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Mouse model of neurodegeneration: Atrophy of basal forebrain cholinergic neurons in trisomy 16 transplants

(Down syndrome/trisomy 21/Alzheimer disease)

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ABSTRACT **Vulnerability of specific brain regions and neuronal populations is a characteristic feature of Alzheimer disease and Down syndrome. Cholinergic neurons of the basal forebrain degenerate in both disorders. The basis for neuronal degeneration is unknown. Mouse trisomy 16 (Ts 16) is an animal model of Down syndrome. We sought an experimental system in which the survival and development of Ts 16 basal forebrain cholinergic neurons could be examined beyond the fetal period. As Ts 16 mice do not survive birth, we transplanted fetal Ts 16 and control basal forebrain into the hippocampus of young adult mice. Transplanted neurons survived and grew neurites in all grafts. Over time, we observed selective atrophy of cholinergic neurons in Ts 16 grafts. Denervation of the hippocampus produced a significant increase in the size of Ts 16 cholinergic neurons. This suggests that hippocampal-derived neurotrophic factors acted to prevent degeneration. β/A4-amyloid-containing plaques were not seen. Ts 16 provides a model of spontaneous, genetically determined neurodegeneration that may be used to understand better the molecular pathogenesis of neuronal dysfunction in Alzheimer disease and Down syndrome.**

The neuropathological and neurochemical hallmarks of the Alzheimer disease (AD) brain include selective degeneration of certain neuronal populations associated with decreases in their neurotransmitter markers, loss of synapses in the neocortex, accumulation of abnormal fibrillar deposits in neurons (neurofibrillary tangles), and the deposition of amyloid-containing plaques in the extracellular space (cerebrovascular, diffuse, and neuritic) (1–4). The principal protein component of plaques is a 39- to 42-amino acid amyloid peptide, β/A4, which is derived from a larger protein, the amyloid precursor protein (APP) (5–7). The pathogenetic events leading to dementia in AD remain unclear. It is of interest that certain neuronal populations are much more severely affected than others (8). Thus, the early memory loss and intellectual decline in AD may result from the vulnerability and dysfunction of specific populations of neurons.

The development of an animal model(s) of AD is critical to understanding the complex neuropathology of the disease. An animal model of Down syndrome (DS)—the trisomy 16 (Ts 16) mouse—may provide a means to study important aspects of the AD phenotype, since 100% of individuals with an extra copy of human chromosome 21 develop AD neuropathology by the fourth decade of life (9–11). Mouse chromosome 16 contains a cluster of genes and loci also located on the long arm of human chromosome 21. These include *APP*, superoxide dismutase (*SOD1*), and markers linked to one form of familial AD (12, 13). Ts 16 mice demonstrate phenotypic features seen in DS including endocardial cushion defects and hematologic and immunologic abnormalities (10). In addition, the brains of these mice, as in persons with DS, are reduced in size and decreased in cortical thickness (12, 14).

Basal forebrain cholinergic neurons are vulnerable in adults with DS and AD, and their dysfunction may contribute to dementia (15, 16). In AD, these neurons show reductions in size and cholinergic markers, contain neurofibrillary tangles, and participate in the formation of senile plaques (2, 17). Eventually, they atrophy and die. Coyle and colleagues (12, 18) have also shown that, in the Ts 16 mouse fetus, hypocellularity of basal forebrain neurons affects cholinergic cells more severely than noncholinergic cells. The reduction in cholinergic neurons may be responsible for the decreased activity in fetal Ts 16 brain of choline acetyltransferase (ChAT), the cholinergic neurotransmitter synthetic enzyme (12). It is possible, however, that these changes reflect abnormalities in the genesis of Ts 16 cholinergic neurons or their growth in an underdeveloped brain (18). We sought an experimental system in which the survival and development of Ts 16 basal forebrain neurons could be examined beyond the fetal period. Because Ts 16 mice do not survive birth, fetal basal forebrain cells were transplanted into the hippocampus of a normal host. In addition, we used a well-studied paradigm, septohippocampal transection (fimbria–fornix lesion), to examine the effects of host target denervation and trophic influences on the transplants. Our results indicate that cholinergic neurons are more vulnerable to the effects of Ts 16 than are noncholinergic neurons and that regulation of host target factors can positively influence cholinergic cell size. They also suggest that this model can be used to examine further at a molecular level the pathogenesis of cholinergic neuronal atrophy and degeneration.

