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Pleiotropic effects of trisomy and pharmacologic modulation on structural, functional, molecular, and genetic systems in a Down syndrome mouse model

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Abstract

Down syndrome (DS) is characterized by skeletal and brain structural malformations, cognitive impairment, altered hippocampal metabolite concentration and gene expression imbalance. These alterations were usually investigated separately, and the potential rescuing effects of green tea extracts enriched in epigallocatechin-3-gallate (GTE-EGCG) provided disparate results due to different experimental conditions. We overcame these limitations by conducting the first longitudinal controlled experiment evaluating genotype and GTE-EGCG prenatal chronic treatment effects before and after treatment discontinuation. Our findings revealed that the Ts65Dn mouse model reflected the pleiotropic nature of DS, exhibiting brachycephalic skull, ventriculomegaly, neurodevelopmental delay, hyperactivity, and impaired memory robustness with altered hippocampal metabolite concentration and gene expression. GTE-EGCG treatment modulated most systems simultaneously but did not rescue DS phenotypes. On the contrary, the treatment exacerbated trisomic phenotypes including body weight, tibia microarchitecture, neurodevelopment, adult cognition, and metabolite concentration, not supporting the therapeutic use of GTE-EGCG as a prenatal chronic treatment. Our results highlight the importance of longitudinal experiments assessing the co-modulation of multiple systems throughout development when characterizing preclinical models in complex disorders and evaluating the pleiotropic effects and general safety of pharmacological treatments.

eLife assessment

This study presents **valuable** findings that examine both how Down syndrome (DS)-related physiological, behavioral, and phenotypic traits track across time, as well as how chronic treatment with green tea extracts 25 enriched in epigallocatechin-3-gallate (GTE-EGCG), administered in drinking water spanning prenatal through 5 months of age, impacts these measures in wild-type and Ts65Dn mice. The strength of the evidence is **solid**, due to high variability across measures, perhaps in part attributable to a failure to include sex as a factor for measures known to be sexually dimorphic. This study is of interest to scientists interested in Down Syndrome and its' treatment, as well as scientists who study disorders that impact multiple organ systems.

Introduction

Down syndrome (DS) is a developmental disorder with structural, functional, molecular, and genetic alterations that show a dynamic onset and severity (Antonarakis et al., 2020; de Moraes et al., 2008; Grieco et al., 2015; Keeling et al., 1997; LaCombe & Roper, 2020; Llambrich, González-Colom, et al., 2022; McCarron et al., 2017; Steingass et al., 2011). The alterations associated with DS simultaneously affect multiple systems that are likely interrelated over development, with changes in one system modulating the others (Llambrich, González, et al., 2022). Many studies have provided evidence of these developmental alterations and have demonstrated the ability of dietary supplements such as epigallocatechin-3-gallate (EGCG) or green tea extracts enriched in EGCG (GTE-EGCG) to modulate these systems separately (Catuara-Solarz et al., 2016; de la Torre et al., 2016; Goodlett et al., 2020; Guedj et al., 2009; Llambrich, González, et al., 2022; Rondal, 2020; Stagni et al., 2015; Starbuck et al., 2021). However, a holistic evaluation of the simultaneous effects of trisomy and GTE-EGCG in those intertwined systems is missing. The variety in experimental setups and readouts obtained in different preclinical studies hinders the comparison and integration of results and, as a result, evidence is usually contradictory, reporting both positive and negative treatment effects. This lack of consistency can lead to biased interpretations about the etiology of the disorder and the potential effect of pharmacological treatments.

Much of this DS research was based on the Ts65Dn mouse model (Davisson et al., 1990; Reeves et al., 1995), as it was one of the first preclinical models available for DS and has been widely used for experimental testing. These mice carry a segment with approximately 120 genes homologous to Hsa21 (starting upstream of Mrpl39 to the telomeric end of Mmu16), translocated to a small centromeric part of Mmu17 (Duchon et al., 2011; Herault et al., 2017; Reinholdt et al., 2011). Ts65Dn mice are trisomic for about two-thirds of the genes orthologous to Hsa21, but also carry genes originating from the Mmu17 that are not related with DS, including about 46 protein-coding genes, 35 nonprotein-coding genes and 35 pseudogenes (Muñiz Moreno et al., 2020). These genetic alterations do not fully represent Down syndrome's aneuploidy and other mouse and rat models have been developed recently that more faithfully represent the trisomic nature of DS (Kazuki et al., 2020; Kazuki et al., 2022). However, in this study we used the Ts65Dn mouse model because it recapitulates the main skeletal, brain, cognitive, brain metabolite, and genetic alterations associated with DS (Blazek et al., 2011; Costa et al., 2010; Dierssen et al., 2002; Escorihuela et al., 1998; Gupta et al., 2016; Huang et al., 2000; Llambrich, González, et al., 2022; Mème, 2014; Starbuck et al., 2021) and the effects of GTE-EGCG pharmacological modulation have been extensively evaluated using this mouse model (Catuara-

Solarz et al., 2016 [\[1\]](#); Goodlett et al., 2020 [\[2\]](#); Jamal et al., 2022 [\[3\]](#); Llambrich, González, et al., 2022 [\[4\]](#); McElyea et al., 2016 [\[5\]](#); Stagni et al., 2016 [\[6\]](#); Starbuck et al., 2021 [\[7\]](#); Stringer et al., 2017 [\[8\]](#)).

At the structural level, people with DS show skeletal and brain alterations that progress through ontogeny (Antonarakis et al., 2020 [\[9\]](#); de Moraes et al., 2008 [\[10\]](#); Fischer-Brandies et al., 1986 [\[11\]](#); Keeling et al., 1997 [\[12\]](#); LaCombe & Roper, 2020 [\[13\]](#); Pearlson et al., 1998 [\[14\]](#); Starbuck et al., 2021 [\[7\]](#)). Children with DS have a decreased buildup of bone mass and a low bone turnover rate, resulting in more osteoclast than osteoblast activity, smaller bone area, lower bone mineral density (BMD), and an increased risk of osteoporosis at adulthood (Carfi et al., 2017 [\[15\]](#); Keeling et al., 1997 [\[12\]](#); LaCombe & Roper, 2020 [\[13\]](#)). Individuals with DS also present midfacial hypoplasia and flattened nasal bridge along with skull malformations resulting in a shorter, wider, and rounder skull (brachycephaly) (Blazek et al., 2011 [\[16\]](#); LaCombe & Roper, 2020 [\[13\]](#); Richtsmeier et al., 2000 [\[17\]](#); Suri et al., 2010 [\[18\]](#); Thomas & Roper, 2021 [\[19\]](#)). Previous studies have shown the potential of GTE-EGCG to modulate craniofacial and postcranial morphology, as well as the microarchitecture and bone mineral density of the long bones, showing positive, negative or no treatment effects (Abeysekera et al., 2016 [\[20\]](#); J. D. Blazek et al., 2015 [\[21\]](#); Jamal et al., 2022 [\[3\]](#); Llambrich, González-Colom, et al., 2022 [\[4\]](#); McElyea et al., 2016 [\[5\]](#); Starbuck et al., 2021 [\[7\]](#); Stringer et al., 2017 [\[8\]](#)). We previously detected dose-, time- and anatomical structure-dependent effects in a study using the same mouse model and the same treatment regime with two different GTE-EGCG doses (Llambrich, González-Colom, et al., 2022 [\[4\]](#)). A dose of 100 mg/kg/day of GTE-EGCG exacerbated facial dysmorphologies (Starbuck et al., 2021 [\[7\]](#)), modified the skeletal dysmorphologies associated with DS without rescuing the bones shape (Llambrich, González-Colom, et al., 2022 [\[4\]](#)), and altered the integration between the skull and the brain (Llambrich, González, et al., 2022 [\[4\]](#)). However, a lower dose of 30 mg/kg/day significantly reduced the facial dysmorphologies (Starbuck et al., 2021 [\[7\]](#)) but did not show additional rescuing effects in other skeletal traits (Llambrich, González-Colom, et al., 2022 [\[4\]](#)).

In DS, the craniofacial size and shape alterations are accompanied with structural brain alterations. People with DS show a reduced overall brain volume from birth, with disproportionately smaller hippocampus and cerebellum, and larger ventricles (Hamner et al., 2018 [\[22\]](#); Movsas et al., 2016 [\[23\]](#); Patkee et al., 2020 [\[24\]](#); Pinter et al., 2001 [\[25\]](#); Rodrigues et al., 2019 [\[26\]](#); Smigielska-Kuzia et al., 2011 [\[27\]](#)). Evidence for the effects of GTE-EGCG on brain anatomy is limited. Our own previous study showed that administration of 100 mg/kg/day of GTE-EGCG altered the brain shape of Ts65Dn mice at adulthood (Llambrich, González, et al., 2022 [\[4\]](#)), while another study administering green tea polyphenols corresponding to 0.6 - 1 mg EGCG per day in YACtg152F7 mice showed reduced thalamic-hypothalamic volume and reduced overall brain weight and volume (Guedj et al., 2009 [\[28\]](#)).

The structural brain alterations in DS are associated with cognitive disabilities, causing functional disability (Guidi et al., 2011 [\[29\]](#); Rodrigues et al., 2019 [\[26\]](#); Stagni et al., 2016 [\[6\]](#)). People with DS show relative impairment in executive function, short-term memory, working memory, and explicit long-term memory (Grieco et al., 2015 [\[30\]](#); Lott & Dierssen, 2010 [\[31\]](#); McCarron et al., 2017 [\[32\]](#); Real de Asua et al., 2015 [\[33\]](#); Steingass et al., 2011 [\[34\]](#); Zis & Strydom, 2018 [\[35\]](#)); together with a neurodevelopmental delay in the acquisition of both gross and fine motor skills during childhood (Begaj et al., 2017 [\[36\]](#); Ferreira-Vasques & Lamônica, 2015 [\[37\]](#); Frank & Esbensen, 2015 [\[38\]](#); Kim et al., 2017 [\[39\]](#); Malak et al., 2015 [\[40\]](#)). Regarding cognition, the therapeutic results of green tea extracts are also contradictory. A study administering 60 mg/kg GTE-EGCG to 3- to 4-month-old mBACtgDyrk1a and Ts65Dn mice for 4 weeks rescued working memory (Souchet et al., 2015 [\[41\]](#)), and interventional studies in humans with DS have shown the ability of (GTE-)EGCG to improve memory recognition, working memory, inhibitory control, and adaptive behavior (de la Torre et al., 2016 [\[42\]](#); De la Torre et al., 2014 [\[43\]](#)). However, other studies found no effect or negative effects of (GTE-)EGCG on cognition. In children aged 6-12 years, a randomized phase Ib clinical trial administering 10 mg/kg/day of EGCG for 6 months combined with a dietary supplement did not improve cognition and functionality (Cieuta-Walti et al., 2022 [\[44\]](#)). In mice, GTE-EGCG at a dose of 30

mg/kg/day had no effect on visuospatial learning and memory, and even resulted in reduced swimming speed in the Morris water maze and increased thigmotaxic behavior in 5- to 6-month-old Ts65Dn mice (Catuara-Solarz et al., 2015 [↗](#)).

Several studies have shown that the cognitive functional alterations observed in DS are related with changes at a molecular level on the concentration of hippocampal metabolites, as people with DS show increased myo-inositol levels when compared to euploid population, and people with DS and dementia show reduced levels of N-acetylaspartate (NAA) when compared to people with DS without dementia (Beacher et al., 2005 [↗](#); Lamar et al., 2011 [↗](#)). These findings warrant further investigation into the potential role of hippocampal metabolites in cognitive function, particularly given that few studies to date have investigated the effects of trisomy on the concentration of the main metabolites in the hippocampus of Ts65Dn mice (Huang et al., 2000 [↗](#); Mème, 2014; Santin et al., 2014 [↗](#)) and there is currently no evidence on the effects of (GTE)-EGCG treatment.

Finally, all these structural, functional and molecular alterations are the result of a complex genetic imbalance involving the triplicated genes in chromosome 21 and the interactions of many genes across the genome (Olson et al., 2004 [↗](#)). Indeed, skeletal malformations can be associated with the dysregulation of genes such as *Regulator of Calcineurin 1 (RCAN1)*, *Superoxide Dismutase 1 (SOD1)*, *Engrailed Homeobox 2 (EN2)*, *ETS Proto-Oncogene 2 (ETS2)*, *Sonic Hedgehog (SHH)*, *Dual-Specificity Tyrosine-(Y)-Phosphorylation Regulated Kinase 1A (DYRK1A)*, *SRY-Box Transcription Factor 9 (SOX9)*, and *Orthodenticle Homeobox 2 (OTX2)* (Arron et al., 2006 [↗](#); Billingsley et al., 2013 [↗](#); McElyea et al., 2016 [↗](#); Roper et al., 2009 [↗](#); Thomas & Roper, 2021 [↗](#); Weisfeld-Adams et al., 2016 [↗](#)), while cognitive impairment and altered brain development may be associated with *RCAN1*, *SOD1*, *Oligodendrocyte Transcription Factor 1 (OLIG1)*, *Oligodendrocyte Transcription Factor2 (OLIG2)*, *SIM BHLH Transcription Factor 2 (SIM2)*, *Down Syndrome Cell Adhesion Molecule (DSCAM)*, *DYRK1A*, *Down Syndrome Critical Region 1 (DSCR1)*, *Synaptojanin 1 (SYNJ1)* and *Potassium Inwardly Rectifying Channel Subfamily J Member 6 (KCNJ6)* (Chang et al., 2003 [↗](#); Hoeffler et al., 2007 [↗](#); Kazemi et al., 2016 [↗](#); Kleschevnikov et al., 2017 [↗](#); Lana-Elola et al., 2011 [↗](#); Stagni & Bartesaghi, 2022 [↗](#); Stagni et al., 2018 [↗](#)). From these genes, *DYRK1A*, a gene involved in both skeletal and neuronal development that is overexpressed by trisomy of chromosome 21 (Joshua D. Blazek et al., 2015 [↗](#); Dierssen & de Lagrán, 2006 [↗](#)), has been proposed as a target gene for therapy (Atas-Ozcan et al., 2021 [↗](#); Becker et al., 2014 [↗](#); Dierssen & de Lagrán, 2006 [↗](#); García-Cerro et al., 2014 [↗](#); Jarhad et al., 2018 [↗](#); Ji et al., 2015 [↗](#); Lee et al., 2009 [↗](#); Rondal, 2020 [↗](#)). The potential of EGCG to modulate gene expression in the Ts65Dn mouse model for DS has limited evidence, with one study administering 200 mg/kg EGCG on embryonic days 7 and 8 twice daily showing decreases in *Protein patched homolog 1 (Ptch)* and *Ets2* RNA expression and significant increases in *Rcan1* and *Shh* RNA expression in the first pharyngeal arch of Ts65Dn mice at embryonic day (E) 9.5 (McElyea et al., 2016 [↗](#)).

Since a holistic evaluation of these systems is missing, we designed a longitudinal experimental setup to follow the simultaneous development of structural, functional, molecular, and genetic alterations in the Ts65Dn mouse model. In this study, we conducted a multi-modal *in vivo* imaging study using micro computed tomography (μCT), magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS) to investigate the integrated development of craniofacial shape, BMD, brain volumes, and hippocampal metabolites in wildtype and Ts65Dn mice. Additionally, we evaluated the changes in body weight and performed a battery of neurodevelopmental and adult cognitive tests to assess cognitive function from birth to adulthood throughout development. At endpoint, we also evaluated tibia microarchitecture from *ex vivo* μCT scans and used RNAseq to analyze cerebellar gene expression in the same mice at eight months old (**Fig. 1** [↗](#)). Furthermore, we evaluated the pleiotropic effects of prenatal chronic GTE-EGCG treatment and explored the effects of treatment discontinuation on these systems, providing the first controlled longitudinal study assessing the simultaneous effects of treatment across different systems.

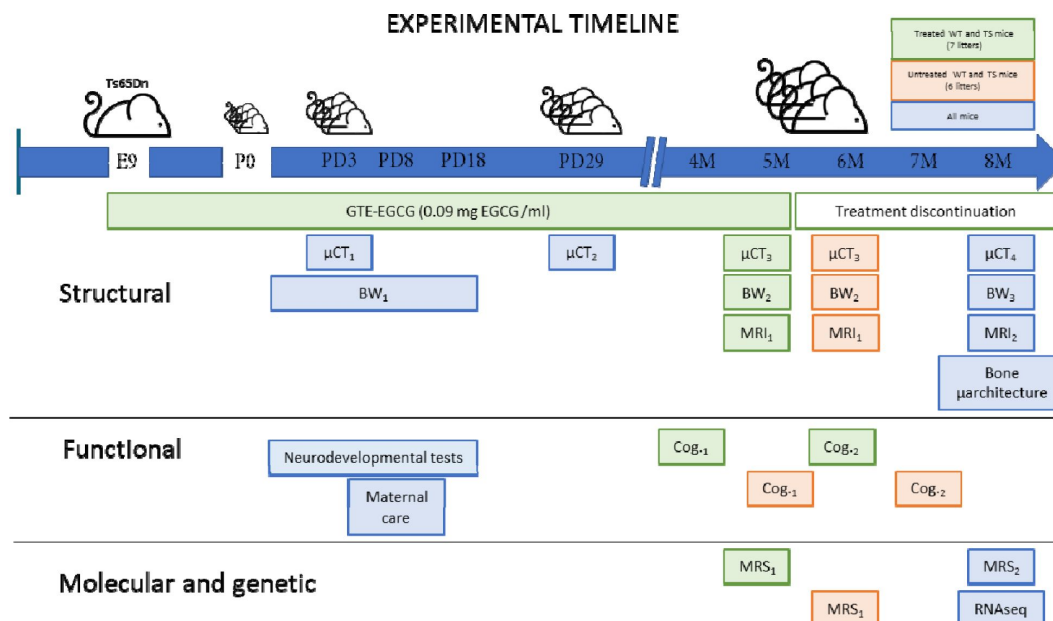


Figure 1.

Experimental design comparing structural, functional, molecular, and genetic characteristics of wildtype (WT) and Ts65Dn (TS) mice over development and evaluating the effects of a prenatal chronic GTE-EGCG treatment and its discontinuation

Ts65Dn and WT littermates were treated prenatally at a concentration of 0.09 mg EGCG/mL, which corresponds to a dose of 30 mg/kg/day, from embryonic day 9 (E9) until mice were 5 months old (5M). A battery of neurodevelopmental tests was performed daily from postnatal day (PD) 1 to PD18 to evaluate early cognitive development. To monitor maternal care, mice home cages were recorded at PD8 for 24h. Mice body weight (BW) was recorded daily from PD1 to PD17 (BW₁), and at two additional times before μCT scanning (BW₂ and BW₃). *In vivo* micro-computed tomography (μCT) scans were performed four times over development to follow skeletal development: μCT₁ at PD3 and μCT₂ at PD29 in all mice; μCT₃ at 5M in treated mice, and 6M in untreated mice; and μCT₄ at 8M in all mice. Additionally, mice were scanned with *in vivo* magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS) before and after treatment discontinuation to quantify brain volumetric changes and metabolite concentrations in the hippocampal region: MRI₁ and MRS₁ at 5M in treated mice and 6M in untreated mice; and MRI₂ and MRS₂ at 8M in all mice. Two batteries of cognitive tests of one-month duration each were performed to evaluate adult cognition: Cog₁ at 4M in treated mice, and 5M in untreated mice; and Cog₂ at 6M in treated mice, and 7M in untreated mice. At endpoint (8M), the tibia of all mice was collected to measure its length using a digital caliper and analyze its microarchitecture using *ex vivo* μCT. At this last stage, cerebellar tissue was also collected to perform RNAseq gene expression analysis.

Results

Our longitudinal experimental setup allowed us to investigate the pleiotropic effects of trisomy, GTE-EGCG treatment and treatment discontinuation at the structural, functional, molecular, and genetic levels in the Ts65Dn Down syndrome mouse model (**Fig. 1**). We compared wildtype (WT) and Ts65Dn (TS) untreated mice to evaluate trisomy effects, and WT and TS treated mice to evaluate treatment effects.

Structural characterization

Structurally, we investigated the longitudinal effects of trisomy, treatment, and treatment discontinuation on body weight, skeletal system, and adult brain volume (**Fig. 2**).

Body Weight

First, we investigated mouse body growth by monitoring body weight over development. TS untreated mice tended to present lower body weights than WT untreated mice over development from birth to adulthood, but these genotype differences did not reach significance at any developmental stage (**Fig. 2A**; $P_{BW1}=0.7630$; $P_{BW2}=0.3688$; $P_{BW3}=0.1094$).

Chronic GTE-EGCG treatment, which started prenatally and was maintained until 5 months, significantly reduced the body growth of TS treated mice from PD1 to PD17 (**Fig. 2A** at BW_1) in comparison with WT untreated ($P<0.0001$), WT treated ($P<0.0001$), and TS untreated mice ($P<0.0001$). At adulthood before treatment discontinuation (**Fig. 2A** at BW_2), these differences were maintained, but only reached significance when compared to WT untreated ($P=0.0322$) and WT treated mice ($P=0.0103$). After three months of treatment discontinuation, both WT and TS treated mice showed a mild increase in body weight, making TS treated mice not significantly different from WT untreated (**Fig. 2A**; $P_{BW3}=0.9284$), and causing WT treated mice to be significantly different from WT untreated (**Fig. 2A**; $P_{BW3}=0.0259$).

Skeletal Development

Since people with DS show craniofacial abnormalities, reduced bone mineral density (BMD), and altered bone microarchitecture (Carfi et al., 2017; Kao et al., 1992; LaCombe & Roper, 2020; Thomas & Roper, 2021; Vicente et al., 2020), we investigated the effects of genotype, treatment, and treatment discontinuation in these systems by performing *in vivo* μ CT scans throughout development and *ex vivo* μ CT scans at endpoint as indicated in **Figure 1**.

Craniofacial morphology

We first performed geometric morphometric analysis on the craniofacial 3D models obtained from *in vivo* μ CT scans to investigate craniofacial shape throughout development. The results confirmed our previous findings (Llambrich, González-Colom, et al., 2022; Llambrich, González, et al., 2022), showing that TS untreated mice presented a significantly different craniofacial shape than WT untreated mice at μ CT₁ ($P<0.0001$) and μ CT₂ ($P=0.0011$) (**Supplementary Table S2**). In this study, we further detected that the craniofacial differences induced by genotype persisted until adulthood (**Fig. 2B**, $P_{\mu CT3}=0.0009$; **Fig. 2C**, $P_{\mu CT4}=0.0055$).

