Volume 313, number 2, 138–142 FEBS 11779 November 1992

Dopaminergic stimulation up-regulates the in vivo expression of brain-derived neurotrophic factor (BDNF) in the striatum

Hitoshi Okazawa, Miho Murata, Masahiko Watanabe, Masaki Kamei and Ichiro Kanazawa

Department of Neurology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

Received 17 September 1992; revised version received 7 October 1992

We investigated the effect of dopamine on the in vivo expression of brain-derived neurotrophic factor (BDNF) in the striatum of mouse. BDNF mRNA expression in the striatum, which was quantified with the reverse transcriptase polymerase chain reaction, was up-regulated from 2 h after oral administration of levodopa, a precursor of dopamine. The increase was sustained for 16 h. Co-administration of haloperidol partially inhibited dopamine-induced BDNF enhancement. These data suggest that dopaminergic stimulation directly promotes the expression of BDNF in the striatum in vivo.

Brain-derived neurotrophic factor; Dopamine; Transcription, Striatum

1. INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is purified as a protein with a property to support embryonic neurons [1,2]. Nerve growth factor (NGF) [3], neurotrophin 3 (NT3) [4–9], neurotrophin 4 (NT4) [10], and neurotrophin 5 (NT5) [11] have similar biological activities and homologous protein structures to BDNF, composing a gene family. The spectra of target neurons of these neurotrophic factors are distinct but partially overlap. For example, both BDNF and NGF have a trophic effect for dorsal root ganglion neurons and cholinergic neurons from basal forebrain nuclei [12]. However, retina ganglion cells [13] respond for BDNF but not for NGF. BDNF supports placode and neural-crest-derived sensory neurons which are not responsive to NGF [13–15]. The selective responsiveness is presumably derived from different expression patterns of neurotrophic receptors.

We are interested in developing therapies for Parkinson's disease (PD) which is caused mainly by neural degeneration in the substantia nigra located in the midbrain. The nigral neurotransmitter, dopamine, is reduced in PD, causing hypoactivity of neurons in the striatum which receives axonal projection from the substantia nigra. Ordinarily PD patients are treated with dopamine supplements. However supplementary therapy is not aimed directly at prevention of nigral degeneration. A candidate substance which could delay the nigral degeneration if BDNF, since BDNF, but not NGF, extends the survival of primary cultured central dopaminergic neurons [16,17]. Therefore, modulating the expression of BDNF in brains of PD patients would be a different type of therapy from the replacement of neurotransmitter of nigral neurons. In this sense, it is important to survey the agents which may influence the expression of BDNF. It is noteworthy that BDNF expression is up-regulated by potassium-induced depolarization in hippocampal neurons [18]. In addition, the application of kainic acid [18–20] and experimental epilepsy [21] seem to promote BDNF expression in the hippocampus through depolarization. It is possible that dopamine induces depolarization on the striatal neurons when released from the terminal of the nigro-striatal fibers and, conversely, neurotrophic factors released from the striatum would support the survival of nigral neurons through the retrograde transport. Therefore, we investigated the effects of dopamine on the expression of BDNF in the striatum.

Correspondence address. H. Okazawa, Department of Neurology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan. Fax: (81) (3) 3813-2129.

2. MATERIALS AND METHODS

2.1. *RNA preparation and RT-PCR*

Balb/c mice were dissected at each interval after the administration of a combined drug of levodopa (3-hydroxy-L-tyrosine)/carbidopa (*S*-α-hydrazino-3,4-dihydroxy-α-methylbenzenepropanoic acid monohydrate) 10:1 orally as a fine suspension. Ordinarily 0.2 mg of levodopa/g body weight of mouse was administered. For the inhibition experiment, 1 μg of haloperidol/g body weight of mouse was co-administered. The mice were observed for at least 1 h after administration to confirm that they did not vomit. Brains were coronally sectioned according to an anatomical atlas. Regions of interest were separated out, rapidly frozen in liquid nitrogen, and kept at −80°C until use.

