RESEARCH ARTICLE

Epigallocatechin-3-gallate, a DYRK1A inhibitor, rescues cognitive deficits in Down syndrome mouse models and in humans

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Scope: Trisomy for human chromosome 21 results in Down syndrome (DS), which is among the most complex genetic perturbations leading to intellectual disability. Accumulating data suggest that overexpression of the dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (DYRK1A), is a critical pathogenic mechanisms in the intellectual deficit.
Methods and results: Here we show that the green tea flavonol epigallocatechin-gallate (EGCG), a DYRK1A inhibitor, rescues the cognitive deficits of both segmental trisomy 16 (Ts65Dn) and transgenic mice overexpressing *Dyrk1A* in a trisomic or disomic genetic background, respectively. It also significantly reverses cognitive deficits in a pilot study in DS individuals with effects on memory recognition, working memory and quality of life. We used the mouse models to ensure that EGCG was able to reduce DYRK1A kinase activity in the hippocampus and found that it also induced significant changes in plasma homocysteine levels, which were

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Abbreviations: DS, Down syndrome; **DYRK1A**, dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A; **EGCG**, epigallocatechin gallate; **GSH-Px**, glutathione peroxidase; **Hcy**, Homocysteine; **HSA21**, human chromosome 21; **IQ**, Intellectual quotient; **oxLDL**, Oxidized LDL; **Tg*Dyrk1A***, Transgenic mice overexpressing *Dyrk1A*

correlated with *Dyrk1A* expression levels. Thus, we could use plasma homocysteine levels as an efficacy biomarker in our human study.
Conclusion: We conclude that EGCG is a promising therapeutic tool for cognitive enhancement in DS, and its efficacy may depend of *Dyrk1A* inhibition.

Keywords:
Cognition / Down syndrome / DYRK1A / Epigallocatechin gallate / Homocysteine

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1 Introduction

Health benefits of the green tea catechins on the prevention of cardiovascular diseases [1], cancer chemoprevention [2], infective [3], and as neuroprotectors in neurodegenerative disease [4] have been postulated. Nevertheless, their in vivo effectiveness and molecular mechanisms are difficult to elucidate and remain a challenging task. Nevertheless, the safety of green tea extracts and of its main active cathechin, the flavonol epigallocatechin gallate (EGCG) should easy the translation from in vitro and animal models to human clinical trials. Recently, the effectiveness of EGCG on the promotion of adult hippocampal neurogenesis has been reported [5] and invites its application in clinical neurodevelopmental and neurodegenerative disorders. In this context a previous report also suggested the beneficial effects of EGCG in animal models of Down syndrome (DS) [6].

Trisomy for human chromosome 21 (HSA21) results in DS (OMIM 190685) and is one of the most common human chromosomal disorders. While the trisomy affects every tissue, in DS reduced cognitive ability is among the most limiting features [7, 8]. Even though there is certainly a contribution of noncoding regions to the phenotype, it is likely that the dosage imbalance of some individual genes on HSA21 directly contributes to some phenotypes. Thus, normalizing expression levels or the function of critical genes could prevent or reverse the deleterious effects of gene overdose. Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (*Dyrk1A*) is a plausible candidate gene to explain some DS phenotypes [reviewed in [9, 10]]. It is localized in the DS critical region of the HSA21 [11, 12] and encodes for a dual kinase phosphorylating both threonine/serine and tyrosine residues. In mice dosage imbalance of *Dyrk1A* in vivo affects brain structure and learning and memory [13, 14]. Reduced *Dyrk1A* expression in heterozygous mice produces microcephaly [15], and compromises neuritogenesis [16] leading to reduced length and complexity of dendritic branches. Heterozygous mice also show visuospatial learning and memory deficits. Similarly, *Dyrk1A* overexpression in transgenic mice (Tg*Dyrk1A*) leads to cognitive impairment and less complex dendritic trees [17, 18]. Intriguingly, cognition impairment and dendritic tree alteration in Tg*Dyrk1A* recapitulates that of Ts65Dn mice, a DS mouse model bearing in trisomy 88 of 161 classical protein coding genes present on HSA21, [19–21]. This suggests *Dyrk1A* as the underlying cause and points its normalization as potential DS therapy.

EGCG is a potent and selective inhibitor of DYRK1A activity [22]. Previous work showed that prenatal EGCG treatment could partially rescue brain alterations in transgenic pups overexpressing *Dyrk1A* and was able to normalize the levels of some synaptic plasticity related proteins in the hippocampus of adult *Dyrk1A* transgenic mice suggesting possible cognitive effects [6]. We here have explored for the first time if EGCG could rescue the cognitive alteration in adult DS mice and in a pilot study in DS subjects. We have used trisomic and transgenic mice to determine the possible effects of short-term EGCG treatment in improving cognitive function, in particular on memory deficits in adults and to identify biomarkers of EGCG efficacy to normalize *Dyrk1A* activity that could then be used in patients. Then, we have carried out a randomized, double-blind, placebo-controlled pilot study evaluating the safety of EGCG and preliminarily its clinical effects in young adults with DS. We conclude that EGCG rescues cognitive deficits in trisomic (Ts65Dn) mice and DS individuals possibly through normalizing DYRK1A activity.