Prenatal chronic GTE-EGCG treatment only showed a significant effect in the craniofacial shape at PD3, where both WT and TS treated mice were different from their untreated counterparts ($P_{WT}=0.0418$; $P_{TS}=0.0005$). However, the treatment never rescued the craniofacial shape in TS treated mice, as these mice were significantly different from WT untreated mice at all stages (**Fig.**

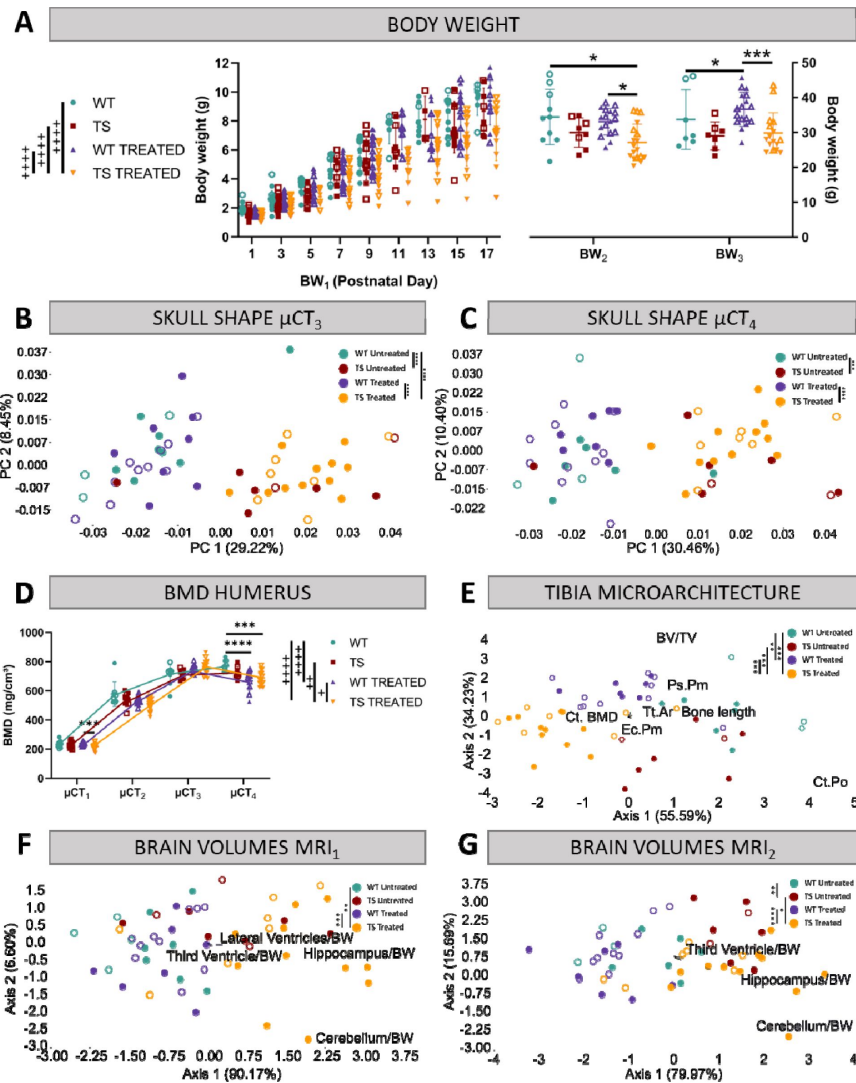


Figure 2

Evaluation of trisomy, prenatal chronic GTE-EGCG treatment and treatment discontinuation effects on structural traits

(A) Body weight measurements over postnatal development in untreated and treated WT and TS mice. (B,C) Skull shape differences between adult WT and TS mice before (B) and after GTE-EGCG treatment discontinuation (C). Skull shape variation explored by a Principal Component Analysis (PCA) based on the 3D coordinates of landmarks recorded on the surfaces of 3D craniofacial reconstructions from *in vivo* μ CT at μ CT₃ (B) and μ CT₄ (C) timepoints. The landmark configuration for each stage is defined in [Supplementary Figure S1](#). Scatter plots are presented along with the morphings associated with the negative and positive extremes of the PC1 axis. Statistical differences between groups were assessed by permutation tests based on the Procrustes distances. (D) Bone mineral density of the humerus over postnatal development. The *in vivo* μ CT scans of the humerus were used to determine the BMD at μ CT₁, μ CT₂, μ CT₃ and μ CT₄ timepoints. (E) Linear discriminant analysis (LDA) based on the results from the tibia microarchitecture tests performed at endpoint (8M), three months after chronic treatment discontinuation. (F,G) LDA based on the brain volumes obtained from *in vivo* MRI before (F) and after GTE-EGCG treatment discontinuation (G). The contribution of each variable to separate groups of mice across Axis1 and Axis2 is represented in each LDA as lines pointing in the direction of each axis, with longer lines indicating higher contributions. All data are presented as mean $\pm$ standard deviation. (+) $p < 0.05$; (++) $p < 0.01$; (****) $p < 0.0001$; Mixed-effects analysis across timepoints; (***) $p < 0.001$; (****) $p < 0.0001$; pairwise comparisons. Mice analyzed may differ across stages due to uncontrollable technical issues inherent to longitudinal studies, such as scanning failure or mouse death during the experiment but represent overall ontogenetic trajectories. Male mice are indicated with empty symbols. Sample sizes used in each test are provided in [Supplementary Table S1](#).

2 B,C; **Supplementary Table S2**). Discontinuing the treatment for three months did not induce any effects (**Fig. 2C**), maintaining the craniofacial dysmorphologies already observed at μCT_3 (**Fig. 2B**).

Bone Mineral Density

The BMD of the humerus was estimated throughout development from the *in vivo* μCT scans. Although we observed that TS untreated mice tended to present lower BMD than WT untreated mice over development and adulthood, the differences did not reach significance at any developmental stage (**Fig. 2D**, **Supplementary Table S3**).

Prenatal GTE-EGCG chronic treatment significantly modified the developmental trajectory of humerus BMD in WT and TS treated mice ($P_{\text{WT}} < 0.0001$; $P_{\text{TS}} = 0.0023$), but never rescued the trisomic phenotype, as TS treated mice remained different from WT untreated ($P < 0.0001$). At PD3, WT and TS treated mice showed similar levels of BMD as compared to untreated mice (**Fig. 2D** at μCT_1) but tended to show lower BMD at PD29 (**Fig. 2D** at μCT_2). This tendency for reduced BMD was rescued at adulthood (**Fig. 2D** at μCT_3), but after three months of treatment discontinuation, both WT and TS treated groups showed a decline in BMD (**Fig. 2D** at μCT_4), which caused WT and TS treated mice to be different than WT untreated control mice ($P_{\text{WT}} = 0.0001$; $P_{\text{TS}} = 0.0002$), suggesting a rebound effect following treatment retrieval.

Tibia Development & Microarchitecture

Finally, we complemented our structural skeletal analysis by evaluating the length and microarchitecture of the tibia from *ex vivo* μCT scans at endpoint, as indicated in **Figure 1**.

To obtain a global view of the genotype and treatment effects, we performed a linear discriminant analysis (LDA) combining the results of all tibia microarchitecture tests into a single analysis that maximized the differences among groups of mice (**Fig. 2E**). The LDA showed limited overlap between groups, as mice separated by treatment along the first axis, and by genotype along the second axis. These results suggested genotype differences and treatment effects that did not rescue the trisomic phenotype (**Fig. 2E**). The multivariate pairwise comparisons after one-way PERMANOVA using Mahalanobis distances detected significant differences between all groups of mice except between WT untreated and TS untreated mice (**Supplementary Table S4**), confirming the treatment effects in both genotypes.

To explore the overall differences detected by the LDA in finer detail, we grouped the results of the tibia microarchitecture tests according to the structural domain they evaluated (cortical bone strength, cortical bone size and trabecular bone), and repeated the multivariate pairwise comparisons (**Supplementary Table S4**). Even though we did not detect any significant difference between WT untreated and TS untreated mice in any structural domain (**Supplementary Table S4**), TS untreated mice tended to show specific reductions in cortical thickness, polar moment of inertia, cross sectional area, and bone area (**Figure 2-figure supplement 1 C,E,F,G**); and the univariate pairwise comparison tests revealed significant differences in the tibia length (**Figure 2-figure supplement 1A**; $P = 0.0170$) and periosteal perimeter (**Figure 2-figure supplement 1I**; $P = 0.0154$).

After GTE-EGCG chronic treatment and discontinuation, WT treated mice were not significantly different than WT untreated mice for any specific structural domain (**Supplementary Table S4**), but the univariate pairwise tests indicated significantly reduced tibia length (**Figure 2-figure supplement 1A**; $P = 0.0006$) and cortical porosity (**Figure 2-figure supplement 1D**; $P = 0.0120$), together with increased cortical bone mineral density (**Figure 2-figure supplement 1B**; $P = 0.0120$). TS treated mice were significantly different than TS untreated mice for the cortical bone strength and cortical bone size domains (**Supplementary Table S4**), showing significantly more mineralized (**Figure 2-figure supplement 1B**; $P = 0.0039$), thicker (**Figure 2-figure**

supplement 1C [↗](#); $P=0.0400$), and less porous (Figure 2-figure supplement 1D [↗](#); $P=0.0005$) cortical bone. However, they appeared different from WT untreated mice in both cortical domains (Supplementary Table S4 [↗](#)) as well as in the cortical BMD (Figure 2-figure supplement 1B [↗](#); $P=0.0097$) and cortical porosity tests (Figure 2-figure supplement 1D [↗](#); $P=0.0107$). These results could be explained either by adverse effects of the treatment during development that were persistent after discontinuation, or by a negative effect following treatment withdrawal.

Brain Development

Finally, as it was reported that people with DS show altered brain volumes (Aylward et al., 1997 [↗](#); Hamner et al., 2018 [↗](#); Movsas et al., 2016 [↗](#); Patkee et al., 2020 [↗](#); Pinter et al., 2001 [↗](#); Rodrigues et al., 2019 [↗](#); Smigielska-Kuzia et al., 2011 [↗](#)), we completed our structural evaluation by assessing the volume of the whole brain, along with the volumes of the hippocampal region, the cerebellum and the ventricles using the *in vivo* MRI scans performed before and after treatment discontinuation (Fig. 1 [↗](#)).

The LDA including the volumes of the subcortical brain regions mentioned above showed genotype separation across Axis1 at MRI₁, which further increased at MRI₂, with WT mice occupying the negative extreme of Axis1 and TS mice occupying the positive extreme (Fig. 2 F,G [↗](#)). The multivariate pairwise comparisons after one-way PERMANOVA using Mahalanobis distances (Supplementary Tables S5 & S6) confirmed genotype differences only at MRI₂, when WT untreated mice were significantly different than TS untreated (Fig. 2G [↗](#); $P=0.0065$). Contrary to previous evidence in Ts65Dn mice (Aldridge et al., 2007 [↗](#); Duchon et al., 2021 [↗](#); Holtzman et al., 1996 [↗](#); Insausti et al., 1998 [↗](#)), adult TS untreated mice tended to present larger volumes than WT untreated mice in all brain regions at both MRI₁ and MRI₂ (Figure 2-figure supplement 2 [↗](#)). The univariate pairwise tests performed at MRI₁ confirmed that TS mice presented a significantly larger brain (Figure 2-figure supplement 2A [↗](#), top; $P=0.0066$), hippocampal region (Figure 2-figure supplement 2B [↗](#), top; $P=0.0254$) and whole ventricles volume (Figure 2-figure supplement 2H [↗](#), top; $P=0.0427$) than WT untreated mice. At MRI₂, these differences did no longer reach significance and significant increases emerged in the volumes of the third ventricle (Figure 2-figure supplement 2E [↗](#), bottom; $P=0.0479$) and fourth ventricle (Figure 2-figure supplement 2F [↗](#), bottom; $P=0.0014$).

Regarding treatment effects, the LDA at MRI₁ showed separation between TS treated mice and the rest of the groups (Fig. 2F [↗](#)), and the multivariate pairwise comparisons after one-way PERMANOVA confirmed significant differences between WT untreated and TS treated mice ($P=0.0047$), but not between TS untreated and TS treated mice ($P=0.4921$). Indeed, TS treated mice tended to show larger volumes than TS untreated mice in all brain regions (Figure 2-figure supplement 2 [↗](#)), but the univariate pairwise comparison tests only detected a significant difference in the hippocampal region (Figure 2-figure supplement 2B [↗](#), top; $P=0.0413$). These effects did not rescue the trisomic phenotype, as TS treated mice were significantly different than WT untreated mice in all brain regions (Figure 2-figure supplement 2 [↗](#), top).

After three months of treatment discontinuation at MRI₂, some TS treated mice tended to occupy the same space as WT untreated mice in the LDA (Fig. 2G [↗](#)), and although the multivariate pairwise comparisons after one-way PERMANOVA did not detect significant differences between WT untreated and TS treated mice ($P=0.0609$), TS treated mice remained significantly different than WT untreated mice in all univariate brain regions except for the volume of the third ventricle (Figure 2-figure supplement 2E [↗](#), bottom; $P=0.0562$), indicating limited rescuing effects at this last stage after treatment discontinuation.

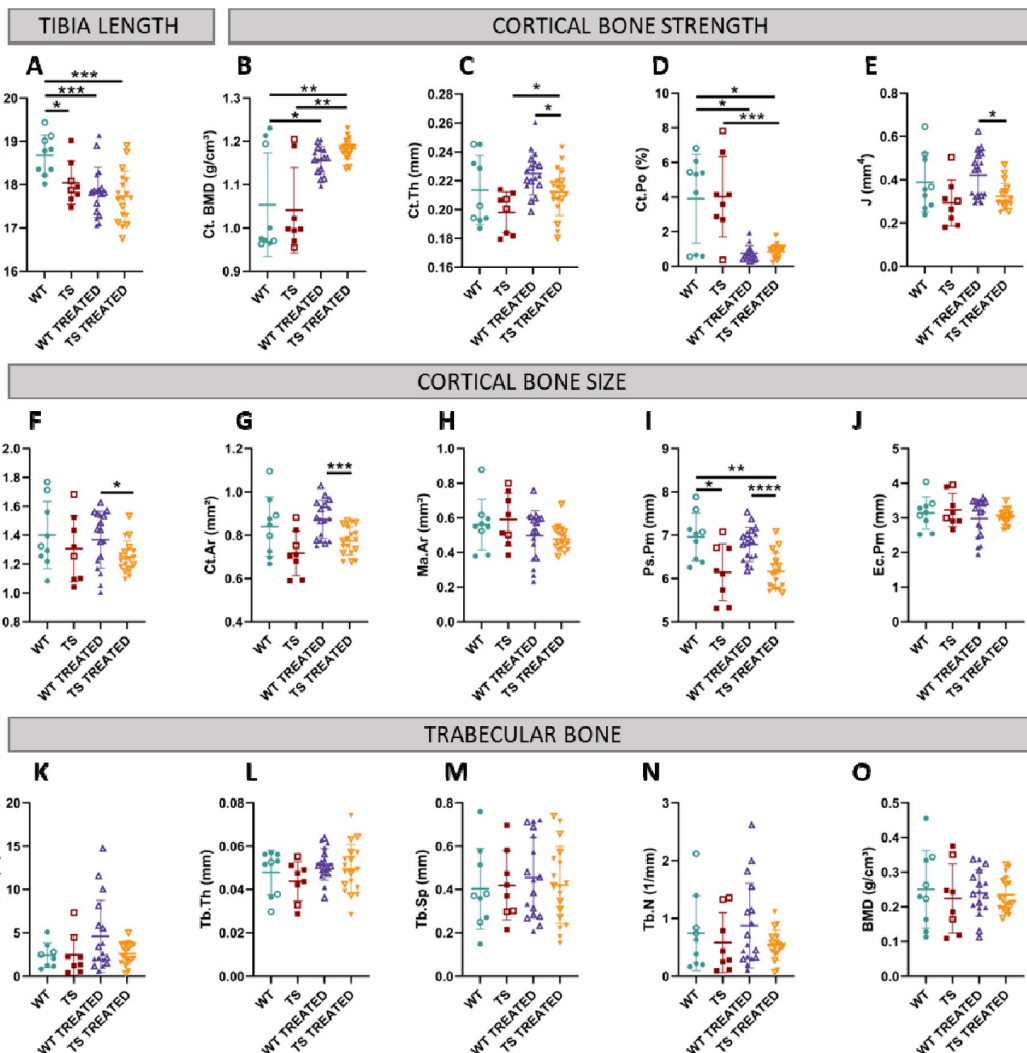


Figure 2-figure supplement 1.

Univariate analysis of tibia microarchitecture in wildtype and trisomic mice at adulthood and effects of prenatal chronic GTE-EGCG treatment and three months of discontinuation

(A) The tibia length was measured using a digital caliper at endpoint. The measurements assessing the microarchitecture of the tibia were obtained from *ex vivo* μ CT scans and grouped in three categories according to structural bone domains: (1) **Cortical bone strength**: (B) cortical bone mineral density, (C) cortical thickness, (D) cortical porosity, and (E) polar moment of inertia; (2) **Cortical bone size**: (F) cross sectional area, (G) bone area, (H) medullary area, (I) periosteal perimeter, and (J) endocortical perimeter. (3) **Trabecular bone**: (K) percentage of bone volume, (L) trabecular thickness, (M) trabecular separation, (N) trabecular number, and (O) bone mineral density. Data are presented as mean $\pm$ standard deviation. (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$; (****) $p < 0.0001$; pairwise tests. Male mice are indicated with empty symbols. Sample sizes for each test are provided in [Supplementary Table S1](#).

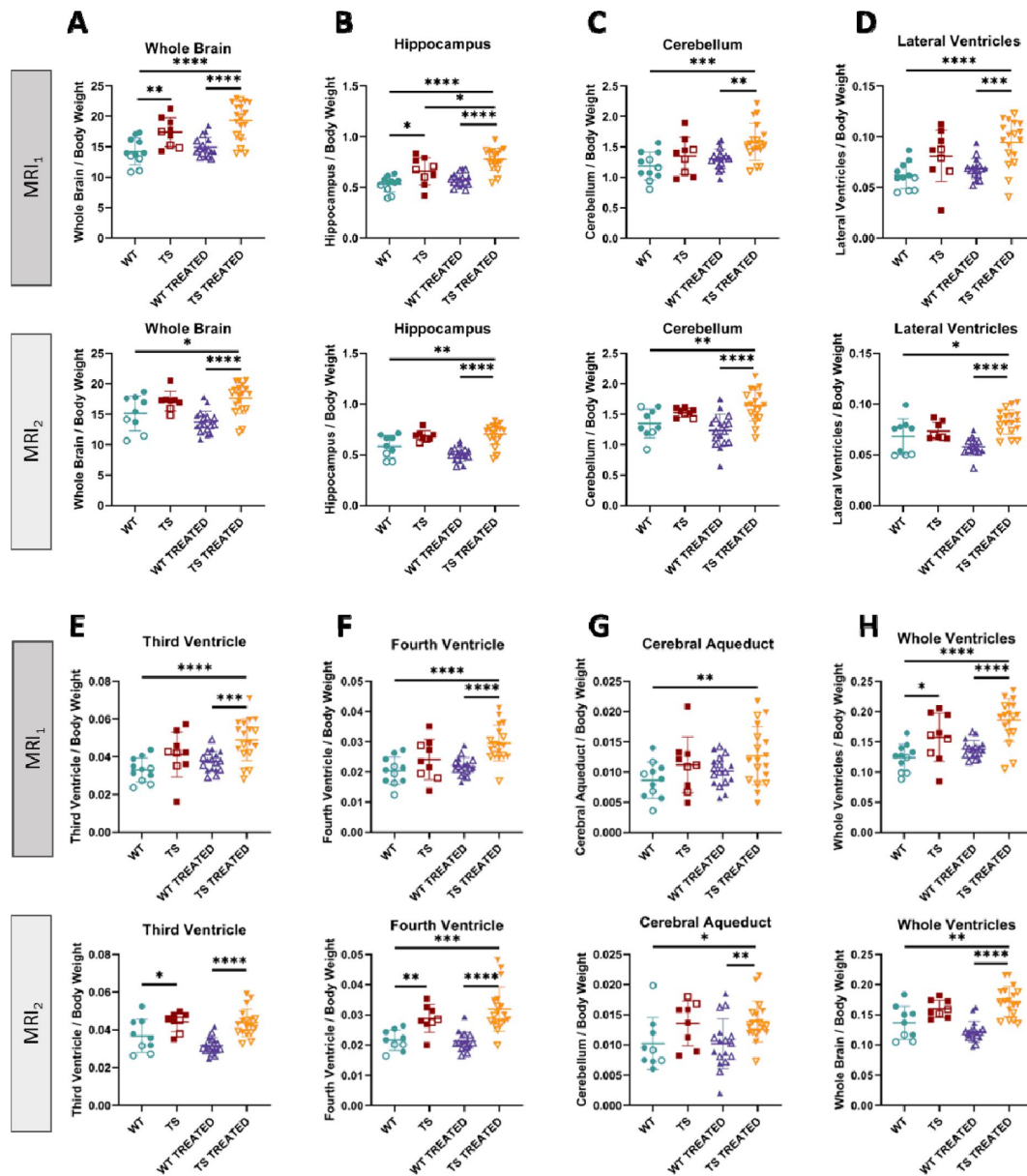


Figure 2-figure supplement 2.

Univariate analysis of brain volumes in wildtype and trisomic mice at adulthood and effects of prenatal chronic GTE-EGCG treatment and its discontinuation

The *in vivo* MRI scans performed at MRS₁ and MRS₂ were used to quantify the volumes of (A) the whole brain, (B) hippocampal region, (C) cerebellum, (D) lateral ventricles, (E) third ventricle, (F) the fourth ventricle, (G) cerebral aqueduct, and (H) whole ventricular system. All volumes were normalized to body weight. For each test, the results at MRS₁ are presented on top and at MRS₂ are presented on the bottom. Data are presented as mean $\pm$ standard deviation. (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$; (****) $p < 0.0001$; pairwise tests. Male mice are indicated with empty symbols. Sample sizes for each test are provided in [Supplementary Table S1](#).

Functional characterization

After evaluating the effects of trisomy and chronic prenatal GTE-EGCG treatment and its discontinuation at a structural level, we investigated its simultaneous effects at a functional level. We performed a battery of cognitive tests to evaluate early neurodevelopment and adult cognition before and after treatment discontinuation ([Fig. 3](#)), at the timepoints specified in [Figure 1](#).

Neurodevelopment

We first evaluated early brain function and neurodevelopment over the first weeks after birth by performing a battery of neurodevelopmental tests from PD1 until PD18 ([Fig. 3A](#)). The LDA with the results from all neurodevelopmental tests combined showed that the treatment was the main differentiating factor among groups of mice, as Axis 1 separated treated and untreated mice ([Fig. 3A](#)). Despite some overlap, Axis2 separated WT and TS mice. These results suggested mild genotype differences and treatment effects that did not rescue the trisomic phenotype ([Fig. 3A](#)). The multivariate pairwise comparisons after one-way PERMANOVA using Mahalanobis distances ([Supplementary Table S7](#)) detected significant differences between TS untreated and WT untreated mice ($P=0.0001$), and between TS treated mice and both TS untreated ($P=0.0001$) and WT untreated mice ($P=0.0001$), confirming these findings.

