QUANTIFYING DYRK1A DURING PERINATAL DEVELOPMENT IN THE HIPPOCAMPUS, CEREBRAL CORTEX, AND CEREBELLUM OF THE Ts65Dn MOUSE MODEL

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Laura Elizabeth Hawley

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STATEMENT OF COMMITTEE APPROVAL

Dr. Randall J. Roper, Chair
Department of Biology

Dr. Charles Goodlett
Department of Psychology

Dr. Teri Belecky-Adams
Department of Biology

Dr. Kathleen A. Marrs
Department of Biology

Dr. Tabitha M. Hardy
Department of Biology

Approved by:
Dr. James D. Marrs

For the little girl playing with minnows in the creek:

Stay curious

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ABSTRACT

The relationship between gene copy number and protein expression levels has not thoroughly been examined in humans or mouse models of Down syndrome (DS) in relationship to developmental changes in the trisomic brain. Found on human chromosome 21 (Hsa21) and triplicated in DS, Dual-specificity tyrosine-phosphorylated regulated kinase 1A (*DYRK1A)* has been linked in DS to neurological deficits by restricting cell growth and proliferation. Little information exists regarding DYRK1A during perinatal development and how its expression may lead to cognitive deficits, and none exists that explores the gene-to-protein relationship during these critical time periods. This study aims to 1) Quantify variable DYRK1A expression across development as a function of age, sex, and brain region in trisomic Ts65Dn mice compared to euploid counterparts and 2) establish that the spatiotemporal pattern of developmental DYRK1A in the brain is not influenced solely by gene copy number, and that reduction of *Dyrk1a* in euploid and trisomic mice does not result in a corresponding global reduction of DYRK1A expression. DYRK1A was quantified in three areas of the postnatal brain at seven ages using the Ts65Dn mouse, the most studied model of DS, and found that trisomic expression is significantly increased on postnatal day ([P]6), declining by the third week to near euploid levels. We also uncovered a sexual dimorphic expression of DYRK1A when comparing animals of different sexes within the same genotype. Data from *Dyrk1a* knockdown mice indicated that reducing only *Dyrk1a* in euploid and in otherwise trisomic animals yields highly variable levels of DYRK1A, dependent on sex and tissue type, supporting the non-intuitive relationship between gene dosage and protein expression. These data emphasize the need to understand the age-dependent regulation of antecedent conditions that are causing changes in *Dyrk1a* expression in the brain.

CHAPTER 1. INTRODUCTION

1.1 Down syndrome

Down syndrome (DS) is caused by the triplication of Hsa21 (TURPIN AND LEJEUNE 1965) and is characterized by transcriptional and translational alterations due to gene dosage imbalance, presenting as a collection of phenotypes affecting every system of the body. Trisomy 21 (Ts21) usually results from a non-disjunction of Hsa21 during meiosis, with older females exhibiting the highest risk, though it can also arise from a Robertsonian translocation (EPSTEIN 2014) or a triplication of part of Hsa21. Individuals with DS display developmental alterations in brain morphology including reductions in sizes of the prefrontal cortex, cerebellum, hippocampus, amygdala, and brain stem in newborns, and structural abnormalities such as reduced dendritic and axonal number and volume, and altered synaptic plasticity (AYLWARD *et al.* 1997; GUIDI *et al.* 2008; DIERSSEN 2012). Deficits in neurogenesis and cell cycle regulation are hypothesized to contribute to the developmental deficits in brain structures in individuals with DS (STAGNI *et al.* 2018). These cellular and structural abnormalities are thought to be the primary causes for impairment of learning, memory, and motor disabilities. Consequently, the brain is one of the most well studied organs in DS.

1.1.1 Down syndrome phenotypes

Classic DS phenotypes include moderate to severe intellectual disability (ID), with a median IQ around 50 with individual values ranging from 30 to 70 (CHAPMAN AND HESKETH 2000). DS results in reduction of brain volume (with the size of the hippocampus and cerebellum being particularly affected), weakened hippocampal dependent memory function, lower dendrite and axon number and volume, and altered synaptic plasticity (BECKER *et al.* 1991; MARTINEZ DE LAGRAN *et al.* 2004; COPPUS *et al.* 2006; LORENZI AND REEVES 2006; GUIDI *et al.* 2008).

Additionally, significant sex differences in developmental phenotypes occur in individuals with DS and in DS mouse models. Unlike neurotypical individuals, median life expectancy is greater in males with DS than females with DS, with increased cardiac deficits in females with DS (GLASSON *et al.* 2002). For individuals with DS, females typically perform in the above-average group on standardized measures of intelligence and adaptive function compared to males

(MARCHAL *et al.* 2016); eight-year-old females with DS had significantly higher developmental age than age matched males with DS (VAN GAMEREN-OOSTEROM *et al.* 2011). In adolescents, males exhibited behavioral problems more often than females (VAN GAMEREN-OOSTEROM *et al.* 2013). When assessed as adults, females show higher cognitive abilities compared to men, and the frequency of profound intellectual disability was twice as high in males as females (MAATTA *et al.* 2006). Males with DS were found to have higher post-mortem synaptic density of synaptophysin than females in areas of brain measured—except for the cerebellum in which females had a marginally higher density (DOWNES *et al.* 2008). These abundant sexual dimorphic expressions of DS traits have not thoroughly been studied in humans or in mouse models.

1.1.2 Ts65Dn mouse model

The Ts65Dn mouse strain is the most studied model of DS, used for testing treatments and therapies to improve the various deficits observed in DS (GARDINER 2015). It contains a segmentally trisomic chromosome, comprised of the distal end of mouse chromosome (Mmu)16 that contains genes orthologous to approximately 50% of genes found on Hsa21, and a centromere with the small proximal end of Mmu17 with ~35 protein coding genes. First characterized in 1990, this mouse displays similar phenotypes to individuals with DS, such as impaired spatial learning and memory, impaired motor coordination, reduced cerebellar volume, as well as impaired neurogenesis in proliferation and differentiation (DAVISSON *et al.* 1990; REEVES *et al.* 1995; BAXTER *et al.* 2000; LIU *et al.* 2015). Ts65Dn mice display spontaneous locomotor hyperactivity and impaired behavioral performance in Morris water maze and novel object recognition tasks (BELICHENKO *et al.* 2007; BELICHENKO *et al.* 2009). Research suggests that excessive inhibition of the dentate gyrus in this mouse model contributes to intellectual disabilities, and that GABA antagonists may be useful therapeutic agents for this disorder (FERNANDEZ *et al.* 2007). High resolution magnetic resonance imaging has demonstrated neuroanatomical parallels between the DS and the Ts65Dn cerebellum, showing that volume is severely reduced in both the internal granule and molecular layers of the cerebellum (BAXTER *et al.* 2000). More research characterizing behavior, development, and phenotype has been conducted in the Ts65Dn than other mouse models of DS and consequently more information is available to use as standard for further experiments. Thus, it was chosen as the most appropriate representation of DS for these experiments.

1.2 DYRK1A

Genes on Hsa21 have been identified as possible regulators of neurogenesis and long-term cognitive function including *Dual-specificity tyrosine-phosphorylated regulated kinase 1A* (*DYRK1A*). DYRK1A is a serine-threonine kinase which regulates many downstream proteins and transcription factors and plays a crucial role during brain development (BRANCHI *et al.* 2004; MARTINEZ DE LAGRAN *et al.* 2004; AHN *et al.* 2006; ARRON *et al.* 2006; CANZONETTA *et al.* 2008; YABUT *et al.* 2010; HAMMERLE *et al.* 2011; PARK AND CHUNG 2013). Homologous to *DYRK1A* in humans is *MNB* in fruit flies, in which mutations cause altered proliferation in the neuroepithelia primordia of the larval central nervous system (CNS), suggesting its role in the regulation of neural proliferation and neurogenesis (TEJEDOR AND HAMMERLE 2011). The relationship between cognitive development and DYRK1A has been recognized in several studies (BECKER AND SIPPL 2011; HAMMERLE *et al.* 2011; TEJEDOR AND HAMMERLE 2011; GUEDJ *et al.* 2012) and has been established as a key component of cell cycle regulation and differentiation.

Developmental differences in *DYRK1A* can affect the developing brain, possibly due to expression in the individual cell and the consequences of a developmental error in downstream cellular progenitors (ROPER AND REEVES 2006). Trisomic *DYRK1A* has been linked to aberrant brain development, brain pathology and impaired cognitive phenotypes in individuals with DS and DS mouse models (ARRON *et al.* 2006; DOWJAT *et al.* 2007; LIU *et al.* 2008; BECKER *et al.* 2014; DUCHON AND HERAULT 2016). *DYRK1A* has been implicated in promoting cell growth, differentiation and tissue patterning by its influence on the Hedgehog pathway (EHE *et al.* 2017) as well as in neuronal proliferation and cell cycle control in individuals with DS and DS mouse models (STAGNI *et al.* 2018).

Because of its involvement in abnormal trisomic neural development, DYRK1A is an attractive target for therapeutic measures to improve cognition in individuals with DS. Little information exists regarding the quantities of DYRK1A in the brain during prenatal and perinatal development, and no information exists regarding sex differences at these ages. Haploinsufficiency of *DYRK1A* caused by mutations, microdeletions or missense variations causes Autosomal Dominant Mental Retardation 7 (MRD7), characterized by prenatal microcephaly, intrauterine growth retardation, feeding problems, developmental delay and febrile seizures/epilepsy (MOLLER *et al.* 2008). Reduction of DYRK1A at times of typical expression could be detrimental to brain development, mimicking the traits of MRD7.

As the overexpression or underexpression of DYRK1A is detrimental to cognitive development, therapies designed to regulate DYRK1A levels in the brain must be carefully controlled. In DS, *DYRK1A* is triplicated, presenting on all three copies of Hsa21. A prevailing dogma of DS research is that when genes are upregulated by 50%, the corresponding protein expression will be upregulated accordingly.

1.3 Gene Dosage Theory

A basic explanation of the effect of trisomic genes is to assume that expression of all genes is upregulated according to gene copy number (1.5-fold) in every cell in the body throughout the lifespan of the individual. Many studies report a 1.5-fold increase of a particular trisomic gene of interest (BONNEY *et al.* 2015; BEACH *et al.* 2017). Although this simplistic trisomic dogma has been disproven in studies of RNA and protein and rejected by many who study the DS genotype-phenotype association, its influence still permeates the interpretation of how DS phenotypes are caused. The non-linear nature of gene-protein dosage correlations are beginning to gain traction but are not yet the prevailing theory among those investigating DS.

There are three prominent theories regarding the gene-protein relationship in DS research: the "few critical genes" hypothesis, the "gene dosage effect" hypothesis, and the "developmental instability hypothesis". The first predicts that a small number of the ~300 triplicated genes in DS cause the growth, developmental, and cognitive abnormalities characteristic of the condition. Researchers proposed the idea that there exists a "Down syndrome critical region", located on the proximal part of 21q22.3, containing genes that when overexpressed play a major role in the pathogenesis of DS (RAHMANI *et al.* 1990), although this theory has been disproven (OLSON *et al.* 2004). Identification of a small number of chromosome sequences commonly duplicated in DS patients led to this theory that duplication or disruption of these few genes will cause notable DS phenotypes.

The "gene dosage effect" hypothesis states that the phenotype is a direct result of the cumulative effect of the imbalance of the individual genes located on the triplicated chromosome or chromosome region. Essentially, that the phenotype results from the overexpression of specific chromosome 21 genes (PRITCHARD AND KOLA 1999). While the distinction between the two hypotheses may seem small, the first points to a small location on Hsa21 that is responsible for the

DS phenotype, while the other postulates that the triplication of any small number of genes on Hsa21 characterize the DS phenotype, but does not identify a region or specific genes.

The third, the "developmental instability" hypothesis, posits that it is the non-specific disturbance of chromosome balance which causes the disruption in homeostasis (PRITCHARD AND KOLA 1999) and that phenotypes arise from the result of cumulative effects of a large number of genes on many different chromosomes (POTIER *et al.* 2006). All three of these theories have been examined and tested with varying levels of evidence, some results of which are dependent on the mouse model being used.

