BDNF-GFP containing secretory granules are localized in the vicinity of synaptic junctions of cultured cortical neurons

Wulf Haubensak, Frank Narz, Rolf Heumann and Volkmar Leßmann*

Lehrstuhl für Molekulare Neurobiochemie, Ruhr-Universität Bochum, NC7/170, 44780 Bochum, Germany
*Author for correspondence (e-mail: volkmar.lessmann@ruhr-uni-bochum.de)

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SUMMARY

The protein family of mammalian neurotrophins, comprising nerve-growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 and -4/5 (NT-3, NT-4/5), supports the survival and the phenotype of neurons from the central as well as the peripheral nervous system (CNS, PNS). In addition, exogenous application of neurotrophins has recently been found to modulate synaptic transmission in the rodent CNS. However, to provide evidence for a role of neurotrophins as endogenous fast acting modulators of synaptic transmission, the synaptic localization and secretion of neurotrophins needs to be shown.

We have now constructed a fusion protein consisting of N-terminal BDNF (the most abundant neurotrophin in the rodent hippocampus and neocortex) and C-terminal green fluorescent protein (GFP) to elucidate the cellular localization of BDNF in cortical neurons. Transient expression of BDNF-GFP in COS-7 cells revealed that the cellular localization in the trans-Golgi network (TGN), the processing of precursor proteins and the secretion of mature BDNF-GFP is indistinguishable from the properties of untagged BDNF.

Upon transient transfection of primary rat cortical neurons, BDNF-GFP was found in secretory granules of the regulated pathway of secretion, as indicated by colocalization with the secretory granule marker secretogranin II. BDNF-GFP vesicles were found in the neurites of transfected neurons with a pattern reminiscent of the localization of endogenous BDNF in untransfected cortical neurons. BDNF-GFP vesicles were found predominantly in the somatodendritic compartment of the neurons, whereas additional axonal localization was found less frequently. Immunocytochemical staining of synaptic terminals with synapsin I antibodies revealed that the density of BDNF-GFP vesicles is elevated in the vicinity of synaptic junctions, indicating that BDNF is localized appropriately to function as an acute modulator of synaptic transmission.

These data suggest that BDNF-GFP will be a useful tool to investigate synaptic release of BDNF during physiological synaptic stimulation, and will thereby allow us to elucidate the participation of neurotrophin release in activity dependent synaptic plasticity.

Key words: Neurotrophin, Synaptic plasticity, Secretogranin II, Transfection, COS cell

INTRODUCTION

The protein family of mammalian neurotrophins comprises NGF (nerve growth factor), BDNF (brain-derived neurotrophic factor), NT-3 (neurotrophin-3) and NT-4/5. Neurotrophins were originally described as homodimeric proteins which are released in limited quantities by target tissues (or target neurons) to support the survival and the function of the innervating neurons (reviewed by Thoenen, 1991).

In recent years, it has been shown that in the central nervous system (CNS) neurotrophins might serve an additional purpose, namely the modulation of the efficacy of synaptic transmission. It was shown that neurotrophins are synthesized in an activity dependent manner by hippocampal and neocortical neurons (Zafra et al., 1992; Patterson et al., 1992; Drägónow et al., 1993; Castren et al., 1992, 1993; Rocamora et al., 1996), and are secreted into the extracellular space following depolarization of hippocampal neurons (Blöchl and Thoenen, 1995, 1996; Goodman et al., 1996). On the other hand, it was shown that exogenously applied neurotrophins are capable of enhancing glutamatergic synaptic transmission in the rat hippocampus (Leßmann et al., 1994; Kang and Schuman, 1995; Levine et al., 1995; Figurov et al., 1996; Scharfman, 1997; Leßmann and Heumann, 1998) and neocortex (Carmignoto et al., 1997; Akaneya et al., 1997). Consequently, it has been speculated that neurotrophins might be released synaptically upon intense excitatory synaptic stimulation, leading in turn to the strengthening of the active synapses. Thus, neurotrophins are hypothesized to function as intercellular synaptic messengers providing positive feedback at intensely stimulated excitatory synapses. This concept is supported by the finding, that long-term potentiation in BDNF knockout mice is impaired (Korte et al., 1995; Patterson et al., 1996). Since activity dependent modulation of synaptic transmission is widely believed to be the cellular correlate of learning and memory formation in mammals, the elucidation...
of the molecular determinants of synaptic plasticity is an important task. However, to establish neurotrophins as endogenously employed synaptic messengers, it is inevitable to directly show the localization and activity-dependent secretion of these proteins from synaptic structures, and evidence for such a synaptic localization/secretion is, as yet, still missing.