MATERIALS AND METHODS

Transplantation Procedure and Production of Ts 16 Embryos. Male mice, doubly heterozygous for two metacentric Robertsonian translocation chromosomes, Rb(16.17)32Lub and Rb(11.16)2H, were mated to superovulated C57BL/6J females as described (19). Both strains of mouse contained the Thy-1.2 allele of Thy-1. Approximately 10–20% of the embryos produced were identified on the basis of their characteristic phenotype as Ts 16 (10). The basal forebrain region (including the septum and diagonal band nuclei) was dissected from five to seven Ts 16 and control fetuses at each transplantation session. After dissociation with 0.1% trypsin

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Abbreviations: AD, Alzheimer disease; DS, Down syndrome; Ts 16, trisomy 16; APP, amyloid precursor protein; ChAT, choline acetyltransferase.

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(GIBCO) in phosphate-buffered saline (PBS, pH 7.4) with glucose (1 mg/ml), cells were resuspended in 10 μl of PBS/glucose per basal forebrain piece. Two-month-old female AKR mice (Thy-1.1) were anesthetized and placed in a modified Kopf (Tujunga, CA) stereotaxic apparatus. Within 2 h of dissociation, two 2-μl cell suspensions were injected into two hippocampal sites per hemisphere: (*i*) 1.5 mm posterior to bregma, 1.7 mm lateral to the midline, and 1.8 mm ventral to the dura and (*ii*) 2.2 mm posterior, 2.2 mm lateral, and 2.0 mm ventral. Ts 16 cells were injected into the right hippocampus and control cells were injected into the left. There was no significant difference in the number of trisomic vs. control cells per microliter of injected cell suspension. Five animals received a bilateral aspirative lesion of the fimbria–fornix within 10 min of transplantation. The cortex immediately overlying the fimbria–fornix was aspirated prior to its removal (20).

Tissue Analysis and Immunohistochemistry. One or 6 months after transplantation, mice were deeply anesthetized (20) and perfused transcardially with 50 ml of PBS (pH 7.4, 4°C) followed by 50 ml of 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate (pH 7.4, 4°C). Brains were removed and post-fixed for 2 h in the same fixative. Three brains were then embedded in paraffin and sectioned at 8 μm. The remaining 17 brains were dehydrated overnight in 20% (wt/vol) sucrose in 0.1 M sodium phosphate (4°C). After freezing in powdered dry ice, 40-μm sections were cut on a freezing-sliding microtome and collected in 0.1 M Tris-buffered saline (TBS, pH 7.6, 4°C), and sections encompassing the entire transplant were processed. In the first of each series of three sections, a monoclonal antibody to ChAT (AB8; gift of B. Wainer, University of Chicago) was used as described (21). The second section was immunostained with a Thy-1.2 antibody (Becton Dickinson). The third section was taken for β/A4 or APP immunostaining (see below) or for staining with Congo red dye or a modified Bielschowsky silver technique. Sections for immunostaining were processed essentially as described (22, 23). Antibodies to Thy-1.2, β/A4, and APP were used at a 1:500 dilution. Some slides were counterstained with Cresyl violet. In each fimbria–fornix-lesioned animal, acetylcholinesterase histochemistry (20) was also performed on at least one hippocampal section to assure the denervating lesion was complete. Two antibodies to β/A4 (residues 1–28) were gifts of (*i*) D. Serban and S. Prusiner (University of California, San Francisco) and (*ii*) R. Siman (Cephalon, West Chester, PA). An antibody to β/A4-(2–43) (gift from K. Beyreuther, University of Heidelberg, and Colin Masters, University of Melbourne) was also used. AD brain was always stained concomitantly with other samples and was used as a positive control. Staining with β/A4 antibodies could be eliminated by preincubating the antibodies with synthetic rodent or human β/A4-(1–28) (23) at 100 μg/ml.

