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Prenatal, but not postnatal, curcumin administration rescues neuromorphological and cognitive alterations in Ts65Dn Down syndrome mice

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Abstract:	Background: The cognitive dysfunction in Down syndrome (DS) is partially caused by deficient neurogenesis during fetal stages. Curcumin enhances neurogenesis and learning and memory. Objectives: We aimed to test the ability of curcumin to rescue the neuromorphological and cognitive alterations of the Ts65Dn (TS) mouse model of DS when administered prenatally or during early post-natal stages, and to evaluate whether these effects were maintained several weeks after the treatment. Methods: To evaluate the effects of prenatal curcumin administration, 65 pregnant TS females were subcutaneously treated with curcumin (300 mg/kg) or vehicle from ED (Embryonic Day) 10 to PD (Post-natal Day) 2. All the analyses were performed on their TS and Control (CO) male and female progeny. At PD2, the changes in neurogenesis, cellularity, and brain weight were analyzed in 30 TS and CO pups. The long-term effects of prenatal curcumin were evaluated in another cohort of 44 TS and CO mice between PD30 and PD45. The neuromorphological effects of early postnatal administration of curcumin were assessed on PD15 in 30 male and female TS and CO pups treated with curcumin (300 mg/kg) or vehicle from PD2 to PD15. The long-term neuromorphological and cognitive effects were assessed from PD60 to PD90 in 45 mice. Data was compared by ANOVAs. Results: Prenatal administration of curcumin increased the brain weight (+45%, $P < 0.001$), the density of BrdU (Bromodeoxyuridine)- (+150%, $P < 0.001$) and DAPI (4′,6-diamidino-2-phenylindole)- (+38%, $P = 0.005$) positive cells, and produced a long-term improvement of cognition in TS (+35%, $P = 0.007$) mice with respect to vehicle-treated mice. Post-natal administration of curcumin did not rescue any of the short- or long-term altered phenotypes of TS mice. Conclusion: The beneficial effects of prenatal curcumin administration to TS mice

	suggest that it could be a therapeutic strategy to treat DS cognitive disabilities.
Additional Information:	
Question	**Response**
Has this manuscript been previously submitted?	No
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Please select a collection option from the list below:	Dietary Bioactive Compounds
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Author Comments:	Dear Editor, Please find attached the revised version of the manuscript entitled “Prenatal, but not postnatal, curcumin administration rescues neuromorphological and cognitive alterations in Ts65Dn Down syndrome mice” by Rueda and coworkers. In this version, we have addressed all the issues and concerns raised by the Reviewer and the Associate Editor. We believe that all these changes have considerably improved the quality of the paper, and we hope that it can now be considered for publicaton in The Journal of Nutrition. Thank you for your time and consideration. Sincerely, Carmen Martínez-Cué Department of Physiology and Pharmacology Faculty of Medicine University of Cantabria Santander, Spain martinec@unican.es

Comments from the Editors and Reviewers:

Reviewer 1: The authors have been responsive to the reviewers' concerns. It does seem that something regarding the interpretation of dose to the human situation would be helpful to have in the manuscript. That said, the authors' answer makes it clear that the situation is complicated so I will leave it up to the Editor decide whether or not any additions are needed. There are no outstanding concerns.

Response: We have added the following paragraph to the Methods section: "This dose of curcumin leads to plasma levels of 2.5 µg/mL in mice (67), while in humans an oral dose of 10 g yields to similar plasma levels (2.3 ± 0.26 µg/mL) (68). In most human studies, curcumin is administered orally at doses ranging from 0.4 to 20 g (7, 67)."

Reviewer 3 (Assistant Editor):

Table 1: explain short -term as part of the title, please.

Response: "Short-term" has been included as a part of the title on Table 1

Letters showing differences should be superscripts.

Response: Letters showing differences are now superscripts

Last 3 columns:

2-factor ANOVA P-values
Karotype Treatment Interaction

<0.001 <0.001 0.19
etc.
Response: The three last columns have been modified following the Editor's instructions

Footnote 1 to the title:
[1]Values are means ± SEMs, n=7-8. Means in a row without a common letter differ, P<0.05 (Fisher's post hoc tests). CO:...

Response: The footnote to the title has been modified following the Editor's instructions.

All aspects of printed figures (fonts, points, bolding, etc.) should be in proportion so that they will be legible when printed in 1-column (< 9 cm) width
or for some complex, multi-panel figures, 2-column width.
REDUCED text should be 6-8 points; enlarge ORIGINAL text as needed.

e.g. values on y axes of figure 1 C-J, figure 3, etc. may need to be larger.

Response: We have enlarged the fonts of the figures so it can be legible when printed including the values on y axis on figures 1-C (now figure 2) and figure 3 (now figure 4).

Panel letters are disproportionately large relative to other text.

Response: We have reduced the size of panel letters and they are now more proportional to the rest of the text in the figures.

Perhaps figure 1 A and B should be a separate figure.

Response: We have separated figures 1A and 1B and figures 1C-1J into two separate figures

All lines and symbols must be easily distinguished from one another; e.g. figure 3.

Response: We have enlarged de size of the symbols and the thickness of the lines in figure 3 (now figure 4).

Prenatal, but not postnatal, curcumin administration rescues neuromorphological and cognitive alterations in Ts65Dn Down syndrome mice

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Footnotes:

i. **Supplemental figures 1**, **2** and **3** and **Supplemental table 1** are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents.

ii. Abbreviations: AD: Alzheimer's disease; ANOVA: Analysis of Variance; BrdU: Bromodeoxyuridine; BDNF: Brain-derived Neurotrophic Factor; BSA: (Bovine Serum Albumin); CO: Control; CO-C: Control Curcumin; CO-V: Control Vehicle; DAPI: 4′,6-Diamidino-2-phenylindole; DG: Dentate Gyrus; DS: Down syndrome; ED: Embryonic Day; GCL: Granular Cell Layer; 5-HT: 5-hydroxytryptamine; LSD: Least Significant Difference; LTP: Long-term Potentiation; ML: Molecular Layer; MWM: Morris Water Maze; PB: Phosphate Buffer; PBS: Phosphate-buffered Saline; PD: Postnatal Day; PFA: Paraformaldehyde; PSD95: Postsynaptic Density protein 95; qPCR: Quantitative Polymerase Chain Reaction; RM: Repeated Measures; SGZ: Subgranular Zone; SVZ: Subventricular Zone; SYN: Synaptophysin; TS: Ts65Dn; TS-C: TS-Curcumin; TS-V: TS Vehicle; TX: Triton X.

iii. Financial support: This study was supported by the "Fondazione Generali e Assicurazioni Generali", Italy; Fundación Tatiana Pérez de Guzmán el Bueno, IDIVAL

(NVAL 19/23), and the Spanish Ministry of Economy and Competitiveness (PSI-2016-76194-R, AEI/FEDER, EU).

iv. Conflict of interests: All the authors declare no conflict of interest.

ABSTRACT

Background: The cognitive dysfunction in Down syndrome (DS) is partially caused by deficient neurogenesis during fetal stages. Curcumin enhances neurogenesis and learning and memory.

Objectives: We aimed to test the ability of curcumin to rescue the neuromorphological and cognitive alterations of the Ts65Dn (TS) mouse model of DS when administered prenatally or during early post-natal stages, and to evaluate whether these effects were maintained several weeks after the treatment.

