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Rapid Communication

Striatal Degeneration Induced by Mitochondrial Blockade Is Prevented by Biologically Delivered NGF

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Consistent with the notion that a defect in cellular energy metabolism is a cause of human neurodegenerative disease, systemic treatment with the mitochondrial complex II inhibitor 3-nitropropionic acid (3-NPA) can model the striatal neurodegeneration seen in Huntington's disease. Previously, we have found that nerve growth factor (NGF), delivered biologically by the implantation of a genetically altered fibroblast cell-line, can protect locally against striatal degeneration induced by infusions of high doses of glutamate receptor agonists. We now report that implantation of NGF-secreting fibroblasts reduces the size of adjacent striatal 3-NPA lesions by an average of 64%. We conclude that biologically delivered NGF protects neurons against excitotoxicity and mitochondrial blockade—both energy-depleting processes—implying that appropriate neurotrophic support in the adult brain could protect against neurodegenerative diseases caused in part by energy depletion.

Key words: 3-nitropropionic acid, neuroprotection, genetically engineered cells, neural transplantation

INTRODUCTION

The mechanism of the neuronal death seen in human neurodegenerative conditions is poorly understood (Maragos et al., 1987; Choi, 1988; Beal, 1992). Indeed, neuronal death throughout development is enigmatic, as are the reasons certain neurons appear fated to survive (O'Leary, 1987; Mattson, 1990; Kater et al., 1989). Central to the problem of neuronal death is the supportive action of multiple neurotrophic molecules (Thoenen and Edgar, 1985; Davies, 1988; Barde, 1988; Snider and Johnson, 1989), such as the members of the nerve growth factor (NGF) family (Leibrock et al., 1989; Hohn et al., 1990; Maisonpierre et al., 1990; Hallbook et al., 1991), which can promote embryonic and adult neuronal survival in vitro and appear to rescue developing neurons destined to die in vivo. The complicated actions of neurotrophic factors, such as NGF, on adult neurons in vivo are only partially understood. The molecular mechanisms mediating neurotrophism are unclear (Thoenen and Edgar, 1985; Davies, 1988; Barde, 1988; Snider and Johnson, 1989), as are the specific effects of NGF on diseased or degenerating neurons (Hefti, 1986; Morgan, 1989; Tuszynski et al., 1990). Previous studies (Schumacher et al., 1991; Frim et al., 1993a) of striatal excitotoxic lesions in adult animals have shown a promising protective effect of biologically delivered NGF. These acute excitotoxic insults appear to be good models of the neurological pathology associated with ischemic lesions (Choi and Rothman, 1990) or trauma (Katayama et al., 1990) and somewhat representative of changes found in adult neural degeneration.

Recent studies on neuronal degeneration implicate energy depletion, specifically mitochondrial dysfunction with age, as a significant contributing factor to neuronal death (Beal, 1992; Wallace, 1992; Simpson and Isacson, 1993). Presumably for this reason, systemic treatment with the mitochondrial complex II (succinic dehydrogenase, succinate Q reductase) inhibitor 3-nitropropionic acid (3-NPA) models the degenerative neural changes seen in Huntington's disease (Hamilton and Gould,

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1987; Ludolf et al., 1991) by causing characteristic bilateral degeneration in the striatum. These changes appear directly related to neuronal energy depletion (Hamilton and Gould, 1987). Here we sought to determine whether NGF, delivered biologically by a fibroblast cell line genetically altered to secrete high levels of NGF, could protect against energy depletion-mediated neuronal degeneration caused by 3-NPA.

MATERIALS AND METHODS

Genetically Engineered Fibroblast Cell-Lines

Cells used for implantation were generated by infection of an immortalized rat fibroblast cell line (Rat 1, provided by M. Rosenberg, UCSD) with a retrovirus vector carrying a mouse cDNA (N.8) encoding a near full-length preproNGF precursor, as previously described (Schumacher et al., 1991; Frim et al., 1993a). The preproNGF cDNA was placed under control of the Moloney murine leukemia virus long terminal repeat promoter (Rosenberg et al., 1988) in a construct containing the Tn5 neomycin resistance gene (Beck et al., 1982) Clones were selected under G418, and then chosen for implantation based on their level of NGF production as determined by a two-site enzyme-linked immunoassay (Boehringer-Mannheim). Cells were injected as a suspension in phosphate buffered saline (PBS), pH 7.4, with 1.0 mg/l $CaCl_2$, 1.0 mg/l $MgCl_2$, and 0.1% glucose. Cell count and viability were assessed by trypan blue dye exclusion before intracerebral injection into rat hosts. Rat 1 cells not altered by retroviral infection were injected into control animals.