When the neurodevelopmental tests were grouped into functional domains (developmental landmarks, neuromotor tests and reflexes) ([Supplementary Table S7](#)), the multivariate pairwise comparisons only indicated significant differences between WT untreated and TS untreated mice for the reflexes ($P=0.0423$). TS untreated mice tended to be delayed in most individual tests ([Figure 3-figure supplement 1](#)), and the univariate pairwise comparison tests confirmed significant delays in the acquisition rate of the vertical climbing ([Figure 3-figure supplement 1E](#); $P=0.0256$) and tactile response ([Figure 3-figure supplement 1I](#); $P=0.0064$), together with the average day of acquisition of the surface righting response ([Figure 3-figure supplement 1C](#); $P=0.0320$). However, we also observed that TS untreated mice tended to be anticipated in the vibrissae placing, blast response, and Preyer reflex ([Figure 3-figure supplement 1 J,M,N](#)), even though these results did not reach significance.

Prenatal chronic treatment with GTE-EGCG caused TS treated mice to be significantly different than TS untreated mice for the domains neuromotor tests and reflexes, and WT treated mice to be different from WT untreated mice for the domain reflexes ([Supplementary Table S7](#)). The univariate tests indicated that treatment significantly modulated the average day of acquisition and acquisition rate of multiple neurodevelopmental tests in both WT and TS treated mice, causing either a delay or an anticipation in most tests ([Figure 3-figure supplement 1](#)). However, the treatment never rescued the trisomic phenotype, as TS treated mice remained significantly different than WT untreated mice for the three functional domains and most of the individual tests ([Supplementary Table S7](#)) ([Figure 3-figure supplement 1](#)).

Finally, we investigated the mothers' behavior during 24h at PD8 to evaluate maternal care and observed that, despite being trisomic, all Ts65Dn mothers performed similarly and there was no large intra-group variation for any of the behaviors analyzed, confirming that maternal care did not influence the results of the neurodevelopmental tests ([Figure 3-figure supplement 2](#)). Furthermore, no significant differences were detected between treated and untreated mothers in any readout ([Figure 3-figure supplement 2](#)), confirming that the GTE-EGCG treatment did not modulate maternal care.

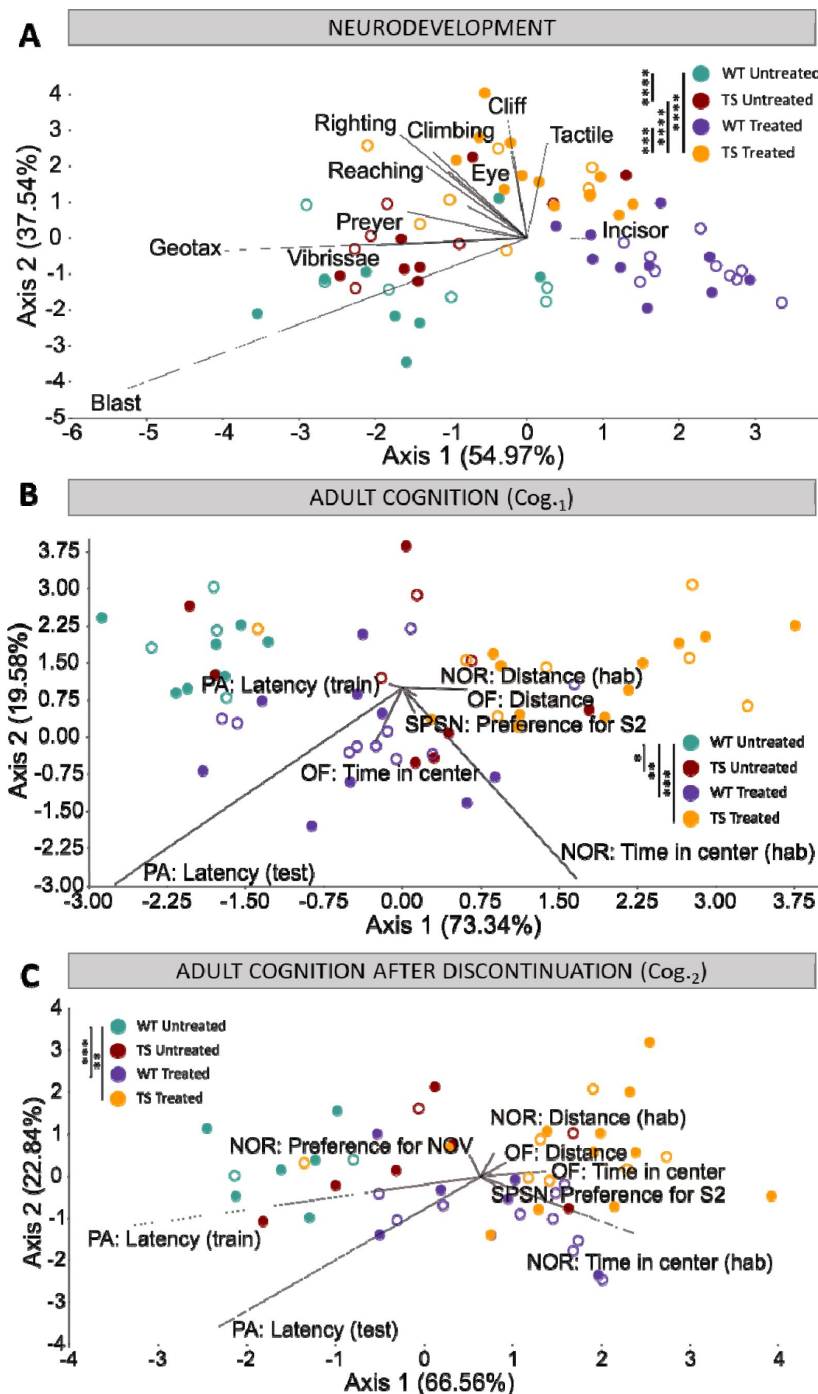
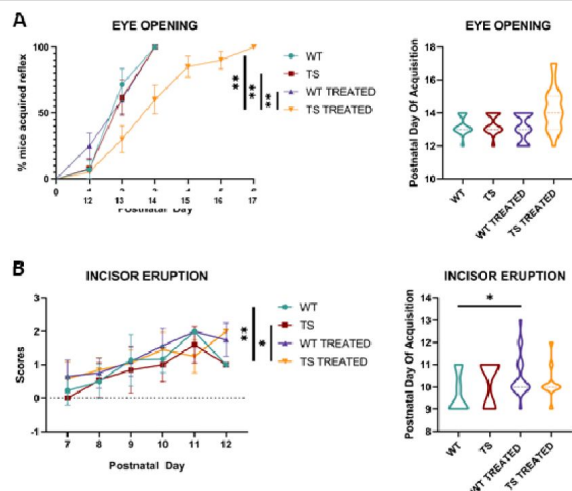


Figure 3

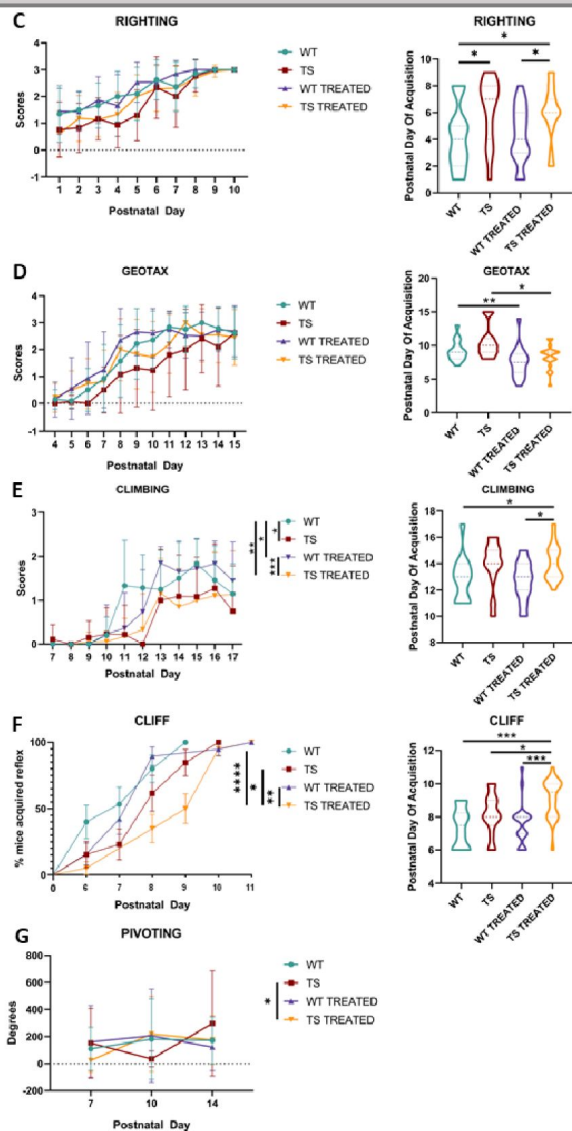
Evaluation of trisomy, prenatal chronic GTE-EGCG treatment and treatment discontinuation effects on cognitive function during development and at adulthood before and after treatment discontinuation

(A) Linear discriminant analysis (LDA) based on the results from the neurodevelopmental tests performed from PD1 until PD18. (B,C) LDA based on the results from the cognitive tests performed at Cog₋₁ (B) and Cog₋₂ (C). The contribution of each variable to separate groups of mice across Axis1 and Axis2 is represented in each LDA as lines pointing in the direction of each axis, with longer lines indicating higher contributions. Male mice are indicated with empty symbols. Sample sizes for each test are provided in [Supplementary Table S1](#).

Neurodevelopmental landmarks



Neuromotor tests



Reflexes

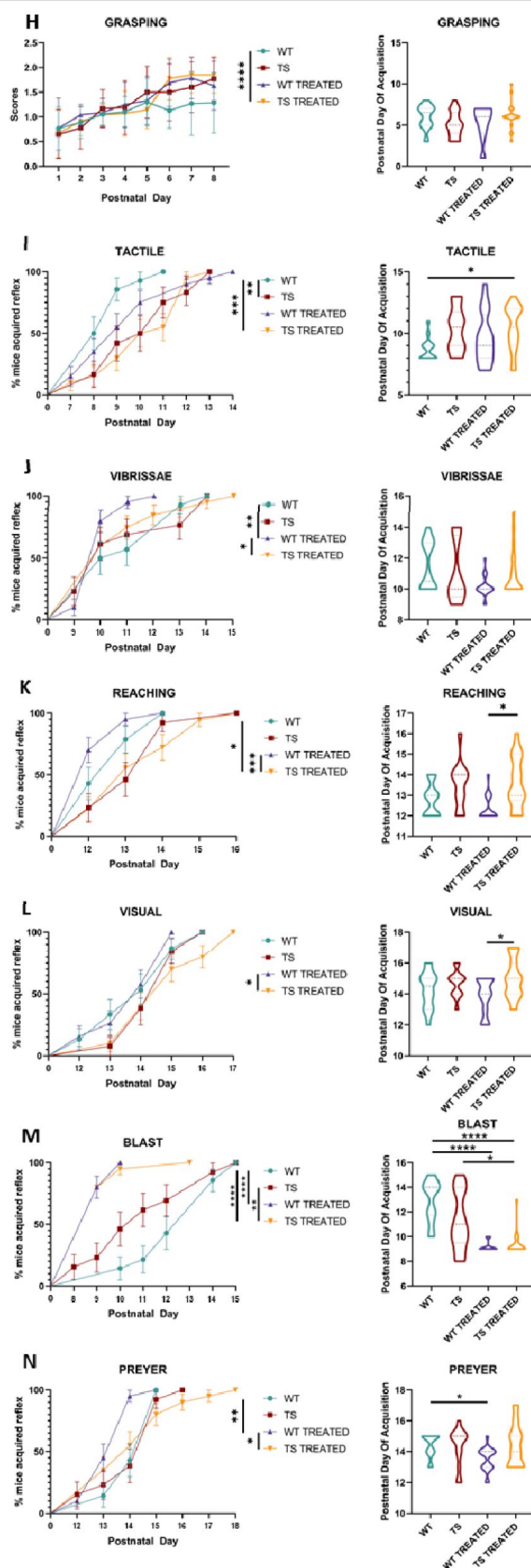


Figure 3-figure supplement 1.

Univariate analysis of early postnatal neurodevelopment in WT and TS mice and effects of pre- and postnatal GTE-EGCG treatment

Neurodevelopment tests were performed from PD1 to PD18 and grouped into three categories according to functional domains. **(1) Neurodevelopmental landmarks:** (A) eye opening, (B) incisor eruption, and pinna detachment (results not shown as no significant differences were detected). **(2) Neuromotor development:** (C) surface righting response, (D) negative geotaxis, (E) vertical climbing, (F) cliff drop aversion, (G) pivoting, homing and walking (results not shown as no significant differences were detected). **(3) Reflexes:** (H) grasping, (I) tactile orientation, (J) vibrissae placing, (K) reaching response, (L) visual placing response, (M) blast response and (N) Preyer reflex. For each test, developmental rate is presented on the left as mean $\pm$ standard deviation and average day of successful test acquisition is presented on the right as violin plots. (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$; (****) $p < 0.0001$. Sample sizes for each test are provided in [Supplementary Table S1](#).

Adult Cognition

Then, we evaluated adult cognitive performance by means of open field (OF), elevated plus maze (EPM), sociability/preference for social novelty (SPSN), novel object recognition (NOR) and passive avoidance (PA) testing at two timepoints, Cog₁ and Cog₂, before and after treatment discontinuation ([Fig. 3 B,C](#)), as specified in [Figure 1](#).

The LDA with the results from all tests combined showed minor overlap between groups at both Cog₁ and Cog₂ ([Fig. 3 B,C](#)), with WT untreated mice occupying the negative extreme of Axis1, WT treated and TS untreated mice presenting an intermediate position, and TS treated mice falling on the positive extreme of Axis1, opposite to WT untreated mice ([Fig. 3 B,C](#)). These results suggested genotype differences between WT and TS untreated mice that were confirmed as significant by the multivariate pairwise comparisons after one-way PERMANOVA at Cog₁ ([Supplementary Table S8](#); $P=0.0125$) but not at Cog₂ ([Supplementary Table S9](#); $P=0.4596$).

When the cognitive tests were grouped into cognitive domains (anxiety, arousal, and memory), the multivariate pairwise comparisons only detected a significant difference between WT untreated and TS untreated mice for the arousal tests at Cog₁ ($P=0.0028$) ([Supplementary Tables S8](#) and [S9](#)). Indeed, TS untreated mice showed a tendency to cover longer distances at higher speed in all arousal tests when compared with WT untreated mice ([Figure 3-figure supplement 3 E-J](#)). These differences reached significance for the distance and speed during OF, as well as for the distance and speed during NOR at Cog₁ ([Figure 3-figure supplement 3 E,F,I,J](#), top). TS untreated mice also tended to show increased exploratory and risk-taking behavior, as they usually presented a tendency of increased time spent in the center of the arena during habituation for NOR at Cog₁ ([Figure 3-figure supplement 3C](#), top), increased percentage of time spent in the open arm during EPM at Cog₁ ([Figure 3-figure supplement 3D](#), top), increased time in the center during OF at Cog₂ ([Figure 3-figure supplement 3A](#), bottom), and reduced time to enter the dark chamber during the training session of PA at Cog₁ ([Figure 3-figure supplement 3M](#), top), although these differences did not reach statistical significance. Finally, TS untreated mice showed impaired memory robustness and formation at the latest stage, with significantly less preference for the novel object during NOR when compared with WT untreated mice ([Figure 3-figure supplement 3K](#), bottom; $P=0.0257$), and a tendency for reduced latency to enter the dark chamber during training and testing sessions for PA at Cog₂ ([Figure 3-figure supplement 3 M,N](#), bottom).

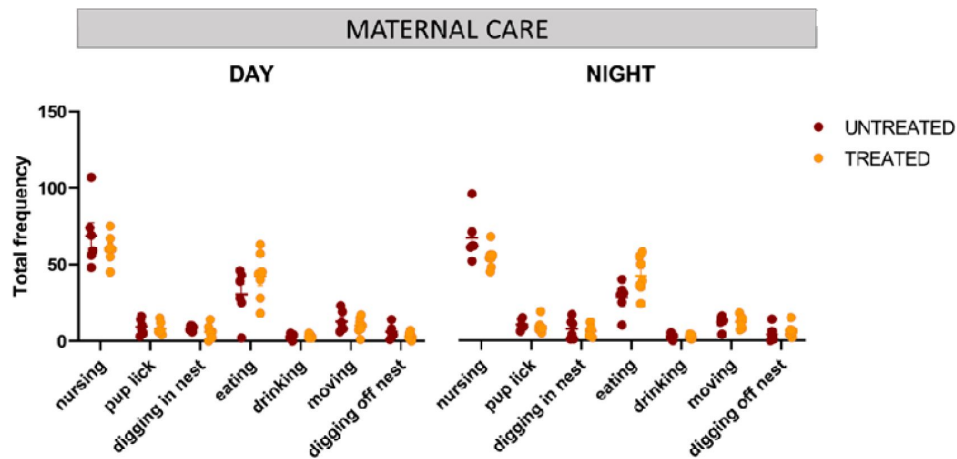


Figure 3-figure supplement 2.

Maternal care evaluation and effects of GTE-EGCG treatment

Frequency of nursing, pup grooming, digging in nest, eating, drinking, moving, and digging off nest behaviors scored manually from 24h videotapes at PD8. Sample sizes for each test are provided in [Supplementary Table S1](#).

Regarding treatment effects, the results from the LDA at Cog₁ suggested that the prenatal chronic treatment induced changes in both WT and TS treated mice (**Fig. 3 B**) that exacerbated the differences in TS treated mice and induced cognitive changes in WT treated mice that resulted in a cognitive performance similar to TS untreated mice (**Fig. 3 B**). Indeed, WT treated mice were significantly different than WT untreated mice when all cognitive tests were considered together as well as for the anxiety and arousal domains (**Supplementary Table S8**). In the case of TS treated mice, the multivariate pairwise comparisons revealed significant differences with WT untreated mice when considering all groups together as well as for the anxiety and arousal domains (**Supplementary Table S8**), confirming the lack of rescuing treatment effects. Both WT and TS treated mice showed significantly increased time in the center during NOR, and increased time in the open arm during EPM at Cog₁ when compared with WT untreated mice (**Figure 3-figure supplement 3 C,D**, top), indicating increased exploratory and risk-taking behavior. Similarly, both WT and TS treated mice showed increased locomotor activity, as they covered significantly more distance at more speed than WT untreated mice for the distance and speed during OF, as well as the distance and speed during NOR at Cog₁ (**Figure 3-figure supplement 3 E,F,I,J**, top). However, in the memory tests (**Figure 3-figure supplement 3 K-N**), the pairwise comparison tests revealed only a significant difference between TS untreated and TS treated mice for the preference for subject 2 (S2) at Cog₁, where treated mice had greater preference for the novel stranger (**Figure 3-figure supplement 3L**, top; $P=0.0137$).

After treatment discontinuation at Cog₂, the LDA (**Fig.3C**) presented a similar pattern as the LDA at Cog₁ (**Fig.3B**), suggesting that discontinuing the treatment for one month did not substantially change the cognitive patterns induced after chronic GTE-EGCG treatment. However, new significant differences emerged between WT untreated and both WT treated and TS treated mice when evaluating the memory domain (**Supplementary Table S9**), suggesting that the treatment discontinuation altered the memory of both WT and TS mice. Furthermore, TS treated mice remained significantly different than WT untreated mice for all tests combined and all cognitive domains (**Supplementary Table S9**), confirming that the effects of treatment discontinuation did not rescue the trisomic cognitive phenotype. Indeed, significant differences between WT untreated and TS treated mice remained for the percentage of time spent in the open arm during EPM at Cog₂ (**Figure 3-figure supplement 3D**, bottom; $P=0.0476$), and new significant differences emerged between WT untreated mice and both WT treated and TS treated mice for the time spent in the center and in the periphery during OF (**Figure 3-figure supplement 3 A,B**, bottom), indicating that treated mice still presented a more explorative and risk-taking behavior. A similar trend was observed for the arousal tests, as even though no significant differences were detected between TS treated and TS untreated mice (**Figure 3-figure supplement 3 E-J**, bottom), both treated groups showed hyperactivity as the univariate pairwise comparison tests confirmed significant differences between TS treated mice and WT untreated mice for all arousal tests (**Figure 3-figure supplement 3 E-J**, bottom); and significant differences were detected between WT treated and WT untreated mice for the distance and speed during SPSN and the distance and speed during NOR (**Figure 3-figure supplement 3 G-J**, bottom). In the memory tests, TS treated mice were significantly different than TS untreated ($P=0.0046$) but not than WT untreated mice for the preference for the novel object during NOR (**Figure 3-figure supplement 3K**, bottom; $P=0.3566$), which could suggest a beneficial treatment effect. Contrary to the other behavioral tests, the results for PA testing at Cog₂ are influenced by the testing at Cog₁ as mice may present a robust contextual fear memory trace created during the testing at Cog₁, preventing them from entering the dark chamber even in the training phase of the test at Cog₂. Indeed, WT untreated mice did not enter the dark box during training at Cog₂, while both WT and TS treated mice showed a reduced tendency to enter the dark chamber which reached significance when comparing TS treated mice with WT untreated mice (**Figure 3-figure supplement 3M**, bottom; $P<0.0001$), indicating less robust contextual memory retention. After re-exposure to the fear conditioning in the training phase of PA at Cog₂, all WT mice avoided the dark chamber while a few TS treated mice still showed a reduced tendency to enter the dark chamber (**Figure 3-figure supplement 3N**, bottom), reflecting altered memory robustness.

