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For the degree of Master of Science

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EFFECT OF EPIGALLOCATECHIN-3-GALLATE ON SKELETAL AND COGNITIVE PHENOTYPES IN A DOWN SYNDROME MOUSE MODEL

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For my amazing husband and family

who have always believed in me and been my greatest support

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LIST OF SYMBOLS

°C	Degrees Celsius
µg	Microgram
µl	Microliter
µM	Micromolar
µm	Micrometer
mL	Mililiter
Mm	Millimolar
M	Molar
g	Gram
mg	Miligrams
mm	Milimeter
N	Newton

ABSTRACT

Abeysekera, Irushi S. M.S., Purdue University, August 2014. Effect of Epigallocatechin-3-gallate on Skeletal and Cognitive Phenotypes in a Down syndrome Mouse Model. Major Professor: Randall J Roper.

Down Syndrome (DS), a genetic disorder that affects ~1 in 700 live births, is caused by trisomy of human chromosome 21 (Hsa21). Individuals with DS are affected by a wide spectrum of phenotypes which vary in severity and penetrance. However, cognitive and skeletal impairments can be commonly observed in all individuals with DS. To study these phenotypes, we utilized the Ts65Dn mouse model that carries three copies of approximately half the gene orthologs found on Hsa21 and exhibit similar phenotypes as observed in humans with DS. Individuals with DS and Ts65Dn mice have deficits in bone mineral density (BMD), bone architecture, bone strength, learning and memory. Over-expression of *DYRK1A*, a serine-threonine kinase encoded on Hsa21, has been linked to deficiencies in DS bone homeostasis and cognition. Epigallocatechin-3-gallate (EGCG), an aromatic polyphenol found in high concentrations in green tea, is a selective inhibitor of *DYRK1A* activity. Normalization of *DYRK1A* activity by EGCG therefore may have the potential to ameliorate skeletal and cognitive deficits. We hypothesized that supplements containing EGCG obtained from health food stores/ online vendors will not be as effective as EGCG from a chemical company in correcting bone deficits associated

with DS. Our results suggest that EGCG improves the bone mineral density of trisomic femurs significantly better than the supplements while the EGCgNOW supplement from NOW FOODS improves trabecular and cortical bone structure. The results from HPLC analysis of supplements showed the presence of other catechins in EGCgNOW and degradation analysis revealed the rapid degradation of supplements. Therefore we hypothesize that the presence of EGCG degradation products and other green tea catechins in supplements may play a role in the differential skeletal effects we observed. We further hypothesized that a three week treatment of adolescent mice with EGCG will lead to an improvement in the learning and memory deficits that are observed in trisomic animals in comparison to control mice. However, our results indicate that three weeks of low-dose EGCG treatment during adolescence is insufficient to improve hippocampal dependent learning and memory deficits of Ts65Dn mice. The possibility remains that a higher dose of EGCG that begins at three weeks but lasts throughout the behavioral test period may result in improvement in learning and memory deficit of Ts65Dn mice.

CHAPTER 1. INTRODUCTION

1.1 Down syndrome

Down syndrome (DS) is the most common genetic cause of mental retardation and results from the triplication of human chromosome 21 (Hsa21) (LEJEUNE 1959). In most instances DS results from the non-disjunction of the Hsa21 during meiosis as a freely segregating chromosome or as a Robertsonian translocation (EPSTEIN 2001a). The rarest form of DS is mosaicism, a condition caused by the presence of trisomic cells together with normal diploid cells. Mosaic phenotypes and severity depend upon the number and distribution of trisomic cells within organs with individuals with lower number of trisomic cells being asymptomatic (EPSTEIN 2001a). The only apparent risk factor associated with DS is maternal age and this becomes clear when studying the rates of non-disjunction during gamete formation. Non-disjunction occurs 86% of the time during female oocyte formation with predominate errors (75%) occurring during meiosis I (EPSTEIN 2001a). There are 552 genes on Hsa21 of which 161 genes are RefSeq protein coding sequences (STURGEON and GARDINER 2011).

DS has an incidence rate of approximately 1 in 700 live births and results in a constellation of phenotypes that affect the cardiovascular, central nervous, and skeletal systems among others (SPENCER 2001; RICHTSMEIER *et al.* 2002; VAN CLEVE SN 2006a; VAN CLEVE SN 2006b; CLEVES *et al.* 2007). Some of the commonly occurring phenotypes are hearing loss (75%), vision loss (60%), obstructive sleep apnea (50-75%),

congenital heart disease (40-60%), refractive errors (50%), infections of the middle ear (50-70%), hypodontia and delayed tooth eruptions (23%), low IQ and an Alzheimer disease like phenotype when individuals are 50-60 years of age (MARILYN J. BULL ; ROUBERTOUX and KERDELHUE 2006; NETZER *et al.* 2010; BULL and GENETICS 2011). There exists however wide variability in phenotypic incidence and severity in individuals with DS (EPSTEIN 2001a; ROUBERTOUX and KERDELHUE 2006).

1.1.1 History of Down syndrome

The first book on mental disorders was published in 1838 by Jean-Etienne-Dominique Esquirol where he identified a collection of phenotypes that we now know as Down syndrome (DS). Esquirol in "*Des maladies mentales considérées sous les rapports medical, hygiénique et medico-légal"* describes a group of patients with physical attributes that ranged from a flat nasal bridge, protruding tongue, oblique eye fissures, short stocky stature, malformed neck and epicanthic eye folds to mental retardation (ESQUIROL 1845). In 1846 Edouard Séguin added to the observations of Esquirol and described characteristics of a small nose, open mouth, tongue morphology and an increase in susceptibility to infections. He also stated that the affected children were mild natured and had the ability to gain language skills and coined the term "furfuraceous cretinism" to describe them (SEGUIN 1866). In 1866, John Langdon Down noted in London Hospital Reports a group of individuals with characteristics similar to those observed in individuals from Mongolia and he coined the term "Mongols" to describe them. He described them to have a flat broad face with straight and scanty hair, round cheeks, narrow palpebral fissures, a small nose and a long thick tongue. He further

described them as being great mimics with a lively sense of humor and speech that is thick and indistinctive (DOWN 1866). He observed that training improved speech, coordination, physical and mental abilities, and that they possessed on average much reduced life expectancies (ROUBERTOUX and KERDELHUE 2006). The term "mongolism" was used to describe these individuals until the mid-nineteenth century. In 1961 the Lancet published a letter penned by a group of 19 scientists who suggested that the term "mongolism" should be discarded as it is "an embarrassing term" and "has no scientific basis" and it should be replaced by a more suitable term. They suggested the use of "Langdon-Down's anomaly" "Down's syndrome or anomaly", "congenital acromicria", or "trisomy 21 anomaly" (HOWARD-JONES 1979). The term "mongolism" was officially removed from use by the World Health Organization (WHO) in 1965 following a request by the Mongolian delegation (HOWARD-JONES 1979). In 1975, the United States Institute of Health (NIH) convened a conference during which it was suggested that the possessive term "Down's syndrome" be replaced by "Down syndrome" (1975).

The genetic cause of DS was not described till the mid-19th century during which time a number of other discoveries that facilitated the identification of the genetic basis of DS were made. In the early part of the 19th century, Thomas H. Morgan and colleagues published a manuscript that presented evidence to support the theory that genes are contained on the chromosome (PATTERSON and COSTA 2005). Joe H. Tjio and Albert Levan used sample spreads of metaphase chromosome cell cultures of fetal lung tissue to positively identify that humans had 46 chromosomes (TJIO and LEVAN 1956). The chromosomal basis of DS was discovered in May 1958 by the French pediatrician and

geneticist Jerome Lejeune who observed an extra copy of the human 21st chromosome (Hsa21) in human fibroblast tissue samples (LEJEUNE 1959).

1.1.2 Genotype- phenotype relationship

The development of therapeutic interventions for individuals with DS relies greatly upon understanding the genotype-phenotype correlation, the role of Hsa21 gene(s) and their interactions with non-Hsa21 genes in the development of various DS phenotypes. There are several methods in which the genotype-phenotype relationship can be studied, including (1) mapping of partial human trisomies (2) use of segmentally trisomic mouse models, (3) studying gene expression in cells and tissues isolated from individuals with DS and mouse models, and (4) triplication of a single trisomic gene in a mouse model (transgenic mouse models) (ALTAFAJ *et al.* 2001; LYLE *et al.* 2009). The discovery of the chromosomal basis of DS by Lejeune in 1959 subsequently lead to the study of the chromosomal structure to pinpoint the exact region of Hsa21 responsible for the myriad phenotypes observed in individuals with DS. The study of individuals with partial trisomy lead to the identification of the distal region of Hsa21, in particular the region of 21q22 as responsible for causing many of the DS phenotypes including mental retardation (MCCORMICK *et al.* 1989). The "Down syndrome critical or chromosomal region" (DSCR) was described to extend for 5.4 Mb on Hsa21 from a proximal boundary between markers *D21S17* and *D21S55* to distal boundary between *MX1* and *BCEI* and carry 33 genes (DELABAR *et al.* 1993; KORENBERG *et al.* 1994). The general consensus at this time was that genes located on the DSCR alone were responsible for causing most of the major phenotypes observed in individuals with DS (KORENBERG *et al.* 1990).

However, experiments conducted in mice that were trisomic for the DSCR (Ts1Rhr) disproved this theory by showing that the genes on the DSCR alone are not sufficient for the generation of the craniofacial phenotype and mental retardation (OLSON *et al.* 2004a). The analysis of DNA from individuals with partial trisomy 21 yielded results showing that some regions on Hsa21 may play a major role in specific phenotypes (KORBEL *et al.* 2009; LYLE *et al.* 2009). The genomic regions of eight phenotypes associated with DS in 30 individuals with partial trisomy 21 were mapped using DNA which was first karyotyped and then analyzed using multicolor fluorescence in situ hybridization (FISH) and array maps (KORBEL *et al.* 2009). The DS-specific congenital heart disease (DSCHD) was one of the phenotypes that Korbel *et al*. studied as it is a major phenotype observed in individuals with DS. DSCHD is associated with abnormal development of the atrioventricular septal defects (AVSD) and an individual with DS has a risk ~1000 fold greater than the general population of developing DSCHD (KORBEL *et al.* 2009). Korbel *et al*. identified a 2.82 Mb region on Hsa21 from DNA obtained from 14 individuals previously diagnosed with DSCHD that were critically associated with the development of endocardial cushion defects which lead to valve and septal defects (KORBEL *et al.* 2009). Further analysis using the mouse model Dp(16)1Yu/+ narrowed the DSCHD critical region down to a 1.77 Mb section with 10 genes of which only the Down syndrome cell adhesion molecule (*DSCAM*) gene was highly expressed in the developing heart and therefore was noted as the likely candidate gene for DSCHD (KORBEL *et al.* 2009). Hirschsprung disease (HSCR) is a congenital gut disease where DS individuals have a 100 fold greater risk than the general population (TORFS and CHRISTIANSON 1998). In the same study the *DSCAM* gene was also implicated as a candidate gene for HSCR,

supporting the results observed in a previous study (YAMAKAWA *et al.* 1998). Individuals with DS have an increased risk of developing Alzheimer disease (AD) and *APP*, a Hsa21 gene has been shown to play a critical role in disease development (ROVELET-LECRUX *et al.* 2006). The region of Hsa21 involved in AD phenotype was narrowed down to a 1.95 Mb region which included *APP* (KORBEL *et al.* 2009). Another study published in 2009 identified 25 phenotypes that were associated with DS in 19 individuals with partial trisomy and 27 phenotypes in 11 monosomy individuals (LYLE *et al.* 2009).

The two studies published by Korbel and Lyle provided key pieces of evidence in the quest for understanding the correlation between genotype-phenotype and provided evidence to further dispel the DSCR theory by showing that different regions on Hsa21 are responsible for the generation of different phenotypes.

1.1.3 Skeletal phenotype in individuals with DS

As average life expectancy of individuals with DS increases due to advances in healthcare, so does the likelihood of the development of age-related diseases like osteoporosis, diabetes, and autoimmune disorders (VAN ALLEN *et al.* 1999; MCKELVEY *et al.* 2012). Osteoporosis is defined as "a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and the consequent increase in fracture risk" (KANIS *et al.* 1994). Individuals with DS possess a number of features which make them more susceptible to the development of an osteoporosis like phenotype. They generally suffer from thyroid abnormalities, hypotonia, and reduced physical activity levels (GUIJARRO *et al.* 2008). It was therefore suggested that the distal portion of Hsa21 may play a role in the development of osteoporosis in DS

(TUMER *et al.* 2005). Guijarro and his colleagues studied the effect of trisomy 21 on bone mineral density (BMD) in individuals (age 19-45 years) with DS who did not suffer from any comorbidity that may influence BMD levels (GUIJARRO *et al.* 2008). They identified that the areal BMD was significantly lower in the spine, femoral neck, total hip, and total body in individuals with DS in comparison to the control group (GUIJARRO *et al.* 2008). Another study conducted by Baptista et al. found that the volumetric BMD in lumbar spine and the femoral neck strength were significantly altered in DS individuals (BAPTISTA *et al.* 2005). A significantly lower level of bone formation serum markers (P1NP) with no accompanying significant change to reabsorption markers were observed in biochemical analysis of blood from young adult DS individuals (MCKELVEY *et al.* 2012). Bone formation and reabsorption mainly occur during childhood and adolescence in a process known as bone turnover where osteoblasts and osteoclasts work together to create and lengthen the skeleton while the same cells help maintain the skeleton throughout adulthood (MCKELVEY *et al.* 2012). McKelvey and colleagues in their study found that individuals with DS have a significantly decreased bone turnover and they concluded that it is the combination of altered bone formation and reabsorption that is the primary cause of low bone mass that is observed in individuals with DS (MCKELVEY *et al.* 2012).

1.1.4 Cognitive phenotype in individuals with DS

DS is the leading genetic cause of mental retardation and results in mild to severe learning and memory deficits (GARDINER *et al.* 2003; ROUBERTOUX and KERDELHUE 2006). Mental retardation in DS is characterized by impairments in hippocampal

dependent memory functions, reduced size of hippocampus and cerebellum, reduced dendritic and axonal number and volume, early occurrence of Alzheimer disease, motor dysfunction and altered synaptic plasticity (BECKER *et al.* 1991; MARTINEZ DE LAGRAN *et al.* 2004; COPPUS *et al.* 2006; LORENZI and REEVES 2006; GUIDI *et al.* 2008). Alterations in brain morphology are apparent in newborns with the amygdala, cerebellum, brain stem, and prefrontal cortex undergoing a reduction in volume (DIERSSEN 2012). The dendrites play a key role in synaptic transmission, and in infants and children with DS a reduction in dendritic spines along with atrophy of the dendritic tree can be observed (TAKASHIMA *et al.* 1989). Within the first few months of life, developmental delays (motor and cognitive) can be observed in infants with the delays becoming more and more significant over time; another noticeable alteration is the decline in intelligent quotient (IQ) which follows a downward trend from infanthood (<1 year) to adolescence (>11 years) (EPSTEIN 2001a). Behavioral problems such as obsessive/compulsive behaviors, low attention span, and stubbornness are a common occurrence in children with DS (4-18 years) which can hamper their personal and social development (CLARK and WILSON 2003). Adults (20-50 years) show a more varied neurological profile with motor function and coordination of movements, language, verbal short-term memory (short term retention of words) and declarative long-term memory (conscious recollection of memories of facts and events) more severely impaired than other forms of memory (LATASH and ANSON 2006; DIERSSEN *et al.* 2009). Furthermore, the structure of the dendrites continue to deteriorate in adults leading to reductions in dendritic branching, length and spine numbers (TAKASHIMA *et al.* 1989).

1.2 Mouse models of DS

The complex disease pathology of DS resulted in the generation of mouse models to study the genotype-phenotype relationship, the role of a chromosomal region or specific genes on the pathogenesis of DS, and possible therapeutic interventions. Mouse models of DS are based upon the orthologous nature between Hsa21 and mouse chromosome 16, 17, and 10 (Mmu16, 17, 10) (Fig. 1.1) (PLETCHER *et al.* 2001; STURGEON and GARDINER 2011). Mouse models can be divided in to three types based upon the method of creation, 1- segmentally trisomic mouse models: Ts65Dn, Ts1Rhr, Ts1Cje, Ts1Yah, *p(10)1Yey/+*, *Dp(16)1Yey/+*, *Dp(17)1Yey/+*, 2- transchromosomic mouse model (Tc1, *Dp(10)1Yey/+; Dp(16)1Yey/+;Dp(17)1Yey/+*), 3- transgenic mouse models (*TgDyrk1a*).