RNA preparation was basically according to the method of Chomzinski and Sacchi [22]. Briefly, 5–10 mg of brain tissue was homogenated in 800 μl of solution D (4 M guanidinium thiocyanate,

Published by Elsevier Science Publishers B.V

25 mM sodium citrate, 0.5% *N*-lauroylsakosyl, 0.1 M 2-mercaptoethanol). After a brief centrifugation, 100 μl of 2 M sodium acetate (pH 4.0) was added to the supernatant and mixed by inversion, 800 μl of phenol saturated with water was added and mixed, and then 200 μl of chloroform–isoamylalcohol (49:1) was added and vortexed vigorously for 1 min. The aqueous phase was recovered after a centrifugation, added with 800 μl of −20°C isopropanol. The solution was divided to three Eppendorf tubes, centrifuged for 30 min at 15,000 rpm at 4°C. The pellet was washed with 70% ethanol and dried. One of the tubes was used for estimation of RNA amounts. Since expression of BDNF in the striatum is very low under physiological conditions [23,24], we used reverse transcriptase polymerase chain reaction (RT-PCR) for the estimation. RT-PCR was performed according to the method described by Zafra et al. [18]. 200 ng of total RNA, 0.1 μM of both directional primers, 1 × RT-PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.1% Triton X-100, 0.25 mM dNTPs, 5 U RNase inhibitor (Boehringer-Mannheim, Germany), 3.2 U AMV-reverse transcriptase XL (Life Science Inc., USA), and 2 U *Taq* polymerase (Perkin-Elmer Cetus Instruments, USA) were mixed in a total volume of 25 μl. PCR primers for neurofilaments were added to this reaction solution when necessary. The solution was incubated at 41°C for 30 min and denatured at 94°C for 3 min and used for PCR. Basically, the thermal cycle condition was as following 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min.

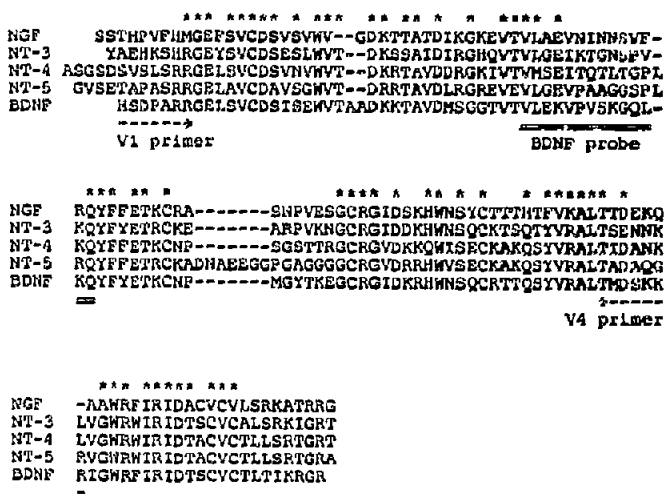

Fig. 1. Positions of the primers used in RT-PCR and the oligonucleotide probe used in Southern blotting are indicated with arrows and a double underline below the sequence comparison of five members of neurotrophins. Amino acid sequences conserved between more than four members are indicated with asterisks. The primers and the probe correspond to the specific sequence of BDNF.

