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From Cell Biology to Tissue Engineering

Morphological alterations in the hippocampus of the Ts65Dn mouse model for Down syndrome correlate with structural plasticity markers

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Summary. Down syndrome (DS) is the most common chromosomal aneuploidy. Although trisomy on chromosome 21 can display variable phenotypes, there is a common feature among all DS individuals: the presence of intellectual disability. This condition is partially attributed to abnormalities found in the hippocampus of individuals with DS and in the murine model for DS, Ts65Dn. To check if all hippocampal areas were equally affected in 4-5 month adult Ts65Dn mice, we analysed the morphology of dentate gyrus granule cells and cornu ammonis pyramidal neurons using Sholl method on Golgi-Cox impregnated neurons. Structural plasticity has been analysed using immunohistochemistry for plasticity molecules followed by densitometric analysis (Brain Derived Neurotrophic Factor (BDNF), Polysialylated form of the Neural Cell Adhesion Molecule (PSA-NCAM) and the Growth Associated Protein 43 (GAP43)). We observed an impairment in the dendritic arborisation of granule cells, but not in the pyramidal neurons in the Ts65Dn mice. When we analysed the expression of molecules related to structural plasticity in trisomic mouse hippocampus, we observed a reduction in the expression of BDNF and PSA-NCAM, and an increment in the expression of GAP43. These alterations were restricted to the regions related to dentate granule cells suggesting an interrelation. Therefore the impairment in dendritic arborisation and molecular plasticity is not a general feature of all Down syndrome principal neurons. Pharmacological manipulations of the levels of plasticity molecules could provide a way to restore granule cell morphology and function.

Key words: PSA-NCAM, BDNF, GAP43, Granule cells, CA1 pyramidal neurons

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Introduction

Down syndrome (DS) is the most common chromosomal aneuploidy, with an incidence of one in 1000 live births (Roizen and Patterson, 2003). Trisomy of the chromosome 21 induces a variable phenotype that may include immune deficiencies, heart defects, increased risk of leukaemia, and early development of Alzheimer's disease. The common feature among all DS subjects is the presence of intellectual disability reflected by impairment in learning and memory. Neural mechanisms underlying this alteration may include defects in the formation of neuronal networks, information processing and brain plasticity.

Several animal models that mimic the alterations in DS are available. One of the most studied is the Ts65Dn mouse. This model is segmentally trisomic for a portion of the mouse chromosome 16 that 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; Sturgeon and Gardiner, 2011; Rueda et al.,

2012). These mice display a delay in the acquisition of a number of sensory and motor tasks (Holtzman et al., 1996; Costa et al., 1999; Martínez-Cué et al., 2013), as well as defects in learning and in the execution of memory tasks mediated by the hippocampus (Reeves et al., 1995; Holtzman et al., 1996; Demas et al., 1998, 1999; Escorihuela et al., 1998; Sago et al., 2000; Hyde et al., 2001), and deficits in long-term potentiation (LTP) (Siarey et al., 1997, 1999; Kleschevnikov et al., 2004, 2012). Many of these manifestations may be the consequence of impairment in structural brain plasticity. Dendritic atrophy is one of the hallmarks of this pathology (Dierssen and Ramakers, 2006). Studies using Golgi techniques have shown dendritic atrophy in the neocortex of adult and young individuals with DS (Marin-Padilla, 1976; Takashima et al., 1981; Becker et al., 1986). Moreover, studies in the Ts65Dn model have demonstrated, among others, alterations in pyramidal neurons of the neocortex (Dierssen et al., 2003) and granule cells of hippocampus (Dang et al., 2014) suggesting that dendritic atrophy is a common feature in the brain of DS individuals and Ts65Dn mice.

Brain plasticity may underlie the impairment observed in dendritic arborisation. Brain plasticity can be defined as the ability to perform adaptive changes related to the structure and function of the central nervous system (Zilles, 1992). Structural plasticity takes place both during development and adulthood. During development, brain structural plasticity is a fundamental element that generates the specificity of connections present in the mature nervous system, allowing morphogenetic processes such as cell proliferation, cell migration, axonal or dendritic growth and remodelling. This plastic ability diminishes with age, becoming reduced during adulthood and limited to some specific regions (Bonfanti, 2006). During adulthood, structural plasticity is reduced to neurogenesis (in the subventricular zone and in the subgranular zone of the dentate gyrus), neuritogenesis and synaptogenesis. These processes are crucial for learning and adaptability (Cotman et al., 1998; Gage, 2000).

One of the cerebral regions where brain structural plasticity remains specially active during adulthood is the hippocampus (Leuner and Gould, 2010). In adult animals, the pyramidal neurons of the CA1 and CA3, and the granule cells of the dentate gyrus are submitted to dynamic modifications of their dendritic profiles, and subjected to synaptic plasticity. The generation of new neurons persists in the dentate gyrus until old age (Altman, 1962; Altman and Das, 1965; Seress, 2007) and the formation of these new neurons implies the growth of axons and dendrites and the formation of new synapses. Hippocampal function and structure is clearly impaired in DS as well as in the Ts65Dn model.

Regarding neurogenesis, a reduction in cell proliferation has been observed in the subgranular zone of the dentate gyrus (Clark et al., 2006; López-Hidalgo et al., 2016), which leads to a reduction in the number of granule cells (Insausti et al., 1998; Lorenzi and Reeves, 2006). Moreover, an impairment in spine formation and maturation has been also observed in cortical pyramidal neurons in DS individuals (Becker et al., 1986).

Synaptically, the expression of synaptophysin (a reliable marker for synapses (Masliah et al., 1990; Eastwood and Harrison, 2001)) in the hippocampus, has shown that the area occupied by this synaptic protein is larger in Ts65Dn mice than in controls, suggesting an increase in the size of the synapses (Kurt et al., 2004; Belichenko et al., 2007, 2009) Similar results were observed by our group in the primary somatosensory cortex (Pérez-Cremades et al., 2010). Our group and others have observed a reduction in the density of excitatory contacts in the hippocampus (Belichenko et al., 2004; Hernández-González et al., 2015) and an increase in the density of inhibitory ones (Hernández-González et al., 2015). Other studies have shown a reduction in number of excitatory contacts in the temporal cortex (Kurt et al., 2000) and an increment of inhibitory contacts in the primary somatosensory cortex (Pérez-Cremades et al., 2010).

Our aim is to correlate the impairment in dendritic arborisation with alterations in the expression of molecules involved in structural plasticity. We analysed the morphology of the dendritic arbour of granule cells and CA1 pyramidal neurons in adult animals, and the expression of the molecules related with structural plasticity: Brain Derived Neurotrophic factor (BDNF), the polysialylated form of the neural cell adhesion molecule (PSA-NCAM) and the Growth Associated Protein 43 (GAP43).

Materials and methods

Experimental mice were generated by repeated backcrossing of Ts65Dn females to C57/6Ei 9 C3H/HeSnJ (B6EiC3) F1 hybrid males. The parental generation was obtained from the research colony of Jackson Laboratory. Euploid littermates of Ts65Dn mice served as controls. For this study, we used four- to five-month-old male mice (18 trisomic mice and 30 euploid mice). The genotypic characterization was established by qRT-PCR using SYBR Green PCR master mix (Applied Biosystems) from genomic DNA extracted from mice tails by means of the phenol-chloroform method. The relative amount of each gene was quantified by the ABI PRISM 7700 (Applied Biosystems). The genes analysed where APP (3 copies) and Apo-B (2 copies) as previously described (Liu et al., 2003). The primers used were: 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'). All animal experimentation was conducted in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and was approved by the Committee

on Bioethics of the Universitat de València (2015/VSC/PEA/00046). Every effort was made to minimize the number of animals used and their suffering.

