AXONAL TRANSPORT IN
MOUSE MODELS OF DOWN SYNDROME

Thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy to the University College London

By Aarti Ruparelia

Department of Neurodegenerative Disease
Institute of Neurology
University College London
DECLARATION

I, Aarti Ruparelia, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
ABSTRACT

Down syndrome (DS) is a complex condition resulting in the most common genetic form of intellectual disability. Trisomy of chromosome 21 in humans (Hsa21) causes DS, likely due to overexpression of some of the 500 genes on this chromosome. People with DS are more susceptible to early-onset Alzheimer Disease (AD), and the histopathological and endocytic perturbations that characterise AD are present at an earlier age in people with DS than the general population with AD. They also display aberrant dendritic spine morphology, which is associated with learning and memory deficits. The Ts65Dn mouse model of DS carries 122 genes on its translocated chromosome and recapitulates these DS-associated phenotypes. Neurodegeneration in these mice may be caused by impaired retrograde axonal transport of essential neurotrophic factors. The triplication of the Hsa21-encoded amyloid precursor protein (APP) gene is proposed to cause enlarged early endosomes and a perturbed endocytic pathway that subsequently leads to axonal transport deficits. However the genetic contribution of other Hsa21 genes to axonal transport deficits remains unknown.

The research in this thesis aimed to recapitulate the axonal transport, endocytic and dendritic phenotypes in Ts65Dn mice, and to elucidate the contribution of Hsa21 genes, to the pathogenesis of these deleterious phenotypes. Live-cell imaging of quantum dot-labelled brain-derived neurotrophic factor (BDNF) in Ts65Dn hippocampal neurons revealed impaired BDNF axonal transport. Neurons from these mice also displayed a greater number of enlarged early endosomes and reduced dendritic surface area and volume. The Ts1Rhr mouse model encodes 31 duplicated genes that are orthologous to the human DS critical region (DSCR), and has disomic APP expression levels. Ts1Rhr hippocampal neurons also revealed impaired BDNF axonal transport, however endosomal and dendritic morphology was spared. This suggests that in addition to APP, one or more genes orthologous to the human DSCR may be necessary for axonal transport deficits but not for the enlarged early endosome phenotype or dendritic abnormalities. Other putative mechanisms, such as perturbed cytoskeleton and motor protein function, may additionally exacerbate impaired axonal transport of neurotrophins.
Dedicated to my family
ACKNOWLEDGEMENT

This thesis would not have been complete without the supervision of Prof. Elizabeth Fisher. Her calmness and superb advice has helped me weather the many storms and struggles during this PhD. I want to thank Lizzy for giving me the opportunity to pursue this PhD, and the Brain Research Trust for awarding me the scholarship. I also want to thank Prof. Giampietro Schiavo for all his support. Thanks also to all my friends in the Fisher lab and the department for all the fun and giggles, the much-needed whinging over coffee, the even-more-needed drinks and the constant encouragement. These lab friends have kept me sane and truly are irreplaceable.

Most of the work herein was conducted in collaboration with Dr. William Mobley at University California, San Diego. I want to extend a massive thank you to Bill for welcoming me into his lab and giving me access to all the resources I needed. A big thank you to Dr. Matthew Pearn for teaching me the “magic” of microfluidic chambers and transport, and for being a great friend. A very special thank you to Dr. Brian Head for being my life-line with his constant support and for believing in me. Without his scientific mentorship and friendship, I truly would have been lost. I also want to acknowledge Brian for his help with the figures in this thesis, and providing access to the AutoNeuron software. I will always be grateful for Brian and Matt’s encouragement through all the tears and mess and their friendly solution to drink all problems away. Thank you for being my family in San Diego.

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ABBREVIATIONS

° C Degrees Celsius
µL Microlitre
µm Micrometre
2N Euploid control
3D Three-dimensional

Aβ Amyloid-β
AD Alzheimer's Disease
AICD APP intracellular C-terminal domain
AKT v-Akt murine thymoma viral oncogene
AMPA 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid
AP2 Adaptor protein-2

BAC Bacterial artificial chromosome
BACE1 β-site APP cleaving enzyme-1
BDNF Brain-derived neurotrophic factor
BFCN Basal forebrain cholinergic neuron
bp Base pairs

Ca²⁺ Calcium
CaM Calmodulin
cAMP Cyclic adenosine monophosphate
CB Cell body
CCD Charge-coupled device
CK1γ Casein kinase 1-γ
cm Centimetre
CNS Central nervous system
CREB cAMP response element-binding protein
CTF C-terminal fragment
<table>
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<th>Description</th>
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<td>DA</td>
<td>Distal axon</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>DIV</td>
<td>Day in vitro</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DS</td>
<td>Down syndrome</td>
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<td>DSCR</td>
<td>Down syndrome critical region</td>
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<td>DYNC1H1</td>
<td>Dynein cytoplasmic heavy chain</td>
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<td>E-I</td>
<td>Excitatory – inhibitory</td>
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<td>Endosomal sorting complex required for transport-1</td>
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<td>FAD</td>
<td>Familial Alzheimer's Disease</td>
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<td>Fascia dentate</td>
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<td>GABA</td>
<td>$\gamma$-aminobutyric acid</td>
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<td>Guanine exchange factor</td>
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<td>GFP</td>
<td>Green fluorescence protein</td>
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<td>GSK3β</td>
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<td>Guanine triphosphate</td>
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<td>Hsa21</td>
<td>Homo sapiens chromosome 21</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
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<tr>
<td>Kb</td>
<td>Kilobase</td>
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<tr>
<td>KLC</td>
<td>Kinesin light chain</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>LCL</td>
<td>Lymphoblastoid cell line</td>
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<td>LTD</td>
<td>Long-term depression</td>
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<td>LTP</td>
<td>Long-term potentiation</td>
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<td>M</td>
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<td>MAP2</td>
<td>Microtubule-associated protein-2</td>
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<td>Mitogen-activated protein kinase</td>
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<td>Megabase</td>
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<td>mBt</td>
<td>Mono-biotinylated</td>
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<td>MGE</td>
<td>Medial ganglionic eminence</td>
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<td>min</td>
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<td>miRNA</td>
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<td>mM</td>
<td>Millimolar</td>
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<td>MMCT</td>
<td>Microcell-mediated chromosome transfer</td>
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<tr>
<td>Mmu</td>
<td><em>Mus musculus</em> chromosome</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>MVB</td>
<td>Multivesicular bodies</td>
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<td>MWM</td>
<td>Morris water maze</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor-κ-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillar tangles</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>nm</td>
<td>Nanometer</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<td>NMJ</td>
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<td>Neuronal precursor cell</td>
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**p75**  p75 neurotrophin receptor

**PCR**  Polymerase chain reaction

**PDMS**  Polydimethylsiloxane

**PFA**  Paraformaldehyde

**pH**  Power of hydrogen

**PI3K**  Phosphoinositol-3-kinase

**PLCγ**  Phospholipase C-γ

**pmol**  Picomoles

**PS**  Presenilin

**PSD**  Postsynaptic density

**Ptch1**  Patched 1

**PtdIns(4,5)P_2**  Phosphatidylinositol 4,5-bisphosphate

**QD**  Quantum dot

**Rab**  Ras-associated binding protein

**RNA**  Ribonucleic acid

**RPM**  Revolutions per minute

**SAD**  Sporadic Alzheimer’s Disease

**sec**  Seconds

**SSH**  Sonic hedgehog

**TBE**  Tris/Borate/EDTA buffer

**TGN**  *trans*-Golgi network

**TIRF**  Total-internal-reflection fluorescent

**Trk**  Tyrosine receptor kinases

**UV**  Ultraviolet

**YAC**  Yeast artificial chromosome
CHAPTER ONE  INTRODUCTION

1.1 Down Syndrome

1.1.1 Trisomy 21

In 1866, John Langdon Down published a seminal paper on his observations of Down syndrome (DS): *Observations on an Ethnic Classification of Idiots*. His description was heavily influenced by Blumenbach’s anthropology, which classified people into 5 races: Ethiopians, Malayans, Caucasians, Aztecs and Mongolians (Ward, 1999). While working with John Conolloy, an ardent ethnologist, Down attempted to ethnically categorise patients with mental retardation. To identify specific facial features, he used skull contour measurements, including the diameter of the head, and photographs he himself took. Although Down’s ethnic classification was never widely accepted, his use of the term “Mongoloids” to characterise people with DS was in keeping with these very limited observations (Ward, 1999). Down also believed DS was not caused by postnatal events. Again, given limited insights into the nature of the disorder, he believed it derived from degeneracy that occurred from parents who suffered from tuberculosis (Ward, 1999). Almost a century after the first observed cases of DS, the development of cytogenetics led to the understanding that chromosomal irregularities may be the cause of intellectual disability and that non-disjunction could result in trisomy or monosomy and may lead to DS. It was only in 1959 that Jerome Lejeune confirmed that DS is, in fact, caused by the trisomy of human chromosome 21 (Hsa21) (Lejeune et al., 1959).

Trisomy 21 comprises complete or whole-chromosome trisomies, partial trisomies, and triplication of single-genes. Complete trisomy of Hsa21 accounts for 95% of the population with DS, with the remaining 5% constituting mosaic or partial trisomies (Dierssen et al., 2009). The additional copy of Hsa21 results in increased expression of many genes encoded on this chromosome, with variations in expression levels in different tissues (Ait Yahya-Graison et al., 2007, Prandini et al., 2007). This increase in gene-dosage has been proposed to cause the plethora of phenotypic alterations that characterise DS. Nevertheless, allelic differences in Hsa21 genes and an
intricate interplay with other non-Hsa21 genes in addition to epigenetic influences and environmental factors can all be envisioned to influence the phenotypes in DS.

The ‘gene-dosage hypothesis’ proposes that a subset of triplicated genes in the gene-rich distal part of Hsa21 (band 21q22), identified as the ‘Down syndrome critical region’ (DSCR), is sufficient to cause specific DS phenotypic traits and that the severity of the syndrome depends on the extent of trisomy of this genomic region (Delabar et al., 1993, Korenberg et al., 1990). However, recent evidence from partial trisomy 21 cases outside of the DSCR that display DS characteristics, argues against the DSCR being sufficient to cause DS-associated phenotypic features (Korbel et al., 2009, Lyle et al., 2009). Nevertheless, a fundamental role for the gene-dosage hypothesis is apparent, and increased dose of specific genes contributes significantly to particular phenotypes (Antonarakis et al., 2004). Trisomy 21 results in the overexpression of Hsa21-encoded microRNAs (miRNAs), which may lead to decreased expression of certain proteins and contribute to the variance in DS-related phenotypes (Kuhn et al., 2008). Current evidence makes a compelling argument for a number of Hsa21 genes and susceptibility regions in causing DS-associated phenotypes; these in turn may be modulated by other Hsa21 loci and elsewhere in the genome, in concert with epigenetic processes, to create a complex network that defines the impact of gene-dosage expression on DS-associated phenotypes (Ruparelia et al., 2012, Ruparelia et al., 2010).

1.1.2 Aetiology and prevalence of DS

DS is a multifaceted genetic condition leading to the overexpression of many of the approximately 550 genes encoded on Hsa21 (Ensembl release 70, including known and novel protein-coding genes but excluding pseudogenes; http://www.ensembl.org/Homo_sapiens/Location/Chromosome?r=21:1-48129895). Several of these coding genes are involved in the regulation of neuronal cell number, transcription, translation and synaptic plasticity; triplication of these genes in DS results in structural and functional defects in multiple systems (Hattori et al., 2000, Letourneau & Antonarakis, 2012, Sturgeon & Gardiner, 2011). DS is characterised by invariable clinical phenotypes such as intellectual disability, hypotonia, craniofacial abnormalities and the presence of Alzheimer’s disease (AD)
in the brains of subjects even before the age of 40 years. Additionally, at least 80 other variable phenotypes affect a subpopulation of DS, including acute megakaryoblastic leukemia, atrioventricular septal heart defects and other neurological complications such as seizures and obstructive sleep apnea (Coppus et al., 2006, Coppus et al., 2012, Freeman et al., 2008, Hassold et al., 1996, Johannsen et al., 1996, Pueschel et al., 1991, Shott et al., 2006, Wechsler et al., 2002).

The incidence of DS is approximately one in 700 live births worldwide (Bittles et al., 2007). It is the most common genetic cause of intellectual disability, accounting for 15% of the population with intellectual disability (Bittles et al., 2002). Chromosomal aneuploidies give rise to approximately 50% of spontaneous abortions before 15 weeks of gestation, with trisomies being responsible for 50% of these spontaneous abortions (Hassold et al., 1996, Hassold et al., 1980). Trisomy 21 is caused by nondisjunction of chromosome 21 during meiosis in either oogenesis or spermatogenesis, resulting in reduced recombination and an imbalance of chromosomes (Sherman et al., 1991). Parental origin of nondisjunction in DS is maternal in almost 95% cases (Antonarakis, 1991), resulting from meiosis I errors in approximately 77% of these cases (Antonarakis et al., 1992). The only well-established risk factor for non-disjunction appears to be advanced maternal age (Penrose, 2009). Even though the rate of recombination remains constant with advancing maternal age, aged oocytes are more susceptible to nondisjunction compared to young oocytes (Lamb et al., 1996).

Due to its high prevalence and multi-system dysfunction, DS is one of the most intensively studied aneuploidy conditions. Remarkable progress in medical treatment and social intervention in developed countries has increased the life expectancy of people with DS from an average of 12 years of age in the 1940s to 60 years of age at present in Western Australia (Bittles et al., 2007). An average annual increase in longevity of 1.7 years between 1983 and 1997 was reported in USA (Yang et al., 2002). Significantly, even with a prenatal diagnosis, the incidence of DS has not diminished, possibly due to relaxed societal pressures of advanced maternal age and reduced stigma of having a child with DS. Indeed, the past 25 years have shown an increase of almost 15% of women above the age of 35 years giving birth in Western Australia (Bittles et al., 2007). Interestingly, males with DS
have an increased life expectancy of more than 3.3 years relative to females with DS (Glasson et al., 2003). This comparative survival advantage over females may be ascribed to a greater incidence of congenital heart defects in females with DS (Morris et al., 1992), and/or menopause occurring at an average age of 46 years old in women with DS, which is approximately 4-6 years earlier than women in the general population (Seltzer et al., 2001). Precocious menopause and the accompanying drop in estrogen levels is a high risk factor for premature mortality through heart disease, stroke, breast cancer and dementia, although this has not been investigated in the DS population (Harlow & Ephross, 1995).

However, increased longevity may be linked to greater prevalence of the disorder and the emergence of new challenges to tackle an ageing population of adults with DS. As individuals with DS age, they are more susceptible than the general population to other age-related conditions, including cardiovascular disorders, thyroid dysfunction, hearing and vision impairments, and depression (Bittles et al., 2007, Glasson et al., 2002). Individuals with DS also display these age-related changes and senescence at an earlier age, as is evident by increased biological age, early mortality, reduced levels of DNA-repair enzymes and early-onset AD (Beacher et al., 2010, Bittles et al., 2007). Precocious ageing observed in DS adults may be caused alterations in early development, which render biological systems vulnerable to other health conditions by genetic influences that emerge during ageing. Thus, in addition to the neurodevelopmental and neurodegeneration research in DS, there is growing importance to provide specialised healthcare and invest in scientific research for the ageing DS population.

1.2 Cognitive and Genetic Insights from Mouse Models of DS

1.2.1 Mouse models of DS

Deciphering the correlation between the effects of trisomic Hsa21 genes to clinical aspects of the syndrome is an important goal in DS research. Examining people with DS who have partial trisomy 21 resulting from translocations or duplications of
genomic segments of Hsa21, has led to crucial discoveries in mapping genes for specific phenotypes (Korbel et al., 2009, Lyle et al., 2009). However, partial trisomy 21 cases are extremely rare, with the largest study to-date examining 30 cases, and the substantial variation of clinical phenotypes within this population limit further analysis. Studies of mouse models of DS have provided a pivotal framework through which the genetic dissociation of genotype-phenotype correlations and the pathological, molecular and neurobiological processes underlying DS-associated phenotypes are unravelled (Ruparelia et al., 2012, Ruparelia et al., 2010). However, much is yet unknown, and further efforts to understand the pathogenesis that leads to the clinical manifestation of DS are greatly needed.

Hsa21 is syntenic to three mouse genomic regions with high conservation of many genes between human and mouse (Hattori et al., 2000, Sturgeon & Gardiner, 2011). Approximately 119 of the Hsa21 orthologous genes are encoded on mouse chromosome 16 (Mmu16), and two smaller regions, Mmu17 and Mmu10, encode 20 and 42 Hsa21 orthologous genes, respectively (Herault et al., 2012). A majority of DS mouse models are segmentally trisomic for various genomic regions on Mmu16 (Figure 1.1). Additionally there are models that are trisomic for the entire, or a portion of the orthologous regions on Mmu17 and Mmu10 (Pereira et al., 2009, Yu et al., 2010b). Several mouse models that are monosomic for these regions have also been generated (Besson et al., 2007, Duchon et al., 2011, Olson et al., 2004a, Olson et al., 2004b, Pereira et al., 2009). These latter trisomic and monosomic models have been instrumental in extending our understanding about DS-associated phenotypes and have been eloquently reviewed elsewhere (Das & Reeves, 2011, Herault et al., 2012, Seregaza et al., 2006). Due to space constraints, only the DS models depicted in Figure 1.1 will be described.

The most widely studied DS mouse model is the Ts(17;16)65Dn (Ts65Dn) mouse, which was selected after X-ray induced irradiation of mouse stocks resulted in a reciprocal segmental translocation between the telomeric end of Mmu16 and the centromeric part of Mmu17 (Davisson et al., 1993). This created trisomy for approximately 122 genes on Mmu16, extending from Mrpl39 to Zfp295 (13.5 Mb), and for approximately 60 genes on Mmu17 (Duchon et al., 2011, Herault et al., 2012, Reinholdt et al., 2011). Ts65Dn mice recapitulate several DS-associated
phenotypes, including perturbed neurodevelopment, structural alterations, and learning and memory deficits. During an experiment to inactivate the \textit{Sod1} gene using the same technique, the Ts(16C-tel)1Cje (Ts1Cje) was generated, which carries a reciprocal translocation between Mmu12 and Mmu16. This partial trisomy extends from \textit{Sod1}, which is inactivated, to \textit{Mx1} (7.5 Mb), and harbours approximately 67 genes on Mmu16 (Herault \textit{et al.}, 2012, Sago \textit{et al.}, 1998).

![Figure 1.1: Mouse models of DS. Hsa21 (green) is syntenic with regions of mouse chromosomes 16 (Mmu16, pink), 17 (Mmu17, blue) and 10 (Mmu10, grey). The transchromosomal Tc1 model carries a freely segregating copy of Hsa21 (O’Doherty \textit{et al.}, 2005). The Ts1Yey;Ts2Yey;Ts3Yey model is syntenic to all homologous regions on Hsa21 (Yu \textit{et al.}, 2010; Yu \textit{et al.}, 2010). Several mouse models are segmentally trisomic for regions of Mmu16, such as the Ts65Dn (Davisson \textit{et al.}, 1993), Ts1Cje (Sago \textit{et al.}, 1998) and Ts1Rhr (Olson \textit{et al.}, 2004) models. (Adapted from Ruparelia \textit{et al.}, 2010).

The use of Cre LoxP technology, which allows rapid and precise generation of duplications or deletions of target regions, has dramatically expanded the availability of mouse models (Brault \textit{et al.}, 2007). The disadvantage of this technique is that the models do not carry an extra chromosome, but harbour a segmental tandem duplication of the region of interest. The first trisomic model to be produced using this technology was the Dp(16Cbr1-ORF9)1Rhr (Ts1Rhr) model, which carries a segmental duplication for 31 genes between \textit{Cbr1} to \textit{Orf9} (4.1 Mb) that is orthologous to the human DSCR (Olson \textit{et al.}, 2004b). Interestingly, although Ts1Cje and Ts1Rhr models exhibit DS-associated neurodevelopmental, structural and memory deficits, the impairments are generally manifest to a lesser extent and severity than in the Ts65Dn mouse model (Belichenko \textit{et al.}, 2009a, Olson \textit{et al.}, 2004a, Olson \textit{et al.}, 2007, Reeves \textit{et al.}, 1995, Sago \textit{et al.}, 1998).
Utilising the same Cre LoxP chromosome-engineering technology, a duplication spanning the entire Hsa21 region syntenic with Mmu16 was recently developed. The Dp(16Lipi-Zfp295)1Yey (Dp(16)1Yey/Ts1Yey) mouse model is homologous to human region 21q11q22.2 extending from Lipi to Zfp295 (22.6 Mb), and carries a triplication of only Hsa21 homologous genes (Li et al., 2007b, Yu et al., 2010b). Although little is known about this model, it demonstrates learning and memory deficits in some behavioural tasks (Yu et al., 2010b). The Dp(17Abcg1-Rrp1b)1Yey (Dp(17)1Yey/Ts3Yey) and Dp(10Prmt2-Pdxk)1Yey (Dp(10)1Yey/Ts2Yey) models carry a duplication of the Hsa21 homologous regions on Mmu17 and Mmu10, respectively (Yu et al., 2010b). Limited behavioural tests in Ts3Yey mice, which harbour 18 genes on a 1.0 Mb region extending from Abcg1 to Rrp1b, reveal relatively preserved learning and memory with some alterations. Surprisingly, Ts2Yey mice, which carry 53 genes (2.3 Mb) extending from Prmt2 to Pdxk, show no deleterious phenotypes in the same behavioural tests (Yu et al., 2010b). Recently, a completely trisomic model was produced, Ts1Yey;Ts2Yey;Ts3Yey, which carries all three mouse genomic regions syntenic to Hsa21. Behavioural tests conducted so far report learning and memory deficits in this model (Yu et al., 2010a).

The first transchromosomic mouse line to carry an almost complete copy of a freely segregating Hsa21 was engineered using a microcell-mediated chromosome transfer (MMCT) technique. The copy of Hsa21 in the Tc(Hsa21)1TybEmcf (Tc1) mouse may better reproduce the DS phenotypes by preserving the integrity of the chromosomal sequence and the complex set of regulatory sequences (O’Doherty et al., 2005). However, this mouse line contains several rearrangements on the Hsa21 transchromosome, in the form of microdeletions and duplications, which were most likely caused by the radiation used during its relocation in murine cells (O’Doherty et al., 2005, Reynolds et al., 2010). Tc1 mice are also mosaic for Hsa21, such that not every cell contains the human chromosome. Nevertheless, the Tc1 mouse is trisomic for approximately 80% of Hsa21 genes and displays DS-associated learning and memory deficits and structural perturbations (Morice et al., 2008, O’Doherty et al., 2005).
1.2.2 Brain anatomy

Altered brain morphology and perturbed neural connectivity may underlie the cognitive deficits in DS. Interestingly, brain structures that mature later in development such as the hippocampus, cerebellum and frontal cortex, display disproportionate impairments (Nadel, 2003). Post-mortem analyses report reduced brain volume and brachycephaly in individuals with DS. These reductions are not universal and are most prominent in the cerebellum and hippocampus. In fact, the average cerebellum in DS is less than 75% the volume of controls (Aylward et al., 1997, Pinter et al., 2001a, Pinter et al., 2001b). The parahippocampal gyrus is reported to be larger in people with DS (Kesslak et al., 1994), however the subcortical areas including the lenticular nuclei and posterior cortical grey matter are relatively preserved (Pinter et al., 2001a, Pinter et al., 2001b).

Mouse models of DS recapitulate these anatomical phenotypes. Morphometric analyses in Ts65Dn mice reveal facial anomalies, altered brain shape, reduced brain volume, cerebellum hypoplasia and smaller ventricles (Aldridge et al., 2007, Baxter et al., 2000, Hill et al., 2007). Ts1Cje mice also demonstrate craniofacial dysmorphology including smaller brains, hypoplasia of the cerebellum and enlarged ventricles (Ishihara et al., 2010, Olson et al., 2004a). Contrary to Ts65Dn and Ts1Cje mice, Ts1Rhr mice are significantly larger than controls, have a larger skull, and do not display brachycephaly. Ts1Rhr mice do not display differences in cerebrum or cerebellum brain volume, but do have a larger posterior hippocampal volume, suggesting that the DSCR genes are not sufficient to produce DS-associated brain anatomy phenotypes (Aldridge et al., 2007, Belichenko et al., 2009a, Olson et al., 2007). Similar to Ts65Dn and Ts1Cje mice, Tc1 mice display alterations in mandible size and reduced cerebellum volume (O'Doherty et al., 2005).

1.2.3 Neurodevelopment

In DS, prenatal brains that are approximately 19 weeks of gestation, display delayed development and disorganised cortical lamination, with reduced dendritic arborisation in cerebral cortex pyramidal cells, accompanied with fewer synapses (Becker et al., 1986, Petit et al., 1984, Weitzdoerfer et al., 2001). Children and adults
with DS show degeneration of the cortical pyramidal neurons, severe dendritic and synaptic impairments, and a reduction in the number of hippocampal neurons and the number of granule cells in the cerebellar cortex (Becker et al., 1986, Contestabile et al., 2009a, Takashima et al., 1989). Abnormal spinogenesis is apparent within the first two years of life in DS, characterized by a failure of dendritic spines to achieve mature morphology and enlarged atrophic spine heads (Marin-Padilla, 1976). The development of the cerebellum is also delayed and precociously terminates (Haydar & Reeves, 2011).

Dendritic perturbations are seen in Ts65Dn and Ts1Cje mice, with reduced dendritic spine density, enlarged boutons and spine heads, and decreased length of spine necks in cortical and hippocampal neurons (Belichenko et al., 2007, Belichenko et al., 2004, Popov et al., 2011). Ts1Rhr mice also show reduced dendritic spine density and increased spine heads (Belichenko et al., 2009a). Additionally, the cerebellum volume in Ts65Dn is decreased, with noteworthy reductions in the molecular layer and the internal granule layer and almost a 20% reduction in Purkinje cell and granule cell density (Baxter et al., 2000). Sonic hedgehog (SHH) growth factor response regulates cell cycle length and neurogenesis in cells; decreased SHH is proposed to elongate the cell cycle length, resulting in increased proliferation rates and impaired neurogenesis (Contestabile et al., 2009a). Ts65Dn mice show a deficient mitotic response to the SHH growth factor, leading to decreased proliferation of the cerebellar granule cells and an alteration in neural crest progenitor cells (Roper et al., 2006, Roper et al., 2009). Neural crest contribute to the correct formation of the craniofacial skeleton, which is necessary for the proper development and functioning of the brain, raising the possibility that altered SHH response may also lead to craniofacial dysmorphism in DS (Le Douarin et al., 2007, Roper et al., 2009). Reduction of cerebellar granule cell density has also been reported in Ts1Cje (Moldrich et al., 2009, Olson et al., 2004b) and Tc1 (O’Doherty et al., 2005) mice. It is likely that increased gene-dosage in DS could affect the signalling involved in neural crest programming during development (Potier et al., 2006, Roper & Reeves, 2006). Defective SHH signalling in neuronal precursor cells (NPC), and the resulting impairments in cerebellar neurogenesis and proliferation, may be caused by overexpression of the AICD (APP intracellular C-terminal domain) fragment of APP (Trazzi et al., 2011). Increased
levels of AICD leads to the enhanced expression of and binding to SHH receptor patched 1 (Ptc1), an inhibitor of the SHH signalling pathway (Trazzi et al., 2011). However, APP is not triplicated in Ts1Cje and Tc1 mice, suggesting a role for other Hsa21 genes and neurobiological pathways in causing increased proliferation and impaired neurogenesis. The cerebellar alterations may also underlie the muscle hypotonia and fine motor control deficits present in DS (Moldrich et al., 2007, Morris et al., 1982).

Excitatory glutamatergic projection neurons comprise approximately 90% of the neocortex and are generated from precursors in the dorsal telencephalic ventricular and subventricular zones. The remaining neurons are inhibitory γ-aminobutyric acid (GABA) interneurons, which are generated in the ventral ventricular zone of the ganglionic eminence (Corbin et al., 2001, Tamamaki et al., 1997). Cortex development involves the neurogenesis of excitatory and inhibitory neurons from their distinct origins of the brain, followed by the migration and differentiation of these neurons within the neocortex (Ang et al., 2003, Corbin et al., 2001). In DS, the mechanisms and processes involved in controlling embryonic production and the allocation of neurons are perturbed (Haydar & Reeves, 2011).

Reduced numbers of excitatory neurons are produced by the dorsal ventricular zone in DS fetal brains and in Ts65Dn mice (Contestabile et al., 2009b). Decreased proliferation and lengthened cell cycle in these neurons results in reduced expansion of maturing cortical layers, leading to delayed development and, possibly, defective synapse formation (Contestabile et al., 2009b). A gene encoded on Hsa21, dual-specificity tyrosine phosphorylation-regulated kinase (DYRK1A), is strongly expressed during embryonic neurogenesis, particularly in NPC, and is implicated in dorsal telencephalic ventricular zone proliferation (Guimera et al., 1996, Hammerle et al., 2002). DYRK1A overexpression has been proposed to lead to premature neuronal differentiation, depletion of NPC available during neurogenesis and inhibition of cell proliferation, presumably through deregulated NOTCH signalling (Hammerle et al., 2002, Tejedor & Hammerle, 2011). DYRK1A also interacts with growth factors, transcription factors and cell-cycle regulatory proteins that are also involved in neural cell proliferation and specification (Park et al., 2009). Furthermore, DYRK1A is involved in neuronal differentiation and
increased levels have been reported to perturb a chromatin-remodelling complex, leading to impaired dendritic growth (Lepagnol-Bestel et al., 2009), and deregulated pluripotency and embryonic stem cell fate (Canzonetta et al., 2008).