The Ts(17^{16})65Dn (hereafter referred as Ts65Dn) mouse model is the most extensively studied and widely used animal model of DS and captures most of the behavioral defects that are seen in humans with DS (DAVISSON *et al.* 1993; LIU *et al.* 2011). Ts65Dn mice carry a genomic fragment of 13 Mb that extends from *Mrpl39* to the telomere of Mmu16 and trisomic for approximately half the gene orthologous found on Hsa21 (DAVISSON *et al.* 1993; REEVES *et al.* 1995; HATTORI *et al.* 2001). Ts65Dn mice replicate many of the DS phenotypes including abnormal dendritic spine density and structure, altered hippocampal functions, severe reduction in LTP, and skeletal abnormalities (BELICHENKO *et al.* 2004; KLESCHEVNIKOV *et al.* 2004; BELICHENKO *et al.* 2007; BELICHENKO *et al.* 2009b; BLAZEK 2011). The Ts1Cje mouse model, generated by Charles J. Epstein's laboratory by gene targeting, carries a ~ 8.1 Mb genomic fragment that is of Mmu16 in origin and orthologous for approximately 67% of the Hsa21 genes

that are triplicated in Ts65Dn mice (SAGO *et al.* 2000; OLSON *et al.* 2004b). These mice display abnormalities in dendritic structure, function, and number with altered synaptic plasticity (BELICHENKO *et al.* 2007). The Ts1Rhr mouse model was generated in the laboratory of Roger Reeves and carries an extra segment of the Mmu16 region from *Cbr1-Fam3b* (~ 33 genes) thus making it an excellent model to study the importance of the DSCR (DELABAR *et al.* 1993; OLSON *et al.* 2004a). The Ts1Rhr mice display learning and memory deficits and abnormalities in dendritic spine morphology and number with accompanying alteration of long term potentiation (LTP), an essential factor of synaptic plasticity (BELICHENKO *et al.* 2009a). The Ts1Yeh mouse model was created recently in Yann Herault's laboratory in France and carries a small Hsa21 homologous 0.59 Mb duplicated region of Mmu17 (PEREIRA *et al.* 2009). The Ts1Yeh mice differ from other mouse models whereby the duplicated region has resulted in the increase of LTP within the hippocampus and thus providing some evidence to the relationship between the three mouse chromosomal regions in phenotype development (PEREIRA *et al.* 2009). The *Dp(10)1Yey/+*, *Dp(16)1Yey/+*,and *Dp(17)1Yey/+* mouse models were created in the laboratory of Eugene Yu and carry duplicated regions of each Hsa21 homologous regions on Mmu10, 16, and 17 respectively (YU *et al.* 2010a). The *Dp(10)1Yey/+* mice showed no impairments in LTP or in behavioral tests while *Dp(16)1Yey/+* mice displayed decreased LTP and cognitive deficits and the *Dp(17)1Yey/+* mice had increased hippocampal LTP (YU *et al.* 2010b).

The first complete transchromosomal mouse model, Tc1 was created by Elizabeth Fisher in 2005 and carries a freely segregating copy of Hsa21 resulting in the triplication of 200 RefSeq genes (O'DOHERTY *et al.* 2005; GRIBBLE *et al.* 2013). The Tc1 mice were

shown to have impairment in short term memory with long term memory and synaptic plasticity been preserved (MORICE *et al.* 2008). These mice performed poorly in Morris water maze (MWM) and novel object learning and memory tasks and displayed reductions in LTP (O'DOHERTY *et al.* 2005; MORICE *et al.* 2008). However one of the major drawbacks of this mouse model is that the freely segregatingHsa21 within the mouse genome results in a high degree of mosicism within tissues (SALEHI *et al.* 2007). Recently the laboratory of Eugene Yu created another transchromosomal mouse model that is trisomic to all Hsa21 homologous regions (YU *et al.* 2010a). The *Dp(10)1Yey/+;Dp(16)1Yey/+;Dp(17)1Yey/+* mouse model was created by normal breeding to generate compound mutant mice carrying two different duplications and then used these compound mutants to cross with the mutant carrying the third duplication to generate mice carrying all three Mmu regions (YU *et al.* 2010a). The mutant mice had a significantly higher latency, slower swimming speed and a longer path length to the hidden platform in the MWM task during training and on the probe day the mutant mice spent a significantly lower amount of time in the target quadrant than control animals (YU *et al.* 2010a). The mutant mice also displayed impairment in grip strength and a significant reduction in hippocampal LTP.

Transgenic mouse models allow the users to study the effect of a specific gene of phenotype development. Transgenic mice overexpressing Dyrk1A (TgDyrk1A) were created to specially study the role of Dyrk1A, a candidate gene for learning and memory deficits in DS (ALTAFAJ *et al.* 2001). These mice showed impairments in cognitive function but had an increased hippocampal LTP (AHN *et al.* 2006).

1.2.1 Skeletal phenotype of Ts65Dn mice

The Ts65Dn mouse model is an excellent predictive animal model of skeletal abnormalities that are observed in individuals with DS. In a previous study conducted in our lab, we identified that bone development and homeostasis was altered in trisomic mice at both 6 and 16 weeks of age (BLAZEK 2011). Blazek and colleagues analyzed femurs and skulls of 6 and 16 week trisomic and euploid mice using dual energy x-ray absorptiometry (DXA), micro CT, histomorphometry, and 3-point bend test. The bone mineral density (BMD), bone mineral content (BMC), percent bone volume (BV/TV) and trabecular number showed significant reductions in trisomic femurs at 6 and 16 weeks while trabecular separation was significantly increased. The trabecular thickness was lower in Ts65Dn mice but this reduction was found to be non-significant at the time points observed. The mid-shaft of the femurs are made up of cortical bone and analysis of this section by micro CT revealed that at 6 and 16 weeks the trisomic mice exhibit a significant reduction in mean total cross-sectional area, mean polar moment of inertia, and mean total periosteal perimeter. The mineralization rate of femurs in trisomic and euploid mice was observed by injecting them with two labels (calcein green and alizarin red) four days apart prior to death. Both trisomic and euploid mice had similar number of active osteoblasts in the femur but at 6 weeks, trisomic mice had a significantly lower mineral apposition rate on the periosteal surface of femur in comparison to age matched euploid littermates. The bone formation rate in the femurs was also shown to be significantly reduced in trisomic mice in comparison to controls. In the final part of the study, the strength of the femurs was analyzed via 3-point bending to study the effect of all of the above changes on the structure of the bones. Ts65Dn mice had a significantly

lower ultimate load and stiffness at 6 and 16 weeks than euploid controls while the energy to failure was reduced but the change was non-significant in 16 week old femurs. All of the results gathered by Blazek *et al*., clearly showed that trisomic mice were affected by a significantly altered skeletal phenotype which was present from adolescence to adulthood (BLAZEK 2011). In a 2012 study Fowler *et al*. studied the skeletal phenotype of Ts65Dn mice at 3 and 24 months of age (FOWLER *et al.* 2012). They observed the structural morphology of the proximal tibia and distal femur of 3 and 24 month old trisomic mice and euploid mice and observed that the trabecular bone underwent significant changes that include reduction of percent bone volume (BV/TV), and trabecular number along with an increase in trabecular separation (FOWLER *et al.* 2012). The cortical bone that makes up the mid-shaft of the femur was found to have a significant reduction in cortical thickness at both time points while in the older mice the periosteal cross section, cortical cross-sectional area, and total cross sectional area also underwent a significant reduction. The significant reductions to the cortical bone in 24 month old mice were also associated with a reduction in mechanical strength. Static histomorphometric analysis of the proximal tibia and distal femurs of 3 month old mice further supported the above observations by showing that a significant reduction in the number of osteoblasts and osteoclasts on the bone surface along with reductions in bone formation and reabsorption markers with an overall reduction in bone turnover was present at this time point (FOWLER *et al.* 2012). The results observed by Fowler *et al*., verified the results observed in our lab and showed that the skeletal phenotype that is present during adolescence persists through adulthood.

1.2.2 Behavioral phenotype of Ts65Dn mice

The Ts65Dn mouse model has been used extensively to explore the deficits in brain structure and function in DS (ESCORIHUELA *et al.* 1995; REEVES *et al.* 1995; BELICHENKO *et al.* 2004; KLESCHEVNIKOV *et al.* 2004; BELICHENKO *et al.* 2007; BELICHENKO *et al.* 2009b). The alterations observed in Ts65Dn mice include spontaneous locomotor hyperactivity, impaired performance in MWM and novel object recognition tasks, reduced dendrite density and enlarged spine heads, and severe impairment of hippocampal LTP (BELICHENKO *et al.* 2007; BELICHENKO *et al.* 2009b). Learning and memory functions are most commonly studied in the hippocampus, a structure tightly linked with cognitive functions (NADEL and BOHBOT 2001). Behavioral tasks have been utilized to study the many deficits that Ts65Dn mice possess due to trisomy. Tasks such as the MWM, novel object recognition (NOR), delayed non-matching to place (DNMP), and locomotor activity are commonly used to study the hippocampus dependent learning and memory deficits (MIZUMORI *et al.* 1999; D'HOOGE and DE DEYN 2001; FERNANDEZ *et al.* 2007). The MWM, developed by RG Morris is used to study spatial learning of a task and the ability to remember said task following a delay (MORRIS *et al.* 1982). The MWM task is used very often in assessment of hippocampal dependent learning and memory functions in Ts65Dn mice (SAGO *et al.* 2000; HYDE *et al.* 2001). Ts65Dn mice have been shown to have significant deficits in the MWM task with an increased latency (time taken to reach the platform) and poorer spatial navigational performance compared to euploid controls (ESCORIHUELA *et al.* 1995; REEVES *et al.* 1995; SAGO *et al.* 2000; HYDE *et al.* 2001). The NOR task is based upon the natural tendency of rodents to explore novel objects and environments (CLARK *et al.* 2000; SQUIRE *et al.* 2007) . It has

been reported in previous studies that Ts65Dn mice were less able to discriminate between a novel and a familiar object and displayed deficits in episodic memory functions (FERNANDEZ *et al.* 2007). The DNMP (T-maze) task is based upon the natural tendency of mice to alternate goal arms and is based upon "working memory", where the decision made the "choice" trial of a trial couplet is based upon what was learned in the "forced" trial (OLTON 1979; DEMBER 1989). Ts65Dn mice showed a severe deficit in working memory in the T-maze with a significantly lower percentage of spontaneous alternation in the maze in comparison to control mice (KLESCHEVNIKOV *et al.* 2012). Several studies have reported that the spontaneous locomotor activity of Ts65Dn mice is significantly increased leading to a higher level of hyperactivity than observed in control mice (HOLTZMAN *et al.* 1996; SAGO *et al.* 2000; KLESCHEVNIKOV *et al.* 2012). The performances of Ts65Dn mice in these hippocampus dependent behavioral tasks points towards the presence of major deficits in this region of the brain.

1.3 *DYRK1A/Dyrk1a*

DYRK1A (dual specificity tyrosine-regulated kinase 1A) is a member of the highly regulated DYRK protein kinase family (TEJEDOR and HAMMERLE 2011). The *DYRK1A* gene is located on Hsa21 and is found in three copies in individuals with DS. Ts65Dn mice have three copies of the Dyrk1a gene which is found on Mmu16. It functions as a master regulator of important processes such as the development of the central nervous system (CNS), cell death, synaptic plasticity, and osteoclastogenesis (LEE *et al.* 2009b; TEJEDOR and HAMMERLE 2011). *DYRK1A* is highly expressed in fetal and adult brains during the development, neurogenesis and differentiation indicating that it

has an important role in the functioning of the CNS (DELABAR *et al.* 1993; GUIMERA *et al.* 1999). The extra copy of *DYRK1A* in humans with DS (older than 3 years) has been shown to lead to a 1.5 fold increase in protein levels in brain tissues which is hypothesized to cause cognitive deficits (DOWJAT *et al.* 2007). The dosage imbalance of *Dyrk1a* is hypothesized to result in the development of a number of phenotypes observed in DS and in mouse models of DS including behavioral and skeletal deficits (KORENBERG *et al.* 1994; ARRON *et al.* 2006). The NFATc1 (Nuclear factor of Activated T-cells 1) pathway is a critical regulator of development in vertebrates and plays a crucial role in bone development and neurogenesis (RICHTSMEIER *et al.* 2000; ARRON *et al.* 2006). It is referred to as a "master" transcription factor for osteoclastogenesis as its induction is essential for cell fate determination (ASAGIRI and TAKAYANAGI 2007). The mechanism of action of NFATc is induced when calcium (Ca^{2+}) enters into the cell which leads to the activation of calcineurin, which dephosphorylates cellular NFATc protein facilitating its nuclear entry and subsequent gene transcription (Figure 1.2). In DS, it is hypothesized that overexpression of *DYRK1A* leads to a significant increase in the phosphorylation of NFATc1, reducing its nuclear presence and preventing it from functioning as a transcription factor (ARRON *et al.* 2006; GWACK *et al.* 2006). The validity of this mechanism has been shown in Nfatc-null mice, where knocking out Nfatc resulted in alterations to the craniofacial, cerebellar granule cell population and muscle development (ARRON *et al.* 2006). Another important gene involved in the NFAT pathways is RCAN (DSCR1) that codes for an inhibitor of the calcineurin/NFAT signaling pathway, the triplication of which in Ts65Dn mice resulted in an increased inhibition of the pathway (ARRON *et al.* 2006).

The generation of transgenic Dyrk1a mice by deleting one copy of the gene ($Dyrk1a^{+/-}$) resulted in developmental delays, neuronal deficits and overall reductions in CNS development. The deletion of both copies of the gene ($Dyrk1a^{-/-}$) resulted in gestational death, indicating that Dyrk1a is a key factor during development (FOTAKI *et al.* 2002). A transgenic mouse model overexpressing Dyrk1a protein was used to test the hypothesis that overexpression of Dyrk1a alone is sufficient to develop motor and cognitive deficits, and the results from this study showed the important role *Dyrk1a* plays during development (ALTAFAJ *et al.* 2001).

1.4 Treatment options

The current accepted protocol for treatment of cognitive and skeletal deficits in individuals with DS is early intervention to aid in learning and social interactions and treatment of symptoms and parathyroid hormone (PTH) treatment. However, as the life expectancy of individuals with DS and our knowledge of the genotype-phenotype association have increased in the past decade or so, so has the need to develop pharmaceutical interventions to help ameliorate the cognitive, skeletal and other phenotypes. Here we focus on treatment options that target various aspects of the cognitive and skeletal deficit. All individuals with DS suffer from some form of mental retardation and therefore there are many different methods of intervention being studied in laboratories all over the world. These include pharmacological targeting of neurotransmitters, environmental enrichment, DYRK1A inhibition and normalization of gene dosage, oxidative stress reduction, and normalization of excitatory/inhibitory balance. Hippocampal dysfunction is believed to be one of the most important factors that

give rise to reduced cognitive function in individuals with DS and in the Ts65Dn mouse model (HYDE *et al.* 2001; PENNINGTON *et al.* 2003). Ts65Dn mice have significantly reduced adult neurogenesis in the dentate gyrus of hippocampal formation (CLARK *et al.* 2006). Therapeutic agents that can therefore enhance development of the hippocampus are being investigated for their validity in mouse models. Ts65Dn mice (neonate and 2-5 month old) when injected with fluoxetine, a serotonin (5-HT) reuptake inhibitor, showed a significant increase in adult neurogenesis in the hippocampus which in some cases were accompanied by improvements in behavioral deficits (CLARK *et al.* 2006; BIANCHI *et al.* 2010). Synaptic plasticity relies on the excitatory/inhibitory balance of the synapses and in Ts65Dn mice there is reduced excitatory synapses which leads to enhanced inhibition (KURT *et al.* 2000; FERNANDEZ *et al.* 2007). Ts65Dn mice (3-4 months of age) injected intraperitoneally with Picrotoxin (PTX), a $GABA_A$ antagonist performed significantly better than untreated mice in an object recognition task and normalized long term potentiation (LTP) (FERNANDEZ *et al.* 2007). Increased oxidative stress may also contribute to impaired neurogenesis, and treatment of Ts65Dn mice with vitamin E, an antioxidant, has been shown to reduce markers of oxidative stress (GARDINER 2010). Another interesting avenue of treatment is maternal choline supplementation and in Ts65Dn mice, supplementation of the mothers diet with extra choline lead to a significant improvement in performance of adult mice in the radial arm water maze, a hippocampal dependent task and in adult neurogenesis (VELAZQUEZ *et al.* 2013). In a study conducted by Fowler *et al*. intermittent treatment of 3 month old Ts65Dn mice with parathyroid hormone (PTH) was shown to result in a significant increase in whole body BMD, bone formation rate, BV/TV, trabecular number and thickness. The results from this study

provided significant evidence to the use of PTH (FDA approved for bone anabolic therapy) in ameliorating the altered skeletal phenotype in DS (FOWLER *et al.* 2012).

A recent study concluded in Spain, studied the validity of Epigallocatechin-3-gallate (EGCG) in improving the learning and memory function in individuals with DS and in Ts65Dn mice (DE LA TORRE *et al.* 2014). In this study the participants were orally administrated an EGCG supplement dose of 9mg/kg/day then subjected to various neurophysiological tests, biochemical analyses and a self-made interview with parents to understand the effect of the treatment. The results revealed that EGCG treatment lead to improvements in episodic and working memory and in social interactions and parents when interviewed reported many positive changes in behavior (DE LA TORRE *et al.* 2014).

1.4.1 Epigallocatechin-3-gallate (EGCG)

Green tea is a popular beverage prepared by drying fresh tender tea leaves (YANG and LANDAU 2000). Polyphenols account for approximately 30-40% of the solid tea leaves of which epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG) and epicatechin (EC) are the most common (Figure 1.3)(SATO and MIYATA 2000). Green tea consists primarily of EGCG (50%) followed by ECG and EGC (KHOKHAR *et al.* 1997). EGCG is a small molecule that has many therapeutic effects such as anti-cancer activity, anti-oxidant activity, anti-bacterial activity, anti-allergic activity and anti-inflammatory activities (LIN and LIN 1997; BROWN 1999; YANG and LANDAU 2000; MAEDA-YAMAMOTO *et al.* 2004; KO *et al.* 2009). EGCG is a selective inhibitor of DYRK1A activity and is thought to function by binding to the ATP binding domain of the protein and inhibiting its activity (BAIN *et al.* 2003; ADAYEV *et al.* 2006). Green tea

catechins undergo rapid degradation following treatment leading to poor bioavailability (FERRUZZI *et al.* 2010). The degradation leads to oxidation, dimerization, and polymerization of catechins leading to the formation of hydrogen perioxide free radicals in aqueous media. The rate of degradation of EGCG in aqueous solution is dependent upon pH, incubation time, and temperature (NAKAYAMA *et al.* 2002). The bioavailability of EGCG was shown to be increased in the presence of ascorbic acid which reduces the solution pH (FERRUZZI *et al.* 2010). Previous studies in which the cognitive phenotype of TgDyrk1a and Ts65Dn mice was studied following oral administration of EGCG have shown that the treatment rescues learning and memory deficits, increases brain volume and rescues synaptic plasticity deficits (GUEDJ *et al.* 2009; DE LA TORRE *et al.* 2014). In another study, treatment with EGCG was shown to inhibit β-Amyloid peptide formation which leads to Alzheimer disease (AD) and lead to an improvement in memory function in a mouse model of AD (LEE *et al.* 2009a).

