Alterations of Neocortical Pyramidal Cell Phenotype in the Ts65Dn Mouse Model of Down Syndrome: Effects of Environmental Enrichment

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Mental retardation in individuals with Down syndrome (DS) is thought to result from anomalous development and function of the brain; however, the underlying neuropathological processes have yet to be determined. Early implementation of special care programs result in limited, and temporary, cognitive improvements in DS individuals. In the present study, we investigated the possible neural correlates of these limited improvements. More specifically, we studied cortical pyramidal cells in the frontal cortex of Ts65Dn mice, a partial trisomy of murine chromosome 16 (MMU16) model characterized by cognitive deficits, hyperactivity, behavioral disruption and reduced attention levels similar to those observed in DS, and their control littermates. Animals were raised either in a standard or in an enriched environment. Environmental enrichment had a marked effect on pyramidal cell structure in control animals. Pyramidal cells in environmentally enriched control animals were significantly more branched and more spinous than non-enriched controls. However, environmental enrichment had little effect on pyramidal cell structure in Ts65Dn mice. As each dendritic spine receives at least one excitatory input, differences in the number of spines found in the dendritic arbors of pyramidal cells in the two groups reflect differences in the number of excitatory inputs they receive and, consequently, complexity in cortical circuitry. The present results suggest that behavioral deficits demonstrated in the Ts65Dn model could be attributed to abnormal circuit development.

Introduction

Down syndrome (DS) is the most common genetic cause of mental retardation, affecting 1 in 1000 newborn children in Europe (Hassold and Jacobs, 1984; Mastroiacovo, 2002). Adult DS brains are smaller than those of normal individuals, particularly the frontal lobes and cerebellum (Pinter *et al.*, 2001), and the depth and number of sulci are reduced. Neuronal density is decreased in distinct cerebral regions and abnormal neuronal morphology is also observed [reviewed in Flórez (Flórez, 1992)]. The fetal DS brain presents a reduction in the width of the cortex, abnormal cortical lamination and reduced synaptic density. In addition, neurons in fetal DS brains are characterized by abnormal dendrites and dendritic spines, abnormal synaptic morphology and abnormal membrane properties (Marin-Padilla, 1976; Suetsugu and Mehraein, 1980; Becker *et al.*, 1986, 1993; Ferrer and Guillota, 1990).

The abnormal development and maturation of the DS brain is believed to be regulated by specific genetic loci in human chromosome 21 (HSA21), those in the DS critical region (DSCR) being considered primary candidates for the phenotypic alteration (Delabar *et al.*, 1993). Thus, mice that are complete or partially trisomic for murine chromosome 16 (MMU16), which has regions of conserved homology with HSA21, have been proposed as models for DS. In the full MMU16 trisomic mouse, Ts16, abnormal embryonic development results in a reduction in the overall number of neurons of the neocortex (Haydar *et al.*, 1996, 2000). The Ts65Dn mouse (Davisson *et al.*, 1990) is a partial trisomic mouse that includes most of the MMU16 region (from *App* to *Mx1*) syntenic to the DSCR of HSA21. Behavioral studies have demonstrated Ts65Dn mice to be poor learners (Escorihuela *et al.*, 1995, 1998; Reeves *et al.*, 1995; Holtzman *et al.*, 1996; Hyde and Crnic, 2001). In addition, morphological studies have demonstrated abnormalities in some brain regions of Ts65Dn mice, including reduction in the volume of the cerebellum and hippocampus, reduction in neuronal density in the dentate gyrus and in the number of excitatory synapses, and synaptic length, in the temporal cortex (Insausti *et al.*, 1998; Baxter *et al.*, 2000; Kurt *et al.*, 2000).

Early implementation of special education programs results in improved cognitive abilities in DS individuals (Connolly *et al.*, 1993; Foreman and Manning, 1986). However, the biological basis of this improvement is yet to be determined. The study of cortical circuitry in the Ts65Dn mouse provides an excellent model to study these mechanisms. Animals reared in enriched environments outperform conspecifics in learning, memory and visual acuity (Fernández-Teruel *et al.*, 1997; Prusky *et al.*, 2000). Structural differences in the cerebral cortex are thought to underpin these behavioral improvements. For example, normal animals raised in an enriched environment have more branched, and more spinous, neurons than those raised in a non-enriched environment (Rosenzweig *et al.*, 1972; Volkmar and Greenough, 1972; Globus *et al.*, 1973; Greenough *et al.*, 1973). However, to date, no study has addressed the issue as to how environment enrichment in DS models may affect neocortical pyramidal cell structure.