The heterologous expression of the green fluorescent protein (GFP) from the jellyfish Aequoria victoria (Prasher et al., 1992; Chalfie et al., 1994) has been shown to yield an autofluorescent protein in many types of cells from different species (for a review see Cubitt et al., 1995). Chimeras consisting of a specific protein of interest and GFP have been used successfully to study the subcellular distribution and the transport of very different types of proteins in mammalian cells (for a review see Gerdes and Kaether, 1996). If the GFP tag (either N-terminal or C-terminal to the protein of interest) is located distantly from targeting signals of the tagged protein, the resulting fluorescent chimeras show identical subcellular localization when compared to the respective untagged proteins (for a review see Cubitt et al., 1995). Thus, GFP-tagged proteins are ideally suited to follow time resolved protein targeting within living cells.

We have now constructed a fusion protein consisting of N-terminal brain-derived neurotrophic factor (BDNF; the most abundant neurotrophin in the rodent hippocampus) and a red-shifted excitation variant of GFP, to investigate the intracellular targeting of this GFP-tagged neurotrophin (BDNF-GFP) in transiently transfected primary cortical neurons and secondary cell lines.

Our results provide evidence that GFP-tagged BDNF is localized and secreted from mammalian cells in a manner that is indistinguishable from endogeneous BDNF and from heterologously expressed untagged BDNF, respectively. As shown by the colocalization with the secretory granule marker secretogranin II, BDNF-GFP is targeted to secretory vesicles which are transported along the neurites of cortical neurons. These BDNF-GFP vesicles are localized in the vicinity of synaptic junctions. Thus, our experiments visualize for the first time directly that BDNF is localized appropriately to function as an acute intercellular synaptic messenger in CNS neurons, and the fusion protein will allow us to follow synaptic release of neurotrophins from living cells upon physiological synaptic stimulation.

MATERIALS AND METHODS

Cell culture

COS-7 cells were grown in culture flasks in DMEM/10% FCS at 37°C and 10% CO₂ in a humidified incubator. For immunocytochemical processing, COS cells were grown on glass coverslips in culture dishes. For staining of living cells (i.e. GFP fluorescence, Bodipy), cells were cultured in glass bottom culture dishes: a hole (1.5 cm in diameter), that was drilled into the bottom of 3.5 cm culture dishes. For staining of living cells (i.e. GFP fluorescence, Bodipy), cells were cultured in glass bottom culture dishes: a hole (1.5 cm in diameter), that was drilled into the bottom of 3.5 cm culture dishes. The conditioned culture medium was replaced by 1 ml Heps-buffered saline (HBS) containing: 135 mM NaCl, 20 mM Hepes, 4 mM KCl, 1 mM Na₂HPO₄, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose. For one culture dish, 5 g of the respective plasmid cDNA (cDNA stock: 1 µg/µl in 10 mM Tris-HCl, 1 mM EDTA; pH 7.9) was dissolved in 60 µl 250 mM CaCl₂, mixed with 60 µl 4X BBS (280 mM NaCl, 15 mM Na₂HPO₄, 50 mM BES, pH 7.1), and added to 1.2 ml of the conditioned medium. HBS in the culture dish was replaced by this transfection solution and 0.9 µl PEI solution (50%, 800,000 kDa, Fluka) per ml H₂O, pH 7.1. After 3-4 days in vitro (DIV) with 10 µM cytosine-β-arabinofuranoside (ARAC) to restrict glial proliferation. Equal results were obtained in hippocampal and neocortical cultures. Thus, we use the term cortical neurons throughout the manuscript.

Construction of expression vectors

The expression plasmid pBDNF-GFP was constructed as follows: the complete sequence of the prepro rat BDNF cDNA in pBluescript was amplified by PCR using a 5′-primer (27 nucleotides) which introduced an EcoRI cleavage site at the N terminus, and a 3′-primer (25 nucleotides) introducing a BamHI cleavage site and removing the stop-codon at the C terminus of the rat BDNF cDNA. The PCR amplification was performed using Vent thermostable DNA polymerase in the buffer supplied by the manufacturer (New England Biolabs). The BamHI-EcoRI-fragment of the PCR product was cloned into the multiple cloning site of the CMV-promotor driven pEGFP-N1 (Clontech) expression vector. The resulting construct pBDNF-GFP codes for C-terminal EGFP-tagged prepro BDNF. The EGFP cDNA contains the S65T chromophore mutation yielding a red shifted excitation spectrum (Heim et al., 1994) and ~190 silent base changes leading to improved protein expression in mammalian cells (Cormack et al., 1996).

The pBDNF expression plasmid was constructed following a similar strategy. The PCR amplification was performed with the same 5′-primer as used for construction of pBDNF-GFP and a 3′-primer (28 nt) introducing two consecutive stop codons and a BamHI restriction site at the end. When cloned into the EGFP-N1 vector, the resulting plasmid (pBDNF) was identical to pBDNF-GFP except for the presence of the two stop codons between the BDNF and the GFP portion of the construct, thus leading to the expression of unfused prepro BDNF.

