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Neurotrophin 4/5 is a trophic factor for mammalian facial motor neurons

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ABSTRACT **The survival of developing motor neurons depends on factors secreted from skeletal muscles and from cells within the central nervous system. Although several members of the nerve growth factor protein family [neurotrophins (NTs)] are able to maintain developing rat motor neurons *in vitro*, only the brain-derived neurotrophic factor has been shown to have significant effects on the survival of motor neurons *in vivo*. In the present study, we demonstrate that NT-4/5 also prevents injury-induced death of facial motor neurons in neonatal rats. Furthermore, facial motor neurons express a functional receptor for NT-4/5, whereas mRNA-encoding NT-4/5 can be detected in their environment throughout embryonic and postnatal life. Thus, both NT-4/5 and brain-derived neurotrophic factor may be physiological survival factors for facial motor neurons and may serve as therapeutic agents for motor neuron disease.**

The survival of developing motor neurons depends on factors secreted by their muscle targets and by cells in the central nervous system (1–6), but the identity of these factors has not yet been fully established. The neurotrophins [NTs, members of the nerve growth factor (NGF) protein family] have been considered to have no direct effects on motor neurons *in vitro* (7, 8). However, a recent study has demonstrated that all known NTs, except NGF, can promote the survival of embryonic rat motor neurons in culture (9). Surprisingly, although NT-3 and brain-derived neurotrophic factor (BDNF) are both potent trophic factors for motor neurons in culture (9), only BDNF has significant effects on the survival of chicken and rat motor neurons *in vivo* (10–13). Furthermore, even the high-affinity receptor for a given NT is not always sufficient to elicit a physiological response (14). Consequently, the physiological importance of putative motor neuron trophic factors must be experimentally determined *in vivo*. The present investigation is designed to examine the physiological role of NT-4/5 on motor neurons. Several complementary approaches are taken, including the demonstration of a pharmacological effect of NT-4/5 on degenerating motor neurons, the localization of functional NT-4/5 receptors on neonatal and adult motor neurons, and the demonstration of the expression of NT-4/5 in the muscle targets of motor neurons at different developmental stages. NT-4/5 is compared to BDNF in all the above measures of physiological significance.

MATERIALS AND METHODS

Histology and Neuronal Counts in Animals with Facial Nerve Axotomies and NT Treatment. Postnatal day 0 (P0) Sprague–Dawley rats were anesthetized by hypothermia and subjected to a unilateral transection of the facial nerve, as described (13). NT-4/5, BDNF, a 1:1 mixture of NT-4/5 and BDNF, or vehicle solution [acetic acid-acidified phosphate-buffered saline (pH 6.0)] (five animals per group) was applied to the proximal nerve stump in a piece of gelfoam (Upjohn) as described (13). Six days after surgery, animals were sacrificed by decapitation, and blocks that contain the pons/medulla were fixed by immersion in chilled Carnoy's solution overnight (4°C). Blocks were subsequently dehydrated in alcohols and embedded in paraffin. Serial coronal sections (7 μm) were stained with cresyl violet. Three sections per case (one through the middle of the facial nucleus and two corresponding to planes at equal anterior and posterior distances from the middle level on each side of the brain) were taken for neuronal counts (six animals per group were counted from vehicle-, NT-4/5-, BDNF-, and NT-4/5-plus-BDNF-treated groups). Motor neurons were identified as Nissl-containing basophilic profiles with nucleoli and were counted at a ×40 magnification by using a computerized image analysis system (software provided by Mark Molliver and adapted by Catherine Fleischman, The Johns Hopkins University School of Medicine).

***trkB in Situ* Hybridization (ISH).** A probe specific for full-length *trkB* was generated by polymerase chain reaction (PCR) amplification of a 1.09-kb sequence whose 5′ end corresponds to nucleotide 2249 of the tyrosine kinase (tk) region of rat *trkB* and whose 3′ end corresponds to nucleotide 3339 of the 3′ untranslated region. Amplification was performed using oligonucleotides 5′-GATAGAATGCATCACCCAGG-3′ (sense) and 5′-TGTGATCGGAAACTGTGTCC-3′ (antisense) as primers and the plasmid that contains the entire full-length rat *trkB* sequence as a template. The amplified product was cloned directly into the pCRII transcription vector (Invitrogen) by using a TA cloning kit (Invitrogen). This vector was then used to generate sense and antisense RNA probes for ISH in neonatal (P3) and adult (3 month old) rats as described (13).