2 Materials and methods

2.1 Preclinical pharmacological studies

2.1.1 Mouse models

Tg*Dyrk1A* were obtained as previously described [13]. Female B6EiC3Sn a/A-Ts (1716) 65Dn (Ts65Dn) mice were obtained from Jackson Laboratories (Bar Harbor, ME) and bred with euploid B6C3BF1 males [23]. Young adult (3 months of age) male mice were randomly distributed in four groups (Tg*Dyrk1A* or Ts65Dn and their corresponding wild-type littermates, on placebo or EGCG). Of interest for the present work, *Dyrk1A* over dosage in Tg*Dyrk1A* yielded similar levels of overexpression than those detected in both the foetal DS brains and the partial trisomic Ts65Dn model [24]. We used the following transgenic (Tg*Dyrk1A*) groups: WT nontreated $n = 13$; TG (Tg*Dyrk1A*)-nontreated $n = 16$; WT-EGCG $n = 16$; TG-EGCG $n = 14$. For the trisomic (Ts65Dn) we used: WT-nontreated $n = 19$; Ts65Dn nontreated $n = 14$; WT-EGCG $n = 22$; Ts65Dn-EGCG $n = 14$.

2.1.2 Ethical statement

Animals were bred and housed under SPF standard environmental conditions, and all experimental procedures were performed in compliance with animal welfare policies and approved by the local ethical committee (Comité Ético de Experimentación Animal del PRBB (CEEA-PRBB); procedure numbers MDS-08–1060P1 and JMC-07–1001P1-MDS). All met local (Spanish law 5/1995 and decrees 214/97, 32/2007; French Ministry of Agriculture law 87/848) and European (EU directives 86/609 and 2001–486) regulations and the Standards for Use of Laboratory Animals n° A5388–01 (NIH). The CRG is authorized to work with genetically modified organisms (A/ES/05/I-13 and A/ES/05/14). The research personnel involved in the experiments were granted the official accreditation to perform the reported experiments.

2.1.3 EGCG administration

Mice were administered EGCG in drinking water for 1 month. EGCG solution was prepared freshly from a green tea extract [Mega Green Tea Extract, Lightly Caffeinated® (0.8% caffeine) Life Extension®, USA; EGCG content of 326.25 mg per capsule] every 3 days (EGCG concentration: 90 mg/mL for a dose of 2–3 mg per day). To test EGCG cognitive effects, Tg*Dyrk1A* or Ts65Dn and their corresponding wild-type littermates, on placebo or EGCG, were tested in a hippocampal-dependent learning using a water maze (WM; see [13, 25]) and a novel object recognition task.

2.1.4 Learning and memory tests

2.1.4.1 Water maze

To test the effect of EGCG on hippocampal-dependent spatial cognition, mice were trained in a water maze (WM) after being administered EGCG in drinking water during 30 days. Tg*Dyrk1A* or Ts65Dn and their corresponding wild-type littermates, on placebo or EGCG were tested. The water maze consisted of a circular pool filled with tepid water (19°C) opacified by addition of white nontoxic paint. A white escape platform was located 1 cm below the water surface in a fixed position. White curtains with affixed black patterns surrounded the maze to provide an arrangement of spatial cues. Mice learned the position of the hidden platform in four training (acquisition) trials per day during 5 or 6 days, depending on the strain [see [9]], until the animals reached the asymptotic performance levels (best execution level). In each trial, mice were placed at one of the starting locations in random order and were allowed to swim until they located the platform. Mice failing to find the platform within 60 s were placed on it for 20 s and were returned to their home cage at the end of every trial. To assess the reference memory a probe session was performed 24 h after last acquisition session. In this session, the platform was removed and mice were allowed to swim for 60 s. All the trials were recorded and traced with an image tracking system (SMART, Panlab, Spain). Escape latencies, swimming speed, percentage of time spent in each quadrant of the pool were monitored and computed. Reference memory was quantified in the probe session comparing the amount of time mice spent in the target quadrant versus the average of the three other quadrants of the pool.

2.1.4.2 Novel object recognition

Mice were tested for learning and memory deficits in the novel object recognition task [25]. This task is based on the innate tendency of rodents to differentially explore novel objects over familiar ones. Tg*Dyrk1A* or Ts65Dn and their corresponding wild-type littermates, on placebo or EGCG, were given a habituation session in a circular arena for 30 min on a single day. In the training session 24 h later, mice were placed in the centre of the arena and allowed to explore two identical objects during 10 min. Following the training period, the rodent was removed from the environment for a delay period which ranged from 1 to 24 h, depending on the type of memory being tested in each strain. After the delay, the rodent is returned to the bin, where a new one replaced one of the original objects. For each mouse, the objects were randomly assigned as either familiar or novel. Also the location of the novel objects (left or right) is counterbalanced between groups. The exploration time for the familiar (TF) and the new object (TN) during the test phase was recorded. Memory was operationally defined by the percentage of exploration of the novel object (discrimination index DI) as the proportion of time that animals spent investigating the novel object minus the proportion spent investigating the familiar one in the testing period [Discrimination Index, DI = [(Novel Object Exploration Time – Familiar Object Exploration Time)/ Total Exploration Time] × 100].

2.1.5. Biochemical analysis

2.1.5.1 DYRK1A activity

Kinase activity of DYRK1A protein was determined from hippocampus (6 mice per group) according to previously published protocol [26].

2.1.5.2 Plasma biomarkers of *Dyrk1A* activity: Homocysteine

The effect of EGCG on plasma homocysteine (Hcy) was evaluated since *Dyrk1A* expression is correlated with plasma Hcy in DS [27] Plasma total Hcy, defined as the total concentration of Hcy after quantitative reductive cleavage of all disulfide bonds, was determined using a fluorimetric HPLC method [28]. We also checked if chronic EGCG administration was able to significantly modify *Dyrk1A* activity in brain samples of Tg*Dyrk1A* transgenic mice.

