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Cerebral cortical astroglia from the trisomy 16 mouse, a model for Down syndrome, produce neuronal cholinergic deficits in cell culture

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ABSTRACT **Trisomy 21 (Down syndrome) is associated with a high incidence of Alzheimer disease and with deficits in cholinergic function in humans. We used the trisomy 16 (Ts16) mouse model for Down syndrome to identify the cellular basis for the cholinergic dysfunction. Cholinergic neurons and cerebral cortical astroglia, obtained separately from Ts16 mouse fetuses and their euploid littermates, were cultured in various combinations. Choline acetyltransferase activity and cholinergic neuron number were both depressed in cultures in which both neurons and glia were derived from Ts16 fetuses. Cholinergic function of normal neurons was significantly down-regulated by coculture with Ts16 glia. Conversely, neurons from Ts16 animals could express normal cholinergic function when grown with normal glia. These observations indicate that astroglia may contribute strongly to the abnormal cholinergic function in the mouse Ts16 model for Down syndrome. The Ts16 glia could lack a cholinergic supporting factor present in normal glia or contain a factor that down-regulates cholinergic function.**

Glia play a supportive role in the life of neurons. Several glia-derived molecules have been identified that are critical for neuronal development (1). To test the hypothesis that some neuropathological conditions are a result of abnormal functioning of the relationship, we utilized the trisomy 16 (Ts16) mouse, a well-established animal model for human trisomy 21 or Down syndrome (DS) (2). The cellular and molecular pathology of DS, which results in developmental delay and mental retardation of over one million individuals in America, is not known (3). It has been suspected that the extra dose of one region of chromosome 21 may be particularly important in the pathogenesis of DS syndrome (4). The homologous trisomy of chromosome 16 in the mouse and the partial trisomy of chromosome 16 (Ts65Dn) have been proven to be useful models for studying the pathology of the human disease (5, 6). Subsequent studies with the Ts65Dn model have shown behavioral abnormalities in trisomic mice, both in the adult and during development, as well as histologic pathology which included cholinergic neuronal loss and astrocytic abnormalities. We prepared dissociated cell cultures of normal (diploid) and Ts16-derived neurons and astroglia separately and grew them together in different combinations. We posed the question, do glia from Ts16 animals produce abnormal development of co-cultured normal neurons? The index of neurodevelopment we used is a cholinergic marker, the enzyme choline acetyltransferase (ChAT), because deficits in cholinergic systems are among the pathological changes that characterize the Ts16 mouse, DS, and Alzheimer disease (7, 8).

METHODS

Trisomic Animals and Cell Cultures. Ts16 mice were produced as described, by breeding mice with Robertsonian translocation of chromosome 16 (9). The identification of trisomic fetuses has been confirmed by analysis of chromosome spreads (10). The dissociated cell cultures were made from the trisomic animals and compared with cultures made from the normal littermates. The cultures were prepared essentially as described (11). Spinal cords were removed from embryonic day 14 mouse embryos for the neuronal population. The cerebral cortices of newborn mice were used to obtain a glia population (12). These preparations have been determined by immunocytochemical and biochemical criteria to be greater than 96% type I astrocytes (13). The glial cells were plated first and the neuronal population was added about 1 week later when the initial glial plating was confluent.

Cell Counts. Neuronal counts were made without knowledge of the nature of the cultures. Fifty to 100 microscopic fields at predetermined locations were made of glutaraldehyde-fixed material with phase contract optics or paraformaldehyde-fixed preparations stained immunocytochemically for neuron-specific enolase. Large multipolar cholinergic neurons with long axons were identified with an antibody against ChAT (14). All cholinergic neurons were counted on each plate used for this determination without knowledge of the type of culture being counted. The counts of ChAT-positive neurons were corrected for any difference in total neuron number between control and the other cultures. For both the ChAT and glial fibrillary acidic protein immunostaining cultures were fixed in 4% paraformaldehyde.