Graft and Neuronal Morphometry. Image analysis was performed with the Bioquant IV image analysis system (R & M Biometrics, Nashville, TN). To measure total graft volume (i.e., volume occupied by the grafted cell bodies) in each animal, every section containing grafted cells, as shown by Thy-1.2 immunostaining, was identified. The two-dimensional area of the grafted cells on each section was determined and the volume was estimated (24). To estimate the number of cholinergic neurons in each graft, all cholinergic neurons in every third section were counted and the correction factor of Abercrombie for cell counts was applied (25). To ensure that measured neurons were of graft origin, no neuron was measured unless it was located (*i*) within an area outlined in an adjacent section with the Thy-1.2 antibody, (*ii*) adjacent to other neurons that did not form an organized cellular grouping characteristic of the host hippocampal formation, and (*iii*) in a region not overlapping the location of host hippocampal neurons. To measure the area of cholinergic neurons, we started in the anterior-most section of each graft and measured every ChAT-immunoreactive cell with a nucleus and at least one process until a minimum of 20 had been analyzed per hippocampus. The area (mean ± SEM) of all measured cells was calculated. All of the sections in which cholinergic neurons were measured were counterstained with cresyl violet. Non-ChAT immunoreactive profiles were identified as neurons when the cells had a large nucleus, an apparent nucleolus, a cell body containing Nissl substance, and an intact cell body. The cross-sectional area of all transplanted non-ChAT-immunoreactive neurons was also measured. Measurements were made with a Zeiss microscope using a ×40 objective. For cresyl violet cells, these measurements were repeated with a ×100 objective (oil immersion) with no difference in results. In frozen sections, we also measured a minimum of 20 neurons per transplant that were immunoreactive with a β/A4 antibody to synthetic human β/A4-(1–28). We interpret this staining to reflect the presence of APP in the cells (see text). Approximately 50% of cresyl violet stained neurons were also APP immunoreactive in both trisomic and control grafts. Student's *t* test was used for all statistical analysis.

RESULTS

Assessment of Transplants in the Intact Hippocampus. At both 1 and 6 months after transplantation into the intact hippocampus, there was evidence of neuronal survival and neurite outgrowth from Ts 16 and control grafts (Fig. 1*A*). Thy-1 is a membrane glycoprotein expressed in neurons and is found on their processes (40). The grafts expressed a different allele of Thy-1 than the host and were easily identifiable. Within the hippocampus, most grafts were injected within or abutting the dentate gyrus. There was no significant difference in graft volume between trisomic and control (diploid) transplants (Table 1). Transplanted trisomic and control neurons were similar in appearance and were not organized in a defined pattern as revealed by cresyl violet staining. Neurite outgrowth from trisomic and control transplants was abundant and most intense throughout the molecular layer of the dentate gyrus (Fig. 1*A*). If the grafted cell bodies contacted the CA1 or CA3 regions, immunostained processes were also seen in the stratum oriens and stratum radiatum. Neuronal outgrowth into the molecular layer and hilus of the dentate gyrus as well as into the stratum oriens and stratum radiatum overlapped that of normal cholinergic projections (41).

