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Brain-derived neurotrophic factor: Subcellular compartmentalization and interneuronal transfer as visualized with anti-peptide antibodies

(nerve growth factor/hippocampus/cholinergic neurons/immunohistochemistry)

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ABSTRACT **The recent cloning of a second member of the nerve growth factor family, brain-derived neurotrophic factor (BDNF), has prompted investigation into the cells that express this factor's mRNA and protein. In the present study, antibodies raised against unique peptide sequences within the porcine BDNF protein detect BDNF-like immunoreactivity in neurons in rat hippocampal and cortical areas consistent with the distribution of BDNF mRNA as detected with *in situ* hybridization. Within these neurons, BDNF-like immunoreactivity was observed in the cytoplasm, dendrites, and nuclei. In addition, BDNF immunoreactivity was observed in the cytoplasm of cholinergic neurons that do not express detectable levels of BDNF mRNA. Thus, anti-peptide antibodies can be used to detect this neurotrophic factor protein in cytoplasmic sites of synthesis and in areas of probable action. We propose that one form of the BDNF protein enters the nucleus and may directly influence transcription, while another fraction of the protein is transported out of the synthesizing cell and can be detected, after retrograde axonal transport, in cytoplasmic granules in the perikarya of cholinergic neurons. These basal forebrain cholinergic neurons project to regions enriched in BDNF-synthesizing cells and are known to be responsive to BDNF *in vitro*. Our data provide information regarding the cellular distribution of BDNF protein *in vivo* and suggest a dendro–axonic interneuronal transfer of BDNF as well as an additional, intracellular signaling pathway not previously thought to occur in postmitotic neurons in brain.**

The purification and cloning of brain-derived neurotrophic factor (BDNF) (1) and the subsequent cloning of neurotrophin 3/hippocampus-derived neurotrophic factor (NT3/HDNF) (2–5) have revealed a family of neurotrophic proteins with a high degree of amino acid sequence homology to the well-known nerve growth factor (NGF) (6–9) and exhibiting somewhat overlapping yet distinct and characteristic patterns of distribution (10).

In situ hybridization and RNA blot-hybridization (Northern blot) analyses from our laboratory and others indicate that the levels of mRNA for all members of the NGF family are relatively high in hippocampus (10, 11). However, BDNF mRNA appears to be more widespread in the brain than either NGF or NT3/HDNF, its distribution being highest in hippocampus, cortex, and other synaptic targets of basal forebrain cholinergic neurons (12–14). Although it has been reported recently that BDNF supports survival and differentiation of septal cholinergic neurons *in vitro* (15, 16) and to some extent dopaminergic neurons *in vitro* (16, 17), little is known regarding the localization, role, and neuronal specificity of BDNF in the central nervous system *in vivo*.

In the present study, our goal was to obtain BDNF-specific antibodies, which do not cross-react with other NGF family members, to compare the distribution of BDNF mRNA and protein and to determine the cellular distribution and fate of BDNF protein. To generate antibodies that can differentiate among the highly conserved NGF family members, one must "select" for unique epitopes within the native protein. We have attempted to restrict the response of the immunized animal to recognize these unique areas by using short peptide immunogens (18).

METHODS

***In Situ* Hybridization.** Oligonucleotide probes complementary to porcine BDNF gene fragments (bases 250–298 and 626–676) (1) were synthesized (Scandinavian Gene Synthesis, Köping, Sweden) and 3′-end-labeled with adenosine 5′-[γ-(^{35}S)thio]triphosphate. An oligonucleotide probe with a sequence complementary to the BDNF-specific oligonucleotide and one with a sequence complementary to the rat NGF receptor mRNA with comparable GC content served as control probes. Procedures for *in situ* hybridization were as described (19, 20). Sections (14 μm) of freshly frozen brain were thawed onto poly(L-lysine)-coated slides and hybridized with specific and control probes for 16 hr at 42°C. Slides were dipped in Kodak NTB2 emulsion, diluted 1:1 in distilled water, and exposed for 6–8 weeks at −20°C.

