Low dose EGCG treatment beginning in adolescence does not improve cognitive impairment in a Down syndrome mouse model

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Down syndrome (DS) or Trisomy 21 causes intellectual disabilities in humans and the Ts65Dn
DS mouse model is deficient in learning and memory tasks. *DYRK1A* is triplicated in DS and
Ts65Dn mice. Ts65Dn mice were given up to ~20 mg/kg/day Epigallocatechin-3-gallate
(EGCG), a Dyrk1a inhibitor, or water beginning on postnatal day 24 and continuing for three or
seven weeks, and were tested on a series of behavioral and learning tasks, including a novel
balance beam test. Ts65Dn as compared to control mice exhibited higher locomotor activity,
impaired novel object recognition, impaired balance beam and decreased spatial learning and
memory. Neither EGCG treatment improved performance of the Ts65Dn mice on these tasks.
Ts65Dn mice had a non-significant increase in Dyrk1a activity in the hippocampus and
cerebellum. Given the translational value of the Ts65Dn mouse model, further studies will be
needed to identify the EGCG doses (and mechanisms) that may improve cognitive function.

Key words: Trisomy 21, Down syndrome, treatment, mouse model, cognition
1. Introduction

Down syndrome (DS) results from the triplication of human chromosome 21 (Hsa21) (Lejeune, 1959) and has a prevalence rate of ~1 in 700 live births (Parker et al., 2010). The most recognizable features of DS are related to cognitive impairment expressed in varying levels of severity among all individuals with DS (Delabar et al., 1993; Roubertoux & Kerdelhue, 2006). Individuals with DS display developmental alterations in brain morphology including reductions in sizes of the prefrontal cortex, cerebellum, hippocampus, amygdala, and brain stem in newborn individuals with DS (Aylward et al., 1997; Dierssen, 2012; Guidi et al., 2008), and structural abnormalities such as reduced dendritic and axonal number and volume, and altered synaptic plasticity (L. Becker, Mito, Takashima, & Onodera, 1991; Coppus et al., 2006; Dierssen, 2012). These brain deficits in individuals with DS are associated with cognitive and intellectual deficits including memory deficiencies, and motor dysfunction.

With increasing numbers of individuals living with DS (Mai et al., 2013; Presson et al., 2013), there is an urgent need to find treatments that can be applied across the life span to improve cognitive function (Gardiner, 2015). Underscoring this priority, 61% of parents of children with DS indicated they would like to reverse the intellectual disability (ID) associated with DS (Inglis, Lohn, Austin, & Hippman, 2014). Caregivers increasingly seek alternatives on their own; a study found that 83% of families were currently using or had used complementary or alternative medicine to alleviate symptoms of DS (Prussing, Sobo, Walker, & Kurtin, 2005). Treatment of DS has been called “an achievable goal” (Underwood, 2014). Rational
development of safe, effective and feasible treatments that provide enduring improvement of
cognitive functions across the life span is one of most pressing public health needs of the DS
community.

Mouse models of DS that recapitulate many of the genetic and phenotypic features
attributed to DS provide key advantages for preclinical development of therapeutic treatments of
DS. The Ts(1716)65Dn mouse (Ts65Dn), the most widely used and well-characterized model in
the study of DS, has a small trisomic chromosome that triplicates about half of the gene
orthologs found on Hsa21 (Reeves et al., 1995; Sturgeon & Gardiner, 2011). Ts65Dn mice
exhibit many of the central nervous system (CNS) phenotypes related to cognitive impairment in
DS including abnormal dendritic spine density and structure, altered hippocampal structure with
reduced number of neurons in the dentate gyrus and CA3 regions, and severe reductions in LTP
(Belichenko et al., 2009; Belichenko, Kleschevnikov, Salehi, Epstein, & Mobley, 2007;
Belichenko et al., 2004; Insausti et al., 1998; Kleschevnikov et al., 2004). Ts65Dn mice also
show a reduction in cerebellum size, as well as the number of cerebral granule cells (Baxter,
Moran, Richardsmeier, Troncoso, & Reeves, 2000). Ts65Dn mice have significant deficits in tasks
that depend on the functional integrity of the hippocampal formation and associated neocortical
circuits, including the Morris water maze spatial learning task and novel object recognition
(Escorihuela et al., 1995; L. A. Hyde, Frisone, & Crnic, 2001; Reeves et al., 1995; Sago et al.,
2000). Locomotor activity of Ts65Dn mice typically is also significantly increased relative to
control mice, consistent with a phenotype of hyperactivity (Holtzman et al., 1996;
Kleschevnikov et al., 2012; Sago et al., 2000).

Recent reports that oral epigallocatechin gallate (EGCG) treatment in mouse models of
DS and in humans with DS may be effective in improving cognitive impairment has ignited
interest in EGCG as a pharmacotherapy with an etiological basis (De la Torre et al., 2014). EGCG treatment (~9mg/kg/day) for 3 months in individuals with DS (age 14-29) significantly improved visual recognition memory and produced borderline improvement in working memory performance, psychomotor speed, and social functioning (De la Torre et al., 2014). EGCG is a known protein kinase inhibitor of Dyrk1a (Dual specificity tyrosine-regulated kinase 1a), one of the genes found in three copies on Hsa21 as well as in Ts65Dn mice (Tejedor & Hammerle, 2011). Dyrk1a protein levels are increased in the frontal, temporal, occipital, and cerebellar cortices as well as cerebral and cerebellar white matter to ~1.5 fold normal in individuals with DS (Dowjat et al., 2007). Dyrk1a protein was also found at ~1.5 control levels in cortex, cerebellum and hippocampus of Ts65Dn mice (Souchet et al., 2014). Genetic perturbance of Dyrk1a levels in humans have been shown to result in microcephaly, seizures, and developmental delay (Moller et al., 2008). Dyrk1a is a promising drug target because small molecules can attach to the ATP binding site of the Dyrk1a protein kinase (W. Becker, Soppa, & Tejedor, 2014).

