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EFFECT OF EPIGALLOCATECHIN-3-GALLATE ON A PATTERN SEPARATION TASK AND HIPPOCAMPAL NEUROGENESIS IN A MOUSE MODEL OF DOWN SYNDROME

For the degree of Master of Science

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EFFECT OF EPIGALLOCATECHIN-3-GALLATE ON A PATTERN SEPARATION TASK AND HIPPOCAMPAL NEUROGENESIS IN A MOUSE MODEL OF DOWN SYNDROME

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Megan Elizabeth Stringer

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For my parents and their unwavering support throughout the years;

Even while chasing my dreams far from home, I have never once felt alone

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ABSTRACT

Stringer, Megan Elizabeth. M.S., Purdue University, December 2015. Effect of Epigallocatechin-3-Gallate on a Pattern Separation Task and Hippocampal Neurogenesis in a Mouse Model of Down Syndrome. Major Professor: Charles Goodlett.

Down syndrome (DS) is caused by three copies of human chromosome 21 (Hsa21) and results in an array of phenotypes including intellectual disability. Ts65Dn mice, the most extensively studied DS model, have three copies of ~50% of the genes on Hsa21 and display many phenotypes associated with DS, including cognitive deficits. *DYRK1A* is found in three copies in humans with Trisomy 21 and in Ts65Dn mice, and is involved in a number of critical pathways including CNS development and osteoclastogenesis. Epigallocatechin-3-gallate (EGCG), the main polyphenol in green tea, inhibits Dyrk1a activity. We have shown that a three-week EGCG treatment (~10mg/kg/day) during adolescence normalizes skeletal abnormalities in Ts65Dn mice, yet the same dose did not rescue deficits in the Morris water maze spatial learning task (MWM) or novel object recognition (NOR). Others have reported that An EGCG dose of 2-3 mg per day (90mg/ml) improved hippocampal-dependent task deficits in Ts65Dn mice. The current study investigated deficits in a radial arm maze pattern separation task in Ts65Dn mice. Pattern separation requires differentiation between similar memories acquired during learning episodes; distinguishing between these similar memories is thought to depend on distinctive encoding in the hippocampus. Pattern separation has

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been linked to functional activity of newly generated granule cells in the dentate gyrus. Recent studies in Ts65Dn mice have reported significant reductions in adult hippocampal neurogenesis, and after EGCG treatment, enhanced hippocampal neurogenesis. Thus, it was hypothesized that Ts65Dn mice would be impaired in the pattern separation task, and that EGCG would alleviate the pattern separation deficits seen in trisomic mice, in association with increased adult hippocampal neurogenesis. At weaning, Ts65Dn mice and euploid littermates were randomly assigned to the water control, or EGCG [0.4 mg/mL], with both treatments yielding average daily intakes of ~50 mg/kg/day. Beginning on postnatal day 75, all mice were trained on a radial arm maze-delayed non-matching-to-place pattern separation task. Euploid mice performed significantly better over training than Ts65Dn mice, including better performance at each of the three separations. EGCG did not significantly alleviate the pattern separation deficits in Ts65Dn mice. After the behavioral testing commenced, animals were given *ad libitum* food access for five days, received a 100mg/kg injection of BrdU, and were perfused two hours later. Coronal sections through the dorsal hippocampus were processed for BrdU labeling, and cells were manually counted throughout the subgranular zone of the dentate gyrus. The euploid controls had significantly more BrdU labeled cells than Ts65Dn mice, however, EGCG does not appear to increase proliferation of the hippocampal neuroprogenitor cells. This is the first report of deficits in Ts65Dn mice on a pattern separation task. To the extent that pattern separation depends on the functional involvement of newly generated neurons in an adult dentate gyrus, this approach in Ts65Dn mice may help identify more targeted pharmacotherapies for cognitive deficits in individuals with DS.

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CHAPTER 1. INTRODUCTION

1.1 Down syndrome

Down syndrome (DS) occurs in approximately 1 in every 700 live births and results from the triplication of human chromosome 21 (Hsa21) (Parker et al., 2010). The genetic basis of DS was discovered in 1958 by John Lejeune who observed an extra copy of human chromosome 21 in human tissue samples (Lejeune, Gautier, & Turpin, 1959). The most common cause of DS is a non-disjunction of Hsa21 during meiosis, where Hsa21 does not completely separate, or a Robertsonian translocation (three copies of long arm Hsa21) (Epstein, 2001). The only known risk factor of DS is advanced maternal age (Yoon et al., 1996).

One of the first observations of individuals with DS was from John Down in London in 1866. He noted that all of these individuals resembled individuals from Mongolia, so he deemed the patients "Mongols." He described a group of patients as having a flat, broad face, long tongue, and a small nose. He described their speech as "thick and indistinct", and their personalities as "humorous." According to Down, the patients improved their speech with training, but their life expectancy was well below the average. In 1961, the journal *The Lancet* published a letter from nineteen researchers, stating that the term "mongol" should no longer be used. The researchers stated that

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“mongol” was an “embarrassing term”, and that the resemblance of these individuals to the Mongolian people was “superficial” and should no longer be used. In 1965, the World Health Organization removed the term “mongolism” and instead “Down’s syndrome” was used (Howard-Jones, 1979). In 1975, the National Institute of Health stated that “Down’s syndrome” should be replaced with “Down syndrome”.

1.1.1 Phenotypes of DS

DS results in an array of phenotypes that affect the cardiovascular, skeletal and central nervous systems. Heart defects affect 40-50% of children with DS (Freeman et al., 2008; Freeman et al., 1998; Tubman et al., 1991). Bone deficits, including a reduction in bone mineral density, and an increased incidence of osteoporosis have also been reported (Center, Beange, & McElduff, 1998; Guijarro et al., 2008). A lower intelligence quotient (IQ) is one of the most common phenotypes, along with hearing loss (75%), obstructive sleep apnea (50%-80%), eye disease (60%), and an Alzheimer-like phenotype in individuals that live over 35-40 years, with plaques accumulating in individuals as young as eight years old (Bull, 2011; Källén, Mastroiacovo, & Robert, 1996; Leverenz & Raskind, 1998; Marcus et al., 1991; Netzer et al., 2010; Shott, Joseph, & Heithaus, 2001; Zigman & Lott, 2007). It is important to note that while these phenotypes are common among individuals with DS, there is a wide range of variability in the incidence and severity of the various phenotypes (Roubertoux & Kerdelhué, 2006).

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1.1.2 Structural and histological deficits observed in DS

Individuals with DS show differences in brain development from typically-developing individuals that emerge early in life. A handful of studies have examined fetuses with DS to determine if histological differences are present during prenatal development. One study found that at 15-22 weeks, there were no differences in the cerebellum or brain stem size, nor were there differences in the size of the hippocampus. However, this study employed qualitative-based analysis (Schmidt-Sidor et al., 1990). More recent studies have employed advanced methods, such as morphometry, stereology and cell phenotyping based techniques. For example, in 17-21 week postmortem fetuses, a reduction in the volume and number of cells in the hippocampus, dentate gyrus (DG) and parahippocampal gyrus was observed. Fetuses with DS also displayed higher levels of cells displaying an astrocyte phenotype versus a neuronal phenotype, possibly due to the observed reduction in hippocampal proliferation (Guidi et al., 2008). Another study found similar results, with 17-21 week fetuses having a smaller proportion of neurons versus astrocytes, together with deficits in cell proliferation (Contestabile et al., 2007).

These histological deficits extend into postnatal development, with 3-5 months old infants with DS displaying a smaller brain stem and cerebellum, as well as a lower brain weight. These infants also display deficits in myelination within the brain stem, cerebellum, spinal cord and posterior horn (Wisniewski, 1989). Abnormal dendritic branching and shorter dendritic length, as well as prefrontal, visual and motor cortex neuron abnormalities have also been observed in individuals with DS (Becker, Armstrong, & Chan, 1986; Marin-Padilla, 1976; Takashima et al., 1981; Takashima et al., 1994; Vukšić et al., 2002). Decreases in visual cortex myelination have also been

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noted (Wisniewski, 1989). Structurally, children with DS show significantly smaller hippocampal and cerebellar volumes (Jernigan et al., 1993; Pinter et al., 2001a; Pinter et al., 2001b). Continuing into adulthood, adults with DS display a range of deficits, including a smaller corpus callosum, and decreases in hippocampal, cerebellar, cerebral cortex and white matter volumes versus age matched controls (Aylward et al., 1997; Aylward et al., 1999; Kesslak et al., 1994; Weis et al.). Individuals with DS have been shown to develop plaques and neurofibrillary tangles, typical of Alzheimer's disease, before the age of 40. These histological changes occur in multiple areas of the brain, including the entorhinal cortex and the DG of the hippocampal formation (Motte & Williams, 1989).

1.1.3 Behavioral deficits observed in DS

DS is the most common genetic cause of an intellectual disability (Yamakawa, 2012). All individuals with DS have an intellectual disability, with an average IQ of 50 and ranging from 30-70 (Chapman & Hesketh, 2000). Accompanying a reduced IQ, individuals with DS display deficits on a range of learning and memory tasks, beginning at an early age. Infants with DS display developmental learning delays in tasks such as the object-concept task and recognition memory tasks (Miranda & Fantz, 1974; Nygaard, Ludvig Reichelt, & Fagan, 2001). As these individuals age, they display deficits in more complex memory tasks, including working memory tests (Jarrold, Baddeley, & Phillips, 2002; Vicari, Bellucci, & Carlesimo, 2005). Other types of memory deficits have been reported, including verbal short-term memory and spatial-spontaneous short-term and long-term memory tasks. (Frenkel & Bourdin, 2009; Lanfranchi et al., 2009; Lanfranchi,

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Cornoldi, & Vianello, 2004; Vicari et al., 2005). Individuals with DS also display performance deficits on tasks such as the virtual Morris water maze (MWM), CANTAB Pattern Recognition Memory Test, and the NEPSY List-Learning Test (Carlesimo, Marotta, & Vicari, 1997; Pennington et al., 2003). These labratory tasks are commonly used to assess memory function because individuals with hippocampal damage show impairments on these tasks (Broadbent, Squire, & Clark, 2004; Goodrich-Hunsaker et al., 2010; Koehler et al., 1998). The poorer performance on these tasks by individuals with DS suggest a phenotype that includes hippocampal dysfunction.

Interestingly, some research suggests that visuospatial memory is somewhat preserved in individuals with DS. For example, multiple studies have reported that children with DS do not display any deficits on a computer version of the Corsi block-tapping task versus controls (Jarrold & Baddeley, 1997; Laws, 2002; Numminen et al., 2001; Vicari, Carlesimo, & Caltagirone, 1995). While these studies report an apparent preservation of visuospatial working memory, these studies do not represent a "real-world" space; there is a lack of environmental and self-integration in solving the task. That is, these computerized assessments rely more on egocentric processing (self to object), rather than allocentric (object to object) processing. Unlike egocentric memory, allocentric memory relies on the integration of multiple sensory systems, including the visual, vestibular and somatosensory systems.

Mangan (1992) implemented a cue and place learning task in infants with and without DS. In the cue learning task, infants were placed on a platform, and were shown two locations where a reward could be hidden. However, only one rewarded location was used, thus, infants learned to always turn a certain direction towards the rewarded

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location, in order to uncover a reward. That reward was always covered with a colored lid, allowing the task to be solved with a visual cue (non-spatial processing). The infants with DS required more trials than controls to solve the task. However, they performed similar to controls in a final probe task. In the place learning task, infants watched the reward being hidden in a hole on the platform, and then were placed in random start positions to search for the reward. This task did not use a colored lid for reward indication, nor were the start positions consistent across training, thus, the infants had to use allocentric processing (position of the reward in relationship to environmental/wall cues). In this task, the infants with DS were significantly impaired compared to controls, requiring more trials to learn the task, as well as a poor search pattern in the probe trial.

