

# Altered Astrocyte Calcium Homeostasis and Proliferation in the Ts65Dn Mouse, a Model of Down Syndrome

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Genes from the Down syndrome (DS) critical region of human chromosome 21, which contribute to the pathology of DS, are also found on mouse chromosome 16. Several animal models of DS with triplication of genes from the DS critical region have been generated, including mouse trisomy 16 (Ts16) and a partial trisomic mouse, Ts65Dn. Using computer-assisted imaging of fura-2 fluorescence, we found an elevation of intracellular cytoplasmic calcium in cortical astrocytes from neonatal Ts65Dn mouse brain, similar to that observed previously in embryonic Ts16 astrocytes. Furthermore, astrocytes from both Ts65Dn and Ts16 cortex fail to respond to the anti-proliferative actions of glutamate. These results suggest that defective regulation of cell proliferation and cellular calcium can result from triplication of DS critical region genes. © 2003 Wiley-Liss, Inc.

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A region of conserved synteny between human chromosome 21 (HSA21) and mouse chromosome 16 (MMU16) encompasses most of the Down Syndrome (DS) critical region (Hattori et al., 2000). This region includes at least 129 orthologous gene pairs (Mural et al., 2002). Triplication of HSA21 (trisomy 21) results in DS, the most common cause of mental retardation (Adams et al., 1981). DS is associated with several brain abnormalities including altered cortical stratification, cellular differentiation, dendritic abnormalities, and astrocytosis (Wisniewski et al., 1984; Becker et al., 1991; Golden and Hyman, 1994). The relationships between the triplication of genes on HSA21 and the reported brain abnormalities and mental retardation of DS are not known.

Mice with either a full triplication of MMU16 (Ts16) or a triplication of only the region in common between MMU16 and HSA21 (Ts65Dn) (Davisson et al., 1993) have been developed to study the cellular basis of the DS phenotype. In Ts16 there is accelerated neuron death, altered regulation of neural progenitor cell prolif-

eration, and a dysregulation of Ca<sup>2+</sup> in both astrocytes and neurons (Haydar et al., 1996, 2000; Bambrick et al., 1997; Muller et al., 1997; Schuchmann et al., 1998). MMU16 includes many genes not found on HSA21, however, and triplication of some of these non-HSA21 genes may contribute to the phenotype of Ts16. Furthermore, Ts16 mice do not survive birth, preventing studies of later brain development and behavior. In contrast, although Ts65Dn mice have not been studied extensively at the cellular level, they survive to adulthood and show deficits in memory (Reeves et al., 1995; Demas et al., 1996, 1998), in tasks requiring an intact hippocampus (Hyde et al., 2001), and in long-term potentiation (LTP) (Siarey et al., 1997). Whether the cellular abnormalities that have been reported in Ts16 are present in Ts65Dn, and therefore could contribute to the reported behavioral abnormalities, is not known. Here we compare two cellular functions (astrocyte proliferation and calcium regulation) in Ts65Dn and Ts16. Defects in these functions would be expected to profoundly affect the development and normal function of the brain.

## MATERIALS AND METHODS

### Generation of Mice

Ts65Dn females were crossed to C57Bl/6Jei × C3H/HeJ (B6EiC3H) F1 hybrid males at each generation (Davisson et al., 1993). The resulting litters contained both Ts65Dn and euploid (2n) mice. Euploid littermates were used as controls in all experiments. Ts16 mice were generated as described previously (Bambrick et al., 1996).