ChAT Measurement. ChAT enzyme activity was determined by the method of Fonnum (15). Cells were scraped from the culture dishes in approximately 200 microliters of PBS. Nonspecific activity was determined and subtracted from the total counts by either omitting choline from the reaction mixture or by doing reactions on tissue from glial cultures. The two methods gave similar results for the net activity.

RESULTS

Spinal cord neuronal cultures prepared from Ts16 embryos were not readily distinguishable microscopically from cultures prepared from normal littermates and, when neurons were counted in several cultures from five platings, the number of

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Abbreviations: Ts16, trisomy 16; DS, Down syndrome; ChAT, choline acetyltransferase.

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Table 1. Cholinergic function in normal and trisomic cell cultures (±S.D.)

	(1)	(2)	(3)	(4)
Neurons	Normal	Ts16	Normal	Ts16
Glia	Normal	Ts16	Ts16	Normal
A. Total neuron number Per 50 fields	450 ± 49.5 (n = 15)	447 ± 111 (n = 14)	403 ± 146 (n = 8)	512 ± 139 (n = 8)
B. ChAT enzyme activity, nM/hr/dish	1.05 ± .41*,† (n = 14)	0.38 ± .30*,‡ (n = 10)	0.63 ± .35† (n = 13)	0.67 ± .25‡ (n = 14)
C. ChAT positive neuron number, % of NN/NG	100 ± 8.5§ (n = 14)	74.2 ± 24§ (n = 13)	86.3 ± 21.5 (n = 11)	87.7 ± 24 (n = 10)
D. ChAT enzyme per cholinergic neuron, B/C × 100	1.05	0.51	0.75	0.76

n refers to the number of individual culture dishes for which determinations were made. Data for total neuron number were obtained from five different experiments, for ChAT enzyme activity from four and for ChAT positive neurons from five. ANOVA showed significant ($P < 0.001$) effect of Ts16 component of either neurons or glia on ChAT enzyme activity. Glial, but not neuronal component had an effect on ChAT positive neuronal number ($P < 0.036$ for glial effect; $P > .058$ for neuronal effect). For pairs of values marked with the same symbols, the significance levels of the differences were as follows: *, $P < 0.001$; †, $P < 0.02$; ‡, $P < 0.05$; §, $P < 0.002$.

neurons in the normal and trisomic cultures were not different (Table 1, row A). When these cultures were compared with respect to both ChAT enzyme activity and cholinergic neuron number, however, highly significant decreases below control