The two different BDNF amplification products were checked for the absence of PCR mutations using an ALFexpress automated sequence analyzer (Pharmacia). As a control for the cellular distribution of unfused GFP, the original pEGFP-N1 vector (Clontech) was used. This plasmid is named pGFP throughout the paper.

Transfection

COS-7 cells were transiently transfected using the polyethylenimine (PEI) protocol (Boussif et al., 1995). The PEI stock solution contained 0.9 mg PEI solution (50%, 800,000 kDa, Fluka) per ml H₂O, pH adjusted to 6.5 using HCl. The transfection solution contained 20 µl plasmid DNA and 60 µl PEI stock per ml 150 mM NaCl. For western blot analysis, COS-7 cells were transfected 24 hours later at a density of 20,000 cells/cm². COS cells for subsequent immunocytochemical analyses were plated at lower densities (10,000 cells/cm²) and were transfected 24 hours later. Transfections were performed using the equivalent of 3 µg plasmid DNA in transfection solution per ml serum-free DMEM. After 4 hours of incubation at 37°C (10% CO₂) the transfection medium was diluted 1:1 by adding fresh culture medium (DMEM/20% FCS). After additional 16 hours of incubation, cells were washed with normal culture medium (DMEM/10% FCS) and cultured for 2 more days.

Primary neuronal cultures were transfected after 7-11 DIV using Ca⁴⁺ phosphate precipitation. The conditioned culture medium was replaced by 1 ml Heps-buffered saline (HBS) containing: 135 mM NaCl, 20 mM Heps, 4 mM KCl, 1 mM Na₂HPO₄, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose. One culture dish, 5 µg of the respective plasmid cDNA (cDNA stock: 1 µg/µl in 10 mM Tris-HCl, 1 mM EDTA; pH 7.9) was dissolved in 60 µl 250 mM CaCl₂, mixed with 60 µl 4X BBS (280 mM NaCl, 15 mM Na₂HPO₄, 50 mM BES, pH 7.1), and added to 1.2 ml of the conditioned medium. HBS in the culture dish was replaced by this transfection solution and 0.9 µl PEI solution (50%, 800,000 kDa, Fluka) per ml H₂O, pH 7.1. After additional 16 hours of incubation, cells were washed with normal culture medium (DMEM/20% FCS) and cultured for 2 more days.
incubated for 3 hours at 37°C and 2.5% CO₂. Cells were washed with HBS and cultured in conditioned medium diluted 1:1 with fresh DMEM/B18. All experiments were performed 24–48 hours posttransfection.

**Immunocytochemistry**

COS-7, hippocampal, and neocortical cultures grown on glass coverslips were fixed for 20 minutes at room temperature (RT) in 4% paraformaldehyde in phosphate buffered saline (PBS), supplemented with 120 mM sucrose. For anti-Tau staining (see below), fixation was performed at 37°C for 40 minutes. After 3 washes in PBS, cells were permeabilized (10 minutes; RT) using 0.25% Triton X-100 in PBS. After washing, cells were incubated (60 minutes; RT) in PBS containing 10% BSA (Serva, fraction V) and 0.1% Triton X-100, to reduce unspecific binding of antibodies. After washing, the cells were incubated (1 hour; RT) with primary antibodies in PBS (1% BSA, 0.1% Triton X-100) at indicated dilutions: anti-BDNF (Santa Cruz, rabbit, 1:400); anti-Golgi-58K protein (Sigma, mouse, 1:50); anti-synapsin I (mouse, 1:500); anti-Tau (Boehringer Mannheim, mouse, 1:200); anti-secretogranin II (rabbit, 1:200). As secondary antibodies, either FITC- or TRITC-conjugated anti-rabbit IgG or biotin-conjugated anti-mouse IgG in PBS (1% BSA, 0.1% Triton X-100; 1 hour at RT) was used. For detection of biotin-conjugated secondary antibodies, Extravidin-TRITC (Sigma, 1:400) was incubated for 1 hour at RT in PBS (as above). Following 3 additional washes, immunofluorescence was viewed through an axioskop fluorescence microscope (Zeiss) using standard TRITC and FITC filter sets. GFP fluorescence was also viewed through a standard FITC filter set.

The number of BDNF-GFP packages in the vicinity of synapsin I immunoreactive terminals was counted. In high power magnifications of photomicrographs (see Fig. 7C,D), the number of green packages was determined in quadrats of 1 μm² in size. The center of these quadrats was defined as the center of the synapsin I immunoreactive spots. Likewise, the density of green packages in neighbouring segments of the same neurites, where synapsin I staining was absent, was determined.

