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Nerve growth factor corrects developmental impairments of basal forebrain cholinergic neurons in the trisomy 16 mouse

(Alzheimer disease/Down syndrome/tissue culture/trophic factors)

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ABSTRACT **The trisomy 16 (Ts16) mouse, which shares genetic and phenotypic homologies with Down syndrome, exhibits impaired development of the basal forebrain cholinergic system. Basal forebrains obtained from Ts16 and euploid littermate fetuses at 15 days of gestation were dissociated and cultured in completely defined medium, with cholinergic neurons identified by choline acetyltransferase (ChAT) immunoreactivity. The Ts16 cultures exhibited fewer ChAT-immunoreactive neurons, which were smaller and emitted shorter, smoother, and more simplified neurites than those from euploid littermates. Whereas the addition of β-nerve growth factor (100 ng/ml) augmented the specific activity of ChAT and neuritic extension for both Ts16 and euploid cholinergic neurons, only Ts16 cultures exhibited an increase in the number and size of ChAT-immunoreactive neurons. Furthermore, Ts16 ChAT-immunoreactive neurites formed varicosities only in the presence of β-nerve growth factor.**

Essentially all individuals with Down syndrome (DS) develop the neuropathologic alterations of Alzheimer disease (AD) by the fourth decade (1). DS results from trisomy of chromosome 21 (HSA 21) or, in rare cases, triplication of the distal portion of the long arm of HSA 21. Synaptic neurochemical studies have demonstrated that, as in AD (2), brains of DS individuals with AD pathology also exhibit significant reductions in choline acetyltransferase (ChAT), the presynaptic marker for cholinergic neurons, in the cerebral cortex (3). Furthermore, studies of the basal forebrain (BF) of young individuals with DS revealed a reduced complement of presumptive cholinergic neurons prior to the onset of AD neuropathology (4), although a quantitative neurochemical study did not demonstrate a reduction in presynaptic cholinergic markers in the cerebral cortex and related structures in young individuals with DS (5).

Gene mapping studies have revealed remarkable genetic homology between a portion of the distal end of long arm of HSA 21 and the distal end of mouse chromosome 16 (MMU 16) (6–8). At least six genes and two anonymous DNA sequences are shared between the two chromosomes. Where the spatial relationships among these genes have been identified, their relationships are preserved between the two species, suggesting that there may also be genetic homology in other genes in this region yet to be mapped. Of particular note are the shared location of the gene encoding the amyloid precursor protein (7) and two anonymous DNA sequences (8) that have been linked to an autosomal dominant hereditary form of AD (9). Because of the genetic homology between portions of MMU 16 and HSA 21, mice with trisomy of MMU 16 (Ts16) may provide a useful genetic model for determining the neurobiologic consequences of triplication of genes shared between HSA 21 and MMU 16, which may contribute to the DS phenotype and its risk for AD (10).

Previous studies have demonstrated significant reductions in the developmental accrual of ChAT, the presynaptic marker of cholinergic neurons, in the brains of fetal Ts16 mice, as compared to their euploid littermates (11, 12). Histochemical studies of the BF have revealed a significantly reduced complement of acetylcholinesterase (AChE)-positive presumptive cholinergic neurons in Ts16 fetuses (13, 14). Analysis of the neurogenesis of these AChE-positive neurons suggest an impairment in their generation in Ts16, although a failure in expression of AChE in subpopulation of later-formed neurons cannot be precluded (13).

Nerve growth factor (NGF) is a well-characterized trophic factor that regulates the differentiation, maintenance, and function of peripheral sympathetic and primary sensory neurons (15). In the central nervous system, cholinergic neurons of the BF have been demonstrated to express NGF receptors (16), retrogradely transport NGF from their terminals to the perikarya (17), and respond to NGF in both *in vivo* and *in vitro* studies (18–20). As the BF cholinergic neuronal atrophy and degeneration induced by axonal transsection can be reversed by exogenously administered NGF, this suggests that NGF may be an essential trophic factor for the metabolic maintenance and survival of BF cholinergic neurons (21, 22). Because of this role, impairment in the synthesis or response to NGF of BF cholinergic neurons could account for selective vulnerability of these neurons in AD (23). Accordingly, in the present study, we have examined the effects of NGF on BF cholinergic neurons in primary disaggregated culture obtained from Ts16. A preliminary report of these findings had been presented (31).