Animals, under pentobarbital overanesthesia, were transcardially perfused using saline followed by a solution containing 4% paraformaldehyde in PB (0.1 M, pH 7.4). Brains (12 trisomic and 20 euploid) were removed and cryoprotected using 30% sucrose. Fifty micron sections (6 subseries for each brain) were obtained using a sliding freezing microtome.

Golgi-Cox impregnation and dendritic arborisation characterization of CA1 pyramidal neurons and dentate granule cells

Some of the animals (6 trisomic and 10 euploid) were transcardially perfused using a solution containing 4% paraformaldehyde in PB (0.1M, pH 7.4). Brains were removed and processed for morphological characterization following the Golgi-Cox method. In brief, the hemispheres were dissected in sections of 3 mm and incubated in an chromating solution containing 5% potassium dichromate and 3% glutaraldehyde for 7 days. After that, samples were impregnated in 1% silver nitrate for 3 days. 150 micron-thick vibratome sections were obtained. Sections were placed in dishes, dehydrated and mounted in EPON resin. For dendritic arborisation the sections were analysed using the Sholl method (Sholl, 1953). In order to be analysed, Golgi-impregnated granule and pyramidal CA1 neurons had to fulfill the following criteria: (1) the cell must not show any truncated dendrites, (2) the dendritic arbour of the cell must show at least a process with a length greater than 120 μm and (3) the soma must be located at least 30 μm deep from the surface of the section. Granule cells and CA1 pyramidal neurons adhering to these criteria were drawn using a camera lucida. The Sholl analysis consists in the number of intersections of the dendrites within annuli (20 μm wide) of increasing radius centred in the soma. We selected only neurons in the same positions within the dentate gyrus granule cell layer (the external part of the upper blade in the dorsal hippocampus), and CA1 (dorsal hippocampus). We analyzed 25 granule cells and 30 pyramidal CA1 neurons from euploid mice and 20 granule and 22 pyramidal CA1 neurons from trisomic mice. In the case of CA1 pyramidal neurons we analysed separately the apical and basal dendrites.

Immunohistochemical procedure

Tissue was processed ''free-floating'' for immunohistochemistry as follows. Briefly, sections were incubated with 10% methanol, 3% H_2O_2 in phosphate-buffered saline (PBS) 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 either in monoclonal mouse IgG anti-GAP43 (1:1000, Novocastra, NCL-GAP43), polyclonal rabbit IgG anti-BDNF (1:100, Santa Cruz Biotech, SC-546), monoclonal mouse IgM anti-PSA-NCAM (1:700, Chemicon Int. Inc., Temecula, CA, USA, MAB5324), monoclonal mouse IgG anti-Gephyrin (1:1000, Synaptic Systems, 147.011) or polyclonal rabbit igG anti-PSD-95 (1:1000, Cell Signaling, 2507) antibodies. After washing, sections were incubated for 2 h with donkey anti-mouse IgM., donkey anti-mouse IgG or donkey anti-rabbit IgG biotinylated antibodies (1:250; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), followed by avidin–biotin–peroxidase complex (ABC; Vector Laboratories, Peterborough, UK) diluted in PBS, for 30 min. Color development was achieved by incubating with 0.05% 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) and 0.033% hydrogen peroxide in PB for 4 minutes. Finally, sections were mounted on slides, dried for one day at room temperature, dehydrated in ascending alcohols and rinsed in xylene. After this, sections were coverslipped using Eukitt mounting medium (PANREAC). All studied sections passed through all procedures simultaneously in order to minimize any difference from the immunohistochemical staining itself. To avoid any bias in the analysis, all slides were coded prior to analysis and remained so until the experiment was completed.

The antibodies had been previously tested in their laboratory of origin; they showed a regional and cellular immunolabelling in the hippocampus similar to previous descriptions for these antigens. In order to confirm that some of the immunostaining was not produced by the secondary antibodies or by the immunohistochemical protocol, we omitted primary antibodies or substituted them by normal donkey serum. These controls resulted in a complete absence of immunostaining in every case.

Densitometrical analysis of expression for the markers

We have analysed the intensity of the immunohistochemical staining for GAP43, BDNF and PSA-NCAM in the different subregions and layers of the hippocampus (strata oriens, pyramidale, radiatum and lacunosum-moleculare CA1; strata oriens, pyramidale, radiatum and lucidum of CA3 and strata moleculare, granulare and hilus of the dentate gyrus. In order to analyse the intensity of the immunostaining we used a previously described methodology (Varea et al., 2007a). Sections of hippocampus (Bregma between -2.06 and -2.30 mm) were examined with an Olympus CX41 microscope under bright-field illumination, homogeneously lit and digitalized using a CCD camera. Photographs were taken at 200X magnification. Images were converted to greyscale. Grey levels were measured using Image J software (NIH). This software can measure the intensity of expression as a increased grey

level. Five measurements were taken for each analysed area. Means were determined for each experimental group (Ts65Dn vs. euploid littermates) and data were subjected to one-way ANOVA followed by Student-Newman-Keuls post hoc tests using the SPSS software package (version 15). Nissl-stained sections adjacent to the measured ones were used to determine the location and borders of the different subregions of the hippocampus.

Results

Dendritic complexity of granule and pyramidal CA1 neurons

We have analysed the dendritic arborisation of Golgi-Cox impregnated granule cells (Fig. 1) and pyramidal CA1 neurons (Fig. 2) using the Sholl method. This method gives an estimation of the complexity of the dendritic arbours. The study of granule cells in euploid and trisomic mice (Fig. 1A,B) revealed a reduction in the total number of intersections (Fig. 1C). The analysis along the dendritic arbour revealed that the reduction is statistically significant in the region between 120 to 160 μm from the cell body (Fig. 1D).

The analysis of dendritic branching of pyramidal CA1 neurons reflected that there was no alteration in the complexity of dendrites in trisomic mice when compared with euploid (Fig. 2A,B). Basal (Fig. 2C,D) and apical (Fig. 2E,F) dendrites showed similar complexity in trisomic and euploid mice.

The difference between granule and pyramidal neurons may be related with alterations in the molecular environment. This could be the result of the different expression of molecules related to neuronal structural plasticity. In order to check this hypothesis we analysed the expression of these molecules (BDNF, PSA-NCAM and GAP43) in the hippocampus of control and trisomic adult mice.

Brain Derived Neurotrophic Factor (BDNF)

The expression of BDNF (Fig. 3) showed a light, diffuse and homogenous staining, as is expected by the nature of this factor, being more intense in the strata pyramidale and oriens of CA1 and the subgranular zone of the dentate gyrus (Fig. 3A).