Retrograde Transport of Iodinated NT-4/5. NT-4/5 was labeled by lactoperoxidase treatment using a modified Enzymobead (Bio-Rad) radioiodination procedure (15). Recovery and radioactivity were determined by trichloroacetic acid precipitation of the labeled protein before and after gel filtration. The specific radioactivity of ^{125}I-labeled NT-4/5 was 100 μCi/μg (1 Ci = 37 GBq). Radiolabeled NT-4/5 was tested for bioactivity in a quantitative rat trigeminal ganglion bioassay. The biological activity of ^{125}I-labeled NT-4/5 determined by parallel dose–response curves was equivalent to that of the unlabeled factor. The iodinated factor was further characterized prior to its *in vivo* utilization on receptor binding assays using TrkB–IgG chimeric receptors. ^{125}I-labeled NT-4/5 binding to these receptors was entirely com-

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Abbreviations: BDNF, brain-derived neurotrophic factor; NT, neurotrophin; NGF, nerve growth factor; E, embryonic day; P, postnatal day; ISH, *in situ* hybridization; tk, tyrosine kinase.

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peted by an excess of unlabeled NT-4/5 but not NGF. ^{125}I-labeled NT-4/5 was injected under the whisker pad of P3 rats (10 μCi per injection site; n = 5 rats) with and without a hundredfold excess of unlabeled NT-4/5 or NGF. Blocks containing the pontomedullary region were fixed 14 h later in phosphate-buffered 4% (wt/vol) formaldehyde for 2–3 days, embedded in paraffin, cut in the transverse plane (15 μm), and processed for emulsion autoradiography (13).

Northern Blot Analysis of *trkB* Expression in Neonatal and Adult Facial Motor Neurons. A probe corresponding to nucleotides 2249–3339 of the rat *trkB* (see above) or the entire *trkB* sequence was gel-purified and labeled with random hexanucleotides to a specific activity of 1×10^9 cpm/μg. Poly(A)$^+$ RNA (10 μg) from pooled facial nuclei of P0 and 3-month-old rats was prepared and analyzed using the above *trkB* probes as described (13). *trkB*-hybridized filters were subsequently stripped and rehybridized with a mouse γ-actin probe (labeled as above), which served as a loading control; actin-hybridized filters were exposed for 2 h at room temperature without intensifying screens.

PCR Analysis of *trkB* Expression in Axotomized Adult Facial Motor Neurons. BDNF expression was studied in parallel to that of NT-4/5 to compare directly the levels of expression of NT-4/5 to those of an established motor neuron trophic factor. Three-month-old Sprague–Dawley rats were anesthetized with enflurane/nitrous oxide/oxygen and subjected to a unilateral transection of the facial nerve 2–3 mm distal to the stylomastoid foramen. Five days later, animals were sacrificed. Facial nuclei from lesioned (n = 3) and control (n = 3) rats were microdissected with a needle puncher from 300-μm-thick fresh frozen sections through the pontomedullary junction. Tissues were subjected to PCR analysis with primers specific for tk-containing rat *trkB* transcripts (sense, aa 395–403; antisense, aa 626–635) (9).

PCR Analysis of NT-4/5 and BDNF Expression in Facial Nucleus and Facial Muscles of Developing and Adult Animals. Facial muscles from animals prepared as above were quickly dissected on ice and frozen [ages examined were embryonic day (E) 10, E15, E20, P2, and adult]. Facial nuclei were micropunched as above (ages examined were P0 and adult). PCR was performed using 10 pmol of rat BDNF-specific sense (aa 12–20) and antisense (aa 59–76) primers or using 10 pmol of rat NT-4/5-specific sense (aa 131–138) and antisense (aa 206–213) primers (9).