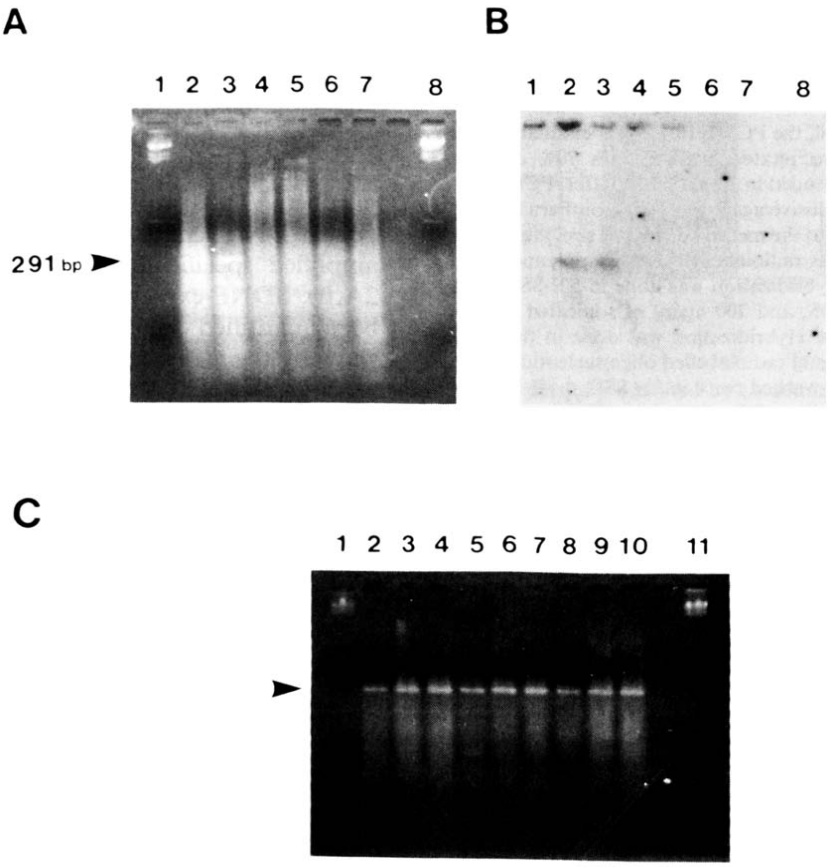

Fig. 2. The BDNF transcript can be detected with RT-PCR in the striatum. (A) 20 rounds of thermal cycles amplified cDNA bands from 1 μg of the hippocampal total RNA independently prepared (lanes 2,3), but not amplified from 1 μg of total RNA prepared from the striatum (lanes 4,5) and a ventral half of the midbrain (lanes 6,7). (B) This agarose gel was blotted to a nylon membrane and hybridized with a BDNF-specific oligonucleotide probe. The probe hybridized with the amplified bands, indicating that our RT-PCR could detect the BDNF transcript. (C) BDNF cDNAs were amplified from total RNA of the hippocampus (lanes 2–4), striatum (lanes 5–7), and a ventral half of the midbrain (lanes 8–10). The number of rounds of thermal cycle are as follows: lane 2, 20; lane 3, 30; lane 4, 40; lane 5, 30; lane 6, 40; lane 7, 50; lane 8, 30, lane 9, 40; lane 10, 50. RT-PCR amplification reached a plateau between 30 and 40 rounds when using striatal RNA.