Previous studies using EGCG (~90 mg/mL) in adult Ts65Dn or TgDyrk1a mice report significant cognitive and brain structural improvements (De la Torre et al., 2014; Pons-Espinal, Martinez de Lagran, & Dierssen, 2013). Previous work in our lab using a three-week low-dose EGCG treatment (~10 mg/kg/day) during adolescence resulted in improvements in skeletal deficits in trisomic mice (Blazek, Abeysekera, Li, & Roper, 2015). A similar dose (~9 mg/kg/day in capsule form) was used in the human study that showed improvements in cognition and more positive caregiver reports (De la Torre et al., 2014). One of the complications, however, with EGCG treatment in the preclinical studies is that it undergoes rapid degradation, resulting in diminishing EGCG concentrations over time when it is delivered via drinking water.
Therefore, it is critical to establish the range of doses and concentrations with oral delivery of EGCG that produce therapeutic effects for specific phenotypes before translating preclinical outcomes to large-scale clinical applications. Given our previous demonstration of significant improvements in skeletal deficits of Ts65Dn mice with the dosage of ~10mg/kg per day, we hypothesized that a similar concentration of EGCG given during adolescence, either for 3 weeks or continuously into young adulthood, would also improve cognitive deficits observed in young adult trisomic mice. We treated Ts65Dn mice beginning in early adolescence at a dose that was similar to that used in the human study and assessed its effects on locomotor activity and a series of learning and memory tasks that are sensitive either to hippocampal and frontal cortical dysfunction [spatial working memory (delayed non-matching to place), place learning and spatial reference memory (Morris water maze) and episodic recognition memory (novel object recognition)] or to cerebellar dysfunction [balance beam performance].

2. Materials and Methods

2.1 Animals

Ts65Dn females (approximately 50% B6 and 50% C3H background with small trisomic marker chromosome) were bred to B6C3F1 males (both obtained from the Jackson Laboratory (Bar Harbor, ME)) in rooms with a standard 12:12 light:dark cycle to generate the mice used in the study. Only male mice were used; it was necessary to retain females as breeders for colony maintenance. On postnatal day (PD) 21, the male mice were weaned and single-housed in standard mouse cages, and randomly assigned to the different EGCG treatment groups. The vivarium containing the mice undergoing treatment was maintained on a reverse 12:12 light:dark cycle with white light off between 0800-2000, during which time only red light was present.
Experiments with animals were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and received prior approval from the IACUC committee at IUPUI.

2.2 EGCG treatment

EGCG (Sigma Aldrich, >95% purity) was prepared by making a stock solution of 15 mg/mL in phosphate buffered saline (PBS). Treatments were delivered via the drinking water in a concentration of 0.124 mg/mL, prepared by diluting the stock solution in tap water. Treatments started on postnatal day (PD) 24, usually 3 days after weaning. For the three-week treatments, all mice were provided water on PD 45; for the continuous groups, the treatments continued throughout the duration of behavioral testing, which ended on PD 70. For the groups given three-week treatments, the initial cohorts were given EGCG or water that was not pH adjusted. However, based on our findings that EGCG significantly degrades under these conditions (see below), the EGCG (and water) treatments of remaining cohorts of the three-week groups and all of the continuous groups were adjusted to an acidic pH (~5.5) by the addition of H₃PO₄, to stabilize the EGCG in solution (see below). The group numbers for the three-week treatments were as follows: Ts65Dn—EGCG + H₃PO₄ n=10, EGCG n= 14, water + H₃PO₄ n=9, water n=9; Euploid—EGCG + H₃PO₄ n=8, EGCG n=13, water + H₃PO₄ n= 8, water n=17. The group numbers for the continuous treatments (all pH adjusted) were as follows: Ts65Dn—EGCG n=8, water n=9; Euploid—EGCG n=12, water n=13. Treatments were placed in drinking tubes and the mice were allowed ad libitum access to its designated treatment as its sole source of fluid. The volumes consumed and animal weights were recorded every 48 hours when the treatments were changed.
2.2.1 Degradation analysis

The potent antioxidant nature of EGCG leads to the rapid degradation of catechins under room temperature conditions, leading to reduced concentration of available EGCG (Ferruzzi, Peters, Green, & Janle, 2010). Therefore, we carried out a HPLC/MS based degradation analysis on EGCG and EGCG + H₃PO₄ (both prepared from the Sigma Aldrich EGCG), and 2 commercial sources of EGCG (Life Extension decaffeinated mega green tea extract, [DC EGCG] and Life Extension lightly caffeinated mega green tea extract, [LC EGCG]) that were used in a previous study (De la Torre et al., 2014). Stock solutions of 10 mg/mL of EGCG were prepared by dissolving the substances in PBS. Samples were diluted to a final concentration of 1 mg/mL EGCG in tap water and kept under room conditions in drinking tubes with exposure to normal room light to correspond to our treatment protocols. The sample dilutions were prepared in triplicates to be tested 1, 24, and 48 hours following their preparation.

Samples were analyzed using an Agilent 1200SL HPLC instrument coupled with an Agilent 6520 quadrupole time-of-flight mass spectrometer (MS). Samples were separated using reverse phase chromatography with a Zorbax Eclipse Plus C18 column (2.1 mm diameter, 50 mm length, 1.8 micron particle size) operating at a temperature of 40°C. Solvents of water with 0.1% formic acid and acetonitrile with 0.1% formic acid were used with a stepwise gradient starting with 10% of acetonitrile with 0.1% formic acid and ending with 95% of acetonitrile with 0.1% formic acid over 5 minutes. Ultra-violet absorption was used to quantify the organic EGCG compound at a wavelength of 230 nm. Calibration curves were prepared using EGCG with concentrations ranging from 0.1-1 mg/mL. MS using electrospray negative ionization operating in auto-MSMS mode was used for confirmation of the EGCG peak and to confirm the exact mass and possible formula assignments of other compounds and their fragment. Results were
analyzed using MassHunter qualitative (mass analysis and formula assignments) and quantitative (UV quantitation) analysis software packages.

2.3 Locomotor activity (LMA)

Ts65Dn and control mice were placed in activity chambers (Med Associates Inc., St. Albans, VT) having plexiglass long walls and aluminum side walls, with each chamber (25.0 × 13.75 × 15.0 cm) equipped with an array of infrared beams positioned 2.5 cm above the floor. The chambers were housed in light- and sound-attenuated cubicles with ventilation fans on one side of the cubicle. Activity testing was conducted in the dark during the dark cycle beginning ~5 hours after light offset, with 30-minute sessions given on two consecutive days. The chambers were thoroughly cleaned with 70% ethanol before each test session, and the mice were transported to and from the testing room in their home cages that were shielded from room light. The LMA sessions occurred on postnatal days (PD) 45 and 46.