A more recent study implemented a similar procedure, a real-world spatial memory task in older, more developed individuals with DS. In this study, participants were asked to locate three rewards hidden among 12 potentially rewarded locations. Cones were used to identify the reward locations by On half of the trials, a cue (red cup) indicated where the reward was located, whereas the other half of trials there was no cue to indicate where the reward was hidden. In the cued setting, individuals with DS were able to discriminate reward from non-reward locations at control levels. However, in the non-cued setting, individuals with DS made fewer correct choices before making an error, visited more locations to find the three rewards, and had fewer correct first choices versus controls (Lavenex et al., 2015).

Research has aimed to tie these memory deficits to the anatomical deficits; yet, this has yielded mixed results. A negative correlation between parahippocampal gyrus size (measured via MRI) and cognitive ability has been suggested, as well as a direct

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correlation between corpus callosum size and cognitive ability (Teipel et al., 2003). However, the same study reported no correlations between cerebral size, cerebellar hemisphere size, or hippocampal size and cognitive ability (Teipel et al., 2003). Connecting these memory deficits with the observed histological abnormalities would allow researchers to better understand the relationship between these two phenotypes of DS.

1.1.4 The Ts65Dn mouse model of DS

Mouse models of DS have been created to study the genotype-phenotype relationship observed in DS. These models are based on the similarities between Hsa21 and mouse chromosomes 16 (Mmu16), Mmu17 and Mmu10 (Pletcher et al., 2001). The most common models include Ts65Dn, Ts1Yey, Ts1Cje, Ts1Rhr, Ts1Yah, and the Tc1 (Gardiner, 2010). The models vary by how the triplicated genomic segment was created, the number of genes involved, as well as the degree of phenotypes exhibited. The Ts65Dn mouse model contains ~140 genes that are highly conserved between Hsa21 and Mmu16 (Yamakawa, 2012). The Ts65Dn mouse model is the most extensively studied and widely used animal model of DS because it replicates many of the DS phenotypes, including craniofacial and appendicular skeletal abnormalities, reduced birth weight, cardiovascular defects, cognitive and behavioral impairments, and brain defects (Baxter et al., 2000; Belichenko et al., 2004; Cooper et al., 2001; Reeves et al., 1995a). Other mouse models, such as the Ts1Cje mouse, have been used for DS research, but its learning deficits are less severe than the Ts65Dn mouse (Sago et al., 2000b). The Tc1 model displays deficits in some learning and memory tasks (NOR) but not others

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(spontaneous alternation t-maze). Furthermore, these mice display variable levels of mosaicsm (O'Doherty et al., 2005; Rueda, Flórez, & Martínez-Cué, 2012). While these and other mouse models are valuable in researching the variable phenotypes that are seen in DS, the Ts65Dn mouse model is the most commonly researched mouse model of DS.

Ts65Dn mice have shown extensive impairments on learning and memory tasks (Demas et al., 1996, 1998; Reeves et al., 1995c). Specifically, in NOR, 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). Ts65Dn mice have also been shown to have significant deficits in the MWM spatial learning task with an increased latency (time taken to reach the platform) and reduced spatial navigational performance compared to euploid controls (Escorihuela et al., 1995; Hyde, Frisone, & Crnic, 2001; Reeves et al., 1995b; Sago et al., 2000a). Ts65Dn mice have also shown an overexpression of the genes *Olig1* and *Olig2*, which leads to an overproduction of inhibitory interneurons in the forebrain (Chakrabarti et al., 2010). Hippocampal long term potentiation (LTP) is used as a model of synaptic plasticity that may engage similar cellular functional modifications, similar to those that occur during learning and memory (Morris, 2003). Kleschevinok et al. (Kleschevnikov et al., 2004) used LTP to observe cell activity in the hippocampus of Ts65Dn mice. They found that excessive inhibition of dentate granule cells (DGCs) limited the activation of NMDA receptors, therefore inhibiting LTP *in vitro*.

Ts65Dn mice display neurohistological deficits during embryonic development. Starting on E13.5, Ts65Dn embryos have smaller telencephalons, but this deficit disappears by E18.5. During this same time period, Ts65Dn mice displayed a decrease in

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the subplate, cortical plate and the intermediate zone, however these layers reached control thickness by E18.5. Despite normal thickness, there were reductions in cell density in all three areas. A reduction in the hippocampal thickness was also observed during E13.5-E18.5. Underlying these morphological deficits, is an observed deficit in cell cycle rate, specifically a longer S phase. Interestingly, the difference in S phase cycle length between the Ts65Dn embryos and controls decreased with age. Furthermore, deficits of proliferation and cell migration were observed (Chakrabarti, Galdzicki, & Haydar, 2007). While some of these deficits normalize, these results suggest that developmental delays are evident in Ts65Dn embryos.

Postnatally, Ts65Dn mice exhibit deficits of the cell cycle in the developing cerebellum, as well as a decrease in cerebellum size, with decreased numbers of both granule and Purkinje cell neurons (Baxter et al., 2000; Contestabile et al., 2009; Roper et al., 2006). Belichenko reported that Ts65Dn mice have synaptic abnormalities, including decreases in spine densities on the dendrites of DGCs (Belichenko et al., 2004). . Deficits in hippocampal neurogenesis are also found in the Ts65Dn mouse. Adult Ts65Dn mice have a decreased density of labeled cells in the cortex and DG, as well as a decrease in the number of neurons in the DG (Chakrabarti et al., 2007; Insausti et al., 1998). Deficits in cell proliferation have been observed at PD2 in Ts65Dn mice (Contestabile et al., 2007); similar results were reported at PD 15 (Bianchi et al., 2010c) and at 20-28 weeks (Insausti et al., 1998). Proliferation deficits have also been seen in older Ts65Dn mice (10-12 months) (Llorens-Martin et al., 2010). Recent research has focused on identifying the gene or set of genes that underlie these behavioral and anatomical deficits that are observed in DS and Ts65Dn mice.

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1.2 <u>The hypothesized role of *DYRK1A* in DS</u>

Dual-specificity tyrosine phosphorylation-regulated kinases (DYRKs) are a family of protein kinases that phosphorylate serine and threonine residues in a number of substrates (Becker & Sippl, 2011). *DYRK1A* is the only Dyrk in the family to be located on Hsa21, and is found in three copies in individuals with DS (Becker & Joost, 1999). An extra copy of *DYRK1A* has been shown to lead to a 1.5 fold increase (measured via Western Blot) in DYRK1A protein expression in various brain tissues of individuals with DS who were 10-40+ years old. However, no differences in expression were observed in the infants with DS (1-3 years old). This discrepancy suggests that *DYRK1A* may only be overexpressed during specific stages of development (Dowjat et al., 2007). *Dyrk1a* is thought to play a key role in development because of its high expression in the CNS, and the severe deficits occurring both with increased or decreased expression levels (Martí et al., 2003). In humans with a deletion of *DYRK1A*, microcephaly, intellectual disability and epilepsy have been observed (Bon & Hoischen, 2011; Courcet et al., 2012; Møller, Kübart, & Hoeltzenbein, 2008).

A recent study examined induced pluripotent stem cells (iPSCs) derived from monozygotic twins discordant for DS and found evidence that DYRK1A contributes to developmental deficits. Specifically, *DYRK1A* was overexpressed by approximately twofold in DS-iPSC-derived neural progenitor cells (NPCs), and when expression was reduced to normal levels (via a DYRK1A inhibitor and short hairpin RNA (shRNA)), previously observed neural proliferation and differentiation deficits were rescued (Hibaoui et al., 2014).

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Dyrk1a protein levels were found to be ~1.5 fold higher than euploid control levels in the cortex, cerebellum and hippocampus of Ts65Dn mice (Souchet et al., 2014). Numerous mouse models have been genetically modified in order to better understand the role *Dyrk1a* plays. *Dyrk1a* knockout mice (*Dyrk1a* -/-) die prenatally, whereas mice with a copy reduction of *Dyrk1a* (*Dyrk1a* +/-) display a wide range of abnormalities, including reduced viability, developmental delays, sensorimotor deficits, reductions in brain size, and deficits in cell structure (Altafaj et al., 2001; Benavides-Piccione et al., 2004; Fotaki & Dierssen, 2002). Interestingly, transgenic mice that overexpress *Dyrk1a* (TgDyrk1a) also express motor and learning deficits, similar to the mice with a reduction of *Dyrk1a* (Martınez de Lagrán et al., 2004). These studies demonstrate that a balance of *Dyrk1a* copy number plays an important role in development. Normalizing the expression level of *Dyrk1a* in mice has yielded convincing evidence for its role in CNS development. For example, normalizing the copy number of *Dyrk1a* in the Ts65Dn mouse rescued deficits on the MWM, contextual fear conditioning, LTP, and proliferation deficits (Garcia-Cerro et al., 2014). Gene targeting in TgDyrk1a and Ts65Dn mice using RNA interference via viral delivery (shRNA) inhibited and normalized *Dyrk1a* levels, and rescued *Dyrk1a*-mediated hyperactivity, motor deficits and some hippocampal-dependent behaviors (Altafaj, Martín, & Ortiz-Abalia, 2013; Ortiz-Abalia et al., 2008). One month of environmental enrichment was also reported to normalize Dyrk1a kinase levels, improve acquisition and probe performance in the MWM, and increase swimming speed in female adolescent TgDyrk1a mice (Pons-Espinal, Martinez de Lagran, & Dierssen, 2013).

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1.3 Treatments targeting cognitive deficits in DS

Treatments that have shown effectiveness in various diseases have been tested in mouse models of DS. Memantine is a NMDA receptor antagonist that has been shown to normalize LTP in human brain slices and is used for the treatment of Alzheimer's disease (Frankiewicz & Parsons, 1999; Reisberg et al., 2003). Ts65Dn mice display abnormal (overactive) NMDA function (Scott-McKean & Costa, 2011). Treatment with memantine (once a week injections for five consecutive weeks beginning at ~4-7 months of age and daily oral administration via drinking water for 3 consecutive weeks beginning at 9 months of age) improves MWM performance, grip strength, and contextual fear conditioning in Ts65Dn mice (Costa, Scott-McKean, & Stasko, 2008; Rueda et al., 2010). In a recent clinical trial called MEADOWS (Memantine for Dementia in Adults Older Than 40 Years With Down's Syndrome), adults received daily oral memantine treatment (10mg/day) for one year. However, memantine did not improve cognitive deficits.

Ts65Dn mice demonstrate increase GABA inhibition, possibly resulting is reduced LTP (Kleschevnikov et al., 2004). Treatment with pentylenetetrazole, a GABA-A antagonist, improved performance on the MWM and restored LTP in the Ts65Dn mouse (Rueda, Florez, & Martinez-Cue, 2008). Selective serotonin reuptake inhibitors have been shown to increase hippocampal neurogenesis (Malberg et al., 2000), and similar findings have been found in the Ts65Dn mouse, along with normalized GABA release, improved spatial memory, and hippocampal plasticity, and improved proliferation (Begenisic et al., 2014; Bianchi et al., 2010a; Bianchi, Ciani, & Guidi, 2010b; Contestabile et al., 2012). However, lithium and PTZ may not be the best potential therapeutic treatments for individuals with DS due to the risk of seizures and

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toxicity (Prasher, 1993). Another alternative treatment is maternal choline supplementation, which significantly improved the offspring's performance in the MWM and hippocampal neurogenesis in the Ts65Dn mouse model (Velazquez et al., 2013).