We have analysed the expression of BDNF using densitometry in adult animals comparing control and trisomic mice (Fig. 3B-J). In the CA1 (Fig. 3B-D) there were no differences between control and trisomic mice (normalized optical density 45.3±2.0 vs 47.9±2.6 in the stratum oriens, 18.9±1.3 vs. 20.4±1.8 in the stratum pyramidale, 39.5±2.8 vs. 40.1±1.7 in the stratum radiatum and finally 20.5±1.5 vs. 20.7±2.3 in the stratum lacunosum-moleculare). In the CA3 (Fig. 3E-G), we only observed a reduction in the stratum lucidum (16.3±1.1 vs. 12.1±1.6, $p<0.05$) of trisomic mice, with no differences in the other strata (16.9±0.7 vs. 17.6±1.0

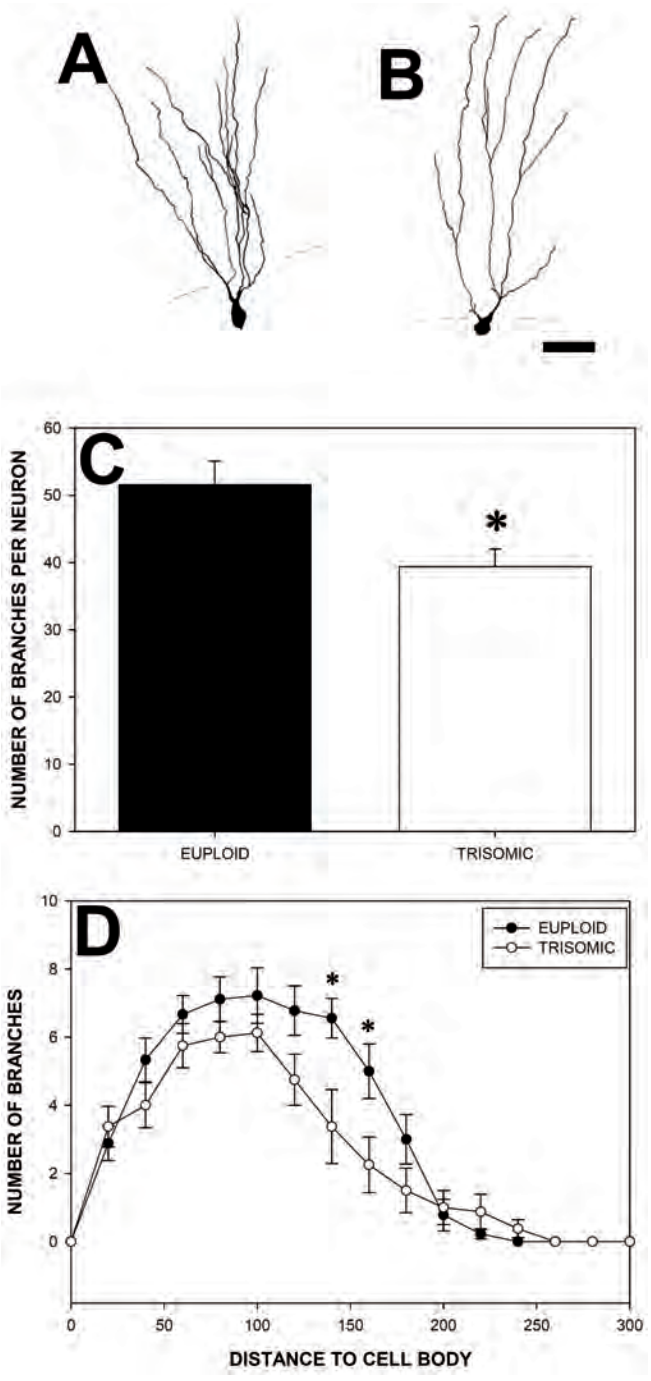

Fig. 1. Alterations in the dendritic branching of hippocampal granule cells of Ts65Dn mice using Sholl analysis. **A, B.** Representative camera lucida drawings from granule cells of euploid and trisomic mice respectively. **C.** Graph showing the total number of intersections in euploid (black bar) and trisomic mice (white bar). **D.** Number or intersections at different distances from the cell somata. (* $p<0.05$). Scale bar: 50 μm.