METHODS OF PROCEDURE

Animals. Ts16 mouse fetuses were obtained by breeding normal C57BL/6J females with males that are doubly heterozygous for Robertsonian translocation, Rb(16.17)7BNR/RB(9.16)9RMA, as described by Gearhart *et al.* (24). Ts16 fetuses were identified phenotypically, and the liver was removed by karyotype analysis. Normal (2N) littermates were used as contemporaneous controls. Thus, to minimize sources of variance, all comparisons were made between cells prepared from Ts16 and those from euploid littermate fetuses that were isolated from the same uteri, prepared for culture and dissociated with the same reagents, and plated at the same density in multiwell plates.

Tissue Culture Procedures. Primary brain-cell cultures were prepared according to the method of Huettner and

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Abbreviations: AChE, acetylcholinesterase; AD, Alzheimer disease; BF, basal forebrain; ChAT, choline acetyltransferase; DS, Down syndrome; HSA 21, human chromosome 21; IR, immunoreactive; MMU 16, mouse chromosome 16; NGF, nerve growth factor; Ts16, trisomy 16.

Baughmann (25). Pregnant dams at embryonic day 15 (the day of vaginal plug = embryonic day 0) were killed by cervical dislocation, and the fetuses were delivered by hysterotomy and placed on ice-cold phosphate-buffered normal saline (PBS) containing glucose (4.5 g/liter). Under sterile conditions, the BF was dissected from each animal and cut into small pieces, placed in 10 ml of Eagle's basic salt solution (EBSS, maintained at 37°C and equilibrated with 95% O_2/5% CO_2) containing 0.05 mM EDTA, 0.1 mM cysteine, and papain (Worthington; 20 units/ml). After a 45-min incubation at 37°C, the enzyme solution was decanted and replaced with EBSS containing bovine serum albumin (1 mg/ml), ovomucoid (1 mg/ml), and 0.01% DNase (Sigma). The pieces of brain were gently triturated with a sterile fire-polished Pasteur pipette. The viability of freshly dissociated cells was determined by exclusion of trypan blue, which routinely exceeded 90%. Dissociated cells were harvested by low-speed centrifugation (10 min, 70 × g) through 5 ml of EBSS containing bovine serum albumin (10 mg/ml) and ovomucoid (10 mg/ml) at room temperature. The cells were resuspended in medium containing 20% (vol/vol) fetal calf serum (26) and plated on poly(D-lysine)-coated 12-well plates (Costar) at a density of 7 × 10^5 cells per ml. Twenty-four hours later the medium was replaced with defined serum-free medium with supplements (26). Homogeneously pure mouse 7S NGF (100 ng/ml) was added to the culture medium at the time of medium replacement. The culture medium was not replaced during the subsequent 7-day period of culture.

Immunocytochemistry. Culture medium was first aspirated and the plates were gently rinsed with PBS, followed by fixation with 4% (vol/vol) formaldehyde in PBS for 30 min at 20°C. After fixation, the cells were incubated for 2–4 hr with 0.1 M sodium phosphate (pH 7.4) containing 5% (wt/vol) sucrose, 5% (wt/vol) bovine serum albumin, and 0.1% Triton X-100. The cultures were then incubated for 12 hr at 4°C with a polyclonal antiserum against human placental ChAT (generously provided by L. Hersh, University of Texas Health Science Center, Dallas), diluted 1:5000 in 0.1 M sodium phosphate (pH 7.4) (27, 28). The cultures were then washed with PBS and incubated with a biotinylated anti-rabbit antibody (diluted 1:200; Vector Laboratories) for 1 hr at room temperature, washed again with PBS, and then incubated with an avidin–biotin-conjugated peroxidase (Vector Laboratories). Peroxidase was visualized by incubation with 0.05% diaminobenzidine and 0.01% hydrogen peroxide for 5 min at room temperature.

Measurement of ChAT Activity. Cultures were washed three times with ice-cold PBS and sonicated in 300 μl of 50 mM Tris·HCl (pH 7.2) containing 0.02% Triton X-100. Homogenate (50 μl) was used for the determination of ChAT activity according to the modification of the method of Fonnum (29). To 50 μl of homogenate was added 50 μl of the mixture: 200 mM NaCl/6 mM choline chloride/50 mM sodium phosphate, pH 7.2/7.5 μM eserine/0.005% bovine serum albumin/36 μM [^{14}C]acetyl coenzyme A (NEN; specific radioactivity, 57.2 mCi/mmol; 1 Ci = 37 GBq). The reaction mixture was incubated for 60 min at 37°C, and the assay was terminated by the addition of 0.4 ml of ice-cold water. The [^{14}C]acetylcholine formed was separated from the substrate on an anion-exchange column (AG 1-X8, 200–400 mesh, chloride form; Bio-Rad). To the column effluent, 0.5 ml of Kalignost solution (1 g of tetraphenylboron in 200 ml of acetylnitrile) was added, the [^{14}C]acetylcholine was extracted into the organic phase, and radioactivity was measured by liquid scintillation spectroscopy. Protein was measured by the method of Lowry *et al.*(30) with bovine serum albumin as the standard.