The purpose of the present study was to determine how cortical circuitry may differ between control and DS models, and how environmental enrichment influences circuit structure. We studied the structure of cortical pyramidal neurons in control and Ts65Dn mice reared in non-enriched and enriched environments. Pyramidal cells in Ts65Dn mice were considerably smaller, less branched and less spinous than those sampled from control animals. In addition, control animals raised in an enriched environment had more branched pyramidal cells that contained considerably more spines (~30% more) than non-enriched controls. However, this effect was not seen for Ts65Dn animals raised in enriched environments. Instead, environment enrichment had little effect on pyramidal cell structure in Ts65Dn mice. These results suggest that the partial trisomy of MMU16 affects the normal development/maturation of cortical circuitry, which may influence the behavioral abilities of these animals and their capability to respond to environmental stimulation.

Materials and Methods

Animals

Experimental mice were generated by repeated backcross of Ts65Dn females to C57/6Ei × C3H/HeSnJ (B6EiC3) F1 hybrid males. The parental

generation was obtained from the research colony at the Jackson Laboratory (Bar Harbor, ME). Mating of Ts65Dn mice was performed in the Laboratory of Developmental Biology (Animal Facility, University of Cantabria, Santander, Spain). All mice were karyotyped and euploid littermates of Ts65Dn mice served as controls. All experiments were performed according to the guidelines on animal welfare as set out in the NIH guidelines for the use and care of experimental animals and the experimental procedures approved by the local Animal Welfare Committee.

Housing and Enrichment Conditions

The experimental design consisted of four groups of 1-year-old female mice. After weaning (21 days of age) the animals were separated by sex, and Ts65Dn and control mice randomly assigned to, and reared under either non-enriched (NE) or enriched (EE) conditions. NE mice were housed two or three per cage (20 × 12 × 12 cm Plexiglas cage; 20 ± 2°C; 12 h light–dark cycle) with unrestricted access to food pellets and water. EE mice were housed eight per cage (42 × 50 × 20 cm split level cage with stainless steel top, floor and front wall, and plastic sides and back; 20 ± 2°C; 12 h light–dark cycle) that was equipped with an activity wheel, wooden swing, and various plastic and wooden toys (paper rolls, blocks, rocks of different colors) that were changed every 3 days. Mice raised under enriched conditions were provided with different types of food, but access was restricted and required the animals learn new methods to retrieve it. EE animals were housed under these enriched conditions from 3 weeks of age to 10 weeks of age, after which they were reared in NE cages.

Processing of Tissue

Animals were overdosed by lethal intraperitoneal injection of sodium pentobarbitone, and perfused intracardially with 4% paraformaldehyde (0.1 M, pH 7.4: PF). The brain was removed and the cortex of the left hemisphere was flattened between two glass slides (Welker and Woolsey, 1974), weighted, and left overnight in PF at 4°C. Sections (150 μm), were cut tangential to the cortical surface with the aid of a Vibratome, and prelabeled with 10^{-5} M 4,6 diamidino-2-phenylindole (Sigma D9542). By focusing through the serial tangential slices we were able to identify the cytoarchitectural differences between cortical layers allowing the identification of the section that contained layer III and subsequent injection of cells at the base of layer III [e.g. see fig. 3 of Elston and Rosa (Elston and Rosa, 1997)]. Cell injection methodology has been described in detail elsewhere (Buhl and Schlote, 1987; Einstein, 1988; Elston *et al*., 1997). Briefly, cells were injected individually with Lucifer Yellow (8% in 0.1 M Tris buffer, pH 7.4) by continuous current. Current was applied until the distal tips of each cell fluoresced brightly. Following injection, the sections were first processed with an antibody to Lucifer Yellow [1:400 000 in stock solution (2% bovine serum albumin (Sigma A3425), 1% Triton X-100 (BDH 30 632), 5% sucrose in 0.1 mol/l phosphate buffer)], then with a biotinylated species specific secondary antibody (Amersham RPN 1004; 1:200 in stock solution), followed by a biotin–horseradish peroxidase complex (Amersham RPN1051; 1:200 in phosphate buffer). DAB (3,3′-diaminobenzidine; Sigma D 8001) was used as the chromogen. Sections were photographed prior to, and following, immunohistochemical processing to determine whether any correction factor should be applied to the data as a result of shrinkage. Cells were injected in the left hemisphere [M2 of Franklin and Paxinos (Franklin and Paxinos, 1997)]. Because of the nature of the experiments, individuals from each litter were overdosed at 3–4 day intervals (i.e. the cell injection may take up to 24 h). As females housed within groups tend to synchronize their estrus cycle, the 3–4 day delay between experiments meant that they were euthanized at different stages during their cycle (both within and between litters). Thus, by selecting animals randomly, we minimized the possibility of an estrus-related effect.