Methods: To evaluate the effects of prenatal curcumin administration, 65 pregnant TS females were subcutaneously treated with curcumin (300 mg/kg) or vehicle from ED (Embryonic Day) 10 to PD (Post-natal Day) 2. All the analyses were performed on their TS and Control (CO) male and female progeny. At PD2, the changes in neurogenesis, cellularity, and brain weight were analyzed in 30 TS and CO pups. The long-term effects of prenatal curcumin were evaluated in another cohort of 44 TS and CO mice between PD30 and PD45. The neuromorphological effects of early postnatal administration of curcumin were assessed on PD15 in 30 male and female TS and CO pups treated with curcumin (300 mg/kg) or vehicle from PD2 to PD15. The long-term neuromorphological and cognitive effects were assessed from PD60 to PD90 in 45 mice. Data was compared by ANOVAs.

Results: Prenatal administration of curcumin increased the brain weight (+45%, $P<0.001$), the density of BrdU (Bromodeoxyuridine)- (+150%, $P<0.001$) and DAPI (4′,6-diamidino-2-phenylindole)- (+38%, $P=0.005$) positive cells, and produced a long-term improvement of cognition in TS (+35%, $P=0.007$) mice with respect to vehicle-treated mice. Post-natal administration of curcumin did not rescue any of the short- or long-term altered phenotypes of TS mice.

Conclusion: The beneficial effects of prenatal curcumin administration to TS mice suggest that it could be a therapeutic strategy to treat DS cognitive disabilities.

Keywords: Down syndrome; Ts65Dn mice; curcumin; neurogenesis; cognition

INTRODUCTION

Curcumin (diferuloylmethane), a polyphenolic compound commonly used as a coloring agent and as a food additive, is obtained from turmeric, the dried rhizome of the plant *Curcuma Longa*. Preclinical studies have demonstrated its therapeutic potential in different inflammatory, cardiovascular, neurological, and neurodegenerative diseases due to its anti-inflammatory, anti-oxidant, and immunomodulatory effects (1-6). Curcumin also stimulates cellular proliferation and neurogenesis in the hippocampi of rodents (1, 7-9), and neural differentiation in rats with cerebral ischemia (10, 11) and in murine models of Alzheimer's disease (AD) (1, 9, 12, 13). Additionally, curcumin improves hippocampal LTP (Long-Term Potentiation) (14), synaptic transmission in mice (14, 15), improves learning and memory in humans and rodents (9, 15-19), reduces the formation of β-amyloid plaques, inhibits tau phosphorylation, and prevents AD-associated cognitive decline (4, 19, 20).

Down syndrome (DS), the most common genetic cause of intellectual disability, affects approximately 1 of every 800 newborns, and is caused by the total or partial triplication of chromosome 21 (21). The most widely accepted model of DS is the Ts65Dn (TS) mouse that carries a partial mutation of 92 chromosome 21 orthologous genes (22). This mouse presents many DS altered phenotypes (23, 24). As in DS, the brain volume of the TS mouse is smaller during embryonic periods (25, 26), and some encephalic structures such as the hippocampus or the cerebellum present a reduced volume in adult TS mice (27-30).

This reduced volume is associated with a smaller cellular density in different brain areas such as the hippocampal granular cell layer (GCL) (26, 31-33). In TS mice, this hypocellularity is caused by alterations in pre- and post-natal neurogenesis in the hippocampal Dentate Gyrus (DG) and in the subventricular zone (SVZ) (26, 28-31, 33-39), which is partially responsible for the cognitive deficits in these mice (23, 40).

Altered synaptic connectivity in TS brains also contributes to their cognitive deficits. TS mice present a reduced synaptic density in the neocortex and in CA1 in early post-natal (26) and adult stages (41), as well as functional and neuromorphological alterations in synapses, dendrites, and spines (42-48).

There is no effective treatment for the cognitive deficits found in DS. Various pharmacotherapies have been proven to reduce neuromorphological alterations and enhance cognition in TS mice (29, 30, 35, 37, 49-56), but many of them either cannot be administered to humans due to their adverse effects, or have not been proven to be effective in different clinical trials (57). Besides, all of them have been tested in adults with DS. Since the neuromorphological alterations that lead to the cognitive deficits in this population appear at pre-natal stages (38, 58, 59), and prenatal diagnosis of DS can be performed from the tenth week of gestation (21), efforts should be made to find compounds that can palliate these alterations and that can be safely administered during these stages.

Because curcumin is a natural compound that is usually taken in the diet and has no adverse effects in humans (60), in this study we evaluated its effects on some of the neuromorphological alterations responsible for the cognitive deficits in TS mice. Curcumin crosses the placental and the blood brain barrier and also reaches the pups through lactation (60-64). Thus, we administered curcumin to pregnant TS females and to newborn pups and evaluated its immediate effects on neurogenesis and synaptogenesis, and assessed whether the neuromorphological and cognitive alterations in TS mice were maintained several weeks after the discontinuation of the pre- or post-natal curcumin treatments.

METHODS

Animals, diets, and treatments

This study was approved by the Cantabria University Institutional Laboratory Animal Care and Use Committee and performed in accordance with the Declaration of Helsinki and the European Communities Council Directive (86/609/EEC).

TS and CO (Control) mice were generated and karyotyped as previously described (65).

Diet

Pregnant and lactating TS females under all treatment conditions were fed with Tekcal 18% protein (#2018, containing 18.6% raw protein, 6.2% fat, and 44.2% carbohydrates) Global Mouse Chow, specially formulated for gestation and lactation (INVIGO, Huntingdon, UK), from ED (Embryonic Day) 0 until the weaning of the pups. For the study of the long-term effects of the prenatal treatments and the short- and long- term effects of the post-natal treatments, all TS and CO mice received Tekcal Mouse Chow 14% protein (#2014, containing 14.3% raw protein, 4.0% fat, and 48.0% carbohydrates), designed to promote normal body weight and longevity in the rodents from weaning (Postnatal Day (PD), 21) to the end of each study.

Treatments

The experimental design of the prenatal and postnatal studies is summarized in **Supplemental figure 1.**

Study I: Prenatal Treatments

A total of sixty-five pregnant TS females were subcutaneously treated with curcumin (300 mg/kg), or vehicle (Bovine Serum Albumin (BSA) 10%) from ED10 until PD2; **Supplemental figure 1**). In humans, these ages correspond to ED38 and ED82,

104 respectively (66). The dose of curcumin was selected because it has been
105 demonstrated to be neuroprotective and/or induce neurogenesis in mice (9). This dose
106 of curcumin leads to plasma levels of 2.5 µg/mL in mice (67), while in humans an oral
107 dose of 10 g yields to similar plasma levels (2.3 ± 0.26 µg/mL) (68). In most human
108 studies, curcumin is administered orally at doses ranging from 0.4 to 20 g (7, 67).

109 Male and female TS and CO pups gestated by 30 TS females under the different
110 treatments were used for the study of the short-term effects, and another cohort of TS
111 and CO mice of both sexes gestated by 35 TS dams under the two treatment
112 conditions was used for the long-term effects study. All the experimental analyses of
113 the effects of the prenatal treatments were performed on the progeny of the pregnant-
114 treated TS mice.