RESULTS

To assess the physiological role and the therapeutic usefulness of NT-4/5, we investigated its ability to prevent the death of neonatal rat facial motor neurons after axotomy. The facial nerves of newborn rats were cut unilaterally, and 20 μg of NT-4/5 or vehicle was applied to the proximal nerve stump in a piece of gelfoam. Six days later, animals were sacrificed, and facial motor neurons ipsi- and contralateral to the lesion were counted. In vehicle-treated animals, there was, on average, a 72% loss of facial motor neurons on the lesioned side; gliosis

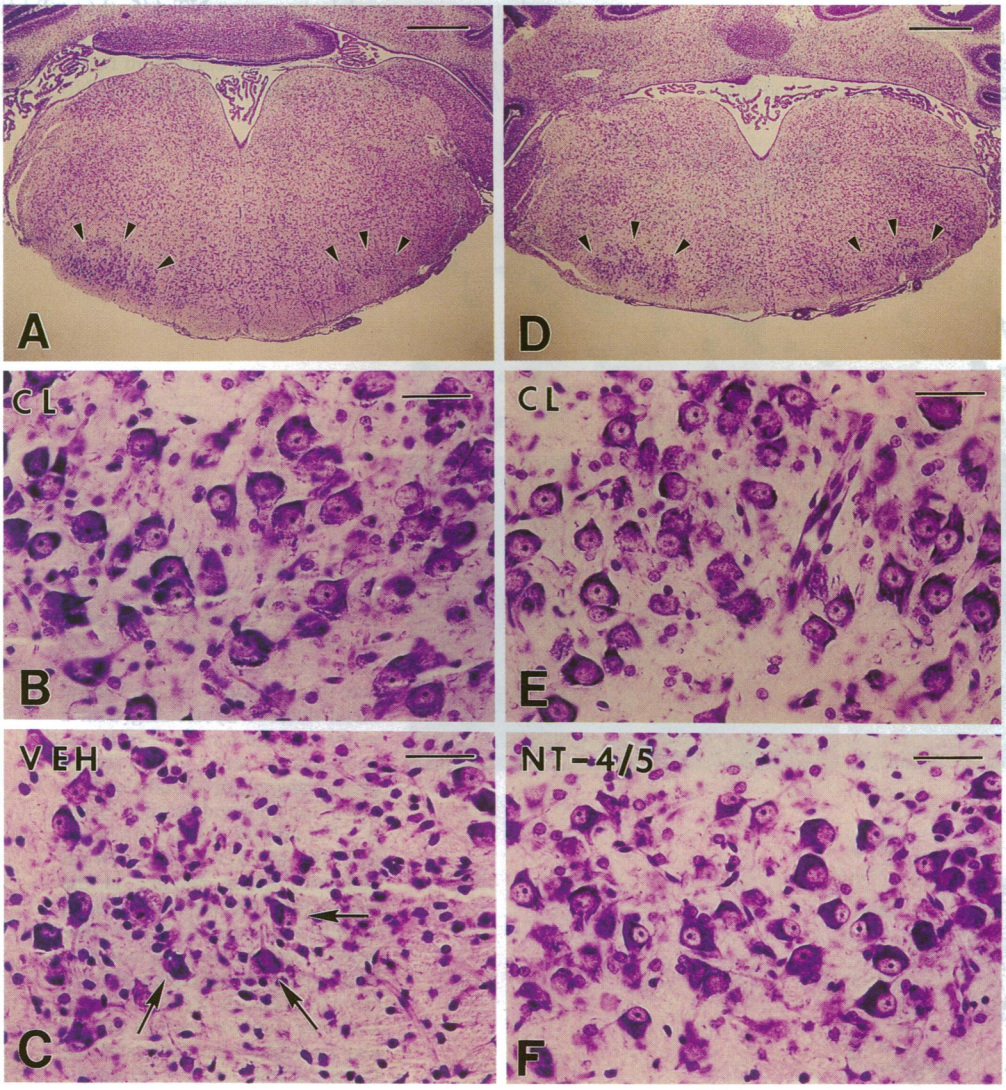


FIG. 1. (*A–C*) Photomicrographs through the axotomized facial nucleus of neonatal rats with and without NT-4/5 treatment. In vehicle-treated animals, profound retrograde degeneration occurs in the facial nucleus ipsilateral to the lesion (right set of arrowheads in *A*; compare with control nucleus indicated with arrowheads on left side). Many remaining motor neurons appear shrunken and chromatolytic and are associated with extensive gliosis (arrows in *C*; compare with healthy neurons in the contralateral facial nucleus in *B*). (*D–F*) In NT-4/5-treated animals, there is a significant preservation of facial motor neurons on the axotomy side (right set of arrowheads in *D*; compare with control nucleus on left side). Rescued motor neurons show intense basophilia, and gliosis is reduced (*F*) (compare with contralateral nucleus in *E* and vehicle-treated axotomized facial nucleus in *C*). (Bars: *A* and *D*, 500 μm; *B*, *C*, *E*, and *F*, 50 μm.).