Morphological Analysis

Cells were only included for analyses if they had a clearly distinguishable apical dendrite, their entire basal dendritic arbor was contained within the section and all dendrites were completely filled. Moreover, as pyramidal cell structure may vary between different cortical regions (Elston, 2000), we only included pyramidal cells that were injected in the same overlapping region of the frontal lobe in all cases. Cells were drawn with the aid of a camera lucida microscope attachment, and their dendritic arbor size was determined by calculating the area contained within a polygon that joined the outermost distal tips of the basal dendrites (Elston and Rosa, 1997). The branching pattern of cells was determined by counting the number of dendritic branches that intersected with concentric circles (centered on the cell body) of increasing radii (25 μm increments) for each cell (Sholl, 1953). The total length of all basal dendrites in each cell was determined with the aid of a digitizing tablet (SummaSketch III) and NIH image software (NIH Research Services, Bethesda, MD). The density of spines on the dendrites of pyramidal cells was determined by counting the number of spines per 10 μm increments of 20 randomly selected horizontally projecting dendrites of different cells (Valverde, 1967). All spine types, including sessile and pedunculate (Jones and Powell, 1969), were included in the spine counts. No correction factors (Feldman and Peters, 1979; Larkman, 1991) were applied to the spine counts as reconstruction of the cells at high power (×100 Zeiss immersion lens) allows the visualization of all spines that issue from the dendrites [i.e. the DAB reaction product is more opaque than the Golgi reaction product: figs 1 and 3 of Elston *et al*. (Elston *et al*., 1999*a*)]. No distinction was made between spine types. The total number of spines found in the basal dendritic arbor of the 'average' pyramidal cell was calculated by multiplying the average number of spines of a given portion of dendrite by the average number of branches for the corresponding region, over the entire dendritic arbor (Elston, 2001). All analysis was performed according to double-blind procedures.

Results

Control and Ts65Dn Mice Reared under Non-enriched (NE) Conditions

(i) Dendritic Arbor Size

One hundred and eighty labeled pyramidal cells (Fig. 1) were included for analysis. Upon initial inspection, it became clear that the basal dendritic arbors of layer III pyramidal cells of Ts65Dn mice differed to those of control animals (Fig. 2). Quantification of the size of the basal dendritic arbors (Fig. 3*A*) revealed that cells in Ts65Dn animals were markedly smaller than those in controls (mean ± SD: 3.0 ± 0.6 × 10^4 μm^2 and 5.05 ± 1.1 × 10^4 μm^2, respectively). A two-way unpaired *t*-test revealed the difference to be significant (t_{81} = 9.9; P < 0.001).

(ii) Branching Pattern and Dendritic Length

Quantification of the number of dendritic branches (Fig. 3*B*) revealed that the peak branching complexity in the arbors of Ts65Dn mice (23.25 ± 4.23) was less than that in controls (25.19 ± 5.9). Statistical analysis (repeated measures ANOVA) of the whole dendritic arbor revealed the difference to be significant [F(1,81) = 14.5, P < 0.001]. The total length of basal dendrites was also analyzed in both groups of animals. As shown in Figure 3*C*, the dendrites in Ts65Dn were significantly shorter than those in control animals (1.92 ± 0.31 × 10^3 μm and 3.35 ± 0.73 × 10^3 μm, respectively; t_{81} = 11.2; P < 0.001).

