Brain-derived Neurotrophic Factor Increases Neurotrophin-3 Expression in Cerebellar Granule Neurons*

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Neurotrophin-3 (NT-3) is a member of the neurotrophin gene family and is highly expressed in the developing rat cerebellum. Here we show that brain-derived neurotrophic factor (BDNF) increased by approximately 10-fold the NT-3 mRNA levels in cultured cerebellar granule neurons isolated from postnatal rats, whereas nerve growth factor (NGF) and NT-3 itself had no effect. The effect of BDNF was additive to that of triiodothyronine (T3), which also increased NT-3 mRNA in these neurons. The drug K252a inhibited the BDNF-mediated stimulation of NT-3 expression, suggesting an involvement of trkB receptors. Nuclear run-on experiments showed that BDNF increased NT-3 transcription, whereas the stability of NT-3 mRNA remained unchanged. The data presented are the first demonstration that one neurotrophin regulates the expression of another and provide evidence that NT-3 production in granule neurons is regulated by both BDNF and T3.

The neurotrophins constitute a family of structurally related neurotrophic factors that act on partly overlapping populations of neurons (1). In brain, NGF, BDNF, and NT-3 are all expressed in the rat hippocampus (1), but BDNF mRNA (2, 3) and NT-3 mRNA are also present in rat cerebellum (3–5). Hippocampal NGF and BDNF mRNA levels are increased by enhanced neuronal activity induced by seizures (6) and by glutamate (7, 8) and muscarinic (9) receptor activation. However, the regulation of NT-3 seems to be different and occurs independently of neurotransmitters. In cerebellum, the NT-3 mRNA levels are developmentally regulated (3–5) and are induced by triiodothyronine (T3) both in vivo and in vitro (5). Here we show that the NT-3 mRNA levels in cultured cerebellar granule neurons are also increased by BDNF. The effect of BDNF was additive to that of T3, indicating a dual regulation of NT-3 expression in the developing cerebellum by T3 and by BDNF. In contrast to BDNF, NGF and NT-3 itself were ineffective in increasing the NT-3 mRNA levels. The regulation of NT-3 expression by BDNF reveals an unexpected interaction of the neurotrophins by which one neurotrophin can regulate the expression of another, which is probably of importance during development and after injury.

MATERIALS AND METHODS

Reagents—Dubecco's modified Eagle's medium and fetal calf serum were obtained from Life Technologies, Inc. NGF was purified from male mouse submandibular glands, and recombinant BDNF and NT-3 were produced in the vaccinia expression system (10). K252a was obtained from Kamiya Co. (Thousand Oaks, CA). All other reagents were from Sigma.

Cell Culture—Cerebellar granule cells were prepared from 6–7-day-old rats as previously described (11, 12). Cerebellar neurons were re-suspended in Dubecco's modified Eagle's medium containing 10% fetal calf serum and plated on Costar culture dishes (2 × 10^6 cells/35-mm dish), which were precoated with poly-o-ornithine (0.5 mg/ml). Three hours after plating the medium was changed to a modified serum-free medium as previously described (7). Cells were used for analysis after 2 or 3 days of incubation.

RNA Analysis—Total cellular RNA was extracted using the guanidinium thiocyanate method (13), fractionated on a 1.3% agarose gel, and transferred to a Hybond-N nylon membrane (14). The filters were hybridized overnight at 65 °C in 50% formamide in a solution described earlier (15) in the presence of a 32P-labeled cRNA probes for NT-3. Filters were washed twice for 10 min in 2 × SSC, 0.1% SDS at room temperature and for 15 min in 0.2 × SSC, 0.1% SDS at 72 °C and then exposed to x-ray film. The amount of RNA was estimated with a laser scanning device. The data were normalized to the amount of β-actin present in the RNA samples.

Mouse NT-3 cDNA (16) was cloned into the Bluescript vector (Promega) and RNA probes prepared by run-off transcription.