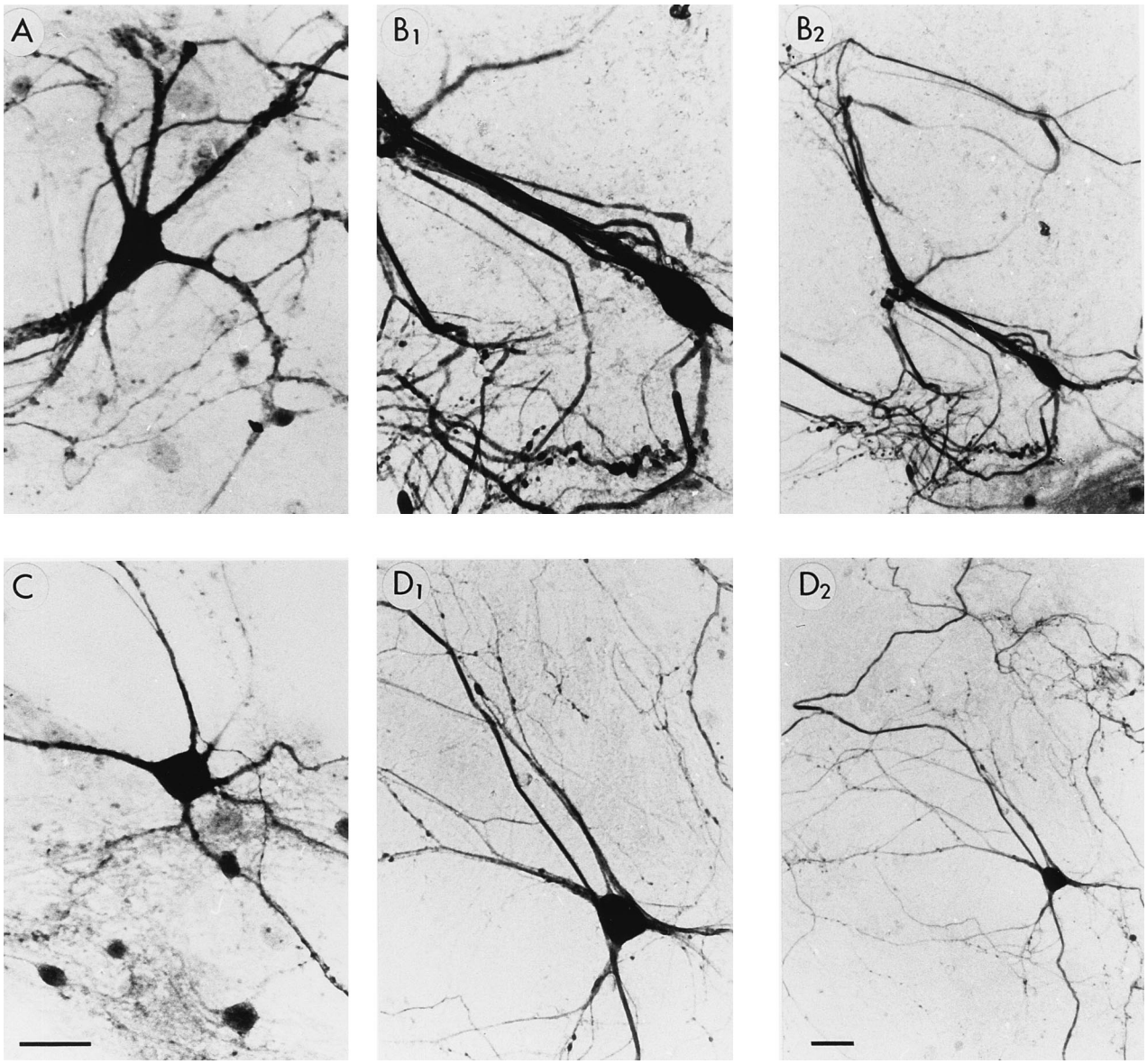

FIG. 1. Cultures from normal (A, B_1, and B_2) and Ts16 fetuses (C, D_1 and D_2) stained with an antibody to ChAT. (Bars = 50 μm; A, B_1, C, and D_1, ×200; B_2 and D_2, ×100). Note the thicker, more elaborate processes in normal than in Ts16 neurons.

values (Table 1, column 1) were evident in trisomic cultures (column 2) for both these indicators of cholinergic function ($P < 0.001$ for ChAT enzyme; $P < 0.002$ for cholinergic neuron number). The decrease in ChAT activity was greater than the decrease in cholinergic neuron number, so the calculated ChAT activity per cholinergic neuron also was decreased in the trisomic cultures (Table 1, row D, columns 1 and 2). Compatible with this calculation, the trisomic cholinergic neurons were generally smaller and less complex morphologically than the normal neurons (Fig. 1 *A* and *B* vs. *C* and *D*). This apparent difference was quantified by measuring the length of neuronal processes in a sample of five neurons from each culture type, which were selected as the largest examples from a larger random sample from two cultures of each condition. Average process length was 3.2 ± 1.2 mm (±SD in this and subsequent comparisons) in the control and 1.7 ± 0.7 mm in the trisomic condition, a difference significant at the 0.05 level. It should be noted that because cholinergic neurons comprise a minority (<5%) of the total neuronal population, a decrease in cholinergic neuron number would not be expected to be detectable as a change in total neuron number.