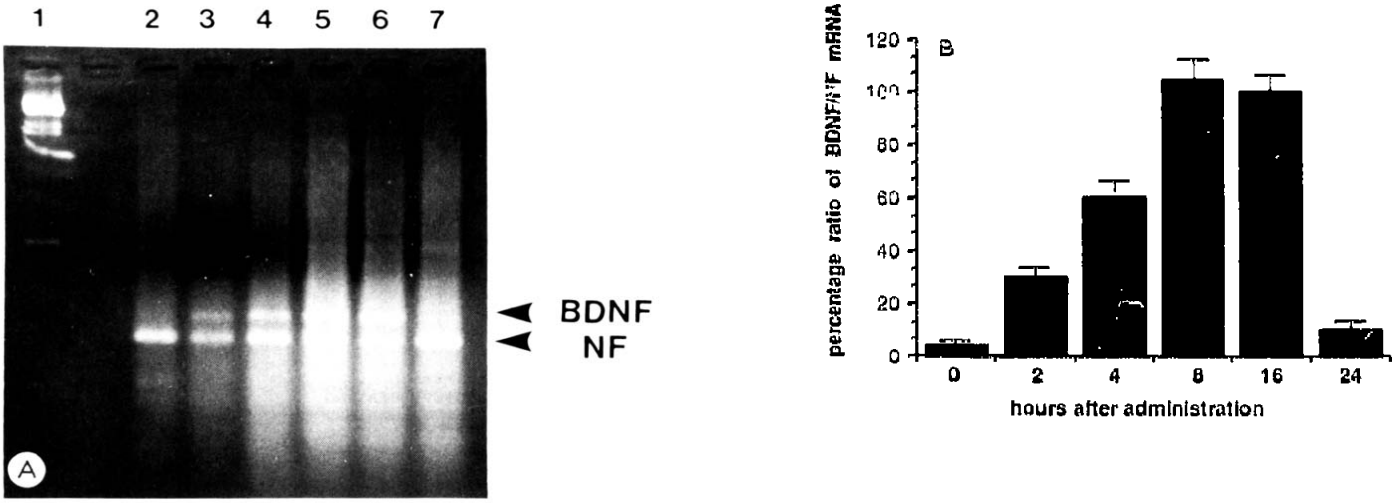

Fig. 3 Time-course of BDNF up-regulation after oral administration of 0.2 mg/g body weight of mouse of levodopa. (A) Brain tissues were dissected without administration (lane 2) or at 2 h (lane 3), 4 h (lane 4), 8 h (lane 5), 16 h (lane 6), and 24 h (lane 7) after administration of levodopa. Lane 1 shows molecular weight markers, λDNA digested by *Hin*dIII. 1 μg of total RNA from the striatum was used in RT-PCR. Amplified cDNAs were separated with a 3% Nusive/agarose gel. cDNA bands of BDNF (291 bp) and neurofilament-M (216 bp) are indicated with arrowheads (BDNF and NF). (B) Amounts of BDNF cDNAs are standardized with those of neurofilament. Values represent the mean ± S.D. of 4 determinations and are expressed as percentages of the internal standard.

2.2. *Southern blotting*

After removal of mineral oil, the PCR product was extracted twice with chloroform, ethanol precipitated, washed with 70% ethanol, dried by lyophilizer, and suspended in 15 μl of TE buffer. PCR products were separated on a 3% Nusive/agarose 3:1 gel. Southern blotting was done basically according to the method described previously [25]. The oligonucleotide probe was radiolabelled with T4 polynucleotide kinase (Takara, Japan). Prehybridization was done in 5 × SSC, 50% deionized formamide, 1% SDS, and 200 ng/ml of sonicated salmon sperm DNA at 42°C for 6 h. Hybridization was done in the same solution by adding about 2 ng/ml radiolabelled oligonucleotide probe at 42°C for 24 h. Filters were washed twice in 5 × SSC, 0.1% SDS, at 40°C for 20 min and sequentially in 0.2 × SSC, 0.1% SDS, at 55°C for 30 min. Filters were exposed to Kodak XOMAT films overnight at −80°C.

2.3. *Quantification of PCR product*

Since the blotting efficiency was sometimes variable, we estimated the amounts of PCR products directly from Polaroid films of agarose gels stained with ethidium bromide. Polaroid films were read with image analyzer (Epson GT8000). Signal areas and mean intensities were analyzed with Color magician III. Signals were standardized with neurofilament PCR product.

2.4. *Primers for PCR and probes for Southern blotting*

PCR primers for the amplification of BDNF were CACTCGGACCCCGCCCGCCG (V1 primer) and TCGTTTTTTGCTATCCATGG (V4 primer). The oligonucleotide probe used in Southern blotting was CTCGAAAAAGTCCCCGTCTCGAAAGGCCAACTGAAGCAG. PCR primers for neurofilament-M were GAAACTCAGGAGAAGGGCAGT and CTCATGCTCTTCAACCTTTTG. The neurofilament expected product is 216 bp in length.

3. RESULTS AND DISCUSSION

In order to develop the specificity of RT-PCR, we made an oligonucleotide probe corresponding to a specific sequence of BDNF in its variable region between PCR primers (Fig. 1) and Southern blotted the PCR products. This probe hybridized to the bands of expected DNA length, indicating that these bands were amplified specifically from the BDNF mRNA (Fig. 2A,B). BDNF expression in the striatum has previously been quantified with Northern blotting by other groups, showing the transcript level is extremely low or almost undetectable [23,24]. In our experiments, with 20 rounds of thermal cycle amplification, cDNA was amplified from hippocampal total RNA but not obviously from total RNA of the striatum and the ventral half of the midbrain, including the substantia nigra (Fig. 2A). However, using 30–50 rounds of thermal cycle amplification, we could clearly detect cDNA bands from striatal total RNA (Fig. 2C). This finding is compatible with the estimation by Northern blotting in the previous reports [23,24].

Using 23 rounds of thermal cycles, which is within the linear amplification range, we investigated the time-course of BDNF expression after oral administration of levodopa (Fig. 3A,B). We used 0.2 mg of levodopa/g body weight of mouse in this experiment. As an internal standard for each RT-PCR reaction, we mixed PCR primers for neurofilament-M, which were selected as a neuronal marker, in order to adjust the number of neurons. BDNF expression is rapidly up-regulated from 2 h after oral administration of levodopa. The amount of transcript did not seem to change obviously at 1 h after administration (data not shown). Considering the nec-