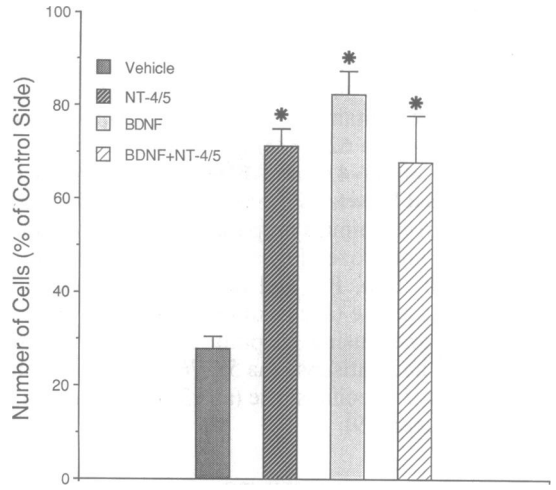


FIG. 2. Numbers of motor neurons in the axotomized facial nucleus from animals treated with vehicle or NTs (NT-4/5, BDNF, and a 1:1 mixture of NT-4/5/BDNF). Numbers are expressed as percentages of motor neurons in the contralateral (nonaxotomized) facial nucleus. Vertical bars on columns indicate the SEM. For statistical evaluation of cell numbers, an analysis of variance was computed using the average percentage value for each of the four groups ($F = 17.02$; $P < 0.001$). The Newman–Keuls multiple range test was used to test for simple effects. All NT groups differed significantly from the vehicle group (asterisk, $P < 0.01$) but not among themselves in any combination.

was severe (Figs. 1 *A–C* and 2). In NT-4/5-treated animals, cell death in the lesioned facial nucleus was reduced to 27% (Figs. 1 *D–F* and 2). Rescued motor neurons maintained good basophilia and Nissl structure and were associated with little gliosis. Thus, in the presence of NT-4/5, 45% more neurons survived the axotomy for 6 days. NT-4/5 was found to be equivalent to BDNF in its ability to prevent death of motor neurons, whereas no further increase in motor neuron survival was observed when BDNF and NT-4/5 were used in combination (Fig. 2). These findings suggest that NT-4/5 prevents motor neuron degeneration and that NT-4/5 and BDNF are likely to act on the same populations of neurons.

The actions of NT-4/5 on cells are mediated via the tk receptor TrkB (8, 16). To determine whether NT-4/5 can act directly on neonatal facial motor neurons, we ascertained whether TrkB receptors present on these cells (13) contain a kinase domain. By ISH, the mRNA for full-length *trkB* isoforms was localized in α-motor neurons of the facial nucleus but not in interneurons (Fig. 3 *A* and *B*). To determine whether these *trkB* transcripts present on neonatal motor neurons encode functional receptors, the ability of facial nuclei to transport NT-4/5 retrogradely was determined. To this end, biologically active ^{125}I-labeled NT-4/5 with appropriate binding characteristics *in vitro* was injected into the whisker pad of neonatal rats with and without a hundredfold excess of unlabeled NT-4/5 or NGF. Animals were sacrificed 14 h later, and blocks containing the facial nuclei were prepared for emulsion autoradiography. Silver grains from transported NT-4/5 were concentrated over cell bodies of facial motor neurons (Fig. 3 *C* and *E*). Retrograde labeling was blocked when NT-4/5 was coinjected with a hundredfold excess of unlabeled NT-4/5 (Fig. 3*D*) but not with NGF. Thus, neonatal facial motor neurons express functional receptors that can retrogradely transport NT-4/5. tk(+) TrkB receptors are also present in facial motor neurons of adult animals, suggesting that motor neurons can retain responsiveness to NT-4/5 and BDNF throughout life (Fig. 4). Northern