1.2.4 Synaptic plasticity

A balanced ratio of excitatory and inhibitory neurons is essential for neuronal development, synaptic plasticity and long-term potentiation (LTP), which is a neural correlate for learning and memory. In addition to the underproduction of cortical excitatory neurons, the excitatory-inhibitory (E-I) ratio is further imbalanced by excessive production of inhibitory interneurons in the Ts65Dn mouse, particularly in the dorsal neocortex and hippocampus. This results in increased neurogenesis and overinhibition, specifically in the medial ganglionic eminence (MGE) (Chakrabarti et al., 2010, Kleschevnikov et al., 2004). During MGE neurogenesis, transcription factors induce MGE precursor cells to differentiate into either inhibitory interneurons or oligodendrocytes (Nery et al., 2002). Transcription factors oligodendrocyte transcription factor 1 (Olig1) and lineage factor 2 (Olig2), are highly expressed during this process. Both these genes are encoded on Hsa1 and have been implicated in the ventral telencephalon inhibitory neuronal phenotype in Ts65Dn mice. Interestingly, normalising expression of these genes to disomic levels in Ts65Dn mice restored MGE neurogenesis and inhibitory neuron production to normal levels (Chakrabarti et al., 2010). Moreover, the overinhibition phenotype was rescued, implicating a pathological gene-dosage role of these genes in causing the E-I imbalance (Chakrabarti et al., 2010). However, these genes may not directly be involved in causing synaptic plasticity deficits, as Ts1Rhr mice demonstrate synaptic deficits, but are not trisomic for Olig1 and Olig2.

Recent electrophysiological data reveal enhanced GABA<sub>A</sub> and GABA<sub>B</sub> receptor-mediated neurotransmission, suggesting an increased presynaptic release of GABA. Enhanced postsynaptic GABA<sub>B</sub> signalling can be attributed to the triplication of KCNJ6/Girk2 (G-protein-coupled inward rectifying potassium channel) that encodes the protein Girk2, which is a subunit of a channel that modulates postsynaptic GABA<sub>B</sub> receptors (Kleschevnikov et al., 2012). In Ts65Dn mice, Girk2 is expressed throughout the brain, particularly in the hippocampus, leading to an almost two-
fold increase in GABA-B-mediated Girk2 current. Increased Kcnj6 gene-dosage is associated with increased channel density, current, and inhibitory GABA B signalling, suggesting a functional role for this gene in E-I imbalance and neuronal transmission (Best et al., 2011, Best et al., 2007, Kleschevnikov et al., 2012). Girk2 channels reduce membrane potential and neuronal excitability; lower excitability is proposed to impede N-methyl-D-aspartate (NMDA)-dependent plasticity, resulting in learning and memory impairments (Ehrengruber et al., 1997).

1.2.5 Contribution of other Hsa21 genes

Additional key Hsa21 genes that may play roles DS-associated neurodevelopment and neurodegeneration are listed in Table 1.1. However, it is likely that other genes encoded on Hsa21, some of which we may not know the role of yet, are also likely to be important for these phenotypes. Down syndrome cell adhesion molecule (DSCAM) plays a critical role in facilitating dendritic morphology and neuronal wiring during neurodevelopment, and contributes to efficient synaptic plasticity in adulthood. Overexpression of DSCAM in Ts1Cje hippocampal neurons was found to inhibit dendritic branching, which may possibly be caused by a loss of NMDA-mediated regulation of DSCAM local mRNA translation, leading to impairments in neurodevelopment and synaptic plasticity (Alves-Sampaio et al., 2010). Synaptojanin 1 (SYNJ1), a presynaptic polyphosphoinositide phosphatase important for membrane trafficking and normal synaptic vesicle recycling, also plays a significant role in maintaining stability of GABAergic neurotransmission (Luthi et al., 2001). The overexpression of SOD1 (Copper/Zinc-superoxide dismutase 1) upregulates GABAergic neurotransmission, and plays additional roles in reducing the number of hippocampal neuronal progenitor cells and LTP, and enhancing sensitivity to degeneration and apoptosis (Gahtan et al., 1998, Levkovitz et al., 1999). Overexpression of the transcriptional repressor, SIM2 (single-minded homolog 2), dramatically reduces expression of DBN1 (Drebrin 1) by directly binding to its promoter. DBN1 is a neuronal expressed gene that affects dendritic spine structure and neuritogenesis and is involved in modulating dendritic spine-cytoskeleton dynamics at postsynaptic terminals (Weitzdoerfer et al., 2001). Significantly, decreased DBN1 levels have been observed in cortices of people with AD and DS (Ooe et al., 2004, Shim & Lubec, 2002).
Table 1.1: Physiological and pathological role of key Hsa21 genes in DS-associated neurodevelopment and neurodegeneration

<table>
<thead>
<tr>
<th>Hsa21 gene</th>
<th>Physiological role</th>
<th>Pathogenic role</th>
</tr>
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<tbody>
<tr>
<td><strong>APP</strong></td>
<td>Cell surface receptor and transmembrane glycoprotein that promotes transcriptional activation. Also implicated in synapse formation and neurite outgrowth.</td>
<td><strong>Neurodevelopment</strong>&lt;br&gt;- Leads to the overexpression of SHH receptor Ptc1, which inhibits SHH signalling pathway leading to impaired cerebellar neurogenesis&lt;br&gt;- Loss of dendritic spines and synaptic plasticity</td>
</tr>
<tr>
<td><strong>DSCAM</strong></td>
<td>Cell adhesion molecule with a crucial role in dendrite morphology and neuronal wiring.</td>
<td><strong>Neurodevelopment</strong>&lt;br&gt;- Inhibits dendritic branching and causes perturbed synaptic plasticity&lt;br&gt;- May lead to aberrant NMDA-mediated regulation of DSCAM local translation</td>
</tr>
<tr>
<td><strong>DYRK1A</strong></td>
<td>Kinase involved in regulating several signalling and cell proliferation mechanism. Also involved in neurogenesis, particularly of neural precursor cells.</td>
<td><strong>Neurodevelopment</strong>&lt;br&gt;- Leads to premature neuronal differentiation, depletion of neural precursor cells and inhibition of cell proliferation&lt;br&gt;- Deregulates genes implicated in dendritic growth, cell pluripotency and embryonic stem cell fate&lt;br&gt;- Reduces dendritic growth and complexity</td>
</tr>
<tr>
<td><strong>ITSN1</strong></td>
<td>Multi-domain adaptor protein implicated in membrane trafficking and synaptic transmission.</td>
<td><strong>Neurodegeneration</strong>&lt;br&gt;- Perturbed retrieval of synaptic vesicle proteins, including SYNJ1 and dynamin, during clathrin-dependent endocytosis&lt;br&gt;- Formation of enlarged early endosomes</td>
</tr>
<tr>
<td><strong>KCNJ6</strong></td>
<td>Effector protein for GABA\textsubscript{A} receptors, that modulates potassium channel current and density.</td>
<td><strong>Neurodevelopment</strong>&lt;br&gt;- Causes GABAergic excitatory-inhibitory imbalance through increased channel density, current and GABA\textsubscript{A} signalling&lt;br&gt;- Reduces membrane potential and neuronal excitability thereby impeding NMDA-dependent plasticity</td>
</tr>
<tr>
<td><strong>OLIG1/OLIG2</strong></td>
<td>Transcription factors implicated in oligodendrogensis and neurogenesis.</td>
<td><strong>Neurodevelopment</strong>&lt;br&gt;- Induces perturbed MGE neurogenesis and inhibitory neuron production&lt;br&gt;- Causes GABAergic excitatory-inhibitory imbalance through an overinhibition phenotype</td>
</tr>
<tr>
<td><strong>PCBP3</strong></td>
<td>Splicing factor important for post-transcription activities.</td>
<td><strong>Neurodegeneration</strong>&lt;br&gt;- Mis-regulates splicing of exon 10 of tau resulting in abnormal ratios of tau isoforms leading to NFTs</td>
</tr>
<tr>
<td>Gene</td>
<td>Function</td>
<td>Neurodegeneration</td>
</tr>
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</table>
| RCAN1 | Inhibits calcineurin-dependent signalling pathways affecting development. | • Upregulates GSK3β to cause enhanced tau phosphorylation  
• Interaction with DYRK1A may aggravate perturbed endocytosis and synaptic vesicle pore kinetics  
• Aberrant dephosphorylation of SYNJ1 during depolarisation of synaptic nerve terminals |  |
| SIM2 | Transcriptional repressor implicated in synaptic plasticity and morphology. | • Reduces DBN1 levels causing morphological cytoskeletal changes at postsynaptic terminals in dendritic spines |  |
| SOD1 | Cytoplasmic protein involved in oxidative stress. | • Decreases hippocampal neuronal progenitors and LTP  
• Increases sensitivity to degeneration and apoptosis  
• Up-regulates GABAergic neurotransmission |  |
| SYNJ1 | Nerve terminal protein implicated in membrane trafficking and synaptic transmission. Also catalyses dephosphorylation of PI(4,5)P₂. | • Inability to maintain stable GABAergic neurotransmission | • Perturbed dephosphorylation of PI(4,5)P₂ and phospholipids during clathrin-dependent endocytosis  
• Formation of enlarged early endosomes |  |

(Adapted from Ruparelia et al., 2012)

### 1.3 Axonal Transport and Endocytic Trafficking

#### 1.3.1 Axonal transport

Unlike other cell types, neurons have a unique architecture to facilitate active communication through long processes called dendrites and axons. To promote neuronal survival and maintenance, neurotrophin-receptor complexes need to be retrogradely transported from axon terminals to the cell body to regulate nuclear and cytosolic activity. Dynamic processes to transmit signals are necessary for long-distance signalling in neurons. The mechanisms underlying the generation, intracellular transport and sorting, and maintenance of long-distance retrograde transmission of neurotrophic signals is an important topic of investigation.
1.3.1.1 Neurotrophins

Neurotrophins are a family of growth factors that are synthesised and released from target tissues. Upon binding to their membrane receptors on axon terminals, neurotrophins activate several signalling pathways that mediate neuronal survival, differentiation and maintenance as well as synapse formation and synaptic plasticity (Bibel & Barde, 2000, Lewin & Barde, 1996, Sofroniew et al., 2001). Pioneering experiments by Levi-Montalcini and colleagues led to the discovery of nerve growth factor (NGF) and the formulation of the neurotrophic factor hypothesis (Levi-Montalcini & Hamburger, 1951). This hypothesis posits that developing neurons compete for a limited supply of target-derived neurotrophic factor and only the neurons that successfully innervate with target tissue are able to survive (Barde, 1989, Yuen et al., 1996). Further investigations have revealed more complex and diverse functions for neurotrophins than initially anticipated (Yuen et al., 1996). Other members of the neurotrophin family include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) (Barde, 1989, Barde et al., 1982).

Neurotrophins are originally synthesised as precursors or pro-neurotrophins, which are cleaved to release mature, active proteins that bind to its receptor (Jing et al., 1992, Lee et al., 2001). Mature neurotrophins bind with either tyrosine receptor kinases (Trk) or p75 neurotrophin receptors (p75), to exert their various cellular effects. They have a specific and high binding affinity with Trk receptors, which include TrkA, TrkB and TrkC; NGF preferentially binds to TrkA, BDNF and NT-4 to TrkB, and NT-3 to TrkC receptors. Additionally, all neurotrophins bind to the low-affinity receptor p75, a member of the tumour necrosis factor superfamily (Figure 1.2). Trk receptor binding promotes signalling cascades for neuronal survival and axonal growth, whereas p75 receptor binding induces neuronal degeneration, apoptosis, collapse of growth cones and the inhibition of axonal regeneration (Dechant & Barde, 2002, Lewin & Barde, 1996, Reichardt, 2006). Neurotrophins stimulate Trk receptor dimerization, and can form heterodimeric receptor complexes, and induce transphosphorylation (Jing et al., 1992). Phosphorylated Trk (pTrk) stimulates the activation of mitogen-activated protein kinase (MAPK), v-Akt murine thymoma viral oncogene (AKT), phosphoinositol-3-
kinase (PI3K) and phospholipase C-gamma (PLCγ) signalling pathways (Huang & Reichardt, 2003, Patapoutian & Reichardt, 2001). The neurotrophin-receptor complex is essential for sustained signalling of these kinases to regulate axonal and dendritic growth, synaptic plasticity and neuronal survival (Reichardt, 2006).

The phosphorylated p75 signalling cascade activates the nuclear factor-κ-light-chain-enhancer of activated B cells (NF-κB) and c-Jun N-terminal kinases (JNK), which are associated with apoptosis (Reichardt, 2006). During cell death and after injury, p75 serves as a pro-apoptotic receptor (Dechant & Barde, 2002). Pro-neurotrophins also trigger p75-induced apoptosis (Lee et al., 2001). Proteolytic cleavage may regulate the physiological activity of neurotrophins with pro-forms preferentially activating p75 to mediate apoptosis, and mature forms activating Trk receptors to promote survival (Lee et al., 2001). This “death receptor” may be crucial for neuronal pruning during developmental cell death or for refinement of target innervation during mis-targeting, when appropriate neurotrophins are not encountered (Chao & Lee, 2004).

Figure 1.2: Neurotrophins and their receptors. NGF preferentially binds to TrkA, BDNF and NT4 to TrkB and NT3 to TrkC. All neurotrophins bind to p75 (Top panel). Mature neurotrophin-receptor binding stimulates receptor dimerisation, and can form heterodimeric receptor complexes to induce transphosphorylation. Different signalling cascade responses are elicited when neurotrophins bind to a specific receptor class. Phosphorylated Trk promotes neuronal survival and differentiation by stimulating MAPK, PI3K and PLCγ signalling cascades. Phosphorylated p75 promotes apoptosis and cell death (through its death domain) and triggers the NFκB and JNK kinases (Bottom panel).
1.3.1.2 BDNF/TrkB and memory processes

In the central nervous system (CNS), the distribution of NGF is restricted and primarily promotes the survival and functioning of basal forebrain cholinergic neurons (BFCN), which project to the hippocampus (Chen et al., 1997). The other neurotrophins are more widely expressed throughout the CNS, with BDNF being the most widely distributed (Tapia-Arancibia et al., 2008). It is highly expressed in the hippocampus, cortex, and basal forebrain and is associated with the survival and functioning of several neuronal populations (Chao & Lee, 2004). All these brain structures are vital to higher cognitive function, implicating an important role for BDNF and its receptor TrkB in learning and memory. The mammalian CNS contains three TrkB isoforms, one full-length and two truncated isoforms. To activate downstream signalling pathways, BDNF binds to the full-length TrkB and forms homodimers, leading to autophosphorylation of tyrosine residues in the TrkB cytoplasmic domains (Huang & Reichardt, 2003, Jing et al., 1992).

In addition to its neurotrophic effects of activating intracellular signalling cascades and controlling gene transcription, a primary function of BDNF is the regulation of synaptic plasticity, and axonal and dendritic growth and guidance (Reichardt, 2006, Yamada & Nabeshima, 2003). Memory acquisition and consolidation are associated with increased BDNF mRNA expression and phosphorylation of TrkB. In hippocampal-dependent contextual learning tasks, such as the Morris water maze (MWM), contextual fear and passive avoidance tests, a rapid and transient increase in BDNF mRNA expression is observed (Hall et al., 2000, Yamada et al., 2002). Bdnf-knockout mice demonstrate learning impairments in the MWM (Llinarsson et al., 1997). Inhibition of BDNF through anti-BDNF antibodies results in memory impairments in the MWM and passive avoidance tests, suggesting a role for BDNF in spatial learning, short-term memory and long-term memory processes (Alonso et al., 2002, Mu et al., 1999).

Through TrkB receptor stimulation, BDNF can enhance glutamatergic synaptic transmission and NMDAR phosphorylation in the hippocampus (Kafitz et al., 1999, Suen et al., 1997). It can activate TrkB receptors through either presynaptic or postsynaptic mechanisms, to modulate LTP enhancement in the hippocampus.
(Figurov et al., 1996, Kovalchuk et al., 2002, Xu et al., 2000). In Bdnf-knockout mice, LTP is impaired but can be rescued by exogenous BDNF (Patterson et al., 1996). BDNF has also been implicated in reference and working memory as demonstrated by tests in radial arm mazes (Mizuno et al., 2000). In a positively motivated radial arm maze test, which assesses spatial reference memory, activation of the TrkB/PI3K signalling pathway and translational processes was associated with spatial memory consolidation (Mizuno et al., 2003). The direct and rapid activation of TrkB in the amygdala is suggested to be responsible for regulating the learning of fear and extinction memories (Jang et al., 2010). In a negatively motivated passive avoidance test, the MAPK signalling pathway was activated during acquisition of fear memory (Alonso et al., 2002). Collectively, BDNF/TrkB is involved in various memory processes including contextual, spatial, fear and emotional learning and memory. Compelling evidence therefore points to the BDNF/TrkB signalling complex in regulating NMDAR-mediated, LTP-associated learning and memory.

1.3.2 Endocytic trafficking

Several models have been put forward for mechanisms underlying long-distance retrograde axonal transport of neurotrophins. The “wave model” postulates that NGF binds to and activates surface TrkA at distal axon terminals, and downstream signalling molecules are subsequently propagated via periodic amplification through signal transducers in the axon to the cell body in a manner similar to a ‘wave’ (Senger & Campenot, 1997). This model does not require endocytosis of NGF after ligand binding. No direct evidence supports this model, instead, evidence shows that it is necessary for the NGF/TrkA signalling complex to be internalised for neuronal survival (Howe & Mobley, 2004, Ye et al., 2003). Building on this model, the “signalling effector model” suggests that second messengers or ionic fluxes, i.e. cytosolic calcium (Ca^{2+}) or downstream kinases, are the primary source of retrograde neurotrophin signalling through amplification effects on nearby channels and kinases (Nguyen et al., 1998). However, this model is limited by the restricted diffusion radius of ionic fluxes and second messengers, which challenges long-distance axonal transport. Furthermore, stimuli other than neurotrophins can stimulate ionic fluxes, thus raising a signalling specificity issue (Wu et al., 2009).
The “NGF-independent retrograde signalling model” is based on the premise that NGF signalling may not necessarily be coupled with retrograde transport of NGF. Despite utilising a mechanism that rendered NGF incapable of internalisation, the activation of NGF persisted to trigger axonal transport of signalling molecules including pTrkA, PI3K and pAKT, which supported neuronal survival for up to 30 hours (MacInnis & Campenot, 2002). NGF-independent retrograde signalling events may be a possible endocytic mechanism used by some neuronal populations to support axonal transport. However, to maintain a sustained NGF-TrkA signal over long-distances probably requires the presence of NGF within a signalling complex to ensure the signal reaches its final destination, as proposed by the “signalling endosome hypothesis”.

1.3.2.1 Signalling endosome hypothesis

The signalling endosome hypothesis may suggest the most efficient mechanism for signalling over long-distances. The hypothesis was built on observations during receptor-mediated endocytosis, that NGF binds to and activates surface TrkA receptors at the axon terminal to facilitate the formation of NGF/pTrkA signalling endosomes (Beattie et al., 1996, Grimes et al., 1997, Ye et al., 2003). This membrane-bound organelle packages the signalling complex to ensure higher fidelity, specificity, and is resistant to interference from noise and crosstalk (Delcroix et al., 2003, Howe & Mobley, 2004). Signalling endosomes serve as retrograde vesicular carriers, which are transported by dynein motor proteins along microtubule tracks, to maintain sustained signalling of NGF/pTrkA along the journey from axon terminal to the cell body. This model is within the realms of perception that signalling pathways within cells are highly organised and compartmentalised to ensure specific and sustained signal transduction (Wu et al., 2009). There is evidence to suggest that these signalling endosomes are in fact, early endosomes (Delcroix et al., 2003).

To maintain sustained signalling that induces transcriptional changes, NGF must be internalised at the axon terminal and retrogradely transported to the cell body to stimulate the phosphorylation of cAMP response element-binding protein (CREB), which is required for neuronal survival (MacInnis et al., 2003). Signalling that is
activated at the plasma membrane as opposed to signalling from ligand-receptor complexes in endosomes activates different sets of signalling molecules. For example, although both NGF and NT3 promote TrkA-mediated axonal outgrowth, only NGF promotes neuronal survival. This functional difference is because unlike NGF, NT3 phosphorylates surface TrkA but does not induce internalisation and thus the neuronal survival transcriptional changes (Kuruvilla et al., 2004).

1.3.2.2 Endocytic pathways

Endocytosis is a mechanism through which cells regulate their developmental and functional programs and communicate across the plasma membrane (Riezman et al., 1997). Neurons require the endocytic pathway to internalise and process extracellular material and neurotrophic factors, recycle, modify and degrade membrane proteins and receptors, and to direct information to intracellular pathways (Clague, 1998, Nixon et al., 2001). Endocytosis is characterised by the internalisation of extracellular molecules from the cell surface into internal plasma membrane-bound compartments that are then trafficked sequentially through numerous vesicular compartments to regulate cell signalling. Internalisation into vesicles can occur through the caveolae, a highly specialised vesicle, or by macropinocytosis, a phagocytic-like non-specific process. However, most internalisation is mediated by clathrin.

Clathrin-mediated endocytosis is responsible for the internalisation of nutrients, pathogens, antigens, growth factors and receptors (Kirchhausen, 2000). This pathway involves the recruitment of soluble clathrin from the cytoplasm to the plasma membrane. The clathrin assembles into a lattice-like structure at the plasma membrane to form coated pits, which bud and pinch off from the membrane through a dynamin-dependent mechanism, to generate clathrin-coated vesicles (Nathke et al., 1992, Schmid, 1997). NGF treatment of PC12 cells results in over a ten-fold increase in the colocalisation of TrkA with the clathrin heavy chain at the cell surface, suggesting that clathrin-coated vesicles transport neurotrophins and their receptors in signalling endosomes (Beattie et al., 1996, Grimes et al., 1997). The NGF/pTrkA signalling complex was later found to recruit clathrin for the formation of clathrin-coated vesicles, discovered in these vesicles (Beattie et al.,
BDNF activation also increases clathrin association with surface membranes of hippocampal neurons (Beattie et al., 2000). Inhibition of clathrin-mediated endocytosis blocks retrograde transport of signalling complexes and transcriptional changes to induce neuronal survival (Deinhardt et al., 2007, Heerssen et al., 2004).

Clathrin-mediated endocytosis is facilitated by adaptor-protein complexes, such as adaptor protein-2 (AP2), which are key components that bind directly to clathrin and other endocytic regulatory proteins and cargo to stimulate the formation of the clathrin coat (Nesterov et al., 1999). Phosphoinositides are second messengers that are also involved in regulating intracellular trafficking, and play a crucial role in assembling and regulating cell-signalling pathways (De Camilli et al., 1996). PtdIns(4,5)P2 is found in clathrin-coated pits and facilitates vesicle formation and budding by binding to clathrin adaptor proteins (Kobayashi et al., 1998). Dynamin plays an essential role in clathrin-coated vesicle formation, by mediating the scission of clathrin-coated pits from the plasma membrane (McNiven, 1998). It interacts with amphiphysin and syndapins, which are proteins that link with the actin cytoskeleton (Qualmann et al., 2000). Amphiphysin recruits dynamin to clathrin-coated pits, and phosphorylation of Amphiphysin 1 inhibits its binding to clathrin and the AP2 adaptor complex (David et al., 1996, Takei et al., 1999).

SYNJ1, encoded on Hsa21, is a polyphosphoinositide phosphatase that directly binds to PtdIns(4,5)P2 (McPherson et al., 1996) and amphiphysin (Micheva et al., 1997) at synaptic nerve terminals. Significantly, clathrin-coated vesicles accumulate at the nerve terminals in Synj1-knockout mice (Cremona et al., 1999). Additionally, phosphorylation of dynamin and SYNJ1 prevents binding to amphiphysin (Bauerfeind et al., 1997, Slepnev et al., 1998). Intersectin 1 (ITSN1), a multiple EH and SH3 domain-containing protein encoded on Hsa21, may link endocytosis with neuronal growth and differentiation through the MAPK pathway (Tong et al., 2000). It binds to dynamin and epsin, which is also involved in increased binding to AP2 (Chen et al., 1999, Sengar et al., 1999). Importantly, Itsn1-knockout mice inhibit endocytosis of the transferrin receptor, which is the archetypical cargo for internalisation through clathrin-mediated endocytosis (Sengar et al., 1999).
Inhibition of the clathrin-mediated pathway stimulates other internalisation pathways, possibly as compensatory mechanisms (Lamaze & Schmid, 1995). These routes are not as well characterised, but can crosstalk with the clathrin-mediated pathway. One pathway involves lipid rafts, which are cell surface microdomains that contain glycosphingolipids and cholesterol (Parton et al., 1994). When associated with caveolin, a raft-resident protein, it triggers the formation of pear-shaped caveolae invaginations at the cell surface that mediates endocytosis (Kurzchalia & Parton, 1999, Parton, 1996). Macropinocytosis is another clathrin-independent pathway that occurs in areas where membranes spread and ruffling is prominent. In this process, vesicles are formed when membrane ruffles move at the tips of actin tails and remodel the actin cytoskeleton in a manner akin to phagocytosis (Merrifield et al., 1999). This route is mediated and used by some bacteria (Francis et al., 1993). It is dependent on signalling pathways that involve Rho GTPases such as RhoA and Rac, which stimulate dendritic maturation and pinocytosis, but inhibit clathrin-dependent endocytosis (Ellis & Mellor, 2000).

1.3.3 Endosomes

Endosomes display unique properties that define its functional specificity. They are an important sorting platform from which specific and sustained long-distance signalling can be maintained (Deinhardt et al., 2006). They are enriched with phosphoinositides and resident proteins that mediate the assembly of vesicles, and have the ability to use microtubule tracks to move long distances towards the cell body. Endosomes nurture an acidic environment, especially in late endosomes, which favours the proteolytic enzymes in the signalling complex (Sorkin & von Zastrow, 2009). After internalisation from entry routes, cell surface molecules and receptors are packaged into a signalling endosome and sorted through various vesicular compartments dependent on their destination. In the case of clathrin-mediated endocytosis, after internalisation, the clathrin-coated vesicle becomes uncoated in the intracellular cytoplasm and fuses with early endosomes (Kirchhausen, 2000, Sorkin & Von Zastrow, 2002). As Figure 1.3 portrays, the internalised molecules can either be recycled back to the plasma membrane from the early endosome or transported to a late endosome or lysosomes (Deinhardt et al., 2006, Lakadamyali et al., 2006).
Figure 1.3: Clathrin-mediated endocytosis at the axon terminal. Neurotrophins bind to their Trk receptors to create a signalling complex. They recruit clathrin at the plasma membrane to facilitate internalisation into clathrin-coated pits (CCP), which then bud and pinch off to form clathrin-coated vesicles (CCV). The CCV becomes uncoated and fuses with a signalling endosome or an early endosome for further sorting. Rab5 and its effector protein EEA1 regulate this process. From this sorting platform, the cargo can be transported to the cell body by dynein motor proteins along the microtubules (MT). The cargo can also be returned back to the plasma membrane through Rab4 and Rab11 regulated recycling endosomes. Additionally, if further proteolysis is required, the cargo can be trafficked to Rab7-regulated late endosomes, or multivesicular bodies (MVB). Subsequently, the cargo can be transported to lysosomes where digestive enzymes proteolyse the remaining cargo.

The dynamics of signalling complexes in endocytic domains are regulated by Ras-associated binding (Rab) GTPases, that cycle between an active and inactive state (Jordens et al., 2005). Once the clathrin-coated vesicle has fused with early endosomes, it may subsequently fuse with other early endosomes through regulation by Rab5 GTPases and its effector proteins including early endosome antigen 1 (EEA1) (Bucci et al., 1992, Gorvel et al., 1991). Many of these cargoes in the early endosome include surface receptors, proteins or lipids, which are returned back to the plasma membrane through Rab4-regulated recycling endosomes (van der Sluijs et al., 1992). Rab11 is also believed to regulate transport along this recycling circuit, but at a later step than Rab4 (Ullrich et al., 1996). Early endosomes also receive cargoes from the trans-Golgi network (TGN) and late endosomes to be sorted back to the plasma membrane.
If the early endosome contains cargo that needs to be proteolysed, such as a downregulated receptor, it is transported to Rab7-regulated late endosomes or lysosomes by either budding off and fusing into a late endosome or maturing into a late endosome (Deinhardt et al., 2006, Griffiths & Gruenberg, 1991, Rink et al., 2005). The transport from early to late endosomes may be facilitated by intermediate vesicles, multivesicular bodies (MVB), which form several internal vesicles through inward budding of the surface membrane (Gruenberg & Maxfield, 1995, Piper & Luzio, 2001). The MVB is responsible for the delivery of lysosomal hydrolases and the downregulation of activated receptors, which are degraded in the lysosome. This process is initiated by ubiquitin and mediated by the ESCRT-1 (endosomal sorting complex required for transport 1) complex to reduce endosome volume and allow degradative enzymes access to the internalised membrane (Katzmann et al., 2001). Degradative enzymes, including the cathepsin family of proteases and other acid hydrolases, are delivered to the late endosome or MVB by the TGN through mannose-6-phosphate receptors or by fusing with a lysosome (Ghosh et al., 2003, Luzio et al., 2000). Late endosomes can also fuse with autophagosomes to reach lysosomes during autophagy, which is a lysosomal pathway that degrades cytosolic proteins and organelles (Levine & Klionsky, 2004).

