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Altered expression of neuropeptides in the primary somatosensory cortex of the Down syndrome model Ts65Dn

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ABSTRACT

Down syndrome is the most common genetic disorder associated with mental retardation. Subjects and mice models for Down syndrome (such as Ts65Dn) show defects in the formation of neuronal networks in both the hippocampus and the cerebral cortex. The principal neurons display alterations in the morphology, density and distribution of dendritic spines in the cortex as well as in the hippocampus. Several evidences point to the possibility that the atrophy observed in principal neurons could be mediated by changes in their inhibitory inputs and, in fact, an imbalance between excitation and inhibition has been observed in Ts65Dn mice in these regions, which are crucial for learning and information processing. These animals have an increased density of interneurons in the primary somatosensory cortex, especially of those expressing calretinin and calbindin D-28k. Here, we have analysed the expression and distribution of several neuropeptides in the primary somatosensory cortex of Ts65Dn mice in order to investigate whether these subpopulations of interneurons are affected. We have observed an increase in the total density of somatostatin expressing interneurons and of those expressing VIP in layer IV in Ts65Dn mice. The typology of the somatostatin and VIP interneurons was unaltered as attested by the pattern of co-expression with other markers. Somatostatin immunoreactive neurons co-express mainly D-28k calbindin and VIP expressing interneurons maintain its pattern of co-expression with calcium binding proteins. These alterations, in case they were also present in subjects with Down syndrome, could be related to their impairment in cognitive profile and could be involved in the neurological defects observed in this disorder.

1. Introduction

Down syndrome, with an incidence of one in 800 live births (Roizen and Patterson, 2003), is one of the most common genetic disorders. The phenotype observed as a consequence of a trisomy on the chromosome 21 may include immune deficiencies, heart defects, increased risk of leukemia and early development of Alzheimer's disease. The principal common feature among all DS individuals is the presence of mental retardation. The substrate for this retardation has not been fully understood and may include defects in the formation of neuronal networks and information processing. Alterations in synaptic plasticity have been related to impaired cognition in different murine models of this genetic alteration (Siarey et al., 2005, 2006).

Among the murine models available to study this genetic alteration, the most widely used is the Ts65Dn mouse, which mimics most of the alterations observed in DS. Ts65Dn mice are segmentally trisomic for a portion of the murine chromosome 16, which is orthologous to the long arm of the human chromosome 21. This segment contains approximately 140 genes, many of which are highly conserved between mice and humans (Gardiner et al., 2003). These mice display delays in the acquisition of a number of sensory and motor tasks (Holtzman et al., 1996; Costa et al., 1999), as well as defects in learning and in the execution of memory tasks mediated by the hippocampus (Reeves et al., 1995; Escorihuela et al., 1995, 1998; Holtzman et al., 1996).

Many studies have shown deficits in the dendritic arborization of the principal cells of the neocortex and hippocampus of DS subjects and murine models for this disorder (Marín-Padilla, 1976; Becker et al., 1986; Vuksic et al., 2002; Takashima et al., 1981, 1989; Kaufmann and Moser, 2000; Dierssen et al., 2003). This atrophy has been related to mental retardation and deficits in cognition (Dierssen and Ramakers, 2006).

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Alterations at the synaptic level have been observed in both DS subjects and murine models. Studies analyzing the expression of synaptophysin (a reliable marker for synapses (Eastwood and Harrison, 2001; Masliah et al., 1990)) have observed that the area occupied by synaptophysin is higher in Ts65Dn mice when compared with euploid mice, suggesting an increase in the size of the synapse (Belichenko et al., 2004, 2007, 2009). The increased expression of synaptophysin was restricted to superficial layers of cortex (Pérez-Cremades et al., 2010). The detailed study of synapse subtypes reflects a reduction in the number of asymmetric (excitatory) synapses per neuron in the hippocampus and in the temporal cortex of Ts65Dn mice (Kurt et al., 2000). Moreover, the inhibitory contacts are redistributed on the dendrites of these principal neurons, resulting in an increased density of those contacting spines and in a decrease of those contacting dendritic shafts (Belichenko et al., 2004). There is also an increased density of inhibitory puncta (observed by immunohistochemistry for GAD-67) in every layer of the cortex (Pérez-Cremades et al., 2010). Altogether, these results suggest an unbalanced level of inhibitory and excitatory inputs in the neocortex and hippocampus in Down syndrome. Supporting this fact, an overactivation of the inhibitory system, causing a general inhibition in the brain, has been observed in Ts65Dn mice (Fernández et al., 2007). This increased inhibition may be responsible, at least to some extent, for the cognitive impairment observed in Down syndrome. In fact, some studies have attempted to reverse the cognitive impairments observed in Ts65Dn mice by using GABAA receptor antagonists (Fernández et al., 2007), obtaining promising results.

Changes in inhibitory puncta density and distribution could be related to changes in the number and types of interneurons in the affected regions. In fact, we have observed an increase in the total number of inhibitory neurons in the primary somatosensory cortex of Ts65Dn mice. The phenotypical characterization revealed that among the increased subpopulations of inhibitory neurons were the calretinin and the calbindin D-28k expressing neurons (Pérez-Cremades et al., 2010).