115 The offspring of these females were assigned to one of four experimental groups,
116 depending on their karyotype and the prenatal treatment that they received: CO pups
117 that were treated prenatally with vehicle (CO-V) or curcumin (CO-C), and TS pups that
118 prenatally received vehicle (TS-V), or curcumin (TS-C). For the long-term effects study,
119 forty-four male and female TS and CO pups gestated by dams under the two
120 treatments were assigned to the same aforementioned experimental groups. Seven to
121 eight pups from each group were used to evaluate the short-term effects (i.e.
122 neurogenesis, cellularity, and brain weight), while 10-12 juvenile mice prenatally
123 treated with curcumin or vehicle (CO-V: n=12, TS-V: n=11, CO-C: n=11, TS-C: n=10)
124 were used to assess the long-term effects of the treatments (i.e. cognition in the Morris
125 Water Maze (MWM), neurogenesis, cellularity, and pre- and post-synaptic markers).

126 *Short-term effects of prenatal treatments*

127 To evaluate the short-term effects of prenatal curcumin treatment on cell proliferation,
128 on PD2 all the pups received an intraperitoneal injection of Bromodeoxyuridine (BrdU)

(150 µg/g). Two hours later, they were euthanized, weighed, and their brains were then removed, processed and cryosectioned as described in (65). Seven series, each containing 6-8 hippocampal sections, were obtained from each animal to perform the histological analyses: GCL volume, cell proliferation (BrdU), and granule cell density (4′,6-Diamidino-2-phenylindole, DAPI staining).

Long-term effects of prenatal treatments

To evaluate the long-term effects of the prenatal treatments on new neuron survival, on PD15, all the pups received an intraperitoneal injection of BrdU (150 µg/g). They were subjected to the behavioral experiments (MWM) between PD30 and PD45, which corresponds to 6 and 8 months of age, respectively, in humans (69). On PD45, the animals were euthanized by decapitation and the brains of 7-8 animals per group were removed, fixed with PFA, and used for the following histological and immunohistochemical analyses: GCL volume, cell proliferation (Ki67 immunohistochemistry), survival (BrdU immunohistochemistry) and pre- and post-synaptic density (Synaptophysin (SYN) and Postsynaptic Density Protein 95 (PSD95) immunohistochemistry). To this end, free-floating 50 µm coronal sections covering the whole hippocampus were cryosectioned. Nine series, each containing 6-8 hippocampal sections, were obtained from each animal.

Study II: post-natal treatments

From PD3 until PD15, which corresponds to ED89 and ED152, respectively, in human neurodevelopment (66), a total of 75 male and female TS and CO pups were subcutaneously treated with curcumin (300 mg/kg) or vehicle (BSA 10%), (**Supplemental figure 1**). Seven- eight animals from each group were used for the short-term effects analyses (i.e. neurogenesis, cellularity, and brain weight), and 10-12 animals per group in the long-term effects experiments (CO-V: n=10, TS-V: n=12, CO-

C: n=11, TS-C: n=12) were used to assess the long-term effects of the treatments (i.e. cognition in the MWM, neurogenesis, cellularity, and pre- and post-synaptic markers).

Short-term effects of post-natal treatments

To evaluate the short-term effects of curcumin administration on cell proliferation, on PD15, 7-8 animals from each group received an intraperitoneal injection of BrdU (150 µg/g). Two hours later, they were euthanized and their brains were then removed, fixed, frozen, and cryosectioned following the same procedure previously described for the study of the short-term effects of prenatal treatments, in order to perform the histological analyses.

Long-term effects of post-natal treatments

To evaluate the long-term effects of postnatal curcumin treatment, another cohort of 45 male and female TS and CO mice was used. On PD60, which corresponds to 10 months of age in humans (69), all the animals received an intraperitoneal injection of BrdU (150 µg/g) in 9% saline, and were then subjected to the behavioral experiments. In order to perform the histological and immunohistochemical analyses, on PD90, which corresponds to 12 months of age in humans (69), the animals were euthanized and their brains were removed, fixed, frozen, and cryosectioned following the same procedure previously described for the study of the long-term effects of prenatal treatments.

Histological and immunohistochemical analyses

Nissl staining

GCL volume was determined on 1 of 7 series for the short-term studies, and on 1 of 9 series for the long-term studies. Nissl staining was performed as previously described

(70). Each coronal section was photographed, and the GCL volume was calculated using the Cavaliery stereological method as previously described (65).

Cell proliferation (Ki67 and BrdU immunofluorescence)

BrdU immunohistochemistry was performed using the same protocols and antibodies described (65). The total number of BrdU+ cells in the GCL and in the SGZ was counted in all sections of a series for the short- and long-term analyses, respectively, using the same method previously described (71). To calculate the density of proliferating cells in each animal, the total number of positive cells per slice was divided by the volume of the GCL, or by the area of the SGZ layer, for the short- and long-term analyses respectively.

Ki67 immunohistochemistry was performed following the same protocols and using the same antibodies previously described (65). Ki67-positive cells were counted in the SGZ using an optical fluorescence microscope (Zeiss Axioskop 2 plus, 40x objective). To determine the density of Ki67+ cells, the total number of these cells was divided by the SGZ area.

DAPI staining

To calculate the number of mature cells, hippocampal sections were counterstained with DAPI (Calbiochem, Billerica, MA, USA; 1:1000) and the cell counts were performed using a previously described physical dissector system coupled with confocal microscopy (72, 73).

SYN and PSD95 immunofluorescence

SYN and PSD95 immunohistochemistry were performed using the same protocol and antibodies previously described (54, 65). Fluorescent images were captured in the Molecular Layer (ML) of the DG, the CA1, and the CA3 using the same parameters and

software previously described (65). For each marker, the number of individual puncta exhibiting SYN or PSD95 immunoreactivity was counted in a 325 μm^2 circle for each image in each hippocampal field.

Cognitive analysis. Morris Water Maze (MWM)

Spatial learning and memory were evaluated using a modified version of the MWM (51). Sixteen consecutive daily sessions were performed: 12 acquisition sessions (platform submerged, in eight of these, the position of the platform changed daily, while in the remaining four it was kept constant), followed by a probe trial, and 4 cued sessions (platform visible). The computerized tracking system Anymaze (Stoelting, Wood Dale, IL, USA) was used to analyze the trajectories of each animal in each trial.

Statistics

Shapiro–Wilk tests were used to test the normality of the data sets. Because all the datasets were normally distributed, parametric tests were used. The water maze data from the acquisition sessions (sessions 1-12) were analyzed using two-way Analysis of Variance (ANOVA) with Repeated Measures (RM) ('session' x 'karyotype' x 'treatment'). The percentage of time spent in each quadrant during the probe trial was analyzed by RM ANOVA ('quadrant'). The rest of the data were analyzed using two-way ('karyotype' x 'treatment') ANOVA or. The mean values of each experimental group were compared *post hoc* using Fisher's LSD (Least Significant Difference) *post-hoc* tests. The differences between groups were considered to be statistically significant when $P<0.05$. All analyses were performed using IBM SPSS (Armonk, New York, USA) for Windows version 22.0.

RESULTS

Short-term effects of pre- and postnatal treatment

1. Body and Brain weight

At PD2, TS-V mice presented smaller body (P<0.05) and brain weights (P<0.01; **table 1**) than their CO-V littermates. Prenatal curcumin increased the brain weight of TS-C mice with respect to TS-V mice (P<0.001), and CO-C mice presented higher body (P<0.001) and brain weights than CO-V animals (P<0.001).

In the postnatal short-term effects study, TS-V mice also presented smaller body (P<0.001) and brain weights (P<0.05) than their CO-V littermates. Postnatal curcumin administration did not modify the body or brain weights of TS or CO mice.

2. Granular cell layer volume

At PD2, the GCL volume of the TS-V animals did not differ from that of CO-V mice (**table 1**). Prenatal curcumin treatment increased the volume of this layer in CO-C mice with respect to CO-V animals (P<0.01).