Cell Implantation and Histology Procedures

Adult male Sprague-Dawley rats (approximately 300–350 g) were implanted unilaterally in the corpus callosum with either Rat 1 fibroblast cells (NGF[−]) found to secrete 6.7 pg NGF/10^5 cells/hr or Rat 1-N.8 no. 2 cells (NGF[+]), found to secrete 177.6 pg NGF/10^5 cells/hr. Using a Kopf rat stereotactic frame, burr holes for grafting into the corpus callosum were made at coordinates calculated from bregma as AP +1.6, L −1.4. Injections were made using a 10 μl Hamilton syringe at V −4.0 calculated from the dura. A total of 10^6 cells was infused over 5 minutes in a volume of 10 μl. All animals received intraperitoneal injections of 20 mg/kg of 3-NPA (Aldrich Chemicals) and 80 mg/kg of succinic acid (Sigma, St. Louis, MO) (to reduce systemic toxicity) between 1600 and 1700 hr on day 7 and on day 8 after graft implantation.

Animals were sacrificed 14 days after graft implantation. Under deep barbiturate anesthesia, animals were perfused by cardiac puncture with cold heparinized saline, followed by 4% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Brains were removed, postfixed overnight in 4% paraformaldehyde in PB at 4°C, and then cryoprotected in 30% sucrose in PB-saline (PBS) until equilibration. Sections through the striatum were cut at 40 μm on a freezing microtome and placed serially into PBS. Every sixth section from each animal was stained with cresyl violet, immunostained (per product instructions; Vectastain ABC, Vector Labs, Burlingame, CA) for choline acetyltransferase (ChAT) (Chemicon) and tyrosine hydroxylase (TH) (East Acres Biologicals), and histochemically stained for acetylcholinesterase, as described (Koelle, 1954, as modified by Geneser-Jansen and Blackstad, 1971).

Morphometric Procedures

Striatal lesions from both sides of each tissue section (grafted and non-grafted) were easily seen by characteristic neuronal loss after cresyl violet staining. The area of these lesions was measured after digitization (Colorcard II, Mass. Microsystems) using the image analysis program IMAGE 1.35 (NIH) on a Macintosh IIx with attached Zeiss axioskop microscope as previously described (Schumacher et al., 1991). Choline acetyltransferase cells were visually counted at 200× magnification in striatum ipsilateral and contralateral to the grafts on sections where NGF[+] grafts were visible.

Statistical Analysis

A ratio of grafted-side lesion to non-grafted-side lesion was obtained for each tissue section. A mean of this ratio (taken from three to five sections per animal on which grafted fibroblasts were visible) was then calculated for each animal. Group values of mean ratios were summed and expressed as mean ± standard deviation; these values were then analyzed by t test (two-tailed) for the two groups using the Statview 512+ program (Brain Power, Inc., CA).

RESULTS

General Histological Observations

Fibroblast grafts were recognized in all animals by the characteristic fibromatous structure present in the corpus callosum (Frim et al., 1993a). To a variable degree, the grafts abutted or extended slightly into the dorsal striatum. Morphology of 3-NPA lesions was distorted by graft placement in several animals; these animals were excluded from analysis. Undistorted striatal lesions were present bilaterally in eight animals implanted with NGF[+] cells and seven animals implanted with NGF[−] cells. The characteristic appearance of the striatal lesions on microscopic examination was distin-

