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Protein levels of genes encoded on chromosome 21 in fetal Down syndrome brain: Challenging the gene dosage effect hypothesis (Part I)

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Summary. Down syndrome (DS) is the most significant genetic disorder with mental retardation and is caused by trisomy 21. The phenotype of DS is thought to result from overexpression of a gene(s) located on the triplicated chromosome (region). An increasing body of evidence that challenge this "gene dosage effect" hypothesis, however, has been reported indicating that this hypothesis still remains to be elucidated. The availability of the complete sequence of genes on chromosome 21 could have an immediate impact on DS research, but no conclusions can be drawn from nucleic acid levels. This made us evaluate protein levels of six proteins, gene products, encoded on chromosome 21 (T-cell lymphoma invasion and metastasis inducing Tiam1 protein, holocarboxylase synthetase, human interferon-regulated resistance GTP-binding protein MxA, Pbx regulating protein 1, autoimmune regulator, and pericentrin) in fetal cortex from DS and controls at 18–19 weeks of gestational age using Western blot technique. None of the investigated proteins showed overexpression in DS compared to controls. Our present data showing unaltered expression of six proteins on chromosome 21 in fetal DS brain suggest that the existence of the trisomic state is not involved in abnormal development of fetal DS brain and that the gene dosage effect hypothesis is not sufficient to fully explain the DS phenotype. We are in the process of quantifying all gene products of chromosome 21 and our first results do not support the gene dosage hypothesis.

Keywords: AIRE – Chromosome 21 – Down syndrome – HCS – MxA – Pericentrin – Prep1 – protein expression – Tiam1

Abbreviations: AIRE, autoimmune regulator; DS, Down syndrome; HCS, holocarboxylase synthetase; Prep1, Pbx regulating protein 1; Tiam1, T-cell lymphoma invasion and metastasis 1

Introduction

Down syndrome (DS) or trisomy 21 is the most common genetic cause of mental retardation with an incidence of 1 in 700–1,000 live births. In addition to mental retardation, individuals with DS suffer from a wide range of abnormalities. In DS, the existence of a trisomic state could result in the production of 50% more gene products present on the unbalanced chromosome segment, and actually these gene dosage effects are considered responsible for the abnormalities of development and function that constitute the aneuploid phenotype. Although such gene dosage effects have been proposed, our group and others showed normal expression of some genes on chromosome 21 in both DS and trisomy 16 mice (Marks et al., 1996; Greber-Platzer et al., 1999; Engidawork et al., 2001) indicating that this hypothesis still remains to be tested.

Until now, molecular biological approaches have been focusing on mapping and gene hunting of chromosome 21. As one of the first triumphs of the Human Genome Project, analysis of the sequence of chromosome 21 has been virtually completed. One of the major challenges in the post-genomic era is to find functional information of the gene products, which serves now as starting point for systematic functional genomics applications. In this aspect, working at the protein level gives more direct information than working at the RNA level, in particular, as the protein is mainly the functioning part and there is a long and unpredictable way from RNA to protein expression. Although a large series of proteins have been found

to be deranged in adult DS brain (confounded by Alzheimer's disease), information about proteins on chromosome 21 in developing DS brain is limited so far. Therefore, we evaluated expression levels of six proteins encoded on chromosome 21 – T-cell lymphoma invasion and metastasis inducing Tiam1 protein (Tiam1; 21q22.11), holocarboxylase synthetase (HCS; 21q22.13), human interferon-regulated resistance GTP-binding protein MxA (myxovirus (influenza) resistance 1; 21q22.3), human homeobox-containing protein or Pbx regulating protein 1 (Prep1; 21q22.3), autoimmune regulator (AIRE; 21q22.3), and pericentrin (21q22.3) (Fig. 1) – in fetal brains from DS and controls at the early second trimester.

Tiam1, originally identified by its ability to induce invasion of T-lymphoma cells (Habets et al., 1994), functions as guanine nucleotide exchange factor for Rac (one of the Rho family GTPase) which can act as a regulator of neural development and its transcripts are mainly expressed in brain and testis (Habets et al., 1995). In brain, Tiam1 expression is restricted to a subset of neurons and the onset of its expression during development correlates well with neuronal differentiation and/or migration (Ehler et al., 1997). This study also suggested that Tiam1 contributes to cytoskeletal reorganization required during cell migration and neurite extension in a specified population of neurons.