**Live Golgi stain with Bodipy TR ceramide**

Defatted BSA (DF-BSA fraction V, Serva) was conjugated with Bodipy TR ceramide according to previously published protocols (Martin and Pagano, 1994). After washing with HBS, cells in glass bottom culture dishes were incubated (20 minutes, 37°C) with 5 μM BSA/Bodipy conjugate. After removal of the dye solution and washing, cells were incubated (20 minutes, 37°C) in dye free HBS, and subsequently in 2% DF-BSA in HBS (3× 10 minutes at 37°C) to adsorb surplus BSA/Bodipy from the cell surfaces. After additional washing, cells were viewed through an inverted fluorescence microscope using a Texas Red filter set.

**Western blot**

Whole cell lysates and culture medium supernatants of BDNF-GFP expressing COS-7 cells were analyzed 48 hours posttransfection. Non-transfected, pGFP-transfected, and pBDNF-transfected COS-7 cells were used as controls. The supernatants were concentrated 8-fold using Centricon tubes (Amicon). Aliquots of cell lysates and supernatants corresponding to 1×10⁴ and 7×10⁴ cells per lane, respectively, were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schüll, 0.2 μm) using standard protocols (Sambrook et al., 1989). After blocking, the nitrocellulose membrane was incubated (1 hour, RT) with an anti-BDNF polyclonal antibody (Santa Cruz; 1:5,000) in Tris buffered saline, containing 10 mM Tris-HCl, 150 mM NaCl, and 0.05% (v/v) Tween-20. After incubation (1 hour, RT) with secondary antibody (HRP-conjugated anti-rabbit IgG, Sigma, 1:5,000), binding of secondary antibodies was detected using an ECL detection kit (Amersham) according to the manufacturer’s instruction.

**RESULTS**

**Expression and localization of BDNF-GFP in COS-7 cells**

In a first set of experiments we determined the intracellular localization of BDNF-GFP upon transient transfection of a secondary mammalian cell line (monkey COS-7 cells). COS cells have been shown previously to be an appropriate model system to study the expression and secretion of heterologously expressed neurotrophins (see e.g. Ernfors et al., 1990; Suter et...
al., 1991; Heymach et al., 1996) and to investigate the intracellular trafficking of GFP-tagged proteins (see e.g. Georget et al., 1997; Presley et al., 1997; Yano et al., 1997). As a control, we transfected COS cells with a GFP cDNA construct without fused BDNF (pGFP), but in all other respects identical to pBDNF-GFP. Forty-eight hours following transfection, we observed a patchy perinuclear distribution of green fluorescence in pBDNF-GFP transfected COS cells, whereas the unfused GFP was evenly distributed throughout the cell body and the nucleus (Fig. 1A,B), indicating that the BDNF sequence contains targeting signals which lead to a specific localization of the fusion protein. In control experiments we confirmed, that, under identical illumination conditions, untransfected COS cells did not show detectable autofluorescence (not shown). A similar distribution of BDNF-GFP fusion protein was obtained in experiments in which a fibroblast cell line (Swiss-3T3) was used for transfection (not shown).

The pattern of green fluorescence in BDNF-GFP transfected COS cells overlapped strikingly with the immunocytochemical detection of the BDNF portion of BDNF-GFP in the same cells (Fig. 1C,D). Immunocytochemical processing of untransfected COS cells did not reveal detectable amounts of endogenous BDNF (not shown). This suggests that the green fluorescence of pBDNF-GFP transfected cells originates from a physically connected BDNF-GFP fusion protein. To investigate whether the GFP tag influenced the intracellular distribution of BDNF-GFP, we also transfected COS cells with an altered BDNF-GFP plasmid that contained 2 stop codons in frame between the BDNF and the GFP coding region (pBDNF, see Materials and Methods). Transfection with this cDNA drives the expression of unfused BDNF protein. As shown by BDNF-immunocytochemistry, COS cells transfected with pBDNF yielded a pattern of BDNF distribution that was indistinguishable from the results obtained with the BDNF-GFP fusion protein (see Fig. 1C,E). Thus, the intracellular distribution of BDNF-GFP seems to be uninfluenced by the GFP tag.

We next sought to determine the identity of the intracellular compartment to which BDNF-GFP was targeted. Secretory proteins like BDNF are expected to transit the trans-Golgi network (TGN) en route to the plasma membrane (for a review see Halban and Irminger, 1994), and BDNF-GFP should colocalize with markers of the TGN. Following transfection of COS cells with pBDNF-GFP, we thus performed immunocytochemistry directed against a 58 kDa TGN marker protein (Bloom and Brashear, 1989). As shown in Fig. 2C,D the BDNF-GFP fluorescence originated from the same cellular compartment as the staining of this TGN marker protein. This finding was substantiated in living COS cells, where BDNF-GFP showed the same subcellular distribution as Bodipy TR ceramide (Fig. 2A,B), which is a vital stain for the TGN compartment (Martin and Pagano, 1994). These results suggest that BDNF-GFP is selectively targeted to the TGN of mammalian cells.