2.4 Novel object recognition (NOR)

The NOR task was performed using a modification of previously described protocols (Babovic et al., 2008; O'Tuathaigh et al., 2010), and was conducted during the dark cycle (1100-1600 hrs) under red light (11-13 lux). Four sets of object pairs were used; across pairs, the objects differed in terms of component materials (e.g., plastic; metal; glass; rubber) and visuo-spatial features (e.g., shape; contrast), but had overall similar dimensions (8.5-11 cm tall; 3-4.5 cm base). Testing occurred over three consecutive days. The first was a habituation day in which the animals were placed individually inside the plywood test box (41 cm × 41 cm × 41 cm, painted medium gray) and allowed to explore the environment for 15 minutes. On the next day
(object exposure day), two matching objects were placed near the NW and SE corners of the arena, 5 cm from each wall, and secured with Velcro on the bottom of the object. Each mouse was placed in the middle of the arena and allowed 15 minutes to explore the arena and objects. On the final day (test day) one object from the previous day and one novel object were placed in the NW and SE corners, with object position assigned randomly, and the mice were given 15 minutes to explore. Activity on each day was recorded with video tracking using ANYMAZE software (Stoelting Co, Wooddale, IL). Video recordings from the exposure and test days were scored for the time spent exploring each object by a minimum of three independent scorers who were blind to the genotype and treatment of the mice. The Discrimination ratios of the time exploring each object on the test day were determined using the following formula:

\[
\text{Discrimination ratio (\%)} = \frac{(\text{Time exploring novel object} - \text{Time exploring familiar object})}{\text{Total exploration time for both objects}} \times 100.
\]

Two NOR tests were given. The first was on PD 50-52 and the second was on PD 68-70 (1 day after water maze testing was completed). For each animal, care was taken to insure that objects used on the second NOR test were not used in its first NOR test.

2.5 T-maze delayed non-matching to place (DNMP)

The first 25 mice of the three-week treatment group were tested on the DNMP task. The mice were gradually food restricted over the course of one week until they reached 85% of free-feeding weight and then were trained to perform the DNMP task using a procedure modified from a previous report (Goodlett, Nonneman, Valentino, & West, 1988). The DNMP apparatus consisted of a wooden T-maze painted flat gray with a start arm and goal arms each 40 cm long, 18 cm high and 12 cm wide, with doors at the start arm (12 cm long) and at the entrance to each
arm. The training was conducted in 3 phases: In phase 1, mice were given three days of
habituation and allowed to freely explore the maze and obtain chocolate milk (Ensure) at the end
of each arm. Phase 2 consisted of alley training in which the mice were given 8 trials of “forced
choices”, half to the left and half to the right in a pseudorandom sequence. In these trials, a
mouse was placed in start area and, after 5 seconds, the door to the start arm was opened and the
mouse was allowed to traverse to the end of a randomly selected goal arm (with the other arm
blocked off) to gain access to the chocolate milk. Once the mice performed Phase 2 consistently
with no hesitation in approaching or drinking the milk, the DNMP training began (Phase 3). In
Phase 3, each mouse was given 8 “trial couplets”; in which the first trial of each couple was a
“forced” run, in which one arm was blocked off and the entry was available to only one arm
from the start arm. After a 10 second period to consume the milk, the mouse was placed back
into the start area, both arms were opened and the milk was made available in the arm opposite
from the previous arm and the mouse was allowed to choose either of the two arms. A self-
correction procedure was followed, such that if the mouse chose the incorrect arm (without
milk), it was allowed to move back through the maze until it entered the goal arm (with milk).
After each trial couplet, the mouse was returned to its home cage for 30 seconds before the start
of the next trial couplet. Across the eight daily trials, the open arm on the forced trail of each
couplet occurred in each direction on half of the trials (4 left, 4 right) in pseudorandom
sequences. Errors on choice trials were scored whenever a mouse entered the incorrect arm (i.e.,
had all four paws in the arm). Latencies to make the initial choice on the forced trail and on the
choice trial were also recorded. Training continued until each mouse reached a criterion of
≥80% correct (at least 7 out of 8) on three consecutive days, or to a maximum of 25 days of
training (reached by one trisomic mouse).
2.6 Balance Beam Task

The balance beam task was performed following a previously described protocol, with modifications (Luong, 2011). The apparatus consisted of 1 m painted wooden beams with a surface width of either 19 mm, 12 mm or 9 mm, situated 58 cm above the floor. The beams were supported by two metal poles, with metal clamps attaching the beams to the rods, and a darkened goal box containing bedding was placed at the end of the beam. The test was conducted in red light (8-10 lux) approximately one hour into the dark cycle. On the first day, a cohort of mice (2-5 mice) was brought into the testing room and left to acclimate to the room for approximately 10 minutes. A handful of bedding (familiar home cage bedding mixed with fresh bedding) was placed into the black goal box, and the mouse was trained to walk from one side of the beam to the goal box. The mouse was first placed about 36 cm from the goal box, and then at increasing distances on subsequent trails, until they were able to traverse the entire length of the beam without stopping. After successfully traversing the entire length of the 19 mm-wide beam, the mouse was allowed to remain in the black box for 30 seconds, and was then returned to its home cage. The beam was wiped with 70% ethanol, and the bedding from the black box was discarded and replaced with new bedding mixture for the next mouse. If the mouse froze or stalled on the beam, the investigator gently prodded it from the back. Training continued on the second day, with the mice being trained to cross the entire length of the 12 mm-wide beam without stopping on three consecutive trials. On the third day, the mice were tested on three trials each on the 12 mm- and 9 mm-wide beams. A Logitech camera at the opposite end of the goal box recorded these trials. The video records were scored by three trained independent scorers, blind to genotype or treatment, to quantify the number of hind paw slips, defined as either the left or right hind paw entirely missing the beam.
2.7 Morris water maze place learning task (MWM)