Changes in copy number of the most dosage sensitive genes on a chromosome are insufficient to drive aneuploid proliferation defects (BONNEY *et al.* 2015). According to the "threshold hypothesis", also called "amplified developmental instability", the DS population can be said to be sensitized, meaning that the trisomic Hsa21 creates a basis of genetic instability for the organism, and small copy number variants on other chromosomes, which would not normally affect an individual, push the acceptable level of variation out of balance and atypical phenotypes present in a disease (LI *et al.* 2012). Aneuploid phenotypes may be the consequence of copy number changes of numerous genes, which have little phenotypic impact when mis-expressed on their own (BONNEY *et al.* 2015). The final phenotype of specific aneuploidies is affected not only by the quantity of genes present but also the alleles or sequence of the genes involved in the relevant chromosome(s) (VEITIA AND POTIER 2015; ROPER *et al.* 2020).

Quantification of both mRNA and protein levels in brains of individuals with DS from fetal to adult stages has shown that not all trisomic genes are dysregulated or expressed at 1.5-fold in all tissues at all times (MAO *et al.* 2003; MAO *et al.* 2005; LOCKSTONE *et al.* 2007; ESPOSITO *et al.* 2008). An approximate 1.5-fold increase in DYRK1A was found in homogenates from adult brains and ~1.5 fold increase in DYRK1A from brain homogenates from 15 month old Ts65Dn mice was observed (LIU *et al.* 2008). Quantification of DYRK1A protein levels in humans with DS ranging from 1-63 years of age found that DYRK1A levels were upregulated 1.3.-1.5 fold in frontal, temporal, occipital, and cerebellar cortices, and 1.7-1.8 fold in the white matter of the corpus callosum and cerebellum (DOWJAT *et al.* 2007). Pooled samples of cerebral cortex found no differences in DYRK1A expression between 1-3 year-old individuals with DS but significantly increased expression of DYRK1A in 10-30 and ≥ 40-year-old individuals with DS. DYRK1A

protein levels were also significantly increased 1.3-1.8 fold in homogenized brains of Ts65Dn as compared to control mice (DOWJAT *et al.* 2007). RNA from brains of 20 week old fetuses with DS showed 1.5 fold upregulation of DYRK1A and RNA from brains of adult Ts65Dn mice showed a 2.1 fold increase of *Dyrk1a* expression (GUIMERA *et al.* 1999).

Not all reports have found DYRK1A expression increased in Ts65Dn mice. The lack of systematic quantitative data for DYRK1A protein and/or mRNA levels in specific tissues makes it difficult to fully ascertain the role of DYRK1A in the development of a specific phenotype (CHOI *et al.* 2009; AHMED *et al.* 2012; STRINGER *et al.* 2017b). It is impossible to overstate the importance of understanding the relationship between dosage imbalance and protein expression in the developing DS brain. With conflicting information regarding critical genes, gene dosage-protein ratios, and sensitized backgrounds influencing trisomic genes, teasing out a map of expression and influence of proteins in DS is vital for untangling the complexities of how trisomy leads to the DS phenotype. It is hypothesized that therapies designed to reduce the influence of imbalanced proteins on transcription and translation should be administered during periods when that expression diverges most significantly from neurotypical development. Developing a map of expression levels at various time points in the brain creates a target for applying treatments (STRINGER *et al.* 2017b). Pharmacological treatments designed to reduce the effects of DYRK1A, in line with the dosage theory of the gene, could be beneficial to trisomic neural development. This study seeks to pinpoint times where this protein of interest is differently expressed during perinatal brain development with the expectation of inhibition for normalization. Inhibition in the incorrect location or time could detrimentally alter the protein concentration in either direction.

1.4 Sexual dimorphism in DS

Across all published preclinical therapeutic studies using DS mouse models, the majority either failed to include sex as a factor or were underpowered to detect interactive effects of sex and genotype (BLOCK *et al.* 2015; GARDINER 2015). Sex had a significant modulatory role in behavioral changes in environmental enrichment of Ts65Dn mice (MARTINEZ-CUE *et al.* 2002). Female but not male Ts65Dn mice showed higher anxiety measures and altered defensive behaviors than control mice (MARTINEZ-CUE *et al.* 2006). The Dp(10)1Yey mouse model, which contains a small number of Hsa21 orthologs in three copies, as compared to control mice showed

significant differences in expression of neuroplasticity-related proteins depending on brain region, with the differences in female Dp(10)1Yey mice most apparent in the cerebellum, whereas the differences in males were most apparent in the hippocampus (BLOCK *et al.* 2015). This research strives to supply evidence that differences between male and female Ts65Dn mice are apparent early in development in the expression of DYRK1A and are present during various timepoints throughout perinatal development. To support other experiments involving DS mouse models, this study aims to identify significant differences between male and female DYRK1A expression in the three brain tissues examined, underlining the necessity to separately investigate both male and female samples. These findings are expected to uncover sexually dimorphic expression of DYRK1A evident during the early stages of brain development, persisting at least until postnatal week three.

1.5 Treatment timelines in DS

As a model organism, mice are integral in studying human growth and have a predictable timeline of development than can be translated across species. Work done by Clancy, Finlay, and Darlington has been instrumental in developing inter-species comparisons of predicted pre- and post-natal dates of neural development (CLANCY *et al.* 2001). In humans, accurate detection of DS can be performed at various points during the pregnancy. Non-invasive prenatal testing (NIPT) can be performed around week 10 of gestation when the fetal fraction (proportion of cell free DNA from the fetus) reaches approximately 4%. A maternal blood sample is taken and analyzed for fetal cell-free DNA from the fetus and used as a pre-screening test which can indicate if further diagnostic testing should be conducted (HOLZGREVE AND HAHN 2000). Non-diagnostic testing can be done by the Triple or Quadruple Screen tests, usually performed around week 16-18 of human gestation. These screenings test for levels of alpha-fetoprotein, human chorionic gonadotropin, and estriol in all three and inhibin A in the latter. Testing has become routine in pregnancies of older mothers, and women with a history miscarriages or previous children with birth defects. Abnormal levels of these markers can indicate abnormalities that may merit further testing, which can confirm DS by techniques such as chorionic villus sampling, amniocentesis, usually around the 20th week of gestation.

The linked regularity of evolutionary and developmental biology shows that the timing and sequence of early events in brain development are conserved across mammals (FINLAY AND

DARLINGTON 1995). While the timing and sequence may be preserved, comparing overall brain development between species does not take into account disparities in relative sizes of mature primate limbic and cortical regions which indicate that these regions develop on a somewhat different timetable in primates (FINLAY *et al.* 2001). One drawback for morphological neurodevelopmental comparisons is that descriptions are based on the features in a "standard" embryo, and the statistical variability of the "standard" is unknown (CLANCY *et al.* 2007). Using the tool, "Translating Time", a database of predictable, "anchor" events has been mapped across species, allowing for a better comparison of brain development parallels between mice and humans (CLANCY *et al.* 2007). Mice present a unique opportunity to study an earlier developed brain outside of the womb, as their gestation period is shorter, and birth occurs when the neonatal brain has not developed as much as that of newborn humans. This allows a prospect to conduct research into how therapies and interventions may affect human development while still in the womb and how earlier interventions may rescue DS phenotypes later in life (OTIS AND BRENT 1954).

Over 20 different potential treatments have been evaluated in preclinical mouse model studies, usually targeting either neurotransmitter systems or aberrant neural pathways and evaluating neurobehavioral phenotypes—and usually in just one sex—with many reporting at least transient improvement (DE LA TORRE AND DIERSSEN 2012; GARDINER 2015). Translation of those therapies to humans with Ts21 has been largely unsuccessful, likely because mechanisms responsible for abnormal developmental trisomic trajectories and sex-specific processes in DS were not modeled in trisomic mice. We recently hypothesized that the effects of trisomic DYRK1A on DS phenotypes would be based upon its overexpression at temporally and spatially specific times (STRINGER *et al.* 2017b). Because neuronal phenotypes are found early in development, it has been hypothesized that intervention during development may be most effective to rescue DS-related brain deficiencies (NAKANO-KOBAYASHI *et al.* 2017).

The neonatal and perinatal brain is highly plastic and can be more easily influenced than a mature and developed brain. Proliferation and maturation take place early in neuronal development, and after differentiation there is limited opportunity to increase the number of new neurons available for tissues and structures. Neonatal therapies may largely shape hippocampal and cerebellar development, and prenatal therapies may have by far the largest impact, by potentially affecting development of the whole brain (STAGNI *et al.* 2015). Therapies administered after the brain has slowed or stopped development may have a more limited effect when attempting

to influence structures and functions that are normally solidified early in life. Late therapies are unlikely to exert drastic changes in the brain (STAGNI *et al.* 2015). Alterations in a differentiated cell may have little to do with the expression of trisomic genes in that cell but could lead to consequences of a developmental error in downstream cellular progenitors (ROPER AND REEVES 2006). The association between trisomic genes and DS phenotypes is complex and likely involves a number of different genes or genetic regions that are associated with DS phenotypes (LYLE *et al.* 2004; KORBEL *et al.* 2009) as well as a temporal basis underlying the altered expression and consequence of trisomic genes (ROPER AND REEVES 2006).

1.6 Thesis hypothesis

This study strives to examine two distinct hypotheses related to DYRK1A expression. First, we hypothesize that DYRK1A expression between euploid and trisomic Ts65Dn mice varies across perinatal development from P6-P24 as a function of age and sex, and in three brain regions (hippocampus, cerebral cortex, and cerebellum). Second, we hypothesize that the spatiotemporal pattern of DYRK1A in three brain regions (hippocampus, cerebral cortex, and cerebellum) of P15 Ts65Dn mice is not influenced solely by gene copy number, and that reduction of *Dyrk1a* in euploid and trisomic mice will not result in a corresponding global reduction of DYRK1A expression in these brain regions. Using the results from this research, we intend to map DYRK1A expression during this perinatal period and accurately predict timepoints where it may be targeted for inhibition with the intent of rescuing aberrant phenotypes.

CHAPTER 2. MATERIALS AND METHODS

2.1 Generation of animals

B6EiC3Sn a/A-Ts(17^{16})65Dn/J (Ts65Dn) females (stock number 001924, the Jackson Laboratory, Bar Harbor, ME), with approximately 50% B6 and 50% C3H background, containing a small trisomic marker chromosome (REEVES *et al.* 1995), were bred to B6C3F1 males to maintain the Ts65Dn colony. Male trisomic Ts65Dn animals are subfertile, necessitating the use of trisomic female offspring from the Ts65Dn × B6C3 matings and continually obtaining (approximately every six months) new trisomic females from the Jackson Laboratory to maintain the colony. Female trisomic Ts65Dn were bred to B6C3 males that carried an inactive *Dyrk1a*$^{fl/fl}$ allele (B6C3.*Dyrk1a*$^{fl/fl}$(F6-F12) mice) (Northwestern University, Evanston, IL) (THOMPSON *et al.* 2015) generating male and female euploid and trisomic mice that had one floxed *Dyrk1a* allele (*Dyrk1a*$^{fl/-}$). The floxed allele was bred into the colony for use in a different study and remained inactive in all animals due to the lack of an appropriate CRE driver necessary to activate the loxP sites. Both male and female trisomic and euploid mice were used in this study. Approximately 23 litters over the course of two years were used to derive the animals for the DYRK1A spatiotemporal development study, with an average of 6.3 pups per litter.

Dyrk1a heterozygous mutant mice (*Dyrk1a*$^{+/-}$) (FOTAKI *et al.* 2002) were backcrossed to B6C3F1 mice for seven generations to parallel the background of Ts65Dn mice. Ts65Dn females were then bred to *Dyrk1a*$^{+/-}$ males to generate four groups of mice used in the study:

- Ts65Dn (Ts +/+/+) with three functional copies of *Dyrk1a*
- Ts65Dn , *Dyrk1a*$^{+/-}$ (Ts +/+/-) with two functional copies of *Dyrk1a*
- Euploid (Eu +/+) with two functional copies of *Dyrk1a*
- Euploid , *Dyrk1a*$^{+/-}$ (Eu +/-) with one functional copy of *Dyrk1a*

All animals were bred and housed in rooms with a standard 12:12 light:dark cycle with lights on at 7:00am in the secure AAALAC-accredited Science Animal Resource Center facility in the IUPUI School of Science. Experiments with animals were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and received prior approval from the IACUC committee at IUPUI (SC255R and SC298R).

