Detection of Brain-derived Neurotrophic Factor in a Vesicular Fraction of Brain Synaptosomes*

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The mRNA encoding brain-derived neurotrophic factor (BDNF) is widely distributed in central nervous system neurons, including in hippocampus and cortex. However, little is known about the physiology of BDNF protein within neurons, including how it is processed or packaged and the mechanisms that control its release. In this study, we have used antibodies to monitor the subcellular distribution of BDNF in cortical extracts from adult rats treated with kainic acid. BDNF immunoreactivity is elevated in rat cortex 12 h after kainic acid treatment. The protein is enriched in a vesicular fraction isolated from lysed synaptosomes, its distribution being similar to that of synaptotagmin, which is associated with synaptic vesicles and large dense core vesicles at nerve terminals. The vesicular pool of BDNF is digested by proteinase K only in the presence of Triton X-100 suggesting localization of BDNF in membrane fractions. Immunocytochemistry detects diffuse and punctate BDNF staining within cell bodies and processes of cortical neurons from kainic acid-treated rats, as well as in mossy fiber terminals of rat hippocampus. Taken together, these data show that BDNF can accumulate axonally within a vesicular compartment of brain neurons. Results support the idea that endogenous BDNF may be transported anterogradely and released by regulated secretory mechanisms.

Neurotrophins promote the development and survival of neurons in both the peripheral and central nervous systems (1–3). In the peripheral nervous system, neurotrophins are produced by non-neuronal cells in peripheral tissues and, following nerve injury, by Schwann cells and fibroblasts surrounding peripheral nerves (4). During development, neurons compete for limiting amounts of neurotrophins in peripheral targets to survive (5, 6). Neurotrophins interact with cell surface receptors on neurons and are thought to mediate survival through retrograde axonal transport of neurotrophin-receptor complexes (7).

Within the central nervous system, the function of neurotrophins is less clear. Neurotrophins are produced by neurons and may function in a paracrine or autocrine fashion to promote nerve cell survival (8, 9). Neurotrophin production is elevated as a result of increased neuronal activity (10, 11) and in response to neuronal injury induced, for example, by anoxia or epileptogenic drugs (12, 13).

Little is known about the mechanisms by which cells process and release neurotrophins. Fibroblasts and Schwann cells associated with peripheral nerves likely release the neurotrophins by constitutive secretion since these cells do not contain a regulated secretory pathway. In contrast, central nervous system neurons contain constitutive as well as regulated secretory pathways (14), the latter being used to package neurotransmitters within synaptic vesicles and neuropeptides within large dense core secretory vesicles (14, 15).

Several studies have suggested that neurons target neurotrophins to the regulated secretory pathway for release at presynaptic nerve terminals. Radiolabeled human recombinant neurotrophin 3 and brain-derived neurotrophic factor (BDNF) injected into the chick eye are anterogradely transported to presynaptic terminals within the tectum. The proteins are subsequently taken up into multivesicular bodies in postsynaptic neurons (16). Also, cultured hippocampal neurons and PC12 cells infected with a herpes simplex virus vector expressing BDNF release the protein following depolarization (17). Pharmacological evidence suggests that nerve growth factor is released from cultured hippocampal neurons by a novel sodium-dependent regulated pathway as well as by constitutive release (18). Despite this evidence, however, virtually nothing is known about the subcellular distribution or transport of endogenous neurotrophins within brain neurons.

In this study, we have treated rats with kainic acid, an epileptogenic drug, and detected BDNF within a microvesicular fraction of lysed synaptosomes from cerebral cortex. The distribution of BDNF is similar to that of synaptotagmin, a protein associated with Ca²⁺-mediated vesicular release from nerve terminals. BDNF appears to be localized within a membrane-bound vesicular structure since it is sensitive to digestion with proteinase K only in the presence of detergent. Immunocytochemistry detects BDNF in the mossy fibers of the hippocampus and in punctate structures within the cell bodies and processes of cortical neurons in kainic acid-treated rats. Taken together, these data suggest that BDNF is associated with vesicles within the regulated secretory pathway of central nervous system neurons. The data are also consistent with the idea that endogenous BDNF is transported anterogradely in central nervous system neurons.

* The abbreviations used are: BDNF, brain-derived neurotrophic factor; hrBDNF, human recombinant BDNF.


**MATERIALS AND METHODS**

*Reagents—* Synaptotagmin antibody was kindly supplied by Dr. Pietro DeCamilli (19). BDNF antibody was obtained from Santa Cruz Biotechnology (for Western blotting) or from AMGEN (for immunocytochemistry). The Santa Cruz antibody was raised against a peptide from the carboxy-terminal region of mature BDNF and affinity purified. The AMGEN BDNF antibody (called RAB) was raised against mature BDNF and has been characterized previously (20). Horseradish peroxidase-conjugated and biotin-conjugated goat anti-rabbit antibodies were obtained from Boehringer Mannheim. Recombinant human BDNF was a gift from Regeneron. All other reagents, unless otherwise noted, were purchased from Sigma.