If correctly processed and targeted, heterologously expressed secretory proteins are released from COS cells into the supernatant (see e.g. Suter et al., 1991). Thus, we collected the supernatant from COS cells transiently transfected with pBDNF-GFP and performed western blot analysis with an anti-BDNF antibody. The pattern of BDNF immunoreactive proteins in the supernatant and in the whole cell lysates was compared with the pattern of equally treated material obtained from pGFP-transfected and untransfected COS cells.

Fig. 2. BDNF-GFP is targeted to the TGN of COS-7 cells. (A,B) COS cells were transfected with pBDNF-GFP and incubated 48 hours after transfection with the vital Golgi stain Bodipy TR ceramide (see Materials and Methods). The green BDNF-GFP fluorescence (A) is localized within the same cellular compartment as the live Golgi stain (B; the Golgi stain at the lower right in B originates from an untransfected COS cell). (C,D) COS cells were transfected with pBDNF-GFP and processed for immunocytochemistry with a monoclonal antibody directed against a Golgi-resident marker protein (anti-58 kDa). The green BDNF-GFP fluorescence (C, FITC filter) coincides with the expression pattern of the Golgi-marker (D, TRITC filter) within the same cell. Bar, 10 μm.
respectively. As shown in Fig. 3A, the supernatant from pBDNF-GFP transfected COS cells contained BDNF immunoreactive bands with relative molecular masses of approximately 58 (double band) and 43 kDa, respectively, which were absent from the supernatant of untransfected COS cells and cells transfected with pGFP. The BDNF-immunoreactive double band at 58 kDa is also specifically detected in the cell lysates of pBDNF-GFP transfected cells. In all cell lysates, an endogenous ~43 kDa protein crossreacts with the BDNF antibody, masking the mature BDNF-GFP in pBDNF-GFP transfected COS cells. The 58 kDa double band corresponds to the fusion protein consisting of unprocessed pro-BDNF (29 kDa) and GFP (27 kDa). The 43 kDa band in the supernatant is close to the expected size for the processed mature fusion protein (i.e. 41 kDa = 14 kDa BDNF + 27 kDa GFP). Although the same BDNF-immunoreactive bands are found in the supernatant and the lysates of pBDNF-GFP transfected COS cells, the relative amounts of the individual bands are different. Whereas the 43 kDa band (corresponding to mature BDNF-GFP) is dominant in the supernatant, the 58 kDa double band (corresponding to unprocessed BDNF-GFP) is dominant in the cell lysates, indicating that correctly processed BDNF-GFP is the prevailing species of the fusion protein which is released from the transfected COS cells. When COS cells were transfected with a plasmid encoding untagged BDNF (pBDNF) a similar pattern of BDNF immunoreactive bands (shifted by 27 kDa, corresponding to the GFP-tag, to lower molecular masses) was observed in the cell lysate and the supernatant (Fig. 3B). BDNF-immunoreactive bands were detected with relative molecular masses of 32 kDa (double band; unprocessed precursor), and 15 kDa (mature BDNF), respectively, and the relative amount of mature BDNF was increased in the supernatant.

Taken together, pBDNF-GFP transfected COS cells express unprocessed and mature fusion proteins of the expected size; the mature protein is the predominant species which is secreted and the GFP tag has no apparent influence on the processing, localization and secretion of BDNF-GFP, when compared with untagged BDNF.

Expression of BDNF-GFP in primary central neurons

At 7-11 DIV, we transfected primary cultures of cortical neurons with pBDNF-GFP and pGFP, respectively, and monitored GFP fluorescence 2 days after transfection. In accordance with the results obtained in COS cells, BDNF-GFP was localized inhomogenously within the cell body and the neurites of the transfected neurons, whereas GFP was distributed evenly throughout the cells (Fig. 4A-C). BDNF-GFP was excluded from the nucleus of the neurons, but this was not apparent when we focussed on the neurites (compare Fig. 4B and C). As seen most obviously in the neurites, the green fluorescence was confined to roundish packages of ~0.3 μm in diameter (see below), and these packages moved along the neurites and eventually stopped or changed the direction of movement during visual inspection. The green packages were often distributed unevenly along the neurites and sometimes accumulated in certain segments of the processes.

We next sought to determine whether the intracellular localization of BDNF-GFP mimics the distribution of endogenous BDNF in cortical neurons. We thus performed immunocytochemistry with an anti-BDNF antibody in...
untransfected cortical neurons after 10 DIV. As shown in Fig. 4D-G, endogenous BDNF was found in the soma and the neurites of cortical neurons, whereas the nucleus was excluded. In the neurites the staining was patchy and reminiscent of the BDNF-GFP packages in the neurons transfected with pBDNF-GFP.