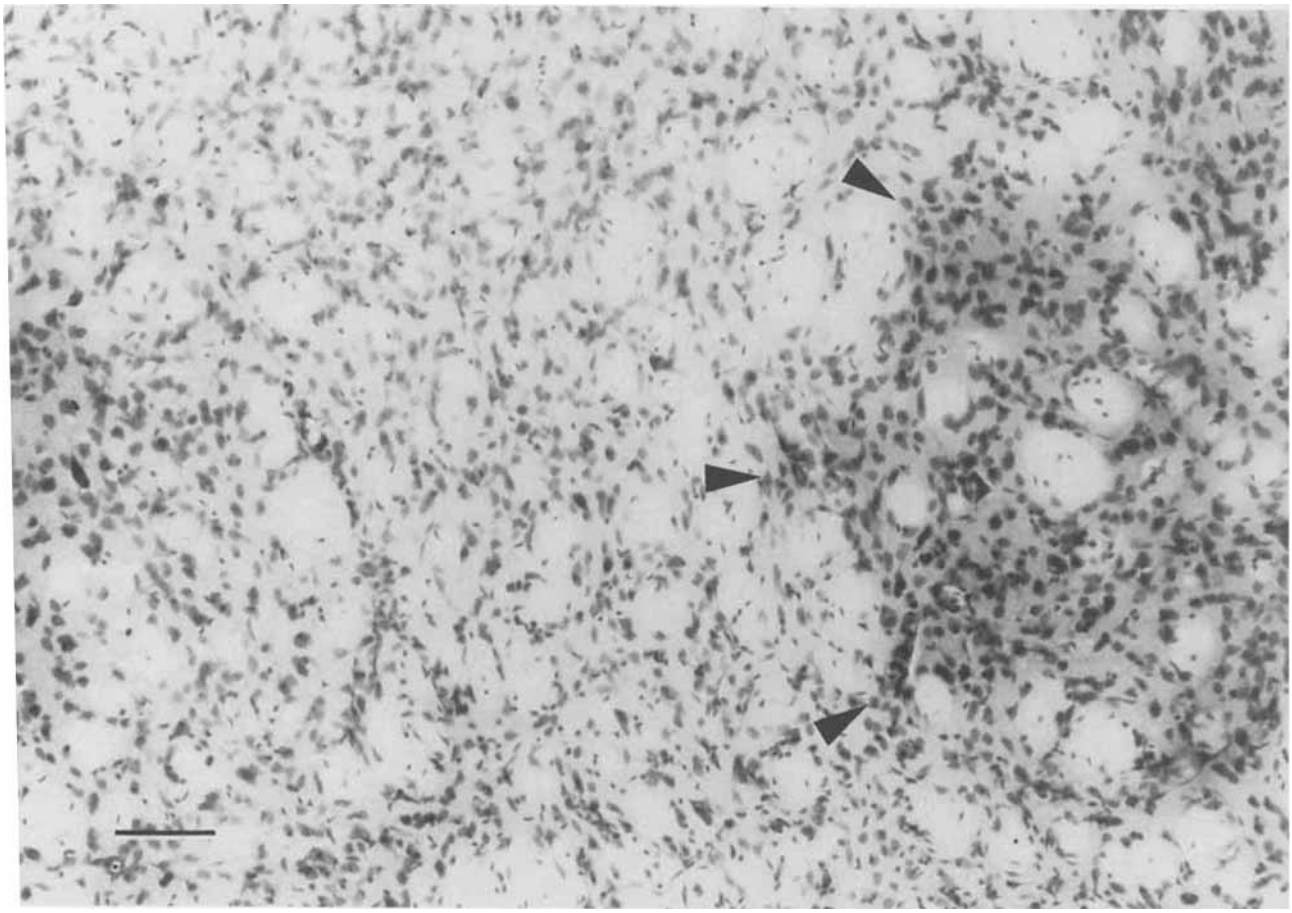


Fig. 1. Photomicrograph of striatal 3-nitropropionic acid (3-NPA) lesion. Depicted is a border area near the edge of a striatal 3-NPA lesion. Note preserved, shrunken fiber bundles in areas of nearly complete neuronal loss. Arrowheads outline boundary of spared area, which shows increased cresyl violet uptake and neurons of more normal morphology. Cresyl violet, scale bar = 160 μm.