Immediately after the postnatal treatments (PD15), TS-V mice presented a smaller GCL volume than CO-V mice (P<0.05). However, postnatal curcumin treatment did not modify the volume of this layer in TS or CO mice.

3. BrdU immunohistochemistry

At PD2, TS-V mice presented a lower density (P<0.01; **figures 1A and 2A**) and a lower total number of BrdU+ cells than their CO-V littermates (P<0.01; **figure 2B**).

Immediately after prenatal curcumin treatment TS-C mice presented an increased density (P<0.001; **figure 2A**) and total number of BrdU+ cells with respect to TS-V mice (P<0.01; **figure 2B**).

At PD15, TS-V mice presented a lower density (*P*<0.05, **figures 1B and 2C**) and a lower total number of BrdU+ cells (*P*<0.05, **figure 2D**) than their CO-V littermates. Postnatal curcumin treatment did not exert any short-term effects on the density (**figure 2C**) or the total number of BrdU+ cells in the hippocampi of TS or CO mice (**figure 2D**).

4. Mature granule cell count (DAPI)

TS-V mice presented a lower total number of DAPI+ cells than CO-V animals at PD2 (*P*<0.05; **figures 1A and 2F**); however, the density of this population of cells did not significantly differ between TS-V and TS-C mice (**figure 2E**). On PD2, TS-C mice presented an enhanced density (*P*=0.005; **figure 2E**) and total number of DAPI+ cells when compared with TS-V animals (*P*<0.01; **figure 2F**).

At PD15, TS-V mice showed a lower total number of DAPI+ cells than CO-V animals (*P*<0.05; **figures 1B and 2H**), although the density of this population of cells did not significantly differ between TS-V and CO-V mice (**figure 2G**). Postnatal curcumin treatment did not affect the density (**figure 2G**) or the total number of DAPI+ cells in TS or CO mice (**figure 2H**).

5. PSD95 and Synaptophysin (SYN)

Immediately after postnatal curcumin treatment, TS-V mice presented a lower number of PSD95+ puncta than their CO-V littermates in the three hippocampal areas analyzed (CA1: *P*<0.05; CA3: *P*<0.01; ML: *P*<0.001; **figure 3A**). TS-C animals presented a short-term enhancement in the number of PSD95+ puncta with respect to TS-V mice in CA3 and the ML (P<0.05), but not in CA1 (**figure 3A**).

At PD15, TS-V mice presented a lower number of SYN+ puncta than CO-V mice in CA1 (*P*<0.05), CA3 (*P*<0.001), and the ML (*P*<0.01; **figure 3B**). Postnatal curcumin administration increased the number of SYN+ puncta in CA1 in TS-C animals with

respect to TS-V mice (*P*<0.05), but it did not exert any effect in CA3 or the ML (**figure 3B**).

Long-term effects of pre- and post-natal treatment

1. Histology: GCL volume, Ki67 and BrdU immunohistochemistry and DAPI staining

Several weeks after the discontinuation of the prenatal or postnatal treatments, TS-V mice presented a smaller GCL volume at PD45 (*P*<0.05; **Supplemental figure 2A**) and at PD90 (*P*<0.05; **Supplemental figure 2B**); a lower density (*P*<0.05; **Supplemental figure 2C**) and total number of Ki67+ cells at PD45 (*P*<0.05; **Supplemental figure 2E**) and at PD90 (density: *P*<0.05, **Supplemental figure 2D**, total number: *P*<0.05; **Supplemental figure 2F**) than CO-V mice. In addition, at PD45, TS-V mice also presented a lower density (*P*<0.01; **Supplemental figure 2G**) and total number of BrdU+ cells (*P*<0.05; **Supplemental figure 2I**) than CO-V mice, and at PD90 (density: *P*<0.05, **Supplemental figure 2H**, total number: *P*<0.05; **Supplemental figure 2J**), and a lower density and total number of DAPI+ cells at PD45 (density: *P*<0.05, **Supplemental figure 2K**; total number: *P*<0.01; **Supplemental figure 2M**) and PD90 (density: *P*<0.05, **Supplemental figure 2L**; total number: *P*<0.05; **Supplemental figure 2N**) than CO-V mice.

However, prenatal or postnatal curcumin administration did not exert any long-term effect on the GCL volume, the density, or the total number of Ki67+, BrdU+, or DAPI+ cells in TS or CO mice (**Supplemental figure 2**).

2. PSD95 and Synaptophysin

TS-V mice presented a lower density of PSD95+ puncta in all hippocampal areas analyzed at PD45 (CA1: *P*<0.05; CA3: *P*<0.05; ML: *P*<0.01; **Supplemental figure 3A**)

296 and at PD90 (CA1: *P*<0.05; CA3: *P*<0.05; ML: *P*<0.05; **Supplemental figure 3B**) when
297 compared to CO-V mice.

298 Although TS-C mice tended to present a higher number of PSD95+ puncta in all
299 hippocampal areas analyzed with respect to TS-V mice at PD45, these effects were not
300 statistically significant (**Supplemental figure 3A**). At PD90, postnatally treated TS-C
301 mice did not differ from TS-V animals in the number of PSD95+ puncta in any of the
302 tested areas (**Supplemental figure 3B**).

303 TS-V mice presented a smaller number of SYN+ puncta than CO-V mice in CA1
304 (*P*<0.01), CA3 (*P*<0.01), and the ML (*P*<0.05; **Supplemental figure 3C**) at PD45, and
305 at PD90 in CA1: (*P*<0.05) and the ML (*P*<0.001; **Supplemental figure 3D**).

306 Curcumin did not exert any long-term effects in the number of SYN+ puncta displayed
307 by TS or CO mice, whether it was administered prenatally (**Supplemental figure 3C**),
308 or postnatally (**Supplemental figure 3D**).

309 **2. Cognition: Morris Water Maze (MWM)**

310 **2.1. Reference learning and memory**

311 Between PD30 and PD45, the four groups of mice prenatally treated with curcumin or
312 vehicle reduced their latency to reach the platform when all sessions were taken into
313 account (sessions 1-12: RM ANOVA 'session': *P*<0.001; **figure 4A, Supplemental**
314 **table 1**), both in the sessions in which the platform position was changed daily
315 (sessions 1-8: *P*<0.001), and in those in which the platform position was kept constant
316 (sessions 9-12: *P*<0.001).

317 The reduction in latency between sessions was significantly different between animals
318 of both karyotypes ('session x karyotype': *P*<0.001), and was also significantly different
319 between animals from the two treatment conditions ('session x treatment': *P*<0.001).

Several weeks after the discontinuation of the postnatal treatment all the mice reduced their latency to reach the platform when all sessions were taken into account (session 1-12: RM ANOVA 'session': $P<0.001$; **figure 4B, Supplemental table 1**), both in the sessions in which the platform position was changed daily (sessions 1-8: $P<0.001$), as well as in those in which the platform position was kept constant (sessions 9-12: $P<0.001$). The reduction in latency between sessions significantly differed between animals of both karyotypes ('session x karyotype': $P<0.001$), but not between both treatment conditions ('session x treatment': $P=0.39$).

When each pair of learning curves was analyzed separately, TS-V mice presented a deteriorated performance when compared with CO-V mice at PD45 ($P<0.001$ **figure 4C**), and at PD90 ($P<0.001$; **figure 4D**).