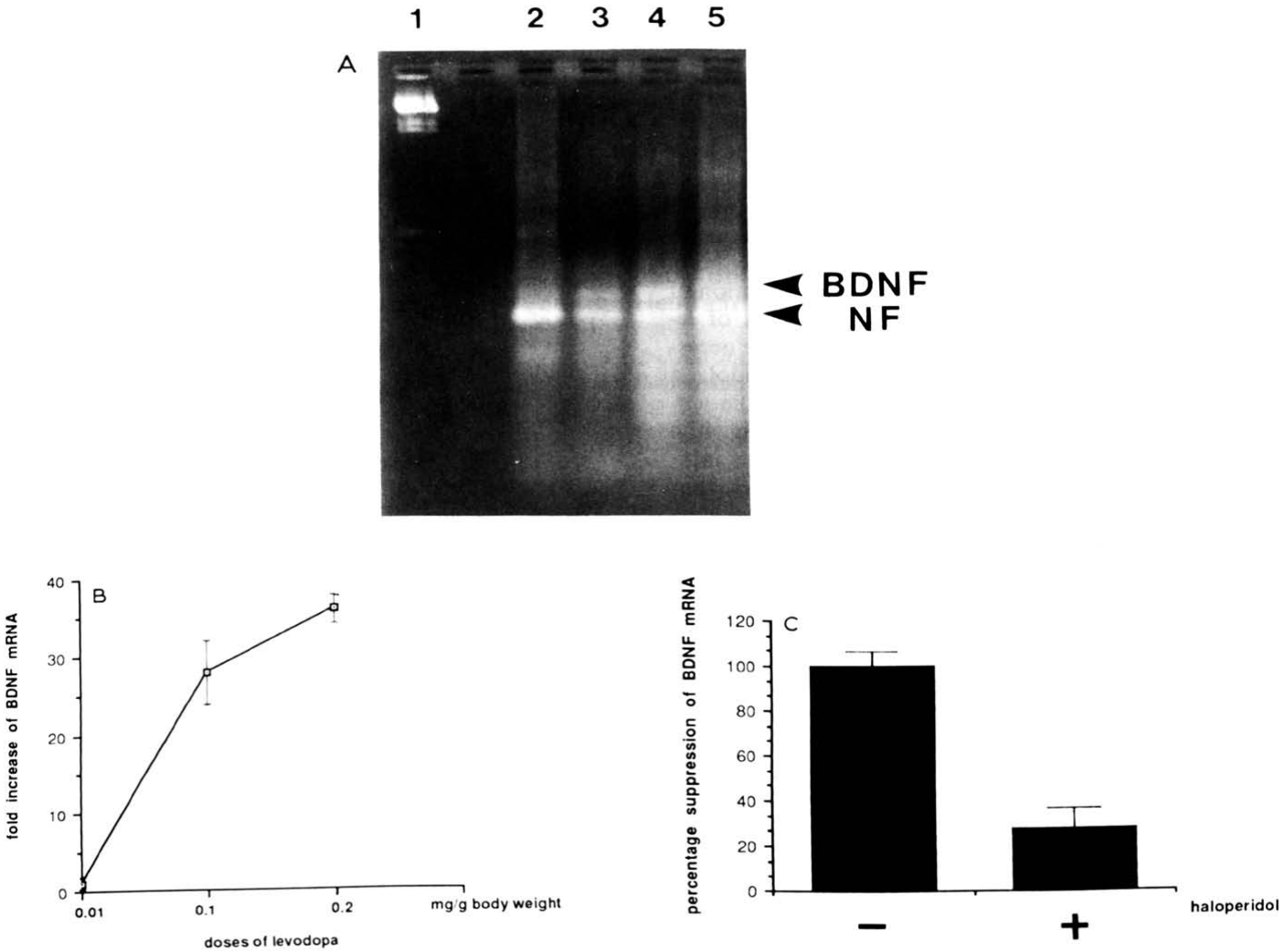

Fig. 4. Up-regulation of BDNF transcription by levodopa is dose dependent and partially inhibited by co-administration of haloperidol (A) Amounts of administration (mg/g body weight of mouse) of levodopa were 0.01 (lane 2), 0.1 (lane 3), and 0.2 (lane 4). 1 μg/g body weight of mouse of haloperidol was co-administrated with 0.2 mg/g body weight of mouse of levodopa (lane 5). Brain tissues were dissected at 6 h after administrations. RT-PCR was done as described before. cDNA were separated in a 3% Nusive/agarose gel. Lane 1 shows molecular weight markers. (B) Fold increase of BDNF transcript. Amounts of BDNF cDNA were standardized with those of neurofilament-M. The values are means ± S.D. ($n = 4$). (C) Inhibition of BDNF expression by haloperidol. BDNF expression was quantified after levodopa (0.2 mg/g body weight) administration alone or co-administration of levodopa and haloperidol (1 μg/g body weight). BDNF expression was standardized with the neurofilament control. The values present are means ± S.D. The value of co-administration is expressed as a relative percentage to the mean of levodopa administration, which is set as 100%.