2.2 PCR

Offspring were genotyped for the Ts65Dn trisomic marker using the breakpoint PCR (REINHOLDT *et al.* 2011) using forward 5'-GTGGCAAGAGACTCAAATTCAAC-3' (Chromosome 17) and reverse 5'-TGGCTTATTATTATCAGGGCATTT-3' (Chromosome 16) primers to amplify a ~275 bp product at the translocation point on 17^{16} murine chromosome and a positive control primer set forward 5'-TGTCTGAAGGGCAATGACTG-3' and reverse 5'-GCTGATCCGTGGCATCTATT-3' that amplifies a 544 bp product. The final PCR mix contained MyTaq (Bioline, Taunton, MA) reaction buffer (1x final), MyTaq polymerase (2.5U/uL final), water, the two sets of primers (0.4uM final) (Life Technologies), and DNA (100ng/rxn). The PCR cycling conditions were set to 94°C for 2 minutes, 94°C for 45 seconds, 58°C for 45 seconds and 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. Samples were separated on a 1.5% agarose gel.

Dyrk1a $^{fl/fl}$/*Dyrk1a* $^{fl/-}$ was determined by PCR on offspring from the appropriate crosses by amplification of a section of the floxed portion of the *D*yrk1a gene and exon 5. Primer sets forward 5'-ATTACCTGGAGAAGAGGGAAG-3' and reverse 5'-TTCTTATGACTGGAATCGCC-3' to amplify a product of 603bp (THOMPSON *et al.* 2015) or 5'-TACCTGGAGAAGAGGGCAAG-3' and 5'-GGCATAACTTGCATACAGTGG-3' to amplify a product of 232bp were utilized to identify this location and confirm floxing of one or both alleles. The final PCR mix for both sets of primers contained MyTaq reaction buffer (1x final), MyTaq polymerase (2.5U/ul final) (Bioline Taunton, MA), water, the two sets of primers (0.4µM final) (Life Technologies), and DNA (100ng/rxn). The PCR cycling conditions were set to 95°C for 3 minutes, 95°C for 20 seconds, 60°C for 20 seconds and 72°C for 15 seconds. The denaturation, annealing and elongation steps were repeated for 35 cycles and were followed by a final elongation step for 1 minute at 72°C, then held for 4°C for 5 minutes. Samples were separated on a 1.5% agarose gel.

Dyrk1a copy number was determined by PCR on offspring from crosses of mice with one wild type copy and one inactivated copy of *Dyrk1a* (*Dyrk1a*$^{+/-}$). The inactivated *Dyrk1a* allele was confirmed using two primers forward 5'-ATTCGCAGCGCATCGCCTTCTATCGCC-3' and reverse 5'-CGTGATGAGCCCTTACCTATG-3' to amplify a product of 287bp (FOTAKI *et al.* 2002). The final PCR mix for both sets of primers contained MyTaq reaction buffer (1x final), MyTaq polymerase (2.5U/ul final) (Bioline Taunton, MA), water, the two sets of primers (0.5µM

final) (Life Technologies), and DNA (100ng/rxn). The PCR cycling conditions were set to 94°C for 2 minutes, 94°C for 15 seconds, 55°C for 15 seconds and 72°C for 10 seconds. The denaturation, annealing and elongation steps were repeated for 35 cycles and were followed by a final elongation step for 1 minute at 72°C, then held for 4°C for 5 minutes. Samples were separated on a 1.5% agarose gel.

Sex was genetically determined by PCR on offspring used in this study (MCFARLANE *et al.* 2013) when visualization could not be performed due to animal age or ambiguous observation. Amplification of *Sly* and *Xly* genes, which reside on the Y and X chromosome, respectively, was accomplished using forward 5'-GATGATTTGAGTGGAAATGTGAGGTA-3' and reverse 5'-CTTATGTTTATAGGCATGCACCATGTA-3' primers. Reactions were performed with a final volume of 25µL containing 1x KCl buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.2µM primers (Life Technologies), 19.05 µL H_20, 1 unit Taq polymerase (Bioline) and 100ng DNA using the following PCR specifications: denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 57°C for 30 s, 55°C for 30 s, and 72°C for 30 s, ending with a 72°C final elongation step for 5 min and 4°C for 5 min. Products were visualized via electrophoresis on a 2% agarose gel. Amplification using the aforementioned primers resulted in a single 280-bp amplicon for males, while female DNA yielded two amplicons of 660-, and 440-bp.

2.3 Selection of animals

Cages were checked daily for births, and postnatal day 6 (P6), P12, P15, P18, P24 male and female euploid and trisomic offspring from (Ts65Dn x B6C3.*Dyrk1a*$^{fl/fl}$) matings were removed from their home cage and euthanized with isoflurane followed by cervical dislocation. P15 male and female euploid and trisomic offspring from (Ts65Dn x *Dyrk1a*$^{+/-}$) matings were removed from their home cage and euthanized with isoflurane followed by cervical dislocation. Only litters containing at least one trisomic mouse were used in all studies to ensure genetic diversity among littermates.

2.4 Dissection and tissue preservation

The hippocampus, cerebral cortex, and cerebellum were rapidly dissected from each mouse after euthanasia, stored in 1.5mL microcentrifuge tubes, separately snap frozen in liquid nitrogen and stored at -80ºC.

2.5 Protein isolation and quantification

Tissue samples were removed from a -80º C freezer, homogenized in RIPA buffer with protease inhibitor [10mM Phosphate Buffer (pH 7.4), 10% Glycerol, 1% NP-40, 0.1% SDS, 4mM EDTA, 0.15M NaCl, 1x Protease inhibitor cocktail (Thermo Scientific)], centrifuged at 10,000 rpm for 10 minutes at 4º C, supernatant slowly extracted (to insure no part of pellet was disturbed), and samples were stored at -80º C. Protein samples were quantified using the Bradford assay, a colorimetric test based on an absorbance shift of a reaction between protein and Coomassie Brilliant Blue G-250. Bradford reagent was made by dissolving 100mg Coomassie Brilliant Blue G-250 (Thermo Fisher) in 100mL of 100% ethanol in a 1L glass bottle. Once dissolved, 80mL of 85% phosphoric acid was added, and then milliQ water added to reach 1L.

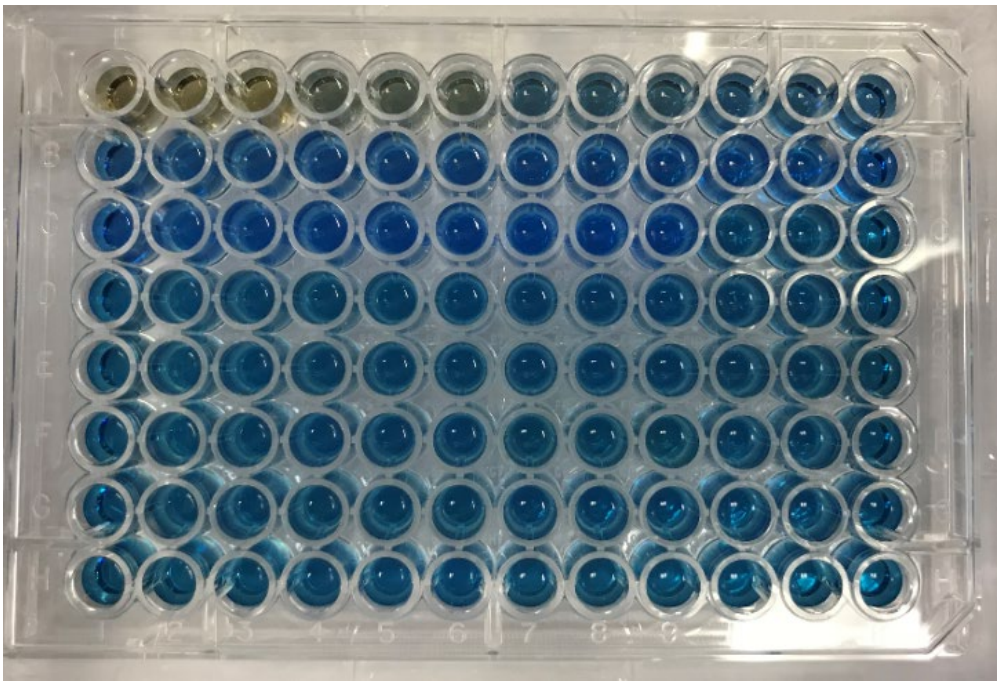

Figure 1 Representative Bradford Assay. The Bradford colorimetric test: Bovine serum albumin concentrations used as controls ranging from 0ug to 100ug, easily identified by the range of intensity in the presence of the Coomassie reagent. All controls and samples run in triplicate

Reagent was filtered to remove any undissolved particulate. 10uL of each protein sample was pipetted to an autoclaved micropipette tube and a pre-determined amount of bovine serum albumin was used as a linear control line to compare the absorbance of the unknown samples. One mL of Bradford reagent was added to each microtube and allowed to incubate for 45-90 minutes before 200uL of sample was pipetted to a 96 well plate for reading in a spectrophotometer. Each sample and control was run in triplicate to ensure accuracy of the reading (BRADFORD 1976).

2.6 DYRK1A protein quantification by Western blot

Isolated protein lysates (20µg) from whole brain (E18.5) or the three brain regions (P6, P12, P15, P18, and P24) were resolved electrophoretically on polyacrylamide gels (Bolt 4-12% Bis Tris Plus Gels), and then transferred to PVDF membranes. Membranes were blocked in 5% milk in Tris Buffered Saline with 0.1% Tween 20 (TBS-T), then incubated overnight at 4°C in primary antibodies diluted in 5% milk-TBS-T (mouse anti-DYRK1A antibody, 1:500 (MO1 Clone 7D10A Abnova)). After 3 washes in TBS-T for 10 minutes each, membranes were labeled with donkey anti-mouse IgG Alexa Fluor 790 secondary antibodies (1:10,000, Jackson Immunoresearch). Fluorescence was detected using a LI-COR CLx Imager, and DYRK1A was quantified using Image Studio. After the membrane was scanned, it was stained with Coomassie blue dye (Coomassie Brilliant Blue R-250 [Thermo Fisher] 0.5mg, 250mL 100% ethanol, 50mL acetic acid, 200mL H_2O) for 2 minutes with gentle agitation. The dye was then drained, and the membrane was gently agitated in a destain solution (500mL 100% ethanol, 400mL H_20, 100mL acetic acid) for ~10 minutes. Then, the destain was drained and the membrane sat in water overnight. After 24 hours, the membrane was scanned using a HP Scanner, and the total protein (measured from ~90kDa to ~40kDa) was quantified in Image J.

Multiple bands were observed at ~90 kDa, the approximate weight of DYRK1A. The first band directly below ~90kDa is likely the first and second isoforms of DYRK1A, which are reported to have a weight of ~86 and ~84kDa. Subsequent isoforms of DYRK1A weigh ~66, 60, 59kDa. The remaining observable faint bands do not appear to match the weights of the DYRK1A isoforms; thus, these residual faint bands are likely due to several methodological variables, including the type of gels and membranes used (Abnova, personal communication). The three

isoforms of DYRK1A were measured independently (labeled as “top”, “middle”, and “bottom”) and compared to total protein levels to determine if these levels were consistent across all animals. No statistical differences were found when measuring individual bands compared to all bands together. To maintain a consistent measurement of all DYRK1A all bands were boxed together and a single fluorescence level from that box was used.