Prenatal treatment with curcumin exerted a long-term benefit in the cognitive abilities of TS animals, as demonstrated by the reduced latency of TS-C mice when compared to TS-V mice ($P=0.007$; **figure 4E**). However, postnatal treatment with curcumin did not exert any long-term benefit in the cognitive abilities of TS animals, as demonstrated by the similar latency to reach the platform displayed by TS-C and TS-V mice across the twelve acquisition sessions (**figure 4F**).

Prenatal curcumin treatment also induced a long-term improvement in the performance of CO-C mice with respect to CO-V mice ($P=0.041$; **figure 4G**). However, postnatal administration of curcumin did not exert any long-term benefit in the performance of CO mice (**figure 4H**).

2.2. Cued sessions

TS and CO mice prenatally treated with curcumin or vehicle did not differ in their latency to reach the platform during the cued sessions (ANOVA 'karyotype': $P=0.063$, 'treatment': $P=0.089$, 'karyotype x treatment': $P=0.25$, data not shown).

TS-V mice postnatally treated with vehicle displayed a longer latency to reach the platform than their CO-V littermates (ANOVA 'karyotype': P=0.006). Curcumin treatment reduced this latency in TS mice, but not in CO mice ('treatment': P=0.20, 'karyotype x treatment': P=0.049).

2.3. Spatial memory

During the probe trial, TS-V mice crossed over the site where the platform was placed during the training sessions a fewer number of times than CO-V mice (prenatally treated: P<0.001; **figure 5A**; postnatally treated: P<0.001; **figure 5B**), and entered in the trained quadrant fewer times than CO-V mice (prenatally treated: P<0.05; **figure 5C**; postnatally treated: P<0.001; **figure 5D**).

Prenatal treatment with curcumin exerted a long-term enhancement in the number of crossings over the platform position performed by TS-C mice with respect to TS-V mice (P<0.05; **figure 5A**), but it had no effect on the number of entries in the trained quadrant (**figure 5C**). Postnatal treatment with curcumin did not exert any effect on the number of crossings over the platform position (**figure 5B**), or the number of entries in the trained quadrant (**figure 5D**).

Exposure to curcumin during gestation increased the percentage of time that TS-C animals spent in the trained quadrant (P<0.001; **figure 5E**) with respect to the rest of the quadrants; while TS-V mice did not exhibit a preference for any of the quadrants (**figure 5E**).

Postnatal curcumin exposure also produced a small long-term improvement in the memory of TS mice, since TS-C mice increased the percentage of time that they spent in the trained quadrant with respect to the rest of the quadrants (P=0.044; **figure 5F**); while TS-V mice spent a similar percentage of time in all quadrants (P=0.98; **figure 5F**).

All groups of prenatally and postnatally treated CO mice, including those that received vehicle, showed a marked preference for the trained quadrant (prenatal CO-C: $P<0.001$; prenatal CO-V: $P<0.001$; **figure 5G**, postnatal CO-C: $P<0.001$; postnatal CO-V: $P<0.001$; **figure 5H**).

DISCUSSION

In this study, prenatal curcumin administration increased the body and brain weights, GCL volume, cell proliferation in the DG, the density of mature granule neurons, and the cognitive abilities of TS and CO mice. These beneficial effects were not observed when curcumin was administered in early postnatal stages to TS or CO mice.

In DS and in the TS mouse, brain development is compromised due to neurogenesis defects, which begin during prenatal stages (26, 58, 59,74). These alterations lead to a pronounced hypocellularity (27, 74-76) that plays an essential role in the cognitive alterations of TS mice and DS individuals. Because of the early onset of these alterations, they ought to be corrected during the critical window of neurodevelopment (i.e. prenatally, or during early postnatal stages) (30, 52, 58, 77).

Because curcumin is usually ingested in the diet, and it does not present toxicity or important side effects (60), its efficacy in treating different pathologies is currently being evaluated in clinical trials (16, 78). However, concerns have been raised about the safety of curcumin administration during pregnancy, because it produces cytotoxicity in mouse blastocysts, resulting in an increased number of spontaneous abortions (79). However, these effects are only observed during the first stages of development after embryonic implantation (ED3 to ED8) (80). Curcumin administration from ED13 to ED16 to female mice with placental inflammatory syndrome reduced the number of spontaneous abortions and increased the number of pups per birth (81). Other studies did not find that curcumin affected the number of dead embryos or the implantation rate (60, 82). Consistent with these results, in the present study, TS females received curcumin or vehicle from ED10 to PD2, and no differences were found between the number of dams aborting, or in the number of pups per birth under curcumin or vehicle treatment.

We performed a preliminary analysis of the data to determine whether there were differences in the phenotypes studied between male and female mice. Because males and females did not differ in any phenotype, in all the experiments we analyzed the data of both sexes together. The few studies that have assessed TS mice phenotypes in both sexes separately show conflicting results. Some of them reported sex differences in cognition (83, 84); however, consistent with our results, other studies did not find differences between them (85, 86).

Consistent with previous reports (28-30, 51, 52), this study found a decrease in the brain weight, GCL volume, reduced neurogenesis, and hypocellularity in TS mice hippocampi at all ages analyzed. Prenatal curcumin administration produced a short-term enhancement in the brain weight, GCL volume, cell proliferation, and the density of mature cells in both TS and CO mice. It is likely that after prenatal curcumin administration, its well-known pro-neurogenic effects were responsible for both the reduction of the neuroanatomical anomalies in TS mice, and for its improvement in CO mice. In fact, curcumin promotes neurogenesis and cell proliferation in different models, including embryonic neural progenitor cells (7, 12, 87-89).

However, the present study did not find any short- or long-term enhancement of cell proliferation or survival when curcumin was administered in early postnatal periods when neurogenesis is still proceeding at a high rate. Conversely, several studies have demonstrated that curcumin is able to promote neurogenesis in adult animals when the rate of neurogenesis is not that high (8, 12, 15, 87). Curcumin exerts its effects on hippocampal neurogenesis mainly by increasing BDNF (Brain-derived Neurotrophic Factor) levels and through the expression of 5-hydroxytryptamine (5-HT) 1A receptors (2, 8, 9). In TS mice, administration of fluoxetine during early postnatal stages rescues neurogenesis through the increase in BDNF and 5-HT_{1A} receptors in the hippocampus at PD45 (30), and these effects are maintained in the adult TS mouse (53). It is unclear

why, if similar mechanisms are implicated in the pro-neurogenic effect of curcumin and fluoxetine, curcumin failed to produce these effects in TS mice in the present study when administered postnatally, or after the discontinuation of the treatment. It also remains to be elucidated why these mechanisms that enhance neurogenesis in normal adult animals failed to do so in CO mice under these conditions.

It is possible that longer duration treatments or continuous administration of curcumin is necessary to induce and maintain its beneficial effects. Curcumin administration to old rats for either 6 or 12 weeks produced higher cell proliferation when the treatment was longer (15). Thus, a cumulative effect of this molecule over long periods of time might be necessary to promote proliferation (15). A longer administration may be necessary to promote neurogenesis in postnatally treated TS and CO mice, and to maintain the pro-neurogenic effects in prenatally treated mice after discontinuation of the treatment.

Prenatal curcumin-treated TS and CO mice displayed a long-term improvement in reference and spatial memory in the MWM. These results are consistent with the pro-cognitive effects of curcumin administration in humans and rodents (9, 15, 16, 18) that have been attributed to the enhancement in neurogenesis and synaptic transmission (14, 15). In the present study, the cognitive effects found several weeks after prenatal curcumin administration cannot be attributed to changes in neurogenesis or cellularity, since these parameters were not modified in TS or CO mice at this time-point.