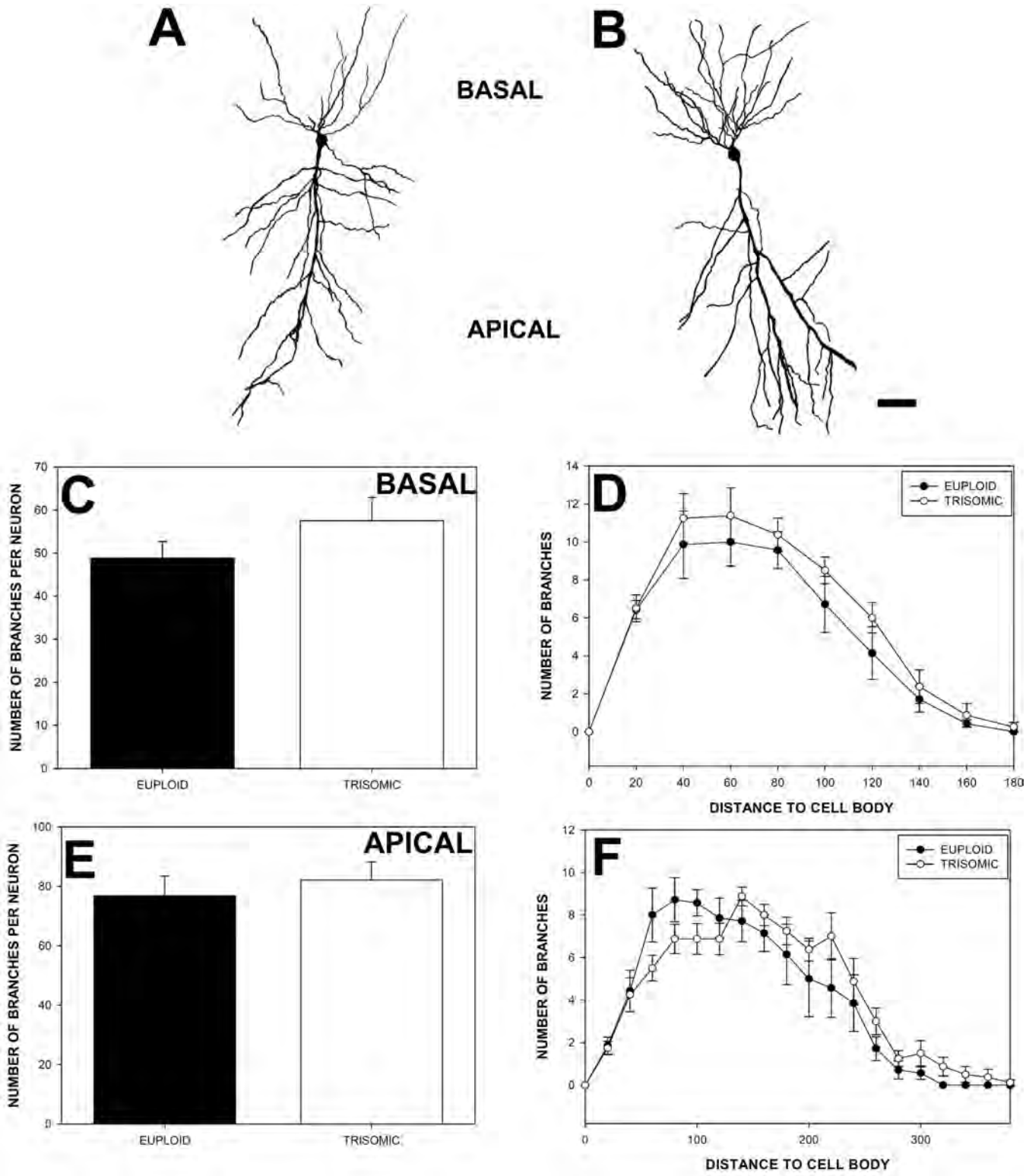

Fig. 2. Alterations in the dendritic branching of hippocampal CA1 pyramidal neurons of Ts65Dn mice using Sholl analysis. **A, B.** Representative camera lucida drawings from CA1 pyramidal neurons of euploid and trisomic mice respectively. **C.** Graph showing the total number of intersections for the basal dendrites in euploid (black bar) and trisomic mice (white bar). **D.** Number or intersections of basal dendrites at different distances from the cell somata. **E.** Graph showing the total number of intersections for the apical dendrites in euploid (black bar) and trisomic mice (white bar). **F.** Number or intersections of apical dendrites at different distances from the cell somata. Scale bar: 50 μm.

in the stratum oriens, 10.1±0.6 vs. 12.2±1.5 in the stratum pyramidale and 9.5±0.6 vs. 9.1±0.6 in the stratum radiatum). In the dentate gyrus (Fig. 3H-J), we observed a statistically significant reduction in the stratum moleculare and hilus (17.9±1.1 vs.13.3±1.1, $p<0.05$ in the stratum moleculare; 17.6±1.1 vs. 14.6±1.9 in the stratum granulare; and 16.9±1.0 vs. 12.8±1.4, $p<0.05$ in the hilus).

Polysialylated form of the neural cell adhesion molecule (PSA-NCAM)

PSA-NCAM in the hippocampus displayed a high intensity of expression (Fig. 4). We have observed the

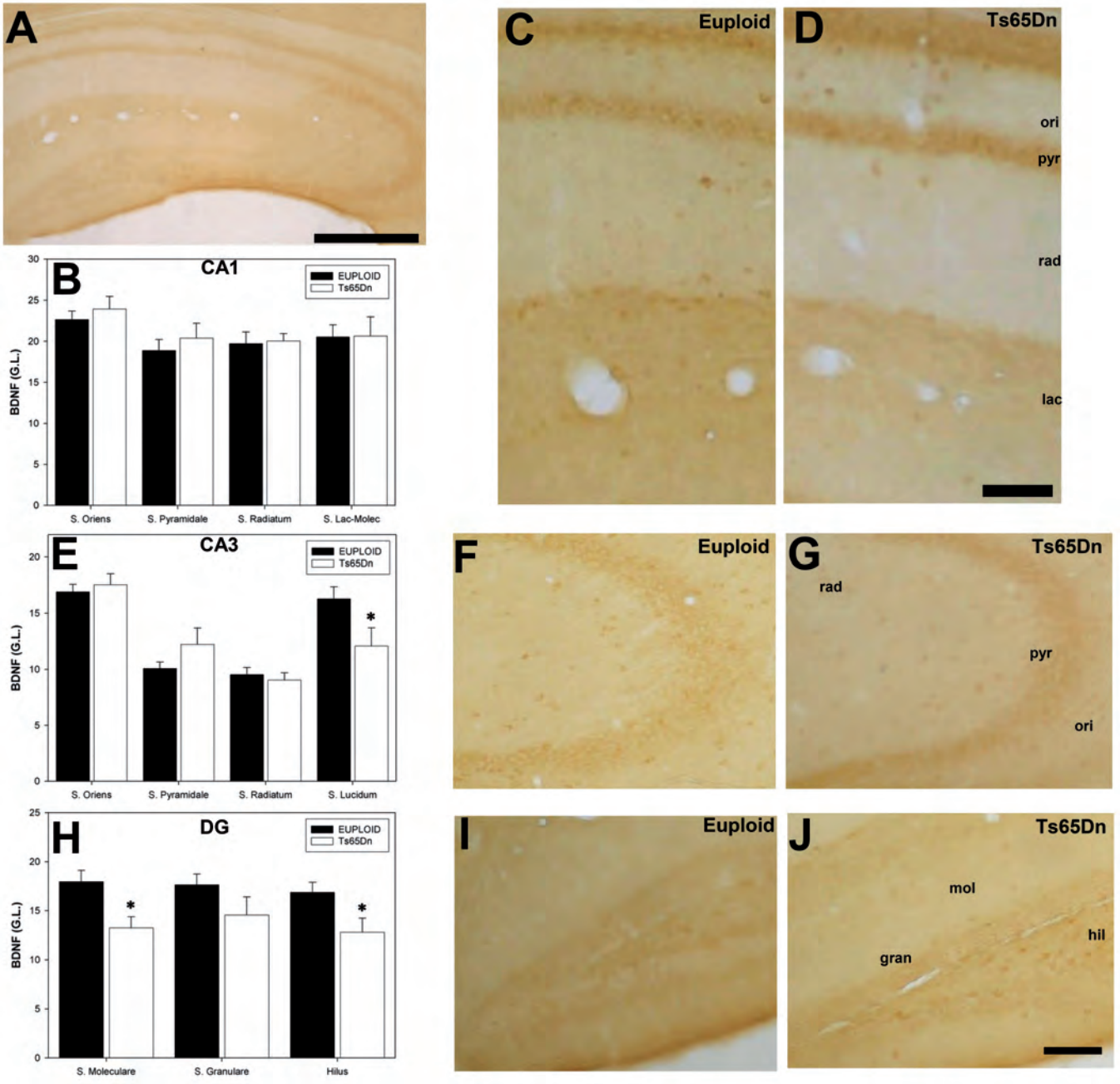

Fig. 3. Alterations in the expression of BDNF in the hippocampus of the Ts65Dn mice. **A.** Panoramic view of the distribution of BDNF in the hippocampus. **B, E, H.** Graphs showing the intensity of expression of BDNF in the different subregions of the hippocampus in euploid mice (black bar) and trisomic mice (white bar). CA1 **(B)**, CA3 **(E)**, dentate gyrus **(H)**. Representative images of the distribution of BDNF in the hippocampus in euploid and Ts65Dn mice respectively, CA1 **(C-D)**, CA3 **(F-G)** and DG **(I-J)**. (* $p<0.05$). Scale bars: A, 500 μm; C, D, 200 μm; F, G, I, J, 100 μm.

highest intensity in the dentate gyrus and stratum lucidum of CA3 (newly generated neurons transiently express PSA-NCAM), followed by a slightly weaker immunoreactivity in the stratum lacunosum of CA1 (Fig. 4A). The staining showed a punctate distribution due to its location in the membrane. Moreover, positive cells could be observed both in the subgranular zone of the dentate gyrus (corresponding to immature granule cells) and in other regions of the hippocampus (corresponding to inhibitory neurons). The analysis of the expression of PSA-NCAM revealed that in trisomic animals there was a general decrease in the CA1 (Fig. 4B-D), although it