Nuclear Run-on Studies—Nuclei were prepared from the cerebellar granule neurons (10^5 cells) and incubated in vitro essentially as described earlier (14). Labeled RNA was hybridized to NT-3 and β-actin cDNA (2 μg) immobilized on nylon filters. After hybridization the filters were washed and exposed to X-ray films.

RESULTS

Effect of Neurotrophins and T3 on NT-3 mRNA Levels in Cerebellar Granule Neurons—Cerebellar granule neurons cultured in a serum-free medium express low levels of NT-3 mRNA. The addition of BDNF (10 ng/ml) to the culture medium strongly (about 10-fold) increased the level of NT-3 mRNA expressed by these neurons (Fig. 1A). Since T3 has been shown to elevate NT-3 mRNA in the granule neurons (5), we compared the effect of T3 and BDNF in this system. Fig. 1B shows that BDNF was more effective than T3 in inducing NT-3 mRNA levels in these cells. In the presence of both T3 (5 ng/ml) and BDNF (10 ng/ml), NT-3 mRNA was elevated about 20-fold, demonstrating a partially additive effect on NT-3 mRNA expression.

Fig. 2 shows the time course of the BDNF effect on NT-3 mRNA. The NT-3 mRNA levels significantly increased after 6 h (4-fold) and were maximal after 24 h. In contrast, T3 elevated NT-3 mRNA level after just 1 h (cf. Ref. 5), providing further evidence that T3 and BDNF act by different mechanisms in enhancing NT-3 expression.

The effect of BDNF on NT-3 expression in this system was dose-dependent. Low concentrations of BDNF (1 ng/ml) gave rise to a distinct elevation in NT-3 mRNA levels (4-fold), and the maximal effect (about a 10-fold increase) was obtained by 10 ng/ml BDNF (Fig. 2B).

Cerebellar granule cells have been shown to express trkB receptors (12), which constitute the essential signal transduc-
The expression of neurotrophins in brain cells is regulated by a great variety of different mechanisms (24–26). For example, the NGF mRNA levels in cultured astrocytes are modulated by various cytokines and growth factors, including fibroblast growth factor (24, 25), interleukin-1 (24–27), and transforming growth factor-β (28). Glucocorticoid hormones have also been shown to regulate NGF mRNA levels in cultured astrocytes (29) and in hippocampal neurons in vivo and in vitro (29, 30). Further studies have demonstrated that neuronal activity is involved in the physiological regulation of NGF and BDNF mRNA levels in the rat hippocampus and cortex (6–9), whereby mRNA expression of these two factors is up-regulated by the glutamate system and down-regulated by the γ-aminobutyric acid system (31). Recently it was also shown that more physiological stimulus, such as light, is able to up-regulate BDNF mRNA levels in the rat visual cortex (15).

In contrast to NGF and BDNF, NT-3 mRNA does not seem to be regulated by neurotransmitters and the NT-3 mRNA levels in hippocampus decreases after seizures (32, 33). It was recently reported that T3 induces NT-3 mRNA expression in cultured cerebellar granule neurons, as well as in the developing rat cerebellum (5). T3 is essential for normal cerebellar development (34), and the induced NT-3 has been shown to promote the differentiation of cultured Purkinje cells (5).

The present study demonstrates that BDNF strongly up-regulates NT-3 mRNA levels in cultured cerebellar granule neurons. BDNF and T3 had a partly additive effect on the NT-3 mRNA level. The time courses of the increase in NT-3 mRNA by BDNF and by T3 are, however, different; T3 rapidly (within 1 h) elevated NT-3 mRNA (5), whereas the effect of BDNF occurred later (Fig. 2). Actinomycin D inhibited the increase in NT-3 mRNA levels.
Previously it was shown that T₃ also supports survival and differentiation of cerebellar granule neurons (11). However, BDNF and T₃ seem to act independently of NT-3 in our system, since NT-3 affected neither survival nor granule neuron differentiation (12). The present results together with our previous observations suggest a dual mechanism for regulating cerebellar granule neuron survival and NT-3 expression, on the one hand by the endocrine system via T₃, and on the other hand by neurotrophins such as BDNF. To determine the potential role of BDNF on cerebellar granule neurons in vivo and the interaction of BDNF with T₃, it is important to study the cellular localization of BDNF expression and the site of its release in the developing cerebellum.