Cognitive function in DS and in the TS mouse is also compromised by excessive oxidative stress and neuroinflammation, both of which are enhanced in mice and human trisomic brains from the embryonic stages and throughout their entire life-span (21, 23, 71, 90, 91). Curcumin exerts antioxidant and anti-inflammatory effects (15, 92-94). Thus, the long-term pro-cognitive effects of curcumin observed in TS and CO mice after prenatal treatments could be due to its antioxidant and/or anti-inflammatory effects (1, 4, 5, 48).

After postnatal curcumin administration, TS or CO mice did not differ from the vehicle treated mice in reference learning and memory, although TS mice displayed a slight enhancement in spatial memory in the probe trial. Post-natal curcumin administration produced a short-term increase (PD15) in the expression of the postsynaptic marker PSD95 in CA3 and the ML, and of the presynaptic marker SYN in CA1, but these effects were no longer evident several weeks after the discontinuation of the treatment. As mentioned above, longer or continuous administration regimens might maintain synaptogenesis and/or produce greater improvement in TS mice cognition.

In conclusion, prenatal curcumin administration enhanced the body and brain weights, GCL volume, cell proliferation, and mature cell density in TS and CO mice, and induced a long-term enhancement of cognition in animals of both karyotypes. However, curcumin administration did not exert any long-term effects in neurogenesis or granule neuron density in prenatally treated mice, or any short-term or long-term effects in postnatally treated mice. Because of the similar effects elicited by curcumin in TS and CO mice, the mechanisms inducing these changes are not likely to be selectively targeting alterations due to trisomy. Some of the properties of this compound could produce similar neuromorphological and cognitive benefits in both normal and pathological conditions. These results suggest that curcumin administration could be a promising strategy to enhance neurodevelopment and cognition in DS and in the normal population. However, further studies are necessary to elucidate the treatment duration and doses required at the different life stages in order to produce and maintain these beneficial effects. Finally, curcumin has a very low oral bioavailability (67), but efforts are being made to increase it using different strategies (95), which would enhance the probability of producing its beneficial effects when administered in the diet.

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Table 1. Short-term effects of prenatal and postnatal curcumin administration on the body weight, brain weight and GCL volume of TS and CO mice

		TS-V	TS-C	CO-V	CO-C	'karyotype'	'treatment'	'karyotype x treatment'
Prenatal Short-term	Body weight (g)	1.37 ± 0.06 [c]	1.52 ± 0.08 [bc]	1.60 ± 0.045 [b]	1.93 ± 0.05 [a]	<0.001	<0.001	0.19
	Brain weight (g)	0.110 ± 0.002 [c]	0.159 ± 0.006 [a]	0.135 ± 0.003 [b]	0.168 ± 0.007 [a]	0.004	<0.001	0.16
	GCL volume (mm^3)	0.32 ± 0.03 [b]	0.39 ± 0.02 [b]	0.40 ± 0.05 [b]	0.59 ± 0.03 [a]	0.10	0.055	0.04
Postnatal Short-term	Body weight (g)	6.79 ± 0.19 [b]	6.43 ± 0.26 [b]	9.25 ± 0.61 [a]	8.31 ± 0.47 [a]	0.001	0.078	0.41
	Brain weight (g)	0.388 ± 0.006 [b]	0.383 ± 0.003 [b]	0.417 ± 0.01 [a]	0.417 ± 0.004 [a]	0.001	0.75	0.75
	GCL volume (mm^3)	0.77 ± 0.03 [b]	0.76 ± 0.03 [b]	0.90 ± 0.05 [a]	0.82 ± 0.049 [b]	0.038	0.26	0.45

[1]Values are means ± SEMs, n=7-8 per group. Means in a row without a common letter differ, $P<0.05$ (Fisher's *post hoc* tests).

[2] The last three columns display the *P* values of the main effects of ‘karyotype’, ‘treatment’ and ‘karyotype x treatment’ after two-way ANOVAs.

[3] CO: Control; CO-C: Control Curcumin; CO-V: Control Vehicle; GCL: Granular Cell Layer; TS: Ts65Dn; TS-C: TS-Curcumin; TS-V: TS Vehicle.

FIGURE LEGENDS

Figure 1. Representative confocal images of BrdU+ immunohistochemistry (upper row), and of DAPI staining (lower row), in the hippocampi of TS and CO mice treated with curcumin or vehicle prenatally (A), or postnatally (B). Scale bars in A and B: 5 µm. CO: Control; CO-C: Control Curcumin; CO-V: Control Vehicle; TS: Ts65Dn; TS-C: TS-Curcumin; TS-V: TS Vehicle.

Figure 2. Density of BrdU+ cells immediately after prenatal (A) or postnatal (C) treatments; total number of BrdU+ cells immediately after prenatal (B) or postnatal (D) treatments; Density of DAPI+ cells immediately after prenatal (E) or postnatal (G) treatments; and total number of DAPI+ cells immediately after prenatal (F) or postnatal (H) treatments. Values are means ± SEMs, n=7-8 per group. Bars without a common letter differ by $P<0.05$ Fisher's *post hoc* tests. BrdU: Bromodeoxyuridine; CO: Control; CO-C: Control Curcumin; CO-V: Control Vehicle; DAPI: 4',6-diamidino-2-phenylindole; LSD: Least Significant Difference; TS: Ts65Dn; TS-C: TS-Curcumin; TS-V: TS Vehicle.

Figure 3. Number of PSD95 (A) and SYN + (B) puncta in the CA1, CA3 areas and ML of the hippocampus of TS and CO mice immediately after postnatal treatment with curcumin or vehicle. Values are means ± SEMs, n=7-8 per group. Bars without a common letter differ by $P<0.05$, Fisher's *post hoc* tests. CA1: *Cornus Ammonis* 1; CA3: *Cornus Ammonis* 3; CO: Control; CO-C: Control Curcumin; CO-V: Control Vehicle; LSD: Least significant Difference; ML: Molecular Layer; PSD95: Postsynaptic Density Protein 95; SYN: Synaptophysin; TS: Ts65Dn; TS-C: TS-Curcumin; TS-V: TS Vehicle.

Figure 4. Latency to reach the platform during the twelve acquisition sessions in the MWM exhibited between by all groups prenatally (A) and postnatally (B)

treated, by TS-V and CO-V prenatally (C) and postnatally (D) treated mice, by TS-C and TS-V prenatally (E) and postnatally (F) treated mice, and by CO-C and CO-V prenatally (G), and postnatally (H) treated mice. Values are means ± SEMs, n=10-12 per group. *: p<0.05, **: p<0.01, ***: p<0.001 vs. CO-V (in C, D, G and H) or vs. TS-V (in E and F), Fisher's LSD *post hoc* tests. On the right side of each figure, the *P*-value of the difference between both learning curves across the twelve sessions (RM ANOVAs) is shown. On top of each figure, the *P* values of the differences between the learning curves of the different groups of mice during the first 8 and the last 4 sessions are shown. CO: Control; CO-C: Control Curcumin; CO-V: Control Vehicle; LSD: Least Significant Difference; TS: Ts65Dn; TS-C: TS-Curcumin; TS-V: TS Vehicle.