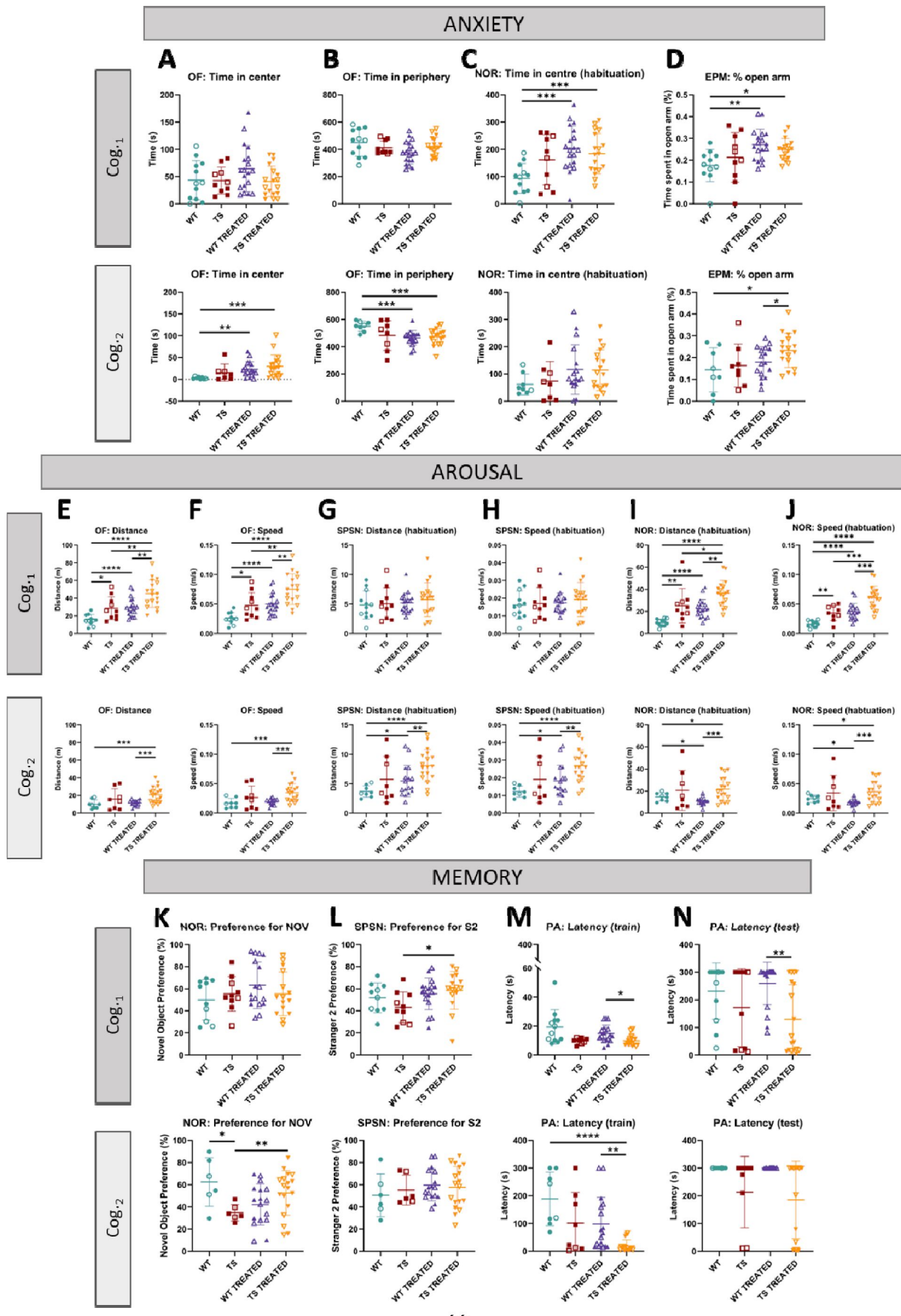


Figure 3-figure supplement 3.

Univariate analysis of adult cognition in WT and TS mice and effects of prenatal chronic GTE-EGCG treatment and its discontinuation

Results for open field (OF), novel object recognition (NOR), elevated plus maze (EPM), sociability/preference for social novelty (SPSN) and passive avoidance (PA) tests performed at Cog.₁ (top) and Cog.₂ (bottom) were grouped into three categories according to functional domains. **(1) Anxiety tests:** **(A)** time in center during OF, **(B)** time in periphery during OF, **(C)** time in center during habituation for NOR, and **(D)** percentage of time in open arm during EPM. **(2) Arousal tests:** **(E)** total distance covered during OF, **(F)** mean speed during OF, **(G)** total distance covered during SPSN, **(H)** mean speed during SPSN, **(I)** total distance covered during habituation for NOR, and **(J)** mean speed during habituation for NOR. **(3) Memory tests:** **(K)** preference for novel object during testing for NOR, **(L)** preference for second stranger during SNS2, **(M)** latency to enter dark chamber during training for PA, and **(N)** latency to enter dark chamber during testing for PA. For each test, the results at Cog.₁ are presented on top and the results at Cog.₂ are presented on the bottom. Data are presented as mean $\pm$ standard deviation. (*) $p < 0.05$; (**) $p < 0.001$; (***) $p < 0.001$; (****) $p < 0.0001$. Male mice are indicated with empty symbols. Sample sizes for each test are provided in [Supplementary Table S1](#).

Molecular and genetic characterization

Finally, to complement the structural and functional evaluation and to obtain a complete holistic characterization of the alterations associated with DS, we performed *in vivo* MRS and *ex vivo* RNAseq at the timepoints specified in [Figure 1](#) to investigate changes in hippocampal region metabolite concentration and cerebellar gene expression respectively ([Fig.4](#)).

Hippocampal Region Metabolites

We performed *in vivo* MRS on a voxel placed in the hippocampal region at two timepoints, MRS₁ and MRS₂, as specified in [Figure 1](#).

We first analyzed global differences in the metabolite spectra by performing an LDA containing the relative integrals of 10 spectral regions between 0.0 ppm and 4.3 ppm ([Supplementary Figure S2](#) and [Supplementary Table S10](#)). The LDA at MRS₁ separated WT untreated and TS untreated mice across Axis1 ([Fig. 4A](#)), and the multivariate pairwise comparisons after one-way PERMANOVA using Mahalanobis distances confirmed significant genotype differences ([Supplementary Table S11](#); $P=0.0136$). After treatment discontinuation at MRS₂, the LDA showed milder genotype separation ([Fig. 4B](#)) and the multivariate pairwise comparisons did not detect any significant difference between WT untreated and TS untreated mice ([Supplementary Table S12](#); $P=0.5790$), confirming these findings.

When evaluating the concentrations of specific main metabolites like N-acetylaspartate (NAA), creatine + phosphocreatine (Cr+PCr), choline + phosphocholine + glycerophosphocoline (tCho), myo-inositol (myo-Inos) and taurine (Tau) ([Figure 4-figure supplement 1](#)), TS untreated mice tended to show lower levels of NAA and tCho at MRS₁ when compared with WT untreated mice ([Figure 4-figure supplement 1 A,C](#)), but the univariate pairwise comparison tests only revealed a significant increase in the concentration of Cr+PCr ([Figure 4-figure supplement 1B](#), top; $P=0.0235$). At MRS₂, the same pattern was observed but the significant differences between WT untreated and TS untreated mice disappeared ([Figure 4-figure supplement 1](#), bottom).

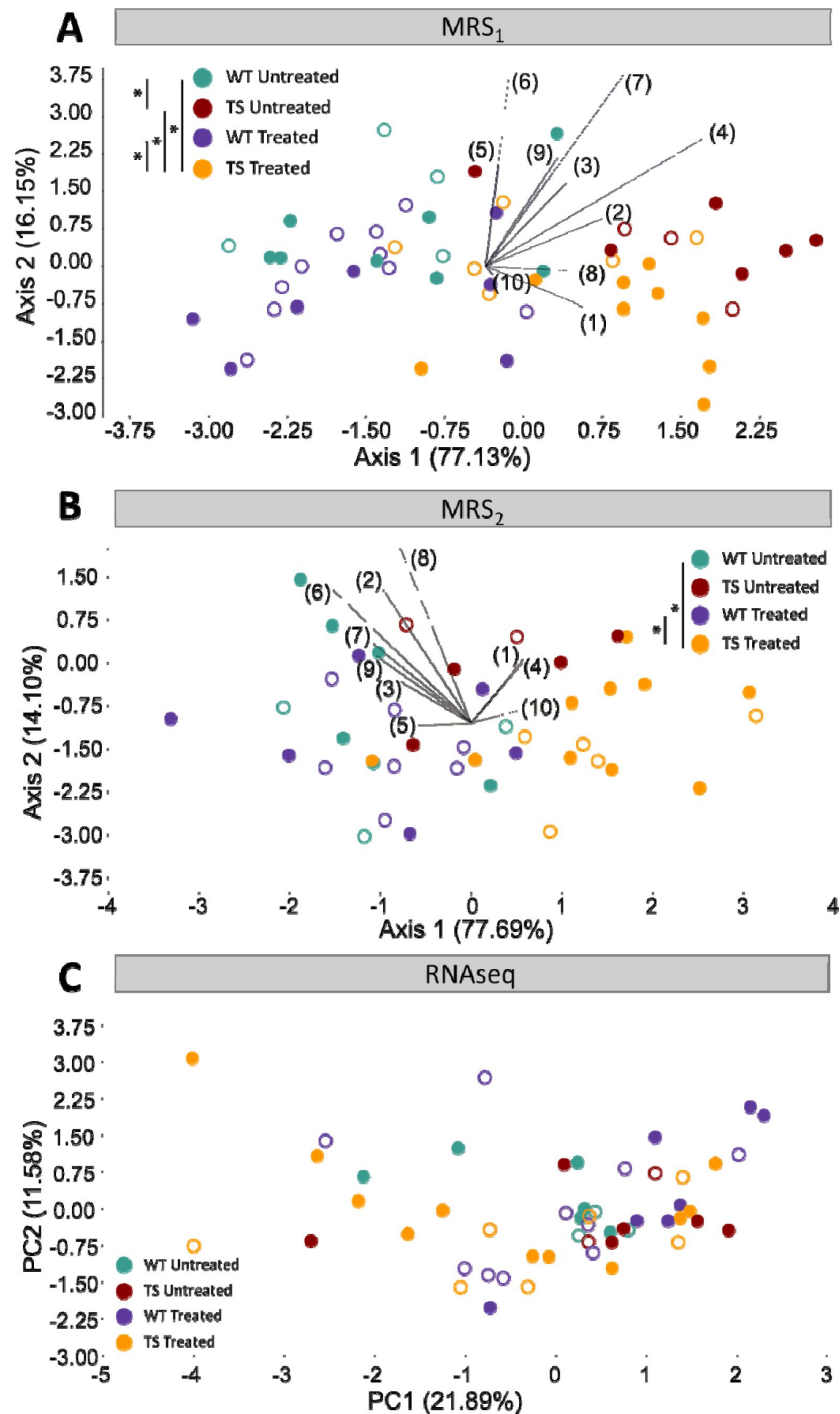


Figure 4.

Evaluation of trisomy, prenatal chronic GTE-EGCG treatment and treatment discontinuation effects on molecular and gene expression parameters

(A,B) Linear discriminant analysis (LDA) based on the relative integrals of 10 spectral regions obtained from MRS performed in the hippocampal region at MRS₁ (A) and MRS₂ (B). The contribution of each variable to separate groups of mice across Axis1 and Axis2 is represented in each LDA as lines pointing in the direction of each axis, with longer lines indicating higher contributions. **(C)** Principal component analysis (PCA) based on the normalized expression of the 125 triplicated genes obtained from RNAseq at 8M. Male mice are indicated with empty symbols. Sample sizes for each test are provided in [Supplementary Table S1](#).

Regarding treatment effects, the LDA at MRS₁ separated treated and untreated mice across Axis2 (**Fig. 4A**), suggesting treatment effects that did not rescue the trisomic phenotype. The multivariate pairwise comparisons after one-way PERMANOVA (**Supplementary Table S11**) showed significant differences between TS treated and both WT untreated ($P=0.0496$) and TS untreated mice ($P=0.0361$), confirming these findings. When evaluating the specific concentrations of NAA, Cr+PCr, tCho, myo-Inos and Tau, both WT treated and TS treated mice tended to show increased levels of Cr+PCr, which reached significance when comparing TS treated mice with WT untreated mice (**Figure 4-figure supplement 1B**, top; $P=0.0317$). Conversely, both WT and TS treated mice tended to show reduced levels of myo-inositol and tCho (**Figure 4-figure supplement 1D**, top), causing TS treated mice to have significantly reduced tCho when compared with WT untreated mice (**Figure 4-figure supplement 1C**, top; $P=0.0296$).

After treatment discontinuation at MRS₂, the LDA showed milder treatment separation across Axis2 (**Fig. 4B**) and the multivariate pairwise comparisons did not detect any significant difference between TS untreated and TS treated mice (**Supplementary Table S12**; $P=0.2111$). However, the differences between TS treated and WT untreated mice remained significant ($P=0.0347$), indicating that discontinuing the treatment for three months did not rescue the trisomic phenotype. When evaluating the specific concentrations of NAA, Cr+PCr, tCho, myo-Inos and Tau, the same pattern was observed and the significant differences between TS treated and WT untreated mice disappeared (**Figure 4-figure supplement 1**, bottom). However, significant differences emerged between WT untreated mice and WT treated mice for the concentration of tCho and myo-inositol (**Figure 4-figure supplement 1 C,D**, bottom; $P_{tCho}=0.0479$, $P_{myo-inositol}=0.0261$).

Gene Expression

Finally, since people with DS show impaired motor skills (Quinzi et al., 2022), we investigated the effects of genotype and chronic treatment after three months of treatment discontinuation in the cerebellar gene expression by performing RNAseq at endpoint (8M).

We first performed a PCA using the normalized expression of the 125 genes that are triplicated in the Ts65Dn mouse model and included in our dataset. The PCA showed major overlap between all groups, even though TS treated mice showed a larger range of variation (**Fig. 4C**). This result suggested that the differences in gene expression of the triplicated genes were minimal and that GTE-EGCG prenatal chronic treatment and three months of discontinuation did not induce major permanent effects when considering all triplicated genes. The multivariate pairwise comparison tests after one-way PERMANOVA using Euclidean distances did not detect any significant difference between groups, confirming these findings (**Supplementary Table S13**).

Then, we analyzed the differentially expressed genes (DEGs) between each pair of experimental groups across the entire transcriptome (**Supplementary Table S14**). When analyzing the entire transcriptome, we only detected 24 DEGs between WT untreated and TS untreated mice even when applying a loose p-adjusted value of 0.1, indicating small differences in cerebellar gene expression (**Supplementary Table S14**). Interestingly, we detected 12 DEGs between WT untreated and TS treated mice, and only 9 of these genes were also found to be differentially expressed between WT untreated and TS untreated mice, which could indicate mild treatment effects in TS mice (**Supplementary Table S14**). However, we did not detect any DEGs between TS untreated and TS treated mice, and only 5 DEGs were detected between WT untreated and WT treated mice, indicating that either the prenatal chronic GTE-EGCG treatment did not largely modulate gene expression or that the treatment had transient effects that were mostly not detected after three months of treatment discontinuation and were not rescuing the trisomic phenotype (**Supplementary Table S14**).

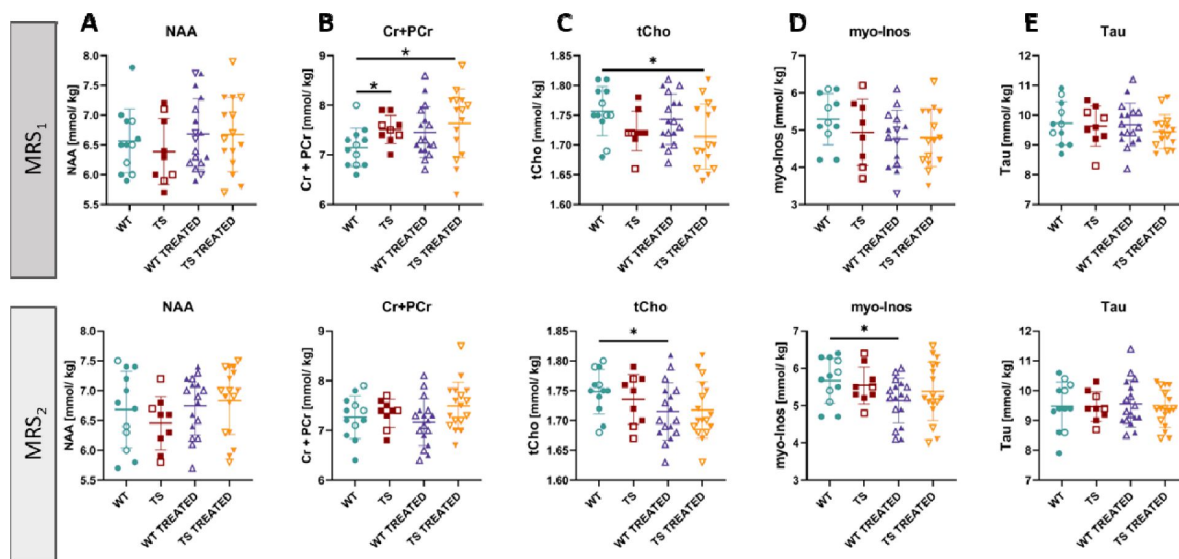


Figure 4-figure supplement 1.

Univariate analysis of hippocampal metabolites in wildtype and trisomic mice at adulthood and effects of prenatal chronic GTE-EGCG treatment and its discontinuation

The *in vivo* MRS scans performed in the hippocampal region at MRS₁ (top) and MRS₂ (bottom) were used to quantify the concentrations of (A) N-acetylaspartate (NAA), (B) creatine + phosphocreatine (Cr+PCr), (C) choline + phosphocholine + GPC (tCho), (D) myo-Inositol (myo-Inos) and (E) taurine (Tau). For each test, the results at MRS₁ are presented on top and results at MRS₂ are presented on the bottom. Data are presented as mean $\pm$ standard deviation. (*) $p < 0.05$; pairwise tests. Male mice are indicated with empty symbols. Sample sizes for each test are provided in [Supplementary Table S1](#).

Discussion

We investigated for the first time the simultaneous pleiotropic effects of trisomy and a chronic prenatal and postnatal treatment with GTE-EGCG on several organ systems and levels throughout development in the Ts65Dn mouse model for DS. Our longitudinal and holistic approach overcomes a main limitation of previous research, in which systems were analyzed independently at single time points using different experimental setups and delivered disparate and contradictory results that could not be generalized. Here, we followed the simultaneous development of structural, functional, molecular, and genetic systems on Ts65Dn mice and provided comparable results of genotype and prenatal chronic GTE-EGCG treatment effects before and after treatment discontinuation.

Detailed comparison of genotype and GTE-EGCG treatment effects across studies

We here summarized and compared our results with previous studies by domain to discuss whether the Ts65Dn mouse model was representative of DS in the human condition, and whether GTE-EGCG treatment produced any effect that could be translated into clinical practice.

Structural domain

Starting with body size, we observed that TS mice tended to weigh less than WT mice. However, as in a recent study (Tallino et al., 2022 [↗](#)), this difference did not achieve statistical significance, and contrasts with previous studies reporting significantly smaller weight of TS mice (Costa et al., 2010 [↗](#); Goodlett et al., 2020 [↗](#); Heinen et al., 2012 [↗](#); Jamal et al., 2022 [↗](#); Roper et al., 2006 [↗](#)). The prenatal chronic GTE-EGCG treatment further reduced the body weight of TS treated mice from PD1 to PD17, but not at adulthood, which is consistent with some studies (Goodlett et al., 2020 [↗](#); Xicota et al., 2020 [↗](#)), but not with others (Jamal et al., 2022 [↗](#); Noll et al., 2022 [↗](#)). Moreover, we observed that WT treated mice showed normal body weight during development, but increased weight after treatment discontinuation, suggesting a possible rebound effect after a prolonged time of treatment administration.

Regarding skeletal morphology, we detected craniofacial alterations that were consistent with previous findings and replicated the human condition (Llambrich, González-Colom, et al., 2022 [↗](#); Llambrich, González, et al., 2022 [↗](#); McElyea et al., 2016 [↗](#); Suri et al., 2010 [↗](#)). We also detected modulatory treatment effects on the craniofacial morphology in treated WT and TS mice at PD3, but these did not remain significant at adulthood, which was an unexpected result considering that our previous findings indicated that GTE-EGCG had modulatory effects from birth that were exacerbated later in development at PD29 (Llambrich, González-Colom, et al., 2022 [↗](#); Llambrich, González, et al., 2022 [↗](#)).

Our multivariate analyses did not detect global tibia microarchitecture alterations associated with genotype and the univariate tests only showed a significant reduction in the length and periosteal perimeter of the proximal tibia, not showing other skeletal alterations previously described (Abeysekera et al., 2016 [↗](#); J. D. Blazek et al., 2015 [↗](#); Blazek et al., 2011 [↗](#); Joshua D. Blazek et al., 2015 [↗](#); Carfi et al., 2017 [↗](#); LaCombe & Roper, 2020 [↗](#); Llambrich, González-Colom, et al., 2022 [↗](#); Thomas et al., 2020 [↗](#); Thomas et al., 2021 [↗](#)). The prenatal chronic treatment and three months of discontinuation did not modulate the trabecular bone and caused general but mild adverse effects in the cortical bone of WT and TS mice, not replicating the modulatory effects of green tea polyphenols previously observed on bone microarchitecture (J. D. Blazek et al., 2015 [↗](#); Goodlett et al., 2020 [↗](#); Huang et al., 2020 [↗](#); Jamal et al., 2022 [↗](#)). Moreover, the GTE-EGCG treatment modified the developmental trajectory of humerus BMD in both WT and TS treated mice with

disparate effects throughout development. These results did not match our previous findings (Llambrich, González-Colom, et al., 2022 [\[1\]](#)) and highlight the variability of treatment responses depending on developmental timing.

In the brain, we detected increased ventricular volumes, which is consistent with the trend observed in our previous study at PD29 (Llambrich, González, et al., 2022 [\[1\]](#)) and the ventriculomegaly typically observed in humans and mouse models for DS (Ishihara et al., 2010 [\[2\]](#); Movsas et al., 2016 [\[3\]](#); Pearson et al., 1998 [\[4\]](#); Raveau et al., 2017 [\[5\]](#)). However, we also detected increased hippocampal, cerebellar, and whole brain volumes in TS untreated mice that do not match clinical reports indicating reduced brain, hippocampal and cerebellar volumes in humans with DS (Aylward et al., 1997 [\[6\]](#); Hamner et al., 2018 [\[7\]](#); Patkee et al., 2020 [\[8\]](#); Pearson et al., 1998 [\[4\]](#); Pinter et al., 2001 [\[9\]](#); Rodrigues et al., 2019 [\[10\]](#); Smigielska-Kuzia et al., 2011 [\[11\]](#)), and other preclinical studies that did not find brain volume differences in Ts65Dn mice (Aldridge et al., 2007 [\[12\]](#); Duchon et al., 2021 [\[13\]](#); Holtzman et al., 1996 [\[14\]](#); Insausti et al., 1998 [\[15\]](#)). The prenatal chronic treatment did not have major effects, but generally increased the volume of all brain regions in TS treated mice, which is contrary to a previous study indicating that administering a green tea infusion corresponding to 0.6–1 mg EGCG per day from mating until adulthood significantly reduced the brain weight and volume in YACtg152F7 mice (Guedj et al., 2009 [\[16\]](#)).

Functional domain

Regarding cognitive function, our results indicated that TS mice showed cognitive delay during early stages of development. As suggested in clinical investigations (Locatelli et al., 2021 [\[17\]](#); J. Luis Olmos-Serrano et al., 2016 [\[18\]](#)), the cognitive alterations continued with development, and adult TS mice showed hyperactivity, impaired memory robustness, and more explorative behavior, in line with previous reports (Aziz et al., 2018 [\[19\]](#); Costa et al., 2010 [\[20\]](#); Coussons-Read & Crnic, 1996 [\[21\]](#); Dierssen et al., 2002 [\[22\]](#); Escorihuela et al., 1995 [\[23\]](#); Escorihuela et al., 1998 [\[24\]](#); Holtzman et al., 1996 [\[14\]](#); Llambrich, González, et al., 2022 [\[1\]](#); J. Luis Olmos-Serrano et al., 2016 [\[18\]](#)). Regarding treatment effects, the combined results from all tests suggested that even though the treatment could have had a positive effect reducing the anxiety of treated mice, it likely altered memory robustness and induced a more hyperactive, explorative, and risk-taking behavior. After treatment discontinuation, when the tests were repeated, all groups of mice presented less locomotor activity and explorative behavior. It is not unlikely that this reduction in activity was due to the stress and anxiety induced by the manipulation and testing during the first round of cognitive evaluation. However, TS untreated mice and both WT and TS treated mice still tended to show increased locomotor activity and explorative behavior compared with WT untreated mice, suggesting that the prenatal chronic treatment could have had permanent adverse effects after one month of discontinuation, or could have reduced the anxiety produced by the first round of testing while mice were treated. These results are in line with previous articles indicating no or negative effects of EGCG on cognition in humans and mice (Cieuta-Walti et al., 2022 [\[25\]](#); Goodlett et al., 2020 [\[26\]](#); Stringer et al., 2015 [\[27\]](#); Stringer et al., 2017 [\[28\]](#)), but are contrary to other articles showing rescuing effects (Catuara-Solarz et al., 2016 [\[29\]](#); de la Torre et al., 2016 [\[30\]](#); De la Torre et al., 2014 [\[31\]](#); Souchet et al., 2019 [\[32\]](#); Stagni et al., 2016 [\[33\]](#); Yin et al., 2017 [\[34\]](#)).