Data Analysis. The number of ChAT-immunoreactive (IR) neurons per plate was determined by counting randomly distributed fields (n = 20) with a light microscopy at ×240.

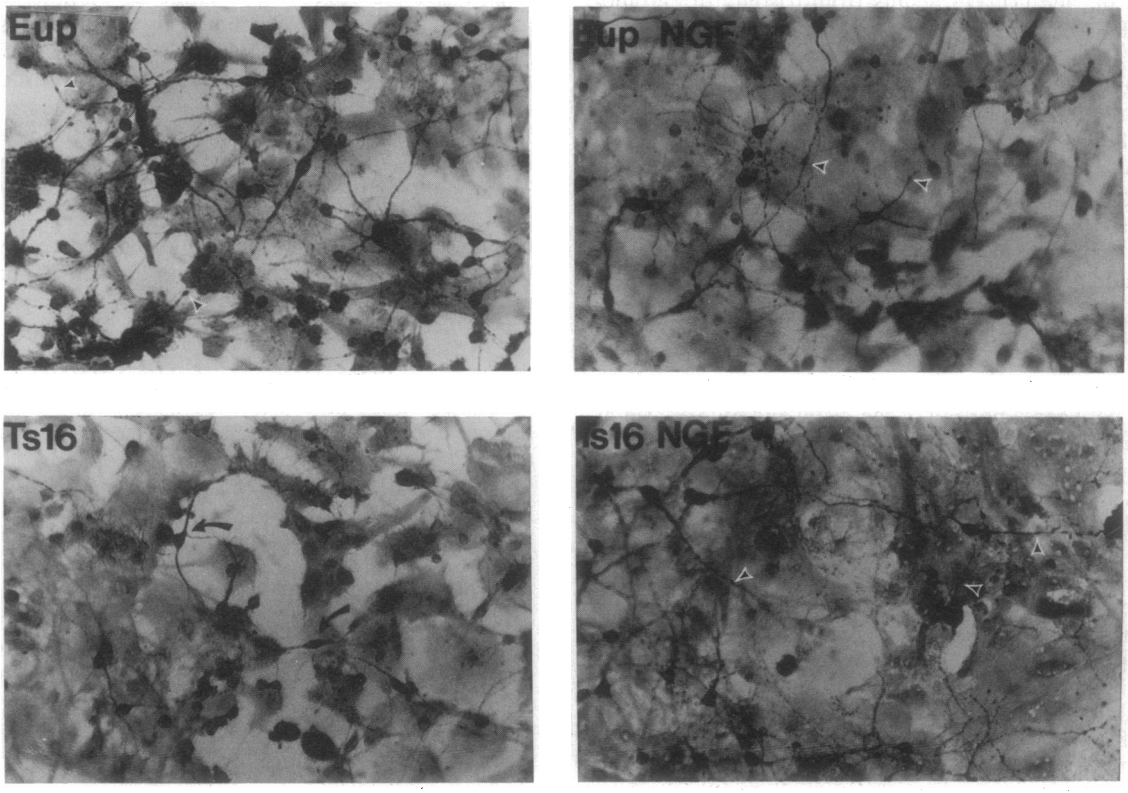

FIG. 1. Effect of NGF on ChAT-IR neurons. Disaggregated cultures of BF from Ts16 and euploid littermates were plated at the same density and cultured for 7 days in completely defined medium in the presence and absence of β-NGF (100 ng/ml). After fixation, the cultures were stained immunocytochemically for ChAT. Note the varicosities on the neuritic processes (arrowheads) in euploid (Eup), euploid plus NGF, and Ts16 plus NGF. In contrast, note the short, thick, and smooth neurites on the ChAT-IR Ts16 neurons grown in the absence of added NGF (arrows).