The Morris water maze (MWM) task was conducted following a previously published protocol (Wagner, Zhou, & Goodlett, 2014). The mice began training in the water maze at ~60 days of age, by an experimenter blind to treatment and genotype. The computer-controlled tracking software (HVS Image, Hampton, UK) recorded and displayed the moment-to-moment position of the animal in the pool. The mice were given 7 days of acquisition training, in which a 9 cm (diameter) white platform was placed in a 125 cm (diameter) pool filled to within 25 cm of the rim of the tank with 26º C water made opaque by adding non-toxic white paint. The top of the submerged platform was 0.5 cm below the surface of the water. Testing was conducted in dim white light (maximum of 32 lux), and various visual cues were prominently placed on the room walls outside the tank. Training consisted of placing the mouse in the pool at one of 7 possible start points (randomly assigned across trials). The animal was allowed to swim for 60 seconds or until it located and climbed onto the platform. If the platform was not found in the 60 seconds, the experimenter gently moved the mouse to the platform. After remaining on the platform for 10 seconds, the mouse was then removed and placed in a heated incubator (30º C) to limit hypothermia during testing. Mice were tested in squads of 3-4, resulting in an inter-trial interval of approximately 3-4 minutes. At the end of each day of testing, the mice remained in their respective incubators for approximately 10 minutes before being returned to the vivarium. Measures obtained during each acquisition trial included latency to find the platform (sec), path length (cm), time spent within 25.4 cm of the wall (thigmotaxis), time spent not moving (floating), and swimming speed (cm/sec). Twenty-four hours after the last training day, the mice were given a 60-sec “probe trial” in which the platform was removed and the animal’s search path was recorded and scored for spatial biases. The HVS Image software superimposed four
virtual counting discs (27 cm diameter) in the center of each quadrant (over the four possible platform positions used). Probe trial measures included time spent in, latency to enter, and numbers of crossings through each of the four virtual counting discs, and these measures were used to quantify the spatial distribution of the search strategy of each animal by comparing the target location (trained during acquisition) to the three non-target locations. Because the pattern and outcomes of the data for number of crossing of each disc were similar to and redundant with the data for time spent in each disc, only the time measure is reported.

2.8 Dyrk1a kinase activity assay

Protein was isolated from the hippocampus and cerebellum of 6 week old mice in RIPA buffer and quantified using a Bradford’s assay (Bradford, 1976). A Dyrk1a kinase activity assay was performed as previously published (Papadopoulos et al., 2011; Pons-Espinal et al., 2013) with modifications. Briefly, the protein sample was cleared of any antibodies by pre-incubation with EZ-view Red Protein affinity gel (Sigma-Aldrich, St Louis, MO). The lysate was immunoprecipitated with a mouse anti-Dyrk1a antibody (7D10, Abnova, Taipei City, Taiwan) and immobilized on glutathione-sepharose beads (4B, bioWORLD, Dublin, OH). To determine the kinase activity of Dyrk1a, 300 μg of the purified protein was incubated at 30° C with 1x kinase buffer, 100 μM ATP, and 200 μM Dyrktide (RRRFRPASPLRGPPK, SignalChem, Richmond, BC) (Himpel et al., 2000) and 2 μCi/sample of [γ-32] ATP. Following incubation, the reaction was arrested by adding 1/3 volume 100 mM EDTA. 10 μL of reaction aliquots were dotted in triplicate onto P81 Whatman paper. After washing extensively (8 × 1mL) with 5% phosphoric acid under vacuum conditions, counts were determined in a liquid scintillation counter (Beckman, Pasadena, CA), 32P levels were detected, and relative kinase activity was
determined by subtracting background activity from euploid and trisomic samples and normalizing kinase activity to euploid levels.

### 2.9 Statistical Analyses

A two way mixed ANOVA was used to examine the degradation (concentration after 1, 24 and 48 hours) of EGCG, with EGCG source as a grouping factor and time as the repeated measure. The discrimination ratios from the NOR data on the test day were analyzed with a two way ANOVA using genotype and treatment as a between subjects variable. A two-way (genotype × treatment) ANOVA was also used to examine the number of trials to criterion on the DNMP task. The average daily latency and path length over the seven days of training were analyzed using a mixed ANOVA with day as a repeated measure and genotype and treatment as between-group factors. For the probe trial, the time spent in the virtual target disc (in the target quadrant) and the average time spent in the 3 equivalent virtual non-target discs (in the other 3 quadrants) were analyzed using a mixed ANOVA with treatment group and genotype as between-group factors and disc location (target vs non-target) as a repeated measure. The number of slips on the balance beam were analyzed using a mixed ANOVA with treatment group and genotype as between-group factors, and beam width (12 mm and 9 mm as a repeated measure. Specific post hoc comparisons between groups to follow up significant ANOVA outcomes were performed using Fisher's least significant difference (LSD) tests ($\alpha=0.05$).

### 3. Results

#### 3.1 EGCG degradation, growth and EGCG intake, and Dyrk1a kinase activity analyses
**EGCG degradation.** As shown in Fig. 1a, the LC/MS analysis revealed that EGCG degrades over 48 hours under room temperature conditions, but the EGCG maintained in the acidified water showed less degradation than EGCG in non-acidified water (reduced to 46% vs. 17% of initial concentrations, respectively). The EGCG in the two Life Extension extracts, also shown in Fig. 1a for comparison, significantly degraded over 48 hours, to 40% and 30% of initial concentrations. A repeated measures ANOVA on the two pure EGCG solutions yielded a significant fluid type × time interaction \([F (6,16)=22.26, p<0.001]\), due to the significantly higher EGCG concentration of the EGCG + H₃PO₄ versus the unstabilized EGCG at both the 24- and 48-hour time points \([(p<0.001) for each]\).

**Growth.** In both the 3-week and in the continuous treatments, the Ts65Dn mice had lower body weights throughout the study. For the 3-week groups, the use of H₃PO₄ in the drinking water to stabilize the EGCG (compared to non-acidified treatments) did not significantly affect body growth or for any of the subsequent behavioral measures, so the data for all measures obtained from the 3-week treatment groups were combined across the subgroups given acidified or non-acidified fluids. Fig. 1b shows the body weights across the entire treatment period for the groups given continuous treatment (stabilized EGCG). There was a significant effect of genotype on growth \([F(1,41)=22.62]\), due to the higher body weights of the euploid mice compared to trisomic mice throughout the experiment. There was no significant main or interactive effect of EGCG treatment on growth.