Cholinergic neurons were consistently identified in grafts from both trisomic and control fetuses (Fig. 1 *B* and *C* and Table 1). One month after transplantation, Ts 16 and control cholinergic neurons were similar in appearance, size, and number (Table 1 and Fig. 2*A*). The morphometry of these neurons was similar to that of basal forebrain (septal) cholinergic neurons *in situ* (i.e., nontransplanted). The size of cholinergic neurons 1 month after transplantation (Fig. 2*A*) did not differ significantly from the size of nontransplanted cholinergic neurons (174.9 ± 3.1 μm^2, $n = 205$; measured in the septum of three 4-month-old C57BL/6J mice). There was, however, a striking difference between the Ts 16 and control grafts noted at 6 months. In all Ts 16 grafts, cholinergic neurons and their proximal neurites frequently appeared shrunken and atrophic (Fig. 1 *B* and *C*). They were significantly smaller than controls (125.9 vs. 166.2 μm^2; Fig. 2*A*). Fig. 2*B* shows that this was due to a decrease in the size of most cholinergic neurons. Control cholinergic neuronal size did not differ between 1 and 6 months. Thus, atrophy of Ts 16 cholinergic neurons occurred during the period from 1 to 6 months after transplantation. Importantly, this process did not affect all neurons, as there was no significant difference in the cross-sectional area of noncholinergic neurons stained by cresyl violet [Ts 16, 125.3 ± 1.6 μm^2, $n = 546$; control, 125.3