1.3.1 Is EGCG a potential treatment for DS?

Epigallocatechin-3-gallate (EGCG) is the most prevalent polyphenol found in green tea (Sato & Miyata, 2000). EGCG is a small molecule hypothesized to improve a wide range of diseases, including cancer, Alzheimer's disease, Parkinson's disease, and multiple sclerosis (Choi et al., 2002; Kostoff, Briggs, & Lyons, 2008; Rezai-Zadeh, Shytle, & Sun, 2005). One of the first studies showing the promise of EGCG was in cancer research. Ahmad et al. documented that EGCG induced apoptosis and cell cycle arrest in cancer cells, without affecting normal cells (Ahmad et al., 1997). EGCG has been shown to affect many factors associated with cell cycle progression, however, its inhibitory effect on kinases is considered a key feature. Specifically, EGCG has been shown to inhibit the mitogen-activated protein kinase and the epidermal growth factor receptor-mediated pathway (Dong et al., 1997; Khan et al., 2006). EGCG has also been shown to be an inhibitor of DYRK1A activity, and is thought to function by binding to the ATP binding domain of the protein and inhibiting its activity (Adayev, Chen-Hwang, & Murakami, 2006; Bain et al., 2003). Consistent with its antioxidative properties, EGCG is known to undergo rapid degradation, with a concentration half-life in water of about 48 hours (Stringer et al., 2015). Nonetheless, research suggests that EGCG has the potential as a therapeutic tool to improve the deficits seen in individuals with DS.

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Oral administration of EGCG in drinking water (0.6-1.2 mg/day and 2-3 mg/day), respectively, to TgDyrk1a and Ts65Dn mice rescues learning and memory deficits, increases brain volume, rescues synaptic plasticity and proliferation deficits, and reduces Dyrk1a kinase activity (De la Torre et al., 2014; Guedj et al., 2009). A three-week, low-dose EGCG treatment (~10 mg/kg/day) during adolescence resulted in improvements in skeletal deficits in trisomic mice, but showed no improvements in a range of learning and memory tasks (Blazek et al., 2015; Stringer et al., 2015). A similar dose (~9 mg/kg/day in capsule form) was used in a human study that showed improvements in cognition and more positive caregiver reports (De la Torre et al., 2014). Administration of EGCG to induced pluripotent stem cells from an individual with DS not only reduced Dyrk1a activity, but rescued cell proliferation and differentiation deficits. Importantly, the iPSC study established at a human cellular level that EGCG has the potential to improve some of the deficits seen in DS, such as cognitive and histological deficits (Hibaui et al., 2014).

1.4 Initial hippocampal neurogenesis findings

In 1913, Santiago Ramon y Cajal hypothesized that neurons in the brain are generated exclusively during prenatal development. This idea was widely accepted until the 1950's and 1960's with the introduction of the use of tritiated thymidine, a radioactive deoxyribonucleoside that incorporates into DNA during the S (synthesis) phase of DNA replication. Through autoradiography of tissues labeled with ^{3}H thymidine, this method can serve as a marker for the timing of generation ("birthdate") of new cells in the brain (Sidman, Miale, & Feder, 1959). Studies from Joseph Altman, Gopal Das, and Shirley Bayer used ^{3}H thymidine labeling and showed that neurons of the

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dentate gyrus and olfactory bulb continued to be generated throughout life (Altman, Brunner, & Bayer, 1973; Altman & Das, 1965; Bayer & Altman, 1974). The full contribution of these studies to postnatal neurogenesis was not fully appreciated until later, when the thymidine method was replaced with Bromodeoxyuridine (BrdU). BrdU is a synthetic nucleotide of thymidine that can be incorporated into DNA and also used as a marker to identify cells that are undergoing replication during the S (synthesis) phase of DNA replication; (Gratzner, 1982; Nowakowski, Lewin, & Miller, 1989). BrdU is more frequently used as it is cheaper, faster and more precisely localized in specific cells via immunocytochemistry and confocal microscopy. When combined with other cell-specific markers, it can confirm a cell's phenotype (Abrous, Koehl, & Le Moal, 2005). The use of BrdU supported Altman's original observations using thymidine (Kaplan & Bell, 1984; Kaplan & Hinds, 1977). When BrdU was detected in neurons in postmortem brain sections in humans that had received an injection of BrdU for cancer treatment, this finding solidified the idea of adult postnatal neurogenesis occurring in both the rodent and mammalian brain (Eriksson et al., 1998).

1.4.1 The hippocampal formation

The hippocampal formation can be divided into four main regions; the DG, the Ammon's horn of the hippocampus proper (CA1, CA2 & CA3), the subicular complex and the entorhinal cortex. There are multiple connected pathways through the hippocampal formation. One of the main projection pathways to the hippocampal formation is the perforant pathway from the entorhinal cortex to the dendrites of the DG granule cells (DGC). The DGCs project to the CA3 region through the mossy fibers, and

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pyramidal cells of the CA3 give rise to axons that either terminate within CA3 or project on to the CA1 region (Amaral & Witter, 1989). The DG is thought of as the first "processing station" in the tri-synaptic loop (Piatti, Ewell, & Leutgeb, 2013). The DG is composed of three main layers, the molecular layer, the granular layer and the polymorphic layer (Amaral & Witter, 1989). The principal cell type in the DG is the granule cell, which gives rise to unmyelinated axons called mossy fibers that project to the CA3 (Amaral, Scharfman, & Lavenex, 2007). While granule cells are excitatory, another cell type found in the DG, pyramidal basket cells, are inhibitory (Ribak & Seress, 1983). A third cell type is found in the DG, the mossy cell which typically resides in the hilus (Wenzel et al., 1997). There are also numerous other connections through the hippocampus, such as direct connections from the entorhinal cortex to CA3 and CA1 and the commissural/associational projections from neurons in the hilus to the inner third of the molecular layer of the DG (Witter & Amaral, 1991). Other studies have shown a direct "back projection" from area CA3 to mature and new granule cells (Vivar et al., 2012). The medial septal nucleus extends cholinergic projections throughout the hippocampus (Dragoi et al., 1999). The hippocampus also receives inputs from the hypothalamus (Segal, 1979), as well as the contralateral hippocampus (Frotscher & Zimmer, 1983).

1.4.2 The process of adult hippocampal neurogenesis

Neurogenesis occurs in two areas of the adult rodent brain, the subventricular zone (SVZ) and the subgranular zone (SGZ) (Alvarez-Buylla & Lim, 2004). Cells generated in the SVZ travel through the rostral migratory stream into the olfactory bulb

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where they become interneurons (Lois, Garcia-Verdugo, & Alvarez-Buylla, 1996). Cells born in the SGZ migrate into the granule cell layer of the DG and can differentiate into neurons, astrocytes, or oligodendrocytes (Mu, Lee, & Gage, 2010). This differentiation is hypothesized to be dependent on astrocytes, as they have been shown to promote differentiation and integration of these progenitor cells *in vitro* (Song, Stevens, & Gage, 2002). As many as 9,000 new cells are generated per day in the rodent DG (Cameron et al., 1993; Kempermann, Kuhn, & Gage, 1997).

There are two main types of cells that play a role in neurogenesis, neural stem cells (Type 1) that are self-renewing and multipotent-capable of giving rise to neurons, astrocytes or oligodendrocytes, and neural progenitor cells (Type II) (Gage & Temple, 2013). These type 1 stem cells give rise to type 2 cells through cell division, cells generated during this process are described as mitotic cells. A subset of these type 2 cells commit to a progenitor cell state (neuroblasts), and can still divide and generate postmitotic immature neurons that are capable of becoming mature neurons. Cells generated during this state are postmitotic cells (Aimone et al., 2014; Hodge et al., 2008). In order to better phenotype cells throughout these various stages of development, a number of immunohistological markers have been developed (und Halbach, 2007).

During the initial proliferation stage, nestin, an intermediate filament protein, is commonly expressed in both neural stem cells and progenitor cells (Abrous et al., 2005). A majority of markers are used in conjunction with BrdU in order to better identify the specific stage of development the cells are undergoing (Abrous et al., 2005). Ki-67 is commonly used in conjunction with BrdU, as this protein is present during all phases of the cell cycle, but is absent in quiescent cells (not dividing or preparing to divide)

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(Scholzen & Gerdes, 2000). Thus, Ki-67 can be used as a marker for proliferation. Another common proliferation marker is glial fibrillary acidic protein (GFAP). Progenitors that express GFAP has been shown to generate the majority of neuroblasts and neurons, thus it can be used as a marker for proliferation (Garcia et al., 2004). However, GFAP is also expressed in mature astrocytes (und Halbach, 2007), so morphological assessments should be taken to distinguish between a progenitor cell, and an astrocyte.

A subset of type 2 cells daughter cells will become post-mitotic and begin to differentiate. Initially, these cells continue to be nestin-postive, but are GFAP-negative (Kronenberg et al., 2003). Shortly thereafter, cells become nestin-negative, and double cortin (DCX) expression, a microtubule binding protein, becomes a prominentmarker. DCX is highly expressed in proliferating progenitors and differentiating neuroblasts, but becomes undetectable approximately two months after labeling with BrdU. However, DCX can be co-expressed in cells that also express NeuN (nuclear neuronal marker), a common marker for mature neurons, for a period of time. Thus, there can be overlap of DCX and NeuN expression and it has been suggested that DCX labels both mitotic and postmitotic (differentiating) neurons (Brown et al., 2003). Poly-sialylated-neural cell-adhesion molecule (PSA-NCAM) is also used, as it is highly expressed in progenitor cells and PSA-NCAM labeled cells commonly express other markers specific to this stage, such as DCX or NeuN (Seki, 2002). During this phase, transcription factors such as Olig2 and Pax6 have been shown to positively regulate progenitor differentiation (Hack et al., 2005). Growth factors, such as brain derived neurotrophic factor (BDNF) and signaling pathways, like the sonic hedgehog pathway (SHH), have both been shown to

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regulate both proliferation and differentiation of cells (Chen et al., 2013; Rowitch et al., 1999). The Wnt pathway has also been shown to regulate progenitor cell proliferation and differentiation, possibly by upregulating NeuroD1 expression, which is a crucial transcription factor for proliferation and differentiation (Lie et al., 2005).

Cells that have a determined neuronal-fate then begin a process of migration and functional integration into the DG. During this cell survival stage, PSA-NCAM and DCX are still commonly used as markers. These cells retain an immature phenotype for approximately four weeks while they undergo both a morphological and physiological maturation process (Zhao, Deng, & Gage, 2008). The migration of these cells to the inner granule cell layer is thought to be regulated by disrupted-in-schizophrenia (DISC1), as downregulation in this protein results in accelerated integration (Duan et al., 2007). Functionally, these newborn cells are initially depolarized by GABA, but this response switches to hyperpolarization around 2-4 weeks of age. At this stage of depolarization, these cells have been shown to have lower thresholds for LTP, resulting in increased potentiation, and plasticity (Ge et al., 2007). Mossy fiber endings establish contact with CA3 pyramidal cells around two weeks of age, and these endings increase in size and complexity over time (Taupin, 2007). While able to become depolarized by GABA, these cells are still relatively inactive, firing few action potentials (Vivar et al., 2012). Around two weeks of age is a crucial time point for these neurons, with around 50% being lost in the maturation process (Dayer et al., 2003). At three weeks of age, the dendrites of these cells reach the outer molecular layer, and GABA has now become hyperpolarizing (Ge et al., 2006). At four weeks of age, dendritic branching and mossy fiber outputs become more complex (Zhao et al., 2006). These cells are considered

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mature around six weeks of age, and are commonly phenotyped using calbindin, a neuronal calcium-binding protein, as well as NeuN, a neuronal specific nuclear protein that is found in the nucleus of postmitotic neurons (Mullen, Buck, & Smith, 1992; Sloviter, 1989; Zhao et al., 2006).

1.4.3 The role of neurogenesis in learning and memory

The process of neurogenesis can be influenced by *ex vivo* factors as well. Environmental enrichment and running have been shown to increase hippocampal neurogenesis (Kempermann et al., 1997; Tashiro, Makino, & Gage, 2007; Van Praag, Kempermann, & Gage, 1999). Conversely, aging and stress have been shown to have a negative effect on neurogenesis (Cameron & Mckay, 2001; Warner-Schmidt & Duman, 2006). Learning can also increase neurogenesis, but this may be limited to specific tasks. Hippocampal-dependent tasks, such as trace eye blink conditioning or spatial navigation tasks, increase the survival of new neurons, specifically those that were generated before training. However, tasks that are not hippocampal-dependent (e.g., delay eyeblink conditioning; visually-cued navigation) do not have an effect (Gould et al., 1999). Interestingly, a study that found a positive effect of running on neurogenesis through promotion of rates of proliferation, did not find the same effect when mice were trained on the MWM, a seemingly hippocampal-dependent task (Van Praag, Kempermann, & Gage, 1999). Importantly, this contrasts with the abovementioned study that did find an increase in survival of newly generated neurons after MWM training. These contrasting studies prompted the idea of a “critical period” for experience-dependent neuron survival, since the studies labeled neurons at different time points. Subsequent studies showed that

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experiences or learning episodes occurring 3-4 weeks after neuronal birth had the greatest effect on neuronal survival and integration into functional hippocampal circuits (Kee et al., 2007; Tashiro et al., 2007).