Fig. 1c shows the average consumption of acid-stabilized EGCG (in mg/kg per day) over the seven weeks of treatment in the continuous groups. Based on the initial concentration of EGCG provided at the beginning of each 2-day interval (0.124 mg/ml) and an average 2-day concentration of 50% of initial concentration (based on the above 2-day degradation studies of
stabilized EGCG), the EGCG consumption was adjusted for loss due to degradation over each 48-hour period using the following formula: 2-day EGCG intake = \{(mls of fluid consumed over 2 days) \times (0.5 \times 0.124 \text{ mg/ml EGCG})\} / \text{bodyweight (kg)}. On a mg/kg basis, the Ts65Dn mice consumed higher quantities of EGCG than did euploid controls (average of 18.1 vs. 15.4 mg/kg/day over the seven weeks), confirmed by a main effect of genotype [F(1,20)=7.735, p=0.012]. In addition, the EGCG intake relative to body weight decreased with age for both groups [main effect of day, F(21,420)=12.185, p<0.001].

*Dyrk1a Activity (see Table 1).* In protein isolated from the hippocampus of six week old Ts65Dn or euploid mice from a subset of the groups given three weeks of treatment, there was a non-significant 1.4 fold increase (p=0.17) in Dyrk1a kinase activity of Ts65Dn (n=6) as compared to euploid (n=7) mice for the groups given water. The EGCG treatment of the Ts65Dn mice (n=4) resulted in Dyrk1a kinase activity of 1.2 fold relative to the euploid-water group (p=0.27), but this was not significantly less than the Ts65Dn + water group. In cerebellum of these same mice, there was a non-significant trend (p=0.065) for the Ts65Dn group given water toward increased Dyrk1a kinase activity relative to euploid-water group, increased by 2.8 fold; the EGCG treatment in Ts65Dn mice (n=3) reduced cerebellar Dyrk1a activity to 1.2 fold as compared to euploid mice, though this did not reach statistical significance relative to the Ts65Dn mice given water.

*{Insert Table 1 Here}*

3.2 Locomotor activity-3 week treatment

Fig. 2 shows locomotor activity as a function of distance traveled per 1-minute bins on each of the two days of testing. The Ts65Dn mice were more active than euploid controls,
confirmed by a significant effect of genotype \( [F(1, 85) = 9.83, p = 0.002] \). There was no significant main or interactive effect of EGCG treatment. The activity of all groups was higher on the first five minutes of testing each day, particularly on the second day of testing \( [\text{day} \times \text{bin interaction}, F(29, 2465) = 20.96, p<0.001] \), and the activity of the Ts65Dn mice was moderately but significantly higher than euploid mice over both testing days \( [\text{Day 1 main effect of genotype}, F(1, 85) = 6.18, p = 0.015; \text{Day 2 main effect of genotype}, F(1, 85) = 11.93, p = 0.001] \).

3.3 NOR-3 week treatment

Fig. 3 shows the discrimination ratios for the first NOR test of the groups given EGCG for three weeks. There was a significant effect of genotype \( [F(1, 79) = 5.617, p = 0.020] \), due to the overall lower discrimination ratios of the Ts65Dn mice relative to euploid controls. There was no significant main or interactive effect of EGCG treatment on this NOR test. In the second NOR test, there were no significant main or interactive effects of genotype or treatment.

3.4 NOR-continuous treatment

For the groups given continuous treatment, there were no significant main or interactive effects of genotype or treatment either on the first NOR test or on the second NOR task for the groups give EGCG treatment throughout behavioral testing (data not shown).

3.5 DNMP task-3 week treatment

As shown in Table 2, the trisomic mice required significantly more trials to reach acquisition criterion compared to euploid controls \( [\text{main effect of genotype}, F(1, 21) = 6.56, p = 0.018] \). There was no significant main or interactive effect of the three-week EGCG treatment
on DNMP acquisition. Latencies to choice on either the forced trials or the choice trials did not differ significantly among groups (data not shown).

3.6 MWM task-3 week treatment

For acquisition of place learning in the MWM, latencies and path lengths to find the hidden platform were strongly correlated (r=0.841, p<0.001), so only the latency data are shown in Fig. 4a. For the groups given treatment for three weeks, all groups showed significant reductions in latencies and path lengths over days [main effect of day, F(6,228)=17.89, p<0.001, for latencies; F(6,228)=15.21, p<0.001, for path lengths]. However, the trisomic mice showed significant acquisition deficits as indicated by longer average latencies [main effect of genotype: F (1,38)=16.99, p<0.001] and average path lengths [main effect of genotype, F (1,38)=9.156, p=0.004] to find the hidden platform, compared to euploid mice. The trisomic mice also had significantly higher thigmotaxis scores [F(1,38)=9.23, p<0.004]. All groups showed an increase in swimming speed over days [main effect of day, F(6,228)=2.44, p=0.027]; however, there were no significant group differences in swimming speed, indicating the euploid and trisomic groups did not differ in this measure of swimming performance.

During the probe trial, the euploid mice spent more time in the target disc than did the trisomic mice, whereas time in the non-target discs did not differ (see Fig. 4b). The impaired spatial search bias, consistent with a deficit in spatial memory in the Ts65Dn mice, was confirmed by a significant interaction of genotype × location [F(1,38)=7.907, p=0.008]. The euploid-water group spent significantly more time in the target disc than in the non-target discs (p=0.004), and the euploid-EGCG groups showed a strong trend that did not reach significance (p=0.057). Neither of the trisomic groups showed a significant bias for the target disc. LSD post
hoc tests between groups showed that the target time for the euploid-water group was significantly higher compared to both the trisomic-water group (p=0.025) and trisomic-EGCG group (p=0.001). The target time of the euploid-EGCG group was significantly higher than the trisomic-EGCG group (p=0.045). There also was a main effect of EGCG treatment on probe trial performance [F(1,38)=4.4, p=0.042], in that the EGCG-treated groups spent less time in the target disc than the water-treated groups, regardless of genotype. Importantly, there were no significant group differences in the time spent in the non-target discs.