Then, images of randomly selected ChAT-IR cells were prepared using the light microscope at ×100 and a microscope drawing tube (camera lucida). These images were subjected to quantitative morphometric assessments with a computerized image analysis system (Bioquant; IBM) to determine cell body surface area, total neurite length, and number of neurite bifurcations. Statistical analysis was performed with the unpaired Student's *t* test. For the cell counts and ChAT activity results, the Wilcoxon rank test was used since there was a non-Gaussian distribution of the data.

RESULTS

Cultures. Fetuses at 15 days gestation were used as a source of BF brain tissue because the majority of BF cholinergic neurons had completed their final division by this stage (13). Modified N2 medium proved to be superior to other tested culture media in sustaining survival and expression of ChAT in the BF cultures. Enzymatic treatment with papain and gentle-mechanical dissociation of fetal BF yielded a high proportion of viable cells with >90% excluding trypan blue. The average total number of dissociated cells obtained from fetal BF at embryonic day 15 was 1×10^6 cells per fetus. Many of the dissociated cells retained short neurites and had recognizable neuronal morphology. The plated cells rapidly adhered to the poly(D-lysine)-coated culture dishes and began to extend processes within a few hours. At the time of plating, most cells were phase-bright with surrounding halos and thin processes consistent with neuronal morphology. By the end of the first week *in vitro*, nonneuronal cells formed a confluent mat upon which well-differentiated neurons lay.

Characteristics of Cholinergic Neurons. The morphology of the BF cholinergic neurons was delineated after immunohistochemical staining for ChAT (Fig. 1). After 7 days in culture, ChAT-IR cells could be divided into two major classes: larger multipolar neurons and a predominant population of bipolar neurons. In the sister Ts16 cultures that were plated at the same density as those prepared from the euploid littermates, there were significantly fewer ChAT-IR bipolar neurons, while the number of multipolar neurons did not differ between the two (Fig. 2). Furthermore, the ChAT-IR neuronal perikarya from Ts16 were smaller than the corresponding cholinergic neurons in the euploid cultures. Quantitative analysis revealed that the neuritic processes on the Ts16 ChAT-IR neurons were shorter, fewer in number, and exhibited fewer branching points than those of the littermate controls (Fig. 3). Notably, the neurites of Ts16 ChAT-IR cells were virtually devoid of varicosities in contrast to their frequent presence on the neurites of euploid cholinergic neurons (Fig. 1).

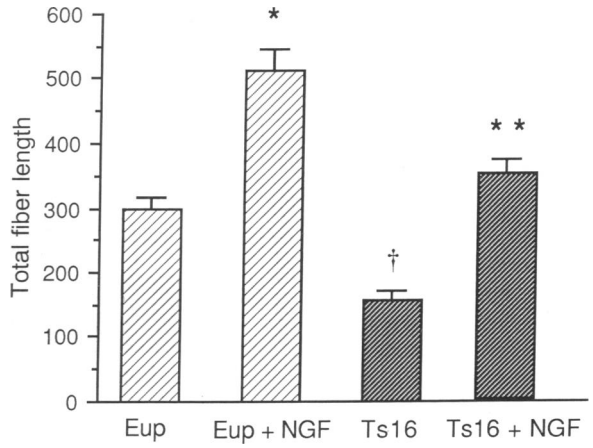

FIG. 3. Neuritic length of bipolar ChAT-IR neurons. The total length of neurites was quantified by image analysis of the ChAT-IR neuritic processes. The results are the mean ± SEM of 80 neurons from six cultures, each under the four conditions. Eup, euploid. *, $P < 0.01$ versus euploid; †, $P < 0.025$ versus euploid; **, $P < 0.01$ versus Ts16.

Addition of β-NGF (100 ng/ml), a concentration reported (18) to exert maximal trophic effects in cultures, exerted some differential effects on the Ts16 cholinergic neurons, as compared to those from euploid littermates. In both preparations, the NGF treatment appeared to increase the intensity of staining for ChAT. Although the number of ChAT-IR bipolar cells in the euploid cultures was not significantly affected by NGF treatment ($P = 0.48$), there was a significant increase in the number of bipolar cholinergic neurons in cultures prepared from the Ts16 mice (Fig. 2). NGF treatment did not affect the size of the ChAT-IR perikarya in euploids but did stimulate elaboration of a more extensive neuritic arbor (Fig. 3). In fact, the average total length of neuritic extensions with NGF treatment doubled. In contrast to the euploids, NGF treatment caused a significant increase in the size of the Ts16 cholinergic perikarya ($P < 0.05$). NGF