Peptide Synthesis and Immunization. Based upon computer-assisted analysis of the amino acid sequences for the NGF family, we have selected several regions within the BDNF protein having the lowest degree of sequence similarity to NGF and NT3/HDNF and lacking significant homology to other known proteins. Swissprot and National Biomedical Research Foundation protein data bases (release 26.0, September 1990) were searched by using the FASTA program and were analyzed for amino acid homology to peptides selected for use in this study (21). Secondary considerations were given to hydrophilicity (22) and predictions of antigenicity and secondary structure (24).

On the basis of these criteria, BDNF-(173–182) decapeptide was synthesized with the carboxyl terminus in the unmodified acid form by automated, solid-phase methods (Microchemical Facility, Institute for Human Genetics, University of Minnesota) and was conjugated to bovine thyroglobulin in 0.8% glutaraldehyde (30 mg of peptide with 90 mg of carrier protein). The conjugate was dialyzed (Spectrapore dialysis tubing, molecular weight cutoff of 6,000–8,000) overnight at 4°C against 0.1 M phosphate-buffered saline (PBS) and diluted with PBS to 1 mg of peptide per ml. Six rabbits (New Zealand White; Estuna, Sweden) were immunized (subcutaneously at six sites along, but beside, the mammary

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Abbreviations: BDNF, brain-derived neurotrophic factor; rmBDNF, recombinant mouse BDNF; NGF, nerve growth factor; NT3, neurotropin 3; HDNF, hippocampus-derived neurotrophic factor.

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line) with a total of 1 mg of peptide emulsified in Freund's complete adjuvant and were administered booster shots of 500 μg in Freund's incomplete adjuvant every 2 weeks for the first 10 weeks and subsequently every 4–6 weeks. Blood was collected 7–10 days after each booster in the first 6 months, and 14–21 days after subsequent boosters.

Immunohistochemical Evaluation. Rat tissues (150 g female, Sprague–Dawley; Alab, Sweden) were processed for immunohistochemistry as described (50). Brains were removed, postfixed in the same fixative for 1.5 hr at room temperature, rinsed four to six times in PBS containing 10% sucrose over 24 hr or until the sucrose wash solution was clear, and frozen, and 14-μm cryostat sections were thawed on gelatin-coated slides. Sera were diluted to 1:1000 to 1:2000 in 0.1 M PBS containing 0.3% Triton X-100. Sections were incubated with primary antisera in a humid chamber at 4°C overnight, rinsed for 10 min three times in 0.1 M PBS, incubated with secondary antisera (fluorescein isothiocyanate-labeled goat-anti-rabbit; Boehringer Mannheim) for 1 hr at room temperature, rinsed in PBS, and mounted in 90% (vol/vol) glycerine in 0.1 M PBS containing 0.01% *p*-phenylenediamine to reduce fading. Epifluorescence microscopy (Nikon-Mikrophot) and scanning laser confocal microscopy (Bio-Rad; MRC-600 equipped with Nikon objective lenses) were used as described (25).

Immunoblot (Western Blot) Analyses. NGF purified from male mouse submandibular glands (26) and recombinant mouse BDNF (rmBDNF) were reduced, alkylated, and treated with SDS. Approximately 1 μg of each prepared protein was loaded onto a 10–15% polyacrylamide/SDS gradient gel and transferred to nitrocellulose filters. Filters were incubated with antipeptide sera (1:500 dilution) and processed as described (27).

RESULTS

Comparison of Cellular Localization of BDNF mRNA and BDNF Protein. *In situ* hybridization studies performed with two BDNF-specific oligonucleotide probes showed strong labeling of neurons in hippocampus, including pyramidal, hilar, and granule cells of the dentate gyrus (Fig. 1*A*). Many scattered cells in amygdala, cingulate, parietal, and entorhinal cortex were also detected as reported (12–14).

Sera from two (R1 and R4) of six rabbits immunized with BDNF-(173–182) generated specific immunostaining patterns in the rat brain (Fig. 1*B*) and fulfilled specificity control criteria (see below), with a cellular distribution in neurons in hippocampus and cortex similar to the distribution of BDNF mRNA as determined by *in situ* hybridization.