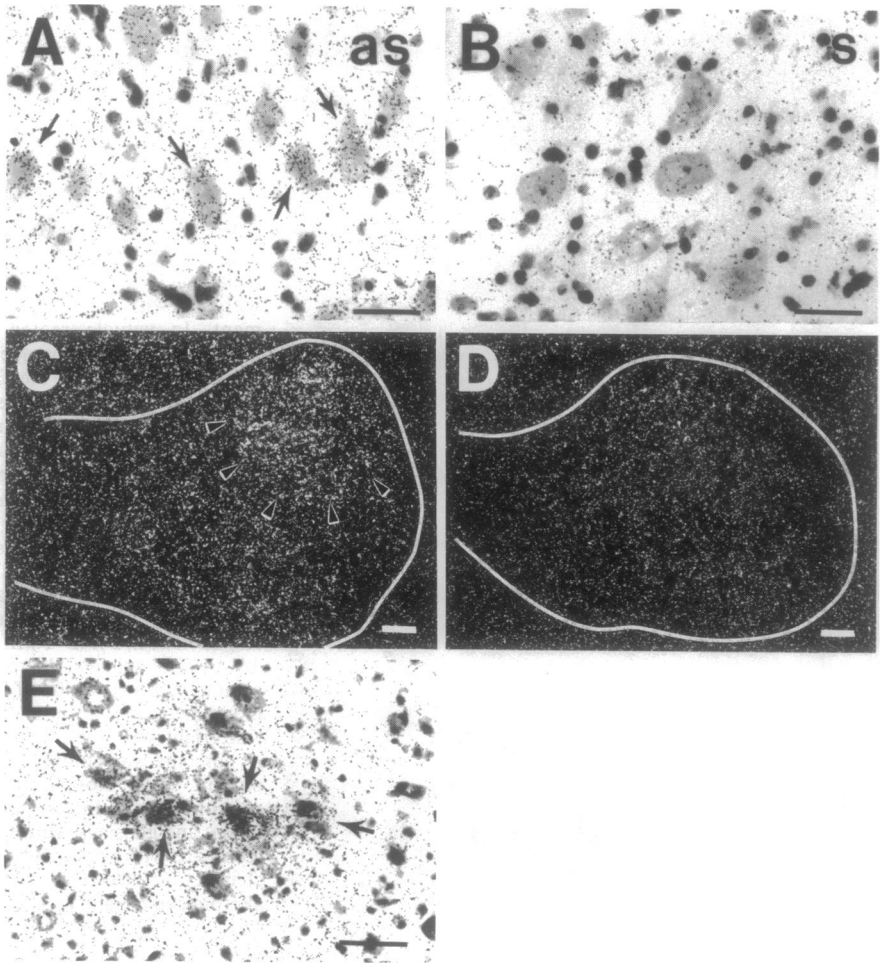


FIG. 3. Expression of functional TrkB receptors in the neonatal facial nucleus. (*A* and *B*) ISH in the facial nucleus of a neonatal rat with an RNA probe specific for tk(+) *trkB* isoforms. Arrows indicate motor neurons in the ventral facial nucleus hybridizing with a probe specific for tk(+) *trkB*. as, antisense; s, sense. (*C–E*) Retrograde transport of ^{125}I-labeled NT-4/5 in the neonatal facial nucleus. (*C*) ^{125}I-labeled NT-4/5 transport from the whisker pad of a P4 rat generates dense labeling in the dorsolateral portion of the facial nucleus (arrowheads) that is competed by coinjection of a hundredfold excess of unlabeled NT-4/5 (*D*) and is clearly localized in cell bodies of α-motor neurons (*E*). (*C* and *D*) White tape delineates the facial nucleus. (Bars = 40 μm.)