3. Pilot clinical trial

3.1 Participants

Thirty-one young adults with DS aged 14 to 29 years old were enrolled from a large cohort of outpatients of the "Fundació Catalana Síndrome de Down" (FCSD, Barcelona).

3.2 Exclusion criteria

Subjects with neurological disease other than DS, relevant medical disease, comorbid mental disorder or currently taking any treatment that could interfere with cognitive function or alter any key biomarkers and biochemical parameters analyzed in the study were excluded. Other common exclusion criteria applied to all the participants were: (i) having suffered from any major illness or undergoing major surgery in the last 3 months before the study; (ii) regular medication in the month preceding the study. Exceptions were made for single doses of symptomatic medication administered up to the week preceding the trial; (iii) current ingestion of vitamin supplements or catechins or AINE in the 2 weeks preceding the study; (iv) history of gastrointestinal, hepatic, renal or any other problems that may alter absorption, distribution, metabolism, or excretion of the drug; (v) subjects under vegetarian diet; (vi) practice of physical exercise for more than 2 h per day or energy consumption of more than 3000 kcal per week.

3.3 Randomization and masking

The study design was randomized, double blind and parallel groups. Subjects were randomly assigned to EGCG ($n = 15$; same preparation as for animal models) or placebo ($n = 16$, identical hard gelatine capsules containing brown sugar).

3.4. Ethical statement

The study was approved by the local ethics committee (CEIC-Parc de Salut Mar, EGCG/DYRC1A/DS/IMIM/1, ClinicalTrials.gov Identifier: NCT01394796) and conducted in accordance with the ethical standards of Helsinki Declaration. Participants, parents and/or legal guardians (in case of legal incapacitation) were informed of the ensuing protocol and gave their written informed consent before enrolment.

3.5. EGCG administration

Participants assigned to the treatment group or placebo received one or two daily capsules depending on body weight, with a mean EGCG oral dose of 9 mg/kg/day (range 6.9–12.7). During the enrolment visit, participants received dietary recommendations to avoid food supplemented with folic acid and raw green vegetables. Medical, biochemical, and neuropsychological explorations were performed at the Hospital del Mar Medical Research Institute (IMIM), and consisted of four evaluations during a 6 months period: baseline, after 1 and 3 months of treatment, and 3 months after treatment discontinuation. Twenty-nine participants completed the 6 months trial: 13 of the EGCG group (two withdrawals) and 16 of the placebo group. Two participants experienced side effects: one in the EGCG group presented increased excitability that required withdrawal of treatment and one in the placebo-treated group required dose reduction due to abdominal pain (from 2 to 1 capsule per day) but continued in the study. Another subject was excluded due to nonreliable compliance of EGCG administration (Supporting Information Table 1; CONSORT flow diagram; http://www.consort-statement.org/, supplementary materials).

3.6 Neuropsychological testing

A comprehensive neuropsychological battery was administered assessing psychomotor speed, attention, episodic memory, executive functions, and visuomotor precision (see Supporting Information Table 3). Tests were presented in a fixed order to allow adequate intertrial intervals on episodic memory measurements. Parallel versions available for episodic memory and executive function tests were used during the follow-up assessments, to control for learning effects. Parents and caregivers completed scales of functional ability in daily living, adaptive behavior and quality of life. Concerning computerized tests, only adult versions of the selected tests from the Cambridge Neuropsychological Test Automated Battery (CANTAB) were used.

3.7 Qualitative data on treatment effects

A brief semistructured self-made interview was conducted with parents at the end of the 6 month trial with the aim to obtain feedback on the performance of the study and to collect qualitative data on their subjective impressions on several parameters of interest related with treatment effects: notion of group assignment (treatment/placebo) before breaking the blind, presence or absence of relevant changes along the study (functional, cognitive, behavioral, clinical, or others) and specific changes observed.

3.8 Biochemical analysis

Blood samples (25 mL) were collected after 10–14 h fasting for general biochemical analyses. Total Hcy concentrations were determined by micro particle enzyme immunoassay (MEIA, Abbott Laboratories, Abbott Park, Ill, USA). Serum aspartate

aminotransferase (AST/GOT) and alanine transaminase (ALT/GPT), glucose, total cholesterol and triglycerides were analyzed by standard enzymatic methods. LDL-cholesterol was calculated by the Friedewald formula whenever triglycerides were <300 mg/dL. Oxidized LDL (oxLDL) in plasma was measured by a sandwich ELISA procedure (oxLDL, Mercodia, Uppsala, Sweden). Plasma glutathione peroxidase (GSH-Px) activity was measured using cumene hydroperoxide as glutathione oxidant (Ransel RS 505, Randox Laboratories, Crumlin, UK). Treatment compliance and bioavailability were analyzed measuring plasma EGCG by HPLC/MS.

3.9 Statistical analysis

For mice experiments, we assessed significant differences using two-way ANOVA followed by Bonferroni/Dunnet post hoc tests. Treatment effect on plasma Hcy was analyzed using two-way (genotype*treatment) ANOVA followed by Bonferroni/Dunnet post hoc test. Differences were considered significant at $p < 0.05$. Spearman correlation analyses of Hcy levels and *Dyrk1a* levels were first performed separately on wild-type and Ts65Dn mice ($p < 0.1$ was considered significant); the two groups were therefore analyzed together. Data were expressed as mean ± SEM. The SPSS statistical software was used for the analysis.