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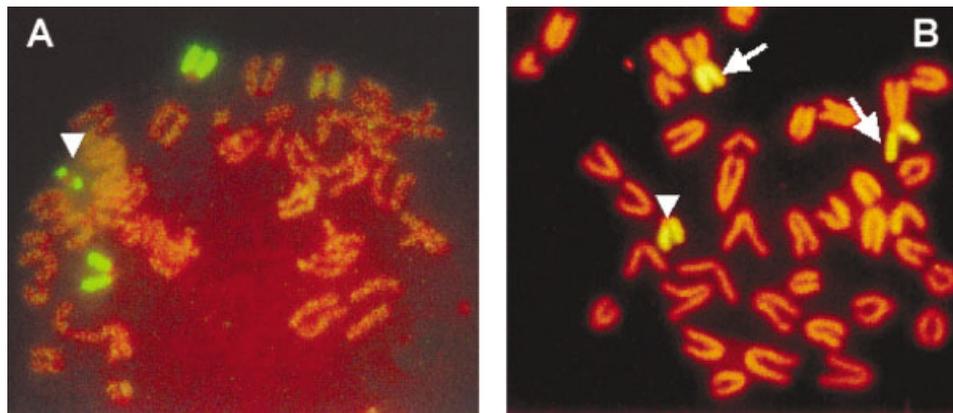


Fig. 1. Determination of genotype of Ts65Dn fibroblasts using FISH. **A:** Metaphase spread from Ts65Dn tail fibroblast hybridized with a mouse chromosome 16 (MMU16) paint-probe (FITC-conjugated). Ts65Dn spreads have three signals with the extra segment of MMU16 indicated by the arrowhead. **B:** Similar spread from Ts16 mouse liver

showing three signals for MMU16, one normal (arrowhead), two on translocations to MMU17 and MMU6 (arrows). DNA was counterstained with propidium iodide (PI, red). Chromosomes co-labeled by the FITC-conjugated MMU16 paint-probe (green) and PI (red) appear green/yellow.

### Genotype Characterization of Ts65Dn Mice

The genotype of each neonate was determined by fluorescence in situ hybridization (FISH) of metaphase spreads from cultured tail fibroblasts using an fluorescein isothiocyanate (FITC)-labeled MMU16-specific, whole chromosome paint probe (Cambio, Cambridge, UK). In some cases, genotype was confirmed using an MMU16-specific probe mapping to the distal portion of the chromosome as described in Strovel et al. (1999).

Tail fibroblasts were prepared by enzymatic digestion of a 3–5 mm section of tail in trypsin/EDTA (0.25% trypsin), followed by collagenase (Type 1A, 0.5mg/ml; Sigma, St. Louis, MO) as described in Strovel et al. (1999). After passage through a #80 mesh screen to isolate single cells, the cell suspension was cultured in MEM/10% fetal bovine serum (FBS) (one tail/35-mm tissue culture dish). Unless otherwise stated, all reagents were from Invitrogen (Carlsbad, CA).

Cultured tail fibroblasts were treated with Karyomax (0.4  $\mu$ g/ml final concentration) and spreads were prepared by standard cytogenetic techniques. Probe preparation was carried out following the manufacturer's specifications (Cambio). Slides were prepared for hybridization by denaturation at 68°C for 2 min followed by ethanol dehydration. Slides were hybridized immediately at 42°C overnight or stored at  $-20^{\circ}\text{C}$  for up to 6 months. After 16 hr hybridization at 37°C, slides were washed in three changes of 50% formamide/2 $\times$  SSC at 45°C for 5 min each and two changes of 0.1 $\times$  SSC at 45°C for 5 min. Propidium iodide (PI) was used as counterstain.

Figure 1A shows a metaphase spread of a cultured tail fibroblast from a Ts65Dn mouse probed with the chromosome MMU16 paint probe. Two normal MMU chromatid pairs and the extra pair of MMU16 segments can be seen. For comparison, Figure 1B shows a metaphase spread from a Ts16 mouse cell with the one normal MMU16 and the two paternal Robertsonian translocations of MMU16 with MMU6 and MMU17.