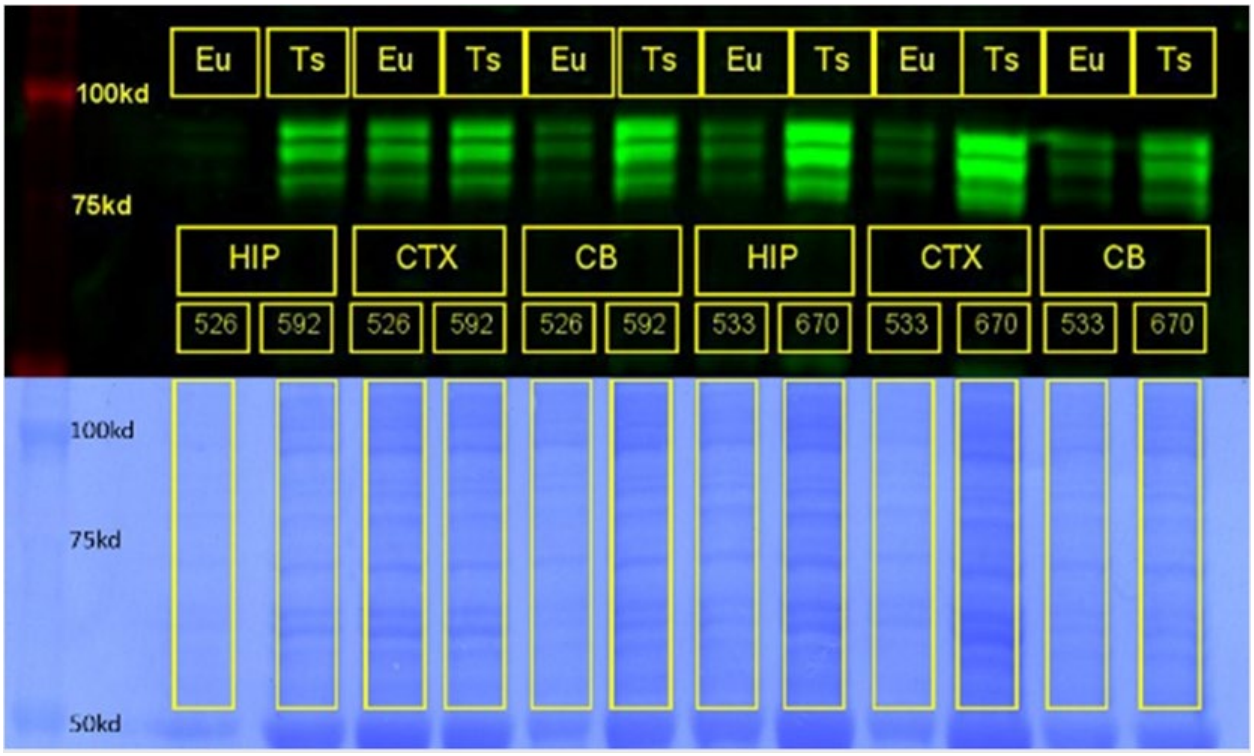

Figure 2 Representative Western Blot Image. Western blot analysis was performed on the three brain regions tested (hippocampus, cerebral cortex, and cerebellum) with trisomic and euploid analyzed on the same blot. Fluorescence differences were detectible between euploid and trisomic animals in most cases with the naked eye and confirmed by chemiluminescence imaging.

When comparing trisomic and euploid brains during development, DYRK1A protein levels were quantified in the prenatal, perinatal, and adolescent mice (P6, P12, P15, P18 and P24). Each membrane contained two controls (Euploid) and two Ts65Dn mice (Trisomic), with all three brain regions from each mouse (total of 12 samples included on each gel). Each normalized value was expressed as a ratio relative to the mean intensity value of the same region for the two controls that were run in the same blot.

When comparing genetic knockdown trisomic and euploid animals, a homogenate brain tissue from each of three regions (hippocampus, cerebral cortex, and cerebellum) from five euploid male animals were combined and protein isolated using the above-mentioned method. This homogenate was run in triplicate on each genetic knockdown blot and used as a standard to compare fold changes between blots. Each blot was run with the three brain regions of one of each of the four genotypes (for a total of 12 samples) plus the triplicate of homogenate. Males and females were run on separate blots. DYRK1A was quantified as a ratio to the standard protein level obtained from the homogenate. First, the optical density of DYRK1A was quantified using a LICOR Odyssey machine with Image Studio software. Using Coomassie blue, the membrane was then stained for total protein, and a ratio of the optical density to total protein established (WELINDER AND EKBLAD 2011). Using an average of the three homogenate protein lanes, individual tissue ratios were normalized to this homogenate ratio. Normalization to this homogenate provided a way to visualize both the trisomic and euploid variation of expression.

2.7 Statistical analysis

For the Western blot analysis, the normalized ratios were first analyzed separately for a given age and sex using all subjects for which data were available from all three brain regions, using 2-way mixed ANOVAs with genotype as a between-group factor and brain region as a within-subjects measure, with Greenhouse-Geisser corrections when assumptions of sphericity were violated. Of the 490 mice contributing to the Western blots in this study, 12 had data missing from one brain region due to technical problems in the Western blot and were excluded from these ANOVAs. At those ages for which the 2-way mixed ANOVA indicated a significant main or interactive effect of genotype for a given sex, follow up post hoc tests within each region (using all available cases for a given age, sex, and region) were used to identify significant increases in DYRK1A in the trisomic group relative to the euploid using directional one-tailed independent groups t-tests, justified by the a priori expectation of 1.5X greater DYRK1A expression in the trisomic mice. When heteroscedasticity was present based on Levene's test of equality of group variances, Welch's t-test was used for the comparison. Group numbers are shown the tables below.

Additional analyses to compare the extent of DYRK1A overexpression across ages and between sexes were performed using Z-score transformations for l. Standard scores (z-scores) were generated for the normalized ratio of each trisomic brain region measure relative to the mean and

standard deviation of the normalized ratios of the respective euploid control group of the same age, sex, and brain region. Individual Z-scores were calculated as follows:

$$Z_{[\text{Ts subject}]} = (\text{Normalized Ratio}_{[\text{Ts subject}]} - \text{Mean}_{[\text{euploid control group}]}) \ / \ \text{StdDev}_{[\text{euploid control group}]}$$

These Z-scores relative to respective controls provide measures of the extent of deviation of the trisomic values from the control mean in terms of standard units, with increasing scores above 0 indicating increasing overexpression, allowing quantitative comparisons of the extent of DYRK1A overexpression across age, sex and brain region. The Z-scores for the trisomic groups were first analyzed with a 3-way ANOVA with Age and Sex as grouping factors and region as a within-subjects factor. Significant main or interactive effects were followed by lower-order ANOVAs for simple main effects done separately for males and females for each brain region, with Age as a grouping factor, followed by Fisher's LSD tests for *post hoc* comparisons (alpha = 0.05). All data were analyzed using SPSS Version 25.

Table 1 Hippocampus Numbers

Hippocampus	Male Euploid	Male Trisomic	Female Euploid	Female Trisomic
P6	n = 8	n = 8	n = 8	n = 7
P12	n = 9	n = 10	n = 7	n = 8
P15	n = 7	n = 6	n = 8	n = 9
P18	n = 8	n = 8	n = 5	n = 5
P24	n = 7	n = 7	n = 6	n = 6

Table 2 Cerebral Cortex Numbers

Cerebral Cortex	Male Euploid	Male Trisomic	Female Euploid	Female Trisomic
P6	n = 6	n = 7	n = 6	n = 7
P12	n = 9	n = 10	n = 8	n = 8
P15	n = 10	n = 8	n = 8	n = 9
P18	n = 8	n = 8	n = 5	n = 5
P24	n = 7	n = 8	n = 6	n = 6

Table 3 Cerebellum Numbers

Cerebellum	Male Euploid	Male Trisomic	Female Euploid	Female Trisomic
P6	n = 7	n = 8	n = 8	n = 8
P12	n = 9	n = 10	n = 7	n = 8
P15	n = 8	n = 8	n = 8	n = 9
P18	n = 8	n = 8	n = 6	n = 5
P24	n = 7	n = 7	n = 6	n = 6

CHAPTER 3. SPATIOTEMPORAL DYRK1A LEVELS DURING PERINATAL BRAIN DEVELOPMENT

3.1 Male DYRK1A Expression Levels Through Early Perinatal Development

In the Ts65Dn male mice expression of DYRK1A was significantly higher on P6 than euploid controls in hippocampus (~5-fold), cerebral cortex (~2.8-fold), and cerebellum (~5-fold), but the differences between genotypes diminished substantially at subsequent ages (P12 and beyond), with trisomic mice typically showing 1-2 fold increases after P6 (see Fig. 3, 4, and 5). The genotype × region ANOVA for P6 males confirmed a significant main effect of genotype [$F(1,11)=18.39$, $p=0.001$], and the elevations in the trisomic groups were significant for all three regions [hippocampus ($p=0.0095$), cerebral cortex ($p=0.0085$), and cerebellum ($p=0.0015$)].

At P12 in males there was a significant difference in expression in the hippocampus and cerebellum, though a much smaller effect than at P6 (less than 2-fold). At P15 the main effect of genotype was again significant [$F(1,9)=38.74$, $p<0.001$], with significantly greater expression of DYRK1A protein in the Ts65Dn males in the hippocampus (~1.5-fold, $p=0.006$), cerebral cortex (~1.75-fold, $p=0.002$) and cerebellum (~1.9-fold, $p=0.004$). At P18 and P24, no significant group differences were present in the males in any brain region.

The trisomic male expression of DYRK1A is highly overexpressed at P6, falls closer to euploid levels at P12, rises again at P15 (though not as high as P6), fails to maintain a significant difference from euploid at P18, and falls again to near euploid levels at P24. This bi-phasic overexpression presents a window of treatment opportunity at P15 where DYRK1A is heightened in all three brain regions and can be targeted for therapeutic treatment and inhibition.

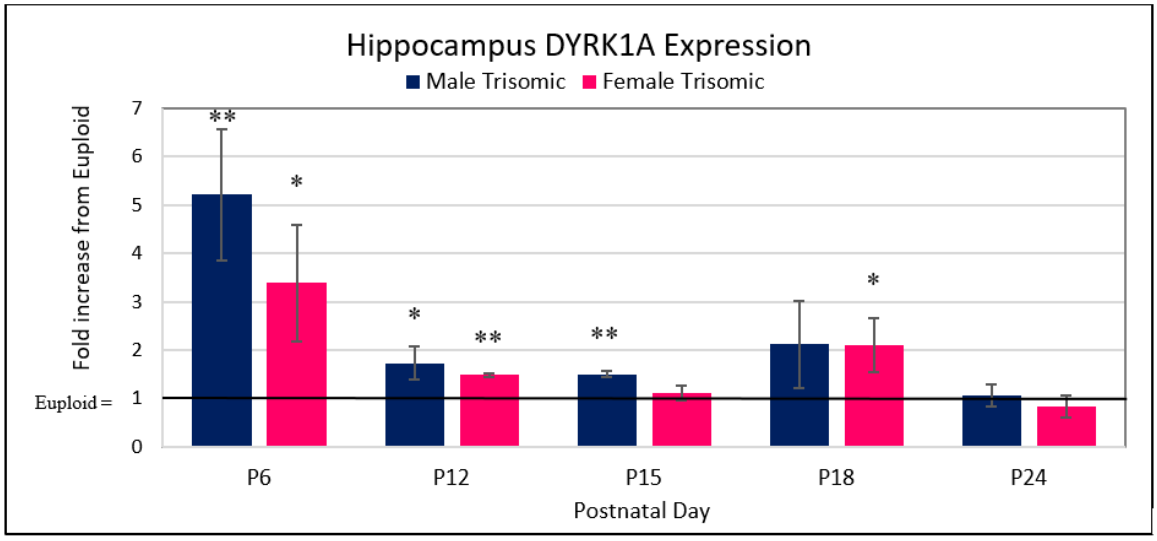

Figure 3 Hippocampus DYRK1A Expression. Typical trisomic expression in the three brain regions tested detected significantly higher DYRK1A levels at P6 than at later ages. The male trisomic P6 hippocampus had the second highest fold difference of any region analyzed. Females experience a biphasic rise in expression at P18 rather than at P15, as seen in males. The hippocampus displays typical patterning of expression also seen in the cerebral cortex and cerebellum of female Ts65Dn trisomic mice.