essary time for absorption of levodopa from the gut, up-regulation begins within 2 h of exposure of neurons to dopamine. The response is equally rapid as that induced by kainic acid in hippocampal neurons [18]. The enhancement of BDNF expression lasted for 16 h after oral administration of levodopa. This period is also compatible with that caused by kainic acid stimulation [18]. The similarity of the time-courses suggests that depolarization by transmitters would induce similar cellular responses which regulate BDNF transcription. In order to comprehend this regulating mechanism of BDNF expression, transcriptional control regions of the chromosome must be analyzed. A part of the BDNF gene, 2.2 kbp of 5′ flanking region, has been analyzed by chloramphenicol acetyltransferase (CAT) assay using a glioma cell line [27]. However, there are no sequences matching the consensus sequences of published enhancer elements within this 2.2 kbp. Therefore transcription factors which act on this portion of the gene and define the level of BDNF expression are not yet known. Furthermore there remains the possibility that some other regions of the chromosome influence

the transcription of BDNF. The up-regulation of BDNF by transmitters might be caused by de novo synthesis of known and/or unknown transcription factors are depolarization, or by modification of some transcription factors, such as an activation of jun by dephosphorylation [28]. These questions should be analyzed in the future.

As the next step, we grossly evaluated the dose–response in order to see whether a clinical application is possible. Administration of 0.01 mg of levodopa/g body weight of mouse is inadequate to elicit up-regulation of BDNF (Fig. 4A,B), suggesting ordinary doses of levodopa used for PD patients are far less than the necessary amount. Co-administration of haloperidol, a dopaminergic antagonist, partially blocked the enhancement of BDNF expression by dopamine (Fig. 4C). The prompt increase of BDNF expression and its inhibition by an antagonist suggest the possibility that dopamine directly promotes BDNF expression.

It is still an open question how neurons are kept alive in physiological and pathological conditions. Neurotrophic factors are clearly candidates for these life-promoting agents. Neurotrophins at least participate in the programmed cell death during development, since application of NGF increases the number of surviving neurons in vivo [30]. Ciliary neurotrophic factor (CNTF), another type of trophic factor, supports the survival of injured or congenitally abnormal motor neurons in vivo [31,32]. BDNF elongates the survival of nigral neurons in primary culture [16,17]. Neurons receive these neurotrophic factors from their target cells, including neurons themselves. Trophic factors are taken up from the axonal terminal and transported retrogradely to the cell body and induce trophic effects (for review see [29]). On the other hand, recent reports revealed that neural activation itself elicits early the gene response of neurotrophic factors [18–20]. These two observations suggest a hypothetical relationship between neurons and their target neurons; target neurons seem to produce neurotrophic factors more abundantly and release them to neurons which send axons to the target neurons, when the neural circuit which connects the two groups of neurons is activated. Our results in the nigro–striatal pathway seem to support this hypothesis. It is possible to speculate that doparminergic stimulation supports nigral neurons through the up-regulation of BDNF expression in the striatum. However, we do not know to what extent BDNF actually participates in the survival of nigral neurons in a physiological or a pathological condition. This question should be clarified in the future in order to develop the application of BDNF for the therapy of neural degeneration.

Acknowledgements: This work was supported by a Grant-in-Aid for Developmental Scientific Research (No. 04557037) from the Ministry of Culture and Education and by a grant from Fujisawa Foundation.