Molecular domain

Regarding hippocampal metabolite concentration, we observed differences between untreated WT and TS mice when evaluating the entire spectra using the LDA, which reflects the analysis of the whole MR spectra rather than selected metabolites, but we only detected a specific significant difference in the levels of Cr+PCr at MRS₁, suggesting that the genotype produced mild general molecular differences rather than large specific disruptions in any metabolite. Our results did not reveal any difference in NAA or myo-inositol, which is consistent with a human study showing no differences in the levels of NAA in the hippocampal region of adults with DS without dementia (Lamar et al., 2011 [\[35\]](#)), but do not replicate the results from another human study indicating increased myo-inositol levels (Beacher et al., 2005 [\[36\]](#)). Other studies in humans with DS reported

decreased NAA levels relative to creatine and myo-inositol in other brain regions, such as the frontal lobes and posterior cingulate cortex (Lin et al., 2016 [\[1\]](#); Smigielska-Kuzia & Sobaniec, 2007 [\[2\]](#)). Studies performed in the Ts65Dn model indicated reduced NAA levels relative to creatine in the hippocampal region (Huang et al., 2000 [\[3\]](#); Mème, 2014), which is contrary to our findings, but another study indicated no differences in NAA absolute concentrations (Santin et al., 2014 [\[4\]](#)). Furthermore, all these mouse studies indicated increased values of inositol or myo-inositol, which we did not detect in this study. These results reflect the large variability of phenotypes associated with DS depending on the developmental stage and region analyzed, as well as the disparity in experimental setups and data analyses between studies, as some studies reported the absolute metabolite concentrations while others reported their relative values normalized to creatine, which could explain the discrepancy among some of the results.

Regarding treatment effects, we detected significant differences between TS treated mice and both TS untreated and WT untreated mice when analyzing the entire spectra using the LDA, but no significant differences between TS untreated and TS treated mice in any specific metabolite. However, TS treated mice presented significantly increased Cr+PCr and reduced tCho levels as compared with WT untreated mice. After treatment discontinuation, these differences were no longer significant, but general differences remained in the LDA between TS treated and WT untreated mice. These results indicated that prenatal chronic GTE-EGCG treatment had mild modulating effects altering the entire hippocampal metabolite profile in TS mice.

Genetic domain

Regarding cerebellar gene expression, we did not detect large differences in the PCA that compared the expression of triplicated genes across groups of mice. Even though these genes presented an extra copy number, their expression was not significantly altered, which is consistent with previous articles (Ait Yahya-Graison et al., 2007 [\[5\]](#); Lyle et al., 2004 [\[6\]](#); J. L. Olmos-Serrano et al., 2016 [\[7\]](#); Saran et al., 2003 [\[8\]](#)).

However, we only detected 24 DEGs between untreated WT and TS mice when analyzing the entire genome, which is a lower number in comparison to other studies (Aziz et al., 2018 [\[9\]](#); Chrast et al., 2000 [\[10\]](#); De Toma et al., 2021 [\[11\]](#); J. L. Olmos-Serrano et al., 2016 [\[7\]](#); Saran et al., 2003 [\[8\]](#); Vilardell et al., 2011 [\[12\]](#)). Contrary to previous findings indicating a transcriptome-wide deregulation (De Toma et al., 2021 [\[11\]](#); Letourneau et al., 2014 [\[13\]](#); Vilardell et al., 2011 [\[12\]](#)), in our study all the 24 DEGs overexpressed in TS untreated mice were mapped to the mouse chromosomes (Mmu) 16 or 17, except for *Ccdc23*, which mapped to Mmu4. Within these 24 DEGs we detected genes typically related with DS and cognitive disability, such as *App*, *Dyrk1A*, *Dscr3*, *Synj1*, *Ttc3*, *Hmgn1*, *Brwd1* and *Usp16*; but we did not detect other genes such as *Sod1*, *Sim2*, *Dscam*, *Rcan1*, *Olig1*, *Olig2* or *Kcnj6* (Aziz et al., 2018 [\[9\]](#); Chrast et al., 2000 [\[10\]](#); De Toma et al., 2021 [\[11\]](#); Ruparel et al., 2012 [\[14\]](#); Vilardell et al., 2011 [\[12\]](#)). These discrepancies with previous results could be explained by methodological reasons, as previous studies investigated other tissues, pooled data from different mouse models, contained only male mice, investigated different developmental timepoints, or used different RNA quantification techniques.

Even though the prenatal chronic GTE-EGCG treatment used in this study was administered continuously from embryonic day 9 until adulthood, the treatment did not cause any large permanent effects in the gene expression of adult TS mice after three months of discontinuation, as we did not detect any DEG between TS untreated and TS treated mice. These results suggest that even though the treatment could have potentially modulated gene expression during a critical window for brain development in TS mice, these effects may have been reverted after treatment withdrawal. In WT mice, five genes were found to be differentially expressed between WT untreated and WT treated mice, of which *Hspa1b* and *Hspa1a* mapped to Mmu17, *Fosl2* mapped to Mmu5, *Actn1* mapped to Mmu12, and *Tm6sf2* mapped to Mmu8; which could indicate permanent

genome-wide treatment effects in a few genes in WT mice. Interestingly, from the list of DEGs, only *Hspa1b* was found to interact with *Dyrk1A* (Rouillard et al., 2016 [DOI](#)), suggesting that GTE-EGCG may have other mechanisms of action than DYRK1A kinase activity inhibition.

The pleiotropic nature of genotype and treatment effects

The combination of multi- and univariate tests performed in our study allowed us to explore in a more comprehensive way the integrated effects of genotype and treatment. Overall, we detected more significant differences when performing multivariate analysis combining the results of different tests together than when we evaluated each test individually, which highlights the pleiotropic nature of DS and GTE-EGCG, as both genotype and treatment caused mild effects in multiple readouts rather than large specific effects in single variables.

For example, we observed that even though TS untreated mice presented mild gene expression alterations in the cerebellum, these mice still showed hyperactivity and increased cerebellar volume. Similarly, TS treated mice presented a larger cerebellum and hyperactivity when compared to WT untreated mice after treatment discontinuation, but only 12 genes were found to be differentially expressed between WT untreated and TS treated mice at that stage. These results can be interpreted as that the altered expression of a few genes was sufficient to alter the brain and cognitive phenotypes, or that the alteration of gene expression during development was no longer detected at adulthood but was sufficient to permanently alter brain volumetry and cognition, which would be in line with previous reports indicating that the altered phenotype in DS was due to small contributions of multiple genes rather than strong effects of a few selected genes (Antonarakis et al., 2004 [DOI](#); Chang et al., 2020 [DOI](#); Chrast et al., 2000 [DOI](#); De Toma et al., 2021 [DOI](#)). Interestingly, trisomy altered the expression of genes related with hypotonia and movement impairment such as *Son*, *Synj1*, *Atp5o*, *Jam2* and *Paxbp1*; but TS mice still showed increased locomotor activity.

Furthermore, we observed that even though there were no significant differences in the concentration of NAA in the hippocampal region of TS untreated mice, these mice showed altered hippocampal region metabolite spectra, increased hippocampal region volume, and altered memory robustness. Similarly, the treatment did not alter the concentration of NAA in the hippocampal region of TS treated mice, but induced general differences in hippocampal region metabolite spectra, mildly increased hippocampal region volume, and altered cognition. These results suggested that the cognitive differences induced by both the genotype and treatment were not related with the concentration of NAA in the hippocampal region but rather with general differences in hippocampal region volume and metabolite spectra.

The advantages of a longitudinal holistic approach

Single holistic and longitudinal experiments evaluating multiple systems simultaneously overcome the limitations of individual analyses and the lack of consistency across studies. The differences between study results highlighted in this study could be due to a large variety of factors, including experimental differences in the mouse model, developmental stage analyzed, sex distribution of the sample, experimental setup, technical differences in data acquisition and analysis, as well as differences in treatment dose, timing, and route of administration. As a result, evidence from different studies is challenging to interpret, and can lead to misinterpretations about the course of the disorder and the effects of pharmacological treatments. With our approach, the differences between experimental setups could be accounted for, analyzing the simultaneous development of different systems in the same mice in a controlled experimental setup, and investigating the related effects of trisomy and treatment in multiple related systems.

Our results support that, overall, the Ts65Dn model reflects the multisystemic nature of DS and recapitulates many characteristics of the trisomic phenotype. As compared to WT mice, Ts65Dn mice presented a trend for reduced body weight over development together with a brachycephalic

skull with facial flatness and a trend for reduced BMD in the humerus. These skeletal alterations co-occurred with cognitive delay during early stages of development, hyperactivity, impaired long-term memory, and increased explorative and risk-taking behavior at adulthood. At the molecular and genetic level, Ts65Dn mice presented alterations in the hippocampal metabolite spectra and differential gene expression in the cerebellum. However, our results also revealed phenotypes that did not match the human condition, as Ts65Dn mice did not show altered tibia microarchitecture and showed increased hippocampal, cerebellar, ventricular, and whole brain volumes. Overall, our longitudinal analyses confirm the validity of the model for the structural, functional, molecular and genetic phenotypes associated with DS, but also support the hypothesis of genotypic and phenotypic drift within the Ts65Dn mouse model (Shaw et al., 2020 [DOI](#)), as we generally observed a decrease in the magnitude of alterations induced by the genotype compared to previous articles including our earlier work.

Regarding the treatment effects, our results confirmed that GTE-EGCG modulated most of these systems simultaneously along development. However, our holistic approach revealed that, in general, the treatment did not rescue the trisomic phenotype and even exacerbated some phenotypes over time, such as body weight, tibia microarchitecture, neurodevelopment, adult cognition, and hippocampal metabolite concentration. Although discontinuing the GTE-EGCG administration reduced the treatment effects, it did not rescue the trisomic phenotype, as TS treated mice remained different from WT untreated mice in most systems. Discontinuing the GTE-EGCG treatment for three months only rescued the body weight and brain volume of a few TS mice but increased the weight of WT mice and reduced the BMD of the humerus in both WT and TS mice. Summing up, our preclinical results warn against a chronic GTE-EGCG treatment initiated prenatally and maintained until adulthood with a dosage of 30 mg/kg/day.

Future directions

Performing specific experiments to evaluate the effects of a potential therapeutic compound on one structure at one timepoint are important first steps to screen for novel therapeutic agents. However, the pleiotropic effects on all involved organ systems over a relevant time course should not be ignored when evaluating its general safety and efficacy, especially in complex disorders like DS.

The experimental pipeline used in this article, from the imaging techniques to the data analysis strategy, are applicable to any rodent model and potential therapeutic compound, allowing the integrated investigation of other therapeutic compounds and DS models that more faithfully replicate the genetic condition of DS, such as the TcHSA21rat and TcMAC21 mouse model (Kazuki et al., 2020 [DOI](#); Kazuki et al., 2022 [DOI](#)). Furthermore, the simultaneous direct and indirect effects of potential therapeutic agents could be investigated in an integrated manner for other disorders and syndromes with multi-systemic alterations such as Apert, Pfeiffer and Crouzon craniosynostosis syndromes (Caputo et al., 2016 [DOI](#); Fernandes et al., 2016 [DOI](#); Monteagudo, 2020 [DOI](#); Nopoulos et al., 2007 [DOI](#); Pirozzi et al., 2018 [DOI](#); Roberts et al., 2012 [DOI](#); Treit et al., 2016 [DOI](#); Vogels & Fryns, 2006 [DOI](#); Wilhoit et al., 2017 [DOI](#); Wozniak et al., 2019 [DOI](#)), considering the associated effects in different systems.

With this holistic approach, the preclinical biomedical research field will be able to solve new challenges and answer new questions, understanding the complexities of systemic diseases in a generalized manner and providing a global context to the contributions of specific genes, proteins, molecules, and compounds.

Materials and methods

Animals, housing, treatment, and experimental design

Ts65Dn (B6EiC3Sn-a/A-Ts (1716)65Dn) females and B6EiC3Sn.BLiAF1/J males (refs. 005252 and 003647, the Jackson Laboratory Bar Harbor, ME, USA) were obtained from the Jackson Laboratory and crossed within six months to obtain F1 trisomic Ts65Dn (TS) mice and euploid wildtype littermates (WT) that were used throughout the experiment. Mice were housed at the animal facility of KU Leuven in individually ventilated cages (IVC cages, 40 cm long x 25 cm wide x 20 cm high) under a 12h light/dark schedule in controlled environmental conditions of humidity (50% - 70%) and temperature ($22 \pm 2^\circ\text{C}$) with food and water supplied *ad libitum*. Date of conception (E0) was determined as the day in which a vaginal plug was present. After birth, all pups were labeled with a non-toxic tattoo ink (Ketchum Animal Tattoo Ink, Green Paste) for identification throughout the longitudinal experiments, as the same mice were used throughout the entire experiment. All procedures complied with all local, national, and European regulations and ARRIVE guidelines and were authorized by the Animal Ethics Committee of KU Leuven (ECD approval number P120/2019).

Mice were genotyped at PD1 by PCR from tail snips adapting the protocol in (Shaw et al., 2020 [↗](#)). Trisomic primers, Chr17fwd-5'-GTGGCAAGAGACTCAAATTCAC-3' and Chr16rev-5'-TGGCTTATTATCAGGGCATT-3'; and positive control primers, IMR8545-5'-AAAGTCGCTCTGAGTTGTAT-3' and IMG8546-5'-GAGCGGGAGAAATGGATATG-3' were used. The following PCR cycle conditions were used: step 1: 94°C for 2 min; step 2: 94°C for 30 s; step 3: 55°C for 45 s; step 4: 72°C for 1 min (steps 2-4 repeated for 40 cycles); step 5: 72°C for 7 min, and a 4°C hold. PCR products were separated on a 1% agarose gel.

We bred a total of 13 litters. Six litters were left untreated and seven litters were treated via the drinking water with GTE-EGCG (Mega Green Tea Extract, Life Extension, USA) at a concentration of 0.09 mg EGCG/mL, as calculated based on the label concentration (45% EGCG per capsule). As EGCG crosses the placental barrier and reaches the embryo (K.O. Chu et al., 2006 [↗](#)), GTE-EGCG treatment started prenatally at embryonic day 9 (E9) via the drinking water of the pregnant dams. After weaning at postnatal day (PD) 21, GTE-EGCG dissolved in water at the same concentration was provided to the young mice *ad libitum* until 5 months (5M), when the treatment was discontinued (Fig. 1 [↗](#)). The treatment was prepared freshly every day and water intake was monitored in each cage. The calculated dosage of EGCG received by an adult mouse would be approximately 30 mg/kg/day considering that, on average, early adult mice weigh 20 g and drink 6 mL of water per day according to our measurements. In developing embryos and pups before weaning, the received dosage was lower since previous studies indicate that maternal plasma concentrations of catechins are about 10 times higher than in placenta and 50–100 times higher than in the fetal brain (K. O. Chu et al., 2006 [↗](#)) and EGCG in milk and plasma of PD1 to PD7 pups was detected at low concentrations (Souchet et al., 2019 [↗](#)).

Mice were allocated to groups according to their genotype and pharmacological intervention: WT and TS mice untreated or treated with GTE-EGCG (Fig. 1 [↗](#)). Investigators were blinded to genotype during animal experimentation, and to genotype and treatment during data analysis. The same mice were longitudinally used throughout the experiment. Sample sizes varied across groups and developmental stages due to uncontrollable technical issues inherent to longitudinal studies, such as scanning failure or mouse death during the experiment. The litter information containing litter number, treatment administration and sex for each mouse is described in **Supplementary Table S15** [↗](#). Detailed information regarding sample sizes for each experiment and analysis is provided in **Supplementary Table S1** [↗](#).

Structural assessment

Body weight

Mice body weight was recorded daily from PD1 to PD17 and before each μ CT scanning ([Fig. 1](#)).

Skeletal development

In vivo μ CT

We performed high resolution longitudinal *in vivo* μ CT at four timepoints from after birth until adulthood to monitor skeletal development ([Fig. 1](#)). Mice were anesthetized by inhalation of 1.5–2% of isoflurane (Piramal Healthcare, Morpeth, Northumberland, United Kingdom) in pure oxygen and scanned *in vivo* with the SkyScan 1278 (Bruker Micro-CT, Kontich, Belgium) for three minutes using the optimized parameters specified in [Supplementary Table S16](#). *In vivo* μ CT data was reconstructed using a beam hardening correction of 10% (NRecon software, Bruker Micro-CT, Kontich, Belgium).

• Skull shape analysis

Skull 3D models were automatically generated from reconstructed *in vivo* μ CT scans by creating an isosurface based on specific threshold for bone using Amira 2019.2 (Thermo Fisher Scientific, Waltham, MA, USA). We compared craniofacial morphology in WT and TS mice with and without GTE-EGCG treatment using Geometric Morphometric quantitative shape analyses ([Dryden & Mardia, 1998](#); [Hallgrímsson et al., 2015](#); [James Rohlf & Marcus, 1993](#); [Klingenberg, 2010](#)). The analysis was based on the 3D coordinates of anatomical homologous landmarks recorded over the skull and face at each developmental stage as described before ([Llambrich, González-Colom, et al., 2022](#)). The landmark configuration for each stage is defined in [Supplementary Figure S1](#) and [Supplementary Table S17](#). Landmarks were acquired using Amira 2019.2.

• Humerus bone mineral density (BMD)

To calculate humerus BMD from the μ CT data, we first computed the humerus mean grey value of each mouse by delimiting a volume of interest of ten slices that was placed right below the deltoid protuberance of the humerus using the CTAn software (Bruker Micro-CT, Kontich, Belgium). Then, we scanned two phantoms with different known densities of hydroxyapatite (100 mg/cm³ and 500 mg/cm³) using the same settings as in the *in vivo* scans ([Supplementary Table S16](#)). A calibration line was obtained between the known hydroxyapatite densities and their corresponding grey values. The resulting equation was applied to calculate the BMD of the humerus of each mouse from their mean grey value.

Tibia length

After sacrifice, the length of the right tibia was measured in all mice using a digital caliper.

Ex vivo μ CT for tibia

The proximal region of the tibia was scanned *ex vivo* using the Skyscan 1272 high-resolution μ CT scanner (Bruker Micro-CT, Kontich, Belgium) with the optimized parameters specified in [Supplementary Table S16](#). After scanning the bones, the raw 2D images were reconstructed using NRecon (version 1.7.3.1, Bruker Micro-CT, Kontich, Belgium) and rotated to a standard position using DataViewer (version 1.5.6.2, Bruker Micro-CT, Kontich, Belgium). The reconstructed images were then analyzed using the CTAn software (version 1.17.8.0, Bruker Micro-CT, Kontich, Belgium) as follows.

• Trabecular analysis

For trabecular bone, a section of 300 slices (1.5 mm) was selected starting 100 slices (0.5 mm) underneath the point in the proximal tibia where the articular condyles met. Then, a region of interest (ROI) was manually defined including the trabecular bone inside the thin cortical outer layer. The descriptions, abbreviations and parameter units are provided in **Supplementary Table S18** [↗](#).

• Cortical analysis

For cortical bone, a section of 100 slices (0.5 mm) was selected in CTAn starting 600 slices (3 mm) underneath the reference point in the proximal tibia. The tissue inside the medullary canal was excluded from the ROI. The descriptions, abbreviations and parameter units are provided in **Supplementary Table S19** [↗](#).

Brain volume

In vivo MRI scanning

Mice were also MR scanned *in vivo* under the same anesthesia (1.5-2% isoflurane, Piramal Healthcare, Morpeth, Northumberland, United Kingdom) at MRI₁ and MRI₂ (**Fig. 1** [↗](#)) with a 9.4 T Bruker Biospec 94/20 small animal μ MR scanner (Bruker Biospin, Ettlingen, Germany; 20 cm horizontal bore) equipped with actively shielded gradients (maximum gradient strength 600 mT m⁻¹). Axial, coronal, and sagittal images were acquired using a 2D T2 weighted Rapid Acquisition with Relaxation Enhancement (RARE) sequence (repetition time (TR)/ echo time (TE): 3781 / 33 ms; RARE factor: 8; averages: 6; field of view (FOV): 20 × 20 mm; matrix 128 × 128; slice number: 35; slice thickness: 0.4 mm; slice gap: 0.1 mm; acquisition time 6 min). A quadrature radiofrequency resonator (inner diameter 7.2 cm, Bruker Biospin) was used for transmission of radiofrequency pulses in combination with and actively decoupled mouse brain surface coil for reception (Bruker Biospin).

Segmentation of brain regions of interest

After MR image acquisition, brain masks were manually delineated on the axial plane for each mouse using 3D Slicer v5.0.2. (<http://www.slicer.org> [↗](#)) (Fedorov et al., 2012 [↗](#)). Then, the masks were fed to the Atlas-Based Imaging Data Analysis (AIDA) pipeline described previously (Pallast et al., 2019 [↗](#)). In brief, the pipeline consisted of a series of preprocessing steps including skull stripping and bias field correction of the MR images before registration with the Allen Mouse Brain Reference Atlas (Sunkin et al., 2013 [↗](#)) through a series of affine and non-linear transformations. The volume of the whole brain was extracted from the manually delineated masks and the volumes of the hippocampal region, cerebellum, and ventricles were extracted from the AIDA segmentations. All brain volumes were normalized to body weight to account for the differences in overall body size between WT and TS mice.

Functional assessment

Neurobehavioral development

Neurobehavioral developmental tests were carried out daily from PD1 to PD18, as previously described (Dierksen et al., 2002 [↗](#); Llambrich, González, et al., 2022 [↗](#)). Mothers were separated from their pups before testing. Pups were then taken out one at a time from their home cage for testing, and mothers were returned into the cage after all pups were evaluated. For each neurobehavioral test, we evaluated the acquisition rate and the average day of successful test completion.

In neurodevelopment tests with a presence/absence binary outcome, such as eye opening, pinna detachment, walking, cliff drop aversion, Preyer reflex, blast response, visual placing, reaching response, vibrissae placing and tactile response; the acquisition rate was scored as the percentage of mice that successfully acquired the landmark or response behavior on each day. The day of successful test completion was considered as the day when there was a positive response.

In those neurodevelopment tests measured with categorical non-binary scores, such as incisor eruption, surface righting response, negative geotaxis, vertical climbing, and grasping; the acquisition rate was scored as the daily average score of each group of mice. The day of successful test completion was considered as the day when the highest score was achieved.