{Insert Fig. 4a and 4b Here}

3.7 MWM task-continuous treatment

In the seven week treatment group, latencies and path lengths to find the hidden platform were strongly correlated (r=0.800, p<0.001), so only latency data are shown in Fig. 5a. All groups showed significant reductions in latencies and path lengths over days [main effect of day, F(6,252)=12.06, p<0.001 for latencies; F(6,252)=15.52, p<0.001 for path length]. However, the Ts65Dn mice were significantly impaired versus controls, as indicated by the day × genotype interaction for latency [F(6,252)=5.282, p<0.001]; as well as path length [F(6,252)=4.44, p<0.001]. The trisomic mice also had significantly higher thigmotaxis scores than euploid controls [main effect of genotype, F(1,42)=7.28, p<0.010; day × genotype interaction, F(6,252)=2.93, p=0.009]. There was a main effect of day on swimming speed [F(6,252)=3.88, p=0.001], however there were no effects of genotype or treatment on swimming speed, again indicating the euploid and trisomic groups did not differ on this measures of swimming performance.

During the probe trial, the euploid mice spent more time in the target disc than did the trisomic mice, whereas time in the non-target discs did not differ (see Fig. 5b). The impaired
spatial search bias, consistent with a deficit in spatial memory in the Ts65Dn mice, was confirmed by a significant interaction of genotype × location \[F(1,42)=8.469, p=0.006\]. Both the euploid-water group and the euploid-EGCG group spent significantly more time in the target disc than in the non-target discs (p=0.001 and p=0.002, respectively). Neither of the trisomic groups, however, showed a significant bias for the target disc. LSD post hoc tests showed that the target time for the euploid-water group was significantly higher compared to both the trisomic-water group (p=0.009) and trisomic-EGCG group (p=0.004). The target time of the euploid-EGCG group was not significantly higher than the trisomic-water group, but was significantly higher than the trisomic-EGCG group (p=0.043). There were no significant group differences in time in the non-target discs.

3.8 Balance Beam-continuous treatment

As shown in Fig. 6, the trisomic mice were impaired on the balance beam task, committing more hind paw slips on the test day, with the impairment being particularly evident on the narrow (9 mm) beam [main effect of genotype, \(F(1,36)=10.258, p=0.003\); genotype × treatment × beam width interaction, \(F(1,36)=4.48, p=0.041\)]. The Ts65Dn mice given water committed significantly more slips on the 9 mm beam compared to the 12 mm beam, and were significantly impaired relative to both euploid groups on the 9 mm beam (p<0.05). The EGCG treatment did not improve the performance of the Ts65Dn mice; in fact, the trisomic-EGCG group was significantly impaired on the 12 mm beam compared to both euploid groups (p<0.05).

4. Discussion
Our results indicate that oral consumption of EGCG failed to significantly rescue learning and memory tasks in young adult Ts65Dn mice given treatments beginning in early adolescence that delivered average daily doses of EGCG up to about 20 mg/kg/day. These results are in contrast to previous reports of cognitive improvement in trisomic mice treated for 1 month with an EGCG supplement with initial concentrations of ~100 mg/kg/day EGCG (De la Torre et al., 2014; Pons-Espinal et al., 2013). The lack of effects in our study, despite using doses intended to model those reported for the clinical trial in individuals with DS, highlight the importance of identifying factors that may determine whether EGCG treatments can rescue deficits in learning and memory in preclinical models of DS. Three important candidate factors that may account for differences are dosage, the developmental timing of the treatment, or interactions with other substances included in some EGCG supplements. We have shown that a three week treatment of ~10 mg/kg/day EGCG improved skeletal deficits in Ts65Dn mice (Blazek et al., 2015). A higher concentration of EGCG may be necessary to significantly improve cognitive deficits in the Ts65Dn mouse. In the current study, EGCG treatment began during adolescence (starting at PD 24) and continued either for 3 or 7 weeks; others used adult mice (3 months olds), suggesting that the age at treatment may play a role. Some of the supplements containing EGCG previously used also contained other green tea catechins and caffeine. EGCG and caffeine have been shown to interact in anxiety and hyperactivity phenotypes (Park, Eun, et al., 2010; Park, Oh, et al., 2010). Finding the treatment dosage, timing, and composition that are reliably effective in preclinical models will be important as EGCG is moved to clinical trials to treat specific deficits associated with DS in humans, and in identifying potential mechanisms of its clinical effectiveness.
Previous studies have shown that Ts65Dn mice display deficits in hippocampal-dependent tasks (Escorihuela et al., 1995; L. A. Hyde et al., 2001; Reeves et al., 1995; Sago et al., 2000). We found similar differences between Ts65Dn and control mice in behavioral tasks thought to depend on the functional integrity of the hippocampal formation. Ts65Dn mice were hyperactive across the two testing sessions in the activity chamber, with moderately but significantly higher levels of locomotor activity. Ts65Dn mice exhibited deficits in acquiring the DNMP task that assesses spatial working memory (Jang, Ahn, Lee, Lee, & Kaang, 2013; Watson, Herbert, & Stanton, 2009) committing more errors and requiring more trials to reach the learning criterion in the DNMP task. They also displayed poorer navigational and spatial memory strategies to find the platform in the Morris water maze spatial learning task (Morris, 1981), now the most common laboratory test of hippocampal-dependent place learning. Interestingly, in the MWM task, in both studies, the Ts65Dn mice showed significantly more thigmotaxic behavior during acquisition training, suggesting that factors such as anxiety or stress reactivity might influence performance of the trisomic mice (Simon, Dupuis, & Costentin, 1994). In probe trials of both studies, there were no significant differences between trisomic and control mice in the time spent in the non-target discs (or in the number of non-target crossings), indicating that the deficit in the search bias toward the target location is likely impaired memory for the location rather than generalized deficits in performance on the probe trial. Our trisomic mice in the three-week treatment groups displayed poor discrimination ratios in the novel object recognition task, indicating a deficit in episodic memory (Fernandez et al., 2007). However, the NOR task was a less reliable indicator of cognitive deficits in our hands, as we did not find significant genotype effects in the continuous treatment groups nor in the second NOR test of either treatment duration. The inconsistencies in obtaining genotype effects in the novel object
task across our studies or relative to other studies may be related to differences in procedures used (lighting conditions, types of objects), to the age at testing (young adult) or to the use of single housing of the mice (beginning shortly after weaning) to provide oral EGCG treatment.