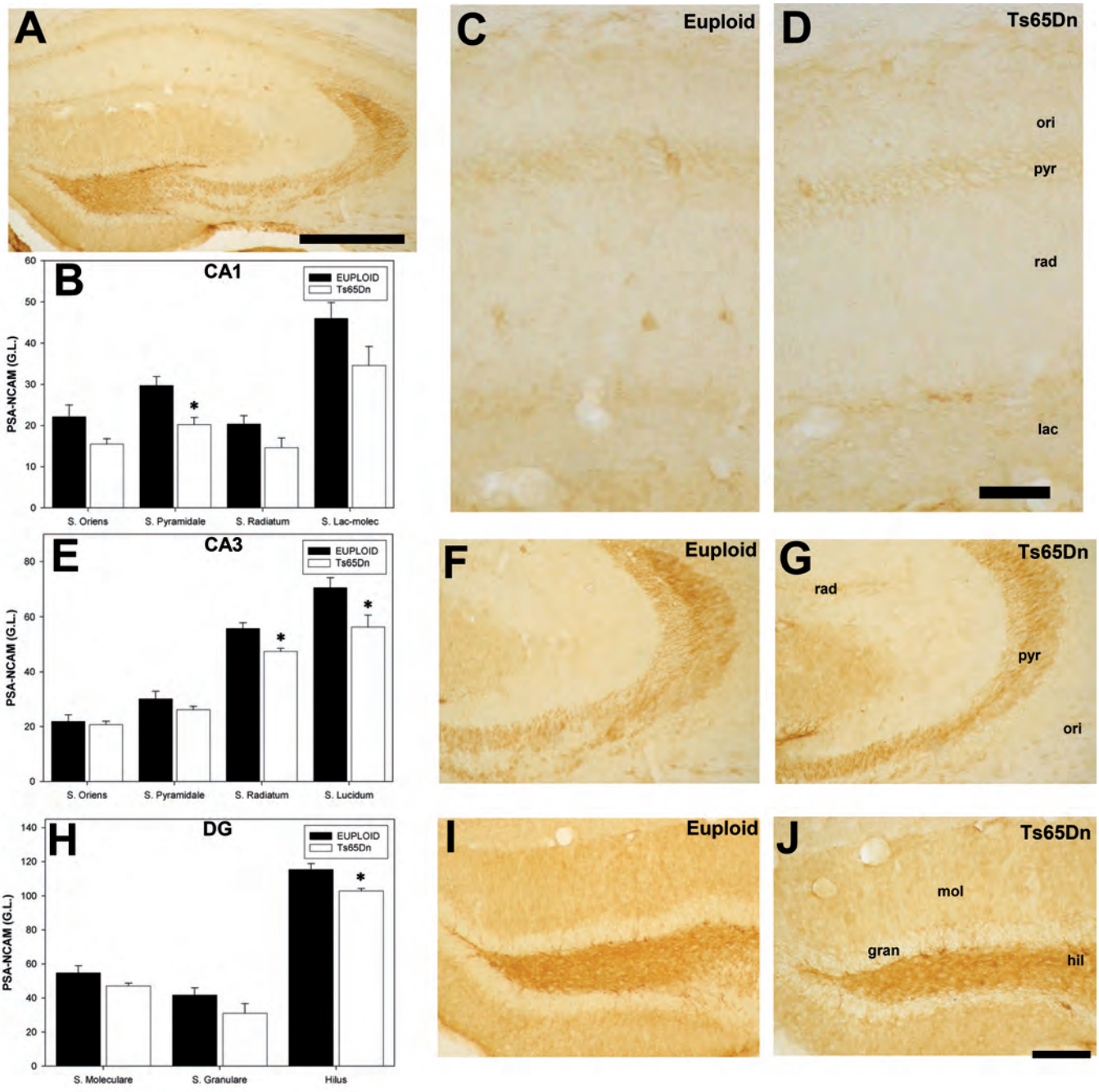

Fig. 4. Alterations in the expression of PSA-NCAM in the hippocampus of the Ts65Dn mice. **A.** Panoramic view of the distribution of PSA-NCAM in the hippocampus. **B, E, H.** Graphs showing the intensity of expression of PSA-NCAM in the different subregions of the hippocampus in euploid mice (black bar) and trisomic mice (white bar). CA1 **(B)**, CA3 **(E)**, dentate gyrus **(H)**. Representative images of the distribution of PSA-NCAM in the hippocampus in euploid and Ts65Dn mice respectively, CA1 **(C-D)**, CA3 **(F-G)** and DG **(I-J).** (* $p<0.05$). Scale bars: A, 500 μm; C, D, 200 μm; F, G, I, J, 100 μm.

was only statistically significant for the stratum pyramidale (22.1±2.5 vs. 15.5±1.3 in the stratum oriens; 29.7±2.2 vs. 20.2±1.7, p<0.05 in the stratum pyramidale; 20.4±2.0 vs. 14.7±2.3 in the stratum radiatum and 45.9±3.9 vs. 34.6±4.6 in the stratum lacunosum-moleculare). In the CA3 (Fig. 4E-G) we also observed a significant decrease in the expression of PSA-NCAM in the strata radiatum and lucidum (21.9±2.4 vs. 20.7±1.2 in the stratum oriens; 30.2±2.7 vs. 26.3±1.2 in the stratum pyramidale; 55.9±2.1 vs. 47.5±1.1, p<0.05 in the stratum radiatum and 70.7±3.6 vs. 56.4±4.3 p<0.05 in the stratum lucidum). In the dentate gyrus (Fig. 4H-J) we have observed a reduced expression of PSA-NCAM in the hilus (54.8±4.2 vs. 47.1±1.6 in the stratum