Early studies focused on identifying a role for the hippocampus in learning and memory tasks. Lesions of the hippocampus result in performance deficits on numerous tasks (delayed nonmatching to sample, MWM, contextual fear conditioning, and trace eye blink conditioning), suggesting that this structure is crucial for these hypothesized hippocampal-dependent tasks (Eichenbaum, Stewart, & Morris, 1990; Salmon, Zola-Morgan, & Squire, 1987; Weiss et al., 1999). However, subsequent studies focusing on ablating neurogenesis, suggested that the integrity of adult hippocampal neurogenesis was not necessary for performance on some hippocampal-dependent tasks. Ablation of postnatal neurogenesis did not impair performance on previously reported hippocampal-dependent tasks, such as contextual fear conditioning, MWM, the Y-maze task, or NOR (Madsen et al., 2003; Saxe et al., 2006; Shors et al., 2002). These results suggested that the generation of new neurons is not necessary for performance on some hippocampal-dependent tasks. For example, the monosynaptic pathway (EC→CA1), bypassing the DG, is sufficient to learn the MWM task (Brun et al., 2002; Nakashiba et al., 2008). These findings were followed by an extensive series of studies aiming to elucidate the specific functional role of adult-born neurons in behavior.

1.5 What role do newborn neurons have in behavior?

DGCs receive direct inputs from the entorhinal cortex, which has been described as a very active network, and its neural representation (approximately 200,000 cells in the

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adult rat) is projected to a much larger number of DGCs (Amaral, Ishizuka, & Claiborne, 1990; West, Slomianka, & Gundersen, 1991). The encoding scheme in the DG has been described as "sparse" because even with a high number of activated neurons, the proportion of active nuerons is relatively low due to the large number of cells in the DG (approximately 1,000,000) (Piatti et al., 2013). Interestingly, only 1-2% of these cells are active during behavioral exploration (Tashiro et al., 2007). These characteristics of the DG are in stark contrast to the entorhinal cortex, which has high levels of active neurons and mean firing rates of neurons (Barnes et al., 1990). The DG has also been described as "sparse" because activity levels in the DG are lower than its main input (EC) as well as its main output (CA3). Thus, the DG has lower activity than the afferent inputs and the target neuronal population (Yassa & Stark, 2011). A sparse representation is hypothesized to be crucial for memory encoding, as this allows information to be encoded in a distinct, non-overlapping manner (Yassa & Stark, 2011). Thus, combined with its unique physiological properties and location within the hippocampal formation, newly generated DGCs are hypothesized to play a key role in this encoding, called "pattern separation" (Yassa & Stark, 2011).

These distinct representations allow, at a behavioral level, discrimination between similar memories acquired during learning (Yassa & Stark, 2011). It is important to note the type of information that the DGCs process during pattern separation. These new granule cells receive input from the lateral entorhinal cortex and perirhinal cortex (PRH), whereas mature cells receive innervation from both the lateral entorhinal cortex and medial entorhinal cortex (Vivar & Van Praag, 2013). The lateral entorhinal cortex and perirhinal cortex are thought to transmit non-spatial information about the environment,

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including familiarity, whereas the medial entorhinal cortex is thought to process more spatial-based information (Deshmukh & Knierim, 2011; Hafting et al., 2005). A diminished PRH has been shown to correlate with impairments in distinguishing between objects with similar, overlapping features (Burke et al., 2011). This suggests that newborn granule cells innervated by the lateral entorhinal cortex/perirhinal cortex may be more specific for processing and separating familiar environmental information versus mature granule cells.

1.5.1 Evidence for the role of newborn neurons in pattern separation

Behavioral pattern separation can be assessed using multiple methodological approaches, including a contextual fear discrimination task. In this task, animals are trained to associate an aversive stimuli (foot shock) with a specific, neutral context. When the animal is placed into a similar context, freezing indicates a lack of context discrimination in the animal, whereas exploration would indicate the recognition of the novel context (Kheirbek et al., 2012). Another method that is commonly used is an automated touchscreen operant chamber. This touchscreen version of pattern separation trains animals to discriminate between locations on a screen, with varying distances as the manipulation. (Josey & Brigman, 2015; McTighe et al., 2009; Talpos et al., 2010). A third method for measuring behavioral pattern separation is a delayed non-matching to sample radial arm maze task. In this task, the animal is first exposed to a start arm and a forced reward arm. After a short delay, the mouse is placed in the same start arm position, with the original and a novel arm (now reward arm) both open. The location of the novel arm is manipulated by varying its distance from the original forced arm. It is

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proposed that it is a more difficult task when the novel arm is close to the forced arm (≤90 degrees), versus a 180 degree separation (Clelland et al., 2009a).

In order to understand the role of DGCs in these pattern separation tasks, multiple studies have examined the relationship between the manipulations of these cells, and subsequent performance on a pattern separation task. Early approaches for ablating neurogenesis included using Methylazoxymethanol acetate (Maeda-Yamamoto et al.), x-irradiation and ionizing radiation (Altman, 1969; Johnston & Coyle, 1979; Peißner et al., 1999). These methods are effective at reducing neurogenesis; however, some animals are still able to learn some of the hippocampal-dependent tasks, and observed impairments may have been confounded by aversive or toxic side effects (Dupret et al., 2005; Groves et al., 2013; Madsen et al., 2003; Saxe et al., 2006; Shors et al., 2002; Urbach et al., 2013; Hernandez-Rabaza et al., 2009). While some of these techniques continue to be used, more genetically targeted techniques have been implicated to study pattern separation. The DNA methyl transferase type 1 (DNMT1) gene is necessary for cell viability. By crossing DNMT1-cKO mice with either DNMT1-loxP/mGFAP-Cre-negative or DNMT1-loxP/mGFAP-Cre-positive mice, half of the offspring would be DNMT1-loxp/mGFAP-Cre-positive. Thus, these animals would lack DNMT1 in cells expressing GFAP (adult neuroprogenitors, thus neurogenesis is prevented) (Cushman et al., 2012). DNMT1-loxP/mGFAP-Cre-positive mice were impaired relative to controls in a spatial pattern separation task. However, they were not impaired versus controls in an object-context recognition or a spatial location recognition task, suggesting that the function of newly generated cells are crucial for pattern separation tasks (Kesner et al., 2014). Ablating neurogenesis using x-irradiation or gene knockout techniques (ablating

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FMRP expression in NSCs) has also been shown to significantly impair pattern separation task performance in the radial arm maze task (Nakashiba et al., 2012), as well as performance in a contextual discrimination task (Nakashiba et al., 2012; Tronel et al., 2012). Conversely, increasing neurogenesis has been shown to improve pattern separation performance. Ablation of *Bax* (a pro-apoptotic gene) significantly increased postnatal neurogenesis and performance on a contextual fear task (Sahay et al., 2011). Physical exercise (via running wheel) improves performance on smaller separations in a touch-screen spatial pattern separation task, and this improvement was also correlated with increased neurogenesis (Creer et al., 2010).

The utilization of a behavioral pattern separation task in the Ts65Dn mouse model could allow researchers to directly correlate a specific Ts65Dn deficit (adult neurogenesis) to a specific behavioral outcome (performance on a pattern separation task). This type of correlation would be invaluable for research that is targeting improvement of the cognitive deficits that are observed in DS. Not only would this task increase the phenotype resolution for the Ts65Dn mouse model, but it would allow researchers to administer targeted treatments (to improve neurogenesis deficits) with the reliance of a direct behavioral measure. With the increasing use of EGCG as a potential therapeutic treatment for the cognitive deficits in DS, examining the effects of EGCG on pattern separation performance, and concurrently on adult neurogenesis in the Ts65Dn mouse model is an important study for DS research.

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1.6 Thesis Hypotheses

HYPOTHESIS 1: Trisomic mice would be impaired on smaller degrees of separation in a spatial pattern separation task versus controls, and that EGCG treatment would ameliorate these deficits.

HYPOTHESIS 2: Trisomic mice would display deficits in adult hippocampal neurogenesis, and that EGCG would increase neurogenesis of Ts65Dn mice by promoting cell proliferation, survival, or both.

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CHAPTER 2. METHODS

2.1 The effect of EGCG on a pattern separation task in the Ts65Dn mouse

2.1.1 Ts65Dn Housing

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, ME). 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 PD 6-10, 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. Ts65Dn and euploid (control) male offspring were weaned on PD21, and singly housed in a climate-controlled room on PD 22. Housing was maintained on a reverse light/dark cycle, so that testing would occur during the active (dark phase) of the circadian cycle.

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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 B6C3F1 males would lead to approximately 25% of all Ts65Dn pups born with this

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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/+), homozygous for the mutation (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 EGCG preparation

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

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dissolving 30 mg of solid EGCG in 2 mL of PBS and was refrigerated to maintain stability. Treatments of 0.4 mg/mL concentration were prepared by diluting the stock solution in tap water. The pH of the EGCG and water (control) treatments were established using phosphoric acid (pH 5-5.5).

On PD22, trisomic and euploid mice were randomly assigned to the EGCG condition (dissolved in drinking water with concentration of 0.4 g/ml EGCG stabilized with phosphoric acid to pH 5.5) or to the control condition (water with phosphoric acid). Fluid consumption was measured every other day when fluids were refreshed. Treatment was ongoing throughout the experiment.

2.1.4 Timeline

Mice were placed into either the cell survival or cell proliferation study. On PD 55, all mice were covered and brought to the injection room. Each mouse was handled for 2 minutes under red light, then was covered and brought back to the housing room. On PD60-PD63, mice were brought back to the same room, and received a 50mg/kg intraperitoneal (i.p.) injection of either BrdU (cell survival study) or saline (cell proliferation study). After injections were complete, mice were covered and brought back to the housing room. Each mouse received a single injection for four consecutive days. On PD64-PD70, mice were weighed, and food restricted daily to 85% body weight. Restricted weights were increased by 2% every week to prevent inhibition of natural growth. Mice were handled during the food restriction week in the pattern separation testing room. After the food restriction week, habituation and testing (20 total days) began in the pattern separation task. At the end of testing, the cell survival animals

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were perfused under deep anesthesia with 4% paraformaldehyde. Brains were carefully removed from the skull and placed in 4% paraformaldehyde. Cell proliferation animals received *ad libtum* access to food for approximately 6 days. They then received a single i.p. injection of 100 mg/kg of BrdU (Sigma, 10mg/ml concentration in 0.9% sterile saline) to label dividing progenitor cells. Two hours later, mice were perfused under deep anesthesia with 4% paraformaldehyde. Brains were carefully removed from the skull and placed in 4% paraformaldehyde. Coronal sections through the hippocampal formation were stored at -20C in a cryoprotectant solution containing glycerol and ethylene glycol in Trisbuffer solution for 48 hours. The slices were then processed for peroxidase immunohistochemistry to label BrdU+ cells. All experimentation was carried in accordance with Indiana University-Purdue University Indianapolis School of Science IACUC.

2.1.5 5-Bromo-2-hydroxyuridine preparation

5-Bromo-2-hydroxyuridine (B5002 Sigma) was stored at 4° C until ready for preparation. 0.9% sterile saline solution was warmed to approximately 50° C. 5-Bromo-2-hydroxyuridine (concentration 10mg/ml) was removed from the freezer and measured on an analytical scale. Bromo-2-hydroxyuridine was then added to a 2ml tube and warm saline was slowly added via syringe. The solution was gently vortexed, and was used immediately for injections.