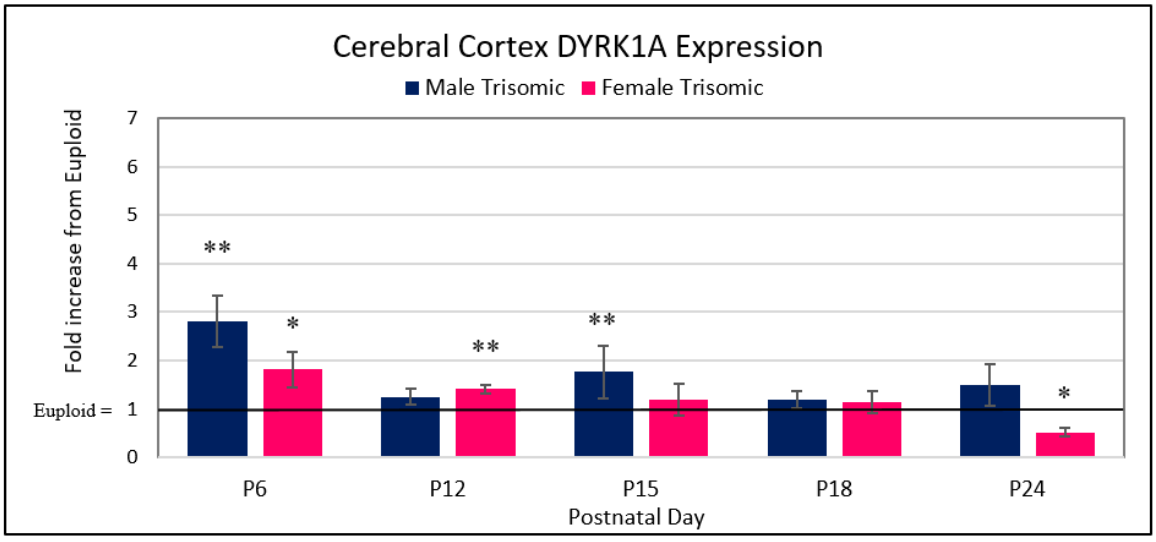

Figure 4 Cerebral Cortex DYRK1A Expression. Only P6 and P15 displayed significant differences between trisomic and euploid DYRK1A expression in the male cerebral cortex. Mimicking male expression, the female cerebral cortex has the lowest fold-increase expression of all areas examined, even falling to below euploid levels at P24.

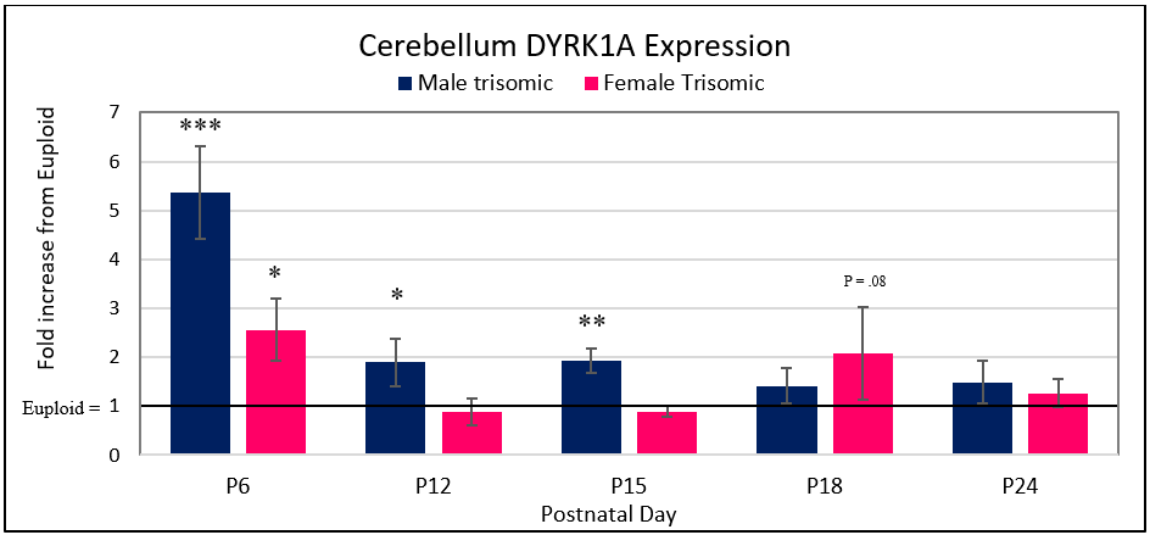

Figure 5 Cerebellum DYRK1A Expression. The trisomic male P6 cerebellum has the highest overexpression of all genotypes tested. DYRK1A levels fall at P12, again rise to significance at P15, then fall again at P18 and P24. The rise at P18 in the female cerebellum did not quite reach significance, with a $P < .08$. The large variations in DYRK1A expression in trisomic animals may account for the lack of power in this effect.

3.2 Female DYRK1A Expression Levels Through Early Perinatal Development

As in the study of male expression, female Ts65Dn mice had no difference between euploid and trisomic expression of DYRK1A at E18.5. Beginning at P6, trisomic mice had higher DYRK1A expression than euploid controls in hippocampus (~3.4-fold), cerebral cortex (~1.8-fold), and cerebellum (~2.5-fold) and the differences between genotypes diminished at subsequent ages, with the exception of a notable elevation on P18 in the hippocampus and cerebellum (see Fig. 3, 4, and 5). At P6, the genotype × region ANOVA confirmed a significant main effect of genotype [$F(1,9)=5.796$, $p=0.039$], with significant increases in the trisomic mice in the hippocampus ($p = 0.0047$) and cerebellum ($p = 0.023$).

At P12 there was a significant genotype × region interaction [$F(1.305, 14.36)=8.308$, $p=0.008$], due mainly to the trisomic females showing significantly higher DYRK1A levels than euploids in the hippocampus ($p=0.0025$) and cortex ($p=0.015$), but no differences in the cerebellum. On P15 and P24, there were no significant group differences in any brain region. However, for P18 females, the main effect of genotype was significant.

Female trisomic expression is similar to the male trisomic pattern of DYRK1A expression, with two distinct differences. First, overall expression across all time points was lower than in

males; even at the peak overexpression of the P6 hippocampus of (~3.5-fold increase), it was lower than the overexpression in males (~5-fold increase). Second, female trisomic animals had their biphasic rise of DYRK1A at P18, rather than at P15 as seen in males.

3.3 Z-Scores

The apparent differences in the extent of DYRK1A overexpression in Ts65Dn mice across age and brain region for males and females were quantitatively compared with the analysis of Z-scores for the male trisomic mice (see Figure 6). The 3-way mixed ANOVA (age × sex × region) confirmed a significant age × brain region interaction [$F(6.279, 92.621)=2.832$, $p=0.013$] and main effect of region [$F(1.57, 92.621)=6.321$, $p=0.005$], along with a significant age × sex interaction [$F(4,59)=2.866$, $p=0.031$] and a main effect of age [$F(4,59)=7.623$, $p<0.001$]. In males, follow-up age × region ANOVAs confirmed that the extent of overexpression varied as a function of region [$F(2,66)=3.863$, $p=0.026$]. The developmental changes in overexpression were confirmed by follow-up age × region ANOVAs. Z-scores confirmed that overexpression varied as a function of region [$F(1.288, 33.486)=5.035$, $p=0.024$], and the developmental changes were confirmed by a significant main effect of age [$F(4,26)=5.064$, $p=0.004$]. For the hippocampus, P6 females had significantly greater relative overexpression than P15 and 24 mice, and P18 mice also had significantly greater relative overexpression than P15 or P24 mice.

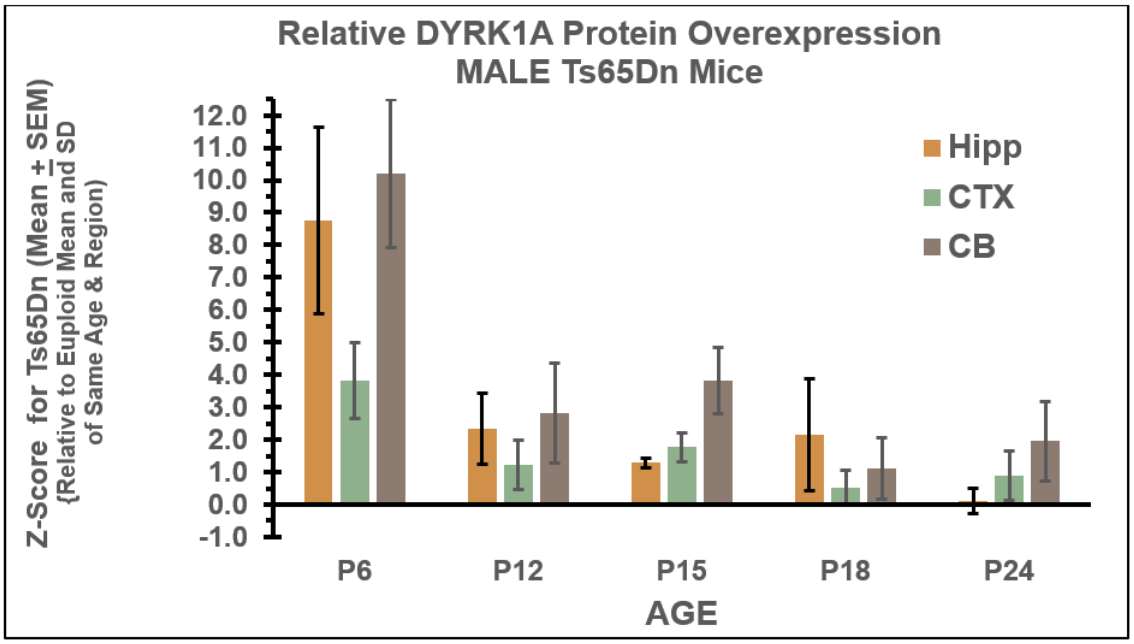

Figure 6 Z-score Male Ts65Dn vs Euploid Ratios of DYRK1A to Total Protein. The 3-way ANOVA confirmed a significant age × brain region interaction and main effect of region, along with a significant age × sex interaction and a main effect of age.

For the cerebral cortex in females, the only significant age differences were the higher levels of overexpression in P6 and P12 mice compared to P24 mice. For the cerebellum, overexpression at P6 was significantly higher than at P12 and P15, and P18 was significantly higher than P12 and P15. Thus, across the perinatal period, the high levels of DYRK1A expression in trisomic mice in the first neonatal week declined significantly after P6, but the extent of changes over development depended on brain region and males and females show different temporal patterns of perinatal changes in DYRK1A expression.