Specificity of Antisera. Antisera were treated overnight at 4°C with specific peptide (dissolved in 50% methanol at 1 mg/ml), peptide–carrier protein conjugate, carrier protein alone, or proteins precipitated from Freund's complete adjuvant. Antisera treated with the same amount of methanol lacking peptide were used as controls. Before application to the tissue, the antisera/protein mixtures were centrifuged at 10,000 × *g* for 15 min to precipitate antibody–protein complexes. Preabsorption with the specific peptide overnight blocked all immunostaining, while preadsorption with the

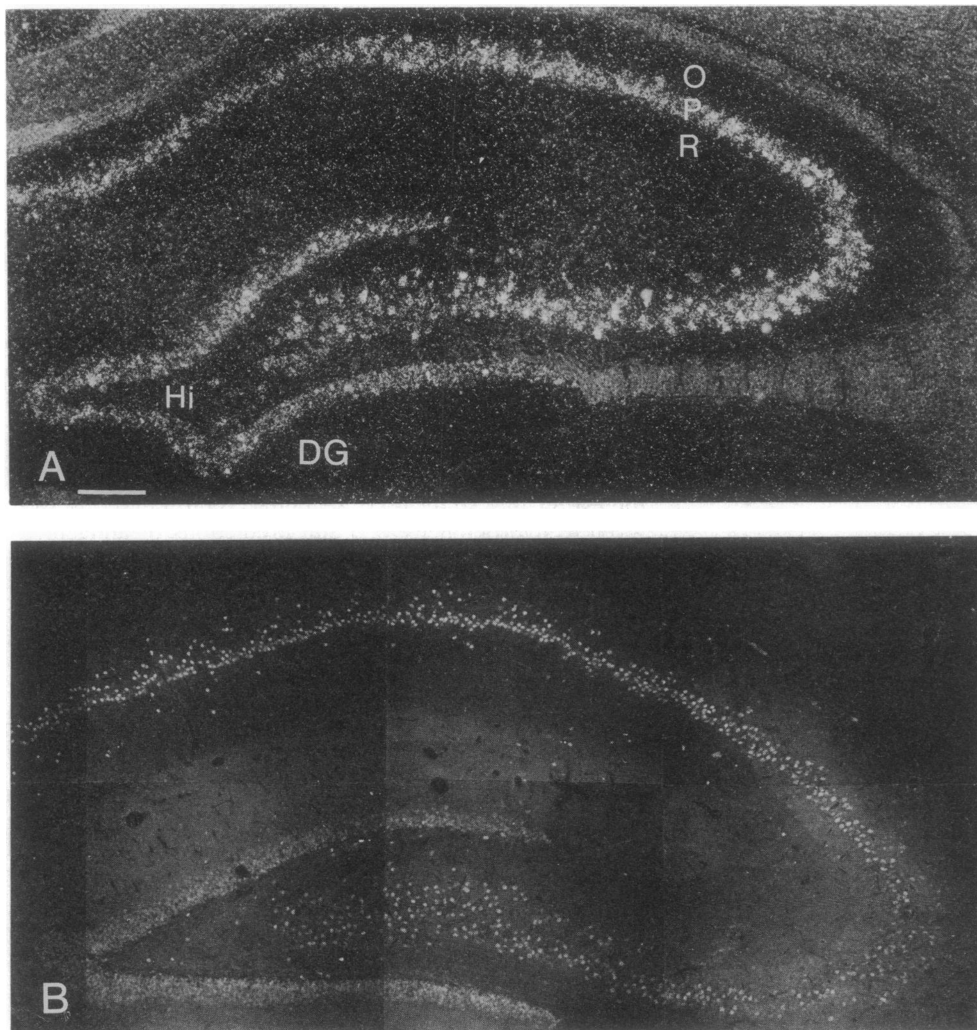

FIG. 1. Coronal sections of adult rat hippocampal formation. (*A*) Emulsion autoradiograph of a section hybridized with an antisense probe to BDNF. Most, but not all, pyramidal cells in areas CA1–CA4 and granule cells in the dentate gyrus (DG) are labeled. Note labeled cells scattered above the pyramidal cell layer (P) in stratum oriens (O), fewer cells in stratum radiatum (R), and strongly labeled neurons in the hilar region (Hi). (*B*) Immunofluorescence localization of BDNF-(173–182) with anti-peptide antiserum from R4 to the nuclei of neurons in the hippocampal formation. Note the strong labeling of many pyramidal cells and scattered cells in stratum oriens and the hilar regions. Granule cells in the dentate gyrus are more weakly labeled. (Bar = 250 μm.)