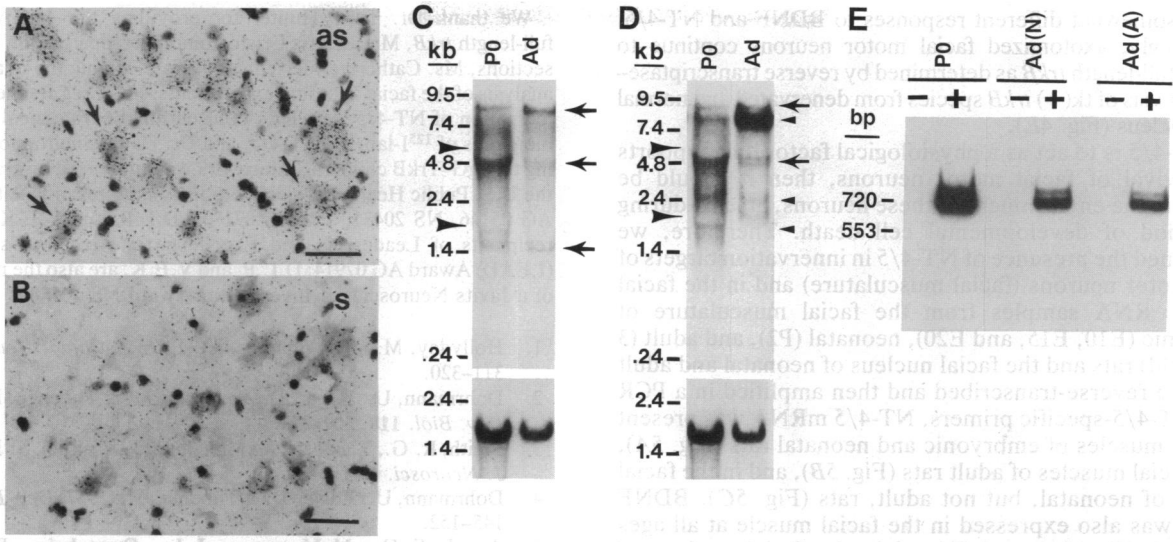
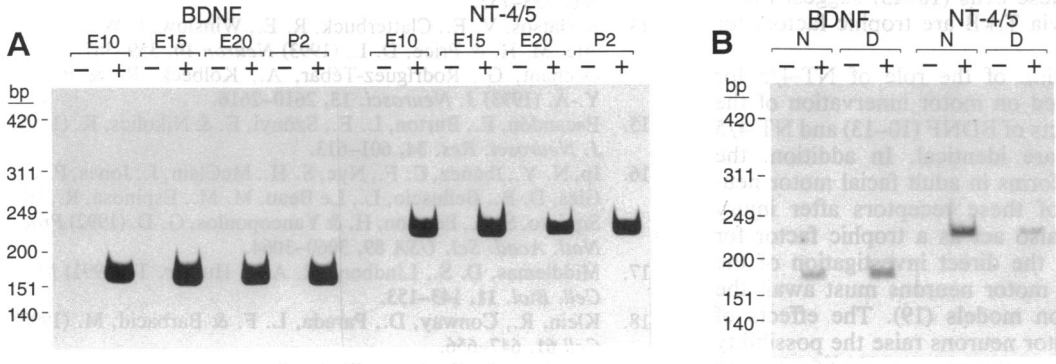


FIG. 4. Expression of TrkB receptors in the adult facial nucleus. (*A* and *B*) ISH of facial motor neurons of an adult rat with the same RNA probe as in Fig. 3 *A* and *B*. Arrows indicate motor neurons in the central facial nucleus of a 3-month-old rat. as, antisense; s, sense. (*C* and *D*) Northern blot analysis of full-length and truncated *trkB* isoforms using poly(A)$^+$-enriched RNA (10 μg) from neonatal (P0) and adult (Ad) facial nucleus. (*C*) A probe specific for tk(+) *trkB* isoforms was used (the same that was used in the synthesis of RNA probes in *A* and *B* and in Fig. 3 *A* and *B*). (*D*) A full-length *trkB* sequence was used as probe to detect both tk(+) and tk(−) *trkB* mRNA species. Right-pointed arrowheads indicate the position of the 28S and 18S bands, which were used as internal markers. Left-pointed arrowheads indicate the major truncated *trkB* isoforms. Left-pointed arrows indicate the catalytic *trkB* isoforms. (*E*) PCR analysis of tk(+) *trkB* expression in the facial nucleus of neonatal and of adult rats before (N) and after (A) axotomy. Lanes: +, RNA samples used in reverse transcription reactions; −, RNA samples from the same tissues incubated in the same buffer but without reverse transcriptase. The amount of cDNA in the PCR was calibrated using PCR primers for glyceraldehyde-3-phosphate dehydrogenase. (Bars = 40 μm.)