(iii) Spine distribution and number

Quantification of the spine density of basal dendrites (Fig. 3*D*) revealed that the maximum number of spines per 10 μm segment was 19.1 ± 4.12 for cells in Ts65Dn mice and 18.1 ± 5.49 for those in control animals. A repeated measures ANOVA on the distribution of spines along the entire length of the dendrites (as a function of distance from the soma to the distal tips) showed the differences to be significantly different between the two groups [F(1,78) = 81.6, P = 0.001]. By combining data from Sholl analyses with that of spine densities, we were able to calculate an estimate of the total number of spines in the basal dendritic arbor of the average cell. These

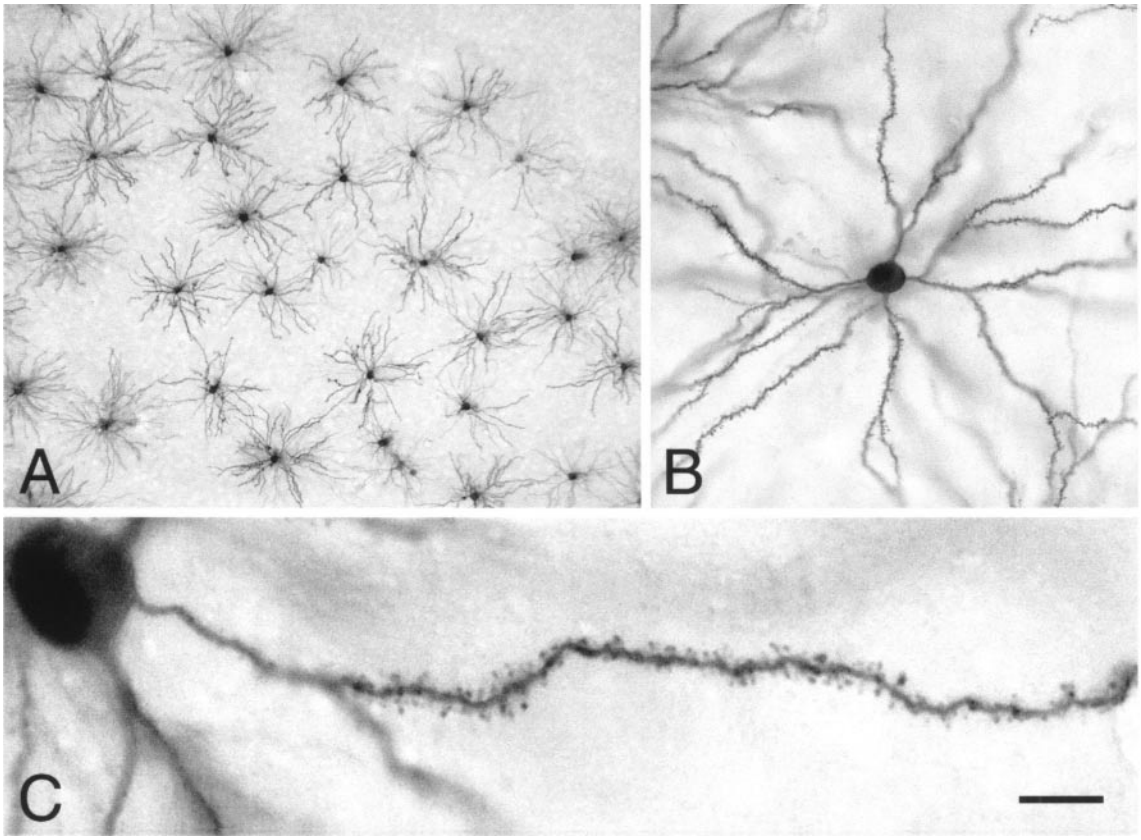
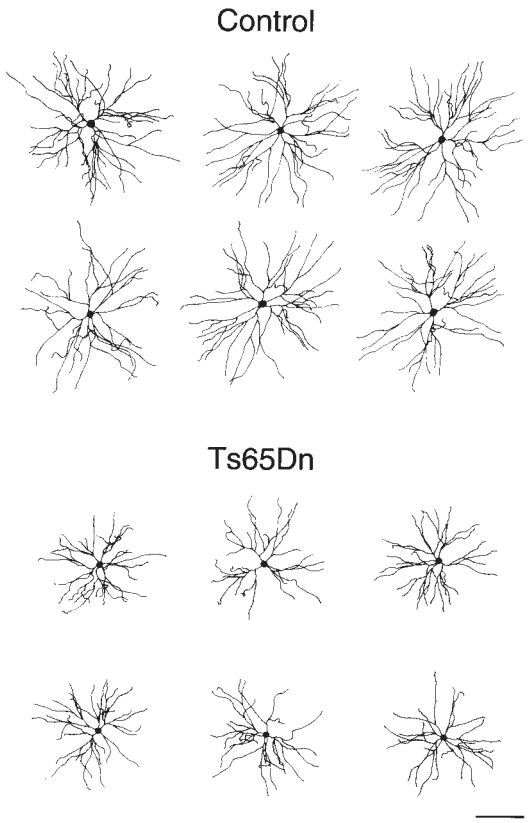