HCS is an enzyme that catalyzes biotin incorporation into carboxylases (Achuta Murthy et al., 1972). In mammalian cells, four carboxylases that require biotin as a prosthetic group are known: acetyl-CoA carboxylase, a rate-limiting enzyme in fatty acid synthesis; pyruvate carboxylase, a key enzyme of gluconeogenesis; propionyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase, an enzyme involved in amino acid catabolism. Biotinylation of these carboxylases is essential for their enzymatic activities and defective function of HCS leads to multiple deficiency of the carboxylases, thereby affecting various metabolic processes in cells.

Interferon-induced human MxA protein that confers resistance to influenza virus is a GTPase, which binds transiently to cellular proteins. It was proposed that expression of MxA in human brain cells is associated with the stimulation of a cellular kinase that is active in phosphorylating both cellular target proteins and viral protein in a study showing the association of the expression of human MxA protein with hyperphosphorylation of viral protein (Schuster et al., 1996).

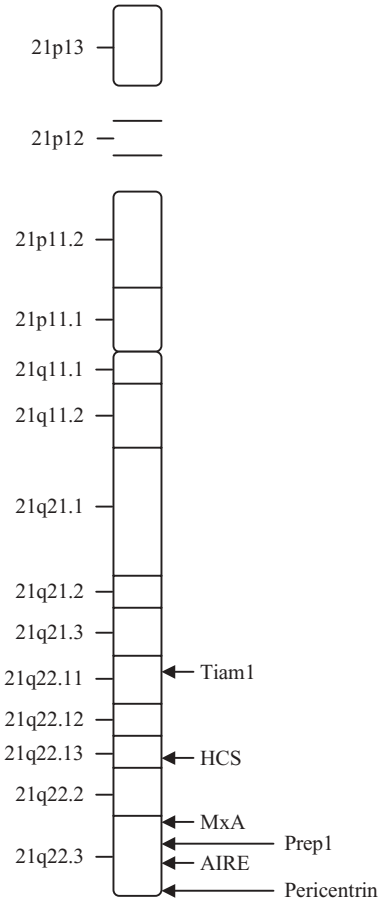

Fig. 1. Giemsa banding (G-bands) of human chromosome 21. Arrows indicate six proteins, products of genes encoded on chromosome 21, used in this study

Prep1 is a member of the three-amino acid loop extension (TALE) class of homeoproteins that constitute a set of transcription factors (Burglin, 1997). This protein is able to specifically interact with Pbx, another member of homeodomain transcription factor. Heterodimeric complexes of Prep1 and Pbx proteins are involved in regulating the activity of the enhancer of urokinase plasminogen activator gene (Berthelsen et al., 1998). Several growth factors and other proteases are also putative target genes for the Prep1-Pbx complex. Pbx in turn is able to form high affinity DNA-binding complexes with several Hox proteins. These Prep1-Pbx-Hox proteins modulate the activity of the somatostatin and collagen V promoter, where they display cooperative functional effects on transcription. Ternary complexes of Prep1-Pbx-Hox protein have been also shown to have important gene regulating properties involved in development and

differentiation of mouse and *Drosophila* embryos (Ferretti et al., 2000).

The AIRE gene, defective in hereditary autoimmune disease APECED (autoimmune polyendocrinopathy candidiasis ectodermal dystrophy) encodes a protein containing several structural domains characteristic of transcriptional regulators (Finnish-German APECED Consortium, 1997). AIRE expression was detected in various tissues suggesting that AIRE might have functions outside the immune system (Halonen et al., 2001). In central nervous system, its immunostaining was widely distributed; the most prominent staining was in neurons, but different types of glial cells were also labeled.