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2.1.6 Pattern Separation Training

The testing apparatus was an eight arm radial maze composed of white Plexiglas. The arms were 76.2 cm long, 7.62 cm high and 0.635 cm thick. The center octagon was 20.9 cm wide, with eight doors for each arm standing 8.25 cm tall. The maze was placed on a sheet of plywood, which rested on top of a cart 87.63 cm above the ground. The center of the maze was placed 114.3 cm from the wall, and visual cues and curtains surrounded the maze. The mice underwent four days of habituation before testing began. A picture of the testing apparatus is seen in Figure 2.1.

1. Habituation Day 1-On the first day, the mice were brought to the testing room and were allowed to individually explore the maze for 10 minutes. Three fruit loops were placed in each arm at 10cm, 20cm and 30cm away from the center octagon. At the end of each arm (30cm), blue 15mL caps held a piece of fruit loop (approximately .05 grams). After ten minutes, the mouse was returned to the homecage, the maze cleaned, and new fruit loops were placed in the maze for the next mouse.
2. Habituation Day 2 and 3-A piece of fruit loop was only placed in the wells at the end of each arm (8 total food pellets). The mice were allowed to explore for 10 minutes, or until all the fruit loops were retrieved. By the third day, all mice were retrieving fruit loops from at least 6 arms.
3. Habituation Day 4- The mice were given four forced trials. Each arm was blocked off except for a start arm and a sample arm. Four random start arms were chosen by the experimenter. The sample arm position (left or right of start arm) was counterbalanced across the four trials. These four days of

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habituation ensured that the mice were motivated by the presence of fruit loops, and that anxiety levels were at a minimum.

On day five, testing began with 6 trials (sample + choice). Mice were tested on a delayed nonmatching to place radial arm maze task, adapted from (Clelland et al., 2009b). Mice were tested on their ability to select, from a choice of two arms, the arm location that had not been presented in the previous trial. During the sample phase, all arms except a start arm and a sample arm were closed off. The mouse was placed in the start arm and was allowed to retrieve a food reward from the sample arm. The mouse was then removed from the maze and placed in its home cage. The start, sample and choice arm were then wiped with 70% ethanol to prevent olfactory cues. The choice arm was no longer blocked off, and now contained the fruit loop. The cleaning took approximately 20-30 seconds. The mouse was then placed in the start arm, and was now forced to choose between the sample (unrewarded) and the choice (reward) arm. The choice arm varied in distance from the sample arm by a separation of 1, 2 or 3 arms. A mouse was determined to make a correct choice by entering and retrieving the fruit loop from the choice arm. If a mouse made an incorrect choice (enters the sample arm), the mouse was allowed to self-correct and retrieve the fruit loop from the choice arm. The mouse was then removed from the maze, and all arms were thoroughly cleaned. All other mice were tested on the same separation before the first mouse began the second trial. Mice underwent six trials (sample plus choice phases) per day of pseudo randomly presented combinations of start plus sample plus correct arms for 18 consecutive days (36 trials of each separation). A summary of the study timeline is seen in Figure 2.2.

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2.1.7 Statistics

For the pattern separation acquisition, the average three-day percentages were analyzed using a mixed analysis of variance (ANOVA) with genotype and treatment as between-group factors and Day (3 day blocks) as a repeated measure. For the separation analysis, a mixed ANOVA with genotype and treatment as between-group factors and separation (3 degrees) as a repeated measure was used.

2.2 The effect of EGCG on proliferation in the dentate gyrus of the Ts65Dn mouse

2.2.1 Tissue collection

Animals assigned to the cell proliferation study received *ad libtum* access to food for approximately 6 days. They then received a single i.p. injection of 100 mg/kg of BrdU (Sigma, 10mg/ml concentration in 0.9% sterile saline) to label dividing progenitor cells. Two hours later, mice were perfused under deep anesthesia with 4% paraformaldehyde. Brains were carefully removed from the skull and placed in 4% paraformaldehyde for 24 hours. Brains were sliced coronally at 40 µm beginning at Bregma -1.22 mm and ending at -2.18 mm (Figure 2.3). Coronal sections through the hippocampal formation were stored at -20° C in a cryoprotectant solution containing glycerol and ethylene glycol in Trisbuffer solution for 48 hours. The slices were then processed for peroxidase immunohistochemistry to label BrdU+ cells.

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2.2.2 Immunohistochemistry

For the peroxidase immunohistochemistry, sections were washed in TBS (0.15 M NaCl and 0.1M Trizma HCl) and treated with 3% H_2O_2 for 10 minutes to block endogenous peroxidases. Then, the slices were washed 5x2 min in TBS, and underwent a 30 minute incubation in 2N HCl at 37ºC. Sections were then washed in 0.1M Boric acid (pH=8.6) in TBS, in TBS alone, incubated for 1 hour in blocking solution (0.5% Triton X-100 and 3% normal goat serum in TBS), and then incubated with the primary antibody solution (rat anti-BrdU, 1:500; Accurate Chemical, OBT0030) for 48 hours at 4ºC. Following primary antibody incubation, sections were washed in the blocking solution, followed by a 1 hour incubation in the blocking solution. The slices were then washed 3 times in TBS, and were incubated in the secondary antibody (biotinylated anti-rat made in goat, 1:250; Vector Laboratories, BA-9400), then for 1 hour in avidin-biotin-peroxidase complex solution (ABC Elite Kit; Vector Laboratories, PK-4000) with nickel-enhanced diaminobenzidine as the chromagen. Slices were mounted to slides and allowed to dry for 48 hours.

2.2.3 Tissue Staining and Counting

Slides were briefly dipped in milli-q water, and then placed in a 0.5% Methyl green solution (Sigma-Aldrich) for seven minutes. This was followed by 2 x 20 second slow dips in water. Slides were then placed in 70% ethanol for approximately 1 minute, with slices being monitored for differentiation. This was followed by a 5 second dip in 95% ethanol, then a 1 minute dip in 100% ethanol. Finally, slides were left in xylene for 5 minutes, and then the counterstained slides were coverslipped with Permount. Slides

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were left to dry for 48 hours. Quantification of BrdU cells was done similar to previous studies (Malberg et al., 2000; Eisch et al., 1999). BrdU+ labeled cells were counted in the subgranular layer of the DG with a bright-field microscopy camera (Nikon), at 1000x magnitude with the 100x oil objective (Figure 2.4). The volume of the granule cell layer was quantified by the 4x objective, using Cavalieri's principle (Stereologer system and software; Systems Planning and Analysis, Inc., Alexandria, VA). Section thickness was measured using a 1000x magnification.

2.2.4 Statistics

Cell counts were analyzed with a 2-way factorial ANOVA. The primary hypothesis would be confirmed if EGCG treatment significantly increased the number of BrdU+ cells in the subgranular zone of the DG of the trisomic mice.

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CHAPTER 3. RESULTS

3.1 The effect of EGCG on pattern separation testing in the Ts65Dn mouse

3.1.1 Ts65Dn growth and EGCG consumption

Trisomic mice weighed significantly less than the euploid mice (Figure 3.1), confirmed by a main effect of genotype [$F(1,61)=31.33$, $p=<0.001$]. However, both groups increased their weight as a function of time [main effect of day, $F(24,1464)= 272.602$, $p=<0.001$]. As shown in Figure 3.2, EGCG consumption for both groups was average approached 70 mg/kg per day in the first week, then decreased their consumption over time to stable levels around 50 mg/kg/day [main effect of day, $F(25,1025)= 16.629$, $p=<0.001$]. Importantly, there were no significant differences in EGCG consumption between the euploid and trisomic mice

3.1.2 Ts65Dn pattern separation performance

Prior to pattern separation, mice either received an injection of saline (cell survival study) or BrdU (cell proliferation study). Of the 65 animals presented here, 44 received an injection of BrdU and 21 received a saline injection. There were no effects of BrdU on subsequent performance in the pattern separation task ($p=0.135$).

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As shown in Figure 3.3, euploid and trisomic groups increased their overall performance over the course of training [main effect of day, $F(5,305)= 4.6$, $p=<0.001$]. However, euploid mice had a higher overall performance versus the trisomic mice, [main effect of genotype. $F(1,61)= 18.435$, $p<0.001$]. No interactive effects were found. For separation performance (Figure 3.4), both euploid and trisomic mice increased their performance as a function of separation, [main effect of separation $F(2, 122)= 20.541$, $p<0.001$]. Euploid mice had an overall higher performance on each separation versus the trisomic mice [main effect of genotype $F(1,61)=21.277$, $p<0.001$]. No main or interactive effects of EGCG treatment were found. To identify differences in performance as a function of the degree of arm separation, the euploid + water (control) animals were assessed with a paired samples t-test between each of the three degrees of separation. As shown in Table 3.1 and Figure 3.4, the euploid animals made significantly fewer correct choices in separation 1 versus separation 2 ($p=0.001$) and in separation 1 versus separation 3 ($p=0.001$). They were not significantly different between separation 2 versus separation 3 performance ($p=0.524$). For the trisomic + water animals, performaning significantly worse in separation 1 compared to separation 2 ($p=0.006$). However, there were no differences in performance when comparing separation 1 versus separation 3 ($p=0.09$), nor when comparing separation 2 versus separation 3 ($p=0.209$) (Table 3.1).

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3.2 The effect of EGCG on proliferation in the dentate gyrus of the hippocampus

3.2.1 Ts65Dn dentate gyrus proliferation

There were no significant differences between euploid and Ts65Dn mice in the density of BrdU-labeled cells [$F(1,18)= 0.896$, $p=0.356$], the average section thickness [$F(1,18)= 0.586$, $p=0.454$], the average volume per section [$F(1,18)=1.10$, $p=0.308$], the number of sections counted [$F(1,18)= 1.88$, $p=0.187$], or the total reference volume [$F(1,18)= 2.149$, $p=0.160$]. There was a main effect of genotype of the total number of labeled cells [$F(1,18) = 8.83$, $p=0.008$], as indicated by the higher number of cells counted in the euploid versus trisomic mice (Table 3.2 & Figure 3.5).

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CHAPTER 4. DISCUSSION

4.1 Ts65Dn performance on a pattern separation radial arm maze task

This study is the first to test the Ts65Dn mouse model in a pattern separation task, and demonstrated that Ts65Dn mice exhibit deficits in acquisition in the pattern separation task that was evident across all three degrees of separation. These deficits were not alleviated with a seven week treatment of EGCG administered via drinking water [0.4mg/ml; ~50mg/kg/day]. Previous work in our and other labs have shown that Ts65Dn mice and individuals with DS exhibit deficits on tasks that are thought to rely on the hippocampus, such as MWM, NOR, and the T-maze task (Pennington et al., 2003; Reeves et al., 1995c; Stringer et al., 2015). The utilization of this pattern separation task provides a novel learning task consistent with the phenotypic profile of Ts65Dn mice.

This task used three degrees of separation, with separation 1 having the highest degree of similarity to the just-experienced forced choice, thus requiring finer-grain pattern separation in order to discriminate and correctly choose the rewarded arms. Separations 2 and 3 were less similar to the just-experienced forced choice, and hypothesized not to rely as much on pattern separation in order to make the correct choice. Ts65Dn mice had an overall deficit on all three separations, rather than a deficit on just the subtly different arm presentations. There are a few factors that could be contributing to these findings. Ts65Dn mice display multiple hippocampal deficits, not

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just in adult hippocampal neurogenesis (Lorenzi & Reeves, 2006). This is important to note because during pattern separation tasks, the DG is not the only structure that is active (Yassa & Stark, 2011). For example, more distinct discriminations (separations 2 and 3) are hypothesized to not require pattern separation encoding, rather, relying on the EC→CA3 pathway (Neunuebel & Knierim, 2014). Ts65Dn mice have also been shown to display deficits in synaptic density and the synapse to neuron ratio in the CA3 area (Kurt et al., 2004; Popov et al., 2011). Thus, this could cause the observed deficits versus controls at the low levels of discrimination (separations 2 and 3). Another study hypothesized that discrimination of distinct stimuli could rely more on mature granule cells, rather than immature cells (Nakashiba et al., 2012). At different stages of development, Ts65Dn mice display premature neuronal differentiation (Kurabayashi & Sanada, 2013) or an elongation of the G1 phase of the cell cycle (de Lagran et al., 2012). Premature neuronal differentiation, as well as a reduction in the number of proliferating cells could both eventually lead to a reduction in the number of mature neurons. (Tejedor & Hämmerle, 2011). While deficits of mature neurons were not examined in the present study, they could explain why Ts65Dn mice display deficits on separations 2 and 3.