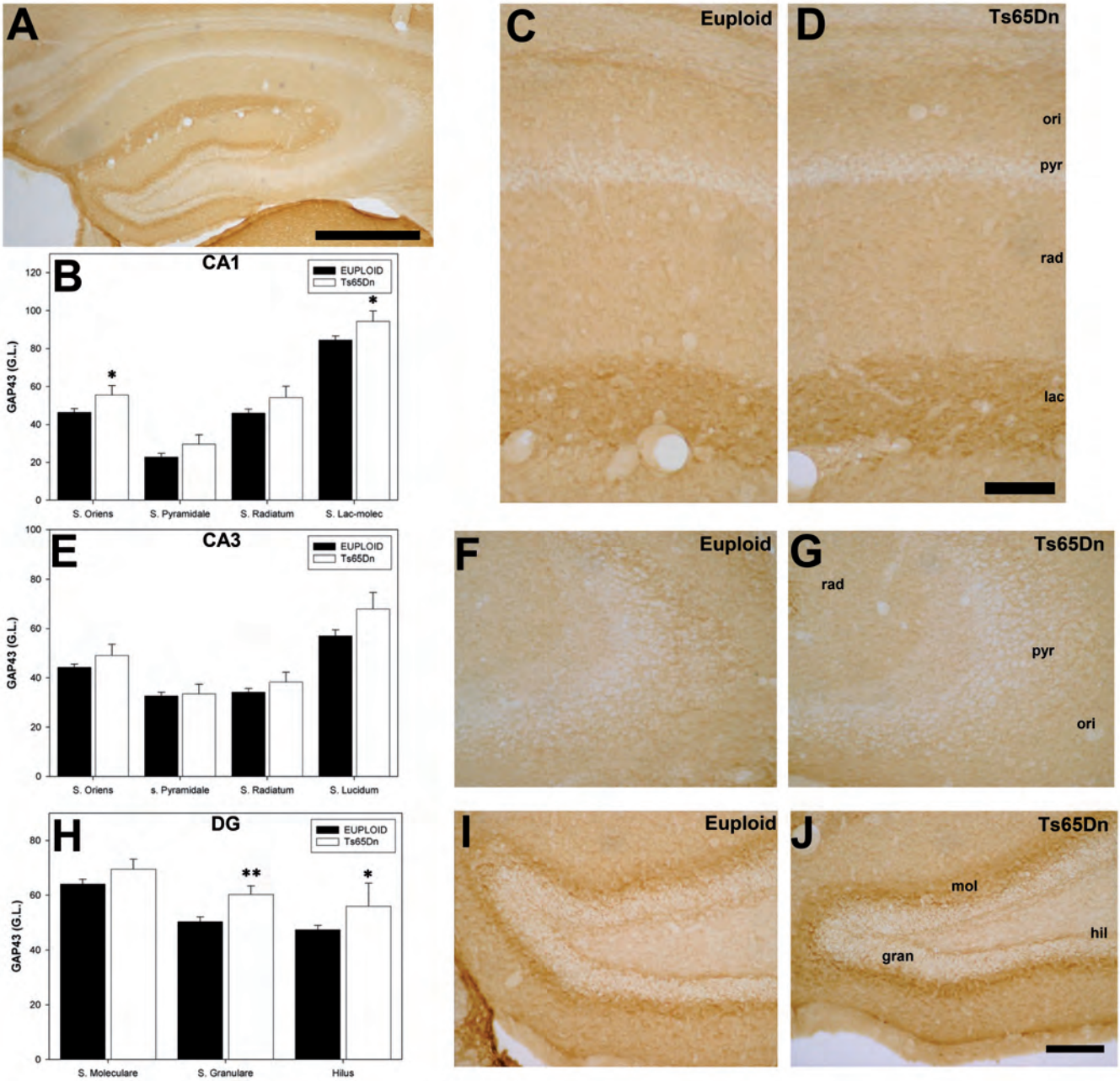

Fig. 5. Alterations in the expression of GAP43 in the hippocampus of the Ts65Dn mice. **A.** Panoramic view of the distribution of GAP43 in the hippocampus. **B, E, H.** Graphs showing the intensity of expression of GAP43 in the different subregions of the hippocampus in euploid mice (black bar) and trisomic mice (white bar). CA1 **(B)**, CA3 **(E)**, dentate gyrus **(H)**. Representative images of the distribution of GAP43 in the hippocampus in euploid and Ts65Dn mice respectively, CA1 **(C-D)**, CA3 **(F-G)** and DG **(I-J)**. (* p<0.05, ** p<0.01). Scale bars: A, 500 μm; C, D, 200 μm; F, G, I, J, 100 μm.

moleculare; 41.8±4.1 vs. 31.0±5.8 in the stratum granulare and 115.5±3.3 vs. 102.9±1.4 p<0.05 in the hilus).

Growth associated protein 43 (GAP43)

GAP43 displayed a pattern of staining more intense than BDNF and presented a punctate appearance (Fig. 5). The highest intensity was observed in the strata lacunosum-moleculare of CA1 and molecular of the dentate gyrus. The subgranular zone of the dentate gyrus also displayed a high intensity of staining (Fig. 5A). The analysis of the expression of GAP43 revealed a general increase in intensity in trisomic mice. In the CA1 (Fig. 5B-D), we observed an increased expression in the strata oriens and lacunosum-moleculare of trisomic mice (46.4±1.9 vs. 55.5±4.9 p<0.05 in the stratum oriens; 22.8±1.9 vs. 29.5±5.0 in the stratum pyramidale;

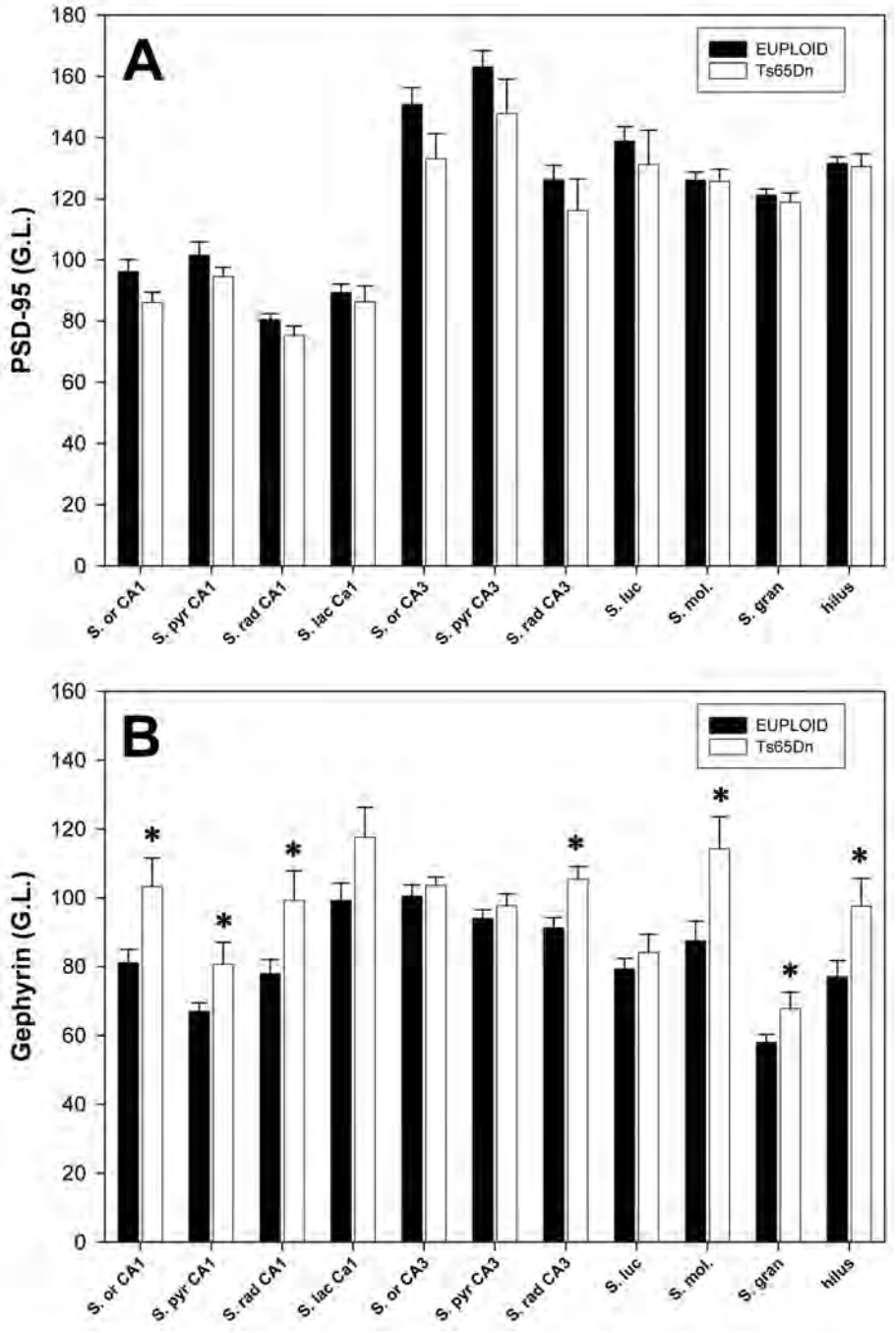

Fig. 6. Alteration in the expression of postsynaptic markers in the hippocampus of the Ts65Dn mice. Graph showing the intensity of expression of PSD-95 **(A)** and Gephyrin **(B)** in the different subregions of the hippocampus (* p<0.05).