Maternal care

For maternal care monitoring, home IVC cages were transferred to a separate light cycle-controlled room with food and water supplied *ad libitum*. Cages were videotaped from the top during 24h using the Foscam C1 camera with night vision and a transparent Plexiglas cover with holes for ventilation. The recordings were manually inspected every 6 minutes for 10 seconds, and maternal behavior was categorized as nursing, pup grooming, digging in nest, eating, drinking, moving, or digging off nest to evaluate the frequency of each maternal behavior.

Adult cognition

Open Field (OF), Elevated Plus Maze (EPM), Sociability/Preference for Social Novelty (SPSN), Novel Object Recognition (NOR) and Passive Avoidance (PA) tests were performed in this order before and after treatment discontinuation ([Fig. 1](#) [↗](#)).

The OF test was performed in a brightly illuminated Plexiglas arena (50 x 50cm) with transparent walls. Dark habituated (30 min) mice were placed in the left bottom corner facing the walls and were left free to explore the arena for 10 minutes. Movements were recorded using a camera and the tracking software ANY-maze™ Video Tracking System software (Stoelting Co., IL, USA). Exploration towards the center of the field was considered a readout for reduced anxiety.

The EPM test took place on a plus shaped maze with 2 open and 2 closed arms (5 cmx30 cm) that was elevated 35 cm from the tabletop. Each mouse was placed in the left closed arm, with the snout pointing away from the crossing. After a 1-minute habituation time, the trial was initiated manually, letting the mouse spend 10 minutes in the arena. Four infrared (IR) beams connected to an activity logger recorded the arm entries of the mouse and one beam recorded the percentage of time that the mouse spent in the open arms per minute.

The SPSN set-up consisted of a Plexiglas box (60 x 15cm) with three compartments separated with perforated Plexiglas walls. The SPSN test involved three trials. At the first stage of habituation, the test mouse was left to explore the middle chamber for 300 seconds, while the left and right chambers were empty and visible from the middle chamber. Next, in the Social Preference stage with Subject 1 (S1), the test mouse was placed in the middle chamber for 300 seconds while one stranger mouse (STR1) was placed in either the left or right chamber, and the other chamber was left empty. Social approach was recorded as time spent close to STR1, and a preference ratio was calculated ($\text{Pref} = 100 * \text{Time close to STR1} / (\text{Time close to STR1} + \text{Time close to Empty})$). Finally, in the Social Novelty stage with Subject 2 (S2) (300 s), a second stranger mouse (STR2) was placed in the previously empty chamber. Social recognition memory was scored as preference towards STR2 (calculated as ratio: $\text{pref} = 100 * (\text{time close to STR2}) / (\text{Time close to STR1} + \text{Time close to STR2})$). The two stranger mice were C57BL/6 wildtype mice of the same sex as the test mouse and had served before as stranger mice in previous SPSN experiments. Explorative social behavior towards stranger mice was measured using ANY-maze™ Video Tracking System software (Stoelting Co., IL, USA).

NOR testing started with a habituation phase where the animals were placed during 15 minutes in a dimly lit open field arena (wooden box 40X40cm painted white). Twenty-four hours later, the animals were reintroduced to the same arena for 10 minutes with two identical falcon tubes filled with colored liquid that were placed in opposite corners equally distant from the mouse. Exploration time involving sniffing in close proximity (<2cm) was recorded by an overhead camera and tracking software. Total exploration time was set at minimally 15 seconds to ensure that object characteristics were encoded. Sixty minutes later, the animals were placed in the arena for 10 minutes, with one object replaced by a novel object, and exploration time was recorded. Preference for novel object was calculated as ratio ($\text{Pref} = 100 \times \text{Time novel object} / (\text{Time familiar object} + \text{Time novel object})$). Objects were randomly assigned as familiar or novel for each mouse, and the left or right position of the novel object was counterbalanced between trials. Exploration time was measured by a camera and ANY-maze™ Video Tracking System software (Stoelting Co., IL, USA).

The PA experimental set-up consisted of a transparent box illuminated with an LED lamp leading to a dark box with an electrifiable grid connected to a shocker (LE 100-26, Panlab Bioseb, Spain) and a lid. Dark habituated mice were placed in the light box and when they entered the dark box (CS), the latency to enter the dark box was recorded, and a mild foot shock was delivered (US 0,5mA, 2s, scrambled). The next day, the trial was repeated (without foot shock presentation) and latency to enter the dark compartment was recorded (maximum 300s). Animals with good memory retention would display a higher latency to enter on day 2. This test was repeated in the same animals after treatment cessation. We noticed that upon re-exposure to the same setup, some animals still remembered the CS-US presentation, and refused to enter the dark box. Therefore, this second testing session reflected the stability of long-term memory.

Molecular and genetic assessment

In vivo MRS

MR spectra were acquired as previously reported using a Bruker Biospec 94/20 MR scanner (Vanherp et al., 2021 [DOI](#); Weerasekera et al., 2018 [DOI](#)). After MRI scanning, MR spectra were acquired from a $2.5 \times 1.25 \times 1.5$ mm voxel placed in the hippocampal region of the brain, using a PRESS sequence with TR/TE 2000/20 ms, 320 averages, and localized shimming with no margin. Water suppression was optimized using VAPOR (Griffey & P. Flamig, 1990 [DOI](#)). An unsuppressed water MR spectrum was acquired before each water-suppressed 1H-MRS spectrum for quantification/referencing. Shimming was performed using FASTMAP, resulting in a final water line width at half height < 20 Hz.

For the multivariate analysis of the spectra, the signals were truncated to retain only the region of interest between 0.0 and 4.3ppm. The msbackadj function in Matlab (Inc., 2022) was applied for baseline and offset correction. The baseline was estimated within multiple shifted windows of width 150 separation units and extracted from the original signal. The baseline corrected signals were further analyzed by segmenting it into 12 spectral regions, which were integrated and normalized to the total integral using peak integration methodology for metabolite quantification (**Supplementary Figure S2** [DOI](#)). The two spectral regions that represent contaminations from macromolecules were excluded (number 11 and 12 in **Supplementary Figure S2** [DOI](#)). Main metabolites present in the respective spectral regions are listed in **Supplementary Table S10** [DOI](#).

For quantification of absolute metabolite concentrations, a similar approach was taken as previously reported (Weerasekera et al., 2018 [DOI](#)). In brief, spectra were processed using jMRUI v6.0 (Stefan et al., 2009 [DOI](#)). Spectra were phase corrected and an HLSVD (Hankel Lanczos Singular Values Decomposition) filter was applied to remove the residual water signal (van den Boogaart, 1994 [DOI](#)). Metabolites were quantified with the QUEST algorithm (Ratney et al., 2004 [DOI](#)) in jMRUI

using a simulated (NMRScopeB) basis set (Starčuk Jr et al., 2009). Results were reported in reference to the non-suppressed water signal. A metabolite data base was used as in (Weerasekera et al., 2018).

Gene expression

Gene expression analysis was performed on a cerebellar tissue homogenate at endpoint (**Fig. 1**). Each cerebellum was dissected and processed independently. Homogenates were obtained with a gentleMACS dissociator (Miltenyi Biotech). Total RNA was then extracted with QIAzol according to the manufacturer's instructions. RNA purity and concentration were assessed by NanoDrop ND-1000 Spectrophotometer and RNA integrity was evaluated by Fragment Analyzer analysis (RIN $\geq$ 8). Illumina TruSeq stranded mRNA kit was used for library preparation, samples were pooled and sequenced on a HiSeq4000, single end, 50bp reads. A minimum of one million reads were obtained per sample. Quality control of raw reads was performed with FastQC v0.11.7 (Andrews, 2010). Adapters were filtered with ea-utils fastq-mcf v1.05 (Aronesty, 2011). Splice-aware alignment was performed with HISat2 (Kim et al., 2019), against the mouse reference genome mm10 using default parameters. Reads mapping to multiple loci in the reference genome were discarded. Resulting Binary Alignment Map (BAM) files were handled with Samtools v1.5 (Li et al., 2009). Quantification of reads per gene was performed with HT-seq Count v0.10.0, Python v2.7.14 (Anders et al., 2014). Count-based differential expression analysis was performed with R-based (The R Foundation for Statistical Computing, Vienna, Austria) Bioconductor package DESeq2 (Love et al., 2014), normalizing absolute counts. Pairwise comparison of the entire genome for all groups was done with default settings and the reported P-values were adjusted for multiple testing with the Benjamini-Hochberg procedure controlling for false discovery rate (FDR) (Supplementary Table S14). Multivariate evaluation of the subset of 125 triplicated genes present in the Ts65Dn mouse model was performed as described below. All mice that survived until endpoint were included in both analyses (**Supplementary Table S1**).

Statistics

Univariate evaluation

The developmental trajectories of the body weight, BMD, and acquisition rate of neurodevelopment tests with categorical non-binary scores were longitudinally analyzed by fitting a mixed-effects model as implemented in GraphPad Prism 8.0, using the Geisser-Greenhouse correction and Restricted Maximum Likelihood (REML) fit as described before (Llambrich, González, et al., 2022). The acquisition rate of neurodevelopment tests with a presence/absence binary outcome was longitudinally analyzed using a log-rank test (Mantel-Haenszel approach), considering the day of appearance of the landmark or response as an event using GraphPad Prism 8.0.

We made five pairwise comparisons for all univariate non-longitudinal data: the body weight at adulthood, the BMD at each developmental timepoint, the variables evaluating tibia microarchitecture, the brain volume of the different regions, the average day of acquisition of the neurodevelopmental tests, the variables evaluating adult cognition, and the concentration of the hippocampal metabolites. We compared WT vs. TS untreated mice to evaluate the genotype effect (1), WT untreated vs. WT treated mice to evaluate the treatment effect in the WT background (2), TS untreated vs. TS treated mice to evaluate the treatment effect in the trisomic background (3), WT untreated vs. TS treated mice to determine whether the treatment had a rescuing effect in trisomic mice (4), and WT treated vs. TS treated mice to evaluate whether the treatment showed different effects in the WT and trisomic background (5). We determined statistical significance for each comparison using univariate statistical tests as previously described (Llambrich, González-Colom, et al., 2022). For the BMD, P-values at each developmental stage were adjusted for multiple comparisons using the Benjamini-Hochberg (Q = 5%) test.

The results for normality, homoscedasticity and statistical tests performed for each variable can be found in **Supplementary Table S20** [\[link\]](#). Mice identified as outliers by the ROUT test ([Motulsky & Brown, 2006](#) [\[link\]](#)) with a Q (maximum desired False Discovery Rate) of 1% were excluded from the analysis. All univariate statistical analysis were performed using GraphPad Prism (v8.02, GraphPad Software, San Diego, California USA).

Multivariate evaluation

We performed multivariate statistics in all tests with multiple variables: craniofacial shape, tibia microarchitecture, brain volumes, neurodevelopmental tests, adult cognitive tests, brain metabolite concentration and gene expression.

We performed a principal component analysis (PCA) for the craniofacial shape and gene expression analysis. The PCA for the craniofacial shape analysis was based on the 3D coordinates of the set of 27 landmarks recorded to capture craniofacial shape. To extract shape information from the 3D landmark configurations, we performed a Generalized Procrustes Analysis (GPA) followed by a PCA at each stage as described before ([Llambrich, González, et al., 2022](#) [\[link\]](#)) using MorphoJ v1.06d ([Klingenberg, 2011](#) [\[link\]](#)). For the gene expression data, the PCA was based on the rlog normalized expression data of the 125 triplicated genes in the Ts65Dn mouse model according to the MGI-Mouse Genome Informatics Database that were present in our dataset and was performed using PAST v4.1 ([Hammer et al., 2001](#) [\[link\]](#)).

As we aimed to maximize differences between groups, we performed a linear discriminant analysis (LDA) for the tibia microarchitecture analysis, brain volumetric analysis, neurodevelopmental tests, adult cognitive tests, and hippocampal metabolite concentration analysis using the results obtained from each test. The variables included in each LDA are shown in Supplementary Table S21. We performed an LDA for each domain considering genotype+treatment as the grouping variable using PAST v4.1. As we compared four groups of mice, the LDA created three new axes that were independent among them and explained 100% of variation across groups. In the LDA, the separation among groups was determined by Mahalanobis distances, which account for correlations between standardized variables, and allow to combine measurements with different units in the same analysis.

If slight or no differences were associated with DS or treatment, the mice groups overlapped in the PCA or LDA scatterplot, showing similar phenotypes. If there were differences, the different groups of mice separated from each other.

To statistically quantify differences between WT, TS, WT treated and TS treated mice and answer the five scientific questions formulated above, we performed pairwise permutation tests with 10.000 rounds following the PCAs and LDAs. For the craniofacial shape analysis, we obtained the P-values from the permutation tests based on the Procrustes distances between the average shape of pairs of groups at each developmental stage using MorphoJ v1.06d ([Klingenberg, 2011](#) [\[link\]](#)). For the gene expression analysis, we obtained the P-values from the pairwise comparisons after a one-way PERMANOVA based on Euclidean distances using PAST v4.1 ([Hammer et al., 2001](#) [\[link\]](#)). For the tibia microarchitecture tests, brain volumetric tests, neurodevelopmental tests, adult cognitive tests, and hippocampal metabolite concentration tests we obtained the P-values from the pairwise comparisons after a one-way PERMANOVA based on Mahalanobis distances using PAST v4.1 ([Hammer et al., 2001](#) [\[link\]](#)).

Finally, to better understand the differences between groups, we selected the variables that were testing for a certain domain and grouped them into categories for the tibia microarchitecture tests, neurodevelopmental tests and adult cognitive tests. We then repeated the multivariate pairwise

permutation analysis. The categories were cortical bone strength, cortical bone size and trabecular bone for the tibia microarchitecture; developmental landmarks, neuromotor tests and reflexes for the neurodevelopmental tests; and anxiety, arousal, and memory for the adult cognitive tests.

Supplementary material

Author Contributions

Conceptualization, SL,NMA,GVV; methodology, LVe,WG,GVV; formal analysis, SL,ES,MA,AC,MP,KW,VVB,LVa,NGF,LGF; investigation, SL,BT,ES; resources, NMA,GVV,ZCV,CA,UH,HP; data curation, SL,NMA, GVV; writing—original draft preparation, SL,NMA,GVV; writing—review and editing, SL,NMA,GVV; visualization, SL,NMA,GVV; supervision, NMA,GVV,ZCV,CA; project administration, GVV; funding acquisition, NMA and GVV. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The animal study protocol was approved by the Animal Ethics Committee of KU Leuven (ECD approval number P120/2019).

Data Availability Statement

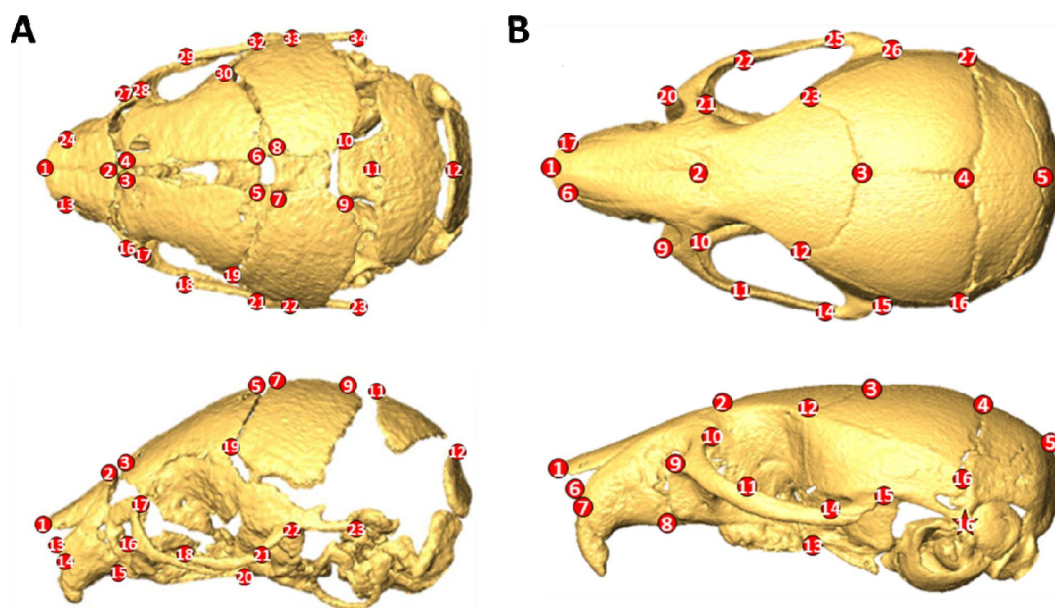
Data is available upon reasonable request to the corresponding authors.

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Conflicts of Interest

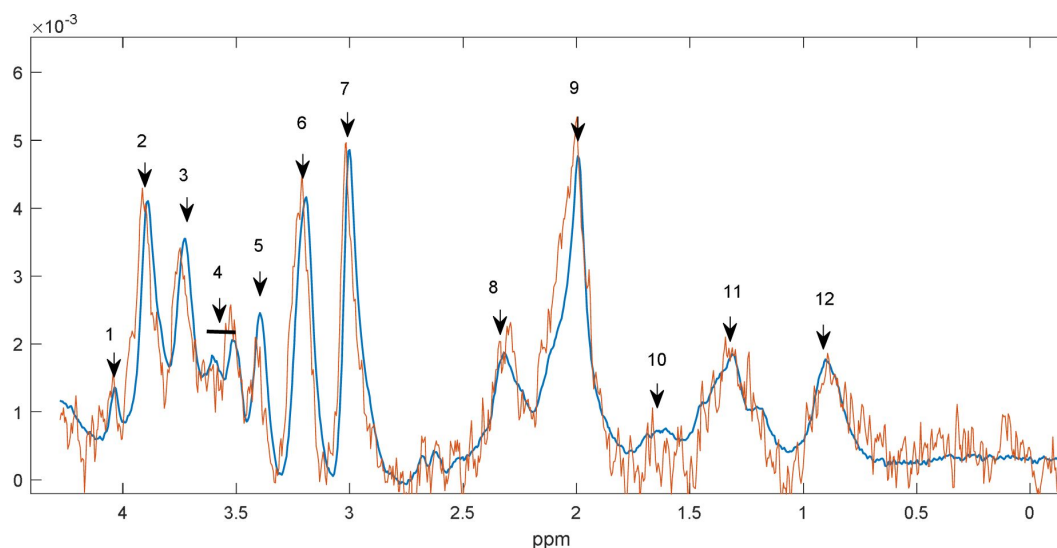
The authors declare no conflict of interest.



Supplementary Figure S1.

Set of anatomical landmarks used to characterize the shape of the skull and face

(A) Set of 34 landmarks characterizing craniofacial shape at μCT_1 from a 3D reconstruction of a μCT scan. (B) Set of 27 landmarks characterizing craniofacial shape at μCT_2 , μCT_3 and μCT_4 from a 3D reconstruction of a μCT scan. See [Supplementary Table S17](#) for precise anatomical definitions.



Supplementary Figure S2.

MR spectra and integrated spectral regions for multivariate MRS analysis

The region from 0 to 4.3 ppm of a representative MR spectrum is shown. The integrated regions and the most concentrated metabolites contributing to those regions are indicated in [Supplementary Table S10](#). Regions 11 and 12, representing contaminations from macromolecules, were not included in the analysis as they showed large variability.

	WT		TS		WT Treated		TS Treated	
	Female	Male	Female	Male	Female	Male	Female	Male
BW1	11	7	11	7	13	10	14	8
BW2	7	3	5	3	7	10	11	7
BW3	5	2	5	2	7	10	9	7
μ CT1	11	7	10	7	9	13	8	8
μ CT2	8	5	6	4	10	9	9	7
μ CT3	8	3	6	2	8	10	11	7
μ CT4	6	3	6	2	7	10	11	7
BMD humerus μ CT1	11	7	10	7	9	13	14	8
BMD humerus μ CT2	4	5	6	4	9	9	11	7
BMD humerus μ CT3	8	3	6	2	7	10	11	7
BMD humerus μ CT4	5	4	6	2	8	8	11	7
Tibia length	6	3	6	2	7	10	11	7
Percentage Bone Volume	6	2	6	2	7	10	11	7
Trabecular Thickness	6	3	6	2	7	10	11	7
Trabecular Separation	6	3	6	2	7	10	11	7
Trabecular Number	6	3	6	2	7	10	11	7
Cross Sectional Area	6	3	6	2	7	10	11	7
Periosteal Perimeter	6	3	6	2	7	10	11	7
Endocortical Perimeter	6	3	6	2	7	10	11	7
Bone Area	6	3	6	2	7	10	11	7
Medullary Area	6	3	6	2	7	10	11	7
Cortical Thickness	6	3	6	2	7	10	11	7
Cortical Porosity	6	3	6	2	7	9	10	7
Polar Moment Of Inertia	6	3	6	2	7	10	11	7
BMD	6	3	6	2	7	10	11	7
Cortical bone mineral density	6	3	6	2	7	9	11	7
Whole Brain (MRI1)	7	4	6	3	7	10	11	7
Hippocampus (MRI1)	7	4	6	3	7	10	11	6
Cerebellum (MRI1)	7	4	6	3	7	10	11	7
Lateral Ventricles (MRI1)	7	4	6	3	7	10	11	7
Third Ventricle (MRI1)	7	4	6	3	7	10	11	7
Fourth Ventricle (MRI1)	7	4	6	3	7	10	11	7
Cerebral Aqueduct (MRI1)	7	4	6	3	7	10	11	7
Whole Ventricles (MRI1)	7	4	6	3	7	10	11	7
Whole Brain (MRI2)	6	3	6	2	7	10	11	7
Hippocampus (MRI2)	6	3	6	2	7	10	11	7
Cerebellum (MRI2)	6	3	6	2	7	10	11	7
Lateral Ventricles (MRI2)	6	3	6	2	6	10	11	7
Third Ventricle (MRI2)	6	3	6	2	7	10	11	7
Fourth Ventricle (MRI2)	6	3	6	2	6	10	11	7

Supplementary Table S1.

Sample size for each analysis, experiment, and developmental stage

Differences in sample size between stages are due to technical reasons such as micro-CT or MR scanner not operating on the scanning day, movement scanning artifacts, or mouse death during the experiment. For the tibia microarchitecture, differences in sample size are due to mice detected as outliers. For the MRS, differences in sample size are due to signal artifacts. For the adult cognitive tests, differences in sample size are due to artifacts during tracking.