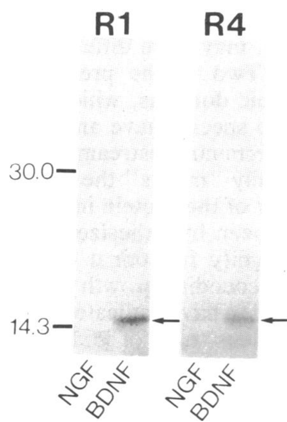

FIG. 2. The reactivity of anti-peptide antibodies R1 and R4 were confirmed by Western blot analysis with purified recombinant mouse BDNF (arrow) and mouse salivary gland NGF. These BDNF-specific antisera showed no detectable amount of reactivity with NGF. Molecular masses are indicated in kDa.

carrier protein or proteins precipitated from Freund's complete adjuvant did not block labeling.

Western blot analyses of rmBDNF with the BDNF-(173–182)-specific antisera indicated that both R1 and R4 antisera specifically recognize the recombinant protein while neither antibody reacts to any detectable extent with NGF (Fig. 2). Moreover, BDNF-specific antisera did not label NGF-containing cells in the male mouse submandibular gland, an organ known to contain extremely high amounts of NGF. The relative molecular mass of rmBDNF as determined in the present study (≈14.5 kDa) appears to be slightly higher than the molecular mass of the factor purified by two-dimensional gel electrophoresis from pig brain (12,300 kDa) (28), and the molecular mass predicted (13,511 kDa) from the deduced amino acid sequence of BDNF (1).

Localization of BDNF-(173–182)-Like Immunoreactivity to Nuclei. At the subcellular level, BDNF-(173–182)-like immunoreactivity, as determined with antisera from R4, was clearly localized in the nuclei of pyramidal cells in hippocampus in the unperturbed adult rat brain (Fig. 3*A*). The nuclei of hilar cells were strongly labeled, while dentate granule cells appeared more weakly immunoreactive (Fig. 1*B*). Strikingly, the nucleoli of neurons were not immunoreactive (Figs. 3*B* and 4*A*). With confocal laser microscopy, immunoreactivity within the nucleus could be resolved as a dense arrangement of fine granules (Fig. 3*B*). Strongly immunoreactive nuclei were detected in other areas coinciding with the expression of BDNF mRNA—e.g., amygdala and cingulate, parietal, and entorhinal cortex (Fig. 3*C*). A faint, diffuse perinuclear cytoplasmic labeling in perikarya and in proximal dendrites was also noted with R4 antisera.

Localization of BDNF-(173–182)-Like Immunoreactivity to Cytoplasmic Compartments. An additional subcellular feature of the distribution of BDNF-(173–182) immunoreactivity was revealed with antiserum from R1, which labeled the cytoplasmic as well as nuclear compartments of neurons believed to express BDNF mRNA. Both R1 and R4 antisera detected similar, if not identical, populations of pyramidal and granule cells in hippocampus, amygdala, entorhinal, parietal and cerebral cortex, and claustrum, which closely correlates with BDNF mRNA distribution, although R1 showed a greater cytoplasmic distribution, clearly labeling cytoplasm of the perikaryon and proximal dendrites (Fig. 4*A*). As observed with antiserum from R4, the nuclei, excluding nucleolar patches, were also clearly labeled with antiserum from R1 (Fig. 4*A*).

BDNF-(173–182)-like immunoreactivity with a strikingly different subcellular compartmentalization was detected with antiserum from R1 in the cytoplasm of cholinergic neurons of nucleus basalis and the medial septal nuclei/diagonal band area. Scattered, large, fluorescent granules were detected in the peripheral cytoplasm of these neurons (Fig. 4*B*), while the cytoplasm of smaller neurons of the medial septum exhibited a more homogeneous immunofluorescence. No nuclear immunoreactivity was detected in neurons containing these clumped, fluorescent cytoplasmic granules, nor was there any hybridization with the BDNF-specific oligonucleotide probes over these neurons, suggesting that they do not, themselves, synthesize BDNF.