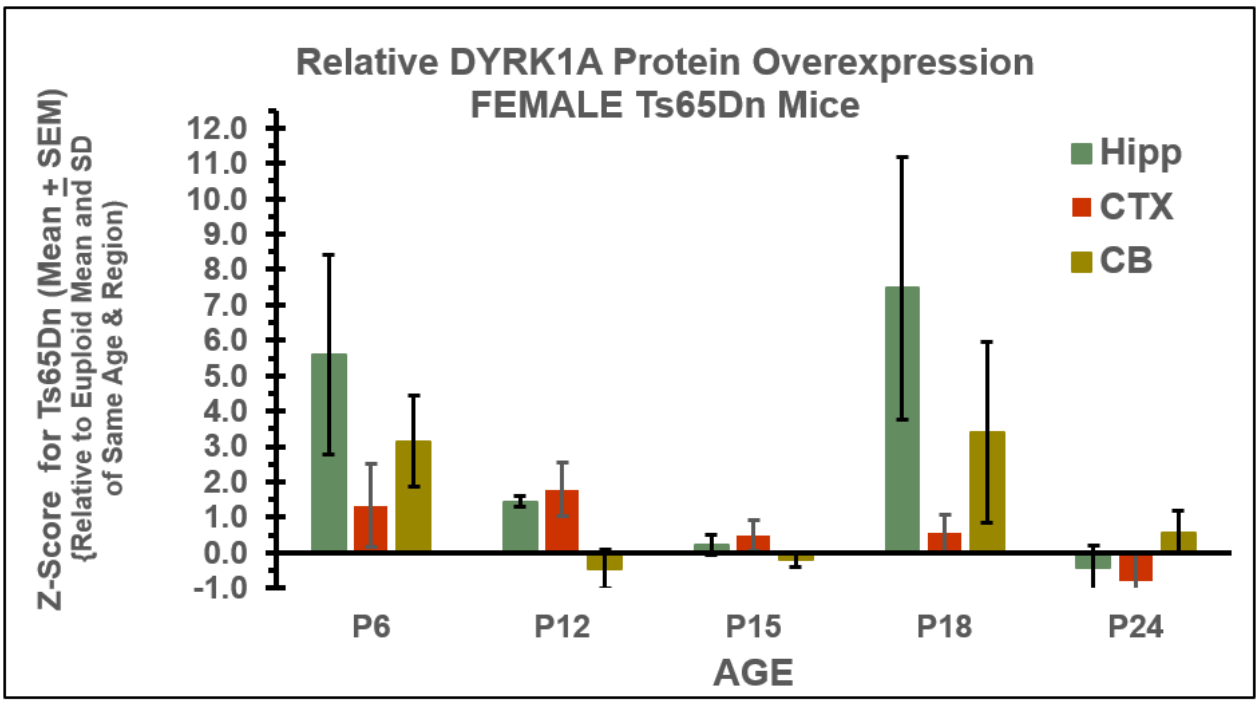

Figure 7 Z-score Female Ts65Dn vs Euploid Ratios of DYRK1A to Total Protein. For trisomic females, follow up age × region ANOVAs on z-scores showed that overexpression varied as a function of region, and the developmental changes were confirmed by a significant main effect of age.

CHAPTER 4. NON-LINEAR GENE TO PROTEIN EXPRESSION

4.1 Gene dosage to protein expression with variable *Dyrk1a* gene copy number in Male Ts65Dn mice

Four specific genotypes of animals were generated to study the influence of *Dyrk1a* gene copy number in trisomic and euploid mice: Trisomic [Ts (+/+/+)], Trisomic, $Dyrk1a^{+/-}$ [Ts (+/+/-)], Euploid [Eu (+/+)], and Euploid, $Dyrk1a^{+/-}$ [Eu (+/-)]. The hippocampus, cerebral cortex, and cerebellum were evaluated at P15, which corresponds with the spike in DYRK1A expression in male Ts65Dn mice. Six animals of each genotype and sex were compared, with females and males separated on different blots. Three *a priori* hypothesis were tested by directional 1 tailed t-tests within sex for each region: Ts (+/+/+) > Eu (+/+); Ts (+/+/+) > Ts (+/+/-); Eu (+/+) > Eu (+/-). We found two significant differences in the hippocampus of the male animals. (See Figure 8). The reduction of *Dyrk1a* from two copies in the otherwise euploid animal resulted in a significant reduction from 1.28-fold to .46-fold. The other significant change in the hippocamps was between the Ts (+/+/+) and the Eu (+/-) with a reduction from 1.79-fold to .46-fold. No other significant changes were seen in the hippocampus. Results from our DYRK1A spatiotemporal development study indicate there is a significant difference to be found between the Ts (+/+/+) and Eu (+/+) at this age between these two genotypes in the hippocampus, however with n=6 we were unable to generate enough power to statistically separate these effects.

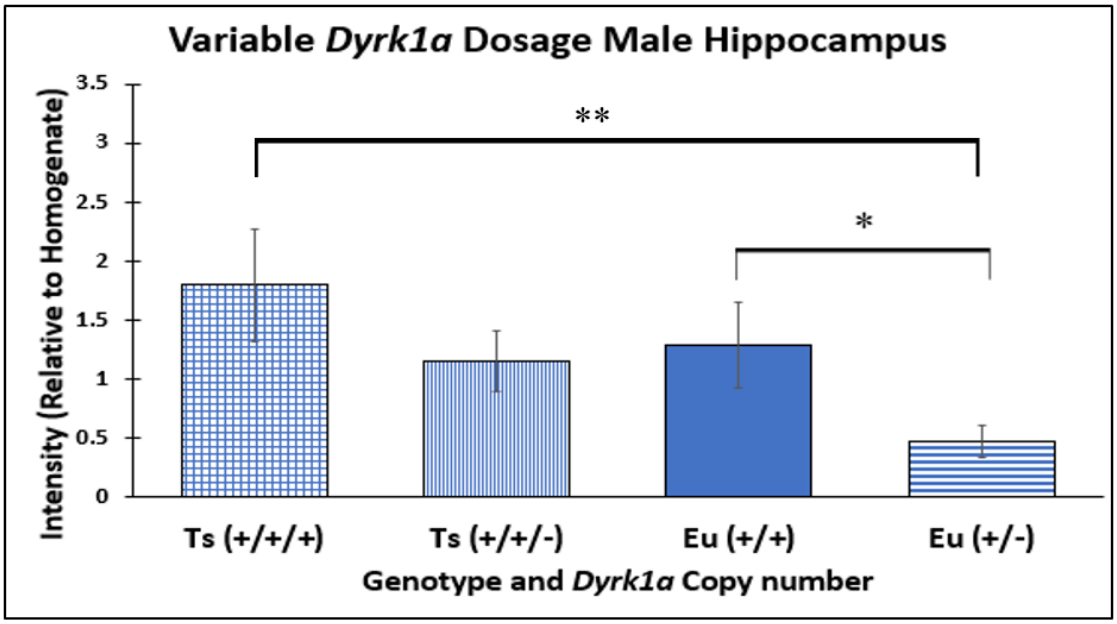

Figure 8 Variable Dyrk1a Dosage Male Hippocampus. In the hippocampus, trisomic knockdown and euploid animals have no significant difference between expression, indicating the reduction of copy number may have normalized expression levels at this age. In our developmental study, there exists a significant difference between trisomic and euploid DYRK1A expression. Due to limited power, this difference could not be reached in this study. Mean ± SEM

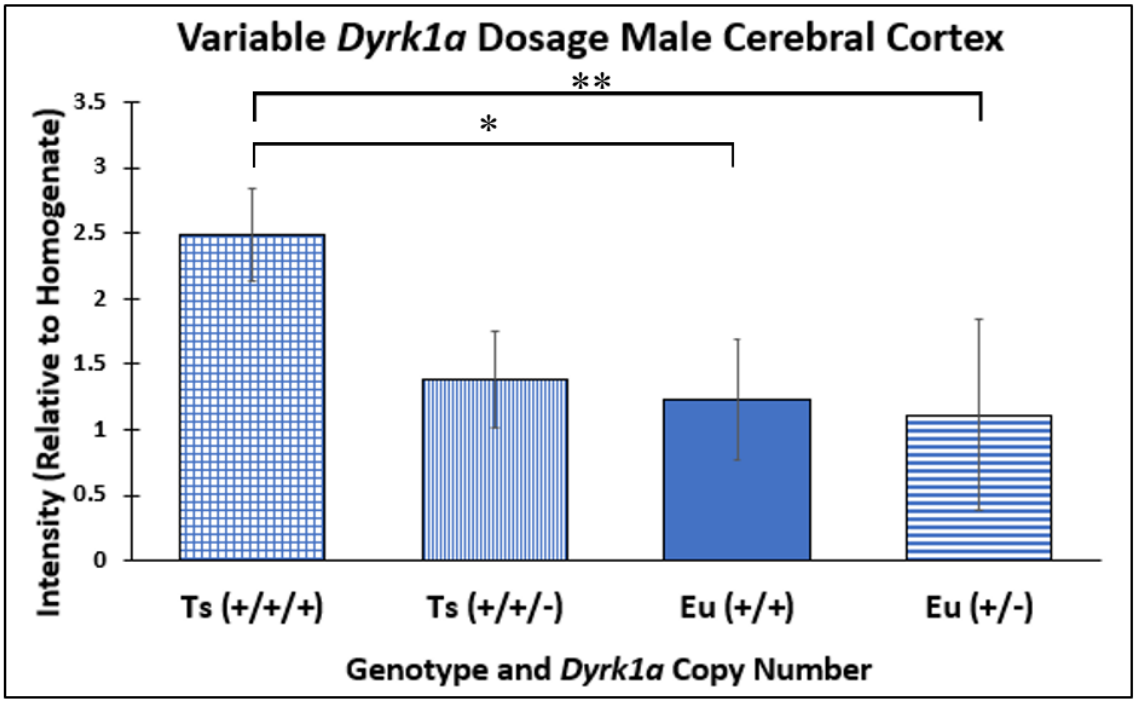

Figure 9 Variable Dyrk1a Dosage Male Cerebral Cortex. Using our three a priori tests, no significant difference was found from any of the four genotypes in the cerebral cortex. Mean ± SEM.

In the male cerebral cortex (see figure 9), two significant differences emerged. The Ts (+/+/+) and Eu (+/-) displayed a significantly decreased expression from 2.48-fold to 1.11-fold, and between Ts (+/+/+) and Eu (+/+). The latter results mimic the results of our developmental test (see figure 4) with the trisomic animal displaying a higher level of DYRK1A than euploid. There is no statistical difference between the TS (+/+/-) and Eu (+/+) animals, whose average intensity levels (1.38 and 1.23 fold of homogenate, respectively), but limited power prevented confirmation of this finding. Reduction of *Dyrk1a* copy number lowered average DYRK1A expression levels in the cerebral cortex under certain circumstances.

The cerebellum of the male showed the greatest number of significant differences between Ts (+/+/+) (2.06-fold) and the three other genotypes (Ts (+/+/-), Eu (+/+) and Eu (+/-)) which exhibited similar expression levels of DYRK1A (.77-fold, .85-fold, and .64-fold). There seems to be a disproportionate effect of trisomy on DYRK1A expression levels in the cerebellum that does not show up in the knockdown or euploid animals.

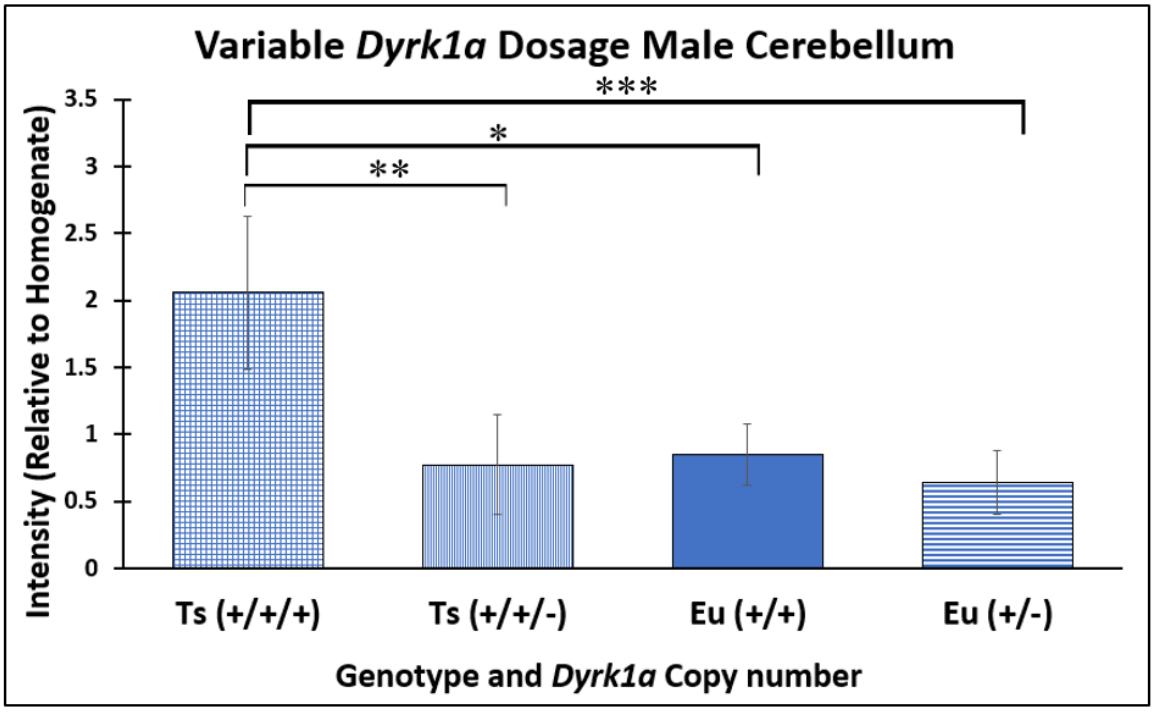

Figure 10 Variable Dyrk1a Dosage Male Cerebellum. The male cerebellum showed the greatest differences, similar to what was seen in the developmental study, of DYRK1A expression in trisomic to trisomic knockdown, euploid, and euploid knockdown. Mean ± SEM.