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REFERENCES
1. Thoenen, H. (1991) Trends Neurosci. 14, 165–170
2. Hoffer, M., Pagliuca, S. R., Hohn, A., Leibrock, J., and Barde, Y.-A. (1990) EMBO J. 9, 2459–2464
3. Maitre, S., Dupuis, P. C., Belluscio, L., Friedman, R. F., Alderson, R. F., Wiegand, S. J., Furth, M. E., Lindsay, R., and Yancopoulos, G. D. (1990) Neuron 5, 501–509
4. Rocamora, N., Garcia-Ladona, F. J., Palacios, J. M., and Mengod, G. (1993) Mol. Brain Res. 17, 1–8
5. Lindholm, D., Casteen, E., Tsuiflidas, P., Kolbeck, R., Berraghi, M., Leistertner, A., Heisenberg, C.-P., Tassorelli, L., Parada, L. F., and Thoenen, H. (1991) J. Cell Biol. 122, 443–450
6. Gall, C. M. and Isackson, P. J. (1989) Science 245, 758–761
7. Zafra, E., Heisenberg, C., Leibrock, J., Thoenen, H., and Lindholm, D. (1990) EMBO J. 9, 3545–3550
8. Effros, R., Bengzon, J., Kokaia, Z., Persson, H., and Lindvall, O. (1991) Neuron 7, 165–176
9. Berraghi, M., Cooper, J., Casteen, E., Zafra, F., Sofroniew, M., Thoenen, H., and Lindholm, D. (1993) J. Neurosci. 13, 3818–3826
10. Götz, R., Kolbeck, R., Lotscheg, F., and Parada, Y.-A. (1992) Eur. J. Biochem. 214, 745–749
11. Heisenberg, C.-P., Thoenen, H., and Lindholm, D. (1992) Neuroreport 3, 685–688
12. Lindholm, D., Dechant, G., Heisenberg, C.-P., and Thoenen, H. (1993) Eur. J. Neurosci. 5, 1455–1464
13. Chomczynski, P. and Sacchi, N. (1987) Analyt. Biochem. 162, 156–159
14. Lindholm, D., Heumann, R., Hengerer, B., and Thoenen, H. (1988) J. Biol. Chem. 263, 16346–16351
15. Casteen, E., Zafra, F., Thoenen, H., and Lindholm, D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9444–9448
16. Hohn, A., Leibrock, J., Bailey, K., and Barde, Y.-A. (1990) Nature 341, 149–152
17. Casteen, E., Sanduri, V., Jing, S., Lamballe, F., Tapley, R., Bryant, S., Carzello, C., Jones, K. R., Reichardt, L. F., and Barde, Y.-A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 13–16
18. Soppet, D., Escandon, E., Marqués, J., Miliard, D., Reid, S. W., Blain, J., Burton, L. E., Stanton, B. R., Kaplan, D. R., Hunter, T., Nikolics, K., and Parada, L. F. (1991) Cell 65, 895–903
19. Squinto, P. S., Stitt, T. N., Aldrich, T. H., Davis, S., Bianco, S. M., Radziejewski, C., Glass, J. G., Masakowski, P., Furth, M. E., Valenzuela, D. M., DiStefano, P. S., and Yancopoulos, G. D. (1991) Cell 65, 885–893
20. Matsuda, Y., and Fukuda, J. (1988) Neurosci. Lett. 87, 295–300
21. Kojima, S., Costerera, M. L., Matsuda, Y., Hama, T., Lazaro, P., and Parada, L. F. (1991) Cell 69, 715–721
22. Tapley, T., Lamballe, F., and Barbadic, M. (1992) Oncogene 7, 371–381
23. Berg, M. M., Sternberg, D. W., Parada, L. F., and Chao, M. V. (1992) J. Biol. Chem. 267, 13–16
24. Spranger, M., Lindholm, D., Bandtlow, C., Heumann, R., Gnahm, H., Näher, Noe, M., and Thoenen, H. (1990) Eur. J. Neurosci. 2, 69–76
25. Yoshida, K., and Gage, F. H. (1992) Brain Res. 589, 14–25
26. Zafra, F., Lindholm, D., Casteen, E., Hartlikk, J., and Thoenen, H. (1992) J. Neurosci. 12, 4793–4799
27. Carman-Kranz, M., Vige, X., and Wise, B. C. (1991) J. Neurochem. 56, 636–643
28. Zafra, F., Lindholm, D., Casteen, E., Hartlikk, J., and Thoenen, H. (1992) NeuroReport 1, 9–12
29. Lindholm, D., Casteen, E., Hengerer, B., Zafra, F., Berninger, B., and Thoenen, H. (1992) Eur. J. Neurosci. 4, 404–410
30. Barbagy, G., and Persson, H. (1992) Eur. J. Neurosci. 4, 396–403
31. Zafra, F., Casteen, E., Thoenen, H., and Lindholm, D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10037–10041
32. Lindvall, O., Effros, R., Bengzon, J., Kokaia, Z., Smith, M.-L., Siesjö, B. K., and Persson, H. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 648–652
33. Rocamora, N., Palacios, J. M., and Mengod, G. (1992) Mol. Brain Res. 13, 27–33
34. Dussault, J. H., and Rozel, J. (1987) Annu. Rev. Physiol. 49, 321–334
35. Matsukawa, I., Meyer, M., and Thoenen, H. (1991) J. Neurosci. 11, 5165–5177
36. Segal, R. A., Takashashi, H., and McKay, R. D. G. (1992) Neuron 9, 1041–1052