DISCUSSION

The presence of BDNF-(173–182) immunoreactivity in cytoplasmic granules and the absence of detectable BDNF mRNA in cholinergic neurons of the basal forebrain suggest interneuronal transfer of BDNF protein from cortical and hippocampal pyramidal cells that produce the protein to cholinergic neurons, which *in vitro* are known to respond to this neurotrophic factor (15, 16). Our data can be interpreted to mean that BDNF is taken up by axon terminals at the site of synthesis in hippocampus, cortex, and/or other regions of

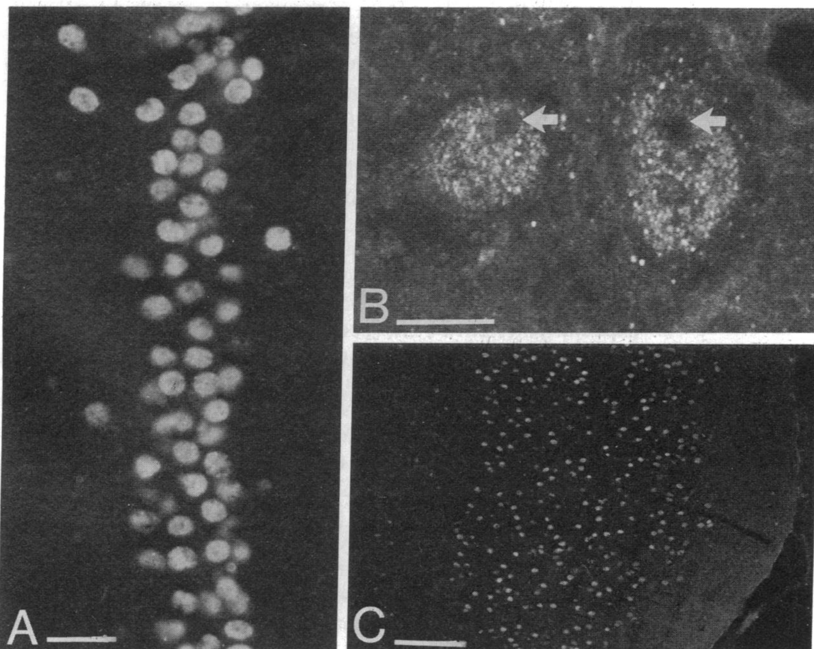

FIG. 3. BDNF-(173–182) immunoreactivity in nuclei as seen with antiserum from R4 in brain areas known to have high levels of BDNF mRNA expression. (*A*) Pyramidal cell layer, including scattered cells in the stratum oriens and radiatum in the hippocampal formation. Note slight patchiness of immunoreactivity and negative nucleoli. (Bar = 30 μm.) (*B*) Clumped, fluorescent particles in the nuclei of pyramidal cells as viewed with a scanning laser confocal microscope. Nucleoli (arrows) are nonimmunoreactive. (Bar = 10 μm.) (*C*) Epifluorescence micrograph of nuclear BDNF-(173–182) immunoreactivity in a horizontal section of entorhinal cortex. (Bar = 130 μm.)