**Detection of BDNF in Rat Cerebellum—** Adult male Harlan Sprague Dawley rats (150–200 g) (Charles River Laboratories, Montreal, Canada) were injected subcutaneously with kainic acid (10 mg/kg) according to an animal care protocol meeting the standards of the Canadian Council on Animal Care. Those animals showing clear seizure activity were sacrificed 12 h after receiving kainic acid. The neocortex and entorhinal cortex were removed and immersed in cold Tris lysis buffer (0.1 M/tris/1 g of tissue), homogenized in a Polytron, incubated for 30 min at 4 °C, and centrifuged at 13,000 rpm for 15 min. The supernatant was centrifuged (100,000 × g), 50 min) in a Beckmann mini-ultracentrifuge, and the supernatant was analyzed. Tissue extracts from BDNF null mice (21) (Jackson Laboratories, Bar Harbor, ME) were processed similarly.

Subcellular fractionation was carried out by differential centrifugation as described previously (22), and samples were analyzed by Western blotting. Briefly, the cortex was removed from kainic acid-treated rats and homogenized (H) in 4 mM Hepes-OH buffer (pH 7.3) containing 320 mM sucrose. Crude synaptosomes (P$_1$) were separated from cytosol (S) and microsomes (P$_2$) by differential centrifugation of a postnuclear supernatant. Synaptosomes were disrupted by hypotonic shock and centrifuged, and the supernatant was centrifuged at 260,000 × $g$ to isolate an LP$_2$ microsomal fraction. Samples containing approximately 50 μg of protein per well were electrophoresed on polyacrylamide gels using standard SDS sample buffer containing 2-mercaptoethanol.

Proteinase K Digestion—Aliquots containing 50 μg of protein from the LP$_2$ fraction were digested on ice with proteinase K (0.4 μg/ml) for 30 min in the presence or absence of 0.5% Triton X-100. The samples were transferred into electrophoresis buffer with pipette tips dipped in 200 mM phenylmethylsulfonyl fluoride in dimethyl sulfoxide, electrophoresed on 15% SDS gels, and analyzed by Western blot.

Immunohistochemistry—Adult rats (normal and kainic acid-treated) as well as control and BDNF null mice were injected with Somnotol and transcardially perfused with heparanized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed, postfixed in the same fixative for 2 h at 4 °C, immersed in 30% sucrose in 0.1 M phosphate buffer for 48 h at 4 °C, and imbedded in OCT freezing medium. Free floating sections (20 μm) cut on a cryostat were washed three times in 0.1 M phosphate buffer followed by incubation in blocking buffer (10% normal goat serum, 0.3% Triton X-100 in 0.1 M phosphate buffer) for 2 h at room temperature. Sections were incubated overnight at 4 °C in the AMGEN (RAB) BDNF antibody diluted 1:5000 in blocking solution. Sections were washed 3 times for 15 min each in blocking solution and incubated for 1 h at room temperature in a biotin-conjugated goat anti-rabbit antibody diluted 1:200 in blocking solution. Tissues were washed as above in blocking solution and incubated 1 h according to the protocol outlined in the VectorStain ABC Elite kit (Dimension Laboratories). Following three additional washes in 0.1 M phosphate buffer, the tissue was bathed in 0.003% 3,3’-diaminobenzidine tetrahydrochloride in 0.1 M phosphate buffer with 0.006% hydrogen peroxide. Sections were rinsed twice, mounted on slides, dehydrated, and coverslipped. In control experiments, the AMGEN (RAB) antibody was incubated overnight in the cold with excess human recombinant BDNF (5 μg), the solution was centrifuged for 15 min at 13,000 rpm, and the supernatant was used for immunocytochemistry as described above.

**RESULTS AND DISCUSSION**

**Detection of BDNF in Rat Cortex—** Fig. 1 is a Western blot showing the specificity of the antibody used in this study for biochemical detection of BDNF (Santa Cruz Biotechnology). The BDNF antibody reacts with human recombinant BDNF as well as with a protein of identical mobility (14 kDa) in extracts of normal mouse brain that is absent from BDNF null mice. BDNF protein levels increased in the cortex of rats treated for 12 h with kainic acid, a result consistent with previous data showing increases in BDNF mRNA (13). The antibody also reacted with two proteins in rat and mouse brain (molecular masses of 20 and 28 kDa). These proteins are also seen in BDNF null mice, suggesting that they are not related to BDNF. In extracts of rat cortex (but not mouse), an immunoreactive protein of 13 kDa was also detected. This same protein was detected by a second antibody raised against a different peptide from the carboxyl terminus of BDNF (kindly provided by Dr. David Kaplan), suggesting that it may contain sequences in common with BDNF (data not shown). Competition experiments using excess BDNF reduced but did not oblate the immunoreactivity of the 13-kDa band (data not shown). Further characterization of this protein is needed. We conclude that the protein migrating at 14 kDa is authentic BDNF, since it is absent from BDNF null mice. It is noteworthy that the mobility on SDS gels of BDNF from rat and mouse is identical to that of human recombinant BDNF. This result differs from the conclusions of others who have suggested that the rat BDNF has a slightly lower molecular mass than human recombinant BDNF (23, 24).