Taken together, these results indicate that in contrast to GFP, BDNF-GFP is targeted to specific compartments of neuronal cells; it is transported in small packages into the neurites, and shows a distribution similar to endogenous BDNF in cortical neurons.

Subcellular localization of BDNF-GFP in primary neurons

The pattern of green fluorescent packages was retained when the BDNF-GFP transfected neurons were fixed with paraformaldehyde, as described previously for GFP fusion proteins (Wang and Hazelrigg, 1994), and we thus performed immunocytochemistry with several antibodies directed against intracellular marker proteins to reveal the neuronal compartments to which BDNF-GFP is targeted.

In agreement with the results obtained in COS cells, BDNF-GFP colocalized with the 58 kDa TGN marker protein in the perinuclear compartment of the neurons (not shown).

Since numerous processes of single pBDNF-GFP transfected neurons showed green fluorescence (although every neuron has only one axon), it is clear from our results that the BDNF-GFP packages were present in dendrites. To investigate whether BDNF-GFP was also transported into axons, we performed immunocytochemistry with antibodies directed against the axon-specific protein Tau (Binder et al., 1985; Mandell and Banker, 1996). As shown in Fig. 5, the axons of neurons transfected with pBDNF-GFP contained in several cases green fluorescing BDNF-GFP packages, in addition to somatodendritic localization within the same cell. However, it clearly emerged from these experiments that there were also numerous neurons with very prominent BDNF-GFP fluorescence in the cell body and many neurites, whereas the affiliated axon was devoid of BDNF-GFP packages (not shown). Thus, although axonal targeting occurs, most of the BDNF-GFP packages were located in the somatodendritic compartment of the neurons, and we never observed exclusive axonal targeting of BDNF-GFP in any neuron investigated.

Secretogranin II (SgII) is a marker protein for secretory granules of the regulated pathway of secretion (for reviews see Huttner et al., 1991; Halban and Irminger, 1994; Ozawa and Takata, 1995). If the green BDNF-GFP packages were identical to secretory granules, SgII should be colocalized in these packages. Indeed, immunocytochemical detection with a polyclonal anti-SgII antiserum (see Fig. 6) revealed that 95.3±5.7% of all BDNF-GFP packages were colocalized with...
Synaptic localization of BDNF-GFP

SgII (average over 366 vesicles from 4 cells; \( \geq 75 \) vesicles per cell analyzed), suggesting that the green BDNF-GFP packages are secretory granules. Judged by the apparent size of green fluorescing packages that colocalized with SgII, we estimated an average diameter of 0.3±0.1 \( \mu \text{m} \) per secretory granule.

Finally we wanted to determine whether the BDNF-GFP vesicles are located close to synaptic structures of the neurons. We therefore performed immunocytochemistry with an antibody directed against the presynaptic marker protein synapsin I in cortical neurons that had been transfected with pBDNF-GFP. Interestingly, synapsin I immunoreactive synaptic terminals were often found close to accumulations of BDNF-GFP vesicles within neurites of the transfected neurons (arrowheads in Fig. 7; density of green vesicles in the vicinity of synaptic terminals: 4.3±0.5 vesicles/\( \mu \text{m}^2 \), \( n=76 \) terminals from 7 cells; density of green vesicles in different segments of the same neurites devoid of synaptic terminals: 1.5±0.3 vesicles/\( \mu \text{m}^2 \)). The presence of BDNF-GFP containing vesicles in the vicinity of synaptic junctions suggests a synaptic function of BDNF in cortical neurons. Whether the BDNF-GFP vesicles are located pre- and/or postsynaptically remains to be shown by immunocytochemical detection at the electron microscopic level.

**DISCUSSION**

In the present study we show that a fused cDNA construct consisting of N-terminal BDNF (including prepro sequences) and C-terminal GFP is expressed, processed, and targeted in mammalian cell lines and primary neurons as expected for a secretory protein, and the intracellular distribution is similar to...
that of endogenous or heterologously expressed BDNF in these cells. As shown by immunocytochemistry, BDNF-GFP is transported in vesicles and targeted into the neurites of transfected neurons where it seems to accumulate in the vicinity of synaptic junctions.

Expression of BDNF constructs in COS-7 cells

Despite the lack of a regulated pathway of secretion, COS cells have been shown previously to yield high levels of expression of heterologously expressed neurotrophins which are released as correctly processed and biologically active proteins into the culture medium (Heymach et al., 1996; Heymach and Shooter, 1995; Suter et al., 1991; Ernfors et al., 1990). In accordance with these previous results, using western blot analysis we also found mature BDNF-GFP (43 kDa) in the supernatant of COS cells that were transiently transfected with pBDNF-GFP. An additional minor BDNF-immunoreactive double band was found in the supernatant, corresponding to uncleaved BDNF-GFP precursor protein (58 kDa). In contrast, this precursor was the dominant BDNF-GFP species in the cell lysates, where the low levels of mature BDNF-GFP were masked by a crossreacting endogenous 43 kDa protein. The relative accumulation of mature BDNF-GFP in the supernatant suggests that processing of the precursor protein precedes the secretion of neurotrophins from COS cells. A very similar pattern of BDNF immunoreactive bands (shifted by ~27 kDa to lower molecular masses), was found in pBDNF transfected COS cells, although the relative amounts of proteins (i.e. proBDNF vs mature BDNF) were slightly different for BDNF and BDNF-GFP (compare Fig. 3A and B). Thus, the processing of BDNF-GFP is largely unaffected by the GFP-tag.