FIG. 4. Stability and transcription of NT-3 mRNA following BDNF treatment. A, neurons were incubated for 6h in the absence or presence of BDNF (10 ng/ml). Actinomycin D (10 pg/ml) was added to control and BDNF-treated cells to inhibit transcription, and the incubation was continued for 3 days. By using an open circle for BDNF-treated cells, closed circles for BDNF-treated cells, and open circles for control cells, the presence of BDNF (10 ng/ml) was added to control and BDNF-treated cells to inhibit transcription, and the incubation was continued for 3 days. C, control; B, BDNF-treated cells.

mRNA both after T₃ (5) and in BDNF-treated cells, suggesting that both compounds increase NT-3 transcription. Nuclear run-on experiments were performed to study the transcription rate of the NT-3 gene directly. The results of these studies supported the notion that BDNF increases NT-3 gene transcription in the granule neurons. In keeping with this finding, there was no apparent change in the stability of NT-3 mRNA after BDNF treatment. The decay rate of the NT-3 mRNA in the granule neurons was about 50 min in both control and BDNF-treated cells. A similar NT-3 mRNA half-life was also noted for T₃-treated cells (not shown). Previous studies have shown that the NGF-mRNA half-life in fibroblasts (14) and Schwann cells (35) is about 30 min, but no data are yet available on neurons.

The stimulation of NT-3 mRNA by BDNF in the granule neurons was specific since neither NGF nor T₃ had any effect. In addition, both the concentration curve and results obtained using the inhibitor K252a indicate that BDNF acts via the trkB receptor. Enhancing NT-3 mRNA expression. Indeed, recent data show a high expression of trkB in granule neurons, as well as the presence of specific BDNF receptor binding sites (12). BDNF was also found to enhance survival of cerebellar granule neurons in culture (12, 36). The observation that BDNF does not act only as a survival factor for cultured cerebellar granule neurons, but also strongly induces the expression of NT-3 mRNA in these neurons, reveals a novel mechanism of neurotrophin interaction by which one neurotrophin regulates the expression of another.