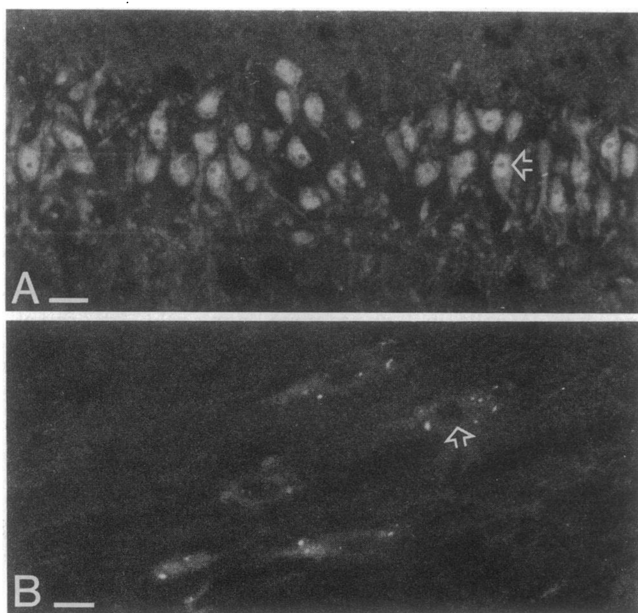

FIG. 4. BDNF-(173–182) immunoreactivity viewed with antiserum from R1. (*A*) Pyramidal cell layer in the hippocampal formation. Note the strongly labeled nuclei (open arrow) and unlabeled nucleoli as seen with antisera from R4. Additionally, note the BDNF-(173–182) immunoreactivity in the cytoplasmic compartment including proximal dendrites. No axonal BDNF-(173–182) immunoreactivity was detected in pyramidal neurons. (*B*) Large fluorescent granules showing BDNF-(173–182) immunoreactivity in the peripheral cytoplasm of nucleus basalis neurons. These neurons show no detectable hybridization to oligonucleotide probes complementary to BDNF mRNA. This BDNF-(173–182) immunoreactivity in cells that do not contain detectable BDNF mRNA suggests that the putative BDNF was made by another neuron, internalized, and transported retrogradely by the nucleus basalis neurons. Note the absence of BDNF-(173–182) immunoreactivity in the nuclei of these neurons in which no BDNF mRNA can be detected. (Bars = 25 μm.)

synthesis and retrogradely transported back to the cell body, as has been reported for NGF (29), where it can be detected in endosomal or lysosomal compartments. Therefore, BDNF may act as a trophic factor for cholinergic systems *in vivo* in the classic, target-derived trophic-factor paradigm described for NGF (6, 30, 31).

Based upon the present *in situ* hybridization and immunohistochemical analyses, it appears that the BDNF translation product may undergo alternative posttranslational modifications that would result in more than one form of the BDNF protein with the potential for differential cellular compartmentalization. In the adult rat brain, the apparent nuclear translocation of BDNF reported here suggests that this neurotrophic factor may enter the nucleus of neurons and directly influence gene expression *in vivo*. There exist precedents for the localization of proteins to the nucleus as well as in secretory pathways of nonneuronal cells. Specifically, although there exists a putative signal sequence for BDNF (1), there also exists a possibility for N-terminal extension of the protein by an alternative CUG initiation codon beginning 60 nucleotides upstream at residue 104, in a manner similar to the fibroblast growth factor (FGF)-related product, int-2. In the case of int-2, the AUG-initiated product contains a signal sequence and is found predominantly in the secretory pathway, while the alternative up-stream CUG-initiated gene product appears in the nucleus (32).

Interestingly, differential splicing of the NGF gene has been reported to result in four different mRNA transcripts with different ratios of distribution in various neuronal and nonneuronal tissues (33). These alternatively initiated transcripts predict closely related NGF precursor proteins that diverge at their N termini and, thus, may have differential subcellular localization and roles. Two of the predicted proteins have N-terminal hydrophobic domains, which are putative signal sequences, while two species have an additional 70 amino acids added to the N terminus upstream of the hydrophobic domain, which essentially "masks" the signal sequence and compromises the entry of the protein into the secretory pathway (32, 33). It has been hypothesized that these structural differences may signify functional differences among the various transcripts encoding growth factor proteins. For example, binding studies have indicated that NGF can accumulate within the nucleus (e.g., of PC12 and dorsal root ganglion cells), binding to specific, saturable receptors on the nuclear membrane or to chromatin within the nucleus of cells bearing the NGF receptor on their surfaces (34–36). It has also been noted that growth factor binding to chromatin is accompanied by a decrease in nuclease sensitivity of the DNA at the growth factor binding region. However, it appears that nuclear translocation by the sensitive cell is not essential for physiological activity of NGF (37).

FGF and platelet-derived growth factor have been detected in the nuclei of nonneuronal cells (38–42), and it recently has been demonstrated that nuclear translocation is essential for maintaining mitogenic activity of acidic FGF (43). While nuclear translocation sequences are not always easily identifiable in protein sequences, they typically consist of 4–10 amino acid residues, rich in the basic amino acids arginine or lysine and often including a proline or a valine (44, 45). Areas within the reported amino acid sequence of BDNF that resemble reported nuclear translocation sequences and could potentially act to effect nuclear translocation of the BDNF protein occur at positions 130–140 (Arg-Val-Arg-Arg-His-Ser-Asp-Pro-Ala-Arg-Arg), 228–237 (Lys-Lys-Arg-Ile-Gly-Trp-Arg-Phe-Ile-Arg), and 249–252 (Lys-Arg-Gly-Arg). However, it remains to be determined by which mechanism BDNF may undergo nuclear translocation in neurons.

It is interesting to note the differential subcellular localization detected with antisera obtained from rabbits immunized with the same decapeptide. Within this restricted sequence to which our polyclonal antisera have been raised, several possible antigenic sites exist. Therefore, one can hypothesize that antiserum from R4 recognizes a more restricted epitope on the BDNF protein, which may only be exposed in the nuclear form of the protein and "hidden" with folding and processing of the protein for secretion. Antiserum from R1 may recognize a less restricted set of epitopes, so that one pool of the antibodies recognizes a sequence of amino acids that is exposed in nuclear translocation of the protein and another population of antibodies recognizes an additional epitope within the 10-amino acid sequence that remains exposed during cytoplasmic processing of the BDNF protein.

When considering potential roles and sites of biological effect for BDNF, including nuclear translocation, it is intriguing to also consider a possible autocrine role for BDNF, especially in light of the recent report that the rat transmembrane receptor-like tyrosine kinase gene ***TRKB*** encodes a functional high-affinity receptor for BDNF (47, 48). While it has been reported that BDNF binds to the low-affinity NGF receptor, low-affinity binding alone does not appear to transduce a biological response (49). The ***TRKB*** gene encodes a transmembrane tyrosine kinase protein closely related to the human ***TRK*** protooncogene product that has been reported to bind NGF with high affinity (51, 52). It appears that ***TRKB*** acts in an analogous manner for high-affinity binding of BDNF as well as of NT3 (47, 48). The expression of ***TRKB*** is largely restricted to the nervous system and TRKB mRNA has been detected at high levels in cortical and hippocampal

pyramidal neurons but not in granule cells of the dentate gyrus (23, 46), which are the same populations of neurons found in this study to express BDNF mRNA and protein-like immunoreactivity. Thus, BDNF-(173–182)-like immunoreactivity found in the nucleus may represent BDNF secreted by the synthesizing neurons, subsequently taken up by those same cells via their *trkB*-encoded high-affinity BDNF receptors to either remain in the cytoplasm or to enter the nucleus and act in an autocrine manner on BDNF-synthesizing neurons.

The BDNF-(173–182) immunoreactivity observed in this study appears to reflect the presence of BDNF protein in the adult rat brain. However, we recognize the limitations of the techniques used. Data from the quenching of BDNF immunostaining with the specific peptide (but not with the carrier protein, Freund's adjuvant, or other molecules with which the rabbits were immunized), immunoblot specificity of antisera for rmBDNF, and the strong correlation of cellular distribution with known location of BDNF mRNA diminish the likelihood of artifactual binding of antibodies to the nucleus. Although BDNF-(173–182) was chosen to minimize the sequence similarities to NGF and NT3/HDNF, the possibility that our antisera are detecting an as-yet-unknown but very similar or even homologous amino acid sequence, perhaps belonging to another member of the NGF family, can not be fully excluded.

In conclusion, the present study reports the cellular distribution of BDNF protein. Using antipeptide antibodies, we present evidence of possible differential posttranslational modifications of the BDNF protein and offer evidence of nuclear translocation and subcellular compartmentalization of a growth factor intrinsic to central nervous system neurons. These results suggest an expansion of concepts regarding the biological role of BDNF *in vivo*, particularly with respect to possible nuclear interactions that may be mediated by nuclear binding proteins or even by BDNF binding directly to nuclear structures and influencing gene regulation as a putative transcription factor. Additionally, our data supporting the possible somatic or dendritic release of BDNF in hippocampus and cortex and the internalization and retrograde transport by basal forebrain neurons suggest that BDNF may be an important candidate trophic molecule to study as a possible etiologic and/or treatment factor in neurodegenerative disease involving cholinergic projections, such as Alzheimer senile dementia.

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