Cerebral Aqueduct (MRI2)	6	3	6	2	7	10	11	7
Whole Ventricles (MRI2)	6	3	6	2	7	10	11	7
Eye Opening	9	5	6	7	10	10	12	8
Vibrissae	9	5	6	7	10	10	12	8
Blast	9	5	6	7	10	10	12	8
Cliff	9	5	6	7	10	10	12	8
Incisor	5	3	2	3	10	7	7	2
Righting	9	6	7	8	10	11	11	8
Negative Geotaxis	9	5	6	6	10	10	11	8
Climbing	8	5	6	6	9	10	11	4
Grasping	6	4	8	9	10	9	12	8
Tactile	9	5	6	6	10	10	12	8
Reaching	9	5	6	7	10	10	11	7
Visual	9	5	6	7	10	10	12	8
Preyer	9	5	6	7	10	10	12	8
Pinna	11	7	9	9	10	13	14	8
Walking	5	4	4	5	6	6	8	7
Homing	6	5	6	6	10	10	12	8
Pivoting	9	5	6	7	10	10	12	8
Nursing	N/A	N/A	6	0	N/A	N/A	7	0
Pup licking	N/A	N/A	6	0	N/A	N/A	7	0
Digging in nest	N/A	N/A	6	0	N/A	N/A	7	0
Eating	N/A	N/A	6	0	N/A	N/A	7	0
Drinking	N/A	N/A	6	0	N/A	N/A	7	0
Moving	N/A	N/A	6	0	N/A	N/A	7	0
Digging off nest	N/A	N/A	6	0	N/A	N/A	7	0
OF: Time in center (Cog1)	8	4	7	3	9	10	11	7
OF: Time in periphery (Cog1)	8	4	7	3	9	10	11	7
NOR: Time in center hab (Cog1)	8	4	7	3	9	10	11	7
EPM: % open arm (Cog1)	7	4	7	3	8	10	9	7
OF: Distance (Cog1)	8	3	7	3	9	10	11	7
OF: Speed (Cog1)	7	4	7	3	9	10	11	7
SPSN: Distance hab (Cog1)	8	4	7	3	9	10	11	7
SPSN: Speed hab (Cog1)	8	4	7	3	9	10	11	7
NOR: Distance hab (Cog1)	8	4	7	3	9	10	11	7
NOR: Speed hab (Cog1)	8	4	6	3	9	10	11	7
NOR: Preference for NOV (Cog1)	7	3	7	3	7	10	8	7
SPSN: Preference for S2 (Cog1)	7	3	7	3	7	10	8	7
PA: Latency train (Cog1)	8	4	5	3	8	10	10	6
PA: Latency test (Cog1)	8	4	7	3	8	10	11	7
OF: Time in center (Cog2)	6	2	5	2	7	10	11	7
OF: Time in periphery (Cog2)	6	2	6	2	7	10	11	7
NOR: Time in center hab (Cog2)	6	2	6	2	7	10	11	7
EPM: % open arm (Cog2)	6	2	6	2	7	10	11	7
OF: Distance (Cog2)	6	2	6	2	7	9	11	7
OF: Speed (Cog2)	6	2	6	2	7	9	11	7
SPSN: Distance hab (Cog2)	6	2	6	2	7	10	11	7
SPSN: Speed hab (Cog2)	6	2	6	2	7	10	11	7
NOR: Distance hab (Cog2)	6	2	6	2	7	9	11	7
NOR: Speed hab (Cog2)	6	2	6	2	7	9	11	7
NOR: Preference for NOV (Cog2)	4	2	4	2	7	10	11	7

Supplementary Table S1. (continued)

SPSN: Preference for S2 (Cog2)	4	2	4	2	7	10	11	7
PA: Latency train (Cog2)	6	2	6	2	7	10	10	5
PA: Latency test (Cog2)	6	2	6	2	5	7	11	7
NAA (MRS1)	8	4	6	3	7	10	9	6
Cr/PCr (MRS1)	8	4	6	3	7	10	9	6
Chol/Pchol/GPC (MRS1)	8	4	6	3	7	10	9	6
myo-Inos (MRS1)	7	4	5	3	5	9	9	6
Tau (MRS1)	7	4	6	3	7	10	9	6
NAA (MRS2)	8	4	6	3	7	10	10	7
Cr/PCr (MRS2)	8	4	6	3	7	10	10	7
Chol/Pchol/GPC (MRS2)	8	4	6	3	7	10	10	7
myo-Inos (MRS2)	8	4	6	3	7	10	10	7
Tau (MRS2)	8	4	6	3	7	10	10	7
RNAseq	6	3	6	2	7	10	11	7

Supplementary Table S1. (continued)

μCT_1	TS Treated	TS Untreated	WT Treated
TS Untreated	0.0005		
WT Treated	<0.0001	N/A	
WT Untreated	<0.0001	<0.0001	0.0418
μCT_2	TS Treated	TS Untreated	WT Treated
TS Untreated	0.3358		
WT Treated	<0.0001	N/A	
WT Untreated	<0.0001	0.0011	0.2719
μCT_3	TS Treated	TS Untreated	WT Treated
TS Untreated	0.1237		
WT Treated	<0.0001	N/A	
WT Untreated	<0.0001	0.0009	0.6242
μCT_4	TS Treated	TS Untreated	WT Treated
TS Untreated	0.2151		
WT Treated	<0.0001	N/A	
WT Untreated	<0.0001	0.0055	0.5590

Supplementary Table S2.

***P*-values resulting from permutation tests (10,000 permutation rounds)
based on Procrustes distances among groups for craniofacial shape**

Bold font indicates statistically significant values. Pairwise comparisons marked as N/A were not calculated since they did not evaluate any relevant scientific question.

Mixed-effects analysis	TS Treated	TS Untreated	WT Treated
TS Untreated	0.0023		
WT Treated	0.0320	N/A	
WT Untreated	<0.0001	0.2397	<0.0001
μCT₁	TS Treated	TS Untreated	WT Treated
TS Untreated	0.0897		
WT Treated	0.0002	N/A	
WT Untreated	0.0225	0.6717	0.3034
μCT₂	TS Treated	TS Untreated	WT Treated
TS Untreated	0.5168		
WT Treated	0.4910	N/A	
WT Untreated	0.0996	0.4346	0.3243
μCT₃	TS Treated	TS Untreated	WT Treated
TS Untreated	0.3721		
WT Treated	0.5098	N/A	
WT Untreated	0.2033	0.9097	0.0735
μCT₄	TS Treated	TS Untreated	WT Treated
TS Untreated	0.0573		
WT Treated	0.2961	N/A	
WT Untreated	0.0002	0.0430	0.0001

Supplementary Table S3.

P-values resulting from the mixed-effects analysis and pairwise tests for humerus BMD

Bold font indicates statistically significant values after Benjamini–Hochberg correction. Pairwise comparisons marked as N/A were not calculated since they did not evaluate any relevant scientific question.

ALL TESTS	TS Treated	TS Untreated	WT Treated
TS Untreated	0.0001		
WT Treated	0.0002	N/A	
WT Untreated	0.0008	0.5589	0.0089
TRABECULAR BONE	TS Treated	TS Untreated	WT Treated
TS Untreated	0.7034		
WT Treated	0.0792	N/A	
WT Untreated	0.8793	0.8944	0.5405
CORTICAL BONE STRENGTH	TS Treated	TS Untreated	WT Treated
TS Untreated	0.0001		
WT Treated	0.0005	N/A	
WT Untreated	0.0002	0.4536	0.0563
CORTICAL BONE SIZE	TS Treated	TS Untreated	WT Treated
TS Untreated	0.0011		
WT Treated	0.0003	N/A	
WT Untreated	0.0007	0.0975	0.3042

Supplementary Table S4.

P-values resulting from the pairwise tests after a one-way PERMANOVA (9,999 permutation rounds) based on Mahalanobis distances for tibia microarchitecture parameters

Bold font indicates statistically significant values. Pairwise comparisons marked as N/A were not calculated since they did not evaluate any relevant scientific question.

ALL TESTS	TS Treated	TS Untreated	WT Treated
TS Untreated	0.4921		
WT Treated	0.0008	N/A	
WT Untreated	0.0047	0.1064	0.4467

Supplementary Table S5.

P-values resulting from the pairwise tests after a one-way PERMANOVA (9,999 permutation rounds) based on Mahalanobis distances for brain volumes before treatment discontinuation

Bold font indicates statistically significant values. Pairwise comparisons marked as N/A were not calculated since they did not evaluate any relevant scientific question.

ALL TESTS	TS Treated	TS Untreated	WT Treated
TS Untreated	0.0472		
WT Treated	0.0001	N/A	
WT Untreated	0.0609	0.0065	0.2808

Supplementary Table S6.

P-values resulting from the pairwise tests after a one-way PERMANOVA (9,999 permutation rounds) based on Mahalanobis distances for brain volumes after treatment discontinuation

Bold font indicates statistically significant values. Pairwise comparisons marked as N/A were not calculated since they did not evaluate any relevant scientific question.

ALL TESTS	TS Treated	TS Untreated	WT Treated
TS Untreated	0.0001		
WT Treated	0.0014	N/A	
WT Untreated	0.0001	0.0001	0.1252
DEV. LANDMARKS	TS Treated	TS Untreated	WT Treated
TS Untreated	0.0557		
WT Treated	0.0163	N/A	
WT Untreated	0.0181	0.8250	0.3925
NEUROMOTOR	TS Treated	TS Untreated	WT Treated
TS Untreated	0.0065		
WT Treated	0.0002	N/A	
WT Untreated	0.0025	0.0555	0.3376
REFLEXES	TS Treated	TS Untreated	WT Treated
TS Untreated	0.1642		
WT Treated	0.0001	N/A	
WT Untreated	0.0001	0.0423	0.0001

Supplementary Table S7.

P-values resulting from the pairwise tests after a one-way PERMANOVA (9,999 permutation rounds) based on Mahalanobis distances for early neurodevelopmental tests

Bold font indicates statistically significant values. Pairwise comparisons marked as N/A were not calculated since they did not evaluate any relevant scientific question.

ALL TESTS	TS Treated	TS Untreated	WT Treated
TS Untreated	0.1792		
WT Treated	0.0507	N/A	
WT Untreated	0.0001	0.0125	0.0050
ANXIETY TESTS	TS Treated	TS Untreated	WT Treated
TS Untreated	0.8212		
WT Treated	0.3742	N/A	
WT Untreated	0.0006	0.1153	0.0035
AROUSAL TESTS	TS Treated	TS Untreated	WT Treated
TS Untreated	0.0337		
WT Treated	0.0017	N/A	
WT Untreated	0.0001	0.0028	0.0004
MEMORY TESTS	TS Treated	TS Untreated	WT Treated
TS Untreated	0.2468		
WT Treated	0.0353	N/A	
WT Untreated	0.0668	0.4824	0.4809

Supplementary Table S8.

***P*-values resulting from the pairwise tests after a one-way PERMANOVA (9,999 permutation rounds) based on Mahalanobis distances for adult cognitive tests before treatment discontinuation at Cog.₁**

Bold font indicates statistically significant values. Pairwise comparisons marked as N/A were not calculated since they did not evaluate any relevant scientific question.

ALL TESTS	TS Treated	TS Untreated	WT Treated
TS Untreated	0.4048		
WT Treated	0.0550	N/A	
WT Untreated	0.0018	0.4596	0.0004
ANXIETY TESTS	TS Treated	TS Untreated	WT Treated
TS Untreated	0.2970		
WT Treated	0.1914	N/A	
WT Untreated	0.0417	0.2898	0.0386
AROUSAL TESTS	TS Treated	TS Untreated	WT Treated
TS Untreated	0.1554		
WT Treated	0.0260	N/A	
WT Untreated	0.0019	0.5638	0.0085
MEMORY TESTS	TS Treated	TS Untreated	WT Treated
TS Untreated	0.3038		
WT Treated	0.2073	N/A	
WT Untreated	0.0087	0.2218	0.0089

Supplementary Table S9.

***P*-values resulting from the pairwise tests after a one-way PERMANOVA (9,999 permutation rounds) based on Mahalanobis distances for adult cognitive tests after treatment discontinuation at Cog₂**

Bold font indicates statistically significant values. Pairwise comparisons marked as N/A were not calculated since they did not evaluate any relevant scientific question.

Region number	Metabolite name	Integration Range [ppm]
1	lactate, myo-inositol	4.00 – 4.20
2	glucose, H α of AA, cre, PCr	3.81 – 4.00
3	myo-inositol, glucose, H α of AA	3.68 – 3.79
4	myo-inositol, gly, glucose	3.45 – 3.62
5	taurine, glucose	3.30 – 3.45
6	PChol, GPC, choline, taurine	3.19 – 3.30
7	cre, PCr, lys, his, cys, tyr	2.91 – 3.10
8	gln, glu, NAA, val, pro	2.18 – 2.45
9	gln, glu, NAA, lys, gaba	1.88 – 2.18
10	ala, leu, lys, arg	1.45 – 1.70
11	lipids, lactate	1.25 – 1.38
12	lipids, ile, leu, val	0.70 – 0.98

Supplementary Table S10.

Most concentrated metabolites contributing to the respective spectral regions in MRS

ala – alanine, arg – arginine, cre – creatine, cys – cysteine, H α of AA – α hydrogen of amino acids, ile – iso leucine, leu – leucine, lys – lysine, gaba – γ -amino butyrate, gln – glutamine, glu – glutamate, gly – glycine, GPC – glycerol phosphocholine, his – histidine, NAA – N-acetyl aspartate, PChol – phosphocholine, PCr – phosphocreatine, tyr – tyrosine, val – valine

ALL TESTS	TS Treated	TS Untreated	WT Treated
TS Untreated	0.0361		
WT Treated	0.0163	N/A	
WT Untreated	0.0496	0.0136	0.4779

Supplementary Table S11.

P-values resulting from the pairwise tests after a one-way PERMANOVA (9,999 permutation rounds) based on Mahalanobis distances for MRS spectra before treatment discontinuation

Bold font indicates statistically significant values. Pairwise comparisons marked as N/A were not calculated since they did not evaluate any relevant scientific question.

ALL TESTS	TS Treated	TS Untreated	WT Treated
TS Untreated	0.2111		
WT Treated	0.0295	N/A	
WT Untreated	0.0347	0.5790	0.5859

Supplementary Table S12.

P-values resulting from the pairwise tests after a one-way PERMANOVA (9,999 permutation rounds) based on Mahalanobis distances for MRS spectra after treatment discontinuation

Bold font indicates statistically significant values. Pairwise comparisons marked as N/A were not calculated since they did not evaluate any relevant scientific question.

ALL TESTS	TS Treated	TS Untreated	WT Treated
TS Untreated	0.2356		
WT Treated	0.1184	N/A	
WT Untreated	0.4473	0.1630	0.5594

Supplementary Table S13.

P-values resulting from the pairwise tests after a one-way PERMANOVA (9,999 permutation rounds) based on Euclidean distances for normalized gene expression data at endpoint

Bold font indicates statistically significant values. Pairwise comparisons marked as N/A were not calculated since they did not evaluate any relevant scientific question.

Mouse number	Litter	Genotype	Treatment	Sex
1	1	TS	Not treated	F
2	1	WT	Not treated	F
3	1	WT	Not treated	M
4	1	TS	Not treated	M
5	1	WT	Not treated	M
6	1	TS	Not treated	F
7	2	TS	Not treated	M
8	2	TS	Not treated	F
9	2	TS	Not treated	M
10	2	TS	Not treated	F
11	3	TS	Not treated	M
12	3	WT	Not treated	F
13	4	WT	Not treated	M
14	4	TS	Not treated	F
15	4	WT	Not treated	F
16	4	WT	Not treated	M
17	4	TS	Not treated	F
18	4	TS	Not treated	F
19	5	WT	Not treated	F
20	5	WT	Not treated	F
21	5	WT	Not treated	F
22	5	WT	Not treated	F
23	6	WT	Not treated	F
24	6	WT	Not treated	M
25	6	TS	Not treated	M
26	6	WT	Not treated	M
27	6	TS	Not treated	F
28	6	TS	Not treated	M

Supplementary Table S15.

Litter information

For each mouse, the litter number, genotype, treatment received and sex are provided.

29	6	TS	Not treated	M
30	7	WT	0.09 mg EGCG/mL	F
31	7	WT	0.09 mg EGCG/mL	M
32	7	WT	0.09 mg EGCG/mL	F
33	7	WT	0.09 mg EGCG/mL	M
34	7	TS	0.09 mg EGCG/mL	M
35	7	TS	0.09 mg EGCG/mL	F
36	8	TS	0.09 mg EGCG/mL	F
37	8	WT	0.09 mg EGCG/mL	F
38	8	WT	0.09 mg EGCG/mL	F
39	8	WT	0.09 mg EGCG/mL	M
40	9	TS	0.09 mg EGCG/mL	M
41	9	WT	0.09 mg EGCG/mL	M
42	9	TS	0.09 mg EGCG/mL	F
43	9	TS	0.09 mg EGCG/mL	F
44	9	TS	0.09 mg EGCG/mL	F
45	9	TS	0.09 mg EGCG/mL	F
46	10	TS	0.09 mg EGCG/mL	F
47	10	WT	0.09 mg EGCG/mL	F
48	10	WT	0.09 mg EGCG/mL	M
49	10	TS	0.09 mg EGCG/mL	M
50	10	WT	0.09 mg EGCG/mL	F
51	10	TS	0.09 mg EGCG/mL	F
52	10	TS	0.09 mg EGCG/mL	F
53	11	WT	0.09 mg EGCG/mL	M
54	11	WT	0.09 mg EGCG/mL	M
55	11	WT	0.09 mg EGCG/mL	F
56	11	WT	0.09 mg EGCG/mL	M
57	11	WT	0.09 mg EGCG/mL	M
58	11	TS	0.09 mg EGCG/mL	M
59	11	TS	0.09 mg EGCG/mL	F
60	12	TS	0.09 mg EGCG/mL	M
61	12	TS	0.09 mg EGCG/mL	M
62	12	WT	0.09 mg EGCG/mL	F
63	12	TS	0.09 mg EGCG/mL	M
64	12	TS	0.09 mg EGCG/mL	F
65	13	TS	0.09 mg EGCG/mL	F
66	13	WT	0.09 mg EGCG/mL	F
67	13	WT	0.09 mg EGCG/mL	F
68	13	WT	0.09 mg EGCG/mL	M
69	13	TS	0.09 mg EGCG/mL	F
70	13	TS	0.09 mg EGCG/mL	M
71	13	TS	0.09 mg EGCG/mL	M

Supplementary Table S15. (continued)

Stage	Source energy (Kv)	Filter	Current (μA)	Exposure time (ms)	Average s	Step increment (°)	Total angle (°)	Time	Voxel size (μm ³)
μCT ₁	35	Al 0.5 mm	500	180	3	1	180°	3 min	51.7
μCT ₂₋₄	55	Al 1 mm	700	80	2	1	360°	3 min	51.7
Tibia (ex vivo)	60	Al 0.5 mm	80	3000	0	0.4	180°	23 min	5

Supplementary Table S16.

μCT scanning parameters used at each stage

Landmark number at μCT_1	Landmark number at μCT_2 , μCT_3 and μCT_4	Anatomical definition
1	1	Tip of the nasal bone
N/A	2	Intersection of nasal and frontal bones
N/A	3	Intersection of frontal and parietal bones
N/A	4	Intersection of parietal and interparietal bones
N/A	5	Intersection of interparietal and occipital bones
13	6	Anterior-most point on intersection of premaxillae and nasal bones (left)
14	7	Center of alveolar ridge over maxillary incisor (left)
15	8	Most inferior point on premaxilla-maxilla suture (left)
16	9	Anterior notch on frontal process lateral to infraorbital fissure (left)
17	10	Intersection of frontal process of maxilla with frontal and lacrimal bones (left)
18	11	Intersection of zygomatic process of maxilla with zygoma (left)
19	12	Frontal-squamosal intersection at temporal crest (left)
20	13	Intersection of maxilla and sphenoid on inferior alveolar (left)
21	14	Intersection of zygoma with zygomatic process of temporal (left)

Supplementary Table S17.

Anatomical definition of craniofacial landmarks

N/A indicates that the landmarks were not acquired at this stage.

N/A	15	Intersection of squamosal body to zygomatic process of squamosal (left)
N/A	16	Intersection of parietal, temporal and occipital bones (left)
24	17	Anterior-most point on intersection of premaxillae and nasal bones (right)
25	18	Center of alveolar ridge over maxillary incisor (right)
26	19	Most inferior point on premaxilla-maxilla suture (right)
27	20	Anterior notch on frontal process lateral to infraorbital fissure (right)
28	21	Intersection of frontal process of maxilla with frontal and lacrimal bones (right)
29	22	Intersection of zygomatic process of maxilla with zygoma (right)
30	23	Frontal-squamosal intersection at temporal crest (right)
31	24	Intersection of maxilla and sphenoid on inferior alveolar (right)
32	25	Intersection of zygoma with zygomatic process of temporal (right)
N/A	26	Intersection of squamosal body to zygomatic process of squamosal (right)
N/A	27	Intersection of parietal, temporal and occipital bones (right)
2	N/A	Posterior-most point on the nasal bone at the midline
3	N/A	Anterior-most point on the frontal bone at the midline (left)
4	N/A	Anterior-most point on the frontal bone at the midline (right)
5	N/A	Posterior-most point on the frontal bone at the midline (left)
6	N/A	Posterior-most point on the frontal bone at the midline (right)
7	N/A	Anterior-most point on the parietal bone at the midline (left)

Supplementary Table S17. (continued)

8	N/A	Anterior-most point on the parietal bone at the midline (right)
9	N/A	Maximum curvature point on the posterior part of the parietal bone (left)
10	N/A	Maximum curvature point on the posterior part of the parietal bone (right)
11	N/A	Anterior-most point on the interparietal bone at the midline
12	N/A	Anterior-most point on the occipital bone at the midline
22	N/A	Joining of squamosal body to zygomatic process of squamosal (left)
23	N/A	Tip of the post-tympanic hook (left)
33	N/A	Joining of squamosal body to zygomatic process of squamosal (right)
34	N/A	Tip of the post-tympanic hook (right)

Supplementary Table S17. (continued)

Parameter	Abbreviation	Description	Unit
Percentage bone volume	BV/TV	Ratio of bone volume to total volume inside the Region of interest	%
Trabecular number	Tb.N	Average number of trabeculae per length unit of bone	1/mm
Trabecular thickness	Tb.Th	Average thickness of the trabecular bone	mm
Trabecular separation	Tb.Sp	Average distance between the trabecular bone	mm
Bone mineral density	BMD	Volumetric density of hydroxyapatite in the trabecular bone	g/cm ³

Supplementary Table S18.

Trabecular bone parameters measured from *ex vivo* micro-CT images at 8M

Parameter	Abbreviation	Description	Unit
Cross sectional area	Tt.Ar	Total area inside the periosteal perimeter	mm ²
Periosteal perimeter	Ps.Pm	Perimeter around the outside of the bone	mm
Endosteal perimeter	Ec.Pm	Perimeter around the medullary canal, inside the cortical bone	mm
Bone area	Ct.Ar	Volume of the cortical bone	mm ²
Medullary area	Ma.Ar	Area of the medullary or bone marrow canal	mm ²
Cortical thickness	Ct.Th	Average thickness of the cortical bone	mm
Porosity	Ct.Po	Ratio of pore volume over the total volume of cortical bone	%
Polar moment of inertia	J	Resistance against torsion	mm ⁴
Cortical bone mineral density	Ct. BMD	Mineral density of the cortical bone	g/cm ³

Supplementary Table S19.