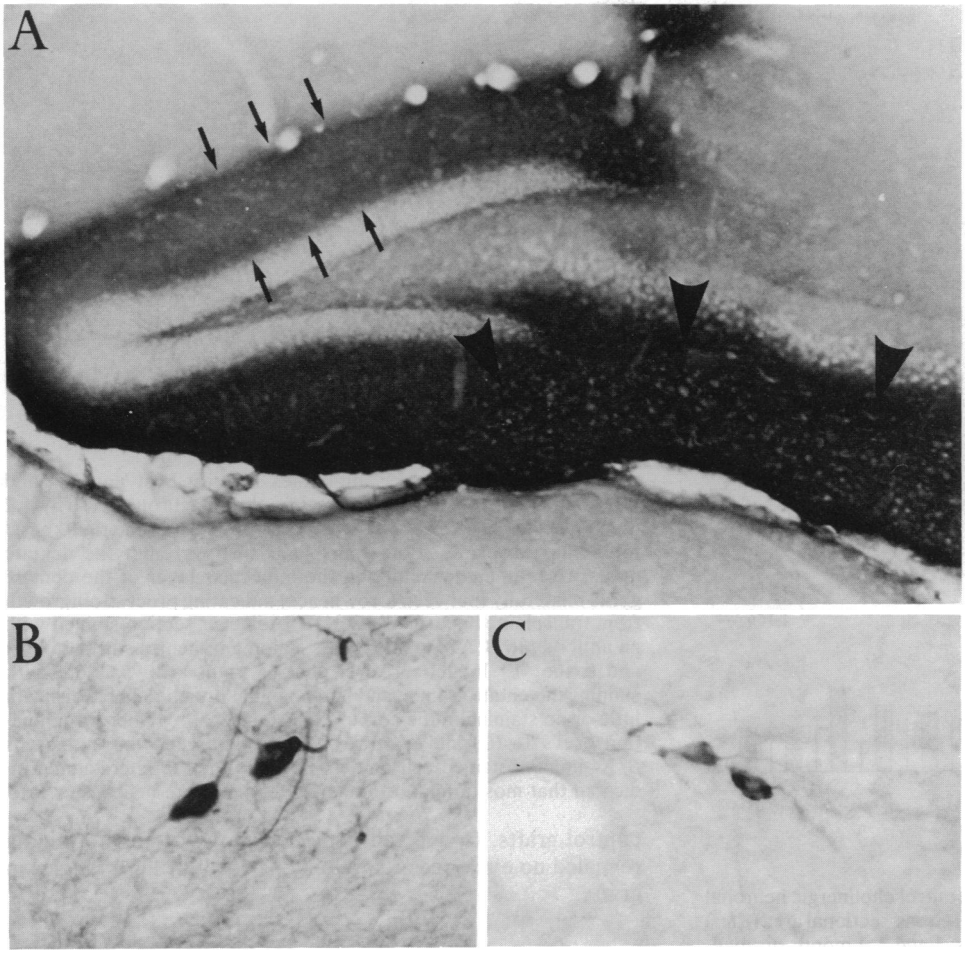

FIG. 1. Morphology of transplanted basal forebrain and cholinergic neurons. (*A*) Immunostaining of Thy-1.2 in a control transplant. The dark area (outlined by the arrowheads) shows grafted cells residing ventral to CA3 pyramidal neurons in the host hippocampus. Antibodies to Thy-1 densely stain neuronal membranes of processes and the circumference of cell bodies. Within this immunostained area, neuronal cell bodies are visible as focal clearings. Immunostained neuronal processes extend from the transplant into the molecular layer of the dentate gyrus (between arrows). Host neurons and neuropil do not stain because they possess a different Thy-1 allele (Thy-1.1), which is not recognized by the antibody (×95). (*B*) Two ChAT-immunoreactive neurons (×720) from a hippocampus immunostained 6 months after transplantation of control basal forebrain cells. The morphology of these neurons resembles that of normal nontransplanted septal cholinergic neurons. (*C*) Two ChAT-immunoreactive neurons (×490) from the opposite hippocampus of the same animal that was transplanted with Ts 16 basal forebrain. Note the shrunken appearance and decreased ChAT immunostaining of these cells.

$\pm$ 1.7 μm^2, n = 507 (mean $\pm$ SEM; P = 1.00, Student's t test)]. This result with cresyl violet was confirmed using antibodies to APP (see below), which identified many grafted trisomic and control neurons (Ts 16, 131.5 $\pm$ 3.7 μm^2, n = 102; controls, 127.4 $\pm$ 3.2 μm^2, n = 102, P = 0.39). Although there was a decrease in the number of cholinergic neurons at 6 months, the change was not statistically significant (Table 1).

Assessment of Transplants in the Denervated Hippocampus. Fimbria–fornix transection denervates the host hippocampus of basal forebrain afferents and has been shown to stimulate hypertrophy of transplanted fetal cholinergic and sympathetic neurons (20, 42). The cause of the hypertrophy is unknown but it may occur through increased availability of trophic factors and of synaptic sites. We asked whether Ts 16 cholinergic neurons would respond to fimbria–fornix transection. As examined 6 months after transplantation, both Ts 16 and control cholinergic neurons were significantly larger in the denervated hippocampus (Fig. 2*A*). Ts 16 neuronal area increased by 49% and controls by 31%. Interestingly, the mean size of Ts 16 cholinergic neurons at 6 months with a fimbria–fornix lesion was somewhat greater than that of control cholinergic neurons in the nondenervated hippocampus (Fig. 2*A*; Ts 16, 187.9 $\pm$ 6.0 μm^2; control, 166.2 $\pm$ 5.1 μm^2; $P < 0.01$). The size distribution of both populations was quite similar (Fig. 2 *B* and *C*). Thus, the Ts 16 cholinergic response was relatively robust. Nevertheless, Ts 16 cholinergic neurons were still smaller than controls in the denervated hippocampus (Fig. 2*A*).

The effect of hippocampal denervation on neuronal size was not selective for cholinergic neurons. Noncholinergic neurons also responded to fimbria–fornix lesion with a smaller increase in size (Ts 16, $P < 0.001$; controls, $P < 0.001$). After fimbria–fornix lesion, the sizes of Ts 16 and control noncholinergic neurons were similar, whether detected by cresyl violet staining (Ts 16, 136.2 $\pm$ 2.0 μm^2, n = 423; control, 134.5 $\pm$ 2.0 μm^2, n = 415, P = 0.548) or by immunostaining for APP (data not shown). Total graft area increased by almost 2-fold after denervation; however, variability between grafts was large and the change was not significant (Table 1).