46.1±2.0 vs. 54.2±5.9 in the stratum radiatum and 84.58±2.05 vs. 94.36±5.52 p<0.05 in the stratum lacunosum-moleculare). In the CA3 (Fig. 5E-G) we observed no differences between control and trisomic mice in GAP43 expression (44.3±1.3 vs.49.1±4.5 in the stratum oriens; 32.6±1.6 vs. 33.5±3.8 in the stratum pyramidale; 34.1±1.5 vs. 38.3±3.9 in the stratum radiatum and 85.6±2.0 vs. 94.4±5.5 in the stratum lucidum). Finally, in the dentate gyrus (Fig. 5H-J), we observed a significant increase in the expression of GAP43 in the stratum granulare and the hilus (64.1±1.7 vs. 69.5±3.6 in the stratum moleculare; 50.4±1.7 vs. 60.3±3.1 p<0.01 in the stratum granulare; and 47.4±1.6 vs. 55.9±3.4 p<0.05 in the hilus). Since the inner molecular layer presents a high expression of GAP43 whereas the outer molecular layer displays a weak staining, we analysed separately the inner and the outer molecular layer. The study revealed that the inner molecular layer of trisomic mice presented a statistically significant increase in the expression of GAP43 (outer molecular layer 44.3±2.7 vs. 51.3±1.9; inner molecular layer 72.5±2.7 vs. 82.4±2.6 p<0.05).

Markers of postsynaptic excitatory and inhibitory densities

In order to correlate the differences in morphology and plasticity markers with alterations in the density of synaptic contacts, we have analysed the expression of markers for the postsynaptic densities for both, excitatory (using PSD-95) and inhibitory (using gephyirin) synaptic contacts. This study complements a previous study where we analysed the expression of synaptic markers (presynaptic) (Hernández et al., 2015).

We observed that the optic density of PSD-95 was not different in trisomic mice in all the regions (Fig. 6A). However the analysis of gephyrin reflected an increase of inhibitory contacts in the hippocampus of trisomic mice in most regions (Fig. 6B).

Discussion

In this report, we have analysed the alterations of granule cells and CA1 pyramidal neurons in the hippocampus of the trisomic murine model for Down Syndrome Ts65Dn. We have observed impairment in the dendritic arbour in granule cells whereas pyramidal CA1 neurons remained unaltered. We have related this alteration with the expression of diverse molecules related to structural plasticity in the hippocampus. The impairment of the dentate granule cell morphology was coupled to a reduction in the expression of BDNF and PSA-NCAM and an increase in the expression of GAP43 only in the dentate gyrus.

Dendritic alteration in the principal neurons of the hippocampus

We have observed an impairment in the dendritic complexity of granule cells. This is similar to other similar impairments previously reported in different brain regions of the Ts65Dn brain (Dierssen et al., 2003; Dang et al., 2014). Similar results have been obtained in individuals with DS (Marin-Padilla, 1976; Becker et al., 1986; Ferrer and Gullotta, 1990). The impairment in granule cell arborisation observed is similar to those observed in 5-6 month old Ts65Dn mice (Dang et al., 2014). Dendritic arborisation of granule neurons is located in the stratum moleculare, where the projections from the entorhinal cortex through the perforant pathway arrives. This constitutes the main input of information into the hippocampus. It has been pointed out that dendritic impairment is a general feature of DS (Dierssen and Ramakers, 2006), however, the study of the complexity of the dendritic arbour in CA1 pyramidal neurons showed that both apical and basal dendrites remain unaltered in the Ts65Dn model. This fact indicates that dendritic alteration is not a general feature of this model and may be limited to specific subtypes of neurons or to specific regions of the brain.

In a previous report (Hernández-González et al., 2015), we showed that in the Ts65Dn model there is a reduction in excitatory contacts (as shown by vesicular glutamate transporter 1, VGLUT1) and an increment in inhibitory contacts (using the isoform of 67 KDa of the glutamate decarboxylase, GAD67). This imbalance between excitation and inhibition has been observed in the hippocampus and other brain regions in DS murine models (Belichenko et al., 2004, 2007, 2009). In this study we have analysed the expression of the postsynaptic markers for excitatory contacts (PSD-95) and for inhibitory contacts (Gephyrin). The results for gephyrin correlate well with the previously reported increment for GAD-67 in the Ts65Dn model. However, regarding the excitatory contacts, with PSD-95 we have not detected the reduction reported using vGLUT1. PSD-95 did not change in trisomic mice. Overall, these results confirm the imbalance between excitation and inhibition previously observed (Belichenko et al., 2009; Hernández-González et al., 2015). However, the discrepancy between the results for VGLUT-1 and PSD-95 indicates that the number of synapses remains unaltered but their size may be smaller. Nevertheless, the efficiency of the excitatory synapses is impaired in the Ts65Dn model. Some studies reflected an impairment in LTP in the Ts65Dn model (Kleschevnikov et al., 2004, 2012; Costa and Grybko, 2005), this impairment could be mediated, at least in part, by this decrease in the presence of VGLUT-1 in the excitatory synapses.

Decreased expression of BDNF in the hippocampus of the Ts65Dn model

BDNF is a crucial molecule for the survival and growth of neurons as well as for the maintenance of their functional activity. This neurotrophic factor participates in the stabilization and maturation of synapses during development as well as in the generation of new synaptic

contacts during adulthood (for a review see Vicario-Abejón et al. 2002), inducing the growth of neurites. Moreover, BDNF has been shown to be necessary for the generation of neurons during adulthood in the subgranular zone of the dentate gyrus and in the subventricular zone (Pinnock and Herbert, 2008). In our studies, we have observed a general decrease in the expression of BDNF in the hippocampus of Ts65Dn mice. Previous studies have reported controversial results: some studies observed a decrease in mRNA for BDNF in the whole hippocampus (Bianchi et al., 2010), whereas others have not observed changes in its expression (Peng et al., 2009). Studies analysing the amount of the protein BDNF in the whole hippocampus have observed reduction (Fukuda et al., 2010) or no change (Pollonini et al., 2008; Lockrow et al., 2011). In our study, we have analysed the expression of the protein using immunohistochemistry and performed a topographical analysis, using densitometry, in order to determine in which regions the expression of BDNF is altered. We observed the reductions affected only some regions of the dentate gyrus which would have been missed when analysing the hippocampus as a whole.