For DS individuals, a descriptive analysis of the sociodemographic, clinical and biochemical parameters of both groups (EGCG and placebo) at baseline was performed by means of absolute and relative frequencies in the case of categorical variables, as well as mean and SD measures for quantitative ones. Treatment effect after 3 months was estimated for clinical, biochemical and cognitive variables within the framework of ANCOVA models. To avoid a possible bias due to group differences at baseline, these models included both baseline values and gender as covariates. Additionally, the intellectual disability level (mild/moderate versus severe) was included in the models for the cognitive variables. We calculated 95% confidence intervals for the treatment effect. The statistical software package R (The R Foundation for Statistical Computing) was used for all the analyses.

4 Results

4.1. EGCG rescues cognitive deficits in Ts65Dn and Tg*Dyrk1A* mice

In the Morris water maze, although nontreated Tg*Dyrk1A* mice overexpressing *Dyrk1A* in an otherwise disomic genetic background showed a slight, though nonsignificant impairment in visuospatial learning and memory, their performance was significantly improved by EGCG (Fig. 1A, ANOVA repeated measures, treatment effect, $p < 0.05$). Poor learning strategies, assessed as percentage of time spent in the periphery of the pool (thigmotaxia), were also observed in nontreated Tg*Dyrk1A* (Supporting Information Fig. 1A, ANOVA with Bonferroni, $p < 0.01$), that were rescued by EGCG (Supporting Information Fig. 1A, ANOVA with Bonferroni, $p < 0.01$). Finally, nontreated Tg*Dyrk1A* showed impaired reference memory (Fig. 1C, ANOVA followed by post hoc Bonferroni analysis, $p < 0.01$) that was again improved by EGCG (Fig. 1C, ANOVA followed by post hoc Bonferroni analysis, $p < 0.05$). Conversely, EGCG did not modify the performance of wild-type mice (Fig. 1A).

To ensure that the effect of EGCG was relevant for DS, we tested the treatment in the Ts65Dn partial trisomic mouse model. Ts65Dn mice showed spatial learning deficits (Fig. 1B, ANOVA repeated measures, genotype effect: $p < 0.0001$), and a marked thigmotactic behavior during the acquisition sessions (Supporting Information Fig. 1B, ANOVA repeated measures, genotype effect: $p < 0.0001$), that were rescued by EGCG (Fig. 1B and Supporting Information Fig. 1B, ANOVA repeated measures treatment effect, $p < 0.05$) without affecting performance in wild-types. In the probe test, Ts65Dn mice showed again a thigmotactic strategy (Fig. 1D, ANOVA with Bonferroni $p < 0.001$) that was recovered by EGCG treatment (Fig. 1D, ANOVA with Bonferroni, $p < 0.05$).

EGCG also rescued the object recognition memory impairment exhibited by Ts65Dn and Tg*Dyrk1A* mice in the novel object recognition test. Both Ts65Dn and Tg*Dyrk1A* mice showed a clear deficit in novelty recognition as shown by a significantly lower recognition index (Fig. 2A and 2B, two-way ANOVA genotype × object interaction with Bonferroni as post hoc: WT nontreated versus Tg*Dyrk1A* nontreated $p < 0.01$; WT nontreated versus Ts65Dn nontreated $p < 0.01$). EGCG significantly restored novel object recognition in both transgenic (Fig. 2A, two-way ANOVA genotype × treatment interaction with Bonferroni as post hoc: Tg*Dyrk1A* nontreated versus Tg*Dyrk1A* EGCG $p < 0.01$) and Ts65Dn (Fig. 2B, $p < 0.01$). Interestingly, EGCG impaired novel object recognition in wild-type mice (Fig. 2A, WT nontreated versus WT EGCG: two-way ANOVA genotype-treatment interaction with Bonferroni as post hoc $p < 0.01$).

4.2 D*yrk1A* activity and plasma biomarkers

DYRK1A kinase activity was increased in the hippocampus of Tg*Dyrk1A* mice (Fig. 3A, one-way ANOVA $p < 0.01$) due to *Dyrk1A* overexpression. EGCG normalized DYRK1A kinase activity in transgenic animals but it did not induce significant modification in wild-type mice (Fig. 3A, two-way ANOVA genotype-treatment interaction with Bonferroni as post hoc $p < 0.01$).

Hcy in plasma were correlated with *Dyrk1A* brain levels (Fig. 3D; Spearman correlation Rho = 0.73; $p = 0.027$). In basal conditions, a decrease of Hcy plasma levels was detected in Tg*Dyrk1A* and Ts65Dn mice (Fig. 3B and 3C), as has also been described in DS patients [29]. One month of