Table 1. Morphologic assessment of basal forebrain grafts after survival of 1 and 6 months

Graft	Graft size, mm^3		Cholinergic neurons, no. per graft	
	Ts 16	Control	Ts 16	Control
No lesion/1-month survival	0.23 $\pm$ 0.08	0.25 $\pm$ 0.06	119 $\pm$ 30	133 $\pm$ 18
No lesion/6-month survival	0.27 $\pm$ 0.06	0.22 $\pm$ 0.11	115 $\pm$ 31	150 $\pm$ 38
Lesion/6-month survival	0.52 $\pm$ 0.19	0.45 $\pm$ 0.24	215 $\pm$ 54	219 $\pm$ 23

Data are mean $\pm$ SEM. There was no significant difference in graft size or number of cholinergic neurons between Ts 16 and control transplants at the time points and conditions listed in the table. The lesion was in the fimbria–fornix. (N = 5 for no lesion, 1-month survival and a fimbria–fornix lesion at a 6-month survival; N = 7 for no lesion and a 6-month survival.)

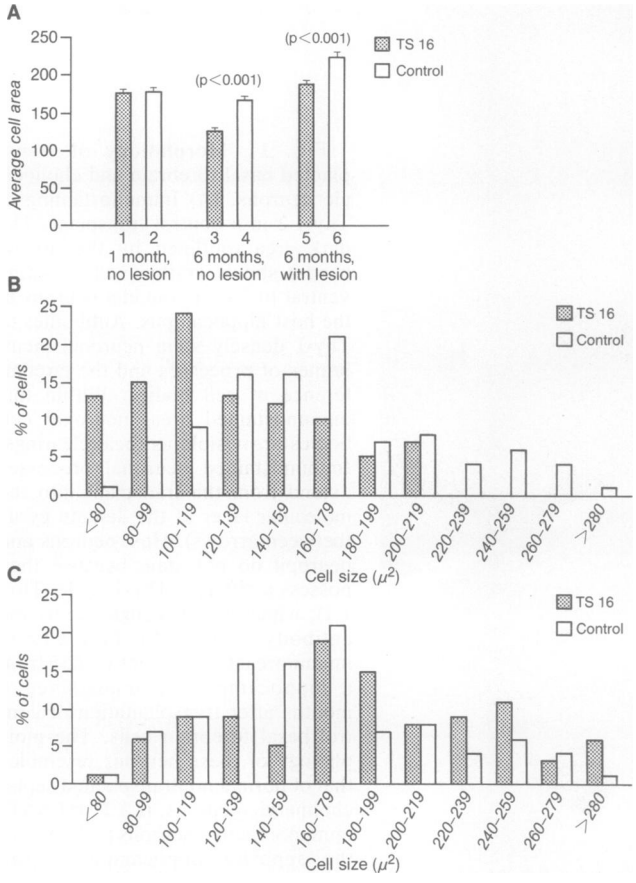

FIG. 2. Examination of trisomic and control cholinergic neuronal size at 1 and 6 months after transplant. (*A*) Cross-sectional area (μm^2) of transplanted cholinergic neurons. Bars: 1 and 2, 1 month survival, no fimbria–fornix lesion; 3 and 4, 6 months survival, no fimbria–fornix lesion; 5 and 6, 6 months survival, with bilateral fimbria–fornix lesion. Data are mean ± SEM. Bars: 1, Ts 16 = 175.9 ± 5.2 μm^2 (n = 106 neurons; N = 5 transplants); 2, control = 178.4 ± 5.3 μm^2 (n = 104; N = 5); 3, Ts 16 = 125.9 ± 4.2 μm^2 (n = 145; N = 7); 4, control = 166.2 ± 5.1 μm^2 (n = 146; N = 7); 5, Ts 16 = 187.9 ± 6.0 μm^2 (n = 102; N = 5); 6, control = 224.3 ± 6.4 μm^2 (n = 104; N = 5). (*B*) Size distribution of trisomic and control cholinergic neurons after 6 months in an unlesioned host. The number of neurons in each of several size classes is presented. Values are derived from the data used in *A* and show that there is a shift in the size distribution of the entire population of Ts 16 cholinergic neurons. (*C*) Size distribution of trisomic cholinergic neurons after 6 months in a lesioned host vs. control cholinergic neurons after 6 months in an unlesioned host.