1.5 Thesis Hypothesis

HYPOTHESIS 1: Effect of different EGCG supplements on correcting skeletal deficits in a Down syndrome mouse model.

We hypothesized that EGCG supplements obtained from different vendors (online and health food stores) will have a differential effect in ameliorating the skeletal phenotype in Ts65Dn mice with chemical grade EGCG from Sigma resulting in the greatest improvement.

HYPOTHESIS 2: Study the effect of a three week EGCG treatment on the learning and memory defects in the Ts65Dn mouse model of Down syndrome.

We hypothesized that a three week treatment of sigma EGCG would result in improvements in cognitive function in trisomic mice when tested on locomotor activity, novel object recognition, T maze, and Morris water maze tasks in comparison to euploid control mice.

CHAPTER 2. MATERIALS AND METHODS

2.1 Effect of different EGCG supplements on correcting skeletal deficits in a Down syndrome mouse model

2.1.1 Ts65Dn husbandry

Female B6EiC3Sn a/A-Ts(17^{16})65Dn (Ts65Dn), C57BL/6J (B6) and C3H/HeJ(C3H) mice were purchased from the Jackson Laboratory (Bar Harbor, MI). The B6C3F1 mice were bred by crossing B6 and C3H males and females. Ts65Dn females (approximately 50% B6 and 50% C3H background with small trisomic marker chromosome) were bred to B6C3F1 males to generate the mice used in the study. Pups were born following 19-21 days of gestation. On postnatal day 6-10 (PD6-10) tissue was obtained and DNA isolated for genotyping by PCR. Only male mice were used in this study due to the sub fertile nature of the Ts65Dn males and the importance of females in colony maintenance. Pups were weaned on PD21, and male mice were single housed and randomly assigned to the different treatment groups

2.1.2 Mouse genotyping by PCR

All mice born into the colony were genotyped using the breakpoint PCR (REINHOLDT *et al.* 2011). The breakpoint PCR makes use of a Chr17fwd primer

(GTGGCAAGAGACTCAAATTCAAC) and Chr16rev primer (TGGCTTATTATTATCAGGGCATTT) set to amplify a ~275 bp product at the translocation point on 17^{16} murine chromosome and a positive control primer set of IMR1781 (TGTCTGAAGGGCAATGACTG) and IMR1782 (GCTGATCCGTGGCATCTATT) that amplifies a 544bp product. The final PCR mix contained 10X reaction buffer, dNTPs, $MgCl_2$, Taq polymerase, water, two sets of primers, and DNA. The PCR cycling conditions were set to 94°C for 2 minutes to initialize the reaction followed immediately by 45 seconds at 94°C to melt the template DNA in a denaturation step that yields single stranded DNA molecules. The template DNA was then cooled down to 55°C for 45 seconds to allow annealing of the primers to the template, and strand elongation to occur through the action of Taq polymerase at 72°C for 1 minute. The denaturation, annealing and elongation steps were repeated for 34 cycles and were followed by a final elongation step for 7 minutes at 72°C. To verify the PCR products, the samples were separated on a 1.5% agarose gel made by dissolving 0.75 g of agarose in 50 mL 1X TAE buffer. The size(s) of PCR products were determined by comparison with a DNA ladder, which contained DNA fragments of known size, run alongside the products on the gel. This is a crucial step in our studies as it is important to positively distinguish trisomic Ts65Dn mice from the euploid mice. The repeated backcrossing of the Ts65Dn females to C3H/HeJ males would lead to approximately 25% of all Ts65Dn pups born with this background to be homozygous for the C3H-derived retinal degeneration mutation *Pde6b*^{rd1} that causes blindness within the first few weeks of life (COSTA *et al.* 2010). Therefore, all mice born in to the colony were screened for retinal degeneration (Rd) (JACKSON 2007). The PCR mix contained 10X KCl reaction

buffer, $MgCl_2$, dNTPs, primers- IMR2093 (AAgCTAgCTgCAgTAACgCCATTT- 560 bp), IMR2094 (ACCTgCATgTgAACCCAgTATTCTATC- 240 bp), IMR2095 (CTACAgCCCCTCTCCAAggTTTATAg- 560 and 240 bp), water and Taq polymerase enzyme. The Rd PCR utilized three separate primers to identify the mice that are heterozygous (rd/+), mutated (rd/rd) or wild type (+/+). The reaction was initialized by holding the samples at 94°C for 5 minutes immediately followed by 30 seconds at 94°C to melt the template DNA in to single strands of DNA. The template DNA was then cooled down to 65°C for 30 seconds to facilitate the attachment of primers to the template, and strand elongation was performed through the action of Taq polymerase at 72°C for 1.5 minute. The denaturation, annealing and elongation steps were repeated for 35 cycles and were followed by a final elongation step for 2 minutes at 72°C. The PCR products were held at 10°C until they were separated on a 1.5% agarose gel. Only heterozygous (rd/+) and wild type (+/+) male mice were utilized in this study.

2.1.3 HPLC analysis of EGCG supplements

EGCG supplements from the following 8 sources were chosen for analysis: EGCG (Sigma Aldrich, St. Louis MO), EGCG (Swanson Health Products, Fargo ND), EGCgNOW (NOW Foods, Bloomingdale IL), EGCG (Source Naturals, Scotts Valley CA), TEAVIGO (Healthy Origins, Pittsburgh PA), Green tea extract 50% EGCG (hardrhino.com), Fluka, and SAFC. An Agilent 1200SL High Performance Liquid Chromatography (HPLC) instrument was used and coupled with an Agilent 6520 quadrupole time-of-flight mass spectrometer (MS). Samples were separated using reverse phase chromatography with a Zorbax Eclipse XD8-C18 (4.6 mm x 150 mm, and 5 µM

particle size). The mobile phase employed a HPLC gradient elution. Solvent A was ammonium acetate and solvent B was acetonitrile with 0.1% methanol, and ammonium acetate. A stepwise gradient was started with 10% B, increased to 40% B after 5 minutes, 100% from 5.50 minutes to 8 minutes, and then decreased to 10% after 2 minutes. Ultra-violet light was used to quantify and identify EGCG at a wavelength of 254 nm.

2.1.4 Degradation analysis of EGCG supplements by HPLC-MS

The potent antioxidant nature of EGCG leads to the rapid degradation of catechins under room temperature conditions, leading to poor bioavailability (FERRUZZI *et al.* 2010). Therefore in addition to purity testing, EGCG, TEAVIGO, EGCgNOW and 2 other compounds (Life Extension decaffeinated mega green tea extract and Life Extension lightly caffeinated mega green tea extract) used in a recently published study (DE LA TORRE *et al.* 2014) were analyzed for their rate of degradation. Stock solutions of 10 mg/mL of EGCG were prepared by dissolving the substances in phosphate buffered saline (PBS). All stock solutions utilized in the degradation analysis were refrigerated to maintain their stability. The supplements EGCG, TEAVIGO, EGCgNOW, Life Extension decaffeinated mega green tea extract (DE EGCG), and Life Extension lightly caffeinated mega green tea extract (LE EGCG) were diluted to a final concentration of 1 mg/mL in tap water and kept under room conditions to correspond to our general treatment protocols. The sample dilutions were prepared in triplicates to be tested 1, 24, and 48 hours following their preparation. Due to degradation within an aqueous solution, a set of EGCG samples stabilized to a pH between 5.00 and 5.55 with phosphoric acid (H_3PO_4) were also analyzed (FERRUZZI *et al.*). All HPLC/MS analyses were performed

in collaboration with Drs. Karl Dria and Tax Georgiades of the Department of Chemistry and Chemical Biology at IUPUI.

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 (A) and acetonitrile with 0.1% formic acid (B) were used with a stepwise gradient starting with 10% B and ending with 95% B 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-MS mode was used to determine the exact mass and possible formula assignments of the compounds and their fragment. Results were analyzed using MassHunter qualitative (mass analysis and formula assignments) and quantitative (UV quantitation) analysis software packages.

2.1.5 EGCG treatment preparation

Following HPLC analysis EGCG and two supplements TEAVIGO and EGCgNOW were chosen to treat Ts65Dn and euploid mice to study the effect of EGCG on the skeletal phenotype following HPLC analysis. TEAVIGO supplement by Healthy Origins (Pittsburgh, PA) contained 90% by weight EGCG per capsule and is sold as a metabolism enhancer while EGCgNOW (Bloomingdale, IL) possessed an EGCG content of 50% by weight per capsule and was marketed as a dietary supplement that provides

antioxidant and cellular health support (Table 2.1). Stock solutions of 10X TEAVIGO and EGCgNOW were prepared by dissolving a capsule in 103 and 161 mL of tap water, respectively. The solutions were briefly (5-10 minutes) placed in a 37°C water bath to ensure that they dissolved completely. The stock solutions were refrigerated at 4°C and diluted as needed. Working solutions of 20-25 mL of 0.124 mg/mL were prepared in tap water and were placed in the drinking tubes every other day.

Trisomic and euploid mice were randomly assigned to treatment groups at PD21and given free access to EGCgNOW, TEAVIGO or water (Eu, water n=18, TEAVIGO n=13, EGCgNOW n=10 and Ts, water n=10, TEAVIGO n=10, EGCgNOW n=10). On PD43 the mice were killed using cervical dislocation following prolonged exposure to isoflurane, USP (Piramal Healthcare, Andhra Pradesh, India). The femurs, mandibles, and skulls were immediately extracted and stored at -20° C until further analyses. All animal use and protocols were approved by the School of Science IACUC committee at IUPUI.

2.1.6 Dual energy X-ray absorptiometry (DXA)

The bone mineral density (BMD) and bone mineral content (BMC) of the femurs and skulls was analyzed using the Lunar Piximus DXA machine (PIXImus Lunar Corp., Madison, WI). The machine was calibrated prior to each use. The femurs and entire skulls were placed caudal side down on the densitometer and scanned using ultrahigh resolution (0.18 mm × 0.18 mm) (Li *et al.* 2009). Lunar Piximus 2 2.0 software was used to assess BMD, bone mineral content (BMC), and total bone area.

2.1.7 Micro CT imaging and analysis

Femurs were imaged using the SkyScan 1172 micro CT (SkyScan, Kontich, Belgium). The femurs from trisomic and control mice were thawed at room temperature and wrapped in parafilm, placed in a Styrofoam mold fitted to the rotating stage in the machine. The scanning parameters were set as follows: voltage 60 kV, resolution 6 μm, binning mode 2 k, and filter Al 0.5 mm. A reconstruction and analysis was conducted on each set of images using CTrecon and CTan software from Skyscan with the following parameters: post alignment variable, smoothing 2, ring artifact reduction 5, beam hardening 20, and threshold 0–0.11. A region of interest was created consisting of the boundaries of trabecular bone in the distal femur and 1 mm of tissue, proximal to the growth plate was analyzed. 3D analysis was conducted on the trabecular bones to obtain percent bone volume, trabecular number, thickness, and separation measurements. The cortical bone analysis of the mid-shaft of the femur was performed by a 2D analysis on a single section to obtain the total and bone areas, periosteal perimeter, and mean polar moment of inertia (MPMI) (calculated from CTan). The anterior–posterior diameter and average cortical thickness of a femur mid-shaft cross-section was measured using CTan and the cross-sectional moment of inertia was calculated from these measures.

2.1.8 Mechanical testing

The strength of the femur was determined by 3-point bending using a miniature materials machine (100 R250 Modular Test System, TestResources Inc, Shakopee, MN) (TURNER and BURR 1993). The femurs were thawed to room temperature, and placed posterior side down on the lower supports of the 3-point bending apparatus held apart at 7

mm. The femur was placed so that the force was applied to the midpoint of the bone at all times. The femur was preloaded using 0.1 N to establish contact with the bone and the displacement rate was set at 0.1 mm/s. Once preloaded, force was applied gradually until the bone was broken. Data were gathered by the program and analyzed using Microsoft Excel. The ultimate force (maximum force sustained by the bone, Figure 4), energy to failure (area under load-deformation curve before bone is broken, Figure 5), and stiffness of the bone (slope of the linear portion of load-deformation curve, Figure 6) were determined using a load-deformation curve as previously defined (MASHIBA *et al.* 2000).

2.1.9 Statistical Analysis

Data were analyzed using a standard two-tailed t-test with significance considered as p-values less than or equal to 0.05.

2.2 Study of the effect of a three week EGCG treatment on the learning and memory defects in the Ts65Dn mouse model of Down syndrome

2.2.1 EGCG preparation and treatment

EGCG obtained from Sigma Aldrich (EGCG, <95%) was chosen as the standard treatment due to its high level of purity. A stock solution of 15 mg/mL was made by dissolving 30 mg of solid EGCG in 2 mL of PBS and was refrigerated to maintain stability. Treatments of 0.124 mg/mL concentration were prepared by diluting the stock solution in tap water. Treatments carried out in the initial part of the study (cohorts 1- 10, Ts= 23, Eu=33) were done using EGCG that was not pH balanced. In the latter part of the study (cohorts 11-13, Ts=12, Eu=5), the EGCG and water (control) treatments were pH balanced using phosphoric acid (pH 5-5.5).

Male mice at PD21 were weaned and randomly assigned to 4 treatment groups that received either EGCG or water, (Ts, EGCG n=17, water n=15 and Eu, EGCG n=12, water n=14). Treatments were placed in drinking tubes and changed every 48 hours. All treatments were changed over to water after three weeks (PD49) and the animals were subjected to a series of behavioral tasks to assess their cognitive abilities.

2.2.2 Locomotor activity

Several groups have previously reported that Ts65Dn mice show a significant increase in hyperactivity in spontaneous locomotor activity tasks when compared to euploid controls (REEVES *et al.* 1995; HOLTZMAN *et al.* 1996; SAGO *et al.* 2000). Ts65Dn and control mice were placed in an activity chamber (Med Associates Inc., St. Albans,

VT) with Plexiglas long walls and aluminum side walls, with each chamber (25.0 × 13.75 × 15.0 cm) equipped with infrared beams along the length of the chamber 2.5 cm above the floor. The chambers were housed in light- and sound-attenuated wooden cubicles with ventilation fans on one side of the cubicle. Testing was conducted in the dark during the dark phase of the animals for 30 minutes on two consecutive days. Following the conclusion of the test on each day, the mice were returned to their home cages and the chambers were thoroughly cleaned with 70% ethanol.

2.2.3 Novel object recognition (NOR)

Rodents have a natural preference towards novel objects than objects they have been previously exposed to and the NOR task can be utilized to study the long term object recognition in trisomic mice in comparison to controls (FERNANDEZ *et al.* 2007; SMITH *et al.* 2014). NOR task was performed following previously described protocols with modifications (BABOVIC *et al.* 2008; O'TUATHAIGH *et al.* 2010). The task was conducted over three days during the dark cycle under red light as described below.

1. Habituation day (day 1) - Subject was placed inside the test arena for 15 minutes and allowed to familiarize itself with the environment. Activity was measured using ANY-maze video tracking systems (Stoelting Co, Wood dale, IL). At the end of the 15 minutes, the subject was returned to the home cage and the test arena was cleaned with 70% ethanol between each animal.
2. Exposure day (day 2) –Two similar objects were attached at the NW and SE corners of the arena, 5 cm from both walls to facilitate exploration around each object. Each

test mouse was placed at the midpoint of the wall opposite to the sample objects; with their nose facing the wall away from objects and allowed to explore the arena for 15 minutes. Time spent interacting with each object was determined as nose or whiskers touching the object or directed toward it within <1cm, or touching the object with a paw. Accidental contact with object (i.e., bumping or using the object as a platform to explore the arena) was not counted as interaction with the object.

3. Test day (day 3) – One object from exposure day and a novel object was assigned to each corner randomly. The animal was placed as described above and given 15 minutes to explore the objects and activity was tracked using ANYMAZE software and returned to the home cage at the end of the experiment.

Video recordings from the exposure and test days were scored for the time spent exploring each object by a minimum of three independent observers who were blind to experimental conditions to ensure accuracy. The data gathered from the videos were used to determine the discrimination ratio using the following formula:

Discrimination ratio (%) = Time exploring novel object- Time exploring familiar object * 100

2.2.4 Delayed non-matching to place (DNMP) task

The DNMP task is based upon the natural tendency of rodents to spontaneously alternate between left and right arm choices in a T- maze (DEACON and RAWLINS 2006; RUBY *et al.* 2013). Mice were food restricted till they reached 80% of free-feeding weight after the completion of NOR test and then trained to run the DNMP task as described in

earlier studies with modifications (GOODLETT *et al.* 1988). The apparatus used was a wooden T-maze painted flat gray with a start arm and goal arms each 40 cm long and 12 cm wide, with guillotine doors at the start arm and the entrance to each arm. The maze was cleaned between each mouse by wiping the alleys with 70% ethanol.

1. Phase 1- The mice were given three days of habituation and allowed to freely explore the T-maze and obtain chocolate milk at each arm.
2. Phase 2- Mice were alley trained to run from the start arm to the end of a randomly selected goal arm with the other arm blocked off.
3. Phase 3- Mice were then trained on the DNMP task to attain a criterion of 80% correct choices on three consecutive days. Each day, they were given 8 "trial couplets"; 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 placed in the arm opposite from the previously arm, and the mouse was allowed to choose either of the two arms. If the mouse chose the incorrect arm, it was allowed to move through the maze till it came to the correct arm and then was blocked in. After each trial couplet, the animal was returned to its home cage for 30 seconds. This training on "0 second" delay between the forced run and choice run of each trial couple continued daily until the mouse reached the 80% criterion on three consecutive days. Half of the trials had the forced run to the left arm, while the other half was forced into the right arm, a schedule randomly assigned by the experimenter each day.