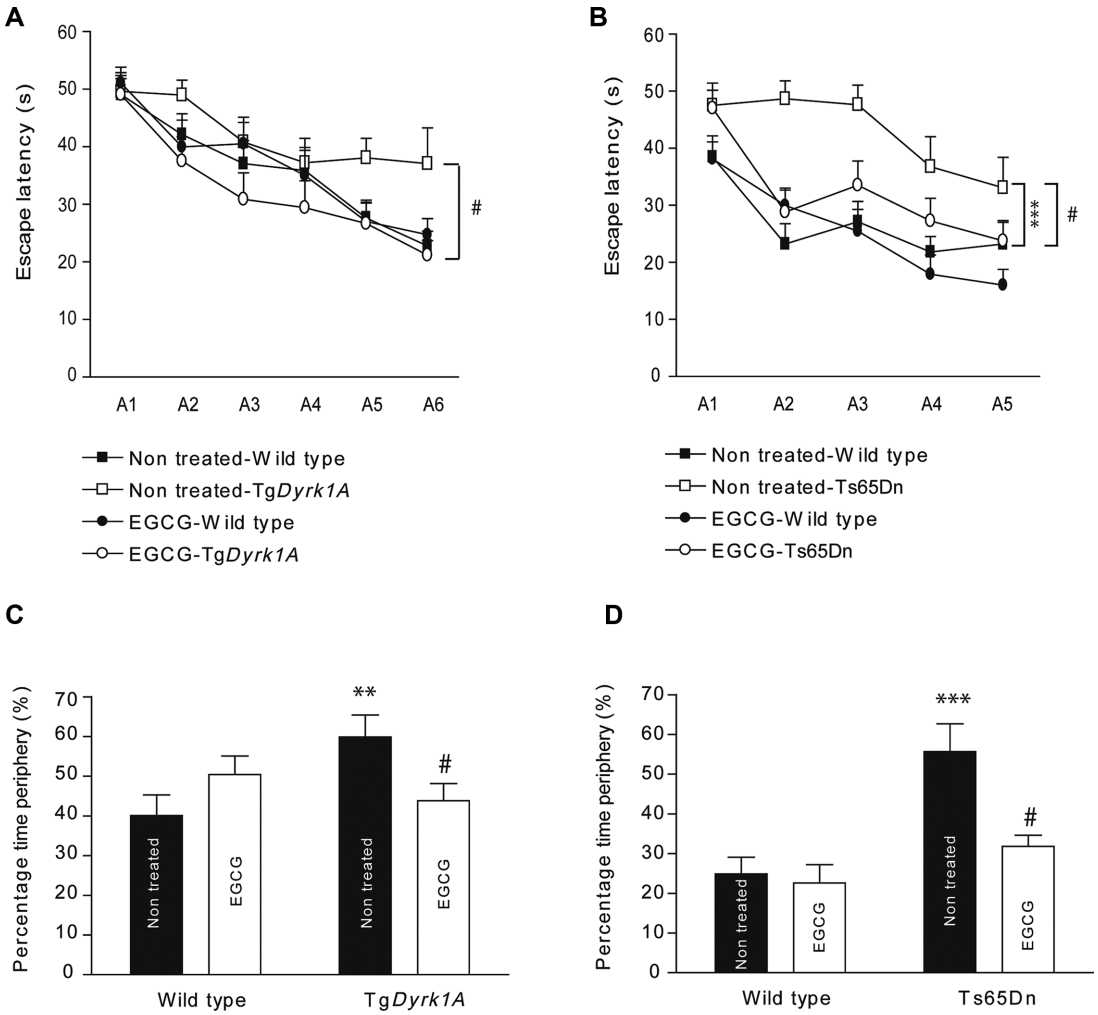

Figure 1. Effects of EGCG on performance in the visuospatial learning and memory task in Tg*Dyrk1A* and Ts65Dn mouse models. (A, B) Time to reach the platform (escape latency) during learning sessions in (A) Tg*Dyrk1A* and (B) Ts65Dn. (C, D) Percentage of time spent in the periphery (thigmotaxis) of the pool during the probe session in (C) Tg*Dyrk1A* and (D) Ts65Dn. Data are represented as mean ± SEM. Genotype effect * $p < 0.05$, *** $p < 0.001$; treatment effect # $p < 0.05$.

treatment with EGCG normalized Hcy plasma levels of both Tg*Dyrk1A* and Ts65Dn to untreated wild-type levels although the effect only reached statistical significance in trisomic mice (Fig. 3B and 3C, two-way ANOVA genotype-treatment interaction with Bonferroni as post hoc: Tg*Dyrk1A* $p = 0.06$; Ts65Dn $p < 0.01$).

4.3 Pilot clinical trial

Twenty-nine participants (Table 1) balanced for gender (51.7% male and 48.2% female) and age (22.2 ± 4.2 years in EGCG and 20.6 ± 2.2 in placebo) between treatment groups, were enrolled. Both groups had similar school attendance in special or standard education centres (14.86 ± 2.6 versus 15.38 ± 2.4 for EGCG and placebo group respectively). Average intellectual quotient (IQ) was similar for both groups, within the range of moderate intellectual disability (IQ = 45.9 ± 7.8 in EGCG versus 42.4 ± 6.4 in placebo). EGCG group showed a higher rate of individuals with trisomy 21 (76·9%) against placebo group (68·7%). In addition, one individual had translocation (7·6%) in the EGCG group whereas one individual was mosaic (6·2%) in the placebo group. DS genetic variations of six individuals remained unknown, two (15·3%) in the EGCG group and four (25%) in the placebo group.

The clinical and biochemical evaluation revealed no significant differences between groups in AST (GOT) or ALT (GPT) values after 3 months of treatment (Supporting Information Table 2). Plasma concentrations of cholesterol (placebo 173.7 ± 26.0 versus EGCG 153.8 ± 22.0 mg/dL, $p = 0.02$), and LDL-cholesterol (111.6 ± 24.8 versus 88.9 ± 22.1 mg/dL, $p = 0.014$) were significantly reduced after 3 months of EGCG treatment. No treatment effects were observed on