Our pattern of BDNF-immunoreactive bands is in good agreement with previous studies of neurotrophin expression in COS cells (Heymach et al., 1996; Suter et al., 1991) and other mammalian cell lines (Edwards et al., 1988), which also revealed a considerable amount of neurotrophin precursor proteins in the cell lysates, and, to a lesser extent in the cell’s supernatants.

As expected for a secretory protein and shown by two different staining procedures (Bodipy TR ceramide and immunocytochemistry vs a 58 kDa TGN marker protein),

Fig. 7. BDNF-GFP is localized in the vicinity of synapses. Neurites of a cortical neuron transfected with pBDNF-GFP at 11 DIV and processed for immunocytochemistry after additional 2 DIV using a monoclonal antibody directed against the presynaptic marker protein synapsin I. Antibody binding was detected using TRITC-conjugated extravidin (see Materials and Methods). The green fluorescing BDNF-GFP vesicles (A; FITC filter set) accumulate close to synaptic junctions marked by synapsin I staining (B; bar, 10 μm). (C and D) The box marked in A at higher magnification for both stainings. Bar, 4 μm. Arrowheads in A-D indicate the position of synapsin I immunoreactive synaptic boutons and the corresponding accumulation of BDNF-GFP vesicles.
BDNF-GFP is located in the trans-Golgi network (TGN) of COS cells. Since unfused GFP protein is distributed uniformly throughout the cytoplasm and nucleus of the COS cells, the TGN specific localization of BDNF-GFP strongly suggests, that targeting signals within the BDNF sequence guided the transportation of BDNF-GFP to the TGN. In addition, the immunolocalization of untagged BDNF in the COS cells (see Fig. 1E) is indistinguishable from the fluorescence pattern of BDNF-GFP.

Taken together, our data demonstrate that in transfected COS cells the processing pattern of BDNF-immunoreactive proteins and the subcellular distribution of BDNF-GFP is indistinguishable from the results obtained with untagged BDNF as well as from other neurotrophins (see references above), suggesting that tagging of BDNF with GFP has no detectable effects on the expression, processing, localization and secretion of this neurotrophin. In addition, preliminary results indicate that BDNF-GFP shows BDNF-like biological activity (W. Haubensak and V. Leßmann, unpublished).

Expression of BDNF-GFP in primary cortical neurons

Our results obtained from primary cortical neurons transfected with pBDNF-GFP demonstrate the selective targeting of BDNF-GFP to TGN-derived SgII containing secretory vesicles, which are transported into the neurites of transfected neurons. Similar to COS cells, GFP alone was distributed uniformly throughout the soma, nucleus and the processes of pGFP transfected neurons, suggesting that the selective localization of BDNF-GFP in neurons was due to targeting signals inherent in the BDNF portion of the fusion protein. Importantly, the subcellular distribution of endogenous BDNF in cortical neurons, as determined here by BDNF-immunocytochemistry, is similar to the pattern of the BDNF-GFP localization, with respect to the granular staining within the cell body, sparing of the nucleus, and the patchy distribution of BDNF-immunoreactivity along the neurites. Thus, it is conceivable that in cortical neurons BDNF-GFP is sorted to the same subcellular compartments as endogenous BDNF.

Using overexpressed GFP-tagged BDNF to investigate the subcellular distribution of this neurotrophin in cortical neurons has several advantages over immunocytochemical detection of BDNF. First, overexpression of BDNF-GFP yields higher amounts of protein and thus stronger fluorescence signals, without detectably influencing the intracellular sorting of the protein (see above). Second, compared to immunocytochemical detection, GFP-tagging of proteins has several important advantages with respect to the spatial resolution, signal strength, and signal/noise-ratio (Wang and Hazelrigg, 1994). Third, GFP shows very low levels of photobleaching during prolonged light exposure. Thus, even in a fixed preparation BDNF-GFP seems to be the most sensitive approach to determine the subcellular distribution of BDNF.