One of our goals was to test the hypothesis that any cholinergic deficit associated with the Ts16 genotype could be attributed at least in part to abnormal glial function. Consistent with this hypothesis, Table 1 shows that normal neurons growing on Ts16 glia (TG+NN) (Table 1, column 3) had significantly ($P < 0.02$) less ChAT activity than NG+NN cultures. On the other hand, the difference between the TG+NN and the TG+TN cultures (Table 1, columns 2 and 3) was not statistically significant. We conclude that trisomic glia can diminish cholinergic expression in normal neurons.

A corollary hypothesis would be that normal glia might increase the cholinergic expression of Ts16 neurons. To test this hypothesis, we grew Ts16 neurons on normal glia (Table 1, column 4) and compared the ChAT activity of such cultures with that of Ts16 neurons grown on Ts16 glia. This test is somewhat ambiguous because the Ts16 neuronal preparations plated on normal glia inevitably include some Ts16 glia. Nevertheless, the experiments did show some "protective" effect of normal glia on Ts16 neurons: the NG+TN cultures expressed 76% more ChAT activity than the TG+TN cultures ($P < 0.05$), although still significantly less than the normal cultures. Total neuronal counts and cholinergic neuron counts were not significantly different in the heterologous preparations (Table 1).

In the heterologous cultures, the preplated glia become mixed with glia introduced with the subsequently plated neurons, and the contribution of these secondarily added glia cells is proportional to the density of the neuronal plating. In cultures with a relatively low density of neurons, the heterologous cultures containing preplated Ts16 glia expressed less cholinergic function (ChAT-positive neuron number or ChAT enzyme activity) than did the heterologous cultures containing normal glia. Fig. 2 shows that the relative expression of cholinergic function in the heterologous cultures was strongly dependent on neuronal density, whereas this dependence was less strong for the homologous cultures. At the lowest neuronal density, the relative cholinergic expression of the two types of heterologous cultures was like the relative expression of the homologous cultures corresponding to the glial component of the heterologous cultures (Fig. 2 *Left*). The ratio of TG+NN/NG+TG was like that of TG+TN/NG+NN. The inverse was true for higher density cultures. Thus, in the cultures with low numbers of neurons, the effect of preplated glia could be seen most clearly. Fig. 3 shows results from experiments with the lowest neuronal number or ChAT expression, demonstrating the effects of the preplated glia. The heterologous cultures containing normal glia (but Ts16 neurons) were very similar to the homologous normal cultures (Fig. 3*A*) and expressed significantly more cholinergic function than did the homologous Ts16 cultures. By contrast, the heterologous cultures containing Ts16 glia (but normal neurons) were like the homologous Ts16 cultures (Fig. 3*B*) and expressed significantly less cholinergic function than did the normal cultures. ANOVA indicated that the glia component of the cultures accounted for ChAT activity and ChAT-positive neuron number ($P < 0.001$), whereas the neuronal component did not ($P > 0.6$).