REFERENCES

[1] Barde, Y.-A., Edgar, D. and Thoenen, H. (1982) EMBO J. 1, 549–553.
[2] Leibrock, J., Lottspeich, F., Hohn, A., Hofer, M., Hengerer, B., Masiakowski, P., Thoenen, H. and Barde, Y.-A. (1989) Nature 341, 149–152.
[3] Levi-Montalcini, R. (1987) EMBO J. 6, 1145–1154.
[4] Ernfors, P., Ibanez, C.F., Ebendal, T., Olson, L. and Persson, H. (1990) Proc Natl. Acad. Sci USA 87, 5454–5458.
[5] Hohn, A., Leibrock, J., Bailey, K. and Barde, Y.-A. (1990) Nature 344, 339–341.
[6] Jones, K.R. and Reichert, L.F. (1990) Proc Natl. Acad. Sci. USA 87, 8060–8064.
[7] Maisonpierre, P.C., Belluscio, L., Squinto, S., Ip, N.Y., Furth, M.E., Lindsay, R.M. and Yancopoulos, G.D. (1990) Science 247, 1446–1451.
[8] Rosenthal, A., Goeddel, D.V., Nguyen, T., Lewis, M., Shih, A. Laramee, G.R., Nikolics, K. and Winslow, J.W. (1990) Neuron 4, 767–773.
[9] Kaisho, Y., Yoshimura, K. and Nakahama, K. (1990) FEBS Lett. 266, 187–191.
[10] Hallbook, F., Ibanez, C.F. and Persson, H. (1991) Neuron 6, 845–858
[11] Berkemeier, L.R., Winslow, J.W., Kaplan, D.R., Nikolics, K., Goeddel, D.V. and Rosenthal, A. (1991) Neuron 7, 857–866.
[12] Alderson, R.F., Alterman, A.L., Barde, Y.-A. and Linsay, R.M. (1991) Neuron 5, 297–306.
[13] Barde, Y.-A. (1989) Neuron 2, 1525–1534.
[14] Davies, A.M. (1987) Development 101, 185–208.
[15] Lindsay, R.M., Thoenen, H. and Barde, Y.-A. (1985) Dev. Biol. 112, 319–328.
[16] Knusel, B., Winslow, J., Rosenthal, A., Burton, L., Seid, D., Nikolics, K. and Hefti, F. (1991) Proc. Natl. Acad. Sci. USA 88, 961–965.
[17] Hyman, C., Hofer, M., Barde, Y.-A., Juhasz, M., Yancopoulos, G.D., Squinto, S.P. and Lindsay, R.M. (1991) Nature 350, 230–232.
[18] Zafra, F., Hengerer, B., Leibrock, J., Thoenen, H. and Lindholm, D. (1990) EMBO J. 11, 3545–3550.
[19] Ballarin, M., Ernfors, P., Lindefors, N. and Persson, H. (1991) Exp. Neurol 114, 35–43.
[20] Gall, C., Murray, K. and Isackson, P.J. (1991) Mol. Brain Res. 9, 113–123.
[21] Ernfors, P., Bengzon, J., Kokaia, Z., Persson, H. and Lindvall, O. (1991) Neuron 7, 165–176.
[22] Chomzinski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[23] Hofer, M., Pagliusi, S.R., Hohn, A., Leibrock, J. and Barde, Y.-A. (1990) EMBO J. 9, 2459–2464.
[24] Maisonpierre, P.C., Belluscio, L., Friedman, B., Alderson, R.F., Wiegand, S.J., Furth, M.E., Lindsay, R.M. and Yancopoulos, G.D. (1990) Neuron 5, 501–509.
[25] Okazawa, H., Okamoto, K., Ishino, F., Ishino-Kaneko, T., Takeda, S., Toyoda, Y., Muramatsu, M. and Hamada, H. (1991) EMBO J. 10, 2997–3005.
[26] Levy, E., Liem, R.K.H., D'Eustachio, P. and Cowan, N.J. (1987) Eur. J. Biochem. 166, 71–77.
[27] Shintani, A., Ono, Y., Kaisho, Y. and Igarashi, K. (1992) Biochem. Biophys. Res. Commun. 182, 325–332.
[28] Boyle, W.J., Smeal, T., Defize, L.H.K., Angel, P., Woodgett, J.R., Karin, M. and Hunter, T. (1991) Cell 64, 573–584.
[29] Thoenen, H. (1991) Trends Neurosci. 14, 165–170.
[30] Hamburger, V., Brunso-Bechtold, J.K. and Yip, J.W. (1981) J. Neurosci. 1, 60–71.
[31] Sendtner, M., Kreuzberg, G.W. and Thoenen, H. (1990) Nature 345, 440–441.
[32] Sendtner, M., Schmalbruch, H., Stockli, K.A., Carrol, P., Kreuzberg, G.W. and Thoenen, H. (1992) Nature 358, 502–504.