Figure 1. (*A*, *B*) Low-power photomicrographs of layer III pyramidal cells injected with Lucifer Yellow in 150μm thick slices, cut parallel to the cortical surface, and processed for a light-stable diaminobenzidine reaction product. (*C*) High-power photomicrograph of a horizontally projecting dendrite illustrating individual dendritic spines. Scale bar: 150μm in *A*; 34μm in *B*; 10μm in *C*.

Figure 2. Camera lucida drawings of layer III pyramidal neurons, as seen in the plane of section tangential to the cortical layers, from control and Ts65Dn mice. Illustrated cells had basal dendritic arbors which approximated the average size for each group. Scale bar = 100μm.



calculations revealed that pyramidal cells in Ts65Dn animals had 24% fewer spines (Fig. 3*E*) in their basal dendritic arbors (2603) than control animals (3447).

Control and Ts65Dn Mice Reared under Environmental Enriched (EE) Conditions

(i) Dendritic Arbor Size

As was the case for animals raised in NE conditions, the basal dendritic arbors of pyramidal cells (Fig. 3*A*) in EE Ts65Dn animals ($3.25 \pm 0.6 \times 10^4$ μm^2) were smaller than those in EE controls ($5.50 \pm 1.1 \times 10^4$ μm^2). A two-way unpaired *t*-test revealed the difference to be significant ($t_{94} = 13.6$; $P < 0.001$). However, environment enrichment had no significant effect on the size of the basal dendritic arbors of pyramidal cells in either control ($t_{74} = 1.6$, $P = 0.053$) or Ts65Dn ($t_{101} = 1.8$, $P = 0.064$) animals.

(ii) Branching Pattern and Dendritic Length

Quantification of the number of branches in the basal dendritic arbor of layer III pyramidal cells (Fig. 3*B*) revealed that the peak branching complexity for cells in enriched Ts65Dn mice (23.8 ± 4.58) was less than that in EE controls (28.5 ± 6.1). Moreover, analysis of the entire dendritic arbor revealed signifi-