**Detection of BDNF in Synaptosomally Derived Vesicular Fractions—** We used differential centrifugation to examine the distribution of BDNF in cortical extracts from kainic acid-treated rats. BDNF immunoreactivity was detectable in the homogenate (H), as well as in the P$_2$ and P$_3$ fractions. Highest levels of BDNF were detectable within the LP$_2$ fraction (Fig. 2, *upper panel*) which is known to contain microvesicles isolated from lysed synaptosomes (22). The distribution of BDNF was similar to that of synaptotagmin (Fig. 2, *lower panel*), a protein involved in Ca$^{2+}$-mediated vesicular release that is present on both synaptic vesicles and large dense core vesicles isolated from synaptic terminals (19, 25). Its presence in the LP$_2$ fraction strongly suggests that BDNF is located presynaptically, as has been reported for synaptotagmin (26), dynamin (26), synapmin I (27), and secretogranin II (27). Another immunoreactive band (16 kDa) was evident in fractions P$_1$, P$_2$, and LP$_1$, all of which are enriched in membranes. This band was evident in samples homogenized in Hepes buffer but was absent from brain extracts homogenized in Tris lysis buffer and centrifuged at high speeds (Fig. 1). The identity of this protein remains to be determined.

**BDNF Is Present in Vesicles—** Fig. 3 (*upper panel*) shows that BDNF in the LP$_2$ fraction is digested by protease K only in...
the presence of Triton X-100 (lane 3), a detergent that disrupts and solubilizes membranes. This result is consistent with BDNF being localized within membrane-bound vesicles. To confirm this result, we probed identical Western blots with an antibody to synaptotagmin. Fig. 3 (lower panel, lane 1) shows that following digestion with protease K in the absence of detergent, synaptotagmin migrates in two forms, a weakly reactive 65-kDa mature form and a more prominent form of 28 kDa. This smaller fragment is related to the unexposed aminoterminal region of synaptotagmin located within vesicles, as reported previously (27). Synaptotagmin is fully digested by protease K in the presence of Triton X-100 (lane 2).

Immunocytochemistry—We attempted to use immunocytochemistry to localize BDNF in brain sections using the antibody from Santa Cruz Biotechnology. However, in test studies, the antibody detected immunoreactivity in the brains of BDNF null mice as well as in sections from control mouse brain treated with antibody that had been preabsorbed with excess hrBDNF. Therefore, this antibody was unsuitable for immunocytochemistry, at least in our hands. In contrast, the RAB BDNF antibody from AMGEN, which in diluted solution failed to detect BDNF on Western blots, is effective for immunocytochemistry, as described previously (20).

Large increases in BDNF mRNA have been reported previ-
ously in dentate granule cells of rat hippocampus following kainic acid treatment (13). Dentate granule cells extend axons (called mossy fibers) through the hilus of the hippocampus to innervate pyramidal CA3 neurons. Fig. 4A shows dense BDNF immunoreactivity in mossy fiber terminals of hippocampus from a kainic acid-treated rat. Staining was also detected in the mossy fibers of control mice, but was totally absent from the hippocampus (or other brain regions) of BDNF null mice (data not shown). Staining was absent from sections of rat hippocampus treated with antibodies to BDNF that had been preabsorbed with excess human recombinant BDNF (Fig. 4A) (inset). The cell bodies of dentate granule cells showed little BDNF immunoreactivity in kainic acid-treated rats suggesting that the protein is concentrated within axons and terminals. BDNF staining within mossy fiber terminals (Fig. 4B) is similar to that of synaptophysin, as reported previously (19).

Within the cortex of kainic acid-treated rats, BDNF immunoreactivity was detectable within the cytoplasm and processes of neurons located predominantly in layers 2 and 5 (data not shown). Staining was either diffuse or, in some cases, punctate (Fig. 4C) and associated with vesicular-like structures in both the cell body and processes of neurons. The precise nature of these punctate staining structures remains to be determined.

Differential centrifugation data in this study show that BDNF is present in microvesicles isolated from a synaptosomal fraction of rat cortex. Furthermore, immunocytochemistry shows that BDNF is present in mossy fiber terminals. In addition, punctate structures containing BDNF immunoreactivity are evident within the cytoplasm and processes of neurons in rat cortex. These data, taken together with the results of others (16–18), are consistent with the idea that endogenous BDNF is transported anterogradely, targeted toward the regulated secretory pathway, and localized within the presynaptic compartment of neurons. Therefore, BDNF and perhaps other neurotrophins may represent an additional class of proteins, like neuropeptides, that are packaged and released from neurons via regulated secretory mechanisms. It will be important to determine the precise nature of the vesicles that contain BDNF and also the controls that regulate its release from neurons.

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