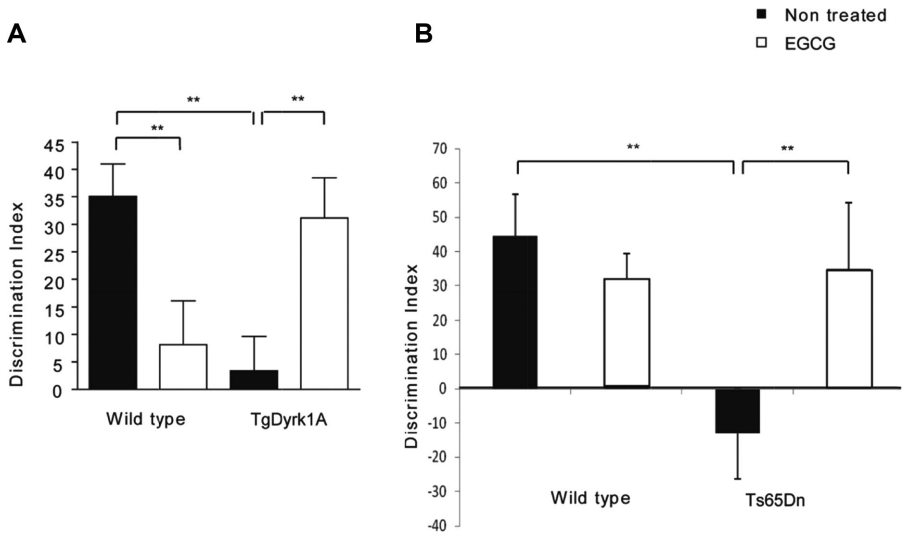

Figure 2. Effect of the EGCG treatment in the novel object recognition test. (A, B) Discrimination index during the test session in (A) Tg*Dyrk1A* and (B) Ts65Dn mice. Data are represented as mean ± SEM. Two-way ANOVA with Bonferroni * $p < 0.05$, ** $p < 0.01$.

triglycerides, HDL-cholesterol, glucose, and GSH-Px activity (Supporting Information Table 2).

Although plasma EGCG concentrations did not differ between genders or time of evaluation (94.4 ± 45.2 versus 89.4 ± 49.3 ng/mL, first versus third month), a significant increase of Hcy was detected after 1 month of treatment, (Treatment effect at 1 month $p = 0.024$, 9.7 ± 1.5 versus 11.6 ± 2.6, paired Student's *t*-test) remaining below the

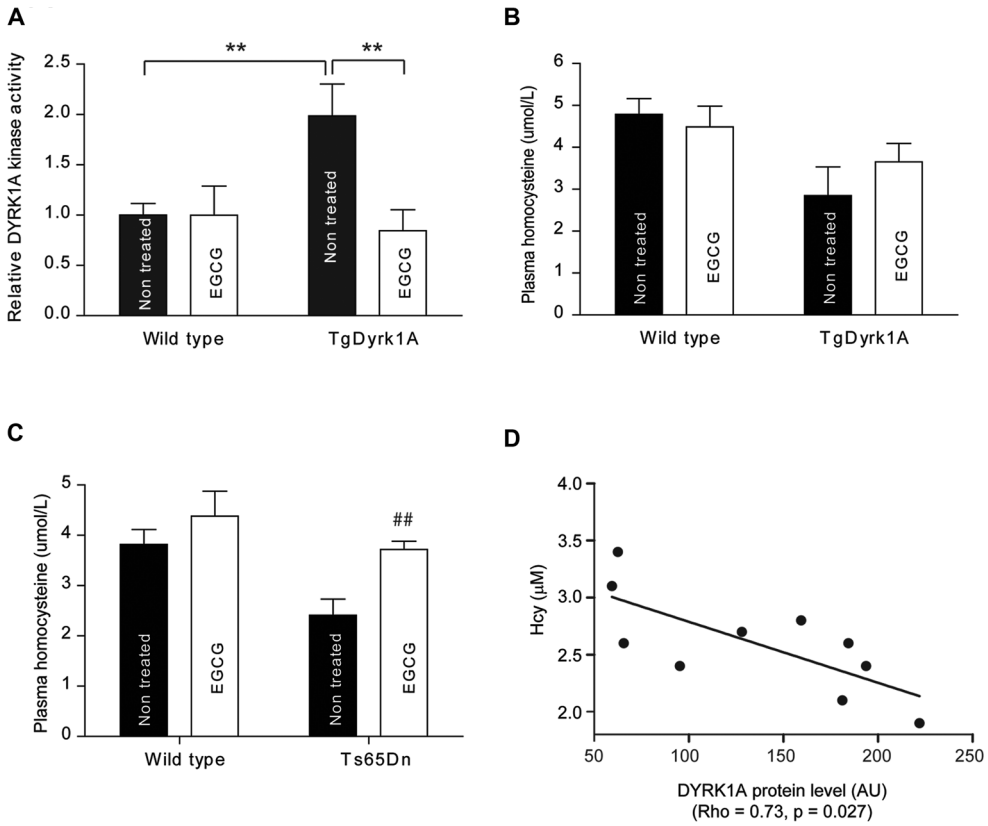

Figure 3. Effect of the EGCG treatment on *Dyrk1A* activity and plasma Hcy levels. (A) DYRK1A kinase activity was normalized after 1 month of EGCG treatment in the hippocampus of Tg*Dyrk1A* mice. Plasma Hcy levels were normalized to wild-type levels in TS65Dn (C) (Two way ANOVA genotype × treatment, followed by Bonferroni/Dunnet post hoc $p < 0.01$) and the same tendency was detected in Tg*Dyrk1A* (B) mice, although it did not reach statistical significance ($p = 0.06$). (D) Brain DYRK1A protein level is negatively correlated with plasma Hcy level in Ts65Dn ($p = 0.027$).