4. Phase 4- Once the mouse reached 80% criterion, an additional five days of testing ensued the next day, with each day having 8 “trial couplets” as described above with a 30 second delay between the forced and choice trial. During the 30 second delay, the animal was placed in a holding cup. After each trial couplet, the animal was returned to its home cage for 30 seconds. Delays were introduced to test spatial working memory function over delay intervals.

2.2.5 Morris water maze (MWM) task

The MWM is a hippocampal dependent learning and memory task which tests the ability of the mouse to use spatial cues to locate a hidden platform. The test consists of 7 consecutive days of training, 4 trials per day, and followed 24 hours later by a probe test. The mice were trained in squads of 4 yielding a delay of 3-5 minutes between trials. The testing apparatus is a 9cm (diameter) platform that was placed in a 125cm (diameter) pool filled to within 25cm of the rim of the tank with 25°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. The testing environment was illuminated and contained visual reference cues (e.g. posters, curtains). The platform position remained constant throughout training; however, the starting location of each series of 4 trials varied each training day. The mice begin training in the water maze at 60 days of age, by an experimenter blind to treatment and genotype. The computer-controlled tracking software displays the moment-to-moment position of the animal in the pool. Dependent variables assessed by this software included escape latency, escape path length, swim speed, initial heading angle and time spent in thigmotaxis (swimming the perimeter of the pool). Training trials

consisted of placing the mouse in the pool (facing the rim of the tank) at one of 8 starting positions. The animal was allowed to swim for 60 seconds or until the platform was found. Different, pseudo-randomly determined, starting points were used for each trial. Animals were not started within the same quadrant as the target, nor were start positions repeated on a given day. If a mouse was unable to find the platform, it was guided to the platform by the experimenter. All animals remained on the platform for 10 seconds before being returned to their respective incubators (30°C), used to limit hypothermia during the waiting interval. Mice were tested in squads of 3 or 4, resulting in a waiting interval of approximately three minutes. At the end of each day of testing, the mice were allowed to remain in the incubators until warm and mostly dry (approximately 10 minutes) before being returned to the vivarium.

On the eighth day of training, animals were subjected to a single 60-second probe trial starting at a pseudo-randomly assigned location. The platform was removed before the probe trial, and the animal's search path was assessed via computerized video tracking superimposing four virtual counting discs over the pool in the location of each of the four possible platform positions used for the place training condition. The video tracking software (HVS Image, Hampton, UK) obtained measures of time spent in, latency to enter, and numbers of passes through each of the four virtual counting discs; these measures were used to quantify the spatial distribution of the search strategy of each animal. The escape platform location used during training was designated as the target counter and compared to the average of the non-target counter variables.

2.2.6 Statistical analysis

Data for the locomotor activity was analyzed with a mixed ANOVA with Genotype and Treatment as grouping factors and Day and Bin as repeated measures. The DNMP task and the NOR task were analyzed using two-way ANOVAs with Genotype and Treatment as grouping factors. The MWM acquisition data (average daily latency to find the platform) were analyzed with a mixed ANOVA with Genotype and Treatment as grouping factors and Day as the repeated measure. For the probe day, time spent in the target quadrant virtual disk counter was analyzed with a two-way ANOVA. Significance was considered as p-values less than or equal to 0.05.

2.2.7 Isolation of protein from brain tissue and quantification

Total protein fraction of brain tissue was determined following a protocol adapted from previously published studies (PAPADOPOULOS *et al.* 2011; PONS-ESPINAL *et al.* 2013). Tissue samples were isolated from brain segments (cortex, hippocampus and cerebellum) in RIPA buffer (10mM Phosphate Buffer (pH 7.4), 10% Glycerol (Fisher Scientific, Fair Lawn, NJ), 1% NP-40 (United States Biological, Swampscott, MA) 0.1% SDS (Fisher Scientific, Fair Lawn, NJ), 4mM EDTA (Fisher Scientific, Fair lawn, NJ), 0.15M NaCl (Fisher Scientific, Fair Lawn, NJ), 1x Protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) of a cohort of mice (trisomic and euploid) that were 6 weeks of age. The tissue samples were snap frozen in liquid nitrogen and stored at -80°C until protein isolation. The isolated protein was quantified using a Bradford assay (BRADFORD 1976). The protein samples were stored at -80°C until further use.

2.2.8 Immunoprecipitation of Dyrk1a protein

The protein sample was cleared of any antibodies by pre-incubation with EZ-view Red Protein affinity gel (30 µl, Sigma-Aldrich, St Louis, MO) for 30 minutes. The protein samples were then centrifuged at 16000g for 10 minutes at 4°C following which the supernatant was removed into fresh tubes. The lysate was mixed with 2 µl of mouse anti-Dyrk1a (7D10, Abnova, Taipei city, Taiwan) antibody, vortexed briefly and incubated for 1 hour at 4°C in a rotator. During incubation, Protein-G sepharose beads (4B, bioWORLD, Dublin, OH) were carefully mixed and dispensed (30 µl) into a fresh set of tubes placed on ice. The beads were gently washed by adding 500 µl of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 15% glycerol, 1 mM EDTA, and protease inhibitor (Roche Diagnostics, Mannheim, Germany) 1 tablet/50 mL added immediately before use and briefly vortexing the samples. The protein samples were then centrifuged for 30 seconds at 8200g. The supernatant was carefully removed and discarded and the beads were washed once more as described above. The tubes were placed on ice and the lysate-antibody mix was added into the beads. The tubes were briefly vortexed and incubated at 4°C overnight to facilitate the attachment of protein-antibody complex on to the sepharose beads. Following the overnight incubation, the samples were centrifuged and placed on ice (1600g, 30 seconds, and 4°C). The supernatant was carefully removed and the beads were washed on ice as follows:

1. 2 x in 500µl of wash buffer with NP-40 (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2mM EDTA, 0.1% NP-40)

2. 2 x in 500µl of wash buffer without NP-40 (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2mM EDTA)
3. 1 x in 500µl of kinase buffer (250 mM HEPES, pH 7.0, 50 mM $MgCl_2$, 5 mM DTT)

After the addition of each buffer, the tubes were vortexed briefly and gently mixed in a water bath (2-8°C) for 5 minutes. The samples were centrifuged (1600g, 30 seconds) and the supernatant was removed on ice. Following the removal of the kinase buffer, the beads were placed on ice and subjected to a kinase assay.

2.2.9 Determination of kinase activity

The kinase activity of the protein samples were determined using a previously published protocol adapted from Pons-Espinal, 2013 and personal communications with Prof. Dr. Walter Becker (PONS-ESPINAL *et al.* 2013; BECKER 2014). A master mix containing 1x kinase buffer, 100 µM ATP, and 200 µM Dyktide (Dyrktide-RRRFRPASPLRGPPK, SignalChem, Richmond, BC) (HIMPEL *et al.* 2000) was prepared and 2 µCi/sample of [γ^{-32}] ATP was added into the master mix immediately prior to use. Trisomic samples were incubated with 2µM Harmine (New Jersey, USA) and EGCG solutions of differing concentrations (10, 25, and 250 µM) to analyze the effect of inhibition on Dyrk1a kinase activity. The beads from the immunoprecipitation assay were incubated at 30°C for 50 minutes with 30 µl of master mix. Following incubation, the reaction was arrested by adding 1/3 volume 100 mM EDTA. 10 µl of each of the samples were blotted on to P81 Whatmann filter paper (GE Healthcare UK Limited, Buckinghamshire, UK) in triplicates and washed extensively (8 x 1mL of 5% phosphoric acid) under vacuum conditions. The filter papers were air dried and placed in plastic

scintillation vials and 5 mL of scintillation liquid (SX20-5 ScintiSafe Econo1 Cocktail, Fisher Chemical, Fair Lawn, NJ) was added into each vial. The vials were placed in the liquid scintillation counter (Beckman, LS 60001C) and the ^{32}P levels were detected.

CHAPTER 3. RESULTS

3.1 Effect of different EGCG supplements on correcting skeletal deficits in a Down syndrome mouse model

3.1.1 HPLC analysis of EGCG supplements

We hypothesized that EGCG supplements obtained from different sources would display differential effects in the amelioration of the skeletal phenotype, with EGCG resulting in the most significant benefits in Ts65Dn mice. To test our hypothesis, we performed HPLC analysis on the different EGCG supplements to identify the level of purity and composition. EGCG possessed a catechin content of >95% and exhibited a large peak on the HPLC-DAD1 (diode array detection) at a retention time of 2.5 minutes that was identified as EGCG and two small peaks at 1.5 and 1.7 minutes that were detected to be contaminants (Figure 3.1). EGCG displayed a relative abundance of *m/z* (mass/charge ratio) 459.0. TEAVIGO supplement had a manufacturer stated EGCG content of 90% of capsule weight and displayed the highest level of purity in comparison to EGCG with a retention time of 2.5 minutes and a single peak at *m/z* 459.0 that corresponded with EGCG (Figure 3.2). EGCgNOW DAD with a manufacturer stated catechin content of 50% by capsule weight showed three small peaks at 1.3, 1.9, and 4.4

minutes which corresponded with EGC, EC/EGCG, and ECG respectively and a larger peak at 2.6 minutes that corresponded with EGCG; with a *m/z* 459.0 (Figure 3.3). Based on the comparisons of purity obtained from HPLC, EGCgNOW was chosen as it appeared to have the least purity in comparison to the other compounds while TEAVIGO exhibited purity closest to that of EGCG and was chosen to compare the differential effects of EGCG compounds obtained from non-chemical companies.

3.1.2 Degradation analysis of EGCG supplements by HPLC-MS

The strong antioxidant nature of EGCG results in its rapid degradation and poor bioavailability (FERRUZZI *et al.* 2010). To study the rate of degradation and the effect of the addition of phosphoric acid to stabilize the catechin content, we conducted a degradation analysis of EGCG supplements by HPLC-MS. The EGCG solutions showed significant reduction in EGCG concentration following 24 and 48 hours incubation when compared to the 1 hour EGCG sample (Table 3.1). LE EGCG had a significantly higher EGCG concentration than EGCG alone after 24 and 48 hour incubations while the addition of phosphoric acid to EGCG resulted in a significant increase in EGCG concentrations at 24 and 48 hours when compared to the same time points of EGCG only samples. DE EGCG had a significantly lower EGCG concentration following 1 hour of incubation but after 24 and 48 hours, DE EGCG showed a significantly higher EGCG concentration than EGCG at same time points (Table 3.1). TEAVIGO and EGCgNOW supplements behaved the poorest with both supplements resulting in significantly reduced EGCG concentrations following 1, 24, and 48 hours of incubation in comparison to EGCG samples at the same time points (Figure 3.4).

3.1.3 Dual energy X-ray absorptiometry (DXA)

To compare the density of the femurs and skulls between mice treated with the different EGCG supplements, DXA analysis was performed following the removal of the bones at 6 weeks. Bone mineral density (BMD), bone mineral content (BMC) and area data were obtained from DXA scanning and analyzed to study the effect of each treatment. BMD of trisomic femurs treated with water (control) were compared to euploid control mice to establish a genotype effect and we observed a significant reduction in BMD of trisomic femurs. The treatment of trisomic mice with EGCG resulted in a significant increase in BMD compared to control trisomic mice (Figure 3.5). However, the increase in BMD imparted by TEAVIGO and EGCgNOW treatment in trisomic mice was non-significantly less than that of EGCG treated trisomic mice. DXA analysis of skulls of EGCG treated trisomic mice showed that EGCG, TEAVIGO, and EGCgNOW treatments led to no significant improvements in BMD (Figure 3.6).

3.1.4 Micro CT imaging and analyses

Micro CT imaging was utilized to study the microstructure and organization of the trabecular bone (distal end) and cortical bone (mid shaft) of femurs. Results from our study agreed with the observations of a previous study conducted in our lab by showing that trisomic mice have a greater trabecular separation, reduced percent bone volume (BV/TV), trabecular number and thickness (BLAZEK 2011). We observed that EGCG treatment lead to a significant increase in percent bone volume and trabecular thickness to control trisomic mice. TEAVIGO supplement significantly reduced trabecular separation in trisomic mice compared to EGCG treated Ts65Dn mice; while EGCgNOW

resulted in a significant increase in percent bone volume and trabecular number in Ts65Dn mice when compared to EGCG treated trisomic mice (Table 3.2).

Blazek *et al.*, identified significant reductions in bone perimeter and mean polar moment of inertia in trisomic cortical bone in comparison to euploid controls (BLAZEK 2011). In our study we observed a similar result with trisomic femurs without treatment showing a significant reduction in bone perimeter and polar moment of inertia (Table 3.3). Treatment of trisomic mice with EGCG resulted in no significant improvement in bone perimeter or polar moment inertia compared to euploid control mice while TEAVIGO and EGCgNOW treatment lead to significant improvements in both cortical bone parameters when compared to EGCG treated trisomic bones (Table 3.3).

3.1.5 Mechanical Testing

To determine the effect of structural changes observed by micro CT and DXA on the bone strength, a three-point bend test was performed on both euploid and trisomic femurs. In a previous study by Blazek and colleagues, it was shown that Ts65Dn femurs had a significantly lower ultimate force (the maximum force that is sustained by the bone), stiffness (area under load-deformation curve before bone is broken) and energy to failure (area under load-deformation curve before bone is broken) than euploid mice (BLAZEK 2011). Our study replicated the results from the Blazek study and showed significant reductions in ultimate force, stiffness, and energy to failure of trisomic control femurs when compared to euploid controls. We observed that EGCG treatment led to a significant improvement in the energy to failure of trisomic femurs compared to trisomic water treated mice (Figures 3.7, and 3.8). TEAVIGO and EGCgNOW treatment of

trisomic mice only resulted in significant reduction in all three mechanical test parameters in comparison to EGCG treated Ts65Dn mice (Figures 3.7, 3.8 and 3.9). Furthermore, we observed that TEAVIGO and EGCgNOW supplements significantly reduced all euploid bone measures while pure EGCG significantly lowered the energy to failure compared to euploid control mice.

3.2 Study the effect of a 3 week EGCG treatment on the learning and memory defects in the Ts65Dn mouse model of Down syndrome

3.2.1 Locomotor activity

The locomotor activity task was utilized to analyze the spontaneous activity patterns of trisomic mice in comparison to controls. Several studies have reported that trisomic mice display a significantly higher level of spontaneous activity in comparison to wild type mice (HOLTZMAN *et al.* 1996; SAGO *et al.* 2000). Figure 3.10A shows locomotor activity as a function of distance traveled per 1-minute bins on each of the two days of testing. There was a significant effect of Genotype [$F(1,67)=7.28$, $p=0.009$], but no significant effect of EGCG treatment. The effect of genotype was due to the higher daily locomotor activity of the trisomic mice compared to the euploid mice, as shown in Fig. 3.10B.

3.2.2 Novel object recognition (NOR)

Rodents have a natural preference to explore novel objects over familiar objects. In the NOR task, testing preference for a novel object over one that has been recently

encountered, can be utilized to study the object recognition memory, and trisomic mice have been found to be impaired relative to euploid controls (FERNANDEZ *et al.* 2007; SMITH *et al.* 2014). The discrimination ratios from the Novel Objet Recognition data were analyzed with a two way ANOVA using Genotype and Treatment as a between subjects variable. A significant effect of genotype was found [F(1,63)=4.289, p=0.042], due to the lower discrimination ratios of the Ts65Dn mice (see Fig.3.11); there was no significant main or interactive effect of EGCG treatment on NOR.

3.2.3 Delayed non-matching to place (DNMP) task

The DNMP task was utilized to analyze acquisition of a spatial working memory task.. A two-way ANOVA was used to examine the number of trials to criterion (number of trials required by the mouse to reach 80% correct choices on three consecutive days in the "0-sec delay" training) in Ts65Dn mice. A significant effect of genotype was found [F(1,21)=6.56, p=0.018] indicating that trisomic mice required more trials to learn the task (Figure 3.12). However, there were no significant main or interactive effects of the three week EGCG treatment on DNMP acquisition.

3.2.4 Morris water maze (MWM) task

The MWM spatial learning task is a commonly utilized test to study hippocampus dependent spatial learning and memory behaviors (MORRIS *et al.* 1982). Multiple studies have reported significant deficits in trisomic mice in both the latency (time taken to reach the platform) and spatial search strategies in probe trials in comparison to control mice (REEVES *et al.* 1995; SAGO *et al.* 2000; HYDE *et al.* 2001). The average daily latency over

the seven days of training was analyzed using a mixed ANOVA with day as repeated measure. There was a significant main effect of Genotype [$F(1,20)=6.96$, $p=0.016$], in addition to the significant main effect of day [$F(6,120) = 11.008$, $p<.001$] (Figure 3.13).

For the probe trial, the trial 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. There was a significant effect of disc location [$F(1,20)=12.586$, $p=0.002$] (Figure 3.14); the lower target search times of the trisomic mice did not reach statistical significance ($p=0.054$). The EGCG treatment did not significantly affect either the acquisition latency measures or the time spent in target quadrant during probe day.

3.2.5 Dyrk1a kinase assay

The triplication of *Dyrk1a* gene dosage and the resulting increase in protein kinase activity levels have been hypothesized to be a major cause of the cognitive phenotype observed in individuals with DS and Ts65Dn mice (KORENBERG *et al.* 1994; ARRON *et al.* 2006). In previous studies, the kinase activity in the brains of trisomic mice was shown to have a 1.5 fold increase in activity than in the euploid samples (GUIMERA *et al.* 1999; DOWJAT *et al.* 2007). In our study the Dyrk1a kinase activity of trisomic hippocampal tissue showed a ~ 1.6 fold increase compared to a euploid tissue (Figure 3.15). The addition of Harmine, a chemical inhibitor of Dyrk1a kinase activity resulted in a ~0.7 fold reduction (BECKER 2014). Furthermore, we observed a dose-dependent

reduction in Dyrk1a kinase activity of 1.2, 0.9, and 0.8 fold with the addition of 10, 25, and 250 μM of EGCG to trisomic hippocampal tissue samples respectively.