1.3.3.1 Early endosomes

The early endosome is a crucial endocytic station that initiates sorting and determines the subsequent destiny of the internalised cargo to be either recycled back to the plasma membrane or degraded in lysosomes (Bucci et al., 1992, de Hoop et al., 1994, Jovic et al., 2010, Stenmark et al., 1994). It is a highly dynamic structure with a great affinity to undergo homotypic fusion (Gruenberg et al., 1989). Early endosomes are pleomorphic structures that are composed of thin tubular extensions (approximately 60 nm in diameter) and large vesicles (approximately 400 nm in diameter), which have membrane invaginations eliciting an appearance of a multi-vesicular compartment (Gruenberg et al., 1989). Conceivably, these morphologically distinct domains are functional whereby the tubular membranes encompass proteins targeted for recycling whereas the multi-vesicular elements cluster proteins directed for degradation. Interestingly, vesicles generated from these distinct morphological structures demonstrate variations in internal
environment. In the multi-vesicular compartment, the pH decreases from 6.2 to 5.5, however it is increased to pH 6.4 in the tubular compartments (Gruenberg et al., 1989). A decrease in acidification may facilitate the initiation of the degradation processes in the lysosome and an increase in acidification may be anticipated for the release back to the plasma membrane. The structural and functional properties of the early endosome ensure tightly regulated and highly specific sorting (Bucci et al., 1992, Stenmark et al., 1994). Active Rab proteins bind to various Rab effector proteins to carry out endocytic trafficking events such as vesicle budding, motility, fusion and tethering (Mills et al., 1998, Simonsen et al., 1998). Rab5 proteins regulate entry of the cargo to the early endosome after internalisation and mediate homotypic fusion, and activate signalling pathways. Its effector protein, EEA1, is specifically localised in early endosomes and is essential for mediating homotypic fusion (Mills et al., 1998, Simonsen et al., 1998).

1.4 Perturbed Axonal Transport and Endocytosis in DS

1.4.1 APP and Alzheimer's Disease in DS

AD is a common neurodegenerative disorder that is characterised by global cognitive decline including progressive loss in memory, orientation and reasoning (Selkoe, 2001). Age-associated dementias are becoming increasingly important to study due to increased life expectancy and greater prevalence of the disease, with AD accounting for 50% to 75% of all persons with dementia (Blennow et al., 2006). Sporadic AD is the major cause of dementia, affecting over 26 million people worldwide (Brookmeyer et al., 2007). The hallmark neuropathological features of AD are distinguished by changes and deposits of misfolded proteins, constituting of hyperphosphorylated Tau in neurofibrillary tangles (NFTs) (Grundke-Iqbal et al., 1986) and extracellular amyloid-β (Aβ) plaques and deposits (Glenner & Wong, 1984b, Masters et al., 1985). AD pathology impacts the entorhinal cortex followed by the hippocampus, parietal and prefrontal cortices (Braak & Braak, 1991).
People with DS above the age of 45 years are highly susceptible to early-onset AD, with the prevalence of dementia increasing exponentially such that by the age of 65 years, over 75% have a clinical diagnosis of dementia (Coppus et al., 2006, Lemere et al., 1996, Masters et al., 1985). Early-onset AD in adults with DS was first recognised over 130 years ago (Fraser & Mitchell, 1876), and subsequent development of some aspects of neuropathology was noted in several important studies (Jervis, 1948, Malamud, 1972, Mann, 1988, Olson & Shaw, 1969). The neuropathology of DS is similar to that for AD in the changes detected, their regional distribution, relative time of appearance and progression over time (Hof et al., 1995). Despite the full-blown clinical manifestation occurring later in life, histopathological hallmark features of AD are seen earlier in people with DS; brain atrophy, Aβ deposits, and accumulation of NFTs are all present in people with DS by the age of 40 (Holtzman et al., 1996, Mann & Esiri, 1989, Wisniewski et al., 1985).

Some variations do exist in the early presentation of AD-associated symptoms in DS compared to the general population. Episodic memory and orientation problems are generally the primary signs of developing AD. In DS however, prefrontal lobe symptoms reflecting emotional changes, may be more common including, indifference, pragnosia, depression and impaired adaptive functioning (Ball et al., 2006, Lott & Head, 2001, Zigman et al., 1996). It is suggested that only in the later stages of the condition do the typical AD-related memory problems, such as executive functioning difficulties, manifest in people with DS (Zigman et al., 1996). Underlying these specific behavioural differences between the two groups may be the sequence of brain regions affected by AD pathology. In people with DS, the dysfunction of the frontal lobes may predispose to AD as this is where Aβ first accumulates in DS, compared to the general population where the hippocampus is initially affected (Braak & Braak, 1991, Lemere et al., 1996). The pathological progression may be dependent on the congenital gross anatomy of the DS brain. Developmental arrest of the frontal brain and underdevelopment of other parts of the cortex have been observed in DS (de la Monte & Hedley-Whyte, 1990, Wisniewski, 1990).
The first chemical relationship between DS and AD was discovered in 1984, when sequence analyses revealed homology between Aβ deposits from DS and that from AD (Glenner & Wong, 1984a). This led to the hypothesis that the genetic defect in AD is localised on Hsa21 (Goate et al., 1989). Indeed, under physiological conditions APP (amyloid precursor protein), an Hsa21-encoded transmembrane glycoprotein, produces Aβ peptides; however overexpression of APP leads to increases in Aβ plaque deposits (Rovelet-Lecrux et al., 2006, Sleegers et al., 2006). Aβ deposition has been observed in brains of children with DS as early as eight years of age (Leverenz & Raskind, 1998). By the age of thirty, Aβ deposition was found in up to 50% of brain specimens (Lemere et al., 1996). A seminal investigation revealing point mutations in APP and co-inherited AD (Goate et al., 1991) provided support for a pathological role of APP in AD. Further evidence is documented in case studies of five rare families with early-onset AD who have small internal duplications of Hsa21 that includes the triplication of APP (Cabrejo et al., 2006). Moreover, in cases of partial trisomy 21, which do not include triplication of APP, no neuropathological or neuropsychological presence of AD is evident (Prasher et al., 1998).

1.4.1.1 APP processing

The abnormal accumulation of Aβ is thought to arise from dysregulated proteolytic processing of its parent molecule, APP (Sisodia et al., 1990). APP is translocated into the endoplasmic reticulum and modified through the secretory pathway (Weidemann et al., 1989). During trafficking, APP can undergo various proteolytic cleavages to release secreted derivatives into vesicles and extracellular space. The first proteolytic cleavage, α-secretase cleavage, occurs 12 amino acids into the transmembrane domain of APP and releases a large soluble ectodomain fragment (α-APP_s) into the extracellular space and retains an 83-residue COOH-terminal fragment (CTF) in the membrane (Esch et al., 1990). As it cleaves within the Aβ domain of APP, it precludes the formation of Aβ and amyloidgenic fragments.

In the amyloidgenic pathway, APP molecules that have not undergone α-secretase cleavage can be subjected to β-secretase cleavage, which cuts 16 residues to the α-cleavage site creating a slightly smaller soluble ectodomain derivative (β-APP_s)
and retaining a 99-residue CTF (C99), which contains the whole Aβ domain (Esch et al., 1990). This may be followed by an inter-membrane cleavage of the C99 fragment by γ-secretase, which releases Aβ isoforms that range from 39-43 amino acid residues. The most common isoform is Aβ40, which is produced by cleavage that occurs in the endoplasmic reticulum. Another common isoform is Aβ42, which is produced by cleavage in the TGN, and is more fibrillogenic and associated with diseased states. Aβ42 fibrils aggregate into oligomers within endosomal vesicles and along microtubules (Takahashi et al., 2004). The amyloid cascade hypothesis postulates that dementia occurs due to the accumulation of cytotoxic Aβ oligomer species, which results in an imbalance between Aβ production and Aβ clearance and the subsequent formation of tau-contain NFTs (Hardy & Allsop, 1991, Hardy & Selkoe, 2002, Hardy & Higgins, 1992).

1.4.2 BDNF and TrkB in AD

Interestingly, the gene encoding TrkB is located on chromosome 9 (band 9q22), which is a region genetically associated to AD (Hamshere et al., 2007, Perry et al., 2007). Decreased BDNF expression and mRNA levels are seen in the hippocampus and cortical areas, which are brain regions that are associated with AD (Connor et al., 1997, Ferrer et al., 1999, Holsinger et al., 2000, Phillips et al., 1991). Post-mortem AD brains also showed reduced BDNF protein levels and up to 50% less BDNF mRNA levels in the nucleus basalis, relative to controls (Fahnestock et al., 2002). Even in the preclinical stage, there is evidence of decreased levels of mature BDNF and its mRNA as well as decreased pro-BDNF levels in the parietal cortex and hippocampus (Michalski & Fahnestock, 2003, Peng et al., 2005). Levels of all Trk receptors, but not the p75 receptor, are also downregulated in AD brain samples. Moreover, Trk mRNA levels correlate with the degree of cognitive impairment, identifying Trk as a potential biomarker of AD progression (Ginsberg et al., 2006).

BDNF and TrkB can modulate APP levels and proteolysis by shifting APP processing towards the α-secretase pathway and thereby promoting accumulation of α-APPs (Holback et al., 2005). However, Aβ is proposed to contribute to reduced neuronal survival in AD brains (Tong et al., 2004). Aβ deposition reduces BDNF/TrkB and
CREB levels and impairs TrkB-mediated signalling, thereby preventing TrkB autophosphorylation and activation of the BDNF/TrkB/CREB signalling pathway (Li & Liu, 2010, Pugazhenthi et al., 2011, Zeng et al., 2010). Scarce evidence exists between the role of BDNF/TrkB and Tau. In NFT-bearing neurons, full length TrkB immunoreactivity is decreased (Ferrer et al., 1999). The physiological role of Tau, a soluble microtubule-associated protein, is to promote the assembly and stability of microtubules; hyperphosphorylated Tau is insoluble, has a low affinity for microtubules and self-assembles (Goedert et al., 2006; Ballatore et al., 2007). BDNF stimulation of P19 neurons induced an immediate and transient dephosphorylation of Tau. This effect was dependent on TrkB-mediated signalling and the activation of the PI3K/AKT signalling pathway (Elliott et al., 2005). It is noteworthy that activation of BDNF/TrkB causes dephosphorylation of Tau, suggesting that reduced levels of BDNF/TrkB in AD may lead to hyperphosphorylation of Tau. Furthermore, BDNF/TrkB activation induces the AKT-dependent phosphorylation of glycogen synthase kinase-3 β (GSK3β), resulting in its inactivation (Li et al., 2007a). GSK3β directly phosphorylates Tau, and reduced BDNF/TrkB levels in AD may additionally increase phosphorylation of Tau through a GSK3β-mediated pathway.

1.4.3 Axonal transport and endosomal perturbations in DS models

1.4.3.1 Contribution of APP

Mouse models of DS recapitulate certain DS-associated neurodegenerative phenotypes. Ts65Dn mice do not exhibit Aβ plaque deposits or NFTs, but they do demonstrate a loss of BFCNs, which is correlated with cognitive decline in these mice (Granholm et al., 2000, Holtzman et al., 1996, Hunter et al., 2003). The BFCNs are particularly vulnerable to neurodegeneration in DS and AD (Holtzman et al., 1996). Ts1Cje mice show no evidence of BFCN neurodegeneration, suggesting that the contribution of one or more of the ~55 extra trisomic genes in Ts65Dn mice are necessary for BFCN neurodegeneration (Sago et al., 1998). Degeneration of BFCNs is suggested to be caused by deficits in NGF retrograde axonal transport (Cooper et al., 2001). Indeed, Ts65Dn mice display a severe impairment of NGF retrograde transport that is six times worse than in Ts1Cje mice (Salehi et al., 2006).
To understand the contribution of specific genes in causing this phenotype, *App* was identified as a triplicated gene that is present in Ts65Dn, but not in Ts1Cje mice. The third copy of *App* in Ts65Dn mice was knocked down to render its expression comparable to disomic levels. Interestingly, in these Ts65Dn*App*++ mice, NGF retrograde transport was restored to that of Ts1Cje mice. Consequently, 60% of the impaired axonal transport was attributed to the triplication of *APP*, with 40% of the deficit still unaccounted for. Significantly, normalising *App* levels prevented the loss of BFCNs in Ts65Dn mice (Salehi et al., 2006). Further support for the role of overexpressed *App* was demonstrated by deficits in retrograde NGF transport in a mouse carrying a human wild-type *APP* transgene and another harbouring a human mutant *APP* transgene (Salehi et al., 2006). *APP* gene-dosage imbalance has also been linked to the degeneration of locus coeruleus neurons, which occurs before the BFCN neuronal loss (Salehi et al., 2009).

BFCN terminal ends in Ts65Dn mice display enlarged early endosomes that contain markers for both NGF and APP, suggesting that the overexpression of *App* causes enlarged early endosomes leading to disrupted retrograde NGF transport and consequently, neurodegeneration (Salehi et al., 2006). Interestingly, Ts1Cje mice do not display enlarged early endosomes and normalising *App* gene dosage in Ts65Dn*App*++ mice rescued the enlarged early endosome phenotype (Cataldo et al., 2003). These studies suggest a gene-dosage effect of *APP* in causing enlarged early endosomes and subsequently, neurotrophin axonal transport deficits (Figure 1.4). However it is important to note that the direct movement of early endosomes, marked by Rab5 antibodies, was not shown in this paper. Endosomal abnormalities develop in neurons of brain regions most severely affected in AD and DS, including the hippocampus and neocortex as well as the basal forebrain. Early endosomes are 32-fold larger in volume in DS pyramidal neurons in lamina III of the prefrontal cortex, compared to controls (Cataldo et al., 2003). Enlarged endosomes and a perturbed endocytic pathway are observed as early as 2 months of age in DS brains and in DS fibroblasts (Cataldo et al., 2008, Cataldo et al., 2003, Jiang et al., 2010). However it is still unclear what causes these endosomal phenotypes.
Figure 1.4: Retrograde axonal transport of neurotrophins in normal and Ts65Dn neurons. (Top panel) Healthy neurons demonstrate normal retrograde transport of neurotrophins in signalling endosomes along the axon. Transport along microtubules (MT) is facilitated by regulatory, scaffolding, accessory and motor proteins including, Rab5, EEA1 and the dynein/dynactin complex. (Bottom panel) Ts65Dn neurons demonstrate impaired axonal transport (based upon findings and conclusions in Saheli et al., 2006). The overexpression of APP in these mice is postulated to cause enlarged early endosomes, which impedes axonal transport of essential neurotrophins required for neuronal survival, leading to neurodegeneration.

The role for APP in causing endocytic deficits was further examined by studying key APP proteolytic enzymes (BACE-1 and γ-secretase) and various APP protelytic fragments (Aβ and βCTF) in DS fibroblasts (Jiang et al., 2010). Morphological and functional endocytic abnormalities in DS fibroblasts were reversed when the expression of APP or BACE-1 was lowered. Overexpression of wildtype APP was sufficient to induce endosomal pathology in control fibroblasts, which were unaltered when transfected with a mutant form of APP that lacked the amino acid
sequence required for the β-site cleavage, thus establishing an importance of the βCTF (Jiang et al., 2010). A γ-secretase inhibitor, which pharmacologically reduces Aβ production but raises βCTF levels, also produced endosome pathology in control fibroblasts and worsened the pathology in DS fibroblasts. Furthermore, control fibroblasts that were transfected with a construct that elevated only the βCTF levels also demonstrated an increase in endosome size similar to that of increased APP expression. Reduced BACE-1 expression, and thus lowered βCTF production rescued endosomal pathology in DS fibroblasts (Jiang et al., 2010). This suggests that endocytic dysfunction is dependent on APP gene-dose and processing and specifically, elevated levels of βCTF, and may be independent of Aβ.

1.4.3.2 Contribution of other Hsa21 genes

Aside from APP, other Hsa21 genes have also been implicated in contributing to DS-associated neurodegeneration, perturbed endocytic pathway and abnormal synaptic circuits (Table 1.1). DYRK1A functionally interacts with and phosphorylates APP, and may thus contribute to the extracellular Aβ plaques seen in AD and DS (Ryoo et al., 2008). None of the available DS mouse models produce NFTs, however Ts1Cje mice display hyperphosphorylation of Tau by the age of 3 months (Shukkur et al., 2006). Interestingly, DYRK1A also encodes a kinase that phosphorylates Tau at a key priming site (Ryoo et al., 2007). Aged Tc1 mice display increased phosphorylation of Tau at the site that is targeted by Dyrk1a (Sheppard et al., 2012). Furthermore, GSK3β is aberrantly dephosphorylated in aged Tc1 mice, which may reduce its activity and additionally lead to increased phosphorylation of Tau (Li et al., 2007a, Sheppard et al., 2012). Triplication of DYRK1A is thus proposed to facilitate the hyperphosphorylation of Tau, in an APP-independent manner, and may lead to NFTs and AD in people with DS (Ryoo et al., 2007).

NFTs can also form through mis-regulated splicing of Tau. Splicing misregulation of exon 10 of Tau has been shown to result in abnormal ratios of tau isoforms leading to tauopathies including AD and early-onset of dementia in DS. A splicing factor on Hsa21, heterogeneous nuclear ribonucleoprotein E3 (PCBP3), was found to activate splicing of exon 10, suggesting a pathogenic role for this gene in forming NFTs and
providing another mechanism through which people with DS may be susceptible to early-onset AD (Wang et al., 2010). A recent study by Lloret and colleagues (Lloret et al., 2011) showed rat cortical neurons incubated with exogenous Aβ-induced oxidative stress, thereby upregulating the expression of RCAN1 (regulator of calcineurin 1), encoded on Hsa21. Calcineurin is a serine-threonine phosphatase that dephosphorylates Tau, and RCAN1 inhibits the phosphatase activity of calcineurin, resulting in increased phosphorylation of Tau. Increased RCAN1 levels also cause the upregulation of GSK3β, which additionally results in the hyperphosphorylation of Tau. Moreover, increased RCAN1 and phospho-Tau levels were found in lymphocytes of people whose ApoE genotype contained the ε4 allele, which has been linked to a higher risk of developing AD, compared to other allelic variations (Lloret et al., 2011).

*ITSN1* and *SYNJ1* are involved in synaptic vesicle endocytosis. *ITSN1* is a multi-domain adaptor protein, which functions with *SYNJ1* and dynamin, to facilitate the retrieval of synaptic vesicle proteins during clathrin-mediated endocytosis (Dittman & Ryan, 2009). *SYNJ1* functions to dephosphorylate phospholipids during this process; this activity is regulated by DYRK1A and includes the phosphorylation of *SYNJ1*, dynamin 1 and amphiphysin (Adayev et al., 2006, Murakami et al., 2006). RCAN1 also interacts with DYRK1A and controls endocytosis and synaptic vesicle fusion pore kinetics (Keating et al., 2008). It dephosphorylates *SYNJ1* during depolarisation of synaptic nerve terminals (Lee et al., 2004). Recently, the overexpression of *SYNJ1* in a neuroblastoma cell line as well as in transgenic mice was associated with the enlarged early endosome phenotype. Interestingly, this phenotype was reduced by silencing *SYNJ1* expression with RNA interference in DS fibroblasts, suggesting a therapeutic target other than *APP* for addressing endosomal morphology and trafficking abnormalities (Cossec et al., 2012). The increased gene-dosage of one or more of these genes may contribute to perturbed endocytic pathways and synaptic functioning in DS, by altering synaptic vesicle morphology, release probability, or the size of available vesicle pools during synapses. In studies using Drosophila, overexpression of the gene homologous to *ITSN1*, *SYNJ1* and *RCAN1* were found to cause abnormal synaptic morphology and impaired vesicle recycling (Chang & Min, 2009).
1.5 Thesis Aim

DS is the most common genetic form of intellectual disability and leads to early-onset AD in a majority of people with DS. Remarkable progress in medical treatment and social intervention has increased life expectancy of people with DS. However, increased longevity has led to the emergence of new challenges to tackle a greater prevalence of the condition, including greater susceptibility to and incidence of early-onset AD. Studies from mouse models of DS suggest that the observed neurodegeneration may occur from impairments in retrograde axonal transport of essential neurotrophic factors. This impairment has been ascribed to enlarged early endosomes that perturb the endocytyc pathway and hinder transport of the signalling endosome containing the neurotrophin-receptor complex, thus impeding transcriptional signals for neuronal survival. Overexpression of the Hsa21-encoded APP gene is suggested to cause enlarged early endosomes, and is attributed to causing 60% of the deficit in impaired retrograde axonal transport of neurotrophins, leading to neurodegeneration (Salehi et al., 2006). However, the identity of the other Hsa21 gene(s) that contribute to the remaining 40% of the deficit are yet unknown.

This thesis aims to help identify the contribution of Hsa21 genes, other than APP, that lead to impaired axonal transport of neurotrophins in DS. The role of these genes will also be investigated in the enlarged early endosome phenotype. Finally, the contribution of trisomic Hsa21 genes will be assessed in causing perturbed dendritic morphology. This will be achieved by:

• Examining Ts65Dn mice to recapitulate retrograde axonal transport deficits and an enlarged early endosome phenotype
• Investigating retrograde axonal transport and early endosomes in the Ts1Rhr model to assess the genetic contribution of the DSCR, which contains 31 genes
• Assessing the functional consequences of overexpressed Hsa21 genes and axonal transport impairments on dendritic morphology
CHAPTER TWO MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

2.1.1.1 DNA

Agarose (Invitrogen, Cat # 16500500)

Ethidium bromide (Sigma, Cat # E1510)

Ethylenediaminetetraacetic acid (EDTA, 0.5M) (Sigma, Cat # EDS)

HyperLadder I (Bioline, Cat # 33053)

HyperLadder IV (Bioline, Cat # 33056)

MeanGreen 2x Taq DNA polymerase PCR master mix (Syzygy, Cat # MMWD-250)

Tris/Borate/EDTA (TBE) buffer solution (10X) (Invitrogen, Cat # 15581-044)

Tris – HCl buffer (1 M, pH 7.5) (Invitrogen, Cat # 15567-027)

2.1.1.2 Cell culture

Alconox detergent powder (Alconox, Cat # 1104)

B-27 serum-free supplement (50X) (Gibco, Cat # 17504-044)

Deoxyribonuclease I (DNase I) (Roche, Cat # 10104159001)
Ethanol (Sigma, Cat # E7023)

Fetal bovine serum (FBS) (Omega Scientific, Cat # FB-02)

D-\(+\)-Glucose solution (45% in ddH\(_2\)O) (Sigma, Cat # G8769)

GlutaMAX supplement (100X) (Gibco, Cat # 35050-061)

Hank’s balanced salt solution (HBSS, 10X) (Gibco, Cat # 14185-052)

N-2-Hydroxyethylpiperazine-N-2-Ethane Sulfonic Acid (HEPES) buffer solution (1 M) (Gibco, Cat # 15630-080)

Hydrochloric acid (HCl, 35%) (Sigma, Cat # H1758)

Isofluorane (Sigma, Cat # CDS019936)

Minimum Essential Medium (MEM) (Gibco, Cat # 11095-080)

Neurobasal medium (1X) (Gibco, Cat # 21103-049)

Penicillin – Streptomycin (Pen-Strep) antibiotics (Gibco, Cat # 15140-122)

Poly-(L)-Lysine (VWR, Cat # 95036-792)

Trypsin (2.5%, 10X) (Gibco, Cat # 15090-046)

2.1.1.3 Immunofluorescence

Albumin from bovine serum (BSA) (Sigma, Cat # A2153)

Dulbecco’s Phosphate-Buffered Saline (DPBS) (Gibco, Cat # 14190-144)

Goat serum (Sigma, Cat # G6767)
Paraformaldehyde (PFA, 4%) (Wako, Cat # 163-20145)

Triton X100 (0.2%) (Sigma, Cat # 93443)

2.1.2 Prepared solutions

2.1.2.1 DNA

NaOH extraction solution (25 mM NaOH, 0.2 mM EDTA)
187.5 μL 2 M NaOH (25 mM)
6 μL 0.5 M EDTA (0.2 mM, pH 8.0)
14.8 mL ddH₂O

Tris neutralisation buffer (40 mM Tris-HCl, pH 5.5)
600 μL 1 M Tris-HCl (40 mM, pH 7.5)
14.4 mL ddH₂O

2.1.2.2 Cell culture

Dissection medium (1X HBSS, 10 mM HEPES buffer, 1% Pen-Strep)
50 mL 10X HBSS
5 mL 1M HEPES buffer
5 mL 100% Pen-Strep antibiotics
440 mL ddH₂O

Plating medium (MEM, 5% heat inactivated FBS, 1X B-27, 1X GlutaMax, 0.6% D-(+)-Glucose)
190 mL MEM
10 mL 100% heat inactivated FBS
4 mL 50X B-27 supplement
2 mL 100X GlutaMax supplement
2.66 mL D-(+)-Glucose solution

Maintenance medium (Neurobasal medium, 2% B27, 1X GlutaMax)
200 mL Neurobasal medium
4 mL 50X B-27 supplement
2 mL 100X GlutaMax supplement

2.1.3 Antibodies

Alexa Fluor 488 Goat Anti-Mouse IgG (H+L) (Molecular Probes, Cat # A-11001)

Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L) (Molecular Probes, Cat # A-11008)

Rabbit polyclonal IgG anti-EEA1 (Cell Signalling, Cat # 2411)

Mouse monoclonal IgG1 anti-MAP2A, 2B, 2C, clone HM-2 (Chemicon, Cat # MAB364)

Qdot 655 streptavidin conjugate (Molecular Probes, Cat # Q10121MP)

2.1.4 Software

AutoNeuron image analysis software (MBF Biosciences)

FluoView image acquisition and analysis software (Olympus Corp)

GraphPad Prism (version 6.0b, GraphPad Software, Inc.)

ImageJ image analysis software (version 1.45, NIH)

Image Lab image acquisition and analysis software (Bio-Rad)
MetaMorph image acquisition and analysis software (version 7.7.7, Molecular Devices)

Volocity image analysis software (Perkin Elmer)

2.1.5 Equipment

300D digital microcentrifuge (Denville Scientific, Cat # C0265-24)

ChemiDoc XRS+ System with UV-transilluminator (Bio-Rad, Cat # 170-8265)

CL2 Centrifuge (Thermo Scientific, Cat # 004260F)

Direct-hea autoflow CO₂ air-jacketed incubator (Sanyo Gallenkamp, Cat # MCO-17AI)

Labculture class II, type A2 biological safety cabinet (ESCO, Cat # LA2-5A2)

Leica DMI6000B inverted microscope (Leica Microsystems)

Microfluidic chambers (Xona Microfluidics, Cat # SND450)

Olympus FluoView FV1000 laser scanning confocal microscope system (Olympus)

PCR thermal cycler (Applied Biosystems, Cat # AB-2720)
2.2 Experimental Animal Methods

2.2.1 Housing and breeding protocols

All animal studies were carried out under the guidance and approved protocol issued by the University California, San Diego (UCSD) Institutional Animal Care and Use Committee (IACUC), Protocol Number S09315. Experiments were in compliance with PHS Policy, USDA Regulations, and UCSD policies for the care and use of animals, the provisions of the ILAR Guide to Care and Use of Laboratory Animals, and all other federal, state, and local laws and regulations governing the use of animals in research. Mouse lines were maintained using the standard husbandry protocol of UCSD Bonner Hall and Skaggs Laboratories. Mice were housed in a controlled environment in the animal facilities. They were subjected to 12 hours light and 12 hours dark with a dusk/dawn phase, in a room with the temperature set at 21°C (± 2 degrees), with 55% relative humidity (± 10%) and with 15 air changes per hour as a ventilation rate for the animal area.

Ts65Dn breeder pairs (Ts65Dn female and C57BL/6J Eicher x C3H/HeSn] F1 (B6EiC3SnF1) male) were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained as an advanced intercross at Bonner Hall Laboratory. This breeding scheme was used because Ts65Dn mice breed very poorly or not at all when inbred, and the B6C3 background appears to be most successful. This scheme creates as close to a genetically homogenous group of mice as is possible to achieve (Davisson et al., 1993, Reeves et al., 1995).

To generate Ts65Dn\textsuperscript{App++} mice, Ts65Dn female mice were mated with male mice hemizygous for an App knockout, in which App was inactivated by deleting the App promotor and its first exon (Zheng et al., 1996). Male hemizygous App mice were maintained on a C57BL/6J background. The product of litters included 2N mice with one copy of App (2N\textsuperscript{App+}), 2N mice with two copies of App (2N\textsuperscript{App++}), Ts65Dn mice with three copies of App (Ts65Dn\textsuperscript{App+++}), and Ts65Dn mice with two copies of App (Ts65Dn\textsuperscript{App++}).
Ts1Rhr mice were F1 animals from C57BL/6J founder chimeras, in which the modified chromosomes were present on a 129S6/SvEv genetic background (Jackson Laboratories). F1 progeny of the founder chimera were backcrossed to (B6 × C3H)F1 mice to produce Ts1Rhr and euploid mice that inherited 50% of their genetic information from B6, 25% from 129 and 25% from C3H strain (Olson et al., 2004a).

2.2.2 DNA genotyping protocols

2.2.2.1 DNA isolation by sodium hydroxide extraction

Tissue biopsies, in the form of tails from embryos, were processed to isolate DNA using a sodium hydroxide (NaOH) rapid DNA extraction protocol. This quick and ‘dirty’ method of DNA extraction is suitable for amplifying DNA products for a polymerase chain reaction (PCR), as highly purified DNA is not required. The tail biopsy (0.5 cm) was first processed by the addition of 75 µL of 25 mM NaOH extraction solution. This was then incubated on a heat-block at 98°C for 1 hour to allow the NaOH to digest through the tissue. To neutralise the solution and prevent further action of the digestion, 100 µL of Tris neutralisation buffer (40 mM, pH 7.0) was then added and briefly vortexed. The solution was then centrifuged at the maximum speed (13,300 RPM, Denville 300D digital microcentrifuge) to pellet the cell debris. The supernatant was extracted into a sterile eppendorf tube and stored at 4°C for future use.

2.2.2.2 Amplification of DNA by PCR

For genotyping purposes, PCR was used to exponentially amplify a specific nucleotide sequence of interest in DNA samples that were processed by the NaOH DNA isolation method. All PCRs were carried out in a total volume of 20 µL unless otherwise stated. A PCR master mix was prepared consisting of DNA (20 ng/µL – 100 ng/µL), MeanGreen PCR mix (a ready-to-use PCR mix containing Taq DNA polymerase, standard taq buffer, MgCl2, stabilizers, 400 µM each dNTP, blue and yellow loading dyes, and a density agent that allows PCR products to be loaded
directly onto an electrophoresis gel), and a mix of primers containing forward and reverse primers for the region of interest in the target DNA and for the control sequence. When conducting a PCR, three extra reactions were carried out in addition to the reactions necessary for the required number of samples. One reaction was a designated non-template control (NTC) to which 1 μL of double distilled water (ddH2O) was added. The other two reactions served as controls to which 1 μL of a known positive and negative DNA sample was added. After transferring the PCR master mix and DNA to PCR tubes, the tubes were subjected to a thermal cycling program on an Applied Biosystems Thermal Cycler. Generally, the thermal cycling involved template denaturation, primer annealing and extension of the annealed primers by DNA polymerase. However the oligonucleotide primer sets and the thermal cycling conditions varied between the different mouse strains.