Table 1. Sociodemographic characteristics and clinical parameters at baseline

	EGCG ($n = 13$)	Placebo ($n = 16$)
Age	22.2 (4.2)	20.6 (2.2)
Sex		
Female	6 (46.2%)	8 (50.0%)
Male	7 (53.8%)	8 (50.0%)
Education (years)	14.86 (2.6)	15.38 (2.4)
Intellectual disability level		
Mild/Moderate	8 (61.5%)	5 (31.2%)
Severe	5 (38.5%)	11 (68.7%)
Intellectual quotient (IQ)		
K-BIT (standardized score)	45.9 (7.8)	42.4 (6.4)
DS genetic variations		
Trisomy 21	10 (76.9%)	11 (68.7%)
Mosaic	0	1 (6.2%)
Translocation	1 (7.6%)	0
Unknown	2 (15.3%)	4 (25.0%)
Weight	60.1 (13.3)	56 (10.9)
Body Mass Index (BMI)	25.4 (4.0)	23.2 (3.8)

Results are presented as mean (SD) for continuous variables and absolute frequency (relative frequency) for categorical variables.

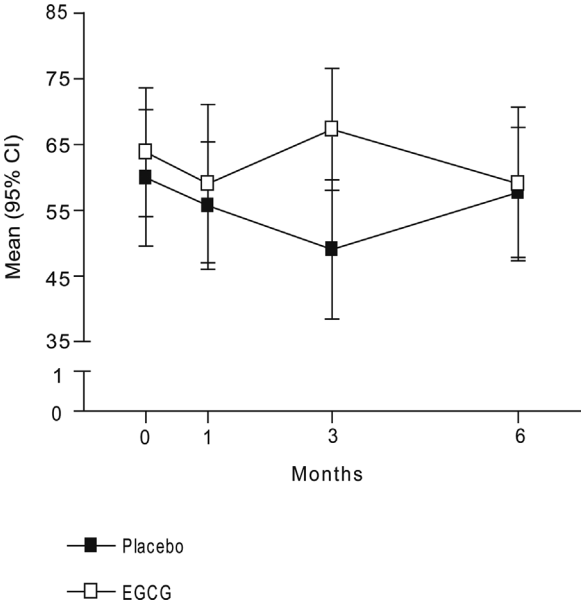

Figure 4. Positive subclinical effects of short-term EGCG treatment on episodic memory. Longitudinal analyses of EGCG treated individuals and placebo group for memory recognition [pattern recognition memory immediate recall percentage correct]. The figure shows 95% confidence interval of the mean score at each evaluation.

critical value (15–20 μM) that was maintained along treatment, and returned to baseline after treatment discontinuation.

Supporting Information Table 3 displays EGCG effects on neuropsychological performance adjusted for gender, IQ, and baseline values. We found significant effects of EGCG on episodic memory and working memory after 3 months of treatment. EGCG-treated individuals showed a significantly higher percentage of correct answers in visual memory recognition (immediate pattern recognition memory-percentage correct; $p = 0.04$) compared to placebo (Fig. 4). A tendency to significant differences was also found for working memory performance between EGCG and placebo groups, the former showing a lower error rate (Spatial Span backwards total errors; $p = 0.08$). Also, the analyses showed a trend to significant EGCG effects on psychomotor speed (MOT) and improved social functioning on the Kidscreen-27 quality of life index (Social Support & Peers; $p = 0.05$; Supporting Information Table 4) compared to placebo. After 3 months of treatment discontinuation, the effects declined and percentage of correct answers in the immediate pattern recognition memory decreased returning to baseline measures (Fig. 4). In addition, the descriptive analysis on treatment effects collected from the parents at the end of the trial (Supporting Information Table 5) showed that most parents correctly guessed the treatment assignment, and parents with EGCG treated children reported a broad range of changes, referring specific positive effects, whereas most parents of placebo condition reported no relevant changes.

5 Discussion

The aim of the present study was to investigate the potential benefits of normalizing the activity of DYRK1A; a kinase overdosed in DS on cognitive performance in mouse models and human DS individuals. We here report improvement of cognitive function both in DS mouse models and humans treated with EGCG, a specific inhibitor of DYRK1A. We also took advantage of the animal models to proof the use of Hcy plasma levels, as a biomarker of hippocampal DYRK1A activity. Our study supports the use of DYRK1A inhibitors, and specifically of EGCG, as promising therapeutic tools for cognitive enhancement in DS.

In previous work, we observed that EGCG could partially rescue some brain morphological alterations in transgenic pups overexpressing *Dyrk1A* [6] and normalized the levels of some synaptic plasticity related proteins in the hippocampus of adult *Dyrk1A* transgenic mice. However, in this initial work, the mice tested only overexpressed *Dyrk1A* in an otherwise disomic genetic context, and besides we did not know the possible cognitive effects, if any. In the present study we used again mice with single overexpression of *Dyrk1A* in a disomic genetic context (Tg*Dyrk1A*) but they were adult, and we also included in the analysis a partial trisomic well-established DS model, the Ts65Dn mice [23]. Our results indicate that 1 month of oral administration of EGCG rescues the hippocampal-dependent learning deficit in both Tg*Dyrk1A* and Ts65Dn without affecting the performance of wild-types.

Besides a general effect on hippocampal dependent learning, inadequate learning strategies (thigmotaxia) were reduced in EGCG-treated Ts65Dn and Tg*Dyrk1A* mice. Thus, we can conclude that EGCG was able to rescue the phenotypes derived of *Dyrk1A* overexpression in transgenic mice and in the partial trisomy model, Ts65Dn. This suggested that the pathological effect driven by *Dyrk1A* overexpression is relevant for DS, and that *Dyrk1A* is a strong candidate for therapy. Our previous work [30] already demonstrated that functional cortico-striatal defects could be corrected through the specific inhibition of *Dyrk1A* expression using adenoviral vectors (AAVshDyrk1A) in Tg*Dyrk1A* mice [31], but here we show that also kinase activity normalization in adult mice can rescue the cognitive phenotypes. However, since *Dyrk1A* is a dosage sensitive gene, the reduction of its kinase activity could be deleterious in wild-type mice. It may be argued that the different brain regions involved in both tasks have differential sensitivity to DYRK1A kinase functionality. However, although EGCG normalized DYRK1A kinase activity in the hippocampus in Tg*Dyrk1A* at the same dose regime that improved hippocampal-dependent tests (Fig. 3A), but surprisingly, the same doses did not modify kinase activity in wild-type mice. This would fit with the lack of effects observed in the Morris water maze in wild-type mice, and may suggest that the deleterious effects in the object recognition test could depend on changes of the *Dyrk1A* activity in cortical regions that we did not test or to other effects of the polyphenol not measured in the present study.