4.2 Gene dosage to protein expression with variable *Dyrk1a* gene copy number in Female Ts65Dn mice

As discussed in our DYRK1A spatiotemporal development study, female trisomic animals exhibit a node of a biphasic expression increase of DYRK1A at P18. In this study, all animals were examined at P15, and our hypothesis included the caveat that expression levels at P18 in our variable dosage animals would be more similar in expression levels due to this developmental difference. As expected, female DYRK1A expression does not vary with gene dosage at P15 as much as male dosage, perhaps because of the somewhat normalized pre-existing expression levels at this age.

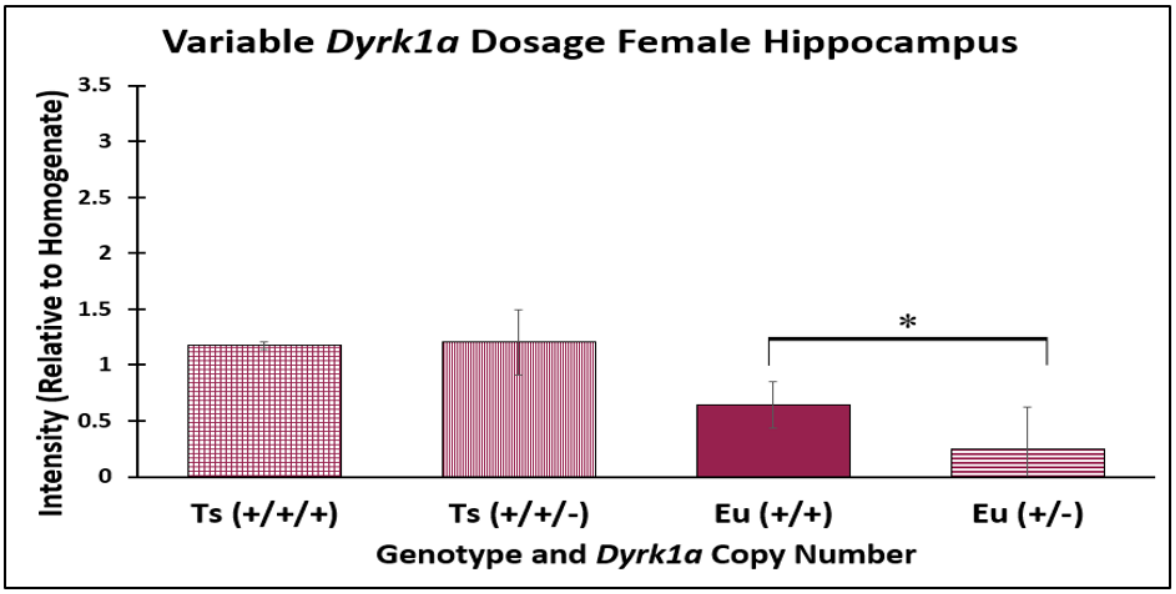

Figure 11 Variable Dyrk1a Dosage Female Hippocampus. The only significant difference in all three regions and in all four genotypes at P15 exist between the euploid and euploid knockdown hippocampus. Mean ± SEM.

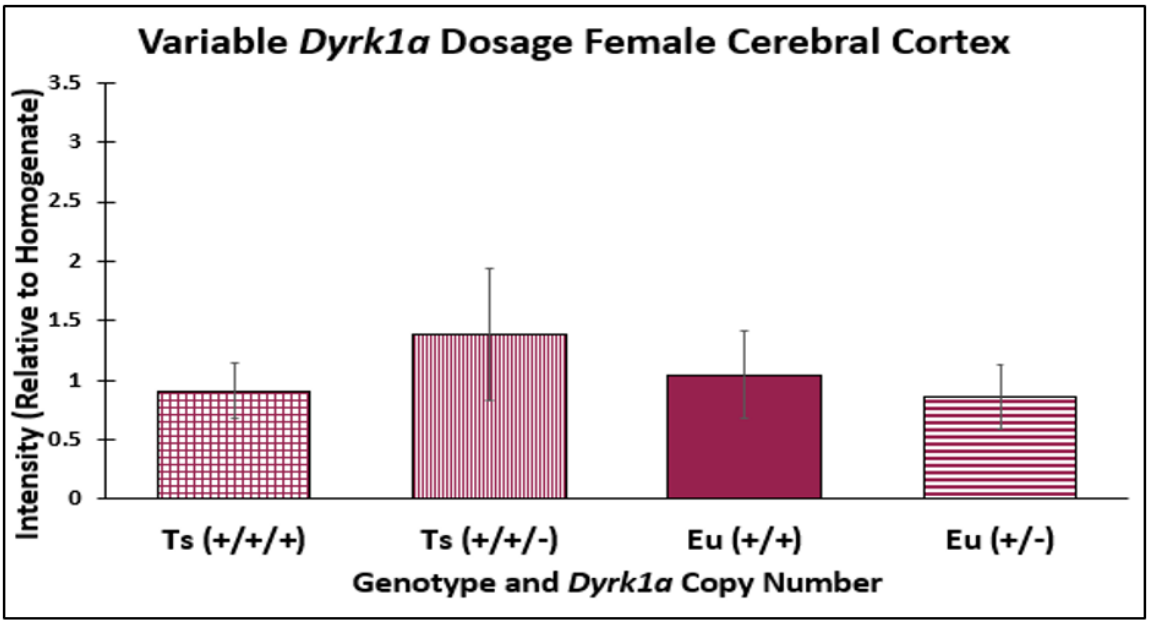

Figure 12 Variable Dyrk1a Dosage Female Cerebral Cortex. The lack of significance between the four genotypes at this age support our hypothesis of copy number reduction only reducing DYRK1A at times of overexpression, as in the P15 males. Mean ± SEM.

In the hippocampus, there exists a significant difference in expression levels between Eu (+/+) and Eu (+/-). However Ts (+/+/+), Ts (+/+/-) and Eu (+/-) display no significant differences in DYRK1A expression in the hippocampus.

There exist no statistical differences between the cerebral cortex or the cerebellum of the P15 *Dyrk1a* female knockdown comparisons.

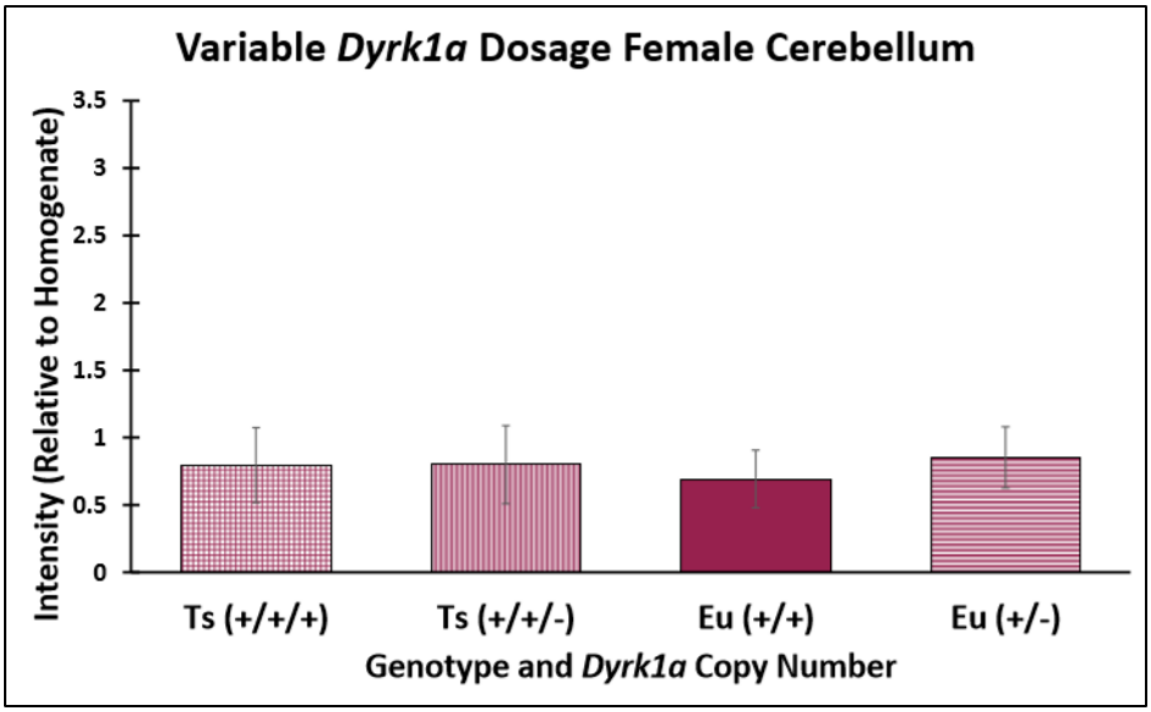

Figure 13 Variable Dyrk1a Dosage Female Cerebellum. Because the female biphasic overexpression of DYRK1A shows up at P18, it was not surprising to observe the lack of differences between the genotypes and their knockdown counterparts. Mean ± SEM.

CHAPTER 5. DISCUSSION

5.1 DYRK1A Spatiotemporal Quantification in Three Brain Tissues

This study has been instrumental in building a knowledge base of DYRK1A spatiotemporal expression in three tissues of the Ts65Dn brain and exploring what type of gene dosage-protein expression relationship exists between the *Dyrk1a* gene copy number and amount of DYRK1A protein expressed. To our knowledge, this is one of the first systematic quantifications of DYRK1A in the Ts65Dn brain and the first to explore its developmental variations over time during the early perinatal period. Our original hypotheses for this study were two-fold: first, we hypothesized that DYRK1A expression varies across perinatal development from P6-P24 as a function of age, sex, and in three brain regions (hippocampus, cerebral cortex, and cerebellum) between euploid and trisomic Ts65Dn mice. Second, we hypothesized that the spatiotemporal pattern of DYRK1A in three brain regions (hippocampus, cerebral cortex, and cerebellum) of P15 Ts65Dn mice is not influenced solely by gene copy number, and that reduction of *Dyrk1a* in euploid and trisomic mice will not result in a corresponding global reduction of DYRK1A. To accomplish these goals, we first mapped DYRK1A protein expression in select tissues of the brain on specific postnatal days of perinatal development, and second, we examined the effects of a genetic knockdown of *Dyrk1a* in the levels of protein expression in these same three tissues at a time of known DYRK1A upregulation in male Ts65Dn animals. This protein has shown itself to be both widely variable between the sexes as well as across the five ages explored here. Mapping the dynamic expression during this short time period and knocking down *Dyrk1a* copy number shows the myriad of factors influencing DYRK1A translation; gene copy number stays the same, thus factors beyond copy number influence the production of protein.

DYRK1A is dysregulated in the trisomic brain of Ts65Dn mice. At P6, both male and female trisomic animals displayed a significant difference in expression from their euploid counterparts, as well as a different pattern of expression between the sexes; illustrating this point, males exhibit their bi-phasic overexpression at P15, and females at P18. The theory that DYRK1A is consistently upregulated 1.5-fold throughout development and between the sexes is directly challenged by the data collected in this study. Our investigations included knocking down one copy of *Dyrk1a* from otherwise trisomic animals, and the variations in protein expression between

the true trisomic and knockdown suggest that factors beyond gene copy number influence the amount of protein translated.