Analysis of Other AD Markers. To ask whether Ts 16 grafts demonstrated other neuropathological markers seen in AD, we examined both frozen and paraffin-embedded transplants for the presence of β/A4 deposition, one of the earliest AD markers to appear in the DS brain (11). In sections of paraffin-embedded (n = 3) or frozen mouse brain (n = 12), we found no definite evidence within grafts or their projections of immunoreactivity resembling either diffuse or neuritic plaques. However, in frozen as opposed to paraffin-embedded sections, host neurons did stain with antibodies to β/A4-(1–28) (Fig. 3*B*), as shown (22). The staining was eliminated if the antibodies were preincubated with synthetic rodent or human β/A4-(1–28) (100 μg/ml). Immunohistochemistry with an antibody to the C terminus of APP gave an identical pattern (23). Therefore, we interpret our β/A4 staining within neurons as due to the presence of the β/A4 region within APP. In frozen sections, β/A4-(1–28) antibodies also stained Ts 16 and control basal forebrain neurons within transplants (Fig. 3 *B–D*). No clear difference was noted in staining intensity or pattern between Ts 16 and control grafts. Congo red and Bielschowsky silver staining revealed no evidence of amyloid or neurofibrillary tangles in grafts.

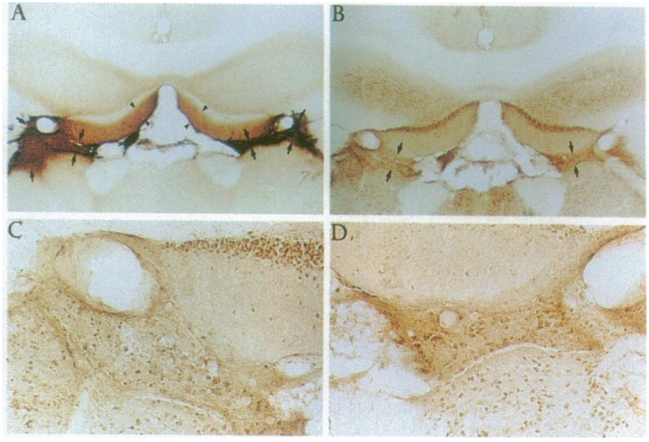

FIG. 3. β/A4 and APP antibodies identify host neurons and transplanted Ts 16 and control basal forebrain neurons. (*A*) Thy-1.2 immunostaining of Ts 16 (on the left) and control (on the right) basal forebrain transplants. Both transplants (arrows) abut the dentate gyrus. Note immunostaining in the molecular layer of the dentate gyrus bilaterally (between arrowheads) indicating process outgrowth from transplanted neurons (×20). (*B*) Adjacent section stained with an antibody to β/A4-(1–28) reveals staining of neurons in both host and tissue. Ts 16, left arrows; control, right arrows. Note dark staining in dentate granule and hippocampal pyramidal neurons with little or no staining in the corpus callosum (×20). Higher magnification of Ts 16 (*C*) and control (*D*) transplants showing numerous β/A4-immunostained cell bodies. Cresyl violet counterstaining indicated that most immunostained cell bodies were neurons (×75).

DISCUSSION

The development of animal models is critical for understanding the pathogenesis of neurodegenerative diseases such as AD. In these studies, mouse Ts 16 was used to examine whether an extra copy of chromosomal material homologous to human chromosome 21 produced aspects of the DS and AD phenotype. We transplanted fetal basal forebrain cells from Ts 16 and control littermates into the hippocampus of normal subjects to compare their survival and growth. Ts 16 transplants demonstrated age-related cholinergic neuronal atrophy. Fimbria–fornix transection produced an increase in the size of these neurons such that they were actually larger than control cholinergic neurons in the nondenervated hippocampus. Our results indicate that Ts 16 basal forebrain transplants provide an animal model of cholinergic neuronal degeneration and raise the possibility that this model can be used to explore the pathogenesis and treatment of cholinergic and other neuronal degeneration in DS and AD.

