Brightfield photomicrographs showing hybridization to the 35S-labeled TrkB and digoxigenin-conjugated BDNF cRNA probes in the dentate gyrus hilus (A and B), the neocortex (C and D), and the piriform cortex (E and F). Examples of cells coexpressing BDNF and TrkB mRNAs are seen in controls (A, C, and E), and with higher levels of both mRNA species, following kainic acid (B and F) or kindling (D). A cell in F expressing TrkB mRNA alone is indicated by arrowheads. (Bar = 16 μm.)

**Fig. 4.** Brightfield color photomicrographs showing hybridization to the 35S-labeled TrkB and digoxigenin-conjugated BDNF cRNA probes in the piriform cortex (A) and dentate gyrus hilus (B) following either kindling stimulations (A) or kainic acid injection (B). Note that the cells are coexpressing high levels of both BDNF (bluish/purple precipitate) and TrkB (black grains) mRNAs. (Bar = 19 μm.)
mRNA expression after brain insults acts to protect the BDNF-synthesizing neuron itself. Elevated levels of both the full-length and the truncated TrkB receptor may amplify the protective effect of BDNF, the truncated receptor by buffering the concentration of the ligand (i.e., maintaining a high local concentration of BDNF), and the full-length receptor by increasing the biological response to BDNF.

Growth factor stimulation via autocrine mechanisms has been implicated in cell transformation and tumor growth (54). Autocrine stimulation may also occur in normal cells—for example, by platelet-derived growth factor (PDGF) in dorsal root ganglia and Schwann cells (55) and by BDNF and NT-3 in developing sympathetic and sensory root ganglia (56, 57). In the brain, synthesis of a trophic factor and its receptor by the same neuron, as shown here for BDNF and TrkB, has not been demonstrated previously, although the overlapping distribution of cells expressing a neurotrophin and a neurotrophin receptor, respectively, has suggested a local mode of action in the hippocampus (29, 41). Both PDGF and PDGF receptors have been detected in central neurons (58–60), though not reported in the same cell, which suggests that autocrine regulation of neuronal function may be a more general principle for trophic factor action in the central nervous system. Multiple trophic factors may be necessary for survival of the synthesizing neuron and its afferent neuronal inputs both under physiological conditions and following brain insults.

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