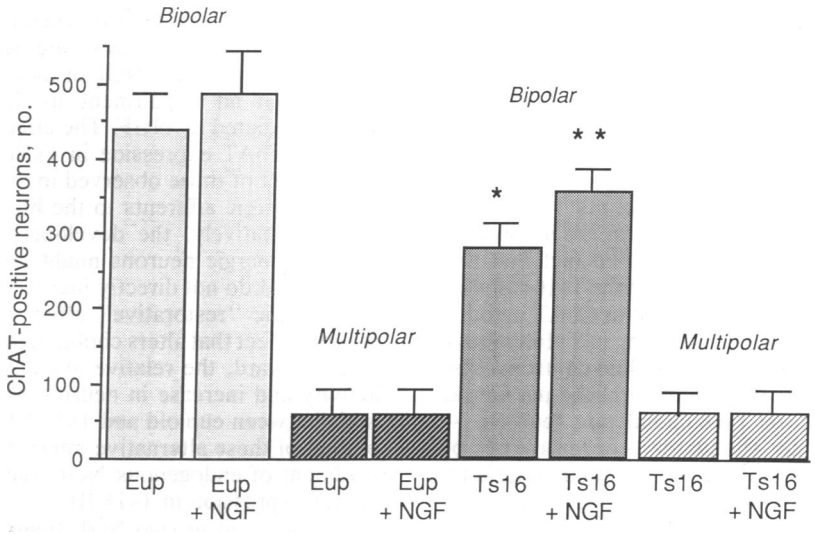

FIG. 2. Number of ChAT-IR neurons. Cultures of disaggregated BF prepared from Ts16 and euploid littermates were plated at the same density and grown for 7 days in the absence or presence of β-NGF (100 ng/ml). Eup, euploid. The results are the mean of six cultures in which 20 randomly selected fields were counted for ChAT-IR neurons. Error bars represent SEM. *, $P = 0.01$ versus euploid bipolar cells; **, $P = 0.04$ versus Ts16 bipolar cells by Wilcoxon signed-rank test.

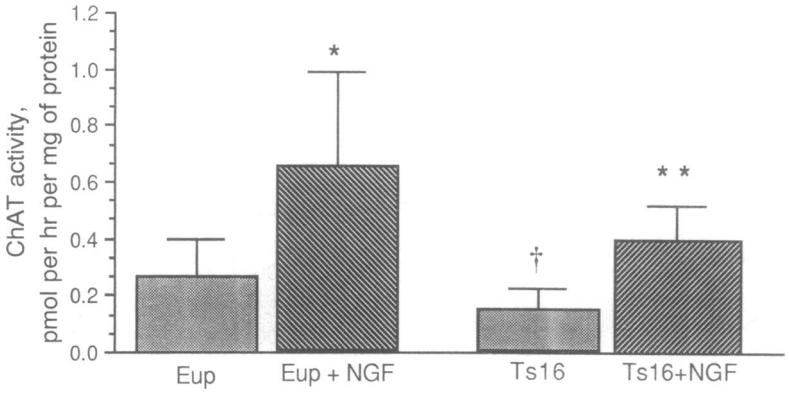

FIG. 4. ChAT activity in Ts16 and euploid cultures. Cultures of disaggregated BF prepared from Ts16 and euploid littermates were plated at the same density and grown for 7 days in the absence or presence of β-NGF (100 ng/ml). The results are the mean of six experiments contemporaneously comparing the four conditions. *, $P < 0.01$ versus euploid; †, $P < 0.05$ versus euploid; **, $P < 0.01$ versus Ts16 by Wilcoxon signed-rank test.

treatment more than doubled the total length of the neuritic processes of ChAT-IR neurons (Fig. 4) and the number of branching points per neuron (data not shown). Notably, NGF-treated ChAT-IR neurons exhibited numerous varicosities on their neurites in contrast to their virtual absence in the untreated Ts16 sister cultures (Fig. 1).

ChAT Activity. Measurement of ChAT activity after 7 days in culture under basal conditions revealed 40% lower activity for the Ts16 cultures, as compared to those prepared from the euploid littermates (Fig. 4). Seven days of NGF treatment resulted in a doubling of the specific activity of ChAT in the euploid cultures and a comparable augmentation of ChAT activity in the Ts16 cultures, resulting in an average value equivalent to that observed in the untreated euploid controls. These effects of NGF treatment appeared to be relatively specific to a subpopulation of neurons since there was no significant differences in the protein content in the four culture conditions ($P > 0.5$).