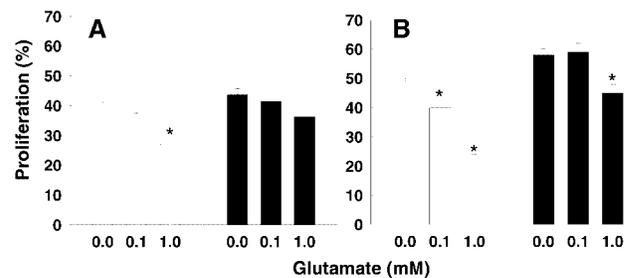
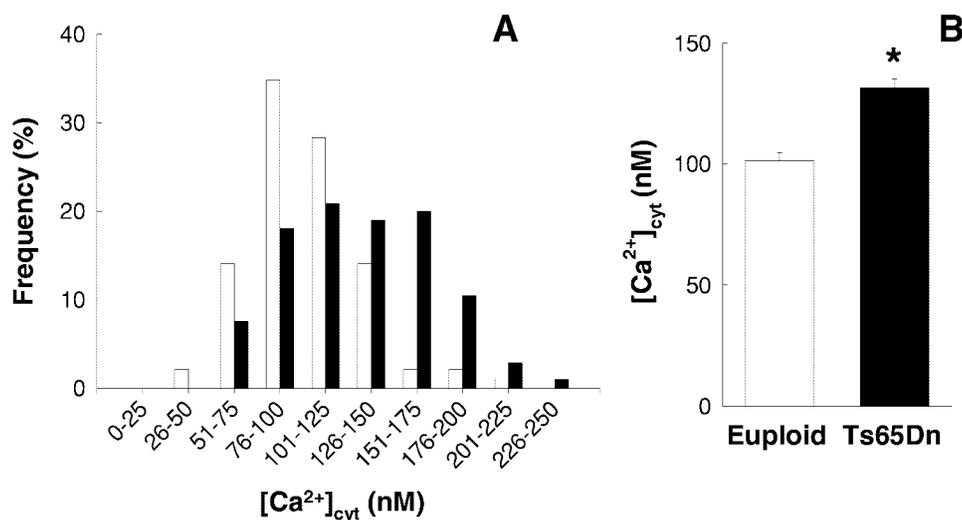


Fig. 2. Glutamate fails to decrease BrdU incorporation in Ts16 and Ts65Dn astrocytes. Bromodeoxyuridine (BrdU) labeling of **(A)** PN1 euploid and Ts65Dn and **(B)** E16 euploid and Ts16 cortical astrocytes in the presence of 0, 0.1, and 1.0 mM glutamate. Cells were incubated for 16 hr in BrdU. Each experiment was done in triplicate. Results are means  $\pm$  SEM for  $n = 3$  separate experiments. \*Significantly different from 0 mM glutamate,  $P < 0.05$ . Euploid astrocytes (open bars) respond to glutamate with a dose-dependent decrease in BrdU incorporation. Ts16 and Ts65Dn astrocytes (solid bars) were  $>10\times$  less sensitive to glutamate.

### Astrocyte Cell Culture

Postnatal Ts65Dn mice (PN1) and embryonic Ts16 (embryonic day 16; E16) mice were sacrificed by decapitation, and the brain from each individual animal was isolated and cultured separately as described previously (Bambrick et al., 1996). Cells were plated at 50,000 cells on 25-mm polylysine-coated glass coverslips in DMEM/F12 (1/1) 10% FBS in a 37°C/5%CO<sub>2</sub>. The medium was changed every 3 days. The viability of the astrocyte cultures was similar for control and trisomic cultures over the 6–8 days for this study and similar initial proliferation rates were found in both cultures by bromodeoxyuridine (BrdU) immunolabeling (Fig. 2). The cultures were  $>90\%$  astrocytes as measured by GFAP staining (data not shown).

Fig. 3. Elevated  $[Ca^{2+}]_{cyt}$  in Ts65Dn astrocytes. **A:** Frequency histogram showing the distribution of  $[Ca^{2+}]_{cyt}$  in astrocytes from euploid (open bars, 92 cells) and Ts65Dn (solid bars, 107 cells) mice. **B:** Mean resting  $[Ca^{2+}]_{cyt}$  was significantly increased ( $P < 0.05$ ) in Ts65Dn astrocytes. Data are from 92 and 102 astrocytes from 10 euploid mice and 7 Ts65Dn mice (3 litters), respectively.