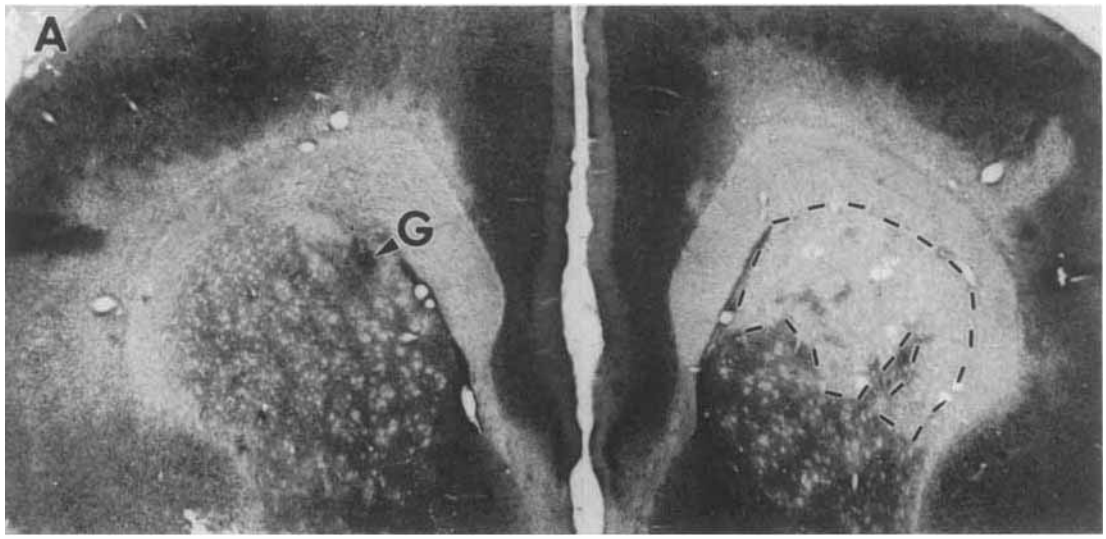
guished by neuronal loss accompanied by fiber bundle sparing analogous to Huntington's disease (Fig. 1).

Within the striatum there was subnuclear architecture to the lesion anatomy: neuronal loss in the anterior striatum was generally dorsomedial, while more posterior in the striatum it was greatest in the ventrolateral zones. This subnuclear striatal lesion anatomy corresponds quite closely to the neuropathological anatomy of Huntington's disease lesions in humans (Vonsattel et al., 1985). Graft placement was dorsal to the anterior part of the striatum, close to areas expected to contain dense neuronal loss after 3-NPA treatment (Fig. 2).

Animals implanted with NGF[+] cells showed marked neuronal sparing in the striatum within areas of expected neuronal loss ipsilateral to the graft implantation site (Fig. 2A). In animals implanted with NGF[−] cells, lesions were generally symmetrical bilaterally in dorsoventral and mediolateral extent (Fig. 2B). This has been noted previously in ungrafted animals lesioned with 3-NPA (Bossi et al., 1993). Also evident in 3-NPA-

Fig. 2. Effect of fibroblast grafts on lesion cross-sectional area. Whole brain cross-sectional view of NGF[+] graft effects **(A)** and NGF[−] graft effects **(B)** is shown. **A:** At an anterior level in the striatum where neuronal loss is usually quite intense after 3-nitropropionic acid (3-NPA) treatment. Note marked sparing on grafted side (graft, G, marked by arrowhead) with only the extreme anterior extent of graft visible. The non-grafted side contains a large intrastriatal area of neuronal loss, seen by decreased cresyl violet uptake and outlined by broken line to show method of cross-sectional area measurement. **B:** Cut from the mid-striatum of an animal implanted with a non-NGF-secreting graft (G) in order to show the extent of the bilateral striatal 3-NPA lesions (outlined) at the level of maximal graft cross-sectional size. Although neuronal loss extends outside the outline, only the areas where neuronal loss was nearly complete were outlined and measured for analysis purposes. There is no sparing ipsilateral to the graft. There is cortical cell loss evident in both A and B; however, the cortex remains relatively intact dorsal to the NGF[+] graft in A.

The effects of NGF[+] and NGF[−] fibroblast implants on grafted side to non-grafted side cross-sectional lesion area ratios are represented graphically below the anatomical sections. Error bars represent standard deviations; *, significant by t-test to $P < 0.0001$.


A
G


B
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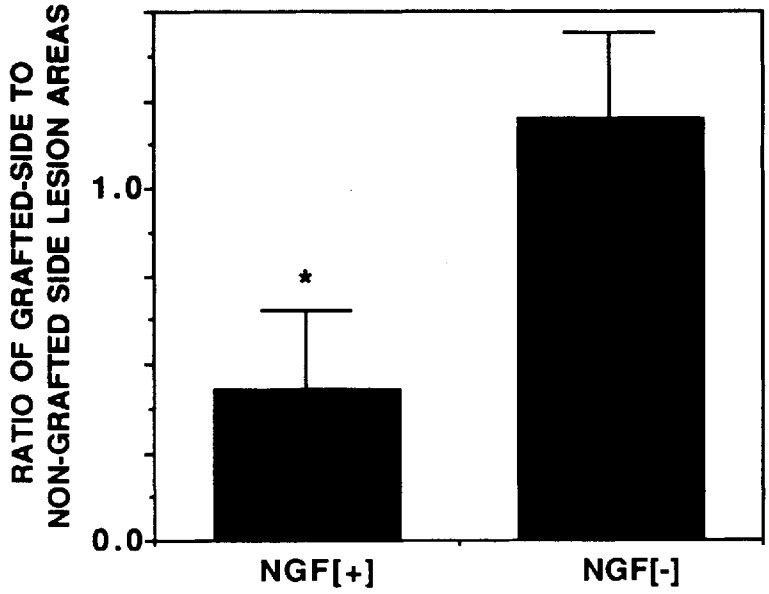
RATIO OF GRAFTED-SIDE TO NON-GRAFTED SIDE LESION AREAS
1.0
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NGF[-]