Another consideration for impaired Ts65Dn performance on a pattern separation task is the overexpression of GABA throughout the hippocampus (Best et al., 2012; Kleschevnikov et al., 2012). A recent study demonstrated that the α5 subunit of GABA-R in the DG is critical for high discrimination trials in a PS task. This study also showed that the neither the α2 GABA-R subunit in the DG nor the α5 GABA-R subunit in the CA1 region was necessary for high discrimination trials, reinforcing that the α5 subunit specifically in the DG is necessary for high-demand discrimination trials (Engin et al.,

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2015). Ts65Dn mice display increases in the number inhibitory interneurons throughout the hippocampus (Hernández-González et al., 2015), as well as deficits of DGCs. Therefore, I hypothesize that these mice have encoding deficits of similar stimuli, as well as deficits in transmitting these representations to the CA3, due to increased inhibition of these DGCs. DS research has shifted towards using inverse agonists in Ts65Dn mice, with studies reporting rescued learning and memory deficits (Braudeau et al., 2011). Recently, the compound RO4938581 was chronically administered for six weeks to Ts65Dn mice and was reported to rescue multiple behavioral and histological deficits (Martínez-Cué et al., 2013). RO4938581 is currently in Phase 2 clinical trials to treat the cognitive deficits observed in DS (Rudolph & Möhler, 2013).

Other studies have found that neurogenesis-deficient animals did poorly on both the low and high discrimination trials (Guo et al., 2011; Zhang et al., 2014). The hyperactive nature of Ts65Dn mice (Stringer et al., 2015) could lead to more exploratory behavior in the maze, thus decreasing the motivation to choose the correct arm in any of the separation trials. It will be critical to gather more data on the latency measures during this task, in order to determine if any motor or motivational effects are seen in the Ts65Dn mice. Another factor that should be taken into consideration with these results in the variability of performances across animals. The Ts65Dn mice used in this study were maintained on a 50% background of C57BL/6 and C3H mice, which could contribute to the wide performance range that is observed. For instance, C57 and C3 mice have been shown to have contrasting performances on a rotarod task (Rogers et al., 1999). In the current task, animals could be trained to a certain criterion to determine if performance above chance levels could be achieved. In this study, all animals as a group averaged

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above chance percentages; however, the previously mentioned procedural change would provide us with a better understanding of the Ts65Dn's ability or inability to learn a pattern separation task.

4.2 The Effect of EGCG on proliferation in the Ts65Dn dentate gyrus of the dorsal hippocampus

There were significant differences in the total number of BrdU-labeled cell counts, with euploid animals having higher cell counts versus trisomic mice. These differences were evident without differences in the section thickness, total sampled volume, average volume per slice, and density of labeled cells in the DG. However, Furthermore, EGCG does not appear to be significantly change the cell counts. These results agree with previous findings reporting proliferation deficits in the Ts65Dn mouse, however the lack of effect of EGCG on proliferation contradicts some previous findings.

Studies have reported that Ts65Dn mice, two to five months old display proliferation and apparent short-term survival deficits (Clark et al., 2006). In this study, BrdU was administered for 9 consecutive days, and the authors examined both cell proliferation and short-term survival. In order to differentiate between these two processes, they measured the percentage of cells that appeared in clusters (indicative of cell proliferation), whereas those migrating would be counted towards short-term survival. Another study used 2-3 month old Ts65Dn mice, and injected BrdU (50mg/kg) once a day for 6 consecutive days. 24 hours after the last injection, the mice were sacrificed. They examined the density of BrdU+ cells in the DG, and found that Ts65Dn had a significantly lower BrdU+ cell density, however, they did not have any significant

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BrdU+ cell cluster densities (Belichenko & Kleschevnikov, 2011). They further examined these clusters of BrdU+ cells and found that Ts65Dn mice had a fewer number of cells per cluster. These findings are in agreement with a Ts65Dn proliferation deficit. As a field, there are few studies using BrdU to analyze adult Ts65Dn cellular proliferation. However, early formula-based findings suggest that adult Ts65Dn mice have decreases in cell counts throughout the hippocampus, and possibly volume deficits in the DG (Insausti et al., 1998; Lorenzi & Reeves, 2006). However, these studies did not utilize a cellular marker, such as BrdU, thus these quantitative cellular deficits observed in Ts65Dn does not necessarily equate to deficits in cell proliferation or cell survival.

The current study did not find any effects of EGCG on proliferation in the DG, although additional subjects need to be added to assure reliability of this conclusion. EGCG and its effect on hippocampal neurogenesis have yielded mixed results from multiple studies. One study examined BALB/cJ mice receiving ~250 mg/kg/day of EGCG via feeding chow for 39 consecutive days, beginning at eleven weeks of age. Also beginning at eleven weeks of age, mice were injected with BrdU (50mg/kg) for 10 consecutive days then sacrificed 30 days after the last injection. EGCG did not have any effects on the number of surviving BrdU+ cells in the DG (Bhattacharya et al., 2015). Another study reported that a single injection of Brdu (50mg/kg), followed by a four week treatment of EGCG (25mg/kg), administered via oral gavage significantly increased the number of cells in the DG (Yoo et al., 2010). Treatment with EGCG increased the number of Ki67+ cells in the subgranular zone of the dentate gyrus by 221% compared to the vehicle group. However, these comparisons were made at an unknown age. Another

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study examined the effect of a two-month administration of EGCG (20mg/kg) via injection, beginning on PD 60, in C57BL/6J mice. For their cell proliferation study, mice were given a single 100mg/kg injection of BrdU after their last EGCG injection, and then sacrificed two hours later. For their cell survival study, four-month old mice were given four daily consecutive injections of BrdU, and then were sacrificed four weeks later. The dose of BrdU or if EGCG treatment continued throughout the four weeks was not reported. They reported that a two-month administration of EGCG increased the number of BrdU+ cells in the subgranular zone of DG approximately 40%. EGCG also increased the density of BrdU-labeld cells in the granule cell layer of the DG. However, EGCG did not increase the number of BrdU/NeuN co-labeled cells, nor did it increase the number of BrdU/DCX co-labeled cells. Thus, administration of EGCG does not appear to be having an effect on the survival of newly generated cells (Wang et al., 2012).

The previous studies showing the positive effect of EGCG on cell proliferation s contradict what was found in the current study. Two stark differences are the strain of mice that was used and the route and dose of EGCG treatment. The studies that used C57BL/6J mice found a positive effect of EGCG on proliferation, whereas the BALB/cj strain did not report any effects. The Ts65Dn mice used in this study were maintained on a 50% background of C57BL/6 and C3H mice. To date, there have been no studies looking at the effects of EGCG administration on C3H mice or hybrid B6/C3H mice. Thus, strain differences could be contributing to the discrepancies in the proliferation results. Interestingly, both studies reporting positive effects of EGCG on cell proliferation used forms of EGCG that were very similar to the one used in the current study (purchased from Sigma-Aldrich). One study used systemic injections to administer

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EGCG, whereas the other used oral gavage to administer EGCG, rather than via drinking water, as administered for approximately eight weeks in the current study . This is a similar administration timeline compared to the two month, 20mg/kg injection of EGCG study. Thus, the route of EGCG administration could be attributing to the lack of effects observed. These differences in administration methodology could be contributing to the lack of effect of EGCG observed in this study.

4.3 Conclusions and future directions

This study demonstrated that Ts65Dn mice display deficits on a spatial pattern separation radial arm maze task in both acquisition, as well as on all degrees of difficulty. This task has not previously been examined in these mice, and results demonstrate a novel cognitive phenotype for the Ts65Dn mice. EGCG does not alleviate any of the deficits that are observed in this task, nor does it increase cell proliferation in these mice. The pattern separation task may prove to be valuable for studies of mouse models of DS, as prior studies suggest that this task could be a direct correlate to adult hippocampal neurogenesis. Pattern separation tasks are currently being used in a clinical setting, with computerized versions of the tasks (Yassa et al., 2011); (Bakker et al., 2008; Stark et al., 2013). Thus, the use of a pattern separation task in Ts65Dn mice to evaluate potential treatments could improve the external and face validity of any future findings.

While not directly examined in this study, *Dyrk1a* could be playing a crucial role in the proliferation and subsequent pattern separation deficits in the Ts65Dn mouse. *Dyrk1a* has been shown to regulate Cyclin D1, a key cell cycle regulator, especially in the G1 phase, where signaling either induces differentiation, or more cellular division

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(Dehay & Kennedy, 2007). Increases in *Dyrk1a* expression has been shown to lengthen the G1 phase, possibly causing a delay in neocortical wall growth, and thus producing fewer neurons early in development (Chakrabarti et al., 2007; Najas et al., 2015). *Dyrk1a* has also been shown to result in premature neuronal differentiation (Yabut, Domogauer, & D'Arcangelo, 2010). Together, these studies suggest that *Dyrk1a* mediates deficits in cell proliferation and differentiation. Furthermore, Ts65Dn mice that express only two copies of *Dyrk1a* display normal performance on memory tasks, as well as normalized synaptic plasticity and cell proliferation (Garcia-Cerro et al., 2014). In order to better understand the role of *Dyrk1a* in Ts65Dn mice, Ts65Dn mice should be crossed with *Dyrk1a* knockdown mice, resulting in four main groups (Euploid +/+, Euploid +/-, Trisomic +/+/+, Trisomic +/+/-). Trisomic (+/+/-) mice will have the normal trisomic complement of genes except for *Dyrk1a* (reduced to two copies), and should illustrate the role of *Dyrk1a* in learning and memory tasks, such as pattern separation.

These findings implicate *Dyrk1a* in playing a role in numerous developmental processes. *In vitro* administration of EGCG, a known *Dyrk1a* inhibitor (Bain et al., 2003) has been shown to restore LTP, rescue proliferation/differentiation deficits, and decrease *Dyrk1a* activity (Hibaoui et al., 2014; Xie et al., 2008). As previously mentioned, EGCG has been found to increase cell proliferation, but did not have an effect in cell survival studies, suggesting that this increase in cell production does not translate to mature integration of newly generated cells into functional circuits in the hippocampus. Moving forward, it will be important to analyze not only proliferation, but cell survival throughout the hippocampus. EGCG has been reported to show a trend towards reducing Dyrk1a activity in the cerebellum of Ts65Dn mice, however, this was not significant

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(p=0.06) (Stringer et al., 2015). One factor that could be contributing to this is EGCG's high solubility, which is hypothesized to result in poor membrane permeability (Smith, 2011). This could causing the rapid degradation that has been observed (Stringer et al., 2015). When mice were injected with either 25mg/kg or 50mg/kg EGCG, and killed 24 hours later, EGCG levels in the brain were one tenth of the amount that was measured in various organs (Smith, 2011). Thus, while EGCG does cross the blood brain barrier, EGCG administered via drinking water (as used in the current study) may not be the best route of administration in order to see histological or behavioral changes in the mouse brain. Studies in humans have increased the bioavailability of EGCG by administering it with ascorbic acid and omega-3 (Mereles & Hunstein, 2011). Another study made EGCG more hydrophobic via acetylation, and reported increased serum levels and an increased half-life of EGCG (Lambert et al., 2006a; Lambert et al., 2006b). In order to deliver a more controlled, higher dose of EGCG, mice should be orally gavaged with EGCG every day. From a pharmacological approach, analysis of EGCG levels in the serum of Ts65Dn mice will allow us to understand how much EGCG is getting into the blood stream. However, even more important is to identify how much EGCG is in the brain, and to determine if it affects *Dyrk1a* activity levels.