CHAPTER 4. DISCUSSION

4.1 Different composition of EGCG supplements results in differential effect in the rescue of skeletal phenotype in Ts65Dn mice

Our study demonstrates that (1) EGCG supplements improved cortical bone and some trabecular bone measures with no concurrent improvement in bone mechanical properties in Ts65Dn mice, (2) EGCgNOW and TEAVIGO undergo rapid degradation under room temperature conditions resulting in poor bioavailability of EGCG polyphenol. Previous studies have shown that individuals with DS have a significantly reduced areal BMD in the femoral neck, spine, hip, total body, total hip, and lumbar spine in comparison to healthy individuals (BAPTISTA *et al.* 2005; GUIJARRO *et al.* 2008). Therefore, it was hypothesized that Trisomy 21 may result in several risk factors that predispose individuals with DS to an osteoporosis like phenotype (GUIJARRO *et al.* 2008). The skeletal deficits observed in humans with DS have been shown to be present in the Ts65Dn mouse model as well. In a previous study conducted in our lab, Blazek *et al*. identified significant reductions in BMD of femurs, percent bone volume (BV/TV), trabecular thickness, number, mineral apposition rate, bone formation rate, and bone strength in adolescent and adult Ts65Dn mice (BLAZEK 2011). The adolescent skeletal deficits together with changes in serum bone markers were also present in adult mice (3 & 24 month) (FOWLER *et al.* 2012). In our study we observed significant reduction of

BMD, percent bone volume, trabecular number, thickness, and bone strength properties together with an increase in trabecular separation in trisomic mice compared to euploid littermates. Epigallocatechin-3-gallate (EGCG), the main polyphenolic compound in green tea was chosen as a therapeutic target as it is a selective inhibitor of Dyrk1a activity and in Fetal Alcohol Syndrome (FAS) studies it has been shown to ameliorate craniofacial and cognitive phenotypes. (BAIN *et al.* 2003; ADAYEV *et al.* 2006; LONG *et al.* 2010; TIWARI *et al.* 2010). FAS and DS share many phenotypic similarities and it is therefore believed that they share some common mechanisms at the cellular and molecular levels (SOLZAK *et al.* 2013). Analysis of femurs following EGCG treatment by Blazek *et al*. showed significant improvement of femoral BMD, percent bone volume, trabecular thickness, and trabecular number compared to euploid littermates. They further observed an increase in mineral apposition rate in Ts65Dn femurs with no positive effects on the energy to failure, stiffness, and ultimate load on the trisomic femurs (BLAZEK).

In our current study, we analyzed the effect of over the counter EGCG supplements on the femurs of Ts65Dn mice. We observed a significant increase in percent bone volume and trabecular number with EGCgNOW supplement and a significant increase in cortical bone measures with both supplement treatments. HPLC analysis of EGCgNOW revealed the presence of EGC and ECG, the 2nd and 3rd most common green tea catechin after EGCG (KHOKHAR *et al.* 1997). EGCG and ECG are the most active green tea catechins with a multitude of biological actions (HAE-SUK KIM 2014). EGC treatment of rat osteoblast-like osteosarcoma cell line have shown that this green tea catechin can promote osteoblast activity while inhibiting osteoclast differentiation in RAW 267.7 cell lines (KO *et al.* 2009). The strong anti-oxidant nature

of EGCG, ECG, and EGC results in their rapid degradation at near-neutral and high pH conditions (QIN YAN ZHU 1997; CHEN *et al.* 2001). Catechins also undergo extensive degradation under digestive conditions (NEILSON *et al.* 2007). Digestion of EGCG in an *in-vitro* digestion model was shown to result in a digesta containing residual EGCG, EGCG C_2'- C_2' homodimers THSN A, THSN D, and EGCG B-ring condensation-elimination homodimer P-2 (NEILSON *et al.* 2007). The EGC digesta contained residual EGC, gallocatechin (GC), and EGC homodimers similar to those observed for EGCG and ECG digesta contained residual ECG and catechin gallate (CG) (NEILSON *et al.* 2007). Therefore, we hypothesize that the presence of other catechins in supplements, degradation of EGCG, ECG, and EGC from exposure to light and room temperature (RT), and intestinal digestion may play a crucial role in the differential skeletal effects observed with EGCG supplements. The analyses of EGCG preparations following 1, 24, and 48 hour incubations by HPLC indicate that pure EGCG and in particular the supplements underwent rapid degradation over time, resulting in very low levels of EGCG at the end of 48 hours of exposure to RT conditions.

We observed that EGCG supplements, in particular EGCgNOW led to an increase of trabecular thickness, trabecular number, percent bone volume, bone perimeter, and polar moment of inertia compared to euploid controls. However, it did not result in an improvement in mechanical strength of the femur. We hypothesize that the improvements in trabecular and cortical bone measures by EGCG supplements does not affect the actual toughness of the bone. Toughness is a measure of resistance to fracture formation and collagen fiber play an integral role in this function (R.K. NALLA 2006; RITCHIE *et al.* 2008). In a normal individual collagen fibrils undergo deformation and elongation under

force but return to normal with the removal of the force (RITCHIE *et al.* 2008). However, in aged collagen a higher number of cross-links are formed which results in a "brittle" bone which is more susceptible to fractures. R.K. Nalla *et al*. demonstrated that fracture toughness of cortical bone is significantly reduced in aged individuals (R.K. NALLA 2006). Individuals with DS undergo accelerated aging and therefore are afflicted with age related diseases such as osteoporosis like bone phenotype. The supplements, EGCg NOW and TEAVIGO may increase cortical and trabecular measures without affecting the collagen structure and therefore we observe no improvements in mechanical strength properties of the bone. EGCgNOW and TEAVIGO were observed to significantly decrease stiffness, ultimate force, and energy to failure for euploid mice which indicates that these supplements alter the mechanical properties of bone. The supplements may be harmful in the absence of overexpression of Dyrk1a and therefore could be harmful if used by a normal individual. Therefore, further studies need to be performed to analyze the effect of EGCG supplements in the absence of trisomy before it can be used to rescue bone deficits in normal individuals.

4.2 Three week treatment of adolescent Ts65Dn mice with low dose EGCG resulted in no improvement of learning and memory deficits.

All individuals with DS are affected by learning and memory deficits which occur early and persist throughout life (EPSTEIN 2001b; GARDINER *et al.* 2003). Ts65Dn mice recapitulate the DS cognitive phenotype and therefore provide an excellent predictive organism to study the effect of trisomy on learning and memory functions (ESCORIHUELA *et al.* 1995; REEVES *et al.* 1995). Our results showed that trisomic mice are significantly

more active, require a significantly higher number of trials to reach criteria in the DNMP task, have a significant lower discrimination ratio in NOR, and have a significantly higher latency in training phase of the Morris water maze. The results from our study support observations from previous studies and show that Ts65Dn mice are affected with learning and memory deficits that center around the hippocampus (REEVES *et al.* 1995; HYDE *et al.* 2001; FERNANDEZ *et al.* 2007; KLESCHEVNIKOV *et al.* 2012).

However, we did not observe a rescue effect in trisomic mice treated with a low-dose EGCG during adolescence in the behavioral tasks performed. In a recently published study, treatment of adult Ts65Dn with a EGCG supplement was shown to improve spatial learning in MWM and object recognition in NOR (DE LA TORRE *et al.* 2014). Two distinct differences exist between our study and De La Torre *et al*.: (1) we treated adolescent mice (aged 3-6 weeks) while De La Torre study used adult (3 months) mice and, (2) we treated mice with 0.124 mg/ mL (0.6-0.7 mg/day) for three weeks while the De La Torre study treated their animals at a concentration of 90 mg/mL (2-3 mg/day) for 1 month.

EGCG is a selective inhibitor of Dyrk1 protein activity and undergoes rapid degradation following administration (BAIN *et al.* 2003; FERRUZZI *et al.* 2010). The plasma levels of EGCG following oral gavage were shown to return to baseline levels in as short as 6 hours in rats (FERRUZZI *et al.* 2010). Therefore, we hypothesize that the short duration of treatment of adolescent mice with a low concentration of EGCG may be insufficient to improve hippocampal dependent learning and memory deficits.

4.3 Future directions

Our studies revealed that ready available EGCG supplements result in confounding effects. We observed that supplements can improve trabecular and cortical bone measures but with no concurrent improvement in mechanical strength. Furthermore, HPLC analysis of the supplements showed the presence of other green tea catechins and rapid degradation in solution. We observed a significant reduction in energy to failure, stiffness, and ultimate force in euploid mice treated with TEAVIGO and EGCgNOW for three week during adolescence. The popularity of EGCG in DS research and wide interest in these published studies from the DS community suggests we as researchers need to be very careful in reporting results from our studies. Therefore, we propose that more studies into the safety of EGCG herbal supplements to normal as well individuals with DS need to be performed before EGCG can be recommended for human use. The rapid degradation under neutral/ basic conditions should be addressed by creating a better transport mechanism for EGCG. EGCG that can be specifically targeted to a particular region of the body will be more effective than a general capsule approach. In another step to elucidate the Dyrk1a-Nfat mechanism, we would begin treatment of three week old Ts65Dn and control mice with an Nfatc inhibitor, cyclosporin A. We hypothesize that if skeletal defects are a result of Dyrk1a- Nfatc pathways as proposed, we will observe reduced BMD, altered trabecular, cortical bone structures, and mechanical properties of bone in euploid and trisomic mice. Previous studies conducted in our lab indicate that the trisomic skeletal deficit is a result of an increase in osteoclasts and therefore increased resorption of bone. In-vitro cell culture of osteoclast cells from euploid and trisomic bone marrow cells will provide us with a convenient method to study the role of trisomy on

osteoclast development. The application of EGCG from chemical as well as over the counter sources will help us further analyze the effect of supplements on bone development.

We observed no EGCG effect on the behavioral phenotype of Ts65Dn mice when treatment was performed during adolescence. We propose moving forward; treatment is continued throughout the behavioral testing period. As EGCG is a selective Dyrk1a kinase inhibitor, it should be present continuously in order to observe improvements in the cognitive defects. The pH of the EGCG treatment should be lowered to more acidic levels (5-5.5) by the addition of phosphoric acid to reduce the rate of degradation that was observed in HPLC-MS analysis. This will produce a more uniform concentration of EGCG during the treatment period and reduce concentration related fluctuations in data. Dyrk1a knock-out mice, with the normal trisomic complement of genes except for Dyrk1a (reduced to two copies) should be incorporated into behavioral studies to observe the role of Dyrk1a in hippocampal dependent learning and memory deficits. Furthermore, we propose that Dyrk1a kinase activity of protein isolated from the hippocampal tissue of EGCG treated mice following the end of behavioral testing be analyzed to study the effect of continuous EGCG treatment on kinase activity. Finally, a study of the different green tea catechins (ECG, EGC, and EC) both as a pilot mouse study and kinase activity study should be performed to analyze the effect of these catechins on both skeletal and behavioral phenotype in the Ts65Dn mouse model.

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TABLES

TABLE

2.1: Percentage of EGCG in different supplements.
The amount of EGCG present in the treatments utilized to study the effect of EGCG on the skeletal phenotype in Ts65Dn mouse model.

Name	Source	Weight of capsule	% of EGCG/capsule
EGCG	Sigma-Aldrich		>95%
EGCgNOW	NOW FOODS	400mg	50% (200mg)
TEAVIGO	Healthy Origins	150mg	90% (135mg)

3.1: Degradation analysis of EGCG supplements by HPLC-MS.
Results showed that LE EGCG showed the most stability over time and that the addition of phosphoric acid results in the increase of EGCG stability while TEAVIGO and EGCgNOW were the most unstable, resulting in significant reduction in EGCG concentration over time.

Treatment	Time point	Expected concentration (mg/mL)	Calculated concentration (mg/mL)
EGCG (3)	1	1	1.001±0.0015
	24	1	0.3349 ± 0.014^{A}
	48	1	0.1699 ± 0.013^{A}
EGCG+Phosphoric acid (3)	1	1	0.9833±0.008
	24	1	0.5068 ± 0.003^{B}
	48	1	0.4637 ± 0.0013^{C}
Lightly caffeinated EGCG- LE EGCG (3)	1	1	1.0567 ± 0.01^{D}
	24	1	0.6038 ± 0.012^{B}
	48	1	0.3975 ± 0.05^{F}
Decaffeinated EGCG- DE EGCG (3)	1	1	0.9294 ± 0.006^{A}
	24	1	0.5624 ± 0.017^{B}
	48	1	0.3083 ± 0.011^{C}

3.1, continued.

TEAVIGO (3)	1	1	0.5376±0.0009[A]
	24	1	0.1247±0.005[B]
	48	1	0.0601±0.002[C]
EGCgNOW (3)	1	1	0.3918±0.004[A]
	24	1	0.1115±0.0005[B]
	48	1	0.0823±0.0004[E]
A $p< 0.001$ when compared to 1 hour EGCG; B $p< 0.001$ compared to 24 hour EGCG; C $p\leq 0.001$ compared to 48 hour EGCG; D ≤ 0.05 compared to 1 hour EGCG; E ≤ 0.05 compared to 48 hour EGCG; F≤ 0.01 compared to 48 hour EGCG			

3.2: Trabecular bone analysis by micro CT.

We observed significant alteration of trabecular bone structure in trisomic mice compared to euploid controls. EGCG improved the percent bone volume and trabecular number of trisomic mice in comparison to untreated Ts65Dn mice. EGCgNOW treatment resulted in improvement of percent bone volume and trabecular number while only the TEAVIGO treatment could significantly improve trabecular separation when compared to EGCG treated trisomic mice.

Genotype	Treatment	Percent bone volume (BV/TV)	Trabecular separation (mm)	Trabecular thickness (mm)	Trabecular number (mm^{-1})
Euploid	Water	16.3±1.02	0.25±0.009	0.053±0.001	3.04±0.2
	EGCG	15.3±0.62	0.27±0.008	0.053±0.001	2.85±0.1
	TEAVIGO	17.1±1.0	0.25±0.01	0.057±0.002	3.0±0.15
	EGCgNOW	18.0±1.44	0.27±0.008	0.06±0.002	3.2±0.2
Trisomic	Water	$9.1\pm1.1^{A,B}$	0.30 ± 0.015^{C}	0.05 ± 0.001^{C}	1.86 ± 0.21^{A}
	EGCG	12.5 ± 1.04^{D}	0.27±0.007	0.053±0.001	2.35 ± 0.16^{D}
	TEAVIGO	13.8±1.4	0.30 ± 0.012^{B}	0.057±0.002	2.13±0.12
	EGCgNOW	15.0 ± 0.95^{B}	0.27±0.013	0.06±0.002	2.73 ± 0.12^{B}

A $p\leq0.001$ compared to euploid water; B $p\leq0.05$ compared to trisomic EGCG; C $p<0.05$ compared to euploid water; D $p<0.05$ compared trisomic water.

3.3: Cortical bone analysis by micro CT.

The bone perimeter and the polar moment of inertia were significantly reduced in trisomic control mice compared to euploid controls. EGCgNOW and TEAVIGO treatment resulted in significant improvement in both cortical bone parameters in trisomic mice compared to trisomic controls.

Genotype	Treatment	Bone perimeter	Polar moment of inertia
Euploid	Water	13.43±1.13	0.47±0.04
	EGCG	12.1±0.66	0.47±0.042
	TEAVIGO	16.41±1.91	0.49±0.05
	EGCgNOW	18.9±2.02	0.57±0.04
Trisomic	Water	9.14±0.23[A]	0.27±0.022[A]
	EGCG	8.88±0.18	0.27±0.021
	TEAVIGO	14.2±1.07[B]	0.43±0.03[B]
	EGCgNOW	14.9±1.92[C]	0.42±0.05[C]
A <0.05 compared to euploid water; B <0.001 compared to trisomic EGCG; C <0.05 compared to trisomic EGCG			

FIGURES

FIGURES

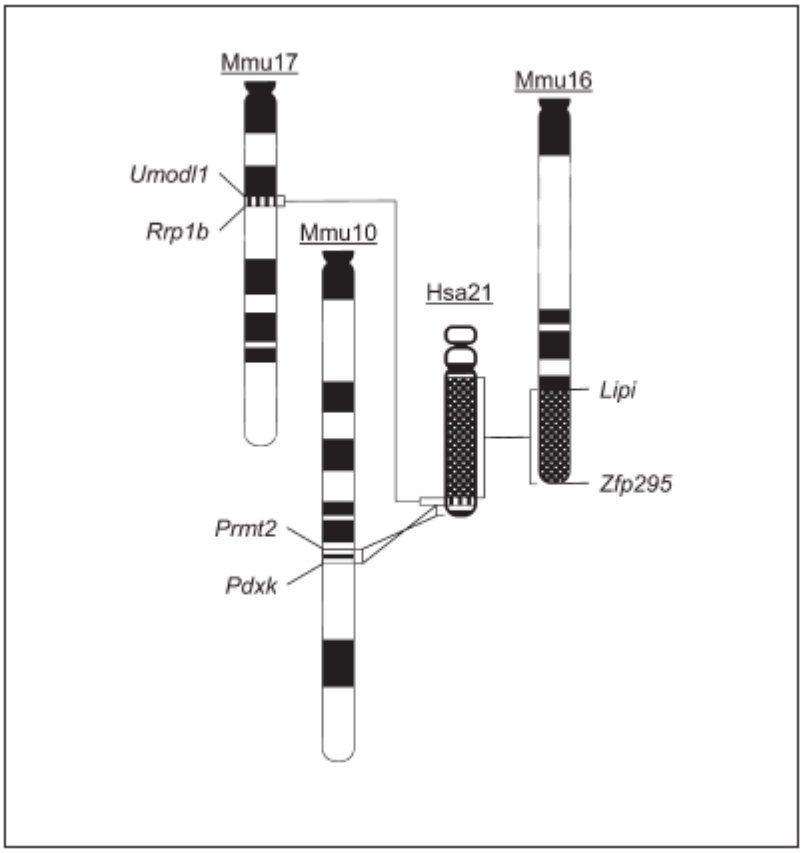


Figure 1.1 Gene orthologs of human chromosome 21 (Hsa21) found on three mouse chromosome, Mmu10, 16, and 17.