To the best of our knowledge, this study is the first to report use of a balance beam task to evaluate motor performance in Ts65Dn mice, showing that Ts65Dn mice displayed increased frequencies of hind paw slips compared to euploid littermates, and the impairment was most evident on the more challenging narrower beam. Delayed motor development is observed in individuals with DS (Carmeli, Kessel, Bar-Chad, & Merrick, 2004). However, Ts65Dn performance in motor dependent tasks has yielded mixed results with some reported impairment in a rotarod task, walking and swimming speed, as well as deficits in hind gait analysis (Costa, Walsh, & Davisson, 1999). However, others have reported that Ts65Dn mice were not significantly impaired on several motor tasks (peg running, accelerating rotarod, and rotating rod) (Lynn A Hyde, Crnic, Pollock, & Bickford, 2001). These discrepancies may be related to procedural differences, as well as the age of the mice. Our results suggest the balance beam may be a more consistent test of motor performance deficits in the Ts65Dn mouse model.

Reducing the activity of the overexpressed *Dyrk1a* has been hypothesized to alleviate cognitive deficits associated with DS. The normalization of *Dyrk1a* copy number in Ts65Dn mice resulted in improvements in some hippocampal-dependent learning tasks (García-Cerro et al., 2014). EGCG has been shown to be an inhibitor of Dyrk1a activity (De la Torre et al., 2014) and EGCG treatment has been shown to improve learning and memory or behavioral aspects in trisomic mouse models and humans with DS, respectively (De la Torre et al., 2014; Pons-Espinal et al., 2013). To the best of our knowledge, this is the first study to report on the effect of EGCG on Dyrk1a activity in Ts65Dn mice. In one previous study using *Dyrk1a* transgenic mice in
which Dyrk1a activity was shown to be increased in the hippocampus, ~90 mg/mL EGCG significantly decreased hippocampal Dyrk1a activity (De la Torre et al., 2014; Pons-Espinal et al., 2013). However, in the Ts65Dn mice of our study with limited group sizes, we found only a non-significant trend toward increased Dyrk1a kinase activity from protein isolated from the cerebellum (less evident in the hippocampus), and the cerebellar Dyrk1a activity in the Ts65Dn mice showed a non-significant trend toward reduction by EGCG treatment. In this kinase activity assay, we observed substantial variability in Dyrk1a activity in both brain regions of Ts65Dn mice that may be attributed to the mixed genetic background of Ts65Dn (as compared to *Dyrk1a* transgenic mice), or it may reflect intrinsically high variability in the assay. Treatment with EGCG non-significantly reduced the Dyrk1a activities in both regions, though to a greater extent in the cerebellum of Ts65Dn mice, to levels nearer that of euploid mice. Conclusive evidence of *in vivo* reductions in Dyrk1a activity by EGCG and potential correlations between Dyrk1a activity and cognitive phenotypes likely will require larger sample sizes or more systematic analysis of Dyrk1a activity across ages of testing. This initial analysis of Dyrk1a activity in Ts65Dn mice suggests that there may be tissue- and brain region-specific variation of Dyrk1 activity, as well as in the ability of EGCG to alter Dyrk1a activity in different tissues. Additional analyses with increased numbers of mice will need to be done to test these hypotheses.

Green tea catechins undergo rapid degradation following treatment, likely leading to reduced bioavailability (Ferruzzi et al., 2010). The bioavailability of EGCG is increased in the presence of ascorbic acid which reduces the solution pH (Ferruzzi et al., 2010). The rate of degradation of EGCG in aqueous solution is dependent upon pH, incubation time, and temperature (Nakayama, Ichiba, Kuwabara, Kajiya, & Kumazawa, 2002). Our findings have
revealed that different EGCG supplements degrade at varying rates which may affect EGCG bioavailability. EGCG containing supplements previously used in trisomic mice have only 30-40% of EGCG left after 48 hours, and this degradation in water may affect previously reported concentrations of EGCG ingested by the mice. The EGCG utilized in our study is ≥ 95% pure and when stabilized with phosphoric acid has about 50% of the initial EGCG concentration available after it is suspended in water for 48 hours. We also noted that in the three week treatment groups, the euploid mice given EGCG stabilized with H₃PO₄ differed in their fluid intake versus euploid mice without H₃PO₄ stabilization. However, this group was not statistically different than the mice who received water with H₃PO₄ stabilization (p=0.11), nor did it affect growth in the seven week treatment group (p=0.745)

Treatments for trisomic cognitive phenotypes have shown promise in mouse models but generally have been less effective in humans (de la Torre & Dierssen, 2012; Gardiner, 2015). Previous reports of EGCG treatment in mice and humans has shown improvements in cognitive phenotypes (De la Torre et al., 2014; Pons-Espinal et al., 2013). Our current work using a pure stabilized form of EGCG in concentrations that produced beneficial effects on skeletal phenotypes failed to produce improvements in cognitive phenotypes. Compared to reports of beneficial effects of EGCG from other investigators, it appears that reliable effects of EGCG on behavioral and cognitive outcomes may depend on the dosages and concentrations delivered, the age of treatments, or interaction with other substances in commercial sources of EGCG. Understanding the impact of these and other variables may be necessary to identify the therapeutic potential of EGCG and its underlying mechanisms in preclinical models of DS.
Acknowledgements

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Figure Captions

**Figure 1: EGCG Degradation.** EGCG degradation analysis (Panel a), Growth of groups given continuous treatment (Panel b), and EGCG consumption of groups given continuous treatment (Panel c).

**Panel a.** Degradation analysis among four different types of EGCG indicated in figure legend: EGCG (≥95% pure powder from Sigma-Aldrich, dissolved in water), EGCG + H$_3$PO$_4$ (≥95% pure powder from Sigma-Aldrich, stabilized to pH 5.5 with phosphoric acid), LC EGCG (Lightly Caffeinated EGCG from Life Extensions); DC EGCG (Decaffeinated EGCG from Life Extensions). Each analysis was performed using 3 samples of each type. Data are represented as mean ± SEM. At 24 and 48 hours, EGCG + H$_3$PO$_4$ had a higher concentration than the unstabilized EGCG.