The aim of this study is to deepen into the changes in interneuron populations in a specific region of the neocortex of Ts65Dn mice, the primary somatosensory cortex. We have chosen this region because previous reports have shown atrophy in principal neurons (Dierssen et al., 2003) and increased density of inhibitory neurons (Pérez-Cremades et al., 2010) We analysed the distribution and density of interneurons expressing four different neuropeptides: cholecystokinin (CCK); somatostatin (SST), neuropeptide Y (NPY) and vasoactive intestinal peptide (VIP) in this cortical region of Ts65Dn mice. The analysis of these neuropeptides allowed us to discriminate the different subtypes of interneurons that were not previously studied in this area. In this way to aim to complete our previous study where we studied calcium binding protein interneurons and observed an increased density of interneurons expressing calretinin (Pérez-Cremades et al., 2010).

2. Experimental procedures

Experimental mice were generated by repeated backcrossing of Ts65Dn females to C57/6Ei 9 C3H/HeSnJ (B6EiC3) F1 hybrid males. The parental generation was purchased from the research colony of The Jackson Laboratory (Ben Harbor, Maine, USA) Euploid littermates of Ts65Dn mice served as controls. The genotypic characterization was established by qRT-PCR using SYBR Green PCR master mix (Applied Biosystems) from genomic DNA extracted of mice tails by mean of the phenol–chloroform method. The relative amount of each gene was quantified by the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The genes analysed where APP (3 copies) and Apo-B (2 copies). The primers used where for APP (APP-F 5′-TGT TCG GCT GTG TGA TCC TGT GAC-3′; APP-R 5′-AGA AAC GAG CGG CGA AGG GC-3′) and for Apo-B (Apo-B-F 5′-TGC CAG GCT TGT GCT GCT GT-3′; Apo-B-R 5′-GGG TGC TGC CTT TCT CTT GGG G-3′).

For this study we used 4–5 month-old male mice (9 trisomic; 12 euploid). All animal experimentation was conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) on the protection of animals used for scientific purposes and approved by the Committee on Bioethics of the Universidad Miguel Hernández of Elche.

Animals were transcardially perfused using a solution containing 4% paraformaldehyde in PB (0.1 M, pH 7.4). Brains were removed and cryoprotected using 30% sucrose. Fifty micron sections (10 subseries for each brain) were obtained using a sliding freezing microtome.

2.1. Immunohistochemical procedure

Tissue was processed "free-floating" for immunohistochemistry as follows. Briefly, sections were incubated with 10% methanol, 3% hydrogen peroxide in phosphate-buffered saline (PBS, pH 7.4) for 10 min to block endogenous peroxidase activity. After this, sections were treated for 1 h with 5% normal donkey serum (NDS) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in PBS with 0.2% Triton- X100 (Sigma–Aldrich, St Louis, MO, USA) and were incubated overnight at room temperature in either monoclonal mouse anti-CCK (Cat No. 9303, Cure (Gastroenteric Biology Center), University of California, USA, 1:1000), polyclonal rabbit anti-VIP (VA 1285, Affiniti, UK, 1:1000); polyclonal rabbit anti-NPY (kindly provided by Dr. T.J. Görcs, 1:1000) (Csiffary et al., 1990) or polyclonal rabbit anti-SST (Cat. No 20067, Diasorin, Stillwater, USA, 1:1000). After washing, sections were incubated for 2 h with donkey anti-mouse IgG or donkey anti-rabbit IgG biotinylated antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA, 1:250), followed by avidin–biotin–peroxidase complex (ABC; Vector Laboratories, Peterborough, UK. 1:200) for 30 min. PBS containing 0.2% Triton-X-100 and 3% NDS was used for primary and secondary antibody dilutions. Color development was achieved by incubating in 0.05% 3,3-diaminobenzidine tetrahydrochloride (Sigma–Aldrich), 0.033% hydrogen peroxide in PB for 4 min.

The antibodies had been previously tested in their laboratory of origin; additionally, they showed a regional and cellular immunolabelling similar to previous descriptions of these antigens and the distribution of neurons is in accordance with previous studies using these antibodies and others specific for these neuropeptides (Chronwall et al., 1985; Forloni et al., 1990; Meziane et al., 1997; Sims et al., 1980). In order to confirm that some of the immunostaining was not produced by the secondary antibodies or by the immunohistochemical protocol itself, we omitted primary antibodies or substituted them by normal donkey serum. Moreover, we have pre-incubated the primary antibodies with the corresponding antigenic peptide. All these controls resulted in a complete absence of immunostaining in every case.

2.2. Density of neuropeptide expressing interneurons in the primary somatosensory cortex of Ts65Dn mice

We have analysed changes in the distribution of specific subpopulations of interneurons (using immunohistochemistry against CCK, SST, NPY and VIP) in the primary somatosensorial cortex of Ts65Dn mice and in their euploid littermates. We counted the number of immunoreactive cells found in 500-μm wide strips (20 strips per group) running perpendicular to the pial surface including all the layers of the primary somatosensory cortex (Berbel et al., 1996; Pérez-Cremades et al., 2010). Our method consisted in counting all the cells in a strip of somatosensory cortex