Figure 5. Number of crossings over the platform position performed by TS and CO mice treated with curcumin or vehicle prenatally (A) or postnatally (B); Number of entries in the trained quadrant performed by TS and CO mice treated with curcumin or vehicle prenatally (C) or postnatally (D) in the probe trial; Percentage of time spent in each quadrant during the probe trial by TS-C and TS-V prenatally (E), or postnatally (F) and by CO-C and CO-V animals treated prenatally (G) or postnatally (H). Values are means ± SEMs, n=10-12 per group. Labeled bars without a common letter differ *P*<0.05, Fisher's LSD *post hoc* tests. The dotted lines in figures C-F represent the chance level, i.e. a probability equal to 25% of the time. CO: Control; CO-C: Control Curcumin; CO-V: Control Vehicle; LSD: Least Significant Difference; TS: Ts65Dn; TS-C: TS-Curcumin; TS-V: TS Vehicle.

Figure 1

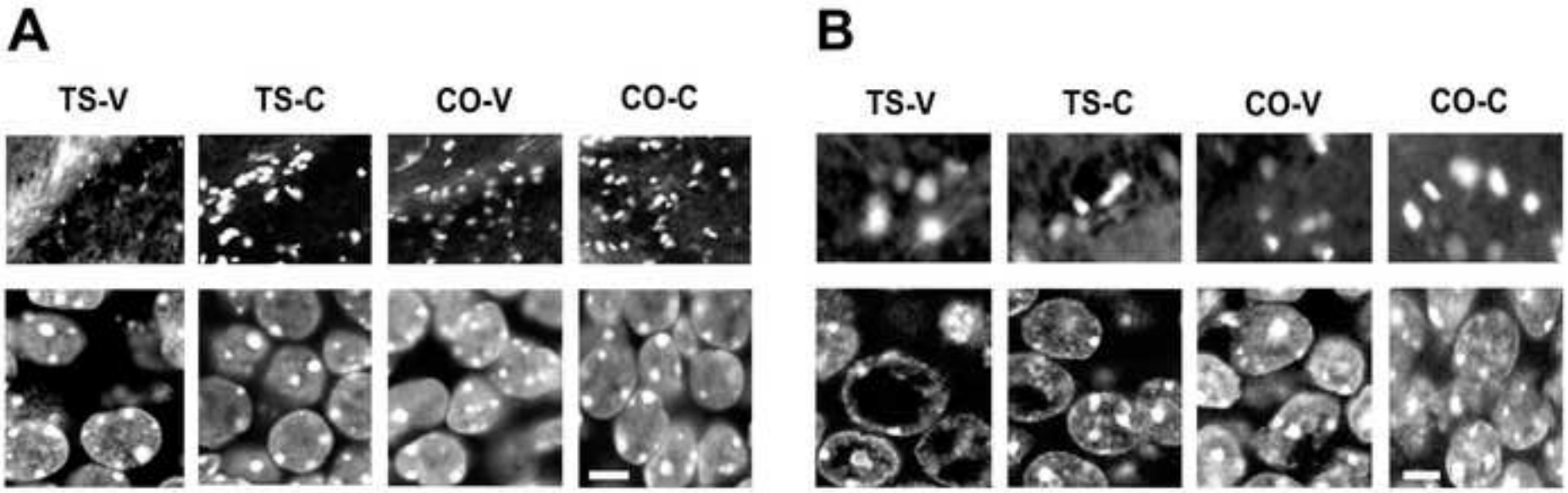

Figure 2

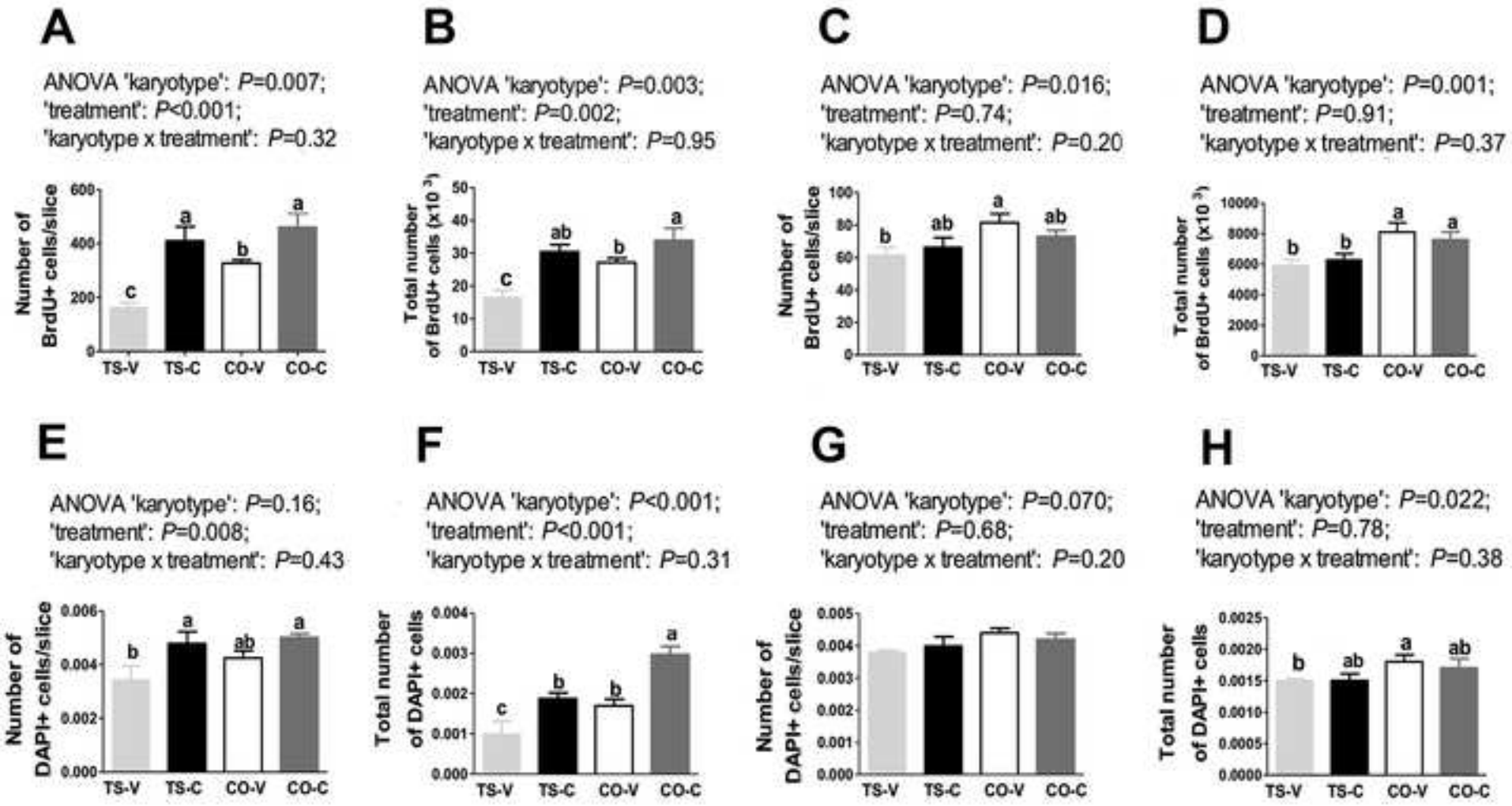

Figure 3

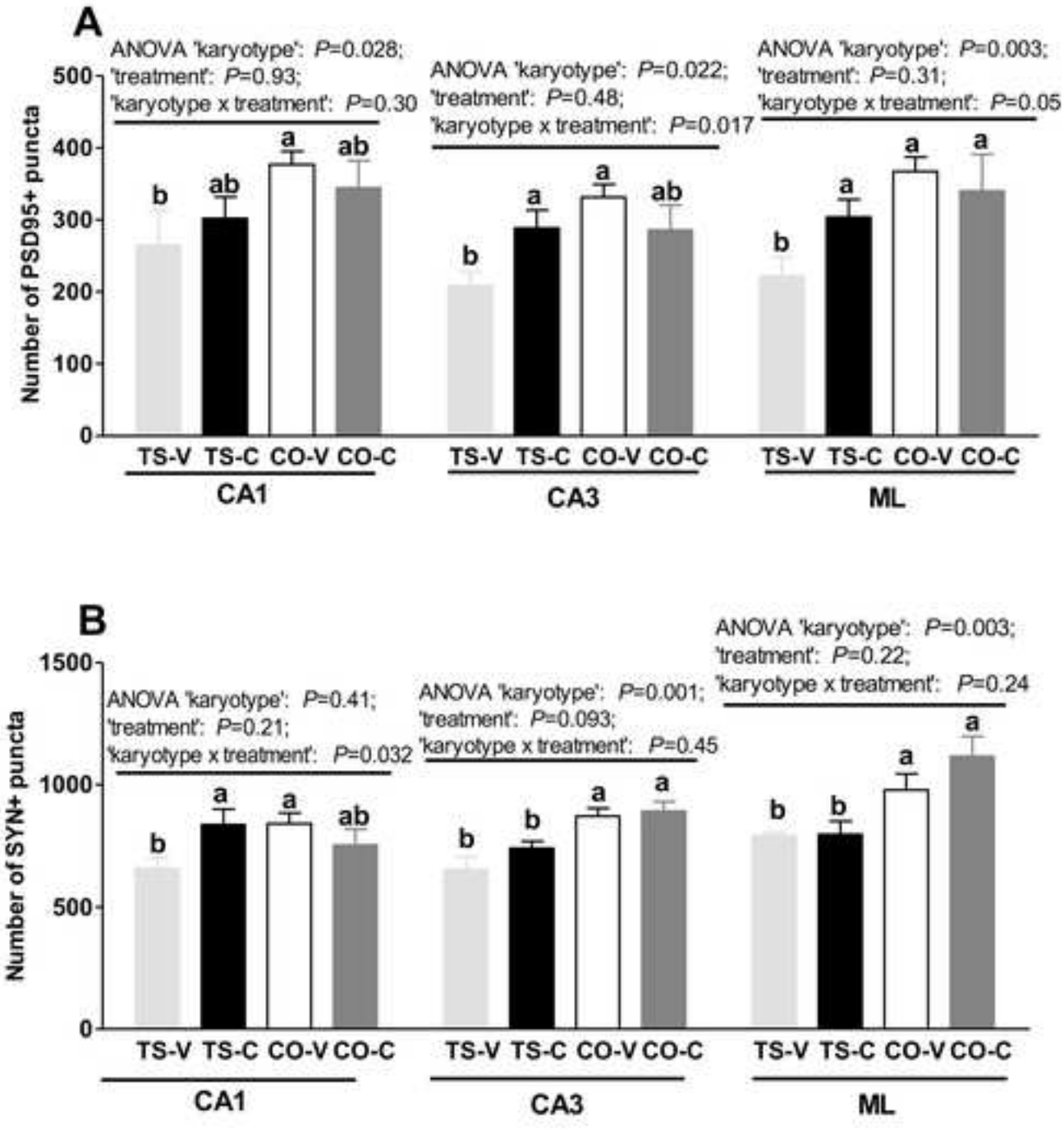

Figure 4

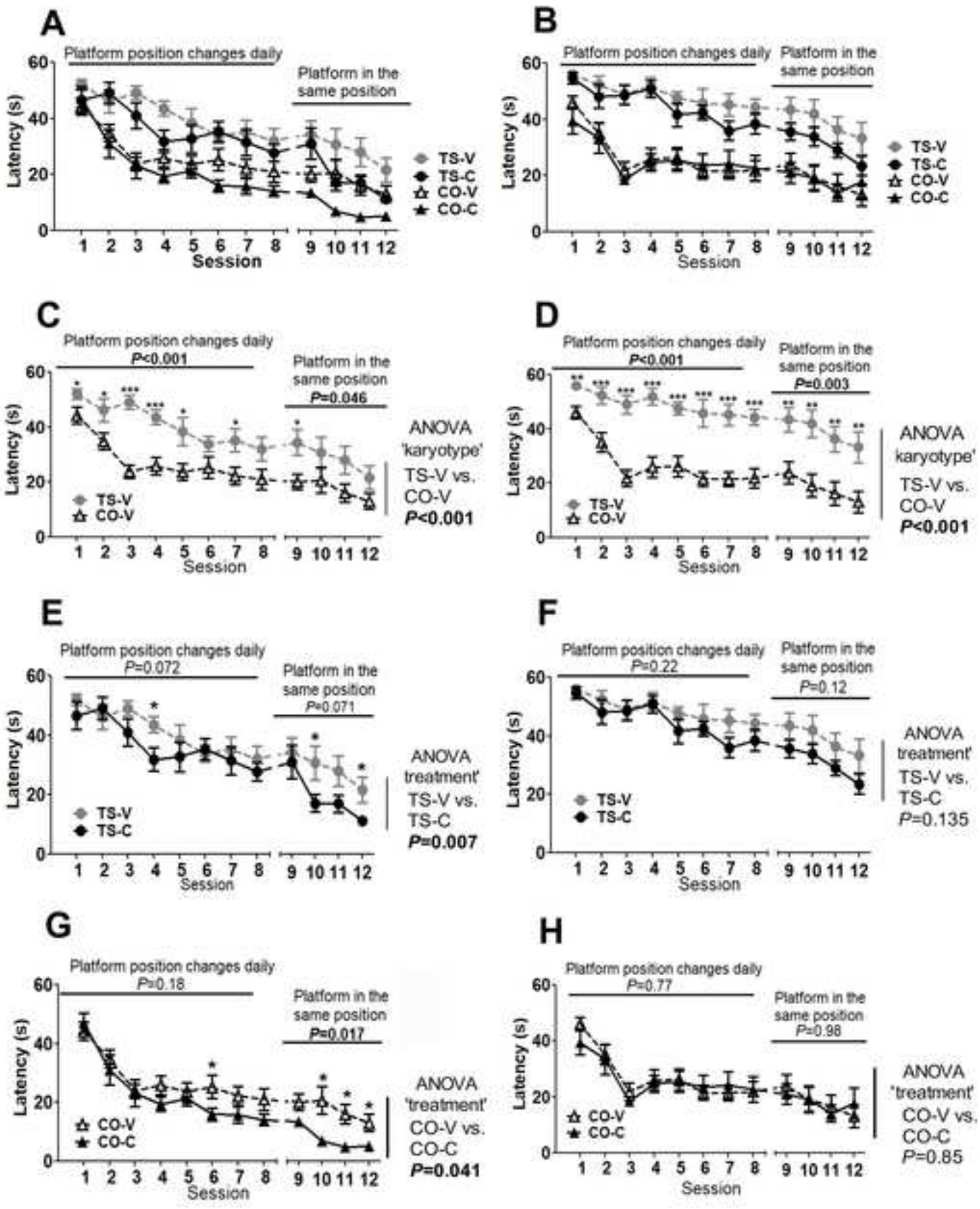

Figure 5

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