Since we wanted to evaluate the effects of the treatment in humans, the next step was to ensure that DYRK1A kinase activity normalization could be measured using a plasma biomarker that could be then detected upon treatment with EGCG in humans. Our previous studies showed that overexpression of *Dyrk1A* is related to decreased Hcy plasma concentrations in Tg*Dyrk1A* and Ts65Dn mice suggesting that Hcy could be a suitable biomarker correlated with *Dyrk1A* in DS [27]. We here demonstrated a significant correlation between Hcy in plasma and brain levels of *Dyrk1A* (Fig. 3D) in Ts65Dn mice. Ts65Dn mice showed decreased Hcy plasma levels similar to what has been described in DS individuals [29]. One month of treatment with EGCG normalized the reduced levels of plasma Hcy of both Tg*Dyrk1A* and Ts65Dn to untreated wild-type levels. These experiments served as a proof of concept that the learning deficits caused by *Dyrk1A* overexpression could be rescued by *Dyrk1A* activity normalization and that plasma Hcy levels could be used as a biomarker.

We then explored the effects of EGCG on cognitive performance in young adults with DS, paying special attention to hippocampal function. Concretely, besides other measures (see Section 4) we used a visual recognition task that measured visuoperceptual processing, the weakest component of visual memory in DS individuals. This measure has proven to be sensitive to hippocampal functioning, in particular of perirhinal cortex, which is critically involved in object recognition memory [32–35] and is reduced in size in DS [36]. EGCG treated individuals showed higher accuracy in visual memory recognition [37, 38] and spatial working memory [39], suggesting a positive effect of this compound also on the prefrontal system, in particular ventromedial, ventrolateral and dorsolateral cortices. Overall, the results from this pilot study are consistent with EGCG effects found in Tg*Dyrk1A* and Ts65Dn mice and tentatively indicate that short-term EGCG treatment is able to induce beneficial effects on cognition in young adults with DS, probably acting on distributed hippocampal-prefrontal functional networks supporting memory abilities.

EGCG administration also induced positive effects on quality of life and social functioning. This was detected by using objective measures such as the Kidscreen battery, but also in a parent/caregivers survey in which subjective positive impressions of mild cognitive enhancement and/or improved behavioral control over a wide array of cognitive/behavioral skills were reported in EGCG treated individuals. Interestingly, while parents reported improved verbal cognitive abilities, that are less preserved in DS, and considered one of the landmarks of global intellectual impairment in these individuals [40, 41] and a significant impact of EGCG cognitive enhancement on everyday living, the neuropsychological testing could not detect these effects. Such discrepancy could respond to a low sensitivity of the functional assessment tools used.

One important aspect was safety and toxicity, since DS individuals may be more sensitive than euploids to some adverse effect. EGCG has been demonstrated to be a safe substance after repeated administration in humans. No alteration of hepatic function markers (AST and ALT) was observed. Animal studies have reported EGCG to be involved in redox cycling and quinone formation having both antioxidant and pro-oxidative activities and being able to induce oxidative stress [42]. We found no increase of biomarkers of oxidative stress (oxLDL and GSH-Px activity), but a reduction of lipid oxidation in subjects treated with EGCG. The improvement on the oxidative/antioxidative status combined with a healthy lipid profile, shown by the total cholesterol and LDL cholesterol concentrations can be considered beneficial.

Importantly, as for mice, in humans the biochemical results allowed to establish a clear relationship between memory improvement and Hcy levels, suggesting a direct dependence of cognitive improvement on *Dyrk1A* activity. Moreover, after 3 months of treatment discontinuation EGCG cognitive effects decline along with a parallel decrease in plasma Hcy levels, suggesting that *Dyrk1A* activity normalization induced cognitive changes through temporary neuronal changes. This is important since *Dyrk1A* has been related to synaptic plasticity in the brain that may lead to stable structural modifications in neural circuits [15, 16, 18]. This has functional consequences, since DS learning and memory problems become considerably more noticeable through childhood and adolescence and appear to be related to an inability to "stabilize" information that is acquired, a problem in memory consolidation that is a function of the hippocampal system (see [10]). Consolidation of labile

information storage requires the translation of short-lasting biochemical synaptic events into more stable functional and structural changes. This has been shown in trisomic mouse models of DS and also in Tg*Dyrk1A* and is proposed to underlie the reduced stability of learning in the human phenotype (see [11] for review). By normalizing *Dyrk1A* activity, EGCG is possibly favoring learning through these mechanisms, and thus it could be speculated that it could be especially beneficial if paired with interventions that also increase plasticity, such as cognitive stimulation.

In conclusion, we here demonstrate the positive effects of EGCG on learning and memory deficits of DS mouse models and humans, thus supporting further research on the use of EGCG as a potential therapeutic agent for improving cognitive deficits in young DS adults. Hcy plasma concentrations are proposed as a good biomarker of efficacy of the treatment.

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