### Cell Proliferation

At 6 days in vitro (DIV), cultures were treated with 0, 0.1, or 1.0 mM glutamate, BrdU (40  $\mu$ M) was added after 4 hr. After a further 16 hr, cultures were fixed in 4% paraformaldehyde. BrdU incorporated into cells synthesizing DNA was detected using an anti-BrdU antibody (Becton-Dickinson, Franklin Lakes, NJ) and a fluorescein-conjugated secondary antibody. Nuclei were counterstained with PI. Proliferation is reported as the percentage of nuclei that were BrdU-positive. At least 400 cells on three coverslips were counted per condition for each cell culture.

### Calcium Imaging

Cytoplasmic free  $Ca^{2+}$  ( $[Ca^{2+}]_{cyt}$ ) was determined by fura-2 imaging (Golovina et al., 1996). Astrocytes were loaded with 3  $\mu$ M fura-2-AM in HEPES-buffered L15 (20 min, 22°C) and then superfused for 30 min with PSS (containing [mM] 140 NaCl, 5.0 KCl, 1.2  $NaH_2PO_4$ , 1.4  $MgCl_2$ , 1.8  $CaCl_2$ , 11.5 glucose, 10 HEPES, pH 7.4) at 34°C to allow esterification of the AM ester. PSS was superfused continuously during the experiment. The fura-2 signal was acquired using a Videoscope CCD200 video camera with a VS4-1845 intensifier. Excitation at 340 and 380 nm was provided by a tunable monochromator (Til Photonics Polychrome II) and emission was measured at 520 nm. The digitized image was analyzed using MetaMorph/Metafluor 3.5 (Universal Imaging). Images were acquired at 0.1 Hz. Determinations of fura-2 signals were made for individual cells, with 5–10 cells per imaged field. The fura-2 signal was calibrated by measuring  $R_{min}$  and  $R_{max}$  in fura-2 solutions containing 0 or 35  $\mu$ M calcium. Cytoplasmic calcium measurements were analyzed by ANOVA. Data were evaluated either by averaging the cytoplasmic calcium values for all the euploid and all the Ts65Dn cells imaged or by determining the average cytoplasmic calcium value for the cells derived from an individual mouse and comparing calcium levels among individuals animals.

### RESULTS

Ts65Dn and euploid littermate neonates appeared similar at postnatal day 1 (PN1), except that the Ts65Dn mice were 17% smaller ( $P < 0.03$ ): total body weight Ts65Dn,  $1.48 \pm 0.05$  g ( $n = 13$ ) versus euploid,  $1.78 \pm 0.06$  g ( $n = 26$ ). This is in contrast to the marked edema, facial abnormalities, and reduction in body weight seen when comparing Ts16 and euploid littermate mice at embryonic day 16 (E16) (Gearhart et al., 1986; Grausz et al., 1991).

Euploid fetal and neonatal astrocytes showed a dose-dependent decrease in BrdU incorporation in the presence of glutamate (Fig. 2, open bars). This is consistent with earlier reports that glutamate decreases astrocyte proliferation in vitro and during early corticogenesis (Nicolletti et al., 1990; LoTurco et al., 1995). The effect of glutamate was greater in the embryonic cultures, possibly reflecting developmental changes in the regulation of glial proliferation.

In contrast, astrocytes from Ts65Dn neonatal mice did not show a significant effect of glutamate (Fig. 2A, filled bars). A similar result was seen for astrocytes derived from embryonic Ts16 brain, which were 10-fold less sensitive to the effect of glutamate on proliferation (Fig. 2B, filled bars) than were cells from euploid littermates.