Pericentrin is a conserved integral component of the filamentous matrix (or the pericentriolar material) of the centrosome which is involved in the initial establishment of organized microtubule arrays (Doxsey et al., 1994). During mitosis, two centrosomes ensure accurate assembly of bipolar spindles by regulating microtubule nucleation, which serves the fidelity of the chromosomal segregation. Spindles in pericentrin-overexpressing cells were disorganized and mispositioned, and chromosomes were misaligned and missegregated during cell division, giving rise to aneuploid cells (Purohit et al., 1999). Pericentrin was co-expressed with cytoplasmic dynein heavy, intermediate, and light intermediated chains, and the dynamitin and p150(Glued) subunits of dynactin. Additionally, centrosomes in cells with reduced levels of pericentrin and gamma-tubulin have a diminished capacity to nucleate microtubules (Young et al., 2000).

Materials and methods

Fetal brain samples

Fetal brain tissues (cerebral cortex) of DS (4 females with 18–19 weeks of gestational age) and controls (4 females with 18–19 weeks of gestational age) used in this study. Brain samples were obtained from Dr. Mara Dierssen (Medical and Molecular Genetics Center-IRO, Hospital Duran i Reynals, Barcelona, Spain) and Dr. Joan Carles Ferreres (Department of Pathology UDIAT-CD, Corporacis Sanit'ria Parc Tauli, Sabadell, Barcelona, Spain). All samples were stored at −70°C and the freezing chain was never interrupted until use.

Antibodies

The details of the preparation and characterization of antibodies have been described previously: HCS (Hiratsuka et al., 1998); MxA (Ronni et al., 1993); Prep1 (Berthelsen et al., 1998); AIRE (Rinderle et al., 1999). Four antibodies for Tiam1 (Santa Cruz, Austria), pericentrin (Covance, USA), actin (Sigma, USA), and neuron specific enolase (NSE; Chemicon, UK) were purchased.

Western blotting

Fetal brain tissues ground under liquid nitrogen were homogenized in lysis buffer containing protease inhibitor cocktail tablet (Rache, Germany) at 4°C and centrifuged at 8,000×g for 10 minutes. The BCA protein assay kit (Pierce, USA) was applied to determine the concentration of protein in the supernatant. Samples (10 μg) were mixed with the sample buffer (100 mM Tris-HCl, 2% SDS, 1% 2-mercaptoethanol, 2% glycerol, 0.01% bromophenol blue, pH 7.6), incubated at 95°C for 15 minutes and loaded onto a ExcelGel SDS homogenous gel (Amersham Pharmacia Biotech, Sweden). Electrophoresis was performed with Multiphor II Electrophoresis System (Amersham Pharmacia Biotech). Proteins separated on the gel were transferred onto PVDF membrane (Millipore, USA) and membranes were incubated in blocking buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 and 2% non-fat dry milk). Membranes were incubated for 2 hours at room temperature with diluted primary antibodies (1:500 for HCS; 1:1,000 for Tiam1, AIRE, and pericentrin; 1:2,000 for MxA; 1:3,000 for actin and NSE; 1:10,000 for Prep1). After 3 times washing for 15 minutes with blocking buffer, membranes were probed with secondary antibodies coupled to horseradish peroxidase (Southern Biotechnology Associates, Inc., USA) for 1 hour. Membranes were washed 3 times for 15 minutes and developed with the Western blot chemiluminescence reagents (NEN™ Life Science Products, Inc., USA).

Statistics

The density of immunoreactive bands was measured by RFLPscan version 2.1 software program (Scanalytics, USA). Between group differences were calculated by non-parametric Mann-Whitney U test using GraphPad Instat2 program and the level of significance was considered at $P < 0.05$.

Results

We evaluated the expression level of six proteins encoded on chromosome 21 (Tiam1, HCS, MxA, Prep1, AIRE, and pericentrin, Fig. 1) in fetal brains with DS compared to controls by Western blot analysis. Two proteins, actin and NSE were used as reference proteins. As shown in Fig. 2, the density of immunoreactive bands of eight proteins investigated in this study was comparable between DS and controls. Protein levels in fetal DS brain showed an increase of approximately 2-fold for Tiam1 and 1.5-fold for HCS, MxA, and pericentrin, but were not reaching statistical significance ($P > 0.05$). On the other hand, expression levels of actin-normalized Prep1 were decreased in DS, although not reaching statistical significance ($P > 0.05$). No difference was observed between DS and controls, when levels of six proteins were normalized with those of actin as shown in Fig. 3.