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TABLES

Table 3.1 Ts65Dn control separation performance

The euploid (Eu) animals performed significantly different in separation 1 versus separation 2 (p=0.001) and separation 1 versus separation 3 (p=0.001). However, they were not significantly different in separation 2 versus separation 3 performance (p=0.524). The trisomic (Ts) animals performed significantly worse in separation 1 versus separation 2. However, there were no performance differences in separation 1 versus separation 3, as well as separation 2 versus separation 3.

Eu+ Water N=18	Pair 1	S1 vs S2	61.67% vs 69.57%	p=0.001
	Pair 2	S1 vs S3	61.67% vs 70.88%	p=0.001
	Pair 3	S2 vs S3	69.57% vs 70.88%	p=0.524
Ts+Water N=11	Pair 1	S1 vs S2	53.67% vs 64.10%	p=0.006
	Pair 2	S1 vs S3	53.68% vs 60.65%	p=0.090
	Pair 3	S2 vs S3	64.09% vs 60.65%	p=0.209

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Table 3.2 Effect of EGCG on cellular proliferation in the dentate gyrus of Ts65Dn mice

There were no significant differences between euploid (Eu) and Ts65Dn (Ts) mice in measures of total slices counted, average thickness of the slices counted, total volume, average volume per slice, density or coefficient of error. There was a significant difference in total cell counts, with Eu mice have higher cell counts than the Ts mice.

	Slice Count	Thickness	Total Volume (mm^3)	Average Volume Per Slice	Total Cell Counts	Density (counts/volume)	Coefficient of Error
Eu+Water, n=6	**14.3333**	**23.2120**	**0.1023 x 10^{-9}**	**7143007.8910**	**358.8333**	**3.6315 x10^{-6}**	**0.0100**
	0.9545	0.6569	0.0129 x 10^{-9}	730622.6161	38.4737	0.3499 x10^{-6}	0.3499
Eu+EGCG, n=6	**15.5000**	**23.8779**	**0.0885 x 10^{-9}**	**5522177.3210**	**287.8333**	**3.6255 x10^{-6}**	**0.0100**
	1.1762	1.5964	0.0151 x 10^{-9}	558953.0661	24.5987	0.5794 x10^{-6}	0.5794
Ts+Water, n=6	**12.5000**	**22.4610**	**0.0713 x 10^{-9}**	**5644649.1870**	**210.3333**	**3.1625 x10^{-6}**	**0.0107**
	1.2042	0.3382	0.0099 x 10^{-9}	526703.6947	30.4014	0.4915 x10^{-6}	0.4915
Ts+EGCG, n=4	**14.2500**	**23.0131**	**0.0819 x 10^{-9}**	**5731911.8270**	**251.7500**	**3.1770 x10^{-6}**	**0.0101**
	0.8539	1.0839	0.0093 x 10^{-9}	449269.4504	13.7257	0.3604 x10^{-6}	0.3604

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FIGURES

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FIGURES

Figure 2.1 Pattern separation maze apparatus

The pattern separation apparatus was an eight arm radial maze. Visual cues were placed around the maze, and included posters, shelves and a curtain.

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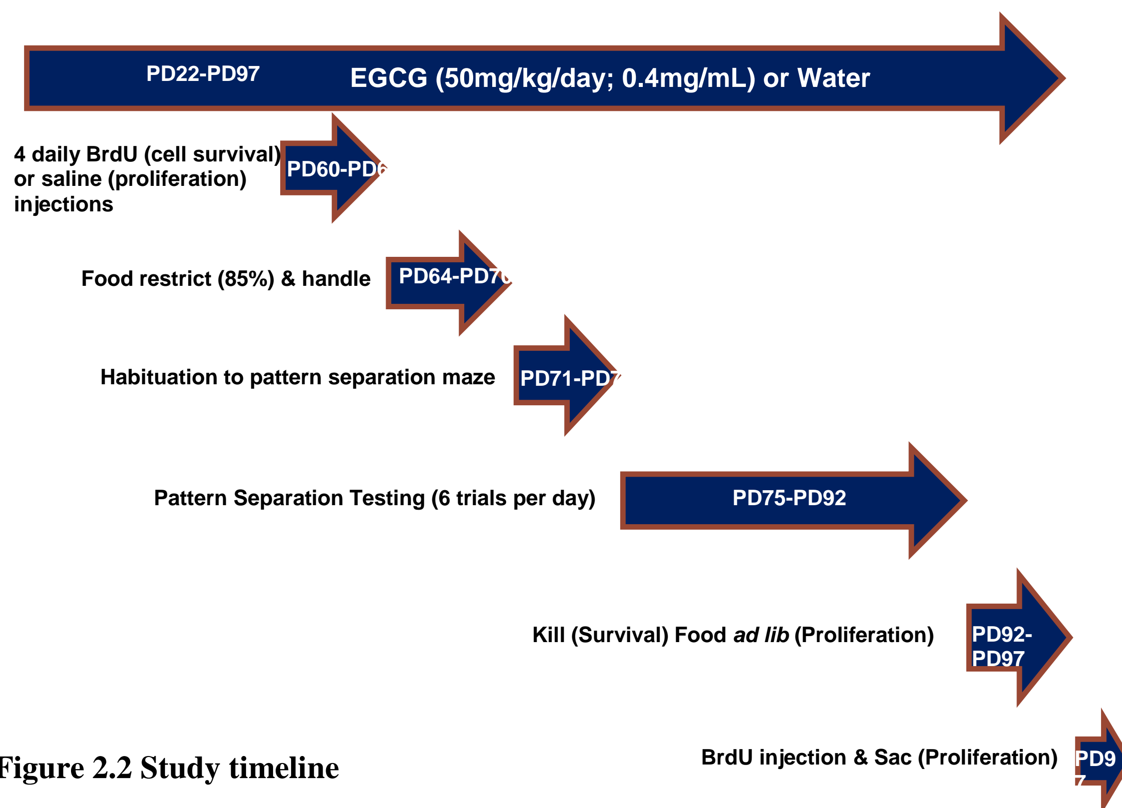

Figure 2.2 Study timeline

Treatment began on PD22 and continued throughout the duration of testing. On PD60, mice received daily injections of either saline or BrdU for four consecutive days. Animals were then food restricted and handled for seven days, followed by four days of habituation to the maze. The animals were then tested for eighteen consecutive days on the pattern separation task. After testing was completed, mice were either sacrificed (cell survival study) or given *ad lib* food for approximately five days (cell proliferation study). Then, the proliferation animals were sacrificed on PD97.

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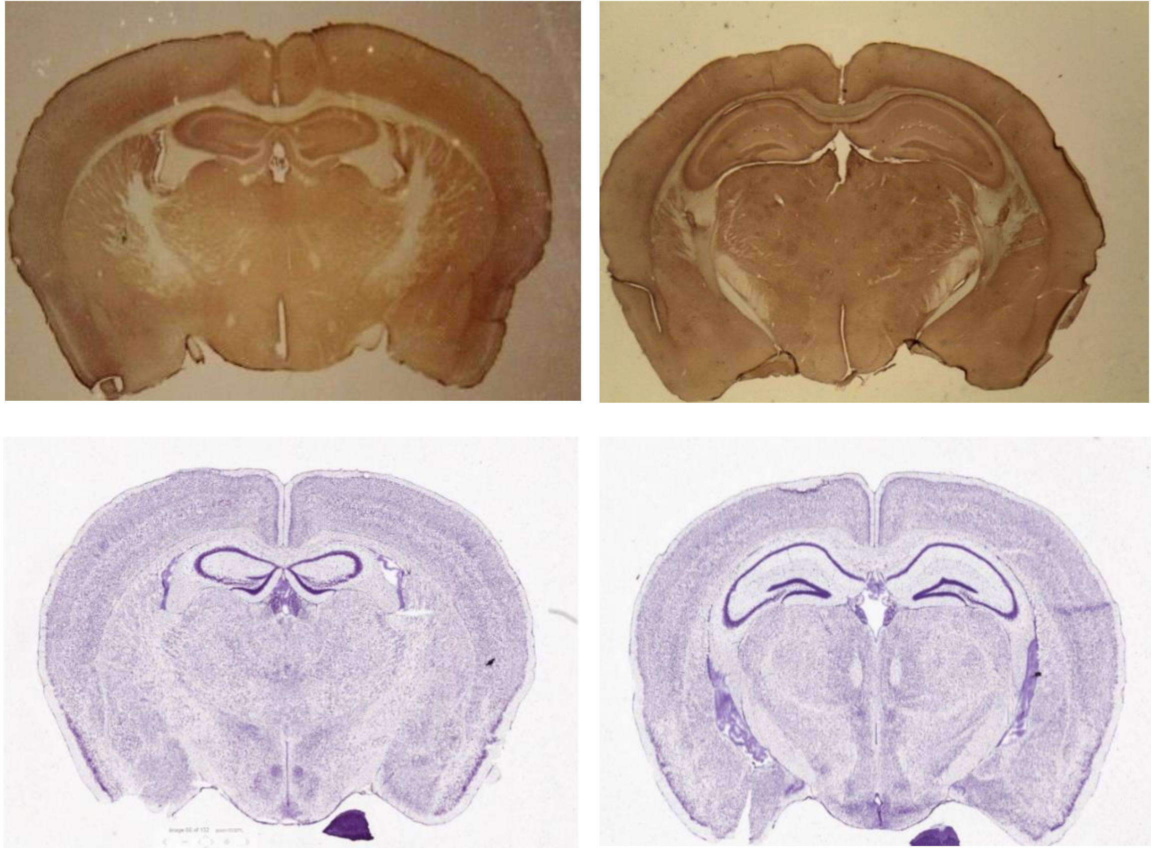

Figure 2. 3 Start and stop points for immunohistochemistry

The first row indicates the average starting (left; case #10631) and stopping (right; case #10525) points for immunohistochemistry processing. The second row are images from the Allen Mouse Brain Atlas that were used as reference points for the experimenter (starting at -1.22mm and ending at -2.18mm).

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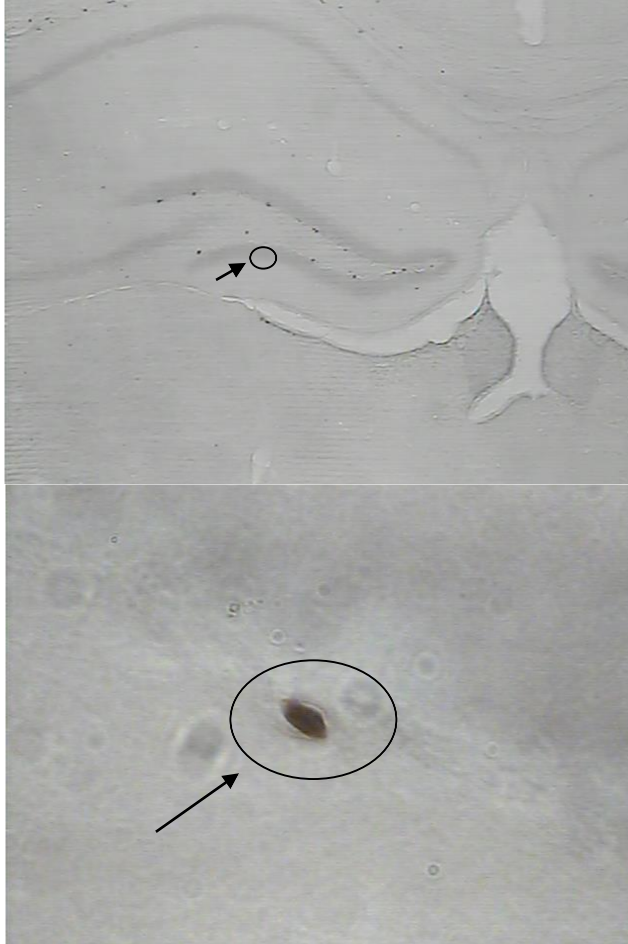

Figure 2.4 Quantification of cells in the dentate gyrus

The top photo represents BrdU stained cells of the left dentate gyrus at 40x. The circle indicates a BrdU stained cell that is magnified to 1000x in the bottom photo.