The analysis of the expression of BDNF in the hippocampus of Ts65Dn model has revealed a reduction in its expression in the stratum moleculare of the dentate gyrus, where the dendrites of granule cells are located. This reduction could be related to the dendritic atrophy observed in granule cells. Moreover, this result may imply a reduction in the presynaptic inputs that these neurons receive, as has been already observed in this model (Belichenko et al., 2009) these projections come mainly from the entorhinal cortex through the perforant pathway and are the main source of information that arrives to the hippocampus. In fact, dendritic atrophy and impaired synapse formation has been observed in individuals with DS (Becker et al., 1986). The hilar region is innervated by granule neuron axons. Adjacent to the hilar region is located the subgranular zone, responsible for adult neurogenesis in the hippocampus, and the reduction observed in BDNF in these areas could be related to the reduction in neurogenesis observed in this model (Bianchi et al., 2010; López-Hidalgo et al., 2016), and in individuals with DS (Wisniewski et al., 1984). In fact, the knockout mice for BDNF showed a reduction of 50% in the adult neurogenesis in this area (Sairanen et al., 2005). BDNF participates in the processes of generation, survival and differentiation of the newly generated neurons (Pinnock and Herbert, 2008). The reduction in the expression of BDNF observed in the stratum lucidum could also be related with the reduction in the number of newly generated neurons in the adult hippocampus, since granule cells project specifically to this stratum of the CA3. The reduction in BDNF induces a loss in synaptic efficiency (Alder et al., 2005; Tyler et al., 2006). It has been observed a reduction in LTP in the slices of the Ts65Dn mice model (Siarey et al., 1997). The deficit in BDNF has been related to impairment in hippocampus-dependent learning and spatial memory (Linnarsson et al., 1997; Mizuno et al., 2003) and similar alterations have been observed in individuals with DS (Lott and Dierssen, 2010) and in Ts65Dn mice (Demas et al., 1998; Escorihuela et al., 1998).

Decreased expression of PSA-NCAM in the hippocampus of the Ts65Dn model

The neural cell adhesion molecule (NCAM) is tightly related to neuronal structural plasticity. This molecule has the ability to incorporate long chains of the sugar polysialic acid (PSA), which confers it antiadhesive properties (Bruses and Rutishauser, 2001). PSA-NCAM is widely expressed during CNS development and decreases with age (Varea et al., 2009). During adulthood, PSA-NCAM is present in brain regions exhibiting high levels of structural plasticity such as the olfactory bulb (Miragall et al., 1988), the hippocampus (Seki and Arai, 1993), the amygdala (Nacher et al., 2002) and the prefrontal cortex (Varea et al., 2005). In these regions the presence of PSA is related to plastic events such as neuronal migration, dendritic extension or retraction, and synaptogenesis (Seki and Rutishauser, 1998; Dityatev et al., 2004). Some of these regions, such as the hippocampus and the olfactory bulb (Altman and Das, 1965; Altman, 1969; Lois and Alvarez-Buylla, 1994) retain the ability to incorporate new neurons. Our results showed a reduction in the expression of PSA-NCAM in some regions of the hippocampus in trisomic mice. A decrease in the expression of PSA-NCAM may induce alterations in synaptogenesis and neurogenesis. We have observed the highest decrease in two regions: a) the pyramidal layer of CA1, in which this reduction could be related to the reduction of LTP observed in the CA1 region of Ts65Dn mice (Costa and Grybko, 2005). And b) the hilus and stratum lucidum, i.e. the regions containing the axons of granular cells. Reductions of PSA-NCAM expression in these regions may affect the connectivity between granule cells and CA3 pyramidal neurons. In fact, the ablation of PSA from PSA-NCAM in the hippocampus induces the appearance of aberrant circuits from granule cells to CA3 pyramidal neurons, and ectopic synaptogenesis (Seki and Rutishauser, 1998). The Ts65Dn model displays alterations in this circuit (Hanson et al., 2007) where there is a reduced number of thorny excrescences on the principal dendrites of CA3 pyramidal neurons throughout the stratum lucidum (Stagni et al., 2013).

Increased expression of GAP43 in the hippocampus of the Ts65Dn model

GAP43 is a protein associated to axonal growth, it is present in the growth cone, as well as in the synaptic terminals undergoing active remodelling (Benowitz and Routtenberg, 1997). During adulthood the expression of GAP43 is generally low, except in neurons involved in

synaptic remodelling, such as those in the associative cortex, the hippocampus and the olfactory bulb (Oestreicher et al., 1997). GAP43 is a presynaptic protein, which in mature neurons is located exclusively in the axons (Gispen et al., 1985; Goslin and Banker, 1990; Goslin et al., 1990). We have observed an overexpression of GAP43 in the stratum oriens of CA1 (where the axons of pyramidal neurons of CA1 are located) and in the stratum granulare and hilus of dentate gyrus (where the axons of granule cells are located).

It has been hypothesized that GAP43 may induce a reactive synaptogenesis in neurons that have undergone a reduction in their dendritic complexity (Shapiro and Whitaker-Azmitia, 2004). Moreover, GAP43 is re-expressed in neurons with injured axons (Frey et al., 2000). Therefore, the alterations in synaptogenesis and dendritogenesis in this model could induce reactive synaptogenesis as reflected by the overexpression of GAP43. A possible alternative or complementary explanation for the increase in GAP43 observed may be the fact that the expression of GAP43 is, at least in part, controlled by the kinase Dyrk1A (Guedj et al., 2012), a gene that is present in three copies in both mice and humans with DS. Therefore, the overexpression of Dyrk1A could induce the overexpression of GAP43.

Cooperative interactions

A reduction in PSA-NCAM could be related to the impaired extension of dendrites observed in the granule cells of the dentate gyrus in the Ts65Dn mouse model, producing a strong alteration of hippocampal structure and more specifically of mossy fibre function, and consequently of hippocampal information processing. A deficit in BDNF could be related to the reduction in neurogenesis and dendritic arbour of granule cells, therefore affecting hippocampal function. On the contrary, GAP43 density is increased. This increment could be related with the deficits observed, among others, in PSA-NCAM and BDNF, or be directly related to the extra copy of Dyrk1A. Reactive dendritogenesis and synaptogenesis (Shapiro and Whitaker-Azmitia, 2004) can be induced by the deficits present in dendritic and axonal profiles of neurons in the Ts65Dn model. Alternatively, it has been observed that NCAM and BDNF signalling converge into GAP43 phosphorylation (Yoshii and Constantine-Paton, 2007), and this modification in GAP43 induces its stabilization and restriction to the axonal and dendritic cone increasing synaptogenesis.

Our results have shown significant differences for different structural plasticity molecules (BDNF, PSA-NCAM and GAP43) in several regions. Impairment or reduction in the expression of molecules related to plasticity induces alterations in cellular migration, axonal pathfinding, neuritic development, active remodelling and integration of newly generated neurons; all these events are present in the Ts65Dn mice. In those regions in which plasticity markers are affected i.e. the dentate gyrus, the principal cells show a reduction in arborisation. On the contrary in those regions in which plasticity markers are not changed, the morphology of the principal cells remains unaltered. Treatments with substances that increases plasticity, like fluoxetine (Varea et al., 2007a,b; Guirado et al., 2009; Stagni et al., 2013) have been demonstrated to be able to increase dendritic arborisation of granule cells (Guidi et al., 2013) suggesting that lack of plasticity underlies deficiencies in morphology.

Conclusion

Alterations in dendritic branching correlate with aberrant expression of molecules related to structural plasticity, with reduction in BDNF and PSA-NCAM, and increases of GAP43. Since this only happens in dentate gyrus, it suggests that impairment in dendritic arborisation is not a general feature of all Down Syndrome principal neurons and every type should be analysed independently. Pharmacological manipulations of the levels of these molecules could provide a way to restore granule cell morphology and restore function.

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Conflict of interest. The authors declare that they have no conflict of interest.

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