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Spatiotemporal development of the forebrain in the Dp(16)1Yey/+ mouse model of Down syndrome

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SCHOOL OF MEDICINE

Dissertation

SPATIOTEMPORAL DEVELOPMENT OF THE FOREBRAIN IN THE
DP(16)1YEY/+ MOUSE MODEL OF DOWN SYNDROME

by

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DEDICATION

I would like to dedicate this work to my parents Bill and Carol, my sister Lee Anne, my brother-in-law Pickett, and my nieces Baylor and Amelia.
ACKNOWLEDGMENTS

I would like to thank all of the members of the Haydar lab, past and present, who have been a vital part of my time in graduate school. To my mentor, Tarik, for all of his support in both research and teaching, for allowing me to gain independence as a scientist, and for helping me to see the significance amongst a lot of negative data. The post-docs, Bill and Luis, taught me all the techniques I learned and what it means to be a good scientist. Thank you for both for being patient teachers and at times, the harshest of critics. To my fellow Haydar lab graduate student, Nadine Aziz, my lab-spouse and better half. It was a privilege to learn alongside you, debate the mouse models, and share in the experience of becoming scientists. I will always be thankful for our ability to work together every day and still foster our amazing friendship outside of the lab. Thank you to Cris, the Haydar Lab Manager, for running our lab like a fine tuned machine and for all of the laughter you bring to work every day.
SPATIOTEMPORAL DEVELOPMENT OF THE FOREBRAIN IN THE
DP(16)1YEY/+ MOUSE MODEL OF DOWN SYNDROME

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ABSTRACT

Down syndrome (DS), or trisomy 21 (Ts21), is the most common genetic
developmental disorder with a prevalence of about one in 700 live births. The triplication
of human chromosome 21 (Hsa21) that characterizes this disorder results in a
constellation of cognitive and physical alterations. The cognitive deficits range from mild
to severe, and persist throughout life. Post-mortem studies of individuals with DS have
revealed various neuropathologic abnormalities that are thought to underlie cognitive
dysfunction, including: disruption of neurogenesis, corticogenesis, synapse formation,
and myelination. However, the etiology of these alterations remains largely unknown. In
order to elucidate the genetic basis of DS-phenotypes, several mouse models have been
developed. The Ts65Dn, Ts1Cje, and Ts16 models, recapitulate DS-related phenotypes
and have extended our knowledge of the associated pathological changes. Despite this
progress, genetic dissimilarities in mouse models may confound phenotypic comparisons
between mouse models and human DS. Specifically, the aforementioned models have a
limited subset of triplicated Hsa-21 homologs or contain non-syntenic genes. Recently, a
novel mouse model, the Dp(16)1Yey/+ (or Dp16), that has the entire Hsa-21 syntenic
region of Mmu16 triplicated and no non-syntenic genes has been developed, suggesting
that Dp16 may present phenotypes more closely matching the human disorder. In this study, we present the first comprehensive analysis of Dp16 embryonic, young and adult brains that includes a focus on the proliferative, inhibitory/excitatory neuronal and oligodendrocyte-lineage phenotypes using histological, immunohistochemical, and behavioral assessments. We hypothesize that due to the larger triplicated segment, the Dp16 mouse model better recapitulates DS-related neuropathologies relative to other mouse models. Despite the extended triplication, Dp16 animals lack DS-related embryonic phenotypes, however, behavioral and cellular phenotypes arise during the 2nd week following birth. The Dp16 is the first model of DS to develop postnatal phenotypes in the absence of changes to embryonic brain development, as such, Dp16 may not be a reliable model to further understand brain development in the DS fetus. However, when used in conjuncture with other models, the Dp16 will be a useful tool in understanding the contribution of aneuploidy and gene dosage to DS-phenotypes in mouse models of DS.
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Morris Water Maze ................................................. MWM
Myelin Basic Protein ............................................... MBP
Neuroepithelium .................................................... NE
Neuropeptide Y ...................................................... NPY
Oligodendrocyte ..................................................... OLG
Oligodendrocyte Progenitor Cell ................................ OPC
Parvalbumin .......................................................... PV
Radial Glial Cell ...................................................... RGC
Somatostatin ........................................................... Sst
Subventricular Zone .................................................. SVZ
Trisomy 21 ............................................................... Ts21
Ventral Germinal Zone .............................................. VGZ
Ventricular Zone ...................................................... VZ
CHAPTER ONE

General Overview

Down syndrome (DS) is the most common genetic cause of intellectual disability (ID) and occurs in 1 of 691 live births and 1 of 549 conceptuses (Parker et al., 2010; Cocchi et al., 2010). The etiological basis of most cases of DS is complete trisomy of human chromosome 21 (Hsa21), which is the smallest human autosome (Lejeune et al., 1959; Hsu, 1998) containing 400-600 protein coding and non-coding genes (Sturgeon and Gardiner, 2011). Despite the relatively small size of Hsa21, trisomy 21 (Ts21) results in a constellation of phenotypes affecting multiple body systems. Congenital heart defects (Ferencz et al., 1989), craniofacial abnormalities (Frostad et al., 1971; Schmidt-Sidor et al., 1990), gastrointestinal problems (Levy, 1991) and an increased incidence of childhood leukemia (Wechsler et al., 2002) have all been reported in individuals with DS. However, these alterations are not seen in all DS cases. Intellectual disability (ID) is a fully penetrant feature of DS, which manifests as deficits in mental capacity including learning and adaptive behaviors. The severity of ID varies across cases (Chapman and Hesketh, 2000) and intelligence quotient (IQ) measures indicate a range of 25-55 (Rynders et al., 1978; Gibson et al., 1988; Vicari, et al., 2004). Individuals with DS also exhibit cognitive deficits (CD) resulting in abnormalities in cognitive domains such as attention, perception, and planning. A major focus of human DS research has been to elucidate the different characteristics and etiologies of ID through comprehensive assessments of behavior, cognition, and executive function.
The Cognitive Deficit and Co-morbidities of DS

Individuals with DS exhibit delays in the acquisition of critical milestones of development during the first year of life (Hartley, 1986; Horovitz and Matson, 2011; Vicari et al., 2013; Cardoso et al., 2015). Motor weakness, rooted in body wide hypotonia (Agiovlasitis et al., 2009), affects motor development and muscle reflexes in babies with DS (Carr, 1970; Melyn and White, 1973; Cardoso et al., 2015). Hypotonia may also contribute to delays in language acquisition (Chapman et al., 1991) as well as sleep apnea, which may exacerbate behavioral and cognitive problems (Bass et al., 2004; Shott, 2006). Infants with DS have a higher incidence of behavioral issues including inattention and impulsivity (Dykens, 2007) as well as Autism Spectrum Disorder, which is found in 18% of DS children (DiGuiseppi et al., 2010). The co-morbidities associated with DS impact development and each require separate intervention to minimize their impact and improve function.

During school years and adolescence, learning and memory impairments become more apparent as the gap between mental age and actual age increases in DS (Lanfranchi et al., 2010). Cognitive studies on school age children with DS have shown that storage of episodic and long term memory is impaired (Carlesimo et al., 1997; Pennington et al., 2003), which may underlie learning disabilities and achieving appropriate mental age. These affected executive functions continue to be compromised during adolescence when individuals with DS exhibit impairments in working memory, planning, inhibition, language, and set shifting (Nelson et al., 2005; Dierssen et al., 2009; Lanfranchi et al., 2010). As evident in children and adolescents with DS, Ts21 has broad effects on many
executive functions that change over time as the brain matures and cognitive domains develop.

In DS, this process of aging is worsened as new co-morbidities arise and previous cognitive impairments persist. Sleep apnea is a large potential contributor to the decline of executive function as sleep apnea impairs working memory and attention (Jackson et al., 2011). Adults with DS continue to exhibit deficits in learning and memory. However, an age-related decline becomes more apparent in adulthood (Nelson et al., 2005). The prevalence of dementia increases with age with 5-22% [DS cases] experiencing dementia by age 40 and 65-80% by age 65 (Prasher and Filer, 1995; Holland et al., 1998; Janicki and Dalton, 2000; Zigman et al., 2002; McCarron et al., 2014). Most often, dementia in DS is associated with the development of Alzheimer’s Disease (AD) and AD pathology is found in the DS brain by age 40 (Wisniewski et al., 1985). Additionally, 25.6% of DS adults have a major psychiatric disorder (i.e. major depressive disorder) (Roizen and Patterson, 2003). The executive function and psychiatric confounds add to the complexity of DS intellectual disability and contribute to the need for modified intervention methods throughout life as function changes. Despite an understanding of the multifaceted ID and CD, the known neuropathological underpinnings of the cognitive presentation are limited. However, \textit{post-mortem} and imaging studies on the human DS brain have considerably advanced our knowledge.

\textit{DS Neuropathology}

Infants with DS present symptomology immediately following birth, suggesting that the fetal development of the central nervous system (CNS) is compromised in the
disorder. Pathologies in the *post-mortem* fetal DS brain indicate that Ts21 causes disruption of many developmental processes leading to a CNS with altered function. As advances in imaging technology proceed, studies have revealed changes during *in utero* development through non-invasive methodology (Bianchi et al., 2014). The DS fetal brain exhibits microcephaly (Guihard-Costa et al., 2006) and reduced cerebellar and hippocampal volume during mid-gestation (Sylvester, 1983; Jernigan and Bellugi, 1990; Raz et al., 1995). Reductions in proliferation in the cortical germinal zone, dentate gyrus (Contestabile et al., 2007), and the cerebellum (Guidi et al., 2011) likely contribute to reductions in brain size as well delays in cortical lamination (Golden and Hyman, 1994) and hypocellularity of the cortex (Wisniewski, 1990; Larsen et al., 2008), dentate gyrus, and cerebellum (Sylvester, 1983; Guidi et al., 2011). The DS brain also exhibits changes in the formation and consolidation of synapses resulting in fewer synapses (Petit et al., 1984; Weitzdoerfer et al., 2011). These morphological and architectonic abnormalities are early alterations that set the stage for a malfunctioning system. As such, this period of *in utero* development may present the first opportunity for treatment of DS neuropathology and subsequent cognitive function.

Reductions in the size of the telencephalon and cerebellum persist into adolescence and young adulthood (Schmidt-Sidor et al., 1990; Jernigan et al., 1993). High resolution imaging studies have isolated changes in different regions of the brain. In particular, the frontal, occipital, and temporal lobes as well as the hippocampus and amygdala are smaller than normal in the DS brain (Schmidt-Sidor et al., 1990; Pinter et al., 2001). These region-specific abnormalities may reflect changes in executive function
as smaller frontal lobes and hippocampi may underlie impairments in episodic memory and long-term memory storage (Carlesimo et al., 1997; Pennington et al., 2003). Surprisingly, reduction in cortical volume is not ubiquitous to the DS brain as the parahippocampal gyrus is enlarged (Kesslak et al., 1994). The functional consequences and etiological basis of sub-region differences have yet to be determined. Despite this, regional differences highlight the spatial variation that exists in neuropathological manifestations within the DS brain.

At the cellular level, hypocellularity of the hippocampus (Sylvester, 1983), cerebral cortex (Schmidt-Sidor et al., 1990, Wisniewski, 1990), and cerebellum (Baxter et al., 2000) persist throughout childhood and adolescents. Several developmental processes, such as myelination and synapse consolidation, begin in prenatal life, but continue following birth and well into the second decade of life (Huttenlocher, 1979; Lidow et al., 1991; Giedd et al., 1999; Brouwer et al., 2012). In DS, dendrites have greater arborization in the neonate brain followed by decreased arbor size in child- and adulthood (Becker et al., 1986; Takashima et al., 1989; Becker et al., 1991; Benavides-Piccioe et al., 2004). Myelination is also delayed in children with DS (Wisniewski, 1990). Together, these deficits likely have profound effects on neuronal communication as the number of sites for synaptic contact and action potential conductance may be affected. Similar to prenatal defects, the etiological basis of postnatal developmental abnormalities remains elusive. However, the postnatal developmental period may present another opportunity for treatment and allow for therapeutics that isolate processes such as myelination and synaptic pruning, which are active during the treatment window.
The adult DS brain also exhibits widespread reductions in the volume of brain structures including the cerebral hemispheres, cerebellum, pons, and mammillary bodies (Kemper, 1991; Raz et al., 1995). In the developing DS brain, changes in the formation of the CNS at morphological and cellular levels are apparent. In adulthood, the cellular abnormalities appear as exacerbation of age-related processes and dementia-related pathologies (Prasher and Filer, 1995; Holland et al., 1998; Janicki and Dalton, 2000; Zigman et al., 2002; McCarron et al., 2014). Specifically, Alzheimer’s pathology is apparent as DS individuals develop the amyloid plaques and neurofibrillary tangles that have been associated with AD (Wisniewski et al., 1985; Zigman et al., 2002; McCarron et al., 2014). The etiological basis of AD neuropathology in DS may partly be caused by the triplication of *APP*, which is localized to Hsa21 (NCBI). AD and dementia in DS are prominent co-morbidities that may require individual assessment and intervention. As such, advanced aging of the DS brain may represent another potential window for pharmacological intervention and such studies would likely add to the understanding of not only DS, but AD as well.

Despite advances in non-invasive methodology to investigate brain abnormalities in DS, relying solely on *post-mortem* tissue limits the advancement of knowledge into the etiology of neuropathology in DS. Human tissue is a limited resource and small sample numbers may result in increased variability. Also, human tissue does not allow for genetic manipulation to investigate the role of individual genes on the development of neuropathology. Additionally, human studies have provided correlative evidence of changes in brain development and structure that likely produce the multifarious CD.
However, the direct link between individual neuropathologies and changes in specific executive and cognitive functions remains elusive and cannot be readily studied within the human system. Further confounding is the genetic complexity of DS as 400-600 coding and non-coding genes have been mapped to Hsa21 (Sturgeon and Gardiner, 2011), and changes in gene expression have been observed on all autosomes in DS (Mao et al., 2003; Mao et al., 2005; Lockstone et al., 2007; Letourneau et al., 2014; Olmos-Serrano et al., 2016). Fortunately, mouse models of DS allow for greater manipulation and experimentation to determine the link between DS genetics, neuropathologies, and cognitive function.

**Mouse Models of Down Syndrome**

Hsa21 genes are conserved in orthologous regions of mouse chromosomes (Mmu) 10, 16, and 17 (Figure 1). The Hsa21 syntenic regions on Mmu10, Mmu16 and Mmu17 vary in gene number; approximately 39 homologs are present on Mmu10, 119 on Mmu16 and 19 on Mmu17 (Figure 1; Das and Reeves, 2011; NCBI). The largest region, located on Mmu16, has been the primary focus of mouse model engineering due to the size of the homologous region and the presence of a subset of genes known as the Down Syndrome Critical Region (DSCR, Rahmani et al., 1990). Mmu16 segmental trisomy is the most prevalent among mouse models of DS and is present in the Ts16, Ts65Dn, Ts1Cje, and Dp16 models, each with varying number of triplicated genes (Das and Reeves, 2011; Figure 1).

The first mouse model of DS, the Ts16, was produced by breeding mice with naturally occurring Robertsonian translocations, which is a translocation event that
occurs at the centromere and is more common among acrocentric chromosomes such as those seen in mice (Figure 1; Gropp et al., 1975). Through this process, a mouse with trisomy for Mmu16 was established (Gropp et al., 1975). Genes localized to Hsa21 were found to be present on Mmu16, bringing the Ts16 into the forefront as a potential model with which to study DS (Reeves et al., 1986). Several disadvantages of the Ts16 were revealed as studies advanced. First, Ts16 embryos die \textit{in utero}, confounding the use of this model for postnatal morphometric and behavioral studies. Second, Mmu16 contains regions of homology to human chromosomes 3, 8, 16 and 21 (Seregaza et al., 2006). Thus, while modeling autosomal trisomy, Ts16 is non-specific to DS as a multitude of genes on Mmu16 are non-homologous to Hsa21.

The Ts65Dn mouse model of DS was engineered using UV irradiation to produce a reciprocal translocation in which the distal portion of Mmu16 attached to the centromeric region of Mmu17 (Figure 1; Davisson et al., 1990). The triplication is carried as a freely segregating extra chromatid, which mirrors a majority of DS cases (Hsu, 1998). The triplicated segment consists of 104 Hsa21 homologs localized to the Mmu16 distal segment and 60 centromeric Mmu17 genes that are non-syntenic to Hsa21 (Figure 1; Duchon et al., 2011; NCBI). As the triplication contains a subset of Hsa21 relevant genes, the Ts65Dn is a model of segmental trisomy. Despite the genetic differences between Ts65Dn and DS, the Ts65Dn mouse model offers distinct advantages over the aforementioned Ts16; specifically, Ts65Dn animals survive following birth and the triplication contains fewer erroneous genes. As such, Ts65Dn is the most widely used
mouse model of DS and its use has elucidated novel neuropathological and etiological aspects of DS.

The Ts1Cje mouse model of DS is a result of a chromosomal translocation of the distal portion of Mmu16 onto the telomeric region of Mmu12 with the translocated region consisting of ~71 Hsa21 homologs that was generated in an attempt to target the gene $\text{Sod1}$, or superoxide dismutase 1, present on Mmu16 and Hsa21 (Figure 1; Sago et al., 1998; NCBI). While the triplicated segment is smaller than that of Ts65Dn, only Hsa-21 homologs are present in the triplicated segment. The translocation onto the telomeric region of Mmu12 resulted in the monosomy of 7 genes downstream of the dynein heavy chain gene $\text{Dnahc11}$ (Figure 1; Duchon et al., 2011) and the inactivation of the $\text{Sod1}$ allele carried on the triplicated segment (Sago et al., 1998). These genetic disturbances that are unrelated to DS, the lack of aneuploidy, and the smaller trisomic segment have limited the use of Ts1Cje.

The Dp16 mouse model of DS is the most recently engineered model of segmental trisomy to Mmu16 (Li et al., 2007; Yu et al., 2007). Long-range Cre-recombinase/loxP-mediated recombination was utilized to target the Hsa21 homologous region on Mmu16 (Figure 1; Li et al., 2007). Specifically, murine embryonic stem cells were transfected with targeting vectors, specific to the Mmu16 proximal gene $\text{D930038D03Rik}$ and distal gene $\text{Zfp295}$, followed by Cre-recombinase. Appropriate recombination conferred resistance to hypoxanthine, aminopterin and thymidine medium allowing for the isolation of successfully recombined stem cells and further confirmed with southern blot analysis. Recombined embryonic stem cells were then microinjected
into albino C57B6/J-Tyc-Brd female mice resulting in chimeric blastocysts containing the Dp16 duplication (Li et al., 2007). This genetic duplication is similar to that observed in Ts1Cje in which the triplicated segment is not passed as an individual chromatid and the mice have a normal number of chromosomes. Genetically, the Dp16 exhibits two advantages over the aforementioned models. First, the triplicated segment of Dp16 contains all Hsa21 homologs located on Mmu16, accounting for 119 genes (Figure 1; NCBI). Second, only Hsa21 homologs are triplicated and no other appreciable genetic differences are present in this model. As such, Dp16 may be the most faithful representation of the genetic landscape of human DS.

These models have greatly contributed to the knowledge of Ts21 with models recapitulating DS-related phenotypes during development despite inherent genetic dissimilarities with the human condition. Mouse models have allowed for greater investigation into the pathological implications and etiological basis of neurologic aberrations in DS. Indeed, mouse models of DS have been invaluable in identifying the cell-type, age, and anatomically-specific effects of Ts21. This includes aberrations in neuro- and corticogenesis, excitatory and inhibitory cell populations, and glial cells. Below, these developmental processes and specific cell-types will be discussed while considering findings in the aforementioned mouse models.

**The Telencephalic Dorsal Germinal Zone: Neuro- and Corticogenesis**

The development of the CNS is initiated with the fusion of the neural tube and formation of the single-cell layer, the neuroepithelium (NE). Cells of the NE undergo interkinetic nuclear migration with nuclei translocating the ventricular zone (VZ),
triggering cellular division upon their return to the apical surface (Caviness and Rakic, 1978; Rakic, 1988). This process is marked by symmetrical divisions, which rapidly increase the cellular density of the VZ. At embryonic day 10 (E10) in mice and E33 in humans, the process of neurogenesis within the dorsal germinal zone (DGZ; Figure 2) begins with NE stem cells undergoing asymmetrical divisions to produce proliferating progenitors, mainly radial glial cells (RGCs) and post-mitotic neurons (Chenn and McConnell, 1995; Rakic, 1995; Noctor et al., 2001; Bystron et al., 2006; Garcia-Moreno et al., 2007). Neurogenesis within the DGZ produces the excitatory neurons of the future cortex (Rakic, 1972). RGCs, the stem cells of neurogenesis, can undergo self-renewing divisions as well as produce neurons, which migrate along the RGC basal process through the intermediate zone (IZ) into the developing cortical plate (CP; Rakic, 1972; 1995; Noctor, 2001; 2002). RGCs can also divide and produce several different neurogenic progenitors which will be discussed below. The period of neurogenesis proceeds from E10 to birth in mice and E33 to gestational week 25 (GW25) in the human fetus (Sidman and Rakic, 1973; Workman et al., 2013). During this time, newly born neurons migrate to their appropriate future cortical layer forming the cortex in a process called corticogenesis. Cortical lamination occurs in an inside-out manner, such that, earlier born neurons (i.e. neurons born on E11 in mice) will reside in the deeper layers (i.e. layer VI) of the neocortex (Angevine and Sidman, 1961; Rakic et al., 1974).

The processes of neurogenesis and corticogenesis are critical for the normal formation of the CNS and both are compromised in mouse models of DS leading to hypocellularity of cortical excitatory neurons. Indeed, the Ts16 brain is significantly
smaller than euploid littermates from mid- to late-gestation (Haydar et al., 1996) and the intermediate zone and cortical plate of the neocortex are smaller in Ts16 until the end of gestation (Haydar et al., 1996). These neuropathologies are comparable to the aforementioned microcephaly and neocortical expansion delays reported in DS fetuses (Schmidt-Sidor et al., 1990; Golden and Hyman, 1994). Additionally, reduced embryonic neurogenesis and increased apoptosis are apparent in Ts16 embryos likely contributing to hypocellularity of the cortical plate (Haydar et al., 2000). During embryonic development, the brain of Ts65Dn fetuses are smaller and exhibit substantial delays in the expansion of the neocortex (Chakrabarti, et al., 2007). These abnormalities most likely arise from reductions in neurogenesis within the DGZ (Chakrabarti, et al., 2007). Similarly, Ts1Cje embryonic development is hallmarked by reductions in neurogenesis and microcephaly (Ishihara et al., 2010). Despite their genetic differences, these three models exhibit phenotypes that appear to mirror findings in the DS fetal brain.

During neurogenesis, RGCs can self-renew and produce neurons as discussed previously. RGCs also divide to produce several sub-types of neural progenitor cells, specifically apical intermediate precursors, basal intermediate precursors, and basal radial glial (aPC, bIPC, and bRG, respectively). Like RGCs, aPCs reside within the VZ and have an apical process (Gal et al., 2006; Mizutani et al., 2007; Stancik et al., 2010; Tyler and Haydar, 2013). The other two sub-types, bIPCs and bRGs, populate the subventricular zone (SVZ) with bRGs maintaining a basal process while bIPCs have an apical process or lose both apical and basal processes (Englund et al., 2005; Kowalczyk et al., 2009; Fietz et al., 2010; Hansen et al., 2010; Shitamukai et al, 2011; Wang et al.,
2010; Elsen et al., 2013; Nelson et al., 2013). While the specific role of the different precursor subtypes is unclear, the size of bRG and bIPC populations increases with cortical complexity (i.e. primates) suggesting that these populations may aid in the expansion and gyrification of the cortex as seen in higher order mammals (Fietz et al., 2010; Hansen et al., 2010; Reillo et al., 2011; Shitamukai et al., 2011; Reillo and Borrel, 2012).

Investigations in the Ts65Dn mouse have shown that progenitor sub-types are differentially affected by segmental trisomy. The aPC population is significantly reduced in the Ts65Dn neocortex at E15.5 (Tyler and Haydar, 2013). The ramification of these population changes is unclear, but the aPC phenotype likely contributes to the reductions in neurogenesis, delays in neocortical expansion, and hypocellularity of the cortex (Chakrabarti et al., 2007). The transcription factor, Tbr2, is expressed exclusively in bIPCs and this population was found to be enlarged in the developing Ts65Dn neocortex at E16.5 (Chakrabarti et al., 2007). This transient increase in bIPCs may be a compensatory mechanism reflecting earlier neurogenesis deficits and may partially explain why the thickness of the Ts65Dn neocortex is normal by the end of embryonic development (Chakrabarti et al., 2007). These studies have elaborated on the known deficits to neurogenesis and corticogenesis in DS by isolating sub-populations that are differentially affected, however these sub-type specific aberrations have not been confirmed in human tissue.
The Telencephalic Ventral Germinal Zone: Interneurons and Oligodendrocytes

While the DGZ gives rise to the excitatory neurons of the cortex, neural progenitors within the ventral germinal zone (VGZ) of the ganglionic eminence (GE) produce a majority of the inhibitory interneurons and oligodendrocytes of the telencephalon (Figure 2; He et al., 2001). The GE is a transient embryonic structure of the ventral forebrain that is subdivided into three distinct regions: the medial ganglionic eminence (MGE), lateral ganglionic eminence (LGE), and caudal ganglionic eminence (CGE). Much like the DGZ, the ventricle is lined with a proliferative VZ in which asymmetrical progenitor cell divisions give rise to RGCs and intermediate progenitors or interneurons (Glickstein, 2007). Within the SVZ of the GE, intermediate progenitors divide symmetrically to produce two interneurons or oligodendrocytes (Ross, 2011). The inhibitory interneurons of the cerebral cortex can be subdivided into several different classes of inhibitory interneurons, which have unique molecular, physiological, and morphological characteristics that are determined by their origin within the GE and birthdate (Wonders and Anderson, 2006; Jovanovic and Thomason, 2011).

Inhibitory Interneurons

The subregions of the GE contribute to interneuron specification through the expression of various transcription factors and morphogen gradients. Sub-types of interneurons can be distinguished by the expression of specific proteins including parvalbumin (PV), somatostatin (Sst), and calretinin (Cr) (Cauli et al., 1997; Xu et al., 2010). The MGE is the birth place of PV, Sst and a small percentage of Cr-expressing interneuron sub-types (Wonders and Anderson 2006; Jovanovic and Thomason, 2011).
Within the MGE, a dorsal to ventral gradient of sonic hedgehog contributes to the specification of this area with Sst and Cr expressing interneurons being born in the dorsal MGE and PV-interneurons in the ventral MGE (Wichterle et al., 2001; Valcanis and Tan, 2003; Xu et al., 2004; Butt et al., 2005; Wonders et al., 2008; Xu et al., 2010). A majority of Cr-expressing interneurons originate in the CGE as well as those expressing neuropeptide Y (NPY), although some NPY and Cr expressing interneurons overlap with Sst expression (Lopez-Bendito et al., 2004; Xu et al., 2004; Butt et al., 2005). The identity of cells born in the LGE remains unknown and these interneurons populate the striatum and olfactory bulb (Anderson et al., 1997; Xu et al., 2004; Waclaw et al., 2006).