Resting  $[Ca^{2+}]_{cyt}$  was 30% higher in Ts65Dn astrocytes (significant,  $P < 0.05$ ) (Fig. 3B). Examination of  $[Ca^{2+}]_{cyt}$  levels in individual cells showed that whereas euploid cell values were distributed symmetrically around a mean of about 100 nM, the Ts65Dn distribution was broader and shifted toward higher  $[Ca^{2+}]_{cyt}$  (Fig. 3A). The distribution of  $[Ca^{2+}]_{cyt}$  was not skewed significantly for either genotype (Kalmogorov-Smirnov normality test,  $P > 0.1$ ), but there was a 29% increase in the standard deviation in Ts65Dn. As a result of the increased mean and spread of the  $[Ca^{2+}]_{cyt}$  values, 35% of the Ts65Dn astrocytes, but only 5% of the euploid cells, had resting

$[Ca^{2+}]_{cyt}$  above 150 nM. Similar differences between  $[Ca^{2+}]_{cyt}$  in Ts65Dn and controls were seen when values were analyzed for individual mice (in nM:  $125 \pm 10$ ,  $n = 7$ ;  $101 \pm 5$ ,  $n = 11$ ; significantly different,  $P < 0.04$ ). Increased resting  $[Ca^{2+}]_{cyt}$  was found in astrocytes from both male (124 vs. 100 nM) and female (125 vs. 101 nM) mice.

## DISCUSSION

We report that glutamate fails to downregulate the proliferation of Ts65Dn and Ts16 astrocytes *in vitro*. Glutamate is known to regulate neural cell progenitor proliferation during the formation of the cerebral cortex (LoTurco et al., 1995). Dysregulation of proliferation could contribute to the delayed exit from the cell cycle of cortical ventricular zone (VZ) neuroblasts in the embryonic Ts16 cortex (Haydar et al., 1996, 2000). Cells from the VZ leave the cell cycle and migrate out to form the cortical plate in a very temporally specific pattern (Caviness et al., 1995). Abnormal regulation of the timing of proliferation could lead to abnormal cortical stratification, as is seen in DS. This insensitivity of Ts16 and Ts65Dn astrocytes to an antimitotic stimulus may underlie the astrogliosis that has been seen in Ts65Dn brain (Holtzman et al., 1996) and to reports of increased astrogliosis in DS and Alzheimer's disease (Jorgensen et al., 1990; Murphy et al., 1992; Ceuterick et al., 1998). The primary function of glutamate in the mature brain is as an excitatory neurotransmitter. As synapses are formed during development, the role of glutamate becomes more restricted and other factors take over as regulators of cell proliferation (Cameron et al., 1998). This could explain the decreased effect of glutamate on astrocytes cultured from postmitotic, as compared to embryonic, mouse brain.

The effect of glutamate on astrocyte proliferation is mediated by metabotropic glutamate receptors (mGluRs) (Condorelli et al., 1989; Nicoletti et al., 1990); mGluR regulation of astrocyte proliferation may be complex. Activation of Group I mGluRs can increase, whereas activation of Group II mGluRs can decrease, thymidine incorporation in cultured astrocytes (Ciccarelli et al., 1997). Because mGluR signaling could be affected by changes in calcium regulation, the defect in calcium homeostasis we report in Ts16 and Ts65Dn could underlie the observed alteration in the regulation of proliferation and affect early brain development.

The altered distribution of mean  $[Ca^{2+}]_{cyt}$  levels in Ts65Dn astrocytes is similar to that reported for Ts16 astrocytes (Bambrick et al., 1997), where there was also a change from the normal bell-shaped to a flattened and right-shifted distribution. Mean  $[Ca^{2+}]_{cyt}$  was also elevated in astrocytes from Ts65Dn neonatal cortex. The 30% increase in mean Ts65Dn astrocyte  $[Ca^{2+}]_{cyt}$  was less than the twofold increase reported for Ts16 astrocytes (Bambrick et al., 1997; Muller et al., 1997). The smaller increase in  $[Ca^{2+}]_{cyt}$  in Ts65Dn astrocytes may reflect a difference between the expression of the phenotype in fetal versus postnatal mice. Alternatively, the differences between Ts16 and Ts65Dn may be due to one or more of

the genes on MMU16, not triplicated in Ts65Dn (Fig. 1). The 30% increase in  $[Ca^{2+}]_{cyt}$  reported here for Ts65Dn astrocytes is similar to that found for Ts16 hippocampal neurons (Schuchmann et al., 1998), where it was reported to be associated with decreased mitochondrial function and increased neuron death.