Discussion

The subcellular distribution of Tiam1 in cultured hippocampal pyramidal neurons was analyzed by

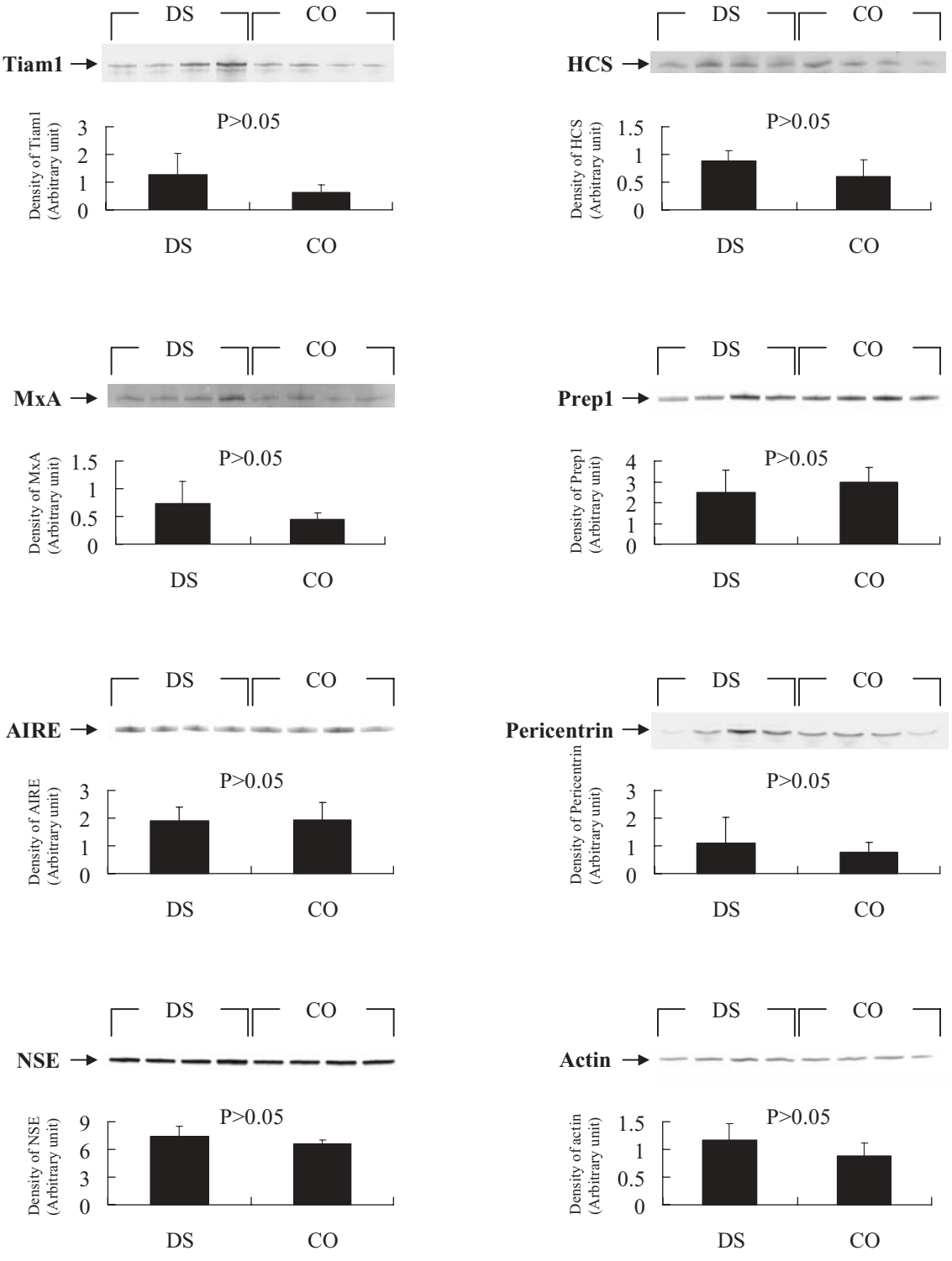

Fig. 2. Western blot analysis for six proteins encoded on chromosome 21 in cerebral cortex from fetal brain with DS and controls. Denatured proteins (10 μg) were loaded, separated on a homogeneous gel and transferred onto PVDF membrane. The membrane was incubated with primary and secondary antibodies. Immunoreactive bands (Tiam1, 205 kDa; HCS, 76 kDa; MxA, 78 kDa; Prep1, 64 kDa; AIRE, 58 kDa; pericentrin, 220 kDa; actin, 42 kDa; NSE, 45 kDa) were detected using chemiluminescence reagents. The density of detected bands was measured and calculated by non-parametric Mann-Whitney U test and the level of significance was considered at $P < 0.05$

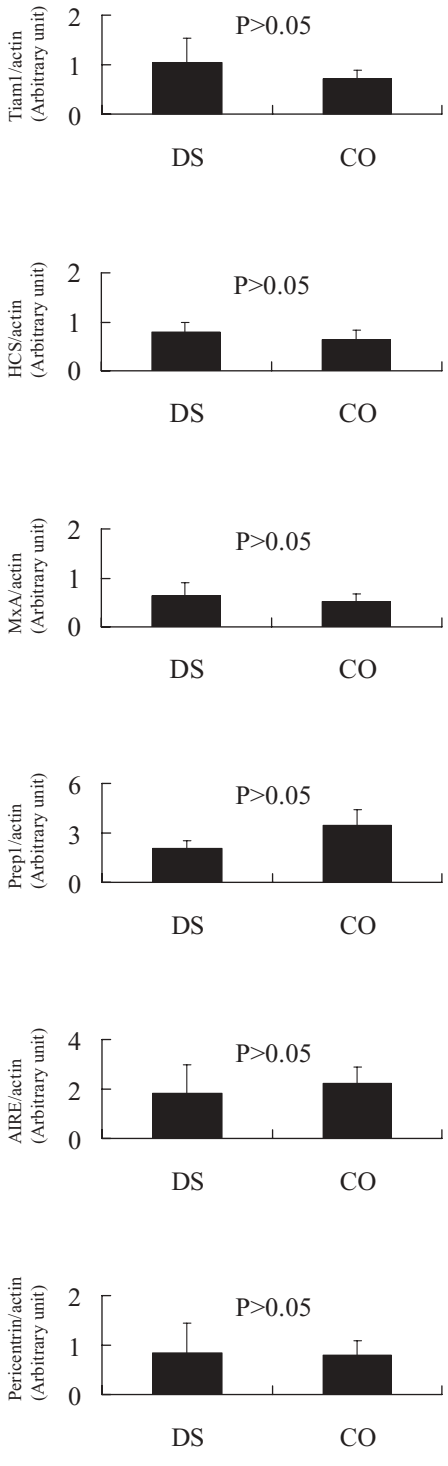

Fig. 3. Expression levels of six proteins normalized with those of actin. The density of immunoreactive band for each protein was normalized with that of actin, which was used as a reference protein

fluorescence microscopy (Kunda et al., 2001). The results obtained clearly revealed that Tiam1 was not only present, but also enriched in neurite growth cones, where it associates with microtubules. In addition, the study suggested that Tiam1 promotes axon formation and hence participation in neuronal polarization by regulating growth cone actin organization and allowing microtubule invasion within selected growth cones. The current data that the expression of Tiam1 was comparable between fetal DS and controls is consistent with our previous result showing unaltered expression of stathmin, a depolymerizing factor of microtubules, in fetal DS brain (Cheon et al., 2001) and do not explain cytoskeleton derangement (Gulesserian et al., 2002) or deficiency of neuronal migration and dendritic outgrowth in fetal DS (Weitzdoerfer et al., 2001).