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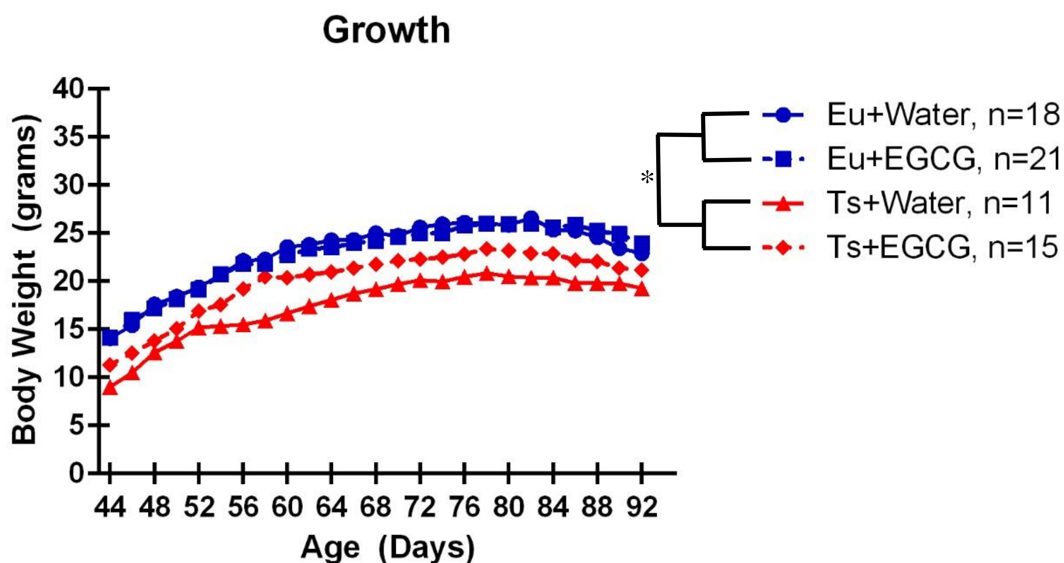

Figure 3.1 Ts65Dn growth

The growth of Ts65Dn (Ts) mice was plotted beginning on PD44, and continuing throughout testing. Euploid (Eu) mice weighed significantly more versus the trisomic (Ts) mice, but both groups increased their weight as a function of time. There was a main effect of day on growth [F(24,1464)= 272.602, p=<0.001], as well as a main effect of genotype (*) [F(1,61)=31.33, p=<0.001].

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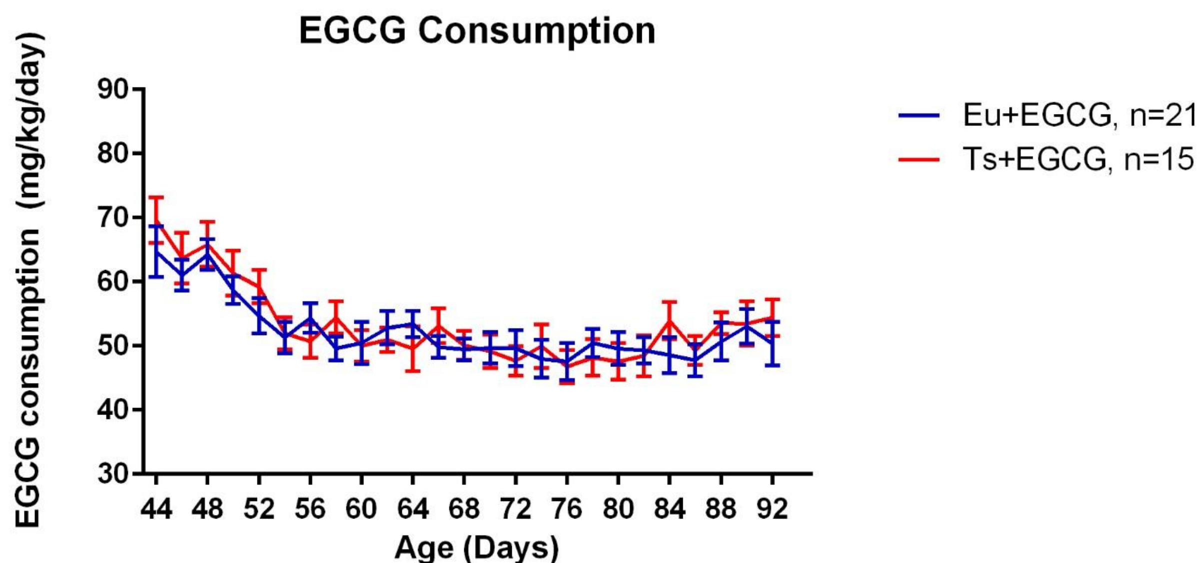

Figure 3.2 EGCG Consumption in Ts65Dn mice

There were no differences in the amount of EGCG consumption between the euploid (Eu) and trisomic (Ts) mice. Both groups decreased their EGCG consumption as a function of time. There was a main effect of day on EGCG consumption [F(25,1025)= 16.629, p=<0.001], but no genotype or interactive effects.

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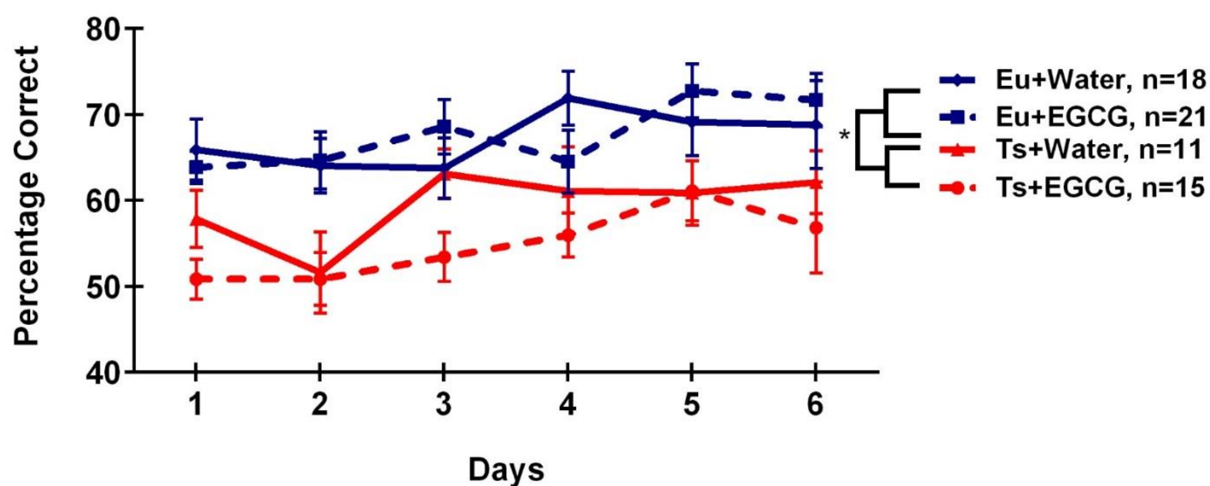

Figure 3.3 Ts65Dn acquisition performance on a pattern separation task

Euploid (Eu) mice displayed a higher overall percentage correct versus the trisomic (Ts) mice. However, both groups improved their performance over time. There were no effects of EGCG on improving performance. For acquisition, there was a main effect of day on performance [F(5,305)= 4.6, p=<0.001], as well as a main effect of genotype (*) [F(1,61)= 18.435, p<0.001], but no interactive effects.

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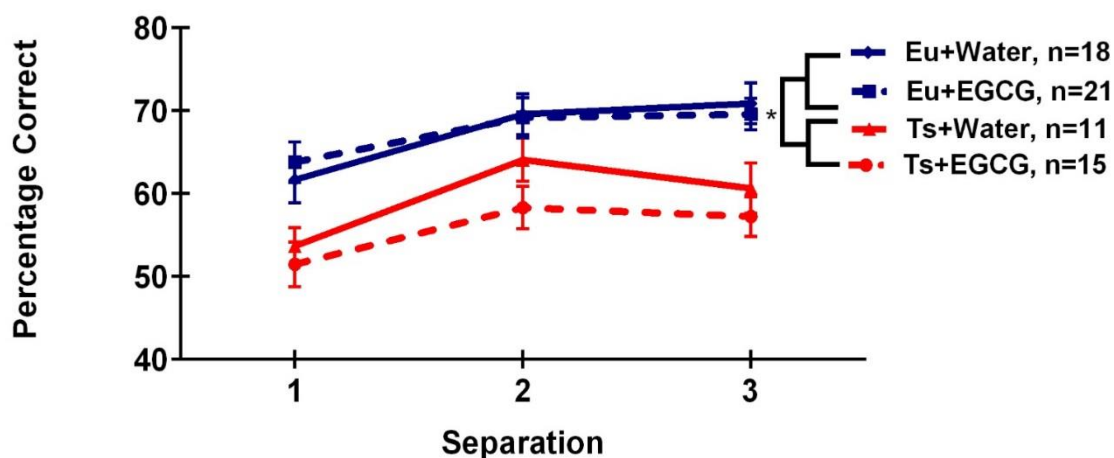

Figure 3.4 Ts65Dn separation performance

Euploid (Eu) mice displayed a higher percentage correct at each degree of separation versus the trisomic (Ts) mice. Both Eu and Ts mice increased their performance as a function of separation. There were no effects of EGCG improving performance. For separation performance, there was a main effect of separation [$F(2, 122) = 20.541$, $p<0.001$] as well as a main effect of genotype (*) [$F(1,61)=21.277$, $p<0.001$], but no interactive effects.

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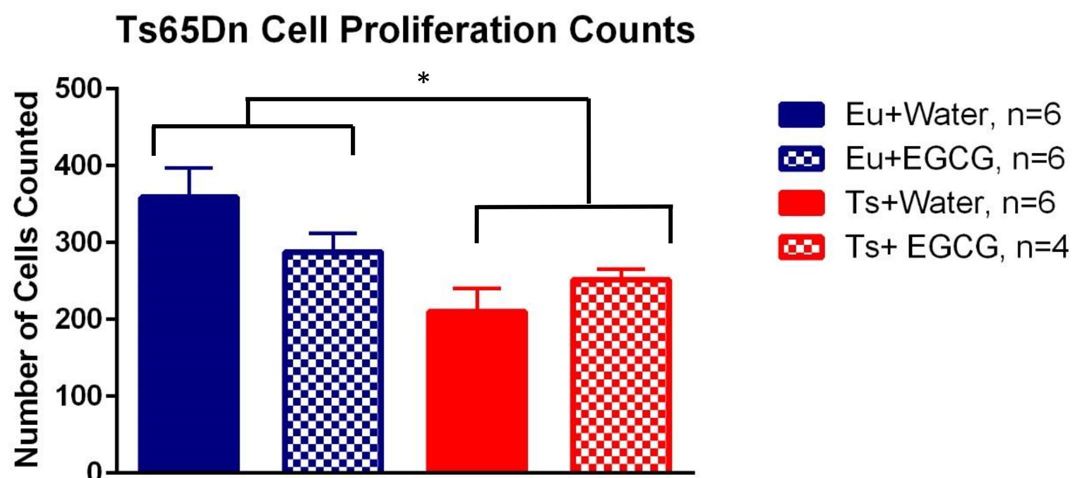

Figure 3.5 Ts65Dn cell proliferation counts

The Euploid (Eu) mice had a higher number of cell counts versus the trisomic (Ts) mice. There were no effects of EGCG on the number of cell counts. There was a main effect of genotype (*) on the total number of cells counted [$F(1,18)=8.83$, $p=0.008$].

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