**Panel b: Growth.** Body weights of euploid and trisomic mice of the groups given continuous treatment with EGCG or water, beginning in adolescence and continuing through the end of behavioral testing in young adulthood. The Ts65Dn mice weighed less throughout the experimental period, and there were no effects of EGCG on growth.

**Panel c: EGCG Consumption (adjusted for degradation).** EGCG consumption (mg/kg per day) of the Ts65Dn and euploid groups, adjusted for loss over each 2-day period due to degradation in the fluid tube (see text for formula). Data are represented as mean ± SEM. The trisomic mice consumed significantly more EGCG (on a mg/kg per day basis) than euploid mice, and the relative intake per day declined over days.

**Figure 2: 3 week Locomotor Activity.** Mean locomotor activity as a function of distance traveled per 1-minute bins on two consecutive days of testing (SEMs not shown because they are smaller than the group symbols). Activity was measured on PD 45-46 in the 3 week treatment groups; euploid (gray shading), Ts65Dn (black shading); water (solid lines); EGCG (broken lines). The Ts65Dn mice were significantly more active than euploid controls on both days as indicated by (*). There was no significant effect of EGCG treatment.

**Figure 3: 3 Week NOR Test 1.** Discrimination ratios of the 3 week (~10mg/kg/day) young adult euploid (Eu) and trisomic (Ts) mice. Bars represent the average discrimination ratio with error bars represented as SEM. The (*) indicates that the euploid mice displayed a higher discrimination ratio than trisomic mice, indicating a preference for the novel object on test day.
Figure 4a: 3 week Latency. Performance on the Morris water maze task by the young adult mice in the 3-week treatment groups. Panel 4a. Data are represented as mean ± SEM. Acquisition in the Morris water maze spatial learning task by the euploid (Eu) and trisomic (Ts) groups. Each line represents the average time (latency) to find the hidden platform for each training day. All groups showed a decrease in latency over training days. However, trisomic mice of both treatment groups displayed deficits versus the euploid groups (main effect of genotype, indicated by *).

Panel 4b: 3 week Probe Trial. Probe trial performance of (Eu) and trisomic (Ts) mice of the two treatment groups on the probe trial (Day 8). Bars represent the average time spent in the target and non-target quadrant, with error bars represented as SEM. The (*) comparing the target and non-target times of the Eu-Water group indicates a significant spatial bias for the target location (Eu+Water, p=0.004; the Eu+EGCG also approached significance, p=0.057); neither trisomic group showed a significant spatial bias for the target area (p’s>0.10). In addition, the Ts+Water group spent significantly less time in the target disc than the Eu+Water group (p<0.05), and the Ts+EGCG group spent significantly less time in the target disc than either of the Euploid groups (#, p<0.05).

Figure 5a: Continuous Latency. Performance on the Morris water maze task by the young adult mice in the continuous treatment groups. Panel 5a. Data are represented as mean ± SEM. Acquisition in the Morris water maze spatial learning task by the euploid (Eu) and trisomic (Ts) groups. Each line represents the average time (latency) to find the hidden platform for each training day. All groups showed a decrease in latency over training days. However, trisomic mice of both treatment groups displayed deficits versus the euploid groups (main effect of genotype, indicated by *).

Panel 5b: Continuous Probe Trial. Probe trial performance of (Eu) and trisomic (Ts) mice of the two treatment groups on the probe trial (Day 8). Bars represent the average time spent in the target and non-target quadrant, with error bars represented as SEM. The (*) comparing the target and non-target times of the euploid groups indicates a significant spatial bias for the target location for both euploid groups (Eu+Water, p=0.001; Eu+EGCG, p=0.002); neither trisomic group showed a significant spatial bias for the target area (all p>0.10). In addition, the Ts+Water group spent significantly less time in the target disc than the Eu+Water group (†, p<0.05), and the Ts+EGCG group spent significantly less time in the target disc than either of the Euploid groups (#, p<0.05).
Figure 6: Continuous Balance Beam Performance. Balance beam performance in young adult euploid (Eu) and trisomic (Ts) Ts65Dn mice given either water or EGCG continuously throughout the experimental period. Data are represented as mean ± SEM. As indicated by the (*), trisomic mice (black lines) displayed more paw slip errors than the euploid mice (grey lines).
Table 1

<table>
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<th>Treatment</th>
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<th>Dyrk1a Activity Hippocampus</th>
<th>Dyrk1a Activity Cerebellum</th>
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<td>3906 (±1270)</td>
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<td>EGCG</td>
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<td>4949 (±1904)</td>
<td>1666 (±104)</td>
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Table 1. Dyrk1a activity in the hippocampus and cerebellum of 6-week old mice given 3 weeks of treatment with water or EGCG (mean ± SEM). The hippocampus and cerebellum was freshly dissected from each mouse, frozen and stored at -80°C until assayed for Dyrk1a activity as described in the text. None of the group differences was significant; there was a non-significant trend for higher Dyrk1a activity in the cerebellum of the Ts65Dn-water group compared to the euploid-water group (p=0.065)
Table 2

<table>
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<td>EGCG</td>
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<td>161 (±19)</td>
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Table 2. Delayed non-matching to place (DNMP) performance of young adult mice of the 3-week treatment groups, based on errors to criterion of 3 consecutive days of >7/8 correct choices (mean ± SEM). The higher number of errors by the Ts65Dn mice was confirmed by a significant main effect of genotype, F(1,21)=6.56, p=0.018.
Figures

Figure 1

(a) EGCG concentration over time for different treatments:
- EGC
- EGC + H3PO4
- LC EGC
- DC EGC

(b) Weight (Grams) over Age (Days):
- Eu+ Water, n=14
- Eu+ EGC, n=13
- Ts+ Water, n=10
- Ts+ EGC, n=8

(c) Adjusted EGCG Consumption over Age (Days):
- Eu+ EGC, n=13
- Ts+ EGC, n=8
Figure 4

a) Change in Seconds over Days for different treatment groups:
- Eu+Water, n=10
- Eu+EGCG, n=11
- Ts+Water, n=10
- Ts+EGCG, n=11

b) Time Spent in Virtual Disc (seconds) for Target and Non-Target groups:
- Eu+Water, n=10
- Eu+EGCG, n=11
- Ts+Water, n=10
- Ts+EGCG, n=11
Figure 6