Several observations suggest that Ts 16 does not affect all neurons. Normal and Ts 16 grafts make extensive and specific neuronal projections. Moreover, neither graft size nor the size of noncholinergic neurons differentiated them. Indeed, atrophy of cholinergic neurons was not apparent 1 month after transplantation. Our studies demonstrated atrophy of cholinergic neurons between 1 and 6 months after transplantation. Cholinergic atrophy and loss of cholinergic markers is a prominent feature of the AD brain and may contribute to dementia (2, 17, 29). Interestingly, morphometric and immunohistochemical studies of the AD basal forebrain using antibodies to ChAT and nerve growth factor receptor have documented shrinkage of immunoreactive cell bodies (26, 27). By using ChAT immunocytochemistry, a 25% decrease was found (26). A similar result, showing a 25% decrease in septal cholinergic neuronal size, has also been

observed in age-impaired rats as compared to young adult subjects (28). As was the case in our model, an exhaustive study by Vogels *et al.* (17) found no decrease in the number of cholinergic neurons in the septum of AD patients. These observations on the size and number of septal cholinergic neurons parallel our findings in the Ts 16 implants. Further study will demonstrate whether cell death and other AD neuropathological features are found after longer survival times. Although other Ts 16 neurons examined in this study were of normal size, we cannot rule out involvement of selected noncholinergic subpopulations among basal forebrain neurons. Further study will demonstrate whether or not Ts 16 affects neuronal populations outside the basal forebrain known to be vulnerable in AD.

Transplantation into the normal hippocampus was used to provide grafts with an equivalent normal target. Although it is possible that control grafts were more successful in eliciting trophic support, the data for the size and number of cholinergic neurons at 1 month and for all other neurons at 1 and 6 months suggest that this was not the case. Thus, it is probable that a factor(s) intrinsic to Ts 16 cells produced cholinergic atrophy. The nature of this factor is unknown. It may have created its effect by actions within grafted neurons or grafted glial cells or through interactions between grafted cells and the host hippocampus. Whatever its cause, its existence must be linked to the presence of an extra copy of one or more genes or regulatory sequences on mouse chromosome 16.

The response of Ts 16 cholinergic neurons to host hippocampal denervation suggests that molecules produced in the denervated target enhanced the trophic state of these cells. Likely candidates are nerve growth factor and brain-derived neurotrophic factor, members of a family of neurotrophic molecules that are produced in hippocampus and act on basal forebrain cholinergic neurons (30–34). Perhaps relevant to observations reported herein is that exogenous nerve growth factor prevents the atrophy of these neurons after axotomy (35, 36) and has been shown to increase ChAT activity in fetal Ts 16 basal forebrain cultures (37). If nerve growth factor prevents or reverses atrophy of Ts 16 cholinergic neurons *in vivo*, then specific neurotrophic molecules may be used to reverse or prevent genetically determined neurodegeneration.

We initiated our studies anticipating that β/A4-containing plaques might be observed in the transplants. This was not the case after 6 months. It is possible that prolonged survival is necessary for β/A4 deposits to develop in this model. Previous studies in the other rodent models of neurodegeneration have not demonstrated amyloid plaques (38). Recently, mice transgenic for human forms of APP have demonstrated extracellular (39) β/A4-immunostained deposits in the cortex and hippocampus. These findings suggest that β/A4 deposits resulted from increased APP expression due to the presence of the transgene or that the structure or processing of human APP differs from rodent APP. Whatever the pathogenesis of β/A4 deposition in AD, our results with Ts 16 show that cholinergic abnormalities occur in the absence of amyloid deposition.

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