2.2.2.3 PCR screening of Ts65Dn and 2N mice

Recently, the breakpoints on Mmu16 and Mmu17 on the translocation chromosome in Ts65Dn mice were identified (Duchon et al., 2011, Reinholdt et al., 2011). Analysis of paired end reads flanking Mmu16 and Mmu17 junction on Mmu1716 and de-novo assembly of the reads directly spanning the junction provided the precise locations of the Mmu16 and Mmu17 breakpoints at 84,351,351 base pairs (bp) and 9,426,822 bp, respectively (Duchon et al., 2011, Reinholdt et al., 2011). This provided an efficient and low cost genotyping method for Ts65Dn. Two primer sets were utilised to identify Ts65Dn positive and euploid control (2N) mice. The first set of primers utilised forward and reverse oligonucleotides that hybridised to opposite strands and flanked the newly discovered breakpoint, an ~275 bp band, in the Ts65Dn mouse (Mmu17Fwd: GTGGCAAGAGACTCAAATTCAAC; Mmu16Rev: TGGCTTATTATTATCGGGCATTT). The other primer set utilised forward (IMR8545Fwd: AAAGTCGCTCTGAGTTGTTAT) and reverse oligonucleotides (IMR8546Rev: GGAGCGGGAGAAATGGATATG) that amplified the Gt(Rosa)26Sor gene encoded on Mmu6, an ~600 bp internal control band present in Ts65Dn and 2N mice. The PCR cocktail used in a total 20 μL reaction with 2 μL DNA template was 10 μL of MeanGreen PCR mix, 4 μL of primer mix (10 pmol reaction, with 10 μL of each primer in 60 μL of ddH2O), 4 μL of ddH2O. The PCR cycling conditions for
the Ts65Dn mouse strain were as follows: a megamix hot start at 95°C for 3 min, followed by 35 cycles of 93°C for 45 sec to denature the DNA, a primer annealing temperature of 55°C for 45 sec and an extension of the annealed primers at 72°C for 1 min. After the last cycle, the reaction mix was incubated at 72°C for 7 min.

2.2.2.4 PCR screening of Ts65Dn x App heterozygous mice

In addition to the Ts65Dn PCR, a PCR screen was conducted on Ts65Dn mice crossed with App heterozygous mice to determine the allele number of knockout or wildtype at the APP locus. The first set of primers utilised forward (APP23Fwd: AGAGCACCGGAGCAAGGCG) and reverse oligonucleotides (APP175Rev: ACCTGCATGTGAACCCAGTATTCTATC) that hybridised to opposite strands and flanked the App allele, a 153 bp band. The other primer set utilised forward (inNeoFwd: GATACTTTTCTCGCCAGGAGC) and reverse oligonucleotides (inNeoRev: GACGCTATTCGGCTATGGG) that amplified an ~300 bp region where the neomycin cassette was inserted at the site of the App deletion, to mark the knockout allele. The PCR cocktail used in a total 20 µL reaction with 2 µL DNA template contained 10 µL of MeanGreen PCR mix, 4 µL of 5 M Betaine, 1.5 µL ddH$_2$O, and 2.5 µL primer mix (20 pmol reaction, with 0.75 µL each of the APP primers and 0.5 µL each of the knockout primers). The PCR cycling conditions for the App allelic variation screen were as follows: a megamix hot start at 94°C for 4 min, followed by 33 cycles of 93°C for 30 sec to denature the DNA, a primer annealing temperature of 62°C for 30 sec and an extension of the annealed primers at 30°C for 1 min. After the last cycle, the reaction mix was incubated at 72°C for 7 min and held at 10°C.

2.2.2.5 PCR screening of Ts1Rhr and 2N mice

Two primer sets were used to identify the duplicated region on Ts1Rhr mice and a internal control band to represent 2N mice. The first set of primers utilised forward and reverse oligonucleotides that hybridised to opposite strands and flanked the recombination between the hygromycin and puromycin antibiotic resistance genes, which were used in the neomycin cassettes to generate the duplicated region in
Ts1Rhr mice, an ~400 bp band (3876Fwd: CCACAAAGAACGGAGCC; 3877Rev: CACCTTTCTCTCCAACCGTC). The internal control forward and reverse primer set amplified the Tcra gene encoded on Mmu14, an ~200 bp internal control band that is present in Ts1Rhr and 2N mice (8744Fwd: CAAATGTTGCTTGTCTGGTG; 8745Rev: GTCAGTGGATGCACAGTTT). The PCR cocktail used in a total 20 µL reaction with 3 µL DNA template contained 10 µL of MeanGreen PCR mix, 4.5 µL of ddH2O, and 2.5 µL primer mix (20 pmol reaction, with 0.75 µL each of primers for the duplicated region and 0.5 µL each of the primers for the control band). The PCR cycling conditions for the Ts1Rhr screen were as follows: a megamix hot start at 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec to denature the DNA, a primer annealing temperature of 66°C for 1 min and an extension of the annealed primers at 72°C for 1 min. After the last cycle, the reaction mix was incubated at 72°C for 2 min and held at 10°C.

2.2.2.6 Gel Electrophoresis

After the amplification reaction, the amplified DNA fragments were separated according to size through electrophoresis on an agarose gel. Out of the total 20 µL of PCR product, 10 µL was loaded onto an UltraPure agarose gel dissolved in 1 x TBE, containing 0.2 µg/ml of ethidium bromide. For resolving Ts65Dn, APP and Ts1Rhr bands, 2%, 1.5% and 1.2% agarose gels were used, respectively. The concentration and size of the amplicons were determined by comparison to a DNA ladder of known concentration and size, which was loaded onto the gel in parallel to the amplicons. HyperLadder I was used for amplicon products ranging in size from 200 bp to 10 kb, and and HyperLadder IV was used for amplicon products ranging in size between 100 bp to 1 kb. Electrophoresis was carried out on the gel containing the amplicons and DNA ladder at a current of 100 volts for 60 min. The amplified products were visualised on a ChemiDoc XRS+ System with an UV-transilluminator.
2.3 Cell Culture Preparation Protocols

2.3.1 Preparation of coverslips and chambers

2.3.1.1 Sterilising coverslips

For immunofluorescence staining purposes, 12-well cell culture plates with round 18 mm diameter (VWR, Cat # 1014-18, Number 1 thickness) coverslips were prepared. For microfluidic chambers (Xona Microfluidics, Cat # SND450), 24 mm x 30 mm coverslips (Corning, Cat # 2975-243, Number 1 thickness) were prepared.

Sterilising coverslips was normally undertaken up to three weeks before dissection. Under a fume hood, 35% hydrochloric acid (HCl) was added to a glass container that had a lid. Using tweezers, coverslips were gently added into the HCl and spread around the bottom of the dish using a glass rod stirrer. This was done to remove any air bubbles and ensure the surfaces of all coverslips were exposed.

Coverslips were left in HCl overnight on a rocking tray. The next day, the HCl was removed and coverslips were washed with ddH2O by placing on the rocking tray for 30 min. This was repeated a total of three times. After the final wash, the ddH2O was removed and 100% EtOH was added to the glass chamber in preparation for flame-sterilisation. Curved forceps were used to pick up a single coverslip, which was subsequently passed through a flame. After the flame quenched the EtOH on the coverslip, and the resulting sterilised coverslip was placed in a cell culture dish with a cover to keep it sterile. Care was taken not to keep the coverslips in the flame for too long, as this would create a crack in the coverslip. After all coverslips had been flame-sterilised, the cell culture dish was sealed with parafilm and stored in a clean environment for later use.

2.3.1.2 Coating of coverslips

Three days to 24 hours prior to dissection, 12-well culture plates were prepared. Coverslips were coated with Poly(L)Lysine (PLL), which serves as an attachment factor that improves cell adherence. Proper coating of the coverslips is crucial, as otherwise it will cause neurons to clump. In a laminar flow hood, one single
coverslip was placed in every well of the 12-well cell culture plate. The coverslips were submerged in PLL by adding 500 µL of PLL to every well, and incubated in PLL for 2-4 hours. The PLL was aspirated off, and the coverslips were rinsed in sterile ddH₂O three times. After the final rinse and aspiration, the coverslips were allowed to air dry in the tissue culture hood. The plates were subsequently sealed with parafilm and stored in a clean environment for later use.

2.3.1.3 Preparation of microfluidic chambers

Two days to 24 hours prior to dissection, the microfluidic chambers were prepared. They were first washed in 10% Alconox detergent solution, and each side of the chamber was gently massaged to remove contaminants. The microfluidic chambers were then thoroughly rinsed in ddH₂O several times until the detergent had completely been washed away. Following this, and immediately before UV exposure, the chambers were immersed in 70% EtOH for up to 10 mins.

Microfluidic chambers and their coverslips were exposed to UV radiation to further sterilise the devices before coating. Using curved forceps, in a laminar flow hood, individual 24 x 30 mm coverslips were placed in a 60 mm tissue culture dish. Washed microfluidic chambers were also laid out in a 15 cm tissue culture dish, and gently pat dry of EtOH. The hood window was closed and coverslips and chambers were exposed to UV radiation for 20 min. Using curved forceps, the microfluidic chambers were flipped around so that the bottom side was exposed, and another round of UV radiation for 20 min was carried out. The dish containing the microfluidic chambers was covered and kept sterile in the hood.

The coverslips were now ready to be coated with PLL, as previously described (Osakada & Cui, 2011, Taylor et al., 2005, Zhang et al., 2011). The coverslips were submerged in PLL by gently adding 600 µL of PLL to every coverslip in every dish, being careful not to spill into the dish. As with the circular coverslips, the rectangular coverslips were incubated in PLL for 2-4 hours. The coverslip was gently lifted with curved forceps, and the PLL was aspirated. The coverslip was then immersed in a 10 cm dish with sterile ddH₂O, and subsequently transferred, using
forceps, to two further dishes filled with sterile ddH₂O. This allowed the coverslip to be rinsed three times in sterile ddH₂O. Care was taken to remember the surface that had been coated. After the final wash, the ddH₂O was aspirated off both sides of the coverslip so that it was completely dry and placed back into its dry 60 mm dish, coated-side up.

Immediately after this, the microfluidic chamber and the coverslip were assembled together in the laminar flow hood. Using curved forceps, the microfluidic chamber was carefully placed, microgroove side down, in the centre of the PLL-coated coverslip. Care was taken to make sure the chamber was securely within the parameters of the coverslip so that wells would not have the opportunity to leak. The chamber was pressed down firmly on the coverslip but carefully as not to break the fragile glass. The curved part of the forceps was used to press down on the device. Careful attention was given to press down on key points of the chamber such as the areas around the main channels, microgrooves and wells, as this is where proper bonding is most needed. The 60 mm dish was then sealed with parafilm and stored in a clean environment for later use.

2.3.2 Isolation of E18 hippocampal neurons

2.3.2.1 Embryo dissection

When a copulation plug is observed, the age of the embryo is determined as embryonic day 0.5 (E0.5). On E18, pregnant female mice were anaesthetised in a container with 250 µL of isoflurane. Approximately 15 sec after movement ceased, the mouse was removed from the container and anaesthesia was confirmed by lack of withdrawal reflex from a toe pinch. The female mouse was sacrificed by cervical dislocation. After soaking the abdomen with 70% EtOH, an incision through the skin was made and sterile blunt forceps and dissection scissors were used to remove the abdominal wall. The left and right uterine horns containing all embryos were dissected out and briefly rinsed in 70% EtOH before immersing in sterile dissection medium on ice. In the dissection hood, using fine watchmakers forceps, each embryo was separated from its placenta and amniotic sac, and placed in a 10 cm culture dish containing dissection medium on ice.
2.3.2.2 Tissue preparation

For every embryo, a 0.5 cm tail snip was made and stored as a biopsy for genotyping purposes. The head of each embryo was removed by cutting at the neck and right above the neural crest. Curved watchmaker forceps were inserted in the olfactory bulb at the front of the brain to keep the head in place. Fine watchmaker forceps were used to remove the skin and skull and expose the brain. The brain was gently scooped out of the cranial cavity and placed in another 10 cm tissue culture dish. Using fine forceps, meninges were removed from the brain tissue, and the hippocampi were rapidly dissected from the brain. The brain was oriented dorsal side up so that the clear midline of two hemispheres was visible. Using a scalpel a sagittal incision was made down the dorsal midline to sever the cerebral commissure and separate the two hemispheres. With a gentle grasp on the midline, one hemisphere was cautiously peeled back using both tips of other forceps, being careful not to damage the hippocampus. An incision was made to sever the hippocampal/septal junction. This lesion was used to grab and further remove the meninges along the curve of the cortex. Cuts between the hippocampus and cortex were made, and the hippocampus was gently scooped out and transferred to a 1.5 mL eppendorf tube with dissection medium on ice. The same steps were conducted on the contralateral hippocampus, and for every embryo.

2.3.2.3 Isolation of cells

All of these steps were conducted in a laminar flow sterile hood. As the genotype of each embryo still needed determining, hippocampi per embryo were treated individually. The hippocampi were rinsed with dissection medium by centrifugation for 1.30 min at a low speed of 1,200 RPM (Thermo Scientific, CL2 centrifuge). The supernatant was removed and hippocampi were resuspended in 400 µL of fresh dissection medium. This was repeated three times. To break up the tissue, 50 µL of 10X Trypsin (2.5%) was added to the dissection media to obtain a final concentration of 0.25%, and incubated in a 37°C water bath for 15 min. After this, 50 µL of 10x DNAse was added to make the solution less viscous and further break up the tissue. To filter out the single cells, a sterilised 9-inch fire-polished
glass Pasteur pipette was used to triturate the solution approximately ten times in about 30 sec. Trituration involves sucking the tissue up into the pipette, without air bubbles, and immediately emptying contents back into the tube, without air bubbles. This is a critical step as triturating too gently will not disrupt the tissue, but triturating vigorously will break the cells. The solution was then subjected to centrifugation at 1,000 RPM (Thermo Scientific, CL2 centrifuge) for 3 min, and the supernatant was carefully aspirated, leaving the cells at the bottom of the tube to be gently resuspended in plating medium. Care was taken to avoid resuspending any blood vessel pellets below the cells.

2.3.2.4 Counting, plating and maintenance of cells

To count cells, 10 µL of cell suspension was aliquoted into a small eppendorf tube containing 10 µL of trypan blue. This was mixed and 10 µL was applied to each side of a hemacytometer (Cascade Biologics). The device was observed under a 10x objective lens with a phase contrast microscope. The number of phase bright spherical cells in the large centre square on both sides of the hemacytometer was counted and added. This sum times $10^4$ is the cell concentration (cells per mL).

For seeding coverslips in 12-well cell culture plates, the desired concentration is about 320 cells per mm² in 50-150 µL of plating media. Based on this density, 100 µL of 37°C plating medium per coverslip was used to resuspend the cells. For each coverslip, 100 µL of the suspension was placed in the centre of the coverslip. The plate was then placed in a humidified 37°C, 5% CO₂ incubator for 1-2 hours. After the cells had adhered and attached to the surface, 400 µL of 37°C plating medium was gently added to each well, to prevent the neurons from over-drying, and returned back to the incubator. Neurons were cultured in plating medium for 2 days and then replaced with 37°C maintenance medium to select out fibroblasts. Neuronal cultures were fed with new maintenance medium every other day by gently aspirating 1/3 of the volume and replacing with fresh maintenance medium.

For seeding in microfluidic chambers, the pelleted cells were resuspended in 40 µL of 37°C plating medium. Out of this, 20 µL was loaded in the top left well of the
chamber. Using suction force from an aspirator placed in the top right well of the chamber, cells were carefully pulled through to ensure even distribution throughout the main channel, that is the width of the cell-body compartment. This was done carefully so as not to aspirate up the cell suspension. The chamber was then placed in a humidified 37°C, 5% CO₂ incubator for 10 min to allow the cells to adhere and attach to the surface of the coverslip. After cells had attached, approximately 200 µL of 37°C plating medium was gently added to fill each well in the device, and the device was placed back into the incubator. Similar to the feeding method used for coverslips, neurons in microfluidic chambers were cultured in plating medium for 2 days and then replaced with 37°C maintenance medium to select out fibroblasts. To feed the cultures, fresh maintenance medium was added every other day by gently aspirating 1/3 of the volume in each well, and then replacing with fresh maintenance medium.

### 2.4 Cell Culture Imaging and Acquisition Protocols

#### 2.4.1 Immunofluorescence of fixed cells

##### 2.4.1.1 Immunofluorescence of early endosomes and dendrites

The 12-well tissue culture plates had been prepared for immunofluorescence studies to examine early endosomes and dendritic morphology. The wells that contained the coverslips of interest were identified and treated on day in vitro 7 (DIV7), which is enough time for the neurons to have grown processes and become mature. All procedures were conducted at room temperature unless otherwise stated. The maintenance media was aspirated off from the wells and the coverslips were rinsed gently twice in PBS to completely remove all maintenance medium and all nonadherent cells. The cells were then fixed in 4% paraformaldehyde (PFA) for 15 min. This fixation procedure immobilises antigens whilst retaining cellular and subcellular structure, allowing antibodies to access all cells and their subcellular compartments. To rupture the cell membrane and allow the antibody access to the inside of the cell to detect the protein, neurons were permeabilised in 0.2% triton
X100 (diluted in PBS) for 10 min. Subsequently, the coverslips were rinsed twice with PBS to clear all fixation and permeabilising detergents, and then blocked in a solution containing 3% BSA and 5% goat serum (as this is the species the secondary antibody was created in), diluted in PBS for 1 hour. The blocking solution was aspirated and the coverslips were incubated with 40 μL of primary antibody overnight at 4°C. To mark early endosomes, rabbit polyclonal anti-EEA1 (Cell Signalling) was diluted 1:200 in blocking solution. To stain for dendrites, a mouse monoclonal anti-MAP2 (Chemicon) was diluted 1:100 in blocking solution. The next day, the coverslips were subjected to three 5 min PBS washes followed by a 1 hour incubation in 40 μL of fluorescent secondary antibody Alexa Fluor 488 goat anti rabbit and Alexa Fluor 488 goat anti mouse (Molecular Probes), which was diluted 1:250 in 1% BSA, for EEA1 and MAP2 primary markers, respectively. The coverslips were then subjected to another three 5 min PBS washes, with the final wash lasting for 30-60 min to ensure a clean signal with minimum background fluorescence. The coverslips were then given a final rinse in ddH₂O and then mounted on a glass microscope slide with mounting media (made in lab, courtesy of Dr Chengbiao Wu), and sealed with clear nail polish. After coverslips had been secured on and dry, they were transferred to 4°C to store until further use.

2.4.1.2 Confocal microscope image acquisition

Confocal fluorescent images for early endosomes and dendritic morphology were acquired using an Olympus FluoView FV100 confocal microscope system (Olympus Corporation), equipped with a Photometrics CCD mounted on a Nikon TE-200 inverted epi-fluorescence microscope. The fundamental advantage of the confocal versus a traditional widefield microscope is the restricted manner in which the emitted light reaches the photomultiplier through a pinhole. The laser scanning confocal microscope gathers the fluorescence emission from the specimen, which is filtered through a confocal pinhole aperture to reject light that originates from regions that are not in the focal plane. Once the emission light passes through the pinhole, it is detected by a photomultiplier, which forms the image in a serial manner.
Fluorescence for early endosome marker EEA1 and for dendritic marker MAP2 was recorded at 488 nm, with the filter bandpass set at 505-530 nm, and the pinhole set at the optimal level and at a set slice position and scaling. Images for early endosomes were acquired using the 100x objective. Z-stack images of dendrites were captured using the 20x objective in 10 optical sections that were spaced by 0.5 mm. Images were acquired at random from several fields on the confocal microscope visual plane. Data sets were acquired and accumulated using the FluoroView software for Olympus.

2.4.2 Live-cell imaging of QD-BDNF endosomes

2.4.2.1 Treatment of neurons with QD655 - mBtBDNF

After plating cells in the cell body compartment in microfluidic chambers, axons started to grow into the microchannels approximately DIV3 and extended through the microchannels into the axonal compartment by DIV7. Experiments were conducted on DIV7 or DIV8, after the axons had sprouted out into the axonal compartment. On the day of the experiment, all compartments (cell body and axonal) were depleted of maintenance medium, and the culture was starved for two hours in serum-free Neurobasal medium in the incubator. Every half hour, the compartments were replenished with fresh serum-free Neurobasal medium.

Mono-biotinylated (mBt) BDNF is routinely produced within the lab, and with the courtesy of Dr Chengbiao Wu, QD-BDNF conjugates were prepared. Streptavidin conjugated QD655 (Molecular Probes) was mixed with mBtBDNF at a molar ratio of 1:1.2 in Neurobasal medium, and incubated on ice for 30 min. For this, 1 µL of QD655 was mixed with 5 µL of mBtBDNF in an eppendorf tube containing 14 µL of serum-free Neurobasal medium. After the QD and BDNF had conjugated, the mixture was added to 4 mL of serum-free Neurobasal medium to make a final concentration of 1 nM QD-BDNF.

Serum-free Neurobasal medium was removed from all compartments in the microfluidic chambers. The axonal compartments were treated with 1 nM QD-BDNF
in serum-free Neurobasal medium. Care was taken not to fill to the top of the well. The cell body compartment was then filled to the top with serum-free Neurobasal medium. The larger volume in the cell body compartment creates hydrostatic pressure between the compartments thereby fluidically isolating each compartment and preventing QD-BDNF from diffusing into the cell body compartment through the microchannels (Taylor et al., 2005). The QD-BDNF was allowed to feed the neurons and be internalised in the axonal compartment for 2 hours in an incubator. Immediately prior to imaging, the QD-BDNF was washed out by removing all media and rinsing all wells with serum-free Neurobasal medium three times. Fresh serum-free Neurobasal medium was added to the wells in preparation for live-cell imaging.

2.4.2.2 Fluorescence live-cell imaging of QD-BDNF

Live-cell imaging of QD-BDNF within the proximal axons was carried out using a Leica DMI6000B inverted microscope (Leica Microsystems), equipped with a CCD camera (Rolera-MGi Fast 1397 from Qimaging) and an environmental chamber that maintained a constant temperature (37°C) and CO2 (5%) during live imaging (Warner Instrument). A set of Texas red excitation/emission filter cubes was used to collect fluorescent QD signals through a 655 (±20 nm) bandpass filter, as previously described (Cui et al., 2009; Zhang et al., 2011). Corresponding DIC images were also captured. Time-lapse images were acquired at the speed of 1 frame/sec over the duration of 120 sec. All data was acquired using the MetaMorph (version 7.7.7, Molecular Devices) software for Leica.

2.5 Data and Statistical Analysis

2.5.1 Early endosome morphology using Volocity

To assess early endosome morphology, image processing and quantitation was carried out using Volocity image analysis software (Perkin Elmer), a powerful tool enabling precise measurements of cellular structures through the generation of
specific analysis protocols that can be applied to different samples. This software allowed a protocol to be created to identify the total number of endosomes per neurons as well as the area of each endosome. A protocol was created to identify the endosomes based on their percentage intensity. The lower limit was set at 25% intensity, and the upper limit was set at maximum. The protocol then excluded any objects smaller that 0.01 µm² as these were determined to be background noise. Following this, the protocol excluded any objects larger than 12 µm² as these were determined to artefacts. In order to determine whether touching objects were discrete endosomes or enlarged endosomes, the protocol separated touching objects based on the average size of 0.5 µm² for an endosome. The protocol was executed and Volocity identified all endosomes that fell within the parameters. The measurement output provided numerical details of the number and area of each endosome.

2.5.1.2 Morphological and statistical analysis

For morphological analysis, the total number of EEA1-positive early endosomes and average area were analysed. For statistical comparisons of endosome distribution, based on endosome number relative to size, each endosome was assigned to one of three groups: small, medium or large. This is commonly practised to ensure accurate group comparisons (Cataldo et al., 2008, Jiang et al., 2010). The ‘small’ endosome category included endosomes that were 0.01 – 0.5 µm² in size. This category included all endosomes from the smallest up to the average endosome size. The ‘medium’ category was defined as containing a distribution of endosomes from 0.51 – 1 µm² in size. Finally, the ‘large’ category contained any endosomes that were larger than 1 µm².

All statistical analyses were conducted on GraphPad Prism (version 6.0b, GraphPad Software, Inc.). To determine the Gaussian distribution of the datasets, a D’Agostino & Pearson omnibus normality test was conducted on every data set, and revealed normal Gaussian distribution. Subsequent two-tailed, unpaired Student’s t-tests were conducted on all data sets to compare trisomic models to their respective 2N controls.
2.5.2 Dendritic morphology using AutoNeuron

To analyse dendritic morphology, image processing and quantitation was carried out using AutoNeuron, which is an extension to the Neurolucida software, an automated interactive tracing tool to measure three-dimensional (3D) volume stacks (MBF Biosciences). It identifies cell bodies and neuronal processes, outlines the cell body volume at each image plane, traces the processes and connects them to form a reconstructed set of trees. After identifying cell bodies of neurons based on defined parameters, the dendritic processes were traced and reconstructed for further quantitative analysis using NeuroExplorer. The following steps were taken to process and quantify the images. The relevant image was opened in Neurolucida and viewed with maximum intensity projection, to ensure all Z-stack slices merged. To identify and trace the cell bodies, AutoNeuron was configured to detect cell bodies above the size of 5 \( \mu m^2 \), with a specified sensitivity of 50 units. Following this, to identify dendritic processes, seed placement was defined with a specified sensitivity of 70 units. After the placement of the seeds had been validated so that the dendritic processes from cell bodies were being followed, the neuron was traced and reconstructed in a new template.

2.5.2.1 Morphological and statistical analysis

For morphological analysis the reconstructed template with traced dendritic processes was opened in NeuroExplorer. A branched structure analysis was conducted to quantify dendritic branching, length area and volume of each dendrite. All statistical analyses were conducted on GraphPad Prism. To determine the Gaussian distribution of the datasets, D’Agostino & Pearson omnibus normality tests were conducted to reveal normal Gaussian distribution. Subsequent two-tailed, unpaired Student’s t-tests were conducted on all data sets to compare trisomic models to their respective 2N controls.
2.5.3 Axonal transport using ImageJ and MetaMorph

To analyse axonal transport and track single molecules in time-lapsed image sequences, image processing was first carried out using MetaMorph software. Every frame captured during live-cell imaging of QD-BDNF in individual axons was collated to create a movie. This movie was initially processed in ImageJ image analysis software (version 1.45, NIH), which dissected the movie into 120 image stacks. Despite extensive efforts to capture movies under exactly the same conditions, the contrast characteristics of original image stacks showed considerable variation, which could be challenging when attempting to detect particle movement. To standardise all image stacks, inter-movie differences in contrast were minimised by applying an automated contrast normalisation to all images in each stack using the ImageJ “Enhance Contrast” function with saturated pixels set at 0.4% (Andrews et al., 2010). This also has the effect of reducing the influence of any photobleaching within each stack. QDs have an intrinsic characteristic of flickering making it possible to identify it as a single QD as opposed to any other background or auto-fluorescence. To remove any stationary objects that did not flicker and further improve signal-to-noise ratio, an average subtraction was calculated on ImageJ. This was done by identifying the average intensity in all the stacks, using the ‘Average Intensity” function under the Z stack projection tab on ImageJ. The “Calculator Plus” plugin was downloaded from the ImageJ website, and the subtraction function was used to subtract the average intensity image from the original image, creating a new series of stacks with all stationary objects removed.

The processed stacks, now devoid of any contrast variations and any stationary objects, were reopened in MetaMorph. To accurately track moving objects involves the use of a spatiotemporal image that detects a temporal trace of particles in a composite \((x,y,t)\) map. As such, kymographs are generated from all time frames along the line of the axon to form a graphical spatiotemporal representation. Constructing the kymograph image involves two steps. The first step involves identifying the outline of the axon on which the kymograph will be computed. The second step automatically assembles the trajectory of the QDs within the identified axon lines in all image stacks onto the kymograph (Zhang et al., 2011).
2.5.3.1 Kymograph and statistical analysis

The advantage of kymographs is the data reduction and graphical representation of movement in a single image. Using the line trace button on MetaMorph, the path of every trajectory was traced. This provided quantitative numerical values for the directionality of movement, distance travelled, as well as being able to identify pauses within the path, for every trajectory. The slopes of the trajectories correspond to their velocity, and dependent on orientation of the axon, it was possible to identify retrograde and anterograde transport directionality. Stationary and micropauased QDs were identified as a single vertical line demonstrating no movement over the time-lapse. All statistical analyses were conducted on GraphPad Prism. To determine the Gaussian distribution of the datasets, D’Agostino & Pearson omnibus normality tests were conducted to reveal normal Gaussian distribution. Subsequent two-tailed, unpaired Student’s t-tests were conducted on all data sets to compare trisomic models to their respective 2N controls.
CHAPTER THREE  AXONAL TRANSPORT IN MOUSE MODELS OF DS

3.1  Introduction

3.1.1  Methods to study axonal transport

Advancements in technology and microscopy have dramatically improved the methods to label and track neurotrophins along their trajectory from the axon to the cell body. The development of radiolabelled $^{125}$I-NGF was crucial for the discovery of NGF retrograde axonal transport and its functional interactions with its receptor (Angeletti et al., 1972). When injected through target organs, such as the iris or salivary gland, $^{125}$I-NGF is retrogradely transported and accumulates in the corresponding neuronal cell bodies (Hendry et al., 1974, Stoeckel et al., 1975). The benefits of radiolabelled neurotrophins include easy quantification of signal and minimal disturbance to neurotrophin function. However, despite its high sensitivity, large amounts of protein are required for reliable detection. Radiation detection also has poor spatial and temporal resolution, which restricts its usage for tracking neurotrophins in live cells (Chowdary et al., 2012).