treated animals was the presence of cortical neuronal loss in the lower layers of the cortex, as seen in Figure 2A and B dorsal to the corpus callosum. Cortical cell loss also appeared to be reduced adjacent to NGF[+] grafts (Fig. 2A, grafted side); however, this observation was not quantifiable, as the cortical cell loss was not as symmetrical or as uniform as was the striatal cell loss. Cortical cell loss can be associated with Huntington's disease neuropathology (Vonsattel et al., 1985; Bruyn, 1973).

Graft Effects on Lesion Cross-Sectional Area

Striatal lesion cross-sectional area was compared side to side for each animal in both the NGF[+] and NGF[−] grafted groups. Three to five cresyl violet sections per animal (representing a span of 0.52 to 1.24 mm) that contained grafted fibroblasts in the corpus callosum and striatal lesions were analyzed bilaterally for lesion cross-sectional area. Sections without visible grafted cells were not analyzed, as we have previously shown that NGF protects only locally in an excitotoxic model (Frim et al., 1993a), and we did not wish to confound our analysis by including lesions too distant to be influenced by the grafts.

NGF[−] grafts had no effect on ipsilateral lesion size (Fig. 2B), with a group mean grafted side to non-grafted side lesion area ratio of 1.20 ± 0.24 (mean ± SD). Animals implanted with NGF[+] cells showed significant reduction in lesion size ipsilateral to the grafts (Fig. 2B), with a grafted side to non-grafted side group mean lesion area ratio of 0.43 ± 0.22. This mean 64% reduction in lesion cross-sectional area ipsilateral to the NGF[+] grafts was significant by t test ($P < 0.0001$). Mean maximal lesion cross-sectional areas contralateral to the grafts for both NGF[−] and NGF[+] groups were not significantly different, showing that NGF[+] grafts had no significant contralateral effects.

Immunocytochemical Staining for Tyrosine Hydroxylase and Choline Acetyltransferase; Histological Staining for Acetylcholinesterase

TH staining for putative dopaminergic fibers was unrevealing in that few fibers were visible in striatum either ipsilateral or contralateral to the grafts, regardless of whether the graft was secreting NGF. Since 3-NPA lesions are known to be fiber sparing in the striatum (Hamilton and Gould, 1987; Ludolph et al., 1991), as are excitotoxic lesions, it is not surprising that there was no difference between NGF[+] and NGF[−] graft effects.

Acetylcholinesterase (AChE) histochemical staining revealed dense fibers throughout the striatum; however, there was some decrease in fiber density in areas where neuronal loss was particularly evident in the cresyl violet-stained sections. As was seen with neuronal sparing, AChE-positive fibers were not visibly decreased in areas adjacent to NGF[+] grafts, and were found growing into or traversing the NGF[+] grafts, consistent with previous observations (Frim et al., 1992).

There were many ChAT-immunopositive neurons evident in areas adjacent to the NGF[+] grafts; however, there were also cholinergic neurons seen scattered throughout the striatum contralateral to the NGF[+] grafts, and bilaterally within the striatum in NGF[−] grafted animals. This observation may be due to the somewhat patchy nature of the 3-NPA striatal lesions (Figs. 1, 2), where occasional small clusters of neurons, some undoubtedly cholinergic, are spared within an otherwise complete lesion. We presume that for this reason no significant numerical difference between cholinergic cells ipsilateral and contralateral to NGF[+] grafts could be found to parallel the neuronal loss ratios. This finding contrasts with that seen in excitotoxic lesion areas protected by NGF[+] grafts (Schumacher et al., 1991; Frim et al., 1993a).