HCS gene was expected to contribute in a gene dosage-dependent manner to the phenotype of DS, with mapping of this gene to the DS critical region of chromosome 21q22, but any experimental confirmation for the association of HCS with DS pathology has not been reported and here we show that HCS was normally expressed in fetal DS brains.

Interestingly, MxA protein has been suggested to play a role in the formation of Lewy bodies in Parkinson's disease and swelling of neuronal processes (Yamada, 1995). Additionally, it has been suggested that increased expression of alpha-interferon in the white matter microglia and appearance of MxA protein in reactive microglia contribute to Alzheimer pathology (Yamada et al., 1994). Considering that DS is associated with increased interferon sensitivity (Tan et al., 1974) resulting in a damaging inflammatory reaction, human brain MxA protein could be used as marker protein to show the interferon-related inflammatory reaction of microglial cells as well as the level of neurodegeneration. Normal expression levels of MxA protein implicate that there is no interferon-related inflammatory reaction in fetal DS brain. Furthermore, based upon the finding that oligodendrocytes, which synthesize, assemble, and maintain myelin in central nervous system express an alpha/beta-interferon-susceptible Mx gene (Szuchet et al., 2002), one could expect that oligodendrocytes in fetal DS brain function normally as in controls.

It was shown that sequestering Prep1 in the cytoplasm leads to extensive loss of rhombomere (r)3- and r4-specific gene expression, as well as defective rhombomere boundary formation in this region (Choe

et al., 2002). These changes in gene expression correlated with impaired neuronal differentiation in r3 and r4 proposing that Prep1 is essential for the specification of r3 and r4 of the hindbrain. Furthermore, various combinations of complexes have been reported to occur among TALE, Pbx, and Hox family (Mann et al., 1998). As a result, the range of DNA targets of this group of homeoproteins is very broad and has the potential of regulating a wide variety of genes during development of embryos and brain. Comparable expression of Prep1 protein in DS and controls suggest that transcription of target genes for Prep1 may be regulated normally in DS brain.

Recently, AIRE as a DNA-binding protein has been reported to activate transcription from a reporter gene when fused to a heterologous DNA binding domain (Bjorses et al., 2000) and it interacts with the co-activator CREB-binding protein (Pitkanen et al., 2000). Considering these results together with its subnuclear localization (speckled sub-domains in the nucleoplasm) and its remarkable structural features, AIRE could play a role in the regulation of transcription. Although the target gene of AIRE has not been identified yet, the transcriptional regulation by AIRE may be unaffected as shown in this report.

Pericentrin may contribute to the assembly, organization and function of centrosomes and mitotic spindles, and function as novel dynein cargoes that can be transported to centrosomes on microtubules. Our result showing the unaltered expression of pericentrin in fetal DS brain suggest that pericentrin may not be responsible for cytoskeleton alterations described previously (Weitzdoerfer et al., 2001; Gulesserian et al., 2002). No difference of cell-cycle kinetics in early or late mitosis between the trisomic and normal cells has been reported (Frias et al., 1983).

There are several hypotheses to explain the DS phenotype including the "gene dosage effect" hypothesis. Such gene dosage effects have been reported in several studies; for example, the concentration of high mobility group protein 14, HMG-14; the overexpression of superoxide dismutase 1, SOD1 in adult brain. However, no differences in mRNA/protein levels of S100β between DS and normal subjects (Marks et al., 1996) and decreased ETS2 transcripts in DS brains have challenged gene dosage effects (Greber-Platzer et al., 1999). Recently, further evidence against gene dosage effect has been reported showing that β-amyloid precursor protein and ETS-2 encoded on chromosome 21 are not overexpressed in fetal DS brains compared to controls at the protein level (Engidawork et al., 2001). Our present data showing unaltered expression of six proteins of chromosome 21 in fetal DS brain suggest that these may not be involved in the abnormal development of fetal DS brain at the stage 18–19 weeks of gestation and that the gene dosage effect hypothesis is not sufficient to fully explain the DS phenotype. The study is of relevance as it shows for the first time levels of functional proteins encoded on chromosome 21 in human fetal brain with DS and we are continuing to quantify proteins whose genes are encoded on chromosome 21.

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