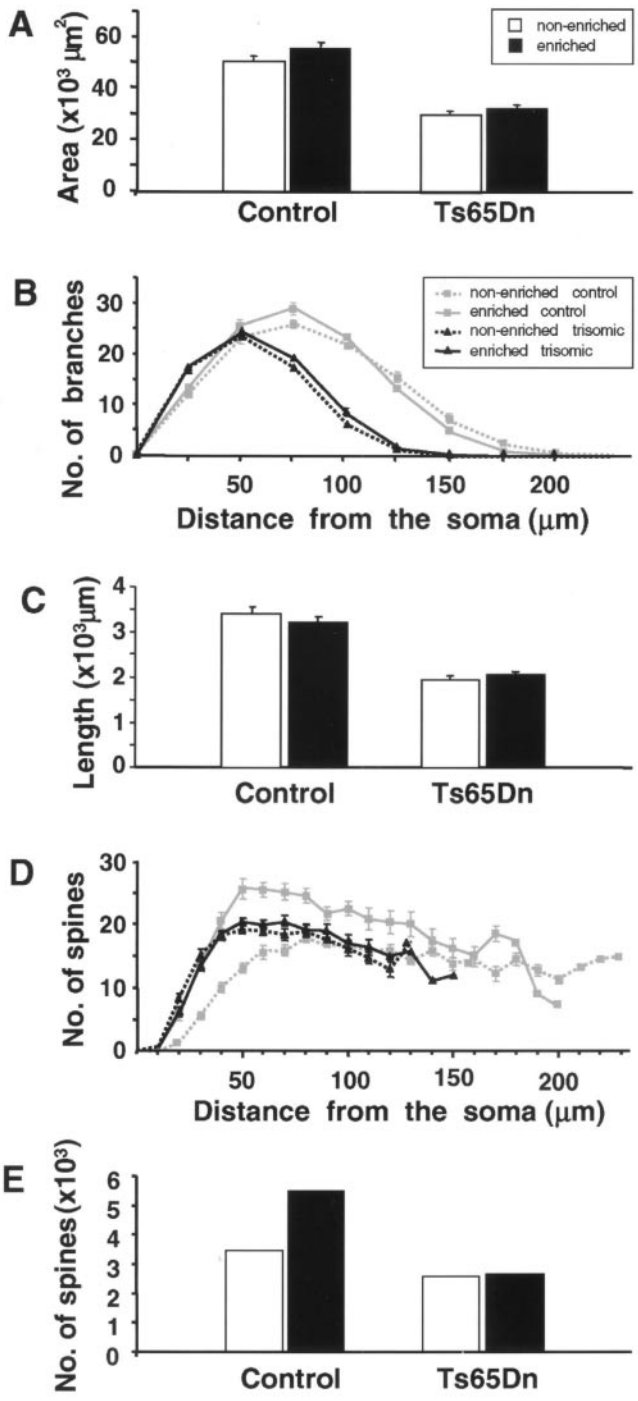


Figure 3. Plots of the areas (*A*), number of dendritic branches (*B*), length of dendritic branches (*C*), spine densities (*D*) and number of spines (*E*) in the basal dendritic arbors of layer III pyramidal cells sampled in enriched and non-enriched Ts65Dn and control mice.

cant differences in their branching patterns [$F(1,94) = 109.8$, $P < 0.001$]. Environment enrichment resulted in a significant increase in the number of dendritic branches of pyramidal cells in control animals [$F(1,74) = 43.3$, $P < 0.001$] but not in Ts65Dn animals [$F(1,101) = 3.8$, $P = 0.052$]. In addition, the total length of the basal dendrites in the basal arbors of pyramidal cells in EE Ts65Dn mice was also found to be significantly shorter than that in EE controls ($2.02 \pm 0.44 \times 10^3$ µm and $3.14 \pm 0.61 \times 10^3$ µm, respectively; $t_{94} = 9.8$, $P < 0.001$) (Fig. 3*C*). However, environment enrichment had no significant effect on the total dendritic length of cells in control or Ts65Dn animals ($t_{74} = 1.3$, $P = 0.2$; $t_{101} = 1.2$, $P = 0.24$, respectively).

(iii) Spine Distribution and Number

Quantification of the spines along the basal dendrites (Fig. 3*D*) revealed that the maximum spine density in EE Ts65Dn animals (20.4 ± 4.4) was less than that in EE control mice (25.7 ± 6.4). An analysis of variance revealed a significant difference the distribution of spines along the entire length of the dendrites [$F(1,58) = 78.4$, $P < 0.001$]. Moreover, environment enrichment resulted in a significant, and dramatic, increase in the spine density in control animals [$F(1,58) = 14.9$, $P < 0.001$] whereas there was no effect in Ts65Dn animals [$F(1,78) = 2.6$, $P = 0.11$]. Environment enrichment resulted in a dramatic increase (>32%) in the total number of spines in the basal dendritic arbor in control animals, but only resulted in a 3% increase in the number of spines in the basal dendritic arbors of pyramidal cells in Ts65Dn animals (Fig. 3*E*). Consequently, pyramidal cells in enriched Ts65Dn mice had fewer spines (2683) in their basal dendritic arbors than those in enriched control animals (5050).

Discussion

In the present study we have demonstrated that the basal dendrites of layer III pyramidal cells in the frontal cortex of Ts65Dn and control animals have markedly different phenotypes. Those in the Ts65Dn mouse are smaller, less branched and less spinous than controls. In addition, whereas environment enrichment had a marked effect on the structure of pyramidal cells in control animals, it had little effect in Ts65Dn mice. These data suggest that normal mechanisms that govern development and maturation of cortical circuits are affected by the partial MMU16 trisomy.