The birthdate of interneurons influences their identity. Similar to excitatory neurons of the DGZ, interneuron birthdate correlates to the inside-out lamination of the cortex (earlier born interneurons typically migrate into the deeper layers of the cortex) (Anderson et al., 2002; Valcanis and Tan, 2003). Within the MGE, PV interneurons are born first (beginning at E10 and ending at E15) and overlap with the birth of Sst interneurons while Cr interneurons are born later (E12.5 to E15.5) (Anderson et al., 2001; Butt et al., 2007). The origin of interneurons is similar in rodents and humans, however there are important differences. First, the inhibitory interneuron population represents 15% of cortical neurons in the rodent while in the monkey and human the population is substantially larger (25-35% of cortical cells) (Defelipe, 2011). Second, 60% of the cortical inhibitory interneuron populations in the rodent are PV- and Sst-expressing cells, while Cr-expressing interneurons are the largest population in the human and non-human primate (Gonchar and Burkhalter, 1997; Kawaguchi and Kubota, 1997; Barinka and
Druga, 2010). Third, the population increase across evolution may be connected to an additional source of interneurons in the SVZ within the cortex of human and non-human primates (Letinic et al., 2002; Petanejk et al., 2009; Hansen et al., 2010). As such, these differences must be considered when comparing inhibitory interneuron phenotypes in the human DS brain and mouse models of DS.

Much like the DGZ, proliferation within the VGZ is affected in mouse models of DS. In the Ts65Dn model, neurogenesis is substantially increased in the MGE and the mitotic population in the MGE SVZ is larger at E14.5 and E15.5 (Chakrabarti et al, 2010). This increase, which opposes a reduction of neurogenesis in the DGZ, results in larger populations of PV- and Sst-expressing interneurons within the adult somatosensory cortex and hippocampus (Chakrabarti et al., 2010; Perez-Cremades et al., 2010; Hernandez-Gonzalez et al., 2015). The etiological basis of the overproduction of inhibitory interneurons was determined using a gene dosage rescue paradigm in which trisomic animals are bred to a mouse heterozygous to a gene within the Hsa21 homologous region. This produces trisomic pups that will only have two copies of the targeted genes. This level of genetic manipulation is a major advantage of using mouse models of DS. Using this strategy, it was determined that the triplication of the transcription factors Olig1 and Olig2, which play a role in interneurogenesis and oligodendrogenesis (Takebayashi et al., 2000; Lu et al., 2002; Zhou and Anderson, 2002), results in the overproduction of interneurons. Normalization of these two genes restored the inhibitory interneuron population in Ts65Dn animals corrected for Olig1/2 triplication (Chakrabarti et al., 2010).
The stark contrast in the DGZ and VGZ phenotypes in the Ts65Dn results in an imbalance in excitation/inhibition within the cortex of Ts65Dn animals. This imbalance is believed to be a major contributor to the cognitive deficit observed in DS and mouse models of DS. In the hippocampus of Ts65Dn, Ts1Cje, and Dp16 animals, long term potentiation (LTP) is substantially reduced and can be restored with the administration of the GABA<sub>\alpha</sub> antagonist, picrotoxin (Kleschevnikov et al., 2004; Hanson et al., 2007; Yu et al., 2010). This error in synaptic plasticity in the hippocampus likely contributes to the deficit in hippocampal-based behavioral paradigms such as the Morris Water Maze (MWM) and Contextual Fear Conditioning (CFC) (Stasko and Costa, 2004; Seregaza et al., 2006; Bianchi et al., 2010; Yu et al., 2010; Olmos-Serrano et al., 2016). While the use of mouse models has revealed electrophysiological and cellular inhibitory-associated phenotypes, the inhibitory-phenotype in human DS is not well defined. The human DS cortex is hypocellular for smaller neurons that were presumed to be inhibitory based on morphology (Ross et al., 1984) however, it is unknown if there are population differences across the inhibitory interneuron subtypes.

**Oligodendrocytes**

The process of oligodendrogenesis exhibits temporal and spatial specificity during embryonic and early postnatal development with oligodendrocyte progenitor cells (OPCs) arising in three distinct waves (Kessaris, et al., 2006). The first two waves are produced from the VGZ and third arises from the DGZ (Kessaris, et al., 2006). The first wave of OPCs are born at E11.5 in the MGE of the mouse, migrate to the cortex by E14.5, and are thought to be replaced postnatally. The second wave arises from the LGE
and CGE at E15 and the final wave of OPCs are generated postnatally in the DGZ of the neocortex (Olivier et al., 2001; Vallstedt et al., 2005; Kessaris, et al., 2006; Ono et al., 2009). Following their generation, OPCs undergo multiple stages of maturation, which can be identified using stage-specific markers. Similar to interneurons, OPCs are produced by an \textit{Olig2}+ expressing progenitor pool (Anderson, 1997; He, 2001; Lavdas, 1999), and cells of the oligodendrocyte (OLG)-lineage continuously express \textit{Olig2}+ throughout their maturation. While in an immature pre-myelinating state, OPCs express the proteoglycan NG2 (Pringle and Richardson, 1993; Pringle et al., 1992; Stallcup and Beasley, 1987) and are maintained in an immature state by \textit{Wnt}-signaling (Fancy et al., 2009; 2011; 2014). Upon their maturation into myelinating OLGs (mOLGs), mOLGs express multiple myelin associated proteins including myelin basic protein (MBP) as well the protein adenomatous polyposis coli (APC), which is labeled using an antibody, CC1 (Verity and Campagnoni, 1988, Jakovcevski and Zecevic, 2005). The process of myelination in postnatal mice peaks 20 days after birth (postnatal day 20, P20; Craig et al., 2003; Dean et al., 2011).

While the origins and maturation of OLGs are similar in mice and humans, the timing of oligodendrocyte production and myelination differ. Oligodendrogenesis is underway by GW15 in the human fetus followed by OPC migration into the cortex by GW20 (Jakovcevski et al., 2009). Myelination begins around mid-gestation in humans, compared to postnatally in mice (Jakovcevski and Zecvic, 2005; Jakovcevski et al., 2009). In humans, the process of myelination is extended with a peak at 2-3 years old and continues well into the second decade of life (Keshavan et al., 2002; Lebel and Beaulieu,
This species-specific temporal shift of developmental processes is important to consider when assessing potential therapeutic targets and DS-related neuropathologies.

In DS, myelination and oligodendrogenesis have received little attention with few studies focusing on the white matter of the brain. DS children and toddlers have significant delays in myelination, however no delay is observable during prenatal myelination (Wisniewski and Schmidt-Sidor, 1989; Koo et al., 1992). To date, no DS mouse model studies on white matter and oligodendrocytes have been done. The Ts65Dn animal exhibits elevated levels of proliferation within the VGZ, which results in a larger inhibitory interneuron population (Chakrabarti et al., 2007). This swell in progenitors may affect the production of OPCs that have the same spatial origin as interneurons. Therefore, cells of the OLG-lineage and myelin formation need to be investigated in the mouse models of DS to allow for further comparison to human phenotypes and greater scrutiny of the models.

Unraveling embryonic and cellular aberrations of DS has been furthered by the use of mouse models. As DS research moves forward, mouse models are being used to assess potential therapeutic efficacy in the amelioration of DS-related pathologies and cognitive deficits. To determine the effects of possible pharmaceuticals, mouse models must be selected with greater scrutiny as to which model best represents the human disorder or phase of life targeted by the treatment. As such, mouse models must be held to a standardized barrage of assessments to compare models and aid in appropriate model selection. Here, we aim to assess the novel Dp(16)1Yey/+ model relative to DS and
mouse model phenotypes. As Dp16 contains the largest Hsa21 syntenic triplication, we hypothesize that Dp(16)1Yey/+ will recapitulate DS-related prenatal phenotypes and postnatal cortical aberrations. To contextualize this novel model, we propose the following aims: (1) To determine the forebrain prenatal phenotype in the Dp(16)1Yey/+ mouse model of Down syndrome, (2) To determine the OLG and white matter phenotypes of the Ts65Dn and DS brain, and (3) To determine the effects of Dp(16)1Yey/+ segmental trisomy on inhibitory neuronal populations and OLGs.
Tables and Figures

Figure 1. Regions of homology between Hsa21 and the mouse genome allow for the generation of mouse models of DS. (Modified and used with permission from Nadine Aziz). Hsa21 is homologous to three regions in the mouse genome localized to Mmu10, Mmu16 and Mmu17. The largest region is present on Mmu16. In mouse models of DS, various methods of engineering results in differing triplicated segments. The Ts65Dn (top left) has 104 homologs translocated onto the centromeric portion of Mmu17 and includes 60 genes non-homologous to Hsa21. In the Ts1Cje (top right) 71 triplicated genes translocated onto Mmu12, resulting in the monosomy of 7 Mmu12 genes. The Ts16 (bottom left) is the triplication of an entire copy of Mmu16. The Dp16 (bottom right) has the largest triplicated segment, 119 genes, attached to the distal end of an endogenous Mmu16
Figure 2. The proliferative germinal zones of the embryonic brain. The germinal zones of the developing brain presented on a coronal representation of the mouse brain. The Dorsal germinal zone (DGZ) is the germinal zone of the neocortex, that produces excitatory cortical neurons, consisting of the VZ and SVZ. The ventral germinal zone (VGZ) is located in the ganglionic eminence where interneuron precursors and the first two waves of oligodendrocytes are produced.
CHAPTER TWO

Absence of prenatal forebrain defects in the Dp(16)1Yey/+ mouse model of Down syndrome

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and immunohistochemical studies.
ABSTRACT

Studies in humans with Down syndrome (DS) show that alterations in fetal brain development are followed by postnatal deficits in neuronal numbers, synaptic plasticity and cognitive and motor function. This same progression is replicated in several mouse models of DS. Dp(16)1Yey/+ (hereafter called Dp16) is a recently developed mouse model of DS in which the entire region of mouse chromosome 16 that is homologous to human chromosome 21 has been triplicated. As such, Dp16 mice may more closely reproduce neurodevelopmental changes occurring in humans with DS. Here we present the first comprehensive cellular and behavioral study of the Dp16 forebrain from embryonic to adult stages. Unexpectedly, our results demonstrate that Dp16 mice do not have prenatal brain defects previously reported in human fetal neocortex and in the developing forebrains of other mouse models, including microcephaly, reduced neurogenesis and abnormal cell proliferation. Nevertheless, we found impairments in postnatal developmental milestones, fewer inhibitory forebrain neurons and deficits in motor and cognitive performance in Dp16 mice. Thus, while this new model does not express prenatal morphological phenotypes associated with DS, abnormalities in the postnatal period appear sufficient to produce significant cognitive deficits in Dp16.

SIGNIFICANCE STATEMENT

Down syndrome (DS) leads to intellectual disability. Several mouse models have increased our understanding of the neuropathology of DS and are currently being used to test therapeutic strategies. A new mouse model that contains an expanded number of DS-related genes, known as Dp16, has recently been generated. We sought to determine
whether the extended triplication creates a better phenocopy of DS-related brain pathologies. To do this we measured embryonic development, forebrain maturation, and perinatal/adult behavior. Our study reveals an absence of prenatal phenotypes in Dp16 fetal brain, but specific cellular and behavioral deficits appear after the first two postnatal weeks. These results uncover important differences in prenatal phenotype between Dp16 animals and humans with DS and other DS mouse models.
INTRODUCTION

Down syndrome (DS), caused by trisomy of 400-600 coding and non-coding genes on human chromosome 21 (Hsa21), occurs with an incidence of 1 in 691 live births (Candfield et al., 2006; Cocchi et al., 2010; Sturgeon and Gardiner, 2011) and leads to intellectual disability (Chapman and Hesketh, 2000). Studies on post-mortem brains of individuals with DS have reported prenatal and postnatal abnormalities, indicating that complex changes to the genetic landscape lead to the cellular defects underlying the morphological and cognitive symptoms of DS (Haydar and Reeves, 2011).

To study the etiology of DS, several mice have been generated by exploiting syntenic regions within the human and mouse genomes. Specifically, Hsa21 genes are conserved in orthologous regions of mouse chromosomes (Mmu) 10, 16 and 17, the largest of which is on Mmu16. Therefore, several mouse models containing a varying number of triplicated Mmu16 genes were created to elucidate the cellular, molecular, physiological, and behavioral deficits in DS (Das and Reeves, 2011). Studies on such mice, namely Ts65Dn, Ts1Cje and Ts16, have reported prenatal brain abnormalities, including reduced proliferation and neurogenesis, delayed neocortical expansion and microcephaly (Haydar et al., 1996; Haydar et al., 2000; Chakrabarti et al., 2007; Ishihara et al., 2010). These reductions in proliferation and brain growth closely match changes found in the brains of human fetuses with DS (Sylvestre, 1983; Golden and Hyman, 1994; Contestabile et al., 2007; Guidi et al., 2008, 2011; Larsen et al., 2008). In both humans and mouse models, forebrain changes have also been found in the immediate postnatal period and during adulthood, including altered ratios of inhibitory and
excitatory neurons, synaptic dysfunction and motor and cognitive behavioral impairments (Schmidt-Sidor et al., 1990; Wisniewski, 1990; Koo et al., 1992; Golden and Hyman, 1994; Belichenko et al., 2004; Kleschevnikov et al., 2004; Best et al., 2007; Chakrabarti et al., 2007, 2010).

In addition to Ts65Dn, Ts1Cje, and Ts16, new mouse models have recently been developed to better replicate the triplication of the genes that map to Hsa21. These mice are designed to carry individual duplications spanning the entire Hsa21 syntenic regions on Mmu10, Mmu16, or Mmu17 [Dp(10)1Yey/+, Dp(16)1Yey/+, and Dp(17)1Yey/+], respectively (Li et al., 2007; Yu, 2010). Among these, Dp(16)1Yey/+ (hereafter called Dp16) offers the following advantages: 1) its trisomic region consists of the entire Hsa21-relevant complement of Mmu16 genes (n=119), ~15 more than Ts65Dn (NCBI), 2) Dp16 males are fertile, eliminating the potential confound of a trisomic maternal uterine environment, 3) in contrast to Ts65Dn, only Hsa21 syntenic genes are triplicated in Dp16 (Duchon et al., 2011). Because of these improvements in genetics and fertility, Dp16 may be a preferred mouse model of DS in future studies (Gardiner, 2014).

Several recent studies examining postnatal development in Dp16 mice have found agenesis of the cerebellum and craniofacial dysmorphism as well as impairments in learning and memory (Yu et al., 2010; Starbuck et al., 2014). These results clearly indicate that Dp16 animals replicate some of the defects found in other mouse models, yet assessments of prenatal brain growth and extensive behavioral phenotyping have not been conducted, preventing a comprehensive comparison to Ts65Dn as well as other mouse models of DS and to humans with DS. Therefore, to further understand the impact
of the larger syntenic region present in Dp16, we performed a comprehensive analysis of prenatal forebrain development combined with neonatal and adult behavioral studies. Unexpectedly, we found Dp16 forebrain growth is not different from euploid littermate controls. Despite the lack of prenatal cellular deficits, we measured significant delays in late-acquisition developmental milestones and changes in the number of inhibitory forebrain neurons. These defects were followed by altered motor function and cognitive performance in adults. Thus, Dp16 may be a good model to investigate the postnatal causes of intellectual disability in DS, but the contribution of prenatal changes in brain development to cognition cannot be studied with Dp16.
MATERIALS AND METHODS

ANIMALS

Embryonic B6129S-Dp (16Lipi-Zfp295)1Yey/NJ

Dp16 animals were received as a gift from Eugene Yu (Roswell Park Institute, Buffalo, NY, USA) and were also purchased from Jackson Laboratory (Stock 013530, Bar Harbor, ME). All Dp16 founders were backcrossed to C57BL/6 mice for at least five generations prior to import into our facility. Our Dp16 breeding colony was maintained by backcrossing Dp16 males with C57BL/6N females obtained from Charles River Laboratories (Wilmington, MA). Breeders were paired every evening between 3-5PM and separated every morning between 9-10AM followed by vaginal plug checks. The presence of vaginal plug was designated embryonic day 0.5 (e0.5) and embryos were collected between e13.5 – e18.5. Pregnant dams were euthanized following approved IACUC guidelines. Embryos were fixed for 24 h in 4% paraformaldehyde (PFA), 1x phosphate buffered saline (PBS) at 4°C. Postnatal day 15 (P15) animals were anesthetized and fixed via intracardial perfusion using 4% PFA, 1x PBS.

Embryonic B6129SC3Sn-Dp (16Lipi-Zfp295)1Yey/NJ x B6/C3Sn F1 Hybrid

To generate a F1 Hybrid, Dp16 animals were bred onto a B6/C3Sn background by crossing Dp16 (on C57BL/6N background) to C3SnHeSnJ animals to obtain Dp16:F1 hybrid animals. Dp16:F1 hybrids were then bred with B6/C3Sn animals to generate timed pregnancies as outlined below. Both male Dp16:F1 hybrids and female Dp16:F1 hybrid animals were used separately in breedings in order to test: 1) the contribution of a
hybrid background and 2) the combined effect of uterine environment and a hybrid background on embryonic brain development. Breeding pairs were established every evening and separated every morning followed by vaginal plug checks as described above. The presence of vaginal plug was designated e0.5 and embryos were collected at e14.5 or e15.5. Pregnant dams were euthanized following approved IACUC guidelines. Embryos were fixed for 24 h in 4% PFA, 1x PBS at 4°C. These animals were used exclusively for gross brain measurements and pallial thickness measurements where noted.

*Adults* B6129S-Dp (16Lipi-Zfp295)1Yey/J

Eleven Dp16 and thirteen euploid 2-3 month-old male mice were purchased from Jackson Laboratory (Stock 013530, Bar Harbor, ME). Standard rodent chow and water were available *ad libitum*. In addition to standard bedding, a Nestlet square was provided in each cage. The colony room was maintained on a 12:12 light/dark cycle, with lights on at 7:00 AM. All experiments were conducted in the light phase, between 8:00 AM and 1:00 PM. To minimize olfactory cues from previous trials, each apparatus was thoroughly cleaned with Sani-Cloth Plus (PDI Healthcare). Each day of testing, mice were left in their home cages in the room used for the experiment at least 1 hour prior to the onset of the study. Water T-maze and Morris water maze tasks were the last experiments in the series. The experimenter was blind to the genotype in all experiments. All experiments involving animals were performed in accordance with institutional, federal and international guidelines.

*Genotyping*
Limb buds (embryos) or tail clippings (postnatal animals) were digested using 0.2 mg/ml proteinase K (Denville Scientific) in DNA digestion buffer (50 mm Tris-HCl, pH 8.0, 100 µm EDTA, pH 8.0, 100 µm NaCl, and 1% SDS) overnight at 55°C followed by phenol/chloroform extraction and DNA was precipitated using ethanol. Genotyping was performed using PCR amplification with primers specific for the Dp16 translocation breakpoint (Forward: 5’-CTG CCA GCC ACT CTA GCT CT-3’, Reverse: 5’-AAT TTC TGT GGG GCA AAA TG-3’) and mFez (Forward: 5’-CTT CGG GAG CAG GTA CCC TA-3’, Reverse: 5’-AAG ATC TGA GGC TCG CCA AG-3’) as an internal positive control. The cycling parameters were: Step 1: 94°C, 3 min; Step 2: 94°C, 45s; Step 3: 58°C, 30s; Step 4: 72°C, 30s (Step 2-4, 40 cycles); Step 5: 72°C, 10 min; Hold at 4°C.

In addition, we confirmed all genotypes using a separate PCR procedure using different primers and protocol (Forward: 5’-TAT TAG GAC AAG GCT GGT GG-3’, Reverse: 5’-GTC AGT GGT TGT GAC TTG TG-3’, gift from Roger H. Reeves). Cycling parameters for the second primer set were: Step 1: 95°C, 1 min; Step 2: 95°C, 15s; Step 3: 60°C, 15s; Step 4: 72°C, 30s (Steps 2-4, 35 cycles); Step 5: 72°C, 7 min; Hold at 4°C.

**Gross Measurements**

All measurements of brain growth were conducted exactly as described previously for Ts65Dn studies in Chakrabarti, et al. (2007). Embryos were imaged and crown-rump measurements were analyzed post-fixation using Axiovision software (Zeiss). All embryo (e13.5-e18.5) crown-rump lengths were measured from the top of the head to the base of the tail. After imaging, embryos were decapitated and brains removed from the cranium. Brains were imaged using Axiovision software (Zeiss). Medial-lateral
lengths were measured from the median longitudinal fissure to the maximal lateral aspect of the telencephalic hemisphere. Rostrocaudal lengths were measured from the frontal pole to the caudal aspect of the occipital pole.

**Tissue Processing - Microarray**

All experiments were conducted according to international ethical standards and approved by the Institutional Animal Care and Use Committee of Tufts University (Protocol B2013-20). Pregnant females were anesthetized with 2.5% isoflurane in a 3/7 O₂/N₂O mixture and euthanized by decapitation at e15.5. Embryos were extracted, identified as e15.5 using Theiler staging (http://www.emouseatlas.org) and decapitated in ice-cold 1x PBS. Embryonic brains were rapidly removed and brain hemispheres dissected on a cold platform and snap frozen in liquid nitrogen before storage at -80°C.

**Tissue Processing - Histology**

After 24 h of PFA fixation, fetal and P15 brains were cryoprotected in 30% sucrose, 1x PBS for 24 h at 4°C followed by freezing in Optimal Cutting Temperature Compound (OCT; Sakura, Torrance, CA), and stored in OCT at -80°C. 16-μm serial coronal sections were obtained using a cryostat (Microm HM 560), mounted on Superfrost ® Plus Microscope slides (Fisher scientific) and stored at -80°C.

**Neocortical and Hippocampal Layer Measurements**

Layer measurements were performed following a similar protocol employed in our previous studies (Chakrabarti et al., 2007, 2010) at the level of the dorsal hippocampus (future sensorimotor cortex). This level corresponds to E13/E14 plate 5 in the work by
Jacobowitz and Abbott (1998). Briefly, embryonic brain sections were incubated in 1 mM TO-PRO®-3 (Invitrogen), 20x PBS for 10 min at room temperature (RT) followed by 2 min washes in 20x PBS. Vectashield with DAPI (Vector Laboratories) was added to all slides that were then mounted and sealed. Slides were then scanned with confocal microscopy as detailed below. We quantified the thickness of the ventricular and subventricular zones (VZ/SVZ), the intermediate zone (IZ), cortical plate (CP), and total pallial thickness. The VZ/SVZ was measured from the ventricular border to the border between densely packed oblong nuclei of the SVZ and the cell sparse IZ. The CP was measured from the border of the IZ to the marginal zone. CP measurements began at e14.5. Measurements of the prenatal hippocampus were collected at e15.5 and e18.5. The thickness of the ventricular zone and hippocampal wall were quantified at both ages, while the pyramidal layer was measured at e18.5 as this layer begins to develop at e16.5. It should be noted that these measurements are specific to the future somatosensory cortex and the dorsal hippocampus and cannot necessarily be extrapolated to the entire telencephalon.

**Immunohistochemistry**

Frozen slides were allowed to come to RT and rinsed in 1x PBS. Antigen retrieval was performed on embryonic slides by microwaving in 10 mM sodium citrate for 1 min at maximum power, followed by 10 min at the minimum power. Slides were incubated in blocking solution (5% donkey serum, 0.2% Triton® X-100, 1x PBS) for 1 h at RT followed by incubation in primary antibody overnight at RT. The next day, slides were washed three times in 1x PBS and incubated with secondary antibody solution for 1 h at
RT followed by three washes in 1x PBS. Slides were mounted in Vectashield with DAPI (Vector laboratories, inc., H-1200). Primary antibodies: rabbit anti-Tbr2 1:500 (abcam 23345), goat anti-Sox2 1:250 (Santa Cruz, sc17320), rabbit anti-pH3 1:500 (millipore 06-570), rat anti-Somatostatin (SOM) 1:50 (Millipore, MAB354), rabbit anti-Parvalbumin (PV) 1:1000 (Swant, PV25), rabbit anti-Calretinin (CR) 1:1000 (Swant, 769913), and rabbit anti-Tbr1 1:1000 (gift from Hevner laboratory). Secondary antibodies: 1:250 for all; donkey anti-rabbit 555 (life technologies, A31572), donkey anti-goat 488 (life technologies, A11055), goat anti-rabbit 546 (life technologies, A11035), goat anti-rabbit 488 1:500 (life technologies, A11008) and goat anti-rat 568 1:500 (life technologies, A11077).

**In vivo Neurogenesis**

At e13.5, pregnant C57BL6/N females carrying offspring from Dp16 males were injected with EdU (50 mg/kg body weight, life technologies, C10425) in 4% saline and sacrificed 24 h later. Embryos were collected and tissue processed as described above. EdU slides were post-fixed (4% PFA in 1x PBS) for 15 min at RT. Fixative was removed and slides were washed in 1x PBS followed by 3% BSA in 1X PBS. Slides were incubated in 0.5% Triton® X-100 in 1x PBS for 20 min at RT followed by a 5 min wash in 1x PBS. Slides were briefly washed in 3% BSA 1x PBS and then incubated in Click-iT® reaction cocktail for 30 min at RT. Click-iT® reaction cocktail was prepared fresh and used within 15 min of preparation and made as follows (amounts per one slide): 438 µl 1x PBS, 10 µl CuSO₄, 2.5 µl fluorescent dye azide (azide-488), 50 µl 1x Reaction Buffer Additive. Reaction cocktail was then removed and slides were washed in 3% BSA
in 1x PBS. Slides were mounted using Vectashield DAPI (Vector laboratories) and sealed.

**Confocal Microscopy and Cell Counting**

All sections were imaged with confocal microscopy using a Zeiss LSM 710 microscope system (Carl Zeiss, Jena). Twelve micron, 20x z-stacks were acquired using Zeiss Zen software (See Appendix for expanded Confocal Microscopy methods). 3-D image volumes of progenitor cell and EdU immunohistochemistry were analyzed using Volocity (Improvision) software using an automated counting algorithm following validation with manual counts on selected sections. All other cell counts were performed manually using ImageJ and LSM Browser software. It should be noted that while these systematic counts were conducted on volumetric images using a counting frame, they were not acquired using randomly sampled unbiased stereological standards and therefore cannot necessarily be extrapolated to the entire telencephalon. The counts are specific to the future somatosensory cortex in embryonic tissue and the somatosensory cortex in postnatal animals. Progenitor population distribution was determined using Volocity software to measure object distances from the ventricular surface and grouped into 20 μm bins. Mitotic (pH3+ labeled) cell distribution in the ganglionic eminence was performed by 20 μm bins from the ventricular surface and cells were counted manually within bins. The experimenter was blind to genotypes.