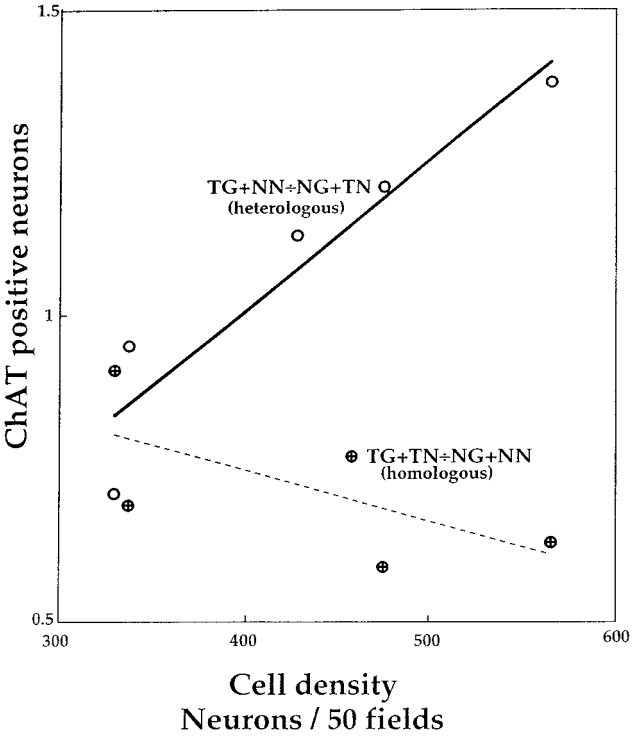

FIG. 2. The relative cholinergic expression (number of ChAT-containing neurons) in homologous and heterologous cultures as a function of the number of neurons in the cultures. The open circles show the cholinergic function as the ratio of normal neurons on trisomic glia to trisomic neurons on normal glia. The circles with crosses show that ratio of expression for trisomic neurons on trisomic glia to that for normal neurons on normal glia. Note that at low neuronal density, the heterologous cultures containing Ts16 glia have relatively low numbers of cholinergic neurons, whereas at high neuronal density the heterologous cultures having Ts16 neurons have the lower number of cholinergic neurons.

Staining of the normal and trisomic cultures with an antibody against the glial fibrillary acidic protein revealed no obvious difference in the appearance of NG+NN and TG+TN astroglia (data not shown).

DISCUSSION

Our results indicate that normal and Ts16 glia differ with regard to their ability to support cholinergic function in neurons. Abnormal Ca^{2+} regulation has been demonstrated in astrocytes from Ts16 cerebral cortex and hippocampal cortex (16, 17). Glial hypertrophy has been described in adult Ts65Dn mice (6). Increased numbers of neuropeptide Y (NPY)-containing neurons (and morphological abnormalities in NPY neurons) in cultures from Ts16 cerebral cortex were shown to be normalized by coculture with euploid glia. Normal neurons grown on Ts16 glia began to resemble trisomic NPY neurons (18).

Ts16 neurons may also be deficient in a number of regards, including the expression of cholinergic function (19). In a transplantation model, Ts16 neurons were normal at 1 month posttransplantation but showed decreases in area (but not