The cause of the dysregulation of  $[Ca^{2+}]_{cyt}$  is not known, but is likely to involve a change in net  $Ca^{2+}$  transport across the plasma membrane. An increase in transient voltage-gated  $Ca^{2+}$  channel currents has been reported in hippocampal Ts16 neurons (Galdzicki et al., 1998). Interestingly, increased current through voltage-gated  $Ca^{2+}$  channels has also been reported in aged neurons (Campbell et al., 1996; Thibault and Landfield, 1996) and may affect learning (Disterhoft et al., 1995) and neuron survival. It is not clear, however, that larger  $Ca^{2+}$  fluxes would lead to an increased resting  $[Ca^{2+}]_{cyt}$ . In a previous study (Bambrick et al., 1997), changes in extracellular  $[Ca^{2+}]$  did not affect the resting  $[Ca^{2+}]_{cyt}$  of Ts16 astrocytes, suggesting that the plasma membrane was not more permeable to  $Ca^{2+}$ . Reduced  $Ca^{2+}$  buffering or efflux are likely candidates for  $Ca^{2+}$  dysregulation. For example, the plasma membrane CaATPase is a well-established target of oxidative damage (Zaidi and Michaelis, 1999). Increased reactive oxygen species have been reported in Ts16 neurons (Schuchmann and Heinemann, 2000) and reduced calcium pump activity could lead to increased  $[Ca^{2+}]_{cyt}$ .

$[Ca^{2+}]_{cyt}$  is normally regulated tightly and even a small, sustained increase in  $[Ca^{2+}]_{cyt}$  may have serious consequences for cell function. Johnson et al. (1992) have reported a bell-shaped response of neuron survival to intracellular calcium with changes above or below the optimum  $[Ca^{2+}]_{cyt}$  decreasing neuron survival (Franklin et al., 1995). Small changes in  $[Ca^{2+}]_{cyt}$  have also been suggested to contribute to increased neuron death in aging (Verkhratsky and Toescu, 1998). It is possible that the dysregulation of  $[Ca^{2+}]_{cyt}$ , associated with triplication of HSA21 or MMU16, contributes to the age-related degeneration of Ts65Dn cholinergic neurons *in vivo* (Holtzman et al., 1996), and the increased rates of cell death of DS and Ts16 neurons *in vitro* (Bambrick et al., 1995; Busciglio and Yankner, 1995; Bambrick and Krueger, 1999). Finally, dysregulation of  $Ca^{2+}$  in Ts65Dn neural cells could impair LTP (a  $Ca^{2+}$ -dependent process) and thus learning and memory. Both reduced LTP (Siarey et al., 1997) and behavioral problems including spatial memory deficits (Reeves et al., 1995; Demas et al., 1996, 1998; Hyde et al., 2001) are present in Ts65Dn.

The present results show that Ts65Dn has a phenotype that can be observed on the cellular level. Astrocyte proliferation and calcium regulation are altered in Ts65Dn, suggesting that these defects could contribute to the gliosis, neuron death, and impaired cognition observed in these animals. Similar abnormalities are present in Ts16 astrocytes, where they may be linked to altered cortical development and impaired neuron survival. The presence of similar changes in both Ts65Dn

and Ts16 is consistent with the alterations arising from triplication of genes on the region of MMU16 conserved on HSA21 and with the possibility that such alterations in cell function contribute to neuropathology in DS.

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