blot analysis reveals three tk-containing mRNA species of 9.0, 4.8, and 1.6 kb. Because the full-length receptor requires 2.4 kb of coding sequence, the two larger species may code for the full-length receptor, whereas the 1.6-kb species may encode a soluble or membrane-associated tk-containing species (17). The 7.5-, 2.4-, and 1.8-kb species detected by full-length *trkB* probes correspond to tk(−) TrkB species as characterized (17). The fact that the 7.5-kb *trkB* mRNA species encoding a truncated TrkB receptor (17, 18) is up-regulated in the adult (Fig. 4*D*) raises the possibility that neonatal and adult motor neurons may



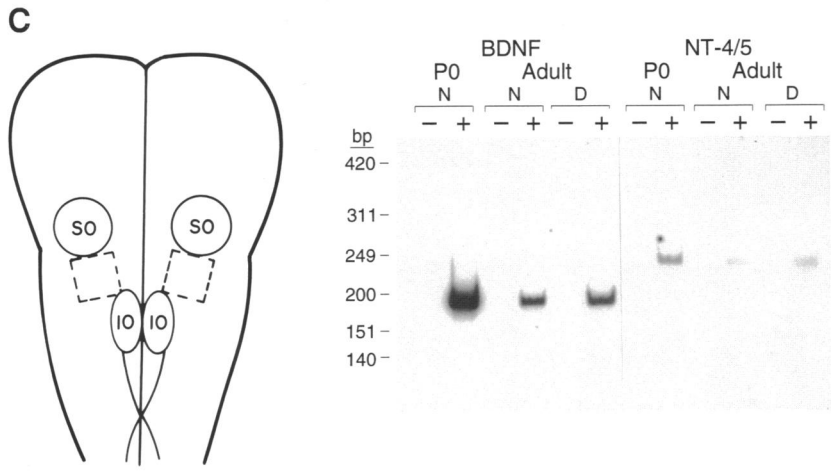


FIG. 5. Expression of NT-4/5 and BDNF in facial muscles and facial nucleus. (*A*) PCR analysis of NT-4/5 and BDNF expression in facial muscles during embryonic development. (*B*) PCR analysis of BDNF and NT-4/5 expression in adult facial muscles before (N) and after (D) denervation. BDNF and NT-4/5 continue to be expressed in denervated adult facial muscles, although they appear to be reciprocally regulated. (*C*) PCR analysis of BDNF and NT-4/5 expression in facial nucleus before (N) and after (D) axotomy. (*Left*) Method of dissection of the facial nucleus in fresh tissues, using as surface landmarks the elevations created at the base of the pons/medulla by the olive nuclei. (*A–C*) Lanes: +, RNA samples used in reverse transcription reactions; −, RNA samples from the same tissues incubated in the same buffer, but without reverse transcriptase—to serve as controls for genomic DNA contamination. Amount of cDNA in PCR was calibrated as in Fig. 4. N, normal; D, denervated (or axotomized); IO, inferior olive; SO, superior olive.

display somewhat different responses to BDNF and NT-4/5. Interestingly, axotomized facial motor neurons continue to express full-length *trkB* as determined by reverse transcriptase–PCR analysis of tk(+) *trkB* species from denervated and normal facial nucleus (Fig. 4*E*).

If NT-4/5 is to act as a physiological factor that supports the survival of facial motor neurons, then it should be present in the environment of these neurons, at least during the period of developmental cell death. Therefore, we ascertained the presence of NT-4/5 in innervation targets of facial motor neurons (facial musculature) and in the facial nucleus. RNA samples from the facial musculature of embryonic (E10, E15, and E20), neonatal (P2), and adult (3 month old) rats and the facial nucleus of neonatal and adult rats were reverse-transcribed and then amplified in a PCR using NT-4/5-specific primers. NT-4/5 mRNA was present in facial muscles of embryonic and neonatal rats (Fig. 5*A*), in the facial muscles of adult rats (Fig. 5*B*), and in the facial nucleus of neonatal, but not adult, rats (Fig. 5*C*). BDNF mRNA was also expressed in the facial muscle at all ages examined (Fig. 5 *A* and *B*) and in the facial nucleus of neonatal and adult animals (Fig. 5*C*). Levels of BDNF mRNA in facial muscle appear to increase, whereas those of NT-4/5 appear to decrease after denervation (Fig. 5*B*). BDNF continues to be expressed in the axotomized facial nucleus in the adult (Fig. 5*C*).