The Trisomic Ts65Dn Mouse As a Model for DS

Most of the genetic, behavioral and anatomical characteristics of the Ts65Dn mouse are consistent with those reported in DS patients [for reviews, see (Hasold and Jacobs, 1984; Carothers *et al.*, 1999; Kaufmann and Moser, 2000; Dierssen *et al.*, 2001)]. Both are characterized by trisomy of a homologous chromosomal region, relatively poor learning and memory, and abnormal brain structure (Escorihuela *et al.*, 1995, 1998; Reeves *et al.*, 1995; Coussons-Read and Crnic, 1996; Demas *et al.*, 1996; Holtzman *et al.*, 1996). In addition, Ts65Dn mice show behavioral disturbances compatible with abnormal function in the frontal cortex (Escorihuela *et al.*, 1995; Coussons-Read and Crnic, 1996). Of particular interest here is the structure of pyramidal cells, the most ubiquitous neuron in the neocortex. The present results on pyramidal cell structure parallel those obtained in humans. By studying autopsy material, several groups have reported that pyramidal cells in DS brains are less spinous than those in controls (Marin-Padilla, 1976; Suetsugu and Mehraein, 1980; Takashima *et al.*, 1981; Ferrer and Guillota, 1990). Here we found that pyramidal cells in Ts65Dn mice are, on average, 24% less spinous than those in controls. Given that trisomy of homologous genes in mice and humans causes similar anatomical and behavioral disturbances, it is likely that they are related. Thus, the Ts65Dn mouse provides means by which to study brain pathology in DS not possible in humans. More specifically, the Ts65Dn mouse provides a vehicle in which to study the microanatomical substrate of mental retardation in DS, and develop new treatment strategies.

Effects of Environmental Enrichment on Ts65Dn Mice

Normal healthy animals reared in an enriched environment outperform those raised in non-enriched environments in learning, memory, and visual acuity tasks (Fernández-Teruel *et al*., 1997; Prusky *et al*., 2000). The improvements in behavioral abilities are believed to result from changes in neural circuitry [for reviews, see (Kintsova and Greenough, 1999; Woolley, 1999)]. For example, improved behavioral abilities that result from various learning paradigms are paralleled by increases in the complexity of pyramidal cell branching structure and synapse to neuron ratio (Greenough *et al*., 1973, 1985; Withers and Greenough, 1989; Kleim *et al*., 1997, 1998; Johansson and Belichenko, 2002). Here we found a dramatic effect of environmental enrichment on cortical pyramidal cell structure in control animals. Three weeks of exposure to enriched stimuli during late development and maturation correlated with a higher number of spines in the dendritic arbors of layer III pyramidal cells in 1-year-old mice: enriched controls had, on average, 32% more spines in their arbors than non-enriched controls. Our findings in trisomic mice, however, revealed that those raised in an enriched environment only had 3% more spines in their dendritic arbors than those raised in a non-enriched environment. Thus, the present results suggest one of two possibilities; either there is a basic difference in cortical development whereby pyramidal cells in the Ts65Dn mouse never become as spinous as those in controls or, alternatively, they lose a higher proportion of spines during normal aging. Further studies are required in young mice soon after environment enrichment to determine the answer.

On the Correlation Between Cortical Structure and Function

As each spine receives at least one asymmetrical glutamatergic synapse (Colonnier, 1968; Jones, 1968; DeFelipe *et al*., 1988; Kharazia *et al*., 1996), more spinous cells may integrate more excitatory inputs than the less spinous cells. Differences in the branching patterns in the dendritic arbors of pyramidal cells have been shown to influence compartmentalization of processing (Koch *et al*., 1982, 1983) and the representational power (Poirazi and Mel, 2001) of cortical neurons. Thus, the structural differences reported here are likely to influence both cellular and systems cortical function [for reviews, see (Jacobs and Scheibel, 2002; Elston, 2003*a*,*b*)]. For example, studies in cortex of higher primates reveal that pyramidal cells in prefrontal cortex, which is thought to be important in cognitive processing (Goldman-Rakic, 1996; Fuster, 1997; Miller and Cohen, 2001), are more branched and more spinous than those in sensory association cortex which, in turn, are more branched and more spinous than those in sensory cortex (Jacobs *et al*., 1997, 2001; Elston *et al*., 1999*a*,*b*, 2001; Elston, 2000). Based on presently available data there appears to be a correlation between cortical function and pyramidal cell structure.

Conclusions

There are close parallels between the human DS and Ts65Dn mouse phenotypes that appear to result from trisomy of homologous genes of conserved function. The present data on the Ts65Dn murine model reveal that the *App* to *Mx1* region of the HSA21 homologue influences the micro-organization of neocortical circuits. It remains to be determined whether the present results reflect differences in the development or maturation of cortical circuitry. Further studies of murine models that overexpress specific subsets of these genes will help to dissect the molecular and cellular pathology of mental retardation in DS.

Notes

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