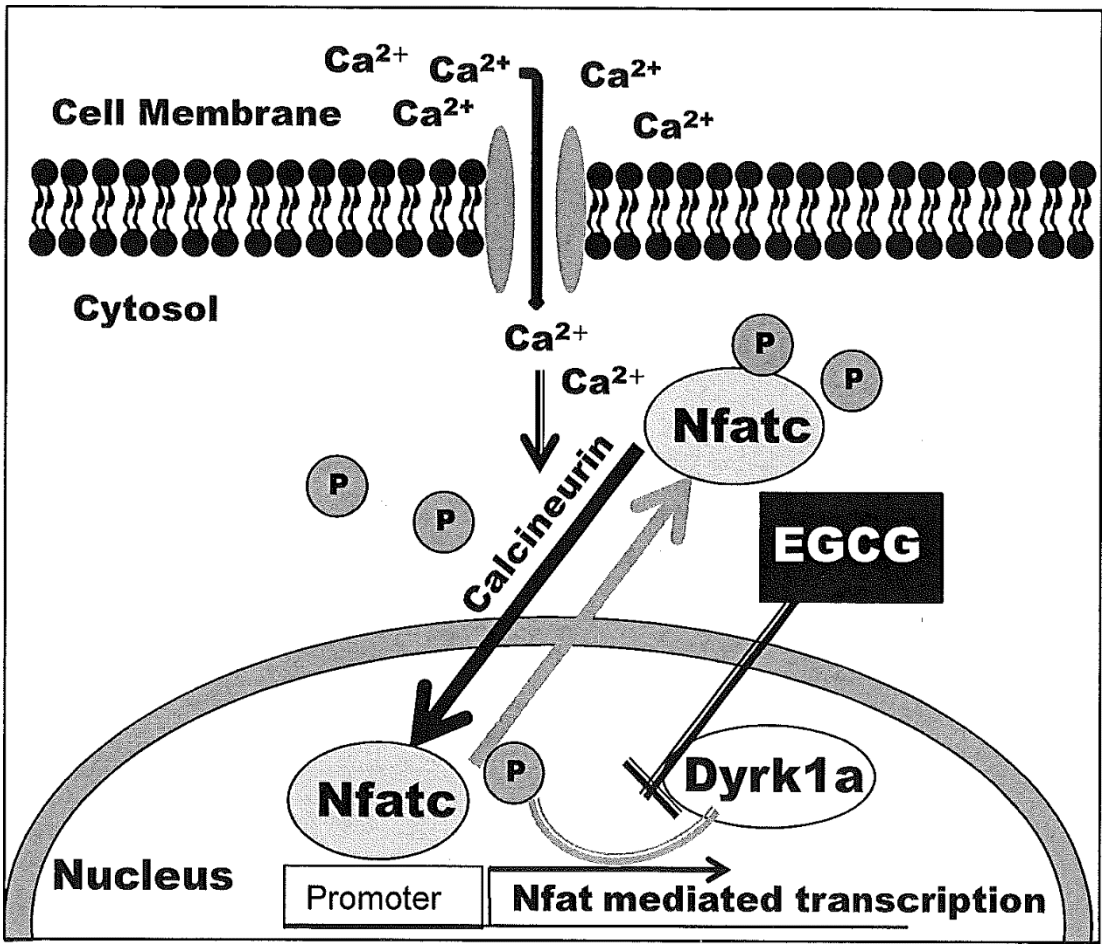
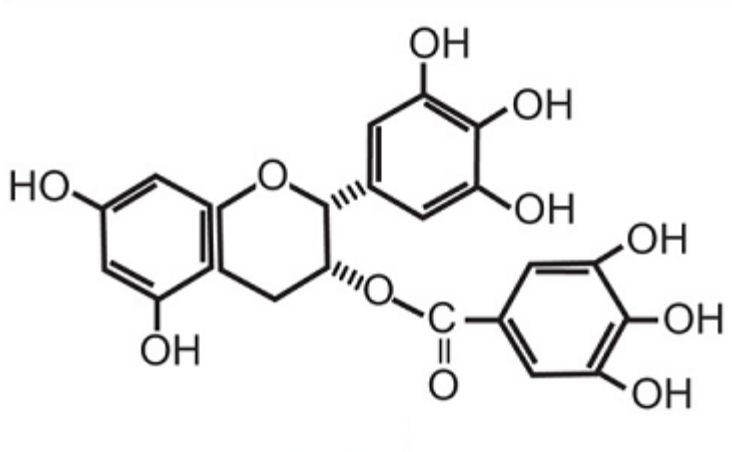
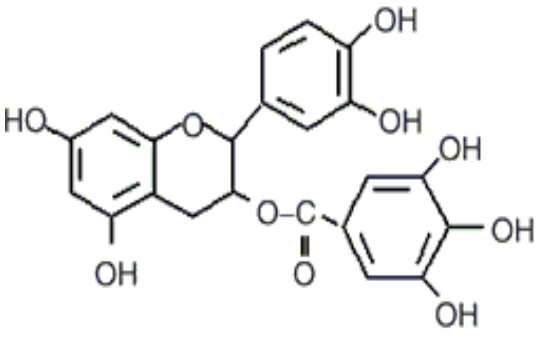


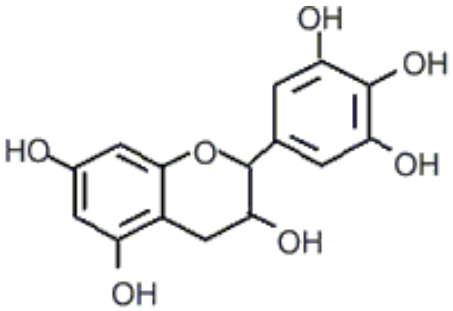
Figure 1.2: Dyrk1a-NFATc1 pathway.
Entry of Ca2+ ions into the cytoplasm activates calcineurin. Activated calcineurin dephosphorylates Nfatc1, moving it from the cytoplasm into the nucleus. Nuclear Dyrk1a phosphorylates Nfatc and thus removes it to the cytoplasm.



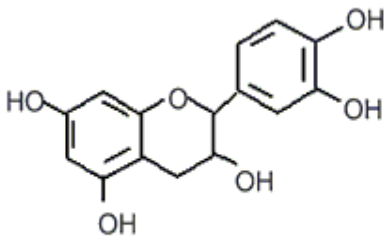
(-)-Epigallocatechin-3-gallate (EGCG)



(-)-Epicatechin-3-gallate (ECG)



(-)-Epicatechin (EC)



(-)-Epigallocatechin (EGC)

Figure 1.3: Chemical structures of green tea catechins

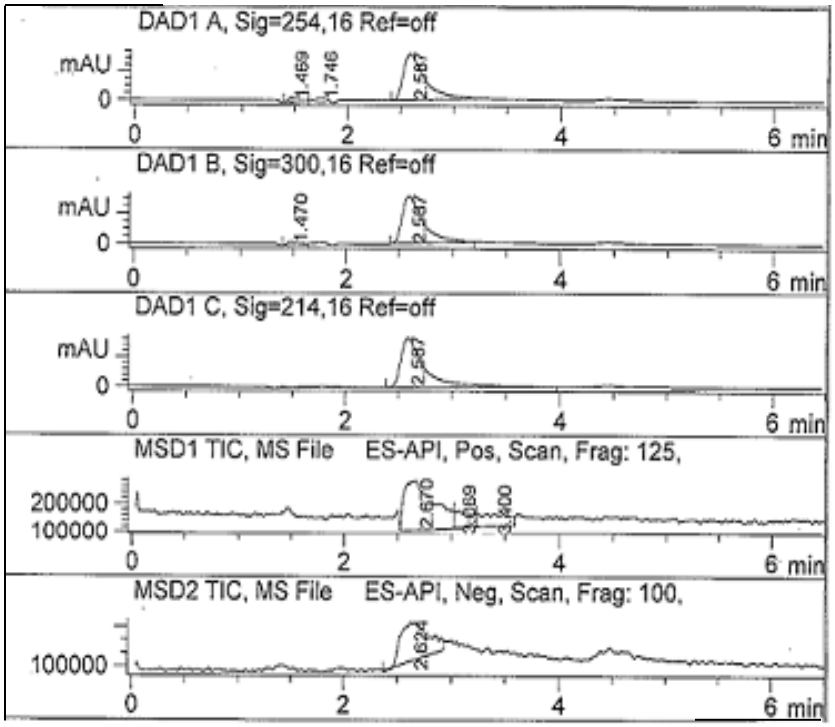


Figure 3.1: HPLC analysis of EGCG
Analysis of sigma EGCG by HPLC revealed a large peak at 2.6 minutes which was identified as EGCG while the two smaller peaks were contaminants.

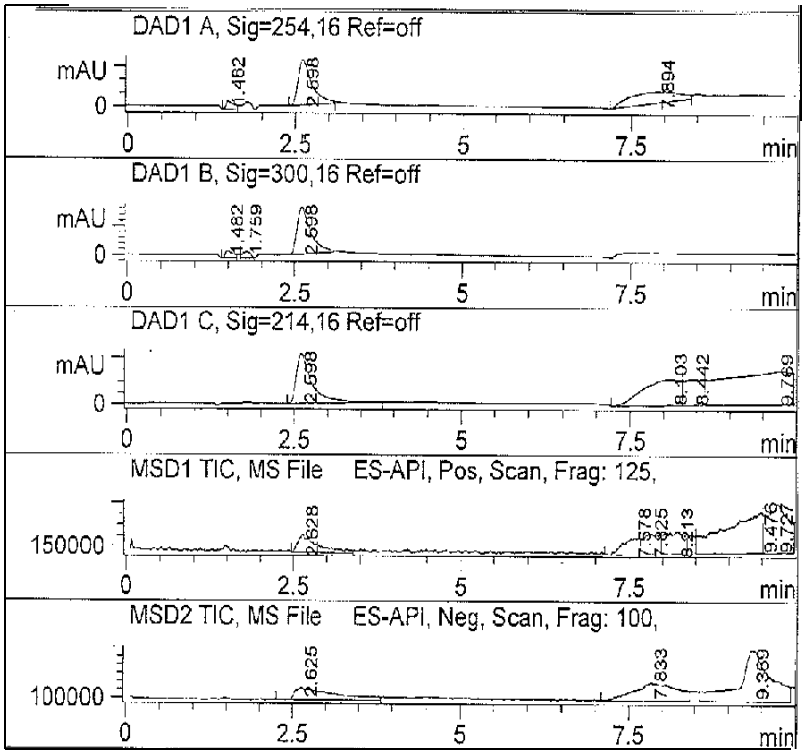


Figure 3.2: HPLC analysis of TEAVIGO
TEAVIGO supplement showed a large peak at 2.6 minutes, identified as EGCG. No other catechins were detected by HPLC and TEAVIGO was chosen for animal treatment as it was the closest to sigma EGCG in purity.

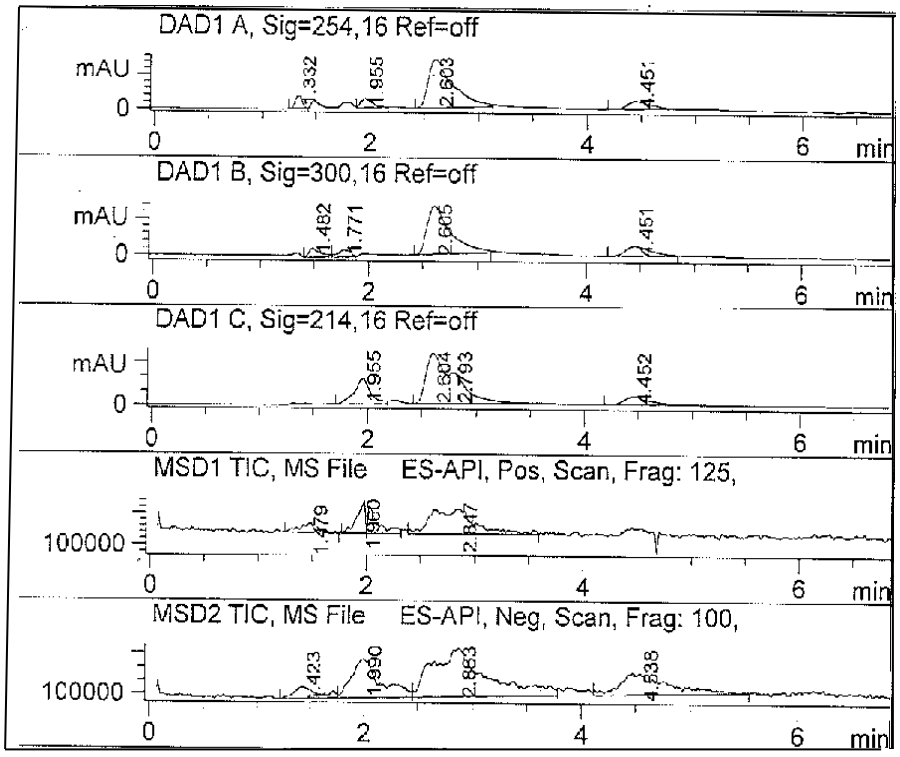


Figure 3.3: HPLC analysis of EGCgNOW
EGCgNOW had the highest level of impurities with small peaks at 1.4, 1.9, and 4.4 minutes that corresponds with EGC, EC/EGCG, and ECG respectively. The large peak at 2.6 minutes was identified as EGCG. EGCgNOW was chosen for animal treatment to study the differential effects of the supplements.

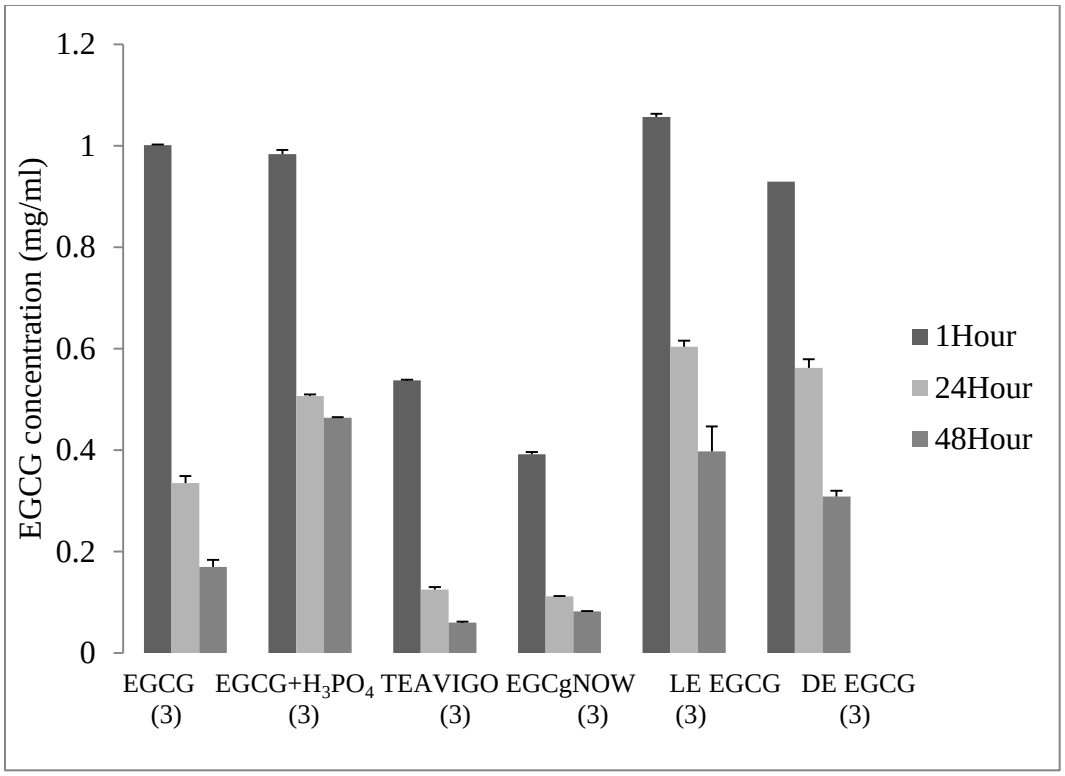


Figure 3.4: Degradation analysis of EGCG supplements by HPLC-MS
Analysis was conducted using HPLC/MS to find the EGCG concentrations at 1, 24 and 48 hours after the preparation. These results showed that lightly caffinated EGCG (LE EGCG) and decaffinated EGCG (DE EGCG) were the most stable. EGCG underwent rapid degradation by 24 and 48 hours but the addition of phosphoric acid (H3PO4) resulted in the stabilization of EGCG concentration. Number within parenthesis indicates the number of replicates used in the study.