DISCUSSION

The ability of NT-4/5 to prevent the death of facial motor neurons after axotomy, the expression of a functional NT-4/5 receptor by facial motor neurons, and the presence of NT-4/5 in the facial musculature during embryonic development, including the period of physiological motor neuron death, indicate that NT-4/5, similar to BDNF, may be a physiological factor for the survival of facial motor neurons. The effects of NT-4/5 on facial motor neurons and the reported *in vivo* effects of BDNF on these cells (10–13) suggest that a subset of NTs transduced via TrkB are trophic factors for motor neurons.

In the present investigation of the role of NT-4/5 for α-motor neurons, we focused on motor innervation of the face. It is likely that the effects of BDNF (10–13) and NT-4/5 on spinal motor neurons are identical. In addition, the presence of tk(+) TrkB isoforms in adult facial motor neurons and the persistence of these receptors after injury suggest that this NT may also act as a trophic factor for mature neurons. However, the direct investigation of the effects of NT-4/5 on adult motor neurons must await the availability of reliable lesion models (19). The effects of NT-4/5 on degenerating motor neurons raise the possibility of the future use of this NT as a therapeutic agent for human motor neuron disease.

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1. Hollyday, M. & Hamburger, V. (1976) *J. Comp. Neurol.* **170,** 311–320.
2. Dohrmann, U., Edgar, D., Sendtner, M. & Thoenen, H. (1986) *Dev. Biol.* **118,** 209–221.
3. Smith, R. G., Vaca, K., McManaman, J. & Appel, S. H. (1986) *J. Neurosci.* **6,** 439–447.
4. Dohrmann, U., Edgar, D. & Thoenen, H. (1987) *Dev. Biol.* **124,** 145–152.
5. Appel, S. H., McManaman, J. L., Oppenheim, R., Haverkamp, L. & Vaca, K. (1989) *Prog. Brain Res.* **79,** 251–256.
6. Bloch-Gallego, E., Huchet, M., El M'Hamdi, H., Xie, F.-K., Tanaka, H. & Henderson, C. E. (1991) *Development* **111,** 221–232.
7. Arakawa, Y., Sendtner, M. & Thoenen, H. (1990) *J. Neurosci.* **10,** 3507–3515.
8. Berkemeier, L. R., Winslow, J. W., Kaplan, D. R., Nikolics, K., Goeddel, D. V. & Rosenthal, A. (1991) *Neuron* **7,** 857–866.
9. Henderson, C. E., Camu, W., Mettling, C., Gouin, A., Poulsen, K., Karihaloo, M., Rullamas, J., Evans, T., McMahon, S. B., Armanini, M. P., Berkemeier, L., Phillips, H. S. & Rosenthal, A. (1993) *Nature (London)* **363,** 266–270.
10. Oppenheim, R. W., Qin-Wei, Y., Prevette, D. & Yan, Q. (1992) *Nature (London)* **360,** 755–757.
11. Sendtner, M., Holtmann, B., Kolbeck, R., Thoenen, H. & Barde, Y.-A. (1992) *Nature (London)* **360,** 757–759.
12. Yan, Q., Elliott, J. & Snider, W. D. (1992) *Nature (London)* **360,** 753–755.
13. Koliatsos, V. E., Clatterbuck, R. E., Winslow, J. W., Cayouette, M. H. & Price, D. L. (1993) *Neuron* **10,** 359–367.
14. Dechant, G., Rodríguez-Tébar, A., Kolbeck, R. & Barde, Y.-A. (1993) *J. Neurosci.* **13,** 2610–2616.
15. Escandón, E., Burton, L. E., Szönyi, É. & Nikolics, K. (1993) *J. Neurosci. Res.* **34,** 601–613.
16. Ip, N. Y., Ibáñez, C. F., Nye, S. H., McClain, J., Jones, P. F., Gies, D. R., Belluscio, L., Le Beau, M. M., Espinosa, R., III, Squinto, S. P., Persson, H. & Yancopoulos, G. D. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 3060–3064.
17. Middlemas, D. S., Lindberg, R. A. & Hunter, T. (1991) *Mol. Cell. Biol.* **11,** 143–153.
18. Klein, R., Conway, D., Parada, L. F. & Barbacid, M. (1990) *Cell* **61,** 647–656.
19. Koliatsos, V. E., Price, W. L., Pardo, C. A. & Price, D. L. (1994) *J. Comp. Neurol.*, in press.