DISCUSSION

The results of these studies are consistent with previous findings concerning the development of the BF cholinergic neurons in the Ts16 mouse *in vivo*. The specific activity of ChAT in the Ts16 fetus is reduced by approximately 40% in the midbrain–brainstem at 18 days of gestation in comparison to the euploid littermates (11). Furthermore, the number of AChE-positive neurons in the BF are also reduced from 30% to 40% in Ts16 fetuses at 16 days gestation as compared to euploid littermates (13, 14). These reductions observed in the intact fetus are comparable to the differences in the specific activity of ChAT in untreated Ts16 BF cultures grown in completely defined medium as compared to those from euploid littermates. Furthermore, cell counts revealed a significant, albeit more modest, reduction in the number of ChAT-IR neurons in the Ts16 cultures. The fact that the reduction in number of ChAT-IR neurons in the Ts16 cultures was not commensurate with the reduction in ChAT activity suggests that individual cholinergic neurons in Ts16 express less ChAT enzyme than do those from euploid controls. This inference is corroborated by the reduction in the perikaryal size and neuritic arbors of the Ts16 cholinergic neurons.

The addition of maximally stimulatory concentrations of β-NGF to the completely defined culture medium exerted a trophic effect on the euploid cholinergic BF neurons, similar to what has been described for cultures of rat BF (20, 21). Although NGF did not affect the number of ChAT-IR neurons or the size of their perikarya, it significantly augmented neuritic elaboration. Consistent with the expansion of the cholinergic neuronal arbor (Fig. 3), NGF treatment more than doubled the specific activity of ChAT in the cultures of BF from euploid littermates (Fig. 4).

In contrast to the effects of NGF on the euploid cholinergic neurons, its actions were more dramatic and pervasive with regard to Ts16 BF cholinergic neurons. Thus, NGF treatment significantly increased the number of bipolar ChAT-IR cells and also significantly increased the number of bipolar ChAT-IR cells and also significantly increased the size of the cholinergic neuronal perikarya. NGF-treated Ts16 cholinergic neurons also exhibited a marked increase in the total length of neurites with more frequent bifurcations and the appearance of neuritic varicosities. These trophic effects were mirrored by the augmentation in specific activity of ChAT in the Ts16 cultures.

The results of these studies suggest that NGF treatment can reverse several of the developmental impairments of cholinergic neurons in the Ts16 BF. Notably, ChAT specific activity, cholinergic perikaryal size, and total neuritic length of the cholinergic neurons approached values indistinguishable from those obtained in euploid cultures not treated with exogenous NGF. These results suggest that developmental defects in the Ts16 BF cholinergic neurons may involve in part alterations in NGF-mediated mechanisms of neuronal differentiation. Since NGF produces effects on the Ts16 neurons (such as increase in the number of ChAT-positive neurons, expansion of neuronal perikarya, and the appearance of varicosities) not observed in the euploid cultures, it is possible that Ts16 cultures are deficient in their ability to synthesize and release NGF or that Ts16 cholinergic neurons suffer from an impairment in the receptor-transduction events mediated by NGF. The effects of NGF on perikaryal size and ChAT expression in Ts16 cholinergic neurons are reminiscent of those observed in the BF after transection of the cholinergic afferents to the hippocampal formation (21, 22). Alternatively, the developmental impairments in the Ts16 cholinergic neurons might involve more fundamental defects that do not directly involve NGF but rather reflect a less-specific "restorative" effect of NGF treatment or an indirect effect that alters cholinergic neuronal differentiation. In this regard, the relative augmentation of ChAT specific activity and increase in neuritic length are roughly comparable between euploid and Ts16 NGF-treated cultures. Resolution of these alternative interpretations will require measurement of endogenous NGF and assessment of NGF receptor expression in Ts16 BF.

The fact that *in vivo* NGF treatment was able to reverse cholinergic neuronal atrophy and/or degeneration in the BF as a consequence of transection of afferents to the cortex and hippocampal formation, a process that resembles the cholinergic pathologic changes in AD, has prompted investigators

to suggest that pharmacologic activation of NGF receptors on these cholinergic neurons might attenuate or reverse the BF cholinergic pathology in AD. In contradistinction to these models, which involve physical disruption of cholinergic afferents, the Ts16 mouse provides a genetic model of selective impaired differentiation of the BF cholinergic neurons based upon genetic homology with DS and its vulnerability to AD. Thus, the present results are consistent with the hypothesis that treatment with NGF or an agent that mimics its effects might have therapeutic benefits in correcting impaired cholinergic differentiation in DS and the delayed cholinergic deficits associated with AD pathology in DS.

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