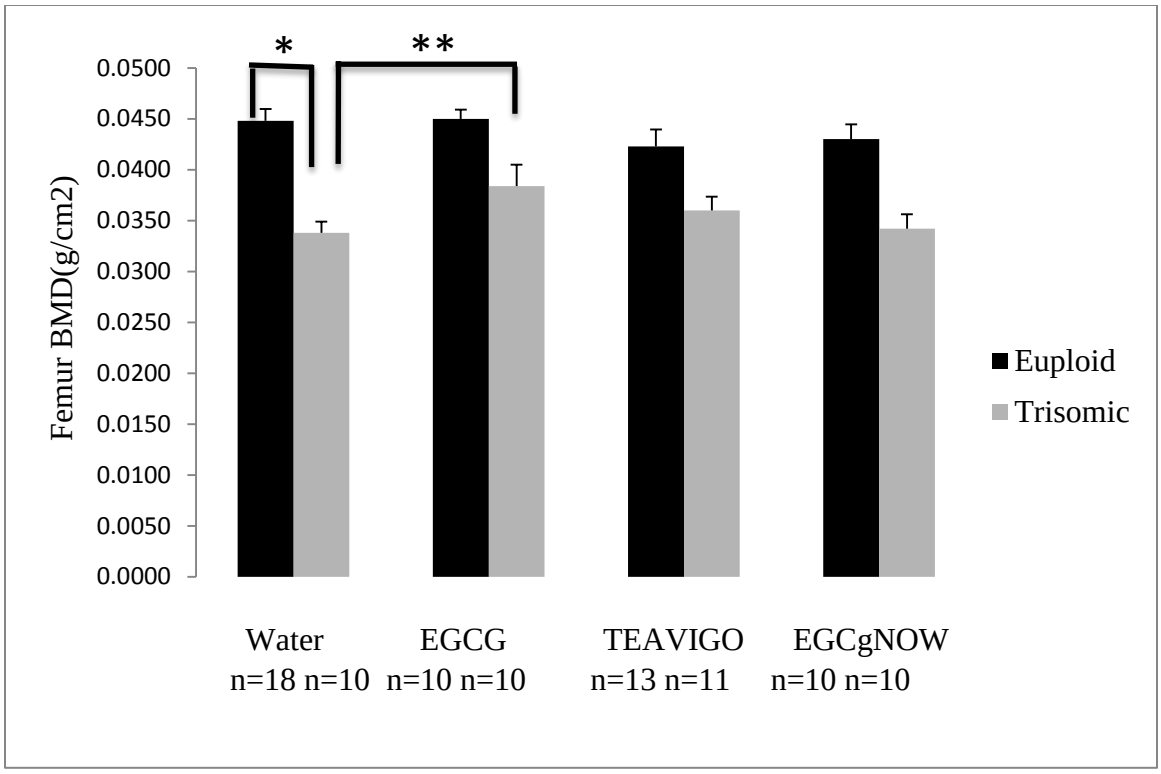


Figure 3.5: BMD analysis of femurs of Ts65Dn and control mice
BMD of femurs were calculated using DXA analysis and results indicated a significant reduction in BMD in trisomic mice treated with water in comparison to euploid control mice (*, $p<0.001$). Treatment o of trisomic mice with EGCG resulted in a significant increase in BMD when compared to trisomic water treated mice (**, $p=0.05$).

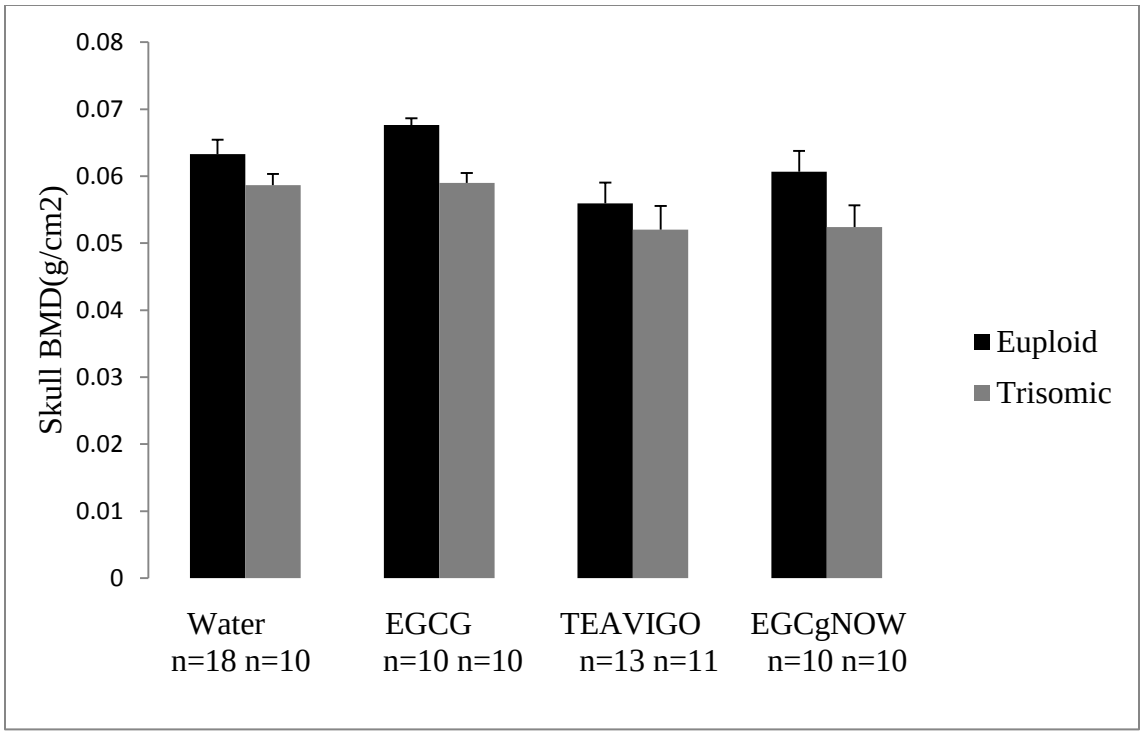


Figure 3.6: DXA analysis of skulls of Ts65Dn and control mice
BMD of the skulls revealed no significant changes with any of the three EGCG preparations used in this study.

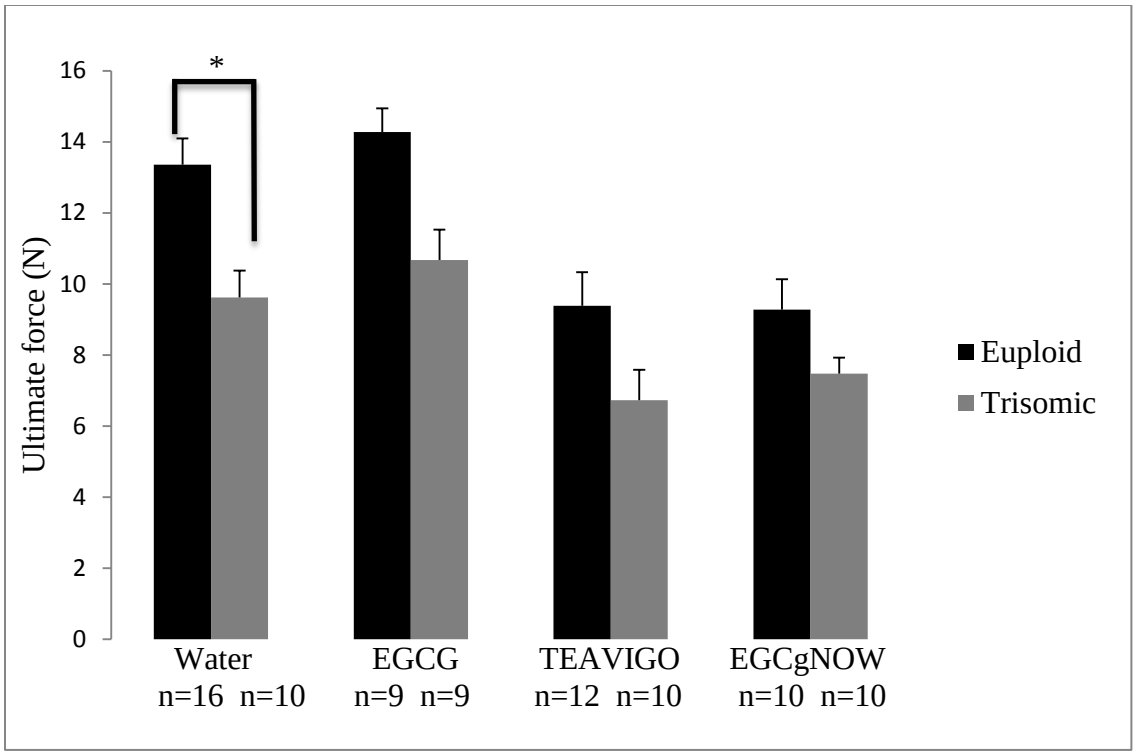


Figure 3.7: Mechanical test analysis of ultimate force
The maximum force that is sustained by the femur is referred to as ultimate force. The results showed that trisomic femurs could bear a significantly lower force than euploid control femurs (*, p=<0.001). None of the EGCG treatments were successful at increasing the ultimate force that could be borne by trisomic femurs.

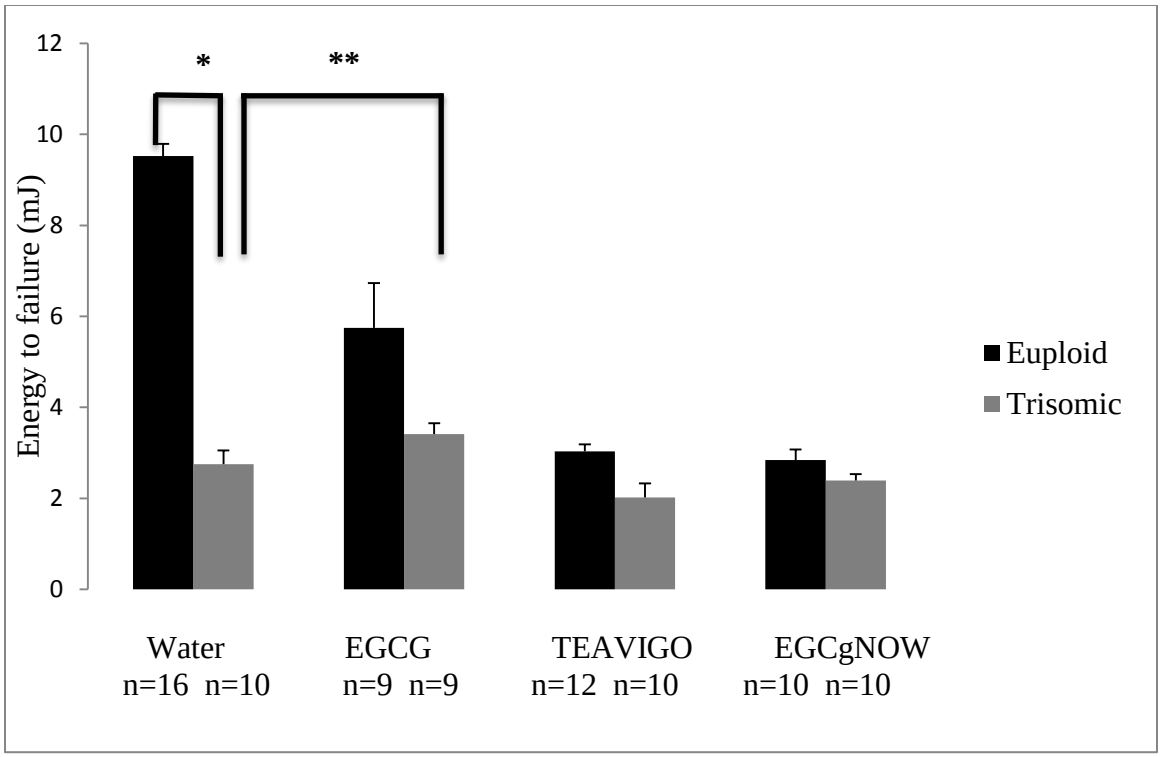


Figure 3.8: Energy to failure analysis of femurs by mechanical test
The energy to failure is described as the area under the load-deformation curve of a bone before it is broken. Trisomic control mice had a significantly lower energy to failure than euploid controls (*, p=≤0.005). The treatment of trisomic mice with EGCG resulted in a significant increase in energy to failure of the femurs in comparison to trisomic mice treated with water (**, p=0.05).

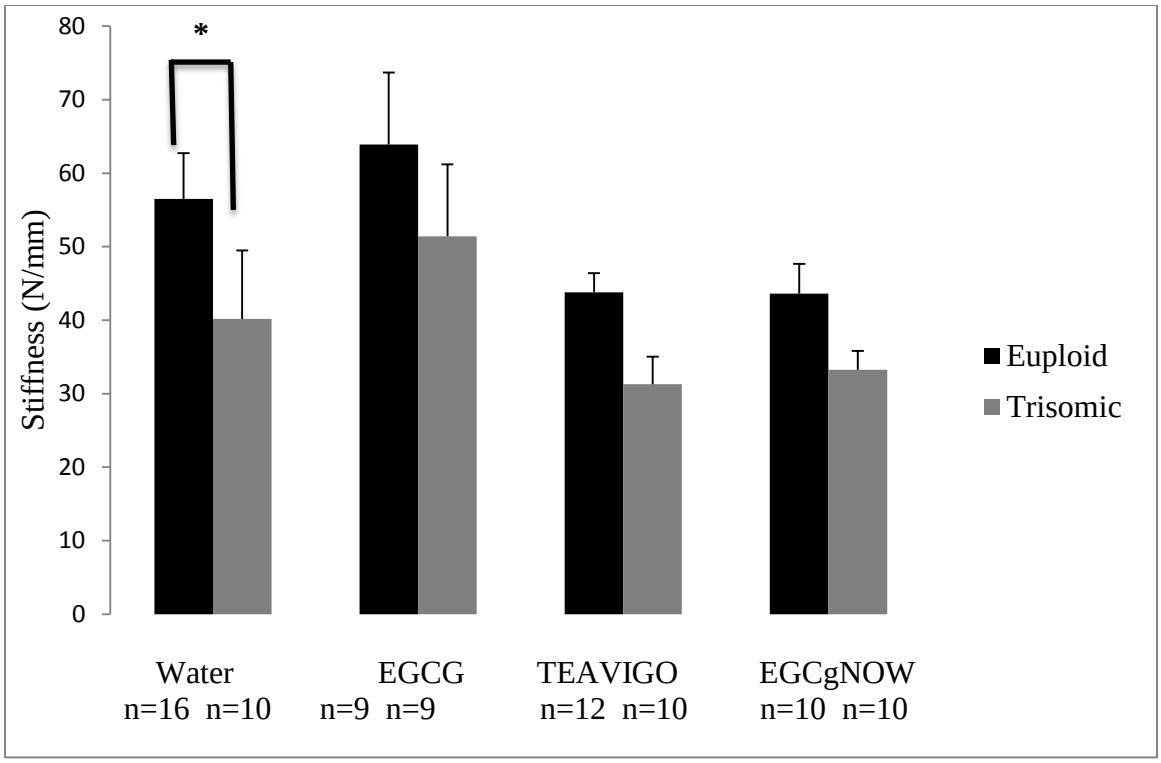


Figure 3.9: Mechanical test analysis of stiffness
The stiffness of a bone is described as the linear portion of the load-deformation curve. We observed a significant reduction in stiffness of trisomic mice compared to euploid control mice (*, p=0.03), but no significant effect of EGCG supplement was observed.

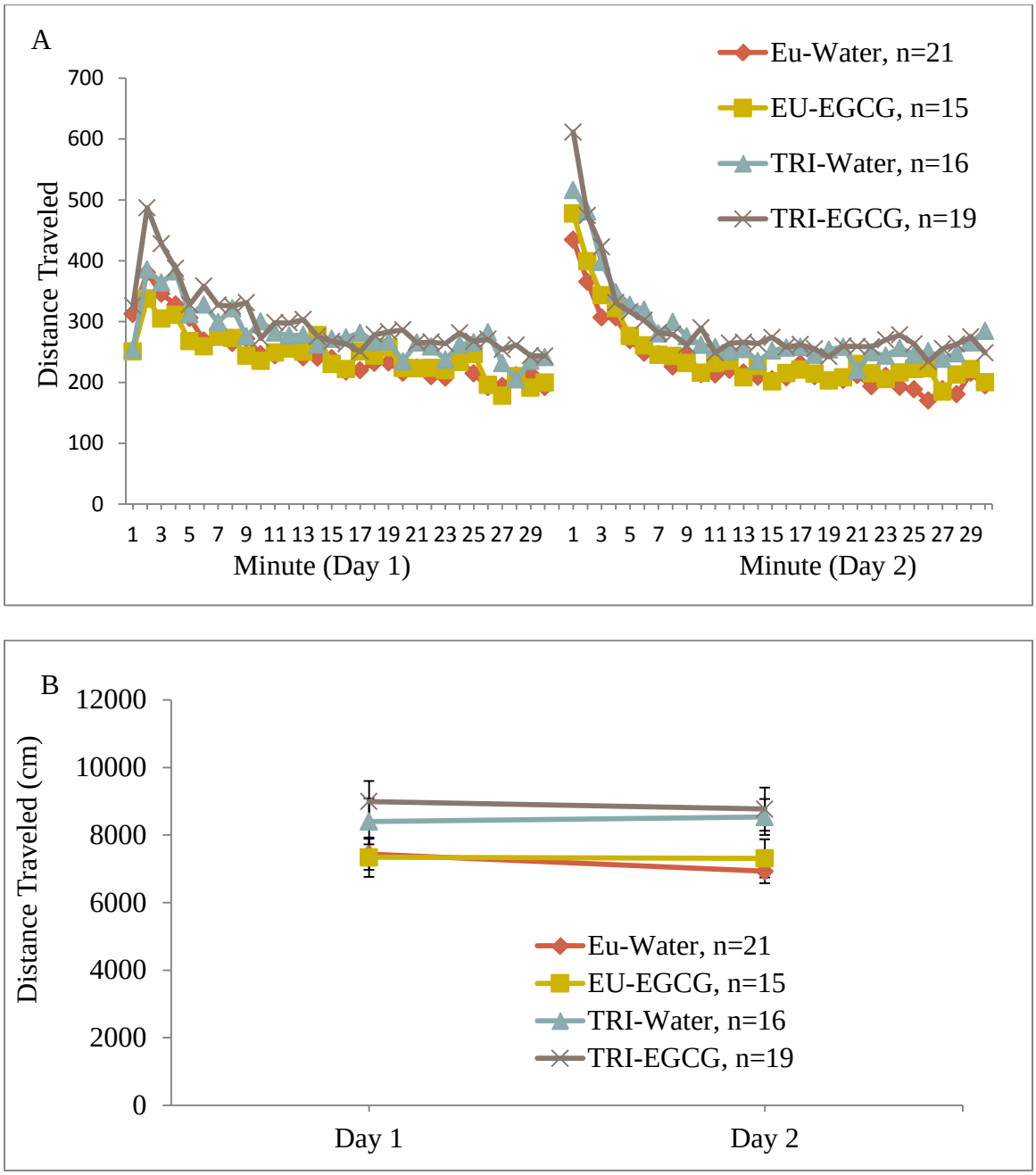


Figure 3.10: Locomotor activity
Activity task was utilized to study the spontaneous activity profile of trisomic mice in comparison to euploid controls and the effect of a three week long EGCG treatment of their activity levels. Trisomic control mice showed a significant increase in activity levels compared to euploid mice (3.10A, p=0.009). We did not observe an EGCG treatment effect. Effect of genotype in day 1 and 2 are shown in 3.10B.