In agreement with our results for BDNF-GFP and endogenous BDNF, a punctate granular immunostaining of endogenous BDNF in the cytoplasm and the proximal processes of cortical neurons (Wetmore et al., 1991), excluding the cell nucleus (Dugich-Djordjevic et al., 1995; Conner et al., 1997; Fawcett et al., 1997) has been described in sections from adult rat brain. Several groups have shown that the BDNF-immunoreactive processes are dendrites (Wetmore et al., 1991, 1994; Dugich-Djordjevic et al., 1995; Goodman et al., 1996), and this correlates with the prominent somatodendritic localization of BDNF-GFP observed in our study. However, in some brain regions BDNF seems to be transported anterogradely into axons (Kawamoto et al., 1996; Altar et al., 1997; Conner et al., 1997; Yan et al., 1997), and axonal targeting of BDNF-GFP was also observed in our study, although this was not a general finding, and there was no case in which BDNF-GFP was found exclusively in the axon of a transfected cell. Whether additional axonal targeting of BDNF-GFP is a celltype specific phenomenon or whether it depends on the developmental stage of a particular neuron remains to be shown by future experiments.

BDNF-GFP was found in discrete, roundish, uniformly sized (~0.3 μm in diameter) packages within the cell body and the neurites of living as well as fixed cortical neurons. This diameter corresponds to the expected size for TGN-derived secretory granules of the regulated secretory pathway (i.e. 200-500 nm in diameter; Ozawa and Takata, 1995). In contrast, secretory granules of the constitutive pathway are much smaller (~50 nm in diameter) and cannot be resolved as such using conventional light microscopy (Halban and Irminger, 1994). Thus, simply judging by size, the observed BDNF-GFP spots in the neurons most likely reflect secretory granules from the regulated secretory pathway. This notion is strongly supported by the colocalization of SgII with BDNF-GFP within these granules (see Fig. 6). Since SgII appears to be a selective marker of secretory granules of the regulated secretory pathway (Huttner et al., 1991; Ozawa and Takata, 1995), our data provide strong evidence that the discrete BDNF-GFP packages within the neurons are indeed secretory granules.

A similar patchy distribution of overexpressed untagged NGF which colocalized with SgII-immunoreactivity has also been described for hippocampal neurons in vitro (Blöchl and Thoenen, 1996), albeit single secretory vesicles were not resolved. Similarly, the BDNF immunocytochemistry in our untransfected cortical neurons did also not yield the same subcellular spatial resolution as is evident from the directly fluorescing BDNF-GFP vesicles (see Fig. 4), stressing one of the advantages of the GFP approach to localize the BDNF protein.

The presence of BDNF-GFP containing secretory granules in the vicinity of synaptic junctions (as shown by synapsin I immunocytochemistry, Fig. 7) suggests a subsynaptic localization of BDNF containing organelles in cortical neurons. Whether these BDNF-GFP vesicles are located pre- and/or postsynaptically cannot be decided from these experiments. However, in the light of the prominent somatodendritic localization of BDNF-GFP observed in our study, it is tempting to speculate that at least some of the BDNF-GFP vesicles in the vicinity of synaptic junctions are located in the postsynaptic cell. In addition, many different neurites of single BDNF-GFP transfected neurons showed accumulations of BDNF-GFP vesicles in the vicinity of synapsin I immunoreactive terminals. Since only one axon is formed by each neuron, the numerous neurites with neighbouring BDNF-GFP fluorescence and synapsin I labeling must include dendritic processes of the BDNF-GFP expressing neuron.
During preparation of this manuscript, Michael et al. (1997) reported the localization of endogenous BDNF in dense core vesicles within axon terminals of PNS neurons (i.e. dorsal root ganglion cells), as shown by immuno electron microscopy, which conforms with the anterograde axonal targeting observed in several brain regions (as discussed above). The data of Michael et al. (1997) confirm our observation of synaptic targeting of BDNF-GFP vesicles in CNS neurons. However, it is not yet clear whether in CNS neurons BDNF is also transported predominantly to presynaptic terminals, and our results suggest at least additional postsynaptic targeting.

Clearly, additional electron microscopic data are needed to reveal the exact ultrastructural localization of BDNF-GFP (as well as of endogenous BDNF) at synaptic junctions in the mammalian brain.

In conclusion our data provide evidence for a vesicular localization of BDNF-GFP in TGN-derived granules of the regulated secretory pathway in cultured cortical neurons. These granules are very prominent in the somatodendritic compartment of the neurons, although additional axonal targeting can also be observed occasionally. The BDNF-GFP precursor is processed, and the mature protein is targeted and released as expected for a secretory protein of the regulated pathway and in a manner that is indistinguishable from the processing of untagged BDNF. The accumulation of BDNF-GFP vesicles close to synaptic junctions supports the view that BDNF is located appropriately to directly influence synaptic transmission.

Thus, the BDNF-GFP approach will enable us for the first time to observe neurotrophin release in real time from living cells, and combined electrophysiological and video imaging studies are in progress to investigate the synaptic release of BDNF-GFP upon physiological synaptic stimulation.

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