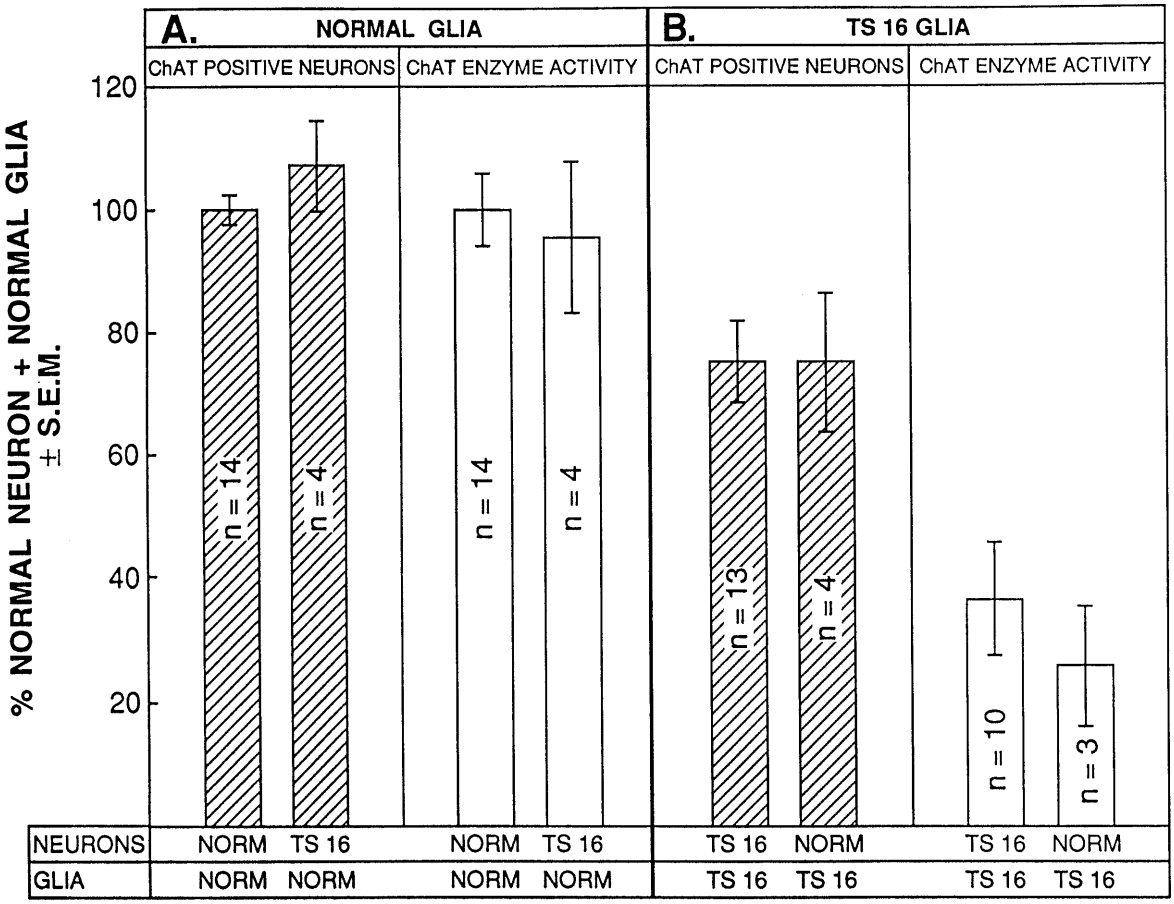

FIG. 3. Plots of the ChAT enzyme levels and cholinergic neuron number in homologous and heterologous cultures expressed as % of NG+NN. For the homologous cultures, the values are taken from Table 1; for the heterologous cultures (TG+NN and NG+TN), the data are taken from cultures with the lowest neuron density in each series of experiments.

number) at 6 months (20). However, the deficits seen in our experiments at 1 month in culture can largely be explained in terms of glial dysfunction. Normal neurons show deficits on trisomic glia and trisomic neurons are partially or completely "cured" of their cholinergic deficits in the presence of normal glia.

The basis for the glia effect on cholinergic function is unknown. Although β amyloid precursor protein (βAPP) mRNA is overexpressed in trisomic tissue (5), in the Ts65Dn trisomic mouse this does not result in βAPP deposition (6), suggesting the involvement of other molecules that might be involved in the pathogenesis of the disease (21). Alternatively, normal glia may provide some positive, supporting factor for cholinergic neurons such as nerve growth factor, which has been demonstrated to elevate cholinergic markers in Ts16 basal forebrain neurons (19) and which may be lacking in the Ts16 glia. Consistent with this latter possibility are observations showing that neurons grown in serum-free medium (in which glia do not survive) show only about 25% as much ChAT activity as similar neurons grown in glia conditioned medium (D. E. Brenneman, personal communication).

In either case, the present results indicate that the abnormalities associated with the Ts16 glia involve both the number of cholinergic neurons and the ChAT expression per cholinergic neuron. This effect is specific in that total neuronal numbers are not differentially affected by the different types of glia. The molecular basis of the glia effect and whether it is due to cell contact or a diffusible substance are experimentally approachable questions.

Regulation of cholinergic function by glia would be expected to influence cerebral cortical electrical activity, because cholinergic inputs to the cerebral cortex play a critical role in maintaining cortical activity and plasticity (22–24). Thus, the glia effects shown here could have a secondary impact on cortical integrity related to activity-dependent neurotrophic phenomena (25). Cholinergic input to the cerebral cortex down-regulates βAPP production (26), and secretion of such trophic materials as activity-dependent neurotrophic factor and nerve growth factor can be induced by stimulation of glia by cholinergic, peptidergic, or catecholaminergic inputs (27, 28). The effectiveness of trophic factors may depend on concurrent neural electrical activation (29). Taken together, these observations and the work reported here suggest that a "vicious cycle" initiated by glia and involving down-regulation of the cholinergic system with an attendant altered cortical electrical activity may contribute to the neurodegenerative process seen in DS and Alzheimer disease.

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