DISCUSSION

Neuronal protection against excitotoxic injury in vivo by biological administration of NGF could involve any of several mechanisms: direct effect on glutamate receptor number of function, induction of protective enzymes [either stress proteins (Rordorf et al., 1991) or enzymes of peroxidative metabolism (Jackson et al., 1990)], or direct effect on ionic balance—specifically, intracellular calcium stores (Johnson et al., 1992), the Na^+/K^+ ATPase (Skaper and Varon, 1983; Sendtner et al., 1988), or chloride fluxes (Rothman, 1985). In this report, we show that biologically delivered NGF is protective against striatal pathology caused by impairment of mitochondrial oxidative metabolism. This finding has important implications in the potential study of the mechanism of NGF protection and in the use of neurotrophic factors to prevent neurodegenerative disorders.

The striatal neuronal lesions caused by the systemic administration of the mitochondrial complex II inhibitor 3-NPA closely mimic those seen in Huntington's disease (HD), both anatomically and histologically (Vonsattel et al., 1985; Hamilton and Gould, 1987; Ludolph et al., 1991). Other areas of the brain are affected by systemic 3-NPA treatment showing a characteristic neuronal loss (i.e., cortex, as seen in Fig. 2). Presumably, this pattern of neuronal loss reflects the baseline energy requirements of neurons in those loci and defines a hierarchy of neuronal energy dependence in the brain of a particular species. Further investigation into specific extrastriatal 3-NPA pathology will determine if all areas affected by 3-NPA have energy demands as high as those of the striatum and if neurotrophic support can ameliorate the

3-NPA-mediated degeneration outside the striatum. Within the striatum, however, the similarity of the 3-NPA-mediated degeneration, taken with other evidence that HD patients manifest mitochondrial impairment (Brennan et al., 1985; Parker et al., 1990), is consistent with a hypothesized energy depletion (or mitochondrial dysfunction) etiology for HD (Beal, 1992; Wallace, 1992).

The significance of impaired energy metabolism as a cause of neuronal degeneration is unclear; however, energy depletion has been theorized as contributing to an excitotoxic neuronal death, presumably due in part to endogenous activity of otherwise sub-toxic glutamate receptor agonist levels in the brain (Zeevalk and Nicklas, 1991; Beal, 1992; Wallace, 1992). Along these lines, we have previously found that 3-NPA lowers the threshold for N-methyl-D-aspartate (NMDA) receptor-mediated neuronal degeneration (Simpson and Isacson, 1993). The selective neuronal loss seen in models of Parkinson's disease based on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), whose active metabolite, MPP^+, is a mitochondrial complex I inhibitor, is likely due to energy impairment and potential excitotoxicity (Bloem et al., 1990; Singer and Ramsay, 1990; Furtado and Mazurek, 1991; Turski et al., 1991).

Observations that NGF has no effect on striatal glutamate receptor binding, glutamate receptor synthesis (D.M. Frim, unpublished observation), or heat shock response to NMDA toxicity (Frim et al., 1993b) but may induce several proteins, including the peroxidative enzyme catalase (Frim et al., 1992), are consistent with an NGF protective mechanism acting distal to blockade of glutamatergic input. For this reason, neurotrophic factor enrichment may be of greater efficacy in protecting against neurodegeneration than glutamate antagonism. Certainly, our observations of NGF-mediated protection against both excitotoxicity and energy impairment define a common pathway of neuronal degeneration caused by these insults that is ameliorated by neurotrophic action.

It has been proposed that the neurotrophic molecules that prevent neuronal death during development might support neuronal survival in the degenerating adult brain (Morgan, 1989). The observation that genetically altered NGF-secreting fibroblasts protect against cell death caused by mitochondrial blockade suggests that an enriched neurotrophic milieu might protect adult neurons against energy-depleting insults. Conversely, neurotrophism (by appropriate neurotrophic factor support) seen during development may be equivalent to protection of neurons from neuronal stress (such as that seen in adult neurodegenerative diseases) resulting from the high energy demands of the embryonic state.

Although the mechanism of NGF-mediated neuroprotection remains unknown, our results imply that some aspect of energy metabolism, either increased efficiency or better protection against depletion, is affected. Regardless of the mechanism, NGF is capable of significant support of energy-impaired adult neurons. This conclusion suggests that the introduction of neurotrophic factor genes into the adult central nervous system, either by neurosurgical implantation of genetically altered cells (Breakefield, 1989; Gage et al., 1990) or by direct gene transfer technology (Breakefield and Deluca, 1991) is a reasonable therapeutic approach to neurodegeneration caused by impaired energy metabolism.

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