Cortical bone parameters measured from *ex vivo* micro-CT images at 8M

	Parameter	Normal according to Shapiro-Wilk?	Equal SD according to F-test?	Test performed
Body growth	BW ₂	No	No	K-S
	BW ₃	No	No	K-S
Bone mineral density	BMD humerus μ CT ₁	Yes	No	Welch's T-test
	BMD humerus μ CT ₂	No	No	K-S
	BMD humerus μ CT ₃	No	No	K-S
	BMD humerus μ CT ₄	Yes	Yes	Welch's T-test
Tibia microarchitecture	Tibia length	Yes	Yes	Welch's T-test
	Percentage Bone Volume	No	No	K-S
	Trabecular Thickness	No	Yes	M-W
	Trabecular Separation	No	Yes	M-W
	Trabecular Number	No	No	K-S
	Cross Sectional Area	Yes	No	Welch's T-test
	Periosteal Perimeter	Yes	Yes	Welch's T-test
	Endocortical Perimeter	No	No	K-S
	Bone Area	Yes	No	Welch's T-test
	Medullary Area	Yes	No	Welch's T-test
	Cortical Thickness	Yes	Yes	Welch's T-test
	Cortical Porosity	No	No	K-S
	Polar Moment Of Inertia	No	No	K-S
	BMD	Yes	No	Welch's T-

Supplementary Table S20.

Normality, homoscedasticity, and statistical tests performed per parameter

If one of the four mice groups was not normally distributed or one pairwise comparison was not homoscedastic, the variable was considered as not normally distributed and/or not homoscedastic.

	Cortical bone mineral density	No	No	test K-S
Brain volumes	Whole Brain (MRI ₁)	Yes	No	Welch's T-test
	hippocampal region (MRI ₁)	Yes	No	Welch's T-test
	Cerebellum (MRI ₁)	Yes	No	Welch's T-test
	Lateral Ventricles (MRI ₁)	Yes	No	Welch's T-test
	Third Ventricle (MRI ₁)	Yes	No	Welch's T-test
	Fourth Ventricle (MRI ₁)	Yes	No	Welch's T-test
	Cerebral Aqueduct (MRI ₁)	Yes	No	Welch's T-test
	Whole Ventricles (MRI ₁)	Yes	No	Welch's T-test
	Whole Brain (MRI ₂)	Yes	Yes	Welch's T-test
	hippocampal region (MRI ₂)	No	Yes	M-W
	Cerebellum (MRI ₂)	Yes	No	Welch's T-test
	Lateral Ventricles (MRI ₂)	No	No	K-S
	Third Ventricle (MRI ₂)	Yes	No	Welch's T-test
	Fourth Ventricle (MRI ₂)	No	No	K-S
	Cerebral Aqueduct (MRI ₂)	Yes	Yes	Welch's T-test
	Whole Ventricles (MRI ₂)	No	Yes	M-W
Neurodevelopmental tests	Eye Opening	No	No	K-S
	Vibrissae	No	No	K-S
	Blast	No	No	K-S
	Cliff	No	Yes	M-W
	Incisor	No	Yes	M-W
	Righting	Yes	Yes	Welch's T-test
	Negative Geotaxis	No	Yes	M-W
	Climbing	No	Yes	M-W
	Grasping	No	Yes	M-W
	Tactile	No	No	K-S
	Reaching	No	No	K-S
	Visual	No	Yes	M-W
	Preyer	No	No	K-S
	Pinna	No	Yes	M-W
	Walking	No	Yes	M-W
Maternal behavior tests	Nursing	No	No	K-S
	Pup licking	Yes	Yes	Welch's T-test

Supplementary Table S20. (continued)

	Digging in nest	Yes	Yes	Welch's T-test
	Eating	Yes	Yes	Welch's T-test
	Drinking	Yes	Yes	Welch's T-test
	Moving	No	No	K-S
	Digging off nest	No	Yes	M-W
Adult cognitive tests	OF: Time in center (Cog ₁)	Yes	Yes	Welch's T-test
	OF: Time in periphery (Cog ₁)	No	No	K-S
	NOR: Time in center hab (Cog ₁)	Yes	Yes	Welch's T-test
	EPM: % open arm (Cog ₁)	Yes	No	Welch's T-test
	OF: Distance (Cog ₁)	Yes	No	Welch's T-test
	OF: Speed (Cog ₁)	Yes	No	Welch's T-test
	SPSN: Distance hab (Cog ₁)	Yes	No	Welch's T-test
	SPSN: Speed hab (Cog ₁)	No	No	K-S
	NOR: Distance hab (Cog ₁)	No	No	K-S
	NOR: Speed hab (Cog ₁)	Yes	No	Welch's T-test
	NOR: Preference for NOV (Cog ₁)	No	Yes	M-W
	SPSN: Preference for S2 (Cog ₁)	No	Yes	M-W
	PA: Latency train (Cog ₁)	No	No	K-S
	PA: Latency test (Cog ₁)	No	No	K-S
	OF: Time in center (Cog ₂)	No	No	K-S
	OF: Time in periphery (Cog ₂)	Yes	No	Welch's T-test
	NOR: Time in center hab (Cog ₂)	No	No	K-S
	EPM: % open arm (Cog ₂)	Yes	Yes	Welch's T-test
	OF: Distance (Cog ₂)	Yes	No	Welch's T-test
	OF: Speed (Cog ₂)	Yes	No	Welch's T-test
	SPSN: Distance hab (Cog ₂)	Yes	No	Welch's T-test
	SPSN: Speed hab (Cog ₂)	Yes	No	Welch's T-test
	NOR: Distance hab (Cog ₂)	Yes	No	Welch's T-test

Supplementary Table S20. (continued)

	NOR: Speed hab (Cog ₂)	Yes	No	Welch's T-test
	NOR: Preference for NOV (Cog ₂)	Yes	No	Welch's T-test
	SPSN: Preference for S2 (Cog ₂)	No	Yes	M-W
	PA: Latency train (Cog ₂)	No	No	K-S
	PA: Latency test (Cog ₂)	No	No	K-S
Hippocampal metabolites	NAA (MRS ₁)	Yes	Yes	Welch's T-test
	Cr+PCr (MRS ₁)	Yes	No	Welch's T-test
	Chol+Pchol+GPC (MRS ₁)	Yes	Yes	Welch's T-test
	myo-Inos (MRS ₁)	Yes	Yes	Welch's T-test
	Tau (MRS ₁)	Yes	Yes	Welch's T-test
	NAA (MRS ₂)	No	Yes	M-W
	Cr+PCr (MRS ₂)	Yes	Yes	Welch's T-test
	Chol+Pchol+GPC (MRS ₂)	Yes	Yes	Welch's T-test
	myo-Inos (MRS ₂)	Yes	Yes	Welch's T-test
	Tau (MRS ₂)	Yes	Yes	Welch's T-test

Supplementary Table S20. (continued)

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Joint Public Review:

Using Ts65Dn - the most commonly used mouse model of Down syndrome (DS) - the goal of this study is two-pronged: 1) to conduct a thorough assessment of DS-related genotypic, physiological, behavioral, and phenotypic measures in a longitudinal manner; and 2) to measure the effects of chronic GTE-EGCG on these measures in the Ts65Dn mouse model. Corroborating results from several previous studies on Ts65Dn mice, findings of this study show confirm the Ts65Dn mouse model exhibits the suite of traits associated with DS. The findings also suggest that the mouse model might have experienced drift, given the milder phenotypes than those reported by earlier studies. Results of the GTE-EGCG treatment do not support its therapeutic use and instead show that the treatment exacerbated certain DS-related phenotypes.

Strengths:

The authors performed a rigorous assessment of treatment and examined treatment and genotypic alterations at multiple time points during growth and aging. Detailed analysis shows differences in genotype during aging as well as genotype with treatment. This study is solid in the overarching methodological approach (with the exception of RNAseq, described below). The biggest strength of the study is its approach and dataset, which corroborate results from a multitude of past studies on Ts65Dn mice, albeit on adult specimens.

Comments on revised submission:

The authors have made numerous changes to address the concerns of the reviewers. The strengths remain: a large, longitudinal data set for the Ts65Dn mouse model across multiple organ systems. The results also clearly show the impact of GTE-EGCG treatment and do not support its therapeutic use.

The authors should report their a priori power calculations that they used when designing their experiment. This should be added to either the Animals or Statistics subsections of the Methods.

<https://doi.org/10.7554/eLife.89763.2.sa0>

Author Response

The following is the authors' response to the original reviews.

This study presents valuable findings that examine both how Down syndrome (DS)-related physiological, behavioral, and phenotypic traits track across time, as well as how chronic treatment with green tea extracts 25 enriched in epigallocatechin-3-gallate (GTE-EGCG), administered in drinking water spanning prenatal through 5 months of age, impacts these measures in wild-type and Ts65Dn mice. However, the strength of the evidence is incomplete, due to high variability across measures, perhaps attributable to a failure to include sex as a factor for measures known to be sexually dimorphic. This study is of interest to scientists interested in Down Syndrome and its' treatment, as well as scientists who study disorders that impact multiple organ systems.

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We deeply appreciate the reviewers' positive feedback. Their acknowledgment of the solid methodological approach and the rigorous assessment of genotypic and treatment effects over various developmental stages resonates with our motivation. Their suggestion to make

eLife assessment

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We deeply appreciate the reviewers' positive feedback. Their acknowledgment of the solid methodological approach and the rigorous assessment of genotypic and treatment effects over various developmental stages resonates with our motivation. Their suggestion to make the dataset available in a public data repository for other researchers is well-taken. We are committed to data sharing and we are creating a dedicated platform to facilitate the accessibility of our research data to the scientific community. Given its size and complexity, we currently hold the dataset available upon reasonable request to the corresponding authors.

Weaknesses:

There are several primary weaknesses, described below:

Sex was not considered in the analyses.

The number of experimental animals of each sex are not clearly represented in the paper, but are buried in supplemental tables, and the Ns for the RNAseq are unclear. No analyses were done to examine sex differences in male/female DS or WT animals with or without treatment. Body measurements will greatly vary by sex, but this was not taken

into consideration during assessments. As such, there is a high amount of variability within each cohort measured for body assessments (tibia, body weight, skeletal development etc.). Supplemental table 14 had the list of each animal, but not collated by sex, genotype or treatment, making it difficult to assess the strength of each measurement.

Our study primarily concentrated on providing a holistic understanding of the impact of trisomy and GTE-EGCG treatment on Down syndrome, and was not explicitly designed to investigate sexual dimorphism. However, instead of reporting on only one sex and thereby obviating sex as a source of variation, as in previously published studies, we decided to include both male and female mice within the study design to represent a more realistic portrayal of the nature of Down syndrome in a heterogeneous population. By encompassing both sexes, we aim to better capture the variability in Down syndrome.

As we do acknowledge the significance of sex bias in scientific research, we considered performing post-hoc analyses to test the effect of sexual dimorphism, but found that our dataset was underpowered to obtain reliable results, since our experiments were not a priori designed to investigate this question and sample sizes for each sex by separate were not large enough. Nevertheless, considering the reviewer's comment, we have taken specific steps to improve the representation of sex-related information and to enhance the clarity of our manuscript.

First, we have redesigned all figures using empty and full symbols to distinguish male from female mice within each analysis, providing readers with an immediate sense of the sex distribution in each experimental group. Moreover, we have modified Supplementary Table 1 to offer a comprehensive breakdown of the number of male and female mice for each test, along with their respective genotypes and treatment groups. This table aims to make the sample size and sex distribution within our study as transparent as possible for our readers. While we acknowledge that our study lacked the statistical power to perform a detailed sex-based analysis, the visual representation of sex in our data shows which systems are mainly affected by sexual dysmorphism. This evidence can guide future investigations directly designed to investigate sexual effects in certain systems or structures.

Key results are not clearly depicted in the main figures

Rigorous assessment of each figure and the clarity of the figure to convey the results of the analysis needs to be performed. Many of the figures do not clearly represent the findings, with authors heavily relying on supplemental figures to present details to explain results. Figure legends do not adequately describe figures; rather, they are limited to describing how the analysis is performed. For example, LDA plots in Figure 4 do not clearly convey the results of metabolite analysis.

Overall, the amount of data presented here is overwhelming, making it difficult to interpret the findings. Some assessments that do not add to the overall paper need to be removed. Clarifying the text, figures and trimming the supplement to represent the data in a manner that is easily understood will improve the readability of the paper. For example, perhaps measures which are not strongly impacted by genotype could be moved to the supplement, because they are not directly relevant to the question of whether GTE-EGCG reverses the impact of trisomy on the measures.

As rightly pointed out by the reviewers, the vast amount of data generated by our holistic and longitudinal approach is one of the primary strengths, but also an important challenge in our study. Our dataset encompasses a comprehensive assessment of the effects of treatment and genotypic alterations at multiple time points during growth and aging. This multi-dimensional evaluation is pivotal to our research, and relegating data to supplementary

material would restrict access to this holistic understanding. Our aim is to provide readers with a complete view of the complex interactions we have explored, and retaining this data in the main text is essential to uphold the integrity of our work.

Indeed, we specifically chose to submit our manuscript to eLife because this journal allows to access supplemental information directly from the text and figures in the main manuscript and best aligned with our approach to data presentation. The eLife format permits us to offer readers a quick and informative overview of all the data within the main figures employing multivariate techniques such as Linear Discriminant Analysis or Principal Component Analysis. Subsequently, we supply more detailed analyses in the supplementary figures for readers who wish to delve deeper into specific aspects. Furthermore, while certain figures may be categorized as supplementary, for us it is crucial, and we would like to emphasize, that every result is comprehensively described in the main text.

Acknowledging the concerns raised about the density of our paper and the potential challenges in interpreting the findings, we have conducted a thorough review of the text and figure legends. We have made revisions with the goal to enhance clarity and readability. We have made dedicated efforts to ensure that readers can readily grasp the significance of our results and appreciate the intricacies of our findings. We firmly believe that with these revisions, our chosen approach is the most effective means of presenting the richness of our data and maintaining the integrity of our findings.

Lack of clarity in the behavioral analyses

Behavioral assessments are not clearly written in the methods. For example, for the novel object recognition task, it isn't clear how preference was calculated. Is this simply the percent of time spent with the novel object, or is this a relative measure (novel:familiar ratio)? This matters because if it is simply the percent of time, the relevant measure is to compare each group to 50% (the absence of a preference). The key measures for each test need to be readily distinguished from the control measures.

There are also many dependent behavioral measures. For example, speed and distance are directly related to each other, but these are typically reported as control measures to help interpret the key measure, which is the anxiety-like behavior. Similarly, some behavioral tests were used to represent multiple behavioral dimensions, such as anxiety and arousal. In general, the measurements of arousal seem atypical (speed and distance are typically reported as control measures, not measures of arousal). Similarly, measures of latency during training would not typically be used as a measure of long-term memory but instead reported as a control measure to show learning occurred. LDA analysis requires independence of the measures, as well as normality. It does not appear that all of the measures fed into this analysis would have met these assumptions, but the methods also do not clearly describe which measures were actually used in the LDA.

We agree with the reviewers' concerns about the clarity of our behavioral analyses and we have thus added information to the methods section to clarify the procedures. Specifically, for SPSN, social approach was recorded as time spent close to STR1, and a preference ratio was calculated as $\text{Pref} = 100 \times \text{Time close to STR1} / (\text{Time close to STR1} + \text{Time close to Empty})$. Social recognition memory was scored as preference towards STR2 and calculated as $\text{Pref} = 100 \times (\text{time close to STR2}) / (\text{Time close to STR1} + \text{Time close to STR2})$. For NOR, preference for novel object was calculated as $\text{Pref} = 100 \times \text{Time novel object} / (\text{Time familiar object} + \text{novel object})$.

With regards to the different variables reported for the behavioral protocols, we agree that some measures, such as path length and speed can be used as control measures. For example, in an open field test, path length is an important control measure to assess whether an animal is engaged in the task. However, if an animal is actively moving, the amount of distance covered can but does not have to correlate with the amount of time that a mouse

spends in the center of the open field. Using the measure of distance covered as a measure for general arousal and time spent in the center as a measure for anxiety related behavior allows a more nuanced evaluation of animal behavior. For instance, two animals spending similar amounts of time in the center may exhibit differences in the distance they cover. In this scenario, we would argue that anxiety related behavior (defined as exploring the center of an open field) would not reflect well a behavioral difference between the two animals, while the aspect of arousal clearly is a differencing factor.

Regarding the PA task and the use of latency during training, we agree that typically latency during training can be used as control measure to show that learning occurred. However, our study involved testing animals at two distinct time points. Contextual fear conditioning creates very robust memory traces that can persist for weeks or even months, and therefore the starting premise is very different when repeating the test. Initially, the animals were experimentally naïve and had not yet experienced a foot shock, leading to a rapid entry into the dark box. However, after experiencing the first CS-US presentation, a robust and persistent contextual fear memory trace is formed. Therefore, the latency observed in the second training phase of the PA reflects in essence long-term contextual fear memory, that is robustly displayed in WT animals but less in treated WT and TS animals. We have included this clarification in the methods and results sections.

Finally, we want to thank the reviewer for noticing the error in the LDAs, as the analysis was indeed performed including dependent variables for some systems. We have re-evaluated the LDAs for the behavioral tests and tibia microarchitecture tests, excluding dependent variables. As a result, the text and significance levels have been adjusted accordingly. To enhance transparency and clarity, we have included Supplementary Table S21, which precisely outlines the variables included in each LDA.

Unclear value of RNAseq

RNAseq was performed in cerebellum, a relatively spared region in DS pathology at an early time point in disease. Further, the expression of 125 genes triplicated in DS was shown in a PCA plot to highly overlap with WT, indicating that there are minimal differences in gene expression in these genes. If these genes are not critical for cerebellar function, perhaps this could account for the lack of differences between WT and Ts65Dn mice. If the authors are interested in performing RNAseq, it would have made more sense to perform this in hippocampus (to compare with metabolites) and to perform more stringent bioinformatic analysis than assessment by PCA of a limited subset of genes. Supplementary Table S14, which shows the differentially expressed genes, appears to be missing from the manuscript and cannot be evaluated. Additionally, the methods of the RNAseq are not sufficiently described and lack critical details. For example, what was the normalization performed, and which groups were compared to identify differentially expressed genes? It would also be worthwhile to describe how animals were identified for RNAseq-were those animals representative of their groups across other measures?

We acknowledge the reviewers' comments on the RNAseq analysis and would like to provide additional insights into our rationale and choices for this analysis. The primary aim of our RNAseq analysis was to offer supplementary evidence in support of the broader context of our paper. Rather than focusing on specific genes, our aim was to assess potential alterations in transcription within genes triplicated in the mouse model and explore differentially expressed genes across the entire genome. Therefore, we conducted a global analysis of the triplicated genes using a PCA and analyzed the differentially expressed genes across the entire genome as shown in Supplementary Table S14. The table was originally included as a separate Excel file but apparently it was not received by the reviewers. We have contacted the eLife editorial to ensure its inclusion in the current version. Furthermore, we have

modified the text to clarify that both the triplicated genes and the entire genome were analyzed.

Regarding the use of cerebellum instead of hippocampus, we agree with the reviewers that the hippocampus is a major tissue of interest in the study of Down Syndrome since it mostly relates to cognition. Trisomic patients, however, also display other typical features such as for example a delay in the acquisition of motor skills. Here we decided to focus on the cerebellum as it is primarily associated to the locomotor system but also plays a role in other cognitive functions such as language processing and memory. Furthermore, at the time of the RNAseq analysis, the mice were 8 months old, equivalent to the adult human stage, and previous studies have shown transcriptomic alterations in this tissue and mouse model (Olmos-Serrano et al., 2016; Saran et al., 2003).

The lack of observable differences between WT and Ts65Dn mice in our PCA analysis may be attributed to several factors as discussed in our article. First, the high variability within each group, inherent to the complexity of DS, may obscure inter-group differences. Additionally, the subtlety of gene expression differences between WT and trisomic mice in the set of triplicated genes, as suggested by other transcriptomics studies on DS (Ait Yahya-Graison et al., 2007; Lyle et al., 2004; Olmos-Serrano et al., 2016; Saran et al., 2003), may contribute to the limited distinctions observed. Furthermore, regarding treatment effects, the timing of the RNAseq analysis should be considered since it was conducted at the endpoint, three months after treatment cessation. This temporal aspect could imply that the effects of the drug are not persistent, and a molecular memory might not be formed and maintained.

Nevertheless, we appreciate the reviewers' constructive comments and acknowledge the potential for more stringent bioinformatic analyses. While our intention was to provide an initial, global perspective, we are eager to support further investigations that delve deeper into the complexities of DS-related molecular mechanisms. Consequently, the dataset is available for other researchers to explore more specific questions upon request.

Finally, we have updated the methods section of the article to offer more detailed information on RNAseq processing and analysis. We have also clarified that all the surviving mice were included in the analysis.

Recommendations for the authors:

(1) Please add power calculations for each of the assessments.

We would like to clarify that we had already conducted power calculations as part of the initial planning and design phase of our study. After data acquisition and analysis, we have utilized appropriate statistical methods to interpret the results based on the data we have collected. Given that we had conducted a priori power calculations prior to data collection and that our analysis is based on the acquired data, we do not see the added value in including post hoc power calculations. Our primary focus has been on performing the correct statistical analyses to accurately interpret the results and draw meaningful conclusions.

(2) Introduction has some excessive references for each statement, which are not necessary. For instance: lines 67-73 are only references for 1 statement and lines 74-76 are references for a 2nd statement in the same sentence.

We have removed redundant references.

(3) Introduction: Lines 136-146 Gene names need to be spelled out, not just the IDs. Were these studies done in human or mouse models of DS?

We have spelled out the names of the genes.

(4) Why was brain volume and brain structure size normalized to body weight, not clearly explained?

The choice to normalize brain volume and brain structure size to body weight was a deliberate decision made to address potential confounding factors in our study. In the case of trisomic (TS) mice, they are generally smaller in size compared to their wild-type (WT) counterparts. The same may hold true for sex-related size differences. Without normalization, assessing brain volume and structure size could be misleading, as it might reflect the differences in overall body size rather than providing insights into the specific aspects of brain structure that we aimed to investigate. We have clarified this in the methods section.

(5) In cognitive tests, some of the WT data represented in Figure 3 does not match supplemental findings. Again power calculations may indicate a higher number of WT mice are needed to clarify this discrepancy.

We appreciate the reviewers' observation regarding the disparities between the data presented in Figure 3 and the supplemental figures. We would like to clarify that these variations are a result of the distinct analytical approaches employed in the two sets of data.

In Figure 3 and all main figures, the data were analyzed using multivariate tests, which consider multiple variables simultaneously and are particularly suited for investigating the collective impact of multiple factors. Conversely, the results shown in the supplementary figures were derived from univariate tests, which focus on individual variables and are well-suited for addressing specific questions related to each variable in isolation. The discrepancies between the data in the main figures and the supplementary figures can be attributed to the differences in the analytical methods chosen.

As for the suggestion of conducting power calculations to address the observed differences, we believe that the differences in data are inherent to the distinct analytical strategies and the specific research questions each analysis intended to answer. Power calculations may not be the most suitable approach in this context, as they pertain to sample size planning for hypothesis testing and may not reconcile the inherent dissimilarity between multivariate and univariate analyses.

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