Further examination is necessary to determine if transcription factors from other chromosomes, number of viable mRNA transcripts, or some other mechanism is causing the dysregulation in DYRK1A translation in the cell. The relationship between mRNA and protein expression correlations have been discussed in detail by previous authors. First, one can correlate the variation of protein and mRNA concentrations originating from the same gene(s) across different individuals, conditions, or time points. Such analyses explore to what extent the variation of mRNA levels is resulting in respective protein concentration changes (LIU *et al.* 2016). Second, correlations can be made on the concentrations of proteins with their respective transcripts, asking what differences between mRNAs are reflected in the proteins, ultimately asking how many protein molecules per transcript are present in a cell (MARGUERAT *et al.* 2012). Increased production or decreased degradation of mRNA transcripts, epigenetic regulation, mRNA silencing, positive feedback loops or limited breakdown of proteins can all contribute to protein levels within the cell, and specific mechanisms contributing to these levels can be individually explored and possibly exploited.

An interesting factor of the DYRK1A expression is revealed when comparing straight ratios of trisomic to euploid protein. In our full analysis, we have normalized proteins to euploid (Figures 3-5) which shows the relative over- and under- expression of the protein. When analyzing DYRK1A as a ratio of total protein within the trisomic animal, the discrepancies are highlighted again in both sexes at P6, in P15 in male, and P18 in female. This is important, as it highlights the finding that not only is DYRK1A overexpressed in the trisomic animal at these ages, but that it is overexpressed in relationship to the total proteome expressed in the trisomic animal. The factors increasing the transcription and translations of DYRK1A are not influencing all proteins within the animal universally.

A previous study quantifying DYRK1A in the forebrains of P5, P10, P20, P25, P30 and P35 animals reported protein quantities that differ from our findings (YIN *et al.* 2017), yet follow the same pattern of expression, starting with a high expression at P5 and falling steadily till P35. A different method of quantification was used as well, comparing DYRK1A expression to β-actin levels. Two different studies have suggested that normalizing to total protein rather than β-actin may be a more accurate method of measuring specific proteins using Western blot analysis, as β-

actin levels in specific tissues may vary among neurological diseases and alterations in cellular processes (ALDRIDGE *et al.* 2008; EATON *et al.* 2013). Differing levels of reported DYRK1A protein in the previous study could also be explained by the combination of male and female data, the combination of multiple brain structures, or a different methodology of investigation. Because of the low fertility rates of male Ts65Dn mice, the common practice is to maintain a robust mouse colony by keeping female trisomic animals for breeding and only use male trisomic animals for research. As we report in this thesis, selecting only male animals for research can eliminate the different expression shown by the opposite sex and may report inaccurate data as representative of both males and females. By identifying DYRK1A levels in three distinct tissues of the brain, data found here reports how structures in close proximity can still have vastly different protein expression. By combining brain tissues, Yin *et al.* used a composite ("forebrain"), and thus have limited the specificity of the investigation, possibly inaccurately representing areas with vastly different expression profiles by combining them. One major methodology change from the previous study is their calculation of DYRK1A to total protein in each sample. Two primary ways are to compare the optical density of DYRK1A to a housekeeping protein, such as actin, or to compare the optical density to total protein levels obtained by Coomassie blue staining. In this study, the choice to use Coomassie blue staining differs from the previous study; housekeeping proteins can be altered by neurological diseases and alterations in cellular processes, both hallmarks of DS (ALDRIDGE *et al.* 2008; EATON *et al.* 2013).

To increase numbers for studies, it is often deemed necessary to combine different ages of animals, such as P0-P6, P7-P12, etc. In a rapidly developing animal, these small windows of time contain vastly different maturity profiles, and expression of a single protein that varies during this time period could lead to highly inaccurate information when combining multiple ages. Additionally, by using different tissues from the same animal, this study highlights that expression is not only dependent on age and sex, but also location within the brain. This work shows significant and dramatic differences between P6 and P12 DYRK1A expression, as well as differences between the three measured tissues at the same age. Though P6 and P12 are less than 1 week apart, the rapidly developing prenatal and perinatal brains are changing expression profiles quickly. Combining different ages of animals and all areas of the brain can result in an inaccurate representation of developmental protein expression.

DYRK1A has become an attractive target for pharmacological therapies designed to increase cognition in individuals with DS. Multiple inhibitors have been identified that will bind with the active site and prevent its function. Drugs and combinations of natural and artificial compounds have been tested and used as inhibitors of DYRK1A to relieve the effects of overexpression (CHEN *et al.* 2005; ZANIN *et al.* 2012; STRINGER *et al.* 2015; DUCHON AND HERAULT 2016). Most recently, several laboratories have presented research that promotes the efficacy of a green tea polyphenol called Epigallocatechin gallatein (EGCG), claiming its ability to increase neurogenesis, increase neural connections, and a rescue of cognitive function (DIERSSEN 2012; DE LA TORRE *et al.* 2014). However, not all these tests were run on pure EGCG, but on green tea extracts with a combination of EGCG and other polyphenols, calling in to question the purity of the substance being tested as well as the reliability of the results. Using the Ts65Dn mouse model in Morris water maze and novel object recognition tests, De La Torre's team found animals treated with Mega Green Tea Extract, Lightly Caffeinated, and their performance improved in both tasks. In experiments from our lab, pure EGCG was administered in the same manner and was shown to have detrimental effects on bone formation and no appreciable effects on cognition in our mouse model (STRINGER *et al.* 2015). When administered P22 to P75, no pattern separation deficits were rescued in the radial arm maze, and no increase of proliferating neuroprogenitor cells in the hippocampus were apparent. Several reasons could account for these pitfalls, notably the purity of the therapeutic and the timing of administration. As the data in this study has shown, there is no singular place, space or time where an inhibitor can be universally administered to normalized DYRK1A protein function. It is an admirable goal to normalize protein function in the trisomic brain with the intent of increasing the quality of life for these individuals, but because DYRK1A is not consistent over the perinatal developmental timeline, universal and generalized inhibition could be highly detrimental to typical development. MRD7, a genetic mutation found in humans where one copy of *DYRK1A* is non-functional, produces injurious phenotypes such as microcephaly, severe mental retardation, anxious autistic behavior, or dysmorphic features (MOLLER *et al.* 2008). Over inhibition of DYRK1A may cause adverse phenotypes, like those seen in MRD7 or those of overexpression. The first step in normalizing DYRK1A production and function is to understand where and when the protein is being expressed and in what quantities.

Mouse development follows a predictable pattern that can be roughly correlated with humans, though on a considerably different timescale (OTIS AND BRENT 1954). Regarding brain growth, the P6 mouse developmental timeline corresponds roughly with human birth, the first point at which therapeutic intervention can be administered independent of the mother. Neurogenesis, neuron migration, structuring and patterning in the brain are set in motion during gastrulation and any abnormalities present will disrupt the delicate balance created by the chemical gradient responsible for coordinating these processes. Early intervention to normalize conditions has the potential for better outcomes, but the risks of intervention must be weighed against the expected results. Our limited knowledge of where and when DYRK1A is being differently expressed restricts our ability to inhibit or enhance its production. Any intervention without a confirmed justification is irresponsible and can significantly harm to the individual. Using the data from this study, a small window of understanding is beginning to open on how manipulation of this protein can be used to modify brain development.

The primary mode of data collection in this study has been the immuno-investigation of DYRK1A quantities through Western blotting. As with any bench technique, Western blots can present variable data if a strict protocol is not followed. A small change in technique or chemistry can lead to inaccurate information. During this study, our laboratory has run approximately 100 Western blots, and as the understanding of how this data can be interpreted changed, our protocol changed for Aim 2. In Aim 1, the technique compared the level of DYRK1A fluoresce from the membrane to a total protein level from the Coomassied membrane. With this ratio, DYRK1A levels were normalized to the euploid counterpart for each brain region. In Aim 2, DYRK1A fluorescence levels were compared to the fluorescence of a homogenate consisting of 3 tissue types from 5 male euploid mice. While using the euploid animals as reference to measure fold increases/decreases of DYRK1A in the trisomic animal is a reliable and accurate measure of DYRK1A, using a third data point as started in Aim 2 allows for a more nuanced view of DYRK1A levels in euploid as well as trisomic animals.

The inconsistent pattern of DYRK1A expression in our knockdown animals highlights an important aspect between protein and gene copy number. According to the prevailing theory of gene dosage, reduction in gene copy number should correlate with a matched reduction of protein in the same tissue. By reducing *Dyrk1a* gene copy number universally in the Ts65Dn trisomic animal and measuring protein levels in the hippocampus, cerebral cortex, and cerebellum, we have

challenged this dogma, introducing data which reiterates support for evidence that reduction of a single gene does not correspond with a linear pattern of protein reduction, and correcting gene copy number will not correct abnormal protein production. The unknown interactions of genes found on Hsa21 or on other chromosomes could account for the differences found with the one fewer copy of *Dyrk1a* causing the differently expressed DYRK1A. These findings suggest multiple mechanisms or interactions that contribute to the trisomic phenotype which must be explored and mapped before attempting to restrict gene or protein function in the trisomic condition. The relationship between spatiotemporal expression and reduction of *Dyrk1a* gene copy number also highlights the importance of sex as a factor of phenotype. Reduction of *Dyrk1a* at P15, which corresponds to an overexpression of protein in males, had different effects than the reduction in females, which display their overexpression at P18. This small difference in the evolving timeline between the two sexes could have major impacts in development.

To our knowledge, this is the first demonstration that trisomic DYRK1A protein overexpression in DS is temporally and spatially specific and is different between males and females. This study was the first to systematically assess perinatal and adolescent levels of DYRK1A protein in the hippocampus, cerebral cortex, and cerebellum of Ts65Dn male and female mice. Given that overexpression of DYRK1A was found before P24 in both male and female mice, these findings are consistent with our previous report of DYRK1A protein at P12, P15, P18, and P24 (STRINGER *et al.* 2017a).

These findings, showing that DYRK1A is highly expressed in these three areas of the trisomic brain early in development and falling to near euploid levels around the third week of development, are important for DS research. Prior understanding of gene-dosage theory postulates that gene dosage matches and corresponds to protein expression, which these results contradict. Understanding and mapping DYRK1A expression during early brain development is essential before introducing any attempts to correct dosage imbalances or therapies targeting abbearent phenotypes associated with DYRK1A expression. Inhibition of any protein during times of typical expression will alter the typical course of development, just as overexpression of these same proteins alter expression in trisomy.

CHAPTER 6. CONCLUSIONS AND FUTURE DIRECTIONS

The wide variance of DYRK1A expression between euploid and trisomic animals at P6 raises an important question: when does this divergence start? Additional testing should be done at earlier timepoints to assess protein levels with the intent to determine when this overexpression starts, and in which tissues it affects the most. Earlier administration of inhibitors has the potential to keep development on a more normalized course, but universal and non-specific inhibition will likely not be beneficial and can have detrimental effects.

Previous data in our laboratory led us to choose P15 as the point to measure DYRK1A protein levels because of the biphasic expression patterns between male and female Ts65Dn mice. After completion of this study, seeing the marked differences between all three brain regions between both sexes at P6, further knockdown testing should be performed at this age of development. Analyzing the patterns of DYRK1A expression to gene copy number during times of greater overexpression should allow for a better picture of protein production patterns.

Having realized the vast difference in expression at P6, it is crucial to test earlier ages to determine when and where the divergence of expression between trisomic and euploid brain tissues starts. It is our intent to extend the testing of protein expression levels to P0, when the transition from the anaerobic to aerobic environments at birth initiates significant changes in the newborn mouse, as well as prenatal embryos at embryonic (E) 18.5, approximately 1-2 days before birth. Using timed matings of trisomic dams and monitoring the weights of successfully bred animals, we will take the brain tissues of the embryos for Western blot analysis of DYRK1A levels.

Overall, this study has helped advance DS research by examining the relationship between DYRK1A and brain region as a dynamic factor of development. It has shown areas where overexpression may influence the cognitive development of individuals with DS and how inhibition could affect some of these changes when a viable pharmacological therapy can be obtained. It has shown that the *Dyrk1a* gene dosage-protein expression relationship is non-linear and is influenced by multiple factors beyond copy number. This body of work will help research that will benefit the DS community by its mapping of the DYRK1A protein as a target of interventive therapy.

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