**Embryonic Brain RNA Isolation and Microarray Experiments**

Total RNA was isolated from brain hemispheres of Dp16 embryos ($n=6$), their euploid littermates ($n=6$), Ts65Dn embryos ($n=5$) and their euploid littermates ($n=6$)
using the RNA II kits, following the manufacturer’s instructions (Macherey-Nagel, Bethlehem, PA). RNA was processed for hybridization to Affymetrix mouse gene 1.0 ST arrays (Affymetrix, Santa Clara, CA) as described previously (Guedj et al., 2015a). Twenty-three arrays were analyzed (6 Dp16, 6 eup, 5 Ts65Dn and 6 eup), and each array corresponded to labeled RNA from one individual embryo. Analyses were performed using Benjamini-Hochberg False Discovery Rate (BH-FDR) of 20% as a cut-off to allow for a direct comparison of differential gene expression in Dp16 embryos to previously published work in the Ts1Cje mouse (Guedj et al., 2015a) and to increase the identification of potentially dysregulated genes for further pathway analysis. It should be noted that this approach may result in a slight increase in the number of false positive results. Statistical analyses were carried out on the normalized data using R software (version 2.13.1). Gene expression data from Dp16 and Ts65Dn tissues were compared to their respective euploid littermates using an unpaired t-test.

**Behavioral Assessment:**

**Developmental Milestones (B6129S-Dp (16Lipi-Zfp295)1Yey/J)**

Dp16 males obtained from Jackson Laboratory were backcrossed to C57BL/6J females for 2 generations. Dp16 pups and euploid littermates from both genders were subjected to a comprehensive set of neonatal behavior tests to measure different sensory and motor developmental parameters between P3 to P21. Specifically, we analyzed fifteen male and sixteen female euploid mice and eight male and eight female Dp16 mice. During the testing period, pups were separated from the dam and placed with nesting material in a small bowl positioned on a heating pad at 37°C. A heat lamp was also placed over the mice
to provide heat from above. We used the Fox scale as described by Hill et al. (2008) to investigate developmental milestones in Dp16 neonates ($n=16$) versus euploid littermates ($n=31$). The Fox scale is a battery of tests that measure: 1) body righting and coordination (surface righting, air righting and negative geotaxis), 2) strength (cliff aversion and forelimb grasp), 3) sensory system maturation (rooting, auditory startle, ear twitch and eye opening), 4) and extinction of rotatory behavior (open field). The amount of time (latency) or presence/absence of reflex was recorded and analyzed by a single experimenter who was blind to animal genotypes.

**Nest-Building**

Mice were individually housed in clean plastic cages supplied with 3g of Nestlet material (pressed cotton squares) at 6pm, one hour prior to the dark phase of the lighting cycle. The next morning (14-15 h later) cages were assessed for nest construction and untorn material. Nest construction was scored as described previously (Deacon, 2006). Briefly, a 5-point scale was used: 1= >90% intact Nestlet, 2= Nestlet partially torn up, 3= Nestlet mostly shredded but no identifiable nest site, 4= an identifiable but flat nest, 5= Nestlet torn >90% and a clear nest crater. The experimenter was blind to the genotype. Euploid $n=13$, Dp16 $n=11$.

**Marble Burying**

We followed the protocol published by Deacon, 2006. Briefly, clean cages (30 x 17 x 14 cm) were filled 5 cm deep with bedding material, lightly tamped down to make a flat, even surface. A regular pattern of glass marbles was placed on the surface, 5 rows
with 3 marbles in each row evenly spaced, about 4 cm apart. The number of marbles buried (to 2/3 their depth) with bedding were counted after 30 min for each animal.

**Spontaneous Alternation**

Spatial working memory was assessed using the continuous variant of the spontaneous alternation procedure, as described previously (O’Tuathaigh et al., 2007; Desbonnet et al., 2012; Olmos-Serrano et al., 2016). Briefly, without prior habituation, animals were placed individually in the center of the Y-maze and allowed to explore freely for 6 min. A video camera mounted above the maze recorded each session and allowed for the analysis of spontaneous alternation, number of arm entries, distance traveled and velocity of movement using Ethovision software (Noldus). Spontaneous alternation was defined as successive entries into the three arms of the Y-maze, in overlapping triplet sets, with arm choices differing from the previous two choices expressed as a percentage of the total number of arm entries: percent alternation = [(number of alternations/total number of arm entries) − 2] × 100 (chance level = 50%).

**Open Field**

Locomotion was measured as the spontaneous activity of mice in a novel open square arena (60cm x 60cm) Mice were tracked using the Ethovision tracking system (Noldus, Actimetrics, Inc., Wilmette, IL). Mice were tracked for 1 h, and the distance traveled and velocity was electronically recorded. We also analyzed the time spent in the border and in the center of the maze within 10 min bins.

**Hind-limb Extension Reflex Test**
To monitor hind-limb extension, mice were suspended by the tail for 10 sec and scored according to the following scale; a score of 2 corresponds to mice that generally place their legs in a wide “V” away from their bodies with splaying of toes. A score of 1 corresponds to an extension reflex in only one hind-limb or extension of both hind-limbs, without splayed toes. A score of 0 corresponds to clenching of toes and hind-limbs to the body. Two tests were performed on consecutive days and a third test was performed one week later. Each test was repeated twice the same day, one h apart. The final score for each mouse was the average of the six tests.

**Hanging Wire**

A standard wire cage lid was used. Duct tape was placed around the perimeter of the lid to prevent the mouse from walking off the edge. The animal was placed on the top of the wire cage lid. The investigator shook the lid lightly three times to cause the mouse to grip the wires, and then the lid was turned upside down. The upside-down lid was held at a height of approximately 20 cm above the cage, high enough to prevent the mouse from easily climbing down but not high enough to cause harm in the event of a fall. A stopwatch was used to measure the latency to fall off the wire lid. A 180-second cutoff time was used for the session.

**Water T-Maze**

The Water T-maze used was constructed from Plexiglas. Each arm was 20 cm long and 7 cm wide. The T-maze was filled with tap water and made opaque with white non-toxic paint (Prang). The temperature of the water was 22°C. The platform is a 5 cm x 5 cm square made from Plexiglas and was submerged during the experiments. Mice were
given ten trials per day with an inter-trial interval (ITI) of approximately 5-8 min. Mice were released from two different locations (N or S) and were trained to find the location of the submerged platform in one of the arms (E). When mice were released from S or N, the other arm was blocked with a Plexiglas rectangle. Mice therefore had only two choices to find the platform in each trial. Mice scored an error (0) when entering two-thirds of the wrong arm while a correct choice (1) was scored when they found the platform. Once mice reached between 80-100% of correct choices, the platform was moved to the opposite arm for reversal learning (W). When mice reached 80-100% of correct choices, the platform was moved again to its first location (E) for assessing double reversal learning. Any errors made into the incorrect arm before the mouse successfully found the platform on its own were counted as perseverative errors. Any errors made after an errorless trial were counted as regressive errors. The time spent to find the platform in the first two trials for reversal and double reversal learning was also measured as an index of cognitive flexibility.

**Morris Water Maze**

Spatial learning and reversal were assessed in the Morris water maze using previously described procedures and equipment (Yang et al., 2012). The apparatus was a circular pool, 125 cm in diameter, filled with 24°C tap water rendered opaque with the addition of non-toxic white paint (Prang). To facilitate spatial learning, room cues made of black and white cardboard were added to the walls surrounding the pool. Trials were videotaped and scored with Ethovision video tracking software (Noldus, Actimetrics, Inc., Wilmette, IL). The order of training was: cued submerged platform trials, hidden
platform trials and a final probe trial with the platform removed. Cued, acquisition and reversal training consisted of four trials a day for 4, 9 and 9 days, respectively. Each training trial began by lowering the mouse into the water close to the pool edge, in a quadrant that was either right of, left of, or opposite to, the target quadrant containing the platform. The start location for each trial was alternated in a semi-random order for each mouse. For cued training, the pool was surrounded by white curtains to occlude sight of extra-maze clues. The platform was cued by means of a metal stick attached to a 5 cm high black ball placed onto it and placed pseudo-randomly in different locations across trials. For the acquisition and reversal training, the hidden platform remained in the same quadrant for all trials for a given mouse. Mice were allowed a maximum of 60 sec to reach the platform. A mouse that failed to reach the platform in 60 sec was guided to the platform by the experimenter. Mice were left on the platform for 15 sec before being removed. After each trial, subjects were put in a cage lined with absorbent paper towels. To confirm that the spatial learning task was acquired after acquisition and reversal training by using distal environmental room cues, subjects were tested in a 60 sec probe trial, 24 h after the completion of the last training session. Reversal training began the next day after the probe test for acquisition training. The hidden platform was moved to the quadrant opposite to its location during acquisition training. Parameters recorded during training days were latency to reach the platform, total distance traveled, swimming speed and thigmotaxis. Parameters recorded during the probe trial were (a) time spent in each quadrant, (b) number of crossings over the trained platform location and (c) proximity as described in Maei et al, 2009.
**Statistical Analysis**

Statistical analyses were performed using Sigmaplot software. For gross measurements and neocortical layer measurements, two-way repeated measure ANOVA were used. For all other histological and immunohistochemical assessments, unpaired $t$-tests were performed to determine statistical significance. For behavioral tests, two-way repeated measure ANOVA and post-hoc Tukey test were used. For developmental milestone analyses, non-parametric Mann-Whitney and Wilcoxon signed-rank tests were used for single and repeated measures, respectively, to determine significant differences between the Euploid and Dp16 groups at a p-value of 0.05. Fisher Exact test (FET) was used to determine differences between data points.
RESULTS

Normal gross embryo and brain size in Dp16 embryonic development

Previous findings in fetuses with DS and from several mouse models, including Ts65Dn and Ts1Cje, reported a significant delay in brain and body growth that are accompanied by decreased neural precursor cell proliferation and slower neurogenesis (Hartley, 1986; Wisniewski, 1990; Chakrabarti et al., 2007; Ishihara et al., 2010). We therefore used the same methods as in previous Ts65Dn studies (Chakrabarti et al., 2007, 2010) to investigate these developmental processes in Dp16. We started by measuring both the crown-rump length as well as expansion of the telencephalon along the rostro-caudal and medial-lateral axes to assess body size and brain growth, respectively (Fig. 3). We found that Dp16 fetuses were similar in size to euploid littermates throughout gestation (Fig. 3A, B). Moreover, Dp16 brain hemispheres were comparable to euploid littermates along both the medial-lateral and rostro-caudal axes (Fig. 3C-E). For all gross morphology metrics there was no difference by genotype. To closely examine Dp16 brain growth, the thickness of the neocortical layers was measured in the prospective somatosensory cortex from e13.5 to e18.5 using the TO-PRO-3 nuclear stain (Fig. 4A, G, H). No differences were found in the thicknesses of the ventricular/sub-ventricular germinal zones (VZ/SVZ), intermediate zone (IZ), cortical plate (CP) nor in overall neocortical thickness across the e13.5-e18.5 time window in Dp16 brains (Fig. 4B-E). We also quantified the thickness of the distinct layers of the developing hippocampus (Fig. 4H-K). Similarly, to the neocortex, hippocampal layer thickness did not vary
between genotypes in all layers considered (Fig. 4I-K). These data demonstrate that prenatal forebrain growth alterations are not observed in Dp16 mice.

The absence of gross anatomical prenatal phenotypes is in stark contrast to previous reports in Ts65Dn (Chakrabarti et al., 2007). However, the Ts65Dn mice in our previous studies were maintained on a hybrid background (C57BL/6 x C3HSnHeSnJ) using trisomic carrier females and F1 hybrid males while Dp16 animals were maintained on a C57BL/6 background and trisomy was passed from the males. To assess whether background strain and trisomic carrier contribute to the lack of prenatal findings in Dp16, we crossed Dp16 animals onto the same hybrid background used in the Ts65Dn study using either male carriers with F1 hybrid females or female carriers with F1 hybrid males. We found no difference in gross brain measurements or neocortical lamination in Dp16 animals on the B6.C3Sn hybrid background at either age observed (e14.5, trisomic father; e15.5 trisomic mother; Fig. 3F, Fig 4F). This suggests that background strain and trisomic inheritance do not influence the lack of embryonic phenotypes observed in Dp16. As such, further histological experiments were performed using the C57BL/6 background.

**Normal development of neural precursors in the dorsal and ventral telencephalon in Dp16**

To more closely evaluate the germinal zones in the developing Dp16 neocortex just as we did previously in Ts65Dn (Chakrabarti et al., 2007; Chakrabarti et al., 2010), we quantified neuronal precursor sub-types in both the dorsal telencephalon (the source of excitatory neurons) and in the medial ganglionic eminence (MGE) area of the ventral
Dorsal telencephalic embryonic sections from e13.5 to e18.5 were immunostained for SOX2 and TBR2 to label apical precursors (aPCs) and basal intermediate precursor cells (bIPC), respectively, and with DAPI to label the entire cell population (Fig. 5A and A’). The numbers of aPCs, bIPCs as well as the combined precursor populations were similar between Dp16 and euploid controls throughout the neurogenesis period (Fig. 5B, C, respectively; combined population data not shown). We also examined the spatial distribution of individual bIPCs and aPCs by measuring their distance from the ventricle and found no difference in either population across genotypes (Fig. 5D, e13.5; data not shown for other embryonic ages). The progenitor populations located in the MGE were immunostained for the transcription factor OLIG2 (Fig. 5E) and with DAPI to label the entire cell population at e14.5. No detectable difference was observed in the numbers of OLIG2+ neural progenitor cells in Dp16 brains (Fig. 5F).

To assess proliferation in the dorsal embryonic neocortex and MGE, brain sections were labeled for actively dividing cells with the M-phase marker phosphorylated histone H3 (pH3, red, Fig. 6A, D). Overall, the actively dividing cell population did not vary across genotypes from e13.5 to e18.5 in both neocortex and MGE (Fig. 6B, C, E, F), indicating that the number of cells undergoing mitosis during various windows of development do not vary between genotypes.

*Normal Neurogenesis in Dp16 embryonic neocortex*
To assess neurogenic output in Dp16 embryonic neocortex as done previously in Ts65Dn (Chakrabarti et al., 2007), e13.5 pregnant C57BL/6 females were injected with the thymidine nucleoside analog 5-ethynyl-2'-deoxyuridine (EdU, 50 mg/kg) which labels cells in S-phase. Embryos were collected 24 h later and EdU-labeled cells were counted in the VZ/SVZ, IZ and CP. The cells outside of the VZ/SVZ were counted to estimate the neuronal output from the germinal zones during the 24 h window (Fig. 6G). The distribution and number of EdU-labeled cells were similar between Dp16 and Euploid indicating that neuronal production defects are not present in the prenatal Dp16 neocortex (Fig. 6H, I). Altogether, these prenatal studies show that the Dp16 forebrain matures without any apparent delays.

**Transcriptome Analysis of Hsa21 Homologs**

In order to determine whether gene expression differences may underlie the disparity between Dp16 and Ts65Dn prenatal phenotypes, we compared gene expression in e15.5 forebrain from Dp16 and Ts65Dn animals. Specifically, we analyzed genes localized to the triplicated segments in both Dp16 and Ts65Dn (Fig. 7). Unexpectedly, the number of differentially expressed (DEX) genes was larger in Ts65Dn (46 DEX genes) than Dp16 (19 DEX genes) (Fig. 7A, B) and only 1 of the 17 triplicated genes unique to Dp16 was differentially expressed at e15.5 (Fig. 7A, B). These results show that, despite having a larger number of Hsa21 syntenic genes, fewer of these genes are dysregulated in the Dp16 fetal brain compared to Ts65Dn.

Given the normal prenatal growth and comparatively normal gene expression profile in Dp16 fetuses as well as the prior demonstration of postnatal morphological,
electrophysiological and behavioral deficits in Dp16 (Yu et al., 2010; Starbuck et al., 2014), we conducted a detailed examination of neonatal and postnatal cognitive and motor function to determine the magnitude and timing of postnatal deficits in Dp16.

**Delayed growth and late-acquisition developmental milestones in Dp16**

Delays in the achievement of developmental milestones in the first year of life are a hallmark of DS (Horovitz and Mason, 2011) and have been previously measured in Ts65Dn (Toso et al., 2008; Olmos-Serrano et al., 2016). To investigate early postnatal development in Dp16 and euploid animals, we performed a daily assessment of growth profiles and developmental milestones in mice of both sexes from P3 to P21 (Fig. 8). These assessments included body weight and body length as well as motor strength, coordination and acquisition of neurological reflexes. Analysis of growth profiles revealed that Dp16 animals displayed a significant postnatal growth delay ranging between 13-29% throughout the pre-weaning period (Fig. 8A). Similarly, tail base and total body length were significantly smaller in Dp16 compared to euploid littermates (**p<.0005, Fig. 8B and data not shown). The growth delay and total body length were also significantly different by sex (data not shown).

The developmental milestone tests showed that Dp16 mice exhibited a significant delay in late-acquisition milestones (primarily after the second postnatal week) relating to gross motor development and neurological reflexes (Fig. 8C-J; FET, *p<0.05). The greatest delays were observed in air righting (AR), auditory startle (AS) and ear twitch (ET) (Fig. 8G, I, J; FET, *p<0.05). The first day of appearance of AR was delayed approximately 3 days for Dp16 while AS and ET were delayed between 1.5-2 days (Fig.
Results were similar when both sexes were examined separately, but a delay in negative geotaxis and eye opening was observed only in Dp16 females (data not shown). We did not find significant differences in the first day of acquisition for the other tests performed.

**Neuronal population abnormalities in P15 Dp16 mice**

Prior work in DS human brain and Ts65Dn mice has elucidated a shift in the excitatory: inhibitory ratio, indicating higher inhibition in the trisomic forebrain. In particular, fewer excitatory neurons and more numerous inhibitory neurons are found in the Ts65Dn neocortex and hippocampus (Chakrabarti et al., 2010). We therefore sought to determine if similar cellular changes are found in Dp16 forebrain when the developmental milestone delays become apparent. We selectively labeled subsets of inhibitory interneuron and excitatory neuron populations in the somatosensory cortex (SSCtx) of P15 Euploid and Dp16 animals. Antibodies to three calcium-binding proteins were used to label subsets of interneurons including, Parvalbumin (PV), Somatostatin (Sst) and Calretinin (Cr) (Fig. 9A, B). We found that the number of PV+ inhibitory interneurons in the Dp16 somatosensory cortex is reduced at P15 (**p=.014; Eup n=4, Dp16 n=5, Fig. 9A, D) and there are also fewer Sst+ labeled cells (*p=.041; Eup n=4, Dp16 n=5, Fig. 9B, D). The Cr+ population density was unchanged in Dp16 animals (Fig. 9B, D). The density of cortical excitatory cells labeled by the transcription factor Tbr1+ was also reduced in Dp16 SSSctx, approaching statistical significance (p=.07; Fig. 9C, E). Thus, despite a lack of prenatal changes in neurogenesis or cortical expansion, Dp16 mice exhibit changes in cortical cell populations at the onset of the developmental
milestone delays. However, these findings are significantly different than in the brains of Ts65Dn mice in which an abnormally large population of forebrain inhibitory neurons is found.

**Impaired adult behavior in Dp16 mice**

Behavioral assessments of adult mouse models of DS have been extensively documented (Escorihuela et al., 1995; Coussons-Read and Crnic, 1996; Sago et al., 1998; Martinez-Cue et al., 2005; Costa et al., 2010; Garcia-Cerro et al., 2014). Morris water maze (MWM) tests have also been performed previously on Dp16 animals (Zhang et al., 2014). This latter study suggests that adult Dp16 mice exhibit similar deficits to other models in hippocampal-based spatial working memory. To gain further clarity on the extent of neurological abnormalities in adult Dp16 mice, we performed a rigorous series of tests to address typical behaviors, motor function and cognitive ability.

**Nest-building and marble burying**

Analysis of overnight nest-building using a detailed nesting scale score and measuring untorn nestlet material revealed that nesting behavior is impaired in Dp16 compared to euploids (Fig. 10A; nest score, \*p<0.05). Interestingly, all animals from both groups tore the entire material provided, but the Dp16 nests were left largely incomplete. To determine whether this difference between groups may be related to slower nest building in Dp16 mice, we examined the time course of this process. We persistently observed abnormal nesting behavior in Dp16 cages overnight (O) + 24 h, O + 96 h and even O + 20 days (Fig. 10A; \*p<0.05, \*\*p<0.01, \*\*\*p<0.01, respectively). While Dp16 mice were able to build nests and showed improvement over time, they did not
reach the nest quality achieved by the euploids, i.e., a rounded nest with high, well 
assembled walls. We also assessed digging behavior with the marble burying test. We 
found that Dp16 animals dug significantly more and buried more marbles than euploid 
mice (*p<0.05), indicating abnormal repetitive behavior (Fig. 10B).

**Spontaneous Alternation**

Spontaneous alternation in a novel environment was assessed using a Y-maze (3 
arm choices). Mice normally have a strong tendency to alternate arm choices (Lalonde, 
2002). Our analysis of spontaneous alternation revealed no significant differences 
between groups (Fig. 10C). Further analysis of arm entries, distance traveled and velocity 
did not reveal significant differences between genotypes (Fig. 10D-F).

**Motor assessment**

Three different behavioral tests were performed to test motor function in Dp16 
mice. For the assessment of general and spontaneous motor activity, both euploid and 
Dp16 adults were observed in a novel open field for one hour. Overall, Dp16 animals 
traveled less distance and exhibited decreased locomotion speed (Fig. 11A, B; p = 
0.0110, p = 0.0124, respectively. Although no difference was seen in distance or speed 
between genotypes during the first 10 min of the test, Dp16 mice traveled less distance 
and exhibited decreased speed from 20 min on (Fig. 11A, B; *p<.05). The time spent in 
the border versus the center of the arena was then used as an indirect measure of anxiety. 
Significant differences between groups were apparent when these different locations were 
analyzed over 10 min intervals (Fig. 11C, F\(_{5,22}\) = 3.038, \(p = 0.013\); F\(_{5,22}\) = 3.038, \(p = 
0.013\), respectively). Dp16 animals spent more time in the border and less time in the
center from 20 min on (Fig. 11C; \( *p<0.05 \)) and had fewer crossings between the border and the center from 20 min on (Fig. 11D; \( *p<0.05 \)). Representative traces of both genotypes during the first 20 min and in the entire hour illustrate this propensity to remain in the border region (Fig. 11E-H).

For further assessment of motor function in Dp16, hind limb extension reflex was tested, revealing a significant impairment in Dp16 animal performance (Fig 11I, \( **p<0.001 \)). Specifically, while Dp16 animals properly extended both hind limbs when lifted by their tails, they failed to properly splay their toes. Lastly, the hanging wire test was performed to test motor strength and balance. Latencies to fall were significantly decreased in Dp16 mice (Fig. 11J, \( ***p=0.002 \)). Altogether, the results from this battery of motor tests indicate that adult Dp16 mice exhibit motor weakness and altered reflexes, as well as a tendency towards anxious behavior.

**Cognitive assessment**

We then used two water mazes to assess learning and memory in Dp16 adults. In the water T-maze experiments, learning and memory were tested during acquisition, reversal (R) and double reversal (DR) periods. We found that while both groups improved their performance over the acquisition period (Fig. 12A; \( F_{3,22}=34.9018, p<0.001 \)), there were significant differences between genotypes in the percentage of correct choices during acquisition (\( F_{1,22}=13.990, p=0.001 \)). In particular, Dp16 animals performed worse than euploids in the first two days of the acquisition period (Fig. 12A; \( p<0.05 \)). Interestingly, when the platform was moved to the opposite arm, both groups improved their performance over the 3-day R period (\( F_{2,22}=64.586, p<0.001 \)) and no
significant differences between genotypes were found ($F_{1,22} = 0.904, p = 0.352$). Both groups also improved their performance over the 3-day DR ($F_{2,22} = 75.572, p < 0.001$) but during this period Dp16 animals had fewer correct choices ($F_{1,22} = 12.291, p = 0.002$), most evidently in the first two days of the DR period (Fig. 12A; $p < 0.05$). We also assessed latencies to find the platform during trial 1 and trial 2 of the first day of the R and DR periods. Interestingly, we found a significant difference between groups on trial 2 of the first day of the DR (Fig. 12B; ** $p<0.01$). Euploid mice improved in performance between trials 1 and 2 of the double reversal ($p < 0.001$) while the Dp16 did not ($p = 0.510$).

Spatial learning and memory was also measured using the MWM. Animals were first tested in a cued learning protocol to assess their ability to swim to a visible goal. Both genotypes learned to swim towards a submerged platform identified by a flag, significantly decreasing their latency over 4 days (Fig. 12 C-F; $F_{3,22} = 98.174, p < 0.001$). Two-way repeated measures ANOVA revealed no significant difference between genotypes in the latency to find the cued platform, distance traveled or ($F_{1,22} = 0.343, p = 0.564$; $F_{1,22} = 1.595, p = 0.220$; $F_{1,22} = 0.0994, p = 0.755$, respectively). Dp16 animals swam more slowly than Euploids to the visible platform ($F_{1,22} = 10.795, p = 0.003$), but this did not affect their performance. Over the following 9 days, mice were tested for spatial learning to a hidden platform (Fig. 12 G-H). Both groups learned the hidden platform location during this period, decreasing their latency and swimming distance (latency, $F_{3,22} = 13.542, p < 0.001$; distance, $F_{3,22} = 14.614, p < 0.001$). However, there was a significant difference between genotypes in these two measures (latency, $F_{1,22} =$
9327, \( p = 0.006; \) distance, \( F_{1,22} = 4.555, p = 0.044 \). Post-hoc Tukey test comparisons indicated that Dp16 performed particularly worse on days 1 and 5 in both latency and distance (Fig. 12G, H; \(*p < 0.05\)). There was no overall difference between genotypes in swimming speed (data not shown), and neither genotype exhibited thigmotaxic behavior (data not shown). Interestingly, learning a second hidden platform location in the reversal phase revealed strong deficits in Dp16 in latency and distance swam (Fig. 12G-H; latency, \( F_{1,22} = 55.569, p < 0.001; \) distance, \( F_{1,22} = 29.364, p < 0.001 \)). Importantly, no difference was seen in swimming speed between groups (\( F_{1,22} = 2.293, p = 0.144 \)). Representative traces of swimming paths clearly show that Dp16 exhibited a greater impairment when searching for the platform during the reversal period (Fig. 12I, J). At the end of both the acquisition and the reversal periods, the platform was removed and mice were allowed to swim freely for 60 seconds in a probe trial test for reference memory. Both probe trials revealed a selective quadrant search indicating proper memory consolidation of the platform location (Fig. 12K, L; \( F_{3,22} = 92.886, p < 0.001; F_{3,22} = 75.616, p < 0.001 \)). In particular, both euploid and Dp16 spent more time in the proper SW and NE quadrants in the acquisition and reversal periods, respectively (Fig. 12K, L; \(*p < 0.05\)). However, Dp16 spent significantly less time in the proper quadrant during the reversal probe trial indicating memory deficits in Dp16. We also found significant differences between genotypes in proximity and the number of virtual platform crossings for both the initial 30 sec and the entire 60 sec during the probe trial in the reversal period (Fig. 12M, N; \( **p < 0.01 \)). Overall, these results demonstrate that Dp16 animals exhibit learning and memory deficits specific to memory extinction and re-learning.
DISCUSSION

In this study, we provide the most comprehensive cellular and behavioral analysis of the Dp16 mouse to date. Strikingly, we show that many key processes of prenatal forebrain development are normal in Dp16. In particular, normal neural precursor proliferation and neurogenesis rates lead to proper forebrain growth during the fetal period. In a comparative transcriptomic study, we also surprisingly found that gene expression in Dp16 embryonic forebrain was significantly less altered than the Ts65Dn forebrain, despite the fact that Dp16 animals harbor more DS-relevant triplicated genes. This unexpected lack of fetal brain morphometric and cellular phenotypes is in stark contrast to the deficits found in humans with DS and in several other mouse models of DS. However, despite this relatively normal prenatal development, we found altered neurological reflexes as early as 2 weeks after birth followed by impaired adult motor and cognitive performance. We also measured significant changes in the numbers of inhibitory forebrain neurons, suggesting that discrete cellular abnormalities, only evident after birth, may underlie the motor and cognitive deficits in Dp16 animals. Therefore, while these results suggest that Dp16 animals may be used to study the contribution of perinatal and postnatal development to intellectual disability in DS, they also raise important questions about the degree to which prenatal deficits contribute to the impaired cognition in mouse models of DS.

Do Dp16 results challenge the developmental dogma of DS?

Transcriptomic and morphometric studies have shown that altered gene expression is present during DS fetal development and is accompanied by changes in
CNS growth and differentiation (Golden and Hyman, 1994; Contestabile et al., 2007; Guidi et al., 2008, 2011; Guedj et al., 2015a, b). Although specific ties between the prenatal phenotypes and the cognitive function have not been elucidated, it is widely accepted that prenatal changes at least partly underlie the intellectual disability in DS (Haydar and Reeves, 2012). This association has been strongly supported by previous studies on several mouse models, particularly Ts65Dn, Ts16 and Ts1Cje, which display defects in neurogenesis and neocortical growth during embryonic forebrain development similar to those seen in fetal DS human brain (Haydar et al., 1996, 2000; Roper et al., 2006; Chakrabarti et al., 2007; Contestabile et al., 2007; Ishihara et al., 2010; Tyler and Haydar, 2013). However, it is important to caution that the aforementioned models of DS are widely different both genetically and phenotypically; also, each one of these models expresses one or more dissimilarities to humans with DS (Das and Reeves, 2011). It is therefore necessary to carefully evaluate the incongruities between the models and their relevance to the human disorder.

This divergence between Dp16 and Ts65Dn prenatal brain growth should be interpreted cautiously as several factors differ greatly between the models and may contribute to the apparent phenotypic differences. First, Ts65Dn is one of few DS models to harbor triplicated genes in a freely segregating chromosome, mirroring the aneuploidy in most cases of DS. The extent to which aneuploidy alone contributes to DS phenotypes is not well understood but it is likely to play a significant role since the presence of any extra, freely segregating chromosome has been shown to alter cellular division and cell cycle kinetics in vitro (Stingele et al., 2012). Furthermore, autosomal trisomies have been
reported to impact social and cognitive development in nonhuman primates (de Waal et al., 1996). Since Dp16 was engineered using the Cre/Lox system to attach its 22.9 Mb duplication to the distal end of one endogenous copy of Mmu16, the resulting genetic triplication is carried as a duplication and not as an extra chromatid. The duplication event in Dp16 may not disturb cell division as much as aneuploidy. The duplication may also impact epigenetic modification or chromatin states, potentially influencing expression of triplicated genes. In considering this latter possibility, it is interesting to note that the triplication in Ts1Cje animals (that do exhibit abnormal brain development and proliferation defects) is caused by a duplication similar to that found in Dp16, except that the duplicated segment is shorter and present on Mmu12. Thus, if the duplication in Dp16 underlies its normal prenatal growth, differences between Dp16 and Ts1Cje in epigenetic regulation are likely to exist. Supporting this possibility, compared to Dp16, Ts1Cje animals differentially express more triplicated genes (39 vs. the 19 in Dp16) in the e15.5 forebrain (present results and Guedj et al., 2015a). Finally, Ts65Dn mice are trisomic for 60 non-syntenic genes near the Mmu17 centromere (Duchon et al., 2011). Although any causal relationship between these non-syntenic genes and the phenotypes observed in Ts65Dn is still unknown, their potential contribution to the prenatal abnormalities in Ts65Dn must be taken into account. Therefore, these findings highlight how different mouse trisomies can result in distinct phenotypes and that each mouse model may replicate different aspects of DS brain development and function.

*The relevance of postnatal deficits in Dp16 to DS cognition*
Studies in infants with DS have reported delays in the achievement of developmental milestones during the first year of life (Hartley, 1986; Wisniewski, 1990; Nilholm, 1999; Vicari et al., 2013; Cardoso et al., 2015) and several mouse models, including Dp16 (present results), exhibit milestone delays (Holtzman et al., 1996; Toso et al., 2008; Guedj et al., 2015; Olmos-Serrano et al., 2016). While our results illustrate some similarities between Dp16 and infants with DS such as lower growth rates, Dp16 neonatal motor development was normal during the first two weeks. Interestingly, the milestones altered in Dp16 were the latest to develop, such as auditory startle and air righting. This differs from observations in the Ts65Dn which exhibit significant delays in both motor and sensory milestones within the first postnatal week (Holtzman et al., 1996; Toso et al., 2008; Olmos-Serrano et al., 2016), indicating that these earliest milestones may be affected by altered prenatal brain development. Our cellular measurements in the postnatal Dp16 neocortex have uncovered significant alterations in neuronal allocation, demonstrating the need to longitudinally profile cellular, molecular and electrophysiological development in the perinatal neocortex in future studies. Together, these findings identify a potential ‘critical window’ after birth during which behavioral and cellular phenotypes begin to manifest in Dp16, and suggest that Dp16 may be a useful model of how perinatal developmental changes caused by trisomy contribute to cognitive dysfunction.

Individuals with DS exhibit cognitive impairment from early infancy into adulthood. Similar to our results in the Dp16, learning and memory tests on several mouse models of DS have consistently demonstrated cognitive impairment in adults
(Stasko and Costa, 2004; Seregaza et al., 2006; Herault et al., 2012). Notably, our study extended these findings by employing two different paradigms and longer testing periods than previous reports, demonstrating that Dp16 impairments become more profound as the task complexity increases. As such, our study uncovers novel aspects of Dp16 memory deficits. This difficulty with complex tasks mimic impairments in cognitive flexibility in individuals with DS (Campbell et al., 2013) and in Ts65Dn adults (Olmos-Serrano et al., 2016) and is indicative of abnormalities in the prefrontal cortex (Li et al., 2015).

It should be noted that while behavioral assessments of model animals are a powerful tool for studying CNS abnormalities, they alone are insufficient to determine the underlying etiological precipitants of behavioral dysfunction. Thus, while the Dp16 deficits in MWM mirror those found in other DS models, the underlying neuropathological mechanisms resulting in this cognitive disability may differ. For example, similar MWM deficits have been reported in other mouse models of developmental and neurodegenerative disorders such as Autism Spectrum Disorder (ASD; Moy et al., 2009; Brielmaier et al., 2012; Speed et al., 2015) and Alzheimer’s Disease (AD; Janus, 2000; Van Dam, 2003; Arendash, 2004; Hartman, 2005; Webster, 2014) despite rather dissimilar genetic underpinnings. Therefore, disparate pathological mechanisms may result in similar deficits in cognitive performance, at least when measured in murine models.

We show here that comprehensive prenatal measurements and extended developmental milestone and cognitive testing provide greater power to elucidate the
developmental timing and magnitude of cognitive sequelae in Dp16. These results highlight the gap that remains in understanding which model is the best representation of the human condition; the longitudinal and rigorous tests conducted here on the Dp16 mice should be followed by similar studies on other models to close this gap.

This study also demonstrates that it is not just the number of triplicated genes that makes a DS mouse model viable, but also that gene expression profiles and chromatin state must also be determined. In the case of Dp16, despite having a genetic landscape nominally closer to the human trisomy, this is not reflected by exacerbated gene expression and a number of key expected developmental defects are missing. These findings indicate that transcriptome correlations between comparably staged human and mouse brains are necessary to identify the degree to which each model replicates the dynamics of DS-specific gene expression throughout development. Once this is accomplished, direct parallels between gene expression, cellular development and cognition can be studied in the appropriate model.
TABLES AND FIGURES

Figure 3. **Gross brain and body size in Dp16 fetuses.** *A*, Euploid (left) and Dp16 (right) fetuses at e14.5. *B*, Dp16 crown-rump length is comparable to euploid throughout prenatal development (e13.5 (Eu=13, Dp=5); e14.5 (Eu=6, Dp=3); e15.5 (Eu=9, Dp=8); e16.5 (Eu=9, Dp=5); e18.5 (Eu=10, Dp=8)). *C*, Euploid (left) and Dp16 (right) brains at e14.5. *D*, Medial-lateral length of cerebral hemispheres is similar between genotypes. *E*, Rostrocaudal length of Dp16 telencephalon is normal throughout the embryonic time window (e13.5 (Eu=13, Dp=5); e14.5 (Eu=9, Dp=7); e15.5 (Eu=11, Dp=8); e16.5 (Eu=11, Dp=7); e18.5 (Eu=10, Dp=8)). *F*, Normal telencephalic size of Dp16 on B6:C3Sn background through paternal (e14.5; Eu=12, Dp=8) and maternal (e15.5; Eu=9, Dp=7) trisomic carriers. In each graph the data points and bars represent the mean±SEM.
**Figure 4. Histology of the Dp16 neocortex and hippocampus.** **A**, TO-PRO-3 stain of coronal neocortical sections at e15.5 (Euploid, left panel; Dp16, right panel). Ventricular/Sub-ventricular zone (VZ/SVZ), Intermediate Zone (IZ), and Cortical plate (CP) are denoted. **B-E**, Expansion of all neocortical layers occurs normally in Dp16 from mid- to late-gestation (e13.5 (Eu=8, Dp=5); e14.5 (Eu=11, Dp=14); e15.5 (Eu=8, Dp=6); e16.5 (Eu=6, Dp=6); e18.5 (Eu=8, Dp=8)). **F**, The thickness of laminae is normal in Dp16 B6:C3Sn animals at e15.5 (Eu=4, Dp=6). **G**, TO-PRO-3 stain of coronal section from an e15.5 brain at the brain level used for quantification of neocortical (solid line white box) and hippocampal (dashed line white box) growth. **H**, Hippocampal TO-PRO-3 stain at e18.5. **I-K**, Hippocampal growth occurs normally in Dp16 animals (e15.5 Eu=4, Dp=4; e18.5 Eu=3, Dp=3). In each graph the data points and bars represent the mean±SEM.
Figure 5. Dorsal and ventral neural progenitor populations are normal in Dp16. A, A’, Immunohistochemistry labeling apical (aPC, Sox2+, green) and basal intermediate progenitors (bIPC, Tbr2+, red) in euploid (A) and Dp16 (A’) e14.5 neocortex (DAPI, blue). B, C, Each cell population contains normal numbers in the Dp16 neocortical germinal zone at all ages studied. D, The spatial distributions of aPC and bIPCs are normal at all embryonic ages in Dp16 (e13.5 shown, e14.5-18.5 not shown; e13.5(Eu=8, Dp=5); e14.5 (Eu=7, Dp=5); e15.5 (Eu=9, Dp=7); e16.5 (Eu=5, Dp=5); e18.5 (Eu=8, Dp=8)). E, F, The number of Olig2+ progenitors (green) in the e14.5 medial ganglionic eminence in the ventral germinal zone is normal in Dp16 animals. In each graph the data points and bars represent the mean±SEM.
Figure 6. Mitosis and Neurogenesis in Dp16 embryonic development. A, Mitotic cells (pH3+, red) in the dorsal germinal zone of euploid e14.5 neocortex. B, C, The number of mitotic events occurring at the ventricular surface (B) and away from the ventricle (C) is consistent between genotypes at all embryonic ages (e13.5 (Eu=8, Dp=5); e14.5 (Eu=7, Dp=5); e15.5 (Eu=9, Dp=8); e16.5 (Eu=5, Dp=5); e18.5 (Eu=9, Dp=8)). D, Representative image of mitotic cells (pH3+, red) in the medial ganglionic eminence (MGE) of an e14.5 euploid animal. E, F, The distribution of mitotic cells in the MGE is normal in Dp16 animals (e13.5 and e15.5 shown; e13.5 (Eu=8, Dp=5); e14.5 (Eu=6, Dp=5); e15.5 (Eu=9, Dp=7); e16.5 (Eu=5, Dp=5)). G, Position of EdU + S-phase cells 24h after labeling (e14.5 euploid image; EdU+, green; DAPI, blue). H, I, The EdU+ population size (H) and distribution (I) is consistent between genotypes (Eu=4, Dp=6). In each graph the data points and bars represent the mean±SEM.
Figure 7. Microarray analysis of Hsa21 homologs in Dp16 and Ts65Dn embryonic brain. A, The fold expression of Hsa21 homologs reveals prominent differences in differentially expressed genes (DEX genes) between Ts65Dn (square) and Dp16 (circle) at e15.5. (red, Ts65Dn DEX genes; blue, Dp16 DEX genes). B, The number of unique DEX genes is higher in Ts65Dn (red, Ts65Dn only DEX genes; blue, Dp16 only DEX genes; red and blue stripes, shared DEX genes). Acknowledgements: Faycal Guedj and Jeroen L.A. Pennings performed the microarray and subsequent analysis.
Figure 8. Mean body weight and length and developmental milestones of Dp16 versus euploid mice. 

A, Daily mean body weights of euploid and Dp16 mice in grams measured from P3 until P21. B, Mean body length of euploid and Dp16 mice from P3 until P14. Both weight and body length are significantly reduced in Dp16 (**p<0.0005).

C-J, Early and late-acquisition developmental milestones of surface righting (C), negative geotaxis (D), forelimb grasp (E), open field (F), ear twitch (G), eye opening (H), air righting (I) and auditory startle (J) of euploid and Dp16. Each data point represents the percentage of animals that acquired the developmental milestone at a specific postnatal day. Fischer Exact test was used to examine significant differences between groups at each postnatal day. (Eu=31, Dp=16, *p<0.05). In each graph the data points represent the mean±SEM. Milestone assessment was performed by Faycal Guedj and analyze by Luis Olmos-Serrano.
Figure 9. Postnatal neuronal population abnormalities in Dp16 somatosensory cortex. A, B, Immunohistochemistry for parvalbumin (A, red), somatostatin (B, red) and calretinin (B, green) expressing interneurons in the P15 somatosensory cortex (left panel, euploid; right panel, Dp16). C, Immunohistochemistry for Tbr1-labeled excitatory neurons (Tbr1, red; DAPI, blue; left panel, euploid; right panel, Dp16). D, A significant reduction in parvalbumin and somatostatin interneuron density in Dp16 somatosensory cortex (PV, **p = .014; Eu=4, Dp=5; Sst, *p = .041; Eu=4, Dp=5), but not calretinin. E, Tbr1+ cell density was reduced in Dp16, but did not reach statistical significance (p=0.07, Eu=4, Dp16=5). In each graph the bars represent the mean±SEM and white scale bars indicate 50 μm.
Figure 10. Innate behaviors of Dp16 versus euploid mice.  

A, Nest building ability. Nesting score was assessed from overnight up to twenty days.  

B, Digging behavior as measured by the number of marbles buried during six minutes.  

C-D, Spontaneous alternation is normal in Dp16. Measures of spontaneous alternation (C), number of arm entries (D), velocity (E) and distance traveled (F) were unchanged from euploids. In each graph, the bars represent the mean± SEM. (Eu=11, Dp=13, *p<0.05, **p<0.01).  

Behavioral assessment and analysis performed by Luis Olmos-Serrano.
Figure 11. Motor activity and function in Dp16 mice. A-D, Locomotor activity was measured in a novel open field arena. Ambulatory activity (A), velocity (B), time spent in the border versus the center (C) and number of transitions between the border and the center (D) identify significant changes in Dp16. Each data point represents the mean± SEM. E-H, Representative traces showing locomotor activity and preferential location during the open field test. I, Hind limb extension reflex is impaired in Dp16. J, Dp16 animals have a reduced latency to fall in the hanging wire test. In each graph, the bars represent the mean± SEM. (Eu=11, Dp=13; *p<0.05, **p<0.001, ***p=0.002). Motor assessment performed by Luis Olmos-Serrano.
Figure 12. Spatial learning and memory performance in Dp16 mice. 

A, Percentage of correct choices using the Water T-maze paradigm. Water T-maze assessment consisted of three phases: a training phase (days 1-4), a reversal phase (days 5 to 7) and a double reversal phase (days 8 to 10). 

B, Mean latencies to find the platform during reversal and double reversal periods. Note that mean latencies are calculated only for trial one and two. 

C-F, Quantitative measures of the visible platform test using the Morris water-maze paradigm. Escape latencies (C), thigmotaxis (D), distance swam (E) and swimming speed (F) are depicted. 

G-H, Quantification of the hidden platform test using the Morris water-maze paradigm. Escape latencies (G) and distance swam (H) are depicted. Note there are an acquisition phase and a reversal phase. 

I-J, Representative traces showing swimming path during acquisition and reversal phases. Each data point represents the mean± SEM. 

K-N, Results of the reference memory (probe trial) test in the Morris water-maze paradigm. Note probe trials were performed the day after acquisition and reversal phases were finished. The percentage of time spent in each of the quadrants after acquisition (K) and after reversal (L) are depicted. Proximity defined as mean average distance to the platform (M) and number of virtually platform crossings (N). Note these parameters were assessed in the initial thirty seconds and in the entire sixty seconds of the test. In each graph, the bars represent the mean± SEM. (Eu=11, Dp=13; *p<0.05; **p<0.01). Learning and memory tasks were conducted and analyzed by Luis Olmos-Serrano.
CHAPTER THREE
Down Syndrome Developmental Brain Transcriptome Reveals Defective
Oligodendrocyte Differentiation and Myelination

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Note: Joseph Goodliffe conducted all immunoblots on human and mouse samples.
ABSTRACT

Trisomy 21, or Down syndrome (DS), is the most common genetic cause of developmental delay and intellectual disability. To gain insight into the underlying molecular and cellular pathogenesis, we conducted a multi-region transcriptome analysis of DS and euploid control brains spanning from mid-fetal development to adulthood. We found genome-wide alterations in the expression of a large number of genes, many of which exhibited temporal and spatial specificity and were associated with distinct biological processes. In particular, we uncovered co-dysregulation of genes associated with oligodendrocyte differentiation and myelination that were validated via cross-species comparison to Ts65Dn trisomy mice. Furthermore, we show that hypomyelination present in Ts65Dn mice is in part due to cell-autonomous effects of trisomy on oligodendrocyte differentiation and results in slower neocortical action potential transmission. Together, these results identify defects in white matter development and function in DS and provide a transcriptional framework for further investigating DS neuropathogenesis.

Highlights

- Genome-wide spatiotemporal dysregulation of gene expression in Down syndrome brains
- Transcriptome changes reflects altered oligodendrocyte development and myelination
- Oligodendrocyte differentiation and myelination altered in Down syndrome model mice
• Speed of action potential propagation is decreased in Ts65Dn neocortical white matter
INTRODUCTION

Trisomy for human chromosome 21 (HSA21) causes Down syndrome (DS) in one out of every 691 live births (Canfield et al., 2006) making it the most common genetic cause of developmental delay and intellectual disability. DS is characterized by a constellation of phenotypes affecting many organ systems, including central nervous system abnormalities such as cognitive and motor impairments, microcephaly, and the early appearance of neuropathological characteristics of Alzheimer’s disease (Hartley et al., 2014; Haydar and Reeves, 2012; Letourneau et al., 2014; Lott, 2012).

Prior insights into DS neuropathology have come from studies of human individuals and trisomic mouse models. Morphometric and cellular studies on human brain indicate that trisomy 21 causes complex spatiotemporal disturbances in neural development. For example, DS brains are smaller than age-matched controls by the mid-fetal period and postnatally exhibit microcephaly and abnormalities in gray and white matter development (Aylward et al., 1997; Colon, 1972; Crome et al., 1966; Golden and Hyman, 1994; Guihard-Costa et al., 2006; Jernigan and Bellugi, 1990; Larsen et al., 2008; Ross et al., 1984; Suetsugu and Mehraein, 1980; Sylvester, 1983; Takashima et al., 1981; Wisniewski, 1990). Mouse models have provided additional insights into the developmental progression of DS, elucidating specific defects in neurogenesis, neuronal differentiation, and impaired synaptic plasticity and learning (Chakrabarti et al., 2010; Chakrabarti et al., 2007; Hyde et al., 2001; Kleschevnikov et al., 2004; Siarey et al., 1999; Siarey et al., 1997; Tyler and Haydar, 2013; Wang et al., 2013). While it is clear that trisomy of HSA21 is the root cause of DS, which biological processes are affected by this
trisomy and which HSA21 and non-HSA21 genes are associated with those processes are not fully understood. Furthermore, the extent to which human postmortem neuropathology is reflected in the mouse models (and vice versa) remains one of the key problems hampering further understanding of DS brain development and function.

Transcriptome profiling has enabled unbiased and genome-wide measurement of the developmental dynamics of gene expression in different regions of healthy and diseased human brains. These approaches have also provided insight into the pathophysiological mechanisms underlying neurodevelopmental brain disorders (Mitchell and Mirnics, 2012; State and Geschwind, 2014; Tebbenkamp et al., 2014). While several reports have investigated gene expression in postmortem human DS brains or derived pluripotent stem cells (Letourneau et al., 2014; Lockstone et al., 2007; Mao et al., 2005; Mao et al., 2003), these studies are limited by sparse genome coverage, numbers of samples or developmental time points, and potential variances between in vitro and in vivo gene regulation. We therefore set out to characterize the spatiotemporal dynamics of gene expression in the DS and matched euploid brain and to subsequently decipher the biological processes affected over the course of prenatal and postnatal development.

We began by carrying out genome-wide transcriptional profiling of different regions of postmortem brains spanning from mid-fetal development to adulthood from male and female DS individuals matched to neurotypical euploid donors. This approach uncovered a highly dynamic disruption of the transcriptome across all chromosomes, with significant and changing alterations in gene expression in different brain regions over time. Bioinformatic analyses revealed transcriptional signatures of biological processes affected
differentially throughout the DS brain. In particular, our gene network analysis uncovered modules of co-expressed genes associated with biological processes previously implicated in neurological dysfunction in DS, such as cell morphogenesis, immune regulation, and neuronal differentiation (Bahn et al., 2002; Lockstone et al., 2007; Sahun et al., 2014; Yoshida et al., 2013; Zampieri et al., 2014), as well as dramatic co-dysregulation of genes associated with key molecular and cellular processes that have not been well characterized in DS brains. Notably, we found transcriptome differences indicating that oligodendrocyte differentiation and myelination, two cellular processes crucial for neurodevelopment and function (Rowitch and Kriegstein, 2010; Zuchero and Barres, 2013), are altered in DS. In a series of validation assays in human brain tissue and in the Ts65Dn mouse model of DS, we demonstrated a spatiotemporal reduction in transcript and protein levels of oligodendrocyte maturation markers which are accompanied by cell-autonomous deficits in oligodendrocyte differentiation and the production of neocortical myelin, both of which are accompanied by slower action potential transmission between cerebral hemispheres. Altogether, the data illustrate disruption of distinct gene networks in DS, providing an extensive framework to identify, prioritize, and test the gene candidates and biological processes underlying the neuropathophysiology of DS. We have also generated a public user-friendly searchable online database to enable gene, tissue, and age related searches of the data (http://medicine.yale.edu/lab/sestan/resources/). To facilitate the usefulness and accessibility of the DS transcriptome, we have also generated a user-friendly, searchable internet database ((http://medicine.yale.edu/lab/sestan/resources/)).
MATERIALS AND METHODS

Human Tissue

This study was conducted using postmortem human brain specimens from tissue collections at the Department of Neuroscience, Yale School of Medicine, and the University of Maryland Brain and Tissue Bank (Baltimore, MD), Brigham and Women’s Hospital Pathology Department, and Boston University Pathology Department. Tissue was collected after obtaining parental or next of kin consent and with approval by the institutional review boards. Tissue was handled in accordance with ethical guidelines and regulations for the research use of human brain tissue set forth by the NIH (http://bioethics.od.nih.gov/humantissue.html) and the WMA Declaration of Helsinki (http://www.wma.net/en/30publications/10policies/b3/index.html).

Appropriate informed consent was obtained and all available non-identifying information was recorded for each specimen. Specimens ranged in age from 14 post-conception week (pcw) to 40 years. The postmortem interval (PMI) was defined as hours between time of death and time when tissue samples were frozen (Table S1).

Human Tissue Dissection and Neuropathological Evaluation

All clinical histories, tissue specimens, and histological sections were evaluated to assess for hypoxia, cerebrovascular incidents, tumors, microbial infections, neurodegeneration, demyelination, and metabolic disease.

Trisomy 21 was confirmed by karyotyping and/or Illumina Omni-2.5 million SNP arrays. The localization of dissected samples was verified by histology in the postnatal brains and across fetal periods using the same anatomical landmarks. The complete list of
periods and samples, including corresponding putative functional brain regions and neocortical areas, analyzed in this study can be found in Table S1, S2A and S2B.

Fresh frozen postmortem brain specimens from donors affected with DS and from neurotypical euploid controls matched on age and sex (see Tables S1 and S2 for the list of brain specimens, tissue samples and brain regions analyzed) were dissected as follows. For fetal brains, the entire neocortical plate and adjacent superficial part of the subplate zone was sampled; for postnatal brains the entire thickness of the cortex, containing all six cortical layers, and the underlying gyral white matter corresponding approximately to white matter segment 4 as defined by von Monakow were sampled. Hippocampal samples were dissected from the middle third of the hippocampus proper. Samples of the cerebellar cortex were dissected from lateral part of posterior lobe of cerebellum and they contain all three layers of cerebellar cortex and underlying white matter, but do not contain cerebellar nuclei. Tissue dissection was done using a dental drill (AnyXing, 300D) with a Lindemann Bone Cutter H162A.11.016 and diamond disk saw (Dental Burs USA; r=11 mm) on an aluminum plate over dry ice.

To prepare tissue sections for microscopic histological and neuropathological examination, small samples (usually the dorsal parietal cortex, striatum with ependymal layer, hippocampus, and the cerebellum) were dissected and fixed in 4% paraformaldehyde and processed for histology and immunohistochemistry as described below. Neocortical cytoarchitecture of each euploid control sample was compared to areal cytoarchitectonic maps to distinguish Brodmann areas (BA). Euploid control specimens with incorrect cytoarchitecture or abnormal microscopical appearance were excluded from the study.
Neocortical areas (see below) were grouped according to the lobes from which they were sampled. The same anatomical landmarks were used to dissect DS brain specimens.

**Frontal lobe**

Dorsolateral prefrontal cortex (DFC) was sampled from approximate border between the anterior and middle third of the medial frontal gyrus. Cytoarchitectonically, DFC corresponds approximately to Brodmann area (BA) 9 and BA46.

Orbital prefrontal cortex (OFC) was sampled from the anterolateral two thirds of the orbital gyri. OFC corresponds approximately to BA11.

Ventrolateral prefrontal cortex (VFC) was sampled from the posterior third of the inferior frontal gyrus, corresponding to the opercular and triangular part of the inferior frontal gyrus. VFC corresponds approximately to BA44 and BA45.

Medial prefrontal cortex (MFC) was sampled from perigenual and subgenual parts of the anterior cingulate gyrus and the anteromedial part of the superior frontal gyrus. MFC corresponds approximately to BA24, BA32 and BA33.

**Parietal lobe**

Primary somatosensory cortex (S1C) was sampled from the ventrolateral part of the postcentral gyrus adjacent to the M1C area. S1C corresponds to BA1, BA2 and BA3.

Posterior inferior parietal cortex (IPC) was sampled from the posterior half of the supramarginal gyrus. IPC corresponds approximately to BA40.

**Temporal lobe**

Posterior superior temporal cortex (STC) was sampled from the posterior third of the superior temporal gyrus. STC corresponds approximately to BA22.
Inferior temporal cortex (ITC) was sampled from the anterior third of the inferior temporal gyrus. ITC corresponds approximately to BA20.

Occipital lobe

Primary visual cortex (V1C) was sampled from the area surrounding the calcarine fissure. Only samples in which the stria of Gennari could be recognized were included. V1C corresponds to BA17. Small pieces of the neighboring BA18 could have been occasionally present in the sample, but the majority of the sample corresponded to BA17.

Hippocampus (HIP) was sampled from the middle third of the retrocommissural hippocampal formation, located on the medial side of the temporal lobe. Sampled areas always contained dentate gyrus and the cornu ammonis. Samples dissected from the frozen tissue may contain small quantities of the neighboring choroid plexus.

Cerebellar cortex (CBC) was sampled from the lateral part of the posterior lobe. The sampled area contained all three layers of cerebellar cortex and underlying white matter but not the deep cerebellar nuclei. CBC approximately corresponds to the lateral pontocerebellum.

Spectral Confocal Reflectance Microscopy

Spectral confocal reflectance microscopy (SCoRe) (Schain et al., 2014) was used to quantify myelinated axon segments in tissue sections of the DFC dissected from 4% paraformaldehyde fixed brains. We analyzed tissue from five pairs of matched brains obtained from the University of Maryland Brain and Tissue Bank, Brigham and Women’s Hospital Pathology Department and Boston University Pathology Department: one pair from 1 year old brains, one pair of 46 year old brains, one pair of 49-50 year-old brains and
two pairs of 70 year old brains. Tissue sections for the 46-50 year old cases were 4µm paraffin sections from Pathology collections at Boston University and Brigham and Women’s Hospital whereas all other cases were 100µm vibratome-cut formalin-fixed sections from the Maryland Brain Bank. We found that SCoRe microscopy parameters did not vary between sections based on how thick they were or how they were processed prior to microscopy. Each pair constituted an experiment and the laser power and detector sensitivity settings were calibrated first to the euploid control brain section to yield a thresholded image highlighting the myelinated axon segments within the image. The same imaging settings were then used for the Ts21 brain of that pair. Images (1024 x 1024; 425 mm x 425 mm) were collected using reflectance from 488nm, 561nm and 633nm laser lines collected with 486-501 nm, 554-563 nm and 630-634 nm filters respectively using a 20X, 0.8NA objective lens. For each brain, we collected overlapping SCoRe images from the DFC in a radial stripe from the pial surface down to the compacted white matter underneath the cortical grey matter. We calculated the myelinated fiber density within each image as the percent of the image pixels containing a myelin reflectance signal.

**RNA Isolation and Exon Array Hybridization**

Total RNA was isolated from 30mg of pulverized frozen brain tissues using a non-phenolic procedure (RNeasy Plus Mini Kit, Qiagen), followed by DNase treatment (TURBO DNase, Ambion). Optical density values at 260/280 were consistently above 1.9 (NanoDrop, Thermo Scientific), and samples with RNA integrity values of at least 5 were used for microarray (RIN>5, Agilent Bioanalyzer). Synthesized cDNA (5.5 µg) using WT Expression kit (Ambion) was labeled and loaded onto individual Affymetrix Human Exon
1.0 ST arrays. Microarrays were hybridized at 45 °C for 16–24 hours, washed and stained using an Affymetrix FS450 fluidics station, according to manufacturer recommendations. Microarrays were scanned on a GeneChip Scanner 3000 and visually inspected for hybridization artifacts. Exon chip analysis was performed using Affymetrix Power Tools 1.12.0. Probe level data was summarized into probe set level data using the Robust Multichip Average (RMA) background correction algorithm in combination with an R-script. The raw image files (.DAT files) were analyzed using Affymetrix GeneChip Operating Software to generate .CEL files.

**Quality Control Measures**

Three QC measures were performed to test the quality of exon array data. First, ratio intensity plots were plotted for all exon arrays to detect spatial artifacts that are defined as severe non-random spatial patterns of exon arrays. The construction of these ratio intensity plots was previously described (Kang et al., 2011). Second, exon array hybridization uniformity was estimated by gene expression uniformity from 5'-end to 3'-end (Figure 21A). Microarrays displaying spatial artifacts and altered hybridization uniformity were excluded for further data analysis. Third, 8 samples were re-tested to evaluate technical reproducibility (Figure 21B). The correlations were high for these technical replicates (Spearman correlation, r² = 0.977; N=16, Figure 21B).

**Data Normalization**

Affymetrix exon array raw data (.CEL files) were normalized using the Partek Genomics Suite version 6.6 to generate probeset-level (exon-level) and transcript cluster (gene-level) intensities. The expression level of a probe set was estimated by averaging the
intensities of all core probe sets within the exon. We applied the following default Partek settings: RMA background correction, exclusion of probes containing SNPs, quantile normalization, mean probe set summarization, and log2-transformation. Only core probe sets defined by Affymetrix were included for the calculation. These core probe sets have reliable sequence annotations. The expression level of a gene (transcript cluster) was estimated using the median of all exons within the gene.

**Transcriptome Data Analyses**

Principal component analysis was applied to visualize the relatedness of DS and their-matched control samples. The first three principal components were calculated using the function “prcomp” in R. All these principal components were plotted using the function “plot3d” in R. Each data point in the picture represents one sample. All samples were colored according to the phenotype of the samples, such as brain region, period, and disease status (Figure 22). To make these pictures clearer, three different directional views are displayed.

A paired t-test was used to identify DEX genes between paired DS and matched control samples across all development periods. FDR-adjusted p-value < 0.1 was used as a cutoff. To see how the DEX genes distribute along the human genome, chromosome location of these DEX genes were determined based on the gene annotations provided by Affymetrix. Chromosomes 1 to 22, X and Y were investigated. The percentage of DEX genes and ratio of up-regulated/down-regulated genes in each chromosome were calculated.
To identify differentially expressed (DEX) genes between paired DS and matched control tissue samples at specific developmental periods, a sliding-window approach and paired t-test were used. The window size was set to 3 periods. For each window, a paired t-test was applied to determine if the expression level of a gene in DS brain samples was significantly different from the expression level in the samples from the matched control. Statistical threshold was set at a p-value < 0.05 and minimum fold difference > 2 between DS and control brains. We performed a 20-cycle permutation test to ensure that our results are robust and are not unduly influenced by any particular sample. For the permutation test, DS samples were randomly matched to control samples within the same stage and with the same sex to identify DEX genes using the same approach and cutoff. The distributions of the number of DEX genes from different match sets were plotted in a boxplot.

Unsupervised signed co-expression networks were constructed using the weighted gene co-expression network analysis (WGCNA) package in R (Zhang and Horvath, 2005). All genes with core probe sets were included in the analysis. A pair-wise correlation matrix was computed, and an adjacency matrix was calculated by raising the correlation matrix to a power. The power was set to 21 according to a scale-free topology criterion (Zhang and Horvath, 2005). For each pair of genes, a robust measure of network interconnectedness (topological overlap measure) was calculated based on the adjacency matrix. The topological overlap based dissimilarity was then used as input for average linkage hierarchical clustering. Modules were generated by hybrid dynamic tree-cutting. To obtain co-expression patterns, we set the minimum module size to 20 genes, deepSplit to 2, and the minimum height for merging modules to 0.15. Each module was summarized by an
eigengene, which is the first principal component of the scaled module expression. To obtain cleaner modules, we defined the module membership measure (also known as module eigengene based connectivity kME) as the correlation between gene expression values and the module eigengene. Genes were iteratively assigned to the module with highest kME as long as they had maximum kME > 0.7. The module membership is also used to rank genes in the module. Top ten genes in the rank were considered as hub genes of module.

For differential expressed genes and co-expression modules, functional enrichment was assessed using the DAVID Bioinformatics Resource 6.7 (http://david.abcc.ncifcrf.gov/).

Analysis of Oligodendrocyte Precursor Cells and Oligodendrocyte Associated Gene Expression

To identify the cell type expression profile of all 121 genes expressed in module (M) 43 (the myelination associated module in Figure 2), we determined if they were highly expressed (FPKM > 20) in acutely purified representative populations of neurons, astrocytes, oligodendrocyte precursor cells (OPCs), newly formed oligodendrocytes, myelinating oligodendrocytes (mOLs), microglia, endothelial cells, and pericytes from mouse cerebral cortex at postnatal day 7 (see Zhang et al. 2014; http://web.stanford.edu/group/barres_lab/brain_rnaseq.html).

Furthermore, to identify genes that are specifically expressed in OPCs and mOLs (Figure 19), but not other cell types, during fetal and early postnatal human brain development we generated a set of genes that were found to both be highly enriched in
OPCs and mOLs using the mouse dataset (Zhang et al. 2014) and in a list of OL and OPC related human genes generated in our previous study that categorizes genes according to their correlations with major cell types and neurodevelopmental processes across human development (Kang et al., 2011). By overlapping these two lists allowed us to create a set of human homologs that are most likely to be selectively enriched in OPCs and mOLs during human fetal and postnatal development. Note that the list in Zhang et al. (2014) specifically tested purified OPCs and mOLs in mouse at one postnatal stage of development. Because OPCs are highly proliferative at this age, the OPC list may contain genes broadly associated with cell proliferation and progenitor states. Therefore, genes that overlap between the datasets in Zhang et al. and Kang et al. provide a highly stringent set of OPC and mOL specific genes that are dynamically expressed over human development (Table S7).

The following methodology was used to derive the lists of genes highly enriched in OPCs and mOLs. The raw RPKM values from Zhang et al. (2014) were first normalized using quantile normalization. Genes enriched in mouse OPC or mOL were identified by the criterion that the fold change of each gene in these respective cell types must be 2 fold greater than in any other cell type (cell types other than OPC, newly formed oligodendrocytes and mOL). These lists were then intersected with the lists of genes highly co-expressed with OPC or mOL markers during human brain development (Kang et al., 2011) to form lists of OPC and mOL genes (Table S7). To test changes in OPC and mOL genes, paired t-test were performed between DS and control for each human developmental
period. Enrichment of OPC and mOL genes in each module was calculated using Fisher’s exact test (Figures 19B and 19D).

**Expression Analysis of Cell Type-Specific Marker Genes Across Tissues**

To confirm the accuracy and reproducibility of our dissection technique within each brain and across individuals, we determined if there was high correlation of the expression of genes enriched in neural cell types between each neocortical region across individual brains, and between samples of DFC and CBC (the brain regions for which the most tissue samples were available) from both control and DS individuals. All analyses were done using adult samples, to avoid discrepancies in developmental age that may confound pairwise analysis. The lists of cell-type enriched genes were obtained from publically available RNA-seq data from purified astrocytes, neurons, and myelinating oligodendrocytes of the mouse cerebral cortex generated by Zhang et al. (2014). For each cell type, genes were ranked by their fold of enrichment (the fold change between this cell type and the maximum expression in the rest of cell types). Human homologs of the top 100 most enriched genes were then defined as cell-type enriched genes. Using the resulting lists of cell-type enriched genes, we calculated the Pearson correlation coefficient of oligodendrocyte, astrocyte, and neuronal human genes between samples of each neocortical area dissected from the same individual brain. If the dissection technique was consistent, high correlation should be observed as the glia/neuron ratio is more consistent throughout the neocortex than between different brain regions (Herculano-Houzel, 2014). In addition, we calculated the Pearson correlation through pairwise comparisons between samples from the CBC and DFC both within and between experimental groups (i.e., control vs control, DS versus DS, and control
versus DS) for astrocytes, neurons, and oligodendrocytes. Note the limited number of samples from other brain regions precluded reliable pairwise analysis. To determine statistically significant differences in the pairwise correlation between each cell type across the experimental groups (e.g. oligodendrocyte versus astrocyte genes in DFC of paired control versus a matched control sample, matched DS versus control, and so on), we used the Wilcoxon signed-rank test (Table S8).

**Droplet Digital PCR**

An aliquot of the total RNA that was previously extracted from each brain region was used for secondary validation by droplet digital PCR analysis. One µg of total RNA was used for cDNA synthesis using oligo dT primers and SuperScript III First-strand synthesis Supermix (Invitrogen), and subsequently diluted with nuclease-free water to 1 ng/µl cDNA. FAM- or VIC-labeled TaqMan® probes were used for detecting copy number of target genes (Applied Biosystems). PCR reactions were conducted on the QX100 Droplet DigitalTM PCR system (Bio-Rad) according to manufacturer recommendations. The reaction mixture containing sample cDNA, primers and probe was partitioned into about 20,000 droplets in oil through the QX100 Droplet Generator. After PCR amplification (95°C 10 min; 40 cycles of (94°C 30 sec, 57°C 60 sec); 98°C 2 min), each droplet provided a positive or negative fluorescent signal indicating the target gene was present or not present after partitioning. Positive and negative droplets were counted in the QX100 Droplet Reader and the software calculated the concentration of target gene as copies per microliter. The copy number of each gene was normalized to the housekeeping
gene GAPDH, which was counted in the same sample. A list of PCR primers listed in Table 1.

**Mice**

Ts65Dn and euploid B6EiC3 mice were generated by backcrossing Ts65Dn females to B6EiC3Sn.BLiAF1/J F1 hybrid (B6EiC3) males. The parental generation was obtained from Jackson Laboratory. Quantitative PCR genotyping was performed on genomic DNA extracted from tail tips (Chakrabarti et al., 2007). All procedures regarding the care and death of these animals was approved by the Institutional Animal Care and Use Committee of Boston University School of Medicine, in accordance with the NIH guide for the care and use of laboratory animal. Ts65Dn and euploid littermates at P7, P15, P30 and P60 were anesthetized by ketamine/xylazine cocktail and intracardially perfused with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer saline (PBS, pH 7.4). Perfused brains were removed and fixed in 4% PFA overnight at 4°C followed by 30% sucrose. All samples were embedded in Tissue-Tek OCT compound (Sakura Finetek), frozen on dry ice and sectioned (16µm) with a Microm HM 560 cryostat (MO BIO Laboratories, Inc.).

**Immunohistochemistry**

All immunohistochemical reactions were performed on 16-µm frozen brain sections. Primary antibodies used were: rabbit anti-Olig2 (1:500, Millipore), guinea-pig anti-NG2 (gift from William B. Stallcup, Sanford-Burnham Medical Research Institute, La Jolla), mouse anti-RIP (1:50, DSHB), mouse anti-CC1 (CalbioChem), rabbit anti-Caspase3 (1:500, Cell Signaling), guinea-pig anti-NF186, rabbit anti-Caspr (1:200, gift from Manzoor Bhat, University of North Carolina at Chapel Hill), mouse anti-MBP (1:1000,
Covance) and mouse anti-MAG (1:1000, Millipore). We used AlexaFluor 488-, AlexaFluor 546- and AlexaFluor 636-conjugated (1:200, Invitrogen) as secondary antibodies. All frozen sections were mounted with Vectashield (Vector Laboratories).

**Immunoblotting**

Dissected cerebral hemispheres were homogenized in RIPA lysis buffer (Santa Cruz Biotechnology). Proteins were loaded into 4-20% gradient gels. Gels were electrotransferred to a 0.2µm nitrocellulose membrane (Millipore Bioscience Research Reagents). Blots were blocked in 5% milk in TBST and incubated in primary antibodies. Bands were detected with appropriate HRP-conjugated secondary antibodies, reacted with chemiluminescent ECL substrate (Pierce) and imaged. Band intensity was measured using Image J program (National Institute of Health).

**Electron Microscopy**

8-9 week-old mice were perfused intra-aortically with a warm solution of 2% of paraformaldehyde and 2.5% of glutaraldehyde in phosphate buffer 0.1M at pH 7.2-7.4. Brains were removed and postfixed in the same fixative. To expose the corpus callosum, cerebral hemispheres were separated in the mid-sagittal plane using a razor blade. This was followed by one longitudinal 3mm cut from the midline to each of the cerebral hemispheres. Each thick section was further trimmed into two blocks with which corpus callosum was clearly seen. Sections were rinsed in 0.1M Phosphate buffer for 30 minutes and then incubated in 1% osmium tetroxide during 2-3 hours. Once blocks were osmicated, blocks were dehydrated in an ascending series of alcohols for 2 hours and finally embedded in Araldite. Embedded corpus callosum was sectioned in the transverse plane, such that the
nerve fibers were cross-sectioned. Semi-thick (1 µm) sections of the entire corpus callosum were first taken and stained with toluidine blue. For electron microscopy, the block was trimmed so thin sections contained only the corpus callosum. All thin sections were stained with uranyl acetate and lead citrate. All thin sections were mounted on 200 mesh grids, and, after they had been examined to ascertain that the quality of preservation was acceptable, electron micrographs were taken using a JEOL 100S electron microscope. All images were taken in a systematic manner, so electron micrographs were taken approximately in the center of the thin section, and thus focusing on the body of the corpus callosum. 6 images per brain were taken at magnification of 3000x and 8000x. 3000x magnification images were used for counting the myelinated axons, while 8000x magnification images were used to assess g-ratios. To calculate g-ratios, the area of axons and axons plus myelin was measured in electron micrographs by first measuring the circumference of each by hand-tracing tool using Image J, then by calculating areas. A minimum of 3500 axons was counted per animal for assessing numbers of myelinated axons while a minimum of 600 axons was analyzed per animal for assessing g-ratios. Statistical analyses were performed by two-tailed Student’s t test analysis. Euploid control, n=3; Ts65Dn, n=3.

**Electrophysiology**

Mice 2-4 weeks old, were anesthetized and decapitated and the brain was rapidly removed and placed in ice-cold (~ 4°C) cutting artificial cerebrospinal fluid (ACSF) containing (in mM) sucrose 206, KCl 2, CaCl2 1, NaH2PO4 1.25, MgSO4 2, MgCl6H2O 2, NaHCO3 26, d-glucose 10, bubbled with a mixture of 95% O2/5% CO2. The
mice were then decapitated and the brains placed in a dish of sACSF on ice for blocking. Coronal slices, 400-µm thick, were cut on a Leica VT1200S and transferred to a warmed (~36°C) solution of normal ACSF (nACSF) NaCl 126, KCl 3, CaCl2 2, NaH2PO4 1.25, MgSO4 2, NaHCO3 26, d-glucose 10, bubbled with a mixture of 95% O2/5% CO2 for 45 minutes. After this recovery period the slices were maintained in the same solution at room temperature for at least 1 hour before recording. All recordings were performed at room temperature (~25 °C). Compound action potentials (CAPs) were evoked by electrical stimulation of the corpus callosum with a bipolar tungsten wire electrode and were recorded with a pulled borosilicate glass pipette (~ 1 MΩ resistance) within the contralateral corpus callosum. Stimulation intensities ranged from 30 to 3000 µA. Input-output curves were generated by recording the amplitudes of N1 and N2 (see inset Figure 13) as a function of stimulation intensity. The amplitude of each response was taken to be the difference between the corresponding trough and a straight line drawn between the adjacent peaks. Three to five responses were averaged for each measurement. The conduction velocities for myelinated and unmyelinated fibers were calculated as the slope of a straight line fitted through a plot of the distance between the recording and stimulating electrodes versus the response latency (time to N1 or N2 respectively). Refractory periods were measured using a paired pulse protocol where two stimuli were applied with a decreasing time interval (10 to 2 ms) between each pulse. Peak amplitudes and onset latencies were calculated using custom written routines in Igor (WaveMetrics, Lake Oswego, OR). Statistical analysis was performed using SigmaPlot (Systat Software Inc. San Jose, CA) and consisted of repeated measure 2-factor ANOVAs for input-output and
refractory period results or a two-tailed Wilcoxon test for conduction velocity data and p < 0.05 was assigned for significance.

**Image analysis**

All fluorescent images were taken on a LSM710 confocal microscope (Carl Zeiss). Three to four brains per genotype were analyzed at each age studied. A minimum of 4 images and up to 6 images per brain were taken for quantitative analysis. 10-µm confocal z-stacks at 40x magnification were analyzed using LSM software to quantify the number of OLIG2+, CC1+, NG2+ and Caspase 3+ cells. 6-µm confocal z-stacks at 63x magnification with zoomX2 were analyzed to quantify the number of nodes using Volocity software (Improvision). For comparative analysis of RIP staining intensity, single plane images were converted to gray-scale. The intensity as the mean gray value obtained from all pixels within a region of interest was quantified using Image J software (National Institutes of Health). All analyses were done blindly to genotype.

**Proliferation and Maturation Assays in Oligodendrocyte Progenitor Cultures**

Mouse oligodendrocyte precursor cells were isolated by immunopanning through positive selection of cells from postnatal day (P) 7 mouse cerebral cortices that bound to PDGFRA (PDGFRα), as previously described with the exception that Papain treatment was carried out in a 37°C, 5% CO2 incubator (Emery and Dugas, 2013; Fancy et al., 2011). Cells were plated on poly-D-lysine coated slide wells and allowed to overnight in proliferation conditions with the addition of CNTF, PDGF, and NTF3 (NT-3) (Peprotech) to base medium. For proliferation analysis, cells were allowed to proliferate for an additional 48 hours. For maturation analysis, the media was switched the next morning to
contain triiodothyronine (T3; Sigma), but not PDGF or CTNF, and cells were maintained in these conditions for 72 hours in a 5% CO2 37°C incubator. Cells were fixed for 15 min in 4% paraformaldehyde at room temperature.

Cells left in proliferative conditions were then immunostained for PDGFRA (PDGFRα) and OLIG2. To count the total number of OPCs an image was taken at 10x of the center of 3 slides wells for each sample and OLIG2+ cells were counted using the Cell counter plug in ImageJ (NIH, Bethesda, MD). Cell counts were additionally assessed for contamination from other cell types. No OLIG2/PDGFRA (PDGFRα)–negative cells were observed. Images of MPB/OLIG2 immunostaining were obtained by the same method and the total number OLIG2 cells, in addition to the number of MBP/OLIG2 double positive cells was quantified. To further assess maturation, the complexity of the morphology of MBP cells was categorized into three groups and quantified: simple (i.e. less mature) in which there were fewer than 6 MBP+ processes, complex in which there were more than MBP+ 6 process, and membranous in which a membranous MBP+ lamella without discernible processes extended from the cell body. Student’s t-tests were conducted to assess statistical significance (defined as p < 0.05) between three experimental replicates.

**Statistical Analysis**

All data are presented as mean ± SEM, unless otherwise noted. Comparisons of mean differences between groups were made by unpaired two-tailed Student’s t-test, except as otherwise noted above in the extended experimental procedures. A probability level of p< 0.05 was considered to be statistically significant.
RESULTS

Genome-Wide Spatiotemporal Alterations of Gene Expression in DS Brains

We used the Affymetrix exon array platform, which features comprehensive genome-wide coverage of the human transcriptome across entire transcripts and each individual exon, to characterize gene expression in different regions of DS brains over the course of development (see Experimental Procedures). Transcriptome profiling was performed using total RNA extracted from eleven regions, including multiple regions of the cerebral neocortex, the hippocampus (HIP), and the cerebellar cortex (CBC) dissected using a standardized protocol from high quality postmortem human brains of DS and clinically and anatomically unremarkable euploid controls (for details on tissue dissections see Extended Experimental Procedures, Figure 21 and Tables S1 and S2). We collected 58 paired tissue samples from 15 euploid control and 15 DS brains ranging in age from 14 post-conception weeks (pcw) to 42 years-old (Table S2) and then allocated them into groups based on a previously described timeline that divides human brain development and adulthood into 15 periods (Kang et al., 2011).

Principal component analysis showed segregation between DS and control samples; however, brain regions and age (developmental periods) contributed more to the global differences in gene expression than disease status (Figure 22), indicating that gene expression in the human brain has a high degree of spatial variability. Table S2). We chose DFC and CBC for our analyses because both regions have been implicated in DS neuropathology (Hartley et al., 2014; Haydar and Reeves, 2012; Letourneau et al., 2014; Lott, 2012) and exhibit diverse transcriptional profiles over the course of development.
(Kang et al., 2011; Tebbenkamp et al., 2014). Of 17,557 mRNA genes profiled, 842 (4.8%) and 571 (3.25%) genes in DFC and CBC, respectively, were defined as significantly differentially expressed (DEX) between paired DS and euploid samples across all periods (Table S3) (FDR = 0.1; see Extended Experimental Procedures). As expected, the percentage of DEX genes on HSA21 was higher than other chromosomes (Figures 13A and 23). While 19.5% (DFC) and 13.9% (CBC) of the genes localized on HSA21 were DEX genes, a median of 4.4% (DFC) and 3.0% (CBC) DEX genes were found on other chromosomes in DS compared to their matched control. However, the majority of DEX genes were non-HSA21 genes (1,337 genes vs 76 genes on HSA21) (Figure 13B). We also found that while all HSA21 DEX genes were up-regulated in DS samples, only 47.7% (DFC) and 45.2% (CBC) of non-HSA21 DEX genes were up-regulated. Conversely, 52.3% (DFC) and 54.8% (CBC) of non-HSA21 genes were down-regulated (Figure 13C), indicating that the triplication of HSA21 leads to both positive and negative regulation of genes on other chromosomes, (Figure 24). This stark difference in the directionality of HSA21 gene expression profiles suggests that the prominent developmental consequence of trisomy 21 is upregulation of genes on HSA21, some of which function as trans factors, and that this is followed by up- and down-regulation of genes distributed throughout the genome. Strikingly, we found that gene expression across all chromosome locations was altered even from the earliest time points measured (i.e., 14 pcw), indicating early and widespread changes to the DS transcriptome.

A combination of sliding-window, paired t-test, and permutation tests was used to investigate temporal dynamics in transcriptional differences between DS and control brains
(see Extended Experimental Procedures). This analysis revealed that the number of DEX genes in DFC increased in an age-dependent manner, with significantly more DEX genes emerging over postnatal development and adulthood (periods 8-14) (Figure 13D, and Table S4). A completely different temporal picture emerged in the CBC, where the number of DEX genes remains constant throughout life (Figure 13E, and Table S4). This dichotomy between the DFC and CBC temporal gene expression profiles extended throughout the genome, with more variability across all chromosomes over developmental time in DFC samples (Figure 23). Gene ontology enrichment analysis indicated potential dysregulated biological categories including transmission of nerve impulse (Benjamini-Hochberg-adjusted (BHA) p=2.9x10-8), synaptic transmission (BHA p=1.4x10-5), cell morphogenesis (BHA p=1.4x10-5), cell-cell signaling (BHA p=6.7x10-5), and axon ensheathment (BHA p=4.8x10-2) (Table S5).

Together, these studies demonstrate that many genes throughout the genome are altered in DS, confirming and extending previous findings that non-HSA21 genes may contribute significantly to trisomy 21 phenotypes (Letourneau et al., 2014; Lockstone et al., 2007). Most importantly, our results clearly show that many of these DEX genes display dynamic temporal and spatial differences in expression and highlight specific biological processes potentially disrupted in developing DS brain.

**Co-Dysregulation of Genes Associated with Oligodendrocyte Differentiation and Myelination in DS**

To integrate the expression differences observed between DS and matched control samples from all 58 paired samples and each brain region into a systems level context, we performed
weighted-gene co-expression network analysis (WGCNA) (Zhang and Horvath, 2005) and identified modules of co-expressed genes. We defined 60 gene modules exhibiting altered expression during DS brain development in the CBC, HIP, and multiple neocortical regions (Table S6 and S7). We then carried out gene ontology enrichment analysis of each module to gain insight into the biological significance of these clusters of co-expressed genes (Table S6). This analysis confirmed a number of cellular and molecular processes previously found to be altered in DS or mouse model brains, including cell morphogenesis (Module [M] 24; Figure 25A), RNA processing (M31), gene transcription (M52), immune responses (M54; Figure 25B), neuronal differentiation (M4 and M45; Figure 26A,D), synaptic transmission and regulation (M10 and M34; Figure 26B,C) and electron transport (M14) (Table S6) (Bahn et al., 2002; Lockstone et al., 2007; Sahun et al., 2014; Yoshida et al., 2013; Zampieri et al., 2014).

WGCNA also identified co-expression modules enriched for genes associated with biological processes previously not well-characterized in DS. Notably, a module of co-expressed genes, number 43 (M43), was enriched for gene ontology categories related to regulation of action potential (BHA p=1.7x10-2) and axon ensheathment (BHA p=3.7x10-2) and was markedly down-regulated throughout the DS neocortex and in the HIP over development (Figure 14A, B and Table S6). Differences in this module accelerated over the course of DS neocortical development (Figure 14A, B), coinciding with the onset and progression of myelination. Note that the absence of a M43 expression phenotype in the CBC of DS brains (Figure 14A, B) is likely because the most numerous cells in CBC, granule cells, have unmyelinated axons.
To further characterize M43 genes, we investigated whether they were associated with the developmental program of specific neural cell types (i.e., astrocytes, endothelial cells, microglia, neurons, oligodendrocyte progenitor cells, newly formed oligodendrocytes or myelinating oligodendrocytes). We queried whether M43 genes were highly expressed (FPKM > 20) in different populations of neural cell types purified from mouse cerebral cortex (Zhang et al., 2014) (see Extended Experimental Procedures). We found that of the 121 mouse homologs of genes in M43, 67 met this cutoff for high expression (FPKM > 20) and 82.1% (55/67) of this subset were found to be highly expressed by oligodendrocyte lineage cells (gene names labeled in red in Figure 14B-D). Importantly, 8 of the top 10 genes with highest intramodular connectivity (hub genes) in M43 were primarily expressed by mature oligodendrocytes and tightly correlated with other oligodendrocyte-associated M43 genes (Figure 14C).

To ensure that DEX genes detected were not due to variability in tissue dissections, we analyzed genes highly enriched in astrocytes, neurons, and oligodendrocytes (Zhang et al., 2014) (see Extended Experimental Procedures). We found that intra-individual gene expression for these three cell types was highly correlated in all samples (see Table S8). We further found by pair-wise analysis that gene expression levels for markers of all three cell types were highly correlated for the same brain regions in all control \((r > 0.94)\) or DS individuals \((r > 0.95)\), indicating uniform tissue collection procedures. However, in agreement with our DEX analysis, expression of oligodendrocyte-specific genes were less correlated and significantly different between DS and control DFC \((r = 0.89, p=0.018\) Wilcoxon signed-rank test; Table S8).
We analyzed individual expression trajectories of several genes within the M43 module known to be expressed in oligodendrocytes and involved in myelination. Lower levels of gene expression spanning from birth to adulthood for *myelin associated glycoprotein* (*MAG*; Figure 15A) and from mid-fetal development to early childhood for *myelin basic protein* (*MBP*; Figure 15B) were observed. To validate the *MAG* and *MBP* exon array data, we assayed the expression of these white matter associated genes and their gene products in the same neocortical samples using digital droplet PCR (ddPCR) (Figure 15C) and immunoblotting (Figure 15D-F). Together, both DEX and WGCNA analyses provide evidence for alterations in neocortical oligodendrocyte differentiation and myelination in developing DS brains at transcript and protein levels.

Given these substantial changes in oligodendrocyte lineage genes and proteins, we sought to confirm previous reports of reduced myelination in the DS forebrain. We imaged tissue sections of the DFC from 5 pairs of human DS and matched control specimens ranging in age from 1-70 years old using the spectral confocal reflectance microscopy (SCoRe) technique (Schain et al., 2014). Consistent with previous reports, we found that the overall density of myelinated fibers is reduced in DS brain across all ages (Figure 28A, B). In addition, by measuring the orientations of fibers, we found that myelinated axons in control brains form a grid-like lattice that resembles a rectilinear grid as described previously (Ang et al., 2003; Wedeen et al., 2012) (Figure 28C). However, DS brains did not exhibit evidence of grid-like myelinated fiber orientations, suggesting reduced complexity of fiber pathways in the trisomic human brain.
**White Matter Abnormalities in a Trisomic Mouse Model of DS**

To determine whether the protein and cellular level consequences of altered oligodendrocyte/myelination associated gene expression in DS are also reflected in mouse models, we examined oligodendrocyte lineage progression, myelination, and neuronal conductivity over development in the Ts65Dn mouse brain. This commonly used DS model is trisomic for ~100 orthologs of the 364 genes on HSA21 and displays many DS-specific phenotypes, including delays in brain development and cognitive defects (Rueda et al., 2012). However, changes in white matter have not been previously described in the Ts65Dn mouse brain. By immunoblotting of neocortex tissue samples (n=10) (G) and immunostaining of the anterior cingulate cortex and underlying corpus callosum (n=9) (H), we found a reduction of MAG (Figure 15G, H) and MBP (Figure 15I, J) protein levels and immunostaining intensity in postnatal day (P) 30 Ts65Dn mice. Reduced immunostaining intensity for 2’,3’-cyclic nucleotide 3’ phosphodiesterase (CNP), an oligodendrocyte and myelin-associated protein, was also observed in the P60 neocortex in Ts65Dn brains (Figure 27). Importantly, these cellular and biochemical changes are first detected during the period of myelin development and refinement and are maintained at later ages (e.g., P60).

**Myelin, Axon and Action Potential Conduction Deficits in Trisomic White Matter**

Alterations in white-matter associated proteins measured within the trisomic human and mouse brains indicate possible changes in the amount or allocation of myelin. We tested this hypothesis with ultrastructural and immunofluorescent studies in Ts65Dn and euploid mice at P60. Electron microscopy revealed that axon profiles in the Ts65Dn corpus
callosum are on average larger than in controls (Figure 16A-B) and there was a trend for fewer myelinated axons in the body of the Ts65Dn corpus callosum ($p= 0.1$, $n=6$; Figure 16C). Furthermore, when myelinated axons were binned by axon area, we found a significant decrease in the number of small-bore myelinated axons (0-0.5 $\mu m^2$; $p= 0.046$; Figure 16D). We also found that myelinated axons in Ts65Dn brains have significantly thinner myelin sheaths ($p< 0.011$; Figure 16E-F). Axons were then binned by size to determine whether a correlation exists between axon diameter and myelin sheath thickness. Specifically, small-bore myelinated Ts65Dn axons within 0-0.5 $\mu m^2$ and 0.5-1 $\mu m^2$ have thinner myelin sheaths than corresponding controls ($p= 0.084$ and $p= 0.076$, respectively; Figure 16G). Altogether, these results indicate a decrease in the percentage of small-bore myelinated axons and myelin sheath thickness in Ts65Dn white matter.

Deficits in myelination and oligodendrocyte maturation can also lead to impaired formation of the nodes of Ranvier (Kaplan et al., 2001; Rasband et al., 1999; Tanaka et al., 2009). Therefore, we immunostained coronal brain sections from Ts65Dn and controls for NF186 (an NFASC isoform) and CNTNAP1 (Thaxton et al., 2010), which mark nodal regions (see extended experimental procedures). Quantification of nodal protein profiles in the Ts65Dn brain at P60 revealed a striking decrease in the number of nodes of Ranvier in the corpus callosum and we found similar decreases in the external capsule (Figure 17A-C; $p<0.005$). Ultrastructural analysis confirmed this result, revealing a trend for fewer paranode cross-sections in Ts65Dn (Figure 17D-E; $p=0.059$). To determine if these findings implicate changes in node formation as a novel human DS brain phenotype, we evaluated expression levels of both NFASC and CNTNAP1 in the exon array dataset. These
genes were significantly down-regulated from birth to adulthood (Figure 17F-G), and subsequent ddPCR analysis confirmed these differences in the DS brain samples (Figure 17H).

To determine the functional consequences of these white matter defects, we measured compound action potentials (CAP) in the corpus callosum of P30-P55 Ts65Dn forebrain slices (Figure 18A). Our results revealed that action potential transmission is significantly slower in myelinated Ts65Dn axons (N1) compared to euploid controls (Figure 18B; \( p=0.04, n=10 \)). In contrast, the conduction velocity in unmyelinated axons (N2) is similar between groups (Figure 18B; \( p=0.4, n=11 \)). Examining the relationship between stimulus intensity and response magnitude (input-output curve) revealed that both types of axons in Ts65Dn are less excitable than those in euploid mice (Figure 18C, D; \( p<0.001 \) for N1, \( p<0.007 \) for N2). However, the action potential refractory periods were not different between groups, indicating that sodium channel kinetics are less likely to be responsible for the shift in the input-output relationship (data not shown). These data, combined with our cellular and electron microscopy results, indicate that white matter abnormalities found in the trisomic human and Ts65Dn mouse brain contribute to structural and functional defects in neurotransmission.

**Alterations in Oligodendrocyte Lineage Progression in DS and Trisomic Mouse**

To gain further insight into possible underlying mechanisms for myelin and white matter defects in DS, we examine the expression of genes associated with development of human oligodendrocyte precursor cells and mature oligodendrocytes by generating sets of genes predicted to be selectively enriched in these cell types during human development.
These gene sets were generated by overlapping lists of oligodendrocyte lineage candidate genes from previous mouse (Zhang et al., 2014) and human (Kang et al., 2011) studies (for details on the gene selection and analysis see Extended Experimental Procedures and Table S9). We then assessed if there was differential expression of the oligodendrocyte precursor and myelinating oligodendrocyte gene sets in DS versus control DFC. We observed that expression of putative human oligodendrocyte precursor cell-related genes gradually increases in DS compared to controls, persisting until periods 12-14 (Figure 19A). In contrast, putative human myelinating oligodendrocyte genes in the DFC are expressed at lower levels from birth into adulthood (Figure 19C). These data support and extend our DEX and WGCNA analyses (Figure 14, Tables S5-S6) suggesting an overall and long-lasting impairment of oligodendrocyte maturation in DS, which is detectable after birth in the neocortex.

To characterize these changes at the gene network level, we determined the overlap between WGCNA modules and sets of genes that we predicted to be selectively enriched in oligodendrocyte precursor cells or myelinating oligodendrocytes (Table S9). We found that human oligodendrocyte precursor cell genes were significantly enriched in modules M9, M36, M43, and M47 (Figure 19B); exon array trajectories for the two most significantly enriched modules (i.e., M9 and M47) indicated elevated expression in DS brain samples over the course of development (Figure 19E). Conversely, human mature oligodendrocyte genes were found to be significantly enriched primarily in two modules, M8 and M43, and their trajectories indicate decreased expression in DS brains during development (Figure 19D, F). Thus, the relevant WGCNA modules exhibit similar
trajectories when compared with the expression changes of individual oligodendrocyte-lineage genes, demonstrating that these cell type-specific defects in oligodendroglial development are associated with complex gene network disturbances in the DS brain. Altogether, these data indicate that aberrations in the process of oligodendrocyte maturation to the myelinating stage may underlie the white matter abnormalities in DS.

To test whether differences observed through our transcriptomic analyses identify changes in the oligodendrocyte lineage at the cellular level, we performed a developmental study of the Ts65Dn corpus callosum from P7 to P60 (Figure 19G-I). We first immunostained for OLIG2 to mark the oligodendrocyte lineage, and CC1 or NG2 to identify myelinating oligodendrocytes or oligodendrocyte precursor cells, respectively. At P7, around the onset of myelination, we found equivalent numbers of OLIG2+, CC1+ and NG2+ cells in Ts65Dn and control brain white matter. However, during the ensuing periods of active myelination and thereafter (after P7 and up to P60), there were fewer OLIG2+ cells in the Ts65Dn white matter compared to controls, reaching significance at P60; this decline in numbers was not due to higher than normal apoptotic rates (data not shown). More importantly, compared to controls and as predicted by the human transcriptome study, there was a higher percentage of OLIG2+/NG2+ oligodendrocyte precursor cells and a decreased percentage of OLIG2+/CC1+ oligodendrocytes in Ts65Dn white matter from P15 on. These results demonstrate that changes in the ratio of oligodendrocyte precursor cells to mature oligodendrocytes develop during the period of active myelination.

Finally, we sought to determine if there were cell-autonomous effects of Ts65Dn chromosomal triplication on oligodendrocyte development by carrying out proliferation
and maturation assays of purified OPCs isolated by immunopanning with an antibody against alpha-type platelet-derived growth factor receptor (PDGFA or PDGFRα) (Figure 20). Effects on OPC proliferation were assayed by plating euploid control and Ts65Dn OPCs at the same density in proliferative conditions for 48 hrs. We found no difference in the number of OLIG2/PDGFRA OPCs in cultures derived from euploid and Ts65Dn mice (Figure 20A, B, & E).

To test for effects of trisomy on oligodendrocyte maturation, OPCs were differentiated for 72 hours. The cells were stained for MBP and OLIG2 (Figure 20C, D). MBP/OLIG2 double positive cells were considered mature, whereas MBP-negative cells were considered immature. We found that the total number of OLIG2 cells was diminished in Ts65Dn cultures after 72 hours, suggesting that Ts65Dn cell viability is reduced in these conditions (Figure 20C, D, F & G). In addition, the percent of OLIG2/MBP double positive cells was significantly diminished, indicative of impaired maturation. To further assess this possibility, we characterized the morphology of MBP+ cells in each culture. We found that a greater proportion of Ts65Dn-derived MBP+ cells displayed a simple morphology compared to euploid cells (Figure 20H). Taken together, the results of these immunopanning experiments indicate that Ts65Dn oligodendrocytes exhibit cell-autonomous impairments in oligodendrocyte maturation and viability, but not proliferation.
DISCUSSION

In this study, we provide the most comprehensive developmental analysis of gene expression in postmortem human DS brains and their respective age-matched controls to date, and thereby establish a novel framework for the study of neural development in DS. This effort has elucidated three key aspects of gene expression differences between DS and euploid control brains. First, dysregulated genes are found throughout the genome and are not present solely on HSA21. Second, many of the dysregulated genes exhibited highly specific temporal and regional expression profiles. Third, these dysregulated genes form distinct co-expression networks associated with distinct biological categories, providing novel and unbiased insight into the multiple biological processes affected in developing and adult DS brains. Viewing DS brain development through this new lens, we have uncovered novel and robust abnormalities in the expression of genes associated with oligodendrocyte development and myelination.

Our assessment of Ts65Dn mice provided strong confirmation of the human oligodendrocyte and white matter defects and identified the cell- autonomous impairments in oligodendrocyte maturation and viability, but not proliferation, as the underlying mechanism. This cross species analysis also allowed us to perform analyses that were not possible with the available fresh, frozen postmortem human DS brains (e.g., immunohistochemistry, electrophysiology and electron microscopy). Moreover, the close similarities in neurodevelopmental defects between DS human and Ts65Dn mouse brains allowed us to further assess the cellular and functional consequences of these abnormalities, identifying changes in action potential conductance velocity in the forebrain.
white matter. These results elucidate a continuum of myelination-associated defects at the molecular, structural and functional levels that likely contribute to developmental delays and life-long intellectual disability in DS.

The chromosomal location of the dysregulated genes identified in our study indicates a scattered, widespread distribution throughout the genome. Interestingly, it has been recently shown by Letourneau et al. (2014) in fibroblasts from monozygotic twins discordant for trisomy 21, that the DS altered gene expression follows a consistent pattern, with increased and decreased gene-expression levels alternating across large chromosomal segments, which they named gene expression dysregulation domains (GEDDs). These GEDDs were not observable when comparing groups of samples due to gene expression differences between individuals (Letourneau et al., 2014), which is consistent with our results (Figure 24). It therefore remains unclear whether and how these individual-specific GEDDs underlie cardinal features of DS brain development and function. Nevertheless, in the present study, we show that dysregulated genes, despite being present throughout the genome, are organized into multiple gene networks that are robust enough to be measured across multiple samples of unrelated individuals. Most importantly, by elucidating the in vivo impact of the white matter-associated gene modules as an example, we show that these systems level changes impact cellular development and play a functional role in brain maturation.

Exemplifying the power of our combined regional sampling and bioinformatics approach, we uncovered substantial differences between brain regions in the number and identities of altered genes and demonstrated temporal changes in these region-specific
expression profiles. Importantly, distinct co-expression modules we identified by WGCNA also exhibited region-specific expression variations between DS and control brains. When considered together, these data indicate that the neurobiology of DS cannot be characterized by static lists of dysregulated genes or gene networks. Instead, the spatiotemporal nature of the disturbance plays a large role over the course of life. Our transcriptome analyses support a “cascade hypothesis” in which dysregulation of genes on HSA21 is likely the first genome-level disturbance and that gene expression changes on other chromosomes follow quickly thereafter. Indeed, HSA21 genes were already robustly upregulated compared to other chromosomes in the youngest fetal DS samples analyzed in our study, while changes in other regions of the genome increase during life with age (Figure 23). Moreover, we found that the temporal dynamics of gene module disruptions track the time periods relevant to specific developmental processes. For example, changes in modules 8, 9, 43 and 47 appear during late fetal development and the first several years of postnatal life (Figures 14 and 19), a time in development when that coincides with dramatic upregulation of oligodendrocyte/myelination genes are dramatically upregulated (Kang et al., 2011) and during a rapid expansion of oligodendrocytes in the human brain (Yeung et al., 2014). The record of these expression changes can now be used for the design and application of therapeutics tailored to particular biological process and brain regions at relevant developmental periods.

Myelination is one of the most prolonged neurodevelopmental processes, continuing until the third decade of life (Benes et al., 1994; Fleschig, 1901; Miller et al., 2012; Yakovlev, 1967). Notably, human studies have shown that development and
maturation of the white matter correlates with increased motor skills and cognitive functions (Casey et al., 2000; Gibson, 1991; Nagy et al., 2004; Paus et al., 1999; Schmithorst et al., 2005). Recent evidence in animal models indicates that ongoing myelin remodeling throughout life may be important for learning, behavior, and cognition throughout adulthood (Liu et al., 2012; McKenzie et al., 2014). Adding to these findings, the present results clearly show a profound deficit in white matter maturation in individuals with DS during infancy and adolescence in DFC, a brain region of late myelination which performs a critical role in the organization of behavioral, linguistic, and cognitive functions (Fuster, 2002; Makinodan et al., 2012). This is in agreement both with previous studies showing that DS children manifest learning and memory problems in late infancy which often worsen in adolescence (Koo et al., 1992; Lanfranchi et al., 2010) and with post-mortem studies that show reduced myelin content (Abraham et al., 2012; Wisniewski, 1990; Wisniewski and Schmidt-Sidor, 1989) and fewer oligodendrocytes in DS striatum (Karlsen and Pakkenberg, 2011).

Our findings raise the intriguing question: Do oligodendrocytes play an important role in the sensorimotor delays and altered cognition found throughout life in DS? This could be determined by rescuing myelination in Ts65Dn, through a cell transplantation and/or pharmacologic approach for instance, to see whether this can reverse cognitive and behavioral phenotypes. Future human neuroimaging studies may be able to lend further insight by querying if the degree of white matter abnormality correlates with the level of cognitive and intellectual impairment in individuals with DS.
Alterations in white matter or myelin components may also play a role in neuropathology in the aging DS brain and early onset Alzheimer’s-like pathology. Nearly all adults with DS develop dementia starting in the third decade of life (Sheehan et al., 2014). While triplication of APP is likely at the root of AD neuropathology in DS, our results raise the intriguing possibility that abnormalities in oligodendrocytes and myelin might contribute to early onset of Alzheimer’s-like pathology in DS. Consistent with this possibility, disturbances in myelin have been associated with an increased rate of Alzheimer’s disease progression (Bartzokis, 2007). Frontal white matter tracts are substantially impacted in the aging DS brain (Powell et al., 2014) and byproducts of homeostatic myelin maintenance can promote the formation of amyloid plaques (Bartzokis, 2011). Characterizing the axon-oligodendrocyte interactions in DS throughout development and aging will thus be an important topic of future work.

The concordance between the human DS and Ts65Dn mouse samples at the gene, protein and cellular levels clearly demonstrates the value of the Ts65Dn mouse in reflecting the cellular and functional consequences of trisomy 21. Our finding that the Ts65Dn white matter phenotype mirrors that occurring in the human DS brain so robustly suggests that it will be a good model to identify neuronal cues that influence the onset and completion of myelination. Both intrinsic and extrinsic factors have been shown to influence myelination. For example, recent studies have shown that electrical activity drives myelination (Gibson et al., 2014; Ishibashi et al., 2006; Wake et al., 2011) and that synaptogenesis and myelination are linked. Indeed, neuronal activity is proposed to promote myelination through NMDA receptor and ERBB3 signaling, among other
pathways (Brinkmann et al., 2008; Makinodan et al., 2012). Notably, genes and modules associated with synaptogenesis and neuronal transmission are downregulated in DS (including ERBB3), suggesting that disrupted neuronal transmission may play a role in impaired myelination. However, we also show that myelin abnormalities in DS are at least in part due to a cell-autonomous phenomenon in oligodendrocyte development. Teasing out the relative contributions of cell-autonomous effects and neuron-glial signaling, or other cell extrinsic mechanisms, will be an important goal of future work. These and other queries can now be approached using the developmental expression profiling and multi-species analysis presented here.

In summary, we profiled the spatiotemporal changes in gene expression that result from trisomy 21 in postmortem human brain. Together with complementary analyses of Ts65Dn segmental trisomy mice, the most extensively studied rodent model of DS, the data reveal a novel role for impaired oligodendrocyte differentiation and myelination, and abnormal white matter tract architecture in DS pathobiology. Our findings indicate that strategies to enhance myelination may therefore serve as therapeutic targets to attenuate the cognitive and neurological symptoms of DS. Our results also implicate spatiotemporal disturbance in other molecular pathways in DS brain, and provide a powerful data set for further computational and functional analyses. We anticipate that the resources provided by this study will inspire and facilitate future studies of the mechanistic basis for impaired neural development in DS and other cognitive disorders, while also lending insight into the genetic and transcriptional underpinnings of previously described DS phenotypes and the relevance of mouse model studies to the human disease.
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Tables S1-S8. SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and eight tables and can be found with this article online. Supplemental figures from the original manuscript are included below. Supplemental tables can be found online with this article.
Figure 13. Genes Dysregulated in DS Brains are Globally Distributed and Developmentally Dynamic. A, The percentage of differentially expressed genes (DEX) on each chromosome indicating global disruptions in gene expression. B, The number of genes on each chromosome that are DEX in Down syndrome (DS). Note that the majority of DEX genes are not located on HSA21. C, The number of up-regulated (white shading) and down-regulated (gray shading) DEX genes per chromosome. D, E, A permutation analysis of DEX genes in the dorsolateral prefrontal cortex (DFC) (D) and cerebellar cortex (CBC) (E) across 4 sliding windows corresponding to periods from mid-fetal development to adulthood. Periods of human brain development and adulthood are defined as previously described (Kang et al., 2011). Note that the number of DEX genes rises over development for DFC but not CBC. No CBC samples were available for periods 5 to 7. All experiments and analyses were performed by members of the Sestan Lab, Yale University.
Figure 14. A Co-Expression Module Enriched in Genes Associated with Oligodendrocyte Development and Myelination is Decreased over Development in DS

A, Gene network analysis identifies distinct modules of co-expressed genes dysregulated in DS, including module (M) 43 that is significantly enriched in genes associated with oligodendrocyte development and myelination. Plots of relative expression of the PC1 of M43 over development in the DFC, primary visual cortex (V1C), hippocampus (HIP), and CBC, indicating higher expression in euploid control versus DS brain that increases in degree over development. B, A heat map showing the expression of M43 genes in DFC (left panel) and CBC (right panel), which confirms and details the gene expression trajectories shown in A. Note that a high proportion of mouse homologs of the M43 genes are expressed in mouse oligodendrocyte lineage cells (gene symbols are highlighted in red). C, A network plot of M43 genes and their intramodular connections (cutoff, Pearson correlation > 0.7). 8 of the 10 hub genes (the top ten genes with highest intramodular connectivity; TMEM63A, MYRF, PLD1, RTKN, ASPA, OPALIN, ERBB3, EVI2A) are primarily expressed by mature oligodendrocytes and tightly correlated with other oligodendrocyte-associated M43 genes. Oligodendrocyte enriched genes are shown in red. Note their central position in the network, suggesting high intramodular connectivity. D, The number of mouse homologs of M43 genes that are highly expressed in major cell types from mouse cerebral cortex. The majority of the M43 genes are highly expressed specifically in oligodendrocytes. Red bars denote the oligodendrocyte lineage; gray bars denote other cell lineages. All experiments and analyses were performed by members of the Sestan Lab, Yale University.
Figure 15. Expression of the Essential Myelin Components MAG and MBP is Diminished in Developing DS and the Ts65Dn Mouse Brains

A, Log2 values of the array signal intensity in human euploid control (Ctrl) and DS DFC show that MAG expression is decreased from birth onwards (periods 8 to 14) in DS. Periods 5-9, p=0.28; periods 8-12, p=0.023; periods 9-13, p=0.025; periods 10-14, p=0.0024; all periods, p=0.014 (paired t-test). B, Log2 values of the array signal intensity in human Ctrl and DS DFC show that MBP expression is decreased from mid-fetal development to early childhood (periods 5-9) in DS. Periods 5-9, p=0.078 (one tailed test, p=0.039); periods 8-12, p=0.192; periods 9-13, p=0.52; periods 10-14, p=0.72; all periods, p=0.10 (paired t-test). C ddPCR analysis of MBP and MAG expression in human Ctrl and DS DFC samples confirming decreased expression in developing DS brains. D, Representative western blots for MAG and MBP in human Ctrl and DS DFC over development (DS samples in red type). E, F, Sliding window analysis of MAG (E) and MBP (F) protein levels identify significant reductions in developing and adult DS brains. *, p≤0.03 (paired t-test).

G, H, Western blotting of neocortex tissue samples (n=10) (G) and immunostaining of the anterior cingulate cortex (n=9) (H) in postnatal day (P) 30 Ctrl and trisomic Ts65Dn mice identifies a reduction of MAG protein expression in Ts65Dn brains. *, p≤0.05; **, p≤0.001 (paired t-test). I, J, Western blotting for MBP in the neocortex (n=3 pairs) (I) and immunostaining for MBP in P30 cingulate cortex (J) of Ctrl and Ts65Dn mice show a trend towards reduced expression of MBP in the white matter of Ts65Dn mice (n=3 pairs). *, p=0.009 (paired t-test). Genetic analyses (A-C) were performed by members of the Sestan Lab, Yale University, and immunostaining of Ts65Dn brains (H, J) by Tarik Haydar.
Figure 16. Diminished Myelin Sheath Thickness in the Ts65Dn Corpus Callosum

A, B, Representative electron micrographs of axon cross sections in the P60 euploid control (A) and Ts65Dn (B) corpus callosum. Scale bar = 1 µm. C, Graph of the numbers of myelinated axons in euploid versus Ts65Dn revealing a trend towards a decrease in the number of myelinated axons in Ts65Dn mice. D, Histogram indicating a decrease in the proportion of small diameter axons and an increase in the proportion of large diameter axons (>1 µm) in Ts65Dn mice. *, p≤0.05; #, p=0.058 (paired t-test). E, Plot of g-ratios (y-axis) and the corresponding diameter for all axons assessed. Black dots = euploid control; red dots = Ts65Dn. F, Bar graph showing there is a significant increase in the mean g-ratio in Ts65Dn mice indicating thinner myelin sheaths around Ts65Dn axons. *, p=0.011 (paired t-test). G, Histogram indicating that the g-ratios of small diameter, but not large diameter axons, are higher in Ts65Dn mice. #, p≤0.08 (paired t-test). Alan Peters and Claire Sethares aided in EM tissue processing and imaging. Imaging and data analysis were performed by Luis Olmos-Serrano.
Euploid control (Corpus callosum)

Ts65Dn (Corpus callosum)

C

Number of myelinated axons

E

Collumus ratio

F

Collumus ratio

D

Percentage of axons

G

Collumus ratio

Axon area (μm²)

Axon area (μm²)
**Figure 17. Impaired Formation of Nodes of Ranvier in Ts65Dn Mice**

*A*, Representative image of nodes of Ranvier in P60 euploid control (Ctrl) corpus callosum identified by immunofluorescent labeling for the paranodal protein NFASC (red) and the internodal protein CNTNAP1 (green) which mark the node of Ranvier. *B*, Representative image of immunostaining for NFASC (red) and CNTNAP1 (green) indicating there are fewer nodes of Ranvier in Ts65Dn mice. *C*, Bar graph demonstrating reduced density of nodes of Ranvier in corpus callosum and external capsule in P60 Ts65Dn brain (n=3 pairs). ***, p≤0.002 (paired t-test). *D, D’,* Electron micrographs showing representative images of paranodes (arrows). *E*, Quantification of paranodes reveals that they are reduced in P60 Ts65Dn corpus callosum (n=3 each). *F*, Log2 values of the array signal intensity in human euploid Ctrl and DS DFC show that NFASC expression is decreased from birth onwards (periods 8 to 14) in DS. Periods 5-9, p=0.23; periods 8-12, p=0.016; periods 9-13, p=0.0092; periods 10-14, p=0.0022; all periods, p=0.0028 (paired t-test). *G*, Log2 values of the array signal intensity for DFC expression of CNTNAP1 show that its expression is decreased from birth onwards (periods 8 to 14) in DS. Periods 5-9, p=0.41; periods 8-12, p=0.018; periods 9-13, p=0.017; periods 10-14, p=0.011; all periods, p=0.012 (paired t-test). *H*, ddPCR analysis of human DFC samples confirming decreased expression of NFASC and CNTNAP1 from childhood onwards in DS. Alan Peters and Claire Sethares aided in EM tissue processing and imaging. Luis Olmos-Serrano performed immunohistochemistry and electron microscopy imaging and data analysis. All genetic analyses were performed by members of the Sestan lab.
Euploid control

Ts65Dn

Mouse

neuronalin (NFASC)

CNTNAP1

Corpus callosum

E

Corpus callosum

Percentage of neurones

Euploid control

Ts65Dn

** p<0.01

F

G

H

dPCR (linear correlation)

y = -0.136x + 2.515
R² = 0.4873

NFASC

CNTNAP1

mRNA fold ratio (OS/CH)
**Figure 18. Slower Axonal Conduction in Ts65Dn Corpus Callosum**

**A**, Experimental setup depicting the stimulating electrode (bottom) and recording electrode (top) within P30 corpus callosum of a coronal section. Compound action potentials (CAP) evoked by stimulation were recorded at multiple sites (red dots) to compute conduction velocity. Inset shows a representative example of an evoked CAP depicting the components arising from myelinated fibers (N1) and unmyelinated fibers (N2).  

**B**, Bar plot showing that conduction velocities for myelinated fibers (N1), but not unmyelinated fibers (N2), were significantly slower in Ts65Dn corpus callosum (*, p=0.04, n=10 for N1; n=11 for N2).

**C**, The input-output relationship for myelinated fibers is right-shifted in Ts65Dn corpus callosum suggesting that this fiber type is less excitable (*, p<0.001 for N1 euploid n=5, Ts65Dn n=4).

**D**, The input-output relationship for unmyelinated fibers is right-shifted in Ts65Dn corpus callosum suggesting that this fiber type is less excitable (*, p≤0.007 for N2; euploid n=5, Ts65Dn n=4). Electrophysiology was performed and analyzed by Nathan Cramer and Zygmunt Galdzicki.
Figure 19. Oligodendrocyte Maturation is Impaired in DS. A, Developmental changes in genes associated with oligodendrocyte progenitor cells expressed as ratio of DS vs. Ctrl. *p≤0.05 (paired t-test). B, -log10 p-values of expression of oligodendrocyte progenitor cell-enriched genes in weighted gene co-expression network modules reveals significant enrichment in the M9, M36, M43 and M47 modules [y-axis = -log10 (p value)]. Lower dashed line corresponds to p = 0.05; upper dashed line corresponds to p = 0.01. C, Developmental changes in genes associated with myelinating oligodendrocytes expressed as ratio of DS vs. Ctrl. *p≤0.05 (paired t-test). D, -log10 p-values of enrichment analysis for expression of mature oligodendrocyte enriched genes in gene network co-expression modules demonstrating significant enrichment in modules M8 and M43 [y-axis = -log10 (p value)]. Lower dashed line corresponds to p = 0.05; upper dashed line corresponds to p = 0.01. E, PC1 plots of the co-expression modules enriched in oligodendrocyte progenitor cell specific genes (M9 and M47) demonstrating that they are increased in DS and that the differences between Ctrl and DS samples increase over postnatal development. F, PC1 plots enriched co-expression modules enriched in specific myelinating oligodendrocyte specific genes (M8 and M43) demonstrating that they are decreased in DS and that the differences between Ctrl and DS samples increase over postnatal development. G, Representative immunofluorescent stains of P60 Ctrl corpus callosum for OLIG2 (purple), CC1 (green), NG2 (red), and nuclei (blue). Yellow arrows point to NG2-labeled OPCs and white arrows point to CC1-labeled mature oligodendrocytes. H, Representative immunofluorescent stains of P60 Ts65Dn corpus callosum for OLIG2 (purple), CC1 (green), NG2 (red), and nuclei (blue). Fewer CC1-labeled mature oligodendrocytes are
apparent. The numbers of OLIG2 immuno-positive cells in the corpus callosum were counted in image volumes from P7-P60 (n=4 pairs at each age). There was a general trend of fewer OLIG2+ cells in the Ts65Dn white matter which becomes significant at P60. In addition, as a proportion of the total OLIG2+ population, the percentage of NG2+ oligodendrocyte progenitor cells is higher in Ts65Dn corpus callosum from P15-P60. In contrast, the percentage of mature CC1+ oligodendrocytes is reduced in Ts65Dn from P15-P60. *, p≤ 0.05; **, p≤ 0.005. Genetic analyses were performed by members of the Sestan lab. Oligodendrocyte immunohistochemistry, imaging, and analysis were performed by William Tyler.
**Figure 20. Impaired Maturation and Viability of Immunopurified Oligodendrocyte Progenitor Cells, In Vitro.**

**A**, Representative micrographs of immunostaining for oligodendrocyte progenitor cells isolated from P7 euploid cortex and proliferated for 48 hours; PDGFRA (green), OLIG2 (red), and nuclei (blue).  

**B**, Representative micrographs immunostaining for oligodendrocyte progenitor cells isolated from P7 Ts65Dn and proliferated for 48 hours; PDGFRA (green), OLIG2 (red), and nuclei (blue).  

**C**, Representative micrographs of immunostaining for oligodendrocyte progenitor cells isolated from P7 euploid cortex and cultured in pro-maturation conditions for 72 hours; MBP (green), OLIG2 (red), and nuclei (blue).  

**D**, Representative micrographs of immunostaining for oligodendrocyte progenitor cells isolated from P7 Ts65Dn cortex and cultured in pro-maturation conditions for 72 hours; MBP (green), OLIG2 (red), and nuclei (blue).  

**E**, Graph indicating there were no differences between euploid control and Ts65Dn in the number OLIG2+ cells observed after 48 hours in proliferative conditions.  

**F**, Graph indicating the number OLIG2+ cells was reduced by ~30% after 72 hours in pro-maturation conditions.  

**G**, Graph indicating the percentage of OLIG2+ cells co-expressing MBP was reduced by ~40% after 72 hours in pro-maturation conditions.  

**H**, Graph indicating a greater percentage of MBP+ cells from Ts65Dn mice cultured in pro-maturation conditions exhibited a simple morphology (<6 processes) than MBP+ euploid cells, which tended to have a more complex (≥ 6 processes) or membranous morphology. *, p≤ 0.05; **, p≤ 0.01 (unpaired Student’s t-test). All experiments and analyses were performed by members of the Sestan lab.
Figure 21. Robustness and Reproducibility of the Exon Microarray Protocol.

(Related to Figure 13). A, Box plots showing the log2 intensity of 100 segments divided along the longest transcript of each gene from the 5’-end to 3’-end compared to the expression of the whole gene indicating array hybridization uniformity. B, Spearman correlation analysis of eight samples, which were re-tested to confirm technical reproducibility. C, D, Hierarchical clustering of all genes (C) or chromosome 21 (HSA21) genes (D) indicating genes cluster most strongly according to region and developmental period rather than disease status or other factors. Disease status (light blue, euploid control; red, Down syndrome), period (blue to red representing younger to old), region (blue, neocortex [NCX; all neocortical areas_regions combined]; white, hippocampus [HIP]; red, cerebellar cortex [CBC]), postmortem interval (PMI; low to high representing blue to red), RNA integrity number (RIN; low to high representing blue to red), ethnicity (African-American, light blue; Caucasian, brown; Hispanic, yellow), and sex (blue, male; pink, female). All experiments and analyses were performed by members of the Sestan lab.
**Figure 22. Principle Component Analysis Reveals that Brain Region and Age Contribute More to Transcriptional Differences than Disease Status. (Related to Figure 13)**

A. Three-dimensional plots of the principle component analysis (PCA) of Down syndrome (DS) samples and their matched euploid controls (Ctrl) rotated in 3 different views for better visualization and colored according to disease status. Note fairly minimal differences between DS versus Ctrl clusters. B, Three-dimensional plots of the PCA of DS samples and their matched controls rotated in 3 different views for better visualization and colored according to brain region (NCX [pooled neocortical regions/areas], HIP, and CBC). Note the large separation of CBC clusters from NCX and HIP. C, Three-dimensional plots of the PCA of DS and Ctrl samples rotated in 3 different views for better visualization and colored according to developmental period. Note that there are clear patterns defining distinct developmental periods, that display larger separation than disease state, but more moderate separation than CBC versus NCX/HIP clusters. All experiments and analyses were performed by members of the Sestan lab.
Figure 23. Developmental Dynamics in the Proportion of Dysregulated Genes Across Chromosomes. (Related to Figure 13) 

A, B, The distribution of differentially expressed (DEX) genes percentages in each chromosome across four sliding window periods calculated from 20 cycle permutations for the dorsolateral prefrontal cortex (DFC) (A) and CBC (B). The percentage of DFC expression rose across the sliding window periods in nearly all chromosomes, while the developmental dynamics in the proportion of DEX genes was variable between chromosomes in the CBC, with most chromosomes exhibiting no change in the percentage of DEX genes across development. For the permutation test, DS samples were randomly matched to control samples within the same stage and with the same sex to identify DEX genes. All experiments and analyses were performed by members of the Sestan lab.
Figure 24. Gene Expression Fold Change is Not Organized in Chromosomal Domains in the Down Syndrome Brain Transcriptome. (Related to Figure 13) Representative plots of a period-specific genome-wide expression variations (log2 fold change [log2FC]) for the DFC indicate a lack of widespread and contiguous genomic regions of up-regulated or down-regulated gene expression in DS brain. A LOWESS smoothing function (red trace) demonstrates minimal deviation from zero. All experiments and analyses were performed by members of the Sestan lab.
Figure 25. Representative Co-Expression Modules with Increased Expression in Down syndrome Brains. (Related to Figure 14) A, Plots of relative expression of the principal component 1 (PC1) of gene co-expression module (M) 24 over development in the DFC, primary visual cortex (V1C), HIP, and CBC, indicating higher expression in DS versus control forebrain that increases over development. Subsequent gene ontology analysis revealed that this module was enriched in genes associated with cell morphogenesis/adhesion. B, Plots of relative expression of the PC1 of M54 over development in the DFC, V1C, HIP, and CBC, indicating higher expression in DS versus control forebrain that increases over development. Subsequent gene ontology analysis revealed that this module was enriched in genes associated with immune responses. All experiments and analyses were performed by members of the Sestan lab.
Figure 26. Representative Co-Expression Modules with Decreased Expression in Down Syndrome Brains. (Related to Figures 14 and 15) 

A, Plots of relative expression of the PC1 of M4 over development in the DFC, V1C, HIP, and CBC, indicating decreased expression in DS versus Ctrl brain over development. Subsequent gene ontology analysis revealed that this module was enriched in genes associated with neuron differentiation. 

B, Plots of relative expression of the PC1 of M10 over development in the DFC, V1C, HIP, and CBC, indicating decreased expression in DS versus Ctrl brain over development. Subsequent gene ontology analysis revealed that this module was enriched in genes associated with synaptic transmission. 

C, Plots of relative expression of the PC1 of M34 over development in the DFC, V1C, HIP, and CBC, indicating decreased expression in DS versus Ctrl brain over development. Subsequent gene ontology analysis revealed that this module was enriched in genes associated with synaptic transmission/regulation. 

D, Plots of relative expression of the PC1 of M45 over development in the DFC, V1C, HIP, and CBC, indicating decreased expression in DS versus Ctrl brain over development. Subsequent gene ontology analysis revealed that this module was enriched in genes associated with neuron differentiation. All experiments and analyses were performed by members of the Sestan lab.
Figure 27. Oligodendrocyte-Related Genes and Proteins are Reduced in Down Syndrome and Ts65Dn Mouse Neocortex. (Related to Figures 15 and 19) A, ddPCR analysis of human euploid Ctrl and DS DFC shows down-regulation of *SOX10* expression in developing and adult DS brain. B, Western blot measurements of SOX10 protein levels in the Ctrl and Ts65Dn mouse corpus callosum (CC) at P30. **, p<0.03 (paired t-test). C, Representative immunofluorescence labeling of the corpus callosum in P60 euploid Ctrl and Ts65Dn mice using the RIP antibody raised against CNP. D, Quantitative analysis of the intensity of RIP immunostaining reveals a significant decrease in Ts65Dn compared to euploid Ctrl mice. *, p<0.02 (paired t-test). ddPCR was performed and analyzed by members of the Sestan lab. Luis Olmos-Serrano performed RIP immunostaining and analysis.
Figure 28. Myelin Laser Reflectance Signatures are Reduced in Down Syndrome Neocortex. (Related to Figure 19) A, Spectral confocal reflectance microscopy of 70 year old Ctrl and DS tissue sections of the DFC. B, Quantification of myelinated fiber density neocortical images from five DS cases captured from the pial surface down to the compacted white matter. Each brain is compared to its respective control brain (black line). C, Representative examples of myelinated fiber orientations at two different depths within 70 year old Ctrl and DS brains. Polar plots depict the fiber orientations. SCoRe imaging and analyses were performed by Tarik Haydar and William Tyler.
CHAPTER FOUR

Static oligodendrocytes and dynamic interneurons: Cell-type specific aberrations in Dp16 cortical populations

Data Unpublished

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Note: All experiments were performed by Joseph Goodliffe.
ABSTRACT

The cognitive deficit in Down syndrome has profound effects on social, behavioral, and emotional development. The etiological basis of neurodevelopmental delays remains widely elusive, however work in mouse models and human tissue have delineated a myriad of neuropathologies that likely contribute to the intellectual disability in DS. The Ts65Dn mouse model of DS exhibits extensive proliferation aberrations and principal among these are reduced neurogenesis in the dorsal germinal zone and elevated rates of apical mitosis in the ventral germinal zone. These spatial changes to embryonic development contribute to hypocellularity of cortical excitatory neurons and an increase in cortical interneurons observed in Ts65Dn adults. A shift in the balance of excitation and inhibition is believed to underlie many neurological disorders including DS and presents as a potential target for therapeutic intervention. Recently, the Dp16 mouse model of DS has been shown to recapitulate adult behavioral phenotypes seen in Ts65Dn, but this model lacks major embryonic DS-related phenotypes. Despite this temporal discrepancy, Dp16 animals begin to exhibit behavioral delays and cellular changes during the second postnatal week of life. In order to understand the temporal changes in the Dp16 model, we assessed interneuron and oligodendrocyte population dynamics and found that while the cortical interneuron population changes with age, oligodendrocyte-lineage populations remain constant.
INTRODUCTION

Down syndrome is the most common genetic intellectual disability and results from trisomy of chromosome 21 (Lejeune, 1959). The triplication of Hsa21 causes a constellation of phenotypes that affects multiple body systems including the central nervous system. The most prevalent phenotype in DS individuals is the cognitive deficit, which is characterized by learning and memory impairments and lower average IQ (Chapman et al., 2000). Pre- and post-natal human studies have shown developmental abnormalities of critical neurological processes including neurogenesis, myelination, neuronal differentiation, and cortical lamination (Becker et al., 1991; Coyle et al., 1986). The etiology of the neurologic abnormalities is not well understood as there are more than 400 genes triplicated in Ts21 (Hattori et al., 2000). Fortunately, several mouse models of DS are available to study the neurologic abnormalities and etiology of the cognitive deficit.

Mouse chromosomes 10, 16, and 17 share Hsa21 syntenic regions; murine chromosome 16 (Mmu16) has the largest syntenic region of 22.9 Mb (Yu et al., 2010). Recently, mouse models have begun to be used to investigate therapeutic interventions that may ameliorate components of the cognitive deficit and improve quality of life in DS individuals. In fact, separate studies on the Ts65Dn mouse model using the anti-depressant, fluoxetine, and epigallocatechin-3-gallate (EGCG), a major component of green tea, have shown significant improvements in cognitive performance during spatial memory tasks as well as recovery of cellular neuropathologies (Bianchi, et al., 2010; Begenisic, et al., 2014; De la Torre, et al., 2014; Guidi, et al., 2014; Souchet, et al., 2015).
While these studies provide important insight into potential treatments for what was once considered an untreatable condition, they also highlight a major question in DS research. Which mouse model best represents the human condition?

The most commonly used mouse model of DS, the Ts65Dn, has a triplication of 104 Hsa21-syntenic genes (NCBI; Davisson, 1990). The Ts65Dn exhibits learning and memory deficits similar to individuals with DS as well as craniofacial abnormalities and atypical development of the heart, cerebellum, hippocampus, and neocortex (Baxter et al., 2000; Chakrabarti et al., 2007; Chakrabarti et al., 2010; Insauti et al., 1998; Lorenzi et al., 2006; Reeves et al., 1995; Richtsmeier et al., 2002). Also, Ts65Dn exhibit cell-type specific changes including an elevation in cortical interneuron populations (Chakrabarti et al., 2010). It has recently been shown that oligodendrocytes and cortical white matter are similarly affected in the Ts65Dn and human DS brain with an overabundance of oligodendrocyte precursor cells and reductions in myelinating oligodendrocytes (Olmos-Serrano et al., 2016; Chapter 3). Despite possessing distinct DS-related phenotypes, the Ts65Dn model carries 60 genes on the extra chromatid that are non-homologous to Hsa21 (Gardiner, 2014). The effect(s) of these non-syntenic genes remains elusive; however, they present a large confound in the use of Ts65Dn as a reliable model of human DS.

A new mouse model of DS, the Dp16, exhibits several advantages over the Ts65Dn including triplication of the entire Hsa-21 syntenic region of Mmu16 and fertile males. Recent work has shown that Dp16 animals have learning and memory deficits, craniofacial abnormalities, and cerebellar phenotypes comparable to Ts65Dn (Yu et al.,
2010; Starbuck et al., 2014) however, the Dp16 model fails to recapitulate many DS-related embryonic phenotypes reported in DS fetuses and the Ts65Dn (Chapter 2). Despite the lack of embryonic pathology, Dp16 develops hypocellularity of cortical interneurons and delays in milestone acquisition by the second week of life (Chapter 2). In order to better understand the cognitive deficit manifested in Dp16, how it may develop over time and how it differs from Ts65Dn, we sought to determine the interneuron and oligodendrocyte phenotypes present in Dp16.
MATERIALS AND METHODS

ANIMALS

Embryonic B6129S-Dp (16Lipi-Zfp295)1Yey/NJ

Dp16 animals were received as a gift from Eugene Yu (Roswell Park Institute, Buffalo, NY, USA) and were also purchased from Jackson Laboratory (Stock 013530, Bar Harbor, ME). All Dp16 founders were backcrossed to C57BL/6 mice for at least five generations prior to import into our facility. Our Dp16 breeding colony was maintained by backcrossing Dp16 males with C57BL/6N females obtained from Charles River Laboratories (Wilmington, MA). Breeders were paired every evening between 3-5PM and separated every morning between 9-10AM followed by vaginal plug checks. Postnatal day 15 and 30 (P15 and P30, respectively) animals were anesthetized and fixed via intracardial perfusion using 4% PFA, 1x PBS.

Genotyping

Limb buds (embryos) or tail clippings (postnatal animals) were digested using 0.2 mg/ml proteinase K (Denville Scientific) in DNA digestion buffer (50 mm Tris-HCl, pH 8.0, 100 µm EDTA, pH 8.0, 100 µm NaCl, and 1% SDS) overnight at 55°C followed by phenol/chloroform extraction and DNA was precipitated using ethanol. Genotyping was performed using PCR amplification with primers specific for the Dp16 translocation breakpoint (Forward: 5’-CTG CCA GCC ACT CTA GCT CT-3’, Reverse: 5’-AAT TTC TGT GGG GCA AAA TG-3’) and mFez (Forward: 5’-CTT CGG GAG CAG GTA CCC TGT GGG GCA AAA TG-3’, Reverse: 5’-AAG ATC TGA GGC TCG CCA AG-3’) as an internal positive control. In addition, we confirmed all genotypes using a separate PCR procedure using
different primers and protocol (Forward: 5’-TAT TAG GAC AAG GCT GGT GG-3’, Reverse: 5’-GTC AGT GGT TGT GAC TTG TG-3’, gift from Roger H. Reeves).

Tissue Processing - Histology

After 24 h of PFA fixation, P15 and P30 brains were cryoprotected in 30% sucrose, 1x PBS for 24 h at 4°C followed by freezing in Optimal Cutting Temperature Compound (OCT; Sakura, Torrance, CA), and stored in OCT at -80°C. 16-μm serial coronal sections were obtained using a cryostat (Microm HM 560), mounted on Superfrost ® Plus Microscope slides (Fisher scientific) and stored at -80°C.

Immunohistochemistry

Frozen slides were allowed to come to RT and rinsed in 1x PBS. Antigen retrieval was performed on embryonic slides by microwaving in 10 mM sodium citrate for 1 min at maximum power, followed by 10 min at the minimum power. Slides were incubated in blocking solution (5% donkey serum, 0.2% Triton® X-100, 1x PBS) for 1 h at RT followed by incubation in primary antibody overnight at RT. The next day, slides were washed three times in 1x PBS and incubated with secondary antibody solution for 1 h at RT followed by three washes in 1x PBS. Slides were mounted in Vectashield with DAPI (Vector laboratories, inc., H-1200). Primary antibodies: rat anti-Somatostatin (SOM) 1:50 (Millipore, MAB354), rabbit anti-Parvalbumin (PV) 1:1000 (Swant, PV25), rabbit anti-Calretinin (CR) 1:1000 (Swant, 769913), rabbit anti-Olig2 1:500 (Millipore), guinea-pig anti-NG2 1:1000 (gift from William B. Stallcup, Sanford-Burham Medical Research Institute, La Jolla), and mouse anti-CC1 1:500 (CalbioChem). Secondary antibodies: 1:250 for all; donkey anti-rabbit 555 (life technologies, A31572), donkey anti-goat 488
(life technologies, A11055), goat anti-rabbit 546 (life technologies, A11035), goat anti-
rabbit 488 1:500 (life technologies, A11008) and goat anti-rat 568 1:500 (life
technologies, A11077).

**Confocal Microscopy and Cell Counting**

All sections were imaged with confocal microscopy using a Zeiss LSM 710
microscope system (Carl Zeiss, Jena). Twelve micron, 20x z-stacks were acquired using
Zeiss Zen software. All cell counts were performed manually using ImageJ and LSM
Browser software. The experimenter was blind to genotypes.

**Statistical Analysis**

Statistical analyses were performed using Sigmaplot software. For all
immunohistochemical assessments, unpaired $t$-tests were performed to determine
statistical significance.
RESULTS

*Persistent hypocellularity of cortical interneurons in Dp16*

Previous findings in Dp16 revealed hypocellularity of parvalbumin (PV) and somatostatin (Sst) inhibitory interneurons in the somatosensory cortex (SSCxt) at postnatal day 15 (P15) (Chapter 2). To determine the timeline of the inhibitory phenotype in Dp16, immunohistochemistry for three interneuron subtypes (PV, Sst, Cr) was performed on P30 cortical slices (Fig 29A, B). At P30, the cortical PV+ population is significantly smaller in Dp16 compared to euploid controls (Fig 29A, C). The Sst+ density was slightly larger, however did not reach significance (Fig 29B, C). No difference was observed for Cr+ labeled cells. This suggests that there is variability in trisomy effects on interneuron subtypes in the Dp16 cortex.

*Oligodendrocyte lineage is widely unaffected in forebrain white matter tracts of Dp16*

Histological studies in Ts65Dn and genetic findings in human DS tissue have revealed a shift in the ratio of immature oligodendrocyte progenitors (OPCs) to mature myelinating oligodendrocytes (mOLGs) (Olmos-Serrano et al., 2016). To assess these cell types, immunohistochemistry was performed on P15 and P30 Dp16 brain slices. Three markers were used to identify the two subtypes of OLGs: Olig2, NG2, and CC1. The transcription factor Olig2 labels the nuclei of all members of the oligodendrocyte lineage while the markers Ng2 and CC1 allow for the differentiation between OPCs (Olig2+/NG2+) and mOLGs (Olig2+/CC1+) as shown in the representative images (Fig 30A, D). Images were collected within the corpus callosum (CC, Fig 30A-C) and external capsule (EC, Fig 30D-F). At P15, the OPC and mOLG populations were comparable in
the CC and EC of both Dp16 and euploid mice (Fig 30B, C, E, F). The similarity between genotypes was also observed at P30 (Fig 30B, C, E, F). As expected, the mOLG population increased in both genotypes and areas over time while the OPC population declined in both genotypes (Fig 30). Together, these data suggest that the OLG lineage is not affected at the population level in the Dp16 mouse in two major cortical white matter tracts.
DISCUSSION

Our present findings reveal two novel features of the Dp16 mouse model of DS: 1) the previously described interneuron phenotype differs from other mouse models and changes over time and 2) the OLG lineage remains unscathed by Dp16 segmental trisomy at a population level.

Changes in the balance of excitation and inhibition is believed to be a major contributor to cognitive abnormalities observed in DS. In the Ts65Dn mouse, the cortex is marked by an overabundance of inhibitory interneurons combined with a reduction in excitatory neurons (Chakrabarti, et al., 2007; 2010). Similarly, changes in synapse structure and synaptic transmission found in the hippocampus of Ts65Dn suggest an excess of inhibition that likely contributes to poor performance in hippocampal based tasks (Kleschevnikov, et al., 2004; Best, et al., 2007; Belichenko, et al, 2009; Best, et al., 2012; Kleschevnikov, et al., 2012). These changes in Ts65Dn have highlighted excessive inhibition as a potential therapeutic target in the treatment of DS (Martinez-Cue, et al., 2014). While this pharmacological intervention may be substantiated by work in the Ts65Dn, findings in Dp16 and human contradict Ts65Dn phenotypes. Indeed, post-mortem findings of the human DS cortex suggest a reduction in small, granular GABAergic cells of layers II and IV (Ross et al., 1984) and DS derived human neural progenitor cell cultures and microarray analysis indicate substantial reductions in GABAergic neurogenesis (Bhattacharyya et al., 2009). Our results indicate hypocellularity of Dp16 interneurons which may better represent findings in human DS
and as such, Dp16 may better model inhibitory phenotypes of DS when compared to Ts65Dn.

Our previous work in Dp16 showed that, while there were no appreciable embryonic phenotypes, there was hypocellularity of parvalbumin and somatostatin cortical inhibitory interneurons at P15 (Chapter 2). At P30, the parvalbumin population remains smaller in Dp16 however, the population is larger at P30 than P15 in the Dp16 brain (Chapter 2 and 4). As there are no changes in proliferation or progenitor populations of the ventral germinal zone, it is unlikely that the inhibitory interneuron phenotype is caused by changes in cell production. The timing of interneuron integration into circuitry is highly dependent on sub-type, birth date and synapse formation. In the case of Dp16, it is possible that the maturation or migration of interneurons is impacted and reflects the changes in populations over time.

As mentioned with interneuron production, the ventral germinal zone of the Dp16 model is widely normal during embryonic development (Chapter 2). The oligodendrocyte population is normal in Dp16 at both ages studied, which is in contrast to previous reports in the Ts65Dn in which there is an overabundance of OPCs and a decline in mOLGs beginning at P15 and persisting to P60 (Chapter 3). This differing phenotype presents another contradiction that sets the Ts65Dn and Dp16 mouse models apart; Ts65Dn findings are not fully recapitulated in Dp16. The underlying genetic differences between models likely underlies the phenotypic opposition, therefore how the genetics of different models contribute to their distinct phenotypes must be determined. This effort is
necessary for the advancement of DS research and the identification of the mouse model that is best for therapeutic assessments.
TABLES AND FIGURES

Figure 29. Hypocellularity in PV-expressing interneurons of the Dp16 P30

Somatosensory cortex. A, Representative image of IHC of the P30 SSCtx (PV-red; DAPI-blue). B, Sst- (green) and Cr- (red) expressing interneurons in the P30 SSCtx (DAPI, blue). C, The density of PV-expressing interneuron is significantly reduced in Dp16 compared to euploid animals (*p<0.05; Eup=4, Dp16=5).
Figure 30. OLG-lineage cells of the corpus callosum (CC) and external capsule (EC).

A, The CC labeled for the OLG-lineage cells (Olig2+, magenta) including OPCs (NG2+, red) and mOLGs (CC1+, green). The white rectangle denotes the area cell counts were conducted (DAPI, blue). B, C, Both the mOLG (NG2+/Olig2+, B) and OPC (CC1+/Olig2+, C) populations are normal in Dp16 compared to euploid littermates at both ages studied. D, Representative image of the EC utilizing the same markers as outlined for the CC (A). E, F, OPC and mOLG populations are normal in the EC at P15 and P30 in Dp16.
CHAPTER FIVE:

General Discussion
SUMMARY OF KEY FINDINGS

Mouse models of DS have been invaluable in understanding how trisomy affects the CNS at the cellular, system, and behavioral level. Innovations in genetic engineering have allowed for the generation of new mice with the goal of obtaining a closer representation of the human condition. As new models are being introduced, DS research has shifted to potential pharmacological interventions that may ameliorate various aspects of this complex disorder. While this exciting move brings hope to a disorder long thought untreatable, mouse models of DS are being used to test potential drugs even though it is unclear which model is the best representation of the human disorder. This major pitfall raises an important question: are drug treatment studies rescuing phenotypes related to DS or are they specific for the model being tested? In this dissertation, the Dp16 mouse model of DS was comprehensively assessed to directly compare to previously established mouse models. The Dp16 model was engineered to carry a triplication of the entire Hsa21 homologous region localized to Mmu16 and is believed to be a more reliable model of DS when compared to the Ts65Dn. In order to determine if Dp16 better represents DS, we sought to (1) characterize the embryonic development and confirm behavioral outcomes in Dp16 (Chapter 2), (2) understand how white matter and oligodendrocytes are impacted in both human DS and the Ts65Dn (Chapter 3), and (3) compare interneurons and oligodendrocyte populations within the Dp16 brain to establish phenotypes in Ts65Dn (Chapter 4).

In Chapter 2, the development of Dp16 was assessed mirroring previous efforts in the Ts65Dn in order to directly compare the two models. Ts65Dn mice have been shown
to exhibit microcephaly and neocortical expansion delays which are present in the DS fetus as well as aberrations in neurogenesis and neural progenitor populations (Chapter 1). Despite possessing an elongated triplicated segment, Dp16 brain development is widely normal in comparison to Ts65Dn as no DS-related phenotypes were found in Dp16 embryos. Normal prenatal development was followed by an onset of behavioral and cellular changes during the second week of postnatal life. Specifically, the Dp16 animals show hypocellularity of interneurons in the cortex and fail to acquire late-stage developmental milestones. These findings, coupled with the confirmation of learning and memory deficits in adulthood, revealed distinct differences between Dp16 and Ts65Dn.

In Chapter 3, a genome wide approach revealed distinct changes in white matter associated genes that prompted the investigation of white matter and OLGs within the human DS brain. Analysis at the genetic and protein level confirmed exon array findings showing that white matter associated genes and proteins were dysregulated in the human tissue and mirrored findings in the Ts65Dn mouse model. Also, temporal changes in the OLG-lineage were found in the Ts65Dn. As Ts65Dn animals aged, there was an increase in the proportion of OPCs relative to the mOLGs of the white matter tracts studied. The loss of mOLGs was mirrored by reductions in the Nodes of Ranvier as well as slower action potential conductance in myelinated axons of the CC. This study provides a strong basis for the use of the Ts65Dn as findings in that model closely mirrored the human data as well as presenting novel findings into white matter deficits in a mouse model of DS.

In Chapter 4, interneurons and OLGs were characterized within the Dp16. Findings in the Ts65Dn have shown an overabundance of interneurons, which may
reflect excessive inhibition in DS, as well as age-related changes to OLGs that was comparable to human data (Chakrbarti et al., 2010; Olmos-Serrano et al., 2016, Chapter 3). Dp16 animals were shown to have persistent hypocellularity of PV-labeled cortical interneurons while the Sst-expressing population appeared normal by P30. The OLG-lineage was found to be unaffected in the EC and CC of Dp16 animals at P15 and P30. These data suggest that interneurons and OLGs are differentially affected in Dp16 when compared to Ts65Dn.

Overall, the major findings of this dissertation have shown that despite possessing a greater level of gene copy similarity to human DS, the Dp16 mouse model fails to recapitulate many DS-related phenotypes both embryonically and postnatally. Nevertheless, the Dp16 model does exhibit distinct changes at the cellular and behavioral level that are consistent with other established models. These data suggest that while Dp16 may be an inappropriate model for studying the embryonic effects of Ts21, it may allow for the isolation of trisomy effects on postnatal developmental processes and their role in cognitive dysfunction.

**GENERAL DISCUSSION**

The phenotypic disparities apparent in the Dp16 mouse model of DS are the first example of a mouse model of DS exhibiting adult cellular and behavioral phenotypes in the absence of prenatal developmental changes. This temporal shift in phenotype manifestation may be caused by the genetic engineering used to induce trisomy in the Dp16. As discussed previously (Chapters 1 and 2), the triplicated segment in Dp16 is attached to an endogenous copy of Mmu16. This presents two confounding factors. First,
the Dp16 is not a mouse model of aneuploidy and does not reflect a majority of DS individuals in which there is a full triplication of a freely segregating Hsa21 (Hsu, 1998). The contribution of aneuploidy to DS phenotypes has yet to be established. For example, it is unknown if changes in neurogenesis are due to the triplication of specific genes within the proliferative zones of the brain or if the presence of an extra chromosome, regardless of the genes triplicated, is sufficient to induce changes in cell cycle dynamics resulting in a reduction in proliferation. Studies in human cell lines have shown that trisomic aneuploidy, regardless of the chromosome, reduces cell proliferation and extends the cell cycle in vitro (Stingele et al., 2012). This gap in knowledge limits the use of many mouse models of DS, including the Dp16. Triplicated genes in an extra segregating chromosome may be necessary to appropriately recapitulate DS prenatal phenotypes in the mouse.

Second, the Dp16 triplicated segment is 22.9 Mb in size (Yu, et al. 2010) and the ramifications of this addition on chromatin structure and stability remains unknown. Microarray data on Dp16 and Ts65Dn (Chapter 2) show that fewer genes are dysregulated in the Dp16 embryonic brain. No studies have investigated epigenetic changes in Dp16. The presented results (Chapter 2) suggest that transcription of triplicated genes is different in Dp16 compared to Ts65Dn. While Dp16 may carry three copies of 119 Hsa21 homologs, the duplication attached to Mmu16 may have inappropriate chromatin remodeling (chromatin condensation) or epigenetic changes (de-acetylation or methylation) that limit transcription of triplicated genes. Further epigenetic and genomic assessment is necessary to determine the mechanism by which transcription
differs in embryonic and adult brains in mouse models of DS. While the lack of embryonic phenotypes limits the use of Dp16 in embryonic studies, the onset of cellular and behavioral abnormalities during perinatal development suggest that Dp16 may be useful in determining how perinatal and postnatal aberrations in models of trisomy contribute to observed cognitive deficits.

Dp16 animals develop delays in the acquisition of late stage developmental milestones as well as reductions in cortical inhibitory interneurons that persists from the onset of milestones delays until at least P30 (Chapters 2 and 4). These phenotypes in Dp16 are in stark contrast to those observed in Ts65Dn. In contrast, Ts65Dn animals exhibit milestone delays immediately following birth and an overabundance of cortical interneurons (Charkrabarti et al., 2010; Olmos-Serrano et al., 2016). The disparities between models is likely due to their inherent genetics. In Ts65Dn, the over production of interneurons is likely due to increases in proliferation within the MGE, where PV- and Sst- interneurons are born (Charkrabarti et al., 2010). In the Dp16, proliferation and progenitor populations are normal (Chapter 2) and yet, there are fewer cortical inhibitory interneurons. As evident in Chapter 2, normal proliferation suggests that the perinatal interneuron phenotype develops following intermediate cell production. However, PV-expressing interneuron production begins at E10.5 and peaks at E12.5, which overlaps with Som-interneuron production, and the proliferative phenotype was assessed in Dp16 embryos beginning at E13.5 (Anderson et al., 2001; Butt et al., 2007; Chapter 2). Therefore, changes to proliferation within the early MGE may contribute to the reduction in PV- and Som-interneuron populations, while the Cr-expressing population is
unscathed as they are born at a different time in the CGE (Anderson et al., 2001; Butt et al., 2007). Further work into earlier proliferation events of the VGZ would confirm whether the interneuron phenotype of the Dp16 initiates during embryonic development. Immature interneurons may also be impacted as they undergo a complicated process of migration and maturation, during which time multiple factors may be affected in models of segmental trisomy resulting in the hypocellular phenotype.

First, the migration of interneurons requires a complicated concert of chemical signals to direct interneuron precursors through tangential migration to the neocortex and subsequent invasion of the appropriate cortical layer (Faux et al., 2012). A critical component of interneuron migration to the cortex is depolarization mediated by GABA<sub>A</sub> receptors (Cuzon et al., 2006; Bortone and Polleux, 2009). Upon reaching the cortex, migration is terminated through the upregulation of the chloride channel KCC2, which reverses the depolarization activity of GABA<sub>A</sub> receptors (RiBortone and Polleux, 2009). In the Ts65Dn mouse, GABA<sub>A</sub> mediated transmission is enhanced in the hippocampus and reduced LTP in CA1 can be normalized when the GABA<sub>A</sub> receptor antagonist, picrotoxin, is applied (Kleschevnikov et al., 2004; Kleschevnikov et al., 2012). Similarly, the administration of a GABA<sub>A</sub> receptor antagonist improves Ts65Dn performance on cognitive tasks (Rueda et al., 2008; Colas, et al., 2013) suggesting that overinhibition mediated by enhanced GABA<sub>A</sub> receptor transmission contributes to cognitive dysfunction. In Dp16, LTP is substantially reduced and Dp16 animals perform poorly in spatial learning tasks (Yu, et al. 2010), however the role of the GABA<sub>A</sub> receptor has not been confirmed. Nevertheless, GABA<sub>A</sub> receptor function may be enhanced in Dp16,
resulting in LTP and cognitive phenotypes similar to Ts65Dn. GABA_A receptor
dysfunction may result in enhanced transmission during interneuron precursor migration,
facilitating their initial migration. However, this increase may induce early termination of
migration preventing interneurons from reaching their appropriate destination. In fact,
interneurons derived from the MGE, the PV- and Sst-expressing interneurons, switch
from depolarization to hyperpolarization earlier than other subtypes (Miyoshi et al.,
2011) and in Dp16 these subtypes are reduced at P15 (Chapter 2). Therefore,
interneurons in Dp16 may be produced in appropriate proportions, but failure to
terminate and populate the neocortex may underlie the population reductions reported
here (Chapter 2 and 4).

Maturation of interneurons is affected by multiple factors including
neurotransmission and neurotrophic factors (Huang et al., 1999; Eggan et al., 2012). The
timing of interneuron maturation is an extended process that occurs during the first
postnatal month of life in the mouse brain (Luhmann and Prince, 1991). During the
second to fourth weeks of postnatal life, interneuron populations integrate into local
circuitry and the formation of appropriate synaptic contacts are necessary for the
maturation of interneurons as well as cortical circuits (Bortone and Polleux, 2009; De
Marco Garcia et al., 2011). During this period of maturation, the expression of
interneuron markers, such as PV, is activity dependent (Patz et al., 2004) suggesting that
successful synapse formation and circuit integration are necessary for maturation of
interneuron sub-types (Le Magueresse and Monyer, 2013). In the Dp16, synapse
formation and cortical neuronal activity have not been investigated. However,
dysfunction in synaptic plasticity in the hippocampus (altered LTP) may mirror changes in cortical synapse formation and activity which may impact interneuron maturation. As such, this change may delay interneuron maturation as suggested by the increase in PV- and SSt-expressing interneurons from P15 to P30 in Dp16. However, the PV-expressing population is still smaller by P30 when compared to euploid animals (Chapter 2 and 4). This temporal change in interneuron population size may be indicative of a shift in interneuron maturation in the Dp16 due to synapse or circuit dysfunction.

The phenotypic variation in interneuron sub-types in Dp16 suggests a complicated interplay of the processes discussed above. The changes in Sst- and PV-expressing populations from P15 to P30 may result from a delay in interneuron migration (Chapter 2 and 4). While the Sst-expressing population meets euploid levels by P30, the PV-population remains reduced (Chapter 4). This may seem incongruous, but differences in sub-type self-programmed apoptosis may result in excessive cell death in PV-interneurons as they have lower survival rates in normal animals (Southwell et al., 2012). In order to better understand the cause of inhibitory interneuron phenotypes in Dp16, migration and apoptosis must be assessed during late embryonic development (migration) and the first two weeks of postnatal life (apoptosis). As the phenotypes vary between Dp16 and Ts65Dn, the question remains: which model is a more accurate representation of the human interneuron phenotype? Unfortunately, little is known about interneurons in the human DS brain, however studies suggest that there may be fewer GABAergic cells in the cortex (Ross et al., 1984). If this is confirmed using IHC for interneuron sub-types in the human brain, then Dp16 may better represent the human condition at the cellular
level. While interneuron phenotypes may suggest that Dp16 is a stronger model than the Ts65Dn, the absence of OLG-lineage phenotypes suggests otherwise.

In Chapter 3, a comprehensive analysis at the gene, protein, cellular, and functional level indicates that the ratio of OPC/mOLGs is shifted in DS and the Ts65Dn, where an overabundance of OPCs correlates with age. What could be contributing to this shift in the OLG-lineage in the Ts65Dn? The maturation of OPCs into mOLGs is highly regulated by the Wnt-signaling cascade which inhibits OPC maturation (Fancy et al., 2009; 2011; 2014). The binding of Wnt to receptors, promotes the stabilization of beta-catenin and subsequent migration into the nucleus to influence gene expression. Recently, OPCs were found to migrate along the vasculature and this interaction, mediated by Wnt-signaling, suppresses OPC maturation (Tsai et al., 2016). In Ts65Dn, beta-catenin is upregulated in the endothelial lining of cerebral blood vessels (Ramakrishna, et al., 2009). The up-regulation of beta-catenin may reflect overactive Wnt-signaling that may prevent OPC maturation in the Ts65Dn mouse model.

This OLG-phenotype is absent in the Dp16, where OPC and mOLG levels were normal from P15 on (Chapter 4). This contrasts with the Ts65Dn OLG-phenotype that begins at P15 (Chapter 3). This phenotypic disparity between models is difficult to reconcile since the basis of the white matter phenotype remains unknown. The triplication of the transcription factors Olig1 and Olig2 contributes to interneuron overproduction in Ts65Dn, however it is unclear how the triplication of these genes impact the OLG lineage (Chakrabarti et al., 2010). It is unlikely that Olig2 triplication induces the observed phenotype as Olig2 null OPCs fail to differentiate while immature
OLGs lacking *Olig2* are pushed towards a myelinating mOLG phenotype (Mei et al., 2013). In Ts65Dn, *Olig2* overexpression results in an overproduction of interneuron precursors within the MGE and based on the pro-differentiation role of *Olig2*, one would expect OPCs to be pushed to differentiate which is not the case as OPCs are overabundant in Ts65Dn white matter (Chakrabarti et al., 2010; Mei et al., 2013; Chapter 3). Therefore, it is unlikely that *Olig1/2* triplication is inhibiting OPC maturation.

Despite an increase in number of triplicated genes, the Dp16 mouse model of DS fails to recapitulate multiple DS-related phenotypes. The lack of embryonic and white matter phenotypes suggests that Dp16 cannot be used to study the effects of trisomy on either process (Chapter 2 and 4). Further, the disparities between Dp16 and Ts65Dn phenotypes requires further work in human tissue to determine which interneuron phenotype (hypocellular, Dp16; hypercellular, Ts65Dn) mirrors the human condition. The contextualization of mouse models of DS requires an assessment utilizing similar methods to allow for direct comparison. Here, assessment of the Dp16 mouse mirrored that in the Ts65Dn mouse. As such, the Dp16 can now be evaluated against other mouse models of DS. However, gaps in the human DS literature confound the ability to claim a single mouse model as a ‘gold standard’. It is unrealistic to expect a mouse model to completely mirror a human condition as brain complexity increases across these two species. As such, mouse models may be selected based on the phenotypes of interest. For example, the Ts65Dn would be favorable for embryonic studies, despite its genetic dissimilarity to human DS, while the closer genetic model, the Dp16, should not be used as such. The data presented here suggest that the use of Dp16 should proceed with
caution until further studies elucidate the underlying differences across mouse models and humans with DS.

**FUTURE DIRECTION**

From this work, several questions have been generated that must be answered in order to further understand the effects of segmental trisomy in the Dp16 mouse. First, the lack of embryonic phenotypes may result from a lack of aneuploidy in Dp16 mice. To determine the contribution of aneuploidy to embryonic phenotypes in mouse models of DS, a model that carries the entire Mmu16 region orthologous to Hsa21 as a freely segregating chromatid must be engineered. A direct comparison of this new model and the Dp16 assessing brain morphology, neurogenesis, and corticogenesis (as performed in Chapter 2) would reveal the relation between aneuploidy and DS-related embryonic phenotypes. Similarly, such a model could be compared at the behavioral level (Chapter 2). The rigorous behavioral assessment in Chapter 2 revealed aspects of the cognitive deficit that had not yet been determined in Dp16, specifically, the deficit in memory extinction and re-learning. This extensive paradigm could be replicated in the ‘aneuploid Dp16’ model to determine the contribution of aneuploidy to the development of cognitive phenotypes. Together, these studies would provide evidence for the role of aneuploidy versus gene dosage in mouse models of Ts21.

The development of unique cellular and behavioral abnormalities in the postnatal Dp16 differ in magnitude and timing when compared to other mouse models of DS requires further assessment of the Dp16 brain from birth until the onset of phenotypes after the second week of postnatal life (Chapter 2 and 4). First, breeding Dp16 animals to
a GAD67-GFP mouse strain would allow for the identification of GABAergic interneurons throughout their maturation and could be coupled with apoptosis assays. Apoptosis can be assessed utilizing the TUNEL assay and IHC for caspase-3 in the Dp16 brain at P0, P7, and P15 within the MGE, the tangential migratory route and neocortex. The combination of these techniques would allow for the identification of apoptotic interneurons which may contribute to the observed phenotypes in Dp16. Interneuron sub-type IHC (Chapter 2 and 4) combined with the above methods would allow for subsequent analysis of apoptotic interneurons and reveal sub-type specific changes in apoptosis. Studies in the human brain are also necessary to further understand the interneuron phenotype. IHC within the GE for the proliferative markers Ki67 and pH3 performed on fetal human DS and control tissue would determine if the human fetal GE is comparable to the highly proliferative Ts65Dn or normal Dp16. Interneuron sub-type specification using IHC or in situ hybridization on human cortex brain samples across the life span is also necessary to determine the state of interneuron populations during their maturation and in their state in the adult brain. Combined, these studies in Dp16 and human tissue would elucidate the interneuron phenotype in DS and aid in selection of the mouse model that best mirrors those findings.

DS research is progressing towards exciting avenues of pharmaceutical interventions never believed possible for a complicated genetic disorder. As these studies move forward, clinical trials in humans are beginning across the world based on evidence from the mouse models. However, conflicting results in the mouse models presented here indicate that phenotypic differences that exist across models and raise caution about the
interpretation of treatment studies and their translation to the human. Many studies have used the Ts65Dn, which best mirrors DS phenotypes, however the genetic dissimilarities to human DS question treatment results as phenotypes in Ts65Dn may be influenced by non-Hsa21 homologs present in this model. The Dp16 mouse model lacks major DS-phenotypes and a lack of knowledge about the Dp16 prevents its use in treatment studies until the basis for the absence of phenotypes is determined. A mouse model of DS may never fully recapitulate every neuropathology of the human brain, yet the mouse models must undergo greater comparative scrutiny to aid in model selection for treatment studies in order to substantiate the move to clinical trials. Despite this, the Dp16, alongside a Dp16-aneuploid mouse, can be a tool with which to study the contribution of aneuploidy and gene dosage to DS-related phenotypes in mouse models of DS.
APPENDIX

Extended Confocal Microscopy Methods

For embryonic and postnatal immunohistochemistry, 12 μm thick z-stacks were collected using the 20x objective of the Zeiss 710 confocal microscope.

For image collection of embryonic tissue, the ventricular surface of the neocortex was oriented to the bottom of the imaging frame, allowing for an imaging area of 250x250x12 μm for each z-stack. Three z-stacks were collected for each embryonic brain in the future somatosensory cortex. The rostral aspect of the dorsal hippocampus and the decussation of the anterior commissure were used as anatomical landmarks to allow for direct comparison across all images. Automated counting algorithms were generated on Volocity software to quantify apical precursors (Sox2+), basal intermediate progenitors (Tbr2+), DAPI nuclei, and EdU-labeled cells. All algorithms were validated by counting labeled cell populations by hand and comparing hand-counts and algorithm generated counts at e13.5 (for progenitor cell counts) or e14.5 (for EdU). Counting algorithms were then used for all subsequent ages. All other embryonic confocal images were analyzed by hand counts. Progenitor cell and EdU images were further analyzed for population distribution using Volocity software. The automated counting algorithm measured the distance of each identified progenitor cell to the ventricular surface. Data was analyzed in Microsoft Excel using 20 micron increments for cell distance to the ventricle for each progenitor population.

Postnatal images were obtained from the boundary of the cortex and sub-cortical white matter to the pial surface. Three to four z-stacks were necessary to span the radial
aspect of the P15 somatosensory cortex and images were stitched into a composite image using Photoshop. Three composite images of the P15 somatosensory cortex were obtained for each brain. All postnatal cell counts were performed by hand and images were collected in the somatosensory cortex using the decussation of the anterior commissure and rostral aspect of the dorsal hippocampus to ensure anatomical accuracy across brains.
BIBLIOGRAPHY


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