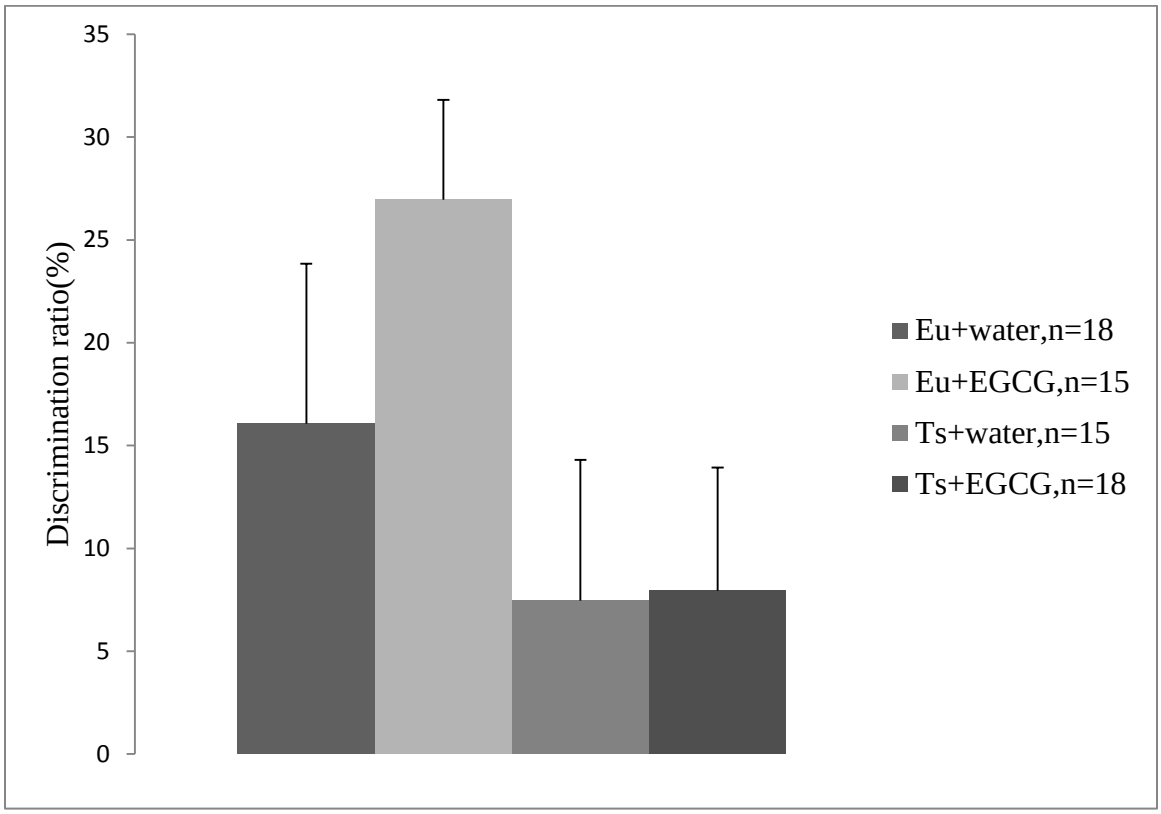


Figure 3.11: Novel object recognition task
The results from the study showed significant reduction in the discrimination ratio (the ratio of time spent exploring the novel object over familiar object) in trisomic mice in comparison to euploid animals (p=0.042). A three week EGCG treatment resulted in no improvements in the discrimination ratio in trisomic mice compared to euploid controls.

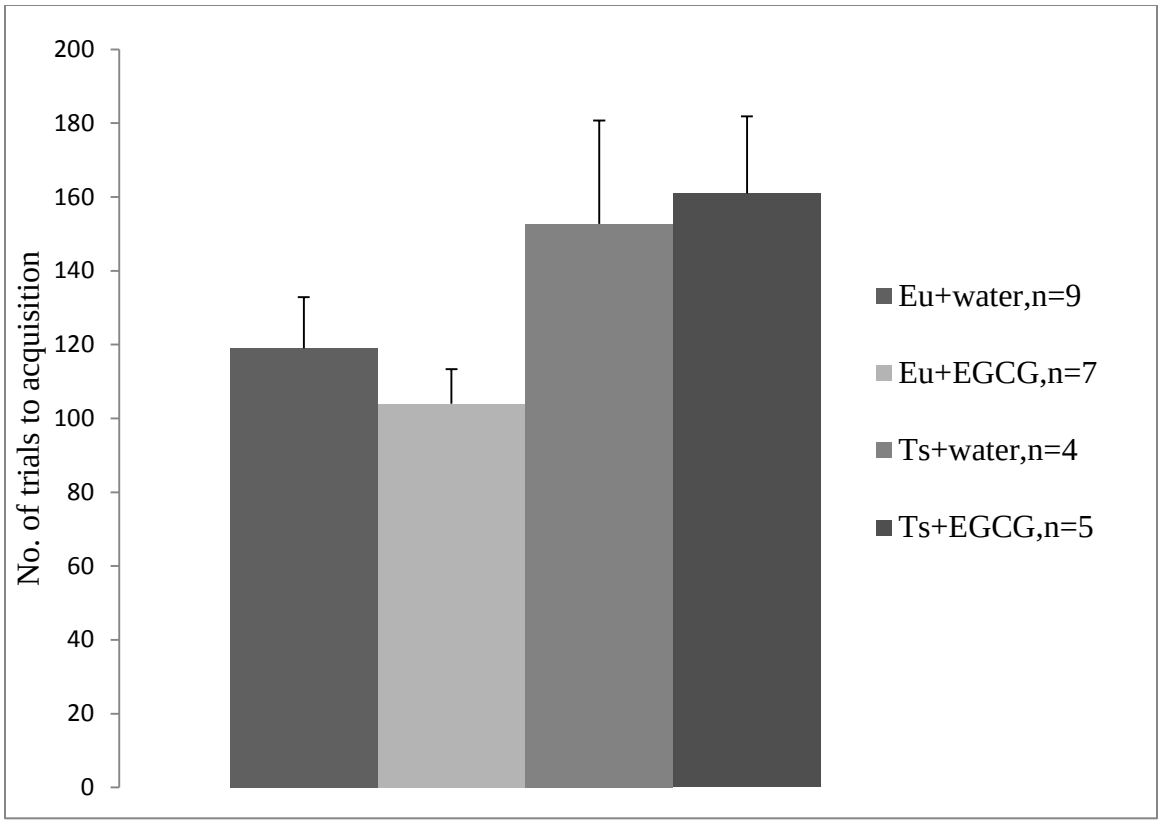


Figure 3.12: Delayed non-matching to place task
DNMP results showed a significant increase in the number of trials required by trisomic mice to make 80% correct arm choices on three consecutive days in comparison to euploid mice (p=0.018). However, EGCG treatment did not rescue this deficit.

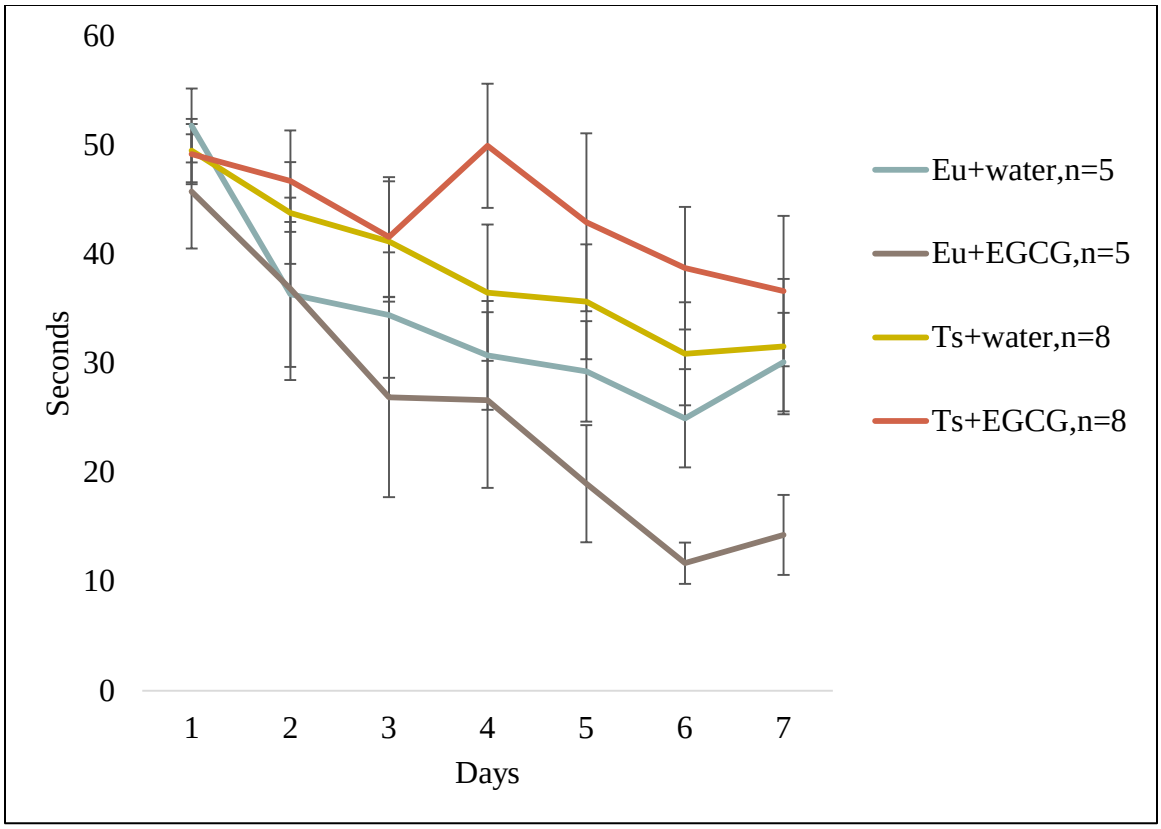


Figure 3.13: Morris water maze training latency
The mice were trained for 7 consecutive days to swim to a hidden platform placed in a previously designated quadrant of the pool. We observed a significant genotype effect ($p=0.016$) with trisomic mice requiring a longer time than euploid mice to find the platform. However, no treatment effect was observed.

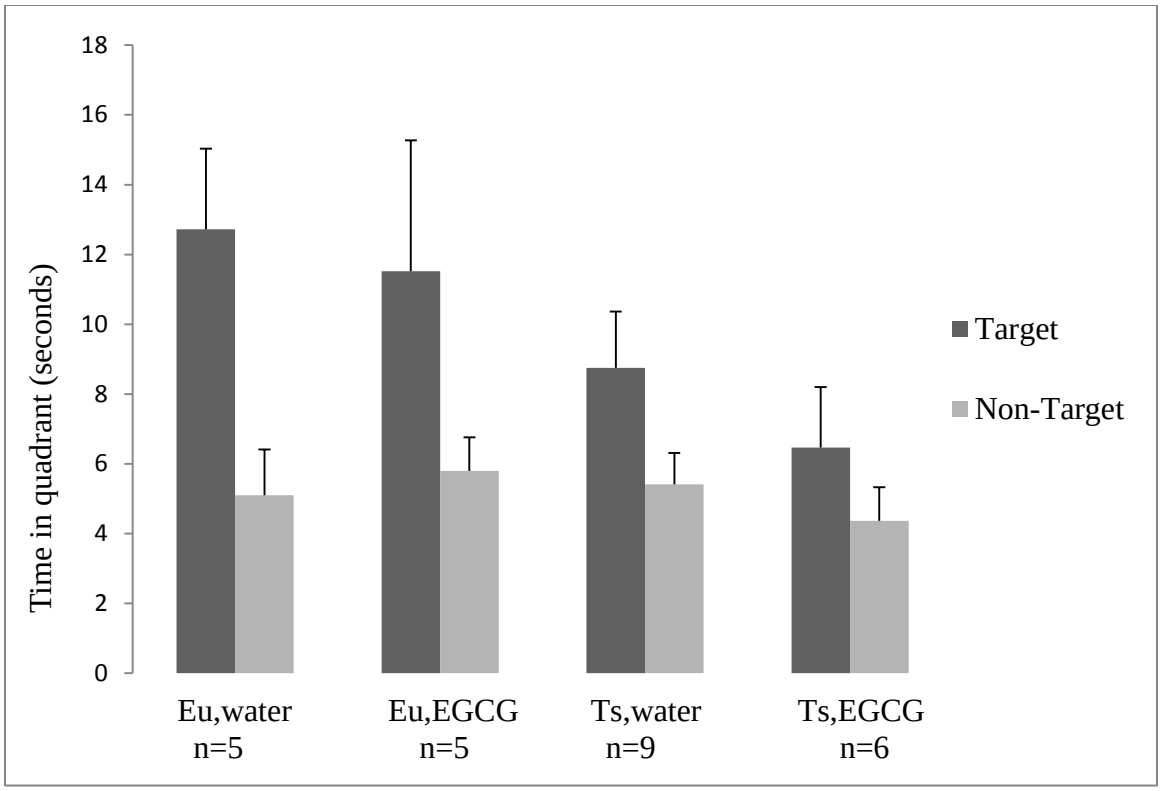


Figure 3.14: Morris water maze probe day
Probe day is utilized to test the ability of the animals to use their training to find the quadrant where the platform was previously hidden. We observed a strong but nonsignificant trend towards a genotype affect in the time spent swimming in/through the target quadrant by trisomic control mice. However no EGCG treatment effect was observed in the trisomic mice in comparison to euploid controls.

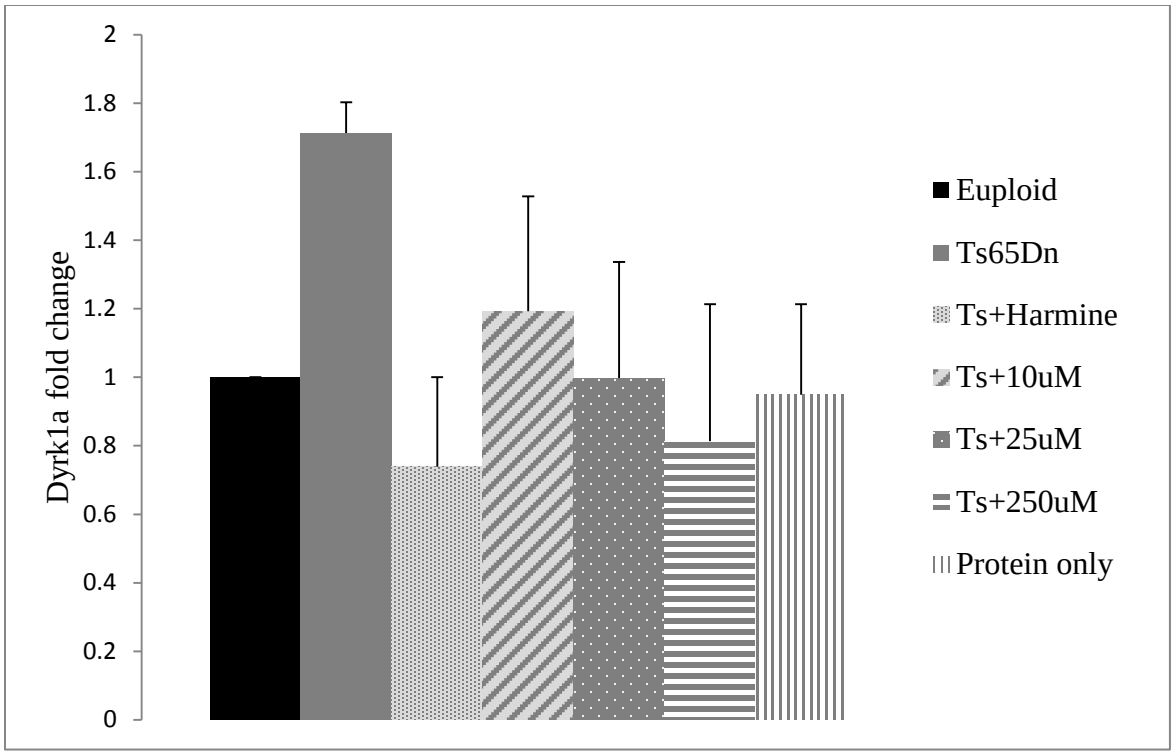


Figure 3.15: Dyrk1a kinase activity assay
Hippocampal tissue samples were analyzed using a ^{32}P kinase assay. We observed a approximate 1.6 fold increase in kinase activity in the trisomic sample in comparison to euploid control samples. The addition of harmine (2µM) to the trisomic sample led to a 0.7 fold reduction in kinase activity. A dose-dependent reduction in kinase activity was observed with the addition of 10, 25, and 250 µM EGCG solutions to trisomic hippocampal tissue samples.