Advances in fluorescence live-cell imaging and the emergence of green fluorescence protein (GFP) made it possible to track neurotrophin transport in live cells. However, GFP is limited by low photostability and has the tendency to rapidly photobleach samples (Hibino et al., 2009). Additionally, a single GFP molecule is difficult to detect in live cells and therefore not used for tracking neurotrophins, as most endosomes are suggested to contain only a single neurotrophin dimer (Cui et al., 2007). Another widely used fluorescent label is the organic cyanine dye Cy3 (Tani et al., 2005). Unfortunately organic labels suffer similar limitations to fluorescent proteins; their insufficient brightness renders it challenging to be used for long-distance neurotrophin trafficking (Chowdary et al., 2012).
The recent emergence of quantum dot (QD)-labelled neurotrophins has transformed our ability to track neurotrophins over long-distances for a sustained period of time. QDs are inorganic, fluorescent probes that have excellent brightness and are extremely photostable (Medintz et al., 2005). Although they are large in size (15-20 nm in diameter), when combined with ultrasensitive optical techniques, QDs facilitate the tracking of individual biomolecules with a high signal-to-noise ratio. Furthermore, QDs contain an electron-dense core that can be useful to identify organelles that contain QD-ligands under electron microscopy (Cui et al., 2007). Neurotrophins can be modified with a biotin moiety and then conjugated to QD through a biotin-streptavidin interaction (Cui et al., 2007, Sung et al., 2011). QD-NGF and QD-BDNF are biologically functional with evidence of receptor binding stimulating neurite outgrowth in PC12 cells, activating Erk and Akt signalling pathways, and maintained retrograde transport of neurotrophin-receptor complexes (Altick et al., 2009, Cui et al., 2007, Vu et al., 2005).

3.1.1.1 Microfluidic chambers and real-time imaging

To mimic the spatial separation of cell bodies and axonal terminals and their different chemical environments in live animals, compartmentalised culture chambers have been designed to create a spatial and chemical separation for cultured sympathetic neurons. This original concept of Campenot chambers (Campenot, 1977, Campenot, 1982, Campenot et al., 2009) was further developed to generate microfluidic chambers (Park et al., 2006, Taylor et al., 2005, Taylor et al., 2003). Microfluidic chambers constitute two or more compartments that are interconnected by microchannels, which allow axonal growth and prevent fluids from intermixing in different compartments (Taylor et al., 2003). The compartmentalised culture enables precise hydrostatic control over small amounts of fluids and variables in neurotrophin axonal transport studies. Additionally they allow for experimental manipulations in various compartments including, drug application and genetic manipulation. Microfluidic chambers are commonly made of polydimethylsiloxane (PDMS) and are optically transparent as well as tolerable to experimental handling, therefore are perfect for live-cell imaging (Park et al., 2006, Taylor et al., 2003).
Single-molecule imaging requires a high signal-to-noise ratio and a reduction of background fluorescence that is generated from diffusive fluorophores in solution, non-specific binding, and autofluorescent molecules in the cell body (Chowdary et al., 2012). Microfluidic chambers are the optimal platform for single-molecule imaging of neurotrophin axonal transport. QD-neurotrophins can be applied to the distal axon compartment and imaged exclusively in the microchannels, which results in a spatial confinement of free QDs only to the distal axon compartment, thereby significantly reducing background fluorescence (Zhang et al., 2010). Total-internal-reflection fluorescent (TIRF) microscopy uses critical-angle illumination to excite fluorophores close to the surface to reduce background fluorescence, but is limited by restricted penetration depth (approximately 150nm). In pseudo-TIRF microscopy, the incidence angle is adjusted to smaller than the critical angle enabling the laser beam to penetrate deeper into the aqueous solution; this facilitates reduced background fluorescence and visualisation of QD-neurotrophins in axons that are located nanometers to a micrometer away (Chowdary et al., 2012, Cui et al., 2007, Zhang et al., 2010).

3.2 Chapter Aim

An abnormal increase in APP gene-dosage has been suggested to disrupt retrograde axonal transport of radiolabelled-NGF, and cause BFCN neurodegeneration in the Ts65Dn mouse model of DS (Salehi et al., 2006). This chapter aims to assess the contribution of other Hsa21 genes in causing the impaired neurotrophin retrograde axonal transport phenotype. The hippocampus is a primary structure affected by DS and AD (Braak & Braak, 1991, Mann, 1988, Wisniewski et al., 1985). BDNF and its TrkB receptor are highly expressed in the hippocampus and heavily implicated in hippocampal-dependent learning and memory processes (Reichardt, 2006, Tapia-Arancibia et al., 2008, Yamada & Nabeshima, 2003). To recapitulate the retrograde axonal transport phenotype, Ts65Dn hippocampal axon terminals were treated with QD-BDNF in microfluidic chambers and imaged using live-cell microscopy. To assess the contribution of Hsa21-encoded genes, other than APP, the Ts65DnApp++- and Ts1Rhr models were also investigated.
3.3 Results

3.3.1 Axonal transport in Ts65Dn mice

3.3.1.1 Axonal transport analysis

To conduct live-cell imaging of QD-BDNF endosomes in microfluidic chambers, DIV7 neurons were first starved of growth factors in serum-free media for two hours and then fed with QD-BDNF in the distal axon (DA) compartment of microfluidic chambers for two hours, as detailed in Chapter 2.4.2. QD-BDNF was internalised by axon terminals in the DA compartment, and subsequently transported along the microchannels towards the cell body (CB) (Figure 3.1).

Figure 3.1: Microfluidic chambers for single-molecule live-cell imaging. (A) Microfluidic chambers (Xona Microfluidics) are made from polydimethysiloxane (PDMS) and are approximately 22 mm in length and 23 mm in width. They constitute of cell body (CB) and distal axon (DA) compartments. (B) The CB and DA are interconnected by microchannels that can be 450µm in length. Neurons were immunostained with a marker for dendrites, MAP2 (top panel), and Tau, a marker for axons (middle panel). The bottom panel shows the merge of both markers, demonstrating the separation of the CB and DA compartments. (C) Schematic depicts a top view of chambers after QD-labelled neurotrophins (red puncta) are incubated in the DA for retrograde transport to the CB. (Figures adapted from Taylor et al., 2005 and Zhang et al., 2010).
QD-BDNF trafficking along the microchannels was visualised using a Leica DMI6000B inverted fluorescent microscope, equipped with an environmental chamber that maintained a constant temperature (37°C) and CO2 (5%) during live imaging (Leica Microsystems). Image stacks were initially processed using NIH ImageJ software. Kymographs were subsequently generated on MetaMorph (Molecular Devices LLC) to follow the transport trajectory of QD-BDNF containing endosomes in Ts65Dn (272 endosomes) and 2N (206 endosomes) hippocampal neurons, which were observed in approximately 30 different axons over three independent experiments for each genotype (Figure 3.2).

![Figure 3.2](image)

**Figure 3.2: Kymographs depicting Ts65Dn QD-BDNF transport trajectory.** Kymographs were generated on MetaMorph software to follow the transport trajectory of QD-BDNF endosomes in (A) 2N and (B) Ts65Dn hippocampal neurons. Kymographs were traced for quantitative analyses to assess several parameters including direction of movement (retrograde, anterograde, stationary), velocity, distance and the number and duration of micropauses. Coloured lines represent trajectory for one single QD-BDNF endosome.

### 3.3.1.2 Velocity, distance and pausing of QD-BDNF endosomes

Kymographs were used to assess several parameters including, average velocity, average distance travelled, time travelled in a retrograde and anterograde direction, time spent stationary, the number of pauses made throughout the movement trajectory (micropauses), and the duration of these micropauses. A two-tailed unpaired Students t-test revealed a slower average velocity of QD-BDNF in Ts65Dn hippocampal neurons (0.16 μm/sec ± 0.01) compared to 2N (0.32 μm/sec
was apparent in both the retrograde ($t=6.32$, $df=476$, $p<0.0001$) and anterograde direction ($t=7.55$, $df=476$, $p<0.0001$). QD-BDNF endosomes in Ts65Dn neurons travelled at an average velocity of $0.19 \mu$m/sec ($\pm 0.01$) in the retrograde direction and $0.16 \mu$m/sec ($\pm 0.01$) in the anterograde direction. In 2N neurons, QD-BDNF endosomes travelled retrogradely at an average velocity of $0.34 \mu$m/sec ($\pm 0.02$) and anterogradely at an average velocity of $0.35 \mu$m/sec ($\pm 0.02$). QD-BDNF endosomes travelled a reduced distance in Ts65Dn neurons (13.33 $\mu$m $\pm$ 0.99) relative to 2N (25.58 $\mu$m $\pm$ 1.37; $t=7.45$, $df=476$, $p<0.0001$).

When examining overall direction of axonal transport (Figure 3.4), QD-BDNF endosomes in Ts65Dn neurons travelled in the retrograde direction for a reduced amount of time ($24.26\% \pm 2.60$) compared to 2N ($42.72\% \pm 3.46$; $t=4.35$, $df=476$, $p<0.0001$). Anterograde axonal transport was also impaired in Ts65Dn neurons ($t=3.24$, $df=476$, $p<0.001$), with QD-BDNF endosomes travelling in the anterograde direction $29.04\%$ ($\pm 2.76$) of the time, compared to 2N that travelled in this direction $43.20\%$ ($\pm 3.46$) of the time. In Ts65Dn neurons, QD-BDNF endosomes spent a greater amount of time stationary ($46.32\% \pm 3.03$) compared to 2N ($14.08\% \pm 2.43$; $t=7.92$, $df=476$, $p<0.0001$).

To further understand reduced velocity of Ts65Dn QD-BDNF endosomes, the number and duration of micropauses throughout the trajectory was examined (Figure 3.5). QD-BDNF endosomes in Ts65Dn neurons did not differ from 2N in the number of micropauses made throughout movement ($t=1.03$, $df=476$, $p=0.30$), with Ts65Dn QD-BNDF endosomes making an average of $1.06 \pm 0.02$ micropauses, and 2N making an average of 1.10 ($\pm 0.05$) micropauses. However, the duration of micropauses did differ significantly between the genotypes ($t=3.07$, $df=476$, $p<0.05$). Whilst trafficking along the axon, Ts65Dn QD-BDNF endosomes micropaused two-fold times longer (13.40% $\pm$ 1.93), whereas QD-BDNF endosomes in 2N only micropaused for 6.46% ($\pm 1.30$) of the time travelled.
Figure 3.3: Average velocity and distance travelled by Ts65Dn QD-BDNF endosomes relative to 2N. (A) Ts65Dn QD-BDNF endosomes in hippocampal neurons display significantly slower movement as demonstrated by approximately two-fold slower total, retrograde and anterograde average velocities compared to 2N. (B) QD-BDNF endosomes in Ts65Dn mice travel half the distance relative to 2N QD-BDNF endosomes (***p<0.0001).
Figure 3.4: Direction travelled by Ts65Dn QD-BDNF endosomes relative to 2N. QD-BDNF endosomes in Ts65Dn hippocampal neurons spent less time trafficking in the retrograde and anterograde direction, and spent a longer time stationary compared to 2N QD-BDNF endosomes (**p<0.0001).

Figure 3.5: Number and duration of micropauses displayed by Ts65Dn QD-BDNF endosomes relative to 2N. (A) QD-BDNF endosomes in Ts65Dn hippocampal neurons did not differ to 2N in number of micropauses made while moving. (B) However they did micropause for a greater duration of time (*p<0.05).
3.3.2 Axonal transport in Ts1Rhr mice

3.3.1.1 Axonal transport analysis

Axonal transport in Ts1Rhr mice was examined using the same procedure outlined above for Ts65Dn mice. Kymographs were generated to follow the trajectory of QD-BDNF endosomes in Ts1Rhr (281 endosomes) and 2N (245 endosomes) hippocampal neurons from over 30 axons across three independent experiments for each genotype (Figure 3.6).

![Figure 3.6: Kymographs depicting Ts1Rhr QD-BDNF transport trajectory.](image)

**Figure 3.6: Kymographs depicting Ts1Rhr QD-BDNF transport trajectory.** Kymographs were generated to individually track and trace the trajectory of QD-BDNF endosomes in (A) 2N and (B) Ts1Rhr hippocampal neurons. Kymographs allowed quantitative analyses of transport parameters including direction of movement (retrograde, anterograde, stationary), velocity, distance travelled and the number and duration of micropauses. Each coloured line represents the trajectory of a single QD-BDNF endosome.

3.3.1.2 Velocity, distance and pausing of QD-BDNF endosomes

To investigate the genetic contribution of the 31 genes orthologous to the Hsa21 DSCR, axonal transport in the Ts1Rhr mouse model was examined. Like Ts65Dn mice, Ts1Rhr mice demonstrated a significantly reduced velocity compared to 2N ($t=5.54$, $df=524$, $p<0.0001$). QD-BDNF endosomes in Ts1Rhr neurons travelled at an average velocity of 0.22 µm/sec ± 0.01 and QD-BDNF endosomes in 2N travelled at an average velocity of 0.32 µm/sec ± 0.01. The slower velocity was prevalent in
both the retrograde ($t=3.29$, $df=524$, $p<0.001$) and anterograde direction of transport ($t=3.67$, $df=524$, $p<0.001$). The average velocity of Ts1Rhr QD-BDNF endosomes travelling in the retrograde direction was 0.28 μm/sec ($±0.02$), and 0.29 μm/sec ($±0.02$) in the anterograde direction. QD-BDNF endosomes in littermate controls travelled at an average velocity of 0.36 μm/sec ($±0.02$) and 0.39 μm/sec ($±0.02$) in the retrograde and anterograde direction, respectively (Figure 3.7). The average distance travelled by QD-BDNF endosomes in Ts1Rhr neurons was 18.76 μm ($±1.09$), which was significantly less than the distance travelled by QD-BDNF endosomes in 2N neurons (28.67 μm $±1.31$; $t=5.88$, $df=524$, $p<0.0001$) (Figure 3.7).

Ts1Rhr neurons displayed a significantly impaired trajectory of axonal transport movement as demonstrated by reduced retrograde ($t=4.60$, $df=524$, $p<0.0001$) transport (Figure 3.8). QD-BDNF endosomes from 2N travelled in a retrograde direction 51.43% ($±3.20$) of the time; QD-BDNF endosomes in Ts1Rhr neurons only travelled in the retrograde direction 32.03% ($±2.79$) of the time. However no significant differences were found in anterograde axonal transport between the genotypes ($t=0.6971$, $df=524$, $p=0.49$), with QD-BDNF endosomes trafficking in the anterograde direction 37.59% ($±3.10$) and 40.57% ($±2.93$) of the time in 2N and Ts1Rhr neurons, respectively. QD-BDNF endosomes from Ts1Rhr neurons spent a greater time stationary (27.40% $±2.67$) compared to controls (10.20% $±1.94$; $t=5.09$, $df=524$, $p<0.0001$).

QD-BDNF endosomes in neurons from Ts1Rhr mice made a greater number of micropauses ($t=2.09$, $df=303$, $p<0.05$) and micropausd for a longer duration of time ($t=6.02$, $df=421$, $p<0.0001$) compared to controls (Figure 3.9). The average number of micropauses made by QD-BDNF endosomes in 2N neurons was 1.23 ($±0.05$), whereas an average of 1.39 ($±0.05$) micropauses were made in Ts1Rhr neurons. During the trajectory of movement, QD-BDNF endosomes micropausd for 11.95% ($±1.45$) of the time in 2N neurons and 26.60% ($±1.97$) of the total movement time in Ts1Rhr neurons.
Figure 3.7: Average velocity and distance travelled by Ts1Rhr QD-BDNF endosomes relative to 2N. (A) Ts1Rhr QD-BDNF endosomes in hippocampal neurons move at a slower velocity than 2N as demonstrated by reduced total, retrograde and anterograde average velocities. (B) These endosomes also travel a lesser distance compared to 2N QD-BDNF endosomes (**p<0.0001).
Figure 3.8: Direction travelled by Ts1Rhr QD-BDNF endosomes relative to 2N. Compared to 2N, Ts1Rhr QD-BDNF endosomes spent less time travelling in the retrograde direction but did not differ in time spent in the anterograde direction. Ts1Rhr QD-BDNF endosomes spent a greater amount of time stationary compared to 2N (**p<0.0001).

Figure 3.9: Number and duration of micropauses displayed by Ts1Rhr QD-BDNF endosomes relative to 2N. (A) QD-BDNF endosomes in Ts1Rhr hippocampal neurons exhibited a greater number of and (B) increased duration of micropauses compared to 2N (*p<0.05; ***p<0.0001).
3.3.3 Axonal transport in Ts65Dn^App++^ mice

Using the same procedure, axonal transport in Ts65Dn^App++^ and Ts65Dn^App+++^ was examined. Kymographs were used to identify and track the QD-BDNF endosome trajectory in Ts65Dn^App++^ (99 endosomes) and Ts65Dn^App+++^ (144 endosomes) hippocampal neurons that were acquired from over 10 axons in one experiment for each genotype.

Quantitative analyses revealed an increase in average velocity in QD-BDNF endosomes from Ts65Dn^App++^ neurons compared to Ts65Dn^App+++^ neurons (t=3.73, df=241, p<0.001) (Figure 3.10). Ts65Dn^App+++^ QD-BDNF endosomes travelled at an average velocity of 0.01 μm/sec (± 0.001), whereas the average velocity of Ts65Dn^App++^ QD-BDNF endosomes was 0.02 μm/sec (± 0.001). This increase in velocity was apparent in both retrograde (t=2.30, df=241, p<0.05) and anterograde (t=8.70, df=241, p<0.001) directions of transport. The average velocity for retrograde transport was 0.29 μm/sec (± 0.02) and 1.37 μm/sec (± 0.10) in Ts65Dn^App+++^ QD-BDNF endosomes. QD-BDNF endosomes in Ts65Dn^App+++^ neurons displayed an average velocity of 0.23 μm/sec (± 0.02) and 0.24 μm/sec (± 0.04) during retrograde and anterograde transport, respectively. Furthermore, the average distance travelled by Ts65Dn^App+++^ QD-BDNF endosomes was increased (23.11 μm ± 1.16; t=3.98, df=241, p<0.0001); the average distance travelled by Ts65Dn^App+++^ QD-BDNF endosomes was 16.11 μm (± 1.29).

Overall direction of movement showed a trend of improved axonal transport in the retrograde direction (t=0.16, df=643, p=0.87). Ts65Dn^App+++^ QD-BDNF endosomes travelled retrogradely 43.74% (± 1.63) of the time, and Ts65Dn^App+++^ QD-BDNF endosomes 44.14% (± 1.86) of the time. Time spent in the anterograde direction was increased significantly relative to Ts65Dn^App+++^ QD-BDNF endosomes (t=2.707, df=643, p<0.001), with Ts65Dn^App+++^ QD-BDNF endosomes trafficking in the anterograde direction 44.68% (± 1.60) of the time, and Ts65Dn^App+++^ QD-BDNF endosomes 38.33% (± 1.70) of the time. Ts65Dn^App+++^ QD-BDNF endosomes spent a decreased amount of time stationary (6.25% ± 2.02) compared to Ts65Dn^App+++^ QD-BDNF endosomes (21.29% ± 4.13; t=3.59, df=24, p<0.001) (Figure 3.11).
Figure 3.10: Average velocity and distance travelled by Ts65Dn\textsuperscript{APP++} QD-BDNF endosomes relative to Ts65Dn\textsuperscript{APP+++} neurons. (A) QD-BDNF endosomes in Ts65Dn\textsuperscript{APP++} hippocampal neurons travel at faster velocity as shown by higher total, retrograde and anterograde average velocities. (B) They also travel a greater distance compared to Ts65Dn\textsuperscript{APP+++} neurons (*p<0.05; ** p<0.001; ***p<0.0001).
Figure 3.11: Direction travelled by Ts65Dn<sup>APP++</sup> QD-BDNF endosomes relative to Ts65Dn<sup>APP+++</sup> neurons. Ts65Dn<sup>APP++</sup> QD-BDNF endosomes did not differ to Ts65Dn<sup>APP+++</sup> QD-BDNF endosomes in time spent trafficking in the retrograde direction. However, they did spend a greater amount of time trafficking in the anterograde direction and decreased amount of time stationary (**p<0.001).

Figure 3.12: Number and duration of micopauses displayed by Ts65Dn<sup>APP++</sup> QD-BDNF endosomes relative to Ts65Dn<sup>APP+++</sup> neurons. (A) QD-BDNF endosomes in Ts65Dn<sup>APP++</sup> hippocampal neurons did not differ to Ts65Dn<sup>APP+++</sup> neurons in the number of micopauses exhibited, (B) but did demonstrate a reduced duration of the micopauses (*p<0.05).
QD-BDNF endosomes from Ts65Dn<sup>App++</sup> neurons did not differ to those from Ts65Dn<sup>App++</sup> neurons in the number of micropauses they made ($t=0.87$, $df=80$, $p=0.39$); Ts65Dn<sup>App++</sup> QD-BDNF endosomes made 1.17 ($\pm$ 0.07) micropauses and Ts65Dn<sup>App++</sup> QD-BDNF endosomes made 1.06 ($\pm$ 0.06) micropauses. However the duration of micropauses did significantly vary between the genotypes ($t=3.20$, $df=586$, $p<0.05$), with Ts65Dn<sup>App++</sup> QD-BDNF endosomes micropausing 7.91% ($\pm$ 1.12) of the time and Ts65Dn<sup>App++</sup>-QD-BDNF endosomes only micropausing 3.81% ($\pm$ 0.71) of the time (Figure 3.12).

3.3.4 Summary of results

The table below (Table 3.1) summarises the BDNF axonal transport results in Ts65Dn and Ts1Rhr mice relative to their controls, and in Ts65Dn<sup>App++</sup> mice compared to Ts65Dn<sup>App++</sup> mice.

**Table 3.1: BDNF axonal transport in Ts65Dn, Ts1Rhr and Ts65Dn<sup>App++</sup> mice***

<table>
<thead>
<tr>
<th>Transport Parameters</th>
<th>Ts65Dn</th>
<th>Ts1Rhr</th>
<th>Ts65Dn&lt;sup&gt;App++&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Velocity</strong></td>
<td>Decreased</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td><strong>Retrograde Velocity</strong></td>
<td>Decreased</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td><strong>Anterograde Velocity</strong></td>
<td>Decreased</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td><strong>Distance Travelled</strong></td>
<td>Decreased</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td><strong>Retrograde Movement</strong></td>
<td>Decreased</td>
<td>Not Significant</td>
<td>Not Significant</td>
</tr>
<tr>
<td><strong>Anterograde Movement</strong></td>
<td>Decreased</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td><strong>Stationary</strong></td>
<td>Increased</td>
<td>Increased</td>
<td>Decreased</td>
</tr>
<tr>
<td><strong>Micropause Number</strong></td>
<td>Not Significant</td>
<td>Increased</td>
<td>Not Significant</td>
</tr>
<tr>
<td><strong>Micropause Duration</strong></td>
<td>Increased</td>
<td>Increased</td>
<td>Decreased</td>
</tr>
</tbody>
</table>

*All results are compared to relative controls.
3.4 Discussion

The combination of advanced techniques and microscopy developments such as QDs, microfluidic chambers and pseudo-TIRF microscopy, has given rise to the ability to identify, track and follow the long-distance trajectory of a single endosome containing a single neurotrophin within a single axon over a long duration. Using these novel techniques, QD-BDNF axonal transport was found to be impaired in Ts65Dn hippocampal neurons. To fully appreciate the complexity of axonal transport, several transport parameters were examined; disruption in Ts65Dn axonal transport was manifest in most of these parameters. Average total velocity of QD-BDNF endosomes from Ts65Dn neurons was decreased and this was evident in both the retrograde and anterograde directions. Throughout the time-lapse of live-cell image acquisition, the distance travelled by Ts65Dn QD-BDNF endosomes was markedly less compared to controls. These data demonstrate that Ts65Dn QD-BDNF endosomes move at a slower rate and move a reduced distance. Decreased anterograde velocity suggests that Ts65Dn QD-BDNF endosomes exhibit global decelerated movement that is not just specific to trafficking towards the cell body. QD-BDNF endosomes from Ts65Dn neurons spent less time travelling in the retrograde direction towards cell body and also less time travelling in the anterograde direction. In fact, they were stationary 50% of the time. To further understand impaired movement, the number and duration of micopauses was examined. Ts65Dn QD-BDNF endosomes did not differ to controls in the number of micopauses they made, however they did pause for almost twice the time duration, thereby partially explaining the hindered movement.

Similar to the findings in the Ts65Dn model, perturbed axonal transport was evident in the Ts1Rhr mouse model. QD-BDNF endosomes from Ts1Rhr neurons moved at a slower rate compared to controls, which was also apparent in both, retrograde and anterograde, directions. The average distance travelled along the axon within a fixed time frame was also significantly reduced, alluding to a global movement impairment that is not direction specific, in QD-BDNF endosomes from Ts1Rhr neurons. Retrograde axonal transport was impaired as demonstrated by QD-BDNF endosomes spending less time travelling towards the cell body, however
anterograde transport was spared, suggesting that triplicated genes in the Ts65Dn region that are not encoded in the Ts1Rhr region may be implicated in the anterograde transport deficits seen in Ts65Dn neurons. Ts1Rhr QD-BDNF endosomes also spent a greater amount of time stationary; they remained stationary approximately 30% of the time compared to littermate controls that were stationary for approximately 10% of the time. QD-BDNF endosomes from Ts1Rhr neurons made a greater number of micropauses and paused for more than twice the amount of time compared to controls, which added to hindered transport movement in this model.

To investigate the specific contribution of APP, preliminary studies of QD-BDNF endosomes from Ts65Dn<sup>App++</sup>-neurons were compared to those from Ts65Dn<sup>App+++</sup>-neurons. Ts65Dn<sup>App++</sup>-neurons demonstrated an improvement in several of the axonal transport parameters examined. The overall, retrograde and anterograde average velocity was increased. This was accompanied with a greater distance travelled within the time frame analysed. Although a trend was apparent for QD-BDNF endosomes from Ts65Dn<sup>App+++</sup>-neurons to spend more time travelling in the retrograde direction, only a significant increase in anterograde transport was seen. In support of improved transport, Ts65Dn<sup>App++</sup>-QD-BDNF endosomes spent almost four times longer stationary compared to Ts65Dn<sup>App+++</sup>-QD-BDNF endosomes. Ts65Dn<sup>App+++</sup>-QD-BDNF endosomes did not vary in the number of micropauses made, however they micropaus ed for more than half the duration of that seen in Ts65Dn<sup>App+++</sup> mice.

Although several QD-BDNF endosomes were examined for Ts65Dn<sup>App++</sup> and Ts65Dn<sup>App+++</sup> mice, all the data for this model was derived from one independent experiment. There is no data from wild-type littermates; therefore general conclusions about the meaning of the results need to be interpreted with caution, as more data from independent experiments is required to validate the findings. As dissections of embryos results in the loss of a valuable transgenic mother, a large amount of time was invested in attempting to dissect postnatal pups (P0-P2) so that the mother could be saved. Failure to obtain a healthy postnatal neuronal preparation meant that this endeavour could not be pursued. The consistency of dissecting hippocampal neurons from embryos was limited by the availability of
transgenic mice; Ts65Dn mice are difficult to breed and widely used within the lab and thus were not always available. Additionally, the Ts65Dn crossed with APP hemizygous mice were extremely difficult to breed and produced very few, if any, viable pups. This resulted in an inability to obtain a large enough sample size to complete the axonal transport studies or conduct any further studies with this model.

Given the importance of BDNF in neuronal survival, synaptic plasticity and memory processes, it is conceivable that improper trafficking of BDNF results in at least some of the neurodegeneration and cognitive deficits seen in DS and AD. The mechanism that has been attributed to underlie the retrograde axonal transport deficit is not the lack of availability of BDNF or its receptor, but rather, enlarged early endosomes that impede the function of early endosomes as a sorting platform and transport cargo efficiently (Salehi et al., 2006). The role of early endosomes will be examined later (Chapter 4), however it does not rule out other mechanisms that may additionally contribute to the neurodegeneration phenotype. For example, it is possible that BDNF axonal transport deficits may trigger the neuron to stimulate the p75 signalling cascade to induce apoptosis and cell death, as a lack of neurotrophic factors may indicate cell injury or death (Dechant & Barde, 2002). However, further studies that examine the role and initiation of the p75 signalling cascade in DS would need to be investigated to support this hypothesis.

The analyses conducted make it difficult to ascertain whether there is a deficit in the number of QD-BDNF endosomes reaching the cell body or whether the same number of endosomes reaches the cell body, albeit slightly delayed. Examining the flux of QD-BDNF endosomes to assess the number that reach the cell body would be informative in understanding whether a neurons requirement for neurotrophins is tightly regulated by temporal dynamics. An initial attempt was made to conduct this analysis, however, it proved to be difficult to count the flux due to the accumulation of bright QDs that emitted large amounts of intractable fluorescence in the cell body compartment. The fluorescence from QDs in the cell body compartment combined with autofluorescence from the neurons also made it difficult to image in the proximal microchannel and produced a very low signal-to-noise ratio. As a result of this, most of the imaging was conducted in the middle-distal microchannel, making
it difficult to determine the number of QD-BDNF endosomes that travelled back to the cell body. The middle-distal microchannel is limited by being extremely busy with the exogenous addition of QD-BDNF; this may, additionally, explain the lowered velocities reported herein.

Moreover, the current experiment is limited by its in vitro nature and the addition of exogenous QD-BDNF to assess BDNF transport in DS. Even though the exogenous BDNF was evidently internalised at the axon terminal and transported along the axon, it is unknown whether the axonal transport of exogenous BDNF is similar to that of endogenous BDNF. Labelling QDs to endogenous BDNF would be the ideal experiment, but this would need to be performed in an in vivo setting.

3.4.1 Axonal transport mechanisms

Previous studies have predominantly focused on retrograde transport of NGF to understand axonal transport deficits in DS (Salehi et al., 2006). However, by only examining velocity and retrograde transport, vital information may be missed that could be informative to deciphering overall axonal transport impairments. As such, this current report investigates several axonal transport parameters to further elucidate the dynamics involved in neurotrophin trafficking impairments including, anterograde transport, stationary and micropausing behaviour. Looking at these specific parameters is particularly important for BDNF as it is heavily involved in synaptic plasticity and dendritic growth in addition to its primary role as a neurotrophic factor. Quantitative analyses of the trajectory of QD-BDNF endosomes suggests there is less likelihood of BDNF reaching the cell body to transmit transcriptional signals and induce critical signalling cascades, due to a slower velocity of QD-BDNF endosomes in DS models that are covering less distance over time. Moreover, this is accompanied with more pausing behaviour that hinders transport further and a greater number of endosomes not moving at all. Thus, it appears that deficits in retrograde and anterograde transport is caused by increased stationary and micropausing behaviour.

The instantaneous speed of QD-NGF has been reported to vary from 0.5 to 5um/s⁻¹, whereas the average speed, which includes moving and pausing phases, ranges
from 0.2 to 0.3μm/s (Chowdary et al., 2012). The direction of QD-NGF transport is predominantly unidirectional with most endosomes moving in the retrograde direction towards the cell bodies. An occasional reversal in direction is reported to be short-lived causing a very small anterograde displacement (Chowdary et al., 2012). However, the velocity of QD-BDNF reported here, even for control mice, is much lower than expected and QD-BDNF endosomes demonstrate bidirectional transport movement. The transport characteristics of QD-BDNF have not been established and it is yet unknown whether physiological differences exist in transport rates between NGF and BDNF, and whether these rates vary in various neuronal types. It is conceivable that the involvement of BDNF in synaptic plasticity and its subcellular localisation in dendrites renders it important to have the ability to traffic in the retrograde and anterograde direction depending on its destination (Kuczewski et al., 2010). Examining QD-NGF using the same protocol would be informative to understand axonal transport of neurotrophins in general and to be able to define and possibly differentiate BDNF transport characteristics.

Both Ts65Dn and Ts1Rhr display impaired QD-BDNF retrograde axonal transport. However, it is yet to be elucidated whether this defect is specific to axonal transport of neurotrophins or whether it is a global defect that affects the axonal transport of other cargoes. Investigating axonal transport of mitochondria or synaptophysin would be informative for understanding the mechanisms underlying axonal transport deficits in DS. The results here indicate a global transport deficit as Ts65Dn mice also demonstrate impairments in anterograde transport. Even though anterograde transport was spared in Ts1Rhr mice, both models display hindered trafficking of QD-BDNF endosomes or no movement at all as is evidenced through increased micropausing and stationary duration. However, the anterograde transport data reported here is not a true representation of the anterograde transport of BDNF, but rather a minor component of anterograde transport of endocytic compartments. To measure the effect of Trisomy 21 on anterograde transport, experiments that involve treating the cell body compartment with QD-BDNF, instead of the distal axon compartment, would need to be conducted. Based on the current findings and previous reports (Cataldo et al., 2008, Cataldo et al., 2003), it is possible that the entire endocytic pathway is disrupted and this would affect axonal transport of not just neurotrophins, but other cargoes as well.
Microfluidic chambers have revolutionised the ability to dissociate axons from cell bodies and enabling experimental manipulations to specific parts of the neurons. These chambers are fragile and extremely susceptible to contamination. Even though the protocol is easy enough to follow, there are several idiosyncrasies with these chambers that take time to master. Microfluidic chambers were not commonly utilised in the lab, hence a large amount of time was dedicated to making this technique successful. Even though QDs are extremely bright and the pseudo-TIRF microscope allows reduction of background fluorescence, technical issues were encountered with achieving a high signal-to-noise ratio. Several attempts were required to create a workable protocol for the lab to follow. An accurate method to analyse kymographs after acquisition of transport of QD-BDNF also needed to be produced. A few publications examined the start and end point of the trajectory (Andrews et al., 2010), however a method to follow every change in direction, the distance travelled and identification of micropauses along the trajectory of QD-labelled molecules has not yet been published.

3.4.2 APP and its interaction with motor proteins

Neurotrophin axonal transport deficits recapitulated in Ts65Dn mice implicate one or more of its 122triplicated genes in contributing to this deleterious phenotype. Previous studies have identified an increase in APP gene-dosage in causing 60% of this impairment (Salehi et al., 2006). In support of this, Ts1Cje mice, which do not carry a trisomy of APP, show a less severe but still significant retrograde transport deficit compared to controls (Salehi et al., 2006). Previous studies show that normalisation of APP to disomic levels leads to improved retrograde axonal transport compared to Ts65DnApp++ mice (Salehi et al., 2006). Preliminary studies in this report with Ts65DnApp+++ mice suggest an improvement in the impaired axonal transport phenotype, however data from this model is inconclusive due to a small sample size and no comparison to a wild-type control.

APP is an enticing candidate gene due to the pathology it generates when overexpressed. Under physiological conditions, APP produces normal Aβ peptides, however when overexpressed, it results in Aβ plague deposits. Evidence for this
comes from families that have small internal duplications of a segment of Hsa21 that includes the triplication of APP (Rovelet-Lecrux et al., 2006), and from partial trisomy 21 cases, which do not include triplication of APP and that do not develop the neuropathological features of AD (Prasher et al., 1998). Additionally, neurodegeneration of BFCNs is reported in Ts65Dn mice, however they are spared in Ts1Cje mice, which do not harbour the third copy of APP. Moreover, enlarged early endosomes are attributed as the underlying mechanism causing axonal transport deficits in Ts65Dn mice. Evidence to support this comes from the colocalisation of APP within enlarged early endosomes (Cataldo et al., 2003, Salehi et al., 2006), and from overexpressing even just the βCTF proteolytic derivate of APP in normal fibroblasts to induce enlarged early endosomes (Jiang et al., 2010).

APP is transported in the fast component of anterograde transport in peripheral and central axons (Buxbaum et al., 1998, Koo et al., 1990), and it is reported that Aβ is released at nerve terminals in the CNS (Lazarov et al., 2002, Sheng et al., 2002). However, much still is unknown about the normal function of APP, whether it is related to γ-secretase proteolytic processing of APP in neurons in vivo, and where this processing occurs. It has been suggested that APP forms a complex with a microtubule motor, kinesin-1, by directly binding with its tandem repeat domain of the kinesin light chain (KLC) subunit (Kamal et al., 2000). This study also demonstrated perturbed anterograde transport of APP in sciatic nerves of KLC1-deficient mice, leading to the hypothesis that APP may be a membrane cargo receptor for kinesin-1 and that KLC is important for APP to be driven into axons by kinesin-1 (Kamal et al., 2000). Further studies identified an axonal membrane compartment that contains APP, β-secretase and PS1, and that fast anterograde axonal transport of this compartment is mediated by APP and kinesin-1. It was also suggested that proteolytic processing of APP may occur in this compartment, and the resulting proteolytic derivatives, Aβ and βCTF, would liberate kinesin-1 from the membrane (Kamal et al., 2001).

Even though the idea that all components for producing Aβ are localised within a single compartment is attractive, these findings were refuted by a study that attempted to replicate the association between APP and kinesin-1 (Lazarov et al.,
In contrast to the mentioned findings, no evidence was found for a direct interaction between APP and kinesin-1. They reported the transport of kinesin-1 in APP-deficient mice was unchanged, and that the two components of APP proteolytic machinery, PS1 and BACE1, are not co-transported with APP in the same membrane compartment in sciatic nerves (Lazarov et al., 2005). Instead, the authors suggest that APP and kinesin-1 could interact indirectly through the mediation of a linker protein, and that APP, PS1, and BACE1 exist in different compartments in peripheral nerves (Lazarov et al., 2005). However, this may not apply to CNS neurons, such as BFCNs, where it is likely that various components of the APP processing machinery are assembled and transported together before encountering membrane-tethered APP-CTFs to generate Aβ.

Recent evidence using advanced microscopy techniques shows that APP levels are correlated with the amount of KLC and heavy chain of cytoplasmic dynein (DYNC1H1) on vesicles (Szpankowski et al., 2012). This is further supported by the genetic reduction of APP, which results in reduced KLC and DYN1H1 levels on APP cargoes. Interestingly, the reduction of KLC led to diminished levels of DYN1H1 on APP vesicles, suggesting that KLC is necessary for the association of DYN1H1 to these cargoes (Szpankowski et al., 2012). Recently, GSK3β was discovered to be a critical negative regulator of bidirectional APP transport, by regulating kinesin-1-mediated and dynein-mediated axonal transport of APP and altering the activity of kinesin-1 motors, but not the binding to their cargo (Weaver et al., 2013).

These data may explain the bidirectional transport deficits seen in Ts65Dn mice due to the overexpression of APP, which may lead to perturbed regulation of the kinesin-1 and dynein motor proteins that carry the APP cargo. It is also noteworthy that BDNF/TrkB activation induces the AKT-dependent phosphorylation of GSK3β, resulting in its inactivation (Li et al., 2007a). GSK3β is a modulator of apoptosis (Li et al., 2007a), and it is feasible that reduced BDNF/TrkB levels, as documented in AD and DS, may lead to greater activation of GSK3β, resulting in increases apoptosis. GSK3β also directly phosphorylates Tau, and reduced BDNF/TrkB levels in AD and DS may additionally increase phosphorylation of Tau through a GSK3β-mediated pathway (Elliott et al., 2005).
3.4.3 Contribution of other Hsa21 genes

It is a reasonable surmise that perturbations in Tau and other microtubule-associated proteins may affect the function of motor proteins to transport their cargo along axons. Another Hsa21 gene, RCAN1, encodes a protein that is a negative regulator of calcineurin, which dephosphorylates Tau. Overexpression of RCAN1 inhibits the phosphatase activity of calcineurin resulting in increased phosphorylation of Tau (Lloret et al., 2011), thus further disrupting the cytoskeleton network. Increased RCAN1 causes the up-regulation of GSK3β, which additionally may aggravate the hyperphosphorylation of Tau (Lloret et al., 2011).

Interestingly, DYRK1A, which is triplicated in the Ts1Rhr model, also interacts with APP and Tau (Ryoo et al., 2008, Ryoo et al., 2007), and it is conceivable that combined overexpression of all these Hsa21 genes may result in similar or even accumulated consequences. To further understand the transport mechanisms, examining the motor proteins and the cytoskeleton network in Ts65Dn and Ts1Rhr mice would have been informative. In addition to an enlarged early endosome phenotype, it is feasible that overexpression of key Hsa21 genes leads to motor protein and cytoskeletal perturbations, mediated by abnormal regulation of GSK3β and Tau, which then results in axonal transport deficits.

The contribution of Hsa21 genes other than APP in axonal transport is further supported by the study conducted by Salehi and colleagues, which examined NGF retrograde transport in Ts65Dn, Ts1Cje and Ts65Dn\textsuperscript{App++} mice (Salehi et al., 2006). Even though Ts65Dn displayed a more severe retrograde transport deficit compared to Ts1Cje mice and this was improved by normalising APP expression in Ts65Dn\textsuperscript{App++} mice, it is noteworthy that both Ts1Cje and Ts65Dn\textsuperscript{App++} mice exhibited a deficit in retrograde transport relative to their controls (Salehi et al., 2006). The study reported that 60% of the impairment could be attributed to the triplication of APP, however 40% of the deficit still remains unaccounted for. Significantly, axonal transport deficits in the Ts1Rhr model suggest a putative role for the 31 triplicated genes that are orthologous to the DSCR on HSA21 in contributing to this phenotype.
Due to their contribution to other DS-associated phenotypes, candidate genes on the Ts1Rhr model were identified as having a possible role in the development of this phenotype including, DSCAM, KCNJ6, SIM2 and DYRK1A. DSCAM is a cell adhesion molecule that is heavily implicated in synaptic plasticity, dendritic morphology and neuronal wiring (Alves-Sampaio et al., 2010). KCNJ6 is an effector protein for GABAB receptors and is involved in maintaining balanced GABAergic neurotransmission by modulating potassium channel current and density (Best et al., 2007, Ehrengruber et al., 1997). SIM2 is a transcriptional repressor implicated in synaptic plasticity and dendritic morphology through modulation of DBN1 levels (Ooe et al., 2004, Shim & Lubec, 2002). Out of this list, DYRK1A, a kinase involved in regulating multiple signalling cascades, was identified as the key candidate gene to start exploring. DYRK1A has been shown to be intricately involved with AD-associated phenotypes through phosphorylation of APP and Tau, and is also required for clathrin-mediated endocytosis (Kim et al., 2010, Ryoo et al., 2008, Ryoo et al., 2007). Attempts were made to lower the expression of DYRK1A using a siRNA and lipofectamine kit to transfected neurons to further understand the contribution of this gene in causing the impairments. However limited time and unsuccessful trials resulted in no data being produced. Even though a particular gene was not identified for causing axonal transport deficits, the findings from this investigation have enabled to narrow down to a genomic region of 31 genes that could contribute to the impairments. As such, it would beneficial to examine other mouse models such as the Ts65Dn<sup>App++</sup> or the Ms1Rhr/Ts65Dn model, which contain genes that are triplicated in Ts65Dn but not Ts1Rhr.
3.5 Conclusion

The discovery of neurotrophins and their essential role in supporting neuronal survival, differentiation, maintenance and even synaptic plasticity has revolutionised our understanding of the physiological and pathological state of neurons. It has led to the notion that neurodegenerative diseases may be caused by perturbed axonal transport of neurotrophins, and this has indeed shown to be an associated mechanism in several neurodegenerative diseases, including AD and DS. Specifically, reduced BDNF protein and mRNA levels have been reported in hippocampal and cortical areas of people with AD. This has severe implications for cognitive deficits as all these brain structures are heavily implicated in learning and memory. Reduced BDNF/TrkB levels have been demonstrated to affect memory acquisition and consolidation and a failure to induce NMDAR-mediated LTP. Importantly, Aβ deposition further reduces BDNF/TrkB levels, thus impairing activation of the BDNF/TrkB/CREB signalling pathway, which is essential for inducing gene transcription. The pathogenesis underlying these deleterious phenotypes in AD and DS may lead to deficits in axonal transport, which results in BDNF unable to reach the cell body and consequently, neurodegeneration. This investigation supports data from previous studies that report axonal transport deficits in Ts65Dn mice. Additionally, axonal transport deficits are observed in Ts1Rhr mice, a mouse model previously not examined. These findings have important implications for the role of APP and the other 31 triplicated genes orthologous to the Hsa21 DSCR, in contributing to axonal transport deficits. It will now be possible to streamline to the putative genes encoded on the Ts1Rhr model to assess their relative contribution in causing impairments in axonal transport. The analysis methodology to assess the trajectory of neurotrophin and endosome complexes has also enabled a deeper understanding of trafficking idiosyncrasies and hindered movement in mouse models of DS. However, understanding the various mechanisms that may underlie axonal transport deficits still remains unknown.
CHAPTER FOUR   EARLY ENDOSONES IN MOUSE MODELS OF DS

4.1 Introduction

Axonal transport deficits in DS have been attributed to the overexpression of APP, which may be mediated by an enlarged early endosome phenotype (Cataldo et al., 2003, Salehi et al., 2006). Key APP proteolytic enzymes including BACE-1 and γ-secretase, and various APP proteolytic fragments including Aβ and βCTF, are all found in early endosomes (Arbel et al., 2005, Rajendran et al., 2008). Therefore, it is conceivable that altered APP processing and early endosomal dysfunction are interrelated processes in AD. Indeed, as already mentioned, it has been shown that the overexpression of APP is necessary, although not sufficient, for inducing endosomal dysmorphology in neurons from mouse models of DS (Cataldo et al., 2003). Additionally, overexpression of the βCTF of APP in DS and control fibroblasts was sufficient to induce enlarged endosomes (Jiang et al., 2010).

In addition to enlarged endosomes found in primary neurons and fibroblasts, enlarged endosomes have recently been discovered in blood cells and lymphoblastoid cell lines (LCLs) from individuals with DS (Cossec et al., 2012). Interestingly, in LCLs that harbour APP microduplications causing autosomal early-onset AD, enlarged endosomes were absent, implicating other Hsa21 genes in causing enlarged endosomes (Cossec et al., 2012). An indepth LCL analysis on eight individuals with partial trisomy 21 revealed enlarged endosomes in only 3 of the individuals. The overlapping triplicated segment identified a 2.56 Mb locus in 21q22.11 containing 33 genes; 14 of the genes are expressed in LCLs, including SYNJ1. Interestingly, this segment extends from TIAM1 to CR626360, which is a region triplicated in Ts65Dn and Ts1Cje mice, but not in Ts1Rhr mice. SYNJ1 overexpression in a neuroblastoma cell line and in transgenic mice leads to enlarged endosomes (Cossec et al., 2012). In the same study, lowering SYNJ1 expression with RNA interference had the effect of reducing the enlarged endosome population in DS fibroblasts.
SYNJ1 catalyses the dephosphorylation of the signalling phospholipid PI(4,5)P$_2$, which controls numerous phenomena at the plasma membrane-cytosol interphase, including regulation of clathrin-mediated endocytosis and the recycling of synaptic vesicles (Cremona et al., 1999, Kim et al., 2002). To facilitate clathrin-mediated endocytosis, PI(4,5)P$_2$ controls the recruitment of clathrin adaptors to the cell surface and other critical endocytic accessory factors such as dynamin (Bauerfeind et al., 1997, McPherson et al., 1996, Slepnev et al., 1998). In Synj1 knock-out mice, fusion of synaptic vesicles was severely impaired, and a persistent accumulation of clathrin-coated vesicles and a back-up of newly reformed vesicles in the synaptic vesicle cluster was observed (Cremona et al., 1999, Kim et al., 2002). At synapses, normal progression of recycling vesicles and a proper PI(4,5)P$_2$ balance is required for effective neurotransmission (Adayev et al., 2006). Overexpression of SYNJ1 perturbs PI(4,5)P$_2$ metabolism and the dyshomeostasis caused by this gene dosage imbalance significantly contributes to cognitive deficits in humans with DS (Arai et al., 2002) and the Ts65Dn mouse model (Voronov et al., 2008). Significantly, restoring Synj1 to disomic levels rescued altered PI(4,5)P$_2$ metabolism in Ts65Dn mice (Voronov et al., 2008).

ITSN1 has also been implicated in causing endosomal and trafficking anomalies. Itsn1 knock-out mice demonstrated a slowing of endocytosis in neurons in addition to enlarged endosomes and reduced NGF levels in the septal region of the brain (Yu et al., 2008). Interestingly, ITSN1 is encoded in the 2.5 Mb region associated with endosomal abnormalities, which was identified during LCL examination of patients with partial trisomy 21 (Cossec et al., 2012). Overexpression of Dyrk1a was found to cause defects in recruitment of endocytic proteins to clathrin-coated pits, thus perturbing clathrin-mediated endocytosis and significantly slowing down synaptic vesicle endocytosis (Kim et al., 2010).
4.2 Chapter Aim

To elucidate the pathological mechanisms underlying axonal transport deficits seen in the Ts65Dn and Ts1Rhr mouse models, the morphology of early endosomes was examined. Even though endosome morphology has been examined in the Ts65Dn model (Cataldo et al., 2003, Cossec et al., 2012, Jiang et al., 2010, Kim et al., 2010, Salehi et al., 2006, Yu et al., 2008), endosomal morphology in the Ts1Rhr mouse model has not yet been characterised. This chapter aims to recapitulate the enlarged endosome phenotype in Ts65Dn hippocampal neurons. Additionally, to assess the genetic contribution of other Hsa21 genes in causing this phenotype, early endosomes will also be examined in the Ts1Rhr mouse model.
4.3 Results

4.3.1 Early endosomes in Ts65Dn mice

Ts65Dn and 2N hippocampal neurons were fixed and immunostained on DIV7 with EEA1, a marker for early endosomes. Images of neurons were captured with an Olympus epi-fluorescence confocal microscope system (Applied Precision) The panel below shows EEA1 immunolabelling in Ts65Dn and 2N neurons (Figure 4.1).

Figure 4.1: Early endosomes in Ts65Dn neurons. Ts65Dn and 2N hippocampal neurons were immunolabelled with an EEA1 (Alexa 488) antibody to assess the presence of enlarged early endosomes.
A total of 16 and 13 neurons were examined for Ts65Dn and 2N genotypes, respectively. Data sets were analysed using the FluoroView software for Olympus. Quantitative morphological analyses were conducted using Volocity 3D image analysis software, a powerful tool for precise measurements of cellular structures through the generation of specific analysis protocols that can be applied to different samples. A protocol was generated that excluded any endosomes smaller than 0.01 μm² and larger than 12 μm², which also separated touching objects based on the average size of 0.5 μm² for an endosome. This protocol was applied to all neurons to maintain a standardised, automated analysis procedure.

Ts65Dn neurons had a significantly greater number of EEA1-labelled early endosomes compared to 2N neurons \((t=3.63, df=27, p<0.001)\). The average number of EEA1-labelled early endosomes in Ts65Dn neurons was 234.30 \((± 21.31)\), compared to an average number of 136.10 \((± 14.57)\) in 2N. EEA1-endosomes in Ts65Dn neurons were on average twice as large relative to EEA1-endosomes from 2N \((t=5.25, df=27, p<0.0001)\). The average area of EEA1-endosomes in 2N neurons was 0.29 μm² \((± 0.03)\), whereas the average size in Ts65Dn neurons was 0.62 μm² \((± 0.05)\) (Figure 4.2).

![Figure 4.2: Total number and mean size of endosomes in Ts65Dn neurons relative to 2N. (A) A greater number of EEA1-positive endosomes was found in Ts65Dn hippocampal neurons. (B) The average size of Ts65Dn EEA1-positive endosomes were approximately twice as large as those found in 2N neurons.](image)
To enable statistical comparisons of the distribution of endosomes, three categories were created based on the size of endosomes, as outlined in Chapter 2.5.1: small (0.01–0.5 µm$^2$), medium (0.51–1 µm$^2$) and large (>1 µm$^2$) endosomes (Figure 4.3). The total number of small endosomes in Ts65Dn neurons did not significantly differ from 2N ($t=1.85$, $df=27$, $p=0.08$), with Ts65Dn neurons displaying an average of 162.10 ($\pm 16.95$) EEA1-labelled small endosomes compared to an average of 120.40 ($\pm 13.76$) small endosomes in 2N neurons. Ts65Dn neurons displayed over 3.5-fold greater number of medium-sized endosomes (36.46 $\pm 3.79$) compared to 2N (10.46 $\pm 1.65$; $t=5.838$, $df=27$, $p<0.0001$). Significantly, there were almost 7-times the number of enlarged early endosomes in Ts65Dn neurons ($t=7.21$, $df=27$, $p<0.0001$), with an average of 35.69 ($\pm 3.73$) large endosomes per cell, compared to 5.23 ($\pm 0.89$) large endosomes per cell in 2N.

**Figure 4.3: Distribution of EEA1-positive endosomes in Ts65Dn neurons relative to 2N.** For statistical comparisons, endosomes were sorted into three categories dependent on their size: small (0.01 – 0.5 µm$^2$), medium (0.51 – 1 µm$^2$) and large (above 1 µm$^2$). Ts65Dn EEA1-positive endosomes did not differ from 2N in the number of small endosomes present, however they displayed approximately 3.5-fold more medium endosomes and 7-fold more large endosomes, compared to 2N neurons.
4.3.2 Early endosomes in Ts1Rhr mice

Similar to the procedure carried out in Ts65Dn mice, Ts1Rhr and 2N hippocampal neurons were fixed and immunostained on DIV7 with EEA1. Images of EEA1 immunolabelling in both genotypes are depicted in the panel below (Figure 4.4).

**Figure 4.4: Early endosomes in Ts1Rhr neurons.** Ts65Dn and 2N hippocampal neurons were immunolabelled with an EEA1 (Alexa 488) antibody to assess the presence of enlarged early endosomes.
A total of 53 and 56 neurons were examined for 2N and Ts1Rhr genotypes, respectively. The same protocol generated for Ts65Dn mice was applied to this model too. Quantitative analyses revealed a fewer number of EEA1-endosomes in Ts1Rhr neurons compared to 2N (t=2.35, df=107, p<0.05), with an average number of 148.60 (± 8.76) EEA1-labelled early endosomes per cell in Ts1Rhr compared to an average number of 177.30 (± 8.41) in 2N. Ts1Rhr EEA1-endosomes did not vary in size (0.31 µm² ± 0.01) relative to EEA1-endosomes in 2N (0.35 µm² ± 0.02; t=1.74, df=107, p=0.09) (Figure 4.5).

Figure 4.5: Total number and mean size of endosomes in Ts1Rhr neurons relative to 2N. (A) Ts1Rhr neurons contained fewer EEA1-positive endosomes, (B) which did not differ in average size compared to 2N.

Ts1Rhr neurons displayed fewer small endosomes (t=2.28, df=107, p<0.05), with an average of 126.50 (± 7.37) small EEA1-endosomes per cell, compared to an average of 149.40 (± 6.80) small endosomes per cell in neurons from 2N. The average number of medium-sized EEA1-endosomes in Ts1Rhr mice (14.16 ± 1.22) also was significantly reduced compared to medium-sized EEA1-endosomes in 2N (18.51 ± 1.70; t=2.10, df=107, p<0.05). Interestingly, the number of large endosomes did not differ between the genotypes (t=1.02, df=107, p=0.31). Ts1Rhr neurons displayed an average of 8.00 (± 0.94) large EEA1-endosomes per cell, compared to an average of 9.36 (± 0.94) large EEA1-endosomes per cell in 2N (Figure 4.6).
Figure 4.6: Distribution of EEA1-positive endosomes in Ts1Rhr neurons relative to 2N. For statistical comparisons, endosomes were sorted to three categories dependent on their size. Ts1Rhr EEA1-positive endosomes displayed a fewer number of small and medium endosomes, however did not differ from controls in the number of enlarged endosomes present.

4.3.3 Summary of results

The table below (Table 4.1) summarises early endosome morphology in Ts65Dn and Ts1Rhr mice, relative to respective controls.

Table 4.1: Early endosome morphology in Ts65Dn and Ts1Rhr mice*

<table>
<thead>
<tr>
<th>Early Endosome Morphology</th>
<th>Ts65Dn</th>
<th>Ts1Rhr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Number of Endosomes/Cell</td>
<td>Increased</td>
<td>Decreased</td>
</tr>
<tr>
<td>Mean Endosome Area</td>
<td>Increased</td>
<td>Not Significant</td>
</tr>
<tr>
<td>Number of Small Endosomes</td>
<td>Not Significant</td>
<td>Decreased</td>
</tr>
<tr>
<td>Number of Medium Endosomes</td>
<td>Increased</td>
<td>Decreased</td>
</tr>
<tr>
<td>Number of Large Endosomes</td>
<td>Increased</td>
<td>Not Significant</td>
</tr>
</tbody>
</table>

*All results are compared to relative controls
4.4 Discussion

Early endosomes are a critical sorting platform at which the destiny of internalised cargo is determined (Gruenberg et al., 1989). They also facilitate the proteolytic processing of certain cargo load, as is the case with APP (Arbel et al., 2005, Rajendran et al., 2008). In support of the existing literature (Cataldo et al., 2003, Salehi et al., 2006), in this study, endosomal anomalies were found in the Ts65Dn mouse model. Not only did Ts65Dn hippocampal neurons demonstrate a greater total number of endosomes, but also the average endosome size was twice that of controls. When the distribution of endosomes was examined, almost 3.5-fold and 7-fold greater number of medium-sized and enlarged endosomes was found, respectively. Interestingly, Ts1Rhr hippocampal neurons displayed fewer early endosomes that did not differ from controls in average size. The number of enlarged endosomes also did not differ from controls, although Ts1Rhr neurons showed fewer small- and medium-sized early endosomes.

Enlarged endosomes are suggested to be caused by increased gene dosage and have been associated with axonal transport deficits and may be a reason why neurotrophins do not reach the cell body (Salehi et al., 2006). Enlarged endosomes may hinder retrograde axonal transport through various mechanisms, which require further examination. Endocytic accessory proteins and dynein motor proteins involved in axonal transport may not be sufficient to carry a larger cargo load and additional recruitment of these proteins may be restricted by availability. Therefore the larger load may not transport as smoothly as it may need to pause several times and may not even move at all, as seen by axonal transport patterns of QD-BDNF endosomes in Ts65Dn hippocampal neurons (Chapter 3). It is also conceivable that endosomes that do not move at all or move slowly may cause a hindrance for other endosomes to get by, i.e. almost like a traffic jam, leading to a perturbed endocytic pathway and consequently an accumulation of aggregated proteins. Indeed, endocytic abnormalities are seen much earlier in DS and AD, even before the accumulation of plaques and tangles (Cataldo et al., 1997, Cataldo et al., 2000, Mann, 1988, Wisniewski et al., 1985).
The Ts65Dn endosome data presented herein is limited by a small sample size as data was obtained from only one experiment. Even though several neurons were examined, the study would have benefitted from data from additional experiments. These final experiments could not be pursued due to contamination issues. During the limited time remaining to complete the experiments, the EEA1 antibody also failed to immunostain successfully, even though it had been optimised and working successfully for the Ts1Rhr neuronal experiments. Additionally, it would have been beneficial to differentiate between axonal and somato-dendritic endosomes to better understand endocytic mechanisms.

4.4.1 APP and APOE in AD-related endosomal pathology

There is compelling evidence for the role of increased gene dosage of APP in causing an enlarged endosome phenotype as demonstrated by the presence of enlarged endosomes in Ts65Dn mice (Cataldo et al., 2003), and in human fibroblasts (Cataldo et al., 2008, Jiang et al., 2010). The absence of enlarged endosomes in Ts1Cje mice, which are not trisomic for App, and a reduced phenotype in Ts65DnApp++ mice, which harbours disomic levels of App, further implicates APP in causing a deleterious phenotype (Cataldo et al., 2003, Salehi et al., 2006). Most AD cases are caused by sporadic instances; familial AD (FAD) constitutes a small portion of cases caused by genetic defects transmitted in an autosomal dominant manner. These are normally caused by mutations in APP at codon 717 or a double mutation at codons 670/671, the presenilin (PS) 1 gene encoded on Hsa1, or the PS2 gene encoded on Hsa14 (Cruts & Van Broeckhoven, 1998). Significantly, enlarged endosomes were present in neurons of patients with APP-linked mutations but absent in patients with PS-linked mutations, thereby further implicating a role for APP in causing this phenotype (Cataldo et al., 2001).

It is therefore tempting to attribute the presence of enlarged endosomes in the Ts65Dn mouse reported in this study to the overexpression of APP, as has been previously documented. However, such a conclusion cannot be made as it is unknown whether APP was encompassed within the enlarged early endosomes. This study would have benefitted from co-immunostaining EEA1 with APP and assessing their colocalisation to determine whether enlarged early endosomes
contained APP in these models. Examining the presence of enlarged endosomes in Ts65Dn<sup>App<sup>++</sup></sup> mice would have also proved ideal to identify the contribution of genes other than APP in the Ts65Dn model that are not on the Ts1Rhr model.

Interestingly, conflicting data suggests that overexpression of APP is not necessary for abnormal endosome morphology. Two mouse models of FAD that overexpress mutant APP and develop pathological features reminiscent of AD were also examined for the presence of enlarged endosomes. Transgenic mice that contain the human Swedish mutation (APP<sub>670/671</sub>) overexpress APP seven-fold, and transgenic mice that harbour the combination of Swedish and London mutations (APP<sub>670/671</sub> plus APP<sub>717</sub>) overexpress APP two-fold. The degree of AD-associated pathology in these models is associated with expression levels and is worse in the Swedish mutation alone (Sturchler-Pierrat et al., 1997). Surprisingly, neurons from both these lines of App transgenic mice had normal-appearing early endosomes and immunolabelling for other endosomal markers, such as EEA1 and Rab4, were similar to those seen in control neurons (Cataldo et al., 2003). This suggests that increased App gene dosage is necessary but not sufficient for endosomal pathology to develop in Ts65Dn mice. However, it is possible that overexpressing a mutated form of human APP in these models causes the absence of enlarged early endosome pathology; it would be interesting to assess whether overexpressing a wild-type form of human APP would result in a similar pathology.

The ApoE gene is encoded on Hsa19 and has been identified as a major susceptibility gene for AD that promotes earlier clinical onset and accentuated neuropathology (Corder et al., 1993). Specifically, inheritance of the ε4 allele of ApoE is associated with increased predisposition for disease in sporadic forms of AD (SAD), as well as in FAD cases harbouring the Swedish mutations alone and those combined with London mutations (van Duijn et al., 1994). Importantly, the endosomal morphology alterations documented in DS and SAD was more severe in individuals at preclinical stages of AD carrying the ε4 allele of ApoE, implicating an important crosstalk between ApoE and the endocytic and secretory pathways (Cataldo et al., 1997, Cataldo et al., 2000). ApoE is not synthesised in neurons and thus intracellular uptake of ApoE is critical. Early endosomes serve as one of the
few intracellular sites in which ApoE can interact with APP and its proteolytic
derivatives (Cataldo et al., 2000). Perturbed endocytosis may influence ApoE
internalisation and function, as is seen by elevated ApoE immunoreactivity in AD
pyramidal neurons (Cataldo et al., 1997). Further support for the crosstalk between
ApoE and the endocytic pathway is demonstrated by the lack of effect of ApoE ε4
genotype on modulating the age of onset in FAD caused by PS mutations (Van
Broeckhoven et al., 1994). This is consistent with the absence of endocytic pathway
impairments and suggests that PS mutations increase Aβ production predominantly
through a non-endocytic route (Xia et al., 1998). Understanding the modulating
effects of ApoE in the development of pathology is significant as increased APP gene
dosage in Ts65Dn, but not in Ts1Rhr mice, may additionally render them more
susceptible to enlarged endosomes through an ApoE-mediated mechanism.

4.4.2 DYRK1A and clathrin-mediated endocytosis

In DS, the mechanism underlying axonal transport impairments strongly suggests
the role of enlarged endosomes (Salehi et al., 2006). However, the current data
report that even though impaired axonal transport is prevalent in Ts65Dn and
Ts1Rhr mouse models, only Ts65Dn mice display enlarged endosomes. The absence
of enlarged early endosomes in the Ts1Rhr model is exciting and significant as it
supports the role of genes outside of the DSCR region, such as APP, SYNJ1 and
ITSN1, in causing enlarged endosomes (Cossec et al., 2012, Salehi et al., 2006, Yu et
al., 2008). Even though DYRK1A, a key candidate gene involved in endocytosis, is
triplicated in Ts1Rhr mice, it may be possible that disomic levels of APP, SYNJ1 and
ITSN1 in this model compensate for any deleterious effects of DYRK1A
overexpression. However, overexpression of DYRK1A has not been reported to
result in abnormal endosome morphology, implicating another mechanism
underlying axonal transport deficits in the Ts1Rhr mice (Kim et al., 2010). As
mentioned earlier, overexpression of DYRK1A causes deficits in clathrin-mediated
endocytosis and slows synaptic vesicle endocytosis by perturbing the recruitment
of endocytic proteins to clathrin-coated pits (Kim et al., 2010). It may be possible
that Ts1Rhr mice display a slowing of the endocytic pathway, such that fewer
neurotrophins are internalised and transported back to the cell body. This is
supported by the current data revealing a fewer number of early endosomes in the Ts1Rhr model compared to controls.

Further support for a putative role of **DYRK1A** overexpression in slowing down endocytosis is associated with its regulation of **SYNJ1** and **ITSN1** during retrieval of synaptic vesicle proteins in clathrin-mediated endocytosis (Adayev *et al.*, 2006, Dittman & Ryan, 2009), and it's regulation of **SYNJ1** during depolarisation of synaptic nerve terminals (Lee *et al.*, 2004). During these processes **DYRK1A** also impacts other endocytic-associated machinery including, dynamin and amphiphysin (Adayev *et al.*, 2006, Murakami *et al.*, 2006). **DYRK1A** also interacts with **RCAN1** to control endocytosis and synaptic vesicle fusion pore kinetics (Keating *et al.*, 2008). Consequently, it is conceivable that overexpression of **DYRK1A** affects retrograde axonal transport of neurotrophins through a mechanism that is independent of enlarged endosomes, and possibly through an effect that limits the availability of endocytic compartments and accessory proteins. However, further studies would need to be conducted to prove this hypothesis.

Additionally, to understand the magnitude of the impairments in endocytosis, investigating components of the early endosome and other endocytic compartments would have been advantageous. It has been reported that **Rab4** recycling endosomes and **Rab7** late endosomes are also enlarged due to a **Rab5** early endosome phenotype (Cataldo *et al.*, 2008). Examining the whole endocytic pathway to identify where the pathology occurs and is initiated would have been informative in understanding the mechanisms underlying the phenotype. This study is also limited by a static look at early endosomes; the endocytic pathway is a dynamic pathway that involves uptake and internalisation of molecules such as neurotrophins and receptors. Conducting a pulse-chase assay that examines re-uptake of other receptors would have been beneficial to assess whether endocytic deficits are specific to certain molecules or whether global deficits are prevalent, and in further understanding the dynamic nature of the endocytic pathway. An ideal experiment for these studies would allow for the morphological analysis of endosomes that contain QD-BDNF and QD-APP whilst live-cell imaging, thus enabling a causative conclusion between the relationship of neurotrophins, enlarged endosomes and the specific contribution of Hsa21 candidate genes.
4.5 Conclusion

Enlarged early endosomes have been suggested to cause the axonal transport deficits seen in DS. Enlarged early endosomes have been found in brains and fibroblasts of people with DS and in the Ts65Dn mouse model, decades before the onset of the neuropathological hallmark features that characterise AD. The absence of enlarged endosomes in Ts1Cje has alluded to the contribution of overexpressed APP in causing enlarged endosomes. Indeed, APP has been found in enlarged endosomes in Ts65Dn neurons and also in DS fibroblasts. The overexpression of APP in normal fibroblast has been sufficient to induce an enlarged endosome phenotype. Recently, the overexpression of SYJN1 was found to also induce an enlarged early endosome phenotype. These findings not only implicate another HSa21 gene in causing this phenotype, but also strengthen the existing suggestion that the pathological role of enlarged early endosomes leads to axonal transport deficits and ultimately, neurodegeneration. In this study, enlarged endosomes were found in Ts65Dn neurons, supporting the role for overexpressed APP and SYNJ1 in causing enlarged early endosomes. Interestingly, no enlarged early endosomes were observed in Ts1Rhr mice, raising the possibility that the BDNF axonal transport deficits in this model are caused by some other mechanism. DYRK1A is a key candidate gene because it is heavily implicated in clathrin-mediated endocytosis and interacts with SYNJ1, ITSN1 and RCAN1 to retrieve synaptic vesicle proteins and control fusion pore kinetics. It interacts with dynamin and amphiphysin to mediate scission of clathrin coats, and additionally associates with the actin cytoskeleton. Thus, it is conceivable that in Ts1Rhr mice, overexpression of DYRK1A affects retrograde axonal transport of neurotrophins through an enlarged endosome-independent mechanism, and possibly through a clathrin-associated machinery effect, which limits the release and availability of endocytic components, motor and accessory proteins. Indeed, in the current investigation a fewer number of early endosomes were displayed in Ts1Rhr mice. Perturbations in the machinery in clathrin-mediated endocytosis may additionally be present in Ts65Dn mice; however further studies are necessary to support this hypothesis.
CHAPTER FIVE          DENDRITIC MORPHOLOGY IN
                       MOUSE MODELS OF DS

5.1 Introduction

Dendrites are branched projections that protrude from the cell body to receive
electro-chemical stimulation from pre-synapses of other neurons. Dendrites of
neurons in most brain regions are covered by small protrusions known as dendritic
spines, which consist of a head that is connected to the dendritic shaft by a narrow
neck. Spines are specialised compartments of the dendritic tree that can locally
control signalling; they are the main site of synaptic input for neurons and are
crucial for receiving and processing synaptic information (von Bohlen Und Halbach,
2009). Spines on hippocampal pyramidal neurons can be subdivided into four
categories based on their morphological, and possibly functional, diversity:
mushroom spines have large heads, thin spines have smaller heads, stubby spines
have no obvious size constrictions, and filopodium spines, named after their hair-
like morphology that frequently make synaptic connections with axons (Peters &
Kaiserman-Abramof, 1970, Skoff & Hamburger, 1974). Spines with large heads are
stable and contribute to strong synaptic connections and hypothesised to represent
“memory spines”, whereas small spines represent “learning spines” as their small
heads are motile and unstable and contribute to weaker or silent synaptic
connections (Kasai et al., 2003).

Dendritic spines are the principal site of excitatory synapses and contain glutamate
receptors and postsynaptic density components, whereas dendritic shafts or cell
soma tend to be where inhibitory GABAergic synapses are located (Megias et al.,
2001, Sorra & Harris, 2000). The spine surface area, spine volume, bouton volume
and the number of presynaptic vesicles correlate with synaptic area and the size of
its excitatory synapse, whereas the length of the spine and the neck diameter are
thought to be independent of the size of the synapse (Fiala et al., 2002). The density
of dendritic spines is correlated with the amount of connectivity that occurs with
axons from other neurons (Geinisman, 2000). A role for spines in synaptic plasticity
is facilitated by compartmentalisation of calcium and other signalling components involved in synaptic efficacy (Fiala et al., 2002). Indeed, it has been shown that some forms of learning, such as spatial learning and associative learning, increases the number of dendritic spines in the hippocampus, suggesting the formation of new synapses (Leuner et al., 2003, Moser et al., 1994). LTP in the hippocampus is associated with increased spine density and insertion of glutamate receptors into the post-synaptic spine membrane, which consequently boosts post-synaptic responsiveness to neurotransmitters (Muller et al., 2000, Toni et al., 2001). The size of the postsynaptic density (PSD) is associated with the size of the spine head and the number of AMPA (a-amino-3-hydroxy-5-methyl-4-isoxazole propionate) glutamate receptors at the spine membrane (Nusser et al., 1998, Takumi et al., 1999).

Synaptic plasticity deficits in neurodegenerative and neuropsychiatric diseases are commonly characterised by alterations in the number and functional properties of synapses, dendrites and dendritic spines (Kasai et al., 2003, Penzes et al., 2011, Selkoe, 2002). These deficits are seen in several neurodegenerative diseases including AD and in intellectual disability disorders including DS. A seminal study of an 18-month-old infant with DS was the earliest to analyse dendritic spines in DS; it revealed that spines were sparse, small and had short stalks in the motor cortex (Marin-Padilla, 1972). Golgi studies on the cerebral cortex of people without DS reveal an increase in postsynaptic spines on the basal dendrites from neonate to 15 years of age, followed by a progressive decrease after 20 years. However in people with DS, there is a shift in pattern displaying a delay in increase and a precocious decrease in postsynaptic spines at the same time points (Takashima et al., 1994). Children with DS show reduced dendritic branching and total dendritic length, which is in stark contrast to the expanding dendritic arborisation seen in age-matched control children (Becker et al., 1986). In adults with DS, the dendritic hypotrophy is exaggerated with severe decreases in dendritic branching, length and spine frequency that is accompanied by degeneration of neurons after the age of 30 years old (Takashima et al., 1989). Individuals with DS that have no evidence of AD, showed a significant reduction in the number of dendritic spines and arbours of hippocampal pyramidal neurons compared to age-matched controls. The phenotype was more severe in individuals with DS that had AD (Ferrer & Gullotta,
1990). This suggests that the dendritic tree atrophies during early childhood in DS and continues throughout their lifespan and progressively degenerates in adulthood, which may also facilitate the early-onset AD seen in DS (Kaufmann & Moser, 2000).

Mouse models of DS also have features resembling the dendritic pathology in DS. Neocortical pyramidal neurons in adult Ts65Dn mice show a reduction in length and arborisation of dendrites (Dierssen et al., 2003). Dendritic spine density is reduced in DG granule cells in the Ts65Dn and Ts1Cje mouse models, accompanied with morphological anomalies including increased spine head size and decreased length of spine necks (Belichenko et al., 2007, Belichenko et al., 2004, Popov et al., 2011). Reduced spine density and enlarged spine heads have been shown in Ts1Rhr mice, however no changes have been found in spine neck morphology (Belichenko et al., 2009a, Belichenko et al., 2007). The reduced density of spines, length and number of branches results in a decreased number of synaptic contacts and altered synaptic plasticity, and could possibly underlie the imbalance between excitatory and inhibitory synapses (Belichenko et al., 2009b, Belichenko et al., 2004, Kurt et al., 2004).

5.2 Chapter Aim

Dendritic atrophy and morphological alterations in dendritic spines have been documented in AD and DS. This has been further associated with functional consequences in synaptic plasticity. This chapter aims to examine whether aberrant dendritic morphology is present in the Ts65Dn and Ts1Rhr models and thus assess the genetic contribution of Hsa21 genes in causing this phenotype. The number of dendritic branching, length, surface area and volume of the dendrites will be examined.
5.3 Results

5.3.1 Dendritic morphology in Ts65Dn mice

Mass cultures of Ts65Dn hippocampal neurons were fixed and immunostained on DIV7 with MAP2, a marker for dendrites. Z-stack images of neurons were captured with an Olympus confocal microscope system (Applied Precision) that included a Photometrics CCD mounted on a Nikon TE-200 inverted epi-fluorescence microscope. For each image, 10 optical sections spaced by 0.5mm were captured at a magnification of 20x. Data sets were analysed using the FluoroView software for Olympus. The panel below shows MAP2 co-immunolabelled with Rab5 in Ts65Dn and 2N neurons (Figure 5.1).

![Image of MAP2 and Rab5 staining](image)

**Figure 5.1: Immunostaining of 2N and Ts65Dn dendrites.** Hippocampal neurons from 2N and Ts65Dn were isolated from E18 pups and grown for 7 days *in vitro*. On DIV7, cells were fixed and prepared for immunofluorescence confocal microscopy. Neurons were stained for MAP2 (488) and Rab5 (568). Scale bar, 20 mm.
To analyse dendritic morphology, the level of branching, length, area and volume of dendrites was assessed using AutoNeuron, which is an extension to the Neurolucida software (MBF Biosciences). AutoNeuron is an automated interactive tracing tool to measure 3D volume stacks. It identifies cell bodies and neuronal processes, outlines the cell body volume at each image plane, traces the processes and connects them to form a reconstructed set of trees. After identifying cell bodies of neurons based on defined parameters, the dendritic processes were traced and reconstructed for further quantitative analysis using Neurolucida. For each genotype, 15 images were captured over 3 independent experiments, with approximately 35 neurons examined per image (Figure 5.2).

Figure 5.2: Tracing of 2N and Ts65Dn dendrites with AutoNeuron. Dendrites on original images labelled with MAP2 were traced after identifying cell bodies within the captured frame. A final traced image was generated from which quantification analyses could be conducted. Scale bar, 20 mm.
Figure 5.3: Quantification of dendrites in Ts65Dn neurons relative to 2N. (A) Ts65Dn neurons did not differ from 2N in the number of dendritic branches that stemmed from each soma (B) or in the length of dendrites. However, there was a significant reduction in (C) dendritic surface area and (D) volume in Ts65Dn neurons compared to 2N (*p<0.05).

Dendrites from Ts65Dn neurons did not vary from 2N in the number of dendritic branches per neuron (t=1.35, df=35, p=0.18) (Figure 5.3A). On average Ts65Dn dendrites had 4.71 (± 0.43) branches compared to 5.88 (± 0.74) branches in neurons of 2N. The length of dendrites in Ts65Dn neurons did not significantly differ from that in 2N neurons (t=1.62, df=35, p=0.11) (Figure 5.3B). The average length of Ts65Dn dendrites was 172.40 µm (± 11.50), and the average length of 2N dendrites was 208.60 µm (± 18.76). Ts65Dn dendrites demonstrated a significantly
reduced dendritic surface area (1061.00 µm² ± 100.50) compared to 2N dendrites (1387.00 µm² ± 110.80; \( t = 2.17, \) \( df = 35, p < 0.05 \)) (Figure 5.3C). The dendritic volume was also significantly reduced in Ts65Dn dendrites (676.80 µm³ ± 92.69) compared to 2N (958.80 µm³ ± 98.01; \( t = 2.09, \) \( df = 35, p < 0.05 \)) (Figure 5.3D).

5.3.2 Dendritic morphology in Ts1Rhr mice

Similar to the Ts65Dn model, mass cultures of Ts1Rhr hippocampal neurons were fixed and immunostained on DIV7 with MAP2. For each image, 10 optical sections spaced by 0.5mm were captured at a magnification of 20x. Data sets were analysed using the FluoroView software for Olympus. The panel below (Figure 5.4) shows Z-stack confocal images of immunolabelled MAP2 and LAMP1, a lysosome-associated membrane protein 1, in Ts1Rhr and wild-type littermate controls neurons.

![Confocal images of MAP2 and LAMP1](image)

**Figure 5.4**: Immunostaining of 2N and Ts1Rhr dendrites. Hippocampal neurons from 2N and Ts1Rhr were isolated from E18 pups and grown for 7 days *in vitro*. On DIV7, neurons were fixed and prepared for immunofluorescence confocal microscopy. Neurons were stained for MAP2 (488) and Lamp1 (568). Scale bar, 20 mm.
AutoNeuron was used to analyse dendritic morphology, the level of branching, length, surface area and volume of dendrites in each image. As with the Ts65Dn protocol, AutoNeuron first identified the cell bodies of neurons and subsequently, dendrites were traced and analyses generated dependent on the reconstructed template. For each genotype, 15 images were captured over 3 independent experiments, with an average of 35 neurons examined per image (Figure 5.5).

![Figure 5.5: Tracing of 2N and Ts1Rhr dendrites with AutoNeuron.](image)

Dendrites in original images labelled with MAP2 were traced after identifying neuron cell bodies within the captured frame. A final traced image was generated from which quantification analyses could be conducted.

The average number of branches per Ts1Rhr neuron was 4.75 (± 0.58), which did not significantly differ to an average of 3.81 (± 0.54) dendritic branches per 2N neurons (t=1.19, df=36, p=0.25) (Figure 5.6A). No significant differences were seen between the average dendritic length of Ts1Rhr neurons (133.30 μm ± 15.78) and 2N neurons (117.60 μm ± 19.25; t=0.63, df=36, p=0.53) (Figure 5.6B). Dendritic surface area also did not vary between the genotypes (t=0.03, df=36, p=0.97). The average surface area of Ts1Rhr dendrites was 660.20 μm² (± 119.50), and the
average surface area of 2N dendrites was 654.10 µm² (± 137.10) (Figure 5.6C). The average dendritic volume of 353.60 µm³ (± 96.67) in Ts1Rhr neurons was not significantly different to the average dendritic volume of 392.10 µm³ (± 102.20) in 2N neurons (t=0.27, df=36, p=0.79) (Figure 5.6D).

Figure 5.6: Quantification of dendrites in Ts1Rhr neurons relative to 2N. Ts1Rhr neurons did not differ to 2N in (A) the number of dendritic branches per soma, (B) in length, (C) surface area or (D) volume of dendrites.
5.3.3 Summary of results

The table below (Table 5.1) summarises dendritic morphology in Ts65Dn and Ts1Rhr mice, relative to respective controls.

Table 5.1 Dendritic morphology in Ts65Dn and Ts1Rhr mice*

<table>
<thead>
<tr>
<th>Dendritic Morphology</th>
<th>Ts65Dn</th>
<th>Ts1Rhr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Branching</td>
<td>Not Significant</td>
<td>Not Significant</td>
</tr>
<tr>
<td>Length</td>
<td>Not Significant</td>
<td>Not Significant</td>
</tr>
<tr>
<td>Surface Area</td>
<td>Decreased</td>
<td>Not Significant</td>
</tr>
<tr>
<td>Volume</td>
<td>Decreased</td>
<td>Not Significant</td>
</tr>
</tbody>
</table>

*All results are compared to relative controls
5.4 Discussion

Dendritic morphology is important for synaptic plasticity and for maintaining effective communication between synapses (Kasai et al., 2003, Newpher & Ehlers, 2009). Dendritic atrophy in DS is associated with synaptic plasticity deficits and neurodegeneration, and has been reported in people with DS as well as mouse models of DS (Becker et al., 1986, Belichenko et al., 2009a, Belichenko et al., 2007, Takashima et al., 1989). In support of abnormal dendritic morphology, the findings herein reveal reduced dendritic surface area and reduced dendritic volume in Ts65Dn mice. However, no deficits were observed in the number of branches per neuron or in dendritic length. Interestingly, no dendritic morphology anomalies were found in Ts1Rhr mice.

Dendritic morphology studies in Ts65Dn mice have shown approximately 18% decrease in spine density in the fascia dentate (FD), a 65% increase in spine head size (defined as above 0.5um² in size), and a 34% decrease in spine neck length, relative to controls (Belichenko et al., 2009b, Belichenko et al., 2007, Belichenko et al., 2004). The same parameters have been affected in Ts1Cje mice with a 12% decrease in spine density in the FD, a 26% increase in spine head size, and a 26% shorter spine neck length, compared to controls (Belichenko et al., 2009a, Belichenko et al., 2007). Ts1Rhr mice showed a 14% decrease in spine density, 7% increase in spine head size, and no differences in spine neck length compared to controls (Belichenko et al., 2009a). No differences in diameter of dendrites were observed in any of the models (Belichenko et al., 2009a, Belichenko et al., 2007, Belichenko et al., 2004).

It is noteworthy that an aggregate severity of phenotype is apparent in these models; Ts65Dn mice, which contain the largest trisomic region, show the most severe spine morphology phenotype. Ts1Cje mice, which contain 78% of the trisomic segment in Ts65Dn mice, demonstrate a less severe phenotype and Ts1Rhr mice, which only contain 31 trisomic genes orthologous to the DSCR, show a markedly less phenotype. The decreased severity in phenotypes amongst these models suggests that genes present in Ts65Dn and Ts1Cje, but not in Ts1Rhr, mice
contribute to the phenotypes. However, the conservation of decreased spine density and enlarged spine head in Ts1Rhr mice is evidence that genes triplicated in this segment contribute to the documented abnormalities (Belichenko et al., 2009a). Conclusively, it seems that for some phenotypes, the contributions of more than one gene are physiologically significant and have an additive effect.

In this study, a similar pattern in severity of phenotype was apparent between Ts65Dn and Ts1Rhr mice. Ts65Dn mice displayed reduced dendritic surface area and dendritic volume, whereas Ts1Rhr mice did not reveal any abnormal dendritic morphology phenotypes. This suggests that triplicated genes present in Ts65Dn mice, but not in Ts1Rhr mice, may be responsible for the dendritic surface area and volume phenotypes. The observation that dendritic branching and length were not affected in either of the trisomic models raises the possibility that genes not triplicated in these models are responsible for preservation of these parameters.

5.4.1 Contribution of Hsa21 genes

Several types of intellectual disabilities have been attributed to genetic defects in scaffolding and adhesion molecules, such as Rho GTPases, that are involved in maintaining dendritic structure, spine morphology and cytoskeletal regulation and consequently, synaptic plasticity (Nadif Kasri & Van Aelst, 2008). Hsa21 encodes several of such genes that may influence dendritic morphology (Sturgeon & Gardiner, 2011). It is feasible that deficits in these proteins might result in smaller spines, which are unable to maintain spine structure and the inability to preserve large spines for the accumulation of activity-dependent plasticity, thereby potentially causing deficits in acquisition of memory processes (Kasai et al., 2003).

Preserved dendritic morphology in Ts1Rhr mice points to triplicated genes not orthologous to the DSCR in causing specific dendritic morphology impairments. A key candidate gene is APP, which amongst other functions, has been suggested to be involved in synaptic plasticity, synapse formation and neurite outgrowth (De Strooper & Annaert, 2000, Turner et al., 2003). In APP knock-out mice, reduced dendritic lengths have been found in hippocampal neurons, leading to impaired LTP and deficits in hippocampal synaptic plasticity (Dawson et al., 1999, Seabrook...
et al., 1999). This was further confirmed in a more recent study of APP knock-out mice, that reported an approximate 35% decrease in spine density in hippocampal neurons in vitro and approximately 15% decrease in vivo (Tyan et al., 2012). The deficit in vitro was partially rescued by sAPPα-conditioned medium, suggesting this specific fragment is necessary for maintenance of dendritic integrity (Tyan et al., 2012).

In AD and mouse models that overexpress APP, proximity to Aβ plaques is associated with dendritic and axonal dystrophy, perturbed sprouting and curvature of dendritic processes, loss of dendritic spines and synaptic loss in hippocampal and neocortical pyramidal neurons (Le et al., 2001, Spires et al., 2005, Tsai et al., 2004). In addition to spine loss and reduced dendritic diameter, axons close to fibrillar Aβ deposits developed large varicosities in the PSAPP transgenic mouse (Tsai et al., 2004). In Tg2756 mice, altered trajectories of dendrites was reported with an approximate 50% deficit in spine density on dendrites close to plaque edges, and almost 25% reduction of dendrites not associated with plaques, suggesting a widespread loss postsynaptic activity (Spires et al., 2005). Collectively, the data strongly implicate the overexpression of APP in Ts65Dn mice in causing dendritic surface area and volume deficits. This does not preclude the involvement of other triplicated genes in Ts65Dn that are trisomic on the Ts1Rhr model. Examining dendritic morphology in the Ts65DnApp++ model would have been extremely insightful in elucidating whether genes other than APP in the Ts65Dn model contribute to this phenotype.

TIAM1 (T lymphoma invasion and metastasis 1) is a Rac1-specific guanine exchange factor (GEF) (Habets et al., 1994). Rac1 is a small GTPase of the Rho family, which are critical regulators of the organisation of the cytoskeleton, and plays a pivotal role in regulating the structure of actin in neuronal growth cones that mediates axonal and dendritic growth. Rho GTPases induce formation of axons and dendrites by transducing signals from guidance cues, such as neurotrophins, to the actin cytoskeleton (Etienne-Manneville & Hall, 2002, Luo, 2000). TIAM1 is widely expressed and is located within growth cones, dendrites and spines to promote the formation of axons and dendrites (Kunda et al., 2001). TIAM1 couples
with the NMDA receptor to control the growth and morphological changes of dendritic arbors and spines (Tolias et al., 2005). Interestingly, BDNF stimulation leads to TrkB directly binding to and phosphorylation of Tiam1/GEF signalling to enhance actin cytoskeleton activity including, lamellipodia formation in vitro, and increased neurite outgrowth from cortical neurons (Miyamoto et al., 2006). TIAM1 was also identified as a mediator of NGF/TrkA-dependent neurite elongation (Shirazi Fard et al., 2010). This implicates TIAM1 as a mediator through which neurotrophin-receptor phosphorylation stimulates Rac1 activity to regulate actin cytoskeleton morphology and induce axonal and dendritic growth. It is therefore conceivable that overexpression of TIAM1, as in DS, may lead to perturbed cytoskeleton activity affecting axonal and dendritic growth.

Several triplicated genes in the Ts1Rhr model are implicated in directly or indirectly regulating dendritic morphology, including DSCAM, SIM2 and DYRK1A (Alves-Sampaio et al., 2010, Ooe et al., 2004, Ryoo et al., 2007, Shim & Lubec, 2002). In primary cortical neurons, Dyrk1a overexpression induced severe reduction of dendritic growth and dendritic complexity (Lepagnol-Bestel et al., 2009). Similarly, in a Dyrk1a transgenic mouse, a transcriptome analysis of embryonic brain subregions identified a concerted deregulation of multiple genes involved in dendritic growth impairments in DS (Lepagnol-Bestel et al., 2009). It is therefore surprising that the overexpression of these key genes do not lead to dendritic morphology deficits in this model. However, as with the persevered dendritic spine neck length morphology in previous studies (Belichenko et al., 2009a, Belichenko et al., 2007), it may be that triplicated genes in Ts1Rhr are not necessary for the dendritic surface area and volume phenotypes seen in this study. Another possibility is that as with the enlarged dendritic spine head and reduced spine density phenotype, a less severe phenotype in dendritic morphology may be present in Ts1Rhr, however the parameters examined may not be sensitive enough to pick up on these deficits. One other possibility is that disomic levels of APP in this model compensates for the overexpression of all these other genes that are involved in regulation of dendrite morphology. However further studies on the Ts65DnApp++ mouse would need to be conducted to confirm this hypothesis.
5.4.2 Synaptic plasticity and memory

The findings reported here are derived from mass cultures of hippocampal neurons and the overall dendritic morphology is examined rather than specifically looking at dendritic spines, which makes it difficult to speculate about functional consequences and dendritic synaptic plasticity. However it does raise the possibility that decreased surface area and volume of dendrites may result in reduced spine density and/or smaller spines, leading to impaired synaptic plasticity and the inability to make strong and frequent synaptic connections. Examination of dendritic morphology at a higher magnification would allow for a more detailed analysis of dendrites and the study of dendritic spines, which would be informative in understanding the contribution of Hsa21 genes in synaptic plasticity deficits seen in the models (Belichenko et al., 2009a, Belichenko et al., 2007, Belichenko et al., 2004). It also would have been advantageous to conduct a Scholl analysis, which examines the order and complexity of dendritic branching thereby providing a deeper understanding of synaptic plasticity deficits and dendritic arborisation in the Ts65Dn and Ts1Rhr mouse models.

When LTP is induced, AMPA receptors are mobilised into dendritic spines, through endosomal-recycling compartments that are localised at the base of dendritic spines, to meet the requirements of increased need for receptors at the membranes of spine necks and heads (Kelly et al., 2011). This implicates a crucial role for endosomal-recycling compartments in generating LTP. Although dendritic spines were not examined in this study and it is unknown whether reduced dendritic surface area and volume leads to synaptic plasticity deficits, it is tempting to postulate that the endosomal abnormalities seen in DS in this report (Chapter Four) and elsewhere (Cataldo et al., 2008, Cataldo et al., 2004, Jiang et al., 2010, Salehi et al., 2006), may hinder the transport of AMPA receptors to dendritic spines in Ts65Dn mice when LTP is induced.
5.5 Conclusion

Dendritic spine morphology is essential for maintaining synaptic plasticity, effective communication between neurons, and for learning and memory. Abnormal dendritic morphology is widely established in people with AD and DS and mouse models that recapitulate these phenotypes. Dendritic dystrophy and arborisation is commonly associated with lowered LTP induction, indicating impaired synaptic plasticity. APP has been suggested to be involved in synaptic plasticity, synapse formation and neurite outgrowth, and mouse models that are App-deficient demonstrate severe impairments in dendritic spine density and morphology. Even proximity to Aβ plaques in mouse models of AD is associated with dendritic and axonal dystrophy, loss of dendrite spines and reduced synaptic plasticity. Significantly, the BDNF/TrkB signaling complex is heavily involved in the regulation of hippocampal spine density and morphology. Mouse models that are Bdnf-deficient or trkB-deficient demonstrate reduced spine density and perturbed morphology. Even though dendritic spines were not examined in this investigation, the current findings support the role of APP in synapse formation, as evidenced by dendritic impairments present in Ts65Dn mice, but absent in Ts1Rhr mice. In Ts1Rhr mice, it may be that disomic levels of APP compensate for potentially reduced levels of BDNF due to axonal transport impairments. However, further studies would need to be conducted to support this hypothesis.
6.1 General Discussion

People with DS are more susceptible to early-onset AD, and the histopathological features and endocytic perturbations that characterise AD are present at an earlier age in people with DS than the general population with AD (Holtzman et al., 1996, Mann & Esiri, 1989, Wisniewski et al., 1985). People with DS, and mouse models of DS, display dendritic atrophy and aberrant dendritic spine morphology, which is associated with synaptic plasticity and learning and memory deficits, and neurodegeneration (Becker et al., 1986, Belichenko et al., 2009a, Belichenko et al., 2007, Takashima et al., 1989). The Ts65Dn mouse model of DS recapitulates certain DS-associated neurodegenerative phenotypes. Even though Ts65Dn mice do not display Aβ plaques or NFTs, they do show cognitive deficits, which is associated with the neurodegeneration of BFCNs (Granholm et al., 2000, Holtzman et al., 1996, Hunter et al., 2003). It is suggested that the degeneration of neurons is caused by impaired retrograde axonal transport of essential neurotrophic factors (Cooper et al., 2001). The triplication of APP, an Hsa21 gene, is proposed to cause enlarged early endosomes and a perturbed endocytic pathway that subsequently leads to axonal transport deficits (Cataldo et al., 2003, Salehi et al., 2006).

The research in this thesis aimed to recapitulate the DS-associated phenotypes found in Ts65Dn mice, and to elucidate the contribution of Hsa21 genes, in addition to APP, to the pathogenesis of these deleterious phenotypes. In support of previous findings, Ts65Dn hippocampal neurons had impaired BDNF axonal transport characterised by endosomes labelled with BDNF moving at a slower velocity and covering less distance in the retrograde direction. Transport in these mice was further hindered by micropauses throughout movement and by stationary endosomes. This impairment may have been caused by enlarged endosomes and upon examining Ts65Dn mice the enlarged early endosome phenotype was indeed recapitulated. However there is no direct evidence that the transported endosomes
are indeed enlarged in size. Ts65Dn neurons displayed a greater number of early endosomes, and a greater number of enlarged early endosomes. Additionally, the average endosome size was twice the size of controls. Similar to previous findings, perturbed dendritic morphology was also prevalent in Ts65Dn mice with dendrites exhibiting reduced surface area and volume. These findings suggest that triplicated genes encoded on the Ts65Dn translocated chromosome, including \textit{APP}, are responsible for impaired BDNF axonal transport, perturbed endocytosis and aberrant dendritic morphology, which may lead to synaptic plasticity deficits and neurodegeneration.

To assess the genetic contribution of other Hsa21 genes in causing these phenotypes, the Ts1Rhr mouse model was examined. Axonal transport and early endosome morphology has not previously been assessed in this model. Ts1Rhr mice encode 31 duplicated genes that are orthologous to the human DSCR, which was once thought to be sufficient to cause most of the DS-associated phenotypes (Delabar \textit{et al.}, 1993, Korenberg \textit{et al.}, 1990, Olson \textit{et al.}, 2004a). Similar to the findings in the Ts65Dn mice, Ts1Rhr mice displayed impaired axonal transport with endosomes containing BDNF moving at a slower rate and travelling a reduced distance. Retrograde transport was also impaired, however anterograde transport was spared. As in Ts65Dn mice, the hindered transport in Ts1Rhr mice was characterised by a greater number and duration of micropauses and more stationary behaviour of BDNF-labelled endosomes. Interestingly, Ts1Rhr did not show any early endosome enlargement or dendritic morphology perturbations. They did however display a reduced number of endosomes. These findings suggest that in addition to \textit{APP}, one or more of the 31 genes encoded on the duplicated segment in Ts1Rhr mice may be responsible for the impaired axonal transport phenotype in the DS mouse models. However, the DSCR may not be sufficient or necessary to cause an increase in number and size of early endosomes or dendritic morphology phenotypes.

The findings from this report have important implications for the mechanisms proposed to underlie axonal transport deficits in Ts65Dn mice. The absence of enlarged endosomes in Ts1Rhr mice supported previous findings that triplicated genes outside the DSCR region, such as \textit{APP} and \textit{SYNJ1}, cause enlarged endosomes.
(Cossec et al., 2012, Salehi et al., 2006). However, other mechanisms may additionally contribute to BDNF axonal transport deficits in DS. The trisomy of *DYRK1A*, which is encoded on the DSCR, suggests the involvement of other endocytic processes including restricted availability of endocytic compartments and accessory proteins, and perturbed clathrin-mediated endocytosis (Adayev et al., 2006, Dittman & Ryan, 2009, Keating et al., 2008). Indeed, a reduced number of endosomes found in Ts1Rhr supports this hypothesis. Moreover, *DYRK1A* phosphorylates Tau, directly and indirectly through GSK3β, raising the possibility that cytoskeleton and motor protein perturbations may additionally contribute to axonal transport deficits (Elliott et al., 2005, Luo, 2000, Ryoo et al., 2007).

6.1.1 Implications for BDNF and memory processes

The current findings support existing literature that shows impairments in retrograde axonal transport of NGF in the Ts65Dn mouse model (Salehi et al., 2006). The data herein extend to impairments in axonal transport of another neurotrophic factor, BDNF, which has not been previously examined in this model, and to a previously unexamined Ts1Rhr mouse model. This suggests that axonal transport deficits are not specific to a neurotrophic factor, but that the trafficking of neurotrophins, in general, is impaired in DS. Perturbations in retrograde transport from the axonal terminal to cell body may compromise the function of BDNF in supporting neuronal survival (Barde et al., 1982). The BDNF/TrkB signalling complex is critical to trigger nuclear transcription of CREB and the stimulation of sustained MAPK/PI3K/PLCγ signalling cascades (Huang & Reichardt, 2003). Perturbed axonal transport of the BDNF/TrkB signalling complex may result in the deleterious phenotypes seen in DS and AD of impaired axonal and dendritic growth, synaptic plasticity deficits and reduced neuronal survival (Becker et al., 1986, Belichenko et al., 2007, Salehi et al., 2006, Spires et al., 2005, Tsai et al., 2004).

BDNF is highly expressed in various brain regions heavily implicated in learning and memory, such as the hippocampus, cortex and basal forebrain, and is essential for not only the survival but also the functioning of these neural populations (Chao & Lee, 2004). Even though BDNF axonal transport was only examined in the
hippocampus, it is conceivable that the deficits are prevalent in all the brain structures that are vital to higher cognitive function, leading to learning and memory deficits. Indeed, decreased BDNF expression and mRNA levels have been reported in the hippocampus and cortical brain regions of people with AD (Connor et al., 1997, Holsinger et al., 2000, Phillips et al., 1991). Memory processes including acquisition and consolidation are associated with increased BDNF mRNA expression and TrkB phosphorylation in hippocampal-dependent contextual learning tasks (Hall et al., 2000, Yamada et al., 2002). Although BDNF and phosphorylated TrkB or their mRNA levels were not assessed in the current study, it would not be surprising if Ts65Dn and Ts1Rhr had decreased expression levels. Deficits in BDNF axonal transport may underlie the cognitive deficits previously documented in behavioural tests with the Ts65Dn and Ts1Rhr mice (Belichenko et al., 2009a, Olson et al., 2007). Support for this hypothesis comes from studies in mouse models showing that inhibition of BDNF leads to deficits in spatial learning, short-term and long-term memory processes (Alonso et al., 2002, Mu et al., 1999).

The activated BDNF/TrkB signalling complex modulates LTP enhancement in the hippocampus by increasing NMDAR phosphorylation (Kafitz et al., 1999, Suen et al., 1997). In Ts65Dn and Ts1Rhr mice, failed LTP induction in the hippocampus and FD has been attributed to an E-I imbalance (Belichenko et al., 2009a, Belichenko et al., 2004, Kleschevnikov et al., 2004). It is possible that impaired BDNF axonal transport may additionally contribute to failed LTP induction and cognitive deficits through decreased NMDAR phosphorylation. However, further studies would need to be conducted to elucidate whether BDNF/TrkB-mediated LTP induction is a contributing mechanism to learning and memory deficits in DS.

BDNF is also involved in the regulation of hippocampal spine density and spine morphology (Chapleau et al., 2008). It is secreted by dendrites and local synthesis of BDNF is demonstrated by dendritic localisation of BDNF mRNA transcripts (Chiaruttini et al., 2009, Gottmann et al., 2009, Kohara et al., 2001, Kolarow et al., 2007, Kuczewski et al., 2010). A study of Bdnf mutant mice found altered spine density and spine head in dendrites of CA1 hippocampal neurons at two months of age (An et al., 2008). In further support of the involvement of BDNF-TrkB signalling cascade in maintenance of dendritic spines, trkB-deficient mice display reduced
dendritic spines in the hippocampus (von Bohlen und Halbach et al., 2008, von Bohlen und Halbach et al., 2006). It is conceivable that deficits in axonal transport of BDNF may result in an inability of BDNF to localise at dendrites and engage in local translation to maintain synaptic plasticity and regulate dendritic morphology. Moreover, perturbed dendritic morphology in Ts65Dn mice may additionally exacerbate suppressed BDNF function. Consequently, the weakened dendritic phenotype seen in Ts65Dn, as characterised by reduced dendritic surface area and volume, accompanied with impaired BDNF axonal transport, may possibly lead to neurodegeneration through lack of activity-dependent synaptic plasticity and reduced ability for neurons to communicate. The absence of dendritic morphology deficits in Ts1Rhr mice may indicate a less severe phenotype compared to Ts65Dn model and may explain the relatively better performance in behavioural tests compared to Ts65Dn mice (Belichenko et al., 2009a, Olson et al., 2007).

6.1.2 Hsa21 genes and additional mechanisms

The role of key candidate Hsa21 genes in causing impaired axonal transport, perturbed endocytosis and aberrant dendritic morphology has been reviewed in Chapters 1, 3-5. The results herein suggest that APP contributes to axonal transport deficits but is not necessary for the impairment, as exhibited in Ts1Rhr mice. However, APP does appear necessary for the early endosome and dendritic morphology perturbations. Other key genes not triplicated in the Ts1Rhr model include, Tiam1, Sod1, Synj1, Olig1, Olig2, Itsn1 and Rcan1. These genes have been implicated in neurogenesis, neurodevelopment, axonal growth and endocytosis. To identify individual genes or loci on Hsa21 that are responsible for producing DS phenotypes, mouse models that overexpress a single gene have been instrumental. The overexpression of some of these genes and the proteins they encode may provide insight into additional mechanisms that lead to neurodegeneration. However, the phenotypes in the transgenic models need to be interpreted with caution, as they may be associated with an uncontrollable overexpression of the transgene and thus may not be a true correlation with DS-associated phenotypes.


*SOD1* plays a crucial role in the metabolism of reactive oxygen species and codes for a key enzyme that catalyses the conversion of superoxide radicals into hydrogen peroxide (Fridovich, 1995). Recently, *SOD1* was found to integrate signals from oxygen, glucose and reactive oxygen, to repress respiration, through a casein kinase 1-gamma (CK1γ)-mediated pathway (Reddi & Culotta, 2013). In addition to reducing hippocampal neuronal progenitor cells, overexpression of *SOD1* has been associated with increased production of reactive oxygen species and apoptosis in DS (Busciglio & Yankner, 1995). Transgenic mice that overexpress human *SOD1* demonstrate spatial memory deficits accompanied with an impaired ability to express LTP in the hippocampus (Gahtan *et al.*, 1998, Levkovitz *et al.*, 1999). Similar to tongue muscles of people with DS, tongue neuromuscular junctions (NMJ) in *SOD1* transgenic mice display withdrawal and destruction of terminal axons and a decreased ratio of terminal axon area to postsynaptic membrane (Avraham *et al.*, 1988). They also show significant changes resembling excessive and premature ageing in the NMJ of the tongue (Yarom *et al.*, 1988). Elevated hydrogen peroxide levels caused by increased *SOD1* gene-dosage affects NMJ membranes and plasticity, and may possibly contribute to the precocious ageing reported in DS and the greater susceptibility to early-onset AD (Beacher *et al.*, 2010, Groner *et al.*, 1990). Proteomic analyses of *SOD1* transgenic cortices reveal reduced proteasome activity, similar to that observed during normal ageing (Le Pecheur *et al.*, 2005). It is possible that premature ageing and neurodegeneration may involve oxidative stress and impairment of proteasome activity.

*RCAN1* is highly expressed in the fetal brain, heart and skeletal muscle, suggesting a crucial role in the development of the CNS (Fuentes *et al.*, 2000, Fuentes *et al.*, 1995). *RCAN1* inhibits calcineurin, a Ca²⁺-activated protein phosphatase that regulates neuronal functions, neurotransmitter release, neurite outgrowth and neuronal cell death (Fuentes *et al.*, 2000). An increase in Ca²⁺ leads to the activation of calcineurin that then dephosphorylates cytosolic NF-AT transcription factor, resulting in its translocation into the nucleus and binding with other transcription factors (Arron *et al.*, 2006, Crabtree, 1999). Overexpression of *RCAN1* inhibits calcineurin-dependent gene transcription by inhibiting translocation of NF-AT to the nucleus, implicating a pathological role for *RCAN1* in perturbed synaptic plasticity (Fuentes *et al.*, 2000). Transgenic mice overexpressing *RCAN1* develop
normally and show no overt phenotype. However, they show reduced levels of exocytosis with decreased number of vesicles fusing with the plasma membrane and at a hindered fusion speed, suggesting RCAN1 regulates exocytosis (Keating et al., 2008). In another transgenic model overexpressing RCAN1, marked deficits in the learning process in a visuospatial learning and memory task was seen, however memory was retained, suggesting a specific deficit in the learning process with intact consolidation of memory (Dierssen et al., 2011). This suggests that the threshold for hippocampal dependent synaptic plasticity and memory storage may be determined by the proper balance between protein phosphorylation and dephosphorylation, mediated by the PKA kinase and calcineurin. The overexpression of RCAN1 and excess inhibition of calcineurin acts as an inhibitory constraint on this balance and significantly affects learning, but not memory (Dierssen et al., 2011).

Recently, transgenic mice overexpressing RCAN1 were found to exhibit structural brain abnormalities in DS-affected areas (Martin et al., 2012). The volume and number of neurons in the hippocampus was reduced, correlating with perturbed adult neurogenesis. Overexpression of RCAN1 altered the ability of NPCs to produce neurons, with proportionately more astrocytes and oligodendrocytes produced in transgenic cultures. This is similar to the phenotype found in mouse models of DS, and it is possible that trisomy of proximal genes, Olig1 and Olig2, may further exacerbate this phenotype (Chakrabarti et al., 2010). Hippocampal pyramidal neurons in the transgenic RCAN1 model also displayed reduced density of dendritic spines, which mirrors the impairments found in DS (Becker et al., 1986, Belichenko et al., 2009a). The mice also demonstrated deficits in hippocampal-dependent learning and memory, with an accompanied failure to maintain LTP. Additionally, they displayed diminished calcium transients and decreased phosphorylation of CaMKII and ERK1/2 proteins, which activate the CREB transcription factor to initiate gene transcription essential for the maintenance of LTP and memory (Martin et al., 2012). These findings implicate an important role for RCAN1 in hippocampal development and therefore, when triplicated, a pathological role in DS-associated brain anomalies, neurogenesis impairments, and synaptic plasticity and learning and memory deficits.
6.1.2.1 DSCR genes

In addition to *DYRK1A*, Ts1Rhr mice encode 30 other genes that are orthologous to the human DSCR including, *DOPEY2, SIM2, KCNJ6, ETS2, PCP4*, which have also been implicated in neurogenesis, neurodevelopment, axonal growth and endocytosis. Transgenic mice that overexpress some of these genes and the proteins they encode may provide further insight into mechanism that lead to neurodegeneration.

*Sim* is a master developmental regulator of Drosophila neurogenesis (Nambu *et al.*, 1991). Mutations in the *sim* gene result in a loss of precursor cells that give rise to the midline cells of the CNS during development (Crews *et al.*, 1988). This implicates *sim* in early midline cell development processes, including synchronised cell division and proper formation of nerve cell precursors (Nambu *et al.*, 1991). The human orthologue of *sim, SIM2*, is expressed in the developing human brain (Rachidi *et al.*, 2005a). During mouse embryonic development, *Sim2* is expressed in facial and trunk cartilage and highly expressed in the ventral diencephalon (Ema *et al.*, 1996, Fan *et al.*, 1996). It is feasible that overexpression of *SIM2* contributes to DS-associated phenotypes including, altered craniofacial morphology, impaired neurogenesis, and an E-I ratio imbalance. Indeed, transgenic mice overexpressing *Sim2* demonstrate mild learning and memory impairments (Ema *et al.*, 1999). In a bacterial artificial chromosome (BAC) transgenic mouse, which overexpresses *Sim2* from its endogenous promoter, no histopathological phenotypes were observed in the brain, but they did demonstrate altered anxiety-related exploratory behaviour and reduced sensitivity to pain (Chrast *et al.*, 2000). The potential role of *SIM2* as a transcriptional repressor and its ability to form heterodimers, suggests that its overexpression may alter dimer formation kinetics leading to abnormal regulation of downstream genes (Chrast *et al.*, 1997).

Purkinje cell protein 4 (*PCP4*), also called brain-specific polypeptide 19 (*PEP19*), is a neuro-specific calmodulin (CaM)-binding protein that inhibits CaM-dependent signalling, which is crucial for synaptic plasticity (Cabin *et al.*, 1996, Chen *et al.*, 1996). The *Pcp4* gene is expressed in postmitotic neuroectoderm cells during mouse embryogenesis and is highly enriched in cerebellar Purkinje cells (Cabin *et al.*, 1996, Chen *et al.*, 1996). CaM kinase-dependent signalling is critical for the
establishment of LTD and for cerebellum-dependent locomotor learning (Ahn et al., 1999). It is likely that the trisomy of PCP4 contributes to synaptic plasticity and cognitive impairments in DS. A transgenic mouse model containing one human copy of PCP4 showed precocious neuronal differentiation and increased the activation of CaMKII, a Ca²⁺-CaM target (Mouton-Liger et al., 2011). Similar modifications were seen in Ts1Cje mice, suggesting that these mechanisms may give rise to abnormal neuronal development in DS (Mouton-Liger et al., 2011). The essential role for PCP4 in synaptic plasticity in the cerebellum is further demonstrated by Pep19/Pcp4-knockout mice, in which synaptic plasticity at excitatory synapses of cerebellar Purkinje cells was dramatically altered from exhibiting LTD to LTP (Wei et al., 2011). Moreover, these mice showed a marked deficit in learning a cerebellum-dependent locomotor task (Wei et al., 2011).

The ETS2 gene is a proto-oncogene that codes for the transcription factor protein C-ets-2 of the ETS family, and is involved in stem cell development, cell death and tumour genesis (Seth et al., 1989). During murine development, Ets2 is expressed in all organs and highly expressed in newly forming cartilage, including skull precursor cells (Kola et al., 1993, Sumarsono et al., 1996). In transgenic mice overexpressing Ets2 less than 2-fold, the mice develop neurocranial, viscerocranial and cervical skeletal abnormalities, similar to those found in people with DS (Sumarsono et al., 1996). Ets2 is also expressed in hippocampal, cortex and cerebellum neurons, which are brain regions in DS that first develop Aβ deposits (Beyreuther et al., 1993, Braak & Braak, 1991, Wolvetang et al., 2003). Ets2 is induced by oxidative stress and overexpression of Ets2 results in neuronal apoptosis (Wolvetang et al., 2003). An increased production of hydrogen peroxide in DS is most likely due to the overexpression of SOD1 (Busciglio & Yankner, 1995), which may further upregulate expression of the triplicated ETS2 gene, leading to apoptosis. Moreover, Ets2 transgenic neurons displayed increased etiolation, shrinkage and neurite fragmentation indicative of cellular degeneration (Wolvetang et al., 2003). Interestingly, brains and primary neuronal cultures from Ets2 transgenic mice display molecular abnormalities seen in DS including, elevated expression of APP, and increased PS1 and Aβ levels (Wolvetang et al., 2003). These findings could explain why the APP expression in DS is three- to four-fold higher
than the expected 1.5-fold increase in gene-dosage, and suggests that Ets2 could further upregulate APP levels. Indeed, Ets2 was found to transactivate APP via specific Ets binding sites in the APP promoter, implicating a role for ETS2 in the pathogenesis of brain abnormalities in DS and possibly AD (Wolvetang et al., 2003).

DOPEY2 (or C21orf5), a member of the Dopey leucine zipper-like family, is expressed in several brain regions, including hippocampus, cerebellum and cortex, which are involved in learning and memory processes and altered in DS (Rachidi et al., 2009). Transgenic mice carrying a human copy of DOPEY2 displayed increased density of cortical cells overexpressing DOPEY2 (Rachidi et al., 2005b). People with DS show a disorganised cortical lamination (Haydar & Reeves, 2011), and the overexpression of DOPEY2 may contribute to the neurological features and cognitive impairments seen in DS. Additionally, in a study that examined eight transgenic mouse lines carrying YACs containing human DNA fragments covering the DSCR, two independent lines carrying the YAC230E8 displayed cerebellar alterations with a significant elongation of the cerebellar antero-posterior axis, a neurological defect in culmen and declivus lobules, increased cortical cell density and mild learning defects (Rachidi et al., 2007). Out of the seven genes on YAC230E8, DOPEY2 was identified as a key candidate gene for causing cerebellar alterations, as it is highly expressed in the brain and its homologous genes in yeast, Caenorhabditis elegans and Drosophila are involved in brain morphogenesis, implicating it as a conserved patterning gene (Rachidi et al., 2007).

In summary, studies from various mouse models of DS and transgenic models overexpressing single, or region of, genes have unravelled the complex contribution of different loci on Hsa21 to DS-associated phenotypes. It is therefore unlikely that a single master gene is responsible for the pathogenesis of specific phenotypes, but rather, a multimodal network of Hsa21 genes interacting together and with other non-Hsa21 genes, that may explain the pathological mechanisms underlying the DS-associated phenotypes. Consequently, a concerted effect of triplicated genes in DS is likely to cause the axonal transport, endocytic and dendritic perturbations that subsequently lead to neurodegeneration.
6.2 Ongoing and Future Work

The findings in this report are informative but not yet complete. Ongoing and future work in the form of a post-doctoral fellowship with Dr Mobley’s lab at University California, San Diego involves assessing the role of APP in axonal transport deficits and endosomal morphology. This involves completing BDNF axonal transport studies in Ts65Dn\(^{App^{++}}\) mice and examining whether neurons from these mice display enlarged early endosomes. Other ongoing experiments include assessing quantitative protein levels of Rab proteins involved in endocytosis, cytoskeletal and motor proteins, and the MAPK signalling cascade to assess the pathways that give rise to perturbed axonal transport, endocytic and dendritic morphology. The protein levels in Ts65Dn, Ts65Dn\(^{App^{++}}\) and Ts1Rhr mice compared to their relative controls will give insight into the genetic contribution to any possible aberrations in protein levels and signalling cascades, and indicate perturbed mechanisms involved.

Further investigations involve examining the putative role of genes in the DSCR in causing BDNF axonal transport deficits. A key candidate gene duplicated in Ts65Dn mice is Dyrk1a. Attempts were made to knock down the expression of this gene, however these efforts were unsuccessful. To assess the contribution of \(DYRK1A\) in causing transport deficits, further attempts will be pursued to knock down its expression in the Ts65Dn, Ts65Dn\(^{App^{++}}\) and Ts1Rhr mouse models. A Dyrk1a-knockout model is also available through collaboration with Professor Tybulewicz at the National Institute for Medical Research, UK. It would be informative to assess axonal transport in this mouse model to investigate the contribution of Dyrk1a in causing impaired transport. Breeding Dyrk1a-knockout mice with Ts65Dn\(^{App^{++}}\) and Ts1Rhr mice could also be conducted to examine whether normalised expression of App and Dyrk1a may rescue axonal transport deficits.
6.3 Conclusions

The triplication of an extra chromosome resulting in the overexpression of over 500 genes affecting multiple organ systems, once suggested that DS would prove an intractable condition to understand and treat. Remarkable advances in medical intervention, progress in scientific research and increased social care has deepened our understanding of DS, facilitated better health care and pointed to potential therapeutic targets to increase longevity. However, an increased life expectancy in people with DS, results in an increased prevalence of DS-associated symptoms, such as early-onset AD, leading to the emerging challenge of a precociously ageing DS population. Despite incredible progress, little is still known about the contribution of specific genes and the neurobiological and molecular pathways they engage to cause the clinical manifestation in DS. Studies assessing individuals with partial trisomy 21 have provided valuable insight into elucidating the contribution of various segments of the chromosome in causing specific DS-associated phenotypes. These findings have been extended and enhanced by studies in mouse models of DS, which have provided insights not only into genotype-phenotype correlations, but also a deeper knowledge of the neurobiological pathways underlying pathogenic mechanisms.

The early-onset AD and neurodegeneration observed in DS may be caused by deficits in axonal transport that impede essential neurotrophic factors from reaching the cell body to induce gene transcriptional changes necessary for neuronal survival. This may be caused by aberrations in the endocytic pathway and an enlarged early endosome phenotype. However, other putative mechanisms, such as perturbed cytoskeleton and motor protein function may additionally exacerbate impaired axonal transport of neurotrophins. Even though the physiological and pathological role of APP implicates it as a key candidate gene responsible for DS-associated phenotypes, it is unlikely that a single master gene contributes to the pathogenesis of all these phenotypes. Rather, a complex and multimodal concert of overexpressed Hsa21 genes synergistically interacting together and with other non-Hsa21 genes is more likely to cause the axonal transport, endocytic and dendritic impairments, leading to neurodegeneration.
REFERENCES


Trisomy for synaptojanin1 in Down syndrome is functionally linked to the enlargement of early endosomes. 

Hum Mol Genet, 21, 3156-3172.


APPENDICES

The appendices below are accessible on the attached CD-ROM:

I  Supplementary video 1 – Axonal transport in Ts65Dn mice
II Supplementary video 2 – Axonal transport in Ts1Rhr mice
III Supplementary video 3 – Axonal transport in Ts65DnApp++ mice
IV  Ts65Dn axonal transport data
V  Ts1Rhr axonal transport data
VI  Ts65DnApp++ axonal transport data
VII  Ts65Dn EEA1-endosome data using Volocity

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X  Ts1Rhr dendritic morphology data using AutoNeuron

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