BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 468–472 (1997)
ARTICLE NO. RC977324

Dopaminergic Transmitter Up-Regulation of Brain-Derived Neurotrophic Factor (BDNF) and Nerve Growth Factor (NGF) Synthesis in Mouse Astrocytes in Culture

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Received August 2, 1997

We developed a highly sensitive enzyme immunoassay (EIA) system for brain-derived neurotrophic factor (BDNF) based on a biotin-streptavidin detection system capable of measuring concentrations as low as 1.0 pg/ml with high reproducibility. Using this EIA system, we examined the effect of dopaminergic transmitters such as dopamine and epinephrine on BDNF synthesis in mouse astrocytes in culture. These drugs had a stimulating effect on BDNF synthesis and showed a stronger promoting activity toward BDNF synthesis than toward nerve growth factor (NGF) synthesis. This is the first reported study in which BDNF synthesis was shown to be strongly stimulated by dopaminergic transmitter in mouse astrocytes. Then, we measured BDNF levels in the developing rat brain (striatum and midbrain). BDNF levels were relatively higher than NGF and NT-3 levels in these tissues. The BDNF level was high at the early stage in which neurons were proliferating, migrating, and differentiating, and it generally decreased as these cells matured. © 1997 Academic Press

A new concept has developed as a result of the discovery of the NGF gene family (1-9). The life of a neuronal cell, from its first differentiation into a specific neuron, through its functional period and aging, and to its death, is controlled by neurotrophins. The neuronal survival and the maintenance of its functions are achieved not by one factor but by a family of factors (1-9).

In the central nervous system (CNS), astrocytes occupy a critical position in the regulation of neural function during development and also in the mature brain. Astrocytes synthesize and secrete a number of diffusable neurotrophic factors (10) and thereby aid in the establishment and maintenance of synaptic connections. Such effects of BDNF on developing basal forebrain cholinergic neurons (11,12), dopaminergic neurons of the substantia (13), and cultures of ventral spinal cord cells (14,15) should soon find clinical application. It is possible that BDNF may be applied therapeutically in Alzheimer's disease (AD), Parkinson's disease (PD), motor neuron disorders such as amyotrophic lateral sclerosis, and in a variety of peripheral neuropathies. Yoshimoto *et al.* (16) reported that *ex vivo* gene therapy with BDNF ameliorates parkinsonian symptoms through a mechanism(s) other than one involving an effect of BDNF on regeneration or sprouting from dopaminergic neurons. Astrocytes and neurons are the sources of NGF and BDNF in the CNS. In order to study the unique biochemical and pathophysiological features of BDNF, it is necessary to develop a highly sensitive and specific assay method for detection of the factor.

The enzyme immunoassay (EIA) system against BDNF reported here is sensitive, reliable, and practical and will allow study of the level of BDNF protein in the CNS and peripheral nervous system (PNS), and determination of the site of biosynthesis and biological functions of BDNF *in vivo.* We have already reported that the secretion of NGF from astrocytes in culture was growth phase dependent (17) and was increased by the addition of catecholamine and its derivatives (18). Although astrocytes have been also shown to express BDNF mRNA (10), BDNF protein has not been detected in the conditioned medium yet. Because the production of neurotrophin in culture is very low, it has not been previously possible to measure the BDNF level specifically. As the amino acid sequences of human, rat, and mouse BDNFs are the same (2), the developed EIA system could be applied to the measurement of the BDNF levels in the samples from all of these species. So, using the EIA system, we first inves-

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0006-291X/97 $25.00

tigated the BDNF level in the conditioned medium of mouse astrocytes and the effects of dopaminergic transmitter on the synthesis of BDNF. The synthesis of the factor was stimulated by the addition of dopamine and epinephrine to the medium, and the stimulating activity of these drugs was stronger toward BDNF than toward NGF.

During brain development, molecules of the NGF family display distinct stage-specific and tissue-specific patterns of expression (19, 20). So, we examined the developmental changes in BDNF level in striatum and midbrain, which areas are innervated by dopaminergic neurons.

This paper deals with the effect of dopaminergic transmitters on the synthesis of BDNF in mouse astrocytes in culture and with the developmental changes in BDNF in the rat brain.

MATERIALS AND METHODS

Enzyme immunoassay for BDNF. Recombinant human BDNF was purchased from Chemicon International Inc. Murine anti-human BDNF monoclonal antibody, used as a primary antibody, was purchased from R&D Systems Inc. Anti-BDNF polyclonal antibody (purchased from Promega) was biotinylated with 5-(N-Succinimidyloxycarbonyl)pentyl D-biotinamide (Dojindo) as described previously (21) and used as the secondary antibody. Aliquots of standard or sample (40 μl) were added to wells of a polystyrene microtiter plate (Falcon) whose surface had been previously coated with 5 μl of 50 μg/ml anti-human BDNF monoclonal antibody. After incubation for 3 hr at room temperature, each well was washed three times with 0.1M Tris-HCl buffer (pH 7.6) containing 0.4M NaCl, 0.1% BSA, 0.1% NaN_3, and 1 mM $MgCl_2$ (buffer W). Then, biotinylated anti-BDNF polyclonal antibody conjugate (20 μl; 50 ng/ml in buffer W) was added, and incubation was carried out overnight at 4 °C. Then each well was washed with buffer W three times, and 30 μl of streptavidin-β-D-galactosidase complex (Sigma) that had been diluted 400-fold from a 0.5 μg/ml stock solution with buffer W was added. After a 1-hr incubation at room temperature, each well was washed three times, and β-D-galactosidase activity bound to the wells was then assayed. The enzyme reaction was started by the addition of 30 μl of buffer W containing 60 mM 4-methylumbelliferyl-β-D-galactosidase (Sigma). After a 4-hr incubation at 37 °C, the enzyme reaction was stopped by the addition of 0.13 ml of 0.1M glycine-NaOH buffer (pH 10.3). The amount of 4-methylumbelliferone formed was measured with a fluorescence spectrometer (model 850, Hitachi) having wavelengths for excitation and emission set at 360 and 450 nm, respectively.

Enzyme immunoassay for NGF and NT-3. The EIA for NGF (or NT-3) was also based on the sandwiching of antigen between an anti-NGF (or NT-3) antibody IgG coating on a polystyrene plate and biotinylated anti-NGF (or NT-3) antibody IgG. The bound antibody complex was measured with streptavidin-linked β-D-galactosidase (22).

Preparation of homogenate from brain. Striatum and midbrain were obtained from Wistar rats and frozen on dry ice and stored at −80 °C. The samples were sonicated in cold 0.1M Tris-HCl buffer, pH 7.6, containing 1M NaCl, 2% BSA, 2mM ethylenediamine tetraacetic acid (disodium salt), 80 trypsin inhibitory units of aprotinin/liter, and 0.02% NaN_3 at 5% wet tissue weight per volume. The solution was centrifuged at 15,000 r.p.m. for 30 min, and the supernatant obtained was assessed for neurotrophin contents.

Cell culture. Astrocytes were cultured from the whole brains of 8-day-old ICR mice and maintained in DMEM containing 10% FCS as described before (23). The preparation of the quiescent astrocytes was done according to the procedures previously reported (23). Namely, astrocyte cells were inoculated into 24-well plates and cultured in DMEM containing FCS until confluence was reached. Then, they were cultured for an additional 10 days in FCS-free DMEM containing 0.5% BSA, with a medium change every 3 days. Cells were cultured in 0.4 ml of DMEM containing 0.5% BSA with or without the test compounds, after which the BDNF, NGF, and NT-3 content in the conditioned medium of each culture was determined by the respective EIAs. Since neurotrophin synthesized by the above cells are secreted rapidly into the conditioned medium (24), the measurements of BDNF, NGF and NT-3 content in conditioned medium reflects the amount of BDNF, NGF, and NT-3 synthesized.

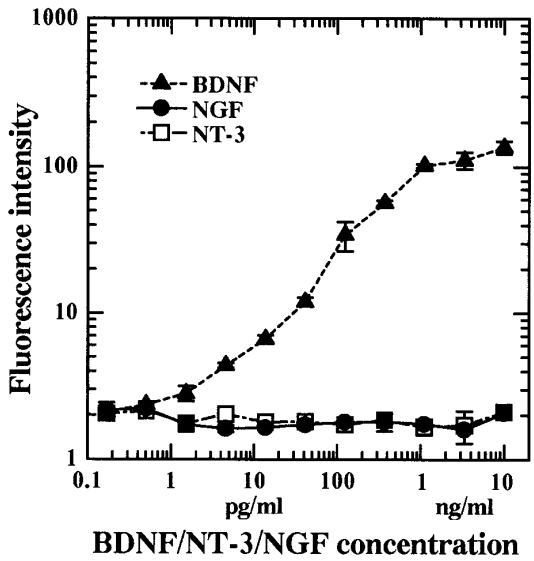

FIG. 1. Comparison of the antigenicity of NTFs by the two-site EIA system for BDNF.

RESULTS

Establishment of enzyme immunoassay. Figure 1 shows a typical dose-response curve of the assay for BDNF. The assay system detected BDNF quantitatively at a concentration as low as 1 pg/ml and did not detect other neurotrophins like NGF or NT-3 when these factors were present below a concentration of 100 ng/ml.

Effect of dopamine and epinephrine on BDNF/NGF synthesis in mouse astrocytes. Dopamine and epinephrine stimulated BDNF/NGF synthesis in astrocytes in culture. As shown in Figure 2a, treatment of the cells with dopamine resulted in a bell-shaped dose-response curve for the stimulation of BDNF synthesis (detected as BDNF in the conditioned medium). The BDNF content in conditioned medium of astrocytes treated with 0.15 mM dopamine corresponded to 3.5 times that of control cultures, whereas that of cells treated with 0.4 mM was the same as the control value. This phenomenon seemed to be caused by the cytotoxic-

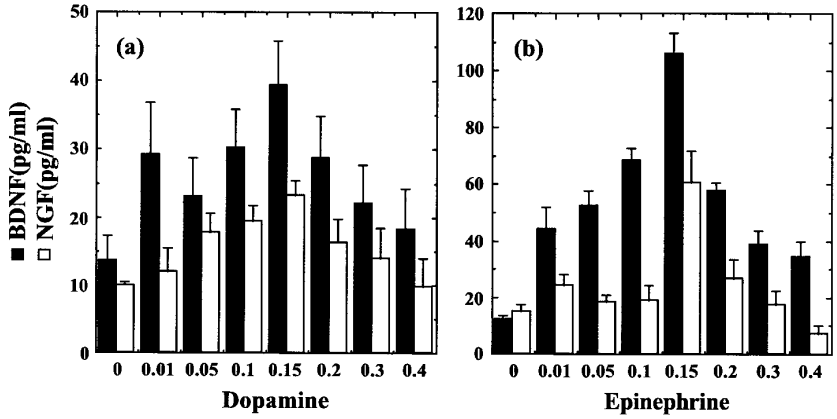

FIG. 2. Effects of dopamine (a) and epinephrine (b) on BDNF content in medium conditioned by mouse astrocytes. Each value is the mean ± S.E. of four determinations.

ity of dopamine, because almost all cells died at a dopamine concentration over 0.4 mM.

Figure 2b shows that epinephrine had a similar stimulating effect on the BDNF synthesis of the cells. The increase in BDNF content brought about by epinephrine at its optimal concentration (0.15 mM) resulted in a level approximately 9-fold over the control. On the other hand, NT-3 was not detected in this conditioned medium (data not shown). In cultured astrocytes, NT-3 may be not synthesized and/or not response to these drugs. Both stimulators showed dose-response characteristics for NGF synthesis that were similar to these for BDNF synthesis.

Developmental changes in neurotrophin levels in striatum and midbrain of rats. Since BDNF is one of the target-derived neurotrophic factors for nigral dopaminergic neurons, changes in BDNF levels in rat brain (striatum and midbrain) during development were investigated (Fig. 3). At postnatal day 0, the BDNF level was relatively low, and then increased, reaching its maximal level at 4 weeks (striatum) or 1 week (midbrain) postpartum. These BDNF levels decreased as the animals matured. Changes in NGF and NT-3 levels in these regions were also investigated. The NGF level was lower than that of other neurotrophins throughout life and did not change dramatically with time in striatum and midbrain. The NT-3 level in the striatum increased sharply after birth and reached its maximal level at 1 week; and then it dramatically decreased to a constant level. Its level in the midbrain gradually decreased after birth.

DISCUSSION

PD is characterized by the progressive loss of dopaminergic neurons of the substantia nigra in the midbrain (25). The nigral neurotransmitter dopamine is reduced in PD, causing hypoactivity of neurons in the striatum, which cells receive axonal projections from the substantia nigra. Ordinarily PD patients are treated with a dopamine precursor or dopamine receptor agonist to activate the postsynaptic target(s) of the

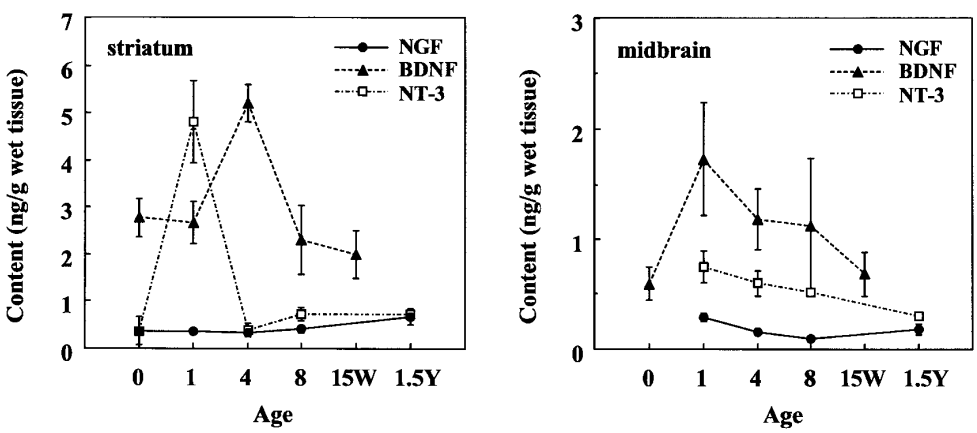

FIG. 3. Developmental changes in BDNF levels in striatum and midbrain of rats.

missing neurons. However, this supplementary therapy is not aimed directly at prevention of nigral degeneration. Neurotrophic factors that slow, halt, or reverse the progression of dopaminergic neural degeneration are thus hopeful candidates for the treatment aimed at the degenerative process in PD. BDNF promotes the survival and differentiation of nigral dopaminergic neurons via several mechanisms. Since BDNF mRNA and the high-affinity receptor, trkB, are expressed in dopaminergic neurons, BDNF may act as an autocrine factor (26). Since BDNF is also supplied by neurons and glia cells around the dopaminergic neurons, BDNF may also function as an paracrine factor (10, 26). Since BDNF is expressed by the target regions such as striatum (neurons and glia cells), BDNF may activate the dopaminergic neurons at the nerve terminal and be retrogradely transported (27).

During brain development, neurotrophins display distinct space-specific and time-specific patterns of expression (19, 20). BDNF- and NGF mRNA are at their lowest level in the embryo and then increase, reaching their maximal level in the adult. In contrast with the change in NGF and BDNF mRNAs, the highest level of NT-3 mRNA is found shortly after birth, and the level dramatically decreases as these regions mature. Earlier we reported developmental changes in the NT-3 protein level in the CNS and PNS (22). These developmental changes in these tissues coincided with the changes in the NT-3 mRNA reported before.

BDNF mRNA has been detected in many regions in the brain, the highest expression being in the hippocampus and cortex (28-30). However, the distribution and regulation of BDNF were mainly investigated at the mRNA level because of the lack of a sensitive, quantitative assay system for BDNF protein. So we developed a two-site EIA for BDNF. Because neurotrophins exhibit a high degree of amino acid homology, cross-reactivity of the antibody is potentially a serious problem for any attempt to use EIA system. However, our assay system was show to be highly specific as well as simple and highly sensitive (Fig. 1).

We have already reported that many compounds including catecholamine (12), its derivatives (31), and coenzymes (32), etc. stimulated the NGF synthesis of mouse astrocytes in culture. So, using our developed EIA system against BDNF, we examined the stimulating effects of dopaminergic transmitters on BDNF synthesis by mouse astrocytes. As a result, these drugs were found to stimulate the synthesis of BDNF protein, measured as BDNF in the cultured medium. Although the stimulation profile of BDNF was similar to that of NGF but not identical, the degree of BDNF synthesis was higher than that of NGF. As we already reported, astrocytes did not synthesize detectable amounts of NT-3; and the NT-3 synthesis was not stimulated by these drugs (22). Our results coincide with those of a previous study showing that dopaminergic stimulation directly promoted the expression of BDNF mRNA in the striatum *in vivo* (33). The expression of neurotrophins changes developmentally and displays a space-specific and time-specific pattern (19, 20). These changes may be regulated by the expression of the high-affinity Trk receptor and by the correspondent neurotransmitters. Recently, it was demonstrated that the expression of NT-3 in cultured rat hippocampus neurons was increased by treatment with BDNF or NT-3 (34). The expression of a neurotrophin may thus be regulated by the neurotrophin itself.

Using our EIA system, we then investigated possible developmental changes in the BDNF protein level in the striatum and midbrain of rats. We reported earlier that hippocampus and cortex were the regions richest in NT-3 protein (22) and that the developmental changes in the protein well coincided with the mRNA expression patterns of NT-3 as above described. However, the changes in the BDNF level in the striatum and midbrain were slightly different from those of NT-3 in the hippocampus and cortex. Dopaminergic neurons in the midbrain innervate the striatum. In these regions, the BDNF levels decreased postnatally after weeks 1∼4. Considering that the period of the first 2 weeks of postnatal development corresponds to the maturation of the dopaminergic innervation of the striatum (35), the demand for BDNF may be decreased after postnatal week 1∼4. However, BDNF is essential for dopaminergic neurons (36), and the level remains higher than that of other neurotrophins throughout life.

In the present study, we found that the synthesis of BDNF in cultured astrocytes was up-regulated by dopaminergic neurotransmitters and that, in the development of the rat brain, BDNF protein levels increased after birth and then decreased as the animals matured.

ACKNOWLEDGMENT

This work was supported in part by a Grant-in-Aid for Scientific Research on Physiology and Pharmacology